Cellular Origin, Life in Extreme Habitats and Astrobiology

Richard Gordon
Joseph Seckbach *Editors*

The Science of Algal Fuels

Phycology, Geology, Biophotonics, Genomics and Nanotechnology

Springer

THE SCIENCE OF ALGAL FUELS

Cellular Origin, Life in Extreme Habitats and Astrobiology

Volume 25

Series Editor:

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ISSN 1566-0400
ISBN 978-94-007-5109-5 ISBN 978-94-007-5110-1 (eBook)
DOI 10.1007/978-94-007-5110-1
Springer Dordrecht Heidelberg New York London

Library of Congress Control Number: 2012952713

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

DEDICATION

Finished and dedicated to Leland Terrell Gordon on his 38th birthday, and to his nephew Raleigh Gordon on his first birthday.
… in Wildness is the preservation of the World.

–Henry David Thoreau, *Walking* (1862)

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BRIEF INTRODUCTION TO *THE SCIENCE OF ALGAL FUELS: PHYCOLOGY, GEOLOGY, BIOPHOTONICS, GENOMICS, AND NANOTECHNOLOGY*

The increasing global demand on fossil petroleum reserves and political manipulation of crude oil have encouraged scientists and politicians to look for alternative sources of energy.

This volume, *The Science of Algal Fuels* (volume 25 of *COLE*), contains 25 chapters dealing with biofuels contributed by experts from numerous countries. It covers several aspects of algal products, one being "oilgae from algae," mainly oils and fuels for engines. Among the prominent algal groups that participate in this process are the diatoms and green algae (*Chlorophyceae*). Their metabolism and breeding play an important role in biomass and extraction of crude oil and algal fuel. There is a strong relation between solar energy influencing algal culture and the photobiology of lipid metabolism.

Currently, many international meetings and conferences on biofuel are taking place in many countries, and several new books and proceedings of conferences have appeared on this topic. All this indicates that this field is "hot" and in the forefront of applied bioscience.

The advantage of extracting natural products from microalgae and seaweeds is that it is easy to culture them on a large scale. They have fast natural growth and yield abundant biomass within a short period. There is also the possibility to grow seaweeds in an "agricultural growing system."

Nanotechnology, one of the techniques for producing biofuels, is described in this volume. Extraction from micro- and macroalgae to produce biofuel is made after harvesting them from their habitats, namely, freshwater, seawater, and wastewater. Such production of biofuel from microalgae and seaweeds via biorefineries occurs in several countries. We believe that there is a great future for extracting high yields of biomass from microalgae and seaweeds for the production of biodiesel, fuel, and oils.

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FOREWORD

The Production of Algal Biofuels

Biofuels from algae are drawing much research, as reflected in this volume. But what about the perspectives for the (commercial) production of such biofuels?

EASY ALGAL OIL?

In recent decades, the world economy has been heavily dependent on "easy oil," mineral oil which is produced easily at low cost and in large amounts (e.g., Dale et al., 2011). As the age of "easy oil" is drawing to a close (e.g., Dale et al., 2011), one of the matters arising is whether there is "easy algal oil" to replace "easy mineral oil."

Commercial cultivation of autotrophic oil producing microalgae ("oilalgae") dates back to the 1950s (Goldman, 1979; Vonshak and Richmond, 1988). The commercial cultivation of autotrophic microalgae has since then served food and feed production, by supplying oil (lipids), algae, carotene, and astaxanthin (Apt and Behrens, 1999; Trautwein, 2001; Spolaore et al., 2006; Sahena et al., 2009; Gachon et al., 2010; Rubio-Rodriguez et al., 2010; Larkum et al., 2012). Commercial cultivation of macroalgae was also significant in the 1950s (Cheng, 1969) and has expanded much since then, serving among others the food, feed, and pharmaceutical industries and the supply of agar and carrageenan (Huguenin, 1976; Bird and Benson, 1987; Renn, 1997; Ask and Azanza, 2002; Lüning and Pang, 2003; Peng et al., 2009; Bezerra and Marinho-Soriano, 2010; Kraan, 2012).

The "oil crisis" of the early 1970s triggered proposals to cultivate autotrophic microalgae as source of oil for energy generation, which in turn did lead to a substantial research effort regarding "oilalgae" (Goldman, 1979). In the same decade, the potential contribution of photosynthetically generated microalgal H_2 to energy supply drew increased attention (Melis, 2002), whereas the production of macroalgal biomass for biofuel (ethanol, methane) production was also researched (Bird and Benson, 1987). Since then, substantial research on algal biofuels has continued (Melis, 2002; Mata et al., 2010; Bernard, 2011; Hallenbeck et al., 2012; Kraan, 2012). Recent years have been characterized by a marked increase in research investigating the energetic potential of algae. This research has regarded both microalgae and macroalgae and has also included isoprenoids and butanol, with research regarding biofuels based on microalgal oil dominating the field (Bernard, 2011; Jones and Mayfield, 2011; Konur, 2011; Kraan, 2012; Lohr et al., 2012; Potts et al., 2012; Wargacki et al., 2012).

Research has so far not led to commercial microalgal oil-based biofuel production nor to the commercial production of other algal biofuels (van Beilen, 2010; Sun et al., 2011). Given present technologies, microalgal oil is characterized by high cost (van Beilen, 2011; Petkov et al., 2012). Algal lipid-based biofuels produced with present technologies appear to be more expensive than similar current terrestrial biofuels, with biofuels from microalgae cultivated in bioreactors being more expensive than biofuels from microalgae grown in open systems such as ponds (Agusdinata et al., 2011; Amer et al., 2011; van Beilen, 2011; Ghasemi et al., 2012; Petkov et al., 2012).

From available research on technologies which put the production of microalgal oil-based biofuels center stage, it would seem that, differently from mineral oil, there is apparently as yet no "easy microalgal oil," which is easily produced on a large scale at low cost.

ENERGETIC RETURN ON (ENERGY) INVESTMENT

A substantial problem for the large-scale production of microalgal oil-based biofuels regards the EROI, the energetic return on (energy) investment over the biofuel life cycle. It has been argued that the EROI for a major energy source should be >5, as this would allow for maintaining quality of life in the absence of readily available abundant fossil energy (Hall et al., 2009). The criterion that the EROI should be >5 is met by several emerging energy technologies such as wind power and photovoltaic power (Kubiszewski et al., 2010; Raugei et al., 2012). However, available life cycle assessments suggest that with present technologies the EROI for algal biofuels is <1 or somewhat larger than 1 (Reijnders, 2012). Increasing oil and algal biomass yields, lowering energy inputs, and aiming at multifunctional algae have emerged in proposals to improve the EROI of microalgal oil-based biofuels (Clarens et al., 2011; Reijnders, 2012). As to the latter: microalgae can be, e.g., used to treat wastes or emissions and as feedstock for biorefineries generating valuable coproducts besides algal oil (e.g., Oswald et al., 1957; Christenson and Sims, 2011; Rosenberg et al., 2011; Ahmed et al., 2012). However, available life cycle assessments studying combinations of such improvements in biofuel life cycles which have an upward effect on the EROI still do not show EROIs >5 (Chowdhry et al., 2012; Reijnders, 2012; Vasudevan et al., 2012). The few available studies which have looked at the life cycles of other algal biofuels such as ethanol from cyanobacteria ("blue-green algae") (Luo et al., 2010), methanol from microalgae (Liu and Ma, 2009), and methane from offshore-cultivated seaweed (Langlois et al., 2012) also suggest EROIs <5.

Moreover, concerns have been raised about the actual achievability of several of the major improvements featuring in life cycle assessments of future technologies for the production of microalgal oil-based biofuels (e.g., van Beilen, 2011).

A case in point is the achievability of major increases in oil and biomass yields from open systems which seem more suitable than photobioreactors for achieving large-scale algal oil production at relatively low cost (Hall and Benemann, 2011; Sun et al., 2011). Yields from open systems in the order of 70–90 Mg dry weight ha^{-1} $year^{-1}$ and 25–40+% oil appear to be important for achieving relatively high EROIs (though not >5) for algal lipid-based biofuels (Reijnders, 2012). One of the reasons for concern about the achievability of such yields is linked to the common difference in "greenness" between surface waters and terrestrial ecosystems with sufficient moisture. The latter tend to be much greener than the former. A main reason for this difference in "greenness" is that phytoplankton (to which "oilalgae" belong) contributes almost half to worldwide photosynthesis but accounts for less than 1% of the photosynthetic biomass present worldwide (Falkowski, 2012). The low share of microalgae in worldwide photosynthetic biomass is linked to grazing by zooplankton and insect larvae and the activity of algal pathogens such as viruses and parasites (Day et al., 2012). Also, infections by competing microorganisms are a major problem in open systems for the cultivation of microalgae. The way to circumvent much of these problems and to produce intended microalgae in commercial open systems is so far to use extreme conditions such as very high pH or salt concentrations (Reijnders, 2012). Such extreme conditions are not conducive to high biomass yields and also necessitate substantial energy inputs in the algal biofuel life cycle for maintaining extreme conditions and for wastewater treatment (Reijnders, 2012).

It has been suggested to increase the microalgal biomass yield by genetically engineered truncated antennae (Ort et al., 2011). However, algae with truncated antennae appear to be less competitive than their counterparts with full antennae, and this may have a negative impact on the yield of intended microalgal biomass in open systems where contamination by full-antenna algae may occur (Ort et al., 2011). And the assumption that high biomass and oil yields can be combined seems to take insufficiently into account that photon demand increases at high lipid contents (Wilhelm and Jacob, 2012). Another problem is the difference between well-controlled field experiments with open ponds, which have been used to support estimates of high future yields, and actual commercial practice. Vonshak and Richmond (1988) found the commercial production of *Spirulina* to be a factor 5–6 lower than in well-controlled field experiments. Whether large-scale production of microalgal oil with an EROI > 5 will emerge from future research and development focusing on algal biofuel production is as yet highly uncertain.

ALGAL BIOFUELS AS COPRODUCT

On the other hand, there is, as pointed out above, the certainty of a current commercial market for macro- and microalgae and constituents thereof. This market may well grow substantially in the future. Algae may in the future be used as a platform for a wider range of substances (e.g., Renn, 1997; Kraan, 2012). And the

presence of the long-chain *n*-3 polyunsaturated fatty acids in microalgal oil is a major asset regarding future food consumption. Long-chain *n*-3 polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are valued as constituents of healthy diets (Graham et al., 2004; Bockisch, 2010; Gogus and Smith, 2010). Ironically these long-chain *n*-3 polyunsaturated fatty acids are problematic in terms of fuel properties because they have relatively poor oxidative stability and ignition quality (Bucy et al., 2012).

The presence of eicosapentaenoic acid and docosahexaenoic acid in the human body is currently to a large extent based on the consumption of fish which in turn ultimately acquire these fatty acids fully or mainly from the consumption of algae (Graham et al., 2004; Sanders, 2009; Bockisch, 2010; Gogus and Smith, 2010). However, in view of the (potential) overexploitation of fish stocks, the supply of fish-based long-chain *n*-3 polyunsaturated fatty acids is limited (Graham et al., 2004; Bockisch, 2010). Also, intervention studies have shown health benefits of algal long-chain *n*-3 polyunsaturated fatty acids (Holub, 2009). Furthermore, one might argue that the direct consumption of microalgal long-chain *n*-3 polyunsaturated fatty acids by humans might be less of an environmental burden and that the problem of co-consumption of toxicants such as methylmercury and PCBs might be reduced if compared with consuming fish (e.g., Oken et al., 2012). Thus, there might well be scope for an expanded production of "oilalgae" for food in the future.

The future production of algae and constituents thereof is likely to be linked with the coproduction of substantial amounts or organic material which might be converted into biofuels. The cultivation of microalgae may be associated with a substantial production of organic compounds leaking into their aquatic environment (Day et al., 2012). And processing of macro- and microalgae might generate substantial amounts of organic compounds that cannot be marketed as food, feed, or ingredients for products. Such organic coproducts from the cultivation and processing of algae might be used for the production of biofuels (e.g., Langlois et al., 2012). As the handling of such coproducts might be argued to rather be in the category of waste treatment, one might moreover argue that the criterion of an EROI > 5 should not necessarily apply. Thus, converting organic coproducts of cultivating and processing algae into biofuels might well make a limited but valuable contribution to future energy supply.

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PREFACE

Success of a book like this depends on many people, the authors, the reviewers, and the publishing staff. Algal fuels made the news during the 2012 US presidential campaign, with remarks by Republican contender Newt Gingrich:

> The President [Barack Obama] says, "the Republicans have three strategies: drilling, drilling drilling." And I want to say tonight, Mr. President this is one of the rare occasions where I can say you are right…. The President said we have to be practical. Drilling won't solve it. And then he offered his practical solution. Anybody here remember what it was? Algae. Algae. I mean I think this summer as gas prices keep going up, one of our campaign techniques should be to go to gas stations with jars of algae and say to people would you rather have the Gingrich solution of drilling and having more oil or would you like to try to put this in your gas tank…. [A]fter he explained drilling doesn't work, the President explains we've had this great breakthrough of natural gas. That we now have thanks to new technology over 100 year supply of natural gas. That in fact we're going to create 600 k new jobs in next decade out of natural gas…. [H]ow does the President think we discovered the natural gas? (Fox News, 2012)

In response to this, RG wrote to Michael Krull, Campaign Manager, Newt 2012:

> Newt's quip of filling up our gas tanks with algae is indeed amusing. However, the USA dropped the ball in 1995 by terminating a 15 year project to develop algal fuels, at a time oil prices dropped, and is now playing catch-up. It's a long range strategy that may deserve some R&D, so a more subtle message that does not alienate the algal fuel community might be worth developing.

Afterward Gingrich added: "By the way I'm pro research into algae" (Calhoun, 2012). But algae entrepreneurs nevertheless got upset (AlgaeIndustry Magazine.com, 2012):

> Before long, Mr. Gingrich will be pumping our product into his car's gas tank; I certainly look forward to seeing that photograph…. This technology enables the production of ethanol for less than $1.00 per gallon using algae, sunlight, carbon dioxide and saltwater. Our novel techniques are projected to produce 6,000 gallons of ethanol per acre per year (compared to corn at 400 gallons/acre/year and sugar cane at 800 gallons/acre/year), plus we have the very important added benefit of consuming carbon dioxide from industrial sources. (Woods, 2012)

Well, maybe. We would all like to project that algae will produce cheap, plentiful biofuels, biodiesel, or ethanol, but despite hundreds of contestants in this race, "we ain't there yet," in terms of having eliminated the need to pump oil out of the ground. In particular, the use of CO_2 from industrial sources (cf. Ladd and Venter, 2008) may prove counterproductive, because CO_2-producing industries may not be considered sustainable and because their total output, when converted to biofuels, may be inadequate for our needs: industrial processes in the USA emit 300×10^6 metric tons CO_2 equivalent per year, which is 5% of that attributed to energy at 6000×10^6 metric tons CO_2 equivalent per year (Table ES-4 in Hockstad

and Cook, 2012). So while we all have a long way to go, we hope that the chapters in this book will be stepping stones to sustainable biofuels for everyone.

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ACKNOWLEDGEMENTS

We acknowledge all our authors for their contributions and specifically the early birds for sending their manuscripts and for their patience. We would like to thank the following 27 reviewers from a dozen countries for volunteering to evaluate the chapters in this volume: Aharon Oren (Israel), Alberto Reis (Portugal), Alvaro Israel (Israel), Angela M. Rocha (Brazil), Anju Dahiya (USA), Christopher J. Rhodes (United Kingdom), Claudio Fuentes (Spain), David J. Chapman (USA), Dinabandhu Sahoo (India), Erika Ortiz (Italy), Ille C. Gebeshuber (Malaysia), Irena Kaczmarska-Ehrman (Canada), Jesse McNichol (Canada), Jian Qin (Australia) John A. Barron (USA), John R. Benemann (USA), Maria Teresa Saraiva Lopes da Silva (Portugal), Mark Edwards (USA), Michael J. Cooney (USA), Pascal Jean Lopez (France), Patrick McGinn (Canada), Raquel Pedrosa Bezerra (Brazil), Raymond J. Ritchie (Australia), Sandeep Kumar (USA), Sunil K. Shukla (India), Supriya Guruprasad (India), and William R. Buckley (USA). If any reviewer's name has been omitted, we apologize for the oversight. Many more people volunteered to be reviewers. We were rather overwhelmed by the response. We wish also to express our appreciation of all our colleagues who assisted us with their good advice for this book. Last but not least, we express our gratitude to Springer's team (Melanie van Overbeek and Dr. Maryse Walsh) for their cooperation, patience, and understanding.

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May 29, 2012

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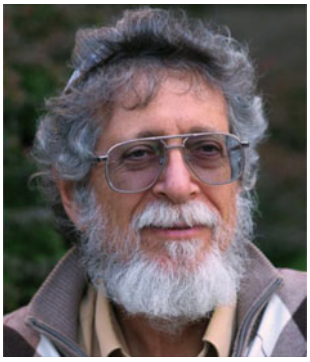

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Professor Joseph Seckbach is the founder and chief editor of *Cellular Origins, Life in Extreme Habitats and Astrobiology* ("COLE") book series. He has coedited other volumes, such as the *Proceeding of Endocytobiology VII Conference* (Freiburg, Germany) and the *Proceedings of Algae and Extreme Environments meeting* (Trebon, Czech Republic) (see http://www.schweizerbart.de/pubs/books/bo/novahedwig-051012300-desc.ht). He coedited the recent volume (with Richard Gordon) entitled *Divine Action and Natural Selection: Science, Faith, and Evolution* published by World Scientific Publishing Company.

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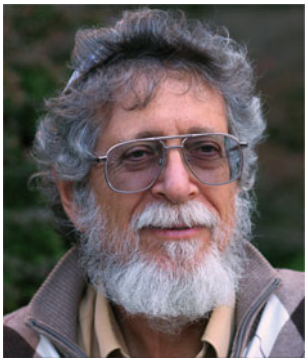

R. Gordon and J. Seckbach (eds.), *The Science of Algal Fuels*,
Cellular Origin, Life in Extreme Habitats and Astrobiology, 3–20
DOI 10.1007/978-94-007-5110-1_1, © Springer Science+Business Media Dordrecht 2012

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QUITTING COLD TURKEY: RAPID OIL INDEPENDENCE FOR THE USA

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1. Introduction

Up until 1955, the United States was completely energy self-sufficient. Afterward, energy imports rose to 19% of total energy consumption by 1980, then to 30% in 2005. The need for 30% additional energy in the USA, all of which was imported, from 1980 to 2005, can be directly attributed to the 30% population growth, from 227 million people in 1980 to 296 million in 2005,since consumption of energy per American held steady since 1980. However, total USA energy production has been roughly constant since 1970, and thus has not kept pace with population increase (Seiferlein, 2009) (cf. Fig. 1).

The USA has enough potential alternative sources of energy to become self-sufficient again. Hydroelectric power could rise from 40,000 to 170,000 MW (Hall et al., 2004). For wind, "even if only 20% of this power could be captured, it could satisfy 100% of the world's energy demand for all purposes and over seven times the world's electricity needs" (Archer and Jacobson, 2005). Since "the land area required to supply all end-use electricity [40% of total energy use (Seiferlein, 2009)] from solar photovoltaics, is about 0.6% of the total land area of the United States" (Kimbis, 2008), solar power could also supply all the energy needed. Extrapolation of cost trends suggests an exponential decrease that will cross a line by the year 2018, after which solar electric power in the USA will be cheaper than other electricity sources (Naem, 2011) (cf. Browning, 2011; Kanellos, 2011; Plumer, 2011; Wesoff, 2010). The $0.5 trillion per year cost of oil import dependence (Greene, 2010) could be put to better use achieving oil independence.

About 85,000,000 barrels of oil are consumed per day worldwide (NationMaster.com, 2011). Tropical sunlight is equivalent to ~4,000 barrels of oil per square kilometer per day. The surface area needed for that much energy would be ~20,000 km^2, possibly in open ocean (Bregman et al., 1995; NREL, 2010). This is 0.004% of the total surface area of the Earth of 500,000,000 km^2. Thus, we may be well on our way to meeting "the terawatt challenge" (Smalley, 2005). On the other hand, as with the now forgotten promise of "unlimited nuclear energy" (Bocking, 1995), we have to proceed with some skepticism. While cheap, solar-powered LEDs now light our way at night, there may yet be some

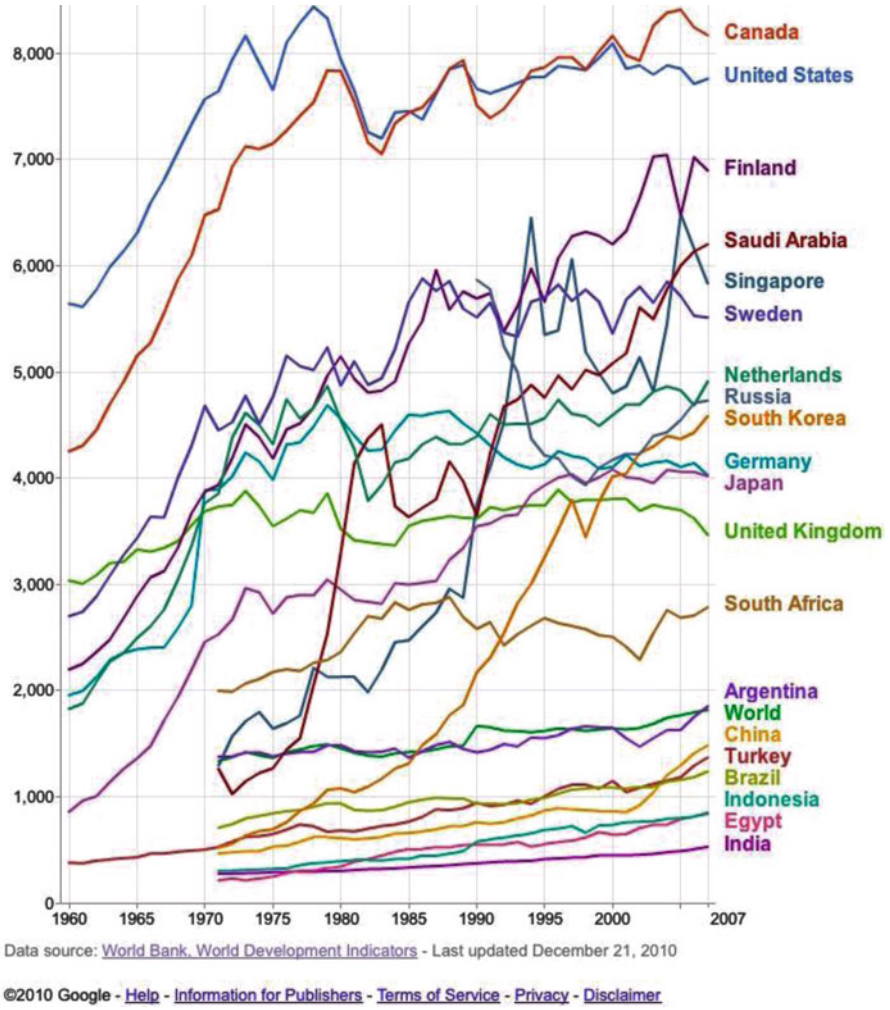

Figure 1. Energy use per capita. Primary energy use (before transformation to other end-use fuels) in kilograms of oil equivalent, per person. USA per capita consumption has been roughly constant since 1970–1980, whereas everyone else appears to be playing catch-up. "Public data" provided by the World Bank and updated periodically (World Bank and World Development Indicators, 2012).

surprises en route to "unlimited solar energy," which is where extrapolation (Naem, 2011) suggests we are going.

Why do we use oil? It is a concentrated energy source and easy to handle, and we have a distribution system in place. We have 100 years of experience of improving oil-burning engines. There is thus a cost of change to alternatives, along with a higher unit cost of alternatives before they reach comparable economies of

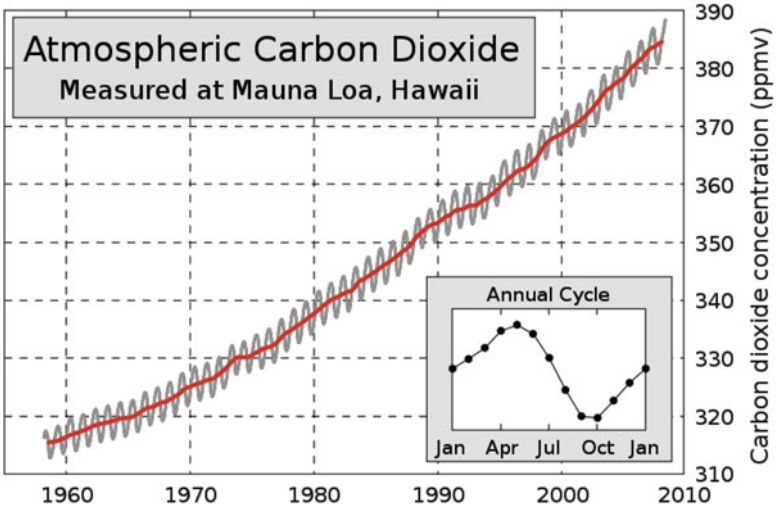

Figure 2. The present measured increase in atmospheric CO_2, with permission (Wikipedia, 2012).

scale. Also "…it's going to be a long time before we run out of oil.… There is plenty of it in Venezuela, Canada, Russia and other parts of the world. At current consumption rates, oil reserve life will be measured in centuries" (Kovarik, 2007). Nevertheless, with many oil fields empirically already past their peak production, the concept of "peak oil" may be a reality "within the next decade or so" (Sorrell et al., 2012), though it would seem that we face a broad peak and a slow decline.

What about all that carbon dioxide? Indeed, atmospheric CO_2 is increasing (Fig. 2). However, the Earth has had 15× higher levels in the past (Fig. 3), and our climate models cannot predict the effects of the present increase (Essex and McKitrick, 2007).

There are thus great practical and financial disincentives to stop using oil and a well-publicized debate between scientists (Essex and McKitrick, 2007; Mann, 2012), let alone between politicians, about whether or not we need to stop consuming oil in the near or distant future.

Current plans to get off oil, including the Pickens Plan, involve a transition taking decades (Cummings, 2010; Greene et al., 2010; Lindenberg et al., 2008; Pickens, 2010). Such models have many problems: (1) the cumulative financial, geopolitical, and security costs associated with prolonged use of imported oil; (2) the ease of backsliding and procrastinating on hard decisions that are difficult to swallow, especially if spread over many presidential terms; (3) the incompleteness of the goal, that is, reducing rather than eliminating oil imports; and (4) the time scale of any such plan that exceeds the persistence time of the model on which it is based (Pilkey and Pilkey-Jarvis, 2007). That is, most long-range projections are

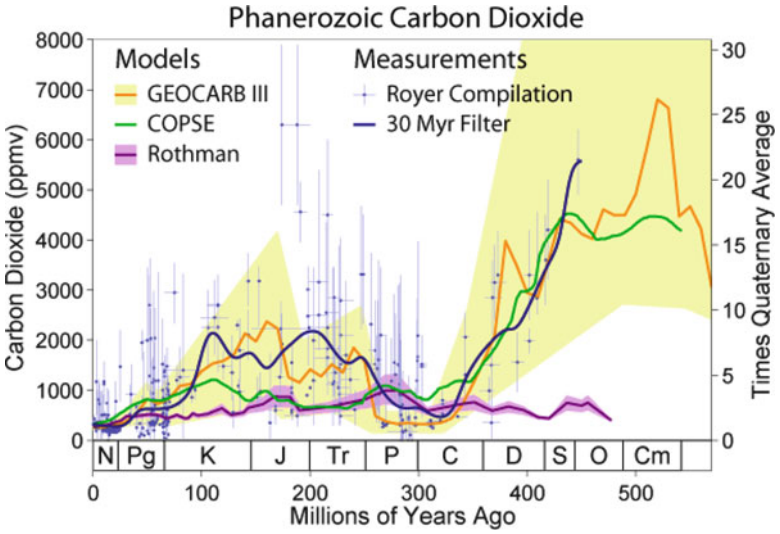

Figure 3. Various estimates or prehistoric CO_2 levels in the Earth's atmosphere. The scale on the *left* is in ppmv = parts per million by volume. The current value is about 400 ppmv (From (Wikipedia, 2012), with permission).

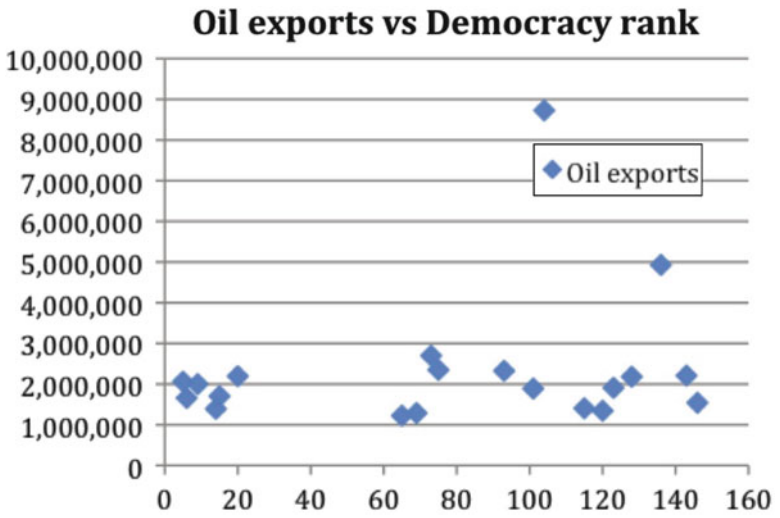

Figure 4. There is a clear split between the democratic countries (*left-hand cluster*) and the undemocratic countries (*right-hand cluster*) that export oil (based on the data in Table 1).

completely unreliable. United Nations 20-year scenarios and the similar spending model of the Gates Foundation for HIV/AIDS led us to formulate new, rapid approaches for halting the AIDS epidemic (Smith? and Gordon, 2009). By the

same token, the claim that "all credible forecasts show America needs [fossil] oil and gas for at least the next several decades" (McNulty, 2010) is itself not credible, and contradictory predictions that we will/will not get off oil spontaneously (Strahan, 2012) are indications of our inability to reliably predict the future. The ecological and economic disaster of the 2010 Deepwater Horizon oil leak in the Gulf of Mexico requires deeper thinking than a battle over oil drilling versus the environment. It is an opportunity to solve both problems, on a much shorter time scale than "several decades," along with the political stalemates that world oil trade prolongs.

It is time for a dramatic political event, either self-initiated by the USA in response to the massive oil spill in the Gulf of Mexico, or debt crisis, or imposed from without, as the OPEC oil embargo in the 1970s (Office of the Historian, 2011), in which all imported oil is suddenly cut off. From a mathematical point of view, this would be called an impulse. It would result in transients to the economic system, that is, possibly bizarre and unnerving changes. In the physical world, familiar transients include hurricanes, tornadoes, floods, earthquakes, volcanoes, etc., phenomena that come suddenly, pass, and settle down. We know the typical consequences of such abrupt changes, and we have learned how to plan for or at least cope with many of them. If we put in the effort to predict or at least present scenarios for the worst problems caused by suddenly ending all oil imports, we could better prepare to get through a self-imposed "oil shock" to a new era of oil independence. The history and models for oil shocks provide a good starting point for predicting and ameliorating effects of a planned oil shock (Aloui and Jammazi, 2009; Apergis and Miller, 2009; Baffes, 2007; Berument et al., 2010; Bodenstein et al., 2011; de Miguel et al., 2003, 2009; Du et al., 2010; Ewing and Thompson, 2007; Guo and Kliesen, 2005; Huang, 2008; Jammazi, 2012; Jones et al., 2004; Ordóñez et al., 2011).

Thus, we propose that the USA simply quit importing oil cold turkey and anticipate and ride through the geopolitical reactions and economic repercussions (Gordon, 2011). The countdown to a date for quitting oil could start with your reading of this article.

2. Case Study: World War II Cutoff from Natural Rubber

Japan's invasion of the South Pacific during World War II resulted in 90% of the sources of natural rubber being inaccessible to the United States at a time when there was "no commercial process to produce a general purpose synthetic rubber" (American Chemical Society, 1998). By analogy to our current situation, despite the earlier incentive to develop synthetic rubber in World War I, importing natural rubber was cheaper: "The fantastic fluctuations in the price of natural rubber led to corresponding fluctuations of the incentive to develop a substitute for this material" (Morawetz, 2000). "By the late 1930s, the United States was using half the world's supply of natural rubber, most of it coming from Southeast Asia"

(American Chemical Society, 1998). The annual consumption rate (600,000 ton/year) was equal to 60% of the US stockpile of one million tons. Quick, effective, massive steps were taken to overcome the Japanese embargo:

1. Plans were made to reach annual production of synthetic rubber (400,000 ton/year), equal to 67% of annual consumption rate, within 18 months, effectively just before the stockpile would run out.
2. The "conflicts arising over the best technical direction to follow" were resolved by appointment of a presidential committee in August 1942 that reported within 1 month. The committee included industry, government, finance, and university participants (American Chemical Society, 1998).
3. A patent and information sharing agreement was struck between all parties involved in synthetic rubber, headed by the Rubber Reserve Company that had been formed earlier by President Franklin D. Roosevelt to deal with rubber as a strategic material (Morawetz, 2000). The cooperation extended to collaborating on improving production and initiating basic research focused on understanding and improving upon the chemistry of synthetic rubber.
4. The President appointed "a rubber director who would have complete authority on the supply and use of rubber" (American Chemical Society, 1998).
5. The "immediate construction and operation of 51 plants to produce the monomers and polymers needed for the manufacture of synthetic rubber" was started (American Chemical Society, 1998).
6. The participants foresaw "development of a large new industry with vast postwar possibilities" (American Chemical Society, 1998). Indeed, one might say that the whole plastics industry took off as a result, in which the USA is still a leader.

"The amazing growth of the American synthetic rubber industry was only overshadowed by the development of the atomic bomb" (Morawetz, 2000) in the parallel Manhattan Project. Despite Germany's 4-year head start, the USA pulled far ahead of Germany in synthetic rubber production: "While the German government made great efforts to be independent of natural rubber in the coming war, neither the political nor the military leaders of the United States seem to have been concerned about the possibility that the import of rubber from Southeast Asia might be interrupted…. Only in June 1940, after German armies had swept over Denmark, Norway, Belgium, The Netherlands and France, did President Roosevelt declare rubber to be a strategic material, forming the Rubber Reserve Corporation" (Morawetz, 2000).

3. Parallels

The parallels of WW I complacency and WW II synthetic rubber production with the current situation for gasoline and other liquid fuels are apparent. For example, the USA dropped the ball in 1995 when a massive project to develop biofuels was

terminated after 15 years due to low oil prices (Sheehan et al., 1998). The lessons of the OPEC embargo had been forgotten. The USA has many ways of producing liquid fuels, some of them sustainably, but has no major commercial venture at present. Possible sources include photosynthetic algae (Gordon and Seckbach, 2012) such as diatoms, perhaps in gasoline-secreting solar panels (Ramachandra et al., 2009), or parts of crops that we do not eat, or sewage, coal and natural gas, these being turned into oil or ethanol by bacteria, yeast, algae, and chemical processing. The USA has a stockpile and reserve of oil that would last a short time if drawn down. Given that the USA can, for now, choose the timing of when it goes off imported oil, greater stockpiles could be accumulated starting immediately.

4. Crude Oil Reserves

The USA presently stores 700 million barrels of crude oil in salt domes around the Gulf of Mexico, with plans to expand to one billion barrels (Fossil Energy Office of Communications, 2009). Proven reserves are 21 billion barrels, while domestic consumption is running at 7.3 billion barrels per year (Energy Information Administration, 2009a). Present storage would thus last 35 days, but proven reserves are enough for 3 years. This is twice as long as it took the USA to achieve independence from natural rubber. The whole storage system, when completed at 1 billion barrel capacity, is estimated to cost $26 billion. That is just $26 per barrel, and even a cost of 50% higher or $39 per barrel is less than current price fluctuations. New ways of storing oil other than salt domes might increase US reserves further.

5. Natural Gas, Uranium, and Coal Reserves

The USA has the largest coal reserves in the world, good for 200 years at current production rates (Bessereau and Saniere, 2010). As coal now represents 22% of US energy consumption (Seiferlein, 2009), if it were solely dependent on it for all energy, that coal could last 40 years. While it may be objectionable from the viewpoint of greenhouse gases, coal converted to liquid fuels, combined with cleaner burning coal technologies similar in environmental result to cleaner burning diesel engines, would provide a longer buffer period than crude oil storage and reserves. This may be preferable to increased offshore drilling or even using Canada's tar sands (Levant, 2010). In a cold turkey scenario for getting off imported oil, the USA would have a transient period that might be temporarily worse for the environment. But the final result could be completely sustainable liquid fuels or a liquid fuel stopgap until alternatives such as solar power take the lead. Coal is perhaps the best stopgap measure.

Natural gas also represents 24% of US energy consumption (Seiferlein, 2009), but the USA has only 5% of world reserves (Energy Information Administration,

2009b). At current consumption rates, US reserves would last 12 years, and imports already are at 17% consumption. However, the practice of extraction of previously uneconomic reserves by fracking (hydraulic fracturing of rock), initiated by smaller companies, has changed this picture (Finkel and Law, 2011) and led to near independence from imported liquefied natural gas (Rogers, 2011) and a drop in price, even in the face of widespread fears and opposition regarding the effects of fracking and CO_2 concerns (Hultman et al., 2011; Wilber, 2012).

Nuclear power represents 8.5% of US present energy use (Seiferlein, 2009), all as electricity. This option would mean we would have to convert to electric cars and trucks, to take advantage of it as a transportation fuel, or to hydrogen-burning vehicles. The USA has only 6% of world reserves of uranium (World Nuclear Association, 2009), and 73% of US uranium oxide is imported. Nuclear power with an imported energy source would not be a good alternative to getting off imported oil.

6. Minimizing Overlaps in Energy Use for Electricity, Transportation, and Food

At the present time transportation energy use in the USA is largely independent of the electric grid. Petroleum use to generate electricity has declined from 15% in 1974 (Pierce et al., 2006) to 1% in 2009 (Energy Information Administration, 2010). The reverse, the use of electricity for transportation, should perhaps remain low, given that no breakthroughs in battery technology are anticipated that would make battery-stored energy density comparable to that of liquid fuels, although mass production may drive down the price (Strahan, 2012). Furthermore, we have over a century of experience with gasoline and diesel engines, so sticking with liquid fuels would allow us to continue using and improving liquid fuel engines (Strahan, 2012). Therefore, a wise policy would continue to make liquid fuels for transportation a separate matter from electric power.

We have been through something like this once before. For a brief period, biofuels from corn and wheat proved promising, until they competed with production of these staples for food and fodder, and the prices of both crops climbed worldwide (Harvey and Pilgrim, 2011). Unless we go in for massive harnessing of solar and wind electric power (which we could do), we had better not tie our whole fleet of vehicles to the electric grid. We've already had enough brownouts in the USA.

One of the overlaps that we had best minimize is intellectual. For example, a recent job posting for research on biofuels required that "microalgal biomass will also be valorized as raw material for the chemical, food and pharmaceutical industry." This is probably due to investors hedging their bets and the higher payoff per gram for pharmaceuticals than cheap biofuel. On the contrary, each of those is a separate problem, perhaps requiring different organisms and approaches. We cannot expect to maximize every goal simultaneously.

7. Maximizing Competition Between Alternative Energy Sources

Competition between alternative fuels is overshadowed by the fluctuations in the price of imported oil, which make and break the fortunes of startup companies, because investors back off when the cost of biofuel exceeds the cost of oil (Bullis, 2008). Oil exporters have no incentives to prevent these price fluctuations:

> …the quota regime… characterises the OPEC agreements. Given that the Saudi oil supply is inelastic in the short term, a shock in the oil market is accommodated by an immediate price change…. Saudi Arabia does not have any incentive for altering the crude oil market equilibrium with either positive or negative supply shocks…. (De Santis, 2003)

In general, all new alternative fuels face the challenge of getting a share of a market presently dominated by cheaper imported oil. If the imports would suddenly stop, alternative fuels could rapidly fill the niche, although higher transitional costs and prices are to be expected in the short term.

Sustainable alternatives could compete and grow in the breathing space with appropriate public policy that avoids usual mechanisms of "…market failure – a circumstance in which pursuit of private interest does not lead to an efficient use of society's resources or fair distribution of society's goods" and government failure: "…the very nature of public agencies makes monitoring difficult and inefficiency likely" (Weimer and Vining, 1999).

Weaning the USA from imported oil will be a difficult process, and yet success is entirely possible, if we but understand the nature of the problem and have the social, political, and economic will to address it. The USA has the technology or certainly can develop it.

8. Who Are We Importing Oil from Now?

We have listed the top 20 oil exporters in Table 1, along with their rankings as democracies. About 70% of these exports are from undemocratic countries.

In summary so far, in the USA and elsewhere, we thus face a number of moral and policy questions:

- Do we stop importing oil from countries that are not democracies?
- Do we judge global warming and/or environmental degradation as of higher concern than lack of democracy, or is such a tradeoff valid?
- Can we stomach higher domestic fossil fuel consumption until we can create infrastructure for alternative fuels?
- Do we value alternative fuels enough to pay a modestly higher price?
- Do we halt our population growth, which seems to be the major driving factor?

Wisely answering all these questions is important and none more so than halting imported oil from undemocratic and often hostile regimes and how this is to be accomplished.

Table 1. The top 20 oil exporting countries.

Country	Oil exports	Democracy rank
Saudi Arabia	8,728,000	104
Russia	4,930,000	136
United Arab Emirates	2,700,000	73
Kuwait	2,349,000	75
Nigeria	2,327,000	93
Iran	2,210,000	143
European Union-UK	2,196,000	2√
Venezuela	2,182,000	128
Norway	2,061,000	5√
Canada	2,001,000	9√
Iraq	1,910,000	123
Algeria	1,891,000	101
United States	1,704,000	15√
Netherlands	1,660,000	6√
Libya	1,542,000	146
Angola	1,407,000	115
United Kingdom	1,393,000	14√
Kazakhstan	1,345,000	120
Singapore	1,289,000	69
Mexico	1,225,000	65
Total of above	47,050,000	

Oil exports are given in barrels/day (CIA, 2012). Democracy rankings (World Audit, 2010) were made before the "Arab Spring" and may change one way or the other as events unfold. Lower rank means more democratic. Those countries appearing in the lower rank cluster in Fig. 4 are being labeled "democratic" here and indicated with a check mark (√)
√ = democratic

9. The Alternative of Importing Oil from Real Democracies

At this writing, during a presidential campaign in the United States, a major issue is the construction of an oil pipeline from Alberta, Canada, to Texas, USA, versus from Alberta through British Columbia and exporting oil to China. The contendents (Levant, 2010; Rooney et al., 2012; Schindler, 2010) argue on the basis of different application of values and on the basis of local concerns. Social, environmental, and economic values need to be optimized, with impacts as follows:

1. Social impacts of the USA trading equally with democratic and undemocratic-dictatorial regimes on citizens of the USA and Canadians
2. Environmental impacts in the USA including the proposed pipeline and offshore drilling on Alberta waterways and land and on British Columbia land and shores with, for example, ports that export oil to China

3. Economic impacts including balance of exports from Canada to the USA versus China, cost of gasoline and its impact on commerce, and related geopolitical consequences
4. Job creation impacts in the USA with foregoing infrastructure links with Canada in the face of the most serious global recession since the great depression of the 1930s
5. Social, environmental, and economic impacts of purchasing oil by the USA from dictatorships including extraordinary costs of maintaining secure supplies at reasonably predictable prices (Delucchi and Murphy, 2008)

Our reading of the literature shows no consideration by any of the parties of how to balance these values and impacts of decisions. This would take leadership exemplified by John F. Kennedy (to the moon by the end of the decade), Franklin D. Roosevelt (in the New Deal), Abraham Lincoln (in building support for the emancipation proclamation), and George Washington (to serve and not serve longer than necessary): to paraphrase James C. Collins (Collins, 2001), having both *humility* – to understand the challenge – *and professional will* to see the challenge carried to completion.

10. Impact of Oil Prices on Labor

Oil prices have major impacts on labor and whole economies, as exemplified by the following quotes:

> We examine the impact of real oil price shocks on labor market flows in the U.S. …oil prices can be considered as a driving force of labor market fluctuations. (Ordóñez et al., 2011)

> This paper analyzes the effects of oil price shocks… on the Spanish economy. The results… reproduce the business cycle path of the Spanish economy…. (de Miguel et al., 2003)

> Overall, we find that the price of oil is a major determinant of economic activity of the country. …the price of oil is a major component of forecast variation for most macroeconomic variables. (Lorde et al., 2009)

> The global economy cannot endure sharp increase in manufacturing costs and product prices. This would reduce consumption and production at the same time, followed shortly by financial market crisis…. rapid increases in oil price are dangerous to world economics. (Chang, 2010)

> …we find that oil prices significantly explain movements in the value of the U.S. dollar (USD) against major currencies from the 1970s to 2008…. Increases in real oil prices lead to a significant depreciation of the USD against net oil exporter currencies, such as Canada, Mexico, and Russia.... the currencies of oil importers, such as Japan, depreciate relative to the USD when the real oil price goes up. (Lizardo and Mollick, 2010)

In getting off imported oil, the USA may have to solve the problem of outsourcing. For example, a US company leads the effort in solar energy, but has done so by establishing "the largest solar research facility in the world" in China (Erwin, 2011).

11. The Benefits of Leading: Public Policy

Presently, the USA is behind in developing and using alternative energy technologies, perhaps precisely because it is so well endowed with its own fossil fuel energy sources. Real leadership requires both transactional and transformational policy changes (Bennis, 1989; Collins, 2001). This kind of leadership requires force of moral vision that is ethical and practical and that works in the short, long, and intermediate terms. Here are five imperatives for leadership to happen, to address the values and impacts listed above, and for the USA to regain the high moral ground it has lost of late, in trying to deal more or less fairly with unfair and hostile regimes:

1. The President of the USA and leaders of the Congress and Senate speak out as one voice on the challenge to go "cold turkey" and, in doing so, dealing fairly with countries and businesses that are themselves acting fairly and not hostile to the interests of the USA, North America, and Europe; this includes securing oil supply now and alternative sources by end of the decade.
2. Look to the environment as the legacy of policy in energy and other matters, following the example of the late great business leader Ray Anderson in working toward a zero environmental footprint, yet realizing it as an ideal that takes his and other's will and effort, together (Anderson, 1998).
3. Be ethical in all major forms, building on a platform of social equity, rights and responsibilities, and practical utility, as in the President leaders mentioned above (Washington, Lincoln, F. D. Roosevelt, Kennedy), who each had their own unique approaches and yet had the good of themselves, their family, their country, and others in their hearts and minds.
4. Look to success of policies in terms of the above and, especially, the number of people employed and the labor wages they make, for this is how Adam Smith also saw progress.
5. Promote the above so that all constituents are brought on board, again as the great leaders of the past have done, always referring to the great task ahead and the progress that is being made.

The current conundrums of oil drilling versus the environment and oil from dictatorships versus worldwide democracy (Levant, 2010; Omgba, 2009) could be solved by the USA. In solving the problem of importing oil, the USA would establish leadership in sustainable energy and provide a strengthened beacon of freedom for the billions of people who still yearn for it, as recently stated:

> The African oil states are often viewed as politically unstable. This political instability is frequently attributed to the presence of oil resources. Our study finds that this instability does not appear in the executive branch of the state. On the contrary, oil causes stability of political power within the executive branch. Given the mechanisms that drive this result, it is very likely that this political characteristic does not contribute to the population's well-being or political development for the states concerned. However, these mechanisms enable us to emphasize that the behavior of public decision makers of oil African states falls under the same logic of their counterparts in the Persian Gulf. (Omgba, 2009)

The above points to the serious need to go beyond one way thinking; strong, committed leadership in voice and action, in going cold turkey within a set date, would signal a better and more dynamic future for us all. The US public may be more ready for such an initiative (Li et al., 2009) than most politicians.

12. Summary

The need for imported oil from 1970 to date is directly attributable to the increase in USA population. There are at least four responses to this situation: increase fossil fuel production, increase alternative fuel production, increase imports, or curb population growth. Whether or not world oil production is peaking or oil is or is not driving global warming, the present geopolitical reality is that about 70% of oil exports are from undemocratic and often hostile countries. Much of the world's turmoil revolves around this fact. A decision by the USA to stop importing oil, especially from such countries, would set in motion a process of oil independence for the USA.

At first, greater oil resource development and/or importation from democracies would occur, and only modest higher costs and prices need be expected in the short term if sound public policy is crafted and followed with adequate monitoring, enforcement, and adjustment. The stability in US oil prices with such policy would set a benchmark against which the many alternative fuel sources could compete with one another, with some reaching their potential to outdo oil via sustained investor support. This would likely result in a drop in oil prices within the medium term. The anticipated drop in oil prices outside the USA might accelerate the development of India and China (Du et al., 2010) and other countries, perhaps even driving China toward democracy and rule of just law. Quitting imported oil cold turkey may depend on US presidential vision and leadership.

13. Acknowledgements

We would like to thank Mark Edwards and William R. Buckley for critical readings.

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R. Gordon and J. Seckbach (eds.), *The Science of Algal Fuels*,
Cellular Origin, Life in Extreme Habitats and Astrobiology, 21–41
DOI 10.1007/978-94-007-5110-1_2, © Springer Science+Business Media Dordrecht 2012

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ALGAL BIOREFINERY: SUSTAINABLE PRODUCTION OF BIOFUELS AND AQUACULTURE FEED?

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1. Introduction

In recent decades, significant efforts have been made toward finding an alternative source of liquid fuel due to the irreversible depletion of fossil reserves. In addition, fossil fuels have been deemed unsustainable based on the fact that they accumulate greenhouse gases (GHG) known to cause global warming. Thus, biofuels derived from plant material appear to be an attractive alternative source of energy. Compared with other forms of renewable energy (e.g., wind, tidal, and solar), biofuels allow energy to be chemically stored and can also be blended with petroleum diesel to different degrees or used directly in existing engines and transportation infrastructures (Singh and Gu, 2010). In the past decade, the biodiesel industry has seen massive growth globally, more than doubling in production every 2 years (Oilworld, 2009). The production of bioenergy from algae has received increased attention, as many species have high lipid contents and some species excrete hydrocarbons (Dismukes et al., 2008; Schenk et al., 2008). Algae have been regarded as possibly the only route to sustainable displacement of high proportions of oil consumption.

With concerns of cost efficiency, the economics of algal biofuel production can be significantly improved by using a biorefinery-based production strategy where all the components of the biomass raw material are used to produce useful byproducts (Singh and Gu, 2010). It is recommended for algal biorefineries to combine and integrate various sectors to maximize economic and environmental benefits, while minimizing waste and pollution (Meher et al., 2006; Singh and Gu, 2010). It is well known that algal meal is a rich source of high-quality protein, vitamins, micronutrients (trace elements), and pigments including carotenoids, which can be used directly in aquafeeds (Naylor et al., 2009; Patnaik et al., 2009). For example, the annual production of microalgae for aquaculture feed reached 1,000 tons (62% for mollusks, 21% for shrimps, and 16% for fish) (Spolaore et al., 2006; Gagneux-Moreaux et al., 2007). As food and fuel production are intricately interconnected, it is anticipated that aquaculture industry may benefit strongly by integrating the growth of algal bioproducts and biofuels (Subhadra and George, 2011). This book

chapter will review the advances in the use of microalgae as part of a multiple product biorefinery for generation of renewable biofuels and aquaculture feed and outline the prospects of algal biorefinery developments for both sectors.

2. Interrelatedness of Biofuels and Aquaculture

Currently, the most widely available form of biodiesel comes from oil crops (e.g., palm, oilseed rape, and soybean). With rapid human population increase on our planet, food security has subsequently become a major concern worldwide. With additional concerns about greenhouse gas (GHG) emissions and competition of biofuel and food crops for arable land, two major criteria have been proposed for biofuels development as key future sustainability principles. As suggested by Hausmann and Wagner (2009), biofuels should contribute to climate change mitigation by reducing GHG in comparison to fossil fuels, and secondly, biofuels need to ensure adequate and improved food security in food-insecure regions.

Environmental impacts of biomass production and carbon sequestration have instigated the need for rapid development of other advanced feedstocks, such as switch grass, woody mass, and algae. However, terrestrial-based bioenergy production systems are facing issues related to indirect emission and carbon debt from land clearance (Fargione et al., 2008; Searchinger et al., 2008) and are becoming a sustainability hurdle for further expansion (Tilman et al., 2009). Moreover, if their lifecycle of biofuel is considered as a whole, the overall savings in energy and GHG emissions are typically below what is normally anticipated (Hill et al., 2006). Based on the unique advantages in productivity and the potential to avoid competition for arable land or biodiverse landscapes, algae have been considered one of the most promising resources for biofuels and biomass.

Although they are not superior to higher plants with respect to photosynthetic efficiency, microalgae have extremely high growth rates and yield more oil than any higher plants (e.g., oil palm; Rodolfi et al., 2009). More than 40% of the Earth's carbon is fixed by algae, and they provide the world with a large proportion of oxygen. Their ecological significance lies in their abundance, extreme biodiversity, and ability to live in a variety of aqueous environments, ranging from very extreme, such as soda lakes and desert soils, to more moderate environments such as fresh-water lakes and oceans (Norton et al., 1996). Different from traditional agriculture, the cultivation of algae does not have to compete with cultivated farmland. Furthermore, algae can make use of waste streams as a nutrient source, which have great potential for waste water bioremediation. More importantly, algae are the basis of the food chain supporting over 70% of the world's biomass (Andersen 1996).

In regard to food security, fish meal has complemented protein-rich food sources from arable land-dependent agriculture to a large extent. However, these seafoods make up the bulk of the diets of carnivorous fish, are obtained from finite sources that are fully exploited, or, in most cases, overfished (PewTrust, 2007). Overfishing of the world's oceans has severely depleted fish populations,

leading to an imbalance in the marine ecosystem. With concerns of sustainability issues for the overutilization of marine resources, aquaculture is regarded as a future sustainable source of quality protein for the planet's growing population. Fish and shellfish mariculture thereby have become an important food sector globally and hold great promise for closing the nutritional gap of many people worldwide (Subhadra and George, 2011). However, at present, the vast majority of aquaculture feed is sourced from wild fish populations. While reducing use of fish oils in aquafeed, an apparent challenge in aquaculture is to produce final products/seafoods with high levels of omega-3 fatty acids, health promoting for the consumers (Subhadra et al., 2006a). This growing concern is also a driving force for the marketing of non-fish omega-3 oils and alternative feed ingredients in aquaculture (Subhadra et al., 2006b). The importance of algae in this domain is compelling as they are the natural food source of most of these animals in their larval stage and naturally produce a substantial amount of omega-3 fatty acids. As algal omega-3 oil can be recovered from the extracted lipids intended for biofuel production, policy initiatives for the meaningful integration of algal biofuel production with aquaculture industries can provide many economic and sustainable outcomes to society. More importantly, this can be coupled with current efforts to produce biodiesel from microalgae, where realistically only up to 30% of dry weight consist of oil suitable for biodiesel production, while it is currently unclear how the remaining 70% of protein-rich biomass can be used for the substitution of fishmeal.

Vice versa, the discharged water from aquaculture could match the requirement for algal growth, similar as has been implemented in aquaponics systems that aim to simultaneously grow fish and vegetables (Tyson et al., 2011). In fact, disposal of aquaculture wastewater has become a major task to further improve production efficiency and decrease the environmental impacts of the aquaculture industry. However, this process is not so straightforward due to the costs associated with nutrient removal. Ironically, fertilizer cost is also one of the major contributors of energy intensiveness and operational cost for algal biofuel production (Subhadra and George, 2011). The effluent from aquaculture production and facilities contains large amounts of nitrates and phosphates which are also essential nutrients for algae growth and production. Therefore, the benefit of the ancillary industries between aquaculture and algal biofuel can offset energy and GHG emission in algal production and also reduce investment for wastewater management in aquaculture. This module becomes more of a tangible reality for mariculture as salty water cannot be used for irrigation.

3. The Concept of an Algal Biorefinery

Based on the IEA Bioenergy Task 42 2009 document, biorefinery is the sustainable processing of biomass into a spectrum of marketable products and energy. The major objective is to produce low-value high-volume and high-value

low-volume products, including fiber residue, biofuel, and some pharmaceutical extracts (Willke and Vorlop, 2004; Huber et al., 2006; Fowler et al., 2006). Three types of biorefineries, phases I, II, and III, have been subsequently described. A phase I biorefinery plant has fixed processing capabilities and uses grain as a feedstock. A phase II involves current wet milling technology and also uses grain feedstock as input. Depending on product demand, price, and contract obligations, phase II has far more processing flexibility to produce various end products, such as starch, high-fructose corn syrup, ethanol, and corn oil (Dyne et al., 1999; Kamm and Kamm, 2004). The most developed biorefinery is phase III, as different technologies can be employed to yield an array of products by using a mix of biomass feedstocks (Kamm and Kamm, 2004). Compared to phase II, it could employ more types of processing methods and produce a mix of higher-value chemicals with a coproduct of ethanol (Tyson et al., 2005). Phase III biorefineries are also referred to as whole-crop, green, or lignocellulose feedstock biorefineries, which are still in research and development (Kamm and Kamm, 2004). By using various technology platforms, an integrated biorefinery is more realistic and cost effective in converting a type of biomass into different desired end products (Balat, 2009a, b). For example, biomass-based biofuel feedstock (e.g., bioethanol from wheat and corn, and biodiesel from rape seed and oil palm) could produce 100 million tons of protein as an additional coproduct in the future (van Haveren et al., 2007). Therefore, any combination of conversion technologies could have the potential to make biofuels from algal biomass competitive.

As algae can produce proteins, carbohydrates, lipids, and other valuable components (e.g., pigments, antioxidants, fatty acids, vitamins, recombinant protein), the concept of an algal biorefinery has to explore the possibility of large-scale parallel and complementary production streams of these bioproducts from industrially grown algae (Fig. 1). The processes applied in an algal biorefinery lead to various products from the algal biomass which if properly integrated makes it a profitable business venture which would not be possible by targeting only one or two products even after significant optimization. It is anticipated that the various products derived from an algal biorefinery can feed various industries and contribute to the co-development of many sectors (Cheng-Wu et al., 2001). For example, as a specialization, recombinant protein (e.g., antibody and antiviral) production from algae (e.g., cyanovirin, griffithsin, interferon) could be used to manufacture drugs and vaccines by pharmaceutical industry. Coproducts from biodiesel production, such as glycerol, can be an input to the industrial chemical sector. The algal biorefinery framework can also yield value-added coproducts which can be used directly or indirectly in the food production sector (Chini Zittelli et al., 1999; Patil et al., 2007). Therefore, synergistic development of algal biofuels with other industries has led to algae being referred to as “biofactories” for future feedstock.

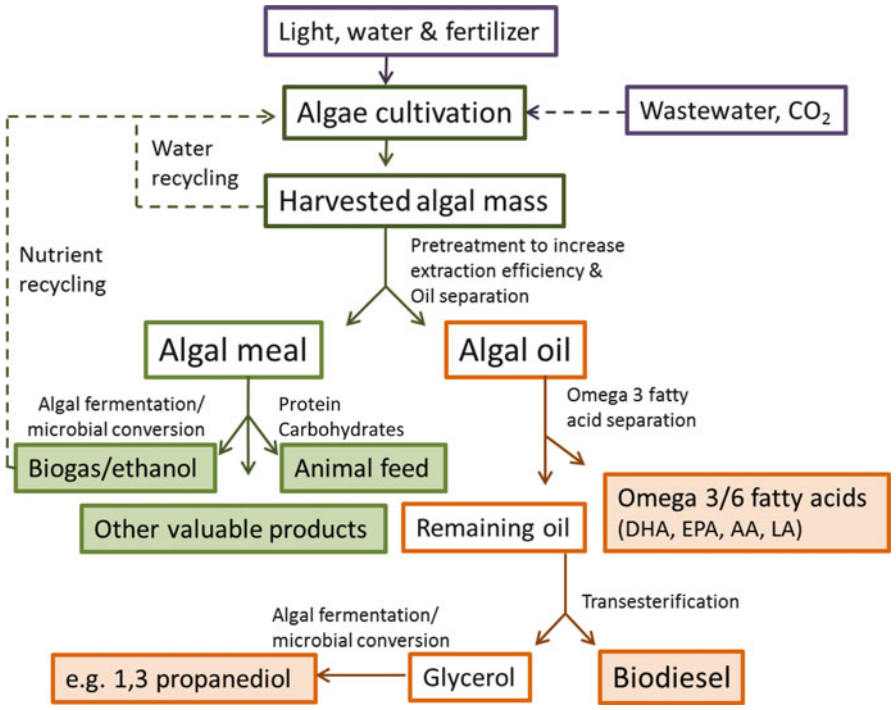

Figure 1. Proposed schematic flow sheet for a microalgal biorefinery aiming at producing omega-3 fatty acids, animal feed, and biodiesel as the main products. If biogas/ethanol is produced instead of animal feed, nutrients can potentially be recycled.

4. The Algal Biorefinery for Algal Biofuels

There are different types of bioenergy outputs from algae, either natural bioproducts or biorefinery products (Fig. 2). Biohydrogen can be produced in small amounts from algae, but all other bioenergy products require a biorefinery process. For example, the non-soluble and non-extractable cellular components of microalgae can be pyrolyzed to produce a hydrocarbon-based fuel (or alkanes). Unlike conventional fatty acid methyl ester (FAME) biodiesel, these hydrocarbon-based fuels can be readily refined to transportation fuels. Certain green microalgae, *Botryococcus braunii,* can produce large amounts of hydrocarbons instead of lipid (Li et al., 2011). These saturated and unsaturated algal hydrocarbons can be used directly or indirectly for substituting gasoline by several cracking process. However, the blooming of *B. braunii* has toxic effect on a variety of aquatic organisms (Chiang et al., 2004), and the utilization of biomass for biofuel

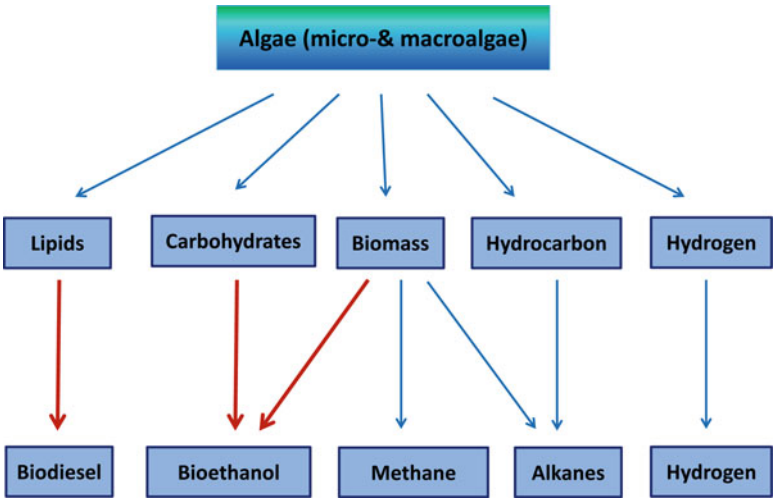

Figure 2. Illustration of routes to different types of bioenergy sources in algae. Area of proposed algal biorefinery highlighted by *red arrows*.

also conflicts with its application for aquaculture. Furthermore, it is worth noticing that carbohydrates (mainly non-starch polysaccharides) in the microalgae residue remain indigestible and cannot be used as an energy source by fish because they do not produce β-glucanases or β-xylanases (Sinha et al., 2011). For this reason, only the biorefineries relevant to biodiesel and bioethanol are discussed in this chapter (Fig. 2).

4.1. ALGAL BIODIESEL

The required rapid growth of carbon-neutral renewable alternatives makes microalgae one of the main future sources of biofuels, which may be the only one that could meet the sustainable displacement of high proportions of oil consumption (Chisti, 2007; Hu et al., 2008b, c; Schenk et al., 2008). Microalgae have a great potential as a future feedstock for biodiesel production because of their high growth rates and high oil content of some species. Many microalgae double their biomass within 24 h under favorable growing conditions. The doubling time during the exponential growth phase for microalgae can be as short as 3.5 h. The oil content of microalgae ranges from 15 to 75% (dry weight). Annual oil production from high-oil microalgae can be in the range of 58,700–136,900 L/ha (Chisti, 2007).

Biodiesel is produced as methyl ester fatty acid (FAME) processed through chemical transesterification of triglycerides from algal oil with alcohol (Fig. 3). Normally the transesterification may need a pretreatment for degumming,

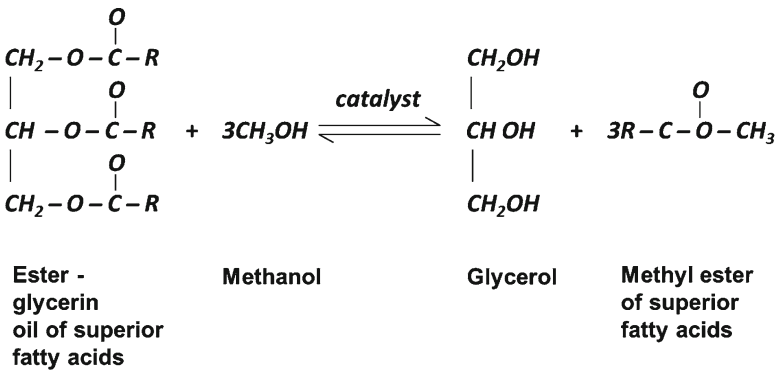

Figure 3. Chemical transesterification of triglycerides for biodiesel production (R- remaining fatty acid groups).

deacidification, bleaching, and dehydration, depending on the composition of the materials. The purpose is to remove phosphatides, free fatty acids (FFAs), water, pigments, and trace metals and reduce oxidation products in raw materials (Cheng and Timilsina, 2011). Alcohol is the other reactant for the transesterification to produce biodiesel. Since the chemical reaction is reversible, alcohol is usually overdosed to improve the biodiesel production efficiency. It also makes it possible to recycle the unreacted alcohol back for reuse to improve the biodiesel production rate and the economics. Either ethanol or methanol can be used, but methanol is commonly used because it is cheaper. Transesterification can be catalyzed by alkalines (NaOH), acids (H_2SO_4), or enzymes (lipase). Alkaline is commonly used as catalyst for oils with high triglycerides content. Acid is usually used in pretreatment of the oils with high FFAs which can be converted to esters. The esters are then converted to biodiesel through transesterification to improve the conversion efficiency. Lipase can convert both triglycerides and FFAs to biodiesel, but it is much more expensive than alkalines or acids (Cheng and Timilsina, 2011). The main products of transesterification are biodiesel and glycerol which can be separated through settling, filtration, and decantation. Refining of both biodiesel and glycerol improves the quality of these products.

4.2. ALGAL BIOETHANOL

Algae have high photon conversion efficiency and can synthesize and accumulate large quantities of carbohydrate biomass for bioethanol production (Subhadra and Edwards, 2010; Packer, 2009). Matsumoto et al. (2003) have screened several strains of marine microalgae with high carbohydrate content and identified a total of 76 strains with a carbohydrate content ranging from 40 to 53%. Microalgae

Chlorella, *Dunaliella*, *Chlamydomonas*, *Scenedesmus*, *Spirulina* contain large amounts (50%) of starch and glycogen which are cheap raw materials for ethanol production (Ueda et al., 1996). The cellulose in microalgae can also be fermented to bioethanol (Chen et al., 2009). Therefore, it has been estimated that 1 ha of microalgae culture could produce 46,760–140,290 L of ethanol per year (Cheryl, 2010). This yield is several times higher than ethanol obtained for other feedstocks (Mussatto et al., 2010).

Generally production of bioethanol from microalgal biomass can be achieved via two methods of fermentation (biochemical process) and gasification (thermo-chemical process) (Singh and Gu, 2010). A two-stage process has been patented for microalgae fermentation (Ueda et al., 1996). In the first stage, microalgae undergo fermentation in an anaerobic environment to produce ethanol. The CO_2 produced in the fermentation process can be recycled for algal cultivation. The second stage involves utilization of remaining algal biomass for methane production through anaerobic digestion. However, a modified fermentation process was recommended wherein yeasts, *Saccharomyces cerevisiae* and *Saccharomyces uvarum*, were added to algae fermentation broth for ethanol production (Bush and Hall, 2006; Harun et al., 2010). If an amylase-producing yeast strain can be used for ethanol fermentation, both saccharification and fermentation processes can be simultaneously carried out in a single step (Harun et al., 2010). The advantage is that utilization of microbial starch-degrading ethanol producers can preclude the cost incurred for acid or enzymatic saccharification of starch. However, research on bioethanol production from microalgal biomass is still in its infancy and not yet commercialized. To date, efforts are still ongoing to optimize conditions to improve bioethanol production (Harun et al., 2010).

5. The Potential of an Algal Biorefinery in Aquaculture

The main applications of microalgae for aquaculture are associated with nutrition as a sole component or as a food additive to basic nutrients. Microalgae are required for larval nutrition during a brief period or even for adults, either for direct consumption in the case of mollusks and penaeid shrimp or indirectly as food for the live prey fed to small fish larvae (Muller-Feuga, 2000). The use of microalgae in fish hatcheries is required for both production of live prey and maintaining the quality of the larvae-rearing medium (Spolaore et al., 2006). A significant growth rate can be obtained with microalgae supplemented diets in many species, such as prawn, oyster, abalone, and scallop (Leber and Pruder, 1988; Moss and Pruder, 1995; Tacon et al., 2002; Burford et al., 2004; Cuzon et al., 2004; Moss et al., 2006; Wasieleski et al., 2006; Dang et al., 2011). The nutritional value of the dietary algae is not only dependent on the chemical composition but also on factors such as the capability of the animal to ingest and digest the algae and to assimilate their nutrients (Lora-Vilchis and Maeda-Martinez, 1997).

As aquafeed additives, algal biorefinery products have been shown to have positive effects on the immune system of developing fish larvae (Reitan et al., 1997) and the regulation of immunity, gene expression and signaling (Bell and Sargent, 2003; Tocher, 2003). For example, feeding guppies with optimal concentrations of a neutral lipid extract of the green microalga *Parietochloris incisa*, containing β-carotene and arachidonic acid (AA) as the major highly unsaturated fatty acids (HUFA), significantly reduced infection with the protozoan parasite *Tetrahymena* sp. (Khozin-Goldberg et al., 2006). An additive effect of *P. incisa*-derived β-carotene and AA-rich triacylglycerols (TAG) was evidenced by increased guppy survival under acute salinity stress (Dagar et al., 2010; Nath et al., 2012). With limited capacity to desaturate and elongate essential C18 fatty acids, it has been indicated that balanced combinations of n-3 HUFA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and n-6 HUFA are needed to optimize the immune response and to obtain the desired immune-stimulatory effect in fish (Fracalossi and Lovell, 1994; Castell et al., 1994; Harel et al., 2001). In fish, carotenoids have been reported to enhance larval growth and survival (Torrissen, 1984). Amar et al. (2000, 2001, 2004) have shown that dietary β-carotene and astaxanthin from synthetic sources increase humoral (serum complement and lysozyme activity) and cellular (phagocytosis and nonspecific cytotoxicity) factors in the innate immune system of rainbow trout. As use of antibiotics in aquaculture is banned in many countries, algal aquafeed could be a more profitable venture, rather than nutrient input alone.

Microalgae are also used for coloring the flesh of salmonids. Astaxanthin and canthaxanthin are the only pigments that can accumulate in the flesh of salmonids whose pinkening represents a 100 million US$, rapidly expanding market (Baker, 2002; Raja et al., 2007). This feed additive is produced by chemical synthesis and available at a price of 3,000 US$/kg. Some companies like Algatec-Sweden, Norbio-Norway, Biotechna-UK, Aquasearch, Cyanotech, Maricultura, Danisco Biotechnology, and Oceancolor-USA have entered the astaxanthin market. In fact, microalgal astaxanthin has been approved in Japan and Canada as a pigment in salmonid feeds (Spolaore et al., 2006). Feeds including 5–20% *Arthrospira* (rich in carotene pigments) enhance the red and yellow patterns in carp. This clarity and color definition increases their value. Another example is the traditional French technique called the greening of oysters. It consists of creating a blue-green color on the gills and labial palps of oysters using the diatom *Haslea ostrearia*. This increases the product's market value by 40% (Gagneux-Moreaux et al., 2007).

The most frequently used aquaculture feed species are *Chlorella, Tetraselmis, Isochrysis, Pavlova, Phaeodactylum, Chaetoceros, Nannochloropsis, Skeletonema* and *Thalassiosira*. Combination of different algal species provides better balanced nutrition and improves animal growth better than a diet composed of only one algal species (Spolaore et al., 2006; Chakraborty et al., 2007). In addition to providing protein and energy, they provide other key nutrients such as vitamins, essential polyunsaturated fatty acids (PUFAs), pigments, and sterols which are

transferred through the food chain (Hemaiswarya et al., 2011). Protein and vitamin content is a major factor determining the nutritional value of microalgae. In addition, omega-3 PUFAs (e.g., EPA, AA, and DHA) content is of major importance.

It has been estimated that the production of microalgae for aquaculture could be more than 1,000 tons (62% for mollusks, 21% for shrimps, and 16% for fish; Spolaore et al., 2006; Gagneux-Moreaux et al., 2007). The worldwide annual production of algal biomass is estimated to be 5 million kg/year with a market value of about 1.25 billion US$/year (Pulz and Gross, 2004). Approximately one fifth of this biomass is used to nourish the fish and shellfish that are cultivated in aquaculture hatcheries (Muller-Feuga, 2004). However, valuable extractable chemicals as above could be obtained from biofuel biorefineries. By producing various coproducts such as omega-3 fatty acids and biodiesel in a sequential biomass processing (Fig. 1), algal biorefineries have a strong potential to match the challenge in aquaculture to generate end products/seafoods with high levels of health-promoting nutrients for human consumption without further depleting naturally occurring fishstocks.

6. Trends for the Algal Biorefinery

With increased demand of second- and third-generation biofuels, private investment has sprung up, now far exceeding public funding. For example, the oil giant Exxon Mobil alone has committed US$600 million toward producing liquid transportation fuels from algae in 2009. Furthermore, the development of algal biorefineries is becoming more the subject of major international collaborations. For example, the biofuel from the Algae Technology (BIOFAT) project is coordinated by Abengoa Bioenergia Nuevas Tecnologias (ABNT). Partners include University of Florence, A4F-AlgaFuel (Portugal), Ben-Gurion University (Israel), Fotosintetica & Microbiologica (Italy), Evodos (Netherlands), AlgoSource Technologies (France), IN SRL (Italy), and Hart Energy (Belgium).

Currently, no algal biorefinery is commercially operating. However, significant improvements toward commercialization have been reported by companies like Sapphire Energy, CEHMM, and Cellana. For example, Sapphire has recently announced the raising of US$300 million private and public funding for their commercial scale plant in Luna County, New Mexico (http://www.sapphireenergy.com/). The company has already participated in a test flight using algae-based jetfuel in a Boeing 737–800 twin engine aircraft. CEHMM claims to have the first algal biorefinery with a capacity to operate 1,000 gal each day. They have also successfully produced beef with high omega-3 from cattle fed with a mixture of microalgae and cattle feed (http://www.cehmm.org/). Cellana has been successfully running their demonstration pilot plant and has raised more than US$100 million of private and public funding for their commercial biorefinery plant in Maui, Hawaii (http://cellana.com/). In the meantime, many countries, such as the USA,

Australia, China, Japan, South Korea, the United Kingdom, Italy, and the Philippines, have also announced plans to invest heavily in research and development on algae-based biofuels. For instance in January 2010, the US Department of Energy announced a $44 million investment in algal biofuels development and demonstration to be carried out by the National Alliance for Advanced Biofuels and Bioproducts (NAABB). Led by the Donald Danforth Plant Science Center (St. Louis, MO), NAABB will develop a systems approach for sustainable commercialization of algal biofuel (such as renewable gasoline, diesel, and jetfuel) and other bioproducts (European Biofuels Technology, 2011). According to their statement, NAABB will integrate resources from companies, universities, and national laboratories to overcome the critical barriers of cost, resource use and efficiency, greenhouse gas emissions, and commercial viability. It aims to develop and demonstrate the science and technology necessary to significantly increase production of algal biomass and lipids, efficiently harvest and extract algae and algal products, and establish valuable certified coproducts that scale with renewable fuel production. Coproducts would include animal feed, industrial feedstocks, and additional energy generation (Donald Danforth Plant Science, 2011).

In the proposed algal biorefinery, the algal oil and meal can be processed for high omega-3 fatty acids and animal feed, respectively. The remaining oil can then be processed for biofuels. The byproduct glycerol can be used to produce chemicals such as 1, 3-propanediol or to produce more algal biomass. Similar to aquaculture, the algal meal can be a direct feed supplement in the livestock industry which amounted to $70 billion dollars in the USA (Subhadra and George, 2011). Recent studies on nutritional values have found that algal supplementation can reduce milk fat content and increase the amount of conjugated linoleic acid (CLA) and DHA in the milk fat composition and also an increase in trans-18:1 isomers (precursors of CLA biosynthesis) in ruminants' tissues (Boeckaert et al., 2008; Or-Rashid et al., 2008). Furthermore, the nutrient-rich wastewater and methane from the livestock industry can be used for algal biomass and biofuel production and can help reduce GHG emissions and environmental pollution. There is also scope for combination of algae and lignocellulosics industries to mutually benefit and obtain economic viability.

Although algae have been recognized as a promising biofuel feedstock, it is worth noting that there are also some obstacles hindering the development of microalgae biofuels. For instance, with current available photobioreactors the production cost of algal biomass is eight times higher than that of fossil fuels (Wijffels et al., 2010). Therefore, maximizing the value derived from algal biomass feedstock seems to be essential for algal biofuel development (Fig. 4). Whereas the transition to a biorefinery economy would require huge investment in new infrastructure to produce, store, and deliver algal biorefinery products to end users, it becomes a vision for a future in which algal biorenewables replace fossil fuels. At present, many other algal products are more profitable than biodiesel, and these products will be important to establish profitable algal biorefineries (Fig. 4). However, it can be anticipated that demand for these products will be

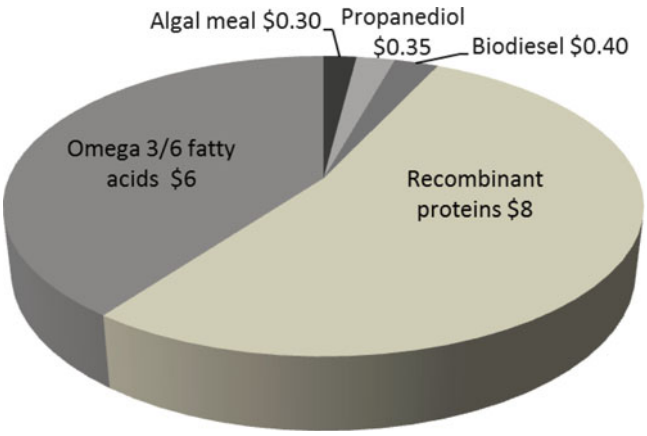

Figure 4. Comparison of values (in US $/kg algal mass) of multiple products from algal biomass (Adapted from Subhadra, 2010).

much more easily satisfied which will influence the price than the demand for biodiesel which aims to replace large proportions of fossil fuels. At present, the productivity of microalgal biomass on average can reach 80 t/ha/year obtained from a microalgae plant (Lundquist et al., 2010). If "biofuel content" accounts for 30% of the biomass, the nonfuel production can easily exceed 50 t/ha/year. *Vice versa*, the ongoing research of microalgae biofuels will likely continue to alleviate the production cost, which is also a desirable criterion for the algal biorefinery.

7. Future Research Directions on Microalgae for Biofuels and Aquaculture Feed

Requirement of high cost and energy for the large-scale production of microalgae has been the major constraint for its commercial utilization. Reduction of excessive power expenditure through optimization of different methods and technologies for agitation, harvesting, and drying of biomass is one of the key directions for future research. Moreover, efficient strain selection, genetic engineering, and utilization of wastewater/CO_2 for biomass production should be taken into consideration.

Microalgae species and strain selection is determined by many factors, such as growth rate, optimal temperature range, lipid accumulation, harvesting properties, and response to nutrient deprivation. These factors affect the performance and productivity of the algae in the adopted culture system. Moreover, identification of differentially expressed genes, proteins, and metabolites that are either directly involved in lipid biosynthesis and degradation or that are coordinately regulated is possible through transcriptomics, proteomics, and metabolomics, respectively (Nguyen et al., 2008; Timmins et al., 2009; Schuhmann et al., 2012; de Oliveira

Dal'Molin et al., 2011). The interpretation of the findings of these methods is challenging as lipid accumulation can be due to an upregulated enzyme downstream or a downregulated enzyme upstream in the metabolic pathway. However, the identification of differentially expressed genes, proteins, or metabolites may lead to the discovery of rate-limiting processes in the cell which can be backed up by the determination of metabolic flux. The study of the metabolic flux by various techniques such as the monitoring of consumption and production of key compounds or the isotopic labeling of key metabolite precursors or intermediates and the monitoring of these isotopes in a time dependent manner is therefore warranted to understand the metabolic dynamics of microalgae (Yang et al., 2000; Fernie et al., 2005; Dong et al., 2006; de Oliveira Dal'Molin et al., 2011). The systems biology approach will allow fine-tuning of algae properties by genetic or metabolic engineering (Mus et al., 2007).

Unsaturated fatty acids constitute the major proportion of lipids in microalgae, raising concerns for storage of biodiesel as the acids are prone to oxidation. One corrective measure is the partial catalytic hydrogenation of the oil (Chisti, 2007). However, higher levels of polyunsaturated fats lower the cold filter plugging point; the temperature at which the fuel starts to form crystals/solidifies and blocks the fuel filters of an engine. It can be seen that the extent of unsaturation in oil lowers its melting point. Therefore, colder climates require a higher unsaturated lipid content to enable the fuel to perform at low temperatures (Knothe, 2005). Microalgae have excellent potential for the genetic modification of their lipid pathways either by upregulation of fatty acid biosynthesis or by downregulation of β-oxidation. By knocking out or modifying enzymes responsible for the synthesis of polyunsaturated lipids in the cell, it should be possible to dramatically increase the proportion of monounsaturated lipids (Schuhmann et al., 2012).

In order to reduce some negative factors, such as light saturation photoinhibition and oxygen toxicity, the photosynthetic efficiency has to be optimized. For example, the reduction of photosynthetic antenna pigments has led to higher cell densities and higher biomass production rates in small-scale cultivation systems (Mussgnug et al., 2007). Other aspects are dependent on the cultivation systems; therefore, though optimization of raceway ponds and photobioreactors, it is desirable that innovative and alternative cultivation systems, that are more efficient and less expensive, would be designed. Linked to the cost of cultivation, the process by-products (e.g., exhausted growth medium and exploited biomass) should be recycled or be considered as new substrates for other processes in an integrated biorefinery system, with higher value and commercial suitability. For example, as fertilizer costs for N and P are likely to increase due to high energy costs and depletion respectively, it can be envisaged that unwanted biomass can be used for biogas (methane) production whereby nutrients from the sludge can be recirculated (Fig. 1). Similarly, fish waste can be used to create additional revenue streams through the growth of algae for biofuel and methane which is called the Aqua-Sphere system (Singh and Gu, 2010).

To this end, the issue of bioaccumulation of heavy metals and bioactive chemicals need to be considered if animal feed is to be produced repeatedly.

Furthermore, it is important to search for native microalgae species, both for biofuel production and aquaculture due to the environmental and economic benefits they offer. In aquaculture, it has been already reported that native species exhibited superior survival than laboratory species in larval *P. magellanicus* (Gouda et al., 2006). However, the process of isolating and testing new species of algae is very labor intensive, and hatcheries are unlikely to adopt new algal species without well-established culture methods and proven long-term success. The enrichment of live algal feeds by altering culture conditions and by adding supplements to the culture water may be a very fruitful area of research. Methods are available for the modification of protein, TAG, and essential fatty acids in live algae (Utting, 1986; Thompson et al., 1990, 1992; von Elert, 2002; Seguineau et al., 2005). However, little is known about the effect of these modified feeds on larval performance. There are reports that modified live feed can improve larval growth while not negatively affecting survival (Leonardos and Lucas, 2000; Pernet et al., 2005). New feeds having a wider spectrum of nutritional components formulated from algal species that are readily ingested and digested by larvae could be developed with the adoption of these methods. Another area of further research is the enrichment of live feeds and its effect on larval growth and survival in numerous aquatic species. Moreover, the direct addition of dissolved organic components such as sugars (Welborn and Manahan, 1990), amino acids (Manahan, 1983), and fatty acids (Jaeckle, 1995) to larval culture tanks may be a very direct and simple way to supply essential nutrients to larvae, although little research has been done in this area.

8. Conclusion

It is quite evident that microalgae are a potential source for a number of very useful products, particularly for biofuel production and aquaculture feed. Now the question arises what should be the ideal configuration of an algal biorefinery? As with other biorefinery ideas, the use of algae presents numerous routes to the future integration of raw materials, processes, and products to create a hybrid biorefinery. As feedstock, algae could fit into most of the integrated biorefinery designs that have already been proposed as its primary components might be optimized to produce more oils, carbohydrates, or proteins. Therefore, a hybrid biorefinery may be a more profitable venture, rather than an exclusively product-based or energy-based biorefinery. By producing various coproducts such as aquaculture feed, recombinant protein, omega-3 fatty acids, biogas, and biodiesel in sequential and parallel biomass processing, the algal biorefinery takes advantage of the various components in raw material and their intermediates, therefore maximizing the value derived from the biomass feedstock (Hu et al., 2008a).

Geographical and climatic conditions in potential algae production areas are ideally sunny and arid on near-flat land with low agricultural or biodiversity value coupled with saline water in the region. These would not only support a strong integrated algal biofuel industry but may also be highly suited for aquaculture (e.g., shrimp production; Subhadra and George, 2011). By cross-feeding products and by-products, such an integrated development would increase the viability and sustainability of both algal bioproducts and aquaculture production while sharing the limited natural resources.

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R. Gordon and J. Seckbach (eds.), *The Science of Algal Fuels*,
Cellular Origin, Life in Extreme Habitats and Astrobiology, 43–62
DOI 10.1007/978-94-007-5110-1_3, © Springer Science+Business Media Dordrecht 2012

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APPROACHES AND PROSPECTIVES FOR ALGAL FUEL

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1. Introduction

Materialistic development of a nation depends upon the kind of fuel resources available in a nation. Most of developing and developed countries are striving hard to maintain or explore new fuel reserves. The current fuel-based demands are mainly fulfilled by fossil fuel/oil reserves. Fossil fuels such as coal, petroleum, and natural gas are hydrocarbons formed by anaerobic decomposition of dead buried phytoplankton and zooplankton. They are nonrenewable energy resources and take millions of years to form. The present energy scenario and increasing demands of petroleum-based fuel has created an imbalance between formation and depletion of these energy resources. According to the US Energy Information Administration (EIA) 2007, world energy consumption is growing about 2.3% per year with 86.4% of energy from fossil fuel including petroleum 36.0%, coal 27.4%, and natural gas 23.0%. EIA estimates that if the current consumption rate is maintained, then all the fossil fuel reserves will be depleted by the year 2057.

The large-scale consumption of fossil fuels globally with its high depletion rate is also becoming one of the major causes of regional and global conflicts over environmental issues. The burning of fossil fuels by human beings is reported as the largest source of emissions of carbon dioxide (a greenhouse gas) that can enhance radioactive forcing and contribute to global warming. The atmospheric concentration of CO_2 is increasing and raising concerns that solar heat will be trapped and the average surface temperature of the Earth will rise in response. Aside from global warming, there are also many other harmful effects of the process of converting fossil fuels to energy. Some of these include air pollution, water pollution, solid waste accumulation, land degradation, and human disease. These major causes are increasing environmental concern and demand to find possible substitutes for fossil fuel energy, forcing researchers globally to think about new possibilities for the generation of renewable energy sources.

The International Energy Agency (IEA, 2002), defined renewable energy as the energy derived from natural processes, which can be replenished constantly. It is derived directly from the sun or from deep within the earth. The definition also includes the electricity and heat generated from solar, wind, ocean, hydropower, biomass, geothermal resources, biofuels, and biohydrogen. But these promises of renewable energy resources in meeting future energy demands are tempered by the fact that most of existing renewable resources (solar, wind, hydroelectric, and geothermal) can only produce electricity, not fuel, and thus cannot act as a direct substitute for fossil fuels.

According to earlier studies, harnessing nature's energy in the form of biomass has proven to be a prominent source of renewable energy (Melis and Happe, 2001). Biomass (plant material) is a renewable energy source as the energy coming from the sun is stored through the process of photosynthesis. When plants are burned or processed to generate biofuel, this energy is released in the form of CO_2, which is reutilized by plants through photosynthesis (Bomani and Center, 2009). Therefore, biofuels are considered as carbon-neutral fuels. By definition, biofuels are described as solid, liquid, or even gaseous fuels, which are in some way derived from biomass (Fatih, 2009). The utilization of biomass for fuel generation not only helps to control global warming but also reduces environmental pollution (Demirbas, 2004). Different sources of biomass have been used for the generation of biofuels, and on the basis of source of biomass origin, biofuels have been categorized as first-, second-, and third-generation biofuels.

The first-generation biofuels are fuels derived from food crops such as corn, soybeans, sorghum, sugarcane, starch, vegetable oil, or animal fats using conventional technologies (Demirbas, 2009). The basic feed stocks for these biofuels are often edible seeds or grains of agricultural plants, which are pressed to yield oils that, subsequently, can be used as raw material for biodiesel. In some countries like Brazil, bioalcohols or alcohol fuels are also used in large amounts. They are produced by fermentation of sugars derived from wheat, corn, sugar beets, sugarcane, or molasses (IEA Report, 2008). But as the global population is rising alarmingly, use of food grains for biofuel production has been criticized for diverting food away from the human food chain. It has been increasingly understood that first-generation biofuels do not have the ability to achieve global targets for oil-product substitution, climate change mitigation, and economic growth. The sustainable production of biofuels from food crops is under review as they create an undue competition for land and water used for food and fiber production. The cumulative impact of these concerns has increased the interest in developing biofuels from nonfood/waste biomass. Feedstock from lignocellulosic materials includes cereal straw, bagasse, forest residues, and purpose-grown energy crops such as grasses and short-rotation forest residue. The second-generation (2G) biofuels use biomass to liquid technology (Inderwildi and King, 2009), including cellulosic biofuels (Carroll and Somerville, 2009).

Many second-generation biofuels are under development such as biohydrogen, biomethanol, Bio-DME, Fischer-Tropsch diesel, biohydrogen diesel, mixed alcohols, and wood diesel.

These second-generation biofuels could avoid some of the concerns facing first-generation biofuels and potentially offer greater cost reduction in the longer term. But some environmentalists have the fear that biofuel production from food/nonfood crops can create biodiversity problems because many animals will lose their habitats, as more and more land plants will be used for biofuel generation. It could also cause even bigger deforestation and soil erosion problems in some developing countries. As without sustainable management, forests could be cleared in these countries to provide way for biofuel production. These disadvantages of first- and second-generation biofuels have made more and more focus toward the third-generation biofuels or algae biofuels.

Algae fuel, also called oilgae or third-generation biofuel, is a biofuel from algae (Demirbas, 2009). Algae represent a large and diverse group of eukaryotic (complex-celled) and prokaryotic photosynthetic organisms. Typically, they are autotrophic organisms, ranging from unicellular to multicellular forms. They are photosynthetic, like plants, and "simple" because they lack the many distinct organs found in land plants. Like higher plants, algae require primarily three components to grow: sunlight, carbon dioxide, and water.

The first distinction that needs to be made is between macroalgae (or seaweed) versus microalgae. Macroalgae are the large multicellular algae often seen growing in ponds and can be seen through naked eyes. These larger algae can grow in a variety of ways. The largest multicellular algae are called seaweed; an example of this is the giant kelp plant, which can be well over 25 m in length. Microalgae, on the other hand, are tiny (±1 to 50 μm) unicellular algae and can be seen with the aid of a microscope (Aresta et al., 2005). Microalgae are responsible for the appearance of cloudiness within a pond or even an aquarium. Both types of algae grow extremely quickly. The largest seaweed, giant kelp, is known to grow as fast as 50 cm/day and can reach a length up to 80 m (Thomas, 2002). Microalgae cells can double every few hours during their exponential growth period (Metting, 1996). The fact that they grow so quickly makes them a promising crop for human use. Microalgae are known to contain large amounts of lipids within their cell structure, and so, they are increasingly becoming of interest as a biofuel feedstock.

The world of algae is so large that it is beyond the scope of this chapter to discuss the biofuel production from all types of algae, so here, we will mainly focus on biofuels from microalgae. These small algae commonly called as "pond scum," comprise the greenish coverings of stagnant ponds and produce a large amount of lipids, close to 50% of their biomass (Waltz, 2009). Microalgae have many different species with widely varying chemical compositions and live as single cells or colonies without any specialization. Biologists have categorized microalgae in a variety of classes, mainly distinguished by their pigmentation, life

cycle, and basic cellular structure. The four most important (at least in terms of abundance) are:

- The blue-green algae (Cyanophyceae) are much closer to bacteria, with prokaryotic structure and organization; these algae play an important role in fixing of nitrogen from the atmosphere. There are approximately 2,000 known species found in a variety of habitats from hot water springs to cold deserts. They are ubiquitous in nature.
- The green algae (Chlorophyceae). These are quite abundant, especially in freshwater. They can occur as single cells or as colonies. Green algae are the evolutionary progenitors of modern plants. The main storage compound for green algae is starch, though oils can be produced under certain stress conditions.
- The diatoms (Bacillariophyceae). These algae dominate the phytoplankton of the oceans but are also found in fresh and brackish water. Approximately 100,000 species are known to exist. Diatoms contain polymerized silica (Si) in their cell walls. All cells store carbon in a variety of forms. Diatoms store carbon in the form of natural oils or as a polymer of carbohydrates known as chrysolaminarin.
- The golden algae (Chrysophyceae). This group of algae is similar to the diatoms. They have more complex pigment systems and can appear yellow, brown, or orange in color. Approximately 1,000 species are known to exist, primarily in freshwater systems. They are similar to diatoms in pigmentation and biochemical composition. The golden algae produce natural oils and carbohydrates as storage compounds.

Microalgae are the most primitive form of plants. While the mechanism of photosynthesis in microalgae is similar to that of higher plants, they are generally more efficient converters of solar energy because of their simple cellular structure. In addition, the cells grow in aqueous suspension and have more efficient access to water, CO_2, and other nutrients (Sheehan et al., 1998).

2. Microalgal Species Considered for Oil Production

According to Solar Energy Research Institute report, the most promising species for biofuel production are *Botryococcus braunii* due to its rich quantities of hydrocarbons, *Nannochloropsis salina* for its high quantities of esters, and *Dunaliella salina* for its high fatty acid content (Feinberg, 1984). The National Renewable Energy Laboratory (NREL) in United States affirms that *Spirulina*, *Dunaliella*, *Scenedesmus*, and *Chlorella* are the most popular strains that have been produced at commercial or large-scale basis (Sheehan et al., 1998). Another advances report has explained that for a sustainable production of algal biomass, the species *Spirulina platensis*, *Dunaliella salina*, and *Chlorella* are the more suitable (Huntley and Redalje, 2006). Some of the microalgal species are grown under stress conditions that increase their lipid quantities. These stresses mainly refer to

Table 1. Microalgae species considered for oil production (Rengel, 2008).

Species	Stress	% Lipids (dry weight)	References
Cyclotella cryptica	Nitrogen deficiency	18	Feinberg (1984)
Dunaliella salina	Osmotic stress and nitrogen deficiency	18.5	Feinberg (1984) and Borowitzka (1999)
	Nitrogen deficiency	14.4	Feinberg (1984)
	Non environmental stress	6	Spolaore et al. (2006)
Nitzschia sp.	Non environmental stress	45–47	Chisti (2007)
Phaeodactylum tricornutum	Non environmental stress	20–30	Molina et al. (2003), Acién et al. (2003) and Chisti (2007)
Botryococcus braunii	Nitrogen deficiency	54.2	Feinberg (1984) and Sawayama et al. (1995)
	Non environmental stress	25–75	Chisti (2007)
Chlamydomonas sp.	Non environmental stress	23	Feinberg (1984)
Chlorella sp.	Non environmental stress	20.7	Feinberg (1984)
	Non environmental stress	28–32	Chisti (2007)
Chlorella vulgaris	Nitrogen deficiency	18	Illman et al. (2000) and Huntley and Redalje (2006)
	Non environmental stress	14–22	Spolaore et al. (2006)
Nannochloris sp.	Non environmental stress	20–35	Chisti (2007)
Nannochloropsis sp.	Nitrogen deficiency	33.3–37.8	Huntley and Redalje (2006)
	Non environmental stress	31–68	Chisti (2007)
Nannochloropsis salina	Nitrogen deficiency	54	Feinberg (1984)
	Non environmental stress	28.6	Feinberg (1984)
Spirulina platensis	Non environmental stress	16.6	Feinberg (1984)
Tetraselmis sueica	Nitrogen deficiency	20–30	Huntley and Redalje (2006)
	Non environmental stress	15–23	Chisti (2007)
Isocttrysis sp.	Nitrogen deficiency	26–45	Feinberg (1984)
	Non environmental stress	25–33	Chisti (2007)

nitrogen or sulfur deficiency. Table 1 shows the most common species found along with literature citations and their respective lipids concentrations.

3. Approaches for Biofuel Production from Microalgae

The idea using microalgae as a source of biofuel is not new, but it is now being taken seriously because of the escalating prices of petroleum and dwindling fossil fuel reserves (Kapdan and Kargi, 2006), which lead to an increasing concerns for national energy securities of the world.

Photosynthetic microalgae are characterized by high growth rates and high population densities (Chisti, 2007; Schneider, 2006). Algae use the CO_2, along

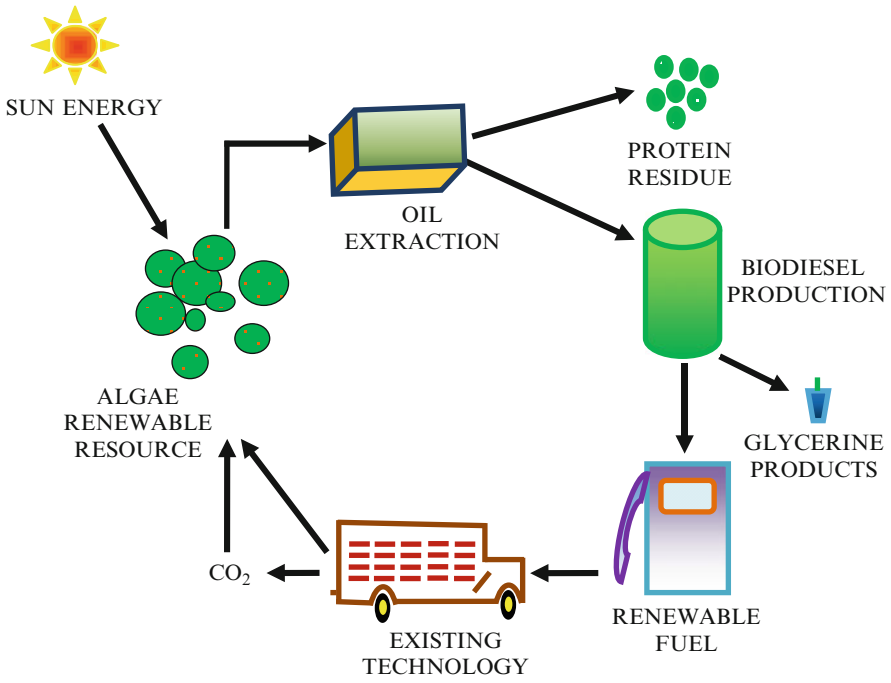

Figure 1. Cyclic representation of biofuel production from algae.

with sunlight and water, to produce sugars by photosynthesis, which are then metabolized into number of compounds such as proteins, carbohydrates, lipids, and nucleic acids in varying proportions (shown in Fig. 1).

While the percentages vary with the type of algae, there are algae types that are comprised up to 40–50% of their overall mass of fatty acids. The chemical composition of various microalgae is shown in Table 2. The lipid and fatty acid contents of microalgae vary in accordance with culture conditions. Algal oil contains saturated and monounsaturated fatty acids. These high proportions of saturated and monounsaturated fatty acids in algae are considered as optimal from a fuel quality standpoint (Sheehan et al., 1998).

Lipid accumulation in algae typically occurs during periods of environmental stress, including growth under nutrient-deprived conditions. Biochemical studies have suggested that acetyl-CoA carboxylase (ACCase), a biotin-containing enzyme that catalyzes an early step in fatty acid biosynthesis, may be involved in the control of this lipid accumulation process. Therefore, it may be possible to enhance lipid production rates by increasing the activity of this enzyme via genetic engineering (Chisti, 2007).

Table 2. Chemical composition of algae expressed on a dry matter basis (%) (Becker, 1994).

Strain	Protein	Carbohydrates	Lipids
Scenedesmus obliquus	50–56	10–17	12–14
Scenedesmus quadricauda	47	–	1.9
Scenedesmus dimorphus	8–18	21–52	16–40
Chlamydomonas reinhardtii	48	17	21
Chlorella vulgaris	51–58	12–17	14–22
Chlorella pyrenoidosa	57	26	2
Spirogyra sp.	6–20	33–64	11–21
Dunaliella bioculata	49	4	8
Dunaliella salina	57	32	6
Euglena gracilis	39–61	14–18	14–20
Prymnesium parvum	28–45	25–33	22–38
Tetraselmis maculata	52	15	3
Porphyridium cruentum	28–39	40–57	9–14
Spirulina platensis	46–63	8–14	4–9
Spirulina maxima	60–71	13–16	6–7
Synechococcus sp.	63	15	11
Anabaena cylindrica	43–56	25–30	4–7

The large bulk of natural oil made by microalgae is in the form of triacylglycerides (TAGs) which is the right kind of oil for producing biodiesel (Danielo, 2005). Fatty acids attached to the TAG within the algal cells can be both short- and long-chain hydrocarbons. The shorter chain-length acids are ideal for the creation of biodiesel, and some of the longer ones can have other beneficial medicinal uses.

Estimated annual oil productivity from algae is found to be much greater than other seed crops. Soybean can only produce about 450 l of oil per hectare. Canola can produce 1,200 l per hectare, and palm can produce 6,000 l. Now, compare that to algae which can yield 90,000 l per hectare (Chisti, 2007; Haag, 2007; Schneider, 2006).

Both physical and chemical processes are applicable in the production of liquid fuels from algal strains of high lipid content. These processes include direct lipid extraction in the production of diesel-oil substitutes, transesterification in the formation of ester fuels, and hydrogenation in the production of hydrocarbons. Oily substances are also produced via liquefaction of microalgal biomass through thermochemical reactions under conditions of high pressure and temperature. In addition to biodiesel, microalgae can also be used to generate energy in several other ways. Some algal species can produce hydrogen gas under specialized growth conditions. The biomass from algae can also be burned similar to wood or anaerobically digested using an anaerobic digester to produce methane biogas which can be used to generate heat and electricity (Campbell, 2008).

4. Advantages of Biodiesel from Algae Oil

Producing biodiesel from algae using efficient and cost-effective methods is considered as the most competent way to make biodiesel fuel. The main advantages of using algae biomass for deriving biodiesel from algae are listed below:

- Some species of algae can be harvested daily for biomass production (Chisti, 2007).
- Algae biofuel is found to be nontoxic, contains no sulfur, is highly biodegradable, and algae can consume carbon dioxide as they grow, so they could be used to capture CO_2 from power stations and other industrial plant that would otherwise go into the atmosphere.
- Algae can be cultivated on wastewater, alkaline water, liquid human sewage, and streams polluted by fertilizer or industrial wastes, which not only help to generate large amounts of algal biomass but also help to reduce or control environmental pollution.
- Algae can also be economically converted into solid fuels, methane gas, or bioethanol (Demirbas, 2005)

5. Algaculture for Biodiesel Production

Algae can be cultivated in two ways – in an open pond system (either naturally occurring or engineered) or in an engineered closed system. The algae strain selected for culture must be resistant to competitors (other aquatic plants) when grown in open pond systems because a natural or open pond, even with monitored growth pattern, does not completely restrict the growth of other phytoplankton species, which can hamper the algaculture.

The modern technique for algae culture like closed growth systems or photobioreactors are generally preferred over traditional/natural culture system. These techniques have several advantages over open pond systems; they not only support the cultivation of specific target alga cultures but also can be fed over CO_2 emitted from different industrial processes. The CO_2 supplied to algal cultures helps to maximize algal growth and simultaneously reduce the environmental pollutions (Iersel et al., 2008).

5.1. Culture Systems

5.1.1. Open Ponds

Open ponds are the oldest and simplest systems for microalgae mass cultivation. In this system, the shallow ponds usually about 1 ft deep are used to culture the algae by maintaining conditions identical to their natural environment. The pond is designed in a raceway configuration in which a paddle wheel provides circulation

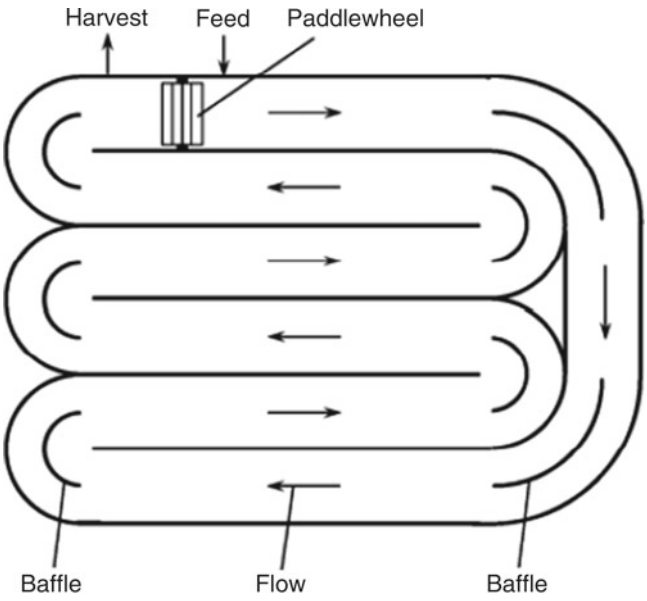

Figure 2. *Aerial view* of raceway pond (Chisti, 2007).

and mixing of algal cells and nutrients. The raceway ponds are typically made from poured concrete, or sometimes, they are simply dug into the earth and lined with plastic to prevent the ground from soaking up the liquid. The system is operated in a continuous mode, that is, the fresh feed containing nutrients including nitrogen, phosphorus, and inorganic salts is added in front of the paddle wheel, and the algal broth is harvested behind the paddle wheel after it has circulated through the loop (Fig. 2).

Depending on the nutrients required by algal species, a variety of wastewater sources can be used for the algal culture, such as dairy/swine lagoon effluent and municipal wastewater. For some marine types of microalgae, seawater or water with high salinity can be used. The utilization of waste CO_2 exhaust from coal-fired power plant is also encouraged; it serves as a growth enhancer for algae and simultaneously reduces the amount of flue gases in environment. Although these open ponds cost less to build and operate than enclosed photobioreactors, this culture system has its intrinsic disadvantages. Since these are open-air systems, they often experience a lot of water loss due to evaporation. Microalgae growing in an open pond do not take up carbon dioxide efficiently, so algal biomass production is limited (Chisti, 2007).

Biomass productivity is also limited by contamination with unwanted algal species as well as other organisms from feed. In addition, optimal culture conditions are difficult to maintain in open ponds, and recovering the biomass from such a dilute culture is expensive (Molina et al., 1999).

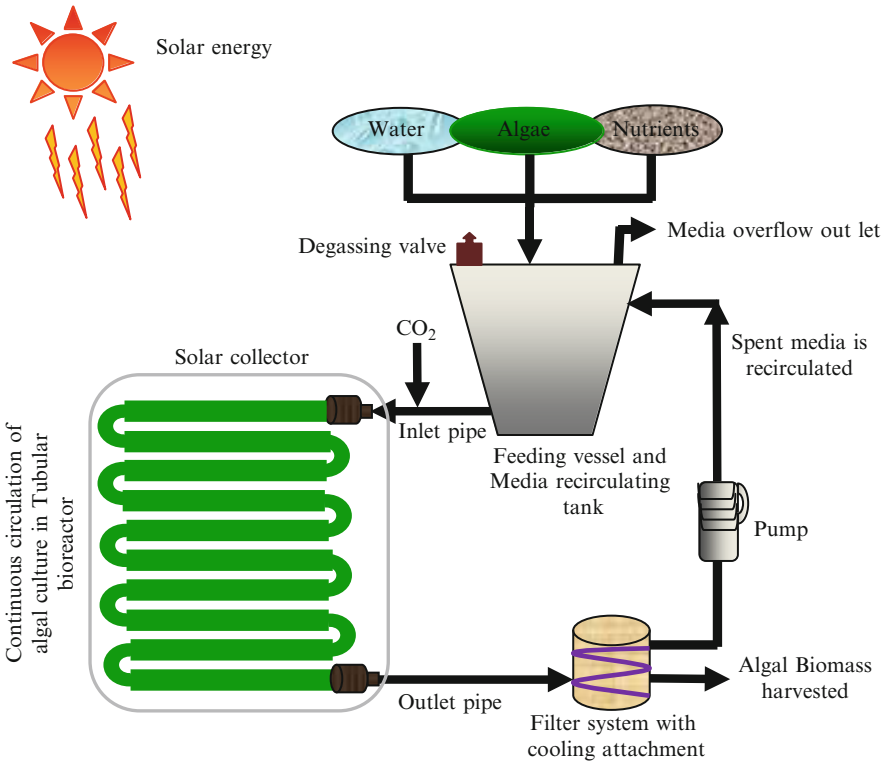

Figure 3. Schematic representation of algal biomass production in tubular photobioreactor.

5.1.2. Enclosed Photobioreactors

Enclosed photobioreactors have been employed to overcome the contamination and evaporation problems encountered in open ponds (Molina et al., 1999). These systems are made of transparent materials and generally placed outdoors for illumination by natural light. The cultivation vessels have a large surface area-to-volume ratio (Fig. 3).

The most widely used photobioreactor is a tubular design, which has a number of clear transparent tubes, usually aligned with the sun rays. The tubes are generally less than 10 cm in diameter to maximize sunlight penetration (Chisti, 2007). The medium broth is circulated through a pump to the tubes, where it is exposed to light for photosynthesis, and then back to a reservoir. The algal biomass is prevented from settling by maintaining a highly turbulent flow within the reactor, using either a mechanical pump or an airlift pump (Chisti, 2007). A portion of the algae is usually harvested after the solar collection tubes. In this way, continuous algal culture is possible (Chisti, 2007). In some photobioreactors, the tubes are coiled spirals to form what is known as a helical

tubular photobioreactor, but these sometimes require artificial illumination, which adds to the production cost. Therefore, this technology is only used for high-value products, not biodiesel feedstock.

The photosynthesis process generates oxygen. In an open-raceway system, this is not a problem as the oxygen is simply returned to the atmosphere. However, in the closed photobioreactor, the oxygen levels will build up until they inhibit and poison the algae. The culture must periodically be returned to a degassing zone, an area where the algal broth is bubbled with air to remove the excess oxygen. Also, the algae use carbon dioxide, which can cause carbon starvation and an increase in pH. Therefore, carbon dioxide must be fed into the system in order to successfully cultivate the microalgae on a large scale. Photobioreactors may require cooling during daylight hours, and the temperature must be regulated at night hours as well. This may be done through heat exchangers, located either in the tubes themselves or in the degassing column (Fig. 3).

The advantages of the enclosed photobioreactors are obvious. They can overcome the problems of contamination and evaporation encountered in open ponds (Molina et al., 1999). The biomass productivity of photobioreactors can be 13 times greater than that of a traditional raceway pond, on average (Chisti, 2007).

Biomass harvesting from photobioreactors is less expensive than that from a raceway pond, since the typical algal biomass is about 30 times as concentrated as the biomass found in raceways (Chisti, 2007). However, enclosed photobioreactors also have some disadvantages. For example, the reactors are more expensive and difficult to scale up. Moreover, light limitation cannot be entirely overcome since light penetration is inversely proportional to the cell concentration. Attachment of cells to the tube walls may also prevent light penetration. Although enclosed systems can enhance the biomass concentration, the growth of microalgae is still suboptimal due to variations in temperature and light intensity.

6. Harvesting

After growing algae in open ponds or photobioreactors, the microalgae biomass needs to be harvested for further processing. Algae may be separated from the medium, and various algal components, such as oil, may be extracted using different methods. For example, algae may be partially separated from the medium using a standing whirlpool circulation or harvesting vortex method. Alternatively, large-industrial scale commercial centrifuges may also be used for algae separation. In addition to this, sedimentation, filtering, or centrifugation techniques are also used to purify oil from other algal components. Separation of algae from the aqueous medium may be facilitated by addition of flocculants, such as clay (e.g., particle size less than 2 μm), aluminum sulfate, or polyacrylamide. In the presence of flocculants, algae may be separated by simple gravitational settling or may be more easily separated by centrifugation. The most commonly used harvest methods are gravity settlement, or centrifuge.

After harvesting, further concentration and oil extraction is required, for which various processes are proposed, including cell breakage and solvent extraction. The residual biomass left after oil extraction can also be used in various ways.

7. Techniques for Oil Extraction from Algal Biomass

Various methods are available for the extraction of algal oil, such as mechanical extraction using hydraulic or screw method, chemical extraction using different organic solvents, ultrasonic extraction, and supercritical extraction using carbon dioxide above its standard temperature and pressure; using mechanical pressing, algal oil can be extracted in a range from 70 to 75% of dry cell mass.

The chemicals like *n*-hexane, benzene, ethanol, chloroform, and diethyl ether are used as solvents in chemical extraction to extract the fatty acids from algal biomass. The most commonly used solvent is *n*-hexane, which is first added into the algal paste and then distilled to obtain the algal oil (Sazdanoff 2006).

Ultrasonic extraction of algae oil involves intense sonication of liquid, which generates sound waves that propagate into the liquid media resulting in alternating high-pressure and low-pressure cycles. During the high-pressure cycle, ultrasonic waves support the diffusion of solvents, such as hexane into the cell structure. As ultrasound breaks the cell wall mechanically by cavitation shear forces, it facilitates the transfer of lipids from the cell into the solvent. Afterward, the oil dissolved in the cyclohexane the pulp/tissue is filtered out. The solution is distilled to separate the oil from the hexane. Ultrasonication not only improves the extraction of oil from the algal cells but also helps in the conversion into biodiesel.

Besides these methods, another advanced method of oil extraction is supercritical fluid extraction method, in which CO_2 is first heated and compressed until it reaches the liquid-gas state. Then, it is added to the harvested algae, acting like a solvent. This technique has been used to obtain hydrocarbons from *Botryococcus braunii* and lipids from the diatom *Skeletonema* (Mendes et al., 1995).

8. Conversion of Algal Oil into Biodiesel

Once the oil is extracted from microalgae or oleaginous crops, the next step is to perform "transesterification" to produce biodiesel. Transesterification is a chemical reaction in which triglycerides of the oil reacts with methanol or ethanol to produce methyl esters and glycerol. It is a reversible reaction of fat or oil (which is composed of triglyceride) with an alcohol to form fatty acid alkyl ester and glycerol. Stoichiometrically, the reaction requires a 3:1 M alcohol to oil ratio, but excess alcohol is (usually, methyl alcohol is used) added to drive the equilibrium toward the product side (West et al., 2008). This large excess of methyl alcohol ensures that the reaction is driven in the direction of methyl esters, that is, toward

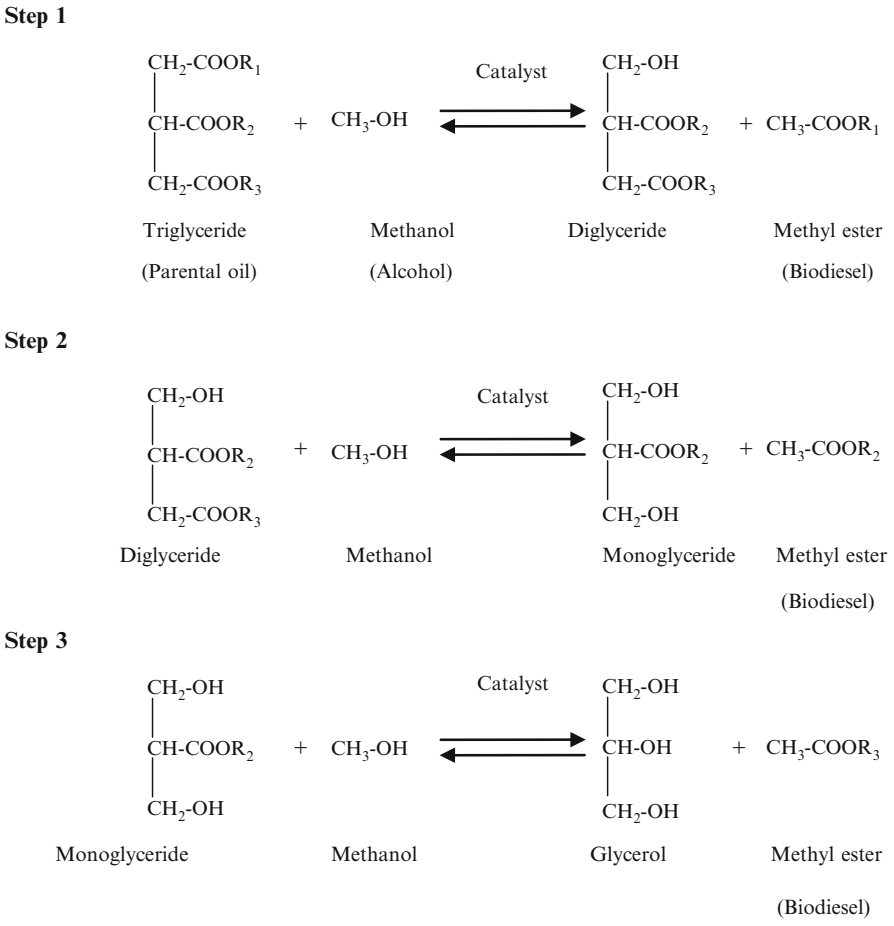

Figure 4. Transesterification of oil to biodiesel. R1–3 are hydrocarbon groups.

biodiesel (Fukuda et al. 2001). The reaction occurs in a stepwise manner: Triglycerides are first converted to diglycerides, then to monoglycerides, and finally to glycerol (Fig. 4). Finally, the biodiesel is recovered by repeated washing with water to remove glycerol and methanol (Chisti, 2007). This process of biodiesel production is found to be most efficient and least corrosive of all the processes as the reaction rate is reasonably high even at a low temperature of 60 °C.

It has been estimated that 1 ton of algal or vegetable oil after transesterification can produce 1 ton of biodiesel (Chisti, 2007). At present, the worldwide production of biodiesel comes mainly comes from rapeseed (84%), sunflower (13%), palm oil (1%), soybean (2%), and others (1%). The European Union is the highest producer of biodiesel, obtained from rapeseed and sunflower oils (Demirbas, 2007). The current scenario of biodiesel production from food/non-

food stuff is changing rapidly; huge efforts have been put to harness the proved potential of algae for biofuel production.

9. Utilization of Algae Leftover After the Extraction of Oil

The biomass residue that remains after extraction of oil could be used partly as high-protein animal feed and, possibly, as a source of small amounts of other high-value microalgal products (Chisti, 2006; Gavrilescu and Chisti, 2005; Molina, 1999). The revenue generated from selling the biomass residues could defray the cost of producing biodiesel. However, the majority of algal biomass residue from oil extraction is expected to undergo anaerobic digestion to produce biogas or bioethanol. This biogas/bioethanol can serve as an energy source for most of the production and processing process of the algal biomass. An additional income could come from the sale of nutrient-rich fertilizer and irrigation water that would be produced during the anaerobic digestion stage (Chisti, 2007). The technology for anaerobic digestion of waste biomass exists and is well developed (Lantz et al., 2007), and the technology for converting biogas to electrical/mechanical power is well established (Gokalp and Lebas, 2004). The carbon dioxide generated from combustion of biogas can be recycled directly for the production of the microalgae biomass (Wyman, 1994). Generation of these products gives further support to biofuel production from algae and simultaneously helps to reduce the waste.

10. Future Prospects and Perspectives of Algae Biofuels

Biofuel is primarily considered as a potentially cheap, low-carbon energy source (Hall et al., 1991). The global biofuel (bioethanol and biodiesel) production tripled from 4.8 billion gallons in 2002 to 16.0 billion in 2007 but still accounts for less than 3% of the global transportation fuel supply (Fig. 5) (Coyle, 2007).

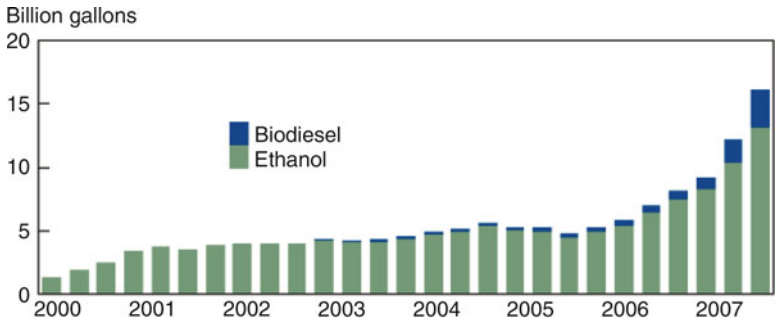

Figure 5. Global biofuel production: 2000–2007 (Source: International Energy Agency; FO Licht; Coyle 2007).

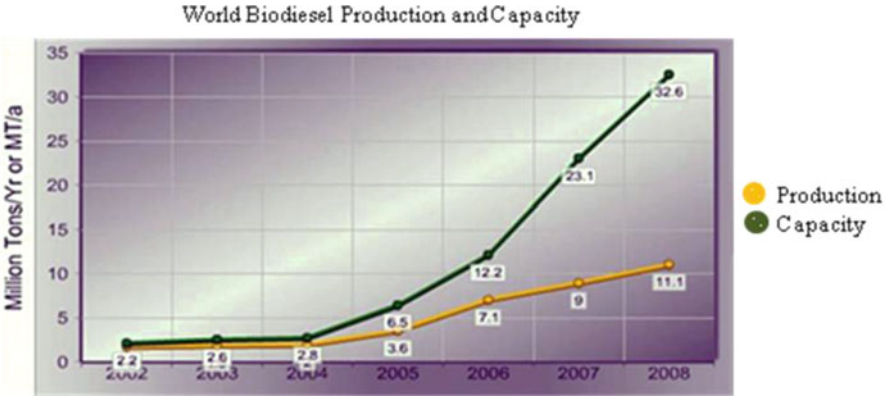

Figure 6. World biodiesel production and capacity (Will, 2008).

Biofuels are more expensive to produce than conventional transport fuels. In spite of this and environmental benefits, some countries have implemented a tax relief system for pilot biofuel/bioethanol production plants. In 2007, the European Union (EU) mandated that 20% of total energy content of petrol and diesel needs to come from renewable fuels. Thailand is aiming for a 10% renewable mix in the next years; India 20% by 2020 (Schubert, 2006). Sweden has targeted to be 100% energy independent by 2020; most of the energy will come through its own nuclear power and renewable biofuels (Schubert, 2006).

With growing infrastructure, efficient technologies, government incentives, and the evolving political issue on climate change, biofuel, especially second- and third- generation (2G, 3G) which does not utilize food as inputs, is considered to be a constant source of renewable energy (Waltz, 2007). Over the past few decades, researchers have been working on producing biofuel from biomass. It had been small-scale exploitation until recently when oil prices began to scale up significantly. The global production of biodiesel increased by 60% in 2005 over 2004, while ethanol increased by 19% (Waltz, 2007).

The global market for biodiesel is expected to increase in the next 10 years (Fig. 6). Europe currently represents 80% of global consumption and production, but the USA is now leading up with a faster rate in production than Europe, from 25 million gallons in 2004 to 450 million gallons in 2007 (Emerging Markets Online, 2008) (Will, 2008).

Brazil is expected to surpass the US and European biodiesel production by 2015. Europe, Brazil, China, and India each have targets to replace 5–20% of total diesel with biodiesel (Kennedy, 2007). If governments continued to invest more in R&D on biofuel exploitation, from second- and third-generation, especially on third-generation biofuel, it would be possible to reach the targets sooner.

11. Challenges of Biofuel Production from Algae

Algae are proven to be most potent source of biofuel production with highest oil production capacity and minimum negative environmental effects. Biofuel from algae can be coupled with flue gas CO_2 mitigation and wastewater treatment and also lead to production of high-value chemical coproducts (Li et al., 2008). The markets for algae biofuel already exist and are growing, but the growth of the market is limited due to underdeveloped/inefficient production technology. The cost of algae biofuel is currently high (Wijffels, 2007). Therefore, to develop biofuels from algae in an industrial process, there are several challenges need to be faced:

- Lack of efficient technology for large biomass production: Currently, the biomass is produced in open or closed ponds. In an open system, low-depth pools give algae greater access to sunlight and help to algae flourish better. But the evaporation and replacement of water, strain contamination (by bacteria or outside organism), and growth condition maintenance such as temperature and pH of water are some issues which greatly affect biomass production. Cultivation of algae in closed photobioreactors is more efficient and reduces some of the problems faced by open culture system. But currently, such photoreactors are not cost-effective and required large investments.
- Production rate: It has been tested by various researches globally that algae produce more lipid/oil in stress/starvation conditions, but these conditions can also affect the growth and reproduction of algae, so more research needs to be done to better understand the metabolic pathways of algae. Simultaneously efficient technologies need to be developed before global commercialization of algal biofuels.

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R. Gordon and J. Seckbach (eds.), *The Science of Algal Fuels*,
Cellular Origin, Life in Extreme Habitats and Astrobiology, 63–81
DOI 10.1007/978-94-007-5110-1_4, © Springer Science+Business Media Dordrecht 2012

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FROM ISOLATION OF POTENTIAL MICROALGAL STRAINS TO STRAIN ENGINEERING FOR BIOFUEL

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1. Introduction

One of most daunting challenges of scientists is to lower the petroleum dependency of the world. Biofuels have emerged as alluring alternatives. Nonedible crops, waste cooking oil, and animal fat have been used as sources of first- and second-generation biofuels. First-generation biofuels involved the use of edible crops such as sugarcane, corn, and canola for the production of ethanol and biodiesel. However, with the maturity of science, second-generation biofuels emerged which involved the use of nonedible crops. Among these the nonedible crop *Jatropha* has been the real focus of scientists all over the world. However, one of the greatest disadvantages of using *Jatropha* is the requirement of a large amount of land and the seasonal nature of these plants. Affording land to meet the world's requirement of petroleum is totally unlikely, and if by any chance agricultural land is put to this use, a definite consequence will be the price hike of food. To overcome this problem, scientists have come up with the idea of third-generation biofuels which involve the use of microalgae. This has the following advantages over the conventional second-generation biofuel: (1) Unlike the crop plants, the microalgae can double their biomass within 24 h (2); oil content may be as high as 66% (3) nonarable land and nonpotable water can be used for their cultivation; and (4) growth is not seasonal and can be harvested daily.

Microalgal biofuels are fast gaining international importance. One of the earliest reports of microalgal biofuel was from *Botryococcus braunii* (Chisti, 1980–1981). Nagle and Lemke (1990) reported methyl ester fuel and Sawayama et al. (1999) oil production from microalgae, while, Hirano et al. (1990) screened microalgae for linolenic acid, and Matsunga et al. (1995) cyanobacteria for high palmitoletic acid production. The research has gained momentum recently, largely due to the pressure to deal with depleting fossil reserves and the danger of oil crops competing with food crops leading to price hike. Recently *Scenedesmus* sp. strain JPCC GA0024 (Matsunga et al., 2009) and *Neochloris oleoabundans* (Gouveia et al., 2009) have been characterized for biofuel production. Marine algae have also been studied for biofuel production (Gouveia and Oliveira, 2008).

Attempts are also being made to increase microalgal lipid production. The traditional attempts include manipulation of nutrients in the media. However,

these manipulations are generally accompanied with a compromised growth. Thus, more recently genetic manipulation techniques have been developed for certain species. Attempts are being made to optimize biofuel production through metabolic engineering. Since all the pathways right from photosynthesis to lipid biosynthesis as well as degradation are interlinked, it is therefore, mandatory to have a thorough understanding, in fact a comprehensive analysis, of all the precursors, their biosynthesis and degradation as well as the metabolic switches. Several studies have used the omic techniques and found a number of proteins with regulatory and important role in biofuel synthesis. Researchers have been able to modify *Synechococcus* to actually secrete fatty acid, thus largely reducing the cost of post harvesting (Liu et al., 2010). As more and more refined tools become available and scientists create algae for specific accumulation of certain metabolites, we are sure to enter into the era of engineered microbes.

Owing to the importance of algal isolation, this chapter starts off with a brief idea of isolation and identification techniques of alga followed by the screening strategies for better biofuel producers. An outline of mass culture techniques is then given followed by the recent trends in the genetic modification of various algal strains.

2. Isolation of Potential Strains

The green algae are an ancient group of aquatic photosynthetic organisms, which gave rise to the land plants. They are a diverse group of simple, mostly autotrophic organisms, occurring both as unicellular to multicellular forms. The largest and most complex marine forms are called seaweeds. They range in size from the microscopic flagellate *Micromonas* to giant kelp that reach 200 ft (60 m) in length. Algae can play a role as biocatalysts for the production of food, chemicals, and fuels, and they are becoming important in the development of solar energy technology, biodegradation, and bioremediation. In addition, some species of algae are eaten directly by humans. The red macroalgae *Porphyra* sp. is a common ingredient in East Asian cuisine. The markets for other algae, like the microalgae *Spirulina* sp., *Chlorella* sp., and *Dunaliella* sp., are expanding as a food supplement in western world health stores.

It is estimated that there are more than 50,000 species of microalga, out of which only a limited number has been analyzed till date (Richmond, 2004). The last decade has shown an explosion in microalgal culture collection round the world. As of today, the freshwater collection of University of Coimbra (http://acoi.ci.uc.pt/) is considered to be the world's largest with more than 4,000 strains and 1,000 species. Similarly the collection of Gottingen University, Germany, that started in early 1920s has about 2,213 strains and 1,273 species (http://epsag.uni-goettingen.de). The University of Texas is yet another huge microalgal collection (www.sbs.utexas.edu/utex/). In Asia, Japan's National Institute of Environmental Studies in Ibaraki holds a collection of 2,150 strains and 700 algal

species (mcc.nies.go.jp/). Australia also holds about 800 strains of different algae (Mata et al., 2010). Algal isolation is generally carried out in three steps: (1) sampling, (2) isolation, and (3) identification.

2.1. SAMPLING

To isolate new strains, a proper sampling is a must. Keeping in mind the diverse habitats of algae, the sampling sites selected for an area may include the following habitats: freshwater, brackish, marine, hypersaline, ponds, lakes, rivers, creeks, estuaries, beaches, salt lakes, lagoons, etc. Further, to enhance the collection efficiency seasonal collection will allow a greater diversity to be collected.

2.2. ISOLATION OF AS MANY UNIALGAL STRAINS AS POSSIBLE

Microalgal isolation is a labor-intensive and patience-requiring task. The first report of algal isolation came way back in 1850 when German-born Ferdinand Cohn succeeded in maintaining the unicellular flagellate *Haematococucs* in pure form. However, he could not maintain a long-term culture of this alga. The first attempt to culture algae by using a combination of salts was made by Famintzin (1871) using the media developed by Knop (1865). One of the first algae to be cultured and grown axenically was *Chlorella* by Beijernick (1890), and Miquel (1892) was the first to isolate and establish axenic cultures of diatoms. Since then, we have come a long way in the isolation and maintenance of algae, but the isolation is still tedious, time-consuming, and at times tricky. Several methods and their modifications have cropped up in recent times for the isolation of algal species. Some of these methods are discussed below:

2.2.1. Serial Dilution

This technique is one of the oldest when algal isolation is concerned. Way back in 1910, Allen and Nelson used this technique with great success to isolate marine planktonic algae (Allen and Nelson, 1910). It still remains one of the most widely used method for the isolation of pure cultures of those microorganisms that have not yet been successfully cultivated on solid media and grow only in liquid media. A microorganism that predominates in a mixed culture can be isolated in pure form by a series of dilutions. The inoculum is subjected to serial dilution in a sterile liquid medium, and a large number of tubes of sterile liquid medium are inoculated with aliquots of each successive dilution. The basic concept behind this dilution can be understood by taking the following example. Suppose we have a culture containing 10 mL of liquid medium, which has 1,000 microorganisms, i.e., 100 microorganisms/mL of the liquid medium. If we take out 1 mL of this medium and mix it with 9 mL of fresh sterile liquid medium, we would then have 100 microorganisms in 10 mL or 10 microorganisms/mL. If we add 1 mL of this

suspension to another 9 mL of fresh sterile liquid medium, each mL would now contain a single microorganism on average. If this tube shows any microbial growth, there is a very high probability (estimable using Poisson statistics) that this growth has resulted from the introduction of a single microorganism in the medium and represents the pure culture of that microorganism (http://www.marine.csiro.au/microalgae/methods/Home-Intro.htm).

2.2.2. Capillary Pipette

In this method described in Stein (1973), a drop of natural collection is placed on an inverted Petri plate. This drop is surrounded by five to six sterile media drops. A capillary pipette is made by heating the narrow end of the Pasteur pipette and pulling both ends of the pipette until the opening approximates 100 μm. Using this capillary pipette, a single algal unit is picked from the drop of natural collection and transferred to one of the sterile drops, and the process is repeated to three to four times until one is satisfied that the sterile drop contains only a single algal unit. To counter check, one can use a compound microscope; the sterile drop can be viewed under a compound microscope. This drop is then transferred to a sterile culture media for growth.

2.2.3. Micromanipulation

If serial dilution uses the concept of diluting the culture to the extent of it having a single cell, how about picking up a single cell from a mixture of algae and culturing it? This is the thought behind the use of micromanipulators. These are devices by which one can pick out a single cell from a mixed culture. This instrument is used in conjunction with a microscope generally from a hanging drop preparation (Frohlich and Konig, 2000). The micromanipulator is equipped with a micropipette which has micrometer adjustments and can be moved right and left, forward, and backward, as well as up and down.

The advantages of this method are that one can be reasonably sure that the cultures come from a single cell and one can obtain strains within the species. The disadvantages are that the equipment is expensive, its manipulation is very tedious, and it requires a skilled operator. This is the reason why this method is reserved for use in highly specialized studies.

2.2.4. Streak Plating

This method is used most commonly to isolate pure cultures of bacteria. A small amount of mixed culture is placed on the tip of an inoculation loop/needle and is streaked across the surface of the agar medium. Streaks are made in such a way that each successive streak dilutes the inoculum sufficiently and the microorganisms are separated from each other. Sometimes streaking out a second plate by the same loop/needle without reinoculation may give a pure microbe culture much more readily. These plates are incubated to allow the growth of colonies. The key principle of this method is that, by streaking, a dilution gradient is established across the face of the Petri plate as bacterial cells are deposited on the agar surface. Because of this dilution gradient, confluent growth does not take place on that part of the medium where few bacterial cells are deposited.

2.2.5. Spray Plating

A derivation of streak spray plating involves the spraying of algal units on agar plates rather than streaking. A drop of natural collection is drawn into a capillary pipette and then sprayed on the agar plate held perpendicular to the spray.

2.2.6. Density Centrifugation

A simple and rapid technique is described for the separation of different microalgae from mixed cultures. The technique relies upon density gradient centrifugation in the silica sol Percoll, thus separation is achieved on the basis of differences in buoyant density. Upon centrifugation in Percoll density gradients, rnicroalgae form discrete bands at particular positions within the gradient. If the banding positions for different algae are sufficiently different, they can be readily separated by fractionation of the gradient. Photosynthetic activity and subsequent growth of microalgae is unaffected by centrifugation in Percoll (Whitelam et al., 1983).

2.2.7. Antibiotics

This should be avoided as far as possible if one is to isolate microalgal strains or any other microbe which will be further used for physiological or ecological studies since there is a good possibility that when isolating strains using antibiotic, certain mutant clones may be produced that do not reflect populations in the wild. However, some normally used antibiotics include antifungal agents such as cycloheximide (= acti-dione), nystatin, or amphotericin B. Germanium dioxide (GeO_2) was used as a media supplement by Lewin (1959) as a means to remove diatoms at a final concentration of 10 mg/L. It acts as a cell division inhibitor rather than a toxin. Markham and Hagmeier (1982) found concentrations as low as 0.045–0.179 mg/L were sufficient to kill diatoms contaminating their kelp cultures.

2.3. SCREENING OF STRAINS FOR LIPID PRODUCTION

Once the microalgal strains have been isolated, it is important to assess their use as biofuel producers. For this generally the lipid content is measured. Several methods have been developed for the extraction and estimation of lipids. It was the famous study of Chevreul on the dissolution of lipids in various solvents which paved the way for the development of lipid extraction and estimation procedures (Bohr, 2009). In 1879, Franz von Soxhlet described the first method based on automatic solvent extraction for milk lipids (Soxhlet, 1879). A further improvement was made in 1914 when a mixture of ethanol/ether (3/1) was used for lipid extraction (Bloor, 1914). In 1957, Folch described the classical procedure of lipid extraction, and this remains the most commonly used procedure by lipidologist around the world (Folch et al., 1957). Some other procedures were proposed by Bligh and Dyer (1959) and Sheppard (1963), which also used solvent mixtures made of chloroform/methanol and ethanol/diethyl ether, respectively.

As of today, dye-based methods for the estimation of lipids are becoming more and more popular. Lipids are not easily stained by water-soluble dye but can

be demonstrated easily using fat-soluble dye. Dyes used are more soluble in lipid than in solvent of the staining solution. Therefore, on application of staining solution, dye moves into lipid material of a cell and is retained there. Smith introduced the use of Nile blue in 1907 for the histochemical detection of tissue lipids (Smith, 1907, 1910). He found that Nile blue and similar dyes of the phenoxazine series had the remarkable property of simultaneously staining acid lipids blue and neutral lipids red. In the same year, Thorpe (1907) examined the composition of Nile blue and other blue phenoxazine dyes and found that they all contained various proportions of oxidation products called phenoxazones. The latter are nonionic and are bright red or yellow. Later Greenspan et al. (1985) used Nile red for the staining of cytoplasmic lipids. For microalgal lipid staining, Gao et al. (2008) have shown that time-domain nuclear magnetic resonance (TD-NMR) method is better than Nile red staining. Wagner et al. (2010) have used FTIR (Fourier transform infrared) spectra while Beal et al. (2010) the NMR spectroscopy. But one of the most recent advances in the lipid-staining methodologies appears to be the dye Bodipy 505/515. Cooper et al. (2010) report that Bodipy 505/515, a green lipophilic fluorescent dye, serves as an excellent vital stain for the oil-containing lipid bodies of live algal cells. Bodipy 505/515 vital staining can be used in combination with fluorescent-activated cell sorting to detect and isolate algal cells possessing high lipid content.

Most of the microalgal species can be induced to accumulate substantial quantities of lipids which have been reported to vary between 1 and 70% of the dry weight. However, the strain having a very high lipid content is often associated with low productivity: as an example, *Botryococcus braunii* has 75% oil content but undergoes slow growth. Most common algae with good productivities and oil levels between 20 and 50 include Chlorella, Crypthecodinium, Cylindrotheca, Dunaliella, Isochrysis, Nannochloropsis, Nannochloris, Neochloris, Nitzschia, Phaeodactylum, Porphyridium, Schizochytrium, and Tetraselmis (Mata et al., 2010). The selection of the most adequate strain must take into account the productivity, lipid content, ability to grow with the nutrients available in the area, and ability to grow under the specific environmental conditions. Also, one must consider the composition of fatty acid. Thomas et al. (1984) analyzed the fatty acid composition of seven freshwater microalga and found that all of them synthesized C 14:0, C16:0, C18:1, C18:2, and C18:3 fatty acids. However, the relative intensity of other individual fatty acids chains is species specific, e.g., C16:4 and C18:4 is found in *Ankistrodesmus* C18:4 and C22:6 in *Isochrysis* and C16:2 and C16:3 in *Nannochloropsis*.

2.4. FURTHER ANALYSIS OF IDENTIFIED STRAINS

For the identification of the algal species isolated, the commonly used keys include Cox (1996), John et al. (2002), Lind and Brook (1980), and Prescott (1970). Cyanobacterial identification is generally done on the basis of the morphological

characters described in traditional keys of Desikachary (1959) and Gietler (1932). Morphological characters are generally prone to environmental changes. For example, if a nitrogen-fixing blue-green alga such as *Anabaena* is grown in the presence of nitrogen, it loses one of its most critical identification marks, i.e., presence of intrafilament heterocyst.

Nowadays, the use of nucleotide and amino acid sequences has become popular to characterize algae (i.e., Saunders and Druehl, 1992; Tan and Druehl, 1994, 1996; Stache-Crain et al., 1997). Nucleotide sequences of the conserved *rbc*L, the highly conserved nuclear small subunit ribosomal RNA gene (18S rRNA), and the highly variable rDNA internal transcribed spacer (ITS) region are now popular regions being examined in algae research (Assali et al., 1990; Siemer et al., 1998; Bailey and Andersen, 1999; Kawaii et al., 2000; Draisma et al., 2001). Currently, algal phylogeny inferred from 18S rRNA sequence comparisons consists of nine separate lineages including the divisions Chlorophyta, Heterokonta, Haptophyta, Cryptophyta, Dinophyta, Euglenophyta, Chlorarachniophyta, Glaucocystophyta, and Rhodophyta (Ariztia et al., 1991; Bhattacharya and Medlin, 1995; Bhattacharya et al., 1992; Saunders et al., 1995). Detection of individual cells using kingdom-level fluorescently labeled rRNA-targeted oligonucleotide probes has been successfully demonstrated (DeLong et al., 1989). Subsequently, whole-cell hybridization has been shown to be a suitable tool for determinative phylogenetic and environmental studies in microbiology (Amann et al., 1990a, b). Simon et al. (2000) have developed group-specific oligonucleotide probes for the division Chlorophyta, the division Haptophyta, and the class Pelagophyceae (division Heterokonta).

3. Algae Cultivation Process

Cultivation of algae is the key process that determines the economic viability of biofuel production. Although microalgae are adapted to grow in all possible environmental condition, in general for biomass growth, they depend on a sufficient amount of carbon and a sufficient light supply. Microalgae can adapt varied metabolisms: autotrophic (capable of synthesizing its own food from inorganic substances, using light or chemical energy), heterotrophic (uses complex organic food synthesized by autotrophs), mixotrophic (capable of both auto- and heterotrophic mode of nutrition), and photoheterotrophic (heterotrophs using light as the source of energy). For example, *Chlorella vulgaris, Haematococcus pluvialis, Arthrospira* and *Spirulina platensis* can grow under photoautotrophic, heterotrophic, as well as under mixotrophic conditions. Other strains such as *Selenastrum capricornutum* and *Scenedesmus acutus* can grow either photoautotrophically, photoheterotrophically, or heterotrophically.

Historically there are two methods for algal cultivation: (1) closed (outdoor and indoor) and (2) open photobioreactors. With recent developments, several

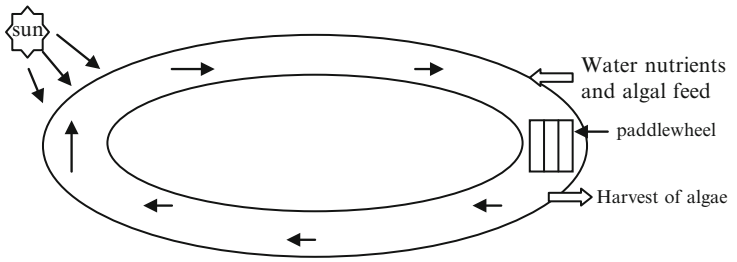

Figure 1. A raceaway pond.

modifications for these systems have also emerged such as hybrid (combined open and closed) cultivation, heterotrophic cultivation (without light), and integrated biofixation systems. In addition, offshore cultivation, aquaculture, and ethanol sweating have also emerged with the growth of industry. In this chapter, we include three systems of algal cultivation: (1) open pond system, (2) closed photobioreactor, and (3) hybrid system, which represent the basics of almost all the variants of open and closed cultivation systems.

3.1. OPEN POND SYSTEM

Open pond systems are the oldest of all methods for the cultivation of algae. These are also the simplest of all the systems. They can be categorized into natural waters (lakes, lagoons, ponds) and artificial ponds or containers. The most commonly used systems include shallow big ponds, tanks, circular ponds, and raceway ponds (Fig. 1). In raceway ponds, the algae, water, and nutrients circulate around a racetrack. With the help of paddle wheels, a continuous flow is maintained, which keeps algae suspended in the water. The ponds are usually kept shallow because the algae need to be exposed to sunlight, and sunlight can only penetrate the pond water to a limited depth. The ponds are operated in a continuous manner, with CO_2 and nutrients being constantly fed to the ponds, while algae-containing water is removed at the other end. These ponds usually operate at water depths of 15–20 cm as at these depths biomass concentrations of 1 g dry weight/L and productivities of 60–100 mg/L/day can be obtained (Pulz, 2001).

One of the major advantages of open ponds is that they are easier to construct and operate than most closed systems, resulting in low production and operating costs. Large ponds have the largest production capacities relative to other systems of comparable cost. Also, open pond cultivation can exploit unusual conditions that suit only specific algae. For instance, *Spirulina sp.* thrives in water with a high concentration of sodium bicarbonate, and *Dunaliella salina* grows in extremely salty water. Economically open pond system of biomass production is ten times less costly in comparison to photobioreactors (Sheehan et al., 1998).

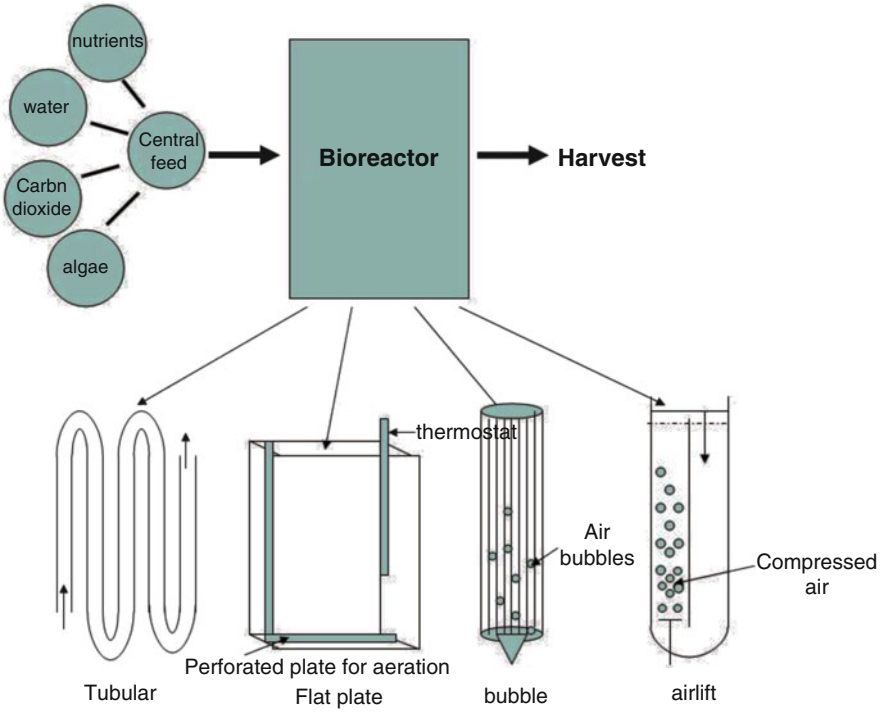

Figure 2. Different types of closed photobioreactors.

However, some of the major disadvantages of this system include poor light utilization by the cells, evaporative losses, diffusion of carbon dioxide into the atmosphere, and a large requirement of land. Bad weather can arrest algae growth. The water in which the algae grow also has to be kept at a certain temperature, which can be difficult to maintain. Moreover, since the pond is open, it is also open for growth of predators and other unwanted species. Contamination from strains of bacteria or other outside organisms often results in undesirable species taking over the desired algae growing in the pond. Thus, the commercial use of this kind of system is as of today limited to the growth of highly robust algae, specifically those that require very specific conditions such as *Spirulina* and *Dunaliella salina* as mentioned above.

3.2. CLOSED PHOTOBIOREACTORS

Closed photobioreactors (Fig. 2) refer to systems closed to the environment having no direct exchange of gases and contaminants with the environment (Tredici, 1999). A photobioreactor can be described as an enclosed (Pulz, 2001)

illuminated culture vessel designed for controlled biomass production of phototrophic liquid cell suspension cultures. Despite their costs, the closed photobioreactors offer advantages such as saving water, energy, and chemicals. They permit essentially single species culture of microalgae for prolonged durations (Schenk et al., 2008).

Closed photobioreactors generally come in the following configurations: (1) tubular systems (glass, plastic, bags), (2) flattened, plate-type systems, and (3) bubble and airlift photobioreactors.

A detailed description of all these different types of photobioreactors as well as their modifications can be found in a review on photobioreactors by Dasgupta et al. (2010). Of all these, the most common type of photobioreactor is tubular and consists of an array of straight transparent tubes made usually of plastic or glass. However, the diameter is generally not more than 100 cm because then sunlight becomes a limiting factor (Khan et al., 2009).

3.3. HYBRID SYSTEM

In hybrid systems, both closed and open systems are used to their respective advantages to give better results. The problem with open ponds is largely contamination. So in hybrid systems, first photobioreactors are used to grow the required species in bulk, and then this is transferred to open ponds in such a large inoculum that unwanted species are not able to establish themselves. This system has been demonstrated by several commercial companies such as Aquasearch (Hawaii, USA) who successfully cultivated *Haematococcus pluvialis* for the production of astaxanthin. Here they first grow *Haematococcus* in bioreactors in nutrient-sufficient conditions and then transfer to open ponds under nutrient-limited conditions to induce astaxanthin production (Schenk et al., 2008a, b). Recently Green Star Products (Montana) have announced the use of the Hybrid Algae Production System (HASP), a combination of closed photobioreactor and open pond system to control the cost and accelerate the growth of algae.

4. Increasing the Lipid Content of Microalgae

The yield of biodiesel from microalgae not only depends on the biomass but also on the oil content in the biomass. That is to say that that an algal strain with a very high growth rate but very low oil content, say 10%, may not suit the needs of the biofuel industry as well as a strain with low growth rate and 40% oil content. Thus, apart from a huge biomass, it is also important to manipulate its oil content. It has been found that in general lipid content is inversely proportional to the stress applied. This happens primarily because nitrogen limitation leads to protein deficiency and the excess carbon from photosynthesis is channeled to storage molecules such as triacyl glycerol (Scott et al., 2010). Another mode of increasing

lipid content is genetic manipulation of the metabolic pathways. Thus, increase in lipid content can be studied under two heads: (a) biosynthetic control and (b) genetic manipulation.

4.1. BIOSYNTHETIC CONTROL

Microalgae can be easily manipulated to produce larger quantities of lipids by altering the nutrient conditions. Several studies have shown that when carbon and light sources are in excess and if one can limit one major nutrient such as phosphorous or nitrogen, the storage of carbon occurs in the form of lipid in place of starch. This enhances the lipid content significantly, which can be translated into biodiesel and is also cost-effective (Mandal and Mallick, 2011; Chisti, 2007; Dismukes et al., 2008). For example, *Chlorella vulgaris* when grown under nutrient-efficient conditions has the oil content up to 14–30% of dry weight, but when nutrient-deficient conditions are applied, the oil content can be raised to the level of 70% (Rodolfi et al., 2009). Similarly *Scenedesmus obliquus* under nitrogen limitation is found to have an enhanced lipid content of 43% as against 12.7% under nitrogen-sufficient conditions (Mandal and Mallick, 2011). Apart from nitrogen limitation, phosphorous limitation was also found to increase the lipid content of *Scenedesmus obliquus* to 29.5% (Mandal and Mallick, 2011). This approach seems to stimulate the breakdown of the phospholipids in the cell membrane into neutral lipids to obtain phosphate, resulting in an accumulation of neutral lipids in the cell (Beer et al., 2009).

While lipid content is increased in the case of nutrient limitation, the growth or biomass production is greatly hampered. To overcome this problem, scientists have come up with the development of two stage bioreactors. Here first of all, the cells are grown to a high density under nutrient-sufficient conditions, and then they are transferred to a nitrogen-deficient medium where lipid content is allowed to increase prior to harvesting of the cells. This strategy has been successfully tested by Rodolfi et al. (2009).

Not only nutrient limitation sometimes nutrient addition also favors the increase of lipid content. For example, Liu et al. (2008) have found that addition of iron (1.2×10^{-5} mol/L) to the growth medium of *Chlorella vulgaris* leads to the accumulation of lipids up to 56.6% dry cell weight.

4.2. METABOLIC ENGINEERING

Although there are only a few algal models which have a routine and established genetic manipulation protocol (e.g., *Chlamydomonas reinhardtii, Volvox carteri,* and *Phaeodactylum tricornutum*), the interest in algal fuels and the knowledge of the genome sequences of several other microalga is surely leading to the development of new model systems. With the advancements in the genetic manipulation

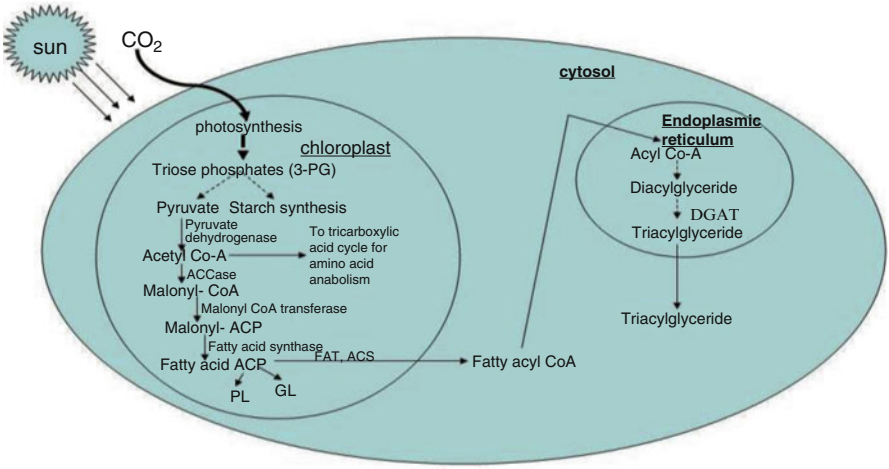

Figure 3. Schematic diagram showing the most direct route of triacylglycerol synthesis from carbon dioxide. Only the major steps are mentioned. Precursor fatty acids are synthesized *de novo* in the chloroplasts using carbon dioxide fixed during photosynthesis. Free fatty acids are then transported to the ER (endoplasmic reticulum) where they come out in the cytosol as oil bodies (triacylglycerides). *PL* phospholipid, *GL* glycolipid, *FAT* fatty acid thioesterases, *ACS* acyl-CoA synthetases, *DGAT* diacylglycerol acyltransferase.

strategies, the possibilities of enhancing lipid content by manipulating the pathways are immense. Several research attempts at genetically modifying *Chlamydomonas reinhardtii* strains have paid rich dividend, and nonhomologous to homologous recombination ratios of 100:1 have been reported (Zorin et al., 2009). Another significant advance in algal genetics is the development of improved gene silencing strategies in *C. reinhardtii*. Bohr (2009) and Zhao et al. (2009) have reported amiRNA (artificial microRNA) for gene knockdown. System level technologies including genomics, transcriptomics, proteomics, and metabolomics are slowly and steadily unraveling the intricacies of the metabolic pathways involved in lipid generation. Figure 3 provides a schematic diagram of the biochemical pathways involved in lipid biosynthesis.

A first look at the pathway to consider an enzyme for overexpression so as to increase the lipid content clearly suggests acetyl-CoA carboxylase (ACCase). As expected, it was in fact one of the first enzymes to be targeted for enhanced lipid production. Contrary to expectations, its overexpression resulted in little success. Dunahay et al. (1995) overexpressed native ACCase in the diatom *C. cryptica* and found that despite a two- or threefold increase in ACCase activity, no increased lipid production could be observed. ACCase from *A. thaliana* has been overexpressed in *B. napus* and *Solanum tuberosum* (potato) (Klaus et al., 2004; Roesler et al., 1997). Overexpression of ACCase in the oleaginous seeds of *B. napus* resulted in a minor increase in seed lipid content of about 6% (384 and

408 mg/g dry weight for wild-type [WT] and transgenic ACCase rapeseed lines, respectively). Courchesne et al. (2009) suggested that unsuccessful accumulation of lipids even after overexpression of ACCase could be due to development of some "secondary bottleneck" that appears after the primary bottleneck (ACCase) is removed.

The next step of the pathway involves the synthesis of fatty acyl ACPs (acyl carrier proteins) from malonyl-CoA by a multi-subunit fatty acid synthase. An attempt to increase expression of 3-ketoacyl-acyl carrier protein synthase III (KASIII) was not successful in increasing lipid production. KASIII from spinach (*Spinacia oleracea*) or *Cuphea hookeriana* was expressed in tobacco (*Nicotiana tabacum*), *A. thaliana*, and *B. napus*, resulting in either no change or reduced seed oil content (Dehesh et al., 2001).

Thus, it seems that increasing the expression of genes involved in lipid biosynthesis does not result in increased fatty acid. Thus, another approach was taken which involved release of the feedback regulation of the pathway. Davis et al. (2000) overexpressed thioesterase which hydrolyzes fatty acyl ACP, thus preventing a saturation signal to the pathway. This resulted in increased fatty acid in *E coli*. Another complementary strategy to enhance lipid production is to decrease lipid catabolism. Kaczmarzyk and Fulda (2010) recently developed a knockout of acyl-CoA synthase in *Synechocystis* and reported increased fatty acid content inside the cell as well as in the media. Another common target for gene manipulation has been DGAT (diglyceride acyltransferase). In both *Arabidopsis* and yeast, 200- to 600-fold increase in DGAT activity has resulted in 3- to 9-fold increase in triacylglycerols (TAGs).

If specifically microalgae are concerned, most of the genetic manipulations are done in *C. reinhardtii*. Moellering and Benning (2010) used N depletion experiments in combination with RNAi suppression to assess changes in the lipid and protein composition in *C. reinhardtii*. They identified a "major lipid droplet protein" (MLDP), which was highly abundant in lipid bodies. RNAi lines of *C reinhardtii* with a 55–60% reduction of MLDP transcript produced 40% larger lipid droplets. Another interesting result is seen in Wang et al. (2009) who have successfully measured increase in the abundance of TAG in *C. reinhardtii* starchless mutant (sta6) deficient in ADP-glucose pyrophosphorylase.

In blue-green algae, attempts to increase lipid production involve those of Deng and Coleman (1999), who expressed pyruvate decarboxylase and alcohol dehydrogenase in cyanobacteria to produce small amounts of ethanol, and that of Kaczmarzyk and Fulda (2010) who generated an acyl-CoA synthase knockout of *Synechocystis*. A very recent work published in PNAS shows a successful strategy to overproduce and secrete fatty acids in genetically engineered *Synechocystis* sp. PCC6803 with manipulation of the fatty acid metabolic pathway and deletion of S-layer proteins, a protective barrier for cyanobacteria cells. The production efficiency can be up to 133 ± 12 mg/L/day at a cell density of 0.23 g of dry weight per liter (Liu et al., 2010).

5. Future Perspectives

The potential of microalgae for use as biodiesel is immense and undoubted. However, it is still some way away from being commercially viable. We have to understand the environmental impacts and the effects on the energy balance and global warming when growing algae on a real commercial scale. Whatever data we have on the economic viability of algal fuels is all hypothetical, and thus, attempts must be made to realize this viability in a realistic experimental condition. Further, we have to understand the biology of algae much better ever had to fully exploit genetic and metabolic engineering. Better methods need to be developed for the genetic manipulation of eukaryotic algae. More and more data on the genome and proteins needs to be generated. Breakthroughs are coming, and the level of interest the present researchers all over the world will undoubtedly provide advances in this area in the coming years. Nonetheless, it will be the integration of biology and engineering that will pave the way for the future of algal biodiesel.

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R. Gordon and J. Seckbach (eds.), *The Science of Algal Fuels*,
Cellular Origin, Life in Extreme Habitats and Astrobiology, 83–100
DOI 10.1007/978-94-007-5110-1_5, © Springer Science+Business Media Dordrecht 2012

INTEGRATED APPROACH TO ALGAE PRODUCTION FOR BIOFUEL UTILIZING ROBUST ALGAL SPECIES

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1. Introduction

Algae as biofuel feedstock provide economically and environmentally beneficial option as follows: (a) algae do not compete with food and water resources; (b) algae grow significantly faster than land crops used for biodiesel and are reported to produce up to 300 times more oil than traditional crops on an area basis; (c) they can treat industrial, municipal, and agricultural wastewaters; (d) they are carbon-neutral and can capture carbon dioxide; (e) low-temperature fuel properties and energy density of algae fuel make it suitable as jet fuel; (f) they ensure a continuous supply; and (g) they can provide valuable by-products like protein-rich feed for farm animals, organic fertilizer, and feedstock for producing biogas.

The algae biomass research community acknowledges that integration of algae biomass production with fuel and waste treatment would be a cost-effective approach. However, algae-oil production at commercial scale is not yet a reality due to the challenges such as the availability of oleaginous (capable of producing oil) algae strains that could be massively cultured to utilize various waste streams as nutrients source. Over 40,000 algal species have been already identified (Hu et al., 2008); however, the list of oleaginous strains is only starting to emerge. To begin with, the Algae Species Program (ASP) sponsored by US Department of Energy (DoE) had identified 300 algae strains suitable for oil production out of the 3,000 collected from different regions in the United States (Sheehan et al., 1998). These strains have been maintained in repositories. Many of the algal strains in algal repository collections (such as the Culture Collection of Algae at the University of Texas, UTEX) have been cultivated for several decades, and as per DoE (2010) it is quite possible that these strains may have lost part of their original wild-type properties necessary for mass culture; for that reason, there is need of isolating novel, native strains directly from unique environments to obtain versatile and robust strains.

The goal of this paper is to look at the concept of algae species *robustness* in context of algae species selection for biodiesel production. Several aspects of species robustness have to be examined: how, for example, does the abundance of a dominating species or algae assemblages can affect many other species co-surviving in the same media; how it can outcompete non/low-lipid wild algae;

how a robust algal strain should respond to changing environmental conditions; and how high-end oleaginous algae species perform in low-quality wastewater streams (municipal, industrial, agricultural: dairy and piggery farms).

2. Robustness Concept as Applied to Algae Biofuel Research

2.1. ROBUSTNESS CHARACTERISTICS

Various desirable characteristics of algae for culturing at massive scales have been identified (Borowitzka, 1992; Chisti, 2007; Hu et al., 2008; Schenk et al., 2008; Griffiths and Harrison, 2009; DoE, 2010) as follows: potential to synthesize and accumulate large quantities of neutral lipids/oil (20–50% DCW); rapid growth rate (e.g., 1–3 doublings per day); large cell size (colonial or filamentous morphology); growth in extreme environments to thrive in saline/brackish water/coastal seawater for which there are few competing demands and tolerate marginal lands (e.g., desert, arid- and semi-arid lands) that are not suitable for conventional agriculture; wide tolerance of environmental conditions; utilize growth nutrients such as nitrogen and phosphorus from a variety of wastewater sources (e.g., agricultural runoff, concentrated animal feed operations, and industrial and municipal wastewaters) providing the additional benefit of wastewater bioremediation; tolerance of contaminants; CO_2 tolerance and uptake to recover carbon dioxide from flue gases emitted from fossil fuel-fired power plants and other; tolerance of shear force; no excretion of auto-inhibitors; high product content to produce value-added coproducts or by-products (e.g., biopolymers, proteins, polysaccharides, pigments, animal feed, fertilizer, and H_2); and grow in suitable culture vessels (photo-bioreactors) throughout the year with annual biomass productivity, on an area basis, exceeding that of terrestrial plants by approximately tenfold.

The important point to note is that robustness is achieved due to combinations of these desirable features. A single algal species is unlikely to excel in all categories; hence, prioritization of desirable features is required (Griffiths and Harrison, 2009). The two major criteria sought in algal culture for mass production are high "algal growth rate" resulting into increased biomass production and "lipid productivity." These points can serve as a foundation for defining first broad criterion of algae species robustness as follows: "an algal species or assemblage that can rapidly grow to produce biomass and accumulate significant amounts of lipid while aggressively out-competing a diverse array of other competing species when grown in cost effective systems."

Studies have been generally using the term "robust" to indicate massive growth of certain algal strains or to indicate high lipid production as in algae biofuel research (e.g., Rodolfi et al., 2009). This term has also been used to indicate the massive growth of algae even leading to blooms. Robustness has been used to indicate algal "biomass–nutrient" relationships as demonstrated in many cross-sectional analyses of lakes and reservoirs (e.g., Smith, 2003). However, it is

important to understand what robustness of a strain means in terms of algae biofuel. In this case, the robustness would be based on "biomass–lipid" relationships in general and "biomass–lipid–nutrients" relationships for integrated systems. It has been proven that in algae, the lipid content may be enhanced by nutrient stress or depletion especially nitrogen (Roessler, 1988), which means combining the algae culturing with nutrients recovery would be a difficult goal to achieve because in the latter case the objective would be to recover maximum nutrients. The algal growth rate is negatively affected by increase in lipid contents under nutrient limitations (Gouveia et al., 2009). To illustrate further, an example of environmental impact on naturally growing algae from a microalgal prospecting experiment follows in the next subsection.

2.1.1. Algal Robustness at Community Level and Lipid Production

Table 1 presents lipid content in algae assemblages including the respective predominant algal species and the suitable media. A comparison of lipid percentages found in different classes of algae shows the higher amount is bulked up by green algae and diatoms (Borowitzka, 1988; Hu et al., 2008). Specifically under the "normal" and the "stress" culture conditions, the oleaginous green algae or chlorophytes show average total lipid contents (dry cell weight) 25.5 and 45.7%, respectively under the two conditions, oleaginous diatoms 22.7 and 44.6%, respectively, and other oleaginous algae (including chrysophytes, haptophytes, eustigmatophytes, dinophytes, xanthophytes, or rhodophytes) 27.1 and 44.6%, respectively, whereas in cyanobacteria considerable amounts of total lipids have not been found, and the accumulation of neutral lipid triacylglycerols has not been observed in naturally occurring cyanobacteria (Hu et al., 2008). The final culture catalog prepared by the Algae Species Program (Sheehan et al., 1998) showed the collection consisted of predominantly green algae (chlorophytes) and diatoms – specifically out of the algae strains present, 26% were chlorophytes, 60% were diatoms, 8% were chrysophytes, and 6% were eustigmatophytes.

A typical algal assemblage varies from environment to environment depending on biotic and abiotic factors, as is shown in Table 1, algae assemblages vary in different sources of water producing different amounts of lipids or fatty acids. A remarkable unity is evident in the global response of algal biomass to nitrogen and phosphorus availability in lakes and reservoirs, wetlands, streams and rivers, and coastal marine waters; most importantly, the species composition of algal communities inhabiting the water column appears to respond similarly to nutrient loading, whether in lakes, reservoirs, or rivers (Smith, 2003).

Here is an example of environmental impact on naturally growing algae community containing lipids. Algae growing in diverse environments representing different nutrients settings, dairy farms (Fig. 1a, d), nondairy farm (Fig. 1c), and a composting site (Fig. 1b), were analyzed for microalgal prospecting (General Systems Research, 2011). The samples collected from these sites on same day were tested for abundance of (a) algae as marked by chlorophyll (Fig. 1) and (b) algae as marked by both chlorophyll and lipids (Fig. 2). Chlorophyll-containing algae

Table 1. Algae (multispecies) and percentage of lipid/fatty content (where available) as growing/cultured in different media.

Algae community	Lipid/fatty acids	Sources/media	Literature
Winter/spring, dominant diatom, *Melosira* sp.; summer/fall, dominant blue-green alga *Lyngbya* sp., green alga *Spirogyra* sp. Rest *Ulothrix, Microspora*, *Claophora*, and numerous pennate diatoms	0.34 ± 0.14	Patuxent River water (Chesapeake Bay)	Mulbry et al. (2010)
Filamentous diatoms and the green alga, *Enteromorpha* sp., dominated year-round	0.65 ± 0.21	Patapsco River water (Chesapeake Bay)	Mulbry et al. (2010)
Diatom *Melosira* sp., blue-green alga, *Lyngbya* sp., and green alga, *Spirogyra* sp., *Ulothrix, Microspora*, *Claophora*, and numerous pennate diatoms	0.51 ± 0.19	Bush River water (Chesapeake Bay)	Mulbry et al. (2010)
Aphanocapsa, *Asterionella, Navicula, Stephanodiscus, Tabellaria*, other diatoms and green algae	0.87–5.33	Enriched lake water (Champlain)	Dahiya et al. (2009, 2010)
Chlorella, *Micractinium*, and *Actinastrum* genera	4.9–11.3	Municipal wastewater	Woertz et al. (2009a)
Scenedesmus, *Micractinium*, *Chlorella*, and *Actinastrum*	10–29	Dairy manure wastewater	Woertz et al. (2009a)
Dominant filamentous *Rhizoclonium hieroglyphicum*. Other *Microspora willeana Lagerh., Ulothrix zonata* (Weber and Mohr) Kütz, *Rhizoclonium hieroglyphicum* (C.A. Agardh) Kütz *and Oedogonium* sp.	0.6–1.5	Dairy manure wastewater	Mulbry et al. (2008)
Chlorella, *Scenedesmus*, *Micractinium*	NA	Sewage (average load)	Oswald (2003)
Euglena, *Chlamydomonas*, and *Oscillatoria*	NA	Sewage (excessive load)	Oswald (2003)

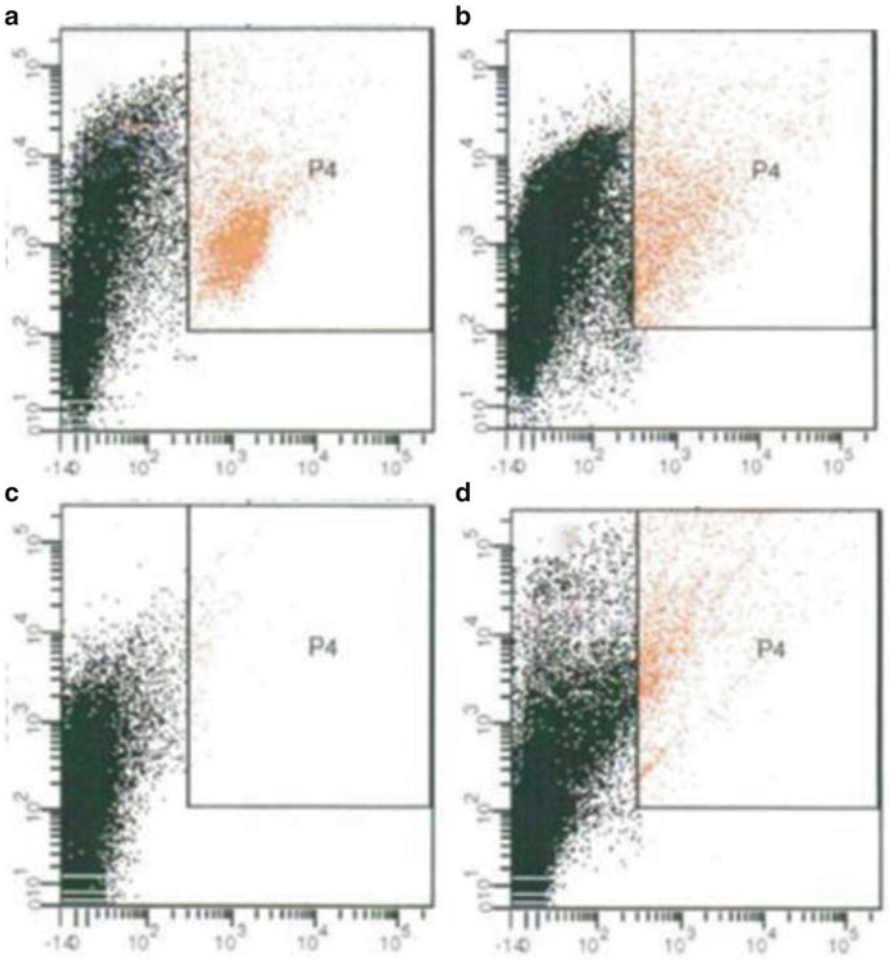

Figure 1. Cytograms showing chlorophyll containing algae (P4) from samples collected from four different sites (Source & © General Systems Research LLC).

populations surviving in these sites showed marked difference between the algal abundance at dairy farm/compost and nondairy farm. The respective samples were set with lipophilic dye and tested for the lipid containing algae populations. From this case, can we infer and generalize that the algae community at higher nutrient-containing sites (dairy farm and compost) is more robust than that at the nondairy farm site, especially for biofuel production, and that the algae cells from the nutrient-rich communities are more robust than the algae at the nondairy farm site? Can we expect the algae cells isolated from high-lipid populations and cultured to produce robust population than the other one? These aspects are important for robust algal strain isolation and selection as discussed in next sections.

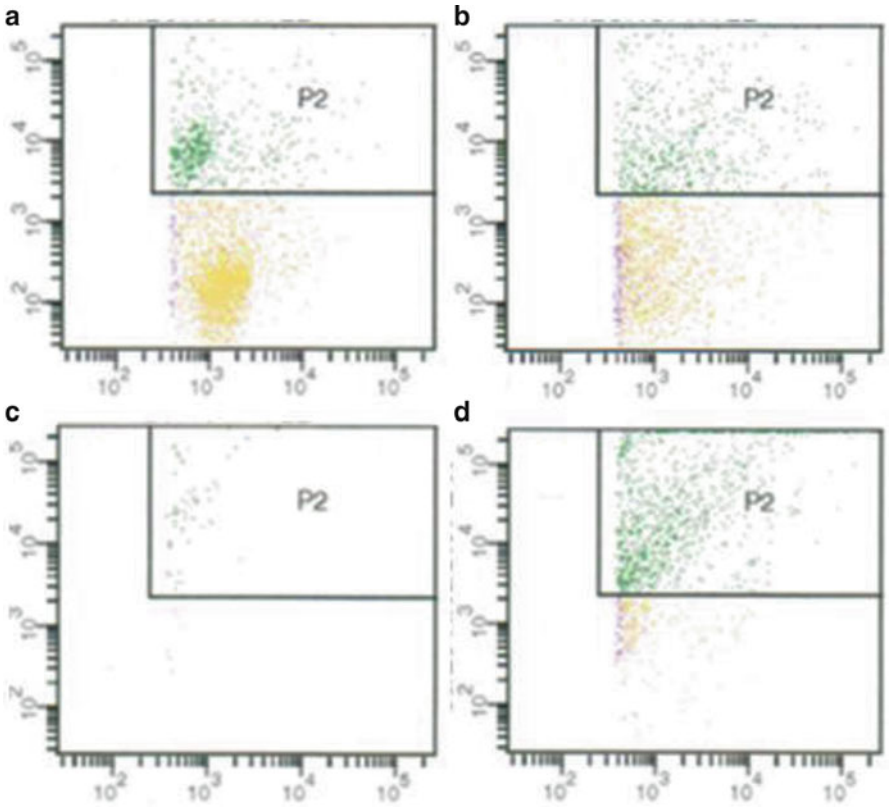

Figure 2. Cytograms showing both chlorophyll and lipid containing algae (P2) from samples collected from four different sites (Source & © General Systems Research LLC).

2.1.2. Algal Robustness at Species Level and High-End Lipid Content

Algal oil production rate at theoretical maximum was found to be 354,000 $L \cdot ha^{-1} \cdot year^{-1}$ (38,000 $gal \cdot ac^{-1} \cdot year^{-1}$) of unrefined oil, while the best cases examined range from 40,700 to 53,200 $L \cdot ha^{-1} \cdot year^{-1}$ (4,350–5,700 $gal \cdot ac^{-1} \cdot year^{-1}$) of unrefined oil (Weyer et al., 2009). The algal biofuel production would require synthesizing and accumulating large quantities of neutral lipids/oil (at least in the range of 20–50% dry cell weight), and the intrinsic ability to produce large quantities of lipid and oil is species/strain-specific rather than genus-specific (Hu et al., 2008).

For culturing algae at massive scales, *high-end* lipid content would be required. Under normal conditions of nutrient regimes, larger than 20% lipid content in algae species has already been found in the algal species growing under laboratory conditions or isolated from naturally growing algae assemblages. This percentage can be increased under stress conditions. The *high-end* oleaginous algae

Table 2. *High-end* oleaginous algae (single species) showing lipid content (percentage dry weight) grown in different media types.

Algae	Lipid %	Media	Literature
Botryococcus braunii	86		Brown et al. (1969) and Wolf (1983)
	80[a]	AM (mod. Chu)	
	>75		Brown et al. (1969)
	63		Banerjee et al. (2002)
	25–75		Metzger and Largeau (2005)
			Chisti (2007)
Schizochytrium sp.	50–77		Chisti (2007)
Nitzschia species:			
N. dissipata	66	AM	Sheehan et al. 1998
N. palea	40	AM	Shifrin and Chisholm (1981)
Boekelovia hooglandii	59	Urea enriched AM	Sheehan et al. (1998)
Monallantus .salina	41–72	AM	Shifrin and Chisholm (1981)
Navicula species:			
N. saprophila	58	AM	Sheehan et al. 1998
N. acceptata	47	AM	Shifrin and Chisholm (1981)
N. pelliculosa	45	AM	Sheehan et al. (1998)
N. pseudotenelloides	42	AM	Sheehan et al. (1998)
Chlorella species:			
C. minutissima	57	AFW	Shifrin and Chisholm (1981)
C. vulgaris	41	AFW	Shifrin and Chisholm (1981)
C. pyrenoidosa	36	AFW	Shifrin and Chisholm (1981)
Dunaliella Sp.	45–55	AM	Sheehan et al. (1998)
Neochloris oleoabundans	35–54	AM	Sheehan et al. (1998)
Monoraphidium sp.	52	AM	Sheehan et al. (1998)
Amphora	51	AM	Sheehan et al. (1998)
Ourococcus	50	AM	Shifrin and Chisholm (1981)
Nannochloris sp.	48	ASW	Shifrin and Chisholm (1981)
	35	ASW ** 45	Sheehan et al. (1998) and Rodolfi et al. (2009)
Nannochloropsis salina	46	ASW	Sheehan et al. (1998)
Scenedesmus	45	AFW	Sheehan et al. (1998)
Scenedesmus obliquus	41	AFW	Shifrin and Chisholm (1981)
Ankistrodesmus	40	AM	Sheehan et al. (1998)
Chaetoceros species:			
C. calcitrans	40	ASW	Rodolfi et al. (2008)
C. muelleri	39	ASW	Sheehan et al. (1998)
Cyclotella cryptica	37	AM	Shifrin and Chisholm (1981)
Amphiprora hyalina	37	AM	Sheehan et al. (1998)
Cylindrotheca sp.	16–37		Chisti (2007)
Pavlova lutheri	36	ASW	Rodolfi et al. (2008)

AM Artificial media, *AFW* Artificial freshwater, *ASW* Artificial seawater f2

[a] Unsaponifiable lipids

** higher content cited by others

species should contain at least 35% lipid content (average of 20–50%). Table 2 presents such species grown in different media types and nutrient conditions including nutrient stress. To illustrate this point, based on the rationale that the oil content of 35% has already been observed in nutrient-sufficient cultures of some species, for mass scale algae biomass and oil production operations in Hawaii, Huntley and Redalje (2007) chose target oil content of 35% even though higher values have been attained.

Many algal species show prominent biomass–lipid relationships. Analysis of microalgal lipid content, biomass productivity, and their combination to yield lipid productivity in 55 species of microalgae (including 17 Chlorophyta, 1 Bacillariophyta, and 5 Cyanobacteria as well as other taxa) showed high lipid productivity ranging from 97 to 160 mg L^{-1} day^{-1} in certain species including *Amphora*, *E. oleoabundans*, *A. falcatus*, *C. sorokiniana*, and *T. suecica* (Griffiths and Harrison, 2009).

The biomass–lipid relationship in the most promising algae strain is not very strong. In terms of lipid production, the green colonial unicellular microalga, *Botryococcus braunii*, is considered to be hydrocarbon-rich alga. It can produce C_{21}–C_{33} odd-numbered n-alkadienes, mono-, tri-, tetra-, and pentaenes, even C_{40} isoprenoid hydrocarbon (Metzger and Largeau 2005; Dayananda et al., 2007), and can reduce nitrate, phosphate, and ammonia–nitrogen concentrations in the waste effluent (seafood processing effluent) by 73, 74, and 79%, respectively (Dumrattana and Tansakul, 2006), and in piggery wastewater effluent ammonia-nitrogen 98% and total nitrogen 43% (An et al., 2003). Very slow rate of growth (Wolf, 1983), with typical doubling time 72 h (Sheehan et al., 1998), makes *B. braunni* unpopular for mass culturing and for integrating this system with wastewater treatment.

In certain species, biomass–lipid–nutrient relationships are strongly demonstrated such as in *Chlorella* species. Since the turn of the twentieth century, *Chlorella* species have been extensively studied for their growth at various nutrients concentrations and/or for food potential, wastewater treatment, etc. (for instance, Eyster et al., 1958; Myers and Graham, 1971; Hills and Nakamura, 1978; Oswald et al., 1953; Oswald, 2003; Huang et al., 1994; Tam and Wong, 1996; Tam et al., 1994), and these species are considered good candidate for biofuel due to their oil accumulation potential. *Chlorella* species are considered robust due to following reasons: (1) they can accumulate lipids up to 50% of their dry weight, thereby making them good candidates for biodiesel production, and as the popular choice because very high biomass production combined with biodiesel production potential so as to produce at the rate of 3,200 GJ/ha/year projected to replace reliance on fossil fuel by 300 EJ/year besides eliminating CO_2 emission by 6.5 Gt/year by the year 2050 (Wang et al., 2008); (2) these species are well-known for use in wastewater treatment (removal of nitrogen and phosphorus) (Oswald et al., 1953; Tam et al., 1994; Gonzalez et al., 1997) as well as for their symbiotic relation with many kinds of naturally occurring bacteria, which is known since 1920s, for example, *Azotobacter chroococcum* (Lipman and Teakle, 1925;

Hills and Nakamura, 1978), *Pseudomonas diminuta*, and *P. vesicularis* (Mouget et al., 1995). *Chlorella pyrenoidosa*, a species commonly found near wastewater treatment ponds, grows vigorously in sterile sewage (Oswald et al., 1953). The *Chlorella* sp. were able to produce more than 3.2 g/m^2-day with lipid contents of about 9% dry weight, while treating dairy farm wastewater and removing upwards of 90% of the total phosphorus and 79% of the total nitrogen contained within the wastewater (Johnson, 2009); (3) addition of an external carbon source induces heterotrophic growth in *Chlorella protothecoides* and increases both growth rate and lipid production, resulting in greater than 50% dry weight lipid (Xu et al., 2006); *Chlorella* has a capacity to produce by-products such as 10–20 times as much protein per unit area per year as cereal crops (Boersma and Barlow, 1975).

2.1.3. Evolutionary Forces to Shape Oil Production by Algae

Can the naturally growing algae assemblages or controlled monocultures be forced toward oil production? Specifically, can the high-end rapidly growing oleaginous strains be evolved to bulk up lipids and compete with the slow-growing hydrocarbon-rich *B. braunii*, or vice versa: can *B. braunii* be evolved to grow rapidly like *Chlorella* species? Importance of these aspects lies in the fact that now geologists and scientists are unanimous about algae being surviving on earth for over 500 million years and that the oil and coal reserves are their direct products. *B. braunii* provides such evidence. The highly resistant nature of the *B. braunii* algae to degradation allows it to be selectively preserved during fossilization, leading to fossil *B. braunii* remains, a major contributor to a number of high oil potential sediments (Simpson et al., 2003).

The evolutionary equilibrium strategies allow the most successful species to follow opportunistic evolutionary pathways (Riley, 1979). The concepts behind evolutionarily stable strategies (ESS) (Maynard Smith and Price, 1973) have been demonstrated by Geritz et al. (1998), who used game theory principles to predict adaptive evolution. Klausmeier and Litchman (2001) modeled these strategies for pelagic and benthic algal systems subject to the light and nutrient competition so that the motile phytoplankton can form a thin layer under poorly mixed conditions, and under the assumption of a thin layer, competition for light from above and nutrients from below can be thought of as a game, with the depth of the phytoplankton layer as the strategy. In this case, the ESS is a depth that prevents growth in the rest of the water column, which is determined in this model. The environmental conditions to force algal evolution to oil production traits require light and dark cycles intertwined with low- and high-nutrient conditions typically found in pelagic ecosystems; however, 50% light and dark regimes tested to evolving the naturally growing lake algae toward producing lipids did not produce immediate results (Dahiya et al., 2010); this is a work in progress.

The effect of light–dark (L–D) cycles on algal photosynthetic activity has been extensively researched for mass culturing of algae for biodiesel production. For instance, Janssen et al. (2001) and Barbosa (2003) found that the light/dark

cycles affect the biomass yield and specific growth rate of algae. Little information is available as to how much time the algal cells should be in the light and dark, and as such there is no consensus on what is an appropriate light/dark cycle (Kommareddy and Anderson, 2004). However, based on the flashing light effect (Laws et al., 1983) tested in ASP studies, the optimized transfer of cells from dark zones to bright zones and vice versa has been tried in the closed bioreactors in order to harness the flashing light effect by inducing low light/high light cycle (Meiser et al., 2004). The frequencies of these cycles is kept at 10 Hz or faster with the dark period lasting up to ten times longer than light period (Janssen et al., 2001). We have a long way to go before the robust oleaginous algae strains or assemblages are evolved for culturing in either open or closed systems. Next section deals with the challenges with integrated systems.

3. Robust Algae and Integrated System Challenges and Solutions

"Integrated systems" are referred to as treatment plants (e.g., sewage) integrated with communities in such a way that maximum benefits are attained by population at least cost without compromising health or welfare (Oswald, 2003). The industries and dairy farms are required to meet regulatory standards for handling and recycling of nutrients including nitrogen and phosphorus, but as per United States Department of Agriculture (USDA) report, the commonly used plants, the anaerobic digesters, for the treatment of wastewaters are effective only in treating the biochemical oxygen demand (BOD) but not nutrient removal (Liebrand and Ling, 2009). The wastewater coming out of biodigester normally needs to be further treated before it can be safely discharged into the water streams. Algae can efficiently utilize the wastewater effluents and recover nitrogen, phosphorus, potassium, heavy metals, and other organic compounds (Oswald et al., 1953; Oswald, 1990; Wilkie and Mulbry, 2002; Kebede-Westhead et al., 2003; Pizarro et al., 2006; Mulbry et al., 2008). This task calls for robust algae strains. Selecting indigenous algae with intrinsic characteristics amenable to bioresource production and waste mitigation – phycoprospecting – is the most sustainable path forward for widespread algae-based bioresource development (Wilkie et al., 2011).

Attempts in growing algal monocultures in high-rate algal ponds for over 3 months have not succeeded primarily due to contamination by wild algae and grazing by zooplankton (Sheehan et al., 1998). Algae-based treatment of wastewater has been shown to be 40% more cost-effective than the best conventional means (Downing et al., 2002), but availability of fast growing oleaginous algae species for treating waste is limited. Integrated algae-oil production and waste treatment (wastewater and/or CO_2) has been identified a cost-effective approach (Sheehan et al., 1998; Lundquist, 2008; DoE, 2010).

Whether monoculture or polyculture, the algae will have to potentially grow symbiotically with other organisms present in the wastewater. An instance of algae assemblage includes *Sargassum natans*, *Ascophyllum rodosurm*, and *Flucus*

vesiculosus growing with the bacteria *Bacillus subtilis* and *Bacillus licheniformis* (Mulligan and Gibbs, 2003), and an instance of algal monoculture is *Chlorella sorokiniana* grown with *Rhodobacter sphaeroides* (Ogbonna et al., 2000). Algae assemblages commonly form in wastewaters. In waste treatment ponds, there is minimum control over the algae species that grow, but some limits could be imposed through pond operations, such as residence time, depth, and mixing, as very often the species of *Chlorella*, *Scenedesmus*, and *Micractinium* are commonly found in these ponds, and other species including *Euglena*, *Chlamydomonas* and *Oscillatoria* may occur in ponds with excessive loadings or long residence times (Oswald, 2003).

3.1. NUTRIENT RECOVERY CORRELATED WITH LIPID CONTENT

"Advanced Integrated Wastewater Pond Systems" designed by Oswald's group have been efficiently used for municipal sewage treatment that could remove over 90% of total nitrogen in the wastewater stream (Oswald, 1990). Natural or wastewater-grown algal assemblage or polyculture is low in lipid contents compared to monocultures (Tables 1 and 2), although it is highly efficient in recovering nutrients. The algal assemblage used in algal turf systems (ATS) for treating dairy and swine wastewater had fatty acid contents ranging from 0.6 to 1.5% of dry weight that recovered over 95% of the nitrogen and phosphorous from agricultural manure wastewater (Mulbry et al., 2008). The fatty acid (FA) content of ATS harvested material from three Chesapeake Bay rivers was 0.3–0.6% of dry weight (Mulbry et al., 2010). Woertz et al. (2009a) reported lipid productivities of 9.7 mg/L/day (air sparged) to 24 mg/L/day (CO_2 sparged), and over 99% of both the ammonium and orthophosphate removed in municipal wastewater, whereas 2.8 g/m^2 per day of lipid productivity in dairy wastewater. Currently, the use of high-end oleaginous species in treatment of wastewater is limited, and lots of research is required in that direction.

3.2. CARBON-DIOXIDE UTILIZATION COMBINED WITH NUTRIENT RECOVERY FOR WASTEWATER TREATMENT

CO_2 addition can also impact lipid production. Gouveia et al. (2009) showed a slight increase in lipid contents when CO_2 was added to the algae culture media. The problem with wastewater treatment ponds is that they are limited in providing carbon for algal growth. The heterotrophic oxidation of organic material by bacteria is one way that CO_2 is made available to algae (Oswald et al., 1953). Addition of CO_2 has been shown to increase algae biomass (Benemann, 2003; Woertz et al., 2009a). High-rate algae ponds fed clarified domestic wastewater and CO_2-rich flue gas are expected to remove nutrients to concentrations similar to those achieved in mechanical treatment technologies, such as activated sludge; however, the

energy intensity of wastewater treatment with CO_2-supplemented high-rate ponds (HRPs) would be less than that of mechanical treatments (Woertz et al., 2009b). This approach would require robust oleaginous strains capable of tolerating high CO_2 concentrations. Some studies have shown that *Cyanidium caldarium* can tolerate 100% CO_2 concentration (Seckbach et al., 1971), *Scenedesmus* sp. 80%, *Chlorella* sp. 40%, and *Eudorina* spp. 20% CO_2 concentrations (Hanagata et al., 1992).

Many studies have utilized either wastewater or CO_2 from the respective point sources as nutrient streams for growing algae biomass. Very few studies have actually taken advantage of both and actually combined CO_2 and wastewater from point sources for algae production. For instance, Yun et al. (1997) cultured *Chlorella vulgaris* (inoculant prepared in 5% (v/v) CO_2) in steel manufacturing wastewater effluent under high concentrations of 15% (v/v) CO_2 supply captured from a power plant and removed 0.92 g. m^{-3} h^{-1} ammonia and 26 g m^{-3} h^{-1} CO_2. One of the possible reasons behind lack of combining CO_2 and wastewater supplies from point sources in culturing algae is the distance between the locations of respective point sources that may not be found close enough to each other, and as such, if an algae production facility is utilizing CO_2/flue gas from a point source, hauling wastewater from a distant location would increase the costs of biomass production. This area of research needs special attention.

3.3. VALUED BY-PRODUCTS

It is estimated that besides integrating algae biomass production for oil with waste treatment, the valued by-products especially from the algae cake leftover from oil extraction, such as feedstock for biogas, organic fertilizer, and proteinaceous feed for animals, can offset the cost of algae biomass production.

The value of algae as food was explored as early as 1950s by Burlew (1953). Later on, Dugan et al. (1972) demonstrated the concept by raising baby chickens to adults on 20% algae-fortified feed, and also he grew the algae used on pasteurized chicken manure. The antibiotic chlorellin extracted from *Chlorella* during World War II marked the start of algae-based pharmaceutical and nutraceutical industry that led to the Japanese *Chlorella* production facilities during 1960s (Oswald, 2003), further leading to current production of *Chlorella*, *Spirulina*, *Dunaliella*, and *Haematococcus* at commercial scales. Fresh dewatered biomass could potentially be mixed in with animal feed (and substituted on a protein basis for soybeans) (Wilkie and Mulbry, 2002). Studies have also focused on use of the dried biomass as an organic fertilizer and demonstrated that it was equivalent to a commercial organic fertilizer with respect to plant mass and nutrient content (Mulbry et al., 2005). Further research is required for utilization of leftover high-end oleaginous algae for fertilizer, animal feed, and possible feedstock for biogas production for systems based on single species or algal assemblages.

To achieve cost-effective algae biomass production for oil research is needed to isolate and test the high-end oleaginous algae species. The robustness of species

is important as the starting point in algae species selection that will yield significant improvements in biomass productivity besides offsetting the production costs when integrated with waste treatment systems and valued by-product production. The continuing efforts to search an ideal robust oleaginous algae strain should be combined with other aspects of algae production.

4. Acknowledgments

Funding provided by Environmental Protection Agency (EPA), Department of Energy (via VT Sustainable Jobs Fund), NASA-EPSCoR (VT), and NSF-EPSCoR (VT) to General Systems Research LLC is greatly appreciated.

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R. Gordon and J. Seckbach (eds.), *The Science of Algal Fuels*,
Cellular Origin, Life in Extreme Habitats and Astrobiology, 101–129
DOI 10.1007/978-94-007-5110-1_6, © Springer Science+Business Media Dordrecht 2012

BIOLOGICAL CONSTRAINTS ON THE PRODUCTION OF MICROALGAL-BASED BIOFUELS

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1. Introduction

Concerns over long-term fossil fuel supply, global warming, and the global human population growth (predicted to grow to between 9 and 11 billion by 2050) are extremely potent stimuli to the development of renewable energy supplies. However, biofuels, particularly first-generation plant-crop-derived fuels, have increased pressure on both agriculturally productive areas and water for irrigation. Further intensification of crop production on arable land for biofuels would place unsustainable demands on already limited freshwater supplies. Furthermore, the implications on food production are obvious, for example, it has been estimated that the grain required to produce the bioethanol needed to fill the fuel tank of any large sports utility vehicle would be sufficient to feed 1 person for 1 year (The World Bank, 2007). On the basis of food politics alone, this approach is not realistic. Obviously, alternative strategies are needed if biofuel production is to be sustainable at the large scale. There are clear advantages of using microalgae over "higher" plants; these include their very high growth rates, their capacity to utilize a large fraction of the solar energy (theoretically ~10% of solar energy can be fixed into biomass), and their ability to grow in conditions that are not favorable for terrestrial biomass growth (Carisson et al., 2007). If one thinks of microalgae as sunlight-driven cell factories, which can convert carbon dioxide to biofuels, the attraction of employing microalgae as a third-generation biofuel producer can seem overwhelming.

To avoid competition for potable or irrigation waters, most future large-scale algal production facilities are likely to use of brackish or salt water in man-made, shallow pond systems. However, it may be feasible to utilize some inland water bodies, with previous successful examples including the harvesting of natural blooms of *Spirulina* from Lake Texcoco and other alkaline lakes (Hu, 2004). It is unlikely that productivity levels in unmanaged natural water bodies will be sufficiently high to be commercially viable. Additionally, the environmental and financial costs of trying to regulate natural water bodies make them an unattractive option. Commercially viable systems will be onshore, almost certainly man-made, or heavily managed in the case of shallow natural water bodies.

Large-scale culturing of algae is a relatively recent phenomenon with the first commercial harvesting and culturing facility of the cyanobacterium *Arthrospira*,

commonly called *Spirulina* (used as a health supplement and for its pigment production) being established in Mexico in the early 1970s (Hu, 2004). Because this is a filamentous organism, harvesting can be achieved relatively cheaply and easily. Additionally, as it is an extremophile, growing in highly alkaline waters, it dominates the ponds in which it is cultivated without the need for frequent reinoculations of the production ponds (Benemann, 2003). The largest production facilities that have been established to date have all employed extremophiles, either on the basis of pH or osmotic potential as is the case for β-carotene production from *Dunaliella salina* (Ben-Amotz, 2004). The product value of the pigments or nutraceutical/health foods produced from *Arthrospira* or *D. salina* is sufficiently high to allow relatively low yields to be commercially viable. However, much higher yields, in the region of 100 t ha^{-1} $year^{-1}$, would be required of an alga suitable for biofuel production in an open pond system to ensure economical viability. This is realistic, as yields 60 t ha^{-1} $year^{-1}$ have already been achieved for *Pleurochrysis carterae* and *D. salina* in field-scale production systems (Moheimani and Borowitzka, 2006).

An Internet search on Algal Biofuels will reveal tens of thousands of websites detailing a bewildering array of information, and noninformation, on the topic. There clearly is a great deal of potential, and in theory, algal biofuels could, for example, replace all the petroleum fuel used in the United States; however, this would require 15,000 square miles (40,000 km^2) of algal production ponds working at high efficiency (Day et al., 2012)! Over the past few years, a large number of academic as well as commercial scientific groups, individual researchers/technologists, and companies have started to work in the field. The best known of the companies involved include Aquaflow Bionomics (New Zealand), Aurora Biofuels (USA), Blue Marble Energy (USA), Cellena (USA), Inventure Chemical (USA), Live Fuels (USA), Petro Sun (USA), Sapphire Energy (USA), Seambiotic (Israel), Solazyme (USA), Solena (USA), and Solix Biofuels (USA). However, the algal biofuel "story" that has caught the public attention, because of the huge potential investment and high media profile of those involved, is the commitment of ExxonMobil, working with the biotech company Synthetic Genomics Inc., to spend $600 million in the sector. The numerous companies and large-scale projects funded by national government agencies, via the EU and other international organizations, working on this topic represent a major financial investment and the development, within a 4–5 year time frame, of a new biotechnological sector. They use, or plan to use, a variety of approaches (photoautotrophic – heterotrophic), technologies (open ponds – closed photobioreactors), and biological materials (wild strains of algae – potential of genetically modified organisms, or GMOs). Although some organizations claim to have their process near market, many are clearly indicating that they are still in a relatively early phase of development. The authors are advocates of the potential of microalgae to become a significant component of future transport fuels, but there are many challenges, which need to be addressed before the production of algal biofuels is technologically realistic and cost-effective. This chapter does not aim

to be a route map or a "how to do it" manual; it outlines some key biological constraints that still require addressing and makes some suggestions to the routes forward.

2. Physiological Constraints

Algae are ancient primitive plants that are *de facto* sunlight-driven factories using a combination of photosynthetic pigments and a relevantly simple structure to adapt to prevailing environmental conditions (Falkowski and Raven, 1997). Algae encompass a huge diversity of different taxa, originating from an equally diverse range of ecological niches. For the purposes of this chapter, cyanobacteria, which are prokaryotic, are considered to be algae in addition to autotrophic protists. The eukaryotic microalgae (protists) are categorized according to their pigmentation, life cycle, and basic cellular structure, and in many cases, phylogenetic data confirms their relationships at higher taxonomic levels (Khan et al., 2009). They include the Chlorophyta (green algae), Rhodophyta (red algae), and Heterokontophyta. Furthermore, they include taxa capable of autotrophic, heterotrophic, or both (i.e., mixotrophic) metabolism. In this chapter, only autotrophic growth is discussed, where the key for survival is photosynthesis and algae absorb carbon dioxide (CO_2) in the presence of light to produce carbon-based biomass and excrete oxygen (O_2) as a waste product (Falkowski and Raven, 1997; Govindjee and Zilinskas-Braun, 1974). Their cultivation could be directly coupled with CO_2 sequestration from power-plant flue gas, or growing them on a large scale could contribute to the stripping of this greenhouse gas from the atmosphere. Other nutrients required for growth include nitrogen, phosphorus as well as silicon in the case of diatoms, and in some cases vitamins and other trace nutrients (Lorenz et al., 2005). Microalgae are one of the most promising sources for renewable biomass production; however, work is needed on various aspects of the topic to ensure that any future process is robust and reproducible. Some of the key issues that need further R&D are outlined below.

2.1. PHOTOSYNTHETIC EFFICIENCY

Photosynthesis at its most basic level can be described as the conversion of light energy to chemical energy, leading to the assimilation of carbon. This photon-driven process is at the basis of the food supply for most life on earth and also the source of all fossil fuels. The availability of light links directly to the growth and production performance of a microalgal cell and will depend upon the distance the cell is from the light source, the density of the culture, and the pigment profile (Camacho et al., 2003; Williams and Laurens, 2010). The initial light reaction, which involves photochemical and redox reactions, occurs in milliseconds, and the light-independent reaction, involving enzyme-driven reactions, occurs in seconds

to hours. This means that the maximum rate of photosynthesis is in reality controlled by the concentration of the Calvin cycle enzymes (Sukenik et al., 1987). Camacho et al. (2003) defined this as the quantity of enzyme catalyzing the step that gets saturated at the lowest concentration of activated photosynthesis. According to Williams and Laurens (2010), it is this mismatch in timescales which gives rise to inefficiencies often linked to photosynthesis, and that is the major problem in maximizing algal yields from mass culture systems.

Photosynthetically active radiation (PAR) refers to the amount of total incoming radiation (approximately 45%), which can potentially be utilized for photosynthesis; from this, the theoretical maximum yield of photosynthetic efficiency (PE) has been calculated to be 13% (Bolton and Hall, 1991). The primary pigment involved in photosynthesis is chlorophyll *a*; it has a low absorption range resulting in only 30–40% of the PAR being captured (Williams and Laurens, 2010). Plants, including algae, use additional pigments, for example, β-carotene, to capture a greater percentage of the available energy. The concentration of these accessory pigments can be increased in algae (photo-acclimation), but this is a slow adaptive process when compared to the timescales involved in the photosynthetic pathways (Prezelin, 1981; Fasham and Platt, 1983; Camacho et al., 2003). Excessive levels of irradiance will result in photoinhibition, photooxidative damage, and potentially death of the cell, but when compared with photosynthesis, this is a relatively slow process occurring over a period of hours (Baroli and Melis, 1996; Williams and Laurens, 2010). Photoinhibition has also been shown to be coupled to an increase in protein synthesis (Raven and Samuelson, 1986). This further reduces the PE to approximately 2–6%, or as low as 1–2% for most terrestrial plants (Vasudevan and Briggs, 2008). However, for some microalgae, the PE has been calculated to be as high as 20% (Tamiya, 1957; Minowa et al., 1995; Acien Fernandez et al., 1998). This is significantly higher than the theoretical maximum (13%) in green plants (Bolton and Hall, 1991) and has been attributed to the simpler structure of unicellular algae (Brennan and Owende, 2010). However, we would suggest that realistic levels of PE that may be achievable in algal pond systems are likely to be closer to 5% than the theoretical maximum.

2.2. LIPID PRODUCTION

To date, the focus for biofuel production has mainly been on the triglycerides; these lipids unlike those that form the membranes of cells and their organelles lack a charge and are thus described as neutral lipids. Despite the obvious interest in these lipids and a considerable amount of existing research, there are still many fundamental questions that need to be answered relating to the biosynthesis and regulation of lipid production in microalgae (Hu et al., 2008). When algal cells are actively growing charged lipids, in the form of phospholipids and glycolipids dominant; however, as the culture enters into stationary phase a change in the overall lipid profile occurs with an increase in the concentration of Triacyl glycerols (TAGs),

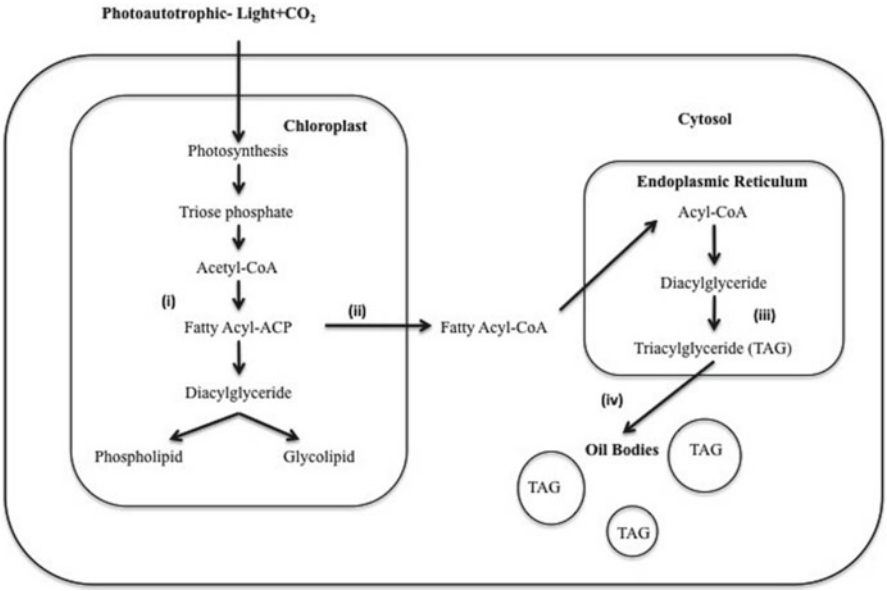

Figure 1. Lipid biosynthetic pathway (Adapted from Scott et al., 2010). Precursors of fatty acids are synthesized in the chloroplast using sunlight and CO_2; these are then exported from the chloroplast and converted in the endoplasmic reticulum into TAGs, which bud off to form oil bodies in the cytosol. Key: (*i*) = acetyl-CoA carboxylase (ACCase) and fatty acid synthase (FAS); (*ii*) = fatty acid thioesterases and acyl-CoA synthetases; (*iii*) = TAG biosynthesis enzymes, including acyl-CoA: diacylglycerol acyltransferase (DGAT); (*iv*) = oil body formation; and (*v*) = ADP-glucose pyrophosphorylase and starch synthase.

which are used as storage products (Hu et al., 2008). It is thought that lipid metabolism occurring within algal cells is very similar to that of terrestrial plants (Fig. 1), but there is limited experimental evidence in support of this, and some broad generalizations have been made by most researchers (Hu et al., 2008).

It is known that fatty acid synthesis occurs in the chloroplast of algal cells with the pathway producing 16 or 18 carbon fatty acids or both (Ohlrogge and Browse, 1995; Guschina and Harwood, 2006; Harwood and Guschina, 2009). These are subsequently used as precursors for the synthesis of chloroplasts and other cellular membranes, plus storage lipids in the form of TAGs. The first step in the pathway is catalyzed by acetyl-CoA carboxylase (ACCase), with the conversion of acetyl-CoA and CO_2 to malonyl CoA in a two-step process (Ohlrogge and Browse, 1995). It is the malonyl CoA that is considered to be the central carbon donor for fatty acid synthesis and is passed onto an acyl carrier protein (ACP), which is involved in all subsequent steps of the pathway (Ohlrogge and Browse, 1995). The end products are produced by four separate condensation reactions to produce saturated 16:0 and 18:0 ACP. Unsaturated fatty acids are produced by the introduction of a double bond by the enzyme stearoyl ACP

desaturase. The enzymes that use the acyl ACPs at the final phase of the synthesis will determine the final composition of fatty acids (Hu et al., 2008).

Currently two ACCase have been purified from two microalgae, and they demonstrate similar structures and size to those found in terrestrial plants (Roessler, 1990a; Livne and Sukenik, 1990). In the diatom *Cyclotella cryptica*, ACCase activity increased two to fourfold in response to silicon deficiency, suggesting that the higher activity may be in part due to covalent modification of the enzyme, but activity was also blocked by protein synthesis inhibitors suggesting the increase in activity could be linked to an upregulation in the enzyme's synthesis (Roessler, 1988; Roessler et al., 1994). The ACCase gene sequence for this diatom also exhibits strong similarity to yeast and animal ACCase in the biotin carboxylase and carboxyltransferase domains and contains a signal peptide at its N-terminus indicating that the enzyme is potentially imported via the endoplasmic reticulum into chloroplast (Hu et al., 2008).

Ratledge (1988) suggested that TAG biosynthesis in algae occurred via the direct glycerol pathway with the fatty acid produced in the chloroplast being transferred from CoA to positions 1 and 2 of glycerol-3-phosphate. The dephosphorylation of the resulting phosphatidic acid (PA) releases a diacylglycerol (DAG) before finally a third fatty acid being transferred to the vacant third position of the DAG. This final step is catalyzed by a diacylglycerol acyltransferase (DGAT), and this enzyme may exhibit a preference for specific acyl molecules giving it a direct role in determining the final acyl composition of the TAG (Hu et al., 2008). The majority of algal TAGs are composed of C14–C18 fatty acids that are saturated or monosaturated (Harwood, 1998; Roessler, 1990b). However, there are exceptions in the form of long-chain PUFAs (>C20) and the partitioning of these fatty acids into TAGs (Iida et al., 1996; Cohen et al., 2000; Bigogno et al., 2002). Kamisaka et al. (1999) proposed that PUFA-rich TAGS were the result of an "acyl shuttle" between diacylglycerol and/or TAG and phospholipids.

2.3. LIPID STORAGE

The majority of microalgae routinely used in most laboratories are model strains. In most cases, they were chosen for their ease of cultivation and not necessarily for their lipid production or their potential for biofuel production (Grossman et al., 2007; Hu et al., 2008). What also appears to be true is that the ability to accumulate lipids is species, or even intraspecies, specific rather than genus specific (Hu et al., 2006). As described by Hu et al. (2008), under various stresses, algae undergo a rapid degradation of the photosynthetic membrane followed by an accumulation of cytosolic TAG-enriched lipid bodies as an energy-storing mechanism. There has been a suggestion by Dahlqvist et al. (2000) of an acyl-CoA-independent mechanism for TAG production in plants and yeast involving a phospholipid: diacylglycerol acyltransferase (PDAT). This enzyme appears to

have a role in the channeling of bilayer polar lipids into the TAG pool (Dahlqvist et al., 2000). Hu et al. (2008) has suggested that if a homologue of the PDAT exists in the algal cell, it could potentially play an important role in the regulation of membrane lipids in response to environmental growth conditions.

TAGs may not only play a role as a carbon and energy store, but possibly act as an electron sink under photooxidative stress. This may occur during periods of high light, or other environmental stress when there is an excess of reactive oxygen species being produced capable of causing inhibition of photosynthesis and general damage to cellular structures and components. The formation of TAGs consumes more energy than that required for the production of carbohydrates or proteins. Furthermore, the production of C18 fatty acids uses twice as much NADPH than is required for the synthesis of a protein or carbohydrate of a similar mass (Hu et al. 2008). This results in a relaxation in the over-reduced electron transport chain under stress conditions. The synthesis of TAGs is also coupled with the secondary carotenoid pathway; the products of this pathway are esterified with the TAGs and sequestered into the cytosolic lipid bodies potentially acting as a "sunscreen" protecting the chloroplast under high light levels (Hu et al., 2008).

Lipid levels for microalgae are quoted in the scientific literature as being in the range of 20–50% and potentially exceeding 80% (Chisti, 2007). These are in the form of different chain lengths of lipids, hydrocarbons, and other complex oils (Banerjee et al., 2002; Metzger and Largeau, 2005; Guschina and Harwood, 2006). This coupled with the high productivity rates quoted for the production of microalgal biomass and the search for new biofuels sources has lead to the renewed interest in algal lipids as a potential source of biodiesel. The conversion of algal TAGs to biodiesel through the chemical process of trans-esterification has already been successfully demonstrated (Belarbi et al., 2000). It has similar properties to petroleum diesel and compares favorably with the international standard EN14214 (Brennan and Owende, 2010). It is nontoxic and contains reduced levels of particulates, carbon monoxide, soot, hydrocarbons, and SOx. However, algae produce higher levels of polyunsaturated fatty acids that tend to decrease the stability of biodiesel, but they also have much lower melting points than monosaturated or saturated lipids (Brennan and Owende, 2010). Therefore, they are still fluid at lower temperatures allowing diesel engines to still be functional, giving algal biodiesel much better cold weather properties than many other bio-feedstocks (Sheehan et al., 1998).

2.4. PRODUCTIVITY

As discussed by Williams and Laurens (2010), the metabolism of these microorganisms will be determined by their surface to volume ratio. This means that their metabolism and growth rates are inversely proportional to their cell diameters. In most cases, as the lipid content increases, other cell components in the form of

proteins and carbohydrates decrease; this is coupled to a reduction in the growth rate (Williams and Laurens, 2010); however, it is not clear how these biochemical groups change as lipid content changes. Nitrogen levels, light intensity, temperature, salinity, CO_2 concentration, and harvesting procedures have all being demonstrated to have an impact on the lipid content of microalgae (Wu and Hsieh, 2008; Chui et al., 2009; Brennan and Owende, 2010). Nitrogen deficiency is often quoted as being necessary to induce the switch to lipid accumulation (Beijerinck, 1904; Spoehr and Milner, 1949; Shifrin and Chisholm, 1981; Cobelas and Lechado, 1989; Roessler, 1990b; Thompson, 1996; Basova, 2005; Merzlyak et al., 2007), but there are few biochemical data available to elucidate what effect nitrogen reduction is having (Roessler, 1990b). It is thought that because storage lipids do not contain nitrogen, as a culture reaches stationary phase and the nitrogen becomes depleted, the synthesis of nitrogen-containing compounds, for example, proteins, is reduced. However, carbon is still assimilated by the cell and is converted into TAGs (Shifrin and Chisholm, 1981; Cobelas and Lechado, 1989; Roessler, 1990b; Thompson, 1996; Basova, 2005; Merzlyak et al., 2007). It has also been observed that during nitrogen limitation, not only do you get an accumulation of storage lipids but that there is a gradual change in the lipid composition from free fatty acids to TAGs (Brennan and Owende, 2010).

Nitrogen is not the only nutrient to play a role in the accumulation of TAGs; the diatom *Cyclotella cryptica* demonstrates increased levels of neutral lipids in response to silicon depletion (Roessler, 1988). Furthermore, phosphorus limitation has been demonstrated to result in increased levels of TAGs in *Monodus subterraneu*s (Khozin-Goldberg and Cohen, 2006), *Phaeodactylum tricornutum*, *Chaetoceros* sp., *Isochrysis galbana*, and *Pavlova lutheri* but decreased lipid content in *Nannochloris atomus* and *Tetraselmis* sp. (Reitan et al., 1994).

Temperature also appears to have a major role to play in fatty acid synthesis; generally, there will be an increase in unsaturated fatty acids with a decrease in temperature, and an increased in saturated fatty acids appears to be coupled with increases in temperatures (Murata et al., 1975; Sato and Murata, 1980; Lynch and Thompson, 1982; Raison, 1986; Renaud et al., 2002). The mechanism for this is unknown, and the overall trend is difficult to establish (Somerville, 1995; Hu et al., 2008). In addition, the effects of light intensity on the composition of lipids produced have been extensively studied, and in general, low light intensities result in mainly membrane polar lipids, whereas high intensities result in an increase in storage TAGs (Spoehr and Milner, 1949; Orcutt and Patterson, 1974; Falkowski and Owens, 1980; Richardson et al., 1983; Post et al., 1985; Sukenik et al., 1987; 1989; Napolitano, 1994; Brown et al., 1996; Khotimchenko and Yakovleva, 2005).

There are clearly a range of interconnected physiological and ecological factors that can be, or have the potential to be, manipulated to enhance productivity. However, it is important for those planning to develop this area that they note that there is not necessarily a correlation between lipid accumulation and biomass productivity. Brennan and Owende (2010) argue that lipid productivity, where both lipid concentration within cells and the biomass produced by these cells are

considered, provides a better measure of the potential biofuel production from a particular microalga.

3. Molecular Constraints

It is increasingly practicable to generate complete genome sequences for eukaryotic algae, and the first eight that became publically accessible included the red alga *Cyanidioschyzon* (Nozaki et al., 2007), the diatoms *Phaeodactylum tricornutum* (Bowler et al., 2008) and *Thalassiosira pseudonana* (Armbrust et al., 2004), the unicellular green alga *Ostreococcus tauri* (Derelle et al., 2006), the flagellate unicell *Chlamydomonas* (Merchant et al., 2007), and the multicellular brown alga *Ectocarpus siliculosus* (Cock et al., 2010). The dilemma of trying to produce algal biomass for conversion to biodiesel is that in order to maximize the production of lipids by the microalgae, one may decrease cell growth and photosynthesis leading to a decrease in overall productivity including lipid production. One possible avenue to solving this is through the genetic engineering of the microalgae in order to enhance lipid production without comprising overall productivity. Compared to the genetic engineering of other organisms, the biotechnology processes involved in producing transgenic microalgae are still in their infancy with *Chlamydomonas reinhardtii* currently the most widely exploited microalgae genetically (Leon-Banares et al., 2004). In order for this area to move forward, there is a need for the appropriate tools, in particular transformation systems, to allow us to move genetic material in and out of these cells. Only a few microalgae have been successfully transformed compared with the large numbers of genetically transformed bacteria, fungi, and terrestrial plants (Leon-Banares et al., 2004). This technique has the potential to give us the ability to express heterologous genes to enhance not only traditional algal products but also as a means of providing new bioactive products for commercial markets (Rochaix et al., 1998; Harris, 2001). However, it must be noted that the work on producing transgenic microalgae has, to date, focused on their suitability to be grown in photobioreactors, and this is not necessarily suitable for the large-scale product of algal biofuels (Zaslavskaia et al., 2001; Richmond, 2004; Leon-Banares et al., 2004). As described by Leon-Banares et al. (2004), the main limitations to the genetic transformation of microalgae are having the correct transformation system, promoter, or reporter, genes and developing mechanisms for the analysis for the stable expression of the introduced gene(s). Significant work is needed before this is a routinely applicable approach for most algal taxa.

3.1. GENETIC ENGINEERING TO ENHANCE PRODUCTIVITY

One of the first reports of genetic manipulation of microalgae was in the area of lipid production (Dunahay, 1993). Others have been in the areas of bioremediation,

recombinant vaccine production, converting obligate photoautotrophs to act as heterotrophic organisms, and the production of hydrogen; in most cases, this has involved the genetic transformation of *C. reinhardtii* (Apt and Behrens, 1999; Cai et al., 1999; Zaslavskaia et al., 2001; Siripornadulsil et al., 2002; Sun et al., 2003). Areas that have been focused on for their potential to increase productivity include improvements to photosynthesis; this includes the amount of pigment present in the organism responsible for capturing photons and the efficiency with which photosystems convert captured photons into carbon compounds (Flynn et al., 2010). An important limitation on productivity is the decrease in the efficient of conversion of sunlight into biomass by microalgae at high light levels. However, many taxa are capable of acclimatization to sunlight quality and quantity by alternating the size and composition of their light-harvesting antenna systems; this has been of considerable interest in terms of genetic engineering (Escoubas et al., 1995; Durnford and Falkowski, 1997; Beckmann et al., 2009). Losses occur during elevated light levels in the conversion of energy into biomass, particular in dense cultures (Beckmann et al., 2009). The energy will mostly be dissipated by the light-harvesting complex (LHC) antenna system of the cells closest to the surface, resulting in light not being able to penetrate deeply into the culture (Polle et al., 2002).

Beckmann et al. (2009) have demonstrated that it is feasible to manipulate the size and composition of the LHC of *C. reinhardtii*'s photosystem II, resulting in an increase in the efficiency of conversion of sunlight to biomass. They linked the importance of this to the differences in light levels a culture of microalgae will be subjected to when grown in mass culture as a means of improving the overall biomass productivity of the culture. A strain of *C. reinhardtii* engineered to have reduced antenna sizes and less chlorophyll outperformed an unmodified strain of the alga at high light levels (Beckmann et al., 2009). It demonstrated an increase in sensitivity against high light, with photoinhibition resulting in less energy captured and sunlight being wasted as heat or fluorescence (Beckmann et al., 2009). Comment has also been made on the fact that a smaller photosystem will occupy less space within the cell potentially allowing more room for commercially useful chemicals (Flynn et al., 2010). It is also worth noting that light-limited cells are generally smaller than their counterparts, so it is as yet unclear how overall productivity and cell size will interact (Falkowski and Raven, 1997). Assuming that genetic manipulation was commercially acceptable, it is unlikely that we would only alter the photosynthetic makeup of an algal strain; almost certainly genes involved in lipid biosynthesis would also be engineered (Dunahay et al., 1996). Flynn et al. (2010) have commented that although GM strains with altered antenna systems are excellent in closed cultivation systems, they are likely to be outcompeted by their native counterparts in an open system. This does not mean that the risk of "escapees" from an open pond system is negligible, even though they may not be competitive in natural niches. It is unlikely that algal strains will be only engineered to increase their photosynthetic efficiency, and it is unclear what added advantage other genetic changes would give these engineered organisms in the natural environment (Flynn et al., 2010).

3.2. GENETIC ENGINEERING OF LIPID PRODUCTION

Genetic transformation has already been used in the area of lipid biosynthesis of microalgae focusing on first part in the pathway, the ACCase enzyme (Dunahay et al., 1995, 1996). The gene, *acc1*, for this enzyme was first isolated from the marine diatom *Cyclotella cryptica* in 1990 by Roessler (1990a) and subsequently transformed by Dunahay et al. (1995, 1996) back into *C. cryptica* and another diatom *Navicula saprophila*. The gene overexpressed in both diatoms by 2–3-fold, but there was no increase in lipid biosynthesis associated with this overexpression (Courchesne et al., 2009). Sheehan et al. (1998) suggested that overexpression of the ACC enzyme alone would not be sufficient to enhance the lipid pathway, and it has been suggested that the committing step catalyzed by ACC is not the rate-limiting step and a secondary rate-limiting step occurs during the overexpression of ACCase (Courchesne et al., 2009).

A range of other genes, either directly or indirectly, have been manipulated in many different organisms with varying degrees of success but not within microalgal species (Courchesne et al., 2009). These have included fatty acid synthetase (FAS) (Verwoert et al., 1995; Subrahmanyam and Cronan, 1998; Dehesh, 2001), lysophosphatidate acyltransferase (LPAT) (Zou et al., 1997), and acyl-CoA:diacylglycerol acyltransferase (DGAT) (Bouvier-Nave et al., 2000; Jako et al., 2001; Galili and Hofgen, 2002). Research has also focused on genes not directly involved in lipid metabolism but those that potentially affect the rate of lipid accumulation by increasing the pool of metabolites that can be converted into lipids, as well as blocking off competing pathways (Courchesne et al., 2009). Very little of this research has been focused on algal pathways, and the results from other systems seem to indicate that this approach may have a negative impact on overall cell growth (Picataggio et al., 1992). A few researchers have suggested a multigene approach (Verwoert et al., 1995; Roesler et al., 1997), but there is not an extensive literature covering this aspect of genetic engineering (Courchesne et al., 2009). Another approach, which has being suggested, is transcription factor engineering, where a metabolic pathway is studied in the context of the whole organism (Courchesne et al., 2009). Here, regulatory transcription factors (TF) are used to control the abundance or activity of multiple enzymes relevant to a particular pathway by either up- or downregulating them in a process referred to as TFE (Capell and Christou, 2004). TFs are proteins classified into some 50 families according to their conserved structure and their DNA binding domains (Courchesne et al., 2009). These proteins interact with the cell's transcription machinery such as DNA polymerase enhancing the rate of transcription for a particular group of genes (Grotewold, 2008), with a combination of TFs regulating a single pathway (Santos and Stephanopoulos, 2008). Courchesne et al. (2009) suggested that TFE could overcome the potential "secondary bottlenecks" associated with genetic engineering approaches taken so far, but this form of genetic engineering is, again, still in its infancy with algae.

3.3. PRODUCTION STRAIN GENETIC STABILITY

The selection of production strains for future biofuel production has been, and continues to be, a major focus of research. It is extremely unlikely that there will be one single tax that is adopted worldwide, as local conditions will dictate the requirement for different characteristics and tolerances. Although the wide-scale use of GMOs is also unlikely, inevitably there will be strain selection to maximize productivity of the desired oils. In the pharmaceutical sector, much of the improvement in yield of antibiotics from fungi has been achieved through the application of conventional mutagenesis, and this approach should be equally applicable to algal oil production. There are already examples of this in algal biotechnology where commercial mutants of *Haematococcus pluvialis* have been produced by chemical means and UV exposure to increase production of the pigment astaxanthin (Tripathi et al., 2001). Irrespectively of whether wild-type, or mutant, strains are employed, there is a fundamental requirement in any industrial process to ensure the genotypic and phenotypic stability of master stock cultures (Day and Stacey, 2008). We envisage that a variety of genetic approaches will be developed capable of confirming the genetic stability of any production strain. Whole genome approaches would be preferable, and in the future, the costs associated with genome mapping will be cheap enough to be used for routine quality assurance. Alternatively, whole genome fingerprinting approaches, such as amplified fragment length polymorphism (AFLP) (Müller et al., 2007), or even molecular probes to monitor potential changes in key sections of the genome may be employed.

Traditionally, algal cultures have been maintained by serial transfer, and although this has successfully maintained some taxa apparently unchanged for many decades (Müller et al., 2005; Day et al., 2010), it cannot guarantee the long-term genotypic security of microbial strains. The only applicable alternative is to employ cryopreservation (storage at ultra-cold temperatures, i.e., <−130 °C) (Day and Stacey, 2008). There are methods available for a range of algae (Day and Brand, 2005); however, it may be necessary to refine methodologies for individual production strains to maximize post-thaw viability levels. It is also a distinct possibility that many of the strains that will be employed will require cryopreservation methods to be developed. A further issue, outwith the scope of this chapter, is ensuring the stability of strains during the inoculum buildup phase. Cryopreserved material generally takes 2–6 weeks to regenerate a 20-ml culture (Day, unpublished), and it can take at least 8–9 weeks to scale up from a 20-ml flask culture to a 10,000-m^2 raceway production pond (Borowitzka, 2005).

4. Interactions with Other Organisms

At present, a range of algae are grown commercially; many, for example, those used in aquaculture, are produced in relatively small quantities (Muller-Feuga, 2004) or for niche applications such as biocide testing. However, a small number of taxa,

most notably *Arthrospira*/*Spirulina* and *Dunaliella*, have been grown in large (1 – >200 ha), open culture systems. Although this has necessitated a considerable amount of R&D as both algae can be cultured under extreme environments, this reduces the potential of contaminant algae or grazers. The *Spirulina* strains that are grown commercially originate from highly alkaline lakes, where the alga commonly forms a virtual monoculture. The extremely high pH (often >pH 10.0) of their culture regime restricts the number of contaminant organisms and grazers. *Dunaliella* is commercially grown in large, managed lagoons, with high osmotic potential. Once again the extreme environment ensures a unialgal culture and restricts the number of grazers, pathogens, etc. capable of reducing productivity. Neither *Spirulina* nor *Dunaliella* are immune to interactions with other organisms, but as discussed above, they are much less susceptible than many other algal taxa. In the following sections, we have outlined some of the negative effects of biological interactions, with in some cases suggestions as to how they can be reduced or avoided.

4.1. COMPETITION BY OTHER ALGAL TAXA

The size of microalgae and their ready dispersability via aerosols, on dust particles, or even by aquatic birds (Atkinson, 1980; Walsh and Steidinger, 2001; Sharma et al., 2007) means that without exception, there will always be the possibility of contamination by other algae where open ponds are employed. Furthermore, many algae are extremely versatile and can survive or even grow well in a variety of ecological niches, for example, one can grow some "marine" *Chlorella* in freshwater medium and "freshwater" *Chlorella* in marine medium (Campbell et al., 2009). This versatility can potentially cause a problem, as many algae can grow well on the same medium (Lorenz et al., 2005). Realistically this will only be an issue where contaminant algae do not have the desired attribute (e.g., oil production) or have health implications (e.g., produce toxins) and are capable of growing much more rapidly or to higher densities than the "production" strain. Production strain selection needs to prioritize rapid growth to minimize the likelihood of overgrowth by a contaminant. However, even within the current relatively small selection of mass-cultured algae, problems may occasionally occur. Despite the high pH of the culture environments employed for *Spirulina* production, the alkaliophilic green alga *Oocystis* may cause significant problems (Belay, 1997). The contamination of *D. salina* ponds by non-carotenoid-producing *Dunaliella* species may also result in lower productivity of the desired alga/product, although this may be controlled by maintaining high levels of salinity (Mitchell and Richmond, 1987). Appropriate management regimes are likely to be the best solution to preventing excessive growth of non-crop, "weed," algae; these will include the use of large inocula (>10%) and employing selective medium and/or selective environmental conditions.

The possibility of "biofouling" (the accumulation of unwanted microorganisms, plants, and animals on man-made surfaces) needs to be considered. Algae adhering to the walls of the culture system can act as a reservoir for contaminating the algal crop. Biofouling can also impede movement/flow of the culture in the pond, or raceway, and impair movement of paddlewheels, pumps, or other mechanical devices. This could have the implications of increasing energy requirements and reducing productivity of the system.

4.2. ALLELOPATHY AND INTERACTIONS WITH BACTERIA

Although the production of axenic algal strains is of particular value for many physiological and genetic studies, it is a relatively artificial and debatably unnatural state. *In vivo* complex microbial interactions are normal for algae, often providing sources of fixed carbon through leakage from healthy cells, as well as though the lysis of dead cells. Many photoautotrophic algae are auxotrophic for vitamins, which they obtain from bacteria in their nature niche (Droop, 1957; Croft et al., 2007) and are often added in artificial media (Lorenz et al., 2005). There is a raft of other positive bacterial/algal relationships including the production of extracellular iron-binding compounds, called siderophores, by specific bacteria associated with phytoplankton (Amin et al., 2009). It is probable that these interactions have been coevolved and in a balanced stable ecosystem bacterial and algal numbers remain stable.

Many applications involve the culturing unialgal, non-axenic cultures, and although there are often bacteria present, during the lag and log phases of growth, on microscopic examination, few bacteria are observed. Older, denser, healthy cultures tend to have higher levels of fixed carbon leakage, with in extreme cases up to 75% of photosynthetically fixed carbon being lost from cells (Wolfstein et al., 2002). This invariably results in the growth of bacteria in non-axenic systems. Furthermore, on senescence, the lysis of the algal cells, with the subsequent release of organic material, may stimulate a bacterial bloom. This is analogous to what happens in nature on the collapse of an algal bloom, when the effects can be ecologically catastrophic (Mhlanga et al., 2006). In addition, it has been demonstrated that certain bacteria can induce lysis of algae. In some cases, the bacteria actively glide toward the alga, attach, and then release a lytic agent that results in the lysis of the alga within 30 min (Daft and Stewart, 1973). Work on this topic is ongoing, and predatory bacteria including members of the genera *Bacillus*, *Flexibacter*, *Cytophaga*, and *Myxobacteria* have been identified (Shilo, 1970; Shi et al., 2006; Toncheva-Panova et al., 2008). These appear to exhibit a variety of predation mechanisms including physical contact between prey and predator, release of extracellular substances, entrapment of prey by the predator followed by antibiosis and endoparasitism, or ectoparasitism of the host by the predator. Relatively recently this approach has been considered as a biological control agent for the toxin-producing bloom cyanobacterium *Microcystis*

aeruginosa (Gumbo et al., 2008), and although there are no reports, that we are aware of, there is the potential for this to be an issue in algal mass culture systems.

In our experience, the cause and effect of bacterial bloom/culture collapse are closely interconnected, but we have seen no evidence of bacteria being actively associated with cell lysis. However, when a bacterial bloom occurs, there is little hope of arresting, or recovering from, the culture collapse. In algal mass cultures, pond and culture management regimes ensuring that the culture remains healthy at all times are needed. The organism and the product will dictate the culture regime employed to avoid catastrophic culture failure. Maintaining the culture in log phase has the advantage of avoiding a lag when the material is used to reinitiate a new culture but may not be optimal if oil production/accumulation is only associated with the stationary phase.

A further issue worthy of consideration is the production by algae of a wide range of secondary metabolites, some of which have been demonstrated to play a role in allelopathy, that is, they have an inhibitory effect against either competitors or predators. Allelopathic compounds include alkaloids, cyclic peptides, terpenes, and volatile organic compounds (Leflaive and Ten-Hage, 2007). Examples of allelopathic interactions include the influencing of algal succession (Keating, 1977; Sharp et al., 1979), the production of the antibiotic cyanobacterin by *Scytonema hofmanii* (Mason et al., 1982), algicides by *Synechococcus* (Gleason and Paulson, 1984), the fungicide aponin by *Nannochloris* (Halvarson et al., 1984; Kirk, 1980), and an extracellular herbivore deterrent from *Anabaena flos-aquae* (Ostrofsky et al., 1983). No allelopathic compound type is associated with a particular phylogenetic group of algae, and their modes of action vary from inhibition of photosynthesis to oxidative stress or even cellular paralysis (Gross et al., 1991; Leflaive et al., 2008; Yamasaki et al., 2009). There is no evidence, as far as the authors are aware, that allelopathy has had any significant influence in algal productivity in algal mass culture systems. However, it is certainly an additional factor to consider when selecting possible candidate strains for mass culture.

4.3. INFECTION BY OTHER MICROORGANISMS

Agricultural crops can be devastated by disease/infection, and huge efforts and resources have gone into developing strategies to breed disease resistant cultivars, treatments such as specific fungicides, and the application of crop management approaches to prevent, or reduce, infection. Unfortunately, many phytoplankton species are susceptible to fungal parasitism. These parasitic fungi mainly belong to the Chytridiomycetes (chytrids), relatively primitive fungi closely related to true fungi, which are found in freshwater, terrestrial, and estuarine environments in particular. Chytrids have been considered to be highly host-specific parasites (Canter, 1984), but *Paraphysoderma sedebokerensis*, a parasitic chytrid that attacks the green alga *Haematococcus pluvialis*, has been demonstrated to be capable of infecting a range of unicellular green algae (Gutman et al., 2009). If a

motile, naked zoospore meets a susceptible algal cell, it may attach to the cell wall, encyst, and subsequently produce a fine germ tube that penetrates the algal wall. Enzymes produced by the chytrid break down the content of the algal cell, which is then transferred back into the encysted spore. As a result, the latter enlarges and eventually in its turn becomes a sporangium. Although in many cases this leads to lysis of the algal cell, infection by a single chytrid does not necessarily lead to the death of the algal host cell. Chytrids may prefer larger host cells, since they would gain more resources, but this suggestion may purely be based on their ease of observation. The dynamics of chytrid epidemics in a number of studies were partly explained by environmental factors such as light, temperature, nutrients, pH, turbulence, and zooplankton grazing (Ibelings et al., 2004; Kagami et al., 2007). In freshwater systems, fungal chytrids have been linked to mass mortalities of host organisms, suppression or retardation of phytoplankton blooms, and selective effects on species composition leading to successional changes in plankton communities (Canter, 1984; Kagami et al., 2007). In the marine environments, massive infections of the brown alga *Pylaiella* by the fungal parasites *Eurychasma dicksonii* (Saprolegniales, Oomycota, Heterokonta) and *Chytridium polysiphoniae* (Chytridiomycota, Eumycota) have been reported (Küpper and Müller, 1999). Clearly marine microalgae may also be susceptible to fungal infection, and an ongoing study has suggested that there are a large number of different marine fungi capable of infecting a wide variety of planktonic algal taxa (Gachon, unpublished observation). Algae are not defenseless against infection, and several mechanisms have been suggested, such as a hypersensitivity response, chemical defense, maintaining a high genetic diversity, and multi-trophic indirect defenses (Kagami et al., 2007).

Another potentially problem causing group are the parasitic dinoflagellates of the genera *Amoebophrya* and *Perkinsozoa.* They are widely distributed in coastal waters, where they commonly infect both photosynthetic and heterotrophic dinoflagellates (Coats and Park, 2002; Park et al., 2004). As is the case with fungal infections, recent work indicates that these parasites can have significant impacts on host physiology, behavior, and bloom dynamics (Salomon et al., 2009).

Viruses are ubiquitous in the aquatic environment, and their associations with both eukaryotic algae and cyanobacteria are well known (see Van Etten et al., 1991; Suttle, 2005; Lawrence, 2008 for overviews). Cyanophages infect cyanobacteria, they are divided between three families of tailed phages (Myoviridae, Podoviridae, and Siphoviridae), and all are double-stranded DNA viruses and are distinguished by their morphology. Most viruses that infect eukaryotic algae are members of the family Phycdnaviridae (Lawrence, 2008). These are also double-stranded DNA viruses. As with other viruses, they are normally specific to a particular host but may be capable of infecting a number of strains within an individual species (Jacobsen et al., 1996; Lawrence et al., 2001). They can use an animal virus-like infection strategy "injecting" their genome into their host via a viral inner-membrane host plasma membrane

fusion mechanism, leaving an extracellular viral capsid (Mackinder et al., 2009); they then use their host as a factory to generate large numbers of new virus particles that are released on lysis of the host cell. The impact of viruses is density dependent, with the most dramatic effects being observed during algal blooms. There have been a large number of reports on the occurrence of viral infections in both natural blooms (Nagasaki et al., 2004; Baudoux and Brussaard, 2005) and mescosm experiments manipulating environmental factors (Martinez et al., 2007; Larsen et al., 2008). The use of algal viruses to control nuisance algae, including *Phaeocystis globosa*, which has bloomed along the Dutch North Sea coast each spring and summer for many decades, has been considered (Anon, 2002), but there remain issues of technical feasibility and public acceptability.

It is clear that viruses can have a dramatic effect on algal blooms; furthermore, algal viruses infect representatives of all major algal taxa, influencing phytoplankton population dynamics, marine food-web interactions, and global biogeochemical cycling (Brussaard, 2004; Lawrence, 2008). As with other potential pathogens of biofuel algae in mass culture systems, the transportation, spread, and persistence of specific viruses remain to be explored fully. However, the potential for algal virus introductions and invasions is clearly evident.

4.4. GRAZING

Grazing by ciliates, amoeba, rotifers, and other zooplankton has a significant influence on natural ecosystems and is instrumental to the effective functioning of the microbial loop (Weisse et al., 1990; Sherr and Sherr, 2002). They are responsible for maintaining equilibrium in natural environments, and the grazing impact of ciliates alone on nanoplankton may account for up to 99% of algal primary production per day (Vargas and Martinez, 2009). There is relatively little published work on this topic that has been derived from studies at algal mass culture facilities, with most researchers focusing on lab-based feeding trials (e.g., James and Hall, 1995; Buskey, 2008), environmental studies (e.g., Sherr et al., 1991; Waterhouse and Welschmeyer, 1995), and a relatively small number of mesocosm environmental manipulation experiments (e.g., Davidson et al., 2007).

Although grazing is a widespread problem, those working in the algal biotechnology field have not published extensively on this topic. There have been reports on loss of algae associated with insect larvae grazing in *Spirulina* ponds (Venkatamaran and Kanya, 1981; Belay, 1997). These can be controlled by netting within the culture and during harvesting (Borowitzka, 2005). Rotifers and other zooplankton may also cause problems; Becker (1994) recommended employing a pH shift to pH 3.0 for 1–2 h to control them. The authors have observed a 90% reduction in the cyanobacterium *Oscillatoria* within 5/6 days, with a corresponding 100-fold increase in the grazing ciliate *Nassula*. This effect is not restricted to freshwater taxa, in *Dunaliella* ponds when the salinity drops

below 20% (w/v) NaCl; amoeba and ciliates can rapidly decimate the algal culture (Post et al., 1983). This has also been reported by Moreno-Garrido and Canavate (2001), who observed that grazing ciliates can clarify dense outdoor mass cultures of *Dunaliella salina* within 2 days.

There are very few publications detailing how to reduce, or prevent, protozoan grazing. Engineering-orientated solutions, such as enclosing the ponds in "polytunnels," could reduce the incidence of contamination. An option that has been used successfully in photobioreactors involves "flushing out" the grazers and then allowing reestablishment of the culture; this would be much more problematic to achieve in open pond systems. As mentioned above, infection by grazers could be managed by the control of salinity and pH for some taxa. Depending on the algal taxa and their susceptibility to environmental perturbations, this approach has potential for more widespread application into the algal biofuel sector. The only specific protozoan inhibitor available, Cytochalasin (Leakey et al., 1994), is prohibitively expensive. However, alternative chemical treatments may be possible, for example, the application of a dose of 10 mg 1^{-1} quinine to contaminated outdoor algal mass cultures completely eliminated ciliates within a short time and allowed the algal population to start recovering (Moreno-Garrido and Canavate, 2001). Biological control is in theory an attractive option. However, the introduction of predators (e.g., copepods) would be problematic as they consume both microalgae and protozoa. The authors are unaware of any cultivatable, target-specific protozoan pathogens, or viruses, which could be introduced, but this is a topic that requires additional research.

5. Concluding Comments

Compared to other groups of microbes, microalgae have not been studied from a biotechnology point of view to any great extent. As discussed above, of the few thousand species kept in culture, only a few have had their chemical content investigated, and a very limited number are grown commercially. However, from previous research, we know that some algae can accumulate high levels of oil and that this can be due to nitrogen, or other key nutrient, deficiency. It is also clear that microalgae, although single celled, have an underlying biochemistry similar to that of more highly organized multicellular systems such as land plants. Because of this, they provide a more accessible means to investigate the processes of lipid production, storage, and utilization than their land-bound counterparts. The knowledge being accumulated on the physiology of algae, and their responses to environmental manipulation, will provide the building blocks, not only for the production of transport fuels but also to higher value products such as lipid-based neutraceuticals and functional foods.

A further key building block to the future success of algal biofuels is the understanding and capacity to control lipid production at a molecular level.

The genomes of a number of algae have now been fully sequenced, and the development of new molecular tools to study these genomes and their functions provides us with the opportunity to understand how these organisms function in terms of oil production at the molecular level. We are confident that it will prove possible to manipulate them to increase yields and to establish what other important value-added chemicals can be obtained from this type of biomass.

Having the "optimal" production strain and the underpinning scientific understanding to develop and optimize productivity are the starting points of future large-scale algal biofuel production. Scale-up of both production and downstream process also involves significant challenges, not least in the management and manipulation of the alga's environment. Although photobioreactors provide a greater level of process control, growth of algal in open ponds system is likely to be more economical than growth in enclosed systems for biofuels production. It is clear that maintenance of a specific species and/or strain of microalgae coupled with high productivity is extremely difficult in an open pond system (Brennan and Owende, 2010; Chisti, 2007), but in our opinion, this remains the single greatest barrier to the success of algal biofuels.

The scientific challenges to overcome biological and chemical engineering constraints are being investigated by scientists and technologists worldwide. The drivers for this work remain, and indeed increase, as concerns over global warming, fossil fuel security, and population growth intensify. The attraction of algal biofuels to help develop a globally sustainable energy portfolio is huge. Productivity remains at the forefront of the most attractive traits of algae. According to some estimates, the yield of oil from algae is over 200 times the yield from the best-performing plant oils, and a yield of 12,000 $1ha^{-1}$ for microalgae compared to 1,190 $1ha^{-1}$ for oilseed rape has been suggested by Schenk et al. (2008). However, these figures may be unrealistic, and analyses based on experimental data estimate that microalgal yield can vary from 20 to 30 times that of temperate oil crops (Tredici, 2004). Even at these levels, the potential remains high.

In conclusion, we are confident that if the scientific and financial investments being currently made are suitably and realistically targeted, they will lead to a new major industry. It is difficult to estimate a time frame, but if one considers that man has been developing terrestrial agriculture for well in excess of 10,000 years and algal mass culturing is <50 years old, then it unsurprising that we have so much to learn. There are, of course, no guarantees that we are at the dawn of a new technological age where algal biofuels play a significant component, but if pushed to pull a figure "out of a hat," the authors estimate that in 15–20 years' time, algal biofuels will make a significant contribution to global transport fuel supply. Time will tell, and hopefully, the above estimate is unduly pessimistic.

6. Acknowledgments

The authors acknowledge funding for the BioMara project (www.biomara.ac.uk). The Biomara project is generously supported by the European Regional Development Fund through the INTERREG IVA Programme, Highlands and Islands Enterprise, The Crown Estate, Northern Ireland Executive, Scottish Government, and Irish Government.

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R. Gordon and J. Seckbach (eds.), *The Science of Algal Fuels*,
Cellular Origin, Life in Extreme Habitats and Astrobiology, 131–146
DOI 10.1007/978-94-007-5110-1_7, © Springer Science+Business Media Dordrecht 2012

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ADAPTING MASS ALGACULTURE FOR A NORTHERN CLIMATE

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1. Uncertainty About the Future of Microalgal Biofuels

Microalgal-based biofuels have been discussed extensively as a 'second-generation' feedstock for biofuel production (Schenk et al., 2008), with many reports claiming fewer negative environmental impacts associated with land use, fossil fuel consumption and water use (Chisti, 2008a). While the theoretical potential is rarely questioned, recent reviews documenting the life cycle of hypothetical microalgal biofuel production note several limitations on production, notably the need to reduce both nutrient and CO_2 inputs to make the process less dependent on fossil fuels (Lardon et al., 2009; Ehimen, 2010). Other reviewers have suggested that unless industrial or municipal waste streams are used to replace synthetic fertilizers and sources of CO_2, the impact of microalgal biofuels (before processing to biodiesel or other fuels) is actually greater than conventional biofuel crops such as corn, canola and switchgrass (Clarens et al., 2010), though doubts have been raised about the relevance of the comparisons used in this study (Subhadra, 2010). Some authors have suggested enclosed 'photobioreactors' as a cost-effective way of producing biofuels. Photobioreactors have many theoretical advantages, including more efficient use of high light intensities in vertically structured systems, a potential increase in areal productivity due to a more controlled environment and the efficient recycling of water (Chisti, 2008b; Subhadra, 2010). Other authors have argued that the energy balance of photobioreactor-based biomass generation is unfavourable and productivities are no greater than open-pond systems (Jorquera et al., 2010; Lee, 2001).

Contributing to the uncertainty on the matter, over-optimistic estimates of biomass productivity have underlain many of the calculations for high productivities. It is noted by many authors that at present, photosynthetic efficiency is not likely to exceed that of irrigated tropical crops (Benemann, 1997; Walker, 2009; van Beilen, 2010), which argues against microalgal culture for biofuels applications, unless the cultivation provides benefits in the form of higher value products and services. With this in mind, our proposed cultivation system would be outdoors, illuminated by natural sunlight and fed by waste nutrients and CO_2 and, if necessary, waste heat.

The majority of the research thus far has considered this type of system in 'ideal' tropical or subtropical climates where irradiance, temperature and land-use

conflict would not be limiting factors (Subhadra and Edwards, 2010; Yang et al., 2011). Despite these arguments, there are disadvantages to the cultivation of biomass for energy in one centralized region if waste resources are to be used, the most obvious being the need to channel nutrients and CO_2 to the cultivation facility. There is a distinct possibility that a large transport infrastructure for the capture of CO_2 from fossil fuels will someday be built, but this infrastructure is not yet developed, except for enhanced oil recovery operations (McCarthy, 2001). In a centralized energy production scenario, it would also be more difficult to utilize agricultural/industrial/municipal wastewater, though it is possible to recover nutrients such as phosphorus in solid form from wastewater by crystallization (Huang et al., 2006) which could then be transported to algal cultivation sites.

On the other hand, many point sources of industrial flue gas are located near major urban centres, which usually also have nutrient-rich wastewater systems that could serve as a growth medium for algae. With this in mind, we believe that an effort should be made to exploit local opportunities for generating bioenergy, due to the benefits – no need for CO_2 compression or extensive pipelines and no transport or use of fossil fuel fertilizers. This is a strategy which can and should be applied to northern climates.

2. Problems Specific to Algal Biofuels in a Northern Climate

In Canada, the area between 40° and 55° latitude corresponds to the most highly populated areas, with large industrial facilities occurring alongside many urban centres. From an algal cultivation perspective, the most significant problems for this area are seasonality of temperature and irradiance. While polar microalgal strains can grow at temperatures below zero degrees Celsius (Aletsee and Jahnke, 1992), it is certain that for reasons of productivity, as well as ease of cultivation and harvesting, temperatures above zero would be required. A possible exception would be the cultivation of psychrophilic halophiles which could grow in a medium that would remain liquid at temperatures below zero.

Analysis of the effect of temperature on growth rates of microalgae reveals a general trend of lower growth rate at lower temperatures (Raven and Geider, 1988; Goldman and Carpenter, 1974), though it remains difficult to compare overall productivities between climates, due to a number of confounding factors. Temperature variability in warm environments has a set of disadvantages that may reduce the productivities otherwise predicted. Problems include high and potentially damaging temperatures to mesophilic algae in photobioreactors (Béchet et al., 2010) which may increase respiration losses (Grobbelaar and Soeder, 1985), as well as nightly drops in temperature contributing to photoinhibition (Vonshak et al., 2001), with all of these factors possibly limiting growth to the morning or late afternoon when lower light levels do not trigger photoinhibition (Richmond et al., 1990). In addition, predicted high productivities in

warm climates partly depend on assumptions of high light conversion efficiency. Microalgae are able to efficiently convert solar energy at low irradiances, but efficiency drops sharply at light intensities equivalent to full sunlight. It may be possible to increase the level of growth efficiency at high light intensities by genetically engineering mutants to have smaller light-harvesting antennae which would allow more efficient light utilization in dense cultures by preventing self-shading (Hunter, 2010), though it should be noted that this technology is far from application and unsuited to open-pond cultivation due to concerns about genetically modified organisms.

Considering the suite of problems facing the application of large-scale algaculture in warm climates, the problems of a northern climate appear less severe. If temperature regulation through waste heat usage were integrated into the system, it would likely reduce the aforementioned problems associated with photochemical adaptation. It is unlikely to be necessary to raise temperatures to more than 10°C, as microalgal strains isolated from cold locations that are adapted to low temperatures can be used. Suzuki and Takahashi (1995) demonstrated that diatom species isolated from several environments with different maximum yearly temperatures were adapted to grow fastest at the temperatures they normally encounter in their environment. Maximal growth rates of warm temperature-adapted species growing at 20–25°C showed only slightly higher growth rates than those adapted to lower temperatures of 10–15°C (1.5 doublings/day vs. 1 doubling/day). Indeed, local strains that thrive in wastewater ponds continue to be active in winter months, simply growing at a reduced rate (Griffiths, 2009).

In terms of low irradiance in winter months, many strains can adapt by accumulating photosynthetic pigments. At high density, however, it is unlikely that low-light-adapted species would be able to use this strategy to their advantage due to self-shading. Some phylogenetic groups such as dinoflagellates and diatoms are known to be better at tolerating low light intensities (Falkowski and LaRoche, 1991), and it is possible that a careful strain selection programme could identify suitable isolates for a northern climate.

Other authors have suggested supplemental lighting to obtain a better biomass productivity during winter months (Baliga and Powers, 2010), and this can be potentially supplied by renewable energy if algae cultivation were integrated with renewable generation as some suggest (Subhadra and Edwards, 2010), though this is likely to be only practical for cultivation of high-value compounds due to the cost of artificial lighting. Other strategies for the cold months could include growing mixotrophic strains of microalgae that have a reduced requirement for sunlight or by deploying heterotrophic strains, which have no requirement for sunlight at all. Wastewater has a significant organic component that contributes to biological oxygen demand, but supplementation with an external waste source such as glycerol is likely. This is fortuitous, as glycerol is currently a low-value, abundant waste carbon source which has been shown to be suitable for microalgal cultivation (Chi et al., 2007; Park et al., 2011).

Bruton et al. (2009), writing on the possibility of producing algal biofuels in Ireland, came to a similar conclusion:

> The biggest unknown in Ireland or other similar climates is whether it is possible to achieve reasonable productivity in view of prevailing natural light and temperatures. For regions at higher latitude, it may be possible to identify local strains requiring low light intensities and lower water temperatures but giving satisfactory growth rates and yields…It is likely that a large seasonality penalty will exist if microalgae are to be cultivated in Ireland where the latitude is 53°N. Despite this limitation, microalgae production for biofuel cannot be ruled out without further research and validation of the concept in Ireland.

We share this optimistic outlook – even if microalgal biofuels do not become a commercial reality in northern climates, such an effort would advance basic research into biorefining, wastewater treatment and CO_2 mitigation.

3. Non-biofuel Advantages of Microalgal Culture: Tertiary Wastewater Treatment

As already mentioned, microalgal cultivation systems need to be first deployed where the cultivation of algae has the potential to offer significant environmental benefits separate from biofuel production. Wastewater remediation to prevent eutrophication is one of the most significant areas where an environmental benefit can be realized. Oceanic 'dead zones' – where excess nutrients have caused algal blooms and a resulting drawdown of oxygen during bacterially mediated decomposition of the accumulated algal biomass – are a growing concern worldwide (Diaz and Rosenberg, 2008). It is likely that increased concern for this problem as well as the economic benefit of recovering energy and resources from wastewater will drive further refinement of wastewater treatment (Williams et al., 2008; Benemann et al., 2002). In addition, algal cultivation coupled to wastewater treatment may play an indirect role in reducing the emissions of greenhouse gases that are associated with agriculture, which comprise some 13.5% of the global greenhouse gas emission load (Bernstein et al., 2007). This emission is due partly to the use of nitrogen fertilizers produced using the Haber-Bosch process, which uses large amounts of energy and hydrogen derived from natural gas. Therefore, by recycling these nutrients, society could significantly reduce the amount of synthetic fertilizers required for agriculture or algaculture.

Microalgae have for some decades now been used in municipal wastewater treatment installations for a variety of purposes. As far back as the 1970s, microalgae have been used in an ingeniously designed biologically mediated wastewater treatment system referred to as Advanced Integrated Wastewater Pond Systems (AIWPS) developed by researchers at the University of California at Berkeley (see Oswald, 2003 for review). In these systems, microalgae are typically grown in a high-rate pond and serve as the primary site of N removal, water oxygenation (to promote aerobic digestion of wastes) and disinfection (due to the high pH caused by preferential CO_2 and NO_3^- removal by algal growth). It is important to

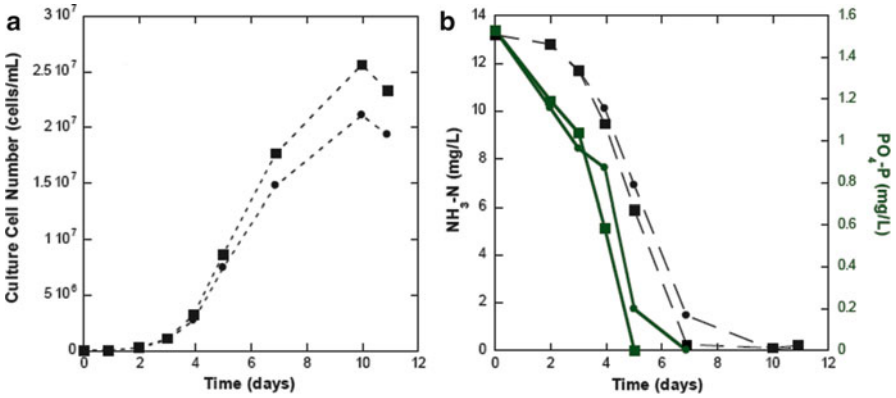

Figure 1. Accumulation of algal cells (**a**) and simultaneous removal of nutrients (**b**) during active growth of replicate laboratory cultures of the freshwater chlorophyte *Neochloris oleoabundans* in municipal wastewater sampled from the Mill Cove secondary wastewater treatment plant (Bedford, NS, Canada).

point out, however, that in the AIWPS, the microalgae are a critical component of primary treatment and so are permanently resident in the high-rate pond and used only to condition the water to promote the treatment process – that is to say that the algal biomass created was never considered as a source of energy. In contemporary wastewater treatment systems using microalgae, however, it may be possible, indeed desirable, to extract the energy from the accumulated algal biomass for useful purposes.

Many algal strains grow well on wastewater substrates (Goldman and Stanley, 1974), and pilot projects have used this technique to reduce effluent nutrients, especially phosphorus (Green et al., 1996; Griffiths, 2009; Chinnasamy et al., 2010). Indeed, in our own laboratory, we have cultured common chlorophytic microalgae on secondary treated wastewater to high densities with no supplemental CO_2 aeration (Fig. 1). Once the culture had entered the exponential phase of growth, rapid removal of ammonia and phosphate to undetectable levels was observed. Some companies have even built an economic model around the use of such resource-rich wastewater for algae cultivation (see http://www.livefuels.com/). The use of microalgae in northern climates is an area of active research – Canadian scientists have reported the use of easily harvestable psychrotolerant cyanobacteria for nitrogen and phosphorus removal at temperatures achievable in a northern climate during spring and fall (Chevalier et al., 2000), temperatures that could be achieved in winter with the application of waste heat. An economic analysis of a similar processing system serving a small population in Scandinavia (60°N) demonstrates theoretical advantages in terms of resource recycling and resource use as compared with traditional wastewater treatment plants with or without managed wetlands to control excess effluent (Grönlund et al., 2004), though the authors offer few insights into solving problems of temperature and irradiance.

Any successful system will likely need to mitigate winter temperature variability to maintain good nutrient removal (Pagand et al., 2000), which would be accomplished by waste heat utilization (discussed further below).

Advanced nutrient recycling is highly relevant for microalgal culture which would otherwise require a large fossil fuel input from synthetic fertilizers (Ehimen, 2010), hence the decision to pursue wastewater treatment synergies for microalgal biofuels. The current strategies for wastewater treatment treat nitrogen as an environmental problem with disposal preferred to environmental release. Sewage sludges are commonly applied to agricultural land in some nations, which helps to recover some nutrients, but considerable losses of nitrogen still occur in some systems by nitrification and denitrification (Bock et al., 1995; Third et al., 2001). If a cost-effective way of recycling the nitrogen present in wastewater were present, it would do much to reduce the fertilizer burden on agricultural and mass algaculture systems.

Based on the well known 'Redfield ratio' for elemental composition of microalgae (Lenton and Watson, 2000), we can predict that the nitrogen content of algal biomass will be approximately 6–7% by weight, on par with many organic fertilizers such as fish meal. With such a high nitrogen content, it is feasible that the microalgal biomass could be applied as a fertilizer for local agricultural production (Rose, 1999). Multiple high-rate ponds may be used if full nutrient removal is desired, in which case it may be possible to cultivate nitrogen-fixing cyanobacteria in the final treatment ponds where nitrogen is limiting. The biomass produced can then be applied to fields to boost production (Benemann, 1979), with some studies even showing that the application of live cyanobacteria to agricultural fields offers the potential benefit of nitrogen fixation after incorporation (Pereira et al., 2008).

Other effective applications of wastewater technology require modifying infrastructure, but could offer significant benefits to both agriculture and algaculture. According to a life cycle analysis of Lundin et al. (2000), the most effective method of recovering nutrients from human waste is by separating urine at the source, since urine contains up to 80% of the nitrogen and about half of the phosphorus contained in wastewater. This recovery has significant advantages over continual resynthesis or mining of mineral nutrients after denitrification and phosphorus removal (Maurer et al., 2003). Other technologies exist to precipitate phosphorus from solution as mineral struvite (Huang et al., 2006), but this process is likely to be more energetically intensive than simple utilization by algae. Recycling of nutrients from wastewater may offer a way of expanding a cultivation area for microalgae beyond the wastewater treatment plant, and if long-term R&D produces a viable system for producing high-quality biofuel or bioactive compounds, it could be easily scaled up without any additional cost for fertilizer.

Naturally, the infrastructure required for wastewater treatment may be impractical in locations where land is insufficient or where flow rates exceed the processing ability of the algal pond, factors that will depend strongly on the nutrient loading and quantity of the wastewater. Nonetheless, this system will

likely prove an attractive option in the future as a way to minimize treatment costs and facility size as well as accelerate nutrient removal and waste processing (Oswald, 1973) ahead of anticipated changes to regulatory requirements in developed nations.

4. CO_2 Abatement and Waste Heat Dissipation

If algae were grown at a sufficient productivity to displace transportation fuels, it is clear that there would be a significant savings in greenhouse gas emissions, provided the production process was carbon neutral. Algae also have a potential role in so-called biological carbon capture by fixing industrially emitted carbon dioxide. Many sources, from fossil fuel generating stations to cement factories to breweries, offer a concentrated stream of carbon dioxide which can potentially displace the current sources of CO_2 for microalgal culture, which would be a significant cost reduction. Microalgae are able to grow using pure flue gas, and cycling flue gas through the growth medium also removes other gases such as hydrogen sulphide (Negoro et al., 1993; Hamasaki et al., 1994; Doucha et al., 2005). There may also be a small nutritive effect to nitrogen and sulphur gases (Kumar et al., 2010).

CO_2 separation from flue gas is achievable using available technology such as membrane separation (Dortmundt and Doshi, 1999; Scholes et al., 2008) and fairly easily introduced into available pond systems (Su et al., 2008). With current achievable productivities of somewhere in the range of 20 g/m^2/day (Schenk et al., 2008; Chisti, 2008a), it follows that the amount of area required to fix all the carbon dioxide emitted from a typical coal generating station emitting approximately 1,000,000 tons of CO_2 per year would be ~7,600 ha (see Appendix for calculations). If theoretically higher biomass yields on the order of 30–60 g/m^2/day are achieved (Schenk et al., 2008), land requirements could be significantly decreased.

Ultimately, however, the actual productivities realized will depend on the local resources available, particularly the quantity and quality of available incident solar radiation. Even with increased productivities, it is fairly clear that biological carbon capture technology is unlikely to be able to remove a large proportion of the fossil CO_2 emitted (Benemann, 1997), but may prove economically viable if conducted in a regulatory environment where carbon is priced (Kadam, 1997, 2001). In this manner, funding from CO_2 capture projects can contribute to essential research and development goals for algaculture. Some long-term R&D goals may include optimizing the cultivation of coccolithophorid algae that convert CO_2 to calcium carbonate ($CaCO_3$) which is more easily sequestered (Jansson and Northen, 2010; Moheimani and Borowitzka, 2007), although this strategy will likely not be practicable in the near future because of the difficulty of maintaining reasonably unialgal species in outdoor ponds. It is also important to bear in mind that the maximum quantity of industrial 'waste' CO_2 that can be fixed by algae is

constrained by the Redfield ratio, as mentioned earlier. Microalgae fix both carbon and nitrogen into organic matter at a fairly fixed ratio of approximately 6.6:1. Currently, the quantity of waste CO_2 being emitted into the atmosphere is far in excess of the corresponding amount of ammonia being discharged in treated wastewater, thereby placing a finite limit on the amount of CO_2 that can be fixed biologically.

Cooling waste streams of water from industrial sources before discharge is a regulatory necessity in many locations due to its potential impact on aquatic ecosystems. Algae have been proposed by some authors to ameliorate the discharge from nuclear and fossil fuel plants by culturing thermotolerant nitrogen-fixing cyanobacteria (Wilde et al., 1991). In a northern climate during winter, algaculture could be used similarly to both regulate the temperature of open ponds as well as accomplish dissipation of waste heat. It is unclear how this could be accomplished with an open-pond model – some minor insulation may be required, depending on the size of the heat source, the size of the algaculture operation and the outdoor temperature, but any insulation could hopefully be supplied using cheap plastic materials. Photobioreactors would potentially be easier to regulate than ponds, but are unlikely to be used for an integrated wastewater plant which needs to deal with a large and variable flux of water. This source of low-grade heat could offer other potential benefits to an algaculture operation, such as pasteurization of ponds or acceleration of other microbial treatment steps such as anaerobic digestion or primary treatment of waste, especially during winter months. Waste heat may also be theoretically exploited from other nonindustrial sources if the algaculture location were coupled to biogas electricity generation or geothermal energy (Subhadra and Edwards, 2010).

5. Downstream Processing: Energy Recovery, Biorefining and Potential Postharvest Nutrient Recycling

As with microalgal culture everywhere, harvesting and energy extraction is likely to be a significant barrier for the commercialization of this form of biofuel production. At present, the production of biodiesel appears to be favoured over other forms of energy generation, due to the theoretical ease with which fatty acids are convertible to a liquid fuel. Microalgae are proposed to be an ideal source for biodiesel, which we will first consider. Processing to methyl ester fuel (biodiesel) most often involves a primary extraction step, which usually consists of both mechanical pressing and solvent extraction. Following extraction of cellular lipids, further processing is undertaken to remove undesirable compounds that were co-extracted (e.g. proteins, pigments), a step commonly known as ‘degumming’, after which the purified lipid can be converted to methyl esters by the addition of methanol with an appropriate catalyst.

It is important to note several things about this procedure – first and foremost, both of these processes consume significant amounts of energy, and the challenges

of extracting microalgal lipids at scale have yet to be met. Energy may be reduced by using a so-called 'in situ' transesterification which combines both extraction and conversion to biodiesel into a single step, with comparable results (Ehimen et al., 2010). Both of these procedures have a requirement for dry biomass – quantities of water greater than 5% ($^{w}/_{w}$) are generally known to inhibit acid-catalyzed transesterification, and even smaller quantities may influence base-catalyzed reactions (Christie, 2003). This is a significant challenge, since dewatering and drying of biomass of microalgae is energy intensive and certain to negatively affect the overall energy balance.

Another consideration is that during lipid extraction, a significant amount of pigment and other contaminating compounds will likely be co-extracted with microalgal biomass. Results from our laboratory indicate that up to 2/3 of some lipid extracts produced by accelerated solvent extraction are not convertible to biodiesel, which suggests that overall biodiesel yields may be significantly lower than reported in the literature for many strains (McNichol et al., 2011). Indeed, it has been long known that lipid biosynthesis and high growth rate are mutually exclusive (Sheehan et al., 1998). Still other results from our own lab indicate that under nutrient replete conditions, most strains produce very little neutral lipid, with the exception of the slow-growing *Botryococcus braunii*. Therefore, we can reasonably expect that in some species, upwards of 80% of the biomass is likely to be waste from the perspective of biodiesel generation.

Given this uncertainty, processing of microalgal biomass is likely to require other energy recovery processes besides oil extraction, even in optimistic scenarios where cultivation of oleaginous, fast-growing algae is possible. One approach that is sure to be essential in supporting microalgal R&D is to develop a so-called 'biorefinery' to maximize high-value compounds that can generate revenue for algaculture operations (Wijffels et al., 2010). Well-known nutritive compounds such as omega-3 fatty acids are produced as a high percentage of total fatty acids by some microalgae (diatoms in particular (Dunstan et al., 1994)), and high quantities of protein may find end uses as animal or human supplements. Despite these possibilities, it is still likely that initial systems will have to deal with large quantities of residual biomass before either a market is established or quality is improved, which is where other methods of energy recovery become attractive.

In situations where lipid content is low, anaerobic digestion for the production of methane (for fuel or electricity) may offer an efficient way of recovering energy. Some authors have suggested that where cell lipid content is less than 40%, anaerobic digestion may be the most energetically favourable process (Sialve et al., 2009), with others demonstrating the same basic concept using 'defatted' biomass for anaerobic digestion (Ehimen et al., 2009). The main advantages of this procedure include greater potential recovery of energy, nutrient recycling in the form of nitrogen and the ability to digest wet biomass. At present, there are considerable limitations to methane production such as energy input (Collet et al., 2011) and lower than expected yields, though this may be offset by economies of scale (Benemann et al., 2002). Part of the difficulty in

digesting microbial biomass in an anaerobic system is the inherently low C/N ratio, which may be countered by adding a high carbon product that can be co-digested (Yen and Brune, 2007), which could consist of lignocellulosic material from agricultural or forestry waste. Production of methane and on-site use for electricity generation offers some other hypothetical benefits, which include the production of waste heat for controlling pond temperature as well as the production of CO_2 in biogas which can then be recycled back into the cultivation system, thereby simultaneously improving the biogas quality (by concentrating methane) and stimulating algal growth (Mann et al., 2009).

Another technique that can be used to extract energy from algae is gasification, which treats wet biomass at high temperature and pressure using a catalyst, producing methane as a product (Elliott et al., 2004). This process has the potential to be significantly simpler than anaerobic digestion, and energetically favourable, recovering some 60–70% of the heating value of biomass (Stucki et al., 2009), though it requires a more advanced infrastructure. In practice, gasification can produce both methane and a liquid substrate for further algal growth since cellular nitrogen is converted into ammonia during the process (Minowa and Sawayama, 1999).

These processes, along with possible co-firing of algal biomass with coal (Kadam, 2002), offer considerable flexibility in biomass utilization. A variety of strategies will undoubtedly be essential to maximize the energy production of such a 'biorefinery' which will likely be required to deal with multiple different feedstocks, from microalgae to agricultural waste, and still generate energy effectively and recycle nutrients (Kamm and Kamm, 2004; Antizar-Ladislao and Turrion-Gomez, 2008).

6. Conclusion

In the short term, microalgal cultivation for biofuel production will not likely prove economical, nor will it ever be a panacea for climate change. Despite the many limitations, opportunities exist for synergy with wastewater and CO_2 abatement that will help to offset initial costs of research, and since any advances in microalgal technology are unlikely to differ between sites, technology developed at one location likely will be broadly applicable to any biofuel operation. It remains unclear whether or not microalgal cultivation will be applicable year-round in northern climates, but the potential for significant advances in cultivation, harvesting and processing technology gives a strong motivation for large R&D projects in Canada and other northern nations.

Another important reason for scaling up production is to evaluate the various claims promulgated by proponents of biofuels. While microalgae theoretically offer a carbon-neutral source of bioenergy, real-life validation of life cycle inputs are sorely needed, with Reijnders (2008) arguing that fossil fuel inputs required to build cultivation facilities are rarely considered. These and other criticisms must

be tackled by real-world validation of all the impacts of microalgal culture, from carbon dioxide release due to land-use change to the energy requirements of all growth and processing steps, before we can be satisfied that microalgae offer a robust contribution to solving either the world's climate crisis or energy needs.

7. Appendix

CO_2 sequestration calculation: Given productivity of 20 g/m^2/day, maximum productivity per year = 20 * 365 = 7.3 kg/m^2/year. Approximately 1.8 t of CO_2 are fixed for each ton of algae produced. 7.3 kg * 1.8 = 13.14 kg CO_2/m^2/year.

Given 1,000,000 metric tons as average value for coal generating station (http://www.ec.gc.ca/pdb/ghg/onlinedata/dataSearch_e.cfm), divide 1,000,000 tons by 0.01314 tons CO_2/m^2/year = ~76,000,000 m^2 = 76 km^2 = 7,600 ha.

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R. Gordon and J. Seckbach (eds.), *The Science of Algal Fuels*,
Cellular Origin, Life in Extreme Habitats and Astrobiology, 147–163
DOI 10.1007/978-94-007-5110-1_8, © Springer Science+Business Media Dordrecht 2012

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NANOTECHNOLOGY FOR ALGAL BIOFUELS

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1. Introduction

In the last two decades, with fast depletion of fossil fuels, the need for renewable and sustainable energy resources has become critical. High prices, nonrenewable nature, and emission of greenhouse carbon have been major drawbacks for fossil fuels (Khan et al., 2009). Currently, there is an urgent and increasing need to improve available methodologies and to develop renewable and revolutionary breakthroughs in the energy field.

Bioenergy technologies can meet these criteria and therefore present an attractive solution. While considering renewable resources and bioenergy production, three factors can significantly impact the final outcome and overall efficiency: (a) the type of biomass and the processes for pretreatment, (b) conversion of the biomass feedstock into high-energy products, and (c) biofuel extraction and separation technologies. For each of these steps, the common requirement is low-cost and low-energy technologies that would enable efficient and sustainable biofuel production.

In the past two decades, several biofuel options have been explored as alternatives to fossil fuels (Korres et al., 2010; Prasad et al., 2007a, b; Singh et al., 2010a, b, 2011; Pant et al., 2010). Biodiesel, an alternative to fossil diesel, is produced via transesterification of oils. Its renewable, nontoxic, and biodegradable nature makes biodiesel an attractive candidate for replacing fossil diesel. Biodiesel can be obtained through various renewable resources. Among the sources under investigation, algal biofuels provide one of the most viable options (Gavrilescu and Chisti, 2005) for biofuel production for the following reasons:

- No competition for cultivable land.
- Algae grow using non-potable water.
- Provides savings for greenhouse carbon (GHC) due to high consumption of CO_2 during algal cultivation.
- Algal biofuel is a carbon neutral energy source (similar to other biomass resources).

Algal biofuels, however, face a few challenges:

- It is difficult to achieve consistent industrial-scale algae production.
- Cost of algae production and harvesting is high.
- Lipid extraction can be energy intensive and may cause pollution problems.

Beyond the advantages listed above, algae have other growth characteristics that make them the only source for biodiesel production with potential to completely displace fossil fuel. In comparison to other energy crops, microalgae grow extremely fast, and many species are oil-rich, making algal biomass an excellent choice for biodiesel production (Singh et al., 2011). In addition to biofuels, algal biomass can be used for the production of (a) protein-rich feed for human (and animal) consumption, (b) ethanol and biogas via fermentation technologies, and (c) polyunsaturated fatty acids (PUFA) as a substitute for fish oil (Burton et al., 2009).

1.1. CHALLENGES IN COMMERCIALIZATION OF ALGAL BIOFUELS

In spite of the advantages and promise of algal biofuels, commercialization of algal biofuels faces several challenges.

- Production of algal strains with high efficiency for lipid production: Genetic modification (GM) is an option to improve efficiency and yield of algal biofuels. Algenol is developing a GM strain of cyanobacteria in Canada that can be used for ethanol production in Mexico.
- Improved technologies for growth of algal cultures: Several reactor designs are in practice for growing algae, but these suffer from one or more limitations with respect to gas transfer, mixing, illumination, and biomass yield. New technologies and improved reactor designs are required to overcome these limitations.
- Improved harvesting technologies: Harvesting is energy-intensive at present. Algae biofuel production can be significantly improved with help from development and adaptation of refined and cost-effective separation technologies that are currently in use for other industries such as food and pharmaceutical.
- Procedures for lipid extraction prior to esterification: Extraction of lipids without the need for drying of biomass and use of solvents would make the procedures highly efficient and cost-effective.
- Alternative esterification process: Existing process of biodiesel production requires separation of lipids free from water and fatty acids, leading to high production costs. Improved technologies for enzymatic esterification with lipase and low temperature processes need to be developed and optimized.

Nanotechnology involves design, characterization, production, and application of structures, devices, and systems by controlled manipulation of size and shape at the nanometer scale. Nanotechnology can potentially provide solutions to many of the challenges faced in commercialization of algal biofuels. The following

sections address nanotechnology solutions for preparation of engineered strains of algae, novel nanotechnologies for growth of algal culture, and improved nanomaterials for harvesting, extraction, and separation of biofuels.

2. What Is Nanotechnology?

The National Science Foundation (NSF) defines nanotechnology as research and technology development at the atomic, molecular, or macromolecular levels, in the length scale of approximately 1–100-nm range, to provide a fundamental understanding of phenomena and materials at the nanoscale and to create and use structures, devices, and systems that have novel properties and functions because of their small and/or intermediate size (www.nano.gov). Nanotechnology allows one to synthesize materials with unique properties and customize their structures for specific applications. It exists in nature in form of all the nanomachinery of cellular systems and viruses. Figure 1 illustrates natural and man-made structures and gives a perspective about their relative sizes.

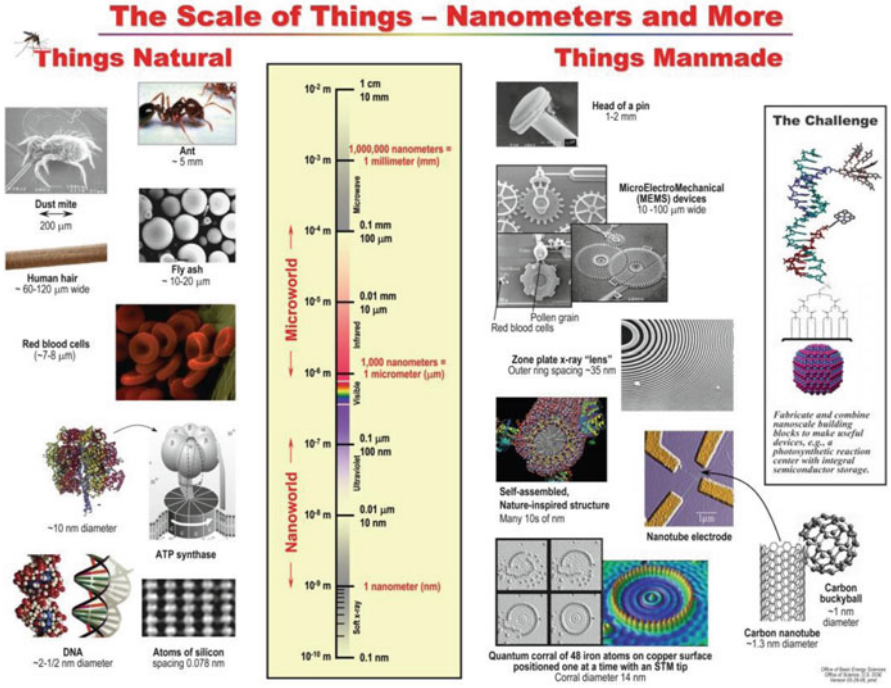

Figure 1. Nanostructures found in nature and man-made nanostructures (From http://science.energy.gov/bes/news-and-resources/scale-of-things-chart/).

Nature uses nanosystems with ultimate precision in molecular fabrication. Knowledge of the molecular architectural concepts applied by nature can be exploited and mimicked to the extent possible, to create new and novel energy-related technologies. Current research efforts in nanotechnology have been dedicated to investigate its use for cost-effective, energy efficient, and environmentally sustainable protocols in the renewable energy, healthcare, agricultural, food, and pharmaceutical industries. Since this is a relatively new field, many of the protocols in nanotechnology are relatively unrefined and need optimization. With advancement in characterization and development tools, highly efficient and eco-friendly nanotechnology protocols would likely emerge in the next few years (Wegner and Jones, 2007). With its ability for molecular fabrication, nanotechnology can provide customized nanomaterials for many aspects of energy-efficient biofuel production. It can provide high-performance materials for biomass conversion, fractionation, and extraction; lightweight materials for use in making vehicles; and safer and more efficient materials for storage of hydrogen fuels. Through the use of nanotechnology green, clean, and improved protocols could be made available for various fields such as geothermal, unconventional natural gas, coal and carbon sequestration, nuclear, solar, wind, and hydraulics. This chapter covers nanotechnology innovations specifically applied to algal biofuels.

Nanotechnology has potential for improving current technologies applied for biochemical as well as thermochemical processes for treatment and conversion of biomass to generate bioenergy in a variety of forms such as liquid biofuels, biohydrogen, biogas, and electricity. These include improved materials for enzyme immobilization, materials with improved enzyme loading capacity, nanocatalysts, materials for storage of bioenergy products, materials for separation and purification of liquid biofuels, and nanomaterials for improved bioreactor design. This chapter covers each of these areas in detail in the following sections.

3. Nanotechnology Applications for Algal Biofuels

Several excellent articles in the recent years have covered nanotechnology-based solutions for optimized and energy-efficient protocols for biofuel production (Khanal et al., 2010; Laudenslager et al., 2010; Pugh et al., 2010). The following sections cover several steps in algal biofuel production that stand to benefit from using nanomaterials. Many of these protocols are still in infancy and would need to be optimized to be economically and commercially viable. Nanotechnology-based solutions for algal biomass growth are restricted to artificial illumination in closed photobioreactors (PBRs) and not for open-pond systems. Nano-enabled technologies for biomass treatment and catalytic upgrading of crude biomass, however, are equally applicable to open-pond systems as well as PBRs.

3.1. NANOTECHNOLOGY FOR BIOREACTOR DESIGN

Growth of algal culture is a critical parameter in deciding the overall efficiency of algal biofuel production. Two major components impacting algal growth are (a) illumination and (b) proper mixing for uniform illumination and nutrient contact.

3.2. NANOTECHNOLOGY AND CULTURE ILLUMINATION

One of the major concerns in algae culture illumination has been the availability of cheap and sustainable light sources. Installation and maintenance of an artificial light source in PBRs is a major concern. As algal biomass density increases, illumination of the culture with uniform light intensity becomes challenging because of self-shading as well as biofilm formation on the reactor surfaces (Chen et al., 2011). The light source cannot be placed close to the PBR either, because it generates considerable heat, which would be undesirable for the culture. Together, uniform and sustainable illumination of the PBR is a major challenge and seriously limits the light conversion efficiency of conventional PBRs.

Light-emitting diodes (LEDs) are a good alternative to conventional lighting for artificial illumination of algal cultures in PBRs. LEDs are easy to fit into a PBR because they are lightweight and small. They consume very little power, generate relatively small amounts of heat, have high light conversion efficiency, and can be switched on and off with high tolerance. Currently, significant research is taking place regarding new nanomaterials for fabrication of various types of LEDs (Pompa et al., 2006). By careful control of the nanomaterial components, it is possible to manipulate their illumination properties. This can significantly impact the photoconversion efficiencies when these LEDs are applied for illumination of algal cultures. Currently, LEDs are being used for artificial lighting of PBRs, but only few references specifically mention use of nanomaterials used for LEDs, such as in case of LEDs made with gallium aluminum arsenide (GAAs) used for illumination of algal cultures (Lee and Palsson, 1994).

Red LEDs with an absorption wavelength of 450–470 nm and blue LEDs with an absorption wavelength of 645–665 nm (Yeh and Chung, 2009) have been used for indoor cultivation of algal cultures. Highest specific growth rate and biomass production resulted from use of red LEDs as reported by Wang et al. (2007). In yet another study, Katsuda et al. (2006) reported that applying flashing light with blue LEDs as an the on-off illumination method provided good biomass yields. More research on this topic is required to optimize the LEDs for algal cultures.

Apart from LEDs, the other method of illumination is the use of optical fibers excited by artificial light. Optical fibers are superthin glass fibers drawn out of ultrapure glass, capable of laser light transmission. Light passing through one meter of an ordinary glass is stopped, but the same light can be transmitted over miles without any appreciable decrease in its intensity by optical fibers. Generally,

these optical fibers are end-illuminated to prevent leakage of light through its sides. Rough surfaces are mechanically introduced on the exterior of the fibers, resulting in the emission of light from the entire surface, and not just the ends. These are called sidelight optical fibers. When these are used in PBR for illumination of algal cultures, these significantly increase the light conversion efficiency without generation of heat (Henshaw and Zeu, 2001; Lee and Kim, 1998). In addition to high light conversion efficiencies, these sidelight optical fibers consume significantly less electricity, making them highly cost-effective and energy efficient for illumination of algal cultures.

The latest in the algal culture illumination approaches is the method that uses metal nanoparticles coupled to localized surface plasmon resonance (LPSR) (Torkamani et al., 2010). Plasmons are collective oscillations of free electron gas density at a metal-dielectric interface. Light absorption/scattering at specific wavelengths can be amplified selectively using resonant interactions between photons (light) and surface plasmons. In the work by Torkamani et al. (2010), strong backscattering of blue light from silver nanoparticle suspensions in a miniature PBR was shown to increase photosynthetic activity of algal cultures to result in almost 30% higher biomass densities. In this set up, by monitoring the concentration and size of the silver nanoparticles, light flux can be controlled to avoid photo-inhibition (Steele et al., 2007). Additionally, the frequency of the light can be controlled to optimize frequencies preferred by algal species as well. Figure 2 represents the experimental set-up for algal growth using nanoparticle-enabled mini-PBR.

3.3. NANOTECHNOLOGY FOR GROWTH OF ALGAL CULTURES

3.3.1. Nanobubbles

Beyond energy efficient illumination of the algal cultures, efficient mixing of the algal cultures is necessary to ensure adequate provision of CO_2 and nutrients for the growing cells. Using a hybrid of nano- and microbubbles in an airlift loop bioreactor (ALB), Zimmerman et al. (2009) demonstrated enhanced algal biomass production. The increase in biomass density was due to faster CO_2 dosing and efficient O_2 stripping from the culture medium enabled by hybrid of micro- and nanobubbles. Nanobubbles remain in the system for a long time because of negligible buoyancy. This ensures high mass transfer efficiency with respect to CO_2 delivery and O_2 stripping because of increased surface area of nanobubbles compared with microbubbles. With improvements in nanobubble generation technology, nanobubbles require less energy for generation than microbubbles. Nanobubbles could also be used in floatation of algal biomass. The bubbles provide a global stirring motion that results in mixing of suspended algal cells around the top and the walls so that they are exposed to light which stimulates photosynthesis and results in a net increase in the biomass (Zimmerman et al., 2011). Uniform nanoporous membranes can be used to generate sub-100-nm bubbles (Kukizak and Goto, 2006).

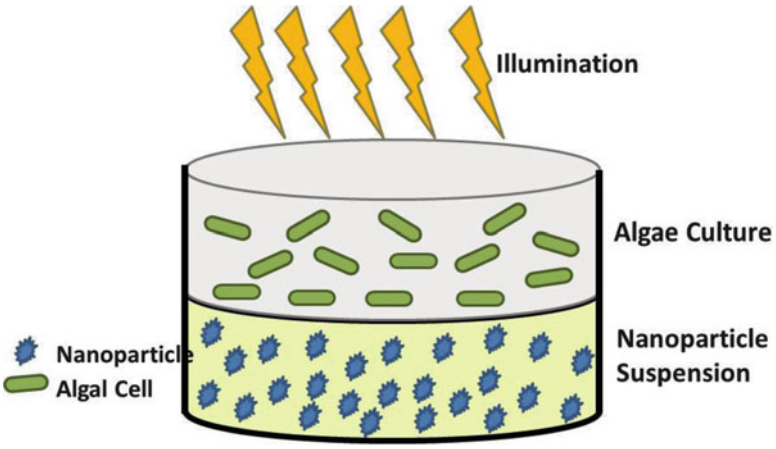

Figure 2. Schematic of mini-photobioreactor using silver nanoparticles (Inspired by Torkamani et al., 2010).

3.4. NANOTECHNOLOGY FOR CONVERSION OF BIOMASS TO BIOFUEL PRODUCTS

While creating sustainable biofuel production procedures, the two critical issues are:

- Effective conversion of algal biomass to biofuel
- Efficient separation and harvesting of the biofuel from the reaction mixtures

At present, the biofuels dominating the bioenergy sector are the short chain, aliphatic alcohols such as ethanol, butanol, iso-butanol, and pentanol (Atsumi et al., 2008; Cann and Liao, 2008). Higher conversions of biomass to these alcohol-based biofuels somehow face a unique situation. Due to the cytotoxicity of the short-chain alcohols, the processes are designed for dilute fermentation, which impacts the downstream processing yields, making the process less sustainable and ineffective with respect to operational costs (Straathof, 2003; Schugerl and Hubbuch, 2005). Additionally, energy costs for dewatering of the biomass and distillation purification of liquid biofuels typically add up to 7–10% of the total production costs (Galbe et al., 2007). For algae, the major processes applied in conversion of biomass into biofuel are anaerobic digestion, use of supercritical fluids, pyrolysis, and gasification. In recent years, several types of nanomaterials such as electrospun nanomaterials (Laudenslager et al., 2010), nanophotocatalysts (Zhong et al., 2005), metal oxides (Crossley et al., 2010), and mesoporous materials (Lucena et al., 2008) have been developed and applied in biofuel production protocols. These materials have shown promise in to providing energy-efficient and cost-effective protocols and are described in the following sections.

3.4.1. Nanotechnology for Biomass Transformation

Use of immobilized enzymes has been a choice for biomass transformation and pretreatment in biofuel production. Nevertheless, these traditional enzyme-based feedstock transformation technologies suffer from limitations of low catalytic efficiencies of enzymes (Li et al., 2007), high cost, poor recovery of catalysts, and a need for extreme conditions such as high temperatures and strong acids (Moxley et al., 2008).

Nanoscaled structures can eliminate many of the above problems. There is a significant increase in the surface area as the size approaches nanoscale. Along with increased surface area for enzyme loading (Cruz et al., 2010; Wang and Hsieh, 2008), nanomaterials help also to increase diffusion of substrates to enzyme molecules, leading to increased production rates (Kim et al., 2004). They offer easier catalyst recovery and recycling (Ashok et al., 2009) along with the ability for a continuous operation (Hongfei et al., 2002). Additionally, use of nanomaterials significantly improves biocatalyst lifetime and stability (Kim and Grate, 2003).

Novel Nanomaterials for Immobilization: In the past, enzyme immobilization applications were limited due to the monolayer adsorption mode of attachment. With the introduction of innovative nanostructured supports, it has been possible to apply enzyme aggregate coatings on the surface of carrier molecules (Kim et al., 2005). These immobilized enzyme aggregates offered several advantages such as high loading capacities, increased enzyme activity and improved stability. The only drawback in the application of the immobilized enzyme aggregate approach is the possibility of reduced overall activity that may result from multilayer structure blocking access of substrate molecules to the enzymes (Pugh et al., 2010). Lipase is an enzyme with significant applications in biocatalysis for algal biofuels production. In his work on immobilized lipase, Sen (2010) immobilized the enzyme in novel silica matrix to create nanocomposites of immobilized enzyme and tested the immobilized lipasae for hydrolysis of p-nitrophenol palmitate to palmitic acid and p-nitrophenol. In another experiment, lipase B from *Candida antarctica* was immobilized on functionalized multiwall carbon nanotubes (MWCNTs) through physical adsorption. The biomaterials retained more than 55% of their initial activity after 6 months at 4°C, while they retained approximately 25% of their initial activity after 30 day of incubation in hexane at 60 °C (Pavlidis et al., 2010).

3.4.2. Nanocatalysts for Cracking/Hydrocracking

Biomass cracking has conventionally applied cobalt-molybdenum catalysts (Tran et al., 2010). Xiao et al. (2008) reported that reduction in the size of free-standing ruthenium catalysts for Fischer-Tropsch reactions from 4 to 2 nm remarkably improved the reaction rates. In most cases, such small metal nanocatalysts require catalytic supports such as transition metals, noble metals, and inorganic oxides (Elliott, 2007). Inorganic oxides such as zeolites and clay carrying numerous Bronsted or Lewis acids on their surfaces can offer catalytic selectivity of cobalt-molybdenum catalysts by varying their Si/Al ratios. The selectivity comes from control of acidic sites available on their surfaces (Leliveld et al., 1999). When used

in catalytic cracking of *Botryococcus braunii*, the ratio of Si/Al seemed to confer selectively controlled production of lower or higher molecular weight hydrocarbons (Tran et al., 2010). Antonokou et al. (2006) investigated novel MCM-41 mesoporous materials for catalytic pyrolysis process. The method resulted in decreased levels of oxygenated fractions in the bio-oil produced in addition to increased stability of the biofuel.

3.4.3. Nanocatalysts for Transesterification

Catalytic transesterification involving nanostructures and nano-sized oxides have shown to significantly impact transesterification in soybean oils. Calcium oxide nanoparticles with crystallite size of 20 nm gave >99% conversion, whereas under the same conditions, commercial CaO nanoparticles with crystallite size of 43 nm resulted in only 2% conversion (Reddy et al., 2006). Thus, it is clear from the above numbers that oxide-nanoparticles may act as better catalysts during transesterification. Even with a 10x scale-up, this process retained almost 91% conversion efficiency (Reddy et al., 2006).

3.4.4. Metal Nanocatalysts for Biogasification of Wet Biomass

Drying of biomass before gasification is not energy efficient. Therefore, metal nanocatalysts for biogasification of wet biomass are being developed by QuantumSphere in California for algal samples from Salton Sea (http://www.qsinano.com/news/releases/2009_02_24.php). Using these metal nanocatalysts, the algal biomass can be converted into hydrogen, methane, and other synthetic gases for subsequent production of liquid fuel.

3.4.5. Zeolites

Zeolites are mesoporous, aluminosilicate minerals commonly used as adsorbents in commercial processes. They have a 3-D silicate structure and depending on various framework types, zeolites can have very open porous structures.

The crystallographic structure formed by tetrahedras of AlO_4 and SiO_4 are the basic building blocks for various zeolite structures, such as zeolites A and X, the most common commercial adsorbents. Typically, due to the alumina-content, these structures have cationic surfaces that can be functionalized for a customized pore size of the particles on nanoscale (4×10^{-10} m) (www.grace.com/EngineeredMaterials/MaterialSciences/Zeolites/ZeoliteStructure.aspx). The cage structure, precise control over pore size, and charge distribution make zeolites a very attractive option as an adsorbent for biofuel separation processes (Fig. 3).

The ethanol produced in the bioenergy sector typically has residual moisture content of 4–6%. Removal of this water by distillation is energy-intensive (Cardona Alzate and Sanchez Toro, 2006), but the hydrophilic zeolites offer a low-energy and cost-effective option with improved ability in selectively removing water from fuels (Wu et al., 2009).

Zeolites have a double advantage when applied to biodiesel production. In the case of biodiesel fuels, the presence of trace amounts of moisture is undesirable

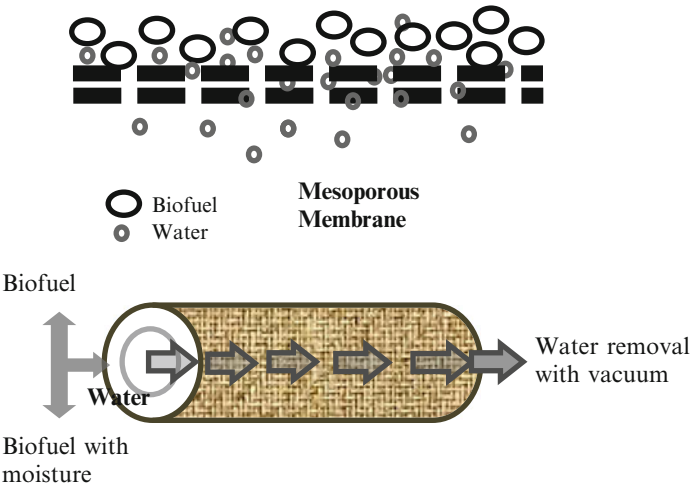

Figure 3. Zeolite membranes for separation of biofuel and moisture (Inspired by http://news.mongabay.com/bioenergy/2007/07/mitsui-engineering-to-use-zeolite.html).

as it may result in production of glycerin as a by-product (Kusdiana and Saka, 2004). The removal of this trace amount of moisture improves the efficiency of the production process. As biodiesel is a combination of methyl esters of fatty acids and water is a by-product of this esterification reaction, effective removal of water from the conversion reactions drives the reaction toward esterification leading to higher efficiency of biodiesel production (Lucena et al., 2008).

Nanofarming for biofuel production using algal culture is an innovative approach currently being explored. In this method developed at the Ames Laboratory (Gibson, 2009), mesoporous nanoparticles were used for continuous harvesting of biofuels from algal cultures without cell lysis (Fig. 4). These particles act as absorbent sponge-like material to selectively remove the lipids from the algal cell membranes, eliminating the need for cell lysis. This opens up possibilities for preparation of *in situ* transesterification of calcium/strontium oxide nanoparticles functionalized with catalytic properties (Liu et al., 2008).

3.4.6. Nanohybrid Catalysts as Emulsion Stabilizers

Upon pyrolysis of biomass, the product liquid obtained is bio-oil that is only partially soluble in water and organic solvents (Huber et al., 2006). Due to such properties, phase-transfer catalysis can be applied for separation and extraction of the biofuel. Generally, in these catalytic processes, a biphasic solvent system comprising two immiscible solvents (generally water and a water-immiscible organic solvent) is used, which is further stabilized by the use of elmusifying surfactants such as quaternary ammonium salts (Stark, 1971). When reactions are carried out in these biphasic catalytic systems, even though a product may be unstable under

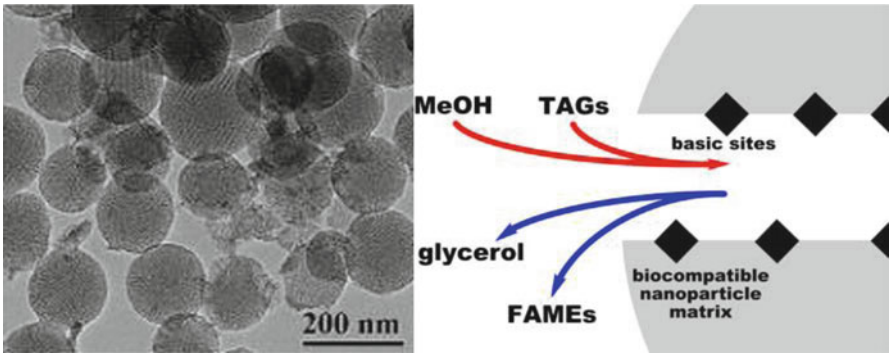

Figure 4. *Left*: A micrograph of mesoporous nanoparticles developed by researchers at Ames Laboratory to harvest biofuel oils from algae. *Right*: Generic scheme for the proposed function of mesoporous nanoparticles as adsorbents in nanofarming (Reprinted with permission from The Canadian Journal of Chemical Engineering, volume 89, February 2011 published by the Canadian Society for Chemical Engineering).

reaction conditions in one solvent phase, upon formation, it can partition into the other phase stably. In such solubility-based partitioning of the products in the phase-transfer catalytic reactions, one can successfully by-pass the heat-intensive distillation processes typically employed for separation of hydrophilic components form the fuel. This greatly simplifies the isolation and purification processes. During the treatment of the complex bio-oils, water content to be removed is almost 30%. The only problem with the biphasic catalysis system is the difficulty in separation of the surfactants from the final products. In the past, applications of solid-particle emulsifiers have been documented in literature (Dinsmore et al., 2002; Dai et al., 1996), but these are not catalytic particles. A combination of solid-phase particles with the emulsion stabilization properties combined with catalytic properties is highly desirable for biofuel production. Various metal oxides are known stabilizers for oil-in-water emulsions (Binks, 2002), whereas due to their inherent hydrophobicity, carbon nanotubes are known to stabilize water-in-oil emulsions (Wang et al., 2004). In their work on nanoparticles, Crossley et al. (2010) synthesized hybrid nanoparticles by fusion of carbon nanotubes with silica. They were able to tune the hydrophilic-hydrophobic proportion of the particles by modifying the composition. Beyond biodiesel production from oils and fats, nanoparticles have also helped improve the technologies for production of hydrogen with chemical reactions. Moreover, they were able to achieve various degrees of hydrogenation activity in the organic phase by varying the formulation of the nanohybrids between SiO_2 and MgO as supporting materials for the nanoparticles. With such solid-stabilized nanohybrid systems, it is possible to design a continuous process with a layered oil-emulsion-water structure where one can achieve full conversion on both sides of the emulsion phase. The reaction happens in the emulsion. Oil-soluble products are removed from the top layer and water-soluble products from the bottom layer.

3.5. NANOTECHNOLOGY AND BIOFUEL ADDITIVES

Due to their application in several processes in biofuel production, solid nanoparticles have impacted the actual process of biofuel production, either as catalytic particles or as carriers for catalytic enzymes. Nevertheless, there are significant applications of liquid nanoparticles or nanodroplets (www.Economist.com/node/16271415). Because of their surface active properties, these liquid nanodroplets help improve the fuel efficiency by monolayer coating on the mechanical parts in contact with the fuel. There are applications of nanoemulsions similar to these liquid nanomaterials in improving heterogeneous catalysis for biomass conversions. These so-called additive-based nanoemulsions result from the interaction of surfactants added to the fuels (fossil fuels as well as biofuels) and trace amounts of moisture content from the fuels (Wulff et al., 2009). For conventional fuels, there is a trade-off between the formation of soot and levels of nitrous oxide formed. By applying these nanoemulsions, that mandate is no longer valid, and both the goals are achieved simultaneously resulting in complete fuel combustion, low fuel emissions, and higher fuel efficiency. Strey et al. (2007) hold a US patent for this application of additive-enhanced fuel performance. According to their hypothesis, proposed for this emulsion-based fuel efficiency, as the first step, the fuels (diesels and biodiesels) readily dissolve fatty acids (oleic acid) and nitrogen-containing compounds (amines). In the subsequent step, these then interact with trace amounts of water from the emulsion without the need for stirring or sonication typically required to solubilized water in a hydrophobic/nonpolar medium. These nanodroplets of water stabilize the interaction of water and hydrophobic factors creating a situation similar to a liquid sponge.

4. Concluding Remarks

Nanotechnology has the potential to impact and improve current methodologies used in production of algal biofuels production. Nanomaterials find applications in several major aspects of algal biofuels from design and operation of photobioreactors, biomass treatment and lipid extraction, and upgrading and refining of crude biofuels. Many of these protocols are still in early stages and are in need of optimization. The potential for use of nanotechnology in overcoming several hurdles faced currently for commercialization of algal biofuels lies in its ability for offering a range of novel, customized nanomaterials for creating cost- and energy-efficient protocols for algal biofuels.

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R. Gordon and J. Seckbach (eds.), *The Science of Algal Fuels*,
Cellular Origin, Life in Extreme Habitats and Astrobiology, 165–175
DOI 10.1007/978-94-007-5110-1_9, © Springer Science+Business Media Dordrecht 2012

FROM ALGAE TO BIOFUEL: ENGINEERING ASPECTS

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1. Introduction

During the past few decades, the average global temperature has risen measurably. The Intergovernmental Panel on Climate Change estimates the average temperature rise during the twentieth century to be 0.74 ± 0.18°C (IPCC, 2007). The major causes of this temperature rise are (a) carbon dioxide emissions from the combustion of coal and petroleum mined from under the earth's surface and (b) the destruction of forests (because forests contribute to the consumption of atmospheric CO_2). Temperature rise has in turn affected weather, biogeochemical cycles, polar ice caps, sea level, sea water quality, and water resources. These physical changes have resulted in impacts on the society as well as human health.

Concern over climate change has prompted governments and private industry around the world to explore and develop nonpetroleum-based fuel sources. Biomass-derived fuels, or biofuels, have consequently received increasing attention during the past decade.

First-generation biofuels include bioethanol or alcohol produced by fermentation of carbohydrates (sugar, starch) from plants and biodiesel produced by transesterification of vegetable oils or animal fats. These fuels resulted in food grains being diverted to biofuel production, leading to food price increases around the world.

Limitations of the first-generation biofuels (Evans, 2007) led to the development of second-generation biofuels, those derived from nonfood crops or nonedible biomass. While these feedstocks do not compete with food, they still need arable land for production, irrigation, and fertilizer input. Moreover, production rates per unit land area are relatively low.

With limitations of second-generation biofuels apparent, focus has now shifted to third-generation biofuels or those derived from algae. Algae can grow on wastewater or saline water, wastelands, and rapidly consume CO_2, a waste gas from industrial stacks. Moreover, biomass productivity per unit area is one to two orders of magnitude higher than first or second-generation biofuel feedstocks. As can be seen from the oil yields from various crops tabulated below (Tickell, 2000; NREL, 1998), algae provide one of the renewable fuel options that can potentially

fulfill the world's energy demand in terms of resource (land, water, light) availability (Brune et al., 2003) (Table 1).

Algae for alternative fuel were investigated extensively for the first time by the United States Department of Energy (DoE) in the early 1970s through its Aquatic Species Program (NREL, 1998). The original aim of the program was to produce hydrogen from algae; it changed to produce lipid-based liquid fuels. The program screened numerous algae species and conducted extensive investigation on the

Table 1. Plant oil yields.

Crop	kg oil/ha/year
Maize (corn)	145
Cashew nut	148
Oats	183
Lupin (lupine)	195
Kenaf	230
Calendula	256
Cotton	273
Hemp	305
Soybean	375
Coffee	386
Flax (linseed)	402
Hazelnuts	405
Euphorbia	440
Pumpkin seed	449
Coriander	450
Mustard seed	481
Camelina	490
Sesame	585
Safflower	655
Rice	696
Tung tree	790
Sunflowers	800
Cacao (cocoa)	863
Peanut	890
Opium poppy	978
Rapeseed	1,000
Oives	1,019
Castor beans	1,188
Pecan nuts	1,505
Jojoba	1,528
Jatropha	1,590
Macadamia nuts	1,887
Brazil nuts	2,010
Avocado	2,217
Coconut	2,260
Chinese tallow	3,950
Oil palm	5,000
Algae (open pond)	80,000

various growth characteristics of algae, their lipid content, the effect of sterile growth environment, and the sustenance of algae cultures. The program investigated growing algae in stagnant open ponds and raceway open ponds. The program concluded in 1998 that it was too costly to grow algae to be cost-competitive with petroleum fuels. Maintenance of the large open pond cultures, ensuring stability by protecting the cultures from external invasive species, and change in weather were the main concerns. Recently there have been renewed efforts to develop algae biofuel technology looking to improve the various facets of the technology.

2. Properties of Algae Biomass: Suitability as a Biofuel Feedstock

Algae contain carbon, hydrogen, oxygen, nitrogen, and phosphorus. Typical ratios of these elements are reported as 106:263:110:16:1, credited to Redfield (1934).

Algae contain both carbohydrates and lipids. Carbohydrates can be converted to bioethanol through fermentation. Glucose is a typical carbohydrate, which is fermented into ethanol as follows:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \quad (1)$$

Thus, two molecules of ethanol are generated from one molecule of glucose, and two molecules of carbon dioxide are released.

Algal lipids can be converted to biodiesel through transesterification as follows:

$$R'OH + R''OOR \rightarrow R''OH + R'OOR \quad (2)$$

Algae biomass can be subjected to anaerobic digestion to produce methane (also referred to as natural gas or biogas). Compressed or liquefied natural gas (CNG or LNG) can also be used as transportation fuels.

From stoichiometric viewpoint, conversion of algae biomass into biofuels takes away carbon, hydrogen, and oxygen, whereas nutrients (N and P) are left behind in the meal. The meal can be utilized as a fertilizer, used as animal feed, or recycled to grow algae biomass.

3. Processing Steps and Design

An algae-to-biofuel process has the following steps: algae biomass growth, harvest, and conversion to biofuel. These are considered separately in this section.

3.1. ALGAE BIOMASS GROWTH

To grow algae, light (energy from photons), water, nutrients, and CO_2 must be available. These conditions can be achieved in open ponds, where sunlight can

provide photons during the day. In industrial settings, controlled growth environment can be created through photobioreactors (PBRs).

All the energy content in algae biomass comes from the light fixed through photosynthesis. Not all the sunlight, however, is photosynthetically active radiation (PAR). Only wavelengths ranging from 400 to 700 nm are absorbed by algae for photosynthesis. This amounts to 45% of total sunlight energy (Hall and Rao, 1999). Algae need 8 mol of photons to fix 1 mol of CO_2 (Hay and Porter, 2006). This means a conversion efficiency of ~27% of absorbed light energy of the photons as shown below:

$$\text{Energy of 1 mol of PAR photons} = 218\ \text{kJ} \tag{3}$$

$$\text{Energy of 8 mol of PAR photons} = 1{,}744\ \text{kJ} \tag{4}$$

$$\text{Energy content of photosynthetic product, glucose} = 16\ \text{kJ/g} \tag{5}$$

$$\text{Amount of glucose fixed from 1mol of } CO_2 = 1/6\text{mol of glucose} = 30\text{g of glucose} \tag{6}$$

$$\text{Energy stored in glucose fixed from 1 mol of } CO_2 = 480\ \text{kJ} \tag{7}$$

$$\text{PAR based photosynthetic efficiency} = (480 \div 1{,}744) \times 100 = 27.5\,\% \tag{8}$$

This (27.5%) is the theoretical maximum efficiency. Experimentally observed values are about 20 mol of photons to fix 1 mol of CO_2 (Hay and Porter, 2006). This means a conversion efficiency of ~11% of absorbed light energy. Considering only 45% of total sunlight energy is photosynthetically active, the real conversion efficiency of falling sunlight to biomass is approximately 5%.

For open pond systems, a daily algal productivity rate of about 25 g/m^2/day has been estimated as an achievable target. This equates to about 90 T biomass/ha/year. Assuming 30% lipid content in the biomass, this would yield 27 T lipid/ha/year.

3.2. ALGAE BIOMASS HARVEST

During the initial stages of algae growth, light, nutrients, and CO_2 are virtually unlimited. As the biomass grows, one of the three becomes limiting. In open ponds, light becomes limiting, as algae cells deeper into the water column and away from light source are shaded by the cells closer to the light source. At this time, some biomass must be harvested and the volume replaced with clear water, so that the remaining biomass multiplies for another harvest cycle. Algae biomass can be harvested simply by pumping the algae slurry and dewatering it. Typical dewatering processes include centrifuging, filtering, or flocculation and sedimentation.

3.3. CONVERSION TO BIOFUEL

Algae biomass harvested in the previous step is converted to biofuel in one of two ways: (1) fermentation of the carbohydrate content of the algae biomass into alcohol and (2) conversion of algal lipid through transesterification into biodiesel.

4. Engineering Challenges and Potential Solutions

While the process flows for algae conversion to biofuel have been fairly well understood, production of biofuel entails engineering challenges. These are briefly discussed in this section.

4.1. ALGAE BIOMASS GROWTH

During the initial stages of exponential growth phase at low concentrations (30–100 mg/L), algae grow rapidly with its own biomass serving as the platform for further growth. The cells grow, divide, and multiply. At these low concentrations, light, CO_2, and supplied nutrients are normally plentiful, and the growth is exponential – limited by algae's own biomass.

As the algae biomass concentration increases, one of the three factors – light, CO_2, or nutrients – becomes limiting. In open pond systems, provided there are enough nutrients and sunlight intensity (1,000–2,000 μmol PAR photons/m^2/s) is well above light saturation values (~200 μmol PAR photons/m^2/s) (Fábregas et al., 2004; Chisti, 2007), CO_2 becomes the first limiting factor. The concentration of CO_2 in air is relatively low (0.039%) (http://www.esrl.noaa.gov/gmd/ccgg/trends/), and the mass transfer rate of CO_2 from air to algae cells is the main limiting factor. The excess supply of CO_2 artificially by locating an algae growth facility near CO_2 emitting sources such as coal-fired power plants is a potential solution.

As with any chemical reaction, the equilibrium can be pushed toward the product side for faster reaction, by increasing the reactant concentration as well as by decreasing the product concentration. Thus, stripping oxygen, the by-product of photosynthesis reaction, also enhances the growth rate.

If CO_2 limitation is addressed by supplying excess CO_2, the following factors can then be considered to enhance the algae growth rate. In natural sunlit systems, as the algae concentration increases, algae cells self-shade each other, and the light stops penetrating to the bottom. Thus, the culture depth should not be appreciably more than the light penetration depth as it would cause biomass loss due to respiration in dark zone. At this point, algae productivity is limited by falling sunlight and the light zone. It must be remembered that sunlight intensity is much more in excess of the saturation light intensity (Fábregas et al., 2004;

Chisti, 2007). The productivity can be increased further by distributing the light excess of saturation intensity to more algae culture. Also, the excess light causes photoinhibition and lowers growth rate (Chisti, 2007). Thus, light distribution could have two-pronged benefits.

Algae growth rate is lowered by the autotoxins released by algae cells during their multiplication (Rodolfi et al., 2003). Apart from these, other factors such as pH, temperature, and sterility also affect algae growth rate; these effects on growth rate vary from species to species. Under ideal growth conditions, all the aforementioned factors would be optimized to achieve the maximum growth rate.

Algae can be grown heterotrophically in dark on external food sources such as waste or cheap sugars for energy instead of light. This approach, however, makes algae very similar to other heterotrophic microorganisms and does not provide the benefit of CO_2 capture.

For open pond systems, the energy needed is mostly for mixing the algae culture (NREL, 1998). The DoE Aquatic Species Program reported that for a 0.1 ha (1,000 m^2) raceway pond, for a reasonable mixing velocity that keeps the algae suspended (15 cm/s), the paddle wheel energy of mixing is 1 kWh/day (3,600 kJ/day) (NREL, 1998). Energy content of the biomass growing in this surface area = 25 g/m^2/day × 1,000 m^2 × 20 kJ/g = 500,000 kJ/day. Thus, the energy balance is favorable for the algae growth stage.

Depriving algae cultures of nutrients (nitrogen and phosphorus) is one common way to increase the lipid content in algae. Though the lipid content is higher, the overall lipid production is lower because the biomass growth itself is lower (NREL, 1998).

Raceway ponds have been used for microalgae cultivation since the 1950s (Chisti, 2007). Among closed PBR designs, there have been attempts to use optical fibers to distribute sunlight. This is presently a costly option. Vertical helical tubular set up and horizontal tubular reactors stacked up one over another are examples of other attempts to distribute sunlight in closed PBR (Chisti, 2007). Vertical flat-plate reactors have been placed adjacent to each other to capture sunlight falling between the plates (Zhang et al., 2001). Light distribution has also been attempted through light scattering by water and algae (Janssen et al., 2001).

Open ponds are affected by diurnal as well as seasonal temperature changes. Open pond cultures could be affected by invasive weeds. Open ponds could also suffer from significant water loss through evaporation. Closed PBRs avoid these problems, but capital costs are higher. Oxygen stripping may be necessary.

4.2. ALGAE OIL EXTRACTION

Separating algae from water and extracting oil are as challenging as growing algae, if not more. The present challenge is to lower the energy expended in the algae separation/oil extraction step so that it does not exceed the energy content of the algae biomass/oil itself. Also, it must be ensured that any attempt in doing so

keeps the algae biofuel business profitable and not lose value of the de-oiled biomass. This means either improving the separation/extraction processes or adopting an algae biofuel technology that avoids this step altogether.

Common conventional separation technologies are drying, filtration, centrifuging, and flocculation using chemicals. Some of the extraction methods are mechanical pressing, hexane extraction, and supercritical extraction. These methods are energy intensive.

To illustrate the energy requirement of conventional separations techniques, let us examine centrifuging. Let us consider an algae facility where 10,000,000 L of algae slurry are harvested daily and the algae biomass concentration is 300 mg/L (0.3 g/L), which is typical of algae ponds.

$$\text{Energy requirement of centrifuge (CDM, 1995)} = 161 \text{ MJ/h for } 22{,}700 \text{ L/h} \quad (9)$$

$$\text{Therefore, energy required to centrifuge } 10{,}000{,}000 \text{ L of algae slurry} = (161 \text{ MJ}/22{,}700 \text{ L}) \times (10{,}000{,}000 \text{ L}) = 70{,}900 \text{ MJ} \quad (10)$$

$$\text{Total biomass in } 10{,}000{,}000 \text{ L of algae slurry} = 10{,}000{,}000 \text{ L} \times 0.3 \text{ g/L} = 3{,}000{,}000 \text{ g} = 3{,}000 \text{ kg} \quad (11)$$

$$\text{Typical energy content of algae biomass (Tredeci, 2010)} = 20 \text{ MJ/kg} \quad (12)$$

$$\text{Therefore energy content of } 3{,}000 \text{ kg of biomass} = 20 \times 3{,}000 = 60{,}000 \text{ MJ} \quad (13)$$

Thus to produce 60,000 MJ of energy value, centrifuging requires over 70,000 MJ of energy, which results in a negative energy balance. Therefore, centrifuging does not appear to be a viable option for dewatering algae biomass harvested as raw slurry. A preliminary, nonenergy-intensive concentration step is necessary.

Some algae species such as *Botryococcus* secrete their lipids outside their cells. Their lipids are in the form of long-chain alkenes. It is relatively easier to extract the lipids since they are outside the cell walls, and they even offer the possibility to extract the lipids without killing the cells. These species, however, grow much slower than the other fast growing algae (Metzger and Largeau, 2005).

If free endless supply of water is available by locating near the sea coast, solar and wind energy could be used to dewater algae by natural evaporation of water, similar to sea salt production. This would require cheap land resources as well.

Energy-efficient separation of oil, water, and biomass has been reported (Eckelberry and Pattarkine, 2010). At the time of writing this document, this technology is being scaled up. Should this succeed at full scale, it shows considerable promise for the algae industry.

Value addition from by-products is one option to make the algae biofuel technology viable. The protein content of algae has good economic value as nutritional product. The de-oiled biomass can also be used for methane production through anaerobic digestion and ethanol/butanol production through fermentation.

5. Concluding Remarks

While algae are the most promising feedstock for biofuel production, considerable engineering challenges remain. Algae growth process is energy-efficient in open ponds exposed to sunlight, but maintenance and weed invasion prevention are challenging. These challenges can be addressed using closed PBRs, but capital costs are high. There are interesting options to increase the growth rate of algae and lipid productivity. The dewatering stage is challenging in terms of energy balance. New solutions that attempt to combine the growth stage with dewatering stage challenges could be of interest. The industry needs a viable dewatering technology. Natural evaporation of water on sea coast is an energy-efficient option. Algae biomass can be converted into valuable by-products, an attractive business model in the short term that can enable research and development on algae conversion into biofuel in the long term.

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R. Gordon and J. Seckbach (eds.), *The Science of Algal Fuels*,
Cellular Origin, Life in Extreme Habitats and Astrobiology, 177–192
DOI 10.1007/978-94-007-5110-1_10, © Springer Science+Business Media Dordrecht 2012

MAKING FUEL FROM ALGAE: IDENTIFYING FACT AMID FICTION

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1. Introduction

Without crude oil, modern civilisation would not exist. In total, some 84 million barrels of oil per day, or in excess of 30 billion barrels a year, are used throughout the world, and 1 quarter of that quantity is consumed alone by the United States of America. The majority of crude oil is refined into fuel for transportation, but it also provides a raw feedstock for a plethora of industries, which produce an almost bewildering number of products ranging from plastics to pharmaceuticals. We are also entirely dependent on oil (and indeed natural gas for fertilisers) to produce the majority of the food consumed in the world. The USA was formerly the world's main oil-producing nation, a mantle that has now been taken by Saudi Arabia which supplies almost 10 million barrels a day to the world oil markets, closely followed by Russia. The price of oil has fluctuated wildly according to world economic and geopolitical influences, and over a 10-year period has varied in the range \$16–\$147 a barrel (Rhodes, 2008a). The issue of peak oil (Hubbert, 1956; Deffeyes, 2005) describes an ultimate situation when world oil production meets a geological maximum, beyond which it must fall inexorably, leading to a gap between rising demand and ultimately falling supply. The term "gap oil" has been coined to describe this situation (Rhodes, 2009). Within a decade or less, the world economies and mechanisms of civilisation will no longer be able to depend on a relentless increase in the output of cheap crude oil nor even on current levels of supply. Recent geopolitical tensions across the Middle East threaten further the secure supply of oil, particularly light crude oil which is most readily refined into petrol (gasoline) to fuel spark-ignition engines, while doubt becomes more urgently insistent over the Saudi Arabian oil reserves, which may prove far less plentiful than has been claimed (Rhodes, 2011). The aspect of carbon emissions, and the consensus that these will lead to unfavourable climate change, further compels the search for low-carbon alternatives to oil since 38% of all the energy used by humans on Earth is derived from oil and fuels refined from it (Rhodes, 2010b), to be compared with 23% from natural gas and 26% from coal. Thus, the origin of the majority of carbon emissions for which humans are responsible is crude oil.

In an effort to address the oil problem, the substitution of oil-based fuels by biofuels has been explored, mainly derived from land-based crops. However, the

area of arable land available to a single country and indeed the world overall is limited, and hence growing fuel crops must inevitably compete with growing food crops. For example, if the United Kingdom were to cease growing food entirely and turn over all of its cropland to rapeseed (canola), it could only match, in the form of biodiesel, around 17% of the fuel used nationally as derived from crude oil. In addition to considerations over their energy content, there are vital differences in the properties of biofuels, for example, biodiesel and bioethanol, from conventional hydrocarbon fuels such as petrol and diesel, which will necessitate the adaptation of engine designs to use them, for example, with regard to high viscosity at low temperatures, in planes flying in the frigidity of the troposphere. Raw ethanol needs to be burned in a specially adapted (high compression ratio) engine to recover more of its energy in terms of tank-to-wheel miles; otherwise, it can deliver only about 70% of the energy content of petrol, kilogram for kilogram in accord with its lower enthalpy of combustion (29 MJ/kg) than is typical for an oil-based fuel like petrol (gasoline) or diesel. The energy content of crude oil is usually reckoned at 42 MJ/kg (Rhodes, 2009). It is important to note that the amount of energy recovered in actual vehicles depends on their engine design. This is normally expressed in terms of well-to-wheel miles efficiency. If the energy originally present in the fuel is accounted against the energy that is actually recovered in terms of how far the fuel will push the vehicle along in terms of miles, this efficiency is about 14% for petrol engines and 20% for diesel engines. The difference is a combination of energy losses when the fuel is actually burned in the vehicle and the energy costs of extracting the crude oil and refining the fuel in the first place.

2. Biofuels

Most biofuels produced in Europe are made from plant oils, such as rapeseed oil, in the form of biodiesel, with a smaller amount of bioethanol that is produced from sugar beet (Duffield et al., 2006). In the USA, the situation is reversed, and huge amounts of corn are turned over to the production of "corn ethanol". The ethanol industry in Brazil is mature, as made from sugar cane which grows well there, with the USA as its major customer for exports. While it is not thought that the Brazilian ethanol industry compromises land on which food crops could be otherwise grown, this is a strong objection made to the diversion of corn grown in the USA from the world food markets to making ethanol. Indeed, part of the huge increases in the price of basic staple foods has been blamed on the use of arable land to produce biofuels rather than to grow food (Reuters, 2008). There are consequently shortages of rice and wheat and a significant reduction in the market stockpile of corn, all of which contributes to a potential food crisis, particularly in developing nations, including China and India. The yields of biodiesel that can be produced from a hectare of land suitable for different "fuel crops" are shown in Table 1.

Table 1. Yield of various plant oils.

Crop	Oil in L/ha
Castor	1,413
Sunflower	952
Safflower	779
Palm	5,950
Soy	446
Canola/rapeseed	1,000
Coconut	2,689
Algae	*80,000*

Data from Becker (1994).

3. Oil from Algae

Of the various means that are being considered to provide alternatives to oil-based fuels, one is making biofuel from algae. There are many advantages claimed as are indicated in the bullet points below, but most noteworthy are the quoted very high yields of oil that might be derived from algae per hectare, compared with that even from high-oil-yielding plants such as palm, which translates to around 6 tonnes of diesel per hectare. In contrast, it is reckoned that some species of high-oil-yielding algae might furnish annually more than 100 tonnes of biodiesel per hectare – an attractive prospect indeed since on this basis an area, say, the size of the United Kingdom, could fuel the entire world (Rhodes, 2009). In principle, the production of algae, to make fuels from, has the following benefits:

- Algae can be grown in tanks to yield over 100 tonne of algal oil per hectare. Hence, the entire fuel demand for the United Kingdom (40 million tonnes of biofuel) could be met on an area of 4,000 km^2.
- No need to use cropland; hence, food production is unaffected.
- Grows well on saline water or wastewater so no demand on freshwater, unlike biofuel crops.
- Can be "fed" nutrients from agricultural run-off water and sewage water, avoiding the need for mineral inputs of N/P fertilisers and cleaning the water/effluent to prevent "algal blooms".
- Can be "fed" CO_2 from power plants, improving algal growth and reducing carbon emissions.
- Easier to process than other biomass, for example, into CH_4, biodiesel, ethanol or hydrocarbons.
- Biodiesel is more biodegradable than petroleum and fuel derived from it.
- Fifty percent of algae can be oil (lipid) c.f. 5–10% for land-based crops (e.g. soya, rapeseed).
- Reduces CO_2 release by replacing oil-based fuels and absorbs CO_2 when it grows through photosynthesis.
- Can be used as a chemical feedstock, plastics.

- Algae (and other biomass) can be processed into organic chemicals, in a "biorefinery", as a basis for a new "bio-organic" chemicals/industry.
- ExxonMobil, Shell, Unilever and many private companies are working on algae to make fuels and other products, therefore, there are serious commercial prospects.
- One recent study shows that growing algae is most efficient as integrated with cleaning CO_2 from power station smokestacks (or a cement plant) and N/P from sewage wastewaters.

3.1. YIELDS OF OIL FROM ALGAE

Claims for the amount of oil that can be "grown" from algae over that from land-based crops like soya tend to vary but range from around 30–182 tonne of oil/ha (Rhodes, 2009). The corresponding figure for rapeseed (canola) is around 1 tonne/ha. Walker has concluded that there is no firm evidence to support the notion that photosynthetic algae are intrinsically more productive than terrestrial plants grown for food (Walker, 2009). He argues that algae may in fact be less productive. Under optimal conditions, all green organisms undergo photosynthesis at the same rate in light of low intensity, and while "sun" species may show differences in full light, these do not necessarily translate into particularly different rates of accumulation of biomass. Thus, whatever the crop, land-based plants or algae, a single acre of land can produce roughly sufficient biomass to either provide fuel for one car or feed several people. Walker concludes that only a very small contribution can be made to road transport by biofuels, but such use of land crops contributes to shortages of food and an escalation of food prices. This indeed we have seen in developing nations such as India and China (Reuters, 2008). Rhodes has challenged some of the claims made for the yields of oil from algae which, in one case once additional biomass is accounted for, appears to exceed the maximum photosynthetic limit (12.7%), and either are exaggerations or imply that some forcing technology has been implemented over the use of simple pond systems (Rhodes, 2009). The issue of which strains of algae should be selected has been discussed by Chisti (2007) who concludes that it is the oil "productivity" that should be maximised not the oil yield nor the growth rate. In an algal culture device, the oil productivity is the mass of oil produced per unit volume of the algal broth in a unit time, that is, the oil yield (gram of oil per gram of biomass) multiplied by the biomass concentration in the algal broth, all divided by the time required to produce the biomass. Productivity is sometimes reported on an area basis, that is, yield (by volume or by mass) per square meter or per hectare. Many algae have been reported to have high oil productivity, for example, by Griffiths and Harrison (2009). Controversy does reign, however, over the matter of whether it is better to grow algae or terrestrial plants to make biofuels, and Chisti has defended his original thesis that making biodiesel from algae is a more energy-effective means to displace petroleum-derived fuels than biodiesel or bioethanol

made from agricultural crops by existing means (Chisti, 2008a) with further hard numbers (Chisti, 2008b), against the conclusions of Reijnders (2008) that algae are net energy-negative sources of fuel (i.e. provide less energy than the fossil fuel energy used to produce them) compared with the overall energy yield of fuels derived from land-based plants such as sugar cane (ethanol) and oil palm (palm oil). Hirano et al. have concluded that methanol can be produced from Spirulina with an energy content that is similar to the fuels derived from the land-based plants (Hirano et al., 1998), but Reijnders stresses that it remains to be seen whether or not the annual yields assumed by Hirano et al. can be obtained in open pond systems in reality. Chisti responds (2008b) that Reijnders' conclusions (2008) are based on two simplistic analyses by Hirano et al. (1998) and Sawayama et al. (1999), which have miscomprehended aspects of large-scale algae production and grossly overestimated the input of fossil energy required to produce algal biofuels. He offers energy costs for all aspects of the process including fertilisers, cultivation, harvesting, oil recovery, energy content of the algal oil and of the biogas produced from the residual biomass. Indeed, as we see later, the production of algae and algal fuel becomes increasingly attractive when it is integrated with the use of other outputs, that is, biogas, and inputs, for example, carbon, nitrogen and phosphorus, that originate from smokestacks (CO_2), and N and P from sewage effluent, such that the production of algae is part of an environmental clean-up strategy (Clarens et al., 2010). A theoretical maximum for algal oil production has been estimated at 354,000 L/ha/year (38,000 gal/ac/year), to be compared with 40,700–53,200 L/ha/year (4,350–5,700 gal/ac/year) determined from a number of actual cases (Weyer et al., 2010).

If algal fuel production is to be carried out on a worldwide scale, rather than merely in tropical and equatorial locations, it is important to consider those aspects pertinent to regions of higher latitude where the ambient solar energy is considerably reduced. In principle, the majority of solar harvesting into algae could be done in sunny desert areas, for example, the Sahara, in analogy with the Desertec project which intends to collect solar energy in the Sahara desert using concentrating solar thermal (CCS) power plants which then convert it to electricity to be exported to southern Europe. It is thought that some 20% of Europe's electrical power demand could be met by this technology (Rhodes, 2010b). Baliga and Powers (2010) have presented a paper which reports a model life-cycle analysis aimed to determine the most effective operating conditions for algae biodiesel production in cold climates intended to minimise impacts on the environment and energy consumption using photobioreactors. It is assumed that the photobioreactor is adjacent to a fossil fuel or biomass power plant that provides excess heat and CO_2 to feed the algae. The model yields a high productivity for biodiesel of 19–25 L/m^2/year (160–210 tonne/ha/year) and a total life-cycle energy consumption of 15–23 MJ/L for algal biodiesel to be compared with 20 MJ/L for soy biodiesel. The energy consumption and air emissions are much lower for algal biodiesel than soy biodiesel when waste heat is utilised.

Chisti has presented a strong case for photobioreactors (Chisti, 2007). Clearly, if these are run with artificial light from lamps, the overall strategy might prove highly energy inefficient (Rhodes, 2009), but if natural light is employed, the yield of "oil" per unit area is greater than can be derived from open pond systems. For example, there are demonstrated biomass productivities leading to 136,900 L/ha of oil from algal strains with a 70%wt% lipid content and 58,700 L/ha from strains containing 30 wt% of oil. Around 80% of the oil that is produced translates into biodiesel, and so these numbers amount to 91 and 39 tonne of algal biodiesel/ha, respectively. Chisti (2007) has pointed out that while feasible technology exists to replace petroleum by algal biodiesel, at present the strategy is not economically viable, mainly due to the costs of harvesting and processing the algae crop. On October 15, 1973, the world faced its first oil crisis when the members of Organization of Arab Petroleum Exporting Countries or the OAPEC (consisting of the Arab members of OPEC plus Egypt and Syria) decided to launch an oil embargo against the West in retaliation for its support of Israel during the Yom Kippur War, also known as the Ramadan War (Bergman and Meltzer, 2004). In an awareness that the United States was particularly vulnerable to vagaries in the supply of imported oil, the Aquatic Species Program (http://www.nrel.gov/docs/legosti/fy98/24190.pdf) was founded. This was a research project implemented in the USA in 1978 under the auspices of the then President Jimmy Carter and was funded by the United States Department of Energy (DoE). The programme ran for the best part of two decades and investigated all aspects of energy production from algae and finally concentrated on the production of biodiesel from them. In excess of 3,000 algal species were evaluated, and from those species that appeared most promising, developmental work was undertaken in the effort to increase their lipid content by reducing the supply of key nutrients, such as nitrogen and silicon. It is an interesting fact that algae, when placed under such conditions of stress, tend to increase their lipid content almost as a self-defence mechanism, probably in an effort to store energy as fat. The open pond system was explored for the mass production of algae, involving the construction of 1,000 m^2 ponds in Roswell, New Mexico, with some success. However, the algal yields were inconsistent, and although 50 g of algae/m^2/day could be achieved, the yields were restricted on those occasions when the ambient temperature fell. To place this in context, this amounts to 50×10^{-6} tonne/(m^2/day) $\times 10^4$ m^2/ha $\times$ 365 day/year = 182.5 tonne/ha/year. If 50% of this mass of oil can be recovered, this amounts to 91 tonne/ha/year which is about two-thirds of the figure claimed in a study by the University of New Hampshire (Rhodes, 2009) but is in the same ballpark and could probably be optimised further. The DoE research staff compiled their work and conclusions into a report that was published in July 1998. In 1995, as part of the overall efforts to lower budget demands, the DoE decided to end the programme. The coincidence between this figure and that deduced from Chisti's result (2007) for the areal yield in a photobioreactor with a 70% oil-yielding algae should be noted, both at 91 tonne of algal biodiesel/ha.

4. Production of Algae

4.1. OPEN PONDS

Raceway-type ponds and lakes are open to the elements and are thus often referred to as "open pond" systems. They are, however, vulnerable to contamination by other microorganisms, such as invasive algal species or bacteria, and so the number of species successfully cultivated in such systems for a specific purpose (such as food, oil or pigment production) is relatively limited. Further disadvantages are that the water temperature and light intensity are not controllable. Since the growing season is largely dependent on location, and mostly limited to the warmer months, open ponds can only be used for less than half the year, unless they are placed in tropical regions. Open ponds are, however, fairly cheap to build since in its simplest form, it is only necessary to dig a trench or a pond. The production output may be very high too, in relation to other systems of comparable size and cost. The approach may be of particular advantage if the desired algae benefits from (or can survive) extreme conditions, say, of cold or salinity, that would kill off other types of algae. As an example, *Spirulina* sp. can grow in water with a high concentration of sodium bicarbonate, and *Dunaliella salina* will grow in extremely salty water. Open culture is also effective if there is a simple and inexpensive system available to select out the desired algae with which to inoculate new ponds with a high starting concentration of it: this has the effect of outcompeting other invasive strains. Some chain diatoms fall into this category because they can be filtered from the outflowing water stream, using a "pillow case" of fine muslin cloth which is tied over the end of the outflow pipe. Most kinds of algae are small enough to pass through the bag, while the chain diatoms are retained and can be used to feed shrimp larvae and to inoculate further tanks or ponds.

4.2. PHOTOBIOREACTORS

Alternatively, a photobioreactor (PBR) can be used to grow algae in, which is generally a closed system fitted with a light source of some kind. The latter may include an artificial lamp, but really any container able to transmit PAR light can act as a PBR. It is possible to vary the basic "open pond" design by covering it with a transparent or translucent barrier or to enclose the pond within a greenhouse. A pond covered with a greenhouse could be considered a PBR. While the system will most likely be smaller if made in this way, it does allow more different species to be grown and those that are being grown to remain dominant. Furthermore, the growing season may be extended, and if the system is heated, it can produce algae all year round. Due to its enclosed nature, it is necessary to introduce all the nutrients essential for the algal growth to the system directly.

A "batch mode" operation is possible for a PBR, in which a continuous stream of sterilised water can be introduced, containing nutrients, air and carbon

dioxide. As the alga grows, it overflows the reactor and is harvested. If sufficient care is not taken, continuous bioreactors often stop working very quickly, but if this induction period is successful, they should continue to operate over a long period. An advantage of this type of algae culture is that algae in the "log phase" are produced which are generally of higher nutrient content than old "senescent" algae. It can be shown that the maximum productivity for a bioreactor occurs when the "exchange rate" (time to exchange one volume of liquid) is equal to the "doubling time" (in mass or volume) of the algae.

Different types of PBRs include:

- Tanks provided with a light source
- Polyethylene sleeves or bags
- Glass or plastic tubes

It is worth noting that if artificial lamps are employed to produce the light with which to irradiate the algae in a PBR, there is the issue of overall energy efficiency to be considered. For example, while an efficiency of perhaps 6% is obtained in algal photosynthesis in terms of usefully absorbed light from the solar spectrum, natural sunlight costs nothing to produce, and whatever is gained may be considered gratis. In contrast, an artificial "sunlamp" is run on electricity that is recovered at only around the Carnot cycle determined efficiency of 36% from a conventional coal-, gas- or nuclear-fired power station, that is, two-thirds of the original fuel energy has already been lost as heat. If therefore we are only to recover 6% of that, then the overall efficiency of the process, intended to make artificial "oil" is around a mere 2%. Put another way, some 98% of the original fossil fuel energy is wasted. Using the electricity directly would be a much better deal. However, such artificially lit PBRs can become economically viable to make "value products", for example, pharmaceuticals, or to provide pure algal strains with which to initiate production in cheaper open pond systems, thus overwhelming invading competitors to grow specific kinds of algae on the large scale fuelled by free sunlight (Rhodes, 2009).

5. Screening Algae for Oil Content Using Near-Infrared (NIR) Spectroscopy

A new method (Lewcock, 2010) has been introduced for investigating the lipid content of algal strains with the view of producing biodiesel from them, based on near-infrared (NIR) spectroscopy. The near-infrared spectrum runs the range of wavelengths 800–2,500 nm and is therefore just below the visible region of the electromagnetic spectrum but above the more usually encountered mid-infrared, at 2,500–30,000 nm. The discovery of infrared radiation is attributed to the British/German Astronomer William Herschel, a polymath who in addition to his astronomical work wrote 24 symphonies. NIR only came to practical use in the 1950s as an analytical device, and while it is less sensitive than normal (mid) IR, NIR

radiation can penetrate samples more easily, meaning they need less analytical preparation and in the case of algae can be examined in their raw state. For the production of biodiesel, the "algal oil" should contain a high level of fatty acids to be converted into biodiesel: triglycerides rather than phospholipids, which are readily distinguished. IR spectroscopy measures the fundamental vibrations (stretching frequencies) of covalent chemical bonds in molecules if they have a dipole moment. NIR measures the "overtones" of fundamental stretching modes, and of coupled vibrations, and thus the spectra are more complex to assign but provide useful fingerprints of particular functional groups. The NIR method is highly specific for the detection of different kinds of fatty acids, and it is intended to develop a database of fingerprints for different fatty acid components in algal biomass, with which to analyse actual algae. The method offers the promise of a rapid and precise screening of algae directly rather than the existing time-consuming, cumbersome and error-prone wet chemical means for analysing algae and may prove pivotal in the development of a putative fuel industry based on algae (Rhodes, 2010a).

6. Processing Algae: Transesterification or Hydrothermal Liquefaction?

6.1. TRANSESTERIFICATION

Most discussions of growing algae for fuel production focus on high-lipid (high-oil-yielding) strains of algae, from which the algal oil is extracted, and this is converted to biodiesel in much the same way as oil from land-based crops like soya by transesterification (Maio and Wu, 2006): refluxing the oil in methanol with a KOH catalyst to convert triglycerides to fatty acid methyl esters plus glycerol (Eq. 1):

$$\begin{array}{l} CH_2OCOR \\ | \\ CHOCOR \\ | \\ CH_2OCOR \\ \text{(triglyceride)} \end{array} + 3CH_3OH \rightarrow 3RCOOCH_3 + \begin{array}{l} CH_2OH \\ | \\ CHOH \\ | \\ CH_2OH \\ \text{(glycerol)} \end{array} \quad (1)$$

There are some truly astounding figures about the amount of biodiesel that might be obtained from farming algae rather than from growing crops. This strategy, however, is beset by the opposing factor that most algae with a high oil content grow more slowly and in lower yield than their less-fatty analogues. The variation in lipid content of algae is illustrated in Table 2. All algae primary comprise proteins, carbohydrates, fats and nucleic acids but in varying proportions.

Table 2. Chemical composition of algae expressed on a dry matter basis (%).

Strain	Protein	Carbohydrates	Lipids	Nucleic acid
Scenedesmus obliquus	50–56	10–17	12–14	3–6
Scenedesmus quadricauda	47	–	1.9	–
Scenedesmus dimorphus	8–18	21–52	16–40	–
Chlamydomonas rheinhardtii	48	17	21	–
Chlorella vulgaris	51–58	12–17	14–22	4–5
Chlorella pyrenoidosa	57	26	2	–
Spirogyra sp.	6–20	33–64	11–21	–
Dunaliella bioculata	49	4	8	–
Dunaliella salina	57	32	6	–
Euglena gracilis	39–61	14–18	14–20	–
Prymnesium parvum	28–45	25–33	22–38	1–2
Tetraselmis maculata	52	15	3	–
Porphyridium cruentum	28–39	40–57	9–14	–
Spirulina platensis	46–63	8–14	4–9	2–5
Spirulina maxima	60–71	13–16	6–7	3–4.5
Synechococcus sp.	63	15	11	5
Anabaena cylindrica	43–56	25–30	4–7	–

Source: Becker (1994).
Algal oil is very high in unsaturated fatty acids. Some UFAs found in different algal species include arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid, gamma-linolenic acid and linoleic acid.

While the percentages differ with the type of algae, some algae contain up to 40% of their overall weight in the form of fatty acids. It is this fatty acid component (oil) that can be extracted and converted into biodiesel.

It is also necessary to dry the algae prior to extraction of the oil, which is a highly energy-intensive process. As an alternative, the method of hydrothermal liquefaction may be employed, which converts all kinds of biomass into potential gaseous and liquid fuels as is discussed in the next section.

6.2. HYDROTHERMAL LIQUEFACTION

As an alternative route to the generation of fuel from microalgae, the method of hydrothermal liquefaction may be employed (Brown et al., 2010; Patil et al., 2008; Stucki et al., 2009; Peterson et al., 2008; Tsukahara and Sawayama, 2005). In effect, the raw algae are heated in the presence of water under pressure, with or without a catalyst being present. The conditions of temperature and pressure may be adjusted so that the water enters the supercritical state (Stucki et al., 2009; Peterson et al., 2008), when it transforms from a polar liquid with a dielectric constant of around 80 under normal conditions to around 3, when it displays properties akin to an organic solvent, for example, n-hexane. The hydrothermal processing may also be carried out in the presence of acids or alkali (Ross et al., 2010). In one report (Dote et al., 1994) of a study in which *Botryococcus braunii*,

an algae with a high water content, was heated under pressure at 300°C, an oil said to be equivalent in quality to petroleum oil was obtained, in a yield of 57–64%, and >95% of which was recovered. A range of liquid and gaseous fuels (e.g. methane) can be obtained from algae using this technology.

7. An Integrated Algae/Environmental Management System

A recent study (Clarens et al., 2010) suggests that overall the CO_2 emissions attendant to producing biofuel from algae may be worse than those from corn, canola (rapeseed) or switch grass. The main problem is the use of carbon dioxide brought from elsewhere in gas bottles and inputs of fertiliser, particularly nitrogen and phosphorus. According to a life-cycle analysis, the land-based crops all were found to sequester more carbon than that emitted in growing them, while the contrary was true for growing algae, meaning that replacing fossil fuels by algal fuels could cause an overall increase in carbon emissions. On closer inspection, the report is in fact very positive about growing algae, particularly in the latter two respects. Read in more detail, the data are only in opposition to making fuel from algae if nitrogen and phosphorus nutrients are added in their mineral forms and if the CO_2 has to be injected into the system (transported as a compressed gas) as made mainly by the process of steam reforming methane (Eq. 2), along with most of the world's available hydrogen:

$$\text{(Overall)}\ CH_4 + 2H_2O \rightarrow CO_2 + 4H_2 \tag{2}$$

H_2 is used to furnish nitrogen (ammonium sulphate and nitrate) fertiliser by reacting it with N_2 via the Haber-Bosch process to make ammonia (NH_3), and so there is in a way a symbiosis between the production of CO_2 and NH_3. The phosphorus would likely be provided by mining "rock phosphate", a process which also requires energy. However, the figures in this "cradle to farm gate" analysis (i.e. they do not include the energy costs of processing the algae or other biomass into fuel *per se*) show that if the production of algae is combined with a wastewater treatment strategy, so that N and P are removed from it by the algae (an otherwise energy-intensive procedure), and fed with CO_2 from smokestacks, most of the environmental burdens attendant to growing algae are offset (i.e. an algae production plant, a power station and a sewage works should all be placed in mutual proximity). Of three possible municipal wastewater effluents evaluated as a source of N and P, the most effective was source-separated urine with a very high content of these elements, in which case growing algae became more environmentally beneficial than the land-based crops. Another life-cycle analysis (Lardon et al., 2009) essentially confirms the conclusions of Clarens et al. (2010). Two different culture conditions, nominal fertilising or nitrogen starvation, in addition to two different extraction options, dry or wet extraction, were examined. The results confirm that microalgae offer considerable potential as an energy

source but highlight the imperative necessity of decreasing the energy and fertiliser consumption during the process. Even if there remains some dispute over the exact figures used, the results emphasise the importance of developing an integrated paradigm of production and recycling for algal fuel production stressed by Rhodes (2008a, b) in the context of rare metals, which are required to maintain the electronics and solar power industries.

8. First "Artificial Cell" May Provide Source of Algal Fuel

A report (Birch, 2010) has been published describing "the first synthetic cell". What has in fact been done is to insert a chemically synthesised genome into a bacterial cell. The *M. mycoides* genome contains over a million letters of genetic code, and current DNA technology delivers perhaps a few thousand units in one go. The team led by Dan Gibson and Craig Venter has exploited the ability of yeast to join together small pieces of DNA using enzymes. Grown in a Petri dish, the synthetic bacterium looks almost identical to the natural version and can similarly self-replicate. For the development of tailor-made life, it is necessary to understand what each gene codes for. The longer run might be that genomes could be designed, but achieving that is some way off. It is more probable that a simple artificial genome could be created that has the essential properties of a living organism. This could permit other gene circuits being introduced, for example, to produce biofuels or fine chemicals. Dr Venter's company, Synthetic Genomics, intends to use the cell synthesis technology to produce modified algae cells from which to make biofuel. The aim is to make a complete algal genome from which "superproductive organisms" could be derived. It is possible that the designer method can overcome some of the drawbacks involved with making fuel from algae, namely, robustness and competitiveness of particular strains over other organisms, enhanced growth rate and yields of algal oil. The method might be the key to the widescale production of fuel from algae, which is thought to be the better option over making fuel from land-based crops such as soya (biodiesel) and corn (ethanol), since the yields are much greater and there is no competition with food-crop production and provide a real alternative to a globalised world that is utterly dependent on supplies of imported crude oil.

9. Conclusions

So, according to the title of this review, have we managed to identify fact amid fiction over algal biofuels? The present summary is highly selective, but it seems to indicate that a yield of around 40–90 tonne/ha of biodiesel can be produced using either a photobioreactor or an open (raceway) pond system, the latter under favourable conditions. If the strategy is to be implemented on the grand scale, it will entail considerable engineering and energy costs. In either case, there will be a

demand on N and P fertilisers, which depend respectively on natural gas and rock phosphate, both of which are in finite supply, and so it will be necessary in the longer term to utilise human and animal wastes to provide these elements as algal nutrients. At present, it is uneconomic to produce algae in quantities to match those of the world's petroleum consumption, which it is intended strategically to replace. If photobioreactors are to be used in earnest, they will require the production of vast amounts of plastic to fabricate them, most probably derived from crude oil. That said, algae are probably the only way forward to a future civilisation which is independent of oil.

10. References

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R. Gordon and J. Seckbach (eds.), *The Science of Algal Fuels*,
Cellular Origin, Life in Extreme Habitats and Astrobiology, 193–214
DOI 10.1007/978-94-007-5110-1_11, © Springer Science+Business Media Dordrecht 2012

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Additional projects combining his interests in archeology and biology were the study of the chemistry and molecular biology of pigments obtained from scale aphids. He identified a species of Kermes oak coccid that grew in Israel, the aphid from which the precious Biblical scarlet dye [*tola'at shani*] was extracted, providing new insights on the ancient dye trade.

Dr. Iluz participated in several research expeditions to Red Sea coral reefs and collected and analyzed bio-optical data from the First Israel-Eritrea Joint Cruise, the GAP-IOLR cruise in the Eastern Mediterranean, and in the First Israel-Seychelles Joint Cruise to the Indian Ocean. He has published 45 scientific papers and is the recipient of several international and Israeli grants.

Dr. Iluz is a popular, enthusiastic teacher and field naturalist inspiring many students to follow in his footsteps and collaborate in his research.

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Dr. Zvy Dubinsky was born in Barcelona, 18/10/1934, emigrated to Israel in 1944 where he served in the army, was member of a Kibbutz, trained as a teacher, and worked as such for a few years. He studied at Bar Ilan University where he was offered a position at the Department of Life Sciences and progressed rapidly to full professor. His contributions revolutionized views in the related fields of the biophysics of photosynthesis, algal biotechnology, and coral reef conservation.

A characteristic of his scientific philosophy has been to develop interdisciplinary approaches and seek for synthesis and integration of results in different fields such as physics, physiology, and ecology. This has resulted in the publication of some 200 scientific papers (68 in the last 10 years) in top journals and the invitation to scores of International symposia, seminars, conferences, research cruises, workshops, all over the world, guest appointments, and sabbaticals in most prestigious institutions in the USA and Japan.

Recently, he was invited by Springer to edit a new volume covering the current views and recent developments in the field of coral and coral reef research: Coral Reefs an Ecosystem in transition (2011), with the leading authorities in the field contributing chapters. An additional volume coedited by him has likewise been published by Springer: "All Flesh Is Grass: Plant-Animal Interactions" (2011).

In 2010, the European Research Council awarded him the prestigious "Advanced Award" (€ 3,300.000, for 5 years) funding his CORALWARM project studying the effects of seawater warming and ocean acidification on corals.

His activity attracted over 60 graduate students; several of them became leaders in their fields as scientists.

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ALGAL OILS: BIOSYNTHESIS AND USES

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1. Introduction

The exponentially growing human population has led to increasing energy demands all over the world. The reported current rate of consumption of petroleum is 105 times faster than the rate that nature can create it (Netravali and Chabba, 2003), and it has more than doubled over a period of 20 years {(Diaz-Tovar et al., 2011; Oil World Annual, 2009) #3580}. The burning of any fossil fuel, gas, oil, or coal adds to atmospheric CO_2, resulting in accelerated global warming and oceanic acidification. Hence, there is growing interest in biofuel, since the plants grown to produce it absorb carbon dioxide during their growth, either from the atmosphere or in aquatic plants – from its dissolved forms in water. Biofuel is a fossil-fuel replacement that is produced from vegetable oils, recycled cooking fats, waste oils, animal fats, or microalgal lipids (Table 1).

Microalgae, like higher plants, besides the ubiquitous structural membrane lipids, produce storage lipid bodies in the form of triacylglycerols (TAGs/TGL), free fatty acids (FFA) (Wang et al., 2009), and various photosynthetic pigments, also classified as lipids.

Lipids are a loosely defined group of organic compounds. The following are the main lipid groups (Fig. 1), functions, and their main biosynthesis pathways (Fig. 2):

Triacylglycerols (TAGs) are neutral lipids that are the major component of many natural oils, such as olive oil (Khandelia et al., 2010). In mammals, TAGs are present mostly inside trafficking lipoprotein particles, which transport cholesterol and TAGs between tissues (Jackson et al., 1976), and in lipid droplets (LDs) (Fujimoto et al., 2008). LDs are also present in other eukaryotes and in some prokaryotic cells that synthesize TAGs for energy and carbon storage (Waltermann et al., 2005).

Fatty acids are the building blocks in various biosynthetic pathways leading to various lipid groups as well as products generated whenever fats are broken down. These acids are not highly soluble in water and can be used for energy by most types of cells. They may be monounsaturated, polyunsaturated, or saturated. Fatty acids are components of cell membranes, hence required for their development,

Table 1. Oil yield of sources of biodiesel (Satyanarayana et al., 2011).

Source	Yield of oil (L ha^{-1} year^{-1})	Required land area (Mha[a])
Corn	172	1,540
Soybean	446	594
Canola	1,190	223
Jatropha	1,892	140
Coconut	2,689	99
Oil palm	5,950	45
Microalgae[b]	70,405	7.6
Microalgae[c]	35,202	15.2

Based on Chisti (2007)
[a]To meet 50% of all transport fuel needs of USA
[b]40% oil (% dry wt) in biomass
[c]20% oil (% dry wt) in biomass

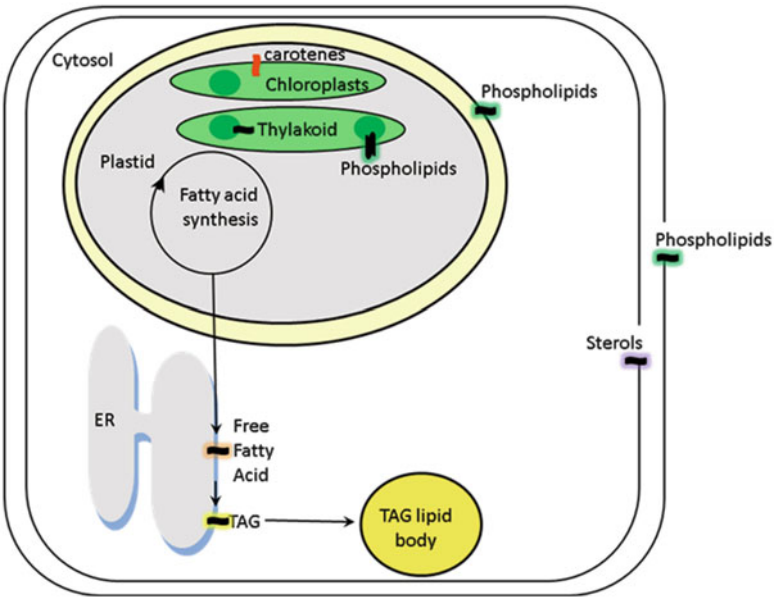

Figure 1. The location of major lipids in the algal cell (After Cowan, 2006; Nabil and Cosson, 1996; Radakovits et al., 2010).

integrity, and function. Fatty acids can be attached to other molecules, such as in triglycerides or phospholipids. When they are not attached to other molecules, they are known as "free" fatty acids (FFA) (Wang et al., 2009).

Phospholipids: This is a general term that includes all lipids containing phosphorus. However, it is a term often mistakenly equated with phosphoglycerides, the most

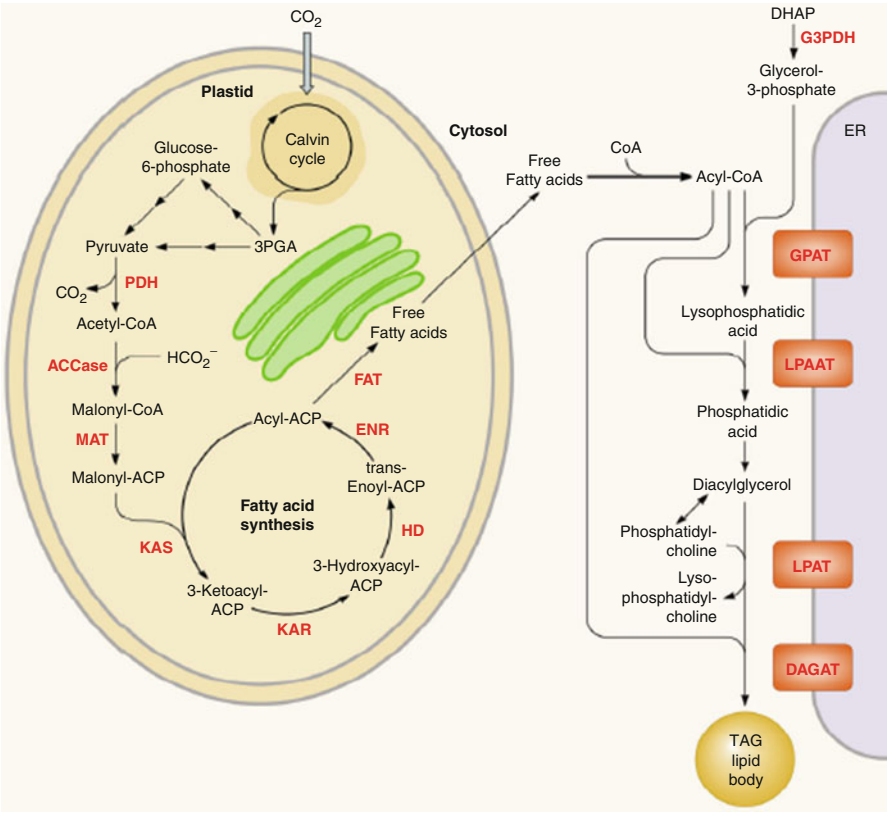

Figure 2. Simplified overview of the metabolites and main pathways in microalgal lipid biosynthesis shown in *black* and key enzymes in *red*. Free fatty acids are synthesized in the chloroplast, while TAGs may be assembled at the ER. *ACCase* acetyl-CoA carboxylase, *ACP* acyl carrier protein, *CoA* coenzyme A, *DAGAT* diacylglycerol acyltransferase, *DHAP* dihydroxyacetone phosphate, *ENR* enoyl-ACP reductase, *FAT* fatty acyl-ACP thioesterase, *G3PDH* gycerol-3-phosphate dehydrogenase, *GPAT* glycerol-3-phosphate acyltransferase, *HD* 3-hydroxyacyl-ACP dehydratase, *KAR* 3-ketoacyl-ACP reductase, *KAS* 3-ketoacyl-ACP synthase, *LPAAT* lyso-phosphatidic acid acyltransferase, *LPAT* lyso-phosphatidylcholine acyltransferase, *MAT* malonyl-CoA:ACP transacylase, *PDH* pyruvate dehydrogenase complex, *TAG* triacylglycerols (Radakovits et al., 2010).

common of the phospholipids. The major phosphoglycerides of animal tissues are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) (Tocher et al., 2008). Phospholipids play a key role in processes such as signal transduction, cytoskeletal rearrangement, and membrane trafficking (Cowan, 2006).

Phosphoglycerolipids (e.g., phosphatidylcholine and phosphatidylethanolamine) are abundant constituents of the plasma membrane, the tonoplast, and the endoplasmic reticulum.

Sterols: glycerolipids and sphingolipids constitute the major lipid classes in plants. Sterol lipids are composed of free and conjugated sterols, i.e., sterol esters, sterol glycosides, and acylated sterol glycosides. Sterol lipids play a crucial role during adaptation to abiotic stresses and plant-pathogen interactions (Wewer et al., 2011).

Sphingolipids are amphipathic molecules with varying degrees of hydrophobic and hydrophilic properties. The sphingoid base is usually 18 carbons in length. Taking into account that there are at least five different sphingoid bases present in mammalian cells, with more than 20 arrangements of fatty acids that differ in alkyl-chain length and the level of both saturation and hydroxylation, and coupled with more than 500 carbohydrate structures reported in the glycosphingolipids, the number of possible structures is considerable (Fuller, 2010).

2. Lipid Functions

2.1. STORAGE (LIQUID/SOLID)

Plant lipids are stored in the crystalline/fluid phase and the solid/gel phase.

The liquid phase is typical for young, normal cells. In this state, the membrane is flexible due to free motility of the fatty acid "tail," resulting in optimal biological functionality. This phase is abundant in unsaturated fatty acids such as linoleic, linolenic, and arachidonic acids. Lipids are also stored during periods when cell doubling is limited by shortage of nutrients such as nitrogen and phosphorus, whereas conditions for photosynthesis remain favorable (Dubinsky and Berman-Frank, 2001). Noteworthy cases are these of hydrocarbon synthesis by *Botryococcus braunii* (Fig. 3) and that of the photoprotective carotenoids β-carotene by *Dunaliella salina var bardawil* and astaxanthin by *Haematococcus pluvialis* (Fig. 9).

The solid phase is typical for senescent and defective cells characterized by tail loss and complete loss of motility. As a result, the membrane becomes rigid (Leshem, 1989).

In most plants, the common storage lipids are in the form of triacylglycerols (Murphy, 1990). There are very few examples of alternative forms of storage lipids in higher plants. Perhaps the most notable of these is the North American desert shrub, Jojoba, which stores its seed lipid in the form of liquid wax consisting of a long-chain fatty alcohol that esterifies into a fatty acid (Weiss, 1983). Another noteworthy exception is that of the green microalgae, *Botryococcus braunii*, which can store up to 75% of its dry biomass as hydrocarbons (Kalacheva et al., 2002).

2.2. STRUCTURE (IN MEMBRANES)

Plant membrane lipids are primarily composed of 16 and 18 carbon fatty acids containing up to three double bonds. Some 300 naturally occurring fatty acids have been described as found in seed oils, and it has been estimated that thousands more

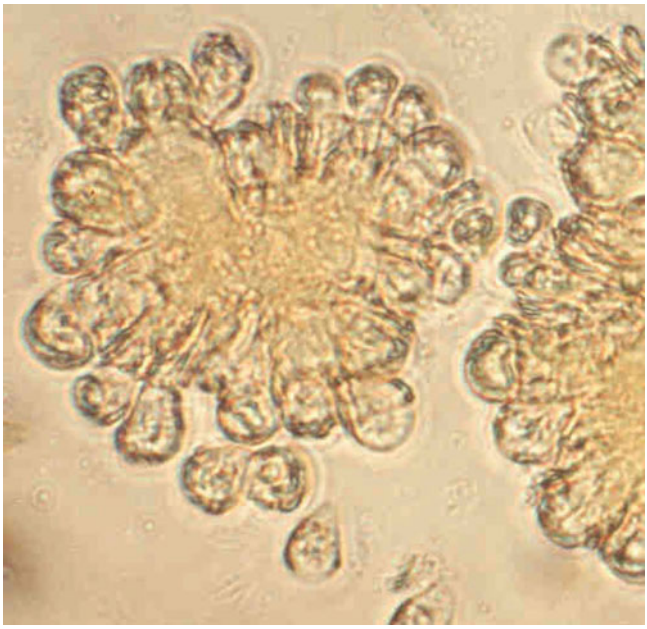

Figure 3. *Botryococcus braunii*. The live cells are seen embedded in a hydrocarbon jelly.

might be present throughout the plant kingdom. The structures of these fatty acids can vary in chain length from 8 to 24 carbons; they can have double bonds in unusual positions, or novel functional groups, such as hydroxy, epoxy, cyclic, halogen, or an acetylenic group on their acyl chain (Millar et al., 2000). Phosphoglycerides are characterized by a common backbone of phosphatidic acid (PA) (Tocher et al., 2008). The phospholipids contain a polar phosphorus head-group and a glycerol chain. In general, phospholipids fulfill structural and signaling functions in algae characterized by continued turnover of their pools (Cowan, 2006).

The thylakoid membrane of chloroplasts consists of the usual cell-membrane components and, as such, is rich in galactolipids (Yamaryo et al., 2003). The thylakoid membrane is the site of four main components of the photosynthetic apparatus, PS1, PS2, cytb6f, and ATPsynthase (Choquet and Vallon, 2000), which contain the various chlorophylls, mostly in their antennae.

2.3. PIGMENTS (SOME OF THESE ALSO BELONG TO THE LIPIDS)

Three major classes of photosynthetic pigments exist among algae and plants in general: chlorophylls, carotenoids (carotenes and xanthophylls), and phycobilins. Only the first two belong to the lipid class. Phycobilins are water-soluble protein-linked chromophores and, as such, do not belong to the lipids.

2.3.1. Chlorophylls

The basic structure of a chlorophyll molecule is a porphyrin ring, coordinated with a central magnesium atom. There are actually three main types of chlorophyll: chlorophyll a, chlorophyll b, and chlorophyll c. The first two are Mg-chlorins, which differ only slightly in the composition of a side chain ($-CH_3$ in chlorophyll a, CHO in chlorophyll b), and the last one is Mg-phytoporphyrins (Fieser and Fieser, 1956; Stryer, 1975; Zapata et al., 2006). The chlorophylls are intimately involved in all aspects of the primary events of photosynthesis: light harvesting, energy transfer, and light energy conversion. The great majority of chlorophyll molecules in the photosynthetic apparatus constitute a light-harvesting apparatus that acts as the initial photoreceptor. Electronic excitation energy that results from absorption of a photon is transferred by the light-harvesting or antenna chlorophyll to a small number of chlorophyll molecules in a photoreaction center, where the electronic excitation energy is trapped and converted to an electron (reducing capacity) and a positive hole (oxidizing capacity) (Katz et al., 1978).

2.3.2. Carotenoids

The photoacclimative plasticity of algal cell, especially that of planktonic species, which are routinely exposed to fast changes in the light intensity to which they are exposed in the course of the vertical mixing of natural water bodies, or the forced mixing in culture ponds or photobioreactors induces dramatic changes in the kind and cellular content of these pigments. In terms of their function in algal cells, they may be divided into two groups having opposite – but complementary – roles. Light-harvesting carotenoids such as peridinin and fucoxanthin expand the light harvesting of algal cells as they efficiently absorb the green wavelengths not absorbed by the chlorophylls. Photoprotective carotenoids include β-carotene and astaxanthin and protect the photosynthetic apparatus from harmful, excessively high light. Most carotenoids contain a linear C_{40} hydrocarbon backbone that includes between 3 and 15 conjugated double bonds (1, 5, 6). The number of double bonds largely determines the spectral properties of a given carotenoid (Armstrong and Hearst, 1996). Carotenoids are found in specific locations and orientations in subcellular structures, and their chemical and physical properties are strongly influenced by other molecules in their vicinity, especially proteins and membrane lipids. In turn, the carotenoids influence the properties of these subcellular structures. Structural features, such as size, shape, and polarity, are essential determinants of the ability of a cartenoid to fit correctly into its molecular environment, which is essential for it to function normally (Britton, 1995).

3. Uses of Lipids

At present, the use of microalgae in aquaculture is increasing, mostly as food for aquatic organisms, such as oysters, shrimp, and fish in artificial food chains, and for direct human consumption as “health food” additives.

Table 2. Lipid compounds and their industrial applications.

Lipid compound	Use/function
Triacylglycerides	Main constituents of edible oil[a]
Diacylglycerides	Food additive: Beneficial effects on obesity and weight-related disorders[a]
Monoacylglycerides	Emulsifiers in food industry[a]
Fatty acids	Pharmaceutical and food industries[a]
Fatty esters	Main constituents of biodiesel[a]
Tocopherols	Main constituents of vitamin E[a]
Phospholipids	Emulsifiers, lubricants, and surfactant[a]
Sterols	Starting materials for synthesis of steroids[a]
Hydrocarbons	Gas and oil[b]
Waxes	Cosmetics, pharmaceuticals, packaging, and plastics[c]
Carotenes	Antioxidants and natural coloring materials[a]

[a]D'iaz-Tovar et al. (2011)
[b]Valero (2010)
[c]Tennant (2004)

Table 3. Oil content in some microalgae (Satyanarayana et al., 2011).

Species	Oil content (% dry wt)
Botryococcus braunii	25–75
Chlorella sp.	28–32
Chlorella emersonii	63
Chlorella minutissima	57
Chlorella protothecoides	23
Chlorella sorokiniana	22
Chlorella vulgaris	40,56.6
Cylindrotheca	16–37
Crypthecodinium	20
Dunalielia primolecta	23
Isochrysis sp.	25–33
Monodus subterraneus	39.3
Monallanthus salina	>20
Nitzschia laevis	69.1
Nannochloris sp.	20–35
Nitzchia sp.	45–47
Parietochloris incisa	62
Phaeodactylum tricornutum	20–30
Schizochytrium sp.	50–77
Tetraselmis suecica	15–23

The algal biomass can be extracted as a source of chemicals for industry, toxins, glycerol, carotene, vitamins, lipids, amino acids, carbohydrates, volatile substances, and the high protein residue fed to poultry or consumed as dried whole cells (Dubinsky and Aaronson, 1982; Pulz and Gross, 2004).

In this review, the emphasis is on lipids, showing the different lipid compounds and their industrial applications (Table 2), and oil content in some major microalgae (Table 3).

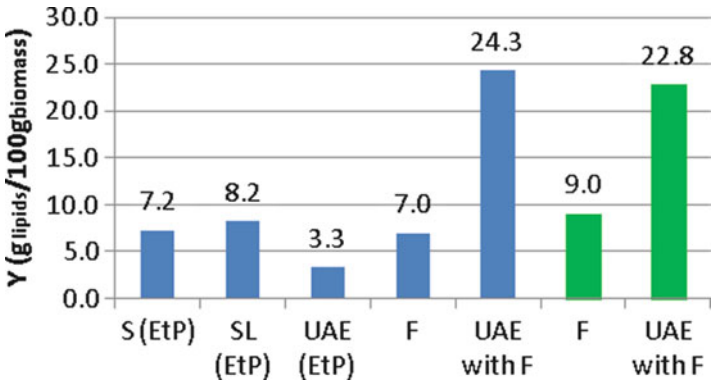

Figure 4. Lipid yield (g_{lipids}/100 g_{mass}) obtained by different techniques of extraction from *Nannochloropsis oculata* grown at 20 °C, 70 µE m^{-2} s^{-1}, and 0.3 g L-1 $NaNO_3$. (■) Wet biomass; (■) dry biomass; S (classic extraction); SL (Soxhlet); UAE (ultrasound-assisted extraction); and F (Folch method).

Vegetable oils and fats play an important role in human nutrition as a source of energy, polyunsaturated fatty acids (PUFA), and fat-soluble vitamins. Chemical industries have focused on the production of renewable sources of energy, notably biodiesel. World production of fats and oils has been growing rapidly over the past few decades and has more than doubled itself from 79.2 million tons in 1990 (Diaz-Tovar et al., 2011) to nearly 165 million tons in the year 2009 {Annual, 2009 #3580}.

3.1. EXTRACTION METHODS

There are a few lipid extraction methods: (a) lipid extraction in solution (Bligh and Dyer, 1959); (b) with ultrasonic bath (Widjaja et al., 2009); (c) Soxhlet (Virot et al., 2007); (d) with petroleum ether (EtP), using ultrasonic bath; and (e) Folch method with ultrasonic bath (Fig. 4) (Converti et al., 2009).

The use of a lipid such as diesel fuel requires a transesterification process. This is costly, and various methods are being explored in order to improve the process's economics. One such method is the production of biodiesel directly from crude dried solid microalgae mixed with methanol-chloroform and a strontium oxide (SrO) catalyst using microwave irradiation (Koberg et al., 2011).

One of the few methods available to analyze lipids according to their different constituent types is by using the Iatroscan TH-10 TLC-FID analyzer. This method – not used routinely today, involves separate analyses of two samples of total lipids in solvents designed to separate neutral and polar lipid classes, together with calibration by a composite standard similar in composition to the sample under analysis. This method does not depend on the degree of unsaturation of the fatty acids present, is rapid, and compares well in accuracy with conventional

combined gravimetric, colorimetric, and densitometric procedures (Fraser et al., 1985). The most widely currently used method is gas chromatography (GC) analysis, in which the sample is dissolved in ethyl acetate and then injected into GC-17A device equipped with a nonpolar column and a flame ionization detector (Widjaja et al., 2009).

4. Factors That Affect Algal Lipid Production

4.1. TEMPERATURE

The effect of temperature on lipid composition in microalgae as well as in bacteria is related to their melting points. The more a lipid is saturated, the higher its melting point, hence at low temperatures, unsaturated lipids are advantageous, and specialized desaturases are activated in several organisms when they encounter low ambient temperatures (Harwood and Guschina, 2009). The effect of growth temperature on lipid content was investigated in the microalgae *Nannochloropsis oculata* and *Chlorella vulgaris*. Both species showed a change in lipid content when temperature was altered. *C. vulgaris* had the highest lipid productivity when the temperature was 25°C (20.22 mg_{lipid}/L^{-1} day^{-1}). When temperature increased, productivity decreased to 8.21 mg_{lipid}/L^{-1} day^{-1} (at 35°C) (Converti et al., 2009). For *N. oculata*, there was a small change in lipid content when the temperature was altered beyond the optimal growth temperature of 20°C (10.01 mg_{lipid}/L^{-1} day^{-1}), and at 15 and 25°C, it was 9.11 and 10.1 mg_{lipid}/L^{-1} day^{-1}, respectively (Converti et al., 2009).

Growth and total lipid content of *Spirulina platensis* (UTEX 1928) were affected by changes in growth temperature from 25 to 38°C. With increased growth rate, total lipid content increased (Fig. 5) (Tedesco and Duerr, 1989).

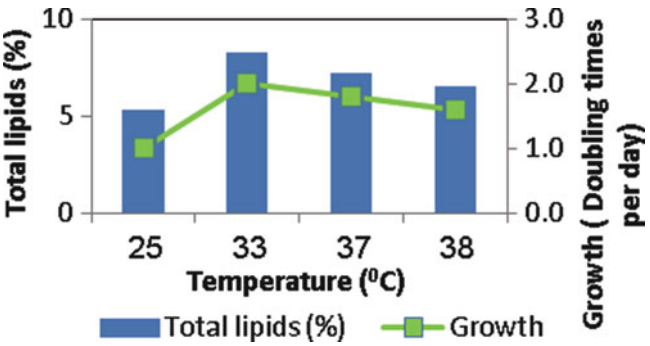

Figure 5. Comparison of the total lipid content and doubling time of *S. platensis* (UTEX 1928) at different temperatures.

Table 4. Comparison of lipids and fatty acids of *S. platensis* (UTEX 1928) at three culture temperatures.

Temp (°C)	Growth	Total lipid	Total fatty acids	Unsaturated/ saturated fatty acids	Major fatty acids (% of total fatty acids)					
					16:0	17:0	18:0	18:1	18:2	18:3
25	1.0	5.3(1.8)	1.8(0.2)	0.81	52.7(0.6)	1.9(0.1)	1.3(0.1)	1.6(0.2)	7.7(0.3)	35.0(0.9)
33	2.0	8.3(1.4)	2.0(0.1)	0.79	54.5(0.5)	*	1.2(0.2)	2.3(0.2)	7.0(0.5)	34.7(0.6)
37	1.8	7.2(0.2)	2.2(0.2)	0.78	54.4(1.0)	*	1.8(0.2)	4.4(0.7)	7.7(1.4)	31.7(1.7)
38	1.6	6.5(5.1)	1.4(0.1)	0.68	56.6(1.1)	*	2.9(0.6)	5.5(0.2)	6.9(0.3)	28.3(1.1)

Growth was measured as number of doublings per day. Total lipid and total fatty-acid levels are listed as % of dry weight. All numbers are the mean of four replicate cultures (except at 33 °C, where $n = 3$). The values in parentheses denote the standard deviation. Trace amounts of fatty acids are indicated with an asterisk *

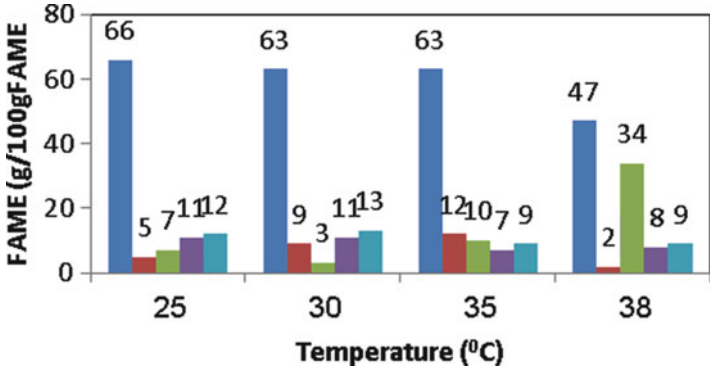

Figure 6. Percentages of individual fatty-acid methyl esters (FAMEs) on the total FAMEs (g/100 gFAME) in *C. vulgaris* at different temperatures.

The lipid composition of *Spirulina platensis* (UTEX 1928) and *C. vulgaris* was investigated and the results given in Table 4 (Tedesco and Duerr, 1989) and Fig. 6 (Converti et al., 2009), respectively.

Lipid composition was investigated at various growth temperatures in the cyanobacterium *Anacystis nidulans*. When growth temperature was changed from 38 to 22 °C, the content of digalactosyldiglyceride decreased, and the contents of monogalactosyl- and sulfoquinovosyldiglycerides increased, while the content of phosphatidylglycerol remained constant (Sato et al., 1979). A similar change in lipid composition with growth temperature was reported in a unicellular alga, *Cyanidium caldarium* (Kleinschmidt and McMahon, 1970).

4.1.1. Light Intensity

The effect of light on total lipid content was investigated in *Spirulina platensis* (UTEX 1928) at several intensities, ranging from 170 to 1,400 µmol photons m^{-2} s^{-1}. It was shown that light intensity affected growth rate and total lipid content, while

Table 5. Total lipid and fatty-acid content (% dry weight) and composition of *S. platensis* (UTEX 1928) under various light intensities.

Light	Growth	Total lipid	Total fatty acids	Unsaturated/ saturated fatty acids	Major fatty acids (% of total fatty acids)				
					16:0	18:0	18:1	18:2	18:3
170	0.9	11.8(2.7)	2.3(2.7)	0.76	55.5(1.1)	1.4(0.7)	3.8(0.9)	6.5(0.5)	33.2(1.7)
330	1.3	8.0(1.0)	1.7(0.1)	0.83	52.5(2.9)	2.2(0.8)	6.3(1.0)	7.8(1.1)	31.0(1.7)
600	1.8	7.2(0.2)	2.2(0.2)	0.78	54.4(1.0)	1.8(0.2)	4.4(0.7)	7.7(1.4)	31.7(1.7)
860	2.1	3.2(0.8)	1.5(0.3)	0.73	54.7(1.6)	3.0(0.5)	3.5(1.0)	7.8(1.0)	31.1(1.1)
1,400	2.2	5.8(1.7)	2.3(0.1)	0.86	52.2(0.8)	1.7(0.2)	2.1(0.1)	8.0(0.3)	36.0(0.8)

The standard light condition was 600 µmole photons m^{-2} s^{-1}. Growth was measured as the number of doublings per day. All numbers are the means of four replicate cultures. The values in parentheses give the standard deviation (Tedesco and Duerr, 1989)

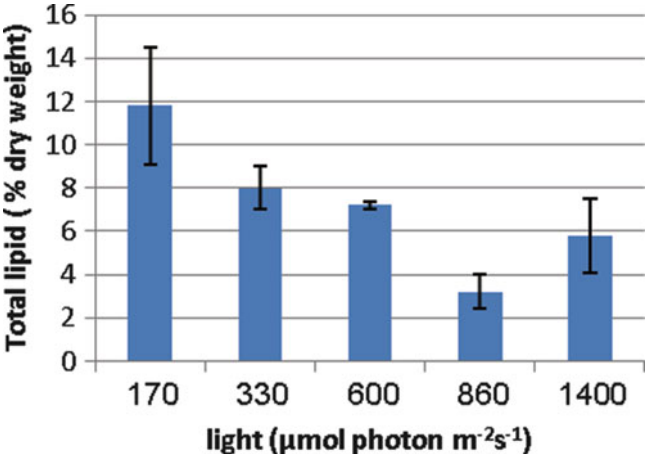

Figure 7 Total lipid (% dry weight) content of *S. platensis* (UTEX 1928) under various light intensities (Tedesco and Duerr, 1989).

growth rates declined with the increasing culture density. Total lipid as a percentage of dry weight decreased as light intensity increased, except at the highest irradiance (Table 5; Fig. 7) (Tedesco and Duerr, 1989).

Another experiment on light intensity was conducted with six strains of marine diatoms: *Cylindrotheca fusiformis* (B211), *Phaeodactylum tricornutum* (B114, B118, and B221), *Nitzschia closterium* (B222), and *Chaetoceros gracilis* (B13). The number of total lipids of B13, B114, and B211 grown at 5,000 lx was lower than those grown at 1,500 Ix. No evident changes were observed in Bl18, B221, and B222 (Table 6; Fig. 8) (Liang et al., 2001).

In Fig. 9, it is shown that different irradiance levels can affect the pigment expression in the green algae *Haematococcus pluvialis.* Above a minimal irradiance

Table 6. The final biomass (dry weight) and total lipids of 6 diatoms strains (Liang et al., 2001).

	DW (g/L)		Total lipids (% DW)	
Microalgal strains	**1,500 lx**	**5,000 lx**	**1,500 lx**	**5,000 lx**
B13	0.35 ± 0.03	0.31 ± 0.22	10.78 ± 2.69	6.97 ± 2.93
B118	0.25 ± 0.00	0.54 ± 0.18	3.64 ± 0.01	4.32 ± 2.33
B221	0.30 ± 0.01	0.46 ± 0.02	5.93 ± 1.03	5.90 ± 4.04
B114	0.40 ± 0.06	0.65 ± 0.13	13.38 ± 1.80	5.18 ± 1.83
B211	0.45 ± 0.39	0.68 ± 0.09	15.93 ± 0.91	13.00 ± 0.28
B222	0.49 ± 0.05	0.72 ± 0.02	4.14 ± 4.00	5.38 ± 1.79

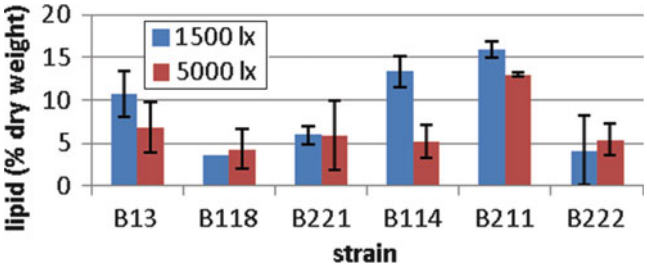

Figure 8. Total lipids (% dry weight) of six diatom strains under various light intensities (Liang et al., 2001).

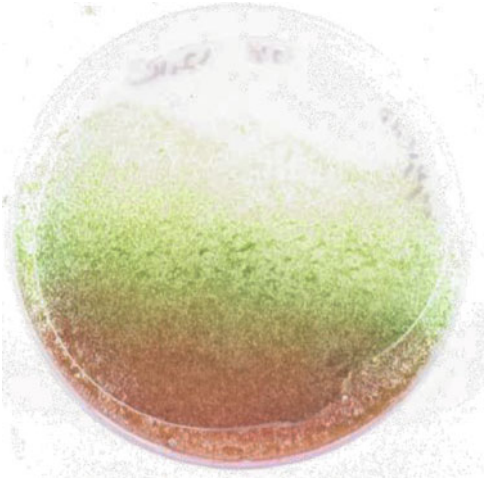

Figure 9. The effect of irradiance level on pigmentation in *Haematococcus pluvialis*. The light gradient went from darkness at the top towards ~1,000 μmol photons m^{-2} s^{-1}. The *red* pigment is astaxanthin.

level, the light-harvesting green chlorophylls (a and b) predominate, whereas under increasing light intensity, their cellular content decreases and the photoprotective carotenoid astaxanthin becomes visible, masking the green color. High light stress is used in the biotechnological production of astaxanthin, mostly as a component in fish feed pellets required in the culture of salmon.

4.1.2. Salinity

The effect of salinity on total lipid content and triacylglycerides (TGs) was investigated in *Dunaliella* cells. An increase of the initial NaCl concentration from 0.5 (equal to seawater) to 1.0 M resulted in higher intracellular lipid content (67.8% are lipids while – of them – 57% are TGs in comparison with 60% being lipids and 41% of the lipids are TG, respectively) for a salt concentration of 0.5 M. The addition of 0.5 or 1.0 M NaCl at the mid-log phase or at the end of the log phase during cultivation with the initial NaCl concentration of 1.0 M further increased the lipid content (71% of the dry weight were lipids, 34% of which are TGs, in comparison with 70% lipids and 32% of them TGs, respectively) (Takagi et al., 2006).

The effect of salinity on *Botryococcus braunii (LB 572)* was investigated. Two-week-old culture of *B. braunii LB 572* grown in modified Chu 13 medium was used as an inoculum at 20% (V/V); sodium chloride was added to the flasks in the range of 17–85 mM and inoculated. The total fat content of the alga grown at different salinities varied in the range of 24–28% (w/w), whereas in the control, it was 20% (Rao et al., 2007).

4.1.3. Nitrogen (N) Starvation

Under nutrient-sufficient conditions, cells synthesize mainly proteins to support growth and division (Myers, 1980). However, when a culture is deprived of an essential nutrient, cell division is stopped, and the fraction of carbon allocated to lipids and carbohydrates can be greatly increased at the expense of protein synthesis (Sukenik and Wahnon, 1991).

The effect of nitrogen starvation on lipid content was investigated in cyanobacterium *Spirulina platensis*. It was shown that the lipids decreased slightly over the first 40 h of N-starvation then increased for the next 40 h. The fatty acids decreased rapidly from approximately 2–1.2% of dry weight with N-starvation (Tedesco and Duerr, 1989).

A green microalga, *Chlorella vulgaris*, was tested for N-starvation effect. It was found that the lipid content was higher with longer incubation time, which led to less nitrogen concentration in the medium. It was also shown that longer time of nitrogen starvation resulted in higher accumulation of lipids inside the cells (Fig. 10) (Widjaja et al., 2009) and different composition (Fig. 11) (Converti et al., 2009).

The effect of N-starvation on lipid content was investigated in *Chlorella* sp. and *Phaeodactylum tricornutum*, which were grown for 7 days in rich media (control) and then harvested and transferred to media without nitrogen.

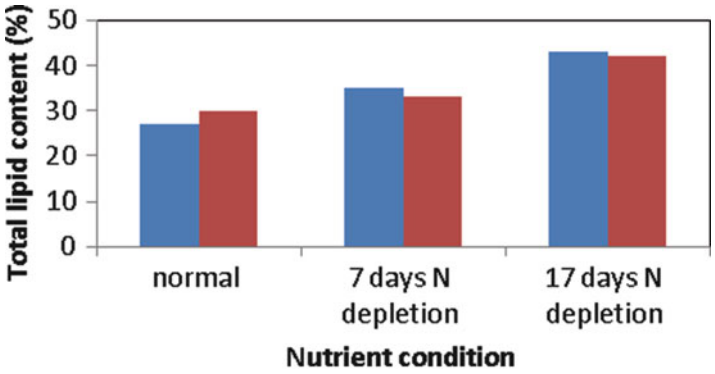

Figure 10. Comparison of total lipid content during normal nutrition and nitrogen starvation at CO_2 flow rate of 20 mL/min. Incubation time under normal nutrition was conducted for 15 days ( ) and 20 days ( ). After normal nutrition, the medium was changed to a nitrogen-depleted one and growth continued for 7 and 17 days. Total lipid content was calculated as the w/w ratio of the chloroform/methanol soluble fraction to dried algal sample. Data were expressed as mean values ($n = 3$) (Widjaja et al., 2009).

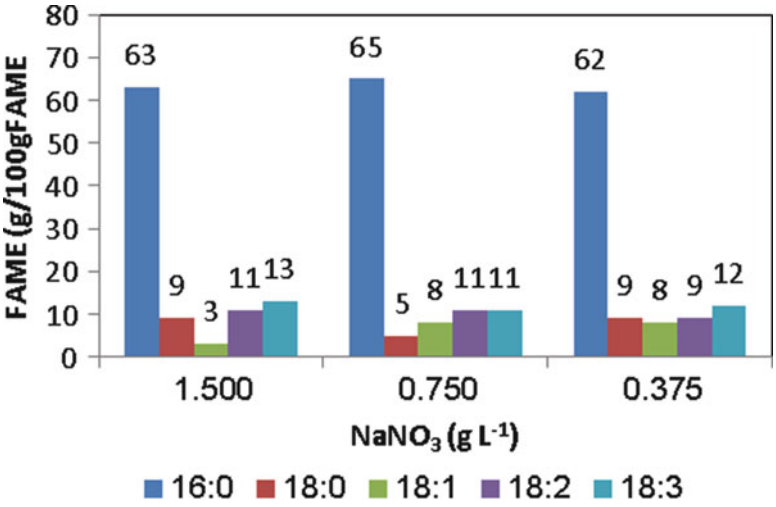

Figure 11. Percentages of individual fatty-acid methyl esters (FAMEs) on the total FAMEs ($g/100g_{FAME}$) in *C. vulgaris* at different concentrations of $NaNO_3$ in the growth medium (Converti et al., 2009).

Samples were stained with Nile red (Doan and Obbard, 2010) and examined in FACS (Gallios Flow Cytometer, Beckman Coulter) (Fig. 12; Table 7). In *chlorella* sp. samples, the mean fluorescence values in the nitrogen-starved samples were more than five times higher than in the control samples. Chemical tests (Koberg et al., 2011) showed a lipid yield of 11% in the control samples compare to 54%

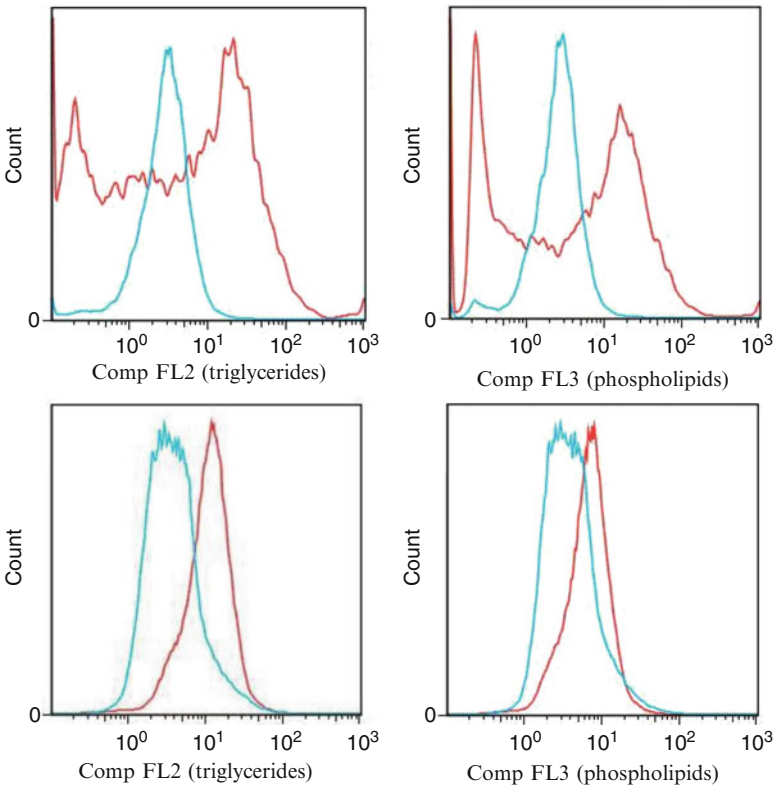

Figure 12. *Nile red* staining of *Chlorella* sp. (**a**, **b**) and *Phaeodactylum tricornutum* (**c**, **d**), as obtained by FACS. Comparison between fluorescence histograms of algal cultures grown under nitrogen starvation (*red*) and control cultures grown in rich media (*blue*). Excitation was at 488 nm; emission in channel FL-2: 575 ± 20 nm for triglycerides; in channel FL3: 620 ± 15 nm for phospholipids (note that the *x*-axis is a $\log^{10}$ scale).

Table 7. Mean fluorescence values of *Chlorella* sp. (a, b) and *P. tricornutum* (c, d).

		Growth medium	Mean FL2-A	Mean FL3-A	Cell no.
a, b		Bristol medium	3.74	3.26	16568
		Bristol medium w/o nitrogen	19.5	17.7	11288
c, d		Sludge water	5.67	5.63	119742
		F2 + Si medium w/o nitrogen	12.9	7.78	137242

Samples stained by Nile red

in microalgae grown under nitrogen-starvation conditions. In the *P. tricornutum* samples, mean fluorescence values of the triglycerides and the phospholipids in the nitrogen-starved samples were about 2.3 and 1.4 times higher than in the control, respectively (Topf and Dubinsky, unpublished).

5. Summary

Microalgae have the potential to be the next "hot item" in many areas, from energy crops to health-food sources. Their growth rates exceed by orders of magnitude those of any high plant "energy crop," combined with unique biochemical plasticity. These properties allow for maximal areal lipid yields and product quality control according to biodiesel specifications. Future lipid production from microalgae are likely to depend on the choice of appropriate algae from among the many not-yet explored and exploited species found in Nature.

Maximization of yields depends on maintaining the right balance between high quantum efficiencies and high photosynthetic rates.

High lipid yields require redirecting biosynthesis away from cell doubling towards lipid accumulation favored by nutrient limitation.

Algae agriculture is a relatively new area that has only emerged on a significant scale during the last century; therefore, we still need to learn the many novel aspects of this field.

Microalgae can be an ideal source of biofuel by using wastewater effluent as a source of essential nutrients while absorbing CO_2 from power stations and industrial smokestacks. Culturing algae on seawater in barren desert areas ensures it will not compete with agricultural food production resources.

The economic feasibility of biodiesel production based on microalgae depends on additional income from wastewater treatment, CO_2 sequestration, and extraction of valuable products from extracted residues.

Algal-oil production costs can be minimized since while generating biodiesel, we also produce valuable fine chemicals, such as vitamins, omega 3, polyunsaturated fatty acids (arachidonic and linoleic), carotenoids (e.g., astaxanthin and β-carotene), vitamin E (alpha-tocopherol), and water- and lipid-soluble antioxidants, besides the proteinaceous extracted meal. These valuable byproducts have great potential in the food, cosmetics, and pharmaceutical industries, while the extracted meal is a high protein component suitable for incorporation in animal feed.

We should not forget that the yield is a product of content and growth rate; hence, finding the best growth rate with the right conditions is an important consideration to keep in mind when aiming at minimizing expenditures while maximizing product yield.

It should be kept in mind that the technology of today will not be the technology of the future, and the algae species will probably be different from what we know nowadays.

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R. Gordon and J. Seckbach (eds.), *The Science of Algal Fuels*,
Cellular Origin, Life in Extreme Habitats and Astrobiology, 215–230
DOI 10.1007/978-94-007-5110-1_12, © Springer Science+Business Media Dordrecht 2012

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BIOFUEL PRODUCTION FROM ALGAE THROUGH INTEGRATED BIOREFINERY

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1. Introduction

Today, all over the world, energy crisis and environmental issues are the most important concern. It is projected that world energy demand will continue to expand by 45% from 2008 to 2030, an average rate of increase in 1.6%/year (World Energy Outlook, 2008). Nearly 81% of the energy supply is from fossil fuels, followed by 16% renewable energy and 2.8% nuclear energy (Fig. 1). Use of fossil fuels as energy is unsustainable due to depleting resources and the accumulation of greenhouse gases in the environment (Demirbas, 2010). Another problem is their uneven distribution in the world where 63% of the global petroleum fuel resources are located in the Middle East with equity, environmental, economic and geopolitical implications (Hacisalihoglu et al., 2009). Biomass-based energy can serve as an alternative energy source to meet the present and future demand, including transportation fuel, although presently only about 0.6% of transportation fuel are supplied as biofuels.

Biofuels include use of solid biomass, biohydrogen, biogas and liquid fuels such as bioethanol and biodiesel. Biological hydrogen production is still in an incipient phase; therefore, the most commonly used biofuels, aside from the burning of solid biomass, are biogas, biodiesel and bioethanol. According to Global Status Report Renewable-2011, about 86 billion litres of bioethanol and 19 billion litres of biodiesel were produced in 2010 compared to 17 billion litres of bioethanol and 0.8 billion litres of biodiesel produced in 2000 (Fig. 2). The USA is the biggest producer of biofuel, followed by Brazil and Germany (Fig. 3). Based on the source of feedstocks, liquid biofuels can be classified into four generations: First-generation biofuels are produced from food crops such as sugar cane, soybeans, cassava, potatoes, maize, etc., and animal fats; second-generation biofuels derived from non-food crops such as *Jatropha*, tobacco, *Miscanthus*, switch grass, wood, wheat straw, waste fruit pulp, etc.; third-generation biofuel from algae; and fourth-generation biofuel from genetically engineered organisms (Demirbas, 2011). In this chapter, we discuss various aspects of algal biofuel production through an integrated biorefinery approach.

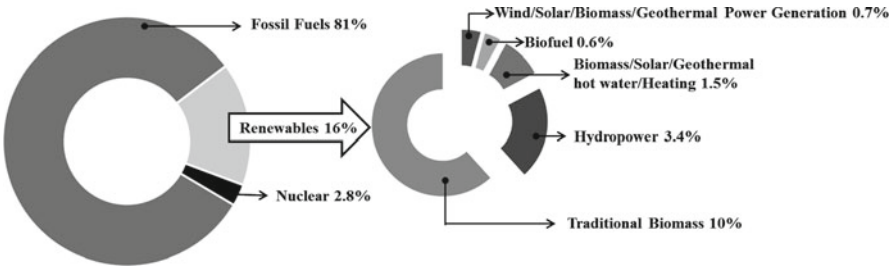

Figure 1. Global energy consumption (Renewables 2011-Global Status Report).

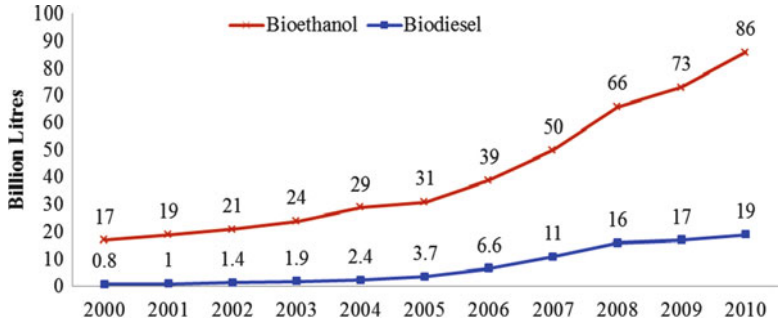

Figure 2. Bioethanol and biodiesel production, 2000–2010 (Courtesy F.O.Licht, Renewables 2011-Global Status Report).

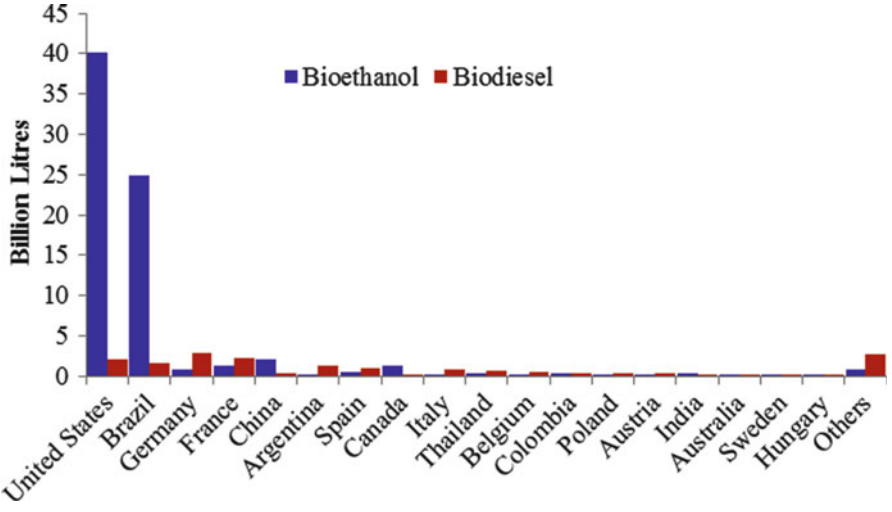

Figure 3. Top 20 bioethanol and biodiesel producer in the world (Reproduced from Biofuels Platform, 2010).

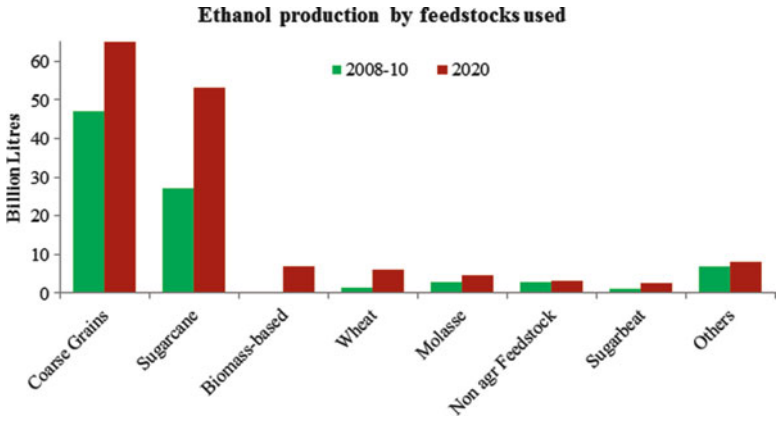

Figure 4. Bioethanol production from various feedstocks (Courtesy Biofuels-OECD-FAO Agricultural Outlook 2011–2020).

1.1. BIOETHANOL

Ethanol is a high-octane fuel which can be used in various combinations with petrol or gasoline such as E10 or E85 containing 10 and 85% ethanol, respectively. When blended with fossil fuel, bioethanol reduces cancer-causing compounds such as benzene, toluene, xylene and ethyl benzene. Bioethanol can be produced either from sugar, starch or lignocellulosic biomass. Sugar can be directly converted into bioethanol by yeast fermentation, whereas starch first needs to be converted to simple sugar like glucose, through a process called saccharification, before being fermented to ethanol. Production of ethanol from lignocellulosic materials is a more complex process, which is not yet at a commercial stage. Various feedstocks such as sugar cane, corn, cereal grains, potato, sweet potato, cassava and other plant materials are being used for the production of bioethanol all over the world (Fig. 4). World ethanol market is projected to reach around 105 billion litres in 2012 (Martin et al., 2010).

1.2. BIODIESEL

Biodiesel is defined as the monoalkyl esters of vegetable oil and animal fats (ASTM, 2008) and is produced by the transesterification of triglyceride with monohydric alcohols. Biodiesel is generally similar to petroleum-derived diesel in its main characteristics such as cetane number, energy content, viscosity and phase changes (Lin and Teong, 2010) and can be blended in any proportion with fossil-based diesel. Therefore, biodiesel has become the most common liquid biofuel in the world after ethanol. Biodiesel are mainly produced from vegetable oil such as,

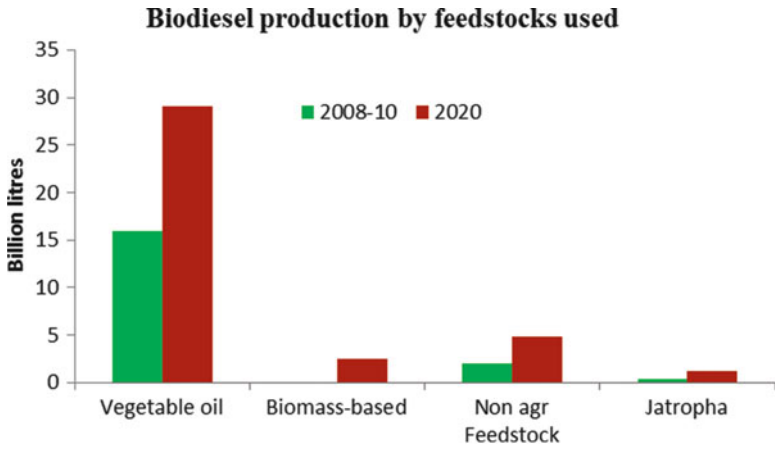

Figure 5. Biodiesel production from various feedstocks (Courtesy Biofuels-OECD-FAO Agricultural Outlook 2011–2020).

soybean, palm, sunflower oil, followed by biomass-based and non-agricultural feedstock (Demirbas, 2009), (Fig. 5). Huang et al. (2010) claim that biodiesel use can decrease by 90% air toxicity and by 95% cancers resulting from fossil diesel use.

2. Need for Alternative Feedstock for Biofuel

Biofuels offer a potential source of renewable energy and possibly large new markets for agricultural producers. But current biofuel programmes are unsustainable from environmental, economic and societal standpoints. The use of corn, sugar cane and vegetable oil has driven the food versus fuels debate because these feedstocks are components of the human food chain (Mata et al., 2010). Large-scale production of biofuels from crop plants usually damages the environment by the use of harmful pesticides and fertiliser, mostly nitrogen, which reduces the fertility of the soil (Fig. 6). Martin et al. (2010) discussed the repercussion of excessive use of agricultural land and water (Table 1). Water requirements also depend on the geographic, climatic variables and type of feedstock used.

3. Algae for Biofuel Production

Algae a novel biofuel feedstocks have several potential advantages including higher area productivity than traditional crops (Posten and Schaub, 2009; Sahoo, 2010), no competition with conventional agricultural land and utilisation of different water sources (e.g. seawater, brackish water and wastewater). Terrestrial plants in

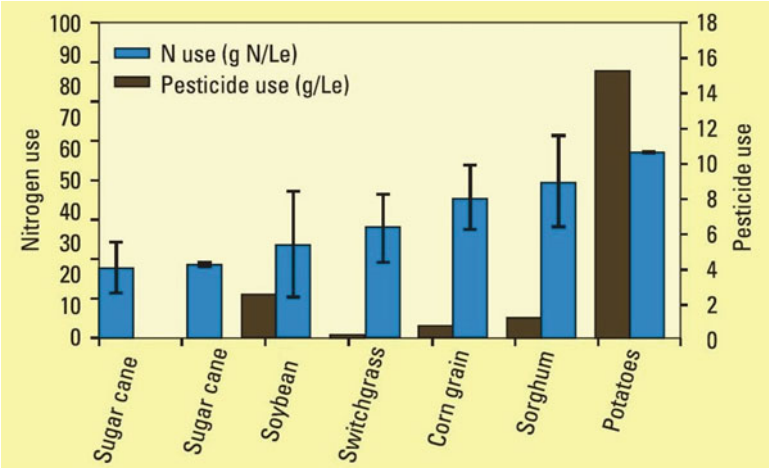

Figure 6. Use of nitrogen and pesticides for various biofuel feedstocks (Courtesy Faus et al., 2009).

Table 1. Water footprint, energy consumption and biofuel production (Courtesy Singh et al., 2011).

	Plant	Water footprint (m^3 GJ^{-1})	Land use (m^3 GJ^{-1})	Energy (GJ $ha^{-1}a^{-1}$)	Biofuel yield (L $ha^{-1}a^{-1}$)
Bioethanol	Cassava	148	79	126	6,000
	Wheat	93	305	33	1,560
	Paddy rice	85	212	47	2,250
	Corn grain	50	133	75	3,571
	Potatoes	105	114	88	4,167
	Sugar cane	50	81	124	5,882
	Sugar beet	46	95	105	5,000
	Sorghum	180	386	26	1,235
	Soybean	383	386	26	1,235
Biodiesel	Soybean	383	689	15	446
	Jatropha	396	162	62	1,896
	Rapeseed	383	258	39	1,190
	Cotton	135	945	11	325
	Sunflower	61	323	31	951
	Oil palm	75	52	192	5,906
	Coconut	49	128	78	2,399
	Groundnut	58	220	45	1,396
	Microalgae	<379	2–13	793–4,457	24,355–136,886

temperate climates can presently achieve a photo conversion efficiency of only about 1%, while microalgae might in the future convert up to 5% of the solar energy into chemical energy (Schenk et al., 2008). Several microalgae such as *Botryococcus, Scenedesmus, Chlorococcum* and *Chlorella* contain significant

Table 2. Biochemical composition of some biofuel feedstock.

	Plant	Carbohydrate	Protein	Lipid	References
	Soybean	25.4	46.7	21.2	Nikolić and Lazić (2011)
Crop plants	*Jatropha*	30.11	32.88	27.36	Azza and Abu-Salem (2010)
	Rapeseed	NA	NA	40–48	Carioca et al. (2009)
	Castor	NA	NA	43–45	Carioca et al. (2009)
	Palm Oil	0.4	0	99.6	Atchley (1984)
	Sugarcane, bagasse	75–80	1.5–2	<1	Han et al. (1983)
	Maize	66–76	5–13	NA	FAO (1993)
	Cassava	80–85	1–2	Trace	Charles et al. (2005)
	Sorghum	65–72	9–13	3–4	Neucere and Sumrell (1980)
Seaweeds	*Caulerpa lentillifera*	44–46	11–12	1–2	Matanjun et al. (2009)
	Ulva lactuca	70	7.06	1.64	Wong and Cheung, 2000
	Eucheuma cottonii	35–36	10–12	1–2	Matanjun et al. (2009)
	Gracilaria cervicornis	63	19.7	0.427	Marinho-soriano et al. (2006)
	Hypnea japonica	57.4	19	1.42	Wong and Cheung (2000)
	Sargassum vulgare	61	13.6	0.491	Marinho-soriano et al. (2006)
	Laminaria hyperborea	50–52	8.9	<1	Horn (2000)
	Ascophyllum nodosum	45–55	4.8–9.8	1–5	Horn (2000)
Microalgae	*Botryococcus braunii*	2	40	33	Eduardo et al. (2010)
	Prymnesium parvum	25–33	28–45	22–38	Singh et al., 2011
	Isochrysis sp.	15–5	29.5	23.4	Renaud et al. (1999)
	Scenedesmus dimorphus	21–52	8–18	16–40	Demirbas (2010)
	Chlorella vulgaris	12–17	51–58	14–22	Demirbas (2010)
	Porphyridium cruentum	40–57	28–39	9–14	Demirbas (2010)
	Spirogyra sp.	33–64	6–20	11–21	Demirbas (2010)

amount of lipids, whereas macroalgae such as *Sargassum, Laminaria, Ascophyllum, Gracilaria* and *Kappaphycus* are higher in their carbohydrate contents which make them possible feedstocks for biodiesel and bioethanol production, respectively (Table 2), (Fig. 7a–d). The use of algae for biofuel was investigated in the USA and Japan as an alternative energy source from the 1970s to 1990s after the oil crisis, but the studies were discontinued when oil prices stabilised (Yokoyama et al., 2007).

3.1. SEAWEEDS FOR BIOETHANOL

Marine macroalgae (seaweeds) lack lignin but contain high amount of carbohydrates which makes them potentially suitable feedstock for the production of bioethanol. The cell wall of algae consists of various forms of complex carbohydrates such as cellulose, hemicellulose, agar, alginate, carrageenan, fucoidan (Kloareg et al., 1986; Goh and Lee, 2010), the latter four being extracted and used in food, personal care products and some industrial applications (Sahoo, 2000). Seaweed industrial wastes, i.e. the remaining pulp after extraction of the high value

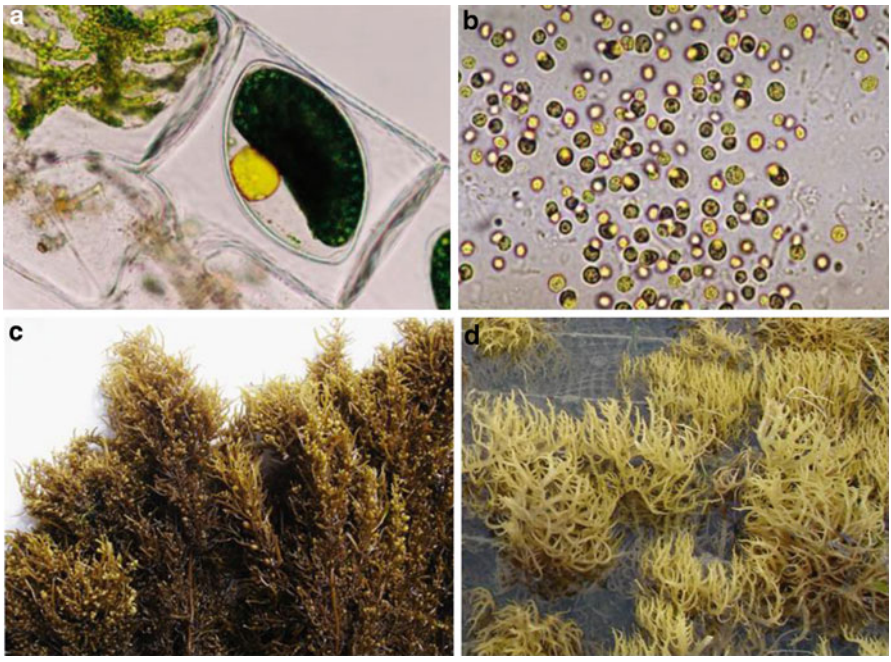

Figure 7. (**a**–**d**) Some of the potential algal species for biofuel production. (**a**) *Spirogyra* sp. showing oil droplets inside the cell, (**b**) *Chlorella* sp. showing accumulation of lipid in the cells, (**c**) *Sargassum* sp. and (**d**) *Kappaphycus* sp. under cultivation.

polysaccharides, still contain high amount of carbohydrate which may be used as a source of raw material for ethanol production (Kumar and Sahoo, 2012). It will also reduce organic load from sea which was washed and deposed into the sea during phycocolloids extraction process (Ge et al., 2011). Utilisations of seaweeds for bioethanol production are only of economic interest when integrated with a utilisation of the higher value components. Seaweeds and waste products can also be used for biogas production through anaerobic digestion (Gunaseelan, 1997).

3.2. PROCESSING OF SEAWEEDS BIOMASS FOR BIOETHANOL PRODUCTION

Seaweed phycocolloids can be converted into bioethanol, but direct use of these phycocolloids for bioethanol production will not be cost-effective. So, after the extraction of phycocolloids, the remaining pulp can be used for bioethanol production.

Since the pulp contains high amount of carbohydrate and other organic materials, these can be converted into bioethanol through saccharification and fermentation. *Saccharomyces cerevisiae*, a common yeast, and *Zymomonas mobilis,*

a bacterium, are the two most important microorganisms used for bioethanol production (Dumsday et al., 1997), but they have a very narrow substrate range. However, *Pichia angophorae* is a more suitable organism for ethanol production from seaweed extract. It can utilise both substrates, mannitol as well as laminaran, simultaneously (Horn et al., 2000).

Apart from alginate, agar and carrageenan, the cell wall of algae also contains cellulose, fucoidan and protein. Anaerobic degradation of fucoidan has not been reported (Forro, 1987), and algal proteins have been reported to have a low digestibility (Michel et al., 1996). This may be due to their cellular localisation or their putative associations with cell wall polysaccharides (Kloareg and Quatrano, 1988). Presence of polyphenols and salt (Ghosh et al., 1981) reduces the biodegradability of algae. For most algae, aspartic and glutamic acids constitute together a large part of the amino acid fraction (Fleurence, 1999). Degradation of cellulose is catalysed by cellulases and occurs both under aerobic and anaerobic conditions. A combined enzymatic attack of agarases, alginate lyases, proteases and cellulases may be necessary to degrade the algal cell wall, as seen in the case of protoplast isolation (Butler et al., 1989).

3.3. MICROALGAE FOR BIODIESEL

Microalgae appear to be one of the important sources to capture solar energy as they are sunlight-driven cell factories that convert carbon dioxide to potential biofuel, food, feeds and high bioactive compounds (Metting and Pyne, 1986; Spolaore et al., 2006). Some species of microalgae contain much higher percentage of oil than conventional oil crops (Table 3). Microalgae can duplicate their biomass in less than 7 days, whereas higher plants take many months or years (Vonshak et al., 1982). Another advantage of microalgae is that their chemical composition can be manipulated by altering the growth environment of the algal species. Carbon dioxide emitted from combustion processes such as power plant, cement plant, steel plant, etc., can be used as a source of carbon for algal growth (Sahoo et al., 2012). Microalgae can be cultivated in seawater or brackish water, raceway ponds on non-arable land and do not compete for resources with conventional agriculture. Microalgal biomass can be harvested during all seasons. Studies on screening of potential microalgae for biodiesel have been reported (Devi, 2008; Devi et al., 2009), but the actual production of biodiesel from microalgae is only in incipient phase.

3.4. PROCESSING OF MICROALGAL BIOMASS FOR BIODIESEL PRODUCTION

The recovery of microalgal biomass requires processes such as dewatering, disruption of the microalgae cells and extraction of the oil fraction. Dewatering mechanisms can be grouped as physical (e.g. centrifugation, spray drying and

Table 3. Comparison of microalgae with other biodiesel feedstocks (Courtesy Mata et al., 2010).

Plant source	Seed oil content (% oil by in biomass)	Oil yield (L oil/ha year)	Land use (m^2 year/kg biodiesel)	Biodiesel productivity (kg biodiesel/ha year)
Corn/maize (*Zea mays* L.)	44	172	66	152
Hemp (*Cannabis sativa* L.)	33	363	31	321
Soybean (*Glycine max* L.)	18	636	18	562
Jatropha (*Jatropha curcas* L.)	28	741	15	656
Camelina (*Camelina sativa* L.)	42	915	12	809
Canola/rapeseed (*Brassica napus* L.)	41	974	12	862
Sunflower (*Helianthus annuus* L.)	40	1,070	11	946
Castor (*Ricinus communis*)	48	1,307	9	1,156
Palm oil (*Elaeis guineensis*)	36	5,366	2	4,747
Microalgae (low oil content)	30	58,700	0.2	51,927
Microalgae (medium oil content)	50	97,800	0.1	86,515
Microalgae (high oil content)	70	136,900	0.1	121,104

filtration), biological (e.g. auto flocculation) or chemical (e.g. alum flocculants). Mechanisms of cell disruption and extraction include grinding, direct solvent extraction, explosive decompression, freeze-drying and supercritical fluids amongst others.

3.4.1. Flocculation

Microalgae are very small so they are very difficult to harvest. Flocculation is the process where the microalgal cells are aggregated in order to increase the particle size. Some flocculating agents such as alum, ferric chloride, ammonium sulphate, ferric sulphate, (Brennan and Owende, 2010) polyacrylamide polymers, (Lee et al., 2009) surfactants, chitosan and other man-made fibres are normally used as flocculating agents (Divakaran and Pillai, 2002).

3.4.2. Filtration

Filtration is another very simple method for harvesting of microalgae. But this method depends largely on the microalgal sizes. During filtration, the pore size of the filter depends on the size of the microalgae and the aggregation rate of microalgae. Culture purity is also important while choosing the filter pore size.

3.4.3. Centrifugation

Centrifugation is also widely used for the harvesting of microalgal biomass. The process is rapid and energy intensive, and biomass recovery depends on the settling characteristics of the cells which are again depending on the density and the radius of the microalgal cells and sedimentation velocity (Brennan and Owende, 2010).

3.4.4. Drying

The harvested microalgal biomass must be processed rapidly for drying. There are various methods for drying which include sun-drying, low-pressure shelf drying, drum drying (Prakash et al., 1997), spray drying (Desmorieux and Decaen, 2006), fluidised bed drying (Leach et al., 1998), freeze-drying (Grima et al., 1994) and Refractance Window™ technology drying (Nindo and Tang, 2007). Sun-drying is the cheapest drying method, but it takes long time to dry, and a large drying surface is required. Spray drying is commonly used for extraction of high value products, but it is relatively expensive and can cause significant deterioration of some algal pigments. Freeze-drying is also an expensive method.

3.4.5. Disruption of Microalgal Biomass

Alternatively to drying, oil can be extracted from wet algal biomass. For this, the algal cells must be broken, or lysed, to extract the oil. Some of the disruption methods for cell rupture include osmotic shock, explosive decompression, mechanical press and mechanical and biological shear. Interestingly, some microalgae degrade through the shearing action of the pumps used in bioreactors, so mechanical shear may also be an option (Shields et al., 2008).

3.4.6. Oil Extraction

Once the cell is ruptured, the lipid fraction, consisting of fatty acids and glycerol, needs to be separated from the remaining cell contents. This can be done by solvent or some other extraction process. Biodiesel is then produce by transesterification in which triglycerides are reacted with methanol to yield glycerol and methyl esters of fatty acids (Mata et al., 2010).

4. Algal Biorefinery for Biofuel Production

According to International Energy Agency (2008), "biorefining is a sustainable processing of biomass into a spectrum of marketable products and energy such as biofuel". A biorefinery is a network of facilities that integrates biomass conversion processes and equipment to produce transportation biofuels, power and chemicals from biomass. This concept is analogous to today's petroleum refinery, which produces multiple fuels and products from petroleum (Cherubini, 2010).

Production of food and fuel is complexly adjoined. Sustainable production of food and fuel is crucial in a carbon-smart society. Integration of the emerging biorefinery concept with other industries in many environmental deliverables while mitigating several sustainability-related issues with respect to greenhouse gas emissions, fossil fuel usage and land use changes for fuel production and future food insufficiency (Subhadra and Grinson-George, 2011).

Production of biofuel from both micro and macro algae is capital intensive energy consuming which involves various chemical and physical processes. Therefore, production of only biofuels from algal biomass will not be cost-effective and environment friendly. Therefore, it is important to produce biofuel

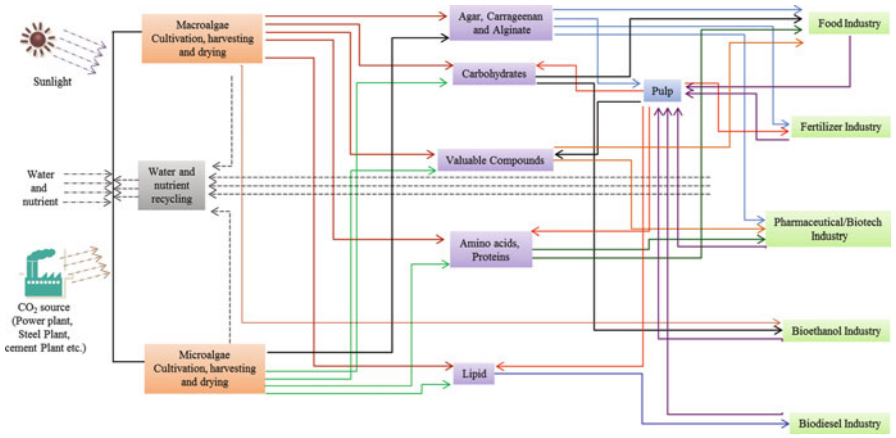

Figure 8. Schematic representation of integrated biorefinery concept.

as co-products along with other by-products through an integrated system of biorefinery approach.

The present biorefinery concept (Fig. 8) emphasises large-scale cultivation of algal biomass (both micro- and macroalgae) and their on-site processing for production of biofuel and other co-products. The most important energy products which can be produced in algal biorefineries are liquid biofuels which include bioethanol, biodiesel, etc. The most important biomass products in algal biorefineries are:

- Biomass – health food, functional food, feed additive, aquaculture, biofertiliser
- Phycocolloids – agar, carrageenan, alginates
- Pigments/carotenoids – astaxanthin, phycocyanin, phycoerythrin, fucoxanthin,
- Vitamin – A, B1, B6, B12, C, E, biotin, riboflavin, nicotinic acid, pantothenate and folic acid
- Other/pharmaceuticals – antifungal, antimicrobial, antiviral, toxins, amino acids, proteins and sterols
- Antioxidants – β-carotene, tocopherol
- Antioxidant extracts – arachidonic acid (ARA polyunsaturated omega-6 fatty acid, docosahexaenoic acid) (DHA omega-3 fatty acid), PUFA extracts (polyunsaturated fatty acids)

Various products and by-products derived from integrated algal biorefinery can feed various industries such as pharmaceutical and food production sector (Subhadra and Grinson-George, 2011). In addition to the above-mentioned products and by-products, the following benefits are also associated with integrated algal biorefinery:

- Net energy gain
- Fulfilling a large portion of world food demand without affecting current food supply

- Can provide livelihood and employment to millions of people worldwide
- Environmental benefits in the form of carbon sequestration and nutrient recycling
- Minimum use of water, energy and land than other plant
- Prospect of setting up in wide range of water

5. Conclusion

Biofuels derived from oil crops, waste cooking oil and animal fats are carbon neutral alternatives to petroleum fuels. However, they cannot realistically satisfy even a small fraction of the existing demand for transport fuels. Therefore, the future of biofuels will depend on the accelerated diffusion of new technologies, with an appropriate and market-friendly regulatory environment. Biofuels from algae can become one of the alternative options for supplementing the petroleum-based fuel without affecting the human food chain and environment.

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PART II:
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R. Gordon and J. Seckbach (eds.), *The Science of Algal Fuels*,
Cellular Origin, Life in Extreme Habitats and Astrobiology, 233–254
DOI 10.1007/978-94-007-5110-1_13, © Springer Science+Business Media Dordrecht 2012

DINOFLAGELLATES AS FEEDSTOCK FOR BIODIESEL PRODUCTION

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1. Introduction

The biotechnological use of microalgae biomass for biofuel production has been developing rapidly over the last few years (Chisti, 2007; Hu et al., 2008). Most of the known microalgae already used for biodiesel production are freshwater microalgae from the chlorophycean group. Target species for biomass production have traditionally been those with a known growth cycle, fast cell growth and those that usually were cultivated for other aims, as a protein source such as *Tetraselmis suecica*, *Spirulina platensis* or those for aquaculture activities such as *Isochrysis galbana, Nannochloropsis oculata* (Rodolfi et al., 2008; Chu et al., 2009) and others that produce special metabolites such as *Haematococcus pluvialis* (Grewe and Griehl, 2008) or *Scenedesmus almeriensis* (Sánchez et al., 2008), which are widely used in industry in the synthesis of pigments and as a food additives. Microalgae are composed, at the cellular level, of varying percentages of lipids, proteins, and carbohydrates. Lipids, especially the polar glycolipids (phospholipids) fraction, function as a structural component of microalgal cell membranes, but the lipids also modulate cellular activity and energy storage. In fact, one of the main biological functions of neutral lipids (triacylglycerol, TAG) is to provide energy for immediate and delayed metabolic requirements (Geider and La Roche, 2002). The values of oil concentration in microalgae vary between 20 and 50% of their total biomass (Chisti, 2007). Accordingly, some microalgae have the potential to synthesize 30 times more oil per hectare than terrestrial plants (Sheehan et al., 2006), and this encourages the use for biofuel production (Hu et al., 2008).

Nowadays, most of the microalgae used for biodiesel production are mainly "green microalgae". These species use principally freshwater as a liquid growth medium, and therefore, strains can be cultivated in countries where this strategic resource is found in abundance. Taking into account that freshwater element would be a problematic resource in the near future (Griffiths and Harrison, 2009;

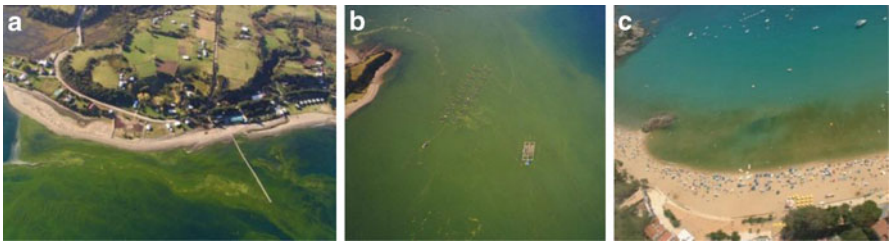

Figure 1. Aerial photograph of phytoplankton blooms. (**a**) and (**b**) *Gymnodinium* cf. *chlorophorum,* south Chile, 42° S, 71° W (photograph courtesy of Plancton Andino Ltda.). (**c**) *Alexandrium taylori*, La Fosca, Catalonia, Spain, 41.5° N, 03° E (Photograph courtesy of PBL group, ICM-CSIC).

Grobbelaar, 2010), it is highly recommended exploring marine strains to significantly reduce the water footprints (Yang et al., 2011). The use of local or "autochthonous" marine microalgae from the same growth location is needed, since this strategy allows the use of the same environmental parameters when cultured in enclosed systems (Morweiser et al., 2010). The use of local marine microalgae avoids the introduction of exotic organisms and any possible ecological problems.

2. Dinoflagellates and Raphidophytes Microalgal Groups

Strains with different growth strategies adapted to live in seawater and with high fatty acids content as neutral lipids or triacylglycerols (TAG) are found naturally in two groups of microalgae, the dinoflagellates and raphidophytes. Most of the dinoflagellates and raphidophytes can grow and produce large blooms under natural conditions. These blooms can cover from up to a few hundred metres (Fig. 1c) to hundreds of kilometres (Fig. 1a, b) and reach cell abundances of million cells per litre. Bloom-forming species have a cosmopolitan distribution, and they can easily be isolated from their environment; this characteristic makes them strategic marine strains because they can grow under local natural conditions in coastal countries around the world. Usually, these microalgae form patches in the upper layers at the surface of the sea, or proliferate in zones where abiotic conditions in the water column such as temperature, density, salinity or nutrient concentration are adequate to trigger rapid growth.

Certain species of dinoflagellates and raphidophytes have been studied for decades from an ecological and physiological point of view (Anderson, 1989; Smayda, 1997) because they are associated in some cases with toxic and noxious events such as massive mortalities of different marine organisms, from small crustaceans (e.g. copepods) to large mammals (e.g. sea lions). The anthropic interest in these noxious organisms has allowed for an extensive development in their ecological research. However, their use for energetic aims is poorly known. In fact, in spite of their negative effect on the environment, these microalgae group could be an interesting and strategic group that can produce natural oils.

It is well established that dinoflagellates tend to have lower growth rates compared to other microalgae taxa with similar cell size (Tang, 1996) and they reflect the lower photosynthetic capacity per unit of biomass of dinoflagellates (Chan, 1980; Tang, 1995). It appears that the low growth rate of dinoflagellates is compensated by their high size and biovolume when these groups are compared with the green algae groups in terms of biomass concentration or cell carbon content (Tang, 1995).

Dinoflagellates and raphidophytes have a huge range of size varying from 8 to 10 μm up to macroscopic size of >0.5 mm. Their biovolume is significantly different from other algae classes. Table 1 shows the range of biovolume for dinoflagellates and raphidophytes compared to other microalgae classes. Dinoflagellates and raphidophytes show biovolume values from 400 up to 88.000 μm^3 (Smayda, 1997; Stolte and Garcés, 2006; Olenina et al., 2006) which are several orders of magnitude higher, even, in the carbon content than the green algae (Tang, 1995). This characteristic could be useful in terms of lipid storage, using the hypothesis that they can accumulate more carbon per cell and they can transform it in lipids. Contrarily, cell density in autotrophic cultures of dinoflagellates and raphidophytes achieves concentration of 10^5*mL^{-1} (in enclosed system or photobioreactor PBR), two orders of magnitude lower than the green algae group (10^7*mL^{-1}) in a similar production system. Although this difference exists in cell abundance, the equilibrium between cell abundance and biovolume leads to a final biomass production (in terms of grams of dry weight per litre) quite similar to both groups of algae. This can be explained due to the differences in biovolumes when comparing green algae against dinoflagellate and raphidophyte classes (Table 1).

3. Strains Growth in Microalgae

In terms of the growth rate, the green algae usually has a duplication time of hours during the exponential phase, and their entire growth curve in batch cultures takes just a few days (between 6 and 20 depending on the species and the culture strategy used). Most of the studies reviewed for dinoflagellates and raphidophytes show growth rates from natural populations where they develop blooms, reaching occasionally fast growth (Stolte and Garcés, 2006). This natural behaviour in the environment is not comparable in controlled culture conditions. In most of the species, it is necessary to determine the abiotic parameters as temperature, salinity, turbulence, quantity and quality of light and sufficient nutrient concentration that regulate growth in controlled culture conditions. In outdoor conditions where the advantage is the natural source of energy provided from the sun, these parameters are almost unknown for dinoflagellates and raphidophytes. Every microalgae species has their own specific requirement in terms of abiotic parameters, culture performance or turbulence regime. The key to a successful microalgal biomass production will be to characterize, test and improve those parameters that affect the growth of autotrophic microalgal cells.

Table 1. Biotic parameters of different dinoflagellate and raphidophyte species compared with those that usually are used for biomass production.

Class	Species	Growth rate div*day^{-1} ± SD	Biovolume (μm^3 ± SD)	Biomass productivity (g*L^{-1}*day^{-1})	Lipid content (% DW)	References
Dinophyceae	*Alexandrium andersoni*	0.10	–	–	–	(3)
Dinophyceae	*Alexandrium catenella*	0.22 ± 0.02	–	–	–	(2)
Dinophyceae	*Alexandrium insuetum*	0.40 ± 0.33	–	–	–	(2)
Dinophyceae	*Alexandrium insuetum*	0.06	–	–	–	(3)
Dinophyceae	*Alexandrium minutum*	0.87 ± 0.14	–	–	–	(2)
Dinophyceae	*Alexandrium minutum*	0.18	3,260 ± 917	0.40 ± 0.1	23	(3)
Dinophyceae	*Alexandrium tamarense*	1.03 ± 0.13	16,485	–	–	(1)
Dinophyceae	*Alexandrium tamarense*	0.23	17,684	–	–	(2)
Dinophyceae	*Alexandrium taylorii*	0.31 ± 0.26	–	–	–	(2)
Dinophyceae	*Amphidinium carterae*	2.89 ± 0.08	432	–	–	(1)
Dinophyceae	*Ceratium furca*	0.44 ± 0.22	50,000 ± 20,000	–	–	(2)
Dinophyceae	*Ceratium tripos*	0.17	88,420 ± 74,540	–	–	(2)
Dinophyceae	*Dinophysis acuminata*	0.36 ± 0.22	16,211 ± 10,037	–	–	(2) (4)
Dinophyceae	*Dinophysis acuta*	0.38 ± 0.04	63,150 ± 33,936	–	–	(2) (4)
Dinophyceae	*Dinophysis norvegica*	0.36 ± 0.23	41,945 ± 26,075	–	–	(2) (4)
Dinophyceae	*Dinophysis tripos*	0.21	37,680	–	–	(2) (4)
Dinophyceae	*Gymnodinium* spp.	0.20 ± 0.11	65,765 ± 52,758	–	13	(2) (4)
Dinophyceae	*Glenodinium halli*	2.19 ± 0.11	–	–	–	(1)
Dinophyceae	*Gymnodinium resplendens*	1.53	8,146	–	–	(1) (4)
Dinophyceae	*Gymnodinium nelsoni*	1.43 ± 0.17	–	–	–	(1)
Dinophyceae	*Heterocapsa triquetra*	0.08 ± 0.06	2,295 ± 1,405	–	–	(2)
Dinophyceae	*Heterocapsa triquetra*	1.34 ± 0.22	419 ± 213	–	–	(1)
Dinophyceae	*Heterocapsa pygmaea*	2.12 ± 0.30	–	–	–	(1)
Dinophyceae	*Karlodinium* sp.	0.60 ± 0.20	–	–	–	(2)
Dinophyceae	*Karlodinium veneficum*	0.39 ± 0.02	494 ± 61	0.21 ± 0.07	27	(3) (5)
Dinophyceae	*Lingulodinium polyedra*	0.86	28,157 ± 12,719	–	–	(1)
Dinophyceae	*Prorocentrum minimum*	0.21	1,240 ± 351	–	–	(2) (4)
Dinophyceae	*Prorocentrum triestinum*	0.14	–	–	–	(2)
Dinophyceae	*Prorocentrum minimum*	2.37 ± 0.12	1,240 ± 351	–	–	(1) (4)
Dinophyceae	*Prorocentrum redfieldii*	2.17 ± 0.22	–	–	–	(1)

Dinophyceae	*Prorocentrum micans*	2.04 ± 0.10	20,292 ± 10,379	–	–	(1) (4)
Dinophyceae	*Scrippsiella trochoidea*	1.25 ± 0.22	5,505 ± 3,873	–	16	(1)
Dinophyceae	*Scrippsiella trochoidea*	0.11	5,505 ± 3,873	–	16	(3)
Raphidophyceae	*Heterosigma akashiwo*	0.17	1,020 ± 249	0.30 ± 0.04	24	(3)
Eustigmatophyceae	*Nannochloropsis* sp.	0.36 ± 0.06	14	0.19 ± 0.02	33	(6) (7)
Prymnesiophyceae	*Isochrysis* sp.	0.22 ± 0.03	69	0.16 ± 0.02	29	(7)
Prasinophyceae	*Tetraselmis suecica*	0.23 ± 0.01	–	0.30 ± 0.02	10	(3) (6)
Chlorophyceae	*Chlorella vulgaris*	0.13 ± 0.01[a]	–	0.18 ± 0.02	19	(7) (8)
Cryptophyceae	*Rhodomonas* sp.	0.28 ± 0.03	112 ± 38	–	13	(4) (5) (6)

Key to references = (1) Smayda (1997), (2) Stolte and Garcés (2006), (3) Fuentes-Grünewald et al. (2009), (4) Olenina et al. (2006), (5) Calbet et al. (2011), (6) Huerlimann et al. (2010), (7) Rodolfi et al. (2008), (8) Converti et al. (2009).

[a]Average of growth rates in cultures under different temperatures.

Among the target microalgae studied in the work of Fuentes-Grünewald et al. (2009), the growth of *Karlodinium veneficum* was the highest, 0.14 day^{-1} in the exponential phase, corresponding to an abundance of 44×10^6 cells L^{-1} at day 30 of culture. For *Heterosigma akashiwo*, maximum abundance was approximately 26×10^6 cells L^{-1} at day 35 of culture, reflecting a growth rate of 0.10 day^{-1} (1 division every 10 days). *K. veneficum* could reach growth rate of 0.47 day^{-1} in photobioreactors. Among the species examined in the study, this raphidophyte was unique in that cell abundance was maintained for more than 6 months (data not shown). Moreover, the cells remained healthy without the addition of fresh medium. This was in contrast to the other cultures, which gradually decayed such that total cell lysis has occurred ~2 months after inoculation. The growth rate of dinoflagellates belonging to the genus *Alexandrium* differed depending on the species. The highest growth rate was that of *A. andersoni*, 0.10 day^{-1} similar to that of the raphidophyte *H. akashiwo*, but the cell abundance of the former (maximum of 9×10^6 cells L^{-1}) was lower than that of the raphidophyte. The growth rates of *A. minutum* and *A. catenella* were two orders of magnitude slower (0.04 and 0.03 day^{-1}, respectively) than those of faster-growing microalgae. In terms of abundance, *A. minutum* reached a maximum of 2.6×10^6 cells L^{-1} and *A. catenella* a maximum of 9.4×10^5 cells L^{-1} at culture days 36 and 35, respectively. Among the Dinophyceae, *K. veneficum* showed the best performance in terms of growth, although the rate measured in this study was much lower than that of wild populations (Stolte and Garcés, 2006). Nonetheless, it was high enough to yield a large biomass in culture within a reasonable period of time. Further studies will be needed to determine whether the growth rate in culture can be improved, e.g. by isolating new strains and/or inoculating the cells in exponential phase before the maximum growth rate is established.

4. Lipids in the Target Microalgae

The approximate elemental compositions of eukaryotic photosynthesizing microalgae show different percentages of carbohydrates (5–45%), proteins (30–65%) and lipids (10–50%) in their cell mass (Geider and La Roche, 2002). This biochemical composition could change with the culture growth phase (culture age) or under environmental changes that cause cell stress. Lipids, the polar and the neutral fraction, are the source of oil in cells. The polar and the neutral fraction have different roles inside the cells, while the polar fraction is composed principally of phosphoglycerides which are use as structural component in cell membranes; neutral lipids serve as energy storage reserves (Geider and La Roche, 2002; Guschina and Harwood, 2006). With regard to lipid content, most of the algae usually utilized for biodiesel purposes (e.g. *Chlorella* species) show concentrations ranging from 22 to 38% of dry weight in standard conditions of growth (Chen and Yeh, 2011). Nevertheless, after a review of the literature, only a few studies presented results in lipid content of dinoflagellates and raphidophytes,

which varied from 13 to 27% of dry weight in standard growth conditions (Table 1). However, strictly for energetic purposes, there is a lack of data on lipid content, lipid and biomass productivity, TAG production in dinoflagellates and raphidophytes cultured in different systems, and conditions for long-term microalgal biomass production.

Contrary to the lack of quantitative data on total lipid content in dinoflagellates and raphidophytes, there are many studies on lipid profile of these groups primarily done as a tool to identify different genera or species, or as a biomarker in the trophic food chain in the ocean (Mansour et al., 1999; Leblond and Chapman, 2000; Reuss and Poulsen, 2002; Marshall et al., 2002; Fiorillo and Rossi, 2010). Data obtained from 62 dinoflagellate species and 11 raphidophyte species showed a long list of fatty acids presented in dinoflagellates and raphidophytes. These studies identified 58 different fatty acids, from short carbon chain as undecaenoic acid (C11:0) to long-chain carbon fatty acid as octacosaenoic acid (C28:0 (Table 2)). In general, the saturated fatty acids (SAFA) are those fatty acids that work as precursor for other monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) (Guschina and Harwood, 2006). Short-chain saturated such

Table 2. List of fatty acids present in the lipid profile of dinoflagellates and raphidophytes.

Fatty acid name	Nomenclature
Undecaenoic acid	C11:0
Lauric acid	C12:0
Tridecaenoic acid	C13:0
Myristic acid	C14:0
Myristoleic acid	C14:1
	C14:2
Pentadecaenoic acid	C15:0
Cis-10-pentadecenoic acid	C15:1
Palmitic acid	C16:0
Palmitoleic acid	C16:1
	C16:1 *n*5
	C16:1 *n*7
	C16:1 *n*13
	C16:2 *n*4
	C16:2 *n*6
	C16:2 *n*7
	C16:3
	C16:3 *n*3
	C16:4 *n*1
	C16:4 *n*3
Heptadecaenoic acid	C17:0
Cis-10-heptadecaenoic acid	C17:1
Stearic acid	C18:0
Oleic acid	C18:1 *n*3
Elaidic acid	C18:1 *n*7

(continued)

Table 2. (continued)

Fatty acid name	Nomenclature
	C18:1 *n*9
	C18:1 *n*13
Linoleic acid	C18:2 *n*6
Linolenic acid	C18:3 *n*3
Gamma-linolenic acid	C18:3 *n*6
	C18:4 *n*3
	C18:5 *n*3
Arachidic acid	C20:0
Cis-11-eicosenoic acid	C20:1
	C20:1 *n*9
	C20:2
Cis-11-14-eicosadienoic acid	C20:2 *n*6
	C20:2 *n*9
Cis-11,14,17-eicosatrienoic acid	C20:3 *n*3
Cis-8,11,14,17-eicosatrienoic acid	C20:3 *n*6
Arachidonic acid (AA)	C20:4 *n*3
	C20:4 *n*6
Cis-5,8,11,14,17-eicosapentaenoic (EPA)	C20:5 *n*3
	C20:6 *n*3
Heneicosaenoic acid	C21:0
Behenic acid	C22:0
Erucic acid	C22:1 *n*9
	C22:1 *n*11
Cis-13,16-docosadienoic acid	C22:2
	C22:5 *n*2
	C22:5 *n*3
	C22:5 *n*6
Cis-4,7,10,13,16,19-docosahexaenoic acid (DHA)	C22:6 *n*3
Tricosaenoic acid	C23:0
Lignoceric acid	C24:0
Nervonic acid	C24:1 *n*9
	C28:8 *n*3
Octacosaenoic acid	C28:y

Data was obtained from 62 dinoflagellate species and 11 raphidophyte species from Fernández-Reiriz et al. (1989), Viso and Marty (1993), Mansour et al. (1999), Leblond and Chapman et al. (2000), Marshall et al. (2002), Leblond et al. (2006), Mooney et al. (2007), Usup et al. (2008), Xu et al. (2006), Giner et al. (2008), Chu et al. (2009), Dorantes-Aranda et al. (2009) and De Boer et al. (2009).

as lauric acid C12:0 or palmitic acid C16:0 are the initial step of the metabolic pathways of fatty acid synthesis and were found to be the main components of neutral lipids (Molina Grima et al., 1994, 1995); by contrast, the PUFA portion or those fatty acids with long-chain carbon are involved in the construction process of cells and work as structural lipids, mainly found in glycolipids and phospholipids fraction (Molina Grima et al., 1995), and their content in autotrophic cells are associated due to the state of growth in microalgae cultures.

Reports indicate that the principal fatty acid portion presented in the dinoflagellates and raphidophytes reviewed is the SAFA fraction, and the main contributors to this fraction were palmitic acid C16:0 and stearic acids C18:0. Other fatty acids identified corresponded to the MUFA fraction; the main contributor to this fraction was the oleic acid C18:1*n*3. It was also observed in the PUFA fraction that the principal fatty acids with a high economical value was for eicosapentaenoic acid or EPA (C20:5*n*3) and docosahexaenoic acid or DHA (C22:6*n*3). The SAFA fraction and especially the palmitic acid (C16:0) and the stearic acid (C18:0) were present in 89% of the reviewed fatty acid profile of dinoflagellate and raphidophyte species, with an average of 18.4% of C16:0 and 6.7% of C18:0. Interestingly, a significant amount of the PUFA, docosahexaenoic acid (DHA) C22:6*n*3, a high-value fatty acid molecule, was found in the 40% of species reviewed, and the average DHA concentration was around of 8.3% of the lipid profile. The polyunsaturated fatty acids C20:5*n*3 or eicosapentaenoic acid (EPA) was present in 48% of the reviewed species with an average of 2.1% of the lipid profile, and the monounsaturated oleic acid (C18:1*n*3) was present in 14% of the species with an average of lipid concentration of 0.6 %. This and other important and essential fatty acids that cannot be synthesized by the human body but are vital for normal metabolism were found in the reviewed dinoflagellates and raphidophytes and are shown in Fig. 2.

A summary of the fatty acid profile of the dinoflagellates and raphidophytes reviewed is shown in Fig. 3. The graph shows specifically the percentage of SAFA, MUFA and PUFA per species. Most of the dinoflagellates have a SAFA concentration of 35% up to 60%. The most abundant fatty acids expressed by dinoflagellates during their growth were those of the C16:0, C18:0 (Fig. 2). The second major group of fatty acids was the MUFA portion, with an average of 20% for all dinoflagellate species reviewed. PUFA portion is high but only in some species. High values of PUFA are not useful for biodiesel production, but some can be valuable as a by-product such as C22:6*n*3 or C20:5*n*3 (Chi et al., 2009). Comparison of the fatty acid profile of the dinoflagellate and raphidophyte strains and terrestrial plants that are commonly used as oil feedstock (palm oil, soybean oil, sunflower oil, olive oil) for biodiesel production shows that most of the dinoflagellate species and some species of raphidophytes have a close fatty acid profile to palm oil (Fig. 3). Based on their fatty acids, the results of comparison among other oil feedstock allow us to infer that these groups of algae have an interesting lipid profile for biodiesel production (Fig. 3).

The fatty acid profile is one of the main characteristics to take into account when we are screening feedstock for biodiesel purposes because, depending on the composition of fatty acids, the biofuel obtained contains different qualities. The requirements of a specific lipid profile for biodiesel purposes have been analysed in the last few years, and according to the American Society for Testing and Materials (ASTM) D6751, (ASTM D6751–08 (2008), to obtain a biodiesel, the raw material has to comply different characteristics such as the cetane number, fatty acid profile and oxidation stability among others. The main desirable

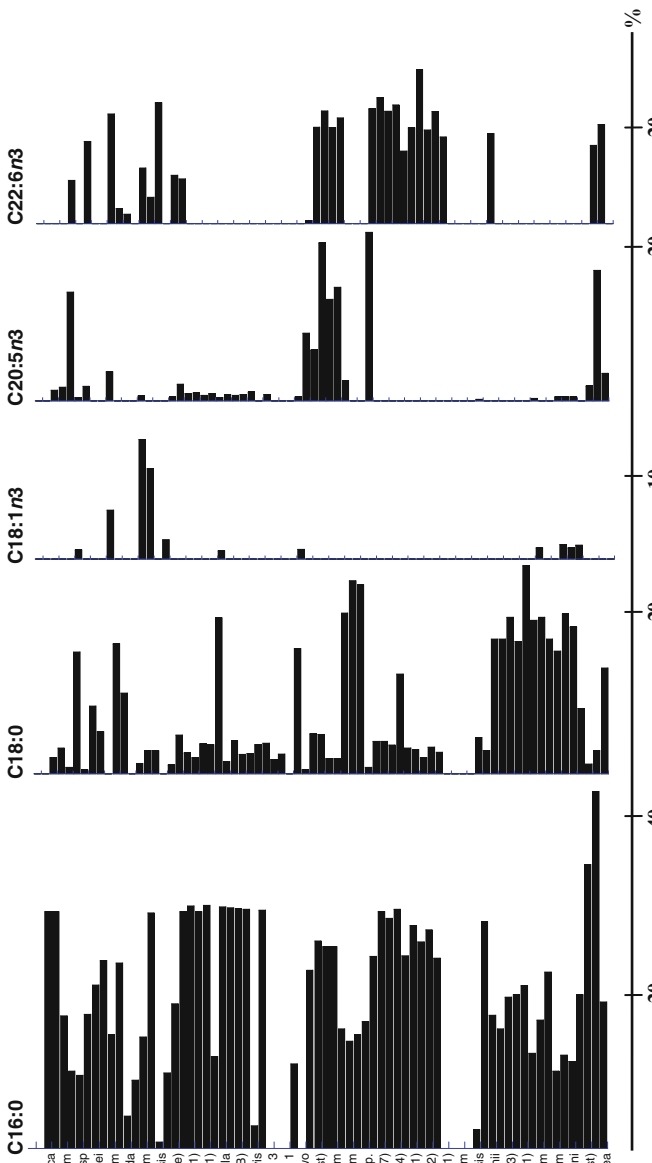

Figure 2. Principal fatty acids found in dinoflagellate and raphidophyte species reviewed. Data was obtained from 62 dinoflagellate and 11 raphidophyte species as in Table 2. C16:0 palmitic acid, C18:0 stearic acid, C20:5*n*3 eicosapentaenoic acid, C18:1*n*3 oleic acid, C22:6*n*3 docosahexaenoic acid (Fernandez-Reiriz et al., 1989; Viso and Marty, 1993; Mansour et al., 1999; Leblond and Chapman et al., 2000; Marshall et al., 2002; Leblond et al., 2006; Mooney et al., 2007; Usup et al., 2008; Xu et al., 2006; Giner et al., 2008; Chu et al., 2009; Dorantes-Aranda et al., 2009; De Boer et al., 2009).

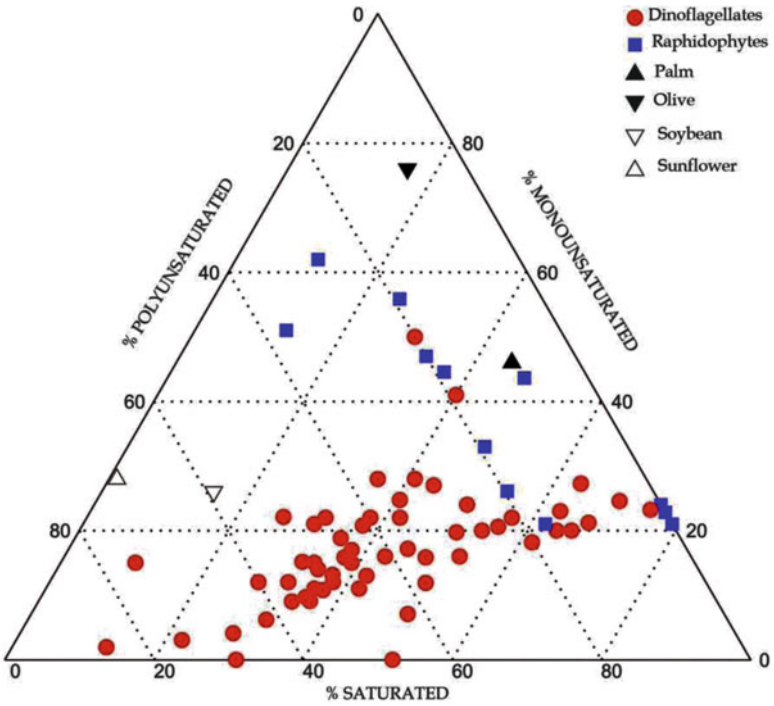

Figure 3. Fatty acid profile of dinoflagellates and raphidophytes, compared with the common oils used for biodiesel production. *Axis* shows the percentage of saturated (SAFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids. References as in Fig. 2.

characteristics of the biofuel obtained from vegetable oils are good oxidation stability (long-term storage) and a high-cetane number (good ignition capabilities). In a recent study, Sanford et al. (2010) compared and evaluated the characteristic of 36 different types of oil feedstock for biodiesel production, including microalgae.

The characteristic of the biodiesel analysed of algae oil meets the allowable limits, except flash point (>93°C) and oxidation stability (>3 h). For flash point, all the oils tested did not pass the test, and in the case of the oxidation stability, their results could be due to the fact that the fatty acid profile of the microalgae utilized in Sanford's work had a high percentage of monounsaturated fatty acids >70% with a consequent high oxidation stability, concluding that the fatty acid profile of the analysed algae was not adequate. But the author did not specify the algae strain used in their study, and the highest percentage presented (70%) probably corresponded to the "green" algae group, the most known group. Therefore, if the objective was to produce biodiesel from microalgae, a low percentage of MUFA and PUFA and high concentration of SAFA portion in the fatty acid profile are required and desirable, and this fatty acid profile is found in several species of dinoflagellates and raphidophytes (Fuentes-Grünewald et al., 2009).

4.1. Enhanced Lipid Production in Target Microalgae

In many studies on Chlorophyceae, Dinophyceae, Raphidophyceae and Eustigmatophyceae, it has been demonstrated that the fatty acid profile and lipid concentration on microalgae, specifically the neutral portion, increase in stationary phase parallel to a nutrient depletion in the culture (Mansour et al., 2003; Li et al., 2008; Liang et al., 2009; Widjaja et al., 2009; Fuentes-Grünewald et al., 2009). The growth curve of microalgae has three well-known steps: condition or lag phase, exponential phase and stationary phase. During the initial lag phase (a few hours or day depending on the species), microalgae cells adapt to culture conditions. The exponential phase is characterized for a high cell division and an increase in the growth rate and biomass production. When nutrient conditions decrease, microalgae cells stop growth and reach the stationary phase; after the stationary phase, the cultures decline and cells die. During the stationary phase and due to the nutrient depletion, the fatty acids in dinoflagellates increase as its showing for *K. veneficum* in Fig. 4.

When the objective of the microalgal biomass production is to produce biodiesel, it is necessary to determine the best harvest time. Also, it is desirable to determine the abiotic variable or a combination that allows us a high biomass production (in a reasonable period of time) and consequently a high lipid concentration in cells. Nowadays, the biochemical engineering using abiotic parameters such as CO_2, light, nutrient depletion, temperature or salinity to obtain lipids from autotrophic microalgae cell is mostly known only for freshwater green algae such as *Chlorella, Nannochloropsis, Neochloris* and *Isochrysis* (Flynn et al., 1993; Li et al., 2008; Converti et al., 2009). However, until now, there is no published information about the use of biochemical engineering strategy that allows an improvement in lipid production in dinoflagellates and raphidophytes.

The main objective in the recent work of Fuentes-Grünewald et al. (2012a) was to determine the lipidic percentage in which the target species (*A. minutum, H. akashiwo* and *K. veneficum*) store neutral lipids, varying abiotic parameters such as temperature (15–20–25°C), aeration and NO_3 concentration (880, 660, 440, 220 μM). The cultures were grown in L1 media, in 2-l Nalgene bottles with a 12:12 (L:D) photoperiod. Cell abundance was monitored by cell counting using inverted microscope, and by measurements, chlorophyll concentration (μg Chl a*L^{-1}) was used as a biomass indicator. Spectrofluorometry readings were obtained dying the cells with Nile red for neutral lipid analysis. The results were verified by lipid extraction for gas chromatography analysis and show an important increase (>200%) with respect to the initial TAG concentration in *A. minutum*, especially in the cultures maintained under higher temperatures >25°C, inorganic dissolved N concentration ≈330 μM and continuous aeration conditions. *A. minutum* cells submitted to the treatment and control cells are shown in Fig. 5. The final lipid production is influenced by the physiological state of the inoculated cells, and it also depends on the number of cells inoculated. To find

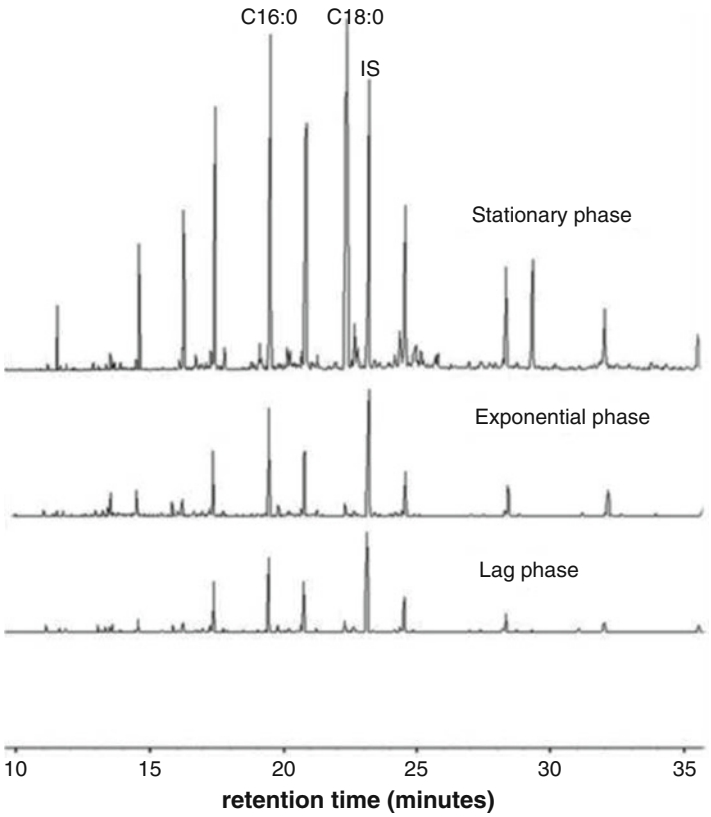

Figure 4. Chromatogram of different culture phases in *Karlodinium veneficum*; at the stationary phase, we observe the increase in their fatty acid profile (C16:0 palmitic acid; C18:0 stearic acid; IS internal standard).

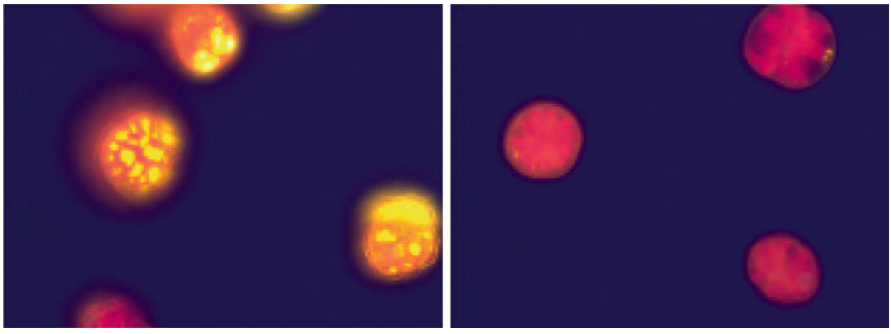

Figure 5. *Left photograph* normal condition (*Alexandrium minutum* cells), *right photograph* stress conditions. *Nile red* stain was used for TAG measurements 488/570 nm read by spectrofluorometer (Photograph from Fuentes-Grünewald et al., 2012a).

the balance between high biomass production and high lipid concentration will allow us to design culturing strategies for TAG production with energetic purposes (Fuentes-Grünewald et al., 2009).

5. Dinoflagellate Cultures: Indoor vs. Outdoor Conditions

Microalgae production used conventionally at outdoor conditions is largely more sustainable than indoor conditions in terms of energy. The main objective in the study of Fuentes-Grünewald et al. (2012b) was to evaluate and compare the growth of strains of dinoflagellate and raphidophyte species using bubble column photobioreactor at indoor and outdoor conditions (Barcelona location 41° 23′ 08.12 N–2° 11′ 45.84 E). To compare both conditions in terms of energy the biotic parameters in the target strains, it was quantified light, temperature, pH, cell yield, growth rate, biomass and lipid production. A bubble column vessel of Plexiglas with a working volume of 30 L in triplicate was used for both strategies. We tested the target strains: *A. minutum, K. veneficum* and *H. akashiwo.* A semicontinuous cultures at outdoor condition (using energy just for air pump) were established for 4 months, harvesting biomass and adding fresh medium depending on the growth rate of the species. Batch culture strategy was used at indoor conditions (using energy for air pump, light and temperature). At outdoor conditions, *H. akashiwo* cell yield was directly influenced by temperature, recording the highest growth rate (0.397 day^{-1}) and biomass (0.97 $g\,L^{-1}$ dry weight) when the range of temperature varied from 20 to 10°C (November–December). The three species decline their production in outdoor condition when a great amplitude of temperature >20°C was recorded or when the minimum temperature was near 0°C. At indoor condition, *H. akashiwo* shows a growth rate of 0.440 day^{-1} and an average biomass production near to 1.17 $g\,L^{-1}$ of dry weight (Fuentes-Grünewald et al., 2012b).

6. Comparison of the Target Species Against the Commonest "Green Algae"

Despite different studies show that dinoflagellates have low growth rate compared with other microalgae taxa (Tang, 1995, 1996), the final biomass production of this group is higher in most of our strains compared with the green algae group (Table 3). This is due to the higher biovolume (total cell volume) of the species. Most of the strains of the chlorophycean group (*Chlorella, Neochloris, Chlamydomonas*), Prasinophyceae as *Tetraselmis* sp. or Prymnesiophyceae as *Isochrysis* spp., have small size and low biovolumes (Olenina et al., 2006). *Chlorella vulgaris* show an average of 13 μm^3, several orders smaller than our *Alexandrium minutum* strain with and an average biovolume of 2,856 μm^3. This can be an advantage for culturing the dinoflagellates in terms of biomass production, lipid accumulation and carbon storage, because, although growth rates and cell concentration usually are lower than green algae, the compared biomass productivity and lipid productivity are higher in *A. minutum* and *H. akashiwo* (Table 3) than the commonest freshwater phototrophic strains *Chlorella vulgaris* (Rodolfi et al., 2008; Gouveia and Oliveira, 2009).

Table 3. Comparison of growth rate, biomass productivity, lipid content and lipid productivity of different microalgae.

Microalgae	Biomass productivity ($g*L^{-1}*day^{-1}$)	μ ($div*day^{-1}$)	Lipid content (% Biomass)	Lipid productivity ($mg*L^{-1}*day^{-1}$)	References
Tetraselmis suecica	0.28	–	12.9	36.4	Rodolfi et al. (2008)
Tetraselmis sp.	–	0.19	10.1	22.7	Huerlimann et al. (2010)
Nannochloropsis sp.	–	0.41	32.7	20.0	Huerlimann et al. (2010)
Nannochloropsis sp.	0.21	–	29.6	61.0	Rodolfi et al. (2008)
Nannochloropsis sp.	0.09	–	28.7	25.8	Gouveia and Oliveira (2009)
Pavlova lutheri	0.14	–	35.5	50.2	Rodolfi et al. (2008)
Skeletonema costatum	0.08	–	21.1	17.4	Rodolfi et al. (2008)
Scenedesmus sp.	0.26	–	21.1	53.9	Rodolfi et al. (2008)
Scenedesmus obliquus	0.09	–	11–22/35–55	19.8/49.5	Gouveia and Oliveira (2009)
Chlorella vulgaris	0.20	–	18.4	36.9	Rodolfi et al. (2008)
Chlorella vulgaris	0.18	–	5.1	9.2	Gouveia and Oliveira (2009)
Isochrysis sp.	–	0.25	28.6	21.1	Huerlimann et al. (2010)
Alexandrium minutum (in – outdoor)	0.16–0.35	0.12–0.37	23–22	36.5–80.7	Fuentes-Grünewald et al. (2012b)
Karlodinium veneficum (in – outdoor)	0.15–0.22	0.13–0.25	27–12	40.3–26.7	Fuentes-Grünewald et al. (2012b)
Heterosigma akashiwo (in – outdoor)	0.20–0.25	0.18–0.28	24–23	48.8–56.1	Fuentes-Grünewald et al. (2012b)

In the work of Rodolfi et al. (2008), the strains were cultivated in 250-mL flasks and incubated at 25°C with continuous illumination in an orbital shaker with CO_2-enriched air. Gouveia and Oliveira (2009) used outdoor raceway ponds for cultures; the lipid content was expressed as % of wet weight. Huerlimann et al. (2010) used aerated batch cultures (10 L) at indoor conditions. In Fuentes-Grünewald et al. (2012b), it was used a photobioreactor (300 L) with injected air, at indoor–outdoor conditions.

High biomass production is not just influenced by the growth rate but also is influenced for cell biovolume. The enhancement of growth rate combined with the high biovolume of the target strains, it was observed that one of the principal production parameter was affected the dry weight biomass production, reaching average values >1.13 ± 0.05 g*L^{-1} at indoor condition and 1.26 ± 0.2 g*L^{-1} for outdoor condition in the same system production, involving a high biomass productivity for dinoflagellates and raphidophytes when were compared with microalgae from the green algae groups (Table 3).

The high biomass productivity of the target microalgae is obtained in a relatively low cell concentration, but this fact could be an advantage in the dewatering stage of the process production, because the energy consumption to extract the same water volume with a filtration technique is higher in those cultures with more cell concentration than those with less cell density. During the dewatering stage or extraction process, the morphology of dinoflagellates and raphidophytes can also be an advantage. The proposed strains *K. veneficum* and *H. akashiwo* are nude tecate that means with any type of cell wall, and *A. minutum* is a tecate with a cell wall composed of numerous plates that can be easily broken comparing with the typical two valves of the diatoms or other microalgae. The strong cell wall in some green microalgae used for biomass production could be an advantage in culture conditions, especially when the cultures are submitted to a higher mechanical or hydrodynamic forces, but can be a disadvantage in terms of cell disruption for lipid extraction, because it implies the use of physical methods (e.g. sonication) that require a high-energy consumption during the extraction process. In *K. veneficum* and *H. akashiwo,* the absence of cell wall implies an advantage in terms of energy during the extraction stage, because it might be easy to break the cell wall of the proposed strains and to perform the extraction process, consequently improving the energetic results of the whole process.

7. Conclusion

Several conclusions have been reached on the production of microalgal biomass of dinoflagellates and raphidophytes to be used as feedstock for biodiesel production reviewed in this chapter:

- Two species of Dinophyceae, *Karlodinium veneficum* and *Alexandrium minutum,* and one Raphidophyceae, *Heterosigma akashiwo*, were found to be a particular interest as a bioresource for biodiesel production based on their lipid content, their net growth rate, their high average in wet biomass and their short period of growth compared with terrestrial plants.
- Dinoflagellates and raphidophytes tested have a closed lipid profile to the commonest terrestrial oil (palm oil) used as feedstock for biodiesel production.
- An increase in the fatty acid content was observed during the transition from exponential phase to stationary phase in the dinoflagellates and raphidophyte tested; this increase was more evident in *K. veneficum* (97%).

- An increase in the TAG portion was observed when the target strains were submitted to a stress condition of growth using abiotic parameters, especially high-temperature and nitrogen depletion.
- There was a significant increase in fatty acid concentration by cells in the strains tested under treatment condition (25°C and 330 μM $NaNO_3$).
- No significant change in fatty acid profile was detected when the strains were submitted to stress condition of high-temperature and low-nitrogen concentration.
- *H. akashiwo, K. veneficum* and *A. minutum* can grow under natural environmental conditions (fluctuations of light, irradiance and temperature) in a bcPBR-enclosed systems for several months in the Mediterranean basin.
- The growth rate, biomass productivity and lipid productivity of dinoflagellate and raphidophyte species were directly affected by irradiance and temperature, reaching higher values of growth in outdoor condition than the indoor condition.
- Biomass productivity, lipid content and lipid productivity of *H. akashiwo* and *A. minutum* were higher to those obtained by the "green algae group" in similar culture conditions.
- No significant change in the fatty acid profile was recorded in those cultures at outdoor conditions, compared with those cultures at indoor conditions.

Finally, dinoflagellates and raphidophytes are widely distributed and readily isolated in many different countries. As shown here, they comprise several strategic species that can be used as a source of raw material for biofuels. An analysis of the biotic characteristics (growth rate, biomass, cell yield, lipid content) of several species of microalgae supports their use as feedstocks for biodiesel production. To make more viable industrial project of biodiesel production from dinoflagellates is mandatory to utilize all the cells as a biorefinery concept, using extraction of special metabolites (pharmaceutical, biomedicine), oil content (for biodiesel production) and the exceeding biomass (for anaerobic digestion to produce methane). More studies are highly recommended in other species of dinoflagellates and raphidophytes in order to obtain more and better knowledge of these organisms and use their natural capacity to form dense and large blooms in the environment and utilize these characteristics in controlled culture conditions in order to obtain biomass for biofuel purposes.

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R. Gordon and J. Seckbach (eds.), *The Science of Algal Fuels*,
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DOI 10.1007/978-94-007-5110-1_14, © Springer Science+Business Media Dordrecht 2012

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BIODIESEL PRODUCTION FROM MICROALGAE: METHODS FOR MICROALGAL LIPID ASSESSMENT WITH EMPHASIS ON THE USE OF FLOW CYTOMETRY

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1. Introduction

Due to the diminishing petroleum reserves and environmental consequences of exhaust gases from fossil diesel, production of alternative fuel materials has attracted wide attention. The current reliance on petro-based fuels and chemicals is no more longer sustainable as the energy demand is projected to grow more than 50% by 2025 (Rudolfi et al., 2009). Renewable, carbon neutral, transport fuels are necessary for environmental and economic sustainability. The ecological footprint of energy generation will reside in a multifaceted approach that includes nuclear, solar, hydrogen, wind, fossil fuels (from which carbon is sequestered), and biofuels. Biodiesel derived from oil crops is a potentially renewable and carbon neutral alternative to petroleum fuels. Unfortunately, biodiesel derived from oil crops, waste cooking oil, and animal fat can realistically satisfy even only a small fraction of the existing demand for transport fuels (Chisti, 2007).

Microalgae, a renewable energy source which has not been fully exploited, have been suggested as very good candidates for fuel production as they appear to be the only renewable biofuel that is capable of meeting the global demand for transport fuels (Chisti, 2008). Autotrophic microalgae are sunlight-driven cell factories that convert carbon dioxide into potential biofuels, foods, feeds, and high-value products. They use a photosynthetic process similar to that of higher developed plants and can complete an entire growing cycle every few days, resulting in a much higher oil productivity as compared to the oil crops. In addition, they are efficient CO_2 fixers. The ability of microalgae to fix CO_2 has been proposed as a method of removing CO_2 from flue gases from power plants and thus can be used to reduce emission of greenhouse gases (Rudolfi et al., 2009). In addition, microalgae production is nonseasonal with the possibility of harvesting daily, and may take place on nonarable land (e.g., desert and seashore lands, saltpans, rocky and sandy areas), and do not compete with food crops, and non-potable water can be used. Raceways may be used in such conditions. Because of their simplicity

and the low investments costs involved, raceway ponds are often used for biodiesel production (Chisti, 2007).

Microalgal lipids have been suggested as a potential diesel fuel substitute with an emphasis on neutral lipids due their lower degree of unsaturation and their accumulation in algal cells at the end of growth stage (Lee et al., 1998). Anyway, if the microalgal oil contains polar lipids, it can be associated with other oils so that a good quality biodiesel can be obtained. In addition, the extent of unsaturation of microalgal oil and its content of fatty acids with more than four double bonds can be reduced easily by partial catalytic hydrogenation of the oil (Chisti, 2007). Therefore, microalgae are promoted as a future source of transportation fuels as they can produce up to ten times more oil per acre than traditional biofuel crops. Screening of microalgae for potential production of oil is therefore of significance. In addition, heterotrophic growth of some microalgae has been used for efficient production of biomass and some metabolites such as lipid which can reduce the cost of microalgal biomass production and microalgal oil production (Miao and Wu, 2006; Silva et al., 2009a).

Despite the enormous potential of algal biofuels, many challenges to making their large-scale production feasible still remain. These challenges include problems posed by the design of optimal bioreactors, the isolation, dewatering, chemical conversion of algal biomass, and the selection of physical and chemical environments needed to attain the optimal algal growth. In fact, microalgal lipid production is strongly dependent on environmental factors. Importantly, a significant component of the design of algal biofuel production process is the screening of lipid content in both naturally occurring and genetically modified algal strains, which will require rapid lipid quantification. Scaling-up the microalgal laboratory cultivation to commercial scale production of biofuel will also require particular attention.

Traditional approaches for the quantification of lipid from microbial biomass rely on time-consuming, labor- and equipment-intensive methods, such as the GC–MS analysis of fatty acid methyl esters (FAME) or the quantification of cellular lipids. Commercial laboratories frequently charge between $50 and $300 per sample for these analyses, making them cost prohibitive if analytical equipment is not readily available (Wawrik and Harriman, 2010). Therefore, the importance of the microalgae as potential fuel sources in future has been emerged the need for the development of methodologies for on-line microalgal lipid production quantification. Nevertheless, most of the published research works and industrial processes still use conventional techniques to monitor cell lipid content.

This chapter will present the main microalgal lipid methods currently used during the algal fuel production process, discussing their advantages and drawbacks. The use of multiparameter flow cytometry to microalgal lipid production process will be also discussed.

2. Conventional Methods

2.1. MICROALGAL DISRUPTION METHODS

There is no doubt that a microalgal cells disruption step is needed before the lipid gravimetric assessment method since the content of the extracted lipids is determined according to the disruption method and device used (Lee et al., 1998, 2010; Cooney et al., 2009).

Lee et al. (1998) compared several disruption methods before *Botryococcus braunii* lipid extraction. The authors used direct efficiency solvents only, sonication, homogenization, high pressure using a French press, bead-beating, and lyophilization of algal cells. They found that most efficient disruption method that produced the highest microalgal lipid content was the bead-beating.

Converti et al. (2009) used the classic Soxhlet extraction with petroleum ether, the Folch method, the ultrasonic extraction using petroleum ether, and the Folch method combined with the use of ultrasounds to assay the lipid production of *Nannochloropsis oculata*. The authors found that the method that extracted the highest lipid amount was the Folch method combined with ultrasounds either using dry or wet biomass.

Later, Lee et al. (2010) using three microalgal species (*Botryococcus* sp., *Chlorella vulgaris*, and *Scenedesmus* sp.) used several methods for microalgal disruption before lipid extraction by the Bligh and Dyer method, which included autoclaving at a high temperature and pressure, 10% NaCl solution (osmotic shock), microwaves, sonication, and bead-beating. The authors found that the efficiency of the lipid extraction differed according to the microalgal species and the cell disruption method. *Botryococcus* sp. produced the highest lipid content when the microwave oven method (at 100°C and 2,459 MHz for 5 min) was used before the lipid extraction step. The bead-beating method produced almost the same lipid content as the microwave disruption method, which is in agreement with the results early reported by Lee et al. (1998). Nevertheless, the authors concluded that the most simple, easy, and efficient cell disruption method for microalgal lipid extraction was the microwave oven method.

2.2. GRAVIMETRIC METHODS

Conventional methods to extract and assess lipids from oilseeds possess little utility for microalgae. For example, the methods of hexane solvent extraction or *in situ* biodiesel production can be complicated by the presence of water and significant cell wall barriers (Haas and Scott, 2007; Hass et al., 2007). Autotrophic and heterotrophic grown microalgae also produce high levels of fat-soluble pigments that further complicate the extraction and follow-on purification of the biofuel. Their presence undermines the utility of organic solvents that are limited

by their low selectivity and capacity to extract the fat-soluble pigments along with the bio-oils (Luque de Castro et al., 1999).

The most popular method for microalgal lipids is the Bligh and Dyer method (1959), which was firstly used to fresh or frozen tissues. The method uses a mixture of three solvents with different polarities (water-chloroform-methanol) extracting a range of lipid compounds with different polarities. However, the structure of algae is different from that of animal tissue, and Ahlgren and Uppsala (1991) stated that microalgal lipids seemed to be more difficult to extract with this method. Nevertheless, Grima et al. (1994) used seven solvent mixtures to extract the lipid fraction of *Isochrysis galbana* microalgal lyophilized biomass following a procedure based on the Bligh and Dyer method and found that the latter rendered the highest yield of lipids.

The Bligh and Dyer method is a laboratory-scale technique and has been extensively used in research works reporting biodiesel production from microalgae processes (Pruvost et al., 2009; Widjaja et al., 2009; Morowvat et al., 2010; Yoo et al., 2010; Vijayaraghavan and Hemanathan, 2009). However, the method seemed to lack in the recovery of nonpolar lipids (Cabrini et al., 1992). Indeed cosolvent systems for extraction of bio-oils are limited by incomplete recoveries owing to multiple factors such as low-solvent-carrying capacity and solvent-lipid polarity mismatch (Cooney et al., 2009).

In order to address this issue, Lepage and Roy (1984) proposed the direct transesterification of human milk and adipose tissue without prior extraction or purification for improved recovery of fatty acids. In general, this approach suggested that a one-step reaction that added the acid catalyst (e.g., acetyl chloride) and alcohol (e.g., methanol) directly to the biomass sample (with heating at 100°C for 1 h under sealed cap) would increase fatty acid concentration extracted (as compared to Bligh and Dyer cosolvent system), giving relatively high recoveries of volatile medium chain triglycerides and eliminate the need to used antioxidants to protect unsaturated fatty acids. Cohen et al. (1988) applied this method to *Porphyridium cruentum* microalgal biomass heating the mixtures at 80°C and added 0.01% butylated hydroxytoluene (BHT) as an antioxidant. Rodriguez-Ruiz et al. (1998) found that the entire reaction could be shortened to 10 min if the mixture was incubated at 100°C under sealed cap. Carvalho and Malcata (2005) reported that when executing the direct transesterification reaction using an acid catalyst (e.g., acetyl chloride), the efficiency of the reaction could be increased when a second "less polar" solvent such as diethyl ether or toluene was mixed with the methanol to modify the polarity of the reaction medium. In general, these findings suggest that the effectiveness of the second cosolvent (i.e., reaction medium) is related to its ability to solubilize the target lipids coupled with its miscibility with methanol.

Despite all these modifications for improvement of the microalgal lipid extraction analytical methods, they have many complicated steps, i.e., extraction, concentration, purification, gravimetric determination, and further characterization

by GC or HPLC, making all these expensive, labor-intensive, and time-consuming determinations difficult to screening large number of microalgal strains or monitoring microalgal cultivations wherein a high number of samples are required to be analyzed. In addition, these procedures must ensure complete extraction and at the same time avoid decomposition and/or oxidation of the lipid constituents. The use of organic solvents generates high amounts of waste which are harmful to the environment if not recycled by distillation and requires extra energy input to recover the solvents. The use of organic solvents may also contaminate the algal solids, thus restricting their use. Furthermore, enough amounts of biomass must be obtained for subsequent lipid extraction and derivatization (Elsey et al., 2007; Chen et al., 2009). However, the biomass available for analysis is often too small in the course of the microalgal growth, which may raise an obstacle to the use of gravimetric methods for lipid assessment. Importantly, lipid content data is usually only available in a considerable time after the sample is taken, too late for alterations to be made to process control.

As a result of all these drawbacks, increasing attention has been focused on *in situ* and fast measurements of lipid content as reported in the following section.

3. At-Line Fast Methods

3.1. NONFLUORESCENT METHODS

There are just a few studies reporting the use of fast nonfluorescence methods for microalgal lipid detection.

Wawrik and Harriman (2010) have reported a simple colorimetric method for quantification of lipid from algal cultures. The technique is based on the hydrolysis of algal lipids to fatty acids and subsequent extraction of their copper salts into chloroform. The amount of copper in the chloroform phase is then colorimetrically determined by adding diethyldithiocarbamate to develop a yellow-colored product (measured by optical density at 440 nm). In this manner, the authors were able to quantify lipid in micro-centrifuge tube format from 1 mL of log-phase algal culture in less than 1 h, and for less than $5 per sample.

Time-domain nuclear magnetic resonance (TD-NMR) has also been used to determine lipid content of *Chlorella protothecoides* for biodiesel production as reported by Gao et al., 2008. In this method, spin-echo NMR pulse sequence is applied to separate the lipid hydrogen nuclei signal from other hydrogen nuclei signals, and lipid content can be assessed after appropriate calibration.

Cheng et al. (2011) developed a colorimetric sulfo-phospho-vanillin (SPV) method for microalgal lipid analysis using a 96-well microplate. The developed method was compared with a macro-gravimetric method, and no difference was found.

3.2. FLUORESCENT METHODS

Most of the reported microalgal lipid quantification fast methods are fluorescent. Fluorescent stains have been used to qualify or quantify the presence of a specific compound. When using animal or microbial cells, the fluorescent stains may indicate the physical or energetical state of the cell components. The stains are chemical compounds that interact with cells or cell components depending on physicochemical interactions, allowing the specific staining of cell wall, membranes, organelles, proteins, and nucleic acids (Cao-Hoang et al., 2008). Fluorescence microscopy and fluorescence spectroscopy can be used to detect fluorescence. While the former only allows visualizing the fluorescence image, the latter allows fluorescence quantification.

The fluorescent stain that has been more widely used to quantify microalgal lipids is the Nile red 9-diethylamino-5H-benzo[α] phenoxazine-5-one. In 1985, Greenspan et al. referred this compound as an excellent stain for detection of intracellular lipid droplets by fluorescence microscopy and cytofluorometry: In aortic smooth muscle cells and peritoneal macrophages, cytoplasmic liquid droplets which are neutral lipids were viewed for yellow-gold fluorescence (excitation, 450–500 nm; emission, >528 nm), and polar lipids were viewed for red fluorescence (excitation, 515–560 nm; emission, >590 nm).

Lee et al. (1998) used for the first time the Nile red to assess the lipid content in the microalgae *Botryococcus braunii* and reported a significant correlation between the stain fluorescence and the gravimetric method.

Liu et al. (2008) quantified the cellular neutral lipid from *Chlorella vulgaris* based on the method of Greenspan et al. (1985), establishing a high correlation between the Nile red fluorescence intensity detected at 500–700 nm and lipid content assessed by the Bligh and Dyer gravimetric method.

Chen et al. (2009) used an improved method using Nile red to detect lipids in several green algal strains. Various physical and chemical treatments were applied to the existing Nile red method in order to improve the dye penetration. When staining the microalgal cells with DMSO (20–30% v/v) at 40°C, they emitted the highest Nile red fluorescence as compared to the other treatments. The authors highlighted that the high neutral lipid content measured in a number of green algal species extracted by the gravimetric method failed to be detected by the commonly used Nile red method due to the composition and structure of the thick and rigid cell walls common in many green algae which might prevent the Nile red dye from penetrating the cell wall and cytoplasmic membrane and subsequently biding with the intracellular neutral lipid and polar lipids to give the desired fluorescence.

In comparison, several authors (Fraser et al., 1987; Cooksey et al., 1987 and de la Jara et al., 2003) have used a Nile red labeling solution made with acetone to vitally stain a variety of algal taxa (e.g., *Scenedesmus obliquus, Neochloris oleoabundans*, *Crypthecodinium cohnii*, *Amphora coffeaeformis*, *Navicula* sp.) prior to fluorescent analysis. Importantly, whereas low concentrations of acetone (0.04%) do not appear to affect cell viability (Cooksey et al., 1987), higher levels of acetone

(5.0%) are toxic to the algae (de la Jara et al., 2003). Owing to the variable rate of Nile red diffusing into the lipid bodies, it has been recommended that algal culture fluorescence be monitored over a time period of 30–40 min after the addition of the stain and that the maximum emission intensity be recorded as opposed to recording the intensity at a predetermined time point (Elsey et al., 2007).

Cooper et al. (2010) used the lipophilic fluorescent BODIPY 505/515 to stain oil-containing lipid bodies of live algal cells in combination with fluorescent-activated cell sorting which can be used to detect and isolate algal cells possessing high lipid content. However, no quantification of the microalgal lipid content was performed.

Fluorescence methods can allow rapid and accurate microalgal lipid content assessment. However, no other information is given beyond the lipid content, such as the microalgae physiological states, which may be a pitfall specially when optimizing the microalgal lipid production process.

3.2.1. Flow Cytometry

Flow cytometry is a rapid method for quantitative measurements of individual cells in a moving fluid. Thousands of individual cells are passed through a light source (lamp or laser), and measurements of light scatter and fluorescence properties are collected simultaneously. The technique also allows the rapid determination of a high number of cell functions by using a great variety of biochemically specific, nontoxic, and fluorescent molecules in conditions close to the *in vivo* status in short-term exposures to high levels of light (Rioboo et al., 2009).

Microalgae are ideal for flow cytometric analysis because they are single celled and contain photosynthetic pigments, such as chlorophyll *a*, which autofluorescence when excited by blue light. This technique has been used to many microalgal ecotoxicity studies (Stauber et al., 2002; Franklin et al., 2004; Adler et al., 2007; Rioboo et al., 2009).

The US National Renewable Energy Laboratory (NREL) through the Aquatic Species Program (ASP) launched a specific R&D program dedicated to alternative renewable fuels, including biodiesel from microalgae that lasted from 1978 to 1996. One of its main objectives was to produce improved algae strains by looking for genetic variability between algal isolates, attempting to use flow cytometry to screen for naturally occurring high lipid individuals, and exploring algal viruses as potential genetic vectors (Sheehan et al., 1998).

In 2003, de la Jara et al. reported a method for *Crypthecodinium cohnii* lipid composition assessment by flow cytometry. The authors used Nile red for the *in vivo* lipid quantification and found that the Nile red fluorescence signal was linearly correlated with the neutral and polar lipid content, detected in the photomultipliers PMT2 and PMT3, respectively, as determined by gravimetric techniques. Therefore, the use of the Nile red dye combined with flow cytometry allowed the lipid class composition assessment, as the stain emitted fluorescence. Indeed, the stain emitted fluorescence at different wavelengths according to lipids in which it is dissolved, as already stated. Fluorescence micrographs shows that

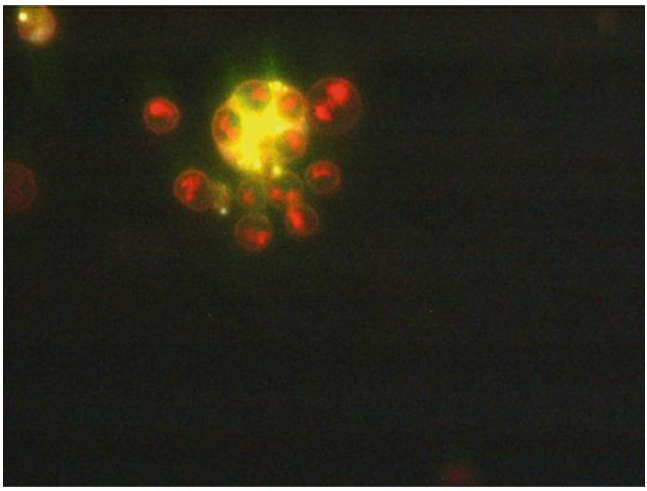

Figure 1. A *Chlorella protothecoides* cell autospore mother cell releasing daughter cells stained with *Nile red*. The *yellow* fluorescence of the cell mother is distinct from the *red* fluorescence emitted from the daughter cells. The fluorescent micrograph was taken using 488 nm excitation and 500 > 505 nm emission wavelength. Magnification 100×.

while the core of the microalga *Chlorella protothecoides* autospore mother cell predominately exhibited yellow fluorescence under 488 nm mainly due to neutral storage lipids, the released daughter cells exhibited red fluorescence mainly due to the structural membrane polar lipids (Fig. 1).

Silva et al. (2009b) also reported linear correlations between the Nile red fluorescence measured by flow cytometry and the total microalgal lipid content during a raceway and sleeve microalgal growth. However, no microalgae physiological states were reported.

However, beyond the use of a rapid lipid detection method, throughout the course of any microbial process for lipid production, it is also essential to monitor other cellular parameters such as cell viability as a high proportion of stressed or dead cells present in any part of the bioprocess will be also detrimental as such cells, although containing lipids in their structure do not accumulate oil as the metabolically active cells do, thus decreasing the process yield.

Nonetheless, the published works aiming at the biodiesel production from microalgae used classical microbiological techniques to monitor lipid content and cell proliferation (Xu et al., 2006; Miao and Wu, 2006; Xiong et al., 2008; Oh et al., 2009; Widjaja et al., 2009; Morowvat et al., 2010) which beset a number of problems. The conventional methods for microalgal lipid assessment show many pitfalls as already pointed out. Optical density cell count and dry-cell weight measurements, although indicative of proliferation, provide no information on cell physiological state and rarely take into account changes in cell size (Hewitt and Nebe-von-Caron, 2001).

Multiparameter flow cytometry coupled with specific fluorescent dyes can provide information about the physiological states of cells and the mechanisms of stress response rapidly, with a high degree of accuracy. For instance, propidium iodide (PI), a fluorescent strain that has widely been used to assess microalgae cell viability (Cid et al., 1996; Franqueira et al., 2000; Silva et al., 2009a; Rioboo et al., 2009), binds to DNA intercalating with double stranded to produce red fluorescence excited by blue light but cannot cross an intact cytoplasmic membrane. In this way, PI can be used to discriminate between viable nonfluorescent cells and nonviable fluorescent cells. Algal metabolic activity can be assessed by esterase activity using fluorescein diacetate (FDA). Healthy cells take up FDA which is then hydrolyzed inside the cell by esterases to produce fluorescent fluorescein, which is hydrophilic and retained by viable cells. Decreased fluorescein fluorescence indicates impaired enzyme activity or loss of cell membrane integrity (Franklin et al., 2001; Prado et al., 2009). Rioboo et al. (2009) also used FDA to measure *in vivo* microalgae cell division. Membrane potential changes in microalgae have been reported as a stress response to exposure to copper, using the lipophilic stain 3,3′-dihexyloxacarbocyanine $DiOC_6(3)$ (Franklin et al., 2004). This technique can also analyze cell density and light scatter signals. Shifts in forward-angle light scatter (<15°) and side-angle light scatter (15–85°) indicate cell size and shape or granularity, respectively.

Silva et al. (2009a) developed a flow cytometric protocol to monitor cell intrinsic light scatter, viability, and lipid content of *Chlorella protothecoides*. Changes in the right-angle light scatter (RALS) and forward-angle light scatter (FALS) were detected during the microalgal growth, which were attributed to the different microalgal cell cycle stages. The microalgal cell viability was assessed using propidium iodide, and the lipid content was determined using Nile red.

Therefore, it is important to assess total cell lipid content, near real time, throughout the microalgal lipid production process. If the cell lipid content information is available during the time course of the bioprocess, decisions on process control strategy can be made (e.g., adjusting the carbon/nitrogen ratio of the medium or increasing the oxygen supply) so that lipid productivity can be rapidly increased.

Multi-staining flow cytometry is proving to be a useful technique for microalgal lipid production process optimization, providing important physiological information, at the individual cell level, about process efficiency that is difficult to obtaining in any other way.

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R. Gordon and J. Seckbach (eds.), *The Science of Algal Fuels*,
Cellular Origin, Life in Extreme Habitats and Astrobiology, 269–282
DOI 10.1007/978-94-007-5110-1_15, © Springer Science+Business Media Dordrecht 2012

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APPROACHES AND PERSPECTIVES ABOUT BIODIESEL AND OIL PRODUCTION USING ALGAE IN MEXICO

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1. Introduction

Oil extraction in México started during the Aztec kingdom with the exploitation of the ground deposits better known as "chapopoteras." It has several uses such as for religious ceremonies, cleaning teeth, and sealing for construction. In 1783, the Spaniard crown declared the mineral rights to use just with a formal notice, but as part of the adjustments occurred after the independence war, several perforations happened in the late nineteenth century, and the transformations of the laws in the country led to the development of private companies to exploit the underground resources found around the Gulf of México, and the first foreign companies were developed (http://petroleo.colmex.mx/index.php/component/content/article/54). However, with the increment of oil production and the taxation of this activity by several Mexicans, a conflictive situation arose which was resolved with the nationalization of all foreign companies in 1938. After that period, the oil production increased in their volume and in the value hitting top values in the late 1970s and at the beginning of the twenty-first century (Marichal, 2008). Oil and gas production represents the venue wherein the Mexican economy has developed over the last century (Gil-Valdivia and Chacon-Dominguez, 2008) in industrial, economic, monetary balance, and in many other political factors.

The actual oil demand in the country is over two million of barrels per day, and the projections are that this will increase up to 3.38 million of barrels per day by the year 2025 with a potential production of 4.7 million of barrels per day (Lajous, 2005). Most of the oil is related to gasoline production for any motor vehicle (car, truck, plane, etc.), and their needs in the future will increase as the calculated economic growth. But other energy-producer resource in the country is natural gas and coal in a less extent. In the case of natural gas, we use it most of the time for electricity production, cooking, and heating. Heavy increases in this

resource are prognosticated for future population growth. As in many countries, there is an urgent need to develop alternative fuels. In the case of México, some legal adjustments are needed to address the issue of the legal rights to explore and use alternative resources. At the present time, plants with high lipid contents are excellent sources for alternative oil and biodiesel production (http://www.bioenergeticos.gob.mx/index.php/biodiesel.html?gclid=CK61nqrg0a8CFaldTAodUFH2gQ), but algae are excellent alternatives for a wider variety of producers. In modern times, the uses of algae have more diversificated (http://www.oilgae.com/algae/use/use.html; Griffiths and Harrison, 2009; Nielsen et al., 2012).

Microalgae are a group of unicellular single-cell organisms that can grow in rivers, lakes, wells, thermal springs, ice, or the ocean. They essentially use the energy of the sun through photosynthesis to convert CO_2 into carbohydrates, protein, and oils (Clarens et al., 2010; Jorquera et al., 2010; Xu et al., 2011). Worldwide microalgae biomass is presently commercially produced primarily for human nutritional products and as feedstuff for early feeding marine larvae. In the 1970s, the DOE started the Aquatic Species Program from 1978 to 1996 looking into microalgal biochemistry and mass production methods for using microalgae as a biofuel (Sheehan et al., 1998). This program was discontinued due to lower petroleum prices at the time. Recently, again escalating oil prices (>$100/barrel) due to depleting resources and high demand have revived interest in microalgae as a sustainable energy alternative (Borowitzka, 2008; Huang et al., 2010). Microalgae are particularly attractive since they can lessen CO_2 emission (Takeuchi et al., 1992); they are a nonfood crop and can produce oil with low land use (Chisti, 2008a, b), in saline or brackish or even wastewater (Christenson and Sims, 2011; Prathima Devi et al., 2012), with a high productivity and high yields since many strains contain up to 20–80% of their weight in triglycerides convertible into biodiesel (Spolaore et al., 2006). For these reasons, there are currently many private companies actively attempting to commercialize biodiesel derived from microalgae; however, none as far as the authors are aware are in production.

Seaweeds are widely distributed in a wide range of habitats from lakes and rivers to the open ocean. They have a similar photosynthetic apparatus than the microalgae (or the land plants), but their photosynthetic efficiency might be higher because of their morphological complexity. Traditional uses of seaweeds are related to hydrocolloid production for several applications, use of pigments for stain clothes, fertilizers, animal/human food source, and uses as support culture for aquaculture of commercial species among other activities (Chapman and Chapman, 1977). There are some physiological challenges in relation to the lipid metabolism in the use of each species (Guschina and Hardwood 2006), but their triglyceride yields are high enough to be considered into biodiesel. It is necessary to develop basic knowledge of concentration of wild populations or develop extensive cultures to produce the necessary biomass and the best methodological process to extract the oil from seaweeds (GBEP, 2009).

Table 1. Comparison EROI of different energy alternatives.

Energy type	EROI
Petroleum (1960s–1970s)	100[a]
Petroleum (today)	20–40
Coal	50–80
Photovoltaic	10–30
Biogas	3–8
Wind	15–25
Nuclear energy	5–15
Hydroelectricity	20–40
Biodiesel from microalgae	1.5–3.0 or <1.0[b]
Ethanol (corn derived)	1.0–1.5

All EROEI numbers from Hall et al. (2009), except [a]Gupta and Hall (2011) and [b]Batan et al. (2010).

There are two alternatives in the use of microscopic (microalgae) species and/or use of macroscopic algae (seaweeds) for oil production (GBEP, 2009) and biogas (Vivekanand et al., 2012). In the recent years, it has been a strong effort in the understanding of oil content (Li et al., 2010) wherein dozens of species have been tested to evaluate their potential use (Griffiths and Harrison, 2009) to determine the quality and quantity of oil yield. The goal of this chapter is to document potential approaches and perspectives about biodiesel and oil production using algae in México as a feasible and economic alternative to help in the future demand.

2. Sites and Methods for Data Gathering

An evaluation of the current knowledge of microalgae advantages, net energy, life cycle assessment, and energy return on investment was made to see if microalgae, or seaweeds, would be reasonable for use as potential oil in biodiesel production. In addition for some seaweed, data was available from two sources to determine the area needed: (1) sampling carried out between 2005 and 2010 along the Baja California Peninsula and (2) published information. For some species, we evaluate their lipid contents using collections made in Bahía Magdalena between 2005 and 2006. Samples were cleaned and dehydrated in the shade for bromatology analysis with the methodology proposed by the AOAC (1995), which includes the determination of lipids. Statistical analysis was performed with Statistica 6.0 software, beginning with *a priori* tests (Normality and Homoscedastic) ($p < 0.05$). Comparison was conducted (one-way ANOVA) between each thallus for all the nutrients and determined if significant differences existed between them across the statistician of test $F(\alpha = 0.05; 8, 18\text{gl})$.

3. General Findings

3.1. MICROALGAE LAND AREA ADVANTAGE

In terms of land use, microalgae have two main advantages for production of biodiesel over seaweed or terrestrial plants: (1) it can use nonarable land, thereby does not compete with food crops and (2) the area needed is much less than oleaginous crops. In a recent paper, Chisti (2008a, b) outlined the production potential of microalgae and the area necessary to replace 50% of all US transportation fuels (Table 1). Chisti (2008a, b) showed that the total area needed of current US crop land is less than 1.1–2.5% (depending on oil content) and uses less area than all other conventional sources of biodiesel. He also discussed the oil content of different microalgae and facility design to produce 100,000 kg of microalgae biomass. He estimated the cost of producing a kilogram of microalgal biomass, if CO_2 is available at no cost, at \$2.95 and \$3.80/kg for photobioreactors and raceways, respectively. These values although encouraging, however, are still a factor 4–5 below the values for producing petroleum at current prices \$0.69/kg (assuming at \$100/barrel), without taking into account the cost of extracting oil from the microalgal biomass; hence, cost will be even higher. Nevertheless, in 2008, Chisti (2008a, b) went further and discussed in a paper that microalgae was not only the better choice but the only choice available to produce biofuels compared to conventional oleaginous plants or seaweeds. The actual costs to produce microalgae have led others to criticize Chisti (2008a, b) that microalgae are not presently a probable replacement for petroleum compared to terrestrial plants (Reijnders, 2008). Some have even gone as far to indicate that microalgae will never be useful as a liquid fuel to replace oil for transportation (Van Beilen, 2011).

3.2. SEAWEED AS COMPLIMENTARY SOURCE OF OIL

The global value of seaweed products is between USD \$5.5 and 6 billion (McHugh, 2003); this comes from a production from wild crop of 1,143,273 metric tons, but from aquaculture activities, another USD\$ 7,187,125 is produced with 15,075,612 metric tons (Global BioEnergy Partnership 2009). México only produces 2.36% of the total wild crop with 27,000 metric tons and no aquaculture efforts. Oilgae (2010), using a production figure of 5,000 dry metric ton per year, estimated that macroalgae grown on 15,000 km^2 is needed to replace 1% of worldwide gasoline consumption. However, new figures strongly suggest that only 10,895 km^2 are needed to replace the 1% of the global production (Roesijadi Jones et al., 2010).

3.3. MICROALGAE AND SEAWEEDS AS NET ENERGY

Presently, the economic viability of microalgae and seaweed farms at prices competitive with petroleum or other conventional energy options has not been proven

(Richardson, et al., 2010; Teixeira et al., 2012). This is undoubtedly due to the bottleneck caused by the need for (1) energy-efficient production of microalgae biomass, (2) efficient process to harvest the algae, and (3) an efficient process to separate the natural oil from the algae has not been created. In order to better understand the problem with energy costs, several similar conceptual methodologies have been developed such as life cycle assessment, net energy ratio, and newer techniques, at least in terms of being employed to study microalgae potential as a biofuels, such as energy return on invested.

3.4. LIFE CYCLE ASSESSMENT (LCA)

Life cycle assessment (LCA) is a tool developed over 20 years ago in the Netherlands for the systematic evaluation of the environmental aspects of a product or service system through all stages of its life cycle. It provides a cradle-to-grave approach for evaluating products performance by considering the potential impacts from all stages from its manufacture, product use, and end-of-life management. The International Organization for Standardization (ISO), a worldwide federation of national standards bodies, has standardized this framework within the ISO 14040 series on LCA (ISO, 1997). There are four basic components involved in carrying out LCA: (1) to define the goal and scope of the study; (2) to identify and quantify environmental loads of the system being analyzed, such as energy and raw materials consumed, the air emissions (e.g., CO_2 greenhouse gases), water effluents, and wastes generated; (3) to evaluate the potential environmental impacts of these loads; and (4) to assess available options for reducing these environmental impacts.

Presently there is not a single uniform approach to LCA at this time, or life cycle impact assessment (LCIA) methodology (Curran, 2004). Also, due to the complexity and scope attempted in some studies and the different metrics utilized to convey the results, comparisons are sometimes difficult. Components frequently studied with LCA analysis are net energy and GHG production. Batan et al. (2010) recently applied LCA methodology in an industrial-scale engineering model for the species *Nannochloropsis* using a photobioreactor architecture to evaluate the net energy ratio (NER) and net greenhouse gas emissions (GHGs) of microalgae biodiesel in comparison to petroleum diesel and soybean-based biodiesel. The resulting NER of the microalgae biodiesel process was found to be 0.93 MJ of energy consumed per MJ of energy produced, which is a ratio of NER of 1.075.

3.5. ENERGY RETURN ON INVESTED (EROI)

Energy return on invested (EROI)—or sometimes referred to as EROEI (energy return on energy invested)—is probably more straightforward to conceptually understand than LCA since its scope only comprehends energy input to energy output.

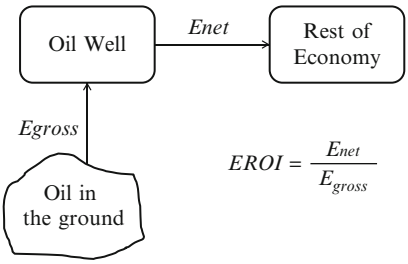

Figure 1. Definition of energy return on invested (EROI) (Adapted from Hall et al., 2008 and Cleveland, 2008).

EROI is simply the ratio of the net energy extracted (*Enet*) to the energy used (*Egross*) in the process (Fig. 1). EROI is a dimensionless number, while energy surplus refers to an actual physical quantity of energy. Suppose the energy extracted in a production process is 100 J, but doing so consumes 1 J. The EROI for that process is 100 (100/1), while the energy surplus delivered is 99 J (100 − 1). It is widely believed that oil EROI in the 1920s–1970s was around 100 (Gupta and Hall, 2011). Net energy analysis was developed in recognition of the importance in understanding energy alternatives due to energy price increases in 1973–1974 and 1980–1981. Recently, interest in net energy analysis, based on EROI, has generated renewed interest due to growing concern of increasing energy prices, its role in climate change, and as a tool for comparing energy alternatives due to prediction of impending deficiency of availability of conventional liquid fossil fuels. The above EROI can be used in general for evaluating any energy system, such as production of microalgae. Recently, Beal et al. (2011) reported the first experimental case results using this energy balance strategy for an integrated algal biocrude production facility. He reported values significantly less than 1.0 for all cases he studied.

3.6. EROEI COMPARISONS

A comparison of EROI of energy alternatives indicates that microalgae and ethanol are currently poor choices for generating surplus energy (Table 1).

3.7. SEAWEED AVAILABILITY, LIFE CYCLE, AND POTENTIAL BIODIESEL CONTENT

Roesijadi Jones et al. (2010) have suggested 10,895 km^2 (1,089.5 ha) of seaweed biomass to substitute 1% of world oil demand; thus, area is a relevant index to consider for potential use of seaweeds as alternatives. In México, there are several

Table 2. Comparative analysis of area (ha) covered by main seaweed and species in México.

Species	Area (ha)	Biomass (wet t tons)
Kelp forest[a]	6,485.05–14,189.28	35,813.1 ± 2,260 87,095.9 ± 4,528.2
Sargassum forest[b]	2,300	45,000
Gelidium robustum[c]	ND	~18,000
Gracilaria beds[d]	~800	~45,000
Eucheuma uncinatum	ND	~650
Chondracanthus sp.	ND	~18,250

[a]Hernández-Carmona et al. (1989a, 1989b, 1991); [b]Aburto-Oropeza et al. (2007); [c]Pesca (2005); [d]Zertuche Gonzalez (1993); Vergara-Rodarte (2008).

ecosystems dominated by seaweed and their covered extensive areas (Table 2). Looking into the actual data, several of these ecosystems/species might play a role in biodiesel production in particular when extensive culture is considered associated with recycle of wastewater (Nielsen et al., 2012). A relevant point represents the knowledge of the life cycle of each species and their limiting factors in relation to lipid production, among them nutrient concentration and temperature range are essential in understanding what controls the quality of lipids. In all the species considered in Table 2, we have a good idea about their life cycle; however, limiting factors in population growth or reproduction are not widely known. Experiments related to ecophysiological trends are crucial in understanding what kind of application might be developed: biodiesel and/or biogas.

In terms of experimental determination of biodiesel among seaweed species, there are big differences. It is necessary to screen each species to select a group of species who might contribute toward the use of species with higher lipid content and less energetic requirements. Table 3 **shows species** with high ether extract (underlined) who reflect high lipid concentration finding out of 14 species surveyed only five have statistically high levels of ether and thus lipids. It is certainly that each species might have higher concentration in relation to the environmental features or culture conditions, but this need to be tested. In particular seasonal variations are relevant to have a sustainable production.

4. Summary

Fossil oil extraction in México started during the Aztec kingdom with the exploitation of the ground deposits better known as "chapopoteras." Fossil oil and gas production represents the venue wherein the Mexican economy has developed over the last century in industrial, economic, monetary balance, and in many other political factors. Most of the oil is related to gasoline production for any motor vehicle (car, truck, plane, etc.), and their needs in the future will increase as the calculated economic growth. At present, plants with high lipid contents are

Table 3. Comparative analysis of the content of ether extract in seaweed species from México.

Species	Ether extract
Codium amplivesiculatum	0.42 ± 0.07
Codium cuneatum	0.41 ± 0.04
Caulerpa sertularoides	0.25 ± 0.02
Ulva lactuca	0.25 ± 0.05
Eisenia arborea	0.9
Macrocystis pyrifera	1.0 + 0.1
Sargassum sp.	2.61 + 0.16
Hypnea johnston	0.48 ± 0.02
Laurencia masonii	1.32 + 0.11
Gelidium robustum	1.3 + 0.12
Gracilaria textorii	0.18 ± 0.05
Gracilaria pacifica	0.19 ± 0.04

excellent sources for alternative oil and biodiesel production. The question remains how would be the most sustainable and profitable way to produce this. In the recent years, it has been a strong effort in the understanding of oil content in each species wherein dozens of species have been tested to evaluate their potential use to determine the quality and quantity of oil yield. The goal of this chapter is to document potential approaches and perspectives about biodiesel and oil production using algae in México as a feasible and economic alternative to help in the future demand. The present bottleneck for microalgae production is its low EROI in comparison to petroleum or other energy sources. Both recent LCA analysis and EROI analysis applied to microalgae—although systems varied, demonstrated values of 1 or less than 1, respectively. EROI for an entire energy profile must be greater than unity for microalgae to be useful as energy replacement for fossil fuels. LCA and EROI studies indicated that as long as important input is not disregarded, such as nutrients, excess heat, and CO_2, that the energy return on investment or sometimes referred to as NER is not competitive with conventional fuels at this time. To compete effectively with petroleum microalgae production, harvest and extract of oil energy efficiency must improve. An EROI of 10 would catalyze the development of whole new energy industry using microalgae. EROI analysis provides an important tool for illuminating the research pathway in developing microalgae as a possible replacement fuel. It is necessary for 10,895 km^2 (1089.5 ha) of seaweed biomass to substitute 1% of world oil demand; thus, area is a relevant index to consider for potential use of seaweeds as alternatives. In México, there are several ecosystems dominated by seaweed and their covered extensive areas and might play a role in oil production in particular when extensive culture is considered associated with recycle of wastewater. We have a good idea about their life cycle; however, limiting factors in population growth or reproduction are not widely known. Experiments related to ecophysiological trends are crucial in understanding what kind of application might be developed: biodiesel and/or biogas.

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R. Gordon and J. Seckbach (eds.), *The Science of Algal Fuels*,
Cellular Origin, Life in Extreme Habitats and Astrobiology, 283–303
DOI 10.1007/978-94-007-5110-1_16, © Springer Science+Business Media Dordrecht 2012

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BIODIESEL PRODUCTION FROM MICROALGAE: A MAPPING OF ARTICLES AND PATENTS

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1. Introduction

The global population crossed the seven billion mark in October 2011 and is expected to reach around 10.5 billion by 2050. This has put increased pressure on food, fuel, and energy supply all over the world. It is now agreed that we are living in an era full of challenges such as climate change, food security, energy security, etc. Energy is going to be the biggest challenge in the twenty-first century. However, current energy supplies are unsustainable from an environmental, economic, and social standpoint. According to World Energy Outlook (2011), international concern about the issue of energy access is growing. Interestingly, the United Nations has declared 2012 to be the "International year of Sustainable Energy for All," and the Rio+ 20 summit represents an important opportunity for action. Furthermore, the report emphasized that providing energy access for all by 2030 is a key global concern. Therefore, governments around the world have been heavily investing in renewable energy resources such as solar, wind, geothermal, tidal, and biomass energies as they are environmentally friendly and can provide long-term solutions. Out of these, biomass-derived fuel (biofuel) can serve as an excellent alternative source to meet the present and future demands.

The term biofuel refers to solid, liquid, or gaseous fuels that are predominantly produced from biorenewable feedstocks (Demirbas, 2009). Biodiesel is defined as the monoalkyl esters of long-chain fatty acids derived from the chemical reaction (transesterification) of renewable feedstocks, such as vegetable oil or animal fats, and alcohol with or without a catalyst (Ahmad et al., 2011). Biodiesel is quite similar to petroleum-derived diesel in its main characteristics such as cetane number, energy contentment, viscosity, and phase changes (Lim and Teong, 2010). Biodiesel does not contain any petroleum product but can be easily blended

with normal diesel, and thus, the vehicle does not require any engine modification. The demand for biodiesel is increasing rapidly. According to the Global Status Report Renewables (2011), world biodiesel production has increased from 0.8 billion l in 2000 to 19 billion l in 2010. The European Union produced nearly ten billion l of biodiesel in 2010 representing 53% of the total production. Germany remains the world's top biodiesel producer at 2.9 billion l in 2010 followed by Brazil, Argentina, France, and United States.

According to the International Energy Agency, biofuels have the potential to meet more than a quarter of world demand for transport fuels by 2050. Depending on the feedstocks used, biodiesel has been categorized into first-, second-, third-, and fourth-generation biodiesel. At present, first-generation biodiesel is mainly produced from feedstocks such as soybean oil, sunflower oil, rapeseed oil, and palm oil. Diversion of these food crops for the distilling of transport fuel has created a serious debate about "food versus fuel." It has also created serious environmental problems in terms of the use of otherwise agricultural land and water and clearing of forests in Indonesia and Malaysia for oil palm cultivation.

Second-generation biodiesel feedstocks such as *Jatropha, Pongamia*, Mahua, waste cooking oil, animal fats, beef tallow, and pork lard present several advantages, but they have not yet been used for large-scale biodiesel production because of limited availability. Third-generation biodiesel can be produced from algae whereas fourth-generation biodiesel from genetically engineered organisms. It has been found that lower aquatic organisms such as microalgae can be used not only for biodiesel production but also for carbon dioxide capture from carbon dioxide emitting industries such as power generation, cement, and steel plants (Sahoo et al., 2012). Microalgae can use water and carbon dioxide to produce biofuels, food, feeds, and high-value bioactive compounds. Algae have several advantages over other crops including *Jatropha* (Sahoo, 2010), and it has already been reported that microalgae can produce 15–300 times more oil than other first-generation oil crops (Chisti, 2007; Schenk et al., 2008). Microalgae oil differs from most vegetable oils in being quite rich in polyunsaturated fatty acids with four or more double bonds (Belarbi et al., 2000; Chisti, 2007). While terrestrial plants in temperate climates can achieve a photoconversion efficiency of only below 1%, microalgae can convert up to 5% of solar energy into chemical energy (Schenk et al., 2008).

The advantages of microalgae over land plants as a source of transport biodiesels are as follows (Sahoo et al., 2012):

- Oil yield per area of microalgae cultures could greatly exceed the yield of the best oilseed crops.
- Microalgae grow in an aquatic medium, but need less water than terrestrial crops.
- Microalgae can be cultivated in sea water or brackish water on nonarable land and do not compete for resources with conventional agriculture.

- Microalgal biomass production may be combined with direct biofixation of waste carbon dioxide.
- Algae cultivation does not need herbicides or pesticides.
- The residual algal biomass after oil extraction may be used as feed, fertilizer, or fermented to process ethanol or methane.
- The biochemical composition of the algal biomass can be modulated by varying growth conditions, and the oil content can be highly enhanced.

Considering the potential of algae for biodiesel production, a large number of research papers, reviews, and articles have been published in recent years. A number of patents have also been filed. This chapter analyzes the past developments and current status of research in algal biodiesel by mapping various articles and patents for future studies.

2. Objective

The objective of this chapter is to critically evaluate and analyze the past developments, current status of research, development, and commercialization of biodiesel production from microalgae alone. Although there are more than 300,000 microalgal species distributed around the world, only 30,000 species have been described in the literature. For this particular study, we chose only four microalgal species: *B. braunii, Chlorella* sp.*, Scenedesmus* sp.*, and Chlorococcum* sp.*,* for their high lipid contents, fast growth, resistance to high temperatures and various other properties, and above all for their potential for commercialization in the near future. This chapter evaluates the potential use of algae for biodiesel production, analyzing various patents and scientific papers. For this study, we used VantagePoint v7 (www.thevantagepoint.com), a powerful text-mining tool, to extract results from patent databases and academic publications.

3. Methodology

3.1. SEARCH FOR ARTICLES

For articles, searches were conducted in Web of Science℠, a product referenced by Thomson Reuter (formerly ISI) Web of Knowledge℠. The Web of Knowledge℠ is a virtual platform for multidisciplinary areas (information about sciences, social sciences, arts, and humanities) containing high-quality information from newspapers, journals, patents, conferences, virtual sites, etc. When academic literature is published, it is analyzed carefully by Thomson Scientific to decide on its inclusion or otherwise on the platform. The criteria are the impact, influence, timeliness, peer review, and geographic representation. Besides the bibliographic

content, there are also tools to access, analyze, and manage research information. It uses multiple databases, which include over 40 million items that can be searched simultaneously (Thomson Reuter Web of Knowledge, 2012). Indexing of content is provided by Science Citation Index Expanded™, Social Sciences Citation Index® Expanded, Conference Proceedings Citation Index℠, and Arts & Humanities Citation Index® (Web of Science, 2012). Initially, we chose the search page of the Web of Science℠ because it is possible to find records using topic terms (TS), author (AU), title (TI), and more. To search one or more search fields, it is necessary to enter words or phrases connected by Boolean search operators such as "and," "or", "not," and "same". The operator "*" is useful to search for plurals and variant spellings. In the search page of the Web of Science℠, we preferred to do our research in the option called "advanced search." Advanced Search query consists of one or more field tags, e.g., AD = Address (searches for institution and/or place names in the Addresses field within a record) or AU = Author (searches for author names in the Author(s) field within a record) and/or other important information, combined with each other, connected by Boolean search operators (Web of Science, 2012). We looked for the document type "articles," combined with the TS (topic). Our TS was the expression "((biodiesel"((biodiesel* or diesel* or biofuel* or fuel* or hydrocarbon * or oil* or (fatty* and acid*))," combined with the other one, containing the names of the algal species in question, (*Botryococcus** *or Chlorella** *or Scenedesmus** *or Chlorococcum**) and the term "product." Using this method, the use of the expression "biofuel" is strategic because many authors publish their articles using the general term "biofuel" instead of "biodiesel." Their intention was to make it easier to find, not restricting only the word "biodiesel." Biofuel includes biodiesel and other kinds of biofuels such as bioethanol and biogas. The search expression was used to ensure all biodiesel from the referred microalgae. We found 501 articles related to algal biofuel, distributed in many Web of Science categories. Then we refined our search, excluding some records of Web of Science Categories, that we considered unrelated to biodiesel production: petroleum engineering, oceanography; materials science biomaterials; spectroscopy; materials science multidisciplinary; meteorology atmospheric sciences; pharmacology pharmacy; nanoscience nanotechnology; geochemistry geophysics; toxicology; physical chemistry; heredity genetics; food science technology; electrochemistry; physical geography; thermodynamics; history and philosophy of science; instruments instrumentation; fisheries; ecology; medicine research experimental; mechanics; optics; nutrition dietetics; paleontology; chemistry analytical; physics nuclear; religion; geosciences multidisciplinary; biophysics; soil science; medicinal chemistry; and veterinary sciences. After further scrutiny, we removed 156 articles which are out of the focus of our research. After this, we found only 345 articles related to algal biodiesel. These articles were then treated with Vantage Point® for data cleaning, analyses, and mapping operations through the application of a thesaurus and Fuzzi logic.

3.2. SEARCH FOR PATENTS

Patents constitute a temporary right of exclusiveness in the exploration of a new technology granted for the state. On the other hand, the concession demands the bearer the revelation of information necessary for the attainment of the technology, protection object (WIPO, 2000). The patents are useful not only for protection but also as a source of information techniques for researches. The patent databases are excellent source of information. They have more advantages than other sources since they are the main channel to present the advances in science and technology but contain informations published in no other medium. In addition, these documents not only show a complete technical information but also data such as the holder and inventor related about questions of commercial type or planning (Gray and Meister, 2006). For patents, we prefer searches in database of gratuitous bases. The European Office of Patents (EPO) was chosen by its range. It covers, beyond its bibliographical documents, data of more than 80 countries. Its database, the Espacenet, offers free access to more than 70 million patent documents worldwide, containing information about inventions and technical developments from 1836 till today (European Office of Patents, 2012). The North American Office of Marks and Patents (USPTO) was not used as it contains only documents deposited or published in the United States, which could limit our search. In the Espacenet site, we went to advanced search. Had to the little volume of patents, we did not use International Patent Classification (IPC) or European Classification System (ECLA) in the search strategy.

After this, the patents were exported from the Espacenet, database of world patents and manually imported into Microsoft Excel®. After reading all the abstracts of the patents, we excluded those out of the target. We emphasize that a detailed study was conducted in order to verify the relevance of each patent with the theme, and we can say with a high degree of certainty that all the patents in this search refer to algae biofuel. In this study, we used the content of abstracts of the patents as the main indicator.

The method for the statistical treatment of data used the priority date as the main indicator. The choice of this indicator was based on the availability of these documents, where only patents filed 18 months before the search could be downloaded.

3.3. TECH MINING

Tech mining makes exploitation of text databases meaningful to those who can gain from derived knowledge about emerging technologies. It begins with the premise that we have the information, the tools to exploit it, and the need for the resulting knowledge. The information provided puts new capabilities at

Table 1. Nine steps of tech mining.

Phase A: Intelligence
1. Issue identification
2. Selection of information sources
3. Search refinement and data retrieval
4. Data cleaning
Phase B: Analysis and design
5. Basic analyses
6. Advanced analyses
Phase C: Choice
7. Representation
8. Interpretation
9. Utilization

the hands of technology managers. Using the material present, these managers can identify and access the most valuable technology information resources (publications, patents, etc.); search, retrieve, and clean the information on topics of interest; and lower the costs and enhance the benefits of competitive technological intelligence operations(Porter and Cunningham, 2005).

Tech mining can compile and analyze information from science and technology databases such as publications and patents. Porter and Cunningham (2005) recommend that tech mining study must be structured in the interactive phases and suggested nine steps as shown in Table 1.

Among tech mining software, we chose VantagePoint v7 for many reasons. It has the capacity of clean downloaded text datasets, aids in discovering previously unrecognized relationships, and enables a range of analyses; repetitive analytical processes can be automated to a degree. Vantage Point 7 provided fuzzy matching capabilities to help clean, identify, associate, and reduce data appropriately (Porter and Cunningham, 2005). For example, handling misspelling, hyphenation, capitalization, and others, VantagePoint v7 removes exact duplicate records in the documents, associating and reducing data. To enhance data reduction, a thesaurus, a grouping of terms into a certain concepts, is useful. VantagePoint v7 works with statistical analyses, particularly Principal Components Analysis (PCA), in order to identify relationships among topics and concepts. When relationships among concepts have been identified, VantagePoint v7 provides assembling the number of relevant documents in clusters for each data set. VantagePoint v7 can construct technology maps to represent relationships graphically. With VantagePoint v7, we can run the Cluster Map, information visualization technology developed by the software Aduna, which allows visualization of sets of categorized objects. Similar in nature to Venn diagrams and Euler diagrams, the Cluster Map shows if and how these sets overlap, through the visualizing instantiated taxonomies or concept hierarchies (Aduna, 2012). After running VantagePoint v7, we found the following results.

4. Results and Discussions

VantagePoint is a powerful text-mining tool for discovering knowledge in search results from patent and literature databases. VantagePoint helps you to rapidly understand and navigate through large search results, giving a better perspective—a better vantage point—on information. The perspective provided by VantagePoint enables to quickly find WHO, WHAT, WHEN, and WHERE, enabling to clarify relationships and find critical patterns—turning information into knowledge (http://www.thevantagepoint.com). Whereas, Aduna develops new ways of exploring data. It gives a visual and rich exploration environment that helps the user to find information better, faster, with more precision and overview. Aduna technology allows the user to express a query in terms of data properties. It shows relations with other properties (http://www.aduna-software.com).

In total, we found 501 articles published related to algal biofuel, which are distributed in many Web of Science categories. After a detailed scrutiny, we removed 156 articles which are out of the focus of our research; thus, we found only 345 articles which are related to algal biodiesel. Similarly, we found a total of 68 patents on algal biofuel related to four taxa as shown in Table 2. Out of these 68 patents, we have listed only 21 patents which are found to be very relevant in this chapter (Table 3).

Figure 1 shows the growth in the number of articles and patents related to the production biodiesel from microalgae. The articles on algal biofuel date back to 1980s to the present (Fig. 1a), whereas the patents are mainly from 2006 to 2011 (Fig. 1b). The number of articles published was minimum until the end of 2006, when it grew rapidly. On the other hand, the first patent related to the production of biofuel from microalgae was registered in 2003. The growth in the number of patents remained constant and moderate until 2008, when the number of deposits increased dramatically. The results demonstrate the incipient and promising character of algae with high lipids for the production of biodiesel. In Fig. 2, the main periodicals and journals where the articles were published are listed.

We believe that the peak in the publication of articles and deposits of patents in 2008 is related to an interest generated by the association of the food crisis with raised commodity prices of petroleum.

This scenario has encouraged the search for green power resources that do not compete with foods.

Table 2. Number of patents on algal biodiesel on four algal taxa.

Keywords	Patents
*Botryococcus**	6
*Chlorella**	54
*Scenedesmus**	6
*Chlorococcum**	2

Table 3. Patents found in our search.

Publication number	Title
WO2011102593 (A2)	Photobioreactor for high-density microalgae culturing and a microalgae culturing and harvesting method using the same
US2011091945 (A1)	Methods of increasing biomass productivity, lipid induction, and controlling Metabolites in algae for production of biofuels using biochemical stimulants
CN101928669 (A)	*Chlorella vulgaris* Yun-32 and preparation method thereof
CN101824386 (A)	*Chlorell*a and application thereof
CN101475823 (A)	Method for preparing biodiesel from sugarcane
US2009298159 (A1)	Method for producing biodiesel from an alga
WO2009062119 (A2)	Microorganisms and methods for increased hydrogen production using diverse carbonaceous feedstock and highly absorptive materials
RU2008137841 (A)	Method for extraction of lipids from biomass
CN101649332 (A)	Production method of biodiesel
WO2009105927 (A1)	A high-density fermentation method of <I
WO2012006302 (A1)	Cultivation of green algae *Chlorococcum pamirum* for production of biofuel
US2011091945 (A1)	Methods of increasing biomass productivity, lipid induction, and controlling Metabolites in algae for production of biofuels using biochemical stimulants
WO2011102593 (A2)	Photobioreactor for high-density microalgae culturing and a microalgae culturing and harvesting method using the same
US2011138682 (A1)	Algal culture production, harvesting, and processing
JP2011068741 (A)	Method for extracting oil and fat from *Scenedesmus* algae and application of oil and fat and degraded residue
MY143383 (A)	A process for outdoor cultivation of microalga *Botryococcus* species for the production of biomass
US2010120111 (A1)	Method of producing hydrocarbon biofuels using genetically modified seaweed
KR20100088958 (A) JP5301097 (A)	Method for preparing hydrocarbon using microalgae
KR20050015233 (A)	Process for producing *Chlorella* containing omega-3 fatty acids by adding EPA and DHA into medium at end of fermentation
US2009087889 (A1)	Methods and compositions for growth hydrocarbons in *Botryococcus* sp.

4.1. COUNTRIES OF ORIGIN OF PUBLISHED PAPERS AND PATENTS

Figure 3 shows the countries of origin of technology and scientific knowledge in algal biofuel. The articles (Fig. 3a) are predominantly published by the United States (22%), followed by China (20%), and India (4%). France (6%), Japan, and South Korea (5%) also stand out in publication numbers. As for patents (Fig. 3b), in terms of country of origin, the United States holds the most (33%), followed by Canada (29%), and South Korea (19%). Other countries have also appropriated their technology, such as Japan (10%), India (5%), and Russia (5%). The great number of North American patents was expected given the high number of articles published in the United States, which indicates a policy investment in this

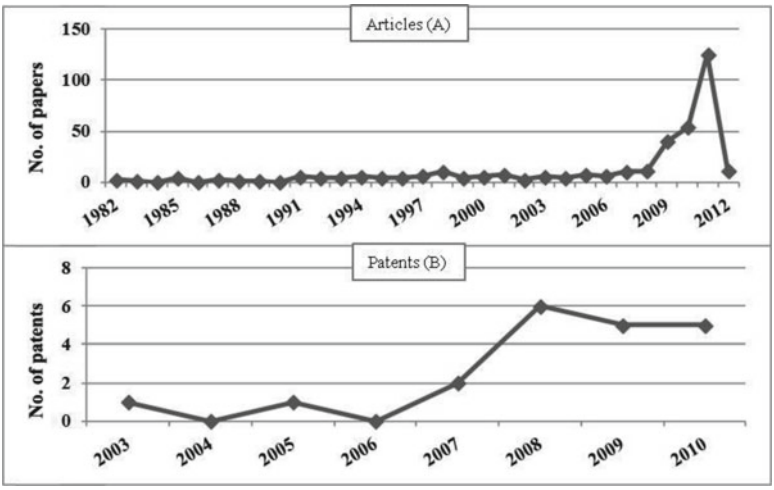

Figure 1. Annual evolution of articles (**a**) and patents (**b**).

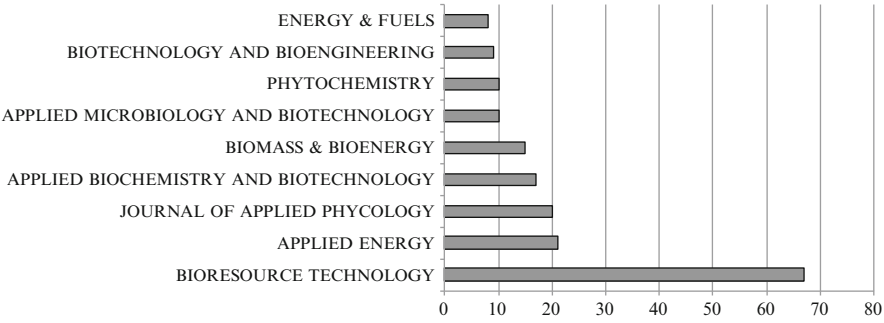

Figure 2. Main referred journals where the articles have been published.

type of technology. Canada, if examined more closely, has one of the highest numbers of patents (29%), while it possesses one of the lowest numbers in article publications (4%), indicating an aggressive policy of appropriation. For the other countries, publication is predominantly in articles, putting the knowledge in the public domain instead of appropriating technology as in the case of the United States and Canada.

We should point out that the great number of articles compared with the number of patents is a pointer that this technology is still incipient in character, with low industrial applicability. However, as already mentioned, on laboratory scale, studies with algae biofuels have been demonstrated to be highly promising.

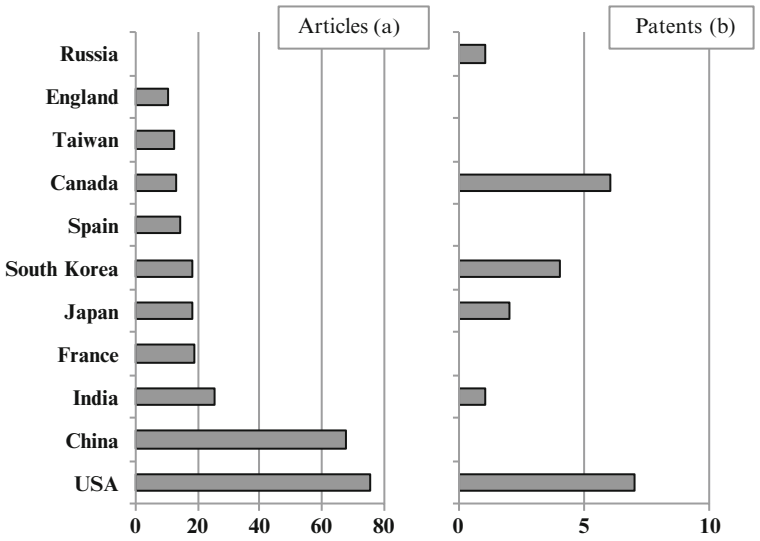

Figure 3. Countries of origin of published papers (**a**) and patents (**b**).

4.2. SECTOR-WISE HOLDERS OF PATENTS AND ITS NETWORKS

A detailed analysis showed that patents have been filed from diversified sectors. Figure 4 shows that the highest number of patents have been filed by private companies, 42% of which shows a great promise for commercialization of algal biodiesel production in near future. Academic sector holds 33% of the patents followed by individual inventors 17%, and governmental sector only 8%. These percentages seem to indicate that the technology is interesting in such a way for the academic sector, being of interest in university projects, as of the enterprise sector, improving or acquiring new technology, what it characterizes the technology as emergent (Quintella et al., 2009a, b).

Figure 5 shows a Cluster Map developed by the company Aduna for visualizing sets of categorized objects. Its main purpose is to show if and how these sets overlap, very similar in nature to Venn diagrams and Euler diagrams. Through the co-title networks, it enters the sectors of society where patents are more published (Fig. 7), making it possible to do a refined analysis of these standards. It was observed that 40% of the deposits with title of private companies are in co-title with independent inventors and academies, which seems to indicate a possible modality of technological transference and/or compensation of entrepreneurial inventors. It was also possible to identify that government institutions appropriated patents without partnerships.

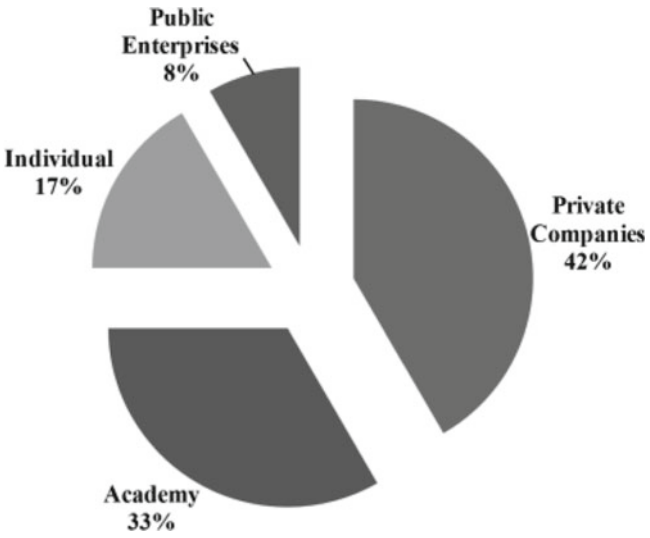

Figure 4 Holders by society sector.

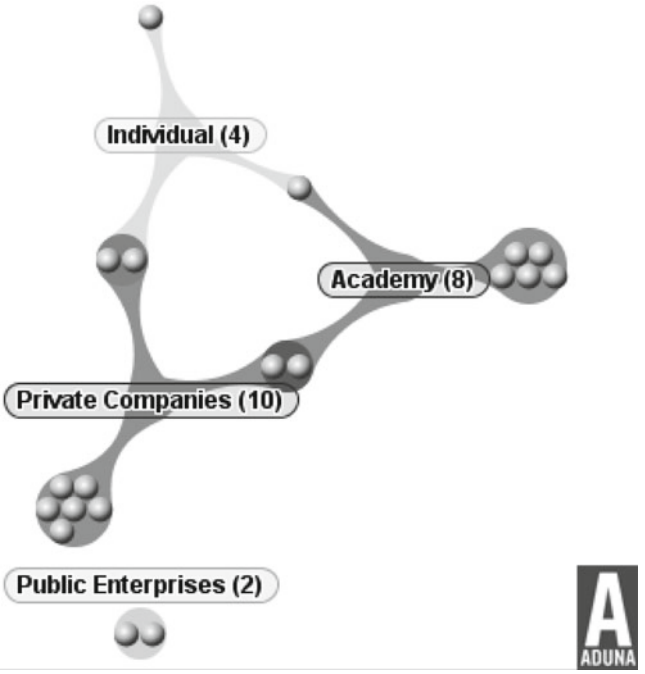

Figure 5. Cluster map of the holder in the sector of the society.

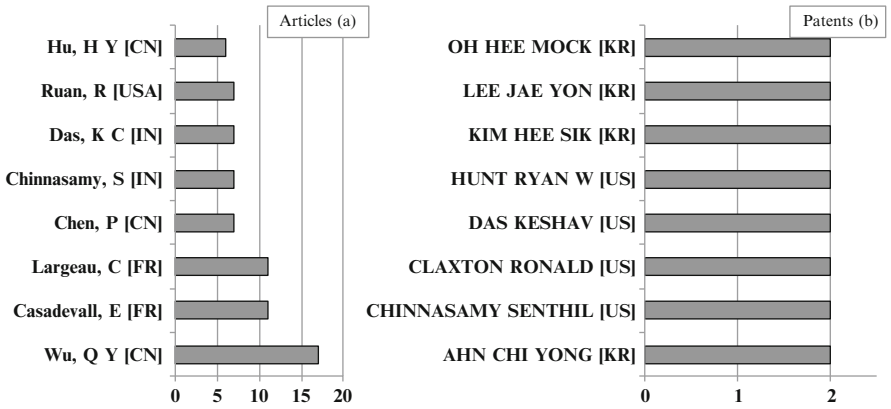

Figure 6. Main authors (**a**) and main inventors (**b**) in biodiesel production from microalgae.

4.3. MAIN AUTHORS AND MAIN INVENTORS

Figure 6 shows the main authors and main inventors for biodiesel production from microalgae. Qingyu Wu from Tsinghua University, Beijing, P.R. China, is the most productive author with 5% share of the published articles in the field, followed by Eliette Casadevall from École Nationale Supérieure de Chimie de Paris, France, with 3%, and Claude Largeau from France with 3%. In terms of patents, the main inventors had been divided between the South Korea and the United States. All the Korean inventors had developed their patents with the Korean Research Institute of Bioscience & Biotechnology, while the American inventors are mainly from University of Georgia. While Fig. 7 shows the cluster map of the authors, Fig. 8 shows the main inventors in biodiesel production from microalgae.

4.4. MAIN APPLICANTS

Figure 9 shows the eight biggest applicants for patents. They are mostly inventors from the Korean Company "Korean Reverse Speeds Institute of Bioscience," which indicates a policy partnership between the company and its collaborators. However, the University of the Georgia and, the Chinese one, Tsinghua University also appeared in our search, with a total of four patents. All the main applicants presented the same amount of deposits, two patents each. Some applicants presented just one patent. This result was weighted in view of the small volume of patents.

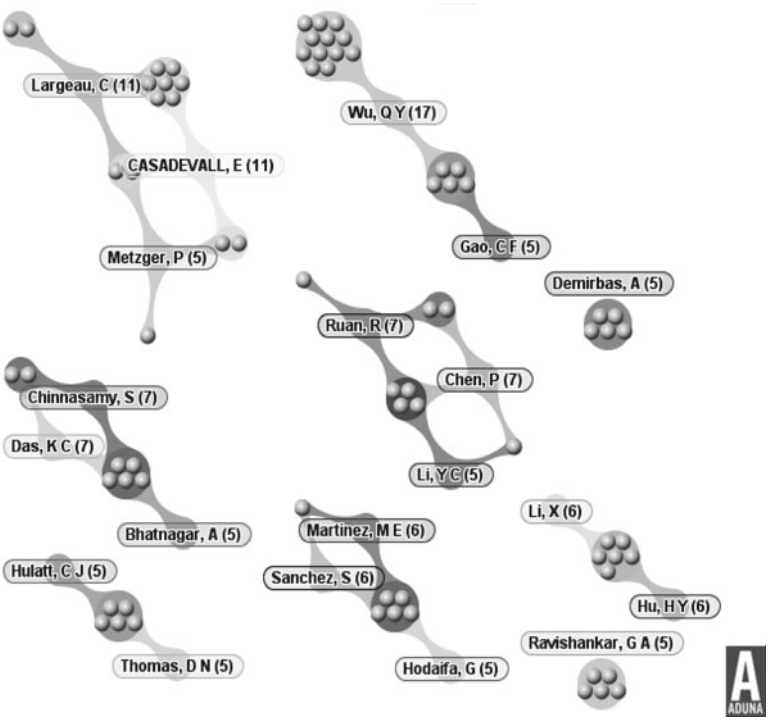

Figure 7. Cluster map of the main authors.

4.5. MICROALGAE: ARTICLES AND PATENTS

Figure 10 shows which algae had been more distinguished, as discussed in articles and patents. We found that the following microalgal species such as *Chlorella* sp. (48%), *Botryococcus* sp. (14%), *Neochloris* sp. (14%), *Scenedesmus* sp. (14%), *Dunaliella* sp., *Chlorococcum* sp. (2%), and *Spirulina* sp. (2%) have the most cited articles. Statistical analysis of patents stood out the species *Chlorella* sp., with approximately 38% of the documents, followed by species *Botryococcus* sp. (21%), and *Scenedesmus* sp. (17%). Yet other species were identified, together totaling 24%.

Among various algal species, highest number of patents and articles have been found on *Chlorella* (Fig. 10). Through the network of this relationship on algae articles are identified (Fig. 11), was possible to more make a refined analysis in search of standards. It was observed that the articles on *Chlorella* sp. Was correlated with other species. The same occurs with the *Botryococcus* sp. and the *Neochloris* sp. evidencing the existence of studies in methodologies standards for algae rich in lipids. The network of relationship identified in

Figure 8. Cluster map of the main inventors.

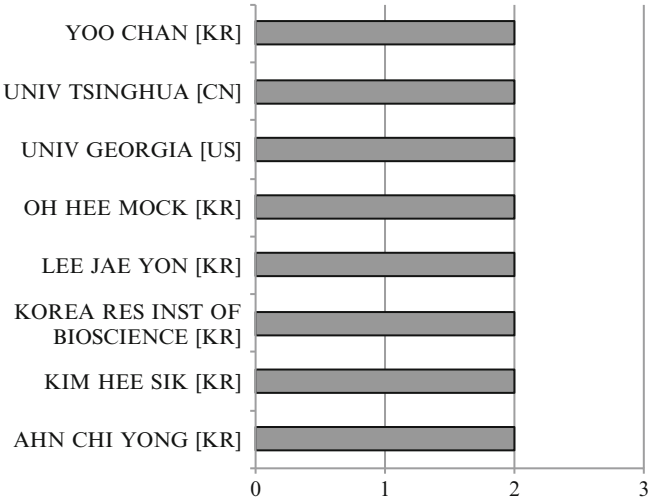

Figure 9. The main applicants found in biodiesel from microalgae.

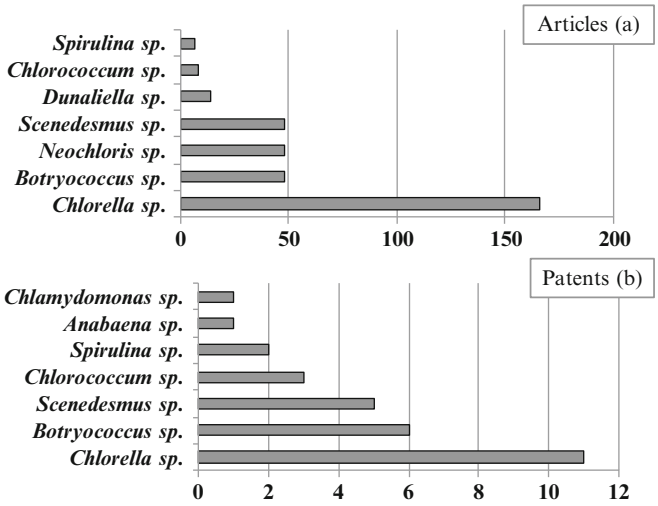

Figure 10. Microalgae species in the articles (**a**) and patents (**b**).

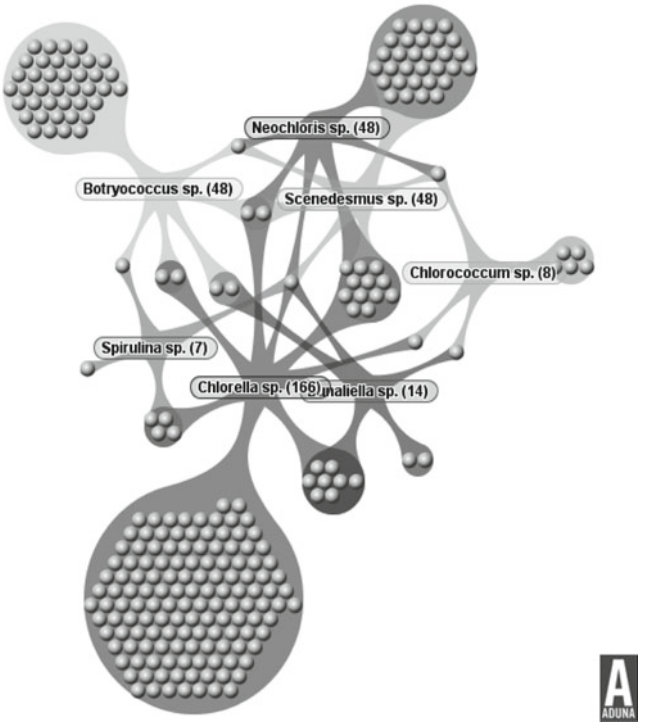

Figure 11. Network of relationships among species of algae found in the articles.

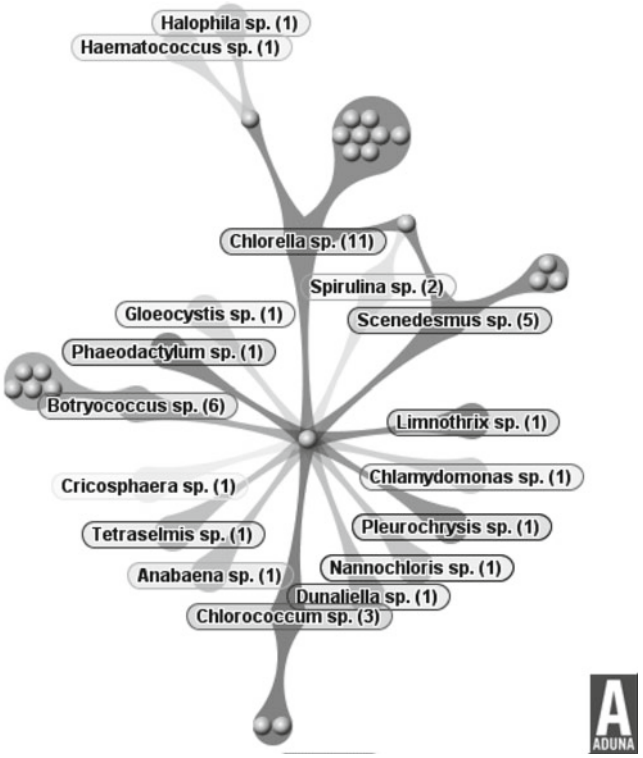

Figure 12. Network of relationships among species of algae found in the patents.

biodiesel production from algal patents (Fig. 12) strengthens the idea of the use of a product, methodology, or process standard for the production of biodiesel from microalgae rich in lipids.

5. Conclusion

As the populations grow in the coming decades, the demand for land, water, and food will increase. Thus, the conversion of agricultural land for energy plantation, excessive use of water, and conversion of food grains to biofuel will not be sustainable in the long term. In the changing global scenario, the demand for biodiesel will continue to grow in a faster pace. Simultaneously, the increase in petroleum price and the rapid depletion of fossil fuels will necessitate use of biodiesel as an alternative option both for consumers and industries. Although algae have shown a lot of potential for biodiesel production, using current methodologies are not commercially viable, and the process is marginal in terms of positive energy balance.

There are several opportunities and challenges which need to be tackled in the field of algal biology, engineering, scale-ups, and finally marketing. Each area will require more in-depth research and innovation. This will produce a large number of articles and patents in the coming years in the field of algal biodiesel production for further mapping and analysis.

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R. Gordon and J. Seckbach (eds.), *The Science of Algal Fuels*,
Cellular Origin, Life in Extreme Habitats and Astrobiology, 305–320
DOI 10.1007/978-94-007-5110-1_17, © Springer Science+Business Media Dordrecht 2012

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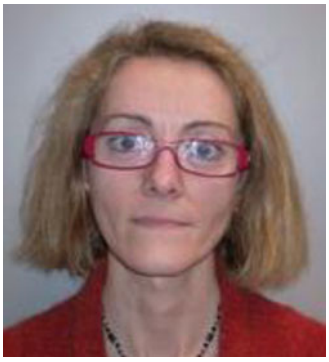

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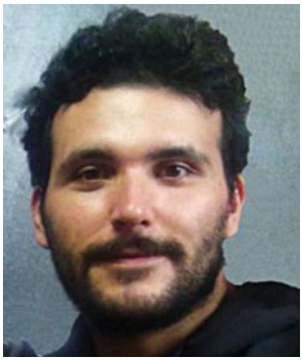

Giannelli Luca

BIOTECHNOLOGY OF HYDROGEN PRODUCTION WITH THE MICROALGA *CHLAMYDOMONAS REINHARDTII*

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1. Introduction

The increase in the cost of energy and the problem of global warming have fostered considerable international efforts to discover a sustainable way to produce energy with zero CO_2 emission. One eco-friendly way of producing energy is the photobiological production of H_2 using the microalga *Chlamydomonas reinhardtii*. In specific conditions, this organism can direct electrons and protons obtained from water biophotolysis toward a specific enzyme, an [Fe]-hydrogenase, so as to obtain molecular H_2. The process was discovered by Gaffron and Rubin (1942), who observed a transient H_2 production with *Scenedesmus*. A way to prolong H_2 production by means of inorganic sulfur deprivation was discovered by Melis and coworkers (Melis et al., 2000). In the past 10 years, considerable progress has been achieved in the photobiological production of hydrogen using *Chlamydomonas* under sulfur starvation conditions, and this has resulted in a number of papers being published on this subject (Melis et al., 2000; Kosourov et al., 2002, 2005, 2007; Tsygankov et al., 2002, 2006; Zhang et al., 2002).

This chapter will briefly summarize the advancements made and the current barriers that need to be overcome for the scale-up to outdoor conditions so as to improve the feasibility of the process.

2. Sulfur-Deprivation Approach

Hydrogen production through direct photolysis according to the reaction:

$$H_2O > PSII > PSI + 4\,photons > Ferredoxin > H_2ase > H_2$$

has recently been at the center of research efforts in biological hydrogen production. However, the initial enthusiasm for this process, which could virtually represent a milestone in the field of the production of renewable energy through algal photosynthesis, greatly cooled as soon as the problem of the inhibition of hydrogenase by O_2 inevitably became apparent. The great challenge of direct H_2O photolysis

lies in solving the problem of how to achieve the simultaneous production of O_2 and H_2. Bubbling inert gas in the culture to avoid the toxic effect of oxygen on the hydrogenase was found to be impractical.

Enthusiasm was renewed after the discovery by Wykoff et al. (1998) that *Chlamydomonas reinhardtii* can lose as much as 75% of the initial photosynthetic capacity (oxygen evolution) within 24 h after the removal of sulfate from the growth medium. Following this discovery, the team at the NRL and the University of California, Berkeley, reported that illuminated *C. reinhardtii* cultures become anaerobic with prolonged sulfur deprivation and express hydrogenase enzyme producing H_2 gas for a few days, reversibly. The reason was that sulfur-starved cells cannot resynthesize the PSII D1 protein, because the repair cycle is blocked due to a lack of sulfur. Sulfur starvation has two important effects on H_2 production: (1) a massive accumulation of starch which acts as a sink for storing excess reducing power and (2) a gradual drop in PSII activity (Wykoff et al., 1998). Once the rate of photosynthetic O_2 evolution drops below the rate of respiration, anaerobic conditions are reached, enabling the induction of hydrogenase and the production of significant amounts of hydrogen for several days. Starch is degraded in parallel to H_2 production (Melis et al., 2000; Melis, 2007). Based on the observation that starchless *C. reinhardtii* mutants *sta6* and *sta7* were strongly affected in their ability to produce hydrogen, Posewitz et al. (2004) suggested that starch metabolism may play a central role in *C. reinhardtii* H_2 production. In fact, two different pathways can supply reductants (i.e., reduced ferredoxin) to H_2ase for hydrogen production in the light: a direct pathway through PSII and an indirect PSII-independent pathway that relies on a non-photochemical reduction of the plastoquinone (PQ) pool (Fouchard et al., 2005; Melis, 2007). It was proposed that starch catabolism plays a role in both pathways (Melis, 2007) by (1) sustaining mitochondrial respiration and permitting the maintenance of anaerobic conditions for the PSII-dependent direct pathway and (2) supplying electrons to the chlororespiratory pathway and to the hydrogenase through a PSI-dependent process along the indirect pathway (Fouchard et al., 2005; Mus et al., 2005; Melis, 2007). In a recent work by Chochois et al. (2009), it was concluded that starch breakdown contributes to the indirect pathway by feeding electrons to the PQ pool, but is dispensable for operation of the direct pathway that prevails in the absence of DCMU (3-(3,4-dichlorophenyl)-1,1dimethyl urea), a herbicide that inhibits photosynthesis.

Typical kinetics of hydrogen production are shown in Fig. 1. It can be seen that the dissolved oxygen concentration increases during the first 24 h of sulfur starvation. Thereafter, the oxygen level starts to decline as a result of a decrease in the photosynthesis rate, which is due to increased PSII photoinhibition. This is clearly detected by a reduction in chlorophyll fluorescence, measured with the $\Delta F/F'_m$, the effective quantum yield of PSII. The H_2 production usually starts after a lag phase of about 16 h (but this greatly depends on the strains) during which no increase either in oxygen or in hydrogen production is observed. Because a reducing environment is established, the redox potential that has a positive value

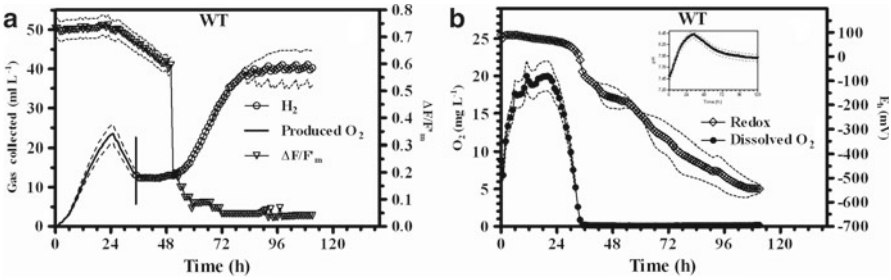

Figure 1. Panel **a**. Time courses in the output of hydrogen (H_2) gas (○) and in the effective quantum yield of PSII ($\Delta F/F'_m$) (▼). Note the initial increase in oxygen produced by the culture (−) during the aerobic phase and detected by the electronic balance. The *vertical line* indicates the time at which the anaerobic conditions started. Panel **b**. Time courses in the redox potential (Eh) (♦), dissolved oxygen (pO_2) (●), and pH behavior (▼), (insert). *C. reinhardtii* WT cultures were incubated under conditions of sulfur starvation and with a photon flux density of 70 μmol photons m^{-2} s^{-1}, which was supplied on both sides of the reactor. *Dashed lines* indicate the standard deviation.

during the aerobic phase becomes negative up to −550 mV. The H_2 production began after a rapid drop in $\Delta F/F'_m$ from 0.6 to 0.2, which is usually observed 50 h after the start of the sulfur deprivation. This drop in the effective quantum yield of PSII during hydrogen production was first reported by Antal et al. (2003), who interpreted it as the consequence of a transition from state 1 to state 2 of the photosynthetic apparatus (Finazzi et al., 1999). State 1 to state 2 transition represents a photoprotective strategy and consists of a partial migration of the LHCII from PSII to PSI, which is known to occur in *C. reinhardtii* in dark anaerobic conditions (Cournac et al., 2002; Cardol et al., 2003; Endo and Asada, 1996).

During the aerobic phase, in the example reported in Fig. 1b (insert), the pH of the culture increases from an initial value of 7.4–8.5 at the end of the anaerobiosis and thereafter declines down to a value of 7.9 during the hydrogen production process. The increase in the pH has been related to the utilization of acetate during the aerobic phase and its decrease, to the consumption of carbohydrate, which causes a release of CO_2.

3. Other Proposed Approaches

3.1. INDIRECT TWO-STAGE BIOPHOTOLYSIS

An indirect two-stage biophotolysis approach has been proposed by Hallenbeck and Benemann (2002). The process is conceptually identical to one that occurs in the cyanobacteria heterocyst system, except that O_2 evolution and H_2 production are separated temporally, with periods of CO_2 fixation and carbohydrate accumulation (and O_2 evolution) alternating with periods during which the carbohydrates

are converted into H_2. Technically, these processes could be carried out either in separate reactors, i.e., open ponds followed by closed photobioreactors, or in a single closed photobioreactor in which H_2 and O_2 are produced in alternating cycles. In outdoor operations, the dark period of the diurnal cycle could enable the development of anaerobic conditions, induction of hydrogenase, and the initiation of H_2 production in the dark, followed by a light-driven H_2 production at sunrise, prior to the new start of the CO_2 fixation. In summary, the process may involve four distinct steps: (1) production in open ponds of a biomass high in storage carbohydrates; (2) concentration of the biomass from the ponds by settling; (3) anaerobic dark fermentation to yield 4 H_2/glucose stored in the algal cells, plus 2 acetates; and (4) a photobioreactor in which the algal cells would convert the two acetates to 8 mol of H_2. However, indirect biophotolysis processes are of questionable economics and remain to be demonstrated even on an experimental level (Hallenbeck and Benemann, 2002).

New approaches to make anaerobiosis partially inactivate PSII have also been proposed. Alternatively, this could be achieved by controlling PSII activity using inducible promoters to switch the PSII activity on and off (Surzycki et al., 2007). Moreover, limitations in the indirect pathway are more likely to rely on enzymatic steps involved in starch breakdown to a non-photochemical reduction of PQs. Recently, a type II NAD(P)H dehydrogenase activity has been evidenced in *C. reinhardtii* chloroplasts that are shown to be involved in non-photochemical reductions of PQs and hydrogen production (Jans et al., 2008). This enzyme, but also enzymes involved in starch breakdown, most of which are still to be identified, may represent good targets for future biotechnological improvements.

Experiments performed with *C. reinhardtii* to downregulate the uptake of sulfate capacity are also in progress (Chen and Melis, 2004; Chen et al., 2005). Mutants affected in the sulfate permease (*SulfP*) gene expression exhibited phenotypes of sulfur-deprived cells that displayed low rates of light-saturated O_2 evolution, a low level of Rubisco, and a low steady-state level of the PSII D1 protein. The mutants showed a capacity to photoproduce H_2 in the presence of up to 150 μM sulfate.

3.2. PRODUCTION OF HYDROGEN USING *CHLAMYDOMONAS REINHARDTII* D1 PROTEIN MUTANTS

Other recent reports describe H_2 production properties of D1 mutants that were less (Makarova et al., 2007) or more (Kruse et al., 2005; Torzillo et al., 2009; Faraloni and Torzillo, 2010) efficient in H_2 production upon sulfur starvation, depending on the specific mutation site. Makarova et al. (2007) generated D1-R323 mutants using the progressively impaired O_2-evolving activity. All the mutants produced H_2 for a shorter time, and exhibited H_2 yields under sulfur starvation. They also accumulated less starch during the growth phase. Moreover, the H_2 production process was more seriously affected in mutants that had a more

Table 1. Phenotypic characteristics of *C. reinhardtii* mutant strains L159I/N230Y, D239-40, D240 compared with their wild type (WT) and CC124 strain.

	Chl	Chl a/b	Chl cell^{-1}	Cell diameter	P^{a}_{max}	R^{b}	R/P^{c} ratio
Strain	**% of dry wt**	**Ratio**	**×10^{-6} μg**	**μm**	**μmol O^2 mg^{-1}**	**Chl h^{-}**	**%**
WT	3.63 (±0.61)	2.90 (±0.01)	3.57 (±0.12)	7.32 (±0.32)	260 (±2.8)	72 (±0.25)	27.70 (±0.21)
L159I/N230Y	1.80 (±0.20)	3.00 (±0.05)	3.32 (±0.14)	9.43 (±0.27)	487 (±36)	190 (±2.5)	39.01 (±2.20)
D240	1.60 (±0.18)	2.89 (±0.04)	1.56 (±0.05)	9.15 (±0.30)	220 (±25.0)	95 (±5.8)	43.18 (±2.04)
D239-40	2.33 (±0.32)	2.99 (±0.05)	2.08 (±0.09)	7.38 (±0.09)	119 (±2.0)	78 (±1.75)	65.55 (±0.36)
CC124	3.40 (±0.43)	2.80 (±0.08)	2.32 (±0.12)	8.23 (±1.22)	172 (±17.4)	104 (±8.6)	60.46 (±1.01)

The standard deviation (SD) for each parameter is reported in parentheses.
[a]Maximum rate of oxygen evolution plus dark respiration.
[b]Dark respiration.
[c]Respiration to oxygen evolution rate.

compromised PSII photochemical quantum yield. In contrast, Torzillo et al. (2009) reported that PSII D1 protein mutants carrying a double amino acid substitution (L159I-N230Y) showed prolonged H_2 production in sulfur-deprived cultures (Table 1). As a result, the mutant H_2 gas production was five times higher than in the commonly used CC124 strains.

Chlamydomonas reinhardtii D1 protein mutants were obtained from wild-type (11/32b) genetic manipulation. They were obtained by removing 4 introns in *psbA* gene encoding for the D1 protein, as previously described (Johanningmeier and Heiss, 1993). Phenotypic characteristics of two amino acid deleted mutants, D240 and D239-40, and a mutant with two amino acid substitutions, L159I/N230Y, are summarized in Table 1 and compared with the wild type and with the CC124 strain.

The deletion of the D240 and D239-40 is located in the D1 protein region involved in the binding of Q_B and D1 degradation, while the mutation of the other mutant strain involves a region implicated in the electron donor capacity to the oxygen-evolving complex (OEC). The phenotypes of the wild type (WT), D1 mutants, and CC124 evidenced differences among these. The amount of chlorophyll per dry weight biomass in all the mutants was 36–56% lower than in the wild type. These results were confirmed when compared on a per cell basis. A reduced chlorophyll content is a desirable requisite for hydrogen production, because (1) the cultures can grow under relatively high cell densities, thus avoiding the problem of a reduction in the light penetration in the culture, due to the self-shading and (2) the cultures can reach anaerobiosis faster, since population density is higher per unit of chlorophyll. Quantification of the photosynthetic parameters revealed that the deleted mutants exhibited values for oxygen evolution

Table 2. Hydrogen output rates measured in wild type (WT) and D1 protein mutant strains.

Strain	Aerobic phase (h)	Lag phase (h)	Production time (h)	H_2 total volume ($mL \cdot L^{-1}$)	Mean H_2 production rates ($mL \cdot L^{-1} \cdot h^{-1}$)	Maximum H_2 production rates[a] ($mL \cdot L^{-1} \cdot h^{-1}$)
WT	34 (±1)	16 (±5)	55 (±5)	26 (±5)	0.47 (±0.11)	1.25 (±0.13)
D240	2 (±1)	26 (±4)	207 (±40)	318 (±23)	1.54 (±0.31)	3.54 (±0.26)
D239-40	3 (±2)	30 (±1)	183 (±30)	475 (±50)	2.60 (±0.18)	7.10 (±1.10)
L159I/N230Y	–	37 (±6)	285 (±53)	504 (±22)	1.81 (±0.35)	6.01 (±0.24)
CC124	20 (±1)	1 (±0.5)	53 (±4)	80 (±8)	1.51 (±0.04)	2.23 (±0.32)

The CC124 is shown as a reference strain being used in laboratories worldwide for hydrogen production studies. The standard deviation (SD) for each parameter is reported in parentheses.
[a]H_2 maximum production rate measured over a period of at least 10 h, during which the rate remained constant.

rates, in addition to respiration rates that were lower and higher, respectively, than those for the wild type.

Both the oxygen evolution and the respiration rate were highest in the L159I/N230Y mutant: 87 and 164% higher than in the wild type, respectively. Despite the fact that the photosynthetic parameters displayed by the mutants were found to be different, the respiration to photosynthesis ratios increased in all the strains, in comparison with the WT. A high respiration to photosynthesis ratio is an appreciated characteristic in strains of *C. reinhardtii* for the production of hydrogen because the higher the respiration to photosynthesis is, the shorter the time that the cultures take to reach anaerobiosis and the onset of the hydrogen production phase.

As compared with the WT, these D1 protein mutants disclosed important peculiarities for the production of hydrogen, such as the following: (1) a greater capability to synthesize carbohydrates and (2) higher sensitivity to photoinhibition. This is because their PSII repair capacity was impaired by the mutations. These differences are translated into a higher hydrogen production output, as compared to the WT. The results regarding the production of hydrogen are shown in Table 2.

In all the mutants, the total volume of biogas was higher than in the wild type and CC124. Compared to the wild type, the H_2 production was 12–19 times greater; moreover, these strains exhibited a better performance in terms of the rate of hydrogen production.

The most productive strain was found to be the D239-40 mutant. This strain combined a great capacity to synthesize carbohydrates with a considerably higher level of fluorescence during the hydrogen production (Fig. 2), thus making PSII's

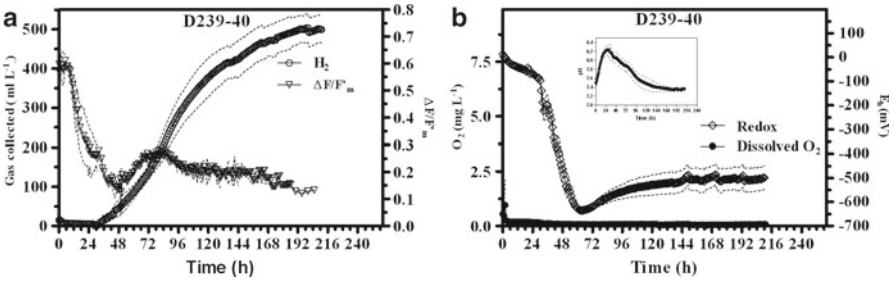

Figure 2. On-line measurements of hydrogen output, effective quantum yield ($\Delta F/F'_m$) (*left panel*), changes in the dissolved oxygen concentration, and pH of cultures (*right panel*) during the hydrogen production process in *C. reinhardtii* cultures of the most productive D239-40 mutant strain. Key symbols as in Fig. 1.

contribution to the global amount of hydrogen more relevant, in comparison with that observed with WT and the other deleted D240 mutant. These findings demonstrated that the contribution of genetic engineering may be very important for improving H_2 production and that D1 protein mutants can play a key role in improving photobiological H_2 production. In particular, the use of Q_B binding and OEC-interaction site D1 protein mutants has been found to be an important step toward improving productivity.

4. Biotechnology of Hydrogen Production

4.1. HYDROGEN PRODUCTION IN LAB-SCALE PHOTOBIOREACTORS

The discovery of the sulfur starvation technique (Melis et al., 2000) represented an effective start-up for the modern biotechnology of H_2 production, because it made it possible to sustain the process for a longer time, leaving room for the optimization of the culture conditions in order to obtain more H_2 biogas. Several studies have been carried out for the purpose of optimizing and prolonging the H_2 production process, such as those on the effect of low residual amounts of sulfur at the onset of the experiment (Laurinavichene et al., 2002; Kosourov et al., 2005); the re-addition of limiting amounts of sulfur to the culture at the end of the production phase (Ghirardi et al., 2000); the use of light-synchronized cultures (Tsygankov et al., 2002); the effect of the initial pH of the medium (Kosourov et al., 2003); the use of different growth conditions (Kosourov et al., 2007); and the effect of light intensity on the production of H_2 (Hahn et al., 2004; Laurinavichene et al., 2004; Tsygankov et al., 2006; Kim et al., 2006). All these experiments were usually conducted in a *Roux type PBR*. One of the weak points of this photobioreactor's design was the poor mixing, which is

achieved with a magnetic bar placed in the bottom of the bottle. In addition, it was difficult to attain full control of the culture's behavior during the hydrogen production process.

The first attempt to improve the culture mixing conditions in order to enhance the H_2 production was carried out in the laboratories of the University of Nantes in France (Pottier et al., 2005; Pruvost et al., 2006; Fouchard et al., 2008). The result of the research was a complex PBR design capable of on-line monitoring and regulation that uses a torus-shaped culture bulk enclosed inside flat transparent walls that allow light inside. The main advancement achieved with this photobioreactor design was an exhaustive fluid dynamics analysis conducted by means of computational fluid dynamics software (CFD), which led the research group to define fully predictable behavior for the fluid inside the reactor.

Another innovative approach to the PBR fluid dynamic design was achieved by Giannelli et al. (2009). A multistage rotating impeller was realized that was embedded inside the existing Roux-like PBR design, which is similar to the one presented by Kosourov et al. (2002). The aim of this innovative stirring system was to create a more ordered light–dark (L–D) cycle within the culture depth, which made it possible to improve its light utilization efficiency by moving cells very rapidly from the core region of the reactor (dark zone) to the external part (light-saturated zone).

The system has been tested in different lights and chlorophyll concentrations, ranging from photolimitation to saturation (Table 3). The maximum light transformation efficiency achieved with the multi-impeller mixing system reached 1.64%, resulting as being about 13 times higher than that reported by Fouchard et al. (2008).

4.2. OUTDOOR HYDROGEN PRODUCTION UNDER SOLAR LIGHT

A first attempt to produce hydrogen outdoors in a 50 L PBR using a sulfur-deprived culture of *C. reinhardtii* (strain CC124) has been reported by Scoma et al. (2010). The experiments were carried out in a fully controlled serpentine tubular PBR (Bocci et al., 1987). Under the climatic conditions of Central Italy, i.e., Florence (latitude 43° North), total light irradiance can reach as much as 2,000 μmol/m²/s, which is almost 30 times higher to that used for laboratory experiments. The strong inhibition of PSII was confirmed by measuring the chlorophyll fluorescence ($\Delta F/F'_m$). This was found to be extremely low in the middle of the day (below 0.1).

Another important constraint in outdoor hydrogen operation is represented by the fact that cultures are exposed to circadian conditions (light/dark cycle) with hydrogen production alternating with exhaustion of the carbohydrate reserves during the dark phase. This strongly affects the maintenance of the

Table 3. Summary of the H_2 production experiments and correspondent energy conversion efficiency (Adapted from Giannelli et al., 2009).

Culture conditions			H_2 induction phase (h)	H_2 production (mL · L^{-1})	Production rates (mL · L^{-1} · h^{-1})	
Light	**Chl (a + b)**	**Mixing**			**Mean value**	**Max. value**
70 + 70	12	Stir bar	22.7 (±1.9)	99.7 (±11.5)	0.99 (±0.11)	2.23 (±0.32)
		Impeller	30.0 (±2.2)	87.2 (±8.1)	1.39 (±0.04)	2.44 (±0.23)
70 + 70	24	Stir bar	17.0 (±1.5)	82.1 (±26.3)	0.98 (±0.34)	1.90 (±0.07)
		Impeller	12.0 (±1.0)	132.9 (±17.8)	2.05 (±0.28)	2.87 (±0.51)
140 + 140	12	Stir bar	32.0 (±5.4)	48.8 (±29.1)	0.78 (±0.39)	1.61 (±0.12)
		Impeller	33.5 (±6.9)	95.0 (±25.7)	1.35 (±0.38)	2.38 (±0.18)
140 + 140	24	Stir bar	13.5 (±1.7)	118.9 (±1.7)	2.49 (±0.04)	3.72 (±0.30)
		Impeller	18.8 (±2.8)	167.6 (±17.1)	4.02 (±0.21)	5.66 (±0.05)

Energy conversion efficiency (%)

Culture conditions				
Light	**Chl (a + b)**	**Mixing**	**Mean value**	**Max. value**
70 + 70	12	Stir bar	0.468	1.260
		Impeller	0.547	1.420
70 + 70	24	Stir bar	0.477	1.100
		Impeller	0.965	1.670
140 + 140	12	Stir bar	0.150	0.460
		Impeller	0.265	0.690
140 + 140	24	Stir bar	0.563	1.080
		Impeller	0.805	1.640

anaerobic condition and limits the total hydrogen output. In some cases, artificial light was supplied at night to sustain hydrogen production (Fig. 3).

The complete recording of an experiment carried out in an outdoor PBR is shown in Fig. 4. It can be seen that most of the hydrogen production was achieved under solar light, while only 27% of the total amount was obtained with artificial light supplied at night, thus confirming that the largest amount of biogas collected indeed originated from metabolism driven by solar light (Fig. 4b).

Results indicated that, in outdoor conditions, the hydrogen production was reduced to about 21% of that attained in the laboratory. According to chlorophyll fluorescence measurements, this scarce hydrogen production was attributed to an overly rapid inactivation of the PSII as the result of extremely high light intensities.

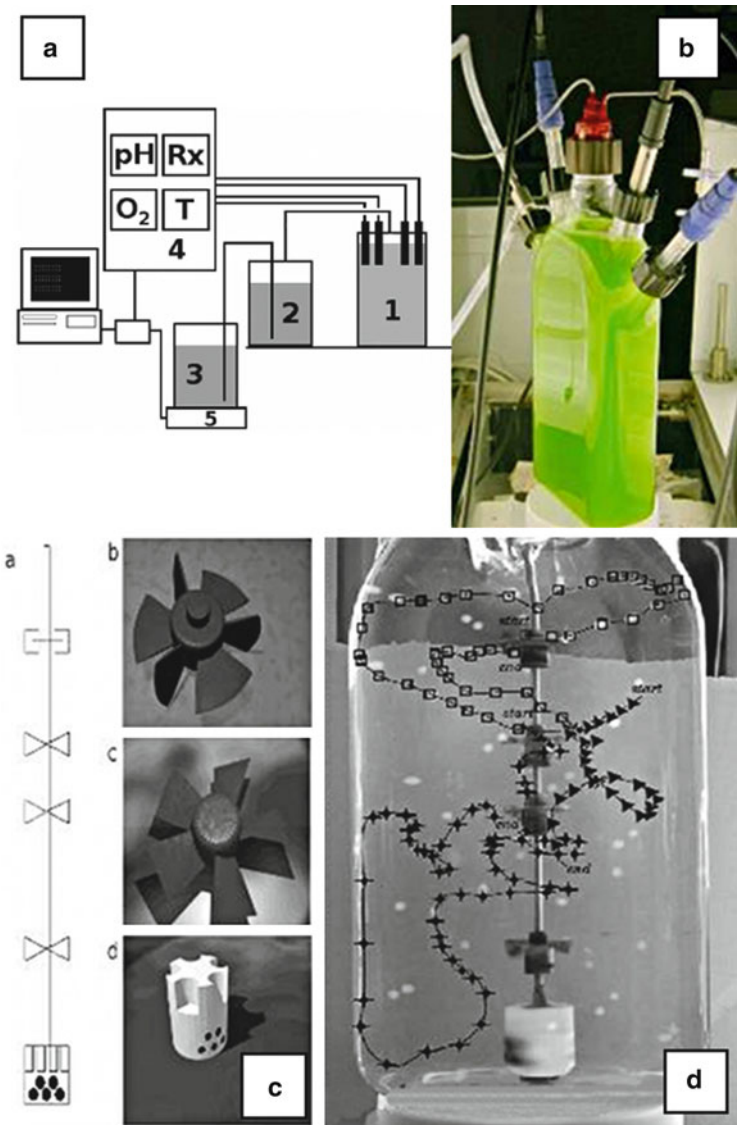

Figure 3. (**a**) Continuous monitoring PBR (Adapted from Kosourov et al., 2002). *Diagram* of the organization of the photobioreactor controlling system: *1* Hydrogen-producing culture; *2* constant pressure gas holder; *3* displaced liquid vessel; *4* analog/digital monitoring devices and interfaces; *5* electronic digital balance for measuring displaced liquid. (**b**) PBR fully equipped with various probes for measuring culture parameters during hydrogen production experiments. (**c**) The scheme of the impeller: *a.* side view, *b, d.* Rushton-like turbines, *c.* pitched blade turbines. (**d**) Examples of traces of some characteristic particles (calculated using the image analysis technique) in which theoretical provisions are well exemplified (From Giannelli et al., 2009, with permission).

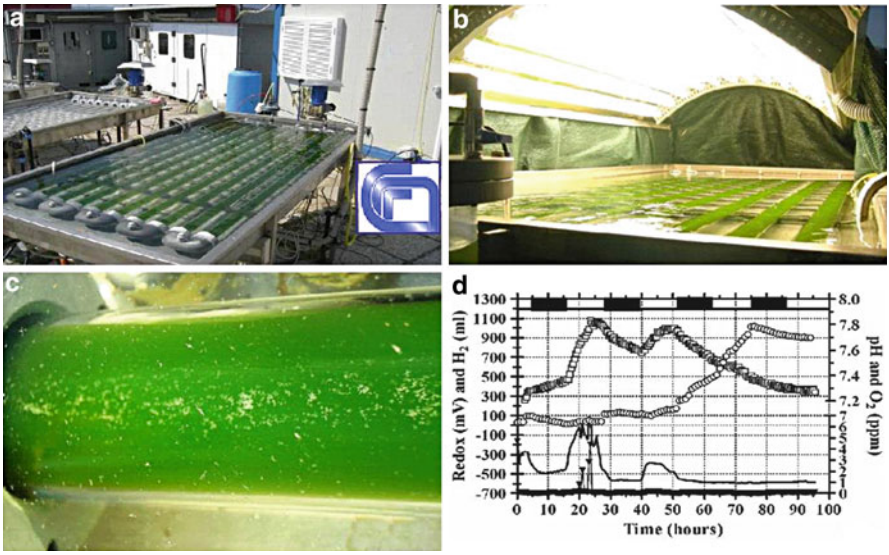

Figure 4. Outdoor PBR setup: (**a**) general view of the reactor immersed in a basic containing thermostated water; (**b**) culture illuminated at night with artificial light; (**c**) hydrogen bubbles produced by cultures; (**d**) continuous recording of culture parameters during the hydrogen production experiment: *squares*, pH; *circles*, hydrogen; *continuous line*, redox potential; *triangles*, dissolved oxygen. *Bar* on top of graph indicates day and night periods.

5. Concluding Remarks

Although the two-phase system carries a penalty in terms of the maximum attainable photon conversion efficiency, it presents the advantage of improving H_2 purity (Hankamer et al., 2007). Further advantages of the two-stage system are that semi-sterile photobioreactors can be used for the hydrogen production phase, thus lowering the overall production cost, particularly since the purity of the H_2 produced is already high enough for direct use in flue cells for electricity production. By eliminating the necessity for sterilizing the materials, it is possible to extend the range of material usable for the production of hydrogen, thus simplifying the reactor design and its cost. Reactor costs significantly affect the cost of hydrogen production (Burgess et al., 2006). According to our laboratory results, with our best strain, a photosynthetic efficiency (PE) of 2.5% (in PAR) is achieved. With this PE, the energy output from the hydrogen production process outdoors would correspond to 0.66 US$/m^2/year. If we assume that the cost of oil is 135 US$/barrel, and that the cost of the photobioreactor is 10 US$/m^2/year (Tredici et al., 1999), the cost of energy from H_2 by biophotolysis assisted by respiration would be ten times higher than that of oil (10 US$/0.99$ = ~10). In order to make the process feasible, it is mandatory to increase the efficiency

of the process close to the theoretical limit, 28.3% in PAR (assuming that eight moles of photons are required to produce two moles of hydrogen, i.e., removing the sensitivity to oxygen of the hydrogenase), or to reduce the cost of the photobioreactor by at least one order of magnitude. An ultimate potential objective might be to increase biohydrogen production as close as possible to about 600.000 m^3/ha/year, which represents an efficiency of total solar light conversion to H_2 of about 10% in sunny areas.

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PART III:
ALGAE FOR BIOFUEL PRODUCTION

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R. Gordon and J. Seckbach (eds.), *The Science of Algal Fuels*,
Cellular Origin, Life in Extreme Habitats and Astrobiology, 323–340
DOI 10.1007/978-94-007-5110-1_18, © Springer Science+Business Media Dordrecht 2012

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HOW TO BREED DIATOMS: EXAMINATION OF TWO SPECIES WITH CONTRASTING REPRODUCTIVE BIOLOGY

Dedicated to the memory of Frank E. Round (1927–2010) and Alexei M. Roshchin (1935–2010), two scientists who contributed greatly to our knowledge of diatoms.

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1. Introduction

A whole series of thought-provoking arguments and ideas have recently been put forward, maintaining that microalgae may have potential as feedstocks for a variety of biofuel applications (e.g. Mascarelli, 2009; Wijffels and Barbosa, 2010; Mata et al., 2010; Radakovits et al., 2010). The diatoms (phylum Bacillariophyta), which are the most speciose and ecologically important group of unicellular algae (e.g. Round et al., 1990; Mann, 1999; Armbrust, 2009), have been a particular focus of attention in this respect (e.g. Sheehan et al., 1998; Bozarth et al., 2009; Radakovits et al., 2010). At present, however, the development of an algae-based biofuel industry is still nascent, and it is clear that intensive R&D efforts will be required over a long period before the 'right' alga or algae are found (or obtained experimentally) and appropriate technologies established for processing biomass on an industrial scale. It seems there is no disagreement that one of the most important R&D priorities is to establish methods and procedures that will allow the controlled genetic manipulations of desirable traits. Currently, in the search for the most promising ways to alter microalgal strains genetically, the focus is usually on advanced molecular methods, primarily transgenesis (genetic engineering) (e.g. Kroth, 2007; Bozarth et al., 2009). However, we also concur with Radakovits et al. (2010) that '…despite the recent advances in biotechnological approaches, the full potential of genetic engineering in some microalgal species, particularly diploid diatoms, can only be fully realized if conventional breeding methods become firmly established thereby allowing useful traits or mutations to be easily combined' (see also Grossman, 2005, 2007).

Conventional breeding involves controlled crossing of selected strains and hence control of reproduction in sexual organisms. Fortunately, a sexual phase is an obligate part of the life cycle in most diatoms, and there is growing body of evidence that it can be controlled and manipulated in the laboratory (Chepurnov

et al., 2004, 2008, but see also earlier significant contributions in this field, e.g. Geitler, 1932; von Stosch, 1965; Roshchin, 1994). Recently, we have attempted to look at sexual breeding in diatoms from the perspective of possible practical applications (Chepurnov et al., 2011). Here we present concrete examples that illustrate how to breed diatoms in practice and demonstrate how gathering knowledge on life-cycle dynamics and reproductive systems is crucially important for increasing the chances of success.

The basic plan of the diatom life cycle is astonishingly uniform in its principal features, that is, gradual diminution of cells in size during vegetative multiplication (according to the principle known as the MacDonald–Pfitzer rule, see e.g. Round, 1972; Pickett-Heaps et al., 1990; Mann, 2011), size-dependent control over sexuality and the link between the sexual event and the restitution of cells in size (via an 'auxospore', which is an expanding cell derived from the zygote: see Figs. 1 and 3). In this chapter, we use two diatoms as examples to illustrate how classical breeding programmes might be established. The choice of two species rather than one reflects the existence of profound differences in the organization of the reproductive system between the so-called centrics and pennates – the two major groups recognized in traditional classifications of diatoms (e.g. Round et al., 1990), although it is now widely accepted that the centric group is unnatural and that the pennates evolved from centric ancestors (e.g. Theriot et al., 2010). These differences include the mode of sexual reproduction, the mechanism by which meiosis is triggered and the mating systems (see Chepurnov et al., 2004), that is, the biological attributes that will have a crucial impact on the selection, development and performance of breeding methods and procedures, the core of the 'classical' manipulation of an organism's genetic material.

2. Justification for the Choice of Species

To illustrate the basic plan of the life cycle in diatoms and the peculiarities of their reproductive systems, we have selected the centric *Cyclotella meneghiniana* Kützing and the pennate *Seminavis robusta* Danielidis & D. G. Mann. Considerations governing the choice were as follows. Both species satisfy the important criterion of laboratory convenience, including ease of stock maintenance and experimental tractability, and both possess life cycles typical of diatoms. So, for instance, in both species, the life cycle involves an alternation of cell size reduction and restitution, restitution involving a special cell, the 'auxospore' that results from sexual reproduction. The capacity of cells to become sexualized is size dependent. And the mode of sexual reproduction is allogamous, fertilization occurring via the union of gametes produced by different individuals (gametangia). On the other hand, *Cyclotella* and *Seminavis* differ in several fundamental characteristics of their reproductive systems, which exemplify the principal distinctions in sexual behaviour between centrics and pennates. Thus, they differ in breeding system (homothally vs. heterothally, though there are also many homothallic pennates),

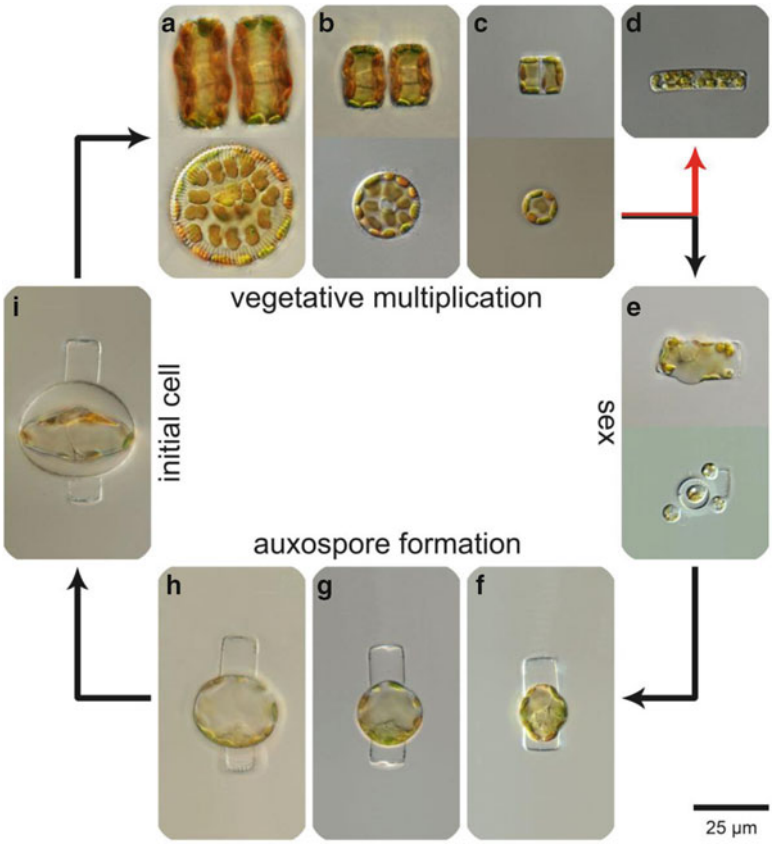

Figure 1. *Cyclotella meneghiniana*: the life cycle. (**a**–**d**) Gradual diminution of cells in size (*upper images* in valve view, *lower* in girdle view except '**d**' shown only in girdle view). (**e**) Oogonium (*upper image*) exposing the membrane of oocyte by partial separation of the thecae and open spermatogonium (*lower image*), with four sperm. (**f**–**h**) Subsequent stages of auxospore expansion. Note that the empty oogonial thecae are still associated with the auxospore, capping it *top* and *bottom*. (**i**) Initial cell has formed but still enclosed in the auxospore membrane.

induction of gametogenesis (cell–cell interaction is required or not) and the mode of sexual reproduction (oogamy vs. morphological isogamy).

The marine diatom *Seminavis robusta* apparently has a chance to become popular as a model species for life-cycle and cell-cycle studies (e.g. Chepurnov et al., 2008; Gillard et al., 2008), following the description of the genus itself by Round et al. (1990) and the first studies of the *S. robusta* life cycle by Chepurnov et al. (2002). So far, the reproductive biology of other *Seminavis* species is little known, but *Seminavis* is closely related to a large genus of freshwater, brackish and marine diatoms, *Navicula* (sensu stricto: see Round et al., 1990), for which there are several studies of the mating system and life cycle (e.g. Roshchin, 1994; Poulíčková and Mann, 2006).

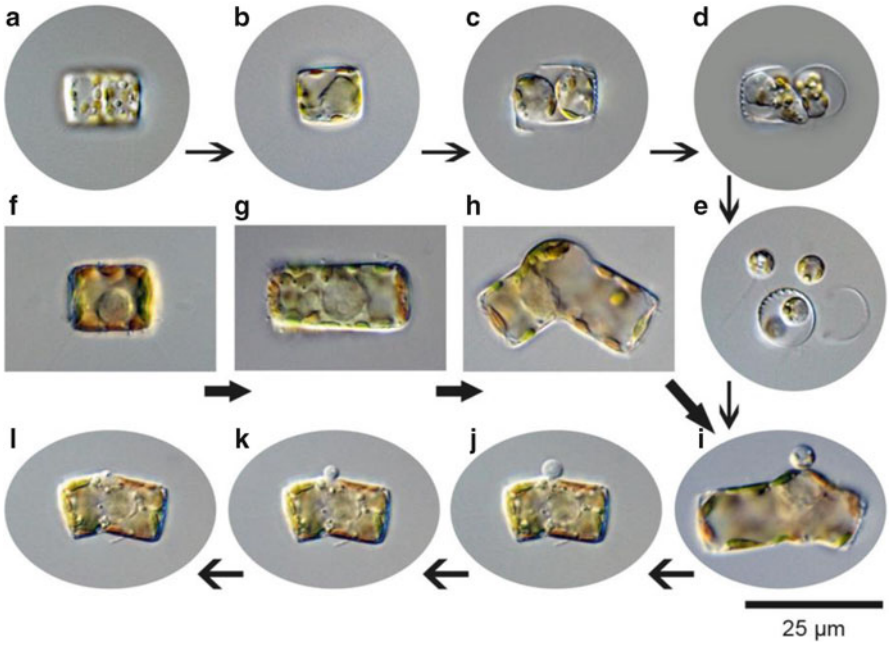

Figure 2. *Cyclotella meneghiniana*: gametogenesis and fertilization. (**a**–**e**) Spermatogenesis. (**a**) Two spermatogonia. (**b**) Primary spermatocyte (note a large nucleus at the prophase of meiosis I). (**c**) Following meiosis I, two secondary spermatocytes have formed. (**d**) Meiosis II. (**e**) Four sperm and two thecae of their gametangium. (**f**–**h**) Oogenesis. (**f**) Early oogonium. (**g**) Oogonium elongated in the pervalvar direction. (**h**) Oogonium partly exposing the membrane of oocyte. (**i**–**l**) Fertilization. (**i**) The sperm has come into contact with egg. (**j**–**l**) Subsequent stages of sperm penetration inside the oogonium.

The reproductive behaviour of *Cyclotella meneghiniana* has been examined repeatedly, but the results obtained by different researchers have not always been similar. For example, in some investigations, auxosporulation has been reported to be associated with oogamous sexual reproduction (e.g. Rao, 1970; Shirokawa et al., 2010), whereas in others, the auxospore is apparently formed after autogamy (sex within a single cell, e.g. Iyengar and Subrahmanyan, 1944; Schultz and Trainor, 1968). This reported variation in the sexual behaviour of *C. meneghiniana* could perhaps reflect developmental plasticity, but it requires further detailed examination and interpretation, especially because it has been shown that there is considerable variation at the molecular level between different isolates of this apparently cosmopolitan centric diatom (Beszteri et al., 2005), possibly indicating the existence of several cryptic or pseudocryptic species within it. We have worked with dozens of monoclonal cultures of this centric diatom, isolated from various locations in Europe and Africa. Where the auxosporulation could be examined in sufficient detail, we observed exclusively oogamy, as illustrated in Figs. 1 and 2. Phylogenetic analyses have also shown that there is a very close relationship between *C. meneghiniana* and the genomics model *Thalassiosira pseudonana*

('*T. pseudonana* is sister to a clade containing the *C. meneghiniana* complex', see Alverson et al., 2011). Hence, *C. meneghiniana* could have great value as an experimental organism, especially given that *T. pseudonana* is aberrant in apparently lacking a sexual cycle. In addition, *Cyclotella* and *Thalassiosira* species have been the focus of attention for various industrial applications (e.g. Lebeau and Robert, 2003), including biofuel production (e.g. Sheehan et al., 1998).

A further important justification for using *C. meneghiniana* and *S. robusta* is that both are available from culture collections. Strains of *C. meneghiniana* can be readily obtained from CCAP (http://www.ccap.ac.uk/), NCMA (https://ncma.bigelow.org/) and UTEX (http://web.biosci.utexas.edu/utex/). Monoclonal cultures of *C. meneghiniana* and *S. robusta* are also maintained in the diatom culture collection of Ghent University, which is currently among the Belgian Co-ordinated Collections of Micro-organisms (BCCM™, http://bccm.belspo.be/). What is unique for this collection is that the life-cycle dynamics of the strains are well-known and protocols are available for reliable control of sexual auxosporulation in experiments. Some of the clones have also been successfully cryopreserved as a number of subcultures that differ in size and hence have the potential to exhibit specific, 'size-dependent' responses to various factors and triggers, primarily in relation to sexual behaviour. The laboratory lineages of *S. robusta* in Ghent represent the first 'long-term' diatom pedigree, created via the development of a breeding programme over several successive generations (Chepurnov et al., 2008).

3. Breeding a Centric Diatom: *Cyclotella meneghiniana*

To exemplify the life cycle of a centric diatom and to illustrate a method for obtaining new genotypes experimentally via sexual reproduction, we used a strain of *Cyclotella meneghiniana* isolated from Schelde River near Ghent (Belgium). *Cyclotella* cells are drum-shaped, and the principal features of the cycle of the Schelde clone most relevant to current considerations were as follows. While the cells were multiplying mitotically, the average size of cells in the clone gradually became smaller (Fig. 1a–d) so that, over the whole cycle, the cells varied between c. 50 and 3 μm in diameter (the size of the valve). When approaching the minimal size (below about 7 μm in diameter), many cells changed morphologically, becoming visibly elongated along the axis perpendicular to the valvar plane (i.e. along the pervalvar axis) (Fig. 1d, see also Desikachary and Rao, 1973; Rao, 1978a). In many diatoms, such critically small cells die. The Schelde clone of *Cyclotella meneghiniana*, however, can apparently be maintained indefinitely, because small cells stopped getting smaller and continued to divide (although slowly). Hence, in old, small-celled cultures, the valve size remained approximately constant or fluctuated within a narrow range (e.g. Geitler, 1932; Roshchin, 1994).

Induction of sexuality became possible when the cells reached c. 22 μm in diameter. Cells of this size and smaller could be triggered to begin gametogenesis (Fig. 1e). Sexual reproduction was oogamous (Fig. 2). The contents of some cells became transformed into a single large non-motile gamete, that is, an 'egg'

(Fig. 2f–h). In gametangia of the opposite sex, the cell contents were shared more or less equally between four motile uniflagellate sperm (Fig. 2a–e).

3.1. SELF-FERTILIZATION

Our *Cyclotella*, like some others studied previously (e.g. Rao, 1970), was homothallic (monoecious): gametes of both the sexes were produced intraclonally. Sex determination is thus not genotypic. Monoclonal cultures of various other centric diatoms have also been found to exhibit homothally, and no centric species has confidently been reported so far to be truly heterothallic (dioecious) (e.g. Drebes, 1977; Chepurnov et al., 2004). We found no evidence of intrinsic barriers to self-fertilization in monoclonal culture of *C. meneghiniana*: sperm and eggs formed intraclonally were able to fuse to produce the viable F1 progeny. However, sperm and eggs were not produced equally at all stages of the life cycle (see below).

It is already well-established that sex in *C. meneghiniana* can be readily induced experimentally in cultures by changing the salinity (e.g. Schultz and Trainor, 1968; Rao, 1978b; Håkansson and Chepurnov, 1999). We maintained stock cultures of the Schelde clone in freshwater 'WC' medium (Guillard and Lorenzen, 1972). In this medium, the clone exhibited sustainable and healthy vegetative growth but with no signs of sexualization even when cells were within the inducible cell size range, apart from infrequent and sporadic spermatogenesis in some small-celled subcultures. However, when seawater was added to the medium, adjusting the salinity to 5–10 ppm, cells responded sexually within a day. During the second day, gametogenesis became vigorous and resulted in a massive burst of fertilization, if gametes of the opposite sex were present in appropriate numbers; a lot of developing auxospores were already present too. F1 cells (some of which were already dividing or had even divided) were observed on the third day.

The first (largest) sexualized cells (c. 22 μm) produced during the life cycle were exclusively oogonia. Since there were no sperm present, eggs were unfertilized and always aborted. Their development was apparently arrested at the prophase of meiosis I, and similar abortion of female gametes during the early stages of meiosis has been reported in *Thalassiosira punctigera* (Chepurnov et al., 2006). Soon, however, below c. 20 μm, oogenesis was joined by spermatogenesis. Consequently, fertilization took place (Fig. 2i–l), and F1 cells started to appear (Fig. 1f–i). Subsequently, as the cells became smaller, the frequency of gametogenesis (gametangia vs. vegetative cells) increased (see also Rao, 1978b), and the ratio between cells undergoing oogenesis and those producing sperm shifted progressively towards spermatogenesis; in small-celled subcultures (close to the minimum, see Fig. 1d), the gametes formed were exclusively or almost exclusively male. This pattern of development, with partly asynchronous production of opposite-sex gametes, is characteristic of sex in the centric group (e.g. von Stosch, 1956; Wiese, 1969; Drebes, 1977).

When cultures were examined directly in the experimental vessel (a Petri dish or a multi-well plate) under an inverted or dissected microscope, it was difficult to distinguish female gametangia from vegetative cells while the gametangia were still at early stages of their development. However, with the aid of high-resolution optical microscopy, young oogonia could be observed to contain an enlarged, spherical nucleus, apparently at prophase of meiosis I (Fig. 2f). The further development of the oogonium involved elongation of the cell (Fig. 2g), followed by gradual separation of the halves (thecae) of the siliceous exoskeleton (the frustule) and partial exposure of the protoplast (Figs. 1e and 2h, i). Cells ready to produce sperm (spermatogonia) could easily be detected microscopically. The chloroplasts they contained were less pigmented and smaller than in the oogonia and vegetative cells (Fig. 2a). Following meiosis (Fig. 2b–d), four uniflagellate spermatozoids were formed per male gametangium (Figs. 1e and 2e). Sperm released by dehiscence of the spermatogonium usually swam actively and readily found oogonia even when cell densities were low or gametogenesis infrequent. In diatoms, the mechanisms of signalling and recognition between compatible gametes (between egg and sperm in *Cyclotella*, or between the isogametes in *Seminavis*, see below) are not yet known, but the success of fertilization we observed shows that these mechanisms are very efficient. In small-celled *Cyclotella* subcultures, in which spermatogenesis was more frequent than oogenesis, we very often observed that numerous sperm surrounded a single oogonium, apparently illustrating 'the strength' of the chemical signal produced by the female cell to attract the males (see Fig. 2B in Chepurnov et al., 2011). Plasmogamy, that is, the penetration of sperm into the egg, was repeatedly seen (Fig. 2i–l). Then, the fertilized egg rounded up and subsequently expanded isodiametrically (= the stage of auxospore development, see Fig. 1f–h) and transformed into a large new silica-walled cell (the 'initial cell') capable of further vegetative multiplication (Fig. 1i). A single, appropriately managed, sexually induced subculture of *Cyclotella* maintained, for instance, in a Petri dish of 50 mm in diameter, could give rise to tens or hundreds of new genotypes (F1).

3.2. CROSS-FERTILIZATION

Until now, there has been very limited practical experience of making *interclonal* sexual crosses in centric diatoms (Chepurnov et al., 2011). One of the most obvious difficulties is that clones are hermaphrodite, producing sperm and eggs simultaneously during part of the life cycle. It is therefore problematic to control sexual reproduction in such a way as to be able to discriminate between F1 cells derived from selfing versus those produced through outcrossing. However, even without appropriate genetic markers, simple practical solutions of overcoming this obstacle can probably always be found, although the approaches are likely often to be species-specific and will require detailed knowledge of the life cycle and sexual reproduction. For example, it may be possible to take advantage of the

shorter or longer periods during which clones are exclusively female (when they are large-celled) or male (when the cells are very small). Alternatively, sexualized cultures could be fractionated by flow cytometry (Sieracki et al., 2005), to separate sperm and eggs (e.g. Vaulot and Chisholm, 1987), which could then be mixed in the desired combinations.

Following intraclonal reproduction in the original Schelde strain of *C. meneghiniana*, we were able to obtain F1 progeny, and these daughter clones were also capable of selfing. However, as we have found in several other *Cyclotella* strains, the vast majority of the F2 cells were not viable and died during auxospore development and initial cell formation. A severe effect of inbreeding has already been reported in species belonging to various diatom lineages, both centric and pennate (e.g. von Stosch, 1965; Roshchin, 1989, 1994; Chepurnov et al., 2004), and may indicate that in nature these diatoms are habitually outcrossing, despite their ability to self in culture. In contrast, when cells of the F1 clones of *C. meneghiniana* were mixed with the parental strain (which was then small-celled and producing sperm vigorously), the offspring derived from the F1 oogonia exhibited high viability. Mating between a hybrid organism and one of its parents (backcrossing) has traditionally been regarded as a very important method in plant agriculture, which is mainly applied to breeding of cross-pollinated plants (e.g. Sleper and Poehlman, 2006).

4. Breeding a Pennate Diatom: *Seminavis robusta*

Seminavis is a representative of a very large, species-rich group of pennates that are characterized by the presence of a 'raphe'. Cells of raphid pennates are capable of gliding movement over a solid substratum (for further explanation, see e.g. Round et al., 1990). The other group of pennate diatoms, the araphids, contains fewer species, though it is phylogenetically more diverse and, indeed, paraphyletic with respect to the raphids; here the valve has no raphe. The life cycle of *Seminavis robusta* is well-understood and can be easily controlled in experiment including the sexual phase (Chepurnov et al., 2002). Other biological characteristics adding to the value of this pennate diatom as a tractable experimental model have been discussed elsewhere (Chepurnov et al., 2008).

4.1. CROSS-FERTILIZATION

Until the last 20 years, most observations of sexual reproduction in pennate diatoms had been made serendipitously, when natural populations were found producing auxospores. This underlay most of the work by Geitler (e.g. literature cited in Geitler, 1973), although a few detailed investigations were made using cultured material, revealing that some pennate diatoms are homothallic and identifying some of the factors that can influence sexualization (e.g. Geitler, 1932; Rozumek, 1968).

More recently, however, it has become common to attempt to induce sexual reproduction through interclonal crosses, because many pennate diatoms, including *Seminavis robusta*, are heterothallic: clones are almost or fully unisexual (i.e. the species is dioecious) (e.g. Roshchin, 1994; Roshchin and Chepurnov, 1994; Davidovich and Bates, 1998; Mann et al., 1999; Chepurnov and Mann, 2004; Chepurnov et al., 2004, 2008). Sex determination is apparently genotypic (as opposed to the phenotypic sex determination of the homothallic centric diatoms), and clones retain essentially the same sexuality throughout the period when they are sexually potent (as opposed to the female to male transition in centrics). Thus, sexual reproduction can only be triggered in the laboratory when clones of opposite mating type are (1) within the 'right' size range, so that they are sexually inducible, and (2) brought together into mixed culture. Heterothallic mating behaviour in pennate diatoms was first reported by von Stosch (1958). However, the prevalence of heterothally in pennates only emerged thanks to the experimental results and interpretations of A.M. Roshchin (summarized in his monograph of 1994; see also Chepurnov et al., 2004).

In order to prepare illustrations and information for the present work, we ordered two clones of *S. robusta*, sexually inducible and belonging to different mating types, from the diatom culture collection of Ghent University. These cultures (their accession numbers were DCG 0115 and DCG 0116: http://bccm.belspo.be/about/dcg.php) were selected from a laboratory lineage, whose pedigree was shown in Chepurnov et al. (2008). The sexual auxosporulation illustrated in Fig. 3 was induced in mixed cultures of these strains.

The largest initial cells of *Seminavis* produced during our experiments were c. 80 μm long. The sexual size threshold is 55 μm: this was the length of the biggest gametangia we were able to find. Vigorous induction of sex, however, occurred only when cells became shorter than 50 μm (see also Chepurnov et al., 2002). Critically small cells were c. 12–15 μm long and continued to divide and get smaller until they eventually died, in contrast to *C. meneghiniana*.

In low-density cultures of *Seminavis*, during the earlier part of the exponential growth phase, the cells do not move much, and we have observed similar behaviour in some other raphid diatoms. As the cultures became denser, the cells moved more and more actively. Pairing of cells occurred soon after cultures of opposite mating type were mixed together in late exponential phase (e.g. Chepurnov et al., 2008), and the efficiency of this process suggests that sexual pheromones exist to help cells of opposite mating type to locate each other (e.g. Chepurnov et al., 2004). However, '… it is unlikely that each mating type would both produce and detect the same pheromone (Dusenbery, 2000, p. 9). Probably, therefore, only one of the mating types provides chemical clues as to its location, while the other responds to this information through directed movement' (Mann and Chepurnov, 2005). So far, there is no direct evidence concerning the nature of diatom pheromones, but *Seminavis* would be an excellent system in which to seek them because of the depth of knowledge about the sexual process [*note added in proof: at the 22nd International Diatom Symposium in August 2012, J. Frenkel et al. reported

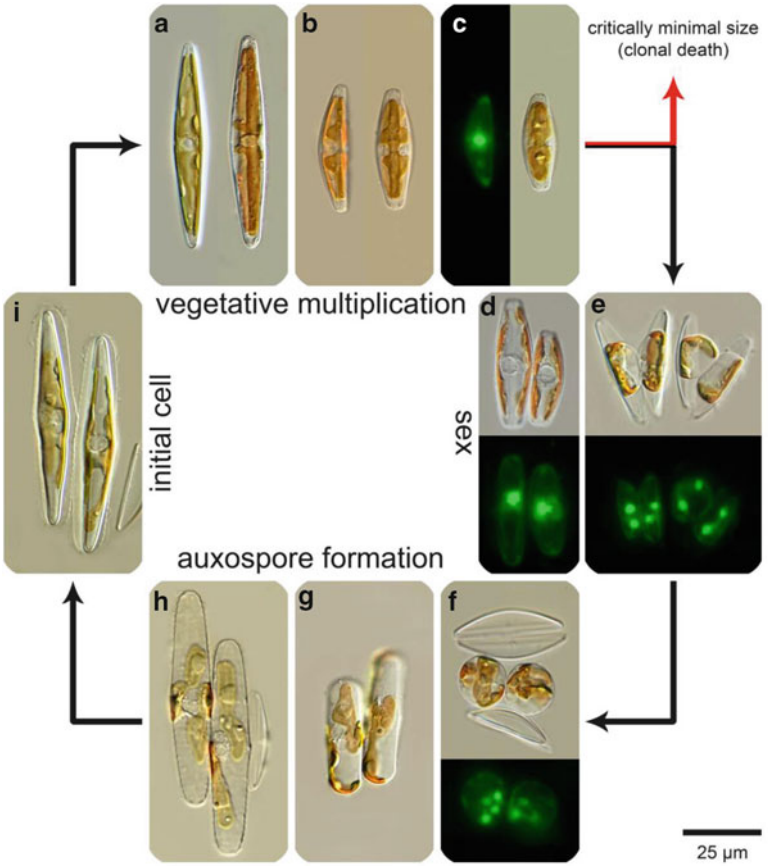

Figure 3. *Seminavis robusta*: the life cycle. (**a**–**c**) Gradual diminution of cells in size (*left images* in *valve* view, *right* in *girdle* view). (**d**) A pair of gametangia before karyokinesis of meiosis I. Note the enlarged spherical nuclei in meiotic prophase. (**e**) Two isogametes per gametangium have formed. Note (*lower image*) the gametes have already passed through karyokinesis of meiosis II and contain two haploid nuclei each. (**f**) Two spherical cells resulted from allogamous fusion of gametes. Note all the four haploid nuclei are still visible in the left cell (*lower image*). On fluorescent images of **c**–**f**, nuclei were visualized via SYBR Green staining. (**g**) Expanding auxospores. (**h**) Expanded auxospores. (**i**) Initial cells, still surrounded by the auxospore cell wall.

that the chemical nature of the *Seminavis* attractant has been determined]. Recently, pheromones have been demonstrated in the araphid pennate *Pseudostaurosira* (Sato et al., 2011): female clones below the critical size threshold constitutively produced a pheromone (ph-1) that caused sexualization of males. Sexualized males in turn secreted a pheromone (ph-2) that induced sexualization of females. However, neither ph-1 nor ph-2 appeared to function as an attractant, and searches for a third chemoattractant pheromone were inconclusive. Neither of the *Pseudostaurosira* pheromones has been characterized chemically.

Once paired, partners signalled each other, apparently via direct surface interactions – contact pairing *sensu* Wiese (1969) – to start meiosis (Fig. 3d). At the end of meiosis I, the protoplast cleaved in the same plane as during mitotic cell division, but the two daughter protoplasts remained enclosed within the parent cell wall and did not form new cell walls of their own. Meiosis II was always acytokinetic so that only two gametes were produced per gametangium (Fig. 3e). The gametangia dehisced by splitting apart of the two halves of the cell wall, and the gametes fused allogamously. The zygotes rounded up (Fig. 3f) and developed into auxospores, which exhibited bipolar expansion (Fig. 3g, h). After expansion was complete, the auxospores transformed into the first vegetative cells of the F1 generation; these cells had a slightly modified morphology (being less angular in transverse section) and are referred to as the initial cells (Fig. 3i). Thus, two parental (gametangial) cells give rise to two cells of a new generation which have something of the same relationship to each other as 'dizygotic twins', except that the pairs of fusing gametes derive from the same two meioses and therefore share the same crossovers. Previously, one of us (VCh) has repeatedly checked mating-type distribution among sibling clones randomly isolated from the same sexually reproducing mixed culture, and the sex ratio did not depart significantly from a 1:1 ratio. Thus, if mating type is determined by a single locus (by invoking the parsimony principle), a 1:1 ratio might indicate that one mating type should be heterozygous at this locus while the other is a homozygous recessive. Mating type distribution was also analysed among a few tens of pairs of the 'dizygotic twins'. In most of the cases, the twins were of opposite mating type. However, infrequently, both cells had the same mating type (see fig. 4 in Chepurnov et al., 2008). We suggest that the appearance and frequency of such cases may depend on how often a DNA fragment embracing the mating type locus is involved in chromosomal crossing over during prophase of meiosis I. We plan to consider this hypothesis in more detail in a forthcoming article on *Seminavis*.

4.2. SELF-FERTILIZATION

Our first experiments on *Seminavis* dealt with a restricted number of clones isolated from nature and did not reveal any that could reproduce intraclonally (Chepurnov et al., 2002). However, as more strains were obtained via the expansion of the laboratory pedigree (Chepurnov et al., 2008), we started to find very infrequent cases of intraclonal auxosporulation, which was associated with the same pattern of sexual reproduction (isogamy) as in mixed cultures of opposite-sex clones. Only a minority of the strains investigated (10 out of c. 80) exhibited intraclonal reproduction and in none of them was it common. Furthermore, all 10 inbreeding clones belonged to the same mating type. Building further on the hypothesis mentioned above that one sex is heterozygous at the mating-type locus, the other homozygous, it would be reasonable to suggest that the mating type capable of sporadic selfing is the one that is heterozygous at the mating-type locus, and that the aberrant behaviour might perhaps arise as a

consequence of mitotic recombination (e.g. Griffiths et al., 1999). Among the F1 cells derived from intraclonal auxosporulation, both mating types were present (we checked this in a few *Seminavis* clones), again consistent with the hypothesis that sporadically selfing clones are heterozygous at the mating-type locus.

Hence, in *Seminavis*, it is more difficult than in *Cyclotella* to self clone, though it is not impossible; backcrossing, on the other hand, is unproblematic. It has also been demonstrated that *Seminavis* is highly tolerant to inbreeding (Chepurnov et al., 2008).

5. Some Concluding Remarks

Currently molecular methods, particularly transgenesis, attract almost all of the attention and funding being given to genetic manipulation of microalgae. It is obvious that transgenic approaches have tremendous potential but they have yet to prove their economic efficiency (e.g. Knight, 2003; Goodman, 2004; Murphy, 2007; Gurian-Sherman, 2009), whereas conventional methods of plant breeding have a long proven record in the improvement of agricultural cultivars. Some experts have therefore expressed concern that the current balance between classical breeding (including all attributes of plant reproductive biology) and molecular technologies may actually slow progress in achieving the principal goal, namely, sustainable growth in the efficiency of genetically controlled selection and improvement of those organisms we exploit economically (e.g. Knight, 2003; Snape, 2004; Gepts and Hancock, 2006).

The position is even worse in diatoms, where the possibilities of classical breeding have been ignored or dismissed as impractical. Curiously, at the very time that the diatom life cycle is receiving renewed attention, as a result of new observations of natural populations and the experimental discovery of heterothally, diatoms have been gaining an undeserved reputation for being difficult to manage sexually (e.g. Grossman, 2005, 2007; Kroth, 2007). For example, in a recent opinion article in *Nature*, Armbrust (2009) wrote 'The sexual cycle of most diatoms cannot be controlled in the laboratory, hindering the development of classical genetic studies. Instead, genetic manipulation of diatoms has relied primarily on the addition of new versions of genes (transformation) or on reduced expression of targeted genes (RNA interference)'. In addition, the prospects for breeding diatoms were probably not helped by the fact that the first two diatoms to be chosen for whole genome sequencing – the centric *Thalassiosira pseudonana* (Armbrust et al., 2004) and the pennate *Phaeodactylum tricornutum* (Bowler et al., 2008) – lack the typical diatom cycle that we have illustrated with *Cyclotella* and *Seminavis*; they may even be asexual.

However, as we have shown here, and also discussed elsewhere (Chepurnov et al., 2004, 2008, 2011; Mann and Chepurnov, 2004), there is no fundamental obstacle to the development of classical breeding in diatoms. An excellent foundation for managing sex in culture, as well as for understanding the visible events of

meiosis, gametogenesis and fertilization, was laid by L. Geitler, H. A. von Stosch and A. M. Roshchin, and others, including ourselves, have built on this foundation (e.g. Amato et al., 2005; Poulíčková and Mann, 2006; Vanormelingen et al., 2008; Davidovich et al., 2009). This body of work shows that the breeding systems of diatoms are very varied, as in higher plants, with all gradations between obligate outbreeders, such as the heterothallic *Seminavis* described here, or *Sellaphora capitata* (Mann et al., 1999), through freely inbreeding diatoms capable of sustained intraclonal mating without loss of vitality over several generations (e.g. *Sellaphora bisexualis*, see Mann et al., 2009), to autogamous or pedogamous forms (Geitler, 1985; Chepurnov et al., 2004; Trobajo et al., 2006), in which diploidy is restored through fusion of two of the haploid products of a single meiosis. Such knowledge is a prerequisite for development of strategies to improve diatom strains. So also is basic information about the induction of sex, and again there are already plenty of pointers to factors that can be important, such as changes in salinity (*Cyclotella meneghiniana*, discussed here) or nutrient status (*Aulacoseira skvortzowii*, *Stephanodiscus* sp.: Jewson, 1992; Jewson et al., 2008), or particular combinations of light and temperature (e.g. Rozumek, 1968), or growth phase (*Seminavis robusta*). Each species probably has its own unique mix of requirements, and, if no previous experimental data are available, hints about which are important may be obtained by considering the habitat in which the diatom grows: for example, nutrient control is more likely in planktonic diatoms than epipelics (because of major annual fluctuations in the availability of N, P, Si or Fe in the water column), salinity effects are unlikely in stenohaline environments, etc. The reproductive biology of diatoms is not mysterious, and controlled crosses are easily made. Perhaps more of a problem than crossing is the isolation and screening of significant numbers of F1, F2, etc. offspring. For planktonic diatoms, flow-cytometric cell sorting may be an important tool, but pennate diatoms are less easily dealt with, because of their tendency to stick rapidly and firmly to surfaces and to each other, forming clumps. All our work with these up until now has been done using manual isolation techniques.

A final point worth making is that, in classical breeding, if we wish to estimate the pool of genes that nature puts at our disposal and use them to the full, it is simply unavoidable that we have to understand the 'real' species boundaries (e.g. Mann, 1999) within which genotypes can be 'mixed'. Thus, improvement of our knowledge of diatom species taxonomy, population biology and biogeography will inevitably impact on the efficiency and success of strain selection and improvement programmes.

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R. Gordon and J. Seckbach (eds.), *The Science of Algal Fuels*,
Cellular Origin, Life in Extreme Habitats and Astrobiology, 341–354
DOI 10.1007/978-94-007-5110-1_19, © Springer Science+Business Media Dordrecht 2012

FUEL FROM SEAWEEDS: RATIONALE AND FEASIBILITY

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1. Introduction

In recent years, governments have taken actions to address global warming and global climate change (UNFCCC, 1992). In spite of accumulating evidence correlating greenhouse gasses (GHG) atmospheric levels and global warming (Broecker, 1975), it was not until 1992 that the United Nations convened to sign the Kyoto Protocol. The objective in this protocol was to achieve "stabilization of greenhouse gas concentrations in the atmosphere at a level that would prevent dangerous anthropogenic interference with the climate system." A time frame was also given: "sufficient to allow ecosystems to adapt naturally to climate change, to ensure that food production is not threatened and to enable economic development to proceed in a sustainable manner."

In the early 1970s, members of the Organization of Arab Petroleum Exporting Countries (OAPEC) decided to slowly raise oil prices, causing a heavy impact on the world economy and posing major threats to the US economy. In response, governments decided to explore new alternatives to gasoline. The USA, through the National Renewable Energy Laboratory (NERL) and the Solar Energy Research Institute (SERI), searched into production of biodiesel from microalgae. Despite significant progress, and because oil prices during the 1980s dropped (due to lower demand caused by higher fuel efficiency in cars and mild winters), the program was abandoned (Mousdale, 2008). In contrast, Brazil, which lay deep in debt partially due to fuel shortage, embarked on a plan to become energy self-sufficient. Sugarcane bioethanol production was the main ingredient in Brazil's plan. Price control over gasoline, ethanol, and their blends, which favored ethanol according to its availability, created an incentive for consumers to buy cars with flex engines so that drivers could swiftly adapt to changing fuel prices. As a result, ethanol automotive technologies continued to develop, rendering Brazil an energy-independent country and a leader in world biofuel production. Although formerly predicted (Kerr, 1998), recent economic events were led by an unprecedented rise in fuel prices reaching a record high of US $140 per barrel during 2008 (Fig. 1).

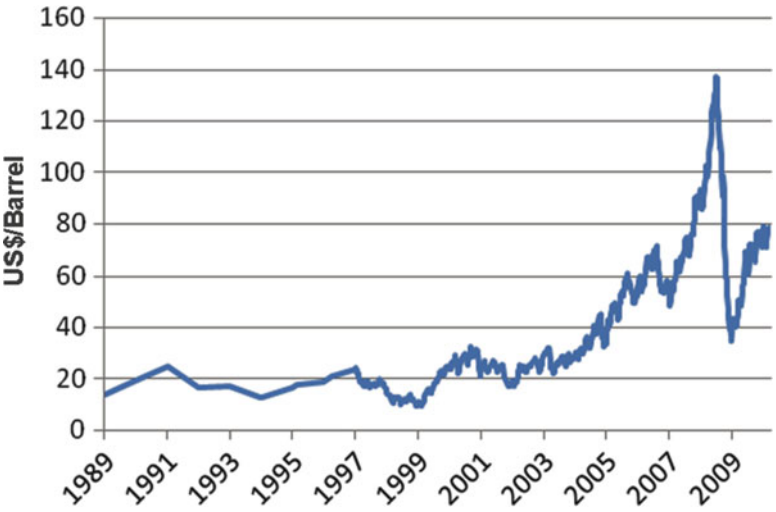

Figure 1. Crude oil prices per barrel (from June 1989 through June 2010). During 1981–1998, oil prices remained constant averaging US$15 a barrel (Adopted from EIA, 2009).

2. Advantages and Disadvantages of the Biofuel Industry

The growing quest for alternative fuel sources is intensifying, with governments of leading countries investing billions of dollars in biofuel technology. Two of the biggest producers, Brazil and the USA, focus on ethanol: Brazil with already a three-decade history of massive production from sugarcane and the USA focusing its resources not only on corn starch ethanol but also on soybean for biodiesel. Evidence of ethanol production (ca. winemaking) gathered from residues found in the Middle East was dated as being as old as 6,000 years ago (Berkowitz, 1996). Similarly, vegetable oils such as olive oil have been used from about that same time in Asia and surrounding areas. Since then, the technology of ethanol production have progressed greatly (Galili et al., 1997), and it may readily be applied. Nevertheless, improvement in process efficiency and search for cheaper and sugar-rich sources still continue. Ever since the creation of the Model T automobile by Henry Ford in the early twentieth century, cars could be run on ethanol as fuel. Furthermore, diesel engines can run on biodiesel made of vegetable oil as was exhibited in 1900 at the Exposition Universelle (Knothe, 2010). In addition, since vegetable oil and ethanol have chemical qualities similar to those of conventional fuel, there is no need for extensive investments in new infrastructures. Flex engines, which are able to switch from gasoline to ethanol, have been available for decades.

Oxidation of organic compounds for the utilization of their chemical energy releases CO_2 and other gasses and particles. Burning fossil fuels releases large amounts of GHG. Using crops as a fuel source will eventually also lead to CO_2

release into the atmosphere. Nevertheless, this will be carbon which had been sequestered by photosynthesis just "recently." Thus, fuel crops would not increment current atmospheric CO_2 levels provided that all processes involved are fueled by renewable energy. This so-called zero CO_2 balance and its approaches have already been implemented for bioethanol production from sugarcane. Here, the plant extract (molasses) is used as a substrate for fermentation, and the burning of its biomass by-products (bagasse) is used as an energy source for the distillation and other processes which take place in the production plant.

The zero CO_2 balance rationale raises a critical question: if the goal of adopting "green fuel" is to diminish threats of global warming by reducing atmospheric CO_2, then how does a "zero CO_2 balance" help? Furthermore, using land crops as a fuel source might indirectly increase GHG. Atmospheric CO_2 might increase, since land-based biofuel farming competes directly and indirectly with rainforests (Koh and Wilcove, 2008). Land plants, mainly rainforests, are responsible for about half of the world's photosynthesis and subsequent CO_2 sequestration (Melillo et al., 1993). A significant amount of the CO_2 sequestered from the atmosphere by rainforests does not return to the atmosphere. Rather, it remains for decades to centuries in the form of cellulose, contained in woody materials in tall land plants. Therefore, growing fuel crops in proximity to rainforest regions is worrisome. Indeed, from its beginning in the sixteenth and seventeenth centuries, sugarcane cultivation along with other crop production has reduced the Atlantic Forest (also in Brazil) to less than 7% of its original size. Current efforts from Brazil to intensify production and export of fuels to other countries are causing much concern. Substitution of pastureland and annual crops by sugarcane had, according to some authors, a beneficial influence on the biodiversity (Margulis, 2004). Much of the land for crop cultivation borders threatened Amazonian rainforest, which might be deforested for planned Brazilian bioethanol production.

Economic considerations also question current biofuel policies. In a brief prepared by the International Food Policy Research Institute for the conference on "Assuring Food and Nutrition Security in Africa by 2020: Prioritizing Actions, Strengthening Actors, and Facilitating Partnerships," held in Kampala, Uganda, April 1–3, 2004, the current availability of food was discussed. In this brief, the importance of strengthening the farming sector in poor regions in the world to increase food security is underlined. In addition, the connection between food availability and prices is stressed. As a rule, using food crops as a fuel source decreased food availability and caused a rise in food prices (Fig. 2).

This scenario is also true when using crops which are intended for livestock feed, such as maize and corn variants used to feed cattle and fowl. Accordingly, using corn as a "fuel crop" (as practiced in the USA) might have caused a rise in the price of basic such as meat, dairy, and eggs as well as increases in land prices (Westcott, 2007), resulting in the economic crisis of 2008 (Fig. 2). These issues are now being addressed, and a whole new field of research of fuel crop cultivation (some genetically engineered) is now on the rise (Vermerris, 2008).

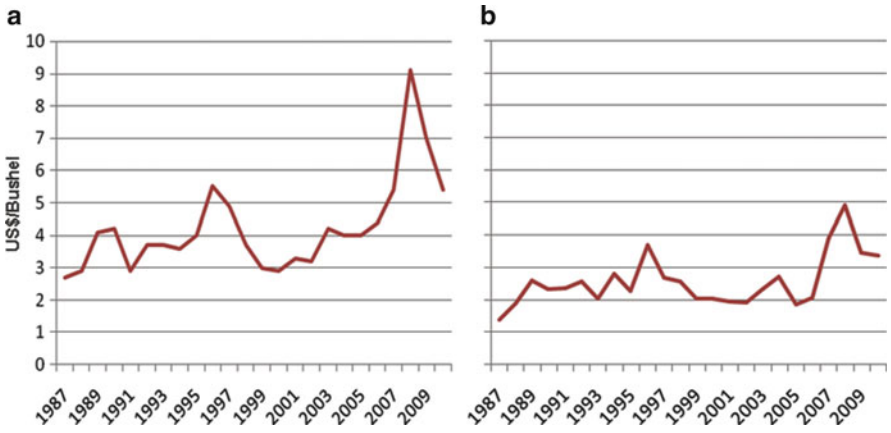

Figure 2. World market food prices (in US$/bushel) for (**a**) wheat and (**b**) corn from 1987 to 2010. (**a**) In both charts, *peaks* seen in 2008 for both major crops closely match world oil prices (The United States Department of Agriculture (USDA), amended).

Unfortunately, the strategy of using nonedible food crops as a source of biofuel does not address the problem at its source since arable land and freshwater are becoming scarce. Many models which simulate future land requirements for fuel crop growing have been constructed (Schubert, 2006). Figure 3 depicts a model which compares the US fuel needs to the size of arable land required to provide for sufficient fuel crops according to current technologies. The large circular section represents the size of arable land that would be needed to provide current US fuel needs using corn ethanol. The small circular section on the right is an approximation of the size of land needed to produce sufficient fuel for the USA using cellulosic feedstock. The small circular section on the left simulates the size of land needed for the USA using microalgal biofuel. Nevertheless, these are all simulations; cellulosic ethanol technology requires energy and funds to reach a point where production can be profitable. At this stage, costs for the production of cellulosic ethanol require energy and funds almost equal to the energy produced and money returned. Although there has been some progress in cellulosic ethanol technology lately, algal biofuel promises a surer approach for the next few decades since the technology to utilize algal biomass has been researched since the 1970s and already exists (Schubert, 2006).

Figure 3 also shows that any attempt to reach sustainability would need transferring a large section of arable land currently used for food crops to fuel crops. Furthermore, considering the fact that quality cultivable land is becoming scarce, and that human consumption rate is also growing rapidly (Pereira et al., 2007), land-based biofuel production as a sustainable energy in the future is highly doubtful. Another relevant human impact resulting from intensive agriculture is

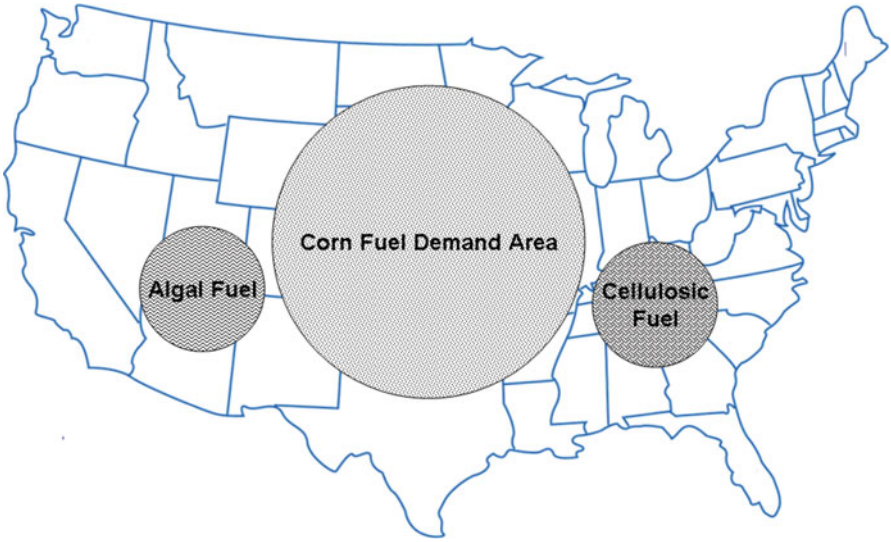

Figure 3. Theoretical surface areas needed for the cultivation of three biomass sources. Each *circle* represents the cultivation area needed to produce sufficient amount of biomass for conversion to liquid fuel needed for 2006 US economy (Dismukes et al., 2008, amended).

land degradation. According to the Millennium Ecosystem Assessment (2005), up to 50% of the agricultural land and 60% of ecosystem services are now affected by some degree of degradation, with agricultural land-use being the chief cause of land degradation. Additional stress on already damaged lands would inevitably cause a serious setback in our vision to achieve a new balance with the environment. Freshwater shortage is another problem which might worsen due to land agriculture intensification and growing urban and industrial land demands. Secondary salinization and water logging are two problems caused by agricultural irrigation, with 20% of this resource already damaged. Furthermore, half of the world's rivers are seriously depleted and polluted, and 60% of the world's largest rivers are significantly fragmented by large dams built mainly for irrigation (Wood and Scherr, 2000; Fraiture et al., 2008).

In addition to water supply constrains, intensive land farming for biofuel might pose another major threat derived from monoculture. Since monoculture lacks biodiversity, it is particularly susceptible to diseases. Recently, the case of the Ug99 "stem-rust" fungus attacking wheat crops across Asia and having the potential to spread to all five populated continents threatened "social destruction" (Singh et al., 2011). The importance of biodiversity in farming and the dangers of monoculture were further established by the work of Zhu et al. (2000), in which rice susceptibility to disease was reduced by 94% by planting mixed strains of rice.

3. Algal Biofuels: Biodiesel and Bioethanol

Considering the above apparent disadvantages in using of terrestrial plants for the production of biofuels, this chapter rather explores the option of aquatic plants as they offer several significant competitive advantages. Algal biofuels can be obtained from both microalgae and macroalgae (so-called seaweeds). Essentially, microalgae may yield biodiesel, and for that reason, they have been quite extensively researched by the American National Renewable Energy Laboratory since the 1970s. The research focused on the production of biodiesel since lipids comprise a high content in these algae. Bioethanol can subsequently be produced from the by-products left after the lipids are extracted (Schenk et al., 2008).

Using algae as feedstock might avoid the economic and environmental strains inherent in terrestrial biomass sources, as discussed above. Massive cultivation of microalgae is generally achieved in land-based raceway ponds. Algae are far more productive than land plants with turnover rates exceeding by an order of magnitude those of any land plant (Dismukes et al., 2008) and can be grown at sustainable rates as a source of feedstock for biodiesel/bioethanol production. Being a nonessential food, rising prices even of edible algae should not pose a threat of famine to developing countries. As shown in Fig. 3, a smaller size of land has to be used for algal biofuel in order to meet US fuel demand. In addition, algae are grown in closed or semi-closed systems, and conventional irrigation is not required and, hence, should not add to freshwater shortages in the long run. Also, growing algae for fuel should not cause land deterioration as might happen as a result of intensive land agriculture of, for example, corn cultivation (Lal et al., 2007). Additionally, algal cultivation does not require the use of insecticides and herbicides which poses the danger of polluting groundwater and nearby rivers and streams. These features as well as the need of seawater rather than freshwater for several attractive species make algal biofuel a promising candidate for steady and sustainable production of energy in the near future.

Currently, in the USA, biodiesel is produced mainly from soybeans. Other sources of commercial biodiesel include canola oil, palm oil, corn oil, animal fat, and waste cooking oil (Felizardo et al., 2006). Standard biofuel production involves a defined chemical process (transesterification) in which triglycerides known to be the main ingredient in vegetable oil react with an alcohol – usually methanol. A catalytic factor is also needed, usually sodium hydroxide or potassium hydroxide (Chisti, 2007). A newer approach is using enzymes as catalysts. Biocatalysts are gaining more attention nowadays and have the potential to outperform chemical catalysts for biodiesel production in the future. Although lipases may become inhibited by the glycerol which is formed in the transesterification process, this problem is being addressed successfully by using acyl acceptor, which prevents the formation of glycerol. As a result, this method becomes significantly advantageous since it allows the recycling of the catalyst. Another advantage present in using biocatalysts is the relatively low reaction

temperature – an optimum of 60 °C when using olive oil as substrate (Vasudevan and Briggs, 2008). These two benefits make the entire process much more cost-effective.

Several of the above-mentioned features for microalgae also apply to macroalgae. Moreover, the cultivation of seaweeds has an important additional advantage over microalgae culture as they can all be grown directly and safely in the sea without putting any pressure on land. Even their processing could be done at sea, as proposed by several project plans worldwide. This last attribute could eliminate entirely environmental and economical concerns since it overcomes most of the hurdles faced when approaching microalgal production, such as harvesting and aeration. In the 1970s and later in 1997, research on methane production from the green seaweed *Ulva* (that used 98,000 m^3 algal biomass harvested off the shores of Brittany and France) was abandoned due to low methane yields and high H_2S contents in the gas (Briand and Morand, 1997). It is only in the last few years, as oil prices have gone up, that researchers returned to work on fermenting algal biomass to produce bioethanol. The high content of proteins and minerals in seaweeds rendered them a good nutritional supplement to fermentation feedstocks of low nutritional value, for example, cellulosic biomass, but still they were not used as the main fermentable feedstock. Although cultivated for many years as "sea vegetables," macroalgae for bioethanol fermentation have yet to emerge as a large industry such as microalgal biodiesel. Lately, another important advantage in seaweed cultivation over other crops was found to be their possible implementation as biofilters of effluents, derived from cultures of marine organisms such as fish and shrimps (Neori et al., 2004). Also known as integrated multi-trophic aquaculture (IMTA), this cultivation strategy is becoming more and more accepted as an ecologically sustainable method for bioremediation of fish mariculture effluents (Kraemer et al., 2004). The ability of seaweeds to absorb heavy metals has also been addressed. Macroalgae grown for these purposes using land-based settings might prove a continuous source of cheap biomass for fermentation and biofuel production.

In one form or another, fermentation is an ancient technology known for thousands of years. From bread rising through grain-based alcoholic beverages (beer, whiskey) to sugar-based alcoholic beverages (wine, rum), the presence of yeast is always needed. Yeasts are the only known organisms which can perform anaerobic fermentation, produce ethanol as a by-product, and tolerate a relatively high concentration of this by-product (up to 18%) in its living environment. Soluble sugars (mainly glucose, fructose, and their derivatives, e.g., sucrose and maltose) provide the main source of energy for yeast. When cultured under anaerobic conditions, these hexoses provide the substrate for ethanol production, as follows:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$$

Starch can also be considered as a substrate since it can be hydrolyzed cheaply into short glucose chains which yeast could easily utilize for consumption.

4. Heightening Lipids and Fermentable Contents in Algae

Since its inception, algal biofuel research has focused on finding the right growing conditions which would cause the organisms to accumulate storage materials in order to obtain high lipid contents, which could then be converted to biodiesel.

The microorganisms *Candida* sp. No. 107 and *Lipomyces starkeyi* accumulate lipids in response to low nutrient availability. When grown in a low glucose and high NH_4 environment, and then abruptly transferred to high glucose and low NH_4 environment, it causes a large increase in intracellular citrate concentration, which precedes an onset of lipid accumulation. This accumulated citrate is transported into the cytosol and cleaved to acetyl-CoA by ATP citrate lyase, an enzyme that does not occur in non-oleaginous species. While the above-mentioned organisms can accumulate up to 5–10% of their biomass when grown in a lipid accumulation-inducing manner, other yeasts such as *Rhodotorula* spp., *Lipomyces starkeyi*, and *Cryptococcus curvatus* (= *Apiotricum curvatum*) can accumulate between 40 and 70% of their biomass as lipid; these fatty acid-accumulating species were dubbed oleaginous species. Some of these organisms are used, or have been used, commercially to produce the various edible oils: *Mucor circinelloides* as a source of g-linolenic acid (18:3, $n-6$), *Mortierella alpina* for producing arachidonic acid (20:4, $n-6$), and *Crypthecodinium cohnii* and *Schizochytrium* spp. for docosahexaenoic acid (22:6, $n-3$) production. In lipid-accumulating microorganisms, two important biochemical responses to nitrogen starvation have been identified: (1) The first identifiable biochemical event following exhaustion of nitrogen from the growth medium is the activation of AMP deaminase, which is considered as a way to release some free ammonium into the cell by cleaving AMP into IMP + NH_4^+. As a consequence, isocitrate (NAD^+-linked) dehydrogenase slows its activity within the mitochondrion. Cessation of isocitrate dehydrogenase activity quickly leads to a buildup of the aforementioned citrate as isocitrate, which is no longer being metabolized. (2) The second biochemical event is the activation of malic enzyme which provides a constant stream of NADPH for fatty acid synthesis (Wynn et al., 2001). Nitrogen starvation in microalgae also stimulates higher starch content as evidenced in electron microscope images (Rodriguez–Lopez and Munoz–Calvo, 1980).

Though much research has been done on heightening lipid content in growing organisms, not much work has been done on the subject of altering culture conditions in order to reach a higher content of starch in macroalgae. In spite of the scarcity of scientific knowledge in this particular field, we can gather evidence from the mechanisms of other autotrophs and their response to different growing conditions.

Rosenberg and Ramus (1982) showed that seasonality was a main factor in determining the C:N ratio in *Ulva* and *Gracilaria*. Furthermore, an inverse C:N relationship was observed under nitrogen limitation. LaPointe and Ryther (1979) have associated a decrease in the C:N ratio in seaweed with increased growth rates at high nitrogen loading. However, under natural conditions, growth

rates in seaweeds cannot be as easily correlated to nutrient availability due to the presence of a flexible loading and storage mechanism. In the red edible seaweed *Porphyra yezoensis*, free amino acids accounted for over 40% of the total nitrogen content (Oohusa et al., 1977). Also, Laycock and Craigie (1977) found that the free amino compound citrullinylarginine comprises 50% of the total nitrogen (more than ten times the level of any of the free amino acids) present in the seaweed *Chondrus crispus*. Taking further these three last-mentioned works' findings, an inverse correlation between storage carbohydrates (including starch) and nitrogen content may be observed, according to nitrogen availability: When nitrogen is limited, carbon is stored in the form of storage carbohydrates. When nitrogen is available, but sunlight is limited, nitrogen is then preferentially stored in the form of specialized amino acids (this process apparently uses up all the stored carbohydrate as carbon skeletons). When sunlight intensifies with the change of seasons, both stored materials, that is, carbohydrates and amino acids, are used by the algae for growth and cell division

5. The Importance of Other Carbohydrates for Ethanol Production

Storage compounds in seaweeds usually include molecules other than starch, depending on the species, such as ulvan in *Ulva* (Lahaye and Robic, 2007) and agar in *Gracilaria* (Hemmingson et al., 1996). Indeed, ulvan and agar are two polysaccharides which have been considered for biofuel application. Additionally, new microorganisms which are able to utilize different sugars as substrate for fermentation are constantly being searched for and genetically engineered to produce greater yields of alcohols. Therefore, attaining greater contents of other polysaccharides in addition to starch is another good attribute for bioethanol feedstock.

6. Estimations of Maximum Yields

At present, the macroalgae species *Ulva lactuca* and *Gracilaria conferta* have been investigated in order to find (1) the growth conditions to attain maximal biomass yields and (2) high carbohydrate contents for the production of algal bioethanol. In a series of experiments performed at the Israel Oceanography and Limnology Research Institute (IOLR), we investigated the effects of high irradiance and macronutrient (N, C, P) starvation and low levels of nutrients on these algal species and found promising results. A long-term research program at IOLR confirmed that 1,000 m^2 outdoor ponds may yield 10 ton of dry weight *Ulva* per year at a production cost of US$1.5 per kg of dry biomass. A larger, 3,000 m^2 pond could lower production costs to US$700/ton dry biomass, including labor, management, and other expenses. At these production rates, approximately 727 liter ethanol/1,000 m^2 can be obtained, assuming 50% starch to ethanol conversion rate, surpassing yields of sugar beet grown in France or sugarcane grown in Brazil (see Table 1) (Brown, 2006). If we consider recent progress in

Table 1. Ethanol yields for various crops and for a *red* (*Gracilaria*) and a green seaweed (*Ulva*).

Crop	Ethanol yield (liter/1,000 m^2)
Sugar beet (France)	667
Sugarcane (Brazil)	618
Cassava (Nigeria)	381
Sweet sorghum (India)	348
Corn (USA)	329
Wheat (France)	258
Gracilaria starch (Hawaii)	353[a]
Ulva starch (Israel)	727[b]
Ulva carbohydrates (Israel)	4,360[c]

Numbers were converted from gallon/acre to liter/1,000 m^2 of land.
[a]Our estimation with data from Glenn and Tollefsen (1991). Data from Brown (2006).
[b]Our estimation assuming 50 % starch to ethanol conversion yield.
[c]Our estimation assuming 50 % carbohydrates to ethanol conversion yield.

marine yeast research, and the possibility of finding yeast that would be able to ferment specific carbohydrates found exclusively in marine algae, the ethanol yield might theoretically reach up to 4,370 liter 1,000 m^2 per year (Table 1). However, calculations using the above data have shown that the production cost of biomass which would yield 1 l of ethanol *Ulva* might reach close to US$10, which is ten times the cost for sugarcane ethanol production cost (Mousdale, 2008). Nevertheless, if we consider the starch fraction of the alga as a mere by-product, as is the case in *Gracilaria* cultivation (McHugh, 2003), the cost of the ethanol produced would only reach as high as the cost of the starch extraction, fermentation, and distillation, without needing to consider algal cultivation costs. For example, in a Southeast Asian Fisheries Development Center's (SEAFDEC, 2010) business proposal for farmers, an economical analysis shows a 110% return on investment in 1 year. A Hawaiian *Gracilaria parvispora* cultivation experiment where the alga was grown in cages in fishponds has yielded a viable continuous crop of 33 ton/ha/year dry weight (Glenn and Tollefsen, 1991). This could translate into 353 l/km^2/year of ethanol in optimum conditions. This impressive yield, which can be compared to the best yields produced from corn in United States and from sweet sorghum in India (Table 1), would be produced solely from the by-products remaining after the agar extraction process. Combining algal mariculture with a bioremediation program, as proposed by SEAFDEC (2010), would not only reduce production costs but also add an ecological benefit to the process.

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R. Gordon and J. Seckbach (eds.), *The Science of Algal Fuels*,
Cellular Origin, Life in Extreme Habitats and Astrobiology, 355–382
DOI 10.1007/978-94-007-5110-1_20, © Springer Science+Business Media Dordrecht 2012

THE CONTRIBUTION OF DIATOMS TO WORLDWIDE CRUDE OIL DEPOSITS

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1. Introduction

Crude oil or petroleum is derived from natural sources which are in the form of organic matter deposited along with the sediments in sedimentary basins from geological past (Hunt, 1863; Hunt et al., 2002; Kvenvolden, 2008). Living organisms of various kinds contribute to production of hydrocarbons as a normal part of their existence in which algae are thought to be one of the principal contributors to production of petroleum (Whitmore, 1944; Oakwood, 1946). They are also considered to yield a large percentage of organic compounds more closely analogous to petroleum as identified through chemical structure of kerogen; therefore, abiogenic origin of petroleum has not been accepted by modern geologists. Brongersma-Sanders (1951) emphasized the impressive local development of abundant plankton in areas of upwelled nutrient-rich ocean waters. It seems that both geologically and geochemically aquatic plant and animal life offer the most likely source material for the bulk of our hydrocarbon reserves.

Petroleum is a complex mixture of hydrocarbons which is readily susceptible to physical and chemical changes, particularly to natural processes involving heat, pressure, filtration, catalysis, microbial action, adsorption, solution, and differential migration. The origin of crude oil has its links to plants which contains porphyrins and are probably derived from chlorophyll. Nitrogenous compounds retained in crude oil are also indicative of their links to living organisms. The crude oil is typically found within the pore spaces of sedimentary rocks with particular age markers (Hedberg, 1964). The detailed theory of petroleum generation has been given by A.E. Kontorovich and summarized by Neruchev (2009).

Diatoms, siliceous algae, are the most dominant and widespread group of eukaryotes on Earth and responsible for 20% of the carbon fixation through photosynthesis (Falkowski et al., 1998; Field et al., 1998). They are abundant in oceanic, coastal, and freshwater habitats and also occur in terrestrial environments, in soils, on rocks, and even in some plants (Round et al., 1990; Mann and Droop, 1996). Being major contributors to global carbon cycling, they collectively

produce 10 km^3 of net hydrated amorphous silica each year (Nelson et al., 1995), but only 1% of the living diatoms in the ocean contribute to the sediment below (Ramachandra et al., 2009).

Many workers have attributed diatoms as major contributors to crude oil deposits (Ehrenberg, 1839; Whitney, 1865, 1867; Anderson, 1926; Tolman, 1927; Hanna, 1928; Levorsen and Berry, 1967; Hunt, 1979, 1996; North, 1985; Mertz, 1989; Aoyagi and Omokawa, 1992; Holba et al., 1998a, b; Gelin et al., 1999; Breger, 2001; Johnson and Grimm, 2001; Gurgey, 2003; Krebs, 1999; Krebs et al., 2011), but the overall total contribution of diatoms to worldwide petroleum reserves has not been estimated (Ramachandra et al., 2009). Keeping in mind that approximately half of the crude oil deposits have been utilized (Guliyev et al., 2001; Fortman et al., 2008), we need to work out an overall guesstimate of total worldwide petroleum deposits continuing natural contributed by diatoms. This will give us a conception that whether we could be dependent on continuing natural crude oil deposits for our next generations or we have to go for biochemical engineering of diatoms to produce crude oil (Gordon, 2008; Ramachandra et al., 2009). In this chapter, we have made an attempt to identify the potential sources of crude oils worldwide based on diatom biostratigraphy and diatom biomarker studies.

The origin of diatoms is considered to be as early as the Early Jurassic as confirmed by findings of a few early diatom frustules (Sorhannus, 2007; Harwood et al., 2007). The relatively rapid evolutionary turnover in marine diatom assemblages occurred near the early Eocene–middle Eocene and Oligocene–Miocene boundaries and during the earliest Oligocene, the early middle Miocene, the latest Miocene, and the late Pliocene (Strelnikova, 1990, 1991; Barron, 1992, 2003; Baldauf, 1993). Diatoms were not significant in numbers until the Eocene (40–35 Ma) (Falkowski et al., 2004; Kooistra et al., 2007) and marine planktonic diatom diversity reached its peak at or immediately before the Eocene–Oligocene transition (Rabosky and Sorhannus, 2009). The burial of diatoms in the seabed over geologic time have uplifted onto the land and contribute diatomaceous earth worldwide which is significant for world's petroleum (Brzezinski, 2008).

2. Worldwide Diatomaceous Oozes/Diatomites: Crude Oil Potentiality

Seafloor sediments comprising of more than 30% diatoms are termed as diatomaceous ooze. Lithified or partially lithified diatomaceous ooze, when very strongly dominated by diatoms (>50%), are generally referred to as diatomites (Scherer et al., 2007). Three major belts of diatomaceous sediments are present in the modern oceans: (a) a southern belt circling the globe between 45° and 60°S latitude, (b) a northern belt within the Pacific (including the Sea of Okhotsk, Sea of Japan, and Bering Sea) and the Atlantic (developed somewhat in the Norwegian Sea),

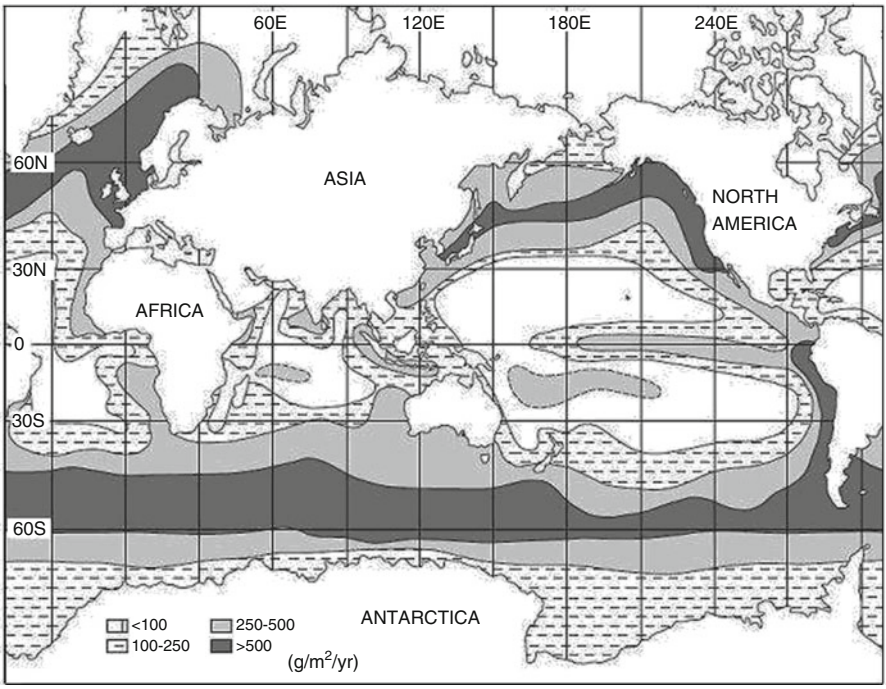

Figure 1. Global variation in the extraction of dissolved silica (g/m^2/year) by phytoplankton in near-surface ocean waters. This represents relative diatom production because diatoms are the dominant phytoplankton group in the oceans. The distribution of diatom production correlates well with belts of diatomaceous sediments on the seafloor (After Lisitzin, 1972). Concentrations along the continental margins and continental shelf of Antarctica are underestimated because sufficient data were unavailable for the initial compilation by Lisitzin (1972).

and (c) an equatorial belt which is well defined in the Pacific and Indian Oceans and less evident in the Atlantic Ocean (Lisitzin, 1972) (Fig. 1).

The southern belt is well developed in the Atlantic, Indian, and Pacific oceans immediately south of the Circum-Antarctic Current which is characterized by siliceous, clayey–siliceous, and siliceous–clayey diatomaceous oozes (Murdmaa, 1987).

The equatorial belt is largely represented by clayey–radiolarian and radiolarian–clayey sediments (Sval'nov, 1991). The northern belt of the dominant silica accumulation is traceable in the Pacific Ocean, while in the Atlantic, it is recognizable based on the bioproductivity and distribution of phytoplankton cells in the surface waters (Volkovinskii et al., 1972). Diagenesis of diatomaceous sediments may have a significant impact on oil reserves by creating fractured siliceous reservoirs, and the diatomaceous sediments may themselves be an important source for petroleum (Mertz, 1984).

Diatom biostratigraphy is used to identify the potential source rocks for petroleum deposits (Krebs, 1999; Krebs et al., 2011), but sometimes it is not feasible as silica cell walls of diatoms are prone to dissolution (Koning et al., 1997; Lončarić et al., 2007). Preservation of diatom frustules in sediments depends on rapid burial, lack of prolonged exposure to alkaline (pH > 7) pore waters, and absence of post-burial temperatures in excess of 35 °C (Calvert, 1974). The dissolution of diatom frustules results from longer exposure to seawater which is undersaturated with respect to silica. It is estimated that only 1–5% of the living diatom assemblage (biocoenosis) comprises the dead assemblage (thanatocoenosis) of seafloor sediments (Lisitzin, 1972), as weakly silicified diatom frustules as part of sidocoenose (sinking assemblages) are highly unlikely to form a part of the seafloor sediments; therefore, diatoms older than 90 Ma are rare (Sims et al., 2006; Harwood et al., 2007). Moreover, the dissolution of the diatom frustule continues even after its preservation due to exposure to bottom waters or interstitial pore water which is depleted in silica (Calvert, 1974). Therefore, most of the diatom deposits are weakly silicified. The presence of heavily silicified diatoms occurs when they have settled rapidly through the water column in aggregates or by incorporation into fecal pellets of zooplankton (Schrader, 1971); therefore, diatom biomarkers are being used extensively to identify the potential source rock for petroleum deposits.

Diatom biostratigraphy has been proven to be a vital tool to correlate siliceous rocks and oil basins with respect to the international geologic timescale (Barron, 1976, 1981; Barron and Keller, 1983). The late middle Eocene through Oligocene diatom biostratigraphy has been established for low latitude: Pacific, Atlantic, and Indian Oceans (Fenner, 1984, 1985; Barron, 1985a, b, c; Fenner and Mikkelsen, 1990; Baldauf and Iwai, 1995; Barron et al., 2004; Barron, 2005).

The North Pacific region is characterized by middle Miocene diatomaceous sediments which include mainly Japan, Kamchatka, Bering Sea, and the Gulf of Alaska. These diatomaceous sediments are due to the synchronous increase in upwelling around the North Pacific (Ingle, 1981). North Pacific diatom biostratigraphy through Miocene to Quaternary has been carried out by Schrader (1973), Koizumi (1985), Koizumi and Tanimura (1985), Barron (1985b, c, 1992, 2003), Fenner (1985), Radionova (1985, 1987, 1991), Akiba (1986), Akiba et al. (1993), Barron and Baldauf (1995), Barron and Gladenkov (1995), Gladenkov and Barron (1995), Gladenkov (1998, 1999, 2005, 2006), and Suto (2005, 2006).

The North Pacific region is represented by diatomaceous oozes and even diatomite sequences (*Thalassiothrix* oozes of the Sea of Japan) (Kazarina et al., 1989) and Miocene *Aulacoseira* diatomites (Pushkar and Cherepanova, 2001; Sancetta, 1982). In the North Atlantic, especially in the Norwegian/Greenland Sea, diatom biostratigraphic zonation details have been worked out by Schrader and Fenner (1976), Scherer and Koç (1996), and Koç and Scherer (1996). Southern Ocean diatom biostratigraphic zonations have been developed by Gombos and Ciesielski (1983), Fenner (1985), Harwood and Maruyama (1992), Gersonde and Bárcena (1998), Ramsay and Baldauf (1999), Censarek and Gersonde (2002),

Zielinski and Gersonde (2002), Olney et al. (2007), Scherer et al. (2007), and Cody et al. (2008). Scherer et al. (2007) have reviewed the application of diatoms in stratigraphic successions worldwide as also distinct biostratigraphic zonations from the tropical, North Atlantic, North Pacific, and Southern Oceans and a developing zonation from the Antarctic continental shelf.

The detailed biostratigraphic zonations thus provided by different workers would provide possible time periods of potential diatom contribution to petroliferous basins. California basin sediments are characterized by fractured silica (diagenesis) which suggests it as a possible site for crude oil (Emery and Rittenberg, 1952).

Eocene marine diatomaceous deposits are well known in Northern Europe, Scandinavia, Eurasia, Siberia, Alaska, New Zealand, Peru, and on the Atlantic and Pacific coasts of North America (Scherer et al., 2007).

3. Geographic Association of Diatomaceous Sediments with Petroleum

3.1. NORTH PACIFIC

The Pacific region is one of most important sources of petroleum due to the abundance of siliceous rocks (Khain and Polyakova, 2008). Major hydrocarbon accumulations were revealed in deepwater zones, at passive margins of the Atlantic, Indian, and Arctic Oceans. The Gulf of Guayaquil near coast of South America is an important petroleum potential which is characterized by a small zone within the subduction margin transformed into the transform zone, which is governed by the specific features of the structure of two pull-apart basins, namely, Talara and Progreso Basins. These basins are filled with Cretaceous and Tertiary rocks up to 9 km thick. Petroleum potential is mainly confined to the Upper Oligocene in Progreso Basin and Eocene in Talara Basin. Sediments are accumulated in the distal part of the delta and prodelta affected by upwelling and contains sandy channels which are in contact with clays. These are mainly dominated by diatom oozes (Khain and Polyakova, 2008).

The basic petroleum potential of the Pacific margin is related to back-arc seas and adjacent mainland where the continental margin, shelf, island arc, and slope oil and gas basins were formed. Most of the basins of the Cenozoic age were initiated on the Paleozoic–Mesozoic and Mesozoic–Lower Paleogene folded basement. The northern seas are characterized by their Eocene–Oligocene sequence of siliceous–clayey–tuffaceous rocks accumulated on the shelf and slopes of deepwater basins (Khain and Polyakova, 2008).

The Monterey Formation of California is a major source of petroleum due to its diatom-rich rocks (Bramlette, 1946; Ingle, 1981; Pisciotto and Garrison, 1981), as base of the Monterey Formation is marked by an increase in diatom-rich sediments (Barron, 1986). The abundance of diatoms in the formation is assigned

by major polar cooling during the middle Miocene (Shackleton and Kennett, 1975; Woodruff et al., 1981) which caused intensification of pole-to-equator thermal gradients and resultant increased upwelling (Isaacs, 1983, 1984). The Santa Barbara Channel area and Naples Beach of the Monterey Formation contain source rocks with high organic matter which is composed of clayey–siliceous, carbonaceous marl, and calcareous–siliceous members (Isaacs, 1983; Peters et al., 2008).

The Sea of Okhotsk incorporates the North Sakhalin oil and gas basins Cretaceous–Paleogene basement composed of turbidites (flysch), cherts, island-arc basalts, and ophiolite sheets. The basin located in the northeastern part of the island and the adjacent shelf includes Oligocene sequences with biogenic siliceous rocks, which overlie sediments of the Neogene Amur prodelta (Khain and Polyakova, 2008).

Clayey–siliceous and siliceous rocks are dominated by diatoms and are one of the major constituents of the oil and gas basins of the Pacific continental margin (Khain and Polyakova, 2008) which include Pilenga Formation (Sakhalin), the Kovacha Group (Kamchatka), Hit Formation (Gulf of Guayaquil), Pil Formation (Sakhalin), and the Temblor, Monterey, Etchigoin, and Sisqouc Formations (California). The siliceous type is mainly represented by shales with authigenic silica and organic matter, i.e., carbonaceous siliceous–clayey rocks. Their formation was significantly promoted by diatom plankton, which is actively produced in cool waters of the Northern Hemisphere and upwelling zones (Khain and Polyakova, 2008). The Pacific Margins and Atlantic Margins contain abundant siliceous rocks which govern specific features of the formation and accumulation of oil and gas (Khain and Polyakova, 2008).

The Akita Basin is an epicontinental back-arc basin in Japan which is one of the most important petroliferous basins (Sumii et al., 1990). This basin developed in the late Oligocene and formed the back-arc region of northeastern Japan during early middle Miocene. It comprises approximately 20 oil and gas deposits (Inaba et al., 2001). The Onnagawa Formation is mainly composed of siliceous, well-lithified shale and mudstone with significant felsic volcaniclastics and marls in which siliceous fossil diatoms and radiolaria are frequent. Their lithologies are mainly represented by marine fine-grained diatomaceous rocks of the Lower Pliocene and Miocene sections of northern Japan (Tada and Iijima, 1983). Diatom and planktonic foraminifera assemblages suggest that the Onnagawa Formation may be correlative to the middle part of the Monterey Formation in California, USA (Aoyagi and Iijima, 1987). Siliceous shales of the Onnagawa Formation are rich in organic matter and represent the principal source rocks in the Akita Basin (Taguchi, 1975; Aoyagi and Iijima, 1987; Hirai et al., 1990; Inaba et al., 2001).

Dogo Island, which is the largest of the Oki Islands in the Sea of Japan (Yamasaki, 1984), is characterized by diatomaceous sediments outcropping in the southern and western parts of the island (Ling and Kobayashi, 1992). The diatomites comprise the *Denticulopsis lauta* or *D. lauta-D. hustedtii* Zone of Koizumi (1973).

The Il'pinskii Peninsula of Kamchatka is represented by two upper formations, namely, Gailkhavilanvayam and Alugivayam. Diatom remains from the Alugivayam Formations were reported by Gladenkov and Gladenkov (2007) which contains Oligocene marine diatom assemblages of moderate to poor preservation, and low abundance and diatom productivity increased in the sea basin after the Eocene–Oligocene transition (Gladenkov, 2009). Diatom fossils have been reported from late Oligocene sedimentary rocks in the Navarin Basin Province, Bering Sea (Baldauf and Barron, 1987).

3.2. CASPIAN SEA

The Caspian Sea region is known for its abundant oil reserves. The South Caspian Basin Province occupies the southern part of the Caspian Sea and adjacent narrow strips of land, of which approximately 45% lie in Azerbaijan, 35% in Turkmenistan, and 20% in Iran. The South Caspian Basin is unique in several respects due to its high sedimentation rate (~4.5 km/m.y.), 5 km of Pliocene sedimentary deposits, relatively low geothermal gradients (1.5 °C/100 m), and abnormally high pressure in the central and southeastern parts of the basin (Abrams, 1996; Tagiyev et al., 1997). Sedimentary strata in the South Caspian Basin are ~20 km in each of the deposits: Azerbaijan offshore, western Turkmenistan onshore and offshore, and Iran offshore. The primary petroleum system of the South Caspian Basin is the Oligocene–Miocene Maykop/Diatom Total Petroleum System (Bagir-Zade et al., 1987; Klett et al., 1997; Smith-Rouch, 2006) which includes the entire South Caspian Basin as well as some adjacent areas, covering a total of some 189,000 km^2. To date, some 105 oil fields have been discovered in the South Caspian Basin, of which 65 are in Azerbaijan, 35 in western Turkmenistan, and the remainder in Iran (Narimanov and Palaz, 1995). The main source rocks in the basin are considered to be Paleogene–Miocene deposits (Bailey et al., 1996; Abrams and Narimanov, 1997; Katz et al., 2000; Huseynov, 2000; Guliyev et al., 2001), but hydrocarbon reservoirs show a wide stratigraphic range, according to which the majority of oil–gas deposits (about 90%) are concentrated in the Lower Pliocene Productive Series with total production (Huseynov et al., 2004). These rocks are overlain by deepwater organic-rich shales and limestones of the Miocene Diatom Formation. There are two source rock units: deepwater black shales of the Oligocene–Lower Miocene Maykop Series and deepwater, anoxic shales of the middle Miocene Diatom Formation. The Diatom Formation directly overlies the Maykop Series, and both units together are generally viewed as a single source rock. However, as revealed by recent geochemical data obtained by western companies, oils generated from each of the stratigraphic units contain a specific set of biomarkers. Both source rocks contain 2% or more total organic carbon (TOC) and mixed Types I and II kerogen (Pigott et al., 1996; Abrams and Narimanov, 1997). The Derbent Basin of the intercontinental Caspian Sea is an important site for petroleum reserves

due to the presence of diatom frustules as also accumulation of diatomaceous oozes (Sval'nov and Kazarina, 2008).

3.3. EASTERN EUROPE

The Polish Flysch Carpathians is one of the largest petroleum provinces of central Europe which contains Cretaceous to Early Miocene strata. The frontal part of Flysch Belt of the Polish Carpathians constitutes one of the oldest petroleum-producing regions in the world (Curtis et al., 2004) in which menilite shales are considered to be the primary source rock (Kruge et al., 1996; Köster et al., 1998a, b). The crude oil samples of menilite source rocks of the Polish Carpathians are characterized by several organic facies within the subbasins like Skole basin, Silesian basin, and Dukla basin (Curtis et al., 2004). These subbasins have high-sulfur organic facies in the western part of Skole which are dominated by diatomaceous shale, diatomite, and chert (Kotlarczyk and Leśniak, 1990; Kuśmierek, 1996). The lower part of the Silesian Unit is dominated by siliciclastic facies similar to Skole basin (Köster et al., 1998a), and the lower part of the Dukla Unit is characterized by siliceous cements and cherts (Ślączka, 1971).

3.4. NORTH SEA

In the North Sea, diatoms are one of the most important microfossil groups used for biostratigraphic correlation to identify the potential source rocks for petroleum. These are found in a Tertiary sequence where the mineral-walled microfossil group of foraminifera and calcareous nanoplankton are absent (Bidgood et al., 1999). The offshore and onshore sediment in the North Sea Basin, *Fenestrella antique,* is suggested to be a marker species for the base of the Eocene (Mitlehner, 1996). North Sea stratigraphy based on diatom assemblages for several fields such as Forth/Harding, Gryphon, and Sedgwick/West Brae has been established (King, 1983; Mudge and Copestake, 1992a, b; Jones et al., 2003; Ahmali et al., 2003). Upper Oligocene–Lower Miocene and Upper Paleocene–Lower Eocene diatom assemblages, as also diatoms from Sele and Balder Formations throughout the North Sea, from west of Shetland to the onshore (Belgium), are useful for correlation (Van Eetvelde et al., 2004).

3.5. TASMANIA

The continental crust off Tasmania is characterized by a pre-Oligocene section consisting largely of shallow marine organic-rich mudstone in which a siliciclastic sequence (abundant diatoms) formed as Tasmania rifted from the surrounding parts of Gondwana. The West Tasmania and South Tasman Rise appear to be

moderately prospective for petroleum as these sites are characterized by organic carbon and bitumen at maturity level, which, therefore, reaches to the petroleum window (Exon et al., 2001). These rocks are overlain by deepwater organic-rich shales and limestones of the Miocene Diatom Formation. There are two source rock units present: deepwater black shales of the Oligocene–Lower Miocene Maykop Series and deepwater anoxic shales of the middle Miocene Diatom Formation. The Diatom Formation directly overlies the Maykop Series and both units together are generally viewed as a single source rock. However, as revealed by recent geochemical data obtained by western companies, oils generated from each of the stratigraphic units contain a specific set of biomarkers. Both source rocks contain 2% or more TOC and mixed Types I and II kerogen (Pigott et al., 1996; Abrams and Narimanov, 1997).

3.6. ASIA

Middle Miocene sediment of the Oman margin (western Arabian Sea) contains diatomaceous silty clay (Debrabant et al., 1991). The Central Indian Basin is characterized by ferromanganese nodules which are buried in siliceous oozes (Pattan and Parthiban 2007). The productivity of the Central Indian Basin during the last 400 kyrs was thought to be increased by siliceous organisms (Pattan et al., 2005). Although, much has not been spoken of diatoms from this area, Banerjee et al. (1999) have reported an abundance of diatoms and radiolarian from east of the Chagos Archipelago, Central Indian Basin, and Indian Ocean. The Barmer Basin of Rajasthan is a potential site for petroleum in India. The Randha Formation of Barmer Basin is characterized by pre-rift sediments (Late Proterozoic) containing siliceous facies shales along with limestone, phosphorites, and dolo-mudstone. Moreover, the Fatehgarh Formation of Fatehgarh is also overlain by siliceous earth of the Bariyara member (Bhowmick, 2008 and references therein). The east coast of India is characterized by several deepwater sedimentary basins which are thought to be typically associated with thick Neogene sediments after separation of India from Gondwana and finally from Antarctica, therefore, characterized by carbonates to siliciclastic rocks and acting as potential sources of crude oil (Bastia, 2006). Indian petroliferous basins have been demarcated largely on the basis of palynomorphs, ostracode zonation (Bhandari et al., 2001), and also foraminiferal zonation. Published reports on diatoms are not available.

4. Diatom Biomarkers: Crude Oil Potential Sites

The dead assemblage of plants and animals during their live stage produce specific molecules which also get preserved in the sediments along with the organism and is generally used to infer their source, called as molecular biomarkers

(Turner, 2002), which are generally useful in petroleum industry to identify the source of crude oil (Moldowan et al., 1985; Curiale, 2007). A.E. Kontorovich pioneered the studies of biomarkers in the organic matter of rocks and petroleum in Siberia, which allowed definite conclusions on the organic origin of petroleum and helped in determining particular petroleum-producing sediments serving as a source for generation of petroleum deposits (Kontorovich et al., 1991, 1994, 1995, 2000; Kashirtsev et al., 1999; Kontorovich, 2004a, b; Kashirtsev and Kontorovich, 2006a, b). Organic biomarkers are the diagenetically altered remains of the products of cellular biosynthesis and may be pertinently termed as molecular fossils. Biomarkers provide a way to determine both parameters: identifying the source rocks from which hydrocarbon accumulations originated and thermal history of source rocks through complementary analyses of sedimentary bitumen in source rocks and their derived oil accumulations. Most biomarkers are derived from lipids and are potentially stable over billion-year timescales under ideal conditions (Brocks and Summons, 2004). Sterols are important membrane lipids, found in all eukaryotic organisms. These are used for reconstruction of the presence and abundance of algal groups (Volkman, 1986) as also for organic geochemical studies for identifying sources of organic matter due to their large variety and diagenetic stability (Volkman, 1986; Volkman et al., 1998). The crude oils and source rock extracts are complex mixtures of organic compounds in which *n*-alkanes are present in a large proportion of the weight of the majority of oils (Hunt, 1979; Tissot and Welte, 1984). The distribution of these biomarkers like steranes, terpanes, isoprenoids and porphyrins are widely used in petroleum exploration programs (Mackenzie, 1984).

Diatoms are one of the most important sources of sterols in the marine environment which are represented by C_{27}–C_{29} sterols in which C_{28} sterols are in abundance (Rampen et al., 2010). A detailed description of secular increase of C_{28} and C_{29} sterols has been described for petroleum systems related with diatoms through the Phanerozoic (Katz et al., 2004). 24-Methylcholesta-5,22E-dien-3β-ol and 24-methylcholesta-5,24(28)-dien-3β-ol are often present as major sterols in diatoms (Orcutt and Patterson, 1975; Gladu et al., 1991; Barrett et al., 1995), whereas cholest-5-en-3β-ol, 24-ethylcholest-5-en-3β-ol and cholesta-5,22E-dien-3β-ol are also frequently present (Volkman, 2003).

Dinosterols and other 4-methyl sterols have also been reported in diatoms (Volkman et al., 1993 and references therein). The other major class of diatom-specific biomarkers is the highly branched isoprenoids (HBI) which are widely used for petroleum exploration due to fact that it is originated 91.5 Ma ago (Sinninghe Damsté et al., 2004) (Fig. 2). 24-Methylcholesta-5,22E-dien-3β-ol is a typical diatom biomarker, and the occurrence of 24-norsterol and its derivatives is also associated with diatoms (Patterson, 1987; Holba et al., 1998b). Petroleum exploration industries have developed and maintained databases of bitumen and oil composition for crude oil samples from petroliferous basins on the globe (GeoMark Research Ltd., www.geomarkresearch.com) and analyzed biomarkers: *n*-alkanes, acyclic isoprenoids, steroids, and triterpenoids.

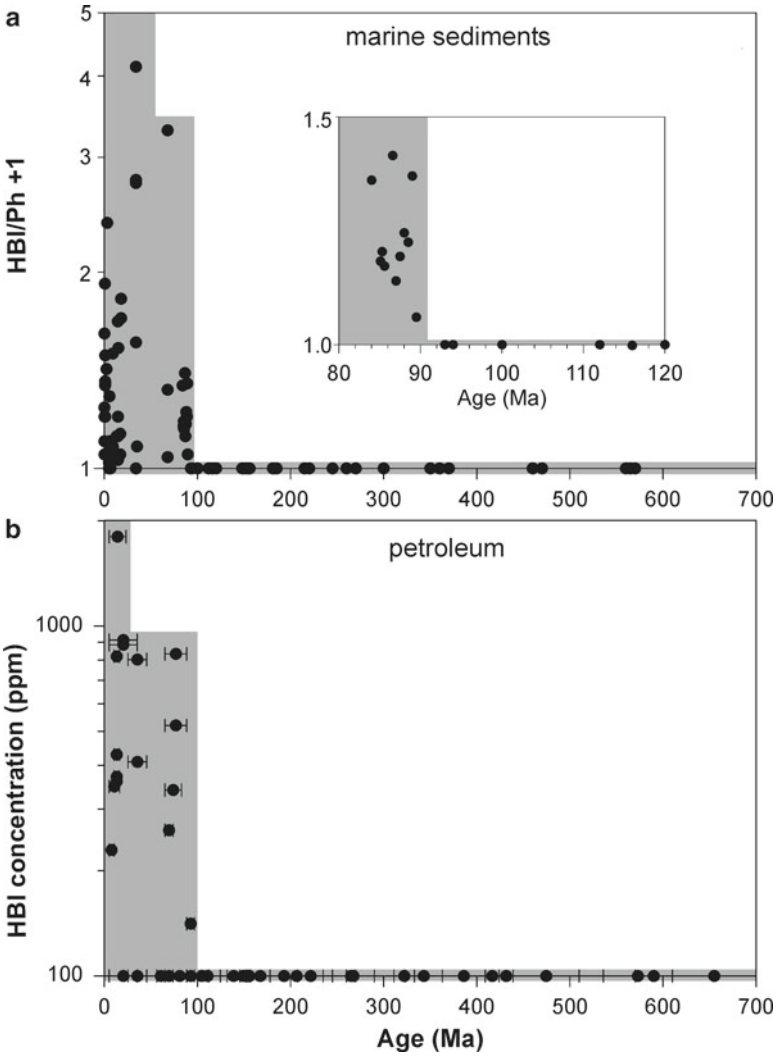

Figure 2. (**a**) The occurrence of the C_{25} HBI skeleton in marine sediments (**b**) and petroleum through geological time based on the analysis of over 400 sediment and 81 petroleum samples. Both plots clearly show that the HBI biosynthetic pathway did not exist before ca. 90 Ma. (*Inset*) shows the more exact timing of this event (After Sinninghe Damsté et al., 2004, reprinted with permission from AAAS).

24-norcholestane, a biomarker of algal origin (Moldowan et al., 1991), is widely used for an age-diagnostic identification of the source rock of petroleum (Holba et al., 1998a, b). These compounds show stepwise increase in relative concentrations in sediments and petroleum from the Jurassic, the Cretaceous, and the Oligocene–Miocene, respectively (Rampen et al., 2007a). The middle to late

Miocene Formations of the north circum-Pacific region is characterized by abundant biogenic silica, Type IIS kerogen, abundant organic sulfur compounds, and diatom-related 24-norcholestane (Suzuki et al., 1993, 1995). 24-Norcholestane is abundant in Onnagawa siliceous sediments of northeast Japan (Suzuki et al., 1993), Miocene Monterey siliceous sediments which are source rocks for Monterey crudes (Curiale et al., 1985; Orr, 1986) and also in its crude oil (Moldowan et al., 1991). The middle to late Miocene Onnagawa Formation of the Akita Basin is a potential site for crude oil (Taguchi, 1975; Tada and Iijima, 1983; Aoyagi and Iijima, 1987; Tada, 1994) and is mainly composed of siliceous mudstone in which diatoms dominate (Tada and Iijima, 1983). The low latitude petroleum contains low concentration of 24-norcholestane, whereas its concentration is high from higher latitudes (Holba et al., 1998a, b), which is substantiated by the presence of a high amount of 24-norsterols in the diatom *Thalassiosira aff. antarctica* (Rampen et al., 2007a). The presence of a high ratio of 24-norcholestane during the Tertiary (Rampen et al., 2007a) is corroborated with the expansion of diatom diversity during the Eocene–Oligocene boundary (ca. 34 Ma) (Tappan and Loeblich, 1973). C_{26} sterols have been reported in a diatom field population of *Thalassionema nitzschioides* (Ballantine et al., 1979) and in sea-ice diatom communities (Nichols et al., 1990). 24-Norsterols have been reported in waters and sediments where *T. antarctica* is abundant, such as the Bransfield Strait, Antarctica (Brault and Simoneit, 1988).

Specific organic compounds, highly branched isoprenoid (HBI) alkenes produced only by diatoms, are useful indicators for petroleum deposits (Sinninghe Damsté et al., 2004). The diatom genera *Rhizosolenia, Haslea, Navicula, Pleurosigma*, and their ancestors are thought to be the major sources of HBI alkanes and HBI thiophenes (Volkman et al., 1994; Belt et al., 1996, 2000a, b, 2001, 2002; Sinninghe Damsté et al., 1999, 2004) (Fig. 3). These biomarkers are present in sediments after evolution of these diatoms; therefore, the occurrence of such compounds in sediments and especially in petroleum can be used to determine their maximum age. The oldest HBI-biosynthesizing diatom, rhizosolenid diatoms evolved ca. 91.5 Ma; thus, sediments or petroleum containing HBIs have a maximum age of 91.5 Ma (Sinninghe Damsté et al., 2004) (Fig. 2). The fossil genus *Rhizosolenia* has been reported globally in the early Oligocene (Baldauf, 1992). The pronounced change in the concentration of HBI biomarkers in the northwest Pacific region strongly suggests that *Rhizosolenia* and related fossil genera prevailed in the diatom community after the E/O transition, making it a potential site for petroleum deposits (Shiine et al., 2008). HBI alkenes have also been reported from coastal marine sediments from the Peru upwelling region (Volkman et al., 1983) and the Eastern North Pacific Ocean (Matsueda and Handa, 1986). C_{25} HBI alkenes were reported from benthic (Belt et al., 2000a, b) as well as planktonic (Belt et al., 2001) species of *Pleurosigma* genus. HBI alkenes are prone to sulfurization during sedimentation or early diagenesis (Kohnen et al., 1990; Sinninghe Damsté et al., 2006). Sulfurized C_{25} HBIs can yield C_{25} HBI alkane through desulfurization during diagenesis (Katsumata and Shimoyama, 2001).

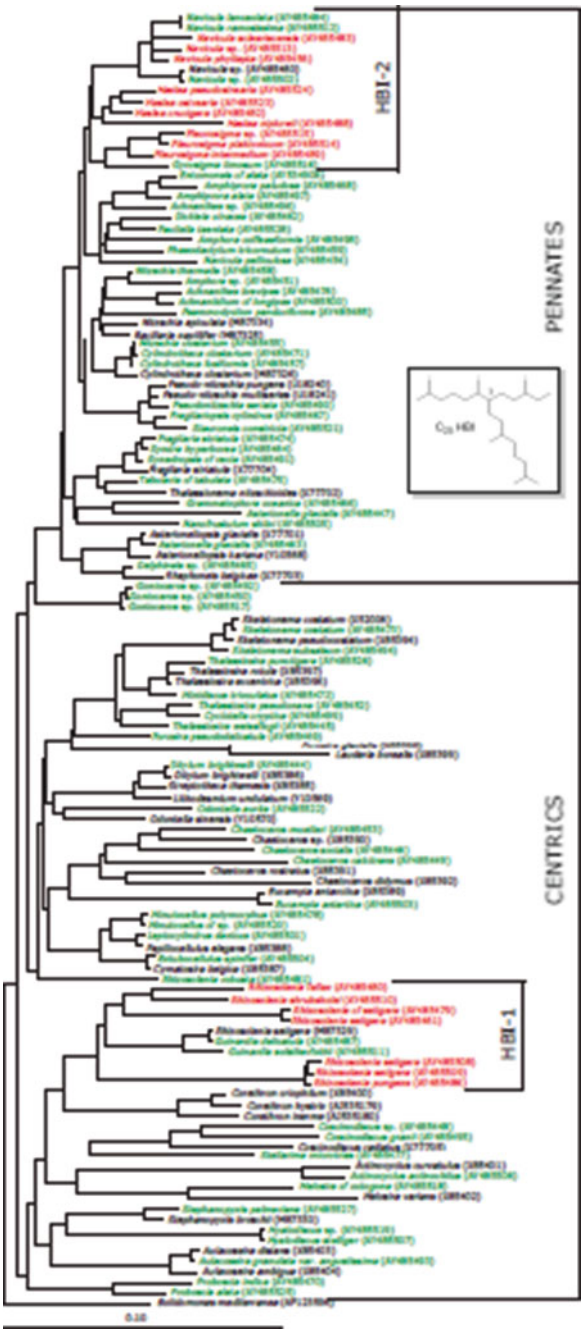

Figure 3. HBI-biosynthesizing strains are indicated in *red*. Diatoms in *green* were tested but did not contain HBI alkenes; diatoms in *black* were not tested for the presence of HBI alkenes (After Sinninghe Damsté et al., 2004, reprinted with permission from AAAS).

The incorporation of sulfur into HBI alkenes to form HBI thiophenes proceeds rapidly under reducing H_2S-rich conditions (Sinninghe Damsté et al., 2006). The Il'pinskii Peninsula of Kamchatka, Russia, is characterized by the highest HBI alkane and HBI thiophene concentrations, indicating a large contribution from diatom-derived organic matter in the sedimentary basin (Shiine et al., 2008). In the Southern Ocean, the diatom *Rhizosolenia oligocaenica* is a Lower Oligocene index fossil (Baldauf and Barron, 1991; Harwood and Maruyama, 1992).

Two diatoms of the genus *Proboscia* are thought to biosynthesize C_{28} and C_{30} 1,14-diols and C27 and C29 12-hydroxy methyl alkanoates (Sinninghe Damsté et al., 2003). *Proboscia* diatoms are the major source of long-chain 1,14-diols and 12-hydroxy methyl alkanoates in nature as these diatoms have been recognized in a wide variety of settings and are often found as quantitatively important members of the phytoplankton in upwelling regions (Sinninghe Damsté et al., 2003). Because of the abundance of *Proboscia* diatoms in upwelling regions, it was suggested (Sinninghe Damsté et al., 2003) that long-chain 1,14-diols and 12-hydroxy methyl alkanoates in sediment cores could be used to indicate high nutrient conditions in the past as well as possible sites of petroleum.

Proboscia alata is a dominant diatom species in the Arabian Sea during the early upwelling season (Smith, 2001), and sediment trap studies in the area (Prahl et al., 2000; Wakeham et al., 2002; Sinninghe Damsté et al., 2003) have shown strong seasonality in the fluxes of long-chain diols and their inferred oxidation products, ketols (Ferreira et al., 2001; Sinninghe Damsté et al., 2003), and mid-chain hydroxy methyl alkanoates.

Long-chain diols and mid-chain hydroxy methyl alkanoates have frequently been reported in Quaternary sediments (Versteegh et al., 1997), and the diatoms genus *Proboscia* is the source of these lipids (Sinninghe Damsté et al., 2003; Rampen et al., 2007b). *Proboscia* species have been recognized in a wide variety of settings ranging from subarctic to tropical environments (Jordan et al., 1991; Hernández-Becerril, 1995; Sunesen and Sar, 2007). Long-chain 1,14-diols and 12-hydroxy methyl alkanoates have been reported from low to high latitudes (Versteegh et al., 1997, 2000; Wakeham et al., 2002; Sinninghe Damsté et al., 2003) which are mainly present in areas of seasonal upwelling such as the Arabian Sea (Rampen et al., 2008, 2009a).

The northwest Indian Ocean is characterized by high upwelling as reported by upwelling indicator diatoms (Koning et al., 2001) and also the presence of *Proboscia* diatom species and 1,14-diols and 12-hydroxy methyl alkanoates (Rampen et al. 2009b).

5. Summary

It is very difficult to accurately assess the quantity of available crude oil under the earth's surface or to have its future predictability (British Petroleum Report, 2010). Here, the plausible potentiality of diatoms in crude oil formation and the quest to guess estimate the fraction of total oil reserves due to diatoms are addressed.

Comparing the global diatomaceous belt (Lisitzin, 1972) and proved oil reserves till 2009 (British Petroleum Report, 2010), it is hypothesized that a large fraction of the crude oil reserves are due to diatoms, but specifically it is still debatable because so far most of the land parts and coastal areas of the world are only explored for oil reserves, and a huge area including the deep sea is still to be explored.

Diatoms collectively produce 10 km^3 of net hydrated amorphous silica each year (Nelson et al., 1995). In comparison, the USA consumption of crude oil is 1.64 m^3/person/year (Gordon et al., 2010). If all 6.9×10^9 people in the world consumed oil at this rate, the total volume needed would be 11 km^3/year. Comparing the diatom sedimentation with possible oil reserves (explored and to be explored), it may be assumed that diatoms ideally contribute to ~90% of oil, however, sedimentation rate of diatoms in the world ocean has varied through the geological timescale due to specific environmental conditions. Therefore, diatom sedimentation can significantly be used for identifying oil reserves with solving the issues of crude oil maturiy.

The quest for petroleum and the changed energy scenario world over led to the intensification of research for petroliferous basins as also the alternative methods for oil generation. Organic matter is believed to be one of the key contributors to the petroleum reserves in the geological past, and the phytoplankton community has played its role. Diatoms, which form an important part, evolved during the Early Jurassic with a rapid evolutionary turnover during the Eocene–Oligocene–Miocene boundaries and also thereafter. This led to their proliferation in the marine realm and led to their contribution to the world petroleum reserves. Many oil basins of the world have been attributed to diatomaceous deposits. Diatom biostratigraphy paved the way for furtherance of research in petroliferous basins with key stratigraphic markers being used for delineation of key zonations. Key locales of petroliferous basins worldwide have been reviewed in the context of the diatoms with detailed reference to the Pacific region. A lot has been researched into the specific diatom biomarkers to locate potential sites for crude oil. An extensive review has been carried out for different regions, with key contributor diatom species discussed.

6. Acknowledgements

We are indebted to editors Prof. Richard Gordon and Prof. Joseph Seckbach for giving us an excellent opportunity to contribute to this chapter. Thanks to the secretary of the Ministry of Earth Sciences and the director of NCAOR for continued encouragement. Special thanks to Dr. John A. Barron, US Geological Survey, and Prof. Noriyuki Suzuki, Hokkaido University, Japan, for providing imperative suggestions and literature for this chapter. Thanks are also extended to Karima Kerkar for her kind help in drafting this manuscript. SKS acknowledges financial support from the University Grants Commission (Dr. D.S. Kothari Postdoctoral Fellowship Scheme). This is NCAOR Contribution No. 31/2012.

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R. Gordon and J. Seckbach (eds.), *The Science of Algal Fuels*,
Cellular Origin, Life in Extreme Habitats and Astrobiology, 383–398
DOI 10.1007/978-94-007-5110-1_21, © Springer Science+Business Media Dordrecht 2012

PHOTOBIOLOGY AND LIPID METABOLISM IN ALGAE

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1. Introduction

On a global scale, roughly half of the annual carbon fixation takes place in the ocean, and in aquatic environments, microalgae is the dominating contributor to primary production. Although the carbon fixation is approximately the same, the standing stock of organic material is much lower in the ocean compared with terrestrial systems. The reason for this is that algae do not build structural components such as wood. This difference is also seen in the turnover time, which, in general, is much faster in the ocean compared with terrestrial systems, and the maximum growth rate of most single cell algae exceeds that of terrestrial plants by far.

The fastest growing algae can under favorable conditions go through several doublings per day. Some of the fast-growing algae also have the ability to build up high lipid reserves (Rai, 1995), which easily can be converted to biodiesel, and the potential of utilizing algae as raw material for biofuel has received a lot of attention dating back to the 1970s (Sheehan et al., 1998). Several reviews have been published recently on the topic of algal lipids (Guschina and Harwood, 2006; Guschina and Harwood, 2009; Harwood and Guschina, 2009), the potential of algal lipids as a raw material for biofuel (Chisti, 2007; Huntley and Redalje, 2007; Hu et al., 2008; Li et al., 2008; Wijffels and Barbosa, 2010), and addressed the question how this would be more sustainable than using traditional energy crop for biofuel production (Smith et al., 2010).

Lipids are a diverse group of compounds. By its simplest definition, lipids are fatty acids and their derivatives such as esters and amides. Lipids play several roles in the physiology of algae, and the two main classes of lipids are polar and neutral lipids (also referred to as nonpolar lipids). Generally, structural lipids found in membranes are polar lipids, whereas storage lipids are neutral lipids.

2. Photosynthesis

The conversion of energy from light to organic material is the key to understanding the metabolism of photosynthesizing organisms such as algae. The light energy fuels the photosynthetic machinery, which produces the chemical energy (ATP) and

reducing power (NADPH) required in the subsequent reactions of carbon fixation and other metabolic processes (Falkowski and Raven, 2007). ATP and NADPH can also be produced by respiration, but the organic material consumed in respiration has primarily been synthesized through photosynthesis. Consequently, all the energy needed for running the metabolism in algae is either directly or indirectly originating from light, driving the electron transport chain in the photosynthetic machinery of the cell (with some exceptions such as mixotrophic algae that can use organic carbon compounds from other sources).

Total solar irradiance in the top of the atmosphere is 1,373 $W m^{-2}$. As the light is transmitted through the earth's atmosphere, this irradiance is reduced by scattering and absorption by water vapor, oxygen, ozone, CO_2, dust, and clouds. This reduction is ranging from approximately 14% in a clean, dry atmosphere to ~90% during a thick cloud cover (Kirk, 1994). Furthermore, the sun elevation greatly affects the solar irradiance at sea level. For a hypothetical clear sky, the seasonal and latitudinal variations in photosynthetically available radiation (PAR) can be modeled accurately (e.g., Kirk, 1994; Williams and Laurens, 2010).

The first step in photosynthesis is light absorption, and photosynthetic pigments in algae and plants have evolved to harvest sunlight efficiently (Kiang et al., 2007). Various pigments of the light-harvesting antenna are complementary in harvesting different wavelengths and are characterized by different absorption spectra. However, they are not able to use the full spectrum of solar irradiation, that is, global irradiance, but only wavelength region from 400 (or 350) to 700 nm, which is termed PAR. The energy of PAR region is approximately 50% of the global irradiance. After absorption, the energy derived from light quanta is transferred rapidly toward the chlorophyll *a* (Chl *a*) molecules in the reaction centers of the two photosystems (Clegg, 2006). These are the sites where the primary photochemical reaction takes place. In the standard light reaction, excitation energy is transferred to photosystem II (PS II) reaction center, to photosystem I (PS I) reaction center, and ends up reducing NADP to NADPH. This linear system is called the *Z*-scheme (Fig. 1).

Absorbed light energy needs to reach the reaction centers in order to contribute to the photochemistry. However, there are loss processes both at the light absorption and the energy transfer steps that affect the energetic efficiency of photosynthesis. For example, some pigments have a photoprotective role acting as sunscreens, preventing damage caused by exposure to too high light energy levels. These pigments dissipate safely the excess energy as heat (Demmig-Adams and Adams, 2006). The amount of energy lost this way may vary from 0% to close to 100% of the absorbed energy, depending on the level and duration of irradiance and on the physiological state of the cells. The light absorbed by the photosynthetic antenna pigments is channeled toward the reaction centers, but the energy transfer is sometimes <100%. At times, the excitation energy cannot be utilized, for example, when all available reaction centers are closed, which may be caused for several reasons. Additionally, when the energy is transferred to the reaction centers, the distribution of excitation energy between PSI and PSII may

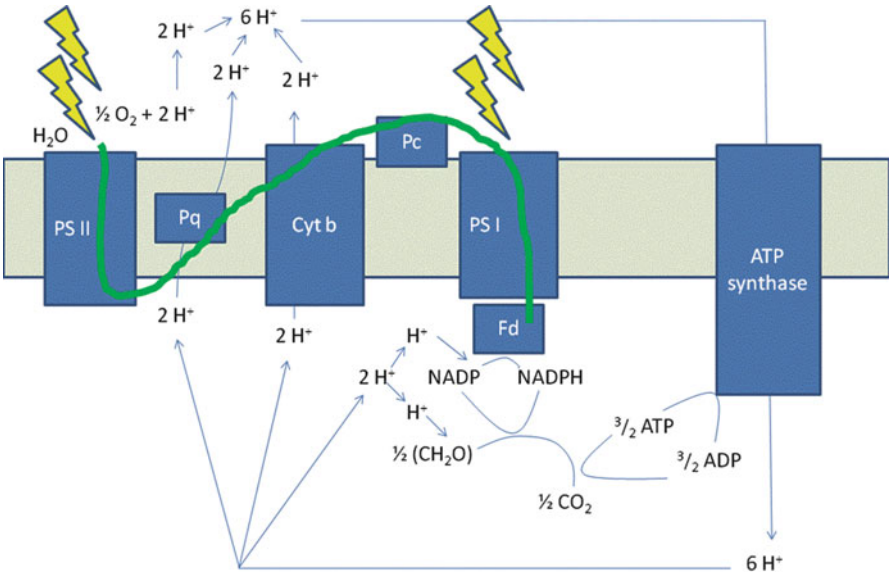

Figure 1. A simplified scheme showing the electron and proton transport across the thylakoid membrane in a classical *Z*-scheme, that is, the two photosystems (PSI and PSII) are connected sequentially. Protons are pumped into the thylakoid lumen and used for generating ATP through the ATP synthase complex. PSI faces the stroma side and provides the reducing power in the form of NADPH through reduced ferredoxin (*Fd*) (More details of the overall process can be found in, for example Falkowski and Raven, 2007).

be sub-optimal. Theoretical minimum quantum requirement for evolution of one O_2 molecule is 8. For the reasons described above, the actual measured requirements are much higher. The lowest quantum requirement obtained in low light is 9–10 mol quanta/mol O_2, and this may be considered the best obtainable efficiency in practice. Such a deviation from the minimum theoretical requirement yields at least 10% reduction in energy efficiency. In high light conditions, or during environmental stress, this value is much higher (e.g., Babin et al., 1996).

There is an additional energy loss because the primary photochemical reactions utilize red photons only. The energy of a light quantum is inversely proportional to its wavelength; blue photons have more energy than red ones. This loss fraction corresponds to the difference in energy between quanta absorbed and that of the lowest excited state of the Chl *a* in the reaction centers (680 nm for PSII and 700 nm for PSI). When calculated for natural sunlight at sea level, and assuming that all light is absorbed by photosynthetic pigments, this loss in energy is roughly 20%. Another loss process is respiration, some of the organic material produced through photosynthesis is used to run the cell metabolism, and subsequent respiration losses are typically 10–20%.

During photochemistry in the light reaction of photosynthesis, electrons originating from water are used for producing ATP and NADPH through the

Table 1. Loss factors affecting energy conversion from sunlight to organic matter by algae.

Loss factors	Reduction in energy (%)
Fraction of sunlight not utilized by algae; whole spectra → PAR[a]	50
Reduction of energy in electron transport chain; PAR → 680/700 nm	20
Reduction of energy due to inefficient light harvesting	10–100
Reduction of energy while converting energy of quanta to chemical energy, carbohydrates	66
Reduction of energy while converting energy of quanta to chemical energy, lipids	72
Respiration losses	10–50
Total, when carbohydrates are the end product[b]	89
Total, when lipids are the end product[b]	91

[a]Photosynthetic active radiation (PAR).
[b]Assuming minimal losses in all processes.

Z-scheme (Fig. 1). During this process, H^+ ions are pumped through the thylakoid membrane, and the ATP is generated as H^+ returns through the ATP synthase machinery. At maximum efficiency, $3H^+$ is pumped across the membrane per excited electron, and $4H^+$ is required per ATP. It is a flexible system, additional ATP can, for example, also be produced by PSI through cyclic electron flow. The NADPH and ATP generated in the light reaction are used in the Calvin cycle to fix carbon. In order to reduce and fix one CO_2 into its sugar equivalent, two NADPHs and three ATPs are consumed. The final product of the Calvin cycle is triose phosphate (3C atoms), which is subsequently combined to form different sugars: different hexoses (6C atoms) or sucrose (12C atoms). Sugars are affecting the osmotic potential of the cell organelle, and the cells need to store carbon in another form to avoid problems with osmoregulation. The most common storage products are lipids and starch, and the preferred storage product is species specific (Rai, 1995). Carbon in these forms can be stored in much higher concentrations than as sugars.

The maximum theoretical energetic yield of photosynthesis is approximately 34%, when calculated using energies of red light quanta, hexose as the end product, and applying maximal conversion efficiencies without any losses. When evident losses of using natural sunlight, losses in photochemical reactions, and requirements of basic cell metabolism are included, the attainable efficiency drops to ~11%, as calculated in Table 1 and also by several other studies (e.g., Melis, 2009; Williams and Laurens, 2010; Weyer et al., 2010; Zemke et al., 2010). This presents an upper limit for conversion of sunlight to biomass using photosynthesis. For the areal-based production of algal biomass, this approximates 70–100 g dry weight m^{-2} day^{-1} using highest available irradiance levels. The obtained values during algal cultivation are generally 5–20 times lower than this (e.g., Sheehan et al., 1998; Williams and Laurens, 2010; Zemke et al., 2010).

For many of the loss processes, little to nothing can be done to increase efficiency. The main loss process that can be reduced is the release of light

energy as heat at high light intensities. The highest photosynthetic efficiency is obtained in the relatively low light region where there is a linear relationship between irradiance and production. As irradiance increases, an increasing proportion of the light energy is dissipated as heat, and to some extent as fluorescence. Much of this loss is due to photoprotective pigments. At high irradiance, the only way to reduce the loss to heat and fluorescence is to have an effective light dilution, meaning that the light energy harvested by individual cells is reduced to a level where maximum efficiency is obtained. This may reduce the production per cell, but light can be distributed to more cells, increasing the total production. Several approaches have been taken for obtaining effective light dilution, from efforts to reducing the individual light-harvesting antennae to different designs of cultivation units (e.g., Sheehan et al., 1998; Zijffers et al., 2008). Developing a way to utilize full sunlight at maximum efficiency will be critical for developing algae as raw material for bulk chemicals and biofuel (Norsker et al., 2011).

3. From Light to Lipid: The Photon Cost of Lipid Synthesis

Algae, and plants in general, have a very limited ability to move to different locations when environmental conditions change. Instead, the metabolic pathways inside the cells have evolved to be very flexible. For example, a simple shift from light to dark requires the cell to be able to run required metabolic processes without photosynthetic products. When light returns, however, the photosynthetic machinery provides another engine to run metabolism through the production of chemical energy (ATP) and reducing power (NADPH). The ATP and NADPH originating from the light reaction are also used for other processes than to produce sugar, for example, to reducing nitrogen and sulfur, and in the synthesis of fatty acids.

As a result of the metabolic flexibility, there is often more than one pathway for synthesizing different compounds and often more than one enzyme that can do the job. This complicates things when trying to understand the specific pathways in a given process, for example, lipid synthesis. Fatty acid synthesis is additionally not a stand-alone process, but several processes need to run in parallel for the cell to function properly. Below we have made an assessment of the theoretical photon cost of lipid production without any loss processes.

CO_2 is one of the most oxidized forms of carbon found in nature, whereas a saturated fatty acid is a highly reduced form of carbon, and the transformation of C from CO_2 into fatty acids requires much energy and reducing power. In order to make one of the most common fatty acids, palmitic acid (16:0), 16C atoms are needed. However, as we will describe below, some carbon will be lost as CO_2 during this process, and in order to make one chain of palmitic acid, 24C atoms are needed in the form of sugar (two sucrose) at the start of the process. The minimum photon cost per C atom is as mentioned 8: 2 photons per NADPH

and 4/3 photons per ATP (Fig. 1). Thus, the total budget for producing the two sucrose molecules, needed for making one chain of palmitic acid, is a minimum of 192 photons (72 ATP and 48 NADPH).

Acetyl CoA is a central starting material in the lipid synthesis (it is central also in many other cellular processes, e.g., feeding into the citric acid cycle). The major pathways producing acetyl CoA involve glycolysis and fatty acid oxidation. There are some uncertainties as to where the acetyl CoA used to form fatty acids originates from (see, e.g., Rawsthorne 2002 for a review), but it is clear that the fatty acids are synthesized in the plastids. Considering lipid synthesis in algae, the likely source of acetyl CoA is through glycolysis via pyruvate. Glycolysis takes part in the cytosol, producing pyruvate that can cross the membrane into the chloroplast, where acetyl CoA is formed (acetyl CoA is most likely not able to cross membranes and has to be produced where there is a need).

In the overall process of glycolysis, sucrose is split up into four pyruvate, providing a net release of four ATP and four NADH, and then further, four NADH is produced when acetyl CoA is formed from pyruvate (Buchanan et al., 2000). In the process, 4C atoms are lost as CO_2 per sucrose molecule, a loss rate of 1/3C. There are alternative routes, for example, the Rubisco enzyme can take part, independent of the Calvin cycle, increasing the C transfer efficiency and reducing the loss to 1/5C (Schwender et al., 2004). Although NADH is also a reducing power, it does not have the same function as NADPH. Generally, NADPH is used to as a reducing power directly, whereas NADH is used to produce ATP; with some exceptions, for example, NADH can be used for nitrate reduction in algae (Syrett, 1982). We will, however, calculate NADH as ATP equivalents, and the exchange rate between NADH and ATP is depending on where the transfer takes place. In the cytosol, NAHD gives rise to 1.5 ATP, and in the mitochondrion, 2.5 ATP is produced per NADH. The ATP and NADH produced during glycolysis do not necessarily directly take part in the fatty acid synthesis, but it is producing chemical energy, which can be seen as sparing photons needed to run other processes. Using NADH = 1.5 ATP equivalents, two sucrose gives rise to 32 ATP in glycolysis, which is ~43 photons worth of ATP. At the start of fatty acid synthesis, with all carbon needed in the form of acetyl CoA, an absolute minimum of 149 photons are needed, when subtracting the 43 photons worth of ATP from the 192 photons needed for making two sucrose molecules. An overview of the process can be seen in Fig. 2.

Every acetyl CoA carries 2C atoms, and during fatty acid synthesis, the carbon needs further reduction in order to make the fatty acid chains. The process starts with the union of one acetyl CoA and one malonyl-ACP (derived from acetyl CoA) molecules. This is the first step in a cycle where 2C atoms are added for every cycle (Fig. 3). The process requires one ATP for transforming acetyl CoA to malonyl-ACP and two NADPH for every turn of the cycle. Each subsequent cycle adds one malonyl-ACP, and this pair-wise addition of C atoms is the reason fatty acids normally have an even number of C atoms. The chemical energy and reducing power needed to form one palmitic acid chain from acetyl

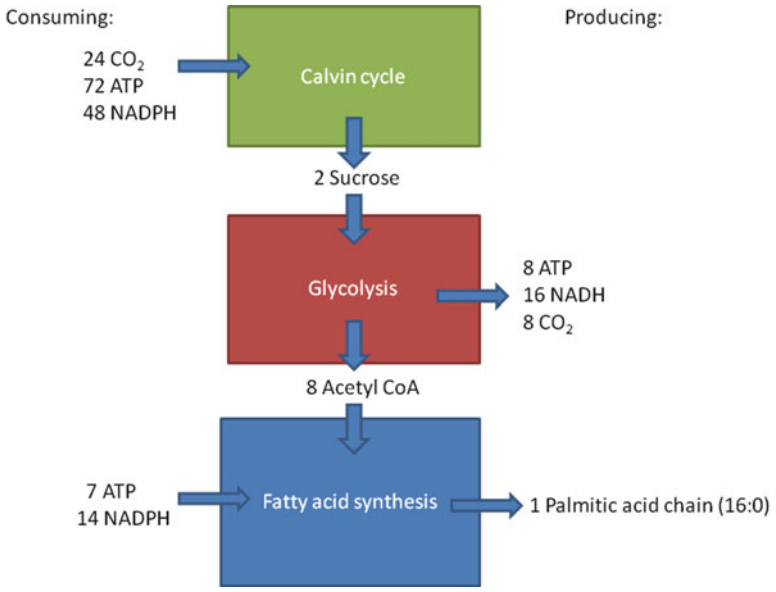

Figure 2. The theoretical stoichiometry of carbon, chemical energy (ATP) and reducing power (NADPH) used in the production of palmitic acid (16:0) when the acetyl CoA is generated through the glycolysis. There are other possible pathways, and this scheme does not take into account any loss processes or parallel processes needed to be run simultaneously (Further details can be found in Buchanan et al., 2000).

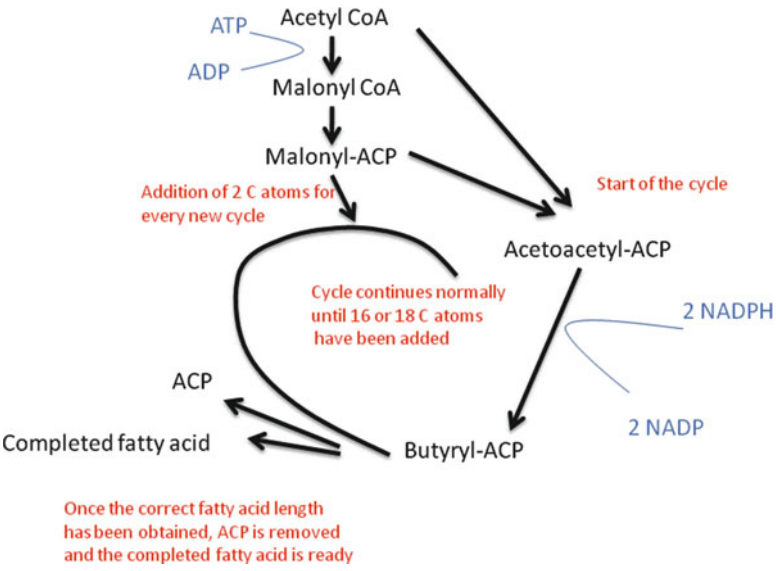

Figure 3. Cycle of fatty acid synthesis in the plastids of plants (Further details can be found in Buchanan et al., 2000).

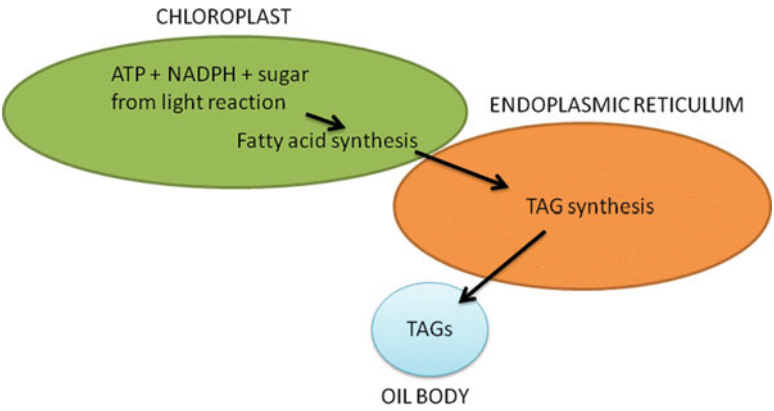

Figure 4. A general schematic of the synthesis of triacylglycerols (*TAGs*). The individual fatty acids are synthesized in the plastids, and the TAGs are combined in the endoplasmic reticulum and released as separate oil bodies into the cytosol.

CoA is consequently 7 ATP and 14 NADPH, which, using our conversion factors above, translates to ~37 photons. The total photon cost for one palmitic acid chain is consequently ~187.

The energy content of palmitic acid is 9.97 MJ mol^{-1}. Taking the requirement of 187 mol photons to produce 1 mol palmitic acid yields energy conversion of ~30% when using red photons or ~22% when using typical underwater light (with 0.24 MJ mol^{-1} photons) (Kirk, 1994). These values are somewhat lower than ones calculated for simple hydrocarbons (34 and 25%, respectively, for glucose), indicating an additional drop of ~12% in energy conversion to the values presented in previous chapter. In practice, however, the conversion factor from light to lipids is much lower. Taking into account the evident loss processes described in Table 1, the best possible energy conversion factor from sunlight to lipid is ~9%.

The fatty acids are synthesized in the plastids, and the process utilizes ATP and NADPH directly from the photosynthetic machinery when there is light. Further processing of the fatty acids takes place in the endoplasmic reticulum where, for example, the triacylglycerols (TAGs) are synthesized and the ready-made storage lipids are budded of in an oil body, which functions as an energy reserve (Fig. 4).

4. Effect of Light Quantity and Quality on Lipid Composition

The effect of light intensity, that is, photon flux density, on the lipid synthesis is not straightforward. There are large differences between different species, and it is clear that the physiological state of the cells is a major factor governing the lipid synthesis. During exponential growth, most of the energy/carbon is put into

growth. For this purpose, structural lipids for, for example, membranes are needed, while the production of storage lipids is at a minimum. During early stationary phase, vegetative growth slows down, and the excess organic carbon produced photosynthetically may be placed into storage lipids. However, not all species use lipids as the main storage product; starch is an alternative storage form used by some species.

In their study of light intensity effect on fatty acid composition under continuous light, Thompson et al. (1990) found large species-specific differences. Correlations between light and fatty acids were found for single species, but there were no consistent correlations over all eight species examined. However, for diatoms, there was a general trend of increasing concentration of palmitic acid (16:0) with increasing irradiance. Also for the diatom, *Thalassiosira pseudonana* did increasing the photon flux density lead to an increasing proportion of saturated fatty acids while decreasing the proportion of polyunsaturated fatty acids (PUFAs) (Brown et al., 1996). A decrease in PUFAs with increasing photon flux can be amplified with increasing temperature (Papina et al., 2007). High light intensities have been suggested to cause oxidative damage to PUFAs (Guschina and Harwood, 2009); however, higher concentration of saturated fatty acids might also reflect that these are the fatty acids stored as triacylglycerols (TAGs) (Khotimchenko and Yakovleva, 2004, 2005; Zhukova and Titlyanov, 2006).

Another aspect that will alter the ratio between saturated and unsaturated fatty acids is photoacclimation. The fatty acids associated with the photosynthetic machinery (e.g., glycolipids in the thylakoid membranes) are mostly polyunsaturated and under low light algae will increase the concentration of photosynthetic pigments and thereby increase the proportion of PUFAs (Walsh et al., 1997; Mock and Kroon, 2002; Zhukova and Titlyanov, 2006). Although there are large species-specific differences and differences due to the physiological state of the algae, the available literature suggests that the proportion of PUFAs increases at low light while the proportion of saturated fatty acids increases at high light intensities. The carbon allocation in different light environments reflects this difference; in low light or when cultures grow dense and cause self shading, low photon flux densities will increase the carbon allocation into light-harvesting pigments high in glycolipids (and PUFAs), but as nutrients are depleted, the fraction of carbon placed into neutral lipids starts to increase (Mock and Gradinger, 2000). A conceptual model of the effect of light on lipid concentration is presented in Fig. 5.

Spectral quality of light may affect composition of algal cell, for example, some pigments like astaxanthin are accumulated more in blue than in red light (Katsuda et al., 2004). However, overall the effect of spectral composition of light on the lipid metabolism has not been very much studied. In the few cases where the quality of light has been a factor, it has mainly been studies of the effect of UV light on cellular processes. Algal cells close to the surface will experience UV light, which has several negative effects on cellular processes. The lipid synthesis seems to be less affected by UV light than many other processes in the cells

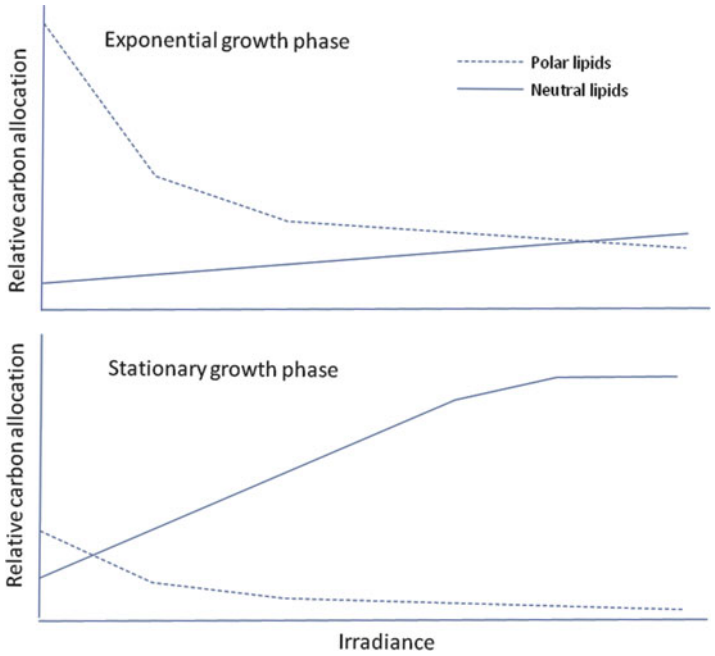

Figure 5. A conceptual, testable model of irradiance effect on the relative contribution of polar and neutral lipids during exponential and stationary growth phase for algal species that use lipids as their main storage product (Details are discussed in the text).

such as the protein synthesis (Smith et al., 1998), but the vulnerability to UV light and its effect on lipid synthesis is species dependent (Arts and Rai, 1997). For *Tetraselmis* sp., Goes et al. (1994) found that UV-B selectively suppressed PUFA synthesis.

5. Effect of Light Cycles on Lipid Composition

5.1. DIURNAL LIGHT-DARK CYCLES

It is clear that the diurnal light cycles directly affect a range of cellular processes such as primary productivity and cell division (Prézelin, 1992). Daily oscillations are commonly governed by biological clocks, and recent development has started to reveal the mechanism governing diurnal cycles down to the genetic level (Bell-Pedersen et al., 2005; Gardner et al., 2006; Monnier et al., 2010).

Generally, the internal C pool inside the cell builds up during the light period, reaching a peak by the end of the light period (Stramski and Reynolds, 1993; Jacquet et al., 2001; DuRand et al., 2002), which is not all that surprising

considering that this is when the photosynthetic production takes place. During the dark period, there is a loss of C due to respiration. For some species, parts of the cellular metabolism, such as protein synthesis, mainly take place during the dark period. Changing the photoperiod normally affects the primary production and thereby the growth rate, for example, increasing the photoperiod increases total production. However, some species of microalgae may need a dark period for running particular metabolic processes, and for these species, there will be an upper limit to how long a photoperiod is tolerated (Sicko-Goad and Andresen, 1991). For example, some species go through cell division mainly or only during the dark period, others, diatoms in particular, seem to go through cell division mainly during the light period (Nelson and Brand, 1979).

Although decreasing day length generally leads to decreased production and growth, microalgae can to some degree compensate for shorter days by increasing the concentration of Chl *a* and other photosynthetic pigments in the cell (Foy et al., 1976; Hobson et al., 1979; Reynolds, 2006). The lipids associated with the photosynthetic pigment would presumably follow the same pattern, and these are synthesized during the light period (Ragni and D'Alcala, 2007).

In one of the few studies on this topic, Brown et al. (1996) investigated the effect of different diurnal rhythms and irradiance on cellular constituents (carbohydrates, proteins, and lipids) of *Thalassiosira pseudonana*. Three treatments were used: 50 μmol photons m^{-2} s^{-1} in 24-h light, 100 μmol photons m^{-2} s^{-1} in 12-h light/12-h dark cycle, and 100 μmol photons m^{-2} s^{-1} in 24-h light. Their results suggested that longer photoperiod would increase growth compared with a shorter photoperiod with the equivalent light energy. The lipid content as percentage of dry weight did not seem to be affected by daily cycles during exponential growth phase, but was somewhat lower in the lowest irradiance during the stationary growth phase.

5.2. EFFECT OF LIGHT FLASHES

It is known from photobioreactors that short-term changes in light-dark cycling also affect primary production (Molina Grima et al., 1999). This effect is caused by turbulent eddies in a dense culture, moving the cells from high light to darkness and back again. This is experienced as light flashes by the microalgae, and the frequency of the light flashes is positively correlated with the productivity (Terry, 1986; Degen et al., 2001). In a classical study, Phillips Jr and Myers (1954) showed that light flashes of 0.001–0.1 s duration with tenfold longer dark periods boosted growth of the green algae *Chlorella pyrenoidosa*. The effect of light flashes can be modeled, and the mechanism is well understood (Rubio et al., 2003; Yoshimoto et al., 2005). The relaxation between light flashes decreases the loss rate, increasing efficiency of the photosynthetic machinery. The effect of distributing the light as light flashes has consequently some of the same effect as light dispersal. Distributing the light energy between cells lowers the production per cell, but increases the

overall production, as more cells receive light energy. The effect of light flashes on lipid metabolism has not been studied in great detail, but the effects could be similar to that of photon flux or diurnal light-dark cycles.

6. Conclusion

The metabolic pathways in algae are very flexible, and although many of the processes are known, it is difficult to precisely know what metabolic route is used when synthesizing lipids. Light is of paramount importance for growth and lipid accumulation in algae, and although a lot of work has been conducted to better understand photosynthesis and the effect of light on growth, there are very few studies on the effect of light on the lipid accumulation in algae. In particular, the effect of spectral quality of light on lipid composition is hardly studied at all.

In this chapter, we have evaluated the different loss processes when transforming light energy to chemical energy in lipids. For many of these loss processes, little can be done to minimize these effects, for example, reduction of irradiance in the atmosphere. The main loss process that can be reduced is the release of light energy as heat at high light intensities. Much of this loss is due to photoprotective pigments, and effectively utilizing high light levels at maximum efficiency is the challenge ahead for developing algae as a raw material for low cost commodities such as fuel.

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R. Gordon and J. Seckbach (eds.), *The Science of Algal Fuels*,
Cellular Origin, Life in Extreme Habitats and Astrobiology, 399–422
DOI 10.1007/978-94-007-5110-1_22, © Springer Science+Business Media Dordrecht 2012

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METABOLIC PATHWAYS IN GREEN ALGAE WITH POTENTIAL VALUE FOR BIOFUEL PRODUCTION

ALGAL FUEL METABOLISM

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1. Introduction

The world is currently using energy at a rate of over 200 million barrels of oil equivalent per day, but with unabated demand in 2050, it could be 2.5 times that amount. To begin to address this challenge in the USA, Congress passed the US Renewable Fuels Standard (RFS) under the Energy Independence and Security Act of 2007. This statute identifies an "*advanced biofuel* as any renewable fuel that meets a 50% life-cycle GHG emissions reduction from the [current] petroleum baseline, and is not derived from corn starch." By 2022, the RFS, included as a part of this law, calls for 21 billion gallons (not including 15 billion gallons of ethanol from corn) of advanced biofuels, including cellulosic ethanol, to be added to our nation's fuel supply. At this point, it is very likely that some of the 21 billion gallons will come from algae, and as such the US Department of Energy released its National Algae Biofuels Roadmap in May of 2010 (http://www1.eere.energy.gov/biomass/pdfs/algal_biofuels_roadmap.pdf) to lay the groundwork for identifying challenges that may need to be resolved technically for algae to be used in the production of economically viable, environmentally sound biofuels. The roadmap was intended to serve as a resource for researchers, engineers, and policy makers by providing a summary of progress and a direction for future algal research activities. This chapter will examine some of the biological pathways found in algae that might be mobilized to provide some of the desired advanced biofuels.

2. Algae as a Platform for Producing Advanced Biofuels

Microalgae are photosynthetic microorganisms capable of harvesting solar energy while converting CO_2 and water to organic macromolecules (e.g., carbohydrates, proteins, and lipids). From a biofuel perspective, the main advantage of algae is that

they are a preferred source or feedstock for high energy density, fungible liquid transportation fuels, including H_2. More specifically, microalgae (a) produce much higher biomass yields per unit area of land than traditional crops; (b) do not compete for arable land or nutrients associated with conventional agriculture; (c) can use waste, saline, or sea water, minimizing the use of freshwater; (d) can recycle waste CO_2 from industrial emission sources; and (e) are amenable to the integrated production of coproducts along with biofuels. As a realistic example, oil yields from algae could be as high as ten times on a per unit land area basis than the highest oil-yielding terrestrial crop plant (oil palm), which in turn is ten times that of corn (Weyer et al., 2010). Actual industrial field trials at this point have demonstrated two times the yield of oil palm per unit land area, which is an encouraging development (Quinn et al., 2012).

3. Biofuel Production Pathways

3.1. PRODUCTION OF ALCOHOLS

3.1.1. The Ethanol Synthesis Pathways

Metabolic pathways that lead to ethanol production are found in many microalgae. In *Chlamydomonas reinhardtii* (a freshwater green alga, hereafter referred to as Chlamydomonas), the formation of ethanol occurs during dark fermentation through two different pathways via the decarboxylation of pyruvate (Fig. 1). Pyruvate (the end product of glycolysis) is used as a substrate by the pyruvate formate lyase (PFL) enzyme, which produces formate and releases acetyl-CoA. Acetyl-CoA is then reduced to ethanol via an alcohol dehydrogenase (ADH1), resulting in the oxidation of two molecules of NADH (Atteia et al., 2006; Dubini et al., 2009; Grossman et al., 2011; Hemschemeier and Happe, 2005; Magneschi et al., 2012). Pyruvate can also be converted to ethanol by an alternate pathway, via a pyruvate decarboxylase (PDC3) and an alcohol/acetaldehyde dehydrogenase enzyme (ADH1/ADH2/ADH3), resulting in the anaerobic production of ethanol and the oxidation of one NADH. Chlamydomonas is rare among eukaryotes in having both pathways for the production of ethanol, but the cellular location of these pathways is not completely resolved (Hemschemeier and Happe, 2005). In fact, Chlamydomonas possesses three distinct enzymes that are potentially important for ethanol production under anoxic conditions: ADH1 and two other putative alcohol dehydrogenases, designated ADH2 and ADH3. Recent data showed that ADH1 is present in chloroplasts; however, the location of ADH2 and three are not known (Terashima et al., 2010). Among the two ethanol production pathways, the pyruvate-acetyl-CoA-ethanol pathway, which is catalyzed by ADH1, seems to be the predominant one (Catalanotti et al., 2012; Magneschi et al., 2012). Furthermore, ADH1 seems to be the dominant enzyme that catalyzes the reduction of acetaldehyde, which is generated by PDC3, to ethanol (Catalanotti et al., 2012; Magneschi et al., 2012).

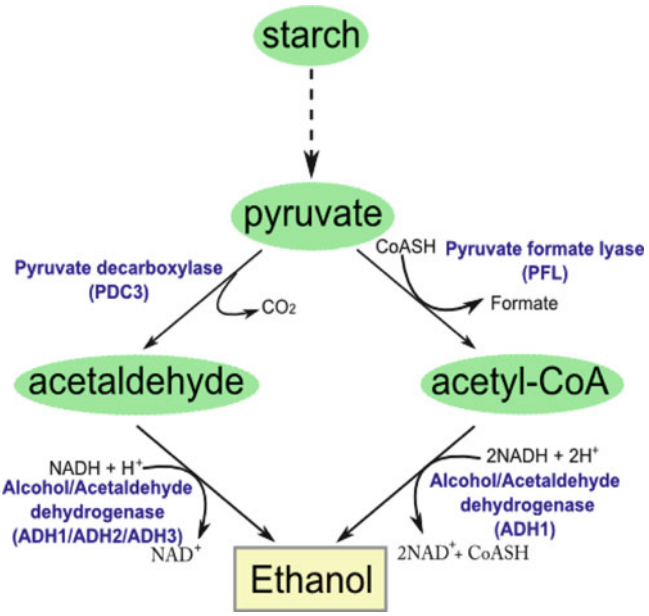

Figure 1. Ethanol synthesis pathways. Pyruvate, originating from the breakdown of starch, is converted to acetyl-CoA and acetaldehyde by the enzymes pyruvate formate lyase (PFL) and pyruvate decarboxylase (PDC3), respectively. Both intermediates are then converted to ethanol by different isoforms of *alcohol/acetaldehyde or alcohol dehydrogenases (ADH): ADH1* from acetyl-CoA and ADH1*/ADH2/ADH3 from acetaldehyde. The text in *blue* represents enzymes.

3.1.2. Other Alcohols

Isopropanol, butanol, and longer-chain alcohols (common in bacterial fermentation processes and potentially useful as biofuels) are not produced naturally in algae. Thus, metabolic engineering would be required to produce these products in algae.

3.2. PRODUCTION OF HYDROCARBONS

3.2.1. The Isoprenoid Biosynthesis Pathway

Isoprenoids consist of a large and diverse group (>40,000) of natural metabolites (hydrocarbons, which contain only hydrogen and carbon atoms), many of which have a plant origin (Buckingham, 1998). In plants, they play important roles in growth and development, primarily in the synthesis of pigments and quinones for photosynthesis as well as in the production of plant hormones like gibberellins, abscisic acid, and cytokinins. In addition, many of these compounds are also involved in survival mechanisms like reproduction, defense, and toxin production. They also serve as membrane components in archaebacteria (prenyl lipids) as

well as sterols in eubacteria. Isoprenoids are synthesized from two different C5 (5 carbons) precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). These C5 precursors can be polymerized further to longer chains that can serve as fuel substitutes. The simplest isoprenoid compounds, such as isoprene, consist of a single C5 unit (molecular formula, C_5H_8) and are called hemiterpenes. Longer-chain isoprenoids with two, four, six, and eight isoprene units are called mono, di, tri, and tetraterpenes, respectively. Chains containing more than eight isoprene units are called polyterpenes. Typically, isoprene units are added either in a "head-to-tail" or "tail-to-head" orientation to yield complex isoprenoid compounds. The importance of isoprenoids has been realized in the pharmaceutical sector as demonstrated by the commercial production of steroids, carotenoids, lycopene, taxol, and artemisinin (Klein-Marcuschamer et al., 2007; Withers and Keasling, 2007). It is known that saturated, unsaturated, branched or cyclic alkanes or alkenes, and several branched-chain alcohols that might serve as alternative fuels can be produced from the same pathway that leads to the production of isoprenoids. It has long been suggested that these hydrocarbons could serve as new sources of biofuels (Metzger and Largeau, 2005; Wake and Hillen, 1980). Compounds, including isopentanol and its acetate ester, which could be used as additives in spark ignition agents, can also be produced through this pathway (Hull et al., 2006).

Isoprenoid synthesis originates from two different pathways, one from pyruvate and glyceraldehyde-3-phosphate (G3P) and the second from acetyl-CoA. Synthesis from the former is called the deoxyxylulose phosphate (DXP) pathway (Fig. 2) and that from the latter is called the mevalonate pathway (Fig. 3). The DXP pathway is also referred to as the methylerythritol phosphate (MEP) pathway or the non-mevalonate pathway. The mevalonate pathway is found primarily in eukaryotes, whereas the DXP pathway is found in prokaryotes as well as in chloroplasts of photosynthetic organisms.

The DXP pathway begins with the condensation of glyceraldehyde-3-phosphate and activated acetaldehyde generated from pyruvate (Lange et al., 2000), a reaction catalyzed by the enzyme DXP synthase (Fig. 2). This leads to the production of DXP. DXP is then reduced by 1-deoxyxylulose-5-phosphate reductoisomerase, using NADPH as the electron donor to 2-*C*-methylerythritol-4-phosphate (MEP). The next step involves conjugation of MEP with cytidine-5-phosphate (CDP from CTP) by MEP cytidyltransferase to form 4-(cytidine-5′-diphospho)-2-*C*-methylerythritol (CDP-ME). The enzyme 4-(cytidine 5-phosphate)-2-*C*-methylerythritol kinase phosphorylates CDP-ME to form 4-(cytidine 5-diphospho)-2-*C*-methylerythritol 2-phosphate (CDP-MEP), which is further converted to 2-*C*-methylerythritol 2,4-cyclodiphosphate (ME-cPP) by 2-*C*-methylerythritol 2,4-cyclodiphosphate synthase. ME-cPP is then converted to hydroxymethylbutenyl 4-diphosphate (HMBPP) by hydroxymethylbutenyl 4-diphosphate synthase, which in turn is finally converted to a mixture of isopentenyl 5-diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) by IPP/DMAPP synthase.

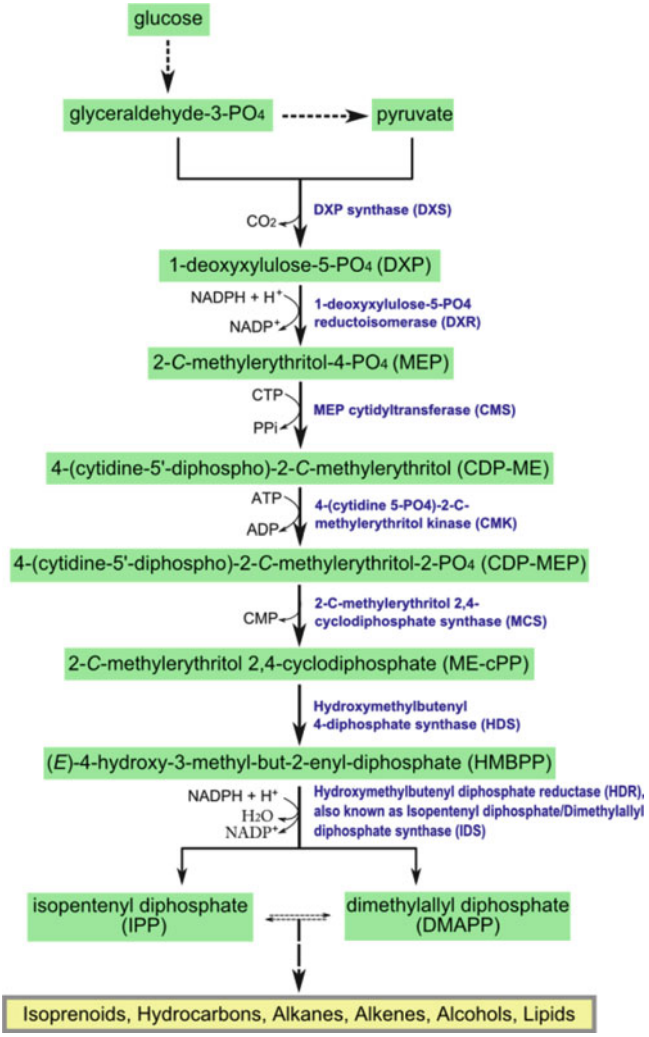

Figure 2. The deoxyxylulose phosphate (DXP) pathway. Glyceraldehyde-3-phosphate and an activated aldehyde, generated from pyruvate, both originating from glycolysis, are converted to DXP by a condensation reaction with the liberation of CO_2. DXP is then converted sequentially to 2-C-methylerythritol-4-phosphate (MEP), 4-(cytidine-5′-diphospho)-2-*C*-methylerythritol (CDP-ME), 4-(cytidine-5′-diphospho)-2-*C*-methylerythritol-2-phosphate (CDP-MEP), 2-*C*-methylerythritol 2,4-cyclodiphosphate (ME-cPP), and hydroxymethylbutenyl 4-diphosphate (HMBPP) before being reduced, in the presence of NADPH, into the C5 precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), by a series of enzymatic reactions. Text in *blue* represents the enzymes that catalyze the individual reactions.

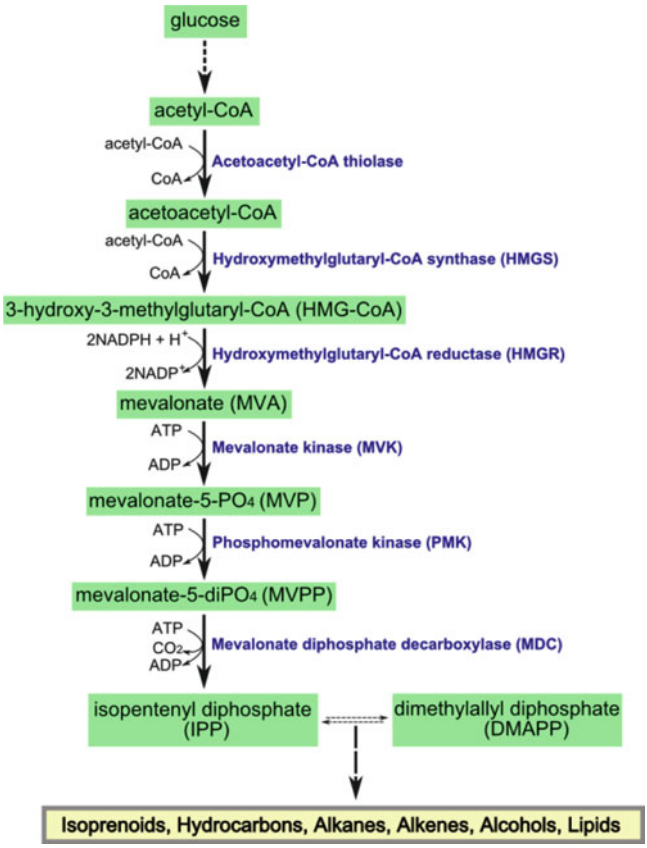

Figure 3. The mevalonate pathway. The substrate for the mevalonate pathway is the acetyl-CoA, which is generated from glycolysis. Acetyl-CoA is converted to the intermediate mevalonate (MVA) via acetoacetyl-CoA and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by two condensation reactions. This is followed by two sequential phosphorylation reactions to yield mevalonate-5-diphosphate (MVPP), which is finally converted to isopentenyl diphosphate (IPP) by an ATP-dependent decarboxylation reaction. IPP can be further converted to dimethylallyl diphosphate (DMAPP) by an isomerase enzyme. Text in *blue* represents enzymes.

The first step in the mevalonate pathway involves two molecules of acetyl-CoA undergoing a Claisen condensation reaction catalyzed by the enzyme acetoacetyl-CoA thiolase to give acetoacetyl-CoA (Fig. 3). The resulting acetoacetyl-CoA undergoes a second round of condensation with acetyl-CoA, catalyzed by HMG-CoA synthase, to give 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). HMG-CoA then undergoes a two-step reduction (using two NADPHs), catalyzed by the membrane-bound enzyme HMG-CoA reductase, forming mevalonate (MVA). MVA is phosphorylated sequentially, first by mevalonate kinase and then by phosphomevalonate kinase to yield mevalonate 5-phosphate (MVP) and

mevalonate 5-diphosphate (MVPP), respectively. Finally, mevalonate diphosphate decarboxylase catalyzes the ATP-dependent decarboxylation of MVPP to yield IPP. IPP can be converted to dimethylallyl diphosphate (DMAPP) by an isomerization reaction.

In higher plants, isoprenoids are synthesized in different cellular compartments (Arigoni et al., 1997; Lichtenthaler et al., 1997; Schwender et al., 1997). In particular, triterpenes and sterols are synthesized via the mevalonate pathway in the cytosol, whereas carotenoids and phytol are synthesized via the DXP pathway in the plastids. On the other hand, the green alga, *Botryococcus braunii*, is known to produce long-chain hydrocarbons in the form of squalenes and lipids (Achitouv et al., 2004; Metzger et al., 2002, 2003; Rager and Metzger, 2000). Depending on the type of hydrocarbons they synthesize, *B. braunii* are classified into three races, A, B, and L. Race A strains are known to produce *n*-alkadiene and triene hydrocarbons (C_{23}–C_{33}) (Metzger et al., 1985a); Race B strains produce triterpenoid hydrocarbons, C_{30}–C_{37} botryococcenes (Metzger et al., 1985b) and C_{31}–C_{34} methylated squalenes (Achitouv et al., 2004; Huang and Poulter, 1989); and Race L strains produce the tetraterpenoid hydrocarbon lycopadiene (Metzger et al., 1990; Metzger and Casadevall, 1987). Similar to other green algae like *Scenedesmus obliquus*, Chlamydomonas, and *Chlorella fusca*, *B. braunii* Race B also synthesizes terpenoids using the non-mevalonate pathway (Disch et al., 1998; Sato et al., 2003). In general, it can be concluded that the phyla, Chlorophyta, exclusively uses the non-mevalonate pathway for synthesis of isoprenoid compounds. In contrast, the red alga, *Cyanidium caldarium,* belonging to the phyla, *Rhodophyta*, produces isoprenoids using the mevalonate pathway. The Euglenophyte, *Euglena gracilis*, is known to produce carotenoids using both pathways (Kim et al., 2004) and sterols and phytols via the mevalonate pathway (Lichtenthaler, 1999).

In cyanobacteria, the genus *Synechocystis* has also been investigated for its ability to synthesize two isoprenoid compounds, phytol and β-carotene, via the DXP pathway (Disch et al., 1998). Direct application of this pathway knowledge, using cyanobacteria (but not algae) for the production of the biofuel precursor, isoprene (a short-chain hydrocarbon), has been reported recently with the transformation of an isoprene synthase gene (*IspS*) from the plant *Pueraria montana* (kudzu) into *Synechocystis* sp. PCC 6803, which lacks the *IspS* gene (Lindberg et al., 2010). The engineered cyanobacterium was then found to be capable of producing low yields of isoprene photosynthetically (50 μg/g dry cell weight/day) from DMAPP, the end product of the DXP pathway. In the future, depending on the type of liquid fuel that is needed, specific enzymes could be engineered into algae to use the isoprenoid pathway for generating different chain-length hydrocarbons that could serve as fuel alternatives to traditional gasoline, diesel, or jet fuels.

3.2.2. The Fatty Acid Synthesis Pathway

One important carbon partitioning mechanism in plants and algae, leading to biofuel precursor production, is the fatty acid synthesis pathway. Fatty acids represent part of the total lipid content of a cell, and they form the building blocks

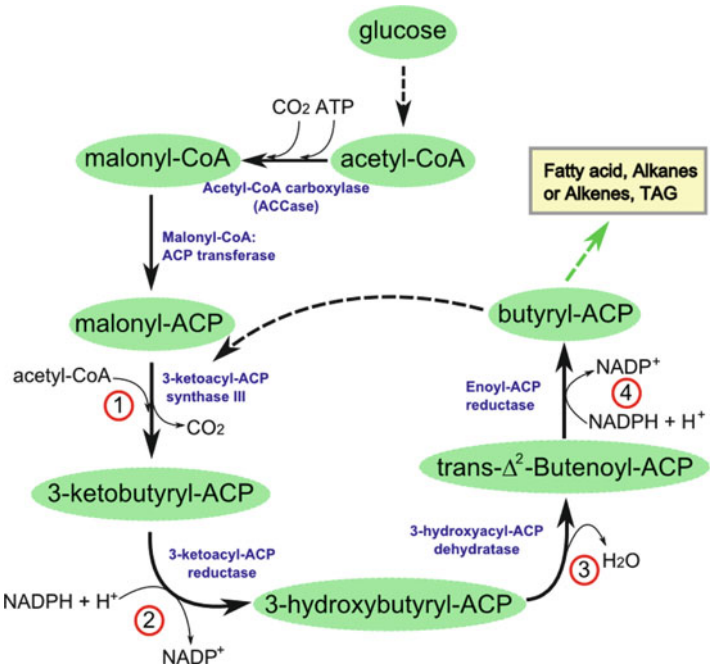

Figure 4. Fatty acid biosynthesis. Acetyl-CoA is converted into malonyl-CoA by the enzyme acetyl-CoA carboxylase. The malonyl group is transferred to an acyl carrier protein to yield malonyl-ACP. Malonyl-ACP undergoes a condensation reaction with acetyl-CoA to yield acyl-ACP with the concomitant release of CO_2 (*marked as reaction 1*). Acyl-ACP then undergoes a series of reactions involving reduction, dehydration, and another reduction reaction to yield longer-chain ACPs (*marked as reactions 2, 3, and 4*, respectively). The three reactions are catalyzed by 3-ketoacyl-ACP reductase, 3-hydroxyacyl-ACP dehydratase, and enoyl-ACP reductase, respectively. Sequential repetition of the last four steps (*marked as reactions 1 through 4*) starting from the condensation (using three different ketoacyl-ACP synthases, 3-ketoacyl-ACP-synthase III (KASIII), KASII (not shown), and KASI (not shown)) with malonyl-ACP yields the 16 or 18-carbon product. The fatty acid is finally released by thioesterases. Text in *blue* represents enzymes.

for various types of lipids. By definition, a fatty acid generally refers to an aliphatic chain carboxylic acid. Aliphatic chain can be saturated or unsaturated and can be of varying lengths. In algae, the *de novo* synthesis of fatty acids occurs primarily in the chloroplast. A schematic representation of the fatty acid biosynthesis pathway is presented in Fig. 4. The first step, involving conversion of acetyl-CoA to malonyl-CoA, is considered to be the commitment step for fatty acid synthesis. This reaction is catalyzed by acetyl-CoA carboxylase (ACCase) in two steps. First, CO_2 is transferred by the biotin carboxylase portion of the ACCase to a nitrogen atom on a biotin prosthetic group that is attached to the ε-amino group of a lysine residue in an ATP-driven reaction. Second, the activated CO_2 is transferred from the biotin to acetyl-CoA to form malonyl-CoA by carboxyltransferase (Ohlrogge

and Browse, 1995). The malonyl group from malonyl-CoA is transferred to an acyl carrier protein to generate malonyl-ACP in a reaction catalyzed by malonyl-CoA:ACP transferase. Malonyl-ACP then undergoes a series of condensation reactions with acyl-ACP (or acetyl-CoA acceptors), resulting in the generation of a carbon-carbon bond along with the release of CO_2. The CO_2 that is regenerated acts as the driving force for the ACCase enzyme to sustain the operation of the cycle in an irreversible manner. The following are a series of four reactions that are repeated in order to produce a saturated 16:0 and 18:0 carbon fatty acid. Malonyl-ACP undergoes a condensation reaction with acetyl-CoA in order to form the four-carbon product, 3-ketoacyl-ACP, with the release of CO_2. This reaction is catalyzed by 3-ketoacyl-ACP synthase III (KAS III) (Jaworski et al., 1989). There are two additional condensing enzymes, KASI and KASII, which are required to produce fatty acids containing 6–16 carbons and 16–18 carbons, respectively. Following each condensation reaction, the enzyme 3-ketoacyl-ACP reductase catalyzes the reduction of 3-ketoacyl-ACP at the carbonyl group using NADPH as the electron donor to produce a hydroxyacyl-ACP. This product undergoes a dehydration reaction catalyzed by the enzyme hydroxyacyl-ACP dehydratase, followed by a second round reduction reaction in the presence of NADH or NADPH that is catalyzed by enoyl-ACP reductase. Each repetition of the above four reactions results in the addition of two carbons to the fatty acid precursor molecule. The final product of this pathway is a saturated fatty acid. In order to introduce unsaturation into the fatty acid, the soluble enzyme stearoyl-ACP desaturase (which is otherwise membrane associated in plants) adds the first double bond (not pictured in Fig. 4). The termination of the elongation process occurs when an acyl-ACP thioesterase hydrolyzes the acyl-ACP (product of reaction 4 in Fig. 4) and releases the fatty acid or when the acyltransferases in the plastid transfers the fatty acid from ACP to glycerol-3-phosphate (see the next section) or to monoacylglycerol-3-phosphate (Ohlrogge and Browse, 1995). The fatty acids can also be converted into linear chain alkanes or alkenes (see the alkane synthesis section). The most commonly found fatty acids in algae have chain lengths of 16–18 carbon atoms. A more detailed analysis of the types of fatty acids found in algae is found elsewhere (Hu et al., 2008).

Fatty acids are primarily synthesized by algae to form membrane-bound lipids, such as glycosylglycerides like monogalactosyldiacylglycerol, digalactosyldiacyl glycerol, and sulfoquinovosyldiacylglycerol found in chloroplasts or phosphoglycerides like phosphatidylethanolamine and phosphatidylglycerol found in plasma membranes (Guckert and Cooksey, 1990; Harwood, 1998; Pohl, 1979; Wada and Murata, 1998). Membrane lipids are known to constitute about 5–20% of the cell's dry weight. It must be noted that localization (chloroplast versus cytosol) of those pathways is still under investigation.

Typically, lipid accumulation in algae occurs under nutrient-deficient conditions. For example, in the diatom *Cyclotella cryptica*, increased activity of ACCase in response to silicon deficiency leads to higher accumulation of triacylglycerol (Roessler, 1988). However, it was also observed subsequently that

the overexpression of ACCase in the same organism did not lead to increased lipid production (Sheehan et al., 1998). On the other hand, modifying factors like light illumination and the type of carbon source affect the level and type of lipids produced by the alga *Porphyridium cruentum* (Oh et al., 2009). Recently, a study has shown that the green microalga *Chlorella zofingiensis*, when grown heterotrophically in the presence of glucose, produces much higher levels of fatty acids and triacylglycerols than when grown photoautotrophically (Liu et al., 2011). This suggests that increasing the supply of acetyl-CoA generated through the glycolytic pathway provides additional substrate for the first step of fatty acid biosynthesis. This type of process is apparently being scaled up for commercial application by Solazyme, Inc., a biotech company in the San Francisco area. Another approach for increasing fatty acid production used knockout mutants of Chlamydomonas with a deficiency in starch accumulation. These mutations blocked starch synthesis, forcing the organism to divert its carbon flux toward triacylglycerols as an alternate storage product (Wang et al., 2009; Work et al., 2010). Proteomic analysis on the *sta6* mutant (a Chlamydomonas mutant strain deficient in starch synthesis) has revealed an upregulation of fatty acid metabolism-related enzymes (Wang et al., 2012). However, a recent report on oil accumulation by Chlamydomonas provides conflicting evidence to show that there may not be a direct correlation between starch accumulation and accumulation of oil bodies in this organism (Siaut et al., 2011). Another example of metabolic engineering that produces increased fatty acids was the heterologous expression of acyl-ACP thioesterases from plants (*Cinnamomum camphora* and *Umbellularia californica*) in the diatom *Phaeodactylum tricornutum*, which led to higher levels of short-chain fatty acids (Radakowits et al., 2010).

3.2.3. The Triacylglycerol Pathway

Triacylglycerols (TAGs) are the main storage compound in many algae exposed to stress conditions, including high light treatment or nutrient starvation. Certain algal species naturally accumulate large amounts of TAG (30–60% of dry weight) and exhibit photosynthetic efficiencies and lipids/oil production potentials at least an order of magnitude higher (on a land area basis) than that observed in oil palm, the highest yielding terrestrial oil crop plant (Hu et al., 2008). The major pathway for the formation of TAGs in plants and algae involves *de novo* fatty acid synthesis in the stroma of the plastids (see the previous section) and subsequent incorporation of the fatty acids into a glycerol backbone. TAG synthesis occurs via three sequential acyl transfers of acyl-CoA (called the direct glycerol or Kennedy pathway) in the endoplasmic reticulum (ER) (Fig. 5). Fatty acids produced in the chloroplast are sequentially transferred from acyl-CoA to positions 1 and 2 of glycerol-3-phosphate, resulting in formation of the central metabolite, phosphatidic acid (PA) (Ohlrogge and Browse, 1995). Dephosphorylation of PA, catalyzed by a specific phosphatase, releases diacylglycerol (DAG) (Dahlqvist et al., 2000). In the final step of TAG synthesis, a third fatty acid is transferred to the vacant

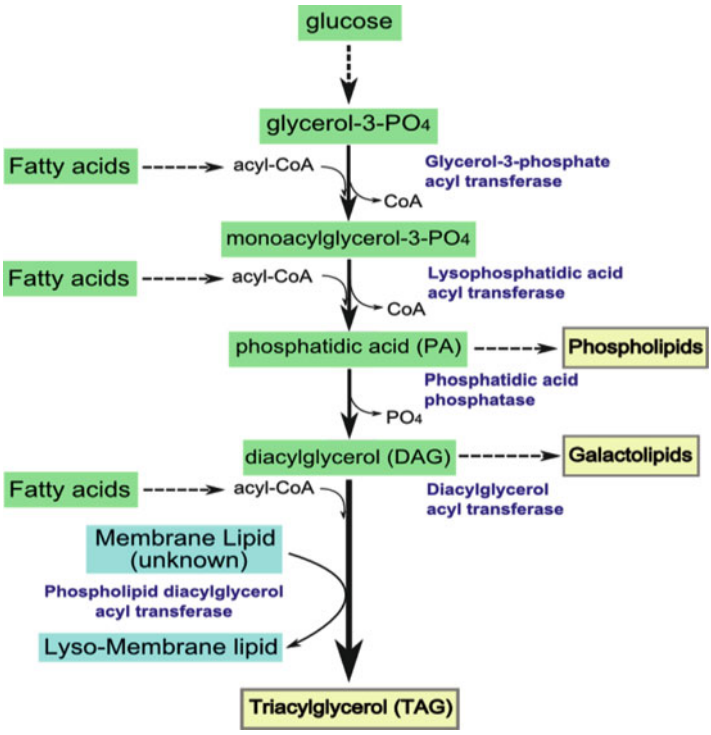

Figure 5. Triacylglycerol (TAG) biosynthesis pathway. Fatty acids are sequentially added to positions 1 and 2 of the glycerol-3-phosphate backbone by two acylation reactions to yield phosphatidic acid (PA). PA loses a phosphate group to form diacylglycerol, which in turn adds another fatty acid to position 3 to form TAG. Phospholipids and galactolipids can be generated from intermediates, PA and DAG, respectively. Text in *blue* represents enzymes. *Green boxes* represent the primary TAG-producing pathway in algae, and the *blue boxes* represent the alternate pathway for TAG production.

position 3 of DAG, and this reaction is catalyzed by diacylglycerol acyltransferase, an enzymatic reaction that is unique to TAG biosynthesis.

PA and DAG can also be used directly as substrates for the synthesis of polar lipids, such as phospholipids and galactolipids. The acyltransferases involved in TAG synthesis may exhibit preferences for specific acyl-CoA molecules (Fig. 5), and thus may play an important role in determining the final acyl composition of TAG. However, recently an alternative pathway to TAG production in Chlamydomonas has also been proposed (Merchant et al., 2011). This route, which is independent of acyl-CoA, is catalyzed by a phospholipid diacylglycerol acyltransferase that uses an unusual fatty acid as substrate to produce TAG (Dahlqvist et al., 2000). In Chlamydomonas phosphoethanolamine is thought to be this fatty acid and the enzyme was identified in oil bodies by proteomic analysis (Nguyen et al., 2011).

The main pathway above is believed to be the major pathway to accumulate TAG in plants and algae. However, the regulation of fatty acids and TAG synthesis in algae is poorly understood at the physiological and biochemical levels. As a result, the lipid yields obtained from algal mass culture efforts, performed to date, fall short of the high values (50–60%) observed in the laboratory, adding to the challenge of achieving cost-competitive algal oil photoproduction (Hu et al., 2008; Pienkos and Darzins, 2009; Sheehan et al., 1998). Current research shows that nitrogen starvation is the main trigger for TAG accumulation, a phenomenon confirmed in many algae, such as *Neochloris oleoabundans*, *Nannochloropsis* sp. QII, and *Parietochloris incisa*. This nutrient stress condition leads to lipid contents of up to 56% (on a dry weight basis), with 80% of that being TAG (Merzlyak et al., 2007; Suen et al., 1987; Tornabene et al., 1983).

It must be mentioned that other culture conditions, such as low light, high light, CO_2, O_2, and temperature, or other nutrient stress conditions, such as sulfur deprivation, can affect the lipid composition, and more specifically, TAG accumulation. For example, increased O_2 concentration has a beneficial effect on neutral lipid accumulation in *Chlorococcum littorale* under N_2-starvation conditions (Ota et al., 2011). It was also observed that light intensity can have a positive effect on lipid composition and TAG accumulation in *Dunaliella bardawil* (Rabbani et al., 1998). Recently, it was suggested that downregulation of oil-body-associated lipases (identified by proteomics analysis), which are involved in TAG degradation, might result in an increase of TAG accumulation in algal cells (Nguyen et al., 2011).

Although the purpose of this chapter is to discuss potentially useful metabolic pathways, it is appropriate to address some application issues in the case of the TAG pathway since there is so much interest in microalgae-based biodiesel. Lipid accumulation and algal growth rates seem to be key parameters that will determine the feasibility of using microalgae for future commercial biodiesel production. However, there are other factors involved, including nutrients, water, CO_2, harvesting, and extraction procedures, which will contribute to the overall cost of algal biodiesel production. Based on a life cycle analysis study, using seawater or wastewater instead of freshwater should reduce the water requirement by 90%, in addition to eliminating the need for all nutrients except phosphate (Yang et al., 2011). Other independent life cycle assessment studies of microalgal biomass production have shown that open pond bioreactors, in comparison to horizontal tubular photobioreactors (PBRs), might prove to be more environmentally sustainable as well as cost-effective, provided that the lipid content reaches around 60% (Jorquera et al., 2010; Stephenson et al., 2010). Furthermore, a combination of closed systems (flat-plate PBRs) and open raceway ponds has been proposed to provide a better alternative than either of these alone (Jorquera et al., 2010). This integrated system could work well for the present-day algal biodiesel production scheme, where TAG accumulation begins only after nutrient deprivation. Hence, the first step involving cell growth and biomass accumulation

via photosynthesis might be achieved in a closed system, and the second step, involving nutrient starvation and production of TAG, could be achieved in open ponds. However, this approach does have the drawback that it cannot support a continuous production process since the cells no longer grow under nutrient-deprived conditions. Nevertheless, neither system (closed bioreactors or open raceway ponds) is cost competitive enough to warrant commercial biodiesel production at this time.

Considering the hurdles that will have to be overcome for a commercially feasible, cost-competitive process (in comparison to fossil-based petroleum), better lipid-producing strains, like *Nannochloropsis* sp. and *Chlorella* sp., will have to be researched intensively, new lipid-accumulating strains will have to be explored, and/or additional genetic engineering approaches will have to be implemented to improve the existing strains. Furthermore, a new approach will have to developed, wherein algal cells actively photosynthesize as well as accumulate TAGs at the same time. However, current knowledge of algal metabolism is insufficient to provide a simple solution to address both these issues simultaneously. Extensive basic research toward understanding the cellular regulation process, activated by nutrient deprivation in algae, has to be undertaken in order to surmount the growth/nutrient-deprivation conundrum and induce continuing growth while concomitantly accumulating TAGs. Algal strains with such altered capabilities could be a possible solution toward improving the efficiency and cost-effectiveness of biodiesel production in a single-stage open pond system.

3.2.4. The Alkane Synthesis Pathway

Synthesis of alkanes is initiated from fatty acids, and hence microalgae that are known for their lipid-producing capabilities might also be used for the generation of alkanes. The primary pathway for generating alkanes involves the reduction of a fatty acid pathway intermediate to generate an aldehyde, which can in turn be converted to an alkane by a decarbonylation reaction (Fig. 6). As mentioned earlier (see the isoprenoid pathway section), the green alga *B. braunii* is known to synthesize high levels of n-alkadiene and triene. The presence of decarbonylation activity was initially detected in *B. braunii* Race A (Dennis and Kolattukudy, 1991). Recently, Schirmer et al. showed that cyanobacteria contain the enzymes required to synthesize alkanes from fatty acids (Schirmer et al., 2010) when they identified the presence of two open reading frames (orf1593 and orf1594) that putatively code for the acyl-ACP reductase and aldehyde decarbonylase enzymes, respectively. Heterologous expression of these orfs in *E. coli* led to the production of C13 and C17 mixtures of alkanes and alkenes. Plants, including *Pisum sativum* and *Arabidopsis thaliana*, have been shown in the past to contain the genetic elements that code for proteins with similar function (Aarts et al., 1995; Cheesbrough and Kolattukudy, 1984). However, only long-chain alkanes are found in plants; hence, they must be further processed before they can be used as fuels.

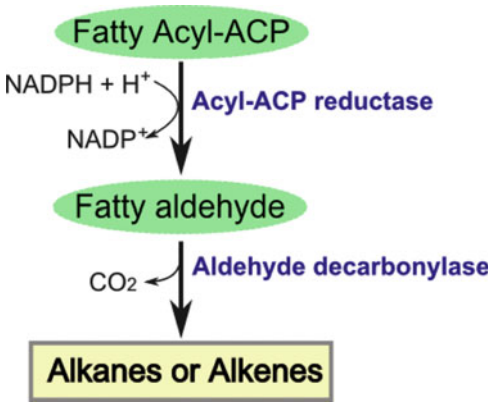

Figure 6. Alkane synthesis pathway. The substrate, fatty acyl-ACP, is reduced by the enzyme acyl-ACP reductase using NADPH as the electron donor. The resulting fatty aldehyde is decarboxylated by aldehyde decarbonylase to yield an alkane or an alkene. Text in *blue* represents enzymes.

3.3. PRODUCTION OF HYDROGEN

While there is a long history documenting the ability of algae to take up and produce H_2, the gas is not usually considered to be a biofuel from the perspective of government-sponsored research. Nevertheless, it is included in this section because biohydrogen is an algal product that can be used as a fuel as well as a substrate for producing carbonaceous fuels biologically, for upgrading poor quality crude oil in the petroleum refining industry, and for producing other valuable coproducts.

3.3.1. The Hydrogen Photoproduction Pathway

Algae normally use photosynthesis to fix CO_2 (for carbohydrate synthesis), but under anaerobic conditions, they can also produce H_2 directly from water in the following reactions: $2H_2O + \text{light energy} \rightarrow O_2 + 4H^+ + 4e^- \rightarrow O_2 + 2H_2$. The phenomenon was first observed in the green alga *Scenedesmus obliquus* (Gaffron and Rubin, 1942). While three pathways for algal H_2 production (two in the light and one in the dark) are now known, the most direct pathway to H_2 from water employs light and both plant-type photosystems (PS) (Philipps et al., 2011), namely, PSII (oxidizes water) and PSI (reduces protons). The latter function is performed by direct electron donation from PSI-associated ferredoxin (FDX) (Fig. 7) to an O_2-sensitive hydrogenase (the enzyme that actually reduces protons and releases molecular H_2). The major type of hydrogenase, common to all eukaryotic algae, is a simple type of iron [FeFe]-hydrogenase. Plants do not contain hydrogenase genes and hence do not produce H_2. The second (light-driven) pathway uses NADH from the glycolytic breakdown of stored carbohydrate as an electron donor but employs only PSI to produce H_2 in the light (Fig. 7). The third pathway uses a pyruvate-ferredoxin-oxidoreductase (PFR) enzyme in a dark, fermentative pathway

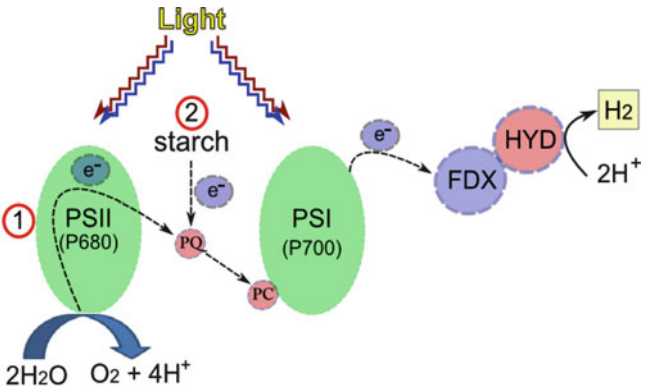

Figure 7. The hydrogen photoproduction pathway. Two different mechanisms by which H_2 can be produced are shown. (1) Electrons generated from the oxidation of water are transferred from activated photosystem II (PSII) to photosystem I (PSI) via the plastoquinone (PQ) pool and plastocyanin (PC). PSI in turn transfers electrons to ferredoxins (FDX) and then onto the hydrogenase (HYD) enzyme to produce H_2. (2) Electrons that are generated from the breakdown of starch are transferred to PSI via the PQ pool. PSII is bypassed in this pathway. A third, dark pathway can also produce H_2 as seen in the next section.

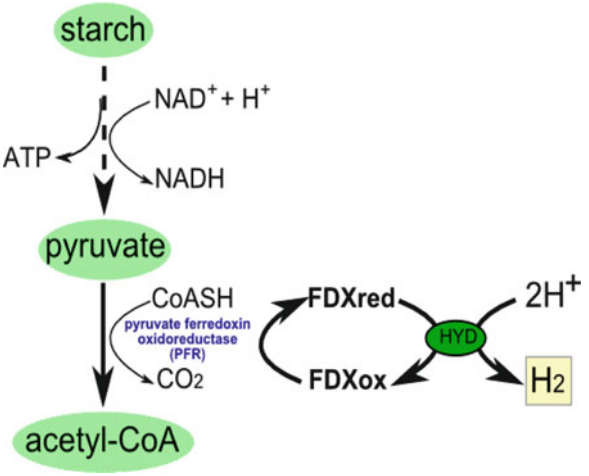

Figure 8. The dark fermentative hydrogen production pathway. Pyruvate, which is generated from the breakdown of starch, is oxidized to acetyl-CoA, along with the formation of CO_2. Ferredoxin (FDX) accepts the electrons released in this process and is reduced. The reduced FDX then transfers electrons to hydrogenases (HYD) to produce H_2. Text in *blue* represents enzymes.

similar to those found in many anaerobic systems (see the next section, Fig. 8). However, the activity of this pathway for H_2 generation is low in *C. reinhardtii* compared to the light-driven processes (Gaffron and Rubin, 1942; Gfeller and Gibbs, 1984; Hemschemeier and Happe, 2005; Meuser et al., 2009, 2012; Miura et al., 1982).

An important advancement in this area was reported 12 years ago with the discovery that depriving *C. reinhardtii* cultures of sulfate decreases the O_2-evolution activity of PSII, while leaving respiratory O_2-uptake activity unaffected (Melis et al., 2000). The net effect was that when O_2 evolution fell below the level of respiration, the culture became anaerobic (inducing the synthesis and activation of the [FeFe]-hydrogenase), and H_2 started bubbling out of the liquid medium after a couple of hours. The activity lasted for about 4 days, much longer than the couple of minutes observed previously (for non-nutrient stressed algae subjected to dark anaerobic treatment) but at a severe cost in terms of photosynthetic efficiency. The mechanism explaining the phenomenon in detail is now known (Ghirardi et al., 2010), and recent research describing H_2 photoproduction by immobilized algae has increased the light conversion efficiencies to about the limit of the system under the nutrient stress condition (Kosourov and Seibert, 2009). But because the hydrogenase enzyme is inactivated by O_2, the real potential for increasing H_2 productivity of algae must await (among other possible advances) the successful engineering of an O_2-tolerant enzyme. This would eliminate the need to severely downregulate photosynthesis (by sulfur deprivation) in order to see H_2 photoproduction. There has been a lot of recent activity in the Systems Biology area examining metabolic pathways associated with algal H_2 production (Catalanotti et al., 2012; Chen et al., 2010; Doebbe et al., 2010; Dubini et al., 2009; Magneschi et al., 2012; Matthew et al., 2009; Mus et al., 2007; Philipps et al., 2011; Terashima et al., 2010), but they have mostly resulted in improved understanding of the dark fermentative reactions as outlined in the next section.

3.3.2. The Dark Fermentation Pathway

Green algae are also capable of producing H_2 through a dark, fermentation pathway. Chlamydomonas, for example, can acclimate to anoxia by shifting from aerobic to fermentative metabolism leading to H_2 production (Gfeller and Gibbs, 1984, 1985; Kreuzberg, 1984; Ohta et al., 1987). Here, pyruvate is oxidized via a pyruvate ferredoxin oxidoreductase (PFR), generating CO_2, acetyl-CoA, and reduced ferredoxin, which in turn can transfer electrons to hydrogenase to produce H_2 (Fig. 8) (Gibbs et al., 1986). During dark fermentation, cellular starch reserves are metabolized to generate ATP, but the reduced pyridine nucleotide that is coproduced must be reoxidized to sustain the activity of glycolysis. The starch breakdown process provides a continuous supply of substrate (pyruvate) to the fermentation pathways (Dubini et al., 2009; Mus et al., 2007), and the reoxidation is linked to both organic acid and H_2 production in *Chlamydomonas* (Gfeller and Gibbs, 1984; Kreuzberg, 1984; Ohta et al., 1987). Although not well studied, this pathway is likely analogous to the H_2-producing heterofermentation pathways described in anaerobic bacteria (Saint-Amans et al., 2001) or in amitochondriate eukaryotes (Dyall et al., 2004). These organisms also couple pyruvate oxidation to ferredoxin reduction via PFR1. Pyruvate can alternatively be oxidized to formate with the release of acetyl-CoA, in a reaction that is catalyzed by pyruvate formate

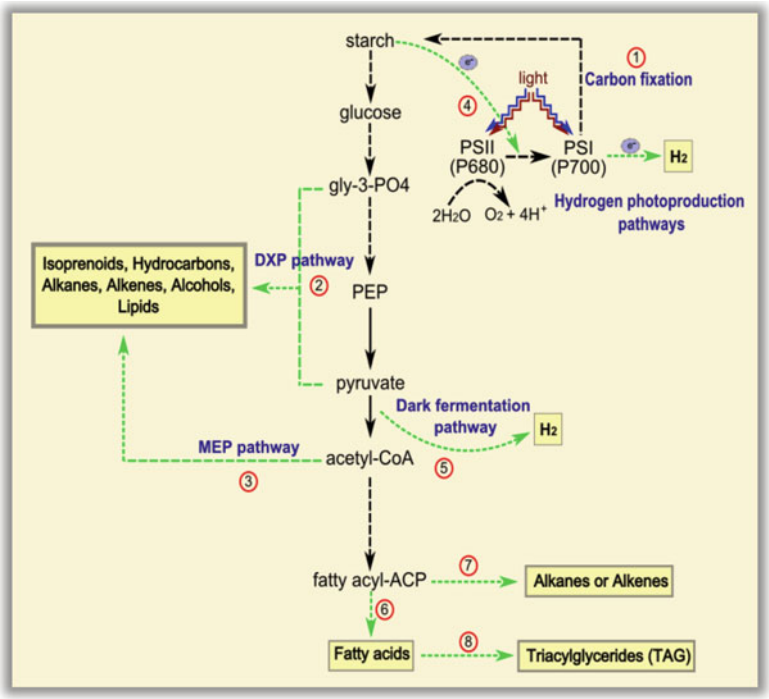

Figure 9. Biofuel production pathways in algae. The intermediates of the glycolysis pathway that originate from the breakdown of starch (stored during photosynthesis, (1)) are used for production of biofuels or their precursors: (2) and (3) synthesis of isoprenoids via the deoxyxylulose phosphate (DXP, from glyceraldehyde-3-phosphate and pyruvate) and mevalonate (MEP, from acetyl-CoA) pathways, respectively; (4) and (5) hydrogen production pathways via hydrogen photoproduction and dark fermentative pathways, respectively; (6) fatty acid synthesis pathway originating from acetyl-CoA; (7) synthesis of alkanes from fatty acyl-ACP; and (8) synthesis of triacylglycerides from fatty acid precursors.

lyase (PFL) in Chlamydomonas (Catalanotti et al., 2012; Hemschemeier et al., 2008; Mus et al., 2007).

3.4. PATHWAY SUMMARY

To put the information discussed in this chapter into a more global perspective, we have assembled a general schematic to highlight all the examined pathways (Fig. 9).

Although a scalable, commercially viable system has not as yet emerged from these pathways, studies that have produced this body of knowledge have provided a foundation to technologies being explored around the world.

4. Future Prospects for Applied Systems

Algae will most certainly be a leading contributor to the production of advanced biofuels over the next 10–15 years. As many as 150 algae companies are engaged in R&D activities aimed at producing cost-competitive, commercially viable technologies for a number of fuels that make use of the metabolic pathways discussed in this chapter. The more that is known about the biology upon which these emerging technologies are based, the quicker they are likely to come online. Many governments around the world are investing heavily in scientific research that will benefit this effort, and the fruits of this work will contribute to a future world with fewer environment problems directly attributable to the current use of fossil fuels.

5. Acknowledgments

The authors would like to thank colleagues in the Photobiology Group at NREL for a stimulating research environment, which led to our opportunity to write this chapter. This work was supported by the Computational Biology Program, Office of Biological and Environmental Research, US Department of Energy (MS), and by the NREL LDRD program.

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PART IV:
FROM WASTE WATER TO FUEL PRODUCTION

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R. Gordon and J. Seckbach (eds.), *The Science of Algal Fuels*,
Cellular Origin, Life in Extreme Habitats and Astrobiology, 425–445
DOI 10.1007/978-94-007-5110-1_23, © Springer Science+Business Media Dordrecht 2012

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WASTEWATER TREATMENT POND ALGAL PRODUCTION FOR BIOFUEL

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1. Municipal Wastewater Treatment Ponds

Municipal wastewater treatment ponds (called facultative ponds) rely on algal photosynthesis to convert sunlight energy, nutrients (N, P) and CO_2 into algal biomass. The algae release O_2 which promotes aerobic bacterial degradation of wastewater organic compounds to release more CO_2 and nutrients that are, in turn, assimilated by the algae (Oswald et al., 1957; Oswald, 1988a). Facultative ponds typically have an organic loading rate of 50–100 kg BOD_5 ha^{-1} day^{-1}, a depth of 1–1.5 m and a hydraulic retention time of 30–60 days. Facultative pond systems (often 2–4 ponds in series) are used at many thousands of municipal, agricultural and industrial wastewater treatment facilities worldwide and provide efficient removal of wastewater solids and BOD. However, nutrient (N, P) and faecal indicator removal is often poor and highly variable, and annual algal biomass productivity (ash-free dry wt) is low at 10–15 t ha^{-1} year (Davies-Colley et al., 1995; Craggs et al., 2003). A major issue with facultative ponds is that the algal biomass is discharged to receiving waters in the pond effluent because current algal harvest technologies are too expensive for use at any but the largest pond systems.

2. Wastewater Treatment High Rate Algal Ponds

High rate algal ponds (HRAPs) are relatively shallow, gently mixed, raceway ponds that were developed by Oswald and colleagues as a more intensive wastewater treatment pond technology that enable much higher removal of wastewater organic compounds, nutrients and faecal indicators than facultative ponds (Oswald et al., 1957; Benemann et al., 1980; Oswald, 1988a; Craggs, 2005). As early as 1960, Oswald also proposed using wastewater treatment HRAPs for the large-scale production of algae for conversion to biofuels (Oswald and Golueke, 1960).

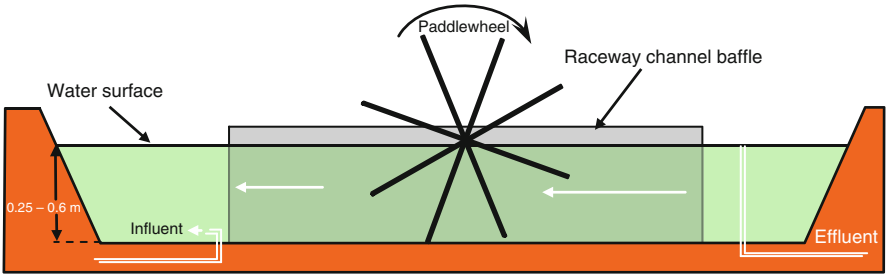

Figure 1. Schematic cross section of a high rate algal pond (HRAP).

Depending on climate, HRAPs are typically designed with an organic loading rate of between 100 and 150 kg BOD_5 ha^{-1} day. HRAP depth, hydraulic retention time and mixing speed are the main operational control variables. HRAP depth varies with wastewater clarity (0.25–0.6 m), and may be related to hydraulic retention time which varies seasonally (with solar radiation and temperature) in temperate climates (3–4 day in summer and 7–9 day in winter).

The channelised raceway design of HRAP enables uniform, low-energy mixing (typically 0.15–0.30 $m\,s^{-1}$) which is usually provided by a paddle wheel (Fig. 1). Mixing velocities higher than 0.3 $m\,s^{-1}$ consume too much power (which increases as a cube function of mixing velocity), and cause scouring of the pond (when clay lined), and thus are not recommended. Horizontal mixing velocities greater than 0.15 $m\,s^{-1}$ select for algal species which form colonies: species that are usually outcompeted in facultative ponds as the colonies settle faster than unicellular algae in quiescent water. Horizontal mixing also causes turbulent eddies that provide a vertical mixing component throughout the pond length and ensures that algal cells are intermittently exposed to sunlight as the depth of light penetration is usually only half to two-thirds of the pond depth depending on algal concentration (100–400 $g\,m^{-3}$) and wastewater clarity.

3. Advanced Wastewater Treatment Pond Systems

Wastewater treatment HRAPs are usually a component of advanced pond systems (APS) that typically include four types of ponds that are arranged in series (Fig. 2): advanced facultative ponds that settle and anaerobically digest wastewater solids, high rate algal ponds, algal settling ponds that harvest algae by gravity sedimentation and maturation ponds that provide additional disinfection mainly through exposure to sunlight UV radiation (Oswald, 1990, 1991; Craggs, 2005). The four ponds have an overall land requirement similar to that of two-pond facultative systems (Oswald, 1996).

APS not only achieve more efficient wastewater treatment than facultative pond systems but recover resources from the wastewater through capture of

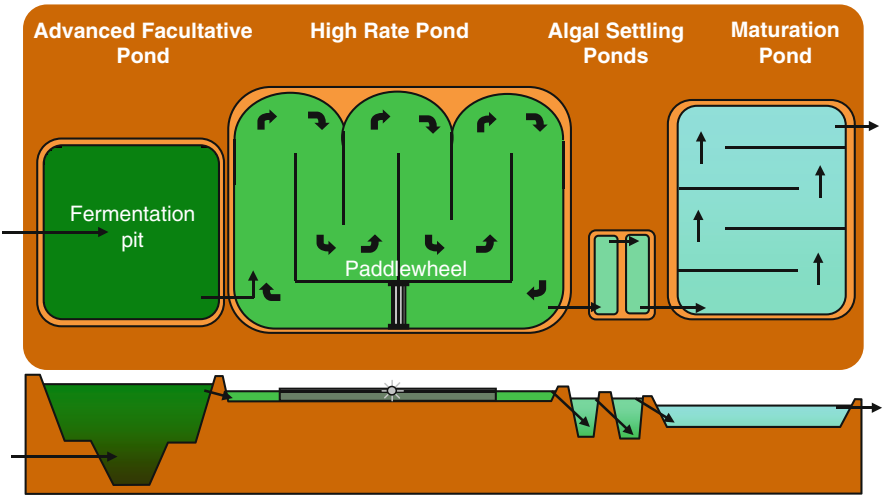

Figure 2. Schematic of municipal wastewater treatment advanced pond system.

Figure 3. Advanced pond systems for wastewater treatment in Northern California.

biogas in the advanced facultative pond and harvest of algae in the algal settling ponds (Oswald, 1991, 1996; Green et al., 1995; Craggs, 2005). However, APS technology has only been applied for wastewater treatment in some small Northern California cities, such as St. Helena (in 1967) and Hilmar (in 2000) (Fig. 3), and a few other installations around the world. There are several reasons for the low uptake of this technology: (1) until recently, nutrient removal has not been a major requirement of the wastewater treatment plants of most cities worldwide; (2) efficient harvest of HRAP algal biomass by gravity settling could not be reliably achieved, and current algal harvest technologies are too expensive; (3) use of harvested algal biomass as a soil amendment has had little economic value due to the low cost of inorganic fertiliser; (4) there is a lack of widespread APS

knowledge and design skills amongst the engineering profession; (5) although cost competitive with electromechanical treatment systems, APS require a relatively large land area; and (6) despite the high power demand of electromechanical treatment systems, operation costs are low due to the current low price of fossil fuel-derived electricity.

4. Commercial Algal Production

HRAP have much lower capital costs than closed photobioreactors but similar productivity and are therefore used to grow the majority (>90%) of current worldwide commercial algal production (for high-value nutritional products, pigments and chemicals). However, even the simplest, lowest cost HRAP (e.g. individual clay-lined ponds of >1 ha) growing algae on nutrient media with CO_2 addition could not produce biofuels economically (at current and near-future fossil fuel prices) without major advances in technology, including much higher algal and biofuel productivities than presently feasible (Benemann, 2003).

5. Wastewater Treatment HRAP with CO_2 Addition

Many of the wastewater treatment issues of APS and the poor economic viability of algal biofuel production using nutrient culture medium fed HRAP could be addressed by returning to the original concept of Oswald and Golueke (1960) in which algal biofuels are produced as a by-product of wastewater treatment in HRAP (Fig. 4). Conventional primary treatment (that removes wastewater solids) is used instead of an advanced facultative pond; the HRAP wastewater treatment

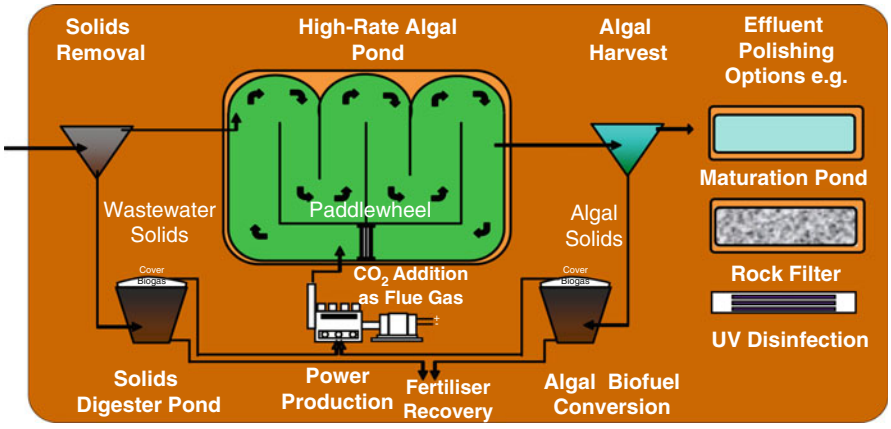

Figure 4. Schematic of a wastewater treatment HRAP with CO_2 addition.

(especially nutrient removal) capability, algal production and algal biomass harvestability are all enhanced by CO_2 addition; and the harvested algal biomass is then converted to biofuels (Benemann et al., 1978, 1980; Benemann and Oswald, 1996; Benemann, 2003).

6. Algal Production in Wastewater Treatment HRAPs

Productivity of wastewater treatment HRAPs varies with climate, local weather, wastewater strength, pond operation (e.g. depth, hydraulic retention time), dominant algal species, invertebrate grazing and infection by fungi, bacteria or viruses. Moreover, the biomass in wastewater treatment HRAP effluents is a combination of algae, bacteria and detritus formed during the wastewater treatment process. This biomass is poorly quantified but, based on microscopic observation, is typically composed of 70–90% algae. At moderate latitudes and Mediterranean climates, annual biomass productivities for wastewater treatment HRAPs are typically 30 t ha^{-1} year which are 2–3 times that of facultative ponds (10–15 t ha^{-1} year). Pilot-scale HRAPs treating domestic wastewater in New Zealand had productivities of about 30 t ha^{-1} year (Craggs et al., 2003), and somewhat higher productivities were reported in California (Benemann et al., 1980). However, algal productivity in wastewater treatment HRAP is depressed by severe carbon limitation, indicated by high daytime pond water pH levels (typically above 10), due to photosynthetic uptake of CO_2 and bicarbonate (Oswald, 1988a; Garcia et al., 2000; Craggs, 2005; Kong et al., 2010; Park and Craggs, 2010).

Carbon limitation is due, in part, to the low C:N ratio of wastewaters (typically 3:1 to 4:1 for municipal wastewater) compared to algal biomass (typically 6:1, ranging from 10:1 to 5:1 depending on whether N is limiting or not) (Benemann et al., 1980; Lundquist, 2008). Thus, domestic wastewaters contain insufficient C to remove all the N (and P) by direct assimilation into algal biomass. More importantly, C limitation, and the concomitant rise in pond water pH above 8.5, severely depresses the growth rates and productivity of algae (Weissman and Goebel, 1987; Kong et al., 2010). Although, by using available bicarbonate, some algal species are able to grow (with low productivity) even above pH 10. The inhibition of algal growth at high pH in wastewater treatment HRAP could also be in part due to high levels of free ammonia at high pH (Azov and Goldman, 1982; Azov et al., 1982; Konig et al., 1987). Further, intense photosynthesis in HRAPs also increases daytime dissolved O_2 levels, typically to 200–300% saturation, while supersaturation of oxygen promotes bacterial degradation of wastewater organic compounds, it can inhibit algal productivity, particularly at high pH and carbon limitation (Weissman et al., 1988). High pond pH, above ~8.5, can also inhibit the growth of aerobic heterotrophic bacteria that oxidise wastewater organic matter to CO_2 (Craggs, 2005). This sets up a feedback loop, which amplifies the effect of high pH and carbon limitation.

Addition of CO_2 to wastewater treatment HRAPs increases carbon availability and enables pond water pH to be maintained at an optimum (pH 7.5–8.5) for both algae and bacteria. The biomass productivity of wastewater treatment HRAPs can potentially be doubled with CO_2 addition to 60 t ha^{-1} year, of which perhaps 20% of the measured volatile suspended solids is non-algal (bacterial and detrital) biomass, as observed in small-scale trials (Benemann et al., 1980; Azov et al., 1982; Lundquist, 2008). Recent pilot-scale research during New Zealand summer conditions has shown that CO_2 addition to wastewater HRAP can increase algal biomass production by up to 100%, with projected productivities of 60 t ha^{-1} year (Heubeck et al., 2007; Park and Craggs, 2010). CO_2 addition also promotes nutrient removal by assimilation into algal biomass.

For wastewater treatment HRAPs, any available source of CO_2 could be used, the most likely being the flue gas from an electricity generator using biogas produced by anaerobic digestion of wastewater solids ("primary sewage sludge") and algal biomass harvested from the HRAPs (Eisenberg et al., 1981; Benemann, 2003). Pond water can also be used to directly purify biogas (e.g. scrub CO_2 and H_2S) (Conde et al., 1993; Mandeno et al., 2005), if the biogas needs to be compressed for use as vehicular fuel or for addition to a natural gas pipeline. However, loss of methane (a potent greenhouse gas) in the scrubbing water is an issue that needs to be resolved for biogas water scrubbing processes.

The algal biomass production potential (t/ML of wastewater) in wastewater treatment HRAPs is directly related to C utilisation, both from the wastewater and any added CO_2. With CO_2 addition, nutrients (N, P) can be assimilated to the maximum extent possible. Productivity and thus nutrient removal are limited mainly by daily solar radiation and temperature, and winter values determine the area necessary for effective year-round wastewater treatment, which increases with increasing latitude (Oswald et al., 1957; Bouterfas et al., 2002; Jeon et al., 2005; Voltolina et al., 2005).

Further algal productivity increases, beyond those that could be achieved through CO_2 addition, are desirable to reduce the area of the wastewater treatment HRAP system and thus improve the economics of both wastewater treatment and wastewater algal biofuels production. It must be noted that further increasing productivity would not increase the amount of algal biomass produced, as that is limited by the amounts of nutrients present in the wastewater and assimilated into the biomass, but increasing productivity will reduce the area of ponds required. One necessary, but not sufficient, approach to increase algal productivity is to select for algal strains that thrive in the HRAP environment – high sunlight, diurnal temperature fluctuations and supersaturated dissolved O_2 (Weissman et al., 1988).

Achieving high productivity also requires dealing with herbivorous zooplankton such as rotifers and cladocerans, which graze on algae and can rapidly proliferate and reduce algal biomass concentrations to low levels within a few days (Benemann et al., 1980; Picot et al., 1991; Cauchie et al., 1995; Nurdogan and Oswald, 1995; Smith et al., 2009). For example, rotifers and cladocerans at

densities greater than 100 L^{-1} were reported to reduce the algal concentration in a wastewater treatment HRAP by 90% within two days (Oswald, 1980), and several days of grazing by a population of the cladoceran, *Daphnia* sp., reduced the chlorophyll *a* concentration of a pond by 99% (Cauchie et al., 1995). Algae are also susceptible to fungal parasitism and bacterial or viral infection which can deplete the pond algal population within a few days and result in changes in algal morphology, species diversity and succession (Wommack and Colwell, 2000; Short and Suttle, 2002; Kagami et al., 2007).

Therefore, to maximise HRAP algal productivity, populations of zooplankton grazers, parasitic fungi and infective bacteria and viruses must be controlled. Zooplankton grazer populations may be limited by application of chemicals or invertebrate hormone mimics or by increasing pond water pH to 11, particularly if the pond water has a high ammoniacal-N concentration (O'Brien and De Noyelles, 1972; Schluter and Groeneweg, 1981; Oswald, 1988b). There are no practical control methods yet for fungal parasitism or bacterial and viral infections, and further research is required to fully understand their influence on algal productivity in wastewater treatment HRAP.

7. Performance of Wastewater Treatment HRAP with CO_2 Addition (HRAP + C System)

The HRAP with CO_2 addition (HRAP + C) system can be used to provide more effective aerobic treatment (oxidation of wastewater organic compounds, BOD) and improved removal of nutrients, faecal indicators and algal biomass than both facultative pond systems and advanced pond systems. Moreover, the HRAP + C system is much more cost-effective and energy efficient than electromechanical wastewater treatment technologies providing an equivalent level of wastewater treatment. A 5 ha wastewater treatment HRAP + C system was successfully operated in Christchurch, New Zealand (Fig. 5), to demonstrate upgrading facultative

Figure 5. HRAP + C system (5 ha) operating in Christchurch, New Zealand.

ponds and production of algae for whole biomass conversion to biofuel using a near critical water reactor (NCWR).

7.1. AEROBIC TREATMENT

Although some aeration is provided by paddle wheel mixing, daytime supersaturated dissolved O_2 levels resulting from algal photosynthesis in the HRAP + C system enable very efficient aerobic treatment (organic matter degradation). The power required for HRAP paddle wheel mixing depends mainly on mixing velocity. For an HRAP with a water depth of 0.3 m and a horizontal flow velocity of 0.15 $m\,s^{-1}$, the power required to operate the paddle wheel is ~15 kW ha^{-1}. The aeration efficiency of HRAP varies between 0.05 and 0.20 $kWh_e\ kg^{-1}\ O_2$ produced depending on season, insolation and other factors (Benemann et al., 1980; Oswald, 1988b; Green et al., 1995). For a wastewater with BOD_5 concentration of 200 $g\,m^{-3}$, this equates to a power requirement of between 15 and 60 $kWh_e\ ML^{-1}$. In comparison, activated sludge requires from 230 to 970 $kWh_e\ ML^{-1}$ (based on the efficiency of different types of aerators: 0.4 to 1.7 $kWh_e\ kg^{-1}\ O_2$) (Owen, 1982; Metcalf and Eddy, Inc., 1991; Green et al., 1995).

7.2. ALGAL NITROGEN REMOVAL

Nitrogen removal by nitrification-denitrification is a common electromechanical nutrient removal process, but it is costly and energy intensive. A typical wastewater primary effluent (after settling) with an organic nitrogen concentration of 40 $g\,N\,m^{-3}$ would require aeration energy of ~400–1,000 $kWh_e\ ML^{-1}$ of wastewater for the nitrification step alone (this is in addition to that required for BOD removal) (Owen, 1982). The HRAP + C system, where sufficient land is available, could provide low-energy tertiary-level nutrient removal, for little more energy than an HRAP designed for BOD removal (Benemann et al., 1978; Eisenberg et al., 1981; Nurdogan and Oswald, 1995; Woertz, 2007; Park and Craggs, 2010). For example, assuming a 3:1 C:N ratio in the wastewater, a 6:1 C:N ratio for algal biomass (48% C, 8% N) and no change in CO_2:O_2 stoichiometry, a doubling (100% increase) in biomass production resulting from CO_2 addition could enable complete nitrogen removal (Heubeck et al., 2007; Park and Craggs, 2010).

7.3. ALGAL PHOSPHORUS REMOVAL

Algal biomass can exhibit N:P ratios ranging from nearly 4:1 (under nitrogen-limiting conditions) to about 30:1. These N:P ratios correspond to algal N and P compositions ranging from a high of 8% N and about 1% P to a low of about 4% N

and 0.35% P (under nitrogen- or phosphate-limiting conditions, respectively). Near-complete assimilation of both N and P into algal biomass from wastewaters with a large range of concentrations of these nutrients is therefore theoretically possible in HRAP with CO_2 addition (Benemann, 2003) and has been recently demonstrated experimentally by Woertz et al. (2009) and at pilot-scale by Park and Craggs (2010,2011). Nutrient assimilation rates can reach 16 kg N ha^{-1} day and 2 kg P ha^{-1} day, based on the typical algal nutrient composition of 8% N and 1% P, and an average productivity of 20 g m^{-2} day of algal biomass. These removals are achieved at much lower capital and operation costs compared to conventional electromechanical treatment technologies (Owen, 1982; Craggs et al., 1999). A key issue for tertiary-level nutrient removal is that the algal cultures have the ability to maintain high productivity when dissolved N has been reduced to low levels (e.g. <1 g m^{-3}). This is based on the fact that it is the internal, not external, nutrient concentration which determines growth rates and productivity, and nutrients are supplied continuously in the influent wastewater (Benemann, 2003; Woertz et al., 2009). In temperate locations, seasonal variation in algal productivity will limit nutrient removal by assimilation into algal biomass during winter.

7.4. ALGAE AUGMENTED NUTRIENT REMOVAL PROCESSES

Nutrient removal processes such as ammonia volatilisation and phosphate precipitation with cations occur in wastewater treatment HRAPs without CO_2 addition, when intense daytime algal photosynthesis results in CO_2 limitation and increases the pond water pH (Nurdogan and Oswald, 1995; Garcia et al., 2000; Craggs et al., 2003; Heubeck et al., 2007). However, these processes are greatly reduced by CO_2 addition to the ponds. For example, Park and Craggs (2011) demonstrated that daytime control of maximum pH to below 8 with CO_2 addition reduced nitrogen loss by ammonia volatilisation from 24% (in a control HRAP without CO_2 addition) to ~9%.

7.5. DISINFECTION

Disinfection of wastewater treatment plant effluent is typically provided by chlorination, ozonation or UV treatment. Chlorination requires 20–540 kWh_e ML^{-1} to generate chlorine, depending on the organic content of the effluent (Owen, 1982). Therefore, if the algal biomass is not efficiently harvested, chlorine requirements are high. Ozonation (100–200 kWh_e ML^{-1}) and UV (20–100 kWh_e ML^{-1}) use less power, but UV requires a very low turbidity effluent and thus a high level of algal removal (Owen, 1982). HRAPs promote natural disinfection mechanisms driven by sunlight, augmented by daytime supersaturated O_2 levels (100–300%) due to algal photosynthesis (Davies-Colley, 2005).

8. Harvesting Wastewater Treatment HRAP Algae

Effective and low-cost removal of algal biomass from HRAP effluent is imperative to achieve both a high effluent quality and an economically competitive wastewater treatment process. HRAP algal harvesting is challenging due to (1) low and varying solid concentration (typically 0.01% to 0.04% solids), (2) cell densities similar to water (1.08–1.13 kg L^{-1}), (3) small cell size (5–25 μm) and (4) strong negative surface charge. The latter (known as "zeta potential") may be associated with exponential growth (Moraine et al., 1979; Lavoie and de la Noue, 1987).

Various harvesting methods have been applied over the years, the main ones being (1) centrifugation (energy intensive, ~1 kWh_e m^{-3} pond water or ~2–3 kWh_e kg^{-1} algae), (2) filtration (ineffective due to clogging of filters and/or high cost), (3) microstraining (only effective for filamentous or large colonial algae) and (4) chemical flocculation followed by sedimentation or dissolved air flotation (DAF) (Oswald and Golueke, 1960; Benemann et al., 1980; Benemann and Oswald, 1996; Shen et al., 2009; Tampier, 2009; Brennan and Owende, 2010; Mata et al., 2010). However, these processes are either not applicable to the algae growing in wastewater treatment HRAP (e.g. filtration) or are too expensive (centrifugation, chemical flocculation).

The process currently employed for algal biomass removal from large facultative oxidation pond effluents is chemical flocculation (using lime, alum, ferric chloride, cationic polyacrylamides, etc.) to form large (1–5 mm) flocs that can be removed by simple settling or by dissolved air flotation (DAF). DAF provides somewhat higher concentration of solids and uses less flocculating chemicals but adds a further ~0.6 kWh_e kg^{-1} algae for air compression. The large amounts of chemical flocculants required are expensive and make it difficult to use the algal biomass, even in anaerobic digestion. Thus, the chemically flocculated algal sludge is typically disposed of either back to the ponds (long-term storage) or to landfill. Centrifugation provides a high-solids biomass, of about 20–25% solids, but the high capital cost and operating energy requirements of centrifugation make this process economically viable only for secondary thickening of harvested algae which already has a 2–4% solid concentration. The challenge is thus to develop a low-cost harvesting method that can produce algal biomass with such a solid concentration.

Wastewater treatment HRAPs select for particular genera of green algae, including *Scenedesmus* sp., *Micractinium* sp., *Actinastrum* sp., *Pediastrum* sp., *Dictyosphaerium* sp. and *Coelastrum* sp., that often form large (50–200 μm) colonies (Benemann et al., 1980; Oswald, 1988a; Banat et al., 1990; Green et al., 1996; Wells, 2005; Heubeck et al., 2007; Park and Craggs, 2010). Microstraining was first proposed as a low-cost harvest method for the large algal colonies (Benemann et al., 1978). However, it was observed that algae removed from the ponds, under quiescent conditions, could self-flocculate, aggregating to form large flocs ("bioflocculation"), and settle with over 90% solid removal (Benemann

et al., 1980; Craggs et al., 2003). This bioflocculation phenomenon is not well understood, but it has been observed with many algae and growth conditions. Bioflocculation can produce a concentrated algal biomass slurry (3–4% DM) and may be promoted by stress conditions, such as nutrient (e.g. N) limitation, or by recycling some of the settled algal biomass (Benemann et al., 1980; Eisenberg et al., 1981; Park and Craggs, 2010). Further research is required to understand and perfect this low-cost harvest process and increase its reliability.

9. Biofuels Production from Wastewater Treatment HRAP Algal Biomass

Conversion of algal biomass harvested from wastewater treatment HRAPs to biofuels could involve one or a combination of four main pathways, discussed briefly below:

1. Anaerobic digestion to produce biogas (methane and CO_2).
2. Extraction and conversion of algal lipids (oils) to biodiesel, green diesel, etc.
3. Fermentation of algal carbohydrates to ethanol.
4. Near-critical water catalytic conversion, gasification or pyrolysis of algal biomass to produce hydrocarbon gases and/or biocrude oils.

9.1. ANAEROBIC DIGESTION TO BIOGAS METHANE

Harvested algal biomass (or the algal residues remaining after oil extraction or ethanol fermentation, see below) can be anaerobically digested to produce biogas (60–80% methane, balance CO_2), with a typical yield similar to that of heated mixed digesters (0.30–0.45 m^3 CH_4 kg^{-1} added algal volatile solids, VS), usually with 50–60% volatile solid conversion (Golueke and Oswald, 1959; Eisenberg et al., 1981; Lundquist et al., 2010; Sukias and Craggs, 2011). Lower yields, compared to other organic substrates, have been attributed to both the relatively refractory nature of algal cell walls and ammonia inhibition. Pretreatment (e.g. heating) of algal biomass has been shown to improve digestibility under mesophylic conditions (Chen and Oswald, 1998). Inhibition of anaerobic digestion can occur at free ammonia concentrations above 4,000–6,000 g NH_3–N m^{-3} (Siegrist et al., 2005). Algal biomass contains typically 8% N of which up to 70% may be released as ammonia during digestion (Golueke and Oswald, 1959). Ammonia toxicity of algal digestion could be overcome by (1) concentrating algal biomass to no more than 5% solids prior to anaerobic digestion (to maintain ammonia levels below 3,000 g m^{-3}), (2) co-digestion with low N organic wastes (e.g. wastepaper, primary sewage sludge) or (3) adaptation of the methanogenic bacterial inoculum to higher ammonia levels. All three options could be applied simultaneously. Co-digestion of HRAP algae biomass with primary sewage sludge can be readily demonstrated (Heubeck et al., 2007; Lundquist et al., 2010) and is similar to electromechanical wastewater treatment plant co-digestion of primary and secondary sludges.

Addition of other available wastes (e.g. wastepaper, Yen and Brune, 2007) increases methane production but would need to be justified economically based on tipping fees received and value of the methane, minus other values of wastepaper use and any additional cost of digestate disposal. Cost-effective anaerobic digestion could be achieved using simple covered digester ponds, which could be fed with algal biomass harvested by bioflocculation (typically 3–4% solids concentration), compared to the 5–10% solids required for conventional, and more expensive, mesophylic heated mixed digesters.

9.1.1. Uses of Biogas

Biogas methane has an energy content of 33.8 MJ m^{-3} (0.67 kg) CH_4 at STP (equivalent to about 1 L of petrol) and can be used directly for heating (9.39 kWh_{heat} m^{-3} CH_4) or for electricity generation at 30% conversion efficiency (2.82 kWh_e m^{-3} CH_4 and simultaneous heat generation ~4.70 kWh_{heat} m^{-3} CH_4). Essentially ~1 kWh_e can be generated from the biogas produced from 1 kg algae assuming a yield of 0.35 m^{-3} CH_4/kg of VS of algal biomass added (Oswald, 1988a, b). This power can be used to displace electricity requirements of the wastewater treatment plant, with any surplus exported to the grid (though this would require additional capital investment for transformers, line upgrades, etc). Biogas can also be cleaned (desulphurised, stripped of CO_2), dried and compressed (>20 MPa) for export into natural gas pipelines or use as transport fuel. However, loss of methane (a potent greenhouse gas) in the scrubbing water is an issue that needs to be resolved. For wastewater treatment plants, power generation is the most widely applicable and lowest cost option.

9.2. TRANSESTERIFICATION OF ALGAL OIL TO BIODIESEL

Biodiesel production from oils extracted from algae grown in HRAPs (though not on wastewaters) was the main research focus of the 1980–1996 US Dept. of Energy Aquatic Species Program (ASP) (Sheehan et al., 1998). The ASP projected that in suitable climates, algae could have higher oil yields than most terrestrial crop plants, due to their potential high productivity, of up to 100 t algae dry matter ha^{-1} year, with up to 50% oil (as triglycerides) content thought to be attainable (Benemann and Oswald, 1996). However, these productivities and oil contents were long-term, speculative projections, with currently achievable values for both productivity and oil content perhaps only half of these values. Moreover, they are dependent on the algal species and even more importantly on strains of species, and with culture conditions, e.g. nitrogen limitation, which often greatly increases oil content of the algae, but not productivity (Feinberg, 1984; Coleman et al., 1987; Cooksey et al., 1987; Benemann and Tillett, 1988; Chelf, 1990; Weyer et al., 2010; Brennan and Owende, 2010). How to simultaneously maximise oil content and productivity is one of the major unresolved problems in algal biofuels production and has not yet been practically shown at scale.

9.2.1. Algal Oil Extraction

Another major issue is the economical extraction of the oil from the algae. Benemann and Oswald (1996) proposed a process involving cell breakage, homogenisation and centrifugation to recover the oil, and much work is ongoing in this area. If drying of the biomass is required, this will add significantly to the overall costs, even for sun or waste heat drying (the only plausible methods). Also, algal oils are not pure triglycerides, but generally contain large amounts of free fatty acids and mono- and diglycerides that are not suitable for direct transesterification (Feinberg, 1984). Further, a high proportion of fatty acids in algae are polyunsaturated, often long-chain, fatty acids that are not suitable for biodiesel production. Therefore, processes that recover and can use all algal lipids classes (mono-, di-, triglycerides, etc.) are of particular interest. In the case of algal biomass grown on wastewaters, harvested algae typically contain 20–30% oil. Maximising oil content, yield or quality would not be a priority, as any oil co-product would only be part of the revenue stream, with the residues anaerobically digested to produce biogas.

9.3. FERMENTATION OF CARBOHYDRATE TO BIOETHANOL

Bioethanol (and biobutanol, although not yet commercialised) could be produced from the fermentable carbohydrate (e.g. starch) portion of algal biomass by conventional yeast fermentation, followed by distillation. However, the carbohydrate content of algal biomass (typically less than 20% of dry matter) is too low for practical ethanol fermentation. As in the case of algal oil production, a higher content of fermentable carbohydrates can be induced by nitrogen (and other nutrient) limitation, depending on the species and strain of algae used. Production of algal biomass with a high starch (e.g. 70%) content, at high productivity, appears to be more feasible than production of algal oils. However, this option has received relatively little attention.

9.4. NEAR-CRITICAL THERMOCHEMICAL CONVERSION

Wet algal biomass (75–95% water content) may be converted to hydrocarbon gases and biocrude oil at high pressure (>20 MPa) and temperature (>300°C) in the presence of a catalyst (Chandler et al., 1998; Yesodharan, 2002; Matsumura et al., 2005). This conversion technology has the similar advantage to anaerobic digestion, in that the algal biomass does not have to be dried, the entire biomass can be converted into biofuels and that the nutrients (in particular N) can be recovered in the process water and are not emitted to the atmosphere as in conventional thermochemical, including combustion, processes. However, more research is required to demonstrate the viability of this technology.

10. Economics of Algal Wastewater Treatment and Biofuels Production with HRAP + C

Capital and operating costs of advanced pond systems for secondary wastewater treatment (BOD_5 removal) (Figs. 2 and 3) are estimated to be only a quarter to a third those of electromechanical secondary-level activated sludge treatment (Green et al., 1995; Downing et al., 2002). Similar or even lower ratios would likely apply in comparing tertiary treatment (nutrient removal) with the HRAP + C system to electromechanical systems that achieve nutrient removal. By replacing the advanced facultative pond and the algal settling ponds of an advanced pond system with conventional primary sedimentation and a bioflocculation settling process for algal removal, respectively, the HRAP + C system would have no more, and possibly less, land area. For the HRAP + C system, the capital and operating costs of algal production and harvesting are essentially fully covered by the wastewater treatment function, with biofuels a relatively minor co-product, which does not significantly impact the overall HRAP + C system economics.

11. Environmental Benefits of HRAP + C Wastewater Treatment and Biofuels Production

Beyond economics, algal wastewater treatment with coproduction of biofuels has fewer environmental impacts ("footprint") in terms of land, water, energy and fertiliser use than schemes for algal biomass production exclusively for biofuels (Borowitzka, 1999, 2005; Benemann, 2003; Tampier, 2009; Clarens et al., 2010). The environmental benefits, from greenhouse gas (GHG) abatement and sustainability in general, also strongly favour HRAP + C systems compared to electromechanical treatment processes (typically advanced activated sludge systems). Algal biofuel production from wastewater treatment HRAP with CO_2 addition abates GHG emissions by several mechanisms (Benemann, 2003; Lundquist et al., 2010):

- Reduction in energy use (mostly electricity and GHG emissions from fossil fuel used for generation) compared with electromechanical wastewater treatment processes. By using sunlight energy and photosynthesis, HRAP + C wastewater treatment systems abate between 100 and 400 kg of CO_2 ML^{-1} treated, compared to fossil energy that would have powered electromechanical treatment (e.g. activated sludge; Green et al., 1995; Benemann, 2003). Nitrogen removal in HRAP + C would abate a further 100–400 kg of CO_2 ML^{-1} treated, compared to conventional processes. The solar disinfection also provided by the HRAP + C system decreases the need for GHG emission from the chemicals and power used by other disinfection processes.
- Substitution of biofuels for fossil fuels (such as biogas-generated electricity) offsets GHG emission from fossil fuel use for generation. GHG abatement

resulting from biofuels replacing fossil fuels depends on the source of power and specific fuel being replaced. For example, generation of electricity from biogas methane abates 0.4 kg CO_2 kWh_e^{-1} from natural gas electricity generation compared to about 0.8 kg for CO_2 kWh_e^{-1} from coal electricity generation (NZMED, 2007). Assuming an intermediate value between natural gas and coal, as well as 1,000 kWh generated from the biogas produced by 1 t of algal biomass, 0.6 t of CO_2 could be abated per tonne of algae produced.

- Use of recovered wastewater nutrients and carbon in algal biofuel residues as fertiliser offsets GHG emissions associated with nitrogenous fertiliser production and phosphate rock mining. Recycling algal biomass (~8% N, ~1% P) or nutrient-rich residues following biofuel conversion for fertiliser use would reduce the need for synthesis of ammonia fertilisers and mining of phosphate rock. The energy required for the manufacture of 1 kg of N fertiliser (as ammonia) is about 16 kWh (mostly natural gas, with emissions of 3.15 kg CO_{2EQV}) and the mining and processing of 1 kg of P (as phosphate) fertiliser requires the equivalent of 4.5 kWh of fuel (mostly liquid fuels, with emissions of 1.4 kg CO_{2EQV}) (West and Marland, 2001; Wood and Cowie, 2004). Therefore, the use of 1 kg of algae (8% N, 1% P) as fertiliser would reduce CO_2 emissions from inorganic fertiliser manufacture by about 0.27 kg CO_{2EQV}.
- Reduced GHGs emitted during conventional electromechanical wastewater treatment, such as methane and nitrous oxide.

12. Conclusions

Municipal wastewater treatment using HRAPs with CO_2 addition, and with algal biofuels as coproducts – the HRAP + C system – provides the potential for energy-efficient and effective tertiary-level wastewater treatment at significantly lower costs compared to electromechanical technologies. Wastewater enriched with flue gas CO_2 is an excellent growth medium (water, nutrients and buffering) for naturally occurring algae. Bioflocculation of algal biomass followed by settling is a very promising low-cost approach to algal harvesting, but further research is required to demonstrate it at a full-scale with year-round reliability. Of the several pathways to convert harvested algal biomass to biofuel, those that use the whole algal biomass and require little or no dewatering of the harvested algae appear to be most appropriate for use in combination with wastewater treatment. In particular, anaerobic digestion of algal biomass along with the settled wastewater solids would be the easiest to apply as the capital and operation costs of anaerobic digestion, and biogas use infrastructure would be funded by the wastewater treatment plant. Harvesting algae from wastewater treatment HRAP effluent enables recovery of wastewater nutrients that can be recycled as fertiliser after biofuel conversion. Wastewater treatment HRAP also provides GHG abatement from a combination of low-energy wastewater treatment, renewable fuel production and fertiliser recovery. Since the HRAP + C system is already a viable technology

for near tertiary-level wastewater treatment, it could provide a "testing ground" to develop and refine full-scale algal production, harvest and biofuel conversion technologies that may be implemented in the future when higher fossil fuel costs make stand-alone HRAP systems for biofuel production economical.

13. Acknowledgements

The authors wish to thank Jason Park, Stephan Heubeck and Ian Woertz who provided valuable contributions to this chapter. NIWA funding was provided by the New Zealand Foundation for Research Science and Technology.

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http://www.uvm.edu/~epscor/index.php?Page=events/2010_algae_for_energy_conference.php

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R. Gordon and J. Seckbach (eds.), *The Science of Algal Fuels*,
Cellular Origin, Life in Extreme Habitats and Astrobiology, 447–466
DOI 10.1007/978-94-007-5110-1_24, © Springer Science+Business Media Dordrecht 2012

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WASTEWATER TREATMENT INTEGRATED WITH ALGAE PRODUCTION FOR BIOFUEL

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1. Introduction

A critical hurdle in algae biomass production is cost-efficiency. Integrated algae production and wastewater treatment would be a cost-effective approach to developing algae biofuels (Benemann et al., 1982; Sheehan et al., 1998; Lundquist et al., 2007; Lundquist, 2008; DoE, 2010) given that algae-based treatment of wastewater has been shown to be 40% more cost-effective than the best conventional means (Downing et al., 2002). The successful role of algae in wastewater treatment has been well documented since the early 1950s in studies by Oswald and his group at the University of California at Berkeley (1953, 1957, 1962, 1990, 2003). A wealth of research on algal treatment of wastewaters has been developing ever since, though not always with functions beyond waste treatment explored.

Wastewater runoff is a known environmental problem contributing to eutrophication of receiving water bodies (Blackburn, 2007). In addition to capital investments required, the cost of wastewater treatment is rising. Various low-quality wastewater streams (municipal, industrial, agricultural: dairy and piggery farms) have been studied as nutrient sources for growing algae (e.g., Oswald et al., 1953; Oswald, 2003; Hills and Nakamura, 1978; Ahn et al., 2001; Wilkie and Mulbry, 2002; Lundquist, 2008; Mulbry et al., 2008; Woertz et al., 2009).

Algae grown in municipal wastewater was reported to successfully remove over 99% of both the ammonium and orthophosphate in a waste stream composed of total Kjeldahl nitrogen (TKN) and total nitrogen 51.0 mg/L, ammonium 39.0 mg/L, organic nitrogen 12.0 mg/L, phosphate 2.1 mg/L, total suspended solids (TSS) 93.0 mg/L, and volatile suspended solids (VSS) 58.0 mg/L at a pH of 7.2 (Woertz et al, 2009). Algal-based "Advanced Integrated Treatment Pond" systems used for municipal sewage treatment have been recorded removing

over 90% of total nitrogen in the wastewater stream (Oswald, 1990). The "Algal Turf Scrubber" system (Adey et al., 1993) has proven that algae fed on a diet of dairy manure can recover over 95% of the nitrogen and phosphorus in agricultural manure wastewater (Mulbry et al., 2005). Anaerobically digested dairy wastewater has been reported to be constituted of TKN 257.0 mg/L, ammonium (as N) 136.0 mg/L, total phosphorus 34.0 mg/L, TSS 1,020 mg/L, and VSS 905.0 mg/L, at a pH of 7.9 (Sooknah and Wilkie, 2004). Total nitrogen in anaerobically digested dairy wastewater tends to be particularly high, with values around 412.0 mg/L reported (Kebede-Westhead et al., 2003). The nutrient composition of brewery wastewater is close to municipal wastewater, with TKN reported as 23.0 mg/L, phosphate (as P) at 9.0 mg/L, TSS of 212.0 mg/L, and VSS of 139.0 mg/L (Ahn et al., 2001). Dahiya et al. have been utilizing highly concentrated brewery wastewater to grow algae for biofuel, with total nitrogen of 49 mg/L and total phosphorus 21.89 mg/L (unpublished data). It is evident from the favorable nutrient compositions of these three common waste streams that wastewater treatment by algae evidently has an existing track record of success. Considering that recent estimates of freshwater resources suggest looming shortage, an additional 2.6 billion people will be sharing the finite resource by 2025, and the global water demand will exceed the supply by 56% (Clarke and Barlow, 2003). Algae-based treatment could provide new streams of treated water for reuse in any number of agricultural, municipal, or industrial systems, thereby reducing the burden on our already stressed water resources.

Integrated systems for wastewater treatment hold great potential for cutting costs and generating new revenue and products for farmers (and especially dairy farms). All farmers are required to meet standards for handling and recycling of manure (nutrients) per guidelines from their State and the US Environmental Protection Agency (EPA). Similar standards are required worldwide in many other countries. According to a United States Department of Agriculture (USDA) report (Liebrand and Ling, 2009), in the United States alone, an estimated 500 billion pounds of manure was produced by the 9.158 million milk cows on 71,510 operations in 2007. According to the USDA, the wastewater by-products of milk production are routinely handled by costly means, such as collecting it, storing it, and spreading it over the land, and only a fraction of it is treated by biodigesters as they tend to be expensive for farmers. Nutrient control (removal of nitrogen and phosphorus) on farms and from other anthropogenic sources is a technical and economical challenge (Pizarro et al., 2006). Waste pretreatment by anaerobic digestion effectively treats biological oxygen demand (BOD) but is poor at removing nutrients (Tchobanoglous et al., 2003). In order to attain the effluent quality thresholds for discharge into surface waters, the treated wastewater effluent coming out of a biodigester needs to undergo further treatment, for which aerobic processes are normally employed. Algae is known to utilize many components found in these effluents, including nitrogen, phosphorus, potassium, heavy metals, and other organic compounds from the wastewaters (Oswald, 1990; Wilkie and Mulbry, 2002; Kebede-Westhead et al., 2003; Pizarro et al., 2006; Mulbry et al., 2008).

An advantage of using agricultural manure wastewater is that unlike municipal wastewater, it does not pose a significant health hazard, as it is devoid of human pathogens, heavy metals, or toxic materials (Abeliovich, 2007).

Algae have also been widely researched and employed to successfully treat a variety of commercial and industrial wastewater streams. Dosnon-Olette et al. (2010) used immobilized cultures of *Scenedesmus obliquus* and *Scenedesmus quadricauda* to phytoremediate the agricultural fungicides dimethomorph and pyrimethanil and the herbicide isoproturon. A microbial treatment consortium of algae and bacteria treating wastewater containing the aromatic compound *p*-chlorophenol included the microalgae species of *Chlorella vulgaris* and *Coenochloris pyrenoidosa* (Lima et al., 2004). Textile wastewaters and their associated dyes have been successfully treated using *Chlorella vulgaris* in high-rate algae ponds (Lim et al., 2010). Carpet mill effluents have been treated by combining 85–90% carpet wastewater with 10–15% municipal wastewater to assess the abilities of a consortium of 15 algae for algal biomass production and biodiesel production. They found that roughly 60% of the oils produced in the consortia could be converted into biodiesel (Chinnasamy et al., 2010). Todd et al. have been using complex ecologically based systems since the 1980s for the treatment of industrial and municipal wastewaters, using technologies based heavily on consortia of algae, bacteria, and higher plants (Todd and Todd, 1993; Todd and Josephson, 1996; Todd et al., 2003).

Many metals can be bioremediated and biosorbed from wastes using algae such as *Chlorella* sp. and *Scenedesmus* sp., among others (Darnall et al., 1986; Greene et al., 1986; Terry and Stone, 2002; Davis et al., 2003; Peña-Castro et al., 2004; Kalin et al., 2004; Muñoz and Guieysse, 2006; Perales-Vela et al., 2006). This attribute has been covered extensively in the literature and so will not be explored at length here. An interesting application of the biosorption of heavy metals by algae would be the potential "mining" of waste streams to recover heavy metals of interest, either to prevent their discharge or for recycling of the metal back into a metallurgical process. In sufficient volume and with a high enough metal concentration, economically valuable metals recovered with algae could provide an additional revenue stream to wastewater and/or algae biofuel producers to offset costs.

A conflict that arises in the integrated system is that maximal algal productivity, crucial to feasible large-scale biofuel production, requires high nutrient levels, whereas optimal wastewater treatment requires stripping of all nutrients from the effluent prior to discharge, which requires longer time periods.

2. Feasibility of an Integrated System

The most feasible way to integrate algae biomass for biofuel production with wastewater treatment is to base the algal culturing process on a symbiotic relationship shared by algae mono-/polycultures along with other (micro) organisms

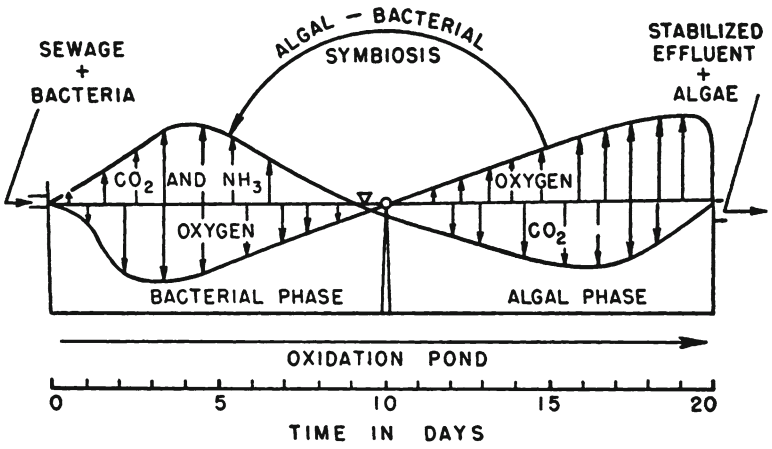

Figure 1. Schematic representation of an oxidation pond (Reproduced from Oswald et al., 1953).

in the wastewater. Oswald et al. (1953) studied this relationship in detail and incorporated their findings into "Advanced Integrated Wastewater Ponds" systems over the last 50 years (Fig. 1). Their system designs are based on the following concept: combining bacteria and algae phases in a wastewater treatment system forces a symbiosis, wherein the algae supply the oxygen demand of bacteria through photosynthesis and bacteria supply the algae with needed carbon, nitrogen, and other products of decomposition. This symbiotic relationship is highly significant: the algal–bacterial combination in wastewater treatment is known for biosorption, which is the removal of effluent components from wastewater via adsorption on living or dead biomass (Oswald, 2003; Mulligan and Gibbs, 2003). Some have suggested that besides supplying CO_2, aerobic bacteria can promote algal growth through reduction of photosynthetic oxygen tension found within the microenvironment of algal cells, which creates more favorable conditions for optimized photosynthetic growth (Mouget et al., 1995). Many ecologically complex wastewater treatment systems rely on these concepts to accomplish treatment.

A consortium ranging from bacteria and algae to plants and lower animals can be assembled to accomplish treatment. Including algae for the production of oxygen in a treatment system is advantageous because it avoids the high levels of volatilization of compounds, odors, volatile treatment intermediates, etc., that might occur during traditional mechanical aeration that is required in the absence of an abundant source of oxygen (Muñoz and Guieysse, 2006). Oxygen produced by algae also reduces the cost of operating the system. In some cases, mechanical aeration of aerobic treatment systems can account for greater than 50% of the energy consumed by the entire wastewater treatment system (Tchobanoglous et al., 2003). In an established symbiotic algae treatment system, the growth of

bacteria would be limited by the photosynthetic production of oxygen by the algal consortium present (Muñoz and Guieysse, 2006), as bacteria can often consume the oxygen through metabolic respiration as quickly as algae can produce it.

Closed photobioreactor systems used for culturing pure populations of algae typically can provide better engineering and biological control and achieve higher algal productivity than open ponds systems (Sheehan et al., 1998; Chisti, 2007; Schenk et al., 2008). Since dissolved oxygen levels much greater than the air saturation values inhibit photosynthesis, a big drawback (in algal monocultures) is that they require "excessive oxygen removal," without concurrently releasing the needed CO_2 (Molina et al., 2001). This is because high concentrations of dissolved oxygen combined with intense sunlight produces photooxidative damage in algal cells (Chisti, 2007). Solving this conundrum remains one of the many challenges for which, many designs have been researched and tested, such as the gas–liquid separator design (Chisti and Moo-Young, 1993; Chisti, 1998) and the gas–liquid transmission device (Su et al., 2008). Regardless of these research innovations, closed algae growth systems continue to be highly expensive, requiring many times higher capital investment than open pond systems.

Overall, currently available algae growth systems are not very efficient (Chisti, 2007; Schenk et al., 2008) for a number of reasons: (1) closed photobioreactors are expensive and (2) open pond systems are relatively cost-effective but have no control over random environmental conditions (contamination by undesirable wild algae and fungi, temperature and light variations, evaporation, pH changes due to rain events, etc.) which affect algae growth. For these reasons, hybrid systems combining the aforementioned (1) and (2) are being researched and tested (Huntley and Redalje, 2007). It has been repeatedly noted that large-scale sterile reactors are not cost-effective due to infrastructure development requirements and that economic solutions will have to include nonsterile open ponds (Huntley and Redalje, 2007; Chisti, 2007; Schenk et al., 2008; DoE, 2010). Designing robust open pond systems or next-generation hybrid systems integrated with wastewater treatment remain unsolved challenges.

3. Integrated System Challenges

3.1. LOCATING FACILITIES

Researchers envisage large-sized algal production facilities on the order of 1,000 ha (Wijffels, 2008). However, finding marginal land of low economic and environmental value may be challenging, especially near metropolitan areas, where major wastewater treatment facilities (especially municipal) are likely located (DoE, 2010). Transportation of wastewater over long distances would not be a cost-effective option. Similarly, in rural settings supporting dairy farms, occupying crop lands with large-scale algae facilities can lead to space conflicts.

Figure 2. Aquaflow Group Inc. integrated municipal wastewater treatment facility.

3.2. SYSTEM DESIGN

3.2.1. Pond Depth

High-rate algal ponds (Oswald, 1990; Benemann et al., 1980) have been used for treatment of municipal wastewater and have been adopted by algae production companies researching biofuel production systems. An example is a modified version patented as the "Partitioned Aquaculture System," developed at Clemson University, which integrates microalgae biofixation of CO_2 with fish aquaculture, promoting a high rate of harvestable algae coupled with fish production (Brune et al., 2001). The New Zealand-based algae-biofuel company Aquaflow Group Inc. claims to operate 60 ha of open oxidation ponds and has been harvesting wild algae from a municipal waste water site (Fig. 2).

A major problem with integrated ponds systems for biofuels is the pond depth. Algae-based wastewater treatment ponds can reach depths of 6 m (though 13 m depths have been tested) (Abeliovich, 2007), since their goal is to treat wastewater and not to optimize high productivity in algae (which is otherwise essential to biofuels production). Algal turf scrubber systems have successfully operated at 1–3 cm of depth (Mulbry et al., 2008). Shallow open ponds (e.g., Fig. 3) normally with water depths of 15–20 cm are found supporting algal biomass concentrations of 1 g/L (dry weight) and productivities of 60–100 mg/L/day (i.e., 10–25 g/m^2/day) (Pulz, 2001), and these ponds can operate efficiently in up to 30 cm of depth (DoE, 2010).

3.2.2. Water Quality and Retention: Pre-/Posttreatment and Algal Production

Optimal wastewater treatment requires an extended retention time so that all of the available nutrients are completely exhausted prior to discharge, whereas

Figure 3. Earthrise Co. CA open pond system. World's largest Spirulina farm, 44 ha.

maximal algae yield requires high organic nutrient concentrations, which means that when wastewater treatment is combined with algae production dual-purpose high-rate oxidation ponds, wastewater treatment tends to remain incomplete, requiring further polishing steps to remove the remaining nutrients, thereby potentially affecting the economics of the process (Abeliovich, 2007). This dichotomy can be overcome in integrated systems, by incorporating effluent polishing steps to treatment coupled to the production of value-added products. These ideas have been described by Todd et al. (2003) in integrated systems ranging from large eco-industrial parks, to smaller scale eco-machines (which we will explore shortly).

The presence of microorganisms, such as fecal coliforms, along with unabsorbed heavy metals could pose serious issues for water reuse or disposal from post algae production. To completely eliminate all human pathogens from contaminated water without resorting to costly chemical inputs, retention of 3–4 months is typically required (Bouhoum et al., 2000). This increases the storage volume with associated increased costs and risks.

The use of chemicals for harvesting algae (e.g., flocculation) would add salts or flocculants to the water, which might additionally contain residual nutrients, heavy metals, or other organic contaminants. This could make disposal of post algae production water more difficult. Another potential complication arises if a treatment system hosted nonnative (i.e., exotic or invasive) algae. In theory at least, these algae they might affect the biodiversity of local ecosystems, including the potential of becoming invasive species. Sterilization of the water used in algae production would be very costly and energy intensive. The process water would therefore require recycling back into the ponds (DoE, 2010).

Figure 4. Monoculture and polyculture algae populations treating brewery wastewater (Sources: General Systems Research LLC & Dahiya et al., 2010).

Non-ionized ammonia in wastewater is known to inhibit the rate of oxygen release in common oxidation pond algae by 50%; combined conditions of 30 mg/L ammonia and pH 8.2 will cause such inhibition (Abeliovich, 2007). In waste stabilization ponds utilizing algae for treatment, phosphorus removal is often low (15–50%) (Picot et al., 1992; Garcia et al., 2000). When all of these problems are considered together, it becomes clear that there are still many challenges before optimal integrated systems can be developed. A fully ecologically engineered and tested integrated system needs to be developed.

3.3. WASTEWATER CONCENTRATION

As noted earlier, maximal algal yield requires high organic nutrient concentrations. Often, highly concentrated wastewaters are dark colored or filled with dissolved/suspended solids. In both instances, light penetration can be obstructed and can negatively affect algal growth. For example, in 100% undiluted dairy wastewater, algal growth was poor as compared to 10 and 25% concentrations, presumably due to the wastewater's high opacity (Woertz et al., 2009). Algal turf systems treating raw or anaerobically digested dairy manure effluent need to be diluted with daily additions of effluent that has been diluted by mixing with freshwater (untreated well water or chlorinated drinking water) (Mulbry et al., 2008). Brewery and municipal wastewaters have been treated at relatively higher concentrations. Brewery wastes have been treated in concentrations of up to 75% brewery waste, and the growth and oil production of algae in both batch polycultures and monocultures is being investigated (Dahiya et al., 2010) (Fig. 4). A 100% concentration of municipal wastewater treatment is reported by Woertz et al. (2009).

Robust ecosystems capable of treating high-concentration wastewaters are created in Eco-Machines™ (Todd and Todd, 1993; Todd and Josephson, 1996;

Figure 5. Eco-machine treating brewery waste (Image: John Todd).

Todd et al., 2005; Todd, 2005) and its numerous variants (Ives-Halperin and Kangas, 2000; Lansing and Martin, 2006; Morgan and Martin, 2008). One pilot system treated 2.6 m^3/day (2,585 L; 683 gal) of full strength dairy/ice cream wastewater, high in BOD, TSS, and FOGs (fats, oils, and greases) (EEA, 1993).

As part of an EPA-funded study, a pilot system in Frederick MD treated 150 m^3 (151,416 L; 40,000 gal) of raw domestic wastewater (100%) per day (US EPA, 1996). This was followed by a scaled up and optimized system in South Burlington, VT, that treated 300 m^3/day (302,833 L; 80,000 gal) of raw domestic wastewater to tertiary standards over several years (Austin et al., 2000; Todd et al., 2003).

Within eco-machines, all the major groups of life are represented, including algae, fungi, bacteria, protozoa, and zooplankton, on upward to snails, clams, and fishes. Higher plants, including shrubs and trees, are grown on adjustable media suspended within the system. The result is an efficient wastewater treatment system that is capable of achieving high-quality water without the need for hazardous chemicals. Eco-machines can be designed in multiple configurations; they can be a tank-based system housed within a greenhouse (e.g., brewery waste treatment in Fig. 5), a combination of exterior constructed algae-based ecologies or wetlands (Fig. 6), or a combination of both of these. The system often includes anaerobic pretreatment, aerobic tanks as the primary treatment approach followed by a final polishing step, either utilizing ecological fluidized beds or small constructed wetlands.

As mentioned, a major component of these systems is suspended and attached algal biomass (growing symbiotically with other organisms), which

Figure 6. Eco-machines treating septage containing EPA priority pollutants, Harwich, MA (Image: John Todd).

serves multiple functions: oxygenation of the system in photosynthetic periods, surface area for treatment and bacterial colonization, uptake of metals and pollutants, as well as providing feed for different consumer species inside the complex system. High volumes of biomass can be rapidly produced inside of the eco-machine, which can be harvested and fed into a number of other systems. It would be feasible to harvest attached algae or microalgae at various points and times in the treatment system for recovery of oils, biomass, or metals. It was noted that in many eco-machines, metals are sequestered by algae and some plants (Todd et al., 2003). This biomass could be recovered, ashed, and treated to recover phycoaccumulated metals, as was suggested earlier. The biomass could also be used as a feed for a biodigestion process to produce biogases and effluent, for fermentation to produce bioethanols (Harun et al., 2009), or for extraction to produce biodiesel.

Integration of robust systems like eco-machines with algal biofuel production could definitely offset the costs. Research and development on adapting the systems to high-strength treatment and high-quality effluent while incorporating and maximizing the growth of algal consortia with high-lipid content is needed.

3.4. ROBUST ALGAL STRAINS OR ASSEMBLAGES AND LIPID PRODUCTIVITIES

Both algae monocultures and polycultures have been grown in various wastewater streams, including dairy and piggery farm wastes and municipal wastewaters. Few studies have reported the oil/lipid productivities for waste-grown high-lipid algal monocultures (e.g., *Botryococcus braunii*; An et al., 2003) and algal polycultures (Woertz et al., 2009; Mulbry et al., 2009) and, in all instances, only at laboratory or pilot scales.

Polycultures (or algal assemblage of many predominating species) are known to thrive in wastewater environments. Algae of genus *Chlorella*, *Scenedesmus*,

and *Micractinium* grow well under average wastewater conditions and *Euglena*, *Chlamydomonas*, and *Oscillatoria* do well under excessive loading conditions or longer residence times (Oswald, 2003). Algal–bacteria combinations including predominately the algae *Sargassum natans*, *Ascophyllum nodosum*, and *Fucus vesiculosus* together with the bacteria *Bacillus subtilis* and *Bacillus licheniformis* are known to support biomass biosorption (Mulligan and Gibbs, 2003). Ogbonna et al. (2000) reported that when together, *Rhodobacter sphaeroides* and *Chlorella sorokiniana* effectively removed nutrients under dark aerobic heterotrophic conditions.

The algal biofuels would require synthesizing and accumulating large quantities of neutral lipids/oil (at least in the range of 20–50% dry cell weight) (see Hu et al., 2008). Wastewater-grown algal polyculture is low in lipids compared to lipid-rich monocultures. The polyculture used in algal turf systems (ATS) for treating dairy and swine wastewater (dominated by *Rhizoclonium* sp.) had very low lipids/oils, with fatty acid contents ranging from 0.6 to 1.5% of dry weight (Mulbry et al., 2008), and also the fatty acid (FA) content of ATS harvested material (from three Chesapeake Bay rivers) was consistently low (0.3–0.6% of dry weight) and varied little between sites (Mulbry et al., 2010). Woertz et al. (2009) reported 2.8 g/m^2/day of lipid productivity from an algal polyculture (*Scenedesmus*>*Micractinium*>*Chlorella*>*Actinastrum*) combined with dairy wastewater treatment and lipid productivities of 9.7 mg/L/day (air-sparged) to 24 mg/L/day (CO_2-sparged) in municipal wastewater (predominately genera *Chlorella*, *Micractinium*, and *Actinastrum*).

The DoE's Algae Species Program studies identified 300 algae strains out of the 3,000 collected which were suitable for oil production from different regions (Sheehan et al., 1998). Among monocultures, algae of the genus *Chlorella* can potentially accumulate lipids up to 50% of their dry weight and can be efficiently grown in wastewater. These are good candidates for biodiesel production because production rates of 3,200 GJ/ha/year have been achieved with these species. It has been suggested that this genus is a potential candidate to help transition our reliance on fossil fuels by 300 EJ/year, as well as reducing CO_2 emissions by 6.5 Gt/year by the year 2050 (Wang et al., 2008). Various *Chlorella* species have been cultured since the 1950s (Eyster et al., 1958; Myers and Graham, 1971) especially in wastewaters (Hills and Nakamura, 1978; Oswald et al., 1953; Oswald, 2003), and it was used as a model genus in DoE studies. Research continues into many other high-lipid potential algae. For details, see Chisti (2007) and Hu et al. (2008). However, the Department of Energy (DoE, 2010) recently stressed that many of the algal strains in algal repository collections (such as The Culture Collection of Algae at the University of Texas at Austin, or UTEX) have been cultivated for several decades, and it was suggested that these strains may have lost part of their original wild-type properties necessary for mass culture. They recommended that researchers and collections isolate new, native strains directly from unique environments to obtain versatile and robust strains that can be used for mass culture in biofuel applications.

4. Cost-Effectiveness and Environmental Impacts of Integrated Systems

The cost of producing algae for oil and the related cost-effectiveness of an integrated system is difficult to estimate because no optimized large-scale operation is yet in existence. Considering the widely quoted estimates by Chisti (2007), the cost of algae biomass produced for providing a liter of oil would be around US$1.40/L for photobioreactors and around US$1.81/L for open ponds. Integrating oil production with wastewater treatment will offset the investment and maintenance costs, which are the main drivers of algae biomass production costs. Calculations from recent studies (Lundquist, 2008) show costs of approximately US$6 per gallon of oil (or US$1.57/L of oil) produced by algae-integrated wastewater treatment (savings 15 kWh per gallon oil produced), which represents a net savings of US$0.24/L for oil produced by open ponds in Lundquist's open pond system as compared to US$1.81/L in Chisti's traditional open pond system.

At the First International Algae Biomass Summit in 2007, Ami Ben-Amotz (National Institute of Oceanography, Israel) presented an interesting analysis which claims that open pond systems yields average 20 g/m^2/day, and Ben-Amotz argued that overall production costs of US$0.34/kg are viable if the algal lipid content is high enough (Schenk et al., 2008). At this same conference, economic target costs for algae hydrocarbons were set at US$2 per gallon (US$0.48/L), with a minimum order of 50 million gallons (210 million liters). Economic figures are available from estimations of algal biomass production based on photobioreactors combined with open pond systems developed by Huntley and Redalje (2007). They calculated algae oil production costs at $84/bbl, with capital cost rates of US$391,211/ha. If we count on the cost-effectiveness of production suggested by Lundquist, even relying on low-lipid production in algae, additional revenue generated from secondary and value-added products generated within the system is possible to offset the costs further. The post-extraction-spent algae can be used as fertilizer or animal feed or cycled into new food chains (along with the postproduction process water) to create further value-added products as was reported by Todd et al. (2003) earlier.

According to a life cycle analysis (LCA) performed by Levine et al. (2009), over 1.552×10^6 L (410,000 gal) of oil could be produced from algae per year in 100 ha of open ponds treating wastewater. However, since no commercial scale facility is producing oil from algae (DoE, 2010), no thorough LCA following the production chain, from algae culturing through to biodiesel, is available. Most of the partial LCA performed have been based on extrapolations of lab-scale or pilot studies and combined with known processes developed for first-generation biofuels (Lardon et al., 2009; Clarens et al., 2010). Clarens et al.'s LCA has been highly criticized by the algal biofuel community due to their overestimation of the negative environmental effects of algae production. The water demand for algae production has not been directly addressed in any of the preliminary algae LCA analyses so far (Aresta et al., 2005). However, based on their LCA model, Clarens et al. (2010) demonstrated that when algae was grown to treat wastewater,

the algae was found to have lower environmental impacts (water, energy use, greenhouse gas emission) than the conventional crops of switchgrass, canola, and corn, as well as compared to algae production fed with clean water and fertilizer, which all showed higher upstream burdens. According to the recent DoE report (2010), one of the major benefits of growing algae is that, unlike terrestrial agriculture, algal culture can utilize water with few competing uses, such as saline and brackish water. This observation is crucial when we consider the water resources needed for the future development and expansion of algal biofuel production.

The recent LCA by one of the leading algae-oil companies, Sapphire Energy, revealed that algae-based fuels emit approximately two-thirds less CO_2 than petroleum-based fuels at scale; and when compared with conventional biofuels, such as corn ethanol and soy biodiesel, algae "green crude" oil has significantly less than half their carbon impact. A report by Sánchez Mirón et al. (1999) states that algal biomass contains approximately 50% carbon by dry weight; and thus, production of 100 tons of algal biomass fixes approximately 183 tons of CO_2 (Chisti, 2007). These numbers make it clear that algae provide a tremendous potential to capture CO_2 emissions from power plant flue gases and other fixed sources. When taken together, all of this suggests that biodiesel from algae can be carbon neutral, because all the power needed for producing and processing the algae could potentially come from biodiesel itself and from methane produced by anaerobic digestion of the biomass residue (or bioethanol from fermentation) left behind after the oil has been extracted (Chisti, 2007).

Clarens et al.'s LCA model (2010) demonstrated that in terms of land use impact, algae offers clear and appreciable improvement over corn, canola, and switchgrass. Their land use estimates indicate that algae cultivation on roughly 13% of the United States' land area using current technologies could meet the nation's total annual energy consumption. In contrast, use of corn would require 41% of the total land area, while switchgrass and canola would require 56 and 66%, respectively. Land use changes implicit in large-scale bioenergy deployment are expected to have important implications for climate change and other impacts, and these so-called "indirect" changes are associated with conversion of arable land into biofuel production. The potential annual oil yields from oil-rich algae are projected to be at least 60 times higher than from soybeans per acre of land, around 15 times more productive than jatropha and around five times more than oil palm (DoE, 2010).

The integrated approach for algae biofuel production involving wastewater treatment has enormous potential. The multiple efficiencies that may be gained through the novel integrations we have explored might unlock the elusive cost efficiencies which are currently holding back the large-scale viability of algae biofuel production. Many of these couplings also offer cost-effective approaches to treating current environmental wastes and problems. Many waste streams contain the nutrients and carbon required to support robust algae populations. As we have noted, integrated systems with multiple species (as opposed to single species systems) are often quite robust and resilient and able to weather lower levels of

engineered control that will be required to make algae biofuel production cost-effective. The possibility of offsetting costs through coupling with waste treatment, as well as the possibilities of creating new products through recovery (mining of waste streams) or production of biomass by-products (fertilizers, animal feed, new food/agricultural food webs, pharmaceuticals, etc.), offers other cost advantages of integrated systems over conventional algae production approaches.

There are still a number of technical challenges which must be researched and engineered. A number of vital parameters for algae biofuel production systems were explored, including facility location, system designs, water depth, water retention, water quality, algal strains/species consortiums, and lipid productivity. Research is needed in all of these areas to address optimization, resilience, and cost-effectiveness. As noted, an often overlooked component of biofuel production is their environmental impacts. Water and energy consumption, infrastructure, CO_2 emissions, and land requirements were all shown to be important considerations in the viable development of algae biofuels and must be accounted for throughout a system's entire life cycle in any development scenario. In an optimum scenario, carbon neutral or carbon negative systems would be the goal for the integrated production of algae biofuels.

It has been said that having the idea is the most important part of any new innovation. In this case, a shift in thinking for algae biofuel production to integrated systems provides the framework for a potential leap forward in both algae biofuels and wastewater treatment. Once the idea of integrated systems for algae biofuel and waste treatment is in place, research and engineering can quickly rise to the challenge of unlocking the biological and technical problems associated with production and put us on a path to carbon neutrality and energy independence.

5. Acknowledgements

Funding provided by NSF-EPSCoR (VT) and Department of Energy (via VT Sustainable Jobs Fund) to Anju Dahiya are greatly appreciated. Anthony McInnis would like to acknowledge the generous support of the University of Vermont's George Washington Henderson Fellowship. The authors also thank Matt Beam for his review of the manuscript.

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R. Gordon and J. Seckbach (eds.), *The Science of Algal Fuels*,
Cellular Origin, Life in Extreme Habitats and Astrobiology, 467–493
DOI 10.1007/978-94-007-5110-1_25, © Springer Science+Business Media Dordrecht 2012

SUB- AND SUPERCRITICAL WATER-BASED PROCESSES FOR MICROALGAE TO BIOFUELS

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1. Introduction

In general, conventional higher land plants are not very efficient in capturing solar energy. Even the fastest growing energy crops can convert solar energy to biomass at a yearly rate of no more than 1 W/m^2 (Yanqun Li et al., 2008). However, the biomass productivity of microalgae, a photosynthetic microorganism, can be 50 times greater than switchgrass (Demirbaş, 2006). Microalgae grow in marine and freshwater environments. Due to their simple cellular structure and submergence in an aqueous environment where they are in the vicinity of water, CO_2, and other nutrients, microalgae are generally more efficient in converting solar energy into biomass. The Department of Energy, USA, supported the Aquatic Species Program (1978–1996) which illustrated the potential of microalgae as a biofuels feedstock. Their study concluded that land is hardly a limitation for sustainable cultivation of microalgae. Microalgae can be used to produce a wide range of advanced biofuels and bioactive compounds (Schenk et al., 2008; Huesemann and Benemann, 2009). Microalgae offer several potential advantages (Schenk et al., 2008; Yanqun Li et al., 2008; Chen et al., 2009) over conventional biomass sources including the following: (1) algal cultivation does not compete with agricultural land, (2) algae can be harvested batchwise all-year round providing a reliable continuous supply; (3) per acre and per gallon of fuel, algal farming utilizes less water than other biofuels; (4) algae can be grown on wastewater or saltwater streams, thereby greatly reducing freshwater use; and (5) algae can capture smokestack CO_2 from coal, gas, or petroleum plants allowing sequestration.

Microalgae primarily comprise of varying proportion of proteins, carbohydrates, lipids, and ash. The percentages vary depending upon the species. Table 1 shows the general composition of different microalgae. What really makes algal biomass feasible for biofuels production is the fact that many forms of algae have very high lipid contents. Biomass fraction that is non-lipid provides a high-value coproduct such as animal feed or fertilizer that offsets the cost of converting the algae to fuels. Growing algae also removes nitrogen and phosphorus from water and consumes atmospheric CO_2.

Table 1. General composition of different algae (% of dry matter) Becker (2007).

Alga	Protein	Carbohydrates	Lipids
Anabaena cylindrica	43–56	25–30	4–7
Aphanizomenon flos-aquae	62	23	3
Chlamydomonas reinhardtii	48	17	21
Chlorella pyrenoidosa	57	26	2
Chlorella vulgaris	51–58	12–17	14–22
Dunaliella salina	57	32	6
Euglena gracilis	39–61	14–18	14–20
Porphyridium cruentum	28–39	40–57	9–14
Scenedesmus obliquus	50–56	10–17	12–14
Spirogyra sp.	6–20	33–64	11–21
Arthrospira maxima	60–71	13–16	6–7
Spirulina platensis	46–63	8–14	4–9
Synechococcus sp.	63	15	11

1.1. MAJOR CHALLENGES IN THE CONVERSION OF MICROALGAE TO BIOFUELS

Biomass can be used for producing solid (biochar), liquid (biodiesel, liquid hydrocarbons, pyrolysis oil), and gaseous fuels (synthesis gas, methane, hydrogen). However, the attractive target is producing fungible fuels such as gasoline, diesel, and jet fuel. Some of the major challenges in accomplishing the objective of cost competitive biofuels production from microalgae are:

1.1.1. Dewatering

It is not uncommon to have less than 1 g of algae per liter of water. Therefore, a cost-efficient harvesting and drying process is needed to produce a biomass suitable for oil recovery. Conversion processes that can process wet biomass are highly desirable for reducing the energy-intensive dewatering cost.

1.1.2. High Nitrogen Content

Microalgae are rich in proteins (Table 1). The elemental composition (carbon, hydrogen, and oxygen) of microalgae is similar to other cellulosic biomass but differs in nitrogen contents. Apart from water, sunlight, and carbon dioxide, nitrogen and phosphorous are the primary nutrients which are required to grow microalgae. Based on the average elemental composition, microalgae can be represented by a general formula as $CH_{1.7}O_{0.4}N_{0.15}P_{0.0094}$. The nitrogen content varies between 4 and 8 wt% of the dry biomass depending upon the physiological state and nutrient limitation condition of microalgae (Greenwell et al., 2009; Wijffels and Barbosa, 2010). Due to the high nitrogen content, NO_x emission and losses of nitrogen fertilizer are matters of great concern besides the high moisture content if the whole microalgae are processed for biofuel (Huesemann and Benemann, 2009).

Organically bound nitrogen in microalgae converts to ammonia in reducing atmosphere and NO_x in combustion/oxidizing atmosphere during the biofuels conversion processes. In biogas production, high nitrogen contents lead to the ammonia toxicity during anaerobic digestion process. Also, high nitrogen contents are reported to inhibit the digestion of algal biomass. Similarly, presence of nitrogen in biomass will cause the formation of NO_x compounds during gasification process which is conducted in the limited supply of oxygen. NO_x is a greenhouse gas and heavily regulated environmental pollutant. Further, gas cleaning also adds to the cost, if synthesis gas or syngas (mixture of CO and H_2) is to be used for liquid fuels production via Fischer-Tropsch (F-T) process. The nitrogen is mainly present as the protein in microalgae. Therefore, it is important to extract high-value protein for the sustainable production of biofuels from microalgae. The major emphasis should be on the value addition to the nonfuel components and the recycle of nutrients as much as possible. The approach for producing biofuels from microalgae can be different compared to the conventional cellulosic biomass processes. The high protein contents of microalgae makes it a potential candidate for extracting protein (Chronakis, 2000), and the lipid contents are best considered for biofuels (John Sheehan et al., 1998; Chisti, 2007; DOE, 2010).

1.1.3. Diverse Composition

More than 50,000 species of microalgae are reported to exist (Mendes, 2007). Algal biomass composition varies depending upon the species (Table 1). The challenge involves developing a process that can tolerate the complex compositions found with different species of algae. In order to make biofuels cost competitive, it is important to develop processes for almost 100% utilization of algal biomass components.

1.2. MICROALGAE TO BIOFUELS CONVERSION PROCESSES

The most attractive target for fuel production from micoalgae is the production of liquid transportation fuels such as gasoline, diesel, and jet fuel (Gouveia and Oliveira, 2009; DOE, 2010). There are several competing pathways for converting the biomass to liquid fuel, chemicals, and/or hydrogen. Figure 1 shows the schematics of generally accepted potential conversion routes for whole algae to biofuels.

1.2.1. Biochemical

Biochemical processes take place at ambient to slightly higher temperature levels using a biological catalyst to bring out the desired chemical transformation. The anaerobic digestion of microalgae, a biochemical process, is a process for production of biogas (Golueke et al., 1957). Biogas mainly contains CH_4 and CO_2 and is an environment friendly clean, cheap, and versatile gaseous fuel (Gupta and Demirbas, 2010a, b). Vergara-Fernandez et al. reported that biogas production

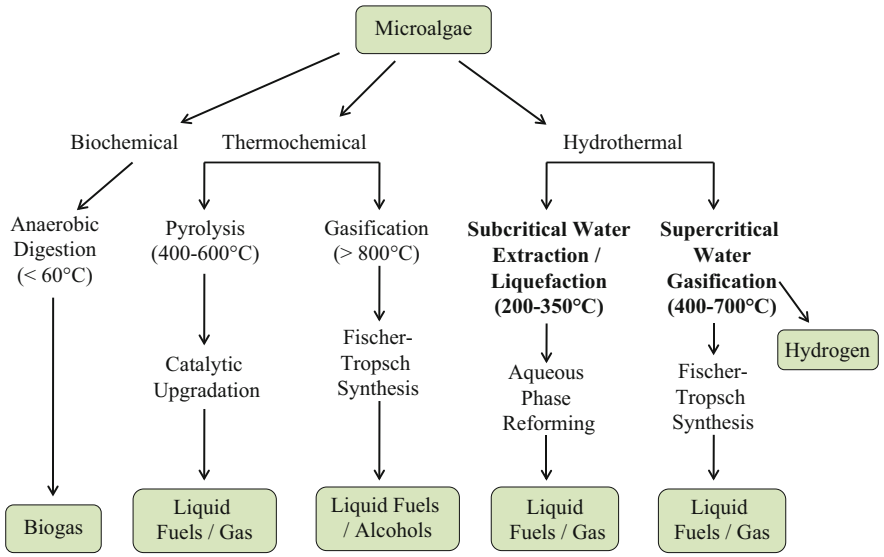

Figure 1. Potential conversion routes for whole algae biomass to liquid and gaseous fuels.

levels of 180.4 ml/g-day of biogas can be realized using a two-stage anaerobic digestion process with different strains of algae, with a methane concentration of 65% (Vergara-Fernandez et al., 2008). The biodegradability of microalgae can be low depending on both the biochemical composition and the nature of the cell wall. The high-cellular protein may cause toxicity due to ammonia release during digestion. Physicochemical pretreatment, co-digestion, and control of gross composition are strategies that can increase the yield of methane (Sialve et al., 2009; Heaven et al., 2011). Unfortunately, biogas garners less importance than the common transportation fuels. The selling cost per unit energy is considerably lower than gasoline. Because the biogas produced is relatively low in value, the anaerobic process must be simple in design, have low operating cost, and produce gas at high rates.

1.2.2. Thermochemical

Thermochemical processes depend on the relationship between heat and chemical action as a means of extracting and creating products and energy. Pyrolysis and gasification are conducted at a temperature of several hundred degrees Celsius.

Pyrolysis is thermal degradation of biomass in the absence of oxygen to produce condensable vapors (bio-oil), hydrocarbon-rich gas mixture, and charcoal; in some instances, a small amount of air may be admitted to promote this endothermic process. Pyrolysis processes typically use dry and finely ground biomass. The oxygen content of bio-oils is usually 35–40 wt%. The presence of oxygen is reason for substantially lower heating value (16–19 MJ/kg) of bio-oils compared

to hydrocarbon fuels. Also of concern is the nitrogen content of algae, because this nitrogen tied up mostly as proteins is transformed to nitrogenous species such as pyridines; removal of such oxygen is possible by decarboxylation, dehydration, and/or catalytic hydrotreating (Venderbosch et al., 2010). Therefore, bio-oils are required to be catalytically upgraded to make them fungible fuels. Considering the high moisture content of microalgae, pyrolysis that requires dried biomass may not be an economical option. An inexpensive dewatering or extraction process will have to be developed (DOE, 2010). In fact, very few studies have been reported on pyrolysis of algae for bio-oil. The bio-oil yield for *Chlorella protothecoides* (a microalga sample) rose from 5.7 to 55.3% as the pyrolysis temperature rose from 250 to 500°C and then gradually decreased to 51.8% and was obtained at 600°C (Demirbaş, 2006). The heating value of bio-oils from algae was in the range of 35 MJ/kg which is relatively higher than that from wood.

Gasification is another transformation pathway for production of liquid fuels from algae. Syngas is produced through the thermal decomposition of organic matter in oxygen-deficient conditions during gasification. The process has several benefits for near-term commercial application due to the inherent advantages over combustion including more flexibility in terms of energy applications, higher economical and thermodynamic efficiency at smaller scales, and potentially lower environmental impact when combined with gas cleaning and refining technologies (Gupta and Demirbas, 2010a, b). Typically gaseous products include CO, H_2, and CH_4. Fischer-Tropsch (F-T) synthesis can be used to convert the gaseous products into liquid fuels through the use of catalysts. Gasification requires feedstock that contains less than 10% moisture (Ni et al., 2006; Jong, 2009). Syngas typically contains undesired impurities derived from biomass feedstock including tar, hydrogen sulfide, carbonyl sulfide, ammonia, hydrogen cyanide, alkali, and dust particles. It is important to clean syngas to avoid F-T catalyst poisoning. Impurities from synthesis gas can be removed through several routes including physical separation, thermal cracking, and catalytic hot gas cleanup (Yung et al., 2009). The significant problem with F-T synthesis is the cost of cleanup and tar reforming. The formation of tars causes coking of the catalyst and must be removed.

Iron and cobalt are the earliest known catalyst used in F-T synthesis. Additionally, group VIII transition metal (Ru, Ni, Pd, etc.) oxides are also regarded as good CO hydrogenation catalysts. The active sites of metal catalysts can be poisoned by the presence of impurities in syngas. In fact, chemical poisoning is one of the most common reasons for the deactivation of F-T catalysts. The presence of sulfur and nitrogen in feedstock can be the cause of impurities in syngas. Sulfur in microalgae is significantly low (<0.1 wt% dry basis) and hence may not be a matter of concern. However, high nitrogen contents of microalgae (discussed earlier) lead to the formation of NH_3, NO_x, and HCN gases. These impurities should be cleaned before syngas is subjected to F-T process for avoiding the catalyst deactivation. It is important to note that the tolerance limits for NH_3, NO_x, and HCN are reported to be 10 ppmv, 0.2 ppmv, and 10 ppb, respectively (Spath and Dayton, 2003).

1.2.3. Hydrothermal

Hydrothermal is broadly defined as the use of water-rich phase above 200°C for conducting chemical reactions. Hydrothermal is also known as sub- and supercritical water (critical point: 374°C, 221 bar) medium. In view of challenges associated with utilizing wet biomass, principally the need for water and nitrogen removal, reactions in sub- and supercritical water medium are considered attractive option for extracting valuable bioactive compounds and producing biofuels from microalgae. Sub- and supercritical water technology utilizes tunable transport and solvent properties of water at elevated temperature for converting biomass to high-energy density fuels and functional materials. The medium is a nontoxic, environmentally benign, and relatively inexpensive for conducting chemical reactions.

2. Theory: Sub- and Supercritical Water

Water has a relatively high critical point because of the strong interaction between the molecules due to strong hydrogen bond. Liquid water below the critical point is referred as subcritical water, whereas water above the critical point is called supercritical water. The density and dielectric constant of the water medium play major roles in solubilizing different compounds. Water at ambient conditions (25°C and 0.1 MPa) is a good solvent for electrolytes because of its high dielectric constant, whereas most organic matter is sparingly soluble. As water is heated, the H-bonding starts weakening, allowing dissociation of water into acidic hydronium ions (H_3O^+) and basic hydroxide ions (OH^-). The structure of water changes significantly near the critical point because of the breakage of infinite networks of hydrogen bonds, and water exists as separate clusters with a chain structure (Kalinichev and Churakov, 1999). In fact, the dielectric constant of water decreases considerably near the critical point, which causes a change in the dynamic viscosity and also increases the self-diffusion coefficient of water (Marcus, 1999).

In the subcritical region, the ionization constant (K_w) of water increases with temperature and is about three orders of magnitude higher than that of ambient water, and the dielectric constant (ε) of water drops from 80 to 20 as shown in Fig. 2 (Tester et al., 1993). A low dielectric constant allows subcritical water to dissolve organic compounds, while a high ionization constant allows subcritical water to provide an acidic medium for hydrolysis reactions. These ionic reactions can be dominant because of the liquid-like properties of subcritical water. Moreover, the physical properties of water, such as viscosity, density, dielectric constant, and ionic product, can be tuned by small changes in pressure and/or temperature in subcritical region (Franck, 1987; Savage, 1999; Miyoshia et al., 2004).

Supercritical water has liquid-like density and gas-like transport properties. Therefore, supercritical water behaves very differently than it does at room temperature. For example, it is highly nonpolar, permitting complete solubilization

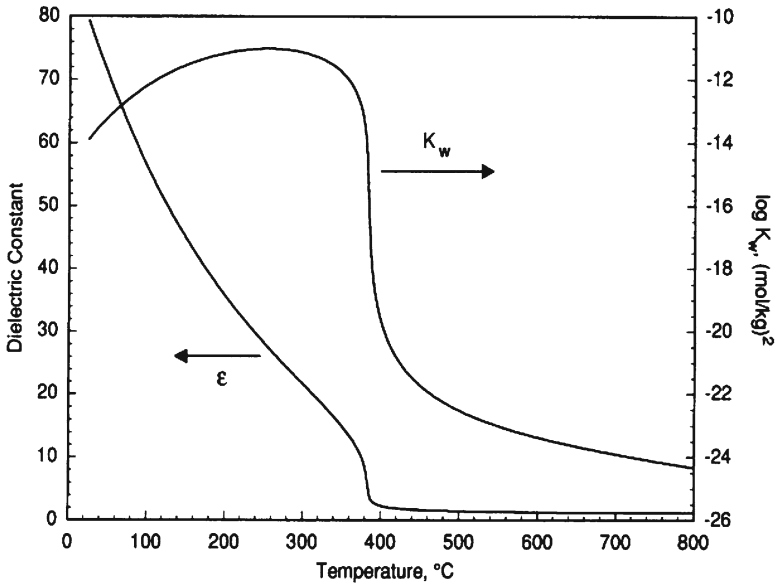

Figure 2. Physical properties of water with temperature, at 24 MPa (Kritzer and Dinjus, 2001).

of most organic compounds. The resulting single-phase mixture does not have many of the conventional transport limitations that are encountered in multi-phase reactors. However, the polar species present, such as inorganic salts, are no longer soluble and start precipitating. The physiochemical properties of water, such as viscosity, ion product, density, and heat capacity, also change dramatically in the supercritical region with only a small change in the temperature or pressure, resulting in a substantial increase in the rates of chemical reactions. It is important to mention that the dielectric behavior of 200°C water is similar to that of ambient methanol, 300°C water is similar to ambient acetone, 370°C water is similar to methylene chloride, and 500°C water is similar to ambient hexane (Kumar, 2010). In addition to the unusual dielectric behavior, transport properties of water are significantly different (Table 2) than the ambient water.

2.1. APPLICATION TO BIOFUELS

Sub- and supercritical water technology offers several advantages over the other biofuels production methods. Some of the major benefits (Peterson et al., 2008) are:

- High-energy and separation efficiency (no phase change, water remains in the liquid phase).
- Versatility of chemistry (can be used to produce solid, liquid, and gaseous fuels).

Table 2. Comparison of ambient and supercritical water.

Properties	Ambient water	Supercritical water
Dielectric constant	78	<5
Solubility of organic compounds	Very low	Fully miscible
Solubility of oxygen	6 ppm	Fully miscible
Solubility of inorganic compounds	Very high	~0
Diffusivity ($cm^2\ s^{-1}$)	10^{-5}	10^{-3}
Viscosity ($g\ cm^{-1}\ s^{-1}$)	10^{-2}	10^{-4}
Density ($g\ cm^{-3}$)	1	0.2–0.9

- Reduced mass transfer resistance in hydrothermal conditions.
- Ability to use wet biomass as well as mixed feedstock.
- Products are completely sterilized with respect to any pathogens including biotoxins, bacteria, or viruses.

The high ionization constant of subcritical water in the range of 200–300°C makes it a suitable medium for extracting proteins and valuable bioactive products from microalgae without the use of any chemicals. With the change of process parameters such as longer residence time or a higher temperature, subcritical water can be used for the liquefaction of microalgae to produce biocrude or liquid fuels via aqueous phase reforming process. In the supercritical water range (>374°C), microalgae can be gasified to produce gaseous fuels such as methane, hydrogen, and synthesis gas. In fact, reactions in subcritical and supercritical water also provide a novel medium to conduct tunable reactions for the synthesis of specialty chemicals from biomass (Matsumura et al., 2006).

2.2. ENERGY BALANCE OF HYDROTHERMAL PROCESSES

In subcritical water-based processes, water is kept in the liquid phase by applying pressure. Thus, latent heat of vaporization required for phase change of water from liquid to vapor phase (2.26 MJ/kg of water) is avoided. This reduces the energy requirement compared to steam-based processes. As an example, 2.869 MJ/kg of energy is required to convert ambient water to steam at 250°C and 0.1 MPa, whereas only 0.976 MJ/kg of energy is required to convert ambient water to subcritical water at 250°C and 5 MPa. This also means that the energy contained in the subcritical water is insufficient to vaporize the water on decompression. Further, it is possible to recover much of the heat from subcritical water. In the case of microalgae, energy required for the dewatering may account for more than 75% of the total energy consumption. Typical thermal dryers use significantly more energy per kilogram of evaporated water (3.3–3.9 MJ/kg). The drying steps lead to large parasitic energy losses that can consume much of the energy content of the biomass. Xu et al. compared the energy balance for wet and dry process for converting microalgae to biofuels (Xu et al., 2011). In this study, biodiesel, glycerol,

pyrolysis oil, and producer gas were considered as products from conventional dry processes, whereas green diesel, hydrogen, producer gas, and CO_2 recycling were considered for the wet process products. The fossil energy ratio (FER), the ratio of higher heating value of biofuel products to fossil energy input, for dry and wet process was reported as 1.50 and 1.37, respectively. The study concluded that the dry route may be more interesting in the short-term basis because of a slightly higher FER, but for the long term, the wet route has more potential because of the opportunity to produce advanced biofuels.

3. Reaction of Microalgae Components in Hydrothermal Medium

Microalgae are relatively small and protected, in many cases, by a thick cell wall as represented in Fig. 3. Typically, very harsh conditions (e.g., mechanical, chemical extraction) are required to break the cell walls for extracting the bioactive compounds. The main structural elements of all plant cell walls are polysaccharides. The resistance of algal cell wall to microbial attack is generally attributed to the discrete structural entities and resistance of cell walls to decompose.

Microalgae cell walls mostly consist of carbohydrates (polymers of glucose, mannose, xylose, galactose, galacturonic acid, etc.) and little protein or lipids (Gretz, 1991; Popper and Tuohy, 2010). Table 3 shows the chemical composition of

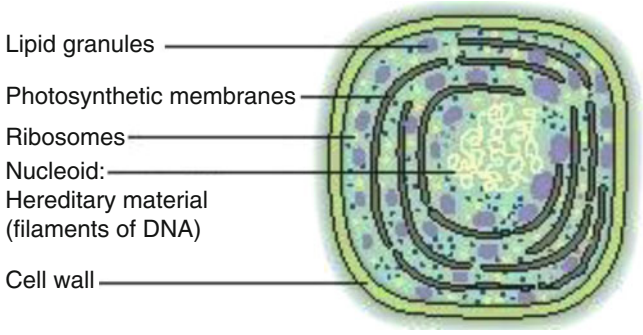

Figure 3. Typical blue-green alga.

Table 3. Chemical composition of the cell walls of three algae species Gunnison and Alexander (1975).

Components	Staurastrum sp	Pediastrum duplex
Protein	0	16.2
Lipid	0	0.7
Carbohydrate	80.0	48.0
Uronic acids	20.0	8.2

the walls of two species. Most algae have a variety of water-soluble polysaccharides (Seigel, 1973). Cellulose, a part of the cell wall in algae, is very widely distributed in the different species (Percival and McDowell, 1981). The wall also consists of alkali-soluble hemicelluloses and alkali-insoluble rigid walls (Abo-Shady et al., 1993). Cell walls, in general, are organized in a conventional framework. The basic framework is highly polymeric. Interspersed within are lower molecular weight polymers and oligomers (often gel like fibers) and inorganic and non-monomeric compounds (Seigel, 1973).

The polymeric components of microalgae, namely, carbohydrates, proteins, and lipids, have different depolymerization kinetics in the subcritical water medium. The hydrolysis rate increases with reaction temperature for these polymers. The hydrolysis of polysaccharides starts above 180°C in subcritical water within a residence time of seconds to a few minutes. The carbohydrates, such as hemicelluloses, starches, and amorphous cellulose, are known to start depolymerizing to water-soluble products in subcritical water above 180°C. In fact, hydrothermal degradation of cellulose is a heterogeneous and pseudo-first-order reaction for which detailed chemistry and mechanisms have been proposed (Schwald and Bobleter, 1989; Bobleter, 1994).

The depolymerization of protein is low or nonexistent at temperatures below 200°C in subcritical water. Peptide bonds of proteins exhibit much higher stability compared to the β-1, 4-, and β-1,6-glycosidic linkages in cellulose and starch, respectively. Protein hydrolysis to amino acids was found to be fairly low even at 230°C in subcritical water after hours of exposure (Rogalinski et al., 2008). Lipids are nonpolar compounds. The reactions of lipids and water strongly depend on the phase behavior (Peterson et al., 2008). The higher temperature causes increased solubility of fat and oils in subcritical water, and ultimately, they become completely soluble by the time water has reached its supercritical state. Earlier studies show that the hydrolysis of triacylglycerols (TAG) and fatty acids, along with their methyl esters, follows the first-order reaction kinetics. The hydrolysis of TAG in subcritical water starts above 280°C, and conversion in excess of 95% was achieved at 340°C within a residence time of 12 min (Kocsisová et al., 2006).

3.1. SUBCRITICAL WATER EXTRACTION

Proteins are natural polyamides. The primary structure of a protein is the sequence of amino acid units that make up the polypeptide chain. Peptide bonds or links (C–N bond between the carboxyl and amine groups) form between amino acid molecules as a result of condensation reactions (Fig. 5). Therefore, amino acids are the building blocks of proteins. Generally, peptides are short chains with 10–50 amino acids, polypeptides are much longer chains with greater than 50 amino acids, and proteins are polypeptides of specific sequence, length, and folded conformation (Abdelmoez et al., 2007). If a polypeptide or protein is subjected to

Table 4. Essential amino acid content in mg of amino acid/g of essential amino acids.

Essential amino acid	Soy	Scenedesmus sp.
Leucine (Leu)	170	123
Lysine (Lys)	160	230
Phenylalanine (Phe)	110	100
Tyrosine (Tyr)	80	74
Methionine (Met)	30	43
Cysteine (Cys)	30	243
Valine (Val)	130	61
Isoleucine (Ile)	120	42

moderately concentrated hydrochloric acid treatment for about 24 h, all of the peptide bonds are hydrolyzed. After neutralization, the free amino acids can be separated by paper chromatography or thin layer chromatography. Amino acids have many uses and applications. They can be used for the synthesis of new materials including medicines, taste enhancers, animal feeds, and even in electronic-related chemicals such as liquid crystals and exposure liquids for color copiers.

Organic nitrogen is mainly present in the proteins of microalgae. Accordingly, it is important to extract this high-value protein to make production of biofuels from microalgae both economic and sustainable. The major emphasis should be to extract as much value from the nonfuel components as possible and to recycle nutrients as much as possible. The approach for producing biofuels from microalgae is quite different from the conventional biomass processing. The high protein contents of microalgae (Table 1) make them ideal candidates for extracting proteins (Chronakis, 2000) and convert the lipids to biofuels (John Sheehan et al., 1998; Chisti, 2007; DOE, 2010). Presently, algae proteins are untapped as a resource. Though information on the utilization of algae proteins in food is very limited, it can be used as a supplement to the basic diet and food products (Chronakis, 2000). As an example, *Scenedesmus* sp. is one of the algal species which is considered a promising feedstock for large-scale biofuel production. A study published by Quevedo et al. shows that not only the nitrogenated compound content is high for *Scenedesmus* sp. but that the protein quality is very good (Quevedo et al., 2008). According to this study, the amino acid profile for *Scenedesmus* sp. is comparable to soy as detailed in Table 4.

The algal proteins and carbohydrates can be extracted by classical methods and/or by employing enzymatic degradation procedures. The classical methods use alkali hydrolysis followed by acid precipitation for protein extraction. The process efficiency is typically limited by the linkages between polysaccharides and proteins. The method appears simple because of the use of conventional chemicals. But, protein yield is typically low due to the degradation at high pH, and undesirable toxic products can be produced. Concentrated alkali causes the breakdown of protein and other valuable compounds (Chronakis, 2000; Sereewatthanawut et al., 2008). Also, this classical process is associated with serious economic and

Table 5. Extraction yields obtained (% dry weight), antimicrobial and antioxidant activities of SWE extracts from *H. pluvialis* obtained at different temperatures.

Extracts (°C)	Yield (%)	*E. coli* MBC (mg/ml)	*S. aureus* MBC (mg/ml)	*C. albicans* MFC (mg/ml)	*A. niger* MFC (mg/ml)	Antioxidant activity TEAC
100	21	3.0	4.0	5.5	15	0.453 ± 0.004
150	32	5.0	5.0	5.5	15	0.366 ± 0.012
200	33	5.0	5.0	5.5	15	1.974 ± 0.012

Adapted from Rodríguez-Meizoso et al. (2010).

environmental constraints due to the heavy use of chemicals. The enzymatic extraction processes are milder than the alkali hydrolysis and produce no toxic chemicals. However, slow conversion rates and high enzyme costs make the process uneconomical (Abdelmoez et al., 2007; Sereewatthanawut et al., 2008). The use of supercritical carbon dioxide has also been reported for extracting active compounds from algae and might be a viable option if dried biomass is used.

The subcritical water extraction process deviates from the conventional alkali hydrolysis process for protein extraction and eliminates the use of expensive and corrosive chemicals. In fact, a study by Sereewatthanawut et al. showed that subcritical water (100–220°C and 30 min) could effectively be used to hydrolyze de-oiled rice bran to produce protein and amino acids (Sereewatthanawut et al., 2008). The amount of protein and amino acids produced was higher than those obtained by conventional alkali hydrolysis (Sereewatthanawut et al., 2008).

In a recent work, Meizoso et al. reported the extraction of compounds with antioxidant and antimicrobial activity from *Haematococcus pluvialis* microalga (Rodríguez-Meizoso et al., 2010). The effect of the extraction temperature (50, 100, 150, and 200°C) and solvent polarity was studied. Results demonstrate that the extraction temperature has a positive influence in the extraction yield and antioxidant activity. Thus, the extraction yield achieved with this process was more than 30 wt% at 200°C and 9 min of reaction time. Moreover, the extract obtained at 200°C presented the highest antioxidant activity by far, while temperature did not seem to significantly affect the antimicrobial activity. Table 5 shows the potential antimicrobial activity of the *H. pluvialis* extracts obtained using subcritical water extraction using four different microbial species, including a gram-negative bacteria (*Escherichia coli*), a gram-positive bacteria (*Staphylococcus aureus*), a yeast (*Candida albicans*), and a fungus (*Aspergillus niger*). The antioxidant activity was measured by Trolox equivalent antioxidant capacity (TEAC) assay and was quantitatively assessed by the determination of the minimum inhibitory concentration (MIC), minimal bactericidal concentration (MBC), and fungicidal concentration (MFC).

Reactions in subcritical water medium are typically very fast (order of few seconds or minutes) where water leads to hydrolysis and rapidly degrades the

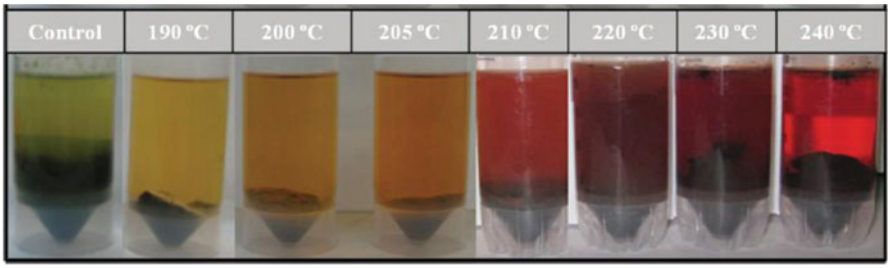

Figure 4. Photograph of *C. fragile* before (control) and after subcritical water treatment as a function of temperature for 10-min residence time (Reproduced from Daneshvar et al., 2011).

polymeric structure of biomass. The subcritical water process has a wide range of applications, such as extraction and wet oxidation of organic compounds. Daneshvar et al. used *Codium fragile* (*C. fragile*) as a model for green microalgae for its conversion to value-added materials (Daneshvar et al., 2011). *C. fragile* belongs to the Codiales order which is widely distributed along the shores of East Asia, Oceania, and Northern Europe. After subcritical water extraction, the products were categorized into three main phases as aqueous-, residual solid-, and hexane-soluble phases. The aqueous phase showed a very high total organic carbon (TOC) content due to the extraction of proteins and carbohydrate fractions of microalgae. Figure 4 shows a photograph of the *C. fragile* samples after subcritical water treatment at different temperatures. Clearly, with increasing temperature, the color of the aqueous phase changed from light green to dark red brown. The solid phase also became darker with temperature. The changes in the color of the aqueous phase are mainly due to the hydrolysis products and decomposition of chlorophyll in the sample.

The above studies show the great potential of subcritical water as an organic solvent for extractive bioactive compounds from microalgae. In view of challenges associated with utilizing wet biomass, principally the need for water and nitrogen removal, reactions in sub- and supercritical water medium provide an attractive option for producing bioproducts and biofuels from microalgae.

3.2. SUBCRITICAL WATER LIQUEFACTION

Subcritical water liquefaction (also termed as hydrothermal liquefaction) process has attracted much attention due its versatility to utilize mixed biomass feedstock without any pretreatment or drying, at a comparatively low temperature. The process is used to convert biomass components to liquid products termed as biocrude. Biocrude sometimes also referred as bio-oil is an aqueous oxygenated solution derived from the direct liquefaction of biomass that can be converted to liquid fuel, hydrogen, or chemicals (Kumar and Gupta, 2009). Liquefaction of

Table 6. Biochemical composition of microalgae and cyanobacteria strains on dry ash free (% daf) basis Biller and Ross (2011).

Strains	Protein	Carbohydrate	Lipid
Chlorella vulgaris	55	9	25
Nannochloropsis oculata	57	8	32
Porphyridium cruentum	43	40	8
Spirulina	65	20	5

biomass in subcritical water proceeds through a series of structural and chemical transformations involving (Chornet and Overend, 1985):

- Solvolysis of biomass resulting in micellar-like structure
- Depolymerization of cellulose, hemicelluloses, and lignin
- Chemical and thermal decomposition of monomers to smaller molecules

Hydrothermal upgrading process was first developed by Shell, where biomass was subjected to subcritical water at 330°C to produce biocrude. Biocrude was further upgraded to liquid fuels via hydrodeoxygenation process (Gourdiaan and Peferoen, 1990). In a conceptual process scheme, it was shown that each ton (dry basis) of biomass can produce 300 kg (or 95 gal) of liquid fuel. There are few studies on liquefaction of algal biomass in subcritical water such as the liquefaction of *Dunaliella tertiolecta* and *Botryococcus braunii* in year 1995 (Minowa et al., 1995; Sawayama et al., 1995; DOE, 2010). Recently, Zhou et al. used marine macroalgae *Enteromorpha prolifera*, one of the main algae genera for green tide for subcritical water liquefaction study in a batch reactor at temperatures of 220–320°C (Dong Zhou et al., 2010). Biocrude yield was 23 wt% at 300°C in presence of 5 wt% Na_2CO_3. The higher heating value (HHV) of the biocrude was reported as 28–30 MJ/kg. It was a complex mixture of ketones, aldehydes, phenols, alkenes, fatty acids, esters, aromatics, and nitrogen containing heterocyclic compounds. Acetic acid was the main component of the water-soluble products. Biller and Ross (2011) compared the bio-oil production from three different microalgae strains and a cyanobacteria conducting hydrothermal liquefaction at 350°C and 20 MPa. Microalgae included *Chlorella vulgaris, Nannochloropsis occulata*, and *Porphyridium cruentum,* and the cyanobacteria was *Spirulina* with biochemical properties given in Table 6.

The yields of biocrude were 5–25 wt% higher than the lipid content of the algae depending upon biochemical composition. The yields of biocrude followed the trend lipids > proteins > carbohydrates. Table 7 shows the HHV of the bio-oil was in the range of 33–39 MJ/kg when microalgae and cyanobacteria were used as feedstock (Biller and Ross, 2011). The HHV of bio-oil from *Nannochloropsis oculata* was highest among these strains which are probably due to its higher lipid contents. The nitrogen content in bio-oil is due to the presence of protein fractions in the feedstock. Biocrude with low nitrogen and high carbon content is desirable. Nitrogen in fuel directly forms NO_x compounds which are undesirable

Table 7. Ultimate analysis, HHV, and energy recovery from microalgae at 350°C, 20 MPa, and 1 h via hydrothermal liquefaction.

Feedstock	C	H	N	S	O	HHV (MJ/kg)	Energy recovery (%)
Chlorella vulgaris	70.7	8.6	5.9	0	14.8	35.1	54.2
Nannochloropsis oculata	68.1	8.8	4.1	0	18.9	34.5	66.1
Porphyridium cruentum	72.8	8.5	5.4	0.3	13.3	35.7	51.6
Spirulina	73.3	9.2	7	0	10.4	36.8	50.7

due to environmental pollution and legislative reasons. The energy recovery is calculated as the ratio of energy contained in biocrude to the energy contained in the feedstock. The study showed that each biochemical component (lipid, carbohydrate, and protein) of feedstock contributes to the bio-oil production which is a distinct advantage of hydrothermal liquefaction compared to conventional physical oil extraction methods.

The products from hydrothermal liquefactions mainly consist of bio-oil, aqueous phase (dissolved organics), light gases, and insoluble residual solids. For the efficient liquefaction process, most of the carbon and hydrogen in the algal biomass should appear in bio-oil. The product separation is one of the most important aspects of hydrothermal liquefaction. In standard laboratory practice, an organic solvent such as dichloromethane, chloroform, hexane, and cyclohexane is used to separate bio-oil from the product mixture by liquid-liquid extraction step. Subsequently, organic solvent is evaporated to recover bio-oil. Figure 5 shows the general schematics of product separation. A typical gas phase composition from hydrothermal liquefaction is CO_2 (66.2%), CH_4 (1.9%), and H_2 (29.7%) along with nitrogen and traces of C_2 and C_3 gases (Brown et al., 2010). Generally, CO_2 consists of more than 85% of gas phase when reaction is conducted at lower temperature (≤300°C) which goes down with temperature, and hydrogen becomes a significant component of the gas phase at higher temperature (≥350°C). Overall, hydrothermal liquefaction of microalgae provides two major advantages over the other liquefaction processes. First, it can utilize biomass with very high water content and thus saves a considerable amount of energy required for dewatering. Secondly, the method is not species (type of feedstock) dependent where only species of high lipid contents can be used. The other polymeric components of microalgae such as proteins and carbohydrates also convert to bio-oil during the process, and so generally higher bio-oil yield is achieved.

3.3. SUPERCRITICAL WATER GASIFICATION (SCWG)

SCWG also termed as hydrothermal gasification is a novel method to process organic matter from biomass (Elliott, 2008). Biomass is gasified to mainly produce hydrogen, methane, and carbon dioxide. The SCWG process can be catalyzed by

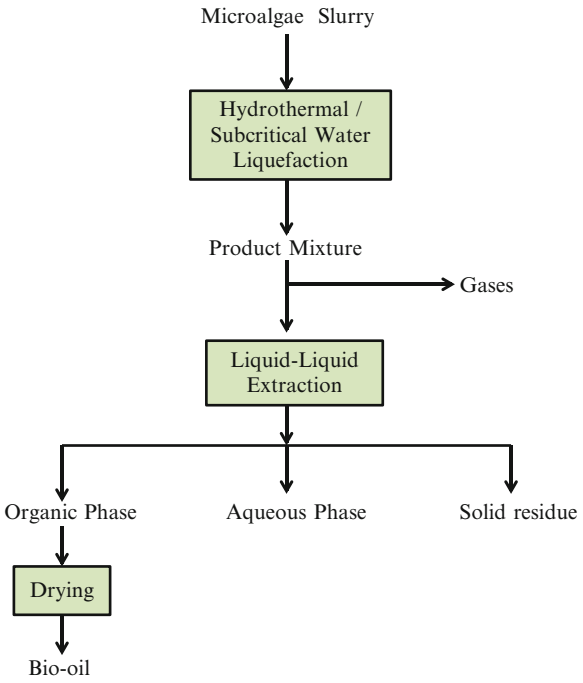

Figure 5. Standard product separation procedure after microalgae liquefaction.

a homogeneous or a heterogeneous catalyst (Kruse, 2009). A relatively fast hydrolysis of biomass in supercritical water leads to a rapid degradation of polymeric structure of biomass. The subsequent reactions also are rather fast, which leads to gas formation at relatively lower temperature compared to dry processes (Kruse, 2009). Above the critical point, the lower density of supercritical fluid favors free radical reactions and makes the reaction conditions conducive for the formation of methane and hydrogen gas (Kruse and Gawlik, 2003). Generally, there are two approaches to gasification in hot compressed water in terms of reaction temperature ranges. Biomass is gasified to mainly methane and carbon dioxide in the presence of an added heterogeneous catalyst in near critical or supercritical water (350–400°C). At higher temperature in supercritical water (500–700°C), biomass is converted to hydrogen-rich gas with or without catalyst or with nonmetal catalysts (Elliott, 2008).

For hydrogen formation from biomass, Watanabe et al. (2002) used Zirconia (ZrO_2) to catalyze the reaction, while Elliott et al. (1993) and Byrd et al. (2007) demonstrated the significant activity of Ru, Rh, and Ni as catalysts. There are numerous studies describing SCWG of cellulosic biomass for methane, hydrogen, or fuel gas production (Matsumura et al., 2005; Peterson et al., 2008). The Pacific Northwest National Laboratory (PNNL) has extensively studied the catalytic hydrothermal gasification process for several feedstocks in the last 30 years.

The principal advantages of SCWG are that process can utilize wet feedstock and can achieve high gasification efficiency (nearly 100% carbon conversion) at comparatively low temperature (400–700°C) (Antal et al., 2000). The homogeneous reaction medium with a minimal mass transfer resistance favors decomposition of organic compounds into gases, decreasing formation of tar and char (Calzavara et al., 2005). Moreover, fuel gas is produced directly at high pressure, which means a smaller reactor volume and lower energy needed to pressurize the gas in a storage tank. The resulting fuel gas is cleaner and less corrosive compared to the conventional dry processes.

The overall biomass gasification for hydrogen production can be represented by a simplified reaction as:

$$\text{Overall}: CH_xO_y + (2-y)H_2O \rightarrow CO_2 + (2-y+x/2)H_2 \quad (1)$$

where x and y are the elemental molar ratios of H/C and O/C in biomass, respectively. Equation (1) is overall reaction, whereas group of intermediate reaction should also be considered for optimizing the SCWG for hydrogen-rich flue gases such as:

$$\text{Steam Reforming}: \quad CH_xO_y + (1-y)H_2O \rightarrow CO + (1-y+x/2)H_2 \quad (2)$$

$$\text{Water - gas shift}: \quad CO + H_2O \leftrightarrow CO_2 + H_2 \quad (3)$$

$$\text{Methanation}: \quad CO + 3H_2 \leftrightarrow CH_4 + H_2O \quad (4)$$

To optimize the hydrogen production from biomass, methanation reaction (4) should be restrained, whereas water-gas shift reaction (3) is desired (Guo et al., 2007). Guo et al. (2007) predicted the thermodynamic equilibrium of wood sawdust ($CH_{1.35}O_{0.617}$) based on minimizing Gibbs free energy. The predicted results show that the product gas includes mainly H_2, CH_4, CO, and CO_2. Figure 6 shows the equilibrium gas composition in the range of 400–700°C at 25 MPa with 5 wt% dry biomass loading. The yield of H_2 and CO_2 increases with temperature, whereas the yield of CH_4 drops sharply. The equilibrium CO yield is very small (order of 10^{-3} mol/kg dry biomass).

During SCWG, the gas yield is $CO_2 > CH_4 > H_2 > CO$ at lower temperatures, whereas it changes to $H_2 > CO_2 > CH_4 > CO$ at higher temperature (above 500°C). The yield of H_2 and CO_2 increases with temperature (Yan et al., 2006). At high temperature, Eq. (3) favors H_2 and CO_2 production. Alkali metals, transition metals (Ni, Pt, Ru, and Rh), and activated carbon have been used to catalyze SCWG process (Guo et al., 2010). The presence of alkali salts catalyzes the gasification processes (Kumar and Gupta, 2008). Due to insolubility of inorganic salts in supercritical water, the alkali salts precipitate out rapidly as fine particles, and these in situ-generated particles provide extra surface area for heterogeneous catalysis (Muthukumaraa and Gupta, 2000). The addition of alkali metal salts (e.g., $KHCO_3$, KOH, Na_2CO_3, K_2CO_3) also reduces coke formation and

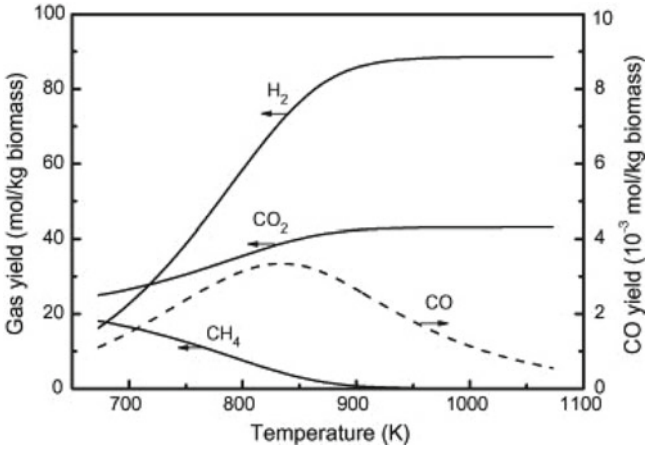

Figure 6. Equilibrium gas yields with temperature at 25 MPa and 5 wt% of dry biomass (Reproduced from Guo et al., 2007).

catalyzes the water-gas shift reaction (Hao et al., 2003). For example, the addition of $KHCO_3$ leads to an increase in gas formation and a decrease in the amount of carbon monoxide (Sinag et al., 2003). For example, the use of K_2CO_3 in the reaction mixture during depolymerization of cellulose in subcritical water substantially enhanced gas formation (Kumar and Gupta, 2008). Ambient water is an excellent solvent (typically several 100 g/l) for most of the salts. On the other hand, solubility of these salts decreases drastically (1–100 ppm) in the low-density supercritical water (Kritzer and Dinjus, 2001). Dielectric constant of water decreases from 78.5 at 25°C to 5 in the near critical region which causes reduced solubility of inorganic ionic compounds in water. Due to insolubility of inorganic salts in supercritical water, the alkali salts precipitate out rapidly as fine particles, and these *in situ*-generated particles provide extra surface area for heterogeneous catalysis (Muthukumaraa and Gupta, 2000).

SCWG has been utilized to gasify coal. Hui et al. (2010) obtained gas containing 70% hydrogen from gasification of a 24 wt% coal-water slurry with 2 wt% sodium carboxymethyl cellulose and 1 wt% K_2CO_3 at 580°C and 25 MPa using a fluidized bed reactor. In SCWG, water acts as a resource of hydrogen. This concept was proven experimentally by Park et al. (when D_2O was used instead of H_2) as reaction medium of SCWG of some organic compound with RuO_2 catalyst. It was found that methane and hydrogen in gas production were not CHD_3 and H_2 or HD, but CD_4 and D_2, which indicated that hydrogen in gaseous product is from water (Guo et al., 2010).

3.3.1. SCWG of Microalgae

Gasification of the algal biomass provides an extremely flexible way to produce different liquid fuels, primarily through Fischer-Tropsch (F-T) synthesis or mixed alcohol synthesis of the resulting syngas. The synthesis of mixed alcohols using

gasification of cellulosic biomass is relatively mature (Phillips, 2007). The major challenge in utilizing algae for the similar application is adjusting the water content of algae (DOE, 2010). If a breakthrough is achieved in algae harvesting and drying, gasification of algae to these biofuels would be comparatively straightforward. Syngas from microalgae has several advantages over other conversion methods. Foremost, it is possible to create a wide variety of fungible fuels with acceptable and known properties. Additionally, syngas is a versatile feedstock and can be used as a building block for producing a number of renewable products, making the process more flexible. Also attractive is the possibility to integrate with existing industrial thermochemical infrastructure (DOE, 2010).

In SCWG, fuel gas is produced directly at high pressure, which means a smaller reactor volume and lower energy needed to pressurize the gas in a storage tank. The nonvolatile inorganic constituents of the coproduct residue are expected to remain in the aqueous solution. This makes the resulting gas cleaner and less corrosive compared to the conventional dry processes.

Ideally, a good molar ratio of H_2 and CO is 2:1 to form alkanes [−CH2−] according to the following reaction (Gupta and Demirbas, 2010a, b):

$$n\mathrm{CO} + 2n\mathrm{H_2} \rightarrow \left[-\mathrm{CH_{2^-}}\right] + n\mathrm{H_2O} \tag{5}$$

Syngas from carbohydrates and other biomass normally has 1:1 M ratio ($C_6H_{12}O_6$ $6CO + 6H_2$). However, H_2 to CO ratio can be adjusted by the use of water-gas shift (WGS) reaction ($CO + H_2O$ $CO_2 + H_2$).

Alkali metal salts in gasification reduces not only the coke formation but also catalyzes the water-gas shift reactions.

Stucki et al. proposed a novel concept on microalgae cultivation using dilute fossil CO_2 emissions and the conversion of the algal biomass to a methane-rich gas (similar to natural gas) through a catalytic (Ru/C and Ru/ZrO_2 catalyst) SCWG (Stucki et al., 2009). The study showed that the complete gasification of microalgae (*Spirulina platensis*) to a methane-rich gas is now possible in supercritical water in the presence of ruthenium catalysts. The process estimated that 60–70% of the heating value of dry algal biomass could be recovered as methane gas in a short reaction time (of the order of several minutes). SCWG of microalgae for methane production is much more efficient than the conventional anaerobic digestion which can be conducted in much shorter time and can be an elegant way to tackle both climate change and dependence on fossil natural gas without competing with food production. Figure 7 represents a simplified system approach for sustainable algae-to-methane process proposed by Stucki et al.

The overall SSCWG reaction of *S. platensis*, approximated by $C_{1.0}H_{1.71}O_{0.48}N_{0.19}S_{0.005}$, was represented by the following simplified reaction:

$$\mathrm{C_{1.0}H_{1.71}O_{0.48}N_{0.19}S_{0.005}} + 0.48\mathrm{H_2O} \rightarrow 0.52\ \mathrm{CH_4} + 0.48\ \mathrm{CO_2} + 0.19\ \mathrm{NH_3} + 0.005\mathrm{H_2S} \tag{6}$$

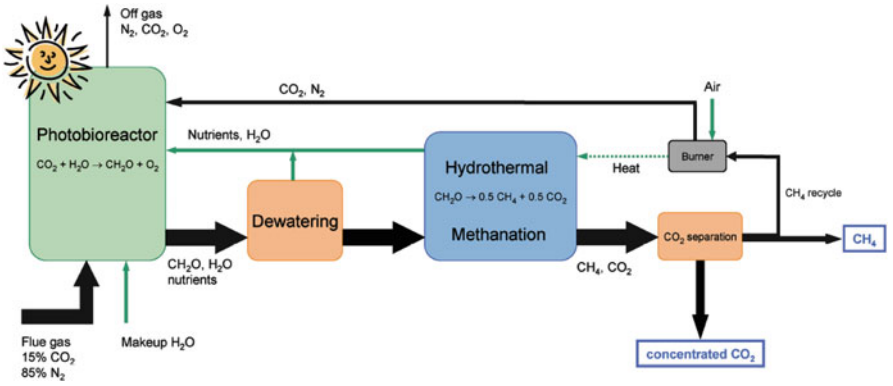

Figure 7. Simplified sketch of the algae-to-methane process via SCWG/hydrothermal gasification. The thickness of the *black arrows* is proportional to the molar flow of carbon (Reproduced from Stucki et al., 2009).

To make the SCWG process thermally self-sufficient, a part of methane gas can be combusted to meet the process heat requirement along with waste heat recovery schemes. In the overall scheme, it was deem that the dewatering of microalgae to 15–20 wt% solid loads for SCWG reactor may only be a critical issue.

In another study, Chakinala et al. (2010) reported that more than 80 wt% of microalgae (*Chlorella vulgaris*) could be gasified in supercritical water. The studies were conducted in quartz capillaries under operating temperatures of 400–700°C, reaction times of 1–15 min in the presence of nickel based catalysts. The dry gas composition (Fig. 8) was mainly comprised of CO_2, CO, CH_4, H_2, and some C_2–C_3 compounds (Chakinala et al., 2010).

The study concluded that the maximum algae gasification efficiency was 75% at 600°C for reaction times of 4 min and higher. The SCWG seemed to be "kinetically determined" and can be increased by increasing temperature, decreasing concentration, and the application of catalysts (Chakinala et al., 2010). Guan et al. (2012) recently conducted a systematic SCWG study of *Nannochloropsis* sp. in the range of 450–550°C for kinetic modeling. The product gases were mainly H_2, CO_2, and CH_4, with lesser amounts of CO, C_2H_4, and C_2H_6. The solid loading strongly affects product gas composition. The yield of hydrogen increased with the decrease of solid loading. The variation of intermediate products indicated that alkanes as an intermediate product reacted quicker than the aromatics. On the basis of this observation and experimental results, a global reaction network (Fig. 9) for algae SCWG was proposed.

The variables such as gasification efficiency, carbon conversion efficiency, gas composition, and energy recovery are generally system specific in SCWG. The types of intermediates mostly govern the products composition, and the

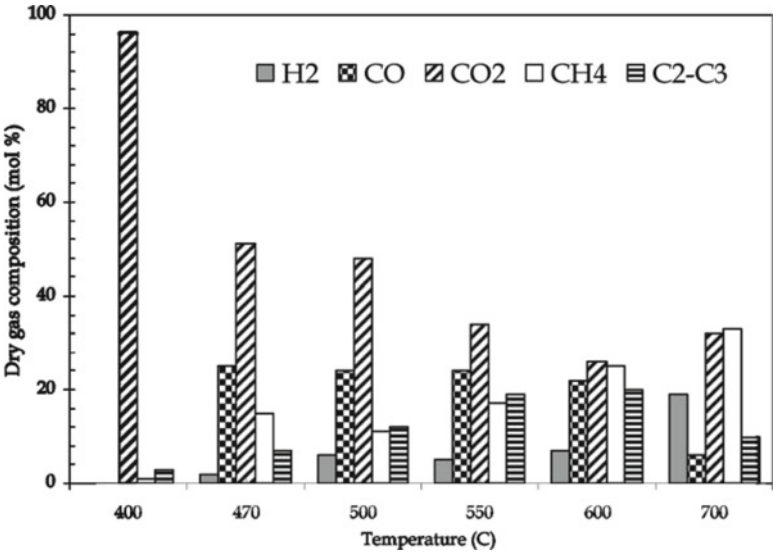

Figure 8. Effect of temperature on dry gas composition at conditions at reaction time 2 min; algae 7.3 wt%; and pressure 24 MPa Reproduced (from Chakinala et al., 2010).

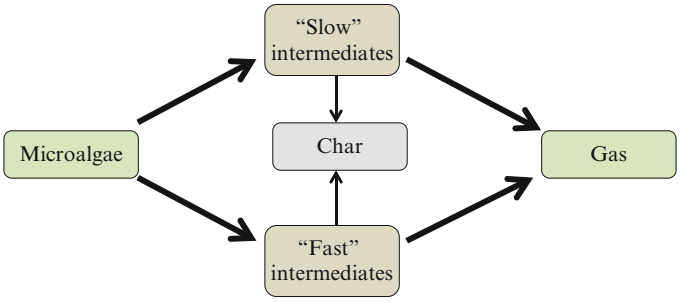

Figure 9. Reaction network for algae gasification in supercritical water (Adapted from Guan et al., 2012).

intermediates that are difficult to gasify are largely responsible for solid residue (char). Proteins are reported to be difficult to gasify and result in coke formation. Protein hydrolyzes to amino acids which degrades subsequently and also forms free stable radical anions via the Maillard reaction of proteins and carbohydrates (Chakinala et al., 2010). The biochemical composition of microalgae (fractions of lipids, carbohydrate, and proteins) is also expected to effect the product gas composition.

4. Challenges of Sub- and Supercritical Water Processing

Although in laboratory experiments excellent results have been achieved and the technology possesses many potential benefits over the conventional methods of processing biomass to biofuels or chemicals, there are certain issues which need to be addressed:

- *Biomass feeding at high pressure*: A solid loading in excess of 15–20 wt% is considered generally economical from a commercial point of view. Feeding slurries at high pressure is always challenging especially for the lab scale studies since low capacity slurry pumps are rarely available. Pumping slurry at large scale is less of a problem, where progressive cavity or similar pumps are commercially available. Microalgae are nonfibrous and oily when compared to cellulosic biomass, and hence, engineering challenges associated with high pressure pumping of algae slurry may not be a significant issue.
- *Salt precipitation*: Plugging of reactors caused by the precipitation of inorganic salts above supercritical temperature (>374°C) and low-density conditions. However, the problem may be used as an opportunity to produce a valuable fertilizer by-product of the process, if managed properly.
- *Corrosion*: The halogens sulfur or phosphorous present in the organic matters is converted to the respective acids, which may cause severe corrosion on the reactor wall under harsh reaction conditions. The corrosion problem can be avoided by selecting the right material of construction and or a slightly modified rector concept. With the successful commercialization of large-scale supercritical water-cooled reactors in nuclear industry, it can be assumed that there are existing materials which can be used for building supercritical water-based reactors for biofuels applications.
- *Coking and deactivation of heterogeneous catalyst*: Some catalyst supports degrades or oxidize in hydrothermal conditions. Decline in catalyst activity is also observed with long period of exposure of catalyst to high-temperature water.

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