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Steven P. Harvey
Editors

Sustainable Biotechnology

Sources of Renewable Energy

Springer

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Editors
Om V. Singh
University of Pittsburgh
Bradford, PA 16701
USA

Steven P. Harvey
U.S. Army Chemical Biological Center
Aberdeen Proving Ground, MD 21010
USA

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The editors gratefully dedicate this book to Daisaku Ikeda in appreciation for his encouragement to us.

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Contributors

F. Gabriel Acién Department of Chemical Engineering, University of Almería, La Cañada de San Urbano, Almería, Spain, facien@ual.es

Cristóbal N. Aguilar Department of Food Science and Technology, School of Chemistry, Universidad Autonoma de Coahuila, Saltillo, Coahuila, México, cristobal.aguilar@mail.uadec.mx

Antonio Aguilera-Carbo Food Science and Nutrition Department, Universidad Autónoma Agraria "Antonio Narro", Buenavista, Saltillo, Coahuila, México, aguilera_carbo@yahoo.com

Rajesh Arora Radiation Biotechnology Laboratory, Division of Radiation Biosciences, Institute of Nuclear Medicine and Allied Sciences, Delhi-110054, India, rajesharoradr@rediffmail.com

Deen Dayal Bansal Radiation Biotechnology Laboratory, Division of Radiation Biosciences, Institute of Nuclear Medicine and Allied Sciences, Delhi-110054, India, bansaldd@gmail.com

Michael S. Briggs Department of Physics, University of New Hampshire, Durham, NH 03824, USA, msbriggs@unh.edu

Anuj K. Chandel Centre for Biotechnology, Jawaharlal Nehru Technological University; Department of Microbiology, Osmania University, Hyderabad-500 007, India, chandel_anujkumar@yahoo.com

Fengjie Cui Department of Food, Agricultural and Biological Engineering, Ohio Agricultural Research and Development Center, Ohio State University, Wooster, OH 44691, USA, cui.43@osu.edu

Stephen R. Decker Chemical and Biosciences Center, National Renewable Energy Laboratory, Golden, CO 80401, USA, Steve.Decker@nrel.gov

Esperanza Del Río Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla-Consejo Superior de Investigaciones Científicas, 41092-Sevilla, Spain, esperanza@ibvf.csic.es

Bryon S. Donohoe Chemical and Biosciences Center, National Renewable Energy Laboratory, Golden, CO 80401, USA, Bryon.Donohoe@nrel.gov

Michael D. Gagnon Department of Chemical Engineering, University of New Hampshire, Durham, NH 03824, USA, mdm4@cisunix.unh.edu

Miguel G. Guerrero Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla-Consejo Superior de Investigaciones Científicas, 41092-Sevilla, Spain, mgguerrero@us.es

Steven P. Harvey US Army Edgewood Chemical Biological Center, AMSRD-ECB-RT-BC, MD 21010-5424, USA, steve.harvey@us.army.mil

Michael E. Himmel Chemical and Biosciences Center, National Renewable Energy Laboratory, Golden, CO 80401, USA, Mike.Himmel@nrel.gov

A. Maarten J. Kootstra Valorisation of Plant Production Chains; Food and Bioprocess Engineering Group, University of Wageningen, 6700 EV, Wageningen, The Netherlands, maarten.kootstra@wur.nl

Raj Kumar Radiation Biotechnology Laboratory, Division of Radiation Biosciences, Institute of Nuclear Medicine and Allied Sciences, Delhi-110054, India, rajkumar790@yahoo.com

Norio Kurosawa Faculty of Engineering, Department of Environmental Engineering for Symbiosis, Soka University, Hachioji, Tokyo, Japan, kurosawa@soka.ac.jp

Yebo Li Department of Food, Agricultural and Biological Engineering, Ohio Agricultural Research and Development Center, Ohio State University, Wooster, OH 44691, USA, li.851@osu.edu

Renpeng Liu Division of Medicinal Chemistry and Natural Product, UNC Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599, USA, renpengl@email.unc.edu

Jian Liu Division of Medicinal Chemistry and Natural Product, UNC Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599, USA, jian_liu@unc.edu

Ron Madl Department of Grain Science and Industry, Kansas State University, Manhattan, KS 66506, USA, rmadl@ksu.edu

Silvia Martins Department of Biological Engineering, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal, smartins@deb.uminho.pt

Marco Mata-Gómez Department of Food Science and Technology, School of Chemistry, Universidad Autonoma de Coahuila, Saltillo, Coahuila, México, matag_24@yahoo.com.mx

James McLaren StrathKirn Inc., Chesterfield, MO 63017, USA, mclaren@strathkirn.com

Diego Mercado Department of Food Science and Technology, School of Chemistry, Universidad Autonoma de Coahuila, Saltillo, Coahuila, México, diego.mercado03@gmail.com

Saurabh Mishra Radiation Biotechnology Laboratory, Division of Radiation Biosciences, Institute of Nuclear Medicine and Allied Sciences, Delhi-110054, India, saurabhrbt@gmail.com

Anis Mohammed Radiation Biotechnology Laboratory, Division of Radiation Biosciences, Institute of Nuclear Medicine and Allied Sciences, Delhi-110054, India, mohdaniskhan@gmail.com

S. Venkata Mohan Bioengineering and Environmental Centre (BEEC), Indian Institute of Chemical Technology (IICT), Hyderabad-500007, India, vmohan_s@yahoo.com; svmohan@iict.res.in

Norio Nagao Faculty of Engineering, Department of Environmental Engineering for Symbiosis, Soka University, Hachioji, Tokyo, Japan, nnagao@soka.ac.jp

Dev Dutt Patel Radiation Biotechnology Laboratory, Division of Radiation Biosciences, Institute of Nuclear Medicine and Allied Sciences, Delhi-110054, India, pateldevdutt@gmail.com

L.V. Rao Department of Microbiology, Osmania University, Hyderabad-500 007, India, vrlinga@gmail.com

Luis Rodriguez Department of Food Science and Technology, School of Chemistry, Universidad Autonoma de Coahuila, Saltillo, Coahuila, México, luisvictor_ibi@hotmail.com

Raul Rodriguez Department of Food Science and Technology, School of Chemistry, Universidad Autonoma de Coahuila, Saltillo, Coahuila, México, rrh961@hotmail.com

Johan P.M. Sanders Valorisation of Plant Production Chains, University of Wageningen, 6700 AA Wageningen, The Netherlands, johan.sanders@wur.nl

Floyd L. Schanbacher Department of Animal Sciences and Environmental Science Graduate Program, The Ohio Agricultural Research and Development Center, The Ohio State University, Columbus, OH 43210, USA, schanbacher.1@osu.edu

Elinor L. Scott Valorisation of Plant Production Chains, University of Wageningen, 6700 AA Wageningen, The Netherlands, elinor.scott@wur.nl

Michael J. Selig Chemical and Biosciences Center, National Renewable Energy Laboratory, Golden, CO 80401, USA, Michael.Selig@nrel.gov

Ashok Sharma Radiation Biotechnology Laboratory, Division of Radiation Biosciences, Institute of Nuclear Medicine and Allied Sciences, Delhi-110054, India, aks_inmas@yahoo.co.in

Rakesh Kumar Sharma Radiation Biotechnology Laboratory, Division of Radiation Biosciences, Institute of Nuclear Medicine and Allied Sciences, Delhi-110054, India, Sharma-rkssharmadrl@yahoo.com

Om V. Singh Division of Biological and Health Sciences, University of Pittsburgh, Bradford, PA 16701, USA, ovs11@pitt.edu, ovs11@yahoo.com

Alexander Steinbüchel Institut für Mikrobiologie der Westfälischen, Wilhelms-Universität Münster, 48149 Münster, Germany, steinbu@uni-muenster.de

Tatsuki Toda Faculty of Engineering, Department of Environmental Engineering for Symbiosis, Soka University, Hachioji, Tokyo, Japan, toda@soka.ac.jp

Rajendra Prasad Tripathi Radiation Biotechnology Laboratory, Division of Radiation Biosciences, Institute of Nuclear Medicine and Allied Sciences, Delhi-110054, India, director@inmas.org

Melvin P. Tucker National Bioenergy Center, National Renewable Energy Laboratory, Golden, CO 80401, USA, Melvin.Tucker@nrel.gov

James J. Valdes Department of the Army, Research, Development and Engineering Command, Aberdeen Proving Ground, MD 21010-5424, USA, james.valdes@us.army.mil

Palligarnai T. Vasudevan Department of Chemical Engineering, University of New Hampshire, Durham, NH 03824, USA, vasu@unh.edu

Sridhar Viamajala Department of Chemical and Environmental Engineering, The University of Toledo, Toledo, OH 43606-3390, sridhar.viamajala@utoledo.edu

Todd B. Vinzant Chemical and Biosciences Center, National Renewable Energy Laboratory, Golden, CO 80401, USA, Todd.Vinzant@nrel.gov

Donghai Wang Department of Biological and Agricultural Engineering, Kansas State University, Manhattan, KS 66506, USA, dwang@ksu.edu

Jerry B. Warner Defense Life Sciences, LLC, McLean, VA 22101, USA, warner@dlsi.com

Keiko Watanabe Faculty of Engineering, Department of Environmental Engineering for Symbiosis, Soka University, Hachioji, Tokyo, Japan, kewatana@soka.ac.jp

Xiaorong Wu Department of Biological and Agricultural Engineering, Kansas State University, Manhattan, KS 66506, USA, xwu3786@ksu.edu

Zhongtang Yu Department of Animal Sciences and Environmental Science Graduate Program, The Ohio Agricultural Research and Development Center, The Ohio State University, Columbus, OH 43210, USA, yu.226@osu.edu

Applications of Biotechnology for the Utilization of Renewable Energy Resources

Om V. Singh and Steven P. Harvey

Introduction

Even given the seemingly unlikely near-term resolution of issues involving atmospheric CO_2 levels and their effect on the climate, the adoption of global conservation measures, and the stabilization of fossil fuel prices, it is still a certainty that global oil and gas supplies will be largely depleted in a matter of decades. That much is clear from even a cursory comparison of the independent estimates of the world's oil and natural gas reserves and the respective data on their consumption, as published regularly on the internet by the US Government Energy Information Administration [1]. Nature of course, offers abundant *renewable* resources that can be used to replace fossil fuels but issues of cost, technology readiness levels, and compatibility with existing distribution networks remain. Cellulosic ethanol and biodiesel are the most immediately obvious target fuels, with hydrogen, methane and butanol as other potentially viable products. Other recent reports have covered various aspects of the current state of biofuels technology [2–4]. Here we continue to bridge the technology gap and focus on critical aspects of lignocellulosic biomolecules and the respective mechanisms regulating their bioconversion to liquid fuels and value-added products of industrial significance.

The lignocellulosic structure does not readily yield its component five- and six-carbon sugars so the efficient biological conversion of biomass typically requires a pretreatment step to render the polysaccharide molecules accessible to enzymes. Several thermochemical or biochemical approaches are currently in various stages of development, and have the potential for major impact on the economics of biofuel

O.V. Singh (✉)
Division of Biological and Health Sciences, University of Pittsburgh, 300 Campus Drive, Bradford, PA 16701 USA
e-mail: ovs11@pitt.edu, ovs11@yahoo.com

S.P. Harvey (✉)
U.S. Army Edgewood Chemical Biological Center, AMSRD-ECB-RT-BC, Bld E3150, 5183 Blackhawk Rd, Aberdeen Proving Ground, MD 21010-5424, USA
e-mail: steve.harvey@us.army.mil

production. In order to derive a stable and cost-effective approach, a greater fundamental understanding is needed of the exact effects of these processes on plant anatomy. These are difficult experiments to conduct and in Chapter 1 "Heat and Mass Transport in Processing of Lignocellulosic Biomass for Fuels and Chemicals", *Viamajala* et al. provide an in-depth report on the effects of heat and mass transport on the efficiency of biomass conversion. Further, *Wu* et al. in Chapter 2 "Biofuels from Lignocellulosic Biomass", give the matter a more detailed consideration by comparing thermochemical and biochemical approaches to the production of biofuel from lignocellulosic biomass.

As compared to gas and oil, relatively greater potential reserves exist for both coal and uranium (probably on the order of a century) but neither is renewable and each is associated with its own environmental conundrum (carbon release and waste storage, respectively). Linus Pauling expressed a particular concern for the destruction of the element uranium, saying "In a thousand or ten thousand years the world may require uranium for a purpose about which we are currently ignorant." [5]. Looking beyond the immediate temporal horizon, we are unavoidably confronted with the need to develop permanently renewable sources of energy.

Earth's most plentiful and renewable energy resources typically include sunlight, wind, geothermal heat, water (rivers, tides and waves), and biomass. All of these are suitable for the generation of electricity but biomass is the current main renewable feedstock for the production of "liquid" fuels - typically ethanol, and biodiesel and possibly to include butanol, hydrogen and methane. These liquid fuels, or energy carriers lie at the heart of the solution to the global energy problem, since they are the materials currently most suitable for use in the transportation sector and for the direct replacement of the immediately endangered fossil resources of oil and gas. *Vasudevan* et al. in Chapter 3 "Environmentally Sustainable Biofuels – The Case for Biodiesel, Biobutanol and Cellulosic Ethanol" provide a detailed discussion of the case for ethanol, butanol and biodiesel. Significantly, a potential technical hurdle confronting the production of biofuels is the efficiency of utilization of hemicellulose-derived sugars. In Chapter 4 "Biotechnological Applications of Hemicellulosic Derived Sugars: State-of-the-Art", *Chandel* et al. examine the challenges associated with the successful utilization of this second most abundant polysaccharide in nature.

Energy-yielding materials are found in various guises, one of which is garbage. Although not always classified as a resource, garbage clearly is renewable (increasingly so, in fact), and processes that convert it into energy are obviously dually beneficial. In Chapter 5 "Tactical Garbage to Energy Refinery (TGER)", *Valdes and Warner* present a hybrid biological/thermochemical system designed for the conversion of military garbage into ethanol and electricity, with clear potential for applications in the civilian sector.

Agricultural waste (e.g. livestock, manure, crop residues, food wastes etc.) is a high impact feedstock with particular utility in the production of biogas. In Chapter 6 "Production of Methane Biogas as Fuel Through Anaerobic Digestion", *Yu and Schanbacher* discuss the anaerobic conversion of biomass to methane. Untreated wastewater also contains biodegradable organics that can

be used to produce hydrogen or methane. In Chapter 7 "Waste to Renewable Energy: A Sustainable and Green Approach Towards Production of Biohydrogen by Acidogenic Fermentation", *Mohan* provides a detailed review of the state of the art with regard to biological hydrogen production using waste and wastewater as substrates with dark fermentation processes.

Many biological processes use mixed cultures operating under non-sterile conditions (e.g. biological hydrogen and methane production, as discussed above). *Watanabe* et al. in Chapter 8 "Bacterial Communities in Various Conditions of the Composting Reactor Revealed by 16S rDNA Clone Analysis and Denaturing Gradient Gel Electrophoresis" demonstrate the utility of 16S rRNA analysis and denaturing gradient gel electrophoresis (DGGE) techniques for tracking microbial communities within a mixed and changing culture. Their work uses a composting process, which offers a typically cost-effective alternative to incineration for the remediation of contaminated soil.

The production of liquid fuel from biomass necessitates the consideration of various issues such as the effects on the food supply, the rainforest, and greenhouse gas production, as well as carbon sustainability certification. Some of these issues may require appropriate regulations and in Chapter 9 "Perspectives on Bioenergy and Biofuels"*, Scott* et al., examine these issues closely.

In addition to its environmental advantages, the use of renewable energy resources offers the potential for stimulation of the economies of the nations where they are produced. The potential products of these renewable materials extend well beyond liquid fuels alone. Owing partly to the enormous volume of their production, fuels are sold for relatively low prices, and the successful implementation of renewable fuels depends, at least initially, on their ability to compete in the marketplace. To this end, it is particularly important to maximize the efficiency of their production in biorefineries where secondary products would be derived from the same feedstock as the fuels. As an example, petroleum refineries have been in operation for over 150 years and now produce lubricants, plastics, solvents, detergents, etc., all from the starting crude oil [6]. Similarly, biomass, in addition to being used for the production of fuels, can be used as a starting material for the production of other value-added products of microbial bioconversion processes such as fermentable sugars, organic acids and enzymes. In Chapter 10 "Perspectives on Chemicals from Renewable Resources", *Scott* et al. describe how, with the aid of biotechnology, Protamylase® generated from starch production, can be used as a medium for the production of a cynophycin polymer, which is a major source of arginine and aspartic acid for the production of many industrially useful compounds including 1,4-butanediamine and succinic acid. In Chapter 11 "Microbial Lactic Acid Production from Renewable Resources", *Li and Cui* describe the production of lactic acid from renewable resources such as starch biomass, cheese whey etc. Lactic acid has recently gained attention due its application to the manufacture of biodegradable polymers. Among other renewable resources, Chapter 12 "Microbial Production of Potent Phenolic-Antioxidants Through Solid State Fermentation", *Martin* et al. describe the role of agroindustrial residues including plant tissues rich in polyphenols for the microbial bioconversion of potent phenolics under solid state

fermentation conditions. Hence, combined with the economy of scale derived from large refineries, secondary products could be key to bridging the price gap between fossil fuels and renewables.

One critical advantage of biofuels is their potential to achieve a reduction in greenhouse gas releases, since the plants from which they are produced derive their carbon from the atmosphere. The overall balance of greenhouse gases however, depends in large measure on the particular feedstocks used and the methods by which they are produced. Corn ethanol for instance, while being potentially carbon neutral, is not likely to achieve an overall reduction in greenhouse gas release due to its requirement for nitrogenous fertilizer and the associated release of nitrous oxide [7]. An interesting approach to the production of biodiesel is the use of algae to synthesize oil from the CO_2 they capture for growth. Algae cultivation offers a potential low-cost alternative to physical methods of carbon sequestration such as pumping liquid CO_2 underground or underwater or chemical methods such as base-mediated capture of CO_2 and subsequent burial of the resulting carbonates. The algae, while using CO_2 as their sole source of carbon for growth, can produce up to 50% of their weight in oil suitable for conversion to biodiesel. Algae are one of the best sources of plentiful biomass on earth; their potential for biosynthesis of astaxanthin, a red carotenoid nutraceutical responsible for the color of salmon flesh, was explored in Chapter 13 "Photoautotrophic Production of Astaxanthin by the Microalga *Haematococcus pluvialis*", *Del Rio* et al.

In a biological system, the biosynthesis of industrially useful compounds has long been recommended. Heparin, a low-molecular weight highly sulfated polysaccharide represents a unique class of natural products, that has long been used as an anticoagulant drug. Due to recent outbreaks of contamination and seizure of heparin manufacturing facilities [8], an efficient bioconversion process of heparin is required. In Chapter 14 "Enzymatic Synthesis of Heparin", *Liu and Liu* describe novel enzymatic approaches for the biosynthesis of heparin sulfate that mimic *E. coli* heparosan.

Discovering new and sustainable resources can help refuel industrial biotechnology. Adverse environmental conditions which normal earth microbiota do not tolerate, offer potential sites to explore specific sets of microorganisms designated as "Extremophiles". The discovery of these microorganisms has enabled the biotechnology industry to innovate unconventional bioproducts i.e. "Extremolytes" [9]. In Chapter 15 "Extremophiles: Sustainable Resource of Natural Compounds-Extremolytes", *Kumar* et al. provide an overview of these extreme habitats. The applications of extremophiles and their products, extremolytes, with their possible implications for human use are also discussed broadly.

This book "Sustainable Biotechnology: Sources of Renewable Energy" is a collection of research reports and reviews elucidating several broad-ranging areas of progress and challenges in the utilization of sustainable resources of renewable energy, especially in biofuels. This book comes just at a time when government and industries are accelerating their efforts in the exploration of alternative energy resources, with expectations of the establishment of long-term sustainable alternatives to petroleum-based liquid fuels. Apart from liquid fuel this book also

emphasizes the use of sustainable resources for value-added products, which may help in revitalizing the biotechnology industry at a broader scale.

We hope readers will find these articles interesting and informative for their research pursuits. It has been our pleasure to put together this book with Springer press. We would like to thank all of the contributing authors for sharing their quality research and ideas with the scientific community through this book.

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Heat and Mass Transport in Processing of Lignocellulosic Biomass for Fuels and Chemicals

Sridhar Viamajala, Bryon S. Donohoe, Stephen R. Decker, Todd B. Vinzant, Michael J. Selig, Michael E. Himmel, and Melvin P. Tucker

Abstract Lignocellulosic biomass, a major feedstock for renewable biofuels and chemicals, is processed by various thermochemical and/or biochemical means. This multi-step processing often involves reactive transformations limited by heat and mass transport. These limitations are dictated by restrictions including (1) plant anatomy, (2) complex ultra-structure and chemical composition of plant cell walls, (3) process engineering requirements or, (4) a combination of these factors. The plant macro- and micro-structural features impose limitations on chemical and enzyme accessibility to carbohydrate containing polymers (cellulose and hemicellulose) which can limit conversion rates and extents. Multiphase systems containing insoluble substrates, soluble catalysts and, in some cases, gaseous steam can pose additional heat and mass transfer restrictions leading to non-uniform reactions. In this chapter, some of these transport challenges relevant to biochemical conversion are discussed in order to underscore the importance of a fundamental understanding of these processes for development of robust and cost-effective routes to fuels and products from lignocellulosic biomass.

Keywords Lignocellulose · Biomass · Biofuels · Heat transport · Mass transport

1 Introduction

The biochemical conversion of lignocellulosic biomass requires several processing steps designed to convert structural carbohydrates, such as cellulose and hemicellulose, to monomeric sugars, which include glucose, xylose, arabinose, and mannose. These sugars can be fermented to ethanol and other products, to varying degrees of effectiveness, by wild type and modified microbial strains. The front end of the process includes feedstock size reduction followed by a thermal chemical treatment, called pretreatment. In practice, this unit operation usually involves the exposure of

S. Viamajala (✉)
Department of Chemical and Environmental Engineering, The University of Toledo, Toledo, OH 43606-3390
e-mail: sridhar.viamajala@utoledo.edu

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biomass to acid or alkaline catalysts at temperatures ranging from 120 to 200°C. Pretreated slurries (the hydrolysate liquor containing soluble sugars, oligosaccharides, and other released solubles plus the residual solids) are then enzymatically digested at 40–60°C to release sugars from the polysaccharides and oligomers remaining after pretreatment [1–9]. In both of these steps, adequate heat, mass, and momentum transfer is required to achieve uniform reactions and desirable kinetics.

Plant cell walls, which make up almost all of the mass in lignocellulosic biomass, are highly variable both across and within plant tissue types. At the macroscopic scale, such as within a stem or leaf, uneven distribution of catalyst (chemical or enzyme) due to the different properties of different tissues results in heterogeneous treatment, with only a fraction of the plant material exposed to optimal conditions [10–13]. Tissues that do not get exposed to sufficient amounts of catalyst during pretreatment are incompletely processed, resulting in decreased overall enzymatic digestibility of pretreated biomass [6]. When pretreatment severity is increased, by increasing temperature, catalyst concentration, or time of reaction, areas of biomass readily exposed to catalyst undergo excessive treatment leading to sugar degradation and formation of toxic by-products (furfural, hydroxymethyl furfural, and levulinic acid) that inhibit downstream sugar fermentation and decrease conversion yields [1]. This problem continues at a microscopic scale due to the compositional and structural differences between middle lamella, primary cell wall, and secondary cell wall. At even smaller scales, intermeshed polymers of cellulose, hemicellulose, lignin, and other polysaccharides present another layer of heterogeneity that must be addressed during bioconversion of plant cell walls to sugars.

Milling to fine particle sizes improves some of these mass transfer limitations, but can add significant costs [14, 15]. Size reduction, however, may not overcome heat transfer limitations associated with short time-scale pretreatments that employ hot water/steam and/or dilute acids. When such pretreatments are carried out at high solids loading (>30% w/w) to improve process efficiency and increase product concentrations, heat cannot penetrate quickly and uniformly into these unsaturated and viscous slurries. It is thought that steam added to high-solids pretreatments can condense on particle surfaces impeding convective heat transfer. Depending on particle and slurry properties, the condensed steam can form temperature gradients within biomass aggregates, resulting in non-uniform pretreatment.

Besides limiting heat transfer rates, biomass slurries can pose other processing challenges. At high solids concentrations, slurries become thick, paste-like, and unsaturated. Limited mass transfer within these slurries can cause localized accumulation of sugars during enzymatic hydrolysis, decreasing cellulase and hemicellulase activity through product inhibition [16–23]. In addition, slurry transport through process unit operations is challenging at full scale. As solid concentrations increase, hydrodynamic interactions between particles and the surrounding fluid as well as interactions among particles increase. At high solids concentrations “dense suspensions” are formed and the resulting multiple-body collisional or frictional interactions and entanglement between particles creates a complex slurry rheology [24–26]. A further complicating aspect is water absorption by biomass, causing the bulk to become unsaturated at fairly low insoluble solids concentrations (~ 30–40%

w/w) and behave as a wet granular material [27]. This material is highly compressible and the wet particles easily "stick" to each other and agglomerate. With no free water in the system, the material becomes difficult to shear or uniformly mix.

At the ultrastructural scale of plant cell walls, catalysts must penetrate through the nano-pore structure of the cell wall matrix to access the "buried" and intermeshed carbohydrate polymers. Based on reported average cell wall pore sizes of 5–25 nm [28–31], small chemical catalysts (<1 nm) may not face as significant a penetration barrier as do enzymes (about 10 nm). The most dominant commercial cellulase component enzyme, cellobiohydrolase I or Cel7A, has dimensions of ~5 × 5 × 12 nm [32, 33] which is roughly the same size as smallest of these reported nano-pores, likely restricting accessibility to primarily surface cellulose chains. Once they have penetrated the cell wall matrix, these enzymes must locate suitable substrates. For Cel7A, this implies that a region of cellulose microfibril has been sufficiently unsheathed from lignin and hemicellulose to expose the cellulose core (Fig. 1). This unsheathing process may be accomplished by the pretreatment or as an ablative effect caused by the system of cellulase enzymes which can peel away microfibrils from the surface layers. Lignin is also a major impediment to cellulase action because it is difficult to remove uniformly or modify through pretreatment. Furthermore, it is entirely unclear at this time if lignin can be effectively removed from cell walls using enzymes.

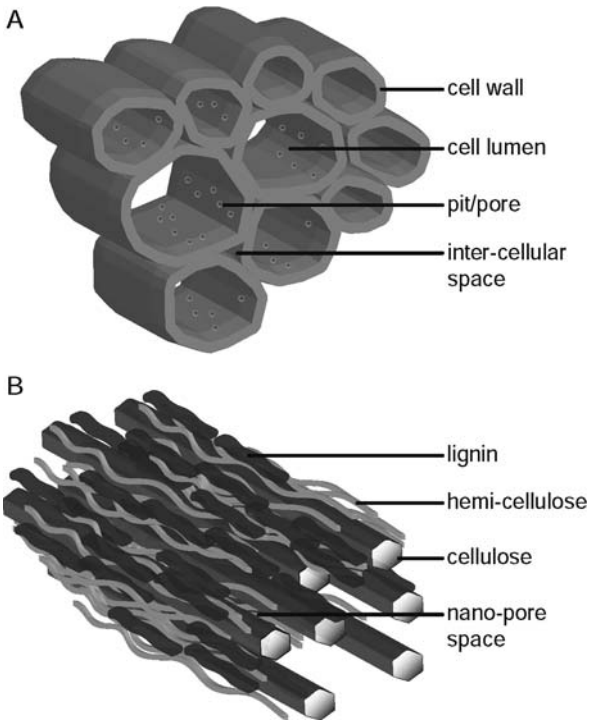

Fig. 1.1 Cartoon depiction of cellular-scale (**a**) and molecular-scale (**b**) obstacles to heat and mass transport in lignocellulosic biomass

Lignin is believed to impede enzymatic hydrolysis of cellulose by interacting with biomass surfaces and either blocking the path of processive hydrolases (e.g. Cel7A), preventing enzymatic access to specific binding sites, or through nonspecific binding of cellulolytic enzymes [34–36] to lignin. Several low-temperature pretreatment protocols, such as alkaline peroxide [37, 38] or lime and oxygen [39], address these issues by removing substantial amounts of lignin. Although these processes are highly relevant to the pulp and paper industry, the fate of lignin and its impact on enzymatic digestibility after high-temperature acidic or neutral pretreatments has largely been neglected until recently [40–42]. Recent observations show that lignin undergoes significant structural changes during high temperature pretreatments. These changes cause it to both mobilize during elevated temperatures and then coalesce upon cooling, both within the cell wall matrix and on the biomass surfaces [40]. This mobilized processed lignin, when redeposited onto cellulose surfaces, can impede enzymatic digestion presumably due to the occlusion of substrate binding sites [42]. All of these transport limitations during lignocellulosic conversion to ethanol impact the overall process performance and thus warrant more detailed further investigation.

2 Macroscopic Transport Through Plant Tissues

In a large-scale process, pre-impregnation of catalyst into large pieces of biomass (>1 cm) is often overlooked; however, milling biomass to reduce this problem can incur large energy and equipment costs [1, 14, 15]. This problem is compounded by the widespread use of process irrelevant biomass sizes for laboratory experiments. Most laboratory studies on biomass to ethanol conversion processes use finely milled materials (20–80 mesh is standard) where the effects of macroscopic transport processes are not easily observed or are masked altogether [43–45]. In larger pilot studies using compression screw feeders, these transport effects can be further masked by the high-shear feeder causing biomass size reduction [6, 8]. Often this size reduction occurs after catalyst impregnation, limiting catalyst effectiveness on pretreatment. A further complication is that compression of the feed stock may cause biomass pore structure collapse, leading to uneven heat and mass transfer during pretreatment [10, 13] as well as limitation of catalyst access to the interior of the biomass.

Before larger biomass particles containing intact tissues are used in processing, it is essential to understand the catalyst transport processes and pathways and the limitations associated with them (Fig. 1). In living plants, vascular tissues such as xylem and phloem are the primary routes for transport of water and nutrients along the length of the plant stem and leaves. Additional transport within tissues and between adjacent cells is carried out through (1) the pits, areas of thin primary cell wall devoid of secondary cell wall between adjacent cells and (2) the apoplast, the contiguous intercellular space exterior to the cell membranes [46]. In dry senesced plants, studies with dyes to visualize fluid movement through tissues showed that the apoplastic space is the major catalyst carrier route, with limited fluid movement

occurring through the vascular tissue [11]. In untreated biomass, the pits do not appear to support significant transport. It is probable that these pits disintegrate and open up during pretreatment allowing fluid to flow through [40]. Thus, new pathways for catalyst penetration are formed either during the drying process that creates fractures in plant tissues or after some degree of biomass degradation.

The primary major barrier to fluid transport into native dry plant tissue appears to be air entrained in the cell lumen. Simple exposure of tissues to high temperature fluids is insufficient to achieve catalyst distribution to all parts of the biomass [11]. The primary escape route for the intracellular air is most likely through pits. However, the small pit openings (approx 20 nm) could be blocked due to cell wall drying and water surface tension may prevent movement through these narrow openings. Forced air removal by vacuum provides additional driving force for the bulk fluid mobility necessary to enhance liquid and catalyst penetration into tissues as demonstrated by Viamajala and coworkers [11]. Heating dry biomass can minimize the amount of entrained air (due to expansion of air by heat) and assist in drawing liquid into the cells by contraction of the entrained air when cooled by immersion in catalyst-carrying liquid. Thus, bulk transport, rather than diffusive penetration, is the dominant mass transfer mechanism into dry biomass.

Although movement of fluids is associated with catalyst transport, the primary goal of catalyst distribution is to deliver the catalyst to cell wall surfaces containing fuel-yielding carbohydrates, rather than to empty cytoplasmic space in dry tissues. In fact, entrainment of fluids in the biomass bulk can be detrimental to small time-scale dilute acid or hot water pretreatments, as the presence of excess water increases the net heat capacity of the material, increasing the heating time needed to achieve desired pretreatment temperatures. Data shown in Fig. 2 support this hypothesis. In this set of experiments, un-milled sections of corn stems

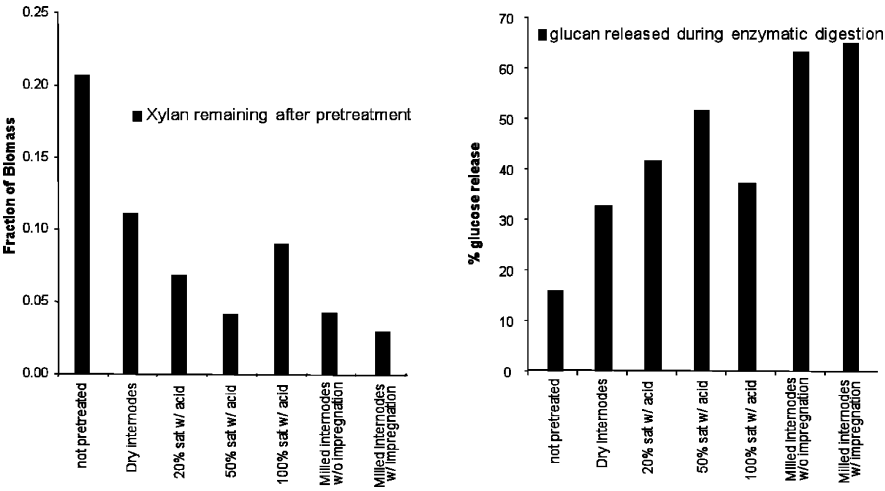

Fig. 1.2 Effect of preimpregnation of corn stover stalks with dilute acid and particle size reduction on (**a**) pretreatment and (**b**) subsequent enzymatic hydrolysis

(~ 1 inch long) were saturated to various degrees with dilute sulfuric acid (2% w/w) and pretreated in 15 mL of the same acid solution at 150°C for 20 min. Milled corn stems (–20 mesh) pretreated under identical conditions served as controls. All pretreatments were performed in 22 mL gold coated Swage-Lok (Cleveland, OH) pipe-reactors, heated in an air-fluidized sand bath [42]. After pretreatment, whole stem sections were air-dried, milled and enzymatically digested for 120 h with a 25 mg/g of cellulose loading of a commercial *T. reesei* cellulase preparation (Spezyme CP, Genencor International, Copenhagen, Denmark) supplemented with an excess loading (90 mg/g of cellulose) of commercial *Aspergillus niger* cellobiase preparation (Novo 188, Novozymes Ltd., Bagsvaerd, Denmark) using procedures described previously [47]. Milled stover pretreated as controls in this experiment was dried and digested similarly, but without any further comminution.

In Fig. 2a, dry internodes pretreated without pre-impregnation of catalyst were poorly pretreated as evidenced by the high amounts of xylan remaining in the biomass after reaction. Stem sections pre-impregnated to achieve 20% saturation showed better reactivity and xylan removal and this trend continued when stem sections pre-impregnated to 50% saturation were pretreated. However, when completely saturated (100%) stem sections were pretreated, xylan conversion was observed to be lower. Milled materials with and without pre-impregnation of catalyst – conditions that would have lowest mass transfer limitations, showed comparable pretreatment performance with each other as well as with the 50% saturated stem sections. These results confirm that only limited catalyst penetration and pretreatment is achieved when air remains entrapped in cytoplasmic spaces such as in dry internodes. Enhanced catalyst distribution and transport dramatically enhances pretreatability up to a certain point, after which excess fluid impedes pretreatment. Similar conclusions on the negative impacts of poor bulk transfer on biomass pretreatability can be inferred from other reported studies also. Tucker and coworkers [10] observed poor pretreatability of biomass during steam explosion of corn stover when materials were not pre-wetted with dilute acid and ascribed their results to mass transport limitations. In another study Kim and coworkers [13] observed poor pretreatment of biomass when the biomass was pressed prior to pretreatment and hypothesized that the mechanical compression of biomass caused pore structure collapse resulting in formation of material that was relatively impervious to heat and mass transfer.

Enzymatic digestion results corresponding to pretreatments shown in Fig. 2a, are presented in Fig. 2b. As expected, release of monomeric sugars from pretreated whole stem sections was proportional to the degree of pretreatment they experienced. Unmilled biomass that was 50% saturated with acid before pretreatment showed better digestibility than the sections that were pre-saturated to lower or higher levels. Milled biomass, however, digested best, demonstrating the importance of enhanced enzyme transport – an outcome of the more thorough and uniform pretreatment of milled materials. With woody feedstocks, milling to fine particle sizes may be impractical and pre-impregnation of biomass with catalyst, as practiced in the pulp and paper industry [48], might need to be utilized to improve conversion efficiencies.

3 Microscopic Transport Through Plant Cell Walls

Enzyme penetration into plant cell wall is widely acknowledged to be a key barrier to economical and effective biochemical conversion of lignocellulosic biomass [5, 49]. In fact, the primary function of pretreatment of lignocellulosic biomass is to assist subsequent enzymatic digestibility by making cell walls more accessible to saccharifying enzymes [1, 4, 44]. However, an accurate description of the methods by which enzymes penetrate cell walls and accomplish cellulose degradation has been lacking. A recent study by Donohoe and coworkers provided, for the first time, direct visual evidence of loosening of plant cell wall structure due to dilute acid pretreatment and the subsequently improved access by cellulases [49]. Figure 3c–f further demonstrate the penetration of cellulases into pretreated cell walls as detected by nano-gold labeled antibodies to Cel7A and other cellulases. This study shows that penetration of enzymes into mildly pretreated cell walls is minimal and that cells stay largely intact even after prolonged exposure to cellulases (Fig. 3a, b). In moderately pretreated cell walls, cellulases are able to partially penetrate and disintegrate the inner secondary layers (S3) only (Fig. 3c, d); whereas the outer layers (S1 and S2) remain impervious to enzymes. In severely pretreated cell walls, enzymes penetrate throughout (Fig. 3e, f). These data suggest that enzymatic digestibility of biomass is restricted by transport of enzymes into cell walls. While not directly evidenced by this study, these results also suggest that thermal pretreatments (and possibly others) "loosen" cell walls in layers providing enzymes access only to these structurally compromised zones of the cell walls. Kinetic data on thermal pretreatments by several research groups also suggests likely mass transfer limited xylan removal that can be modeled as parallel fast and slow reactions [44, 50, 51] and the fundamental observations made by Donohoe and coworkers [49] support this hypothesis.

4 Lignin Mobility and Impact on Biochemical Conversion

Lignin is a polymeric material composed of phenylpropanoid units derived primarily from three cinnamyl alcohols (monolignols): ρ-coumaryl, coniferyl, and sinapyl alcohols. Polymer formation is thought to occur via oxidative (radical-mediated) coupling between monolignols and the growing oligomer/polymer [52, 53] and is commonly believed to occur in a near-random fashion [54], although some recent studies suggest an ordered and protein-regulated lignin synthesis [55]. In any case, the resulting polymer is complex, heterogeneous, and recalcitrant to biological degradation. Although lignin loss is minimal during thermal-acidic/neutral pretreatments, it can undergo structural and chemical changes [56] that significantly influence downstream enzymatic conversion.

Although enzymes thoroughly penetrate cell walls after high severity pretreatments [49], incomplete cellulose conversion by cellulases suggests additional barriers exist at the ultrastructural level. One potential barrier is occlusion of the cellulose microfibrils by residual lignin or hemicellulose that would sterically prevent

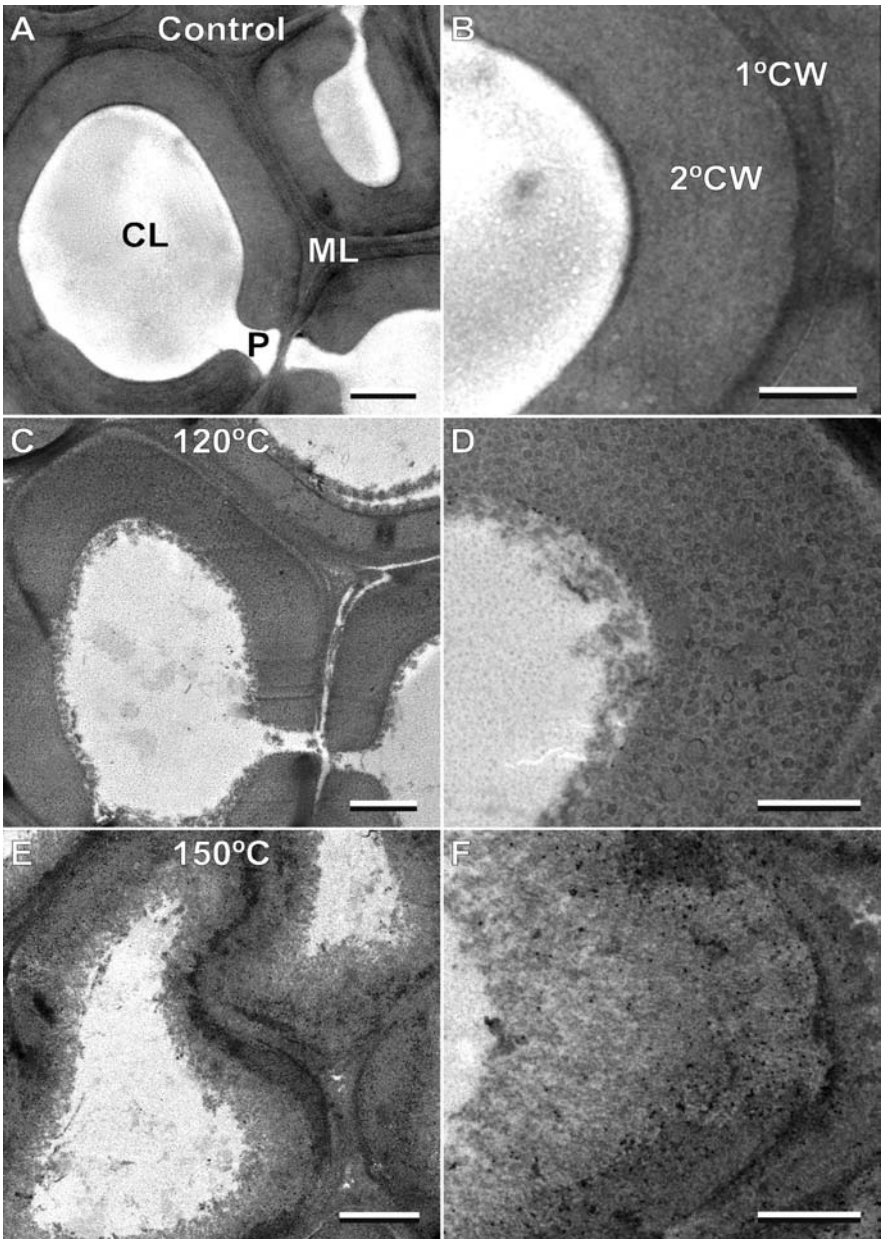

Fig. 1.3 Immuno-labeled electron micrographs of pretreated, digested corn stover cell walls. Gold particles (visible as dark dots especially in **d** and **f**) mark the location of Cel7A enzymes digesting through cell walls following dilute acid pretreatment of varying severity (120°C **c**, **d**; 150°C **e**, **f**). CL, cell lumen; ML, middle lamella; P, pit; 1° CW, primary cell wall; 2° CW, secondary cell wall. Scale bars = 1 μm **a**, **c, e**; 500 nm **b, d, f**

cellulases from binding to cellulose [42]. Other indirect mechanisms that impede complete cellulose hydrolysis are also possible such as non-productive binding of cellulases to lignin [34–36], however reports that contradict this theory also exist [57].

Enzymatic hydrolysis of biomass pretreated under alkaline conditions, which hydrolyzes less xylan than acidic pretreatments, supports the steric hindrance concept. Elevated cellulolytic activity is observed on alkaline pretreated biomass when cellulases are supplemented with xylanases and other hemicellulose degrading enzymes, likely a function of removing additional barriers to cellulose accessibility [58, 59]. A study in pretreatment variability by Selig and co-workers suggested that cellulose digestibility is improved directly by xylan removal, but only indirectly by lignin removal [47]. Removal of lignin by pretreatment appeared to increase enzymatic removal of xylan, which in turn increased cellulose digestibility. Lignin removal alone had little impact on cellulose digestion. Lignin modifying enzymes, however, have been shown to synergistically work with cellulases during digestion of steam-pretreated biomass, improving sugar yields through at least partial removal of the lignin barrier [60]. In spite of a general consensus in the scientific community about the significance of the lignin barrier to cellulose digestibility, only limited attention has been given to the fate of lignin during widely used high temperature dilute acid, hot water, and steam pretreatments which only partially remove lignin [1, 8].

A recent study investigated the fate of lignin during high temperature acid and neutral pretreatments using electron microscopy and spectroscopy techniques [40]. This study revealed that lignin could be mobilized within the cell wall matrix at temperatures as low at 120°C during both neutral and low pH pretreatments, and appears to be, at least in part, dependent on pretreatment severity. On a relatively macro scale, part of the mobilized lignin deposits back on to biomass surfaces as spherical bodies, suggesting that lignin undergoes the following sequence of events during these pretreatments – phase-transition or melting, mobilization into bulk solution, coalescence, and deposition onto solid surfaces. Scanning- and transmission electron microscopy (SEM and TEM) of pretreated cell walls shows that the lignin droplets (stained with $KMnO_4$) take a wide range of sizes (<50 nm to 2 μm) and shapes (Fig. 4a, b and Fig. 5), though the "free" shapes are uniformly spherical. Other shapes observed appear to be dictated by the physical constraints of the structures surrounding them. In addition to redeposition, there also appears to be a reorganization of lignin structure within the cell walls. A fraction of the lignin remains within the walls during pretreatment. This fraction apparently melts, but is unable to escape into the bulk liquid phase before coalescing back into droplets, as evidenced by the $KMnO_4$ stained lignin droplets that appear between layers in the cell wall (Fig. 4b–d).

Aside from the obvious implications of lignin mobility, coalescence, and redeposition observed during high temperature pretreatments, chemical modification of the lignin should also be considered. These may range from covalent bond breakage and formation to changes in inter- and intramolecular interactions. Although FTIR and NMR studies did not distinctly show chemical changes in the mobilized

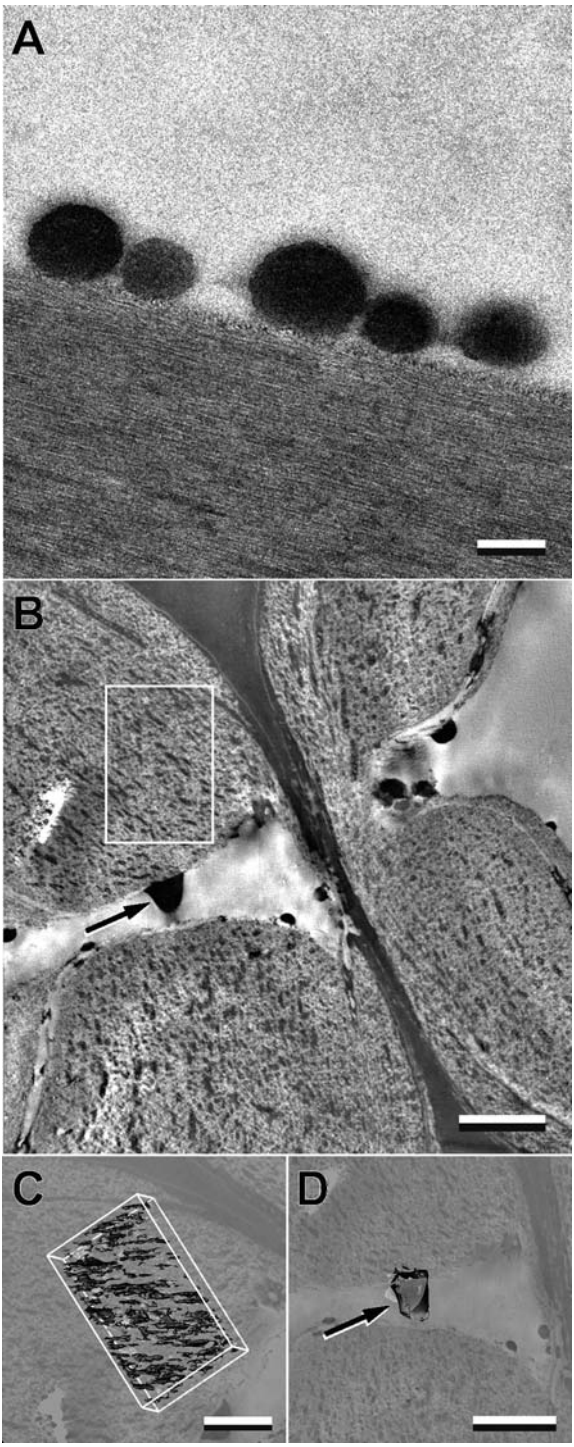

Fig. 1.4 TEM micrograph of lignin droplets re-deposited on cellulose surfaces after being transported from the cell wall matrix during high temperature pretreatments (**a**). Electron tomograph images of coalesced lignin within cell walls. The *boxed region* in **b** has been segmented to show the 3D volume of coalesced lignin (**c**). Large lignin globules can form in openings like pits (*arrow* **b**, **d**). Scale bars = 200 nm **a**; 500 nm **b**, **c**; 200 nm **d**

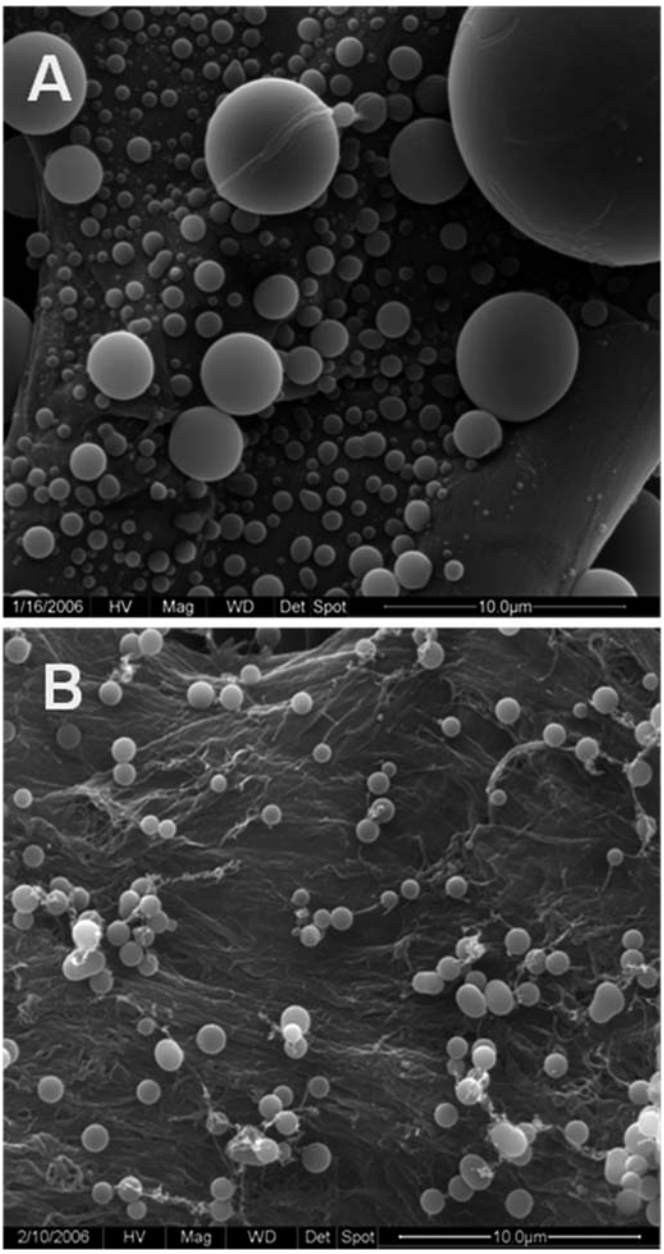

Fig. 1.5 Example SEM micrographs showing lignin droplets redeposited on to cellulose surfaces following exposure to high temperature pretreatment conditions

lignin in this study, it is possible that chemical alteration could be part of the lignin removal and transport process because lignin can partially dissolve and react in acid solutions under appropriate conditions [56]. It is further possible that part of this mobilized lignin could contain lignin-carbohydrate complexes that might sequester cellulases as observed in some studies [34, 36].

Another recent study [42] showed that purified lignin preparations as well as native lignin from corn stover could be redeposited onto clean cellulose surfaces such as filter paper. More severe pretreatments (higher temperature or acid concentrations) resulted in finer redeposited droplets. Under these conditions, digestibility of filter paper was lower by up to 15% in comparison with treatments that did not contain lignin. Since these digestions were performed at very high enzyme loadings to circumvent issues related to non-productive binding to lignin, it appears that physical blockage of the cellulose surface by lignin resulted in lower digestibility. Although redeposited lignin inhibited digestion of pure cellulose substrates in the study by Selig and coworkers [42], it is also probable that the mass transport of lignin could enhance enzymatic cellulose degradation in biomass. For example, we could visualize that as a result of lignin mass transport, the lignin sheath coating cellulose surfaces gets concentrated into droplets rendering a greater cellulose surface area available for enzymatic attack. Removal of lignin could also improve cell wall porosity allowing enzymes better access for penetration. Much work needs to be done to completely understand the nature and implications of lignin transport.

5 Rheology of Biomass Slurries and Implications for Mixing

Uniform distribution of heat, chemical catalysts, and enzymes as well as absence of product gradients within conversion reactors are all dependent on the mixing properties of biomass slurries being processed, which in turn are determined by rheological characteristics. Biomass rheology poses several challenges because of the fibrous nature of the particles, their ability to absorb water and become unsaturated at relatively low solid concentrations of 25–35% (w/w), and the continually changing particle chemical/physical properties during flow through the process. Free water content appears to be the largest factor contributing to slurry rheology. This is especially true at the high solid concentrations that are desired to make the overall process economical by lowering equipment volume and thereby cost [27]. At solid concentrations beyond the point of unsaturation, the slurries become wet granular material that agglomerate and can compact under their own weight if not adequately mixed. At lower concentrations, adequate mixing is still required to prevent settling. To further complicate matters, as biomass gets broken down into its constitutive sugars, changes occur in particle size as well as chemical properties. Water retaining polymers, such as hemicellulose and pectin, are broken down and the previously hygroscopic biomass has lower capacity for water absorption resulting in an increased amount of free water, and thereby altered slurry rheology. These dynamic changes in solid properties necessitate studies to understand rheological behavior of slurries through various process treatments.

In simplest terms, biomass slurries can be described as non-Newtonian pseudoplastic (shear-thinning) fluids [27, 61, 62]. Whereas the exact mechanism leading to pseudoplasticity in biomass slurries is unknown, a possible explanation of the behavior can be ascribed to formation of three dimensional network structure of the fibrous particles and subsequent breakdown of this structure under shear [63]. Previous studies show that while free water is present, apparent viscosity values under continuous shear increase with increasing solid concentrations. These measured apparent viscosities can be modeled with simple Casson, Bingham or Power Law models [27, 61, 62]. Thick slurries with little or no free water do not exhibit a further increase in apparent viscosity with increasing solid concentrations under continuous shear [27]. Other viscoelastic properties, such as storage and loss modulii could continue to change; however, these measurements have not yet been reported for biomass slurries.

The relatively sparse data and lack of fundamental understanding of rheological properties of biomass slurries makes calculations on mixing requirements for biomass conversion processes uncertain. Also, transport properties within biomass slurries, such as convective/conductive heat transport and convective/diffusive mass transport, and their effects on conversion are hard to discern or estimate. For example, Fig. 6 shows enzyme digestibility data obtained during digestion of pretreated corn stover at high solids pretreatments (>15% solids). Each data point was generated as a single measurement from triplicate reactors after 5 days of digestion. As can be seen from Fig. 6a, conversion of cellulose to glucose decreases steadily as solids concentrations increase suggesting inhibition of enzymes, possibly due to poor mass transfer resulting in localized accumulation of sugars as suggested by Hodge and coworkers [22]. Clearly, slurry properties will play a major role in determining these transport parameters that are crucial to determine optimal process performance across multiple scales. As another example, Fig. 7 shows experimental data from tests performed to evaluate heating time in a closed reactor containing biomass slurries of varying concentrations. These data show significant retardation of heat transfer, even with the moderate density slurries containing 10% solids (w/w). Simple heat transfer simulation models have been developed for biomass slurries assuming conductive heat transfer and a one-dimensional system; however, their validity has not been verified with experimental data [64, 65]. In unsaturated biomass slurries containing discrete aggregates, the accurate determination and prediction of transport properties might be a challenging exercise.

6 Outlook for Challenges Associated with Transport Processes in Biochemical Conversion of Lignocellulosic Biomass

Significantly greater research and development effort in the conversion of lignocellulosic biomass, spurred by economic, national security and climate change concerns over the past few years have led to significant strides in development of a fundamental understanding of transport processes that could appreciably

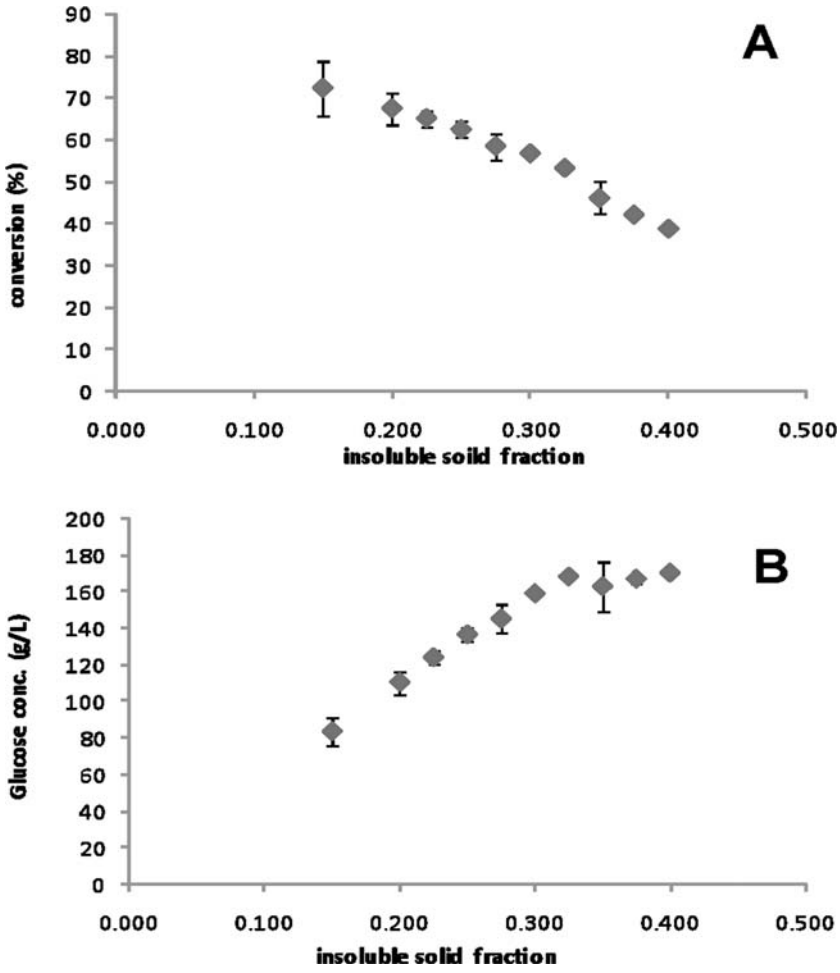

Fig. 1.6 5-day enzymatic digestibility data for pretreated corn stover showing (**a**) decrease in conversion with increasing solids concentration and (**b**) Plateau in glucose release after a solids concentration of 30%

improve overall performance and make renewable liquid transportation fuels sustainable and affordable. A thorough understanding of fundamental issues related to transport processes and the development of predictive models that integrate heat, mass and momentum transport are essential to the design, development and implementation of scale-independent processes. Continued synergism between science and engineering disciplines along with participation by industry is crucial to the development of cost-effective alternative motor fuels by 2012 and the significant displacement of fossil-derived fuels specified by the DOE (Energy Independence and Security Act of 2007) EISA for 2022. Improvements in process equipment,

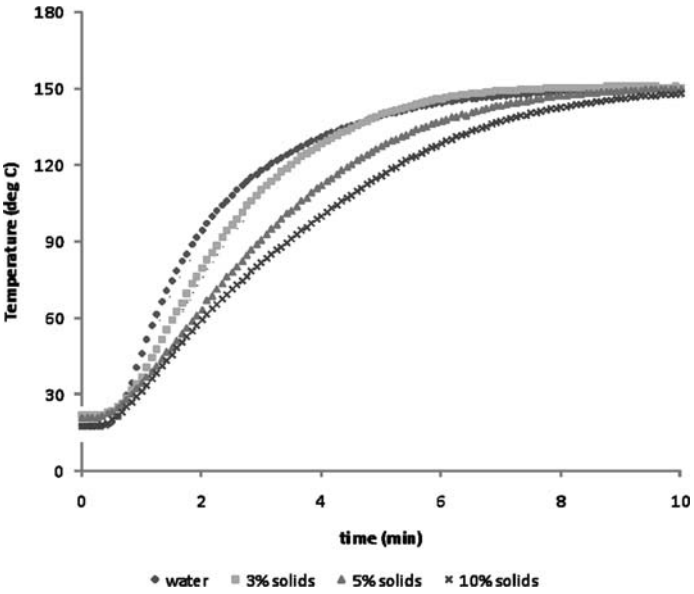

Fig. 1.7 Effect of solid concentrations on heat up time of pretreatment reactor containing biomass slurries

enzymes and microbial systems, as well as improved understanding of the basis for biomass recalcitrance are critical determinants of the successful implementation of biorefineries.

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Biofuels from Lignocellulosic Biomass

Xiaorong Wu, James McLaren, Ron Madl, and Donghai Wang

Abstract Biomass feedstock, which is mainly lignocellulose, has considerable potential to contribute to the future production of biofuels and to the mitigation of carbon dioxide emissions. Several challenges exist in the production, harvesting, and conversion aspects of lignocellulose, and these must be resolved in order to reach economic viability. A broad array of research projects are underway to address the technical hurdles, however, additional research may be required to reach commercial sustainability. Gasification and enzymatic hydrolysis are the main technologies being investigated for the conversion of lignocellulosic biomass into material for the production of biofuels. While each approach has pros and cons, both are being explored to determine the optimum potential commercial method for particular feedstock situations, and to better understand the requirements for the massive scale required to contribute to biofuel volume.

Keywords Lignocellulosic biomass · Biofuels · Syngas · Enzymatic hydrolysis · Pretreatment · Fermentation · Gasification

1 Introduction

As the world population increases from the current 6.7 billion to over 8 billion by 2030 [1], and supporting economic growth expands, energy consumption is projected to increase by 42% to 695 quadrillion (10^{15}) British thermal units (Btu, 1 Btu = 1055 joule) in 2030 [2]. Most of the required energy will still be acquired from fossil fuels, with around 6% being from nuclear sources and about 8% from other renewable energy sources. Carbon dioxide (CO_2) emission from such widespread industrial consumption of fossil fuels (coal, oil, and natural gas) is

D. Wang (✉)
Department of Biological and Agricultural Engineering, Kansas State University, Manhattan, KS 66506, USA
e-mail: dwang@ksu.edu

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likely to continue to be a major contributor to anthropogenic greenhouse gases [3, 4]. Mitigation of CO_2-based contributions to the global warming process requires specific actions, including capture and sequestration of CO_2 during the consumption of fossil fuels and expanded utilization of carbon-neutral and carbon negative renewable energy sources (wind, solar, nuclear, geothermal, and various biomass sources) [3–6]. Most of the types of renewable energy (wind, solar, etc.) can be utilized to generate electricity, but not liquid transport fuels. Consequently, biomass has received much attention as a feedstock for biofuels, both in the existing commercial industry (e.g. ethanol from grains or sugar) and in the research realm where lignocellulose is the current focal feedstock material [7–11]. To avoid confusion, we adapt the common definition for biomass and biofuels as follows:

- *Biomass*: Organic, non-fossil material of biological origin (plant parts including grains, tubers, stems/leaves, roots/tubers, agricultural residues, forest residues, animal residues, and municipal wastes arising from biological sources) potentially constituting a renewable energy source (basically originating from primary capture of solar energy).
- *Lignocellulosic biomass*: Organic material derived from biological origin which has a relatively high content of lignin, hemicellulose, cellulose, and pectin combined into a molecular matrix with a relatively low content of monosaccharides, starch, protein, or oils. Typically refers to plant structural material with high cell wall content. Sometimes referred to as "cellulosic" biomass, which is technically inaccurate, but is (mis)used due to the typical 40%+ cellulose content in lignocellulose.
- *Biofuels*: Liquid fuels and blending components produced from biomass (plant) feedstocks, used primarily for transportation. Technically, biogas (e.g. methane from anaerobic digestion of biological residues) is a "biofuel" but tends to be utilized in stationary combustion units and is typically referred to separately as biogas.

Survey reports suggest that the annual world biomass yield contains sufficient inherent energy to contribute 20–100% of the world's total annual energy consumption of 500 EJ (1 EJ $= 1 \times 10^{18}$ Joule), with annual and regional variations [4, 10, 12]. Currently, commercial biofuels are generated from harvestable components of known crops (starch, sucrose, and oils), while a relatively small amount of the lignocellulosic biomass is used for combustion (cooking/heating fires or cofiring to create steam for electricity generation). The large potential of lignocellulose as an energy feedstock remains to be utilized, and is dependent on the development of economic, sustainable production, and processing systems [11].

Two platforms have been set up to transform the energy in lignocellulosic biomass into liquid fuels or chemicals: the sugar platform and thermochemical platform. In the sugar platform, the lignocellulosic material is first pre-treated to facilitate separation into the major components, then the polymeric celluloses and hemicelluloses are enzymatically hydrolyzed into sugars (hexoses and pentoses), after which these sugars can be fermented into biofuels or converted into other valuable intermediate chemicals. The residual lignin may be utilized as a specialty

intermediate or, more commonly, is combusted for heat or power. In the thermochemical platform, biomass is degraded into small gas molecules (hydrogen, carbon monoxide, carbon dioxide, methane, etc.) under high temperature and certain pressure conditions, then these gas molecules are converted chemically or biologically into Fischer-Tropsch (FT) liquid fuel, alcohols, or other intermediate chemicals. This chapter focuses on the processes, potential, and challenges associated with each of these platforms.

2 Background Research

2.1 Natural Resource Limitation and Economic Security

Although the potential adverse environmental effects of CO_2 emission is a major factor pressuring governments to steer their energy policy away from fossil fuels, the global decline of fossil fuel reserves is also a major driver for public and private organizations around the world to develop technologies to use renewable energy sources. Various estimates exist for the current proved reserves (Rp), and the Rp:consumption ratio (Rp:c), with units of years. For example, the global Rp:c of coal, oil, and natural gas have been estimated as 140, 40, and 60 [3, 13]. Using the widely-recognized global energy database provided in the British Petroleum (BP) energy report [14], we calculated Rp:c for coal, oil and natural gas as 133, 35, and 60, respectively. For coal and natural gas, the Rp:c value is similar to the previously published estimates and indicates that issues may arise later in this century. However, for oil, our Rp:c value of 35 (years) is even less than that published previously, indicating a serious situation with near-term pressure building to replace oil reserves either with new discoveries (perhaps some, but unlikely to be major) or with new alternatives (biofuels can play a role).

The earliest fuel ethanol production from lignocellulose biomass began in Germany, in 1920s [15], using sulfuric acid to hydrolyze wood. The ethanol yield was low at approximately 75–130 L (20–34 gallons) of ethanol per ton of wood hydrolyzed. From 1945 to1960s, several acid-hydrolysis ethanol plants were built in Europe, the USA, and the former Soviet Union. The capacities of these plants ranged from 10,000 to 45,000 tons of wood materials a year. Ethanol yield reached 190–200 L (50–53 gallons) per ton of wood. Subsequently, almost all of these wood-based ethanol plants were closed due to competition from the rapid development of the petroleum industry and relatively inexpensive crude oil feedstock.

The first gasification of biomass can be dated back to the 1800s, when wood was gasified to generate “town gas” for lighting and cooking. Although there are around 140 large gasification facilities in operation around the world today [16], these gasifiers are basically used to generate heat and/or electricity from coal (55% of total 140 large gasification facilities), oil, or natural gas, with a few plants using residues from the wood/pulp industry. The current main products generated from gasifier syngas are power (18%), chemicals (44%), and FT fuel (38%) [16]. To-date, there are no commercial scale gasification or pyrolysis facilities dedicated for

biofuels production from lignocellulosic biomass. However, many research units have been built to investigate the mechanism, kinetics, and economical feasibility of biofuel production via syngas from biomass gasification.

2.2 Limitation of Mainstream Agricultural Crops for Biofuels

In recent years, fuel ethanol production has been revived for use in gasoline transport fuel markets. The main driver for fuel ethanol expansion use has been the need for a gasoline oxygenate, following the issues that were uncovered concerning the previous widespread petroleum industry oxygenate, methyl *tert*-butyl ether (MTBE). Ethanol is biologically safer, biodegradeable, renewable, and carries 88% more oxygen than MTBE (especially useful in the higher compression modern gasoline engines). A secondary, but nonetheless important, driver for ethanol expansion has been to reduce dependence on foreign oil for those countries that import large volumes of crude oil. The success of ethanol to-date has relied on the harvested portions of mainstream agricultural crops, where modern-technology yield increases have allowed increasing harvest volumes [17].

The global production of crop-based renewable ethanol is projected at around 20 billion gallons (77 B liters) for 2008. Figure 1 shows the breakdown by country and main feedstock. In Brazil, fuel ethanol displaces ~20–50% of the transportation petroleum gasoline, with the volume depending on the world price of sugar. Projections are for additional areas to be planted with sugarcane to meet the demand for sugar and fuel, and there are plans to utilize more biotechnology to increase

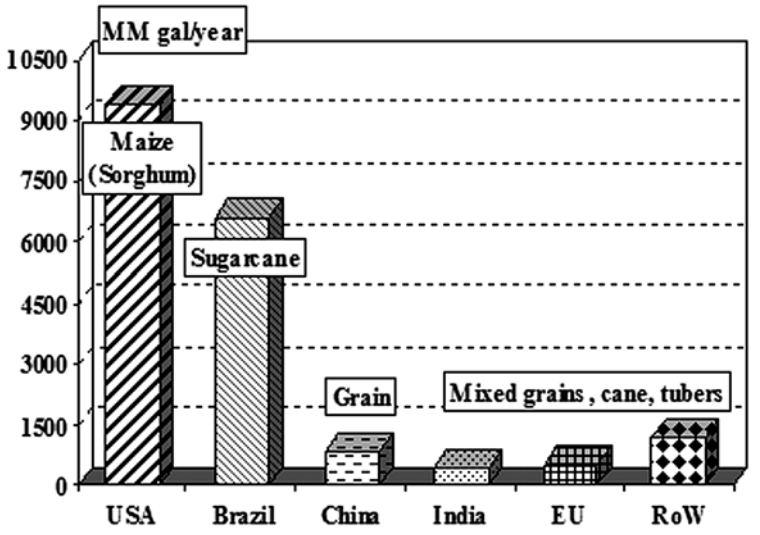

Fig. 1 Estimates of fuel ethanol for 2008, based on production year-to-date and data sourced from the Renewable Fuel Association, USDA-FAS, and StrathKirn Inc.; RoW – Rest of the world

sugarcane yields by over 10% [2]. The fuel ethanol industry in the USA has grown rapidly since 2000, with over 95% of the ethanol being blended into gasoline as an oxygenate (called E10). Current 2008 production is uncertain due to the volatile economy and sharp commodity fluctuations; however, we project the final volume to be around 9.6 billion gallons (equal to about 7% of the US gasoline volume). The majority of the feedstock for US ethanol is corn (maize) grain, with a small amount (~4%) being generated from sorghum. Unlike sugarcane, which cannot be stored and for which the mills must close for several months each year, grains are easily stored for over a year and can be managed and transported in the existing infrastructure. Another advantage of grains is that only the starch is consumed in ethanol fermentation. The protein and oil are carried through in the distillers grains (DG) and are available to go back into the livestock feed system. Nevertheless, there will be an upper limit on the land and farm resources that can be used for grain-based ethanol before impacting other commodity food markets (e.g. today the amount of grain exported from the US is about the same as that used for ethanol). Some analysts suggest that there is an impact today, others project that the maximum amount of corn that can be used for ethanol production is approximately 25–30% of the annual corn production [12]. We estimate that the upper limit will depend on how fast the expected biotechnology-driven yield increase is achieved [11, 17]. For example, we can calculate the mathematical outcome for various scenarios:

- Yield is somehow frozen today at 12 B bushels grain. E10 (oxygenate additive value) used in all US gasoline would require 15 B gal ethanol = 5 B bu grain. This would require 41% of the current corn harvest. However, 30% of that goes back into the feed system as DG so the net utilization is 29% of the available corn grain.
- Yields are projected to continue to increase due to various new technologies, with some industry experts projecting 300 bu/acre in 10–15 years: this would generate 24 B bu grain. Again assuming E10 use at 15B gal ethanol = 5 B bu grain, this would result in only 20% of the crop harvest being taken in. Accounting for the DG return, the net corn grain use would be 14%.

In reality, there are many factors which will impact the final scenario. Irrespective of the exact scenario, it seems that corn grain can provide for existing market demands plus enough grain for future oxygenate use (e.g. E10). While this is an excellent contribution, it does not meet requirements for majority replacement of gasoline volume. Obviously, to achieve further energy independence and further reduce import of foreign oil, additional renewable feedstocks are required to contribute to the total liquid fuel demand.

3 Potential of Lignocellulosic Biomass

A 2005 USDA and DOE joint report [12] showed that a combination of crops, agricultural residues, trees, forest residues, and bringing conservation reserve land into production could generate up to 1.3 billion dry tons of biomass each year. Given

the assumptions regarding a viable conversion process, the energy inherent in this biomass could produce enough biofuels to replace 30–50% of the annual transportation gasoline in US. Thus, biomass represents considerable potential as a feedstock for biofuels, which is reflected in the Renewable Fuel Standard (RFS) contained in the Energy Independence and Security Act of 2007 [18]. Specific targets are mandated for lignocellulosic-derived ethanol in the RFS: the initial goal is 0.1 billion gallons by 2010, with increasing milestone targets that reach 16 billion gallons by 2020. The RFS also calls for 15 billion gallons of ethanol from grain, and the mandate then caps that volume from 2015 onwards [2]. Thus, corn and lignocellulosic ethanol plants will coexist and since there are common processes on the back-end, it is possible that integrated biorefineries (Fig. 2) may emerge to handle both starch and lignocellulosic feedstocks. The integration of cellulosic and traditional dry grind ethanol plants may reduce the per gallon capital investment of lignocellulosic plants, will certainly smooth the risk of lignocellulosic ethanol, and may also improve ethanol yield on a per acre basis [19, 20].

Besides fuel ethanol or butanol, many other chemicals and value-added products may be produced from lignocellulosic biomass. Once the technologies for biorefineries are established and commercialized, a wide range of chemicals (e.g. olefins, plastics, solvents, many chemical intermediates) and biofuels (e.g. biogasoline, alcohols, biodiesel, JP-8, and FT liquids) could be produced from lignocellulosic biomass.

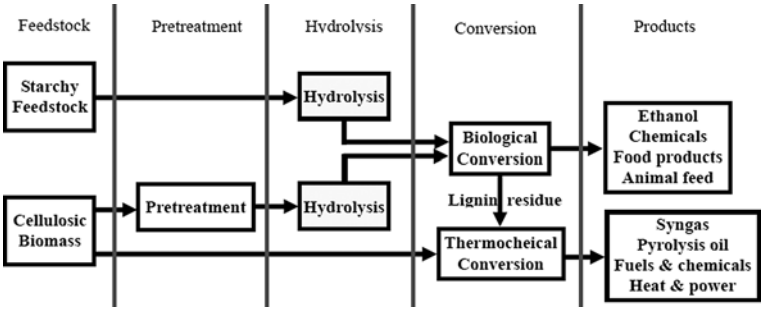

Fig. 2 Possible integration of different biorefineries

4 Technical Issues at Present

Currently, technologies for both biochemical and thermochemical conversions of lignocellulosic biomass are being investigated at research and small pilot plant levels. Demonstration facilities are being built with financial inputs from the DOE (Table 1).

Irrespective of conversion technology, there are several feedstock production and logistics (transportation and storage) issues to be addressed to ensure a usable and consistent supply. For the biochemical conversion process, the major technical

Table 1 Current DOE funded commercial and demonstration scale cellulosic biofuel projects

Company	Project Location	Capacity (MMG)	Feedstock	Conversion Technology	Startup
Abengoa Bioenergy	Hugoton, KS	Ethanol 11.4	Wheat straw, sorghum stubble, switchgrass; 700 t/d	Biochemical and Thermochemical	2011
BlueFire Ethanol, Inc.	Corona, CA	Ethanol 19	Green waste, wood waste, municipal cellulose waste; 700 t/d	Concentrated acid	2009
Mascoma Corp.	Kinross, MI	Ethanol 40	Wood chips and waste	Biochemical	2011
POET	Emmetburg, IA	Ethanol ~30,	corn fiber, cob, stover; 842 t/d	Biochemical	2011
Range Fuels	Soperton, GA	Ethanol 40; methanol, 9	Wood residue, chips. 1200 t/d	Gasification, catalytic reaction	2011
Ecofin LLC	Nicholasville, KY	Ethanol 1; others	corn cob	Solid fermentation	2010
Flambeau River Biofuels LLC	Park Falls, WI	Diesel 6	paper mill and forest residues	Gasification + Fischer-Tropsch	2010
ICM	St. Joseph, MO	Ethanol 1.5	Corn fiber, corn stover, switchgrass and sorghum	Biochemical + thermochemical	2010
Lignol Innovations	Grand Junction, CO	Ethanol 2.0; Lignin, furfural	Soft and hard wood residue	Organolv-biochemical	2012
NewPage	Wisconsin Rapids, WI	Diesel 5.5	Mill residues, wood chips	Gasification + Fischer-Tropsch	2010
Pacific Ethanol	Boardman, OR	Ethanol 2.7; H_2, methane etc.	Wheat straw, corn stover and poplar residue	DTU biogasol technology	2009
RSE Pulp and Chemical LLC	Old Town, ME	Ethanol 2.2	Extracted hemicellulose during pulping	Fermentation	2010
Verenium	Jennings, LA	Methanol 1.4	Bagasse, wood waste, , energy crops, etc.	Dilute acid, biochemical	2009

Sources: http://www.energy.gov/media/ProjectOverview.pdf and http://www.energy.gov/media/Biofuels_Project_Locations.pdf

barriers are pretreatment technology, function and cost of hydrolytic enzymes, mitigation of inhibitors, and fermentation of C6 and C5 sugars [11]. For the thermochemical conversion process, the major technical barriers include understanding the kinetics of gasification, syngas clean-up techniques, and advanced catalyst development (selectivity and longevity) for the FT process [16].

5 Technical Details

5.1 Gasification of Lignocellulosic Biomass

5.1.1 Overview

Gasification is a process where carbonaceous feedstocks react with oxygen and steam at elevated temperatures (500–1500°C) and pressures (up to 33 bar or 480 psi) to yield a mixture of gasses. The mixed-gas product is called synthesis gas or "syngas," consisting primarily of hydrogen (H_2) and carbon monoxide (CO), with varying amounts of carbon dioxide (CO_2), water (H_2O), methane (CH_4), and other elements, depending on the feedstock, gasifier type and conditions [21].

5.1.2 Gasification Process

Depending on how heat is generated, gasification technology can be classified as either directly- or indirectly-heated gasification. For directly-heated gasification, pyrolysis and gasification reactions are conducted in a single vessel, with heat arising from feedstock combustion with oxygen. The syngas generated from this method has low heating values (4–6 MJ/m^3 or ~100–140 Btu/ft^3). For indirectly-heated gasification, the heat-generating process (combustion of char) is separated from the pyrolysis and gasification reactions, which generates high heating value syngas (12–18 MJ/m^3 or ~300–400 Btu/ft^3). Low heating value syngas is usually used to generate steam or electricity via a boiler or gas turbine, while high heating value syngas can also be used as a feedstock for subsequent conversion to fuels and chemicals [22]. According to the flow direction of the feedstock material and oxidant, gasifiers can basically be classified into five types (Table 2, Fig. 3).

Although a portion of the feedstocks are converted to heat during gasification, conversion efficiencies of biomass to syngas are relatively high: e.g. 50–75% on weight basis [22]. This gasification efficiency is mainly due to the utilization of lignin and other organic substances, which cannot be used directly in acid or enzymatic hydrolyzing processes.

5.2 Syngas Generation

Biomass gasification is basically a two-step process, pyrolysis at lower temperature followed by gasification at a higher temperature. Pyrolysis is an endothermic process during which the biomass is decomposed into volatile materials (majority)

Table 2 Characteristics and types of gasifiers [21, 22]

Gasifier Type	Flow Direction		Heating source and major features
	Biomass	Oxidant	
Updraft fixed-bed	Down	Up	Combustion of char; simple process but high tar in syngas, minimal feed size
Downdraft fixed-bed	Down	Down	Partial combustion of volatiles; simple process, low tar in syngas, minimal feed size and limit ash content
Bubbling fluidized-bed (BFB)	Up	Up	Partial combustion of volatiles and char; high CH_4, excellent mixing, heat transfer, and C conversion, extensively tested with biomass
Circulating fluidized-bed (CFB)	Up	Up	Partial combustion of volatiles and char; high CH_4, possible corrosion and attrition problem, not extensively tested with biomass
Entrained flow-bed	Up	Up	Partial combustion of volatiles and tar; very low in tar, CO_2, low in CH_4, biomass has to be pulverized, fluid ash

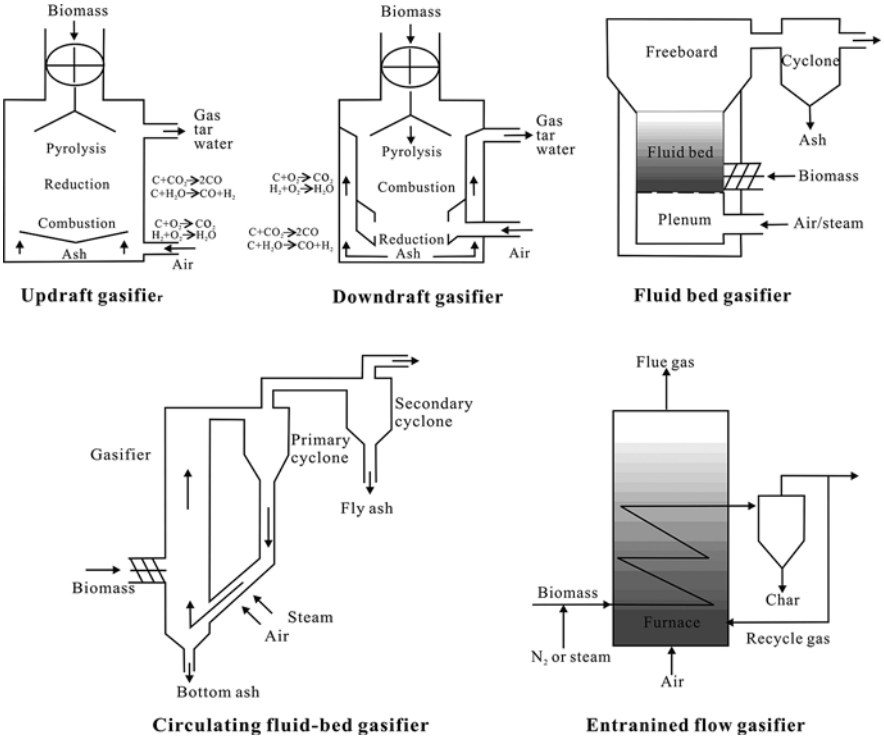

Fig. 3 Illustrative structures of different types of gasifiers (modified from Dr. R. L. Bain's 2004 presentation at DOE/NASCUGC Biomass and Solar Energy Workshop)

and char. Volatiles and char from the pyrolysis process are further converted into gases during the gasification process. Although the exact chemical reactions and kinetics are complex and not yet fully-understood, biomass gasification includes the following:

(1) Combustion	(biomass volatiles/char) + $O_2 \rightarrow CO_2$
(2) Partial oxidation	(biomass volatiles/char) + $O_2 \rightarrow CO$
(3) Methanation	(biomass volatiles/char) + $H_2 \rightarrow CH_4$
(4) Water-gas shift	$CO + H_2O \rightarrow CO_2 + H_2$
(5) CO methanation	$CO + 3H_2 \rightarrow CH_4 + H_2O$
(6) Steam-carbon reaction	(biomass volatiles/char) + $H_2O \rightarrow CO + H_2$
(7) Boudouard reaction	(biomass volatiles/char) + $CO_2 \rightarrow 2CO$

The major components of typical syngas generated from wood are listed in Table 3, and it is evident that output variation occurs, even in the same type of gasifier as gasification conditions (temperature, pressure, O_2, and steam levels) typically impact the syngas composition.

5.3 Liquid Fuels – FT Liquids (Diesel), Ethanol or Butanol, Chemicals

Technically, a variety of different liquid fuels and chemicals can be made from high quality syngas (Fig. 4). The production of liquid fuel, either a thermochemical-catalyzed conversion or a microbial fermentation process (under development),

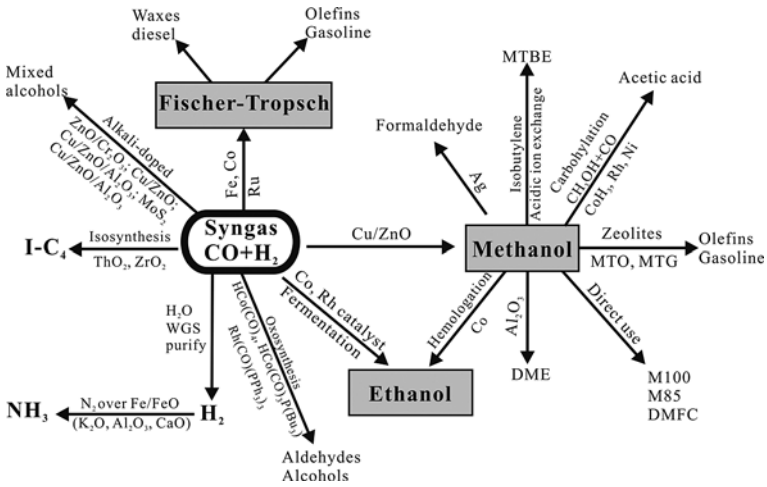

Fig. 4 A diversity of chemicals can be produced from syngas (from page 3 of Drs. Spath and Dayton's 2003 NREL Technical Report, NREL/TP-510-34929, with modification)

Table 3 Major components of wood syngas by direct and indirect heated BFB and CFB

	EPI	GTI	SEI	Sydkraft	Foster Wheeler	BCL/FERCO	MTCI
Type	BFB	BFB	BFB	CFB	CFB	CFB-indirect	BFB-indirect
Feedstock	Wood	Wood	Wood	Wood	Wood	Wood	Pulp
H_2	5.8	14.8	12.7	11.0	15–17	14.9	43.3
CO	17.5	11.7	15.5	16.0	21–22	46.5	9.22
CO_2	15.8	22.4	15.9	10.5	10–11	14.6	28.1
H_2O	dry	dry	dry	12.0	dry	Dry	5.57
CH_4	4.65	10.8	5.72	(in C2+)	5–6	17.8	4.73
C2+	2.58	0.13	2.27	6.5	–	6.2	9.03
Tars	–	0.27	–	(in C2+)	–	–	Scrubbed
N_2	51.9	40.3	47.9	44	46–47	0	0
H_2/CO ratio	0.3	1.6	0.8	0.7	0.7	0.3	4.6
HV (MJ/m^3)	5.6	13.0	5.6	5.0	7.5	18.0	16.7

Data source [21]. EPI: Energy Products of Idaho, GTI: Gas Technology Institute, SEI: Southern Electric International, BCL/FERCO: Battelle Columbus Laboratory/ Future Energy Resources Corporation, MTCI: Manufacturing and Technology Conversion International.

may be used to convert syngas into liquid fuels (methanol, ethanol, gasoline, and FT diesel). The catalytic conversion of syngas to ethanol can occur under high-temperature and high-pressure conditions (~250°C, 60–100 atm) with a molar ratio of H_2 to CO at 2–3:1. However, most syngas (Table 3) does not contain such a high H_2/CO ratio. Also, the catalysis reaction is not specific, resulting in a final mixture of methanol, ethanol, some other higher alcohols, and reactant gases. Considerable technical progress is required to generate ethanol from syngas at a viable commercial scale and various projects continue to explore possible options. For example, Range Fuels in Georgia (Table 1), is in the process of building a 20 million gallon pilot plant to evaluate using this approach for lignocellulose to ethanol conversion. Syngas can also be converted into gasoline or diesel through the so called MTG (methanol-to-gasoline) or the more common FT process. While these methods have been utilized for many years in the fossil fuel industry (coal or natural gas feedstocks), the utilization of lignocellulosic biomass is not yet viewed as being commercial [23]. Two DOE-funded companies (Table 1) are in the process of building demonstration scale plants to further explore the feasibility of the gasification-FT process for biofuel production.

In the microbial fermentation process, anaerobic bacteria such as *Clostridium ljungdahlii* are used to convert cleaned syngas into ethanol [24]. Reactions involved in the biological conversion process are as the follows:

$$CO + 3H_2O \rightarrow C_2H_5OH + 4CO_2$$

$$6H_2 + 2CO_2 \rightarrow C_2H_5OH + 3H_2O$$

In general, conditions for microbial conversion of syngas to ethanol are mild and specific, and the H_2:CO ratio is not critical. However, microbial tolerance to ethanol concentration in the fermentation broth is currently a limitation. Several public and private R&D projects are underway to address the issue (e.g. http://www.coskata.com; http://www.ineosbio.com).

6 Biochemical Conversion of Lignocellulosic Biomass

6.1 Overview

Theoretically, the basic process for biochemical conversion of lignocellulosic biomass into ethanol or other biofuels is relatively straightforward. First, the lignocellulosic matrix must be treated to gain access to and/or separate the main components: lignin, cellulose, hemicellulose, and pectin. The polysaccharides (cellulose and hemicelluloses) are then hydrolyzed to sugars, which are fermented to ethanol. This hydrolytic conversion process for lignocellulosic biomass contributes to the technical barriers that currently limit commercial operations. The fermentation process for ethanol production from lignocellulosic biomass is also more

complex than for corn-based ethanol production. Hydrolysates of lignocellulosic biomass typically contain significant amounts of pentoses (e.g. xylose and arabinose). These C5 sugars are not readily fermented to ethanol by the commonly-used yeast (*Saccharomyces cerevisiae*). Efficiently converting both glucose and pentoses (xylose and arabinose) into ethanol or other biofuels and at reasonably high concentrations (8–12%) is another challenge for the fermentation microorganisms.

6.2 Pretreatment Methods

Many pretreatment processes have been tested for the capability to facilitate lignocellulosic biomass component separation and to aid in subsequent access for the hydrolytic enzymes [25, 26]. The more extensively studied methods are listed in Table 4, which includes AFEX (ammonia fiber explosion) and ARP (ammonia recycle percolation) [27, 28], lime [29], organosolv [30], liquid hot water, ionic liquid [31], dilute acid and steam explosion [32, 33], and enzyme treatment [34]. Additional information on pretreatments is available from Taherzadeh and Karimi [35] and Jorgensen, Kristensen, and Felby [27].

Table 4 Features of some pretreatment processes

Pretreatment	Conditions and solid load	Effects on biomass	Xylose yield (%)	Digestibility of cellulose (%)
Dilute acid	0.5–2% H_2SO_4 140–210°C, 1–30 min; ≤40% solid	Remove and hydrolyze hemicelluloses, redistribute lignin, form furfural and HMF	75–90	<85
Steam explosion	170–250°C, 1–20 min; ≥50% solid		45–65	90
Liquid hot water	190–260°C min-h, 1–10% solid	Remove of some hemicelluloses, lignin	80–98	80–90
AFEX and ARP	Anhydrous or 15% ammonia, 90 or 170°C; >50% solid	Remove lignin, partially hydrolyze hemicelluloses and cellulose, decrystalize cellulose	>90	>80–90
Lime	~0.1 g CaO/g biomass, 55°C a few weeks, 20–40% solid	Remove lignin	>90	>90
Alkaline peroxide	1–7.5% H_2O_2, pH 11.5, 30–85°C 45 min–24 h; 15% solid	Solublize and oxidize lignin	>90	>95
Organosolv	Methanol, ethanol, acetone etc. +<0.1 M acid as catalyst, 160–200°C, h; 25% solid	Remove of lignin and some hemicelluloses	>70	90–100

An effective practical pretreatment process should meet the following standards for use in future commercial facilities: (a) allow excellent cellulose digestibility by commercial cellulases, (b) good recoveries of cellulose and pentoses from hemicelluloses, (c) minimal or no microbial inhibitory by-products, (d) good separation of lignin, (e) be easily managed at large volumes, (f) be relatively inexpensive (capex and opex), (g) not require large energy inputs, and (h) have environmentally acceptable features.

Published economic analysis has suggested that the MESP (minimal ethanol selling price) for cellulosic ethanol from corn stover, using different pretreatment technologies, ranges from $1.41/gallon for the AFEX process to $1.7/gallon for hot water treated corn stover [36]. More recently, Sendich et al. [37] indicated that the MESP for AFEX treated corn stover could be as low as $0.81/gallon due to reduced ammonia concentration and a simplified ammonia recycle process. However, we believe the assumptions used are perhaps overly-optimistic. For example, a feedstock cost of $30/ton is very low, especially given the alternative nutrient and soil texture improvement values for corn stover. More recently, the DOE reported a 2007 cellulosic MESP of $2.43/gallon [38]. In any case, and despite many years of R&D, it is difficult to validate the assumptions since none of the conversion processes have been evaluated at practical scale.

6.3 Cellulose Hydrolysis

Three methods are possible for hydrolyzing cellulose into glucose (C6 sugar for fermentation): 1. dilute acid hydrolysis (<1% H_2SO_4, 215°C, 3 min with 50–70% glucose yield) which is no longer a viable candidate; 2. concentrated acid (30–70% H_2SO_4, 40°C, a few hours, >90% glucose yield), which has been used in Japan and will be evaluated in a DOE-funded pilot facility (Table 1); and 3. enzymatic hydrolysis (cellulase mixture, ~50°C several days, 75–95% glucose yield).

The efficient enzymatic hydrolysis of cellulose by cellulases requires a coordinated and synergistic action of three groups of cellulases: endoglucanase (EG, E.C. 3.2.1.4), exoglucanases like cellodextrinase (E.C. 3.2.1.74) and cellobiohydrolase (CBH, E. C. 3.2.1.91), and β-glucosidase (BG, E. C. 3.2.1.21). EGs and CBHs act on insoluble cellulose molecules [39]. EGs randomly act internally on the amorphous regions of a cellulose polymer chain and generate oligosaccharides of various lengths and additional free ends (reducing and non-reducing ends) for CBH action. CBHs usually hydrolyze both amorphous and crystalline cellulose and cellooligosaccharide chains from the non-reducing ends in a sequential way with cellobiose as the major product, but some CBHs can hydrolyze cellulose chains from both reducing and non-reducing ends [40–42]. The hydrolysis products of these two groups of enzymes include cellodextrins, cellotriose, cellobiose, and glucose. β-glucosidases hydrolyze soluble cellodextrins and cellobiose into glucose from the non-reducing end and remove the product feedback inhibitory effect of cellobiose on EG and CBH (Fig. 5).

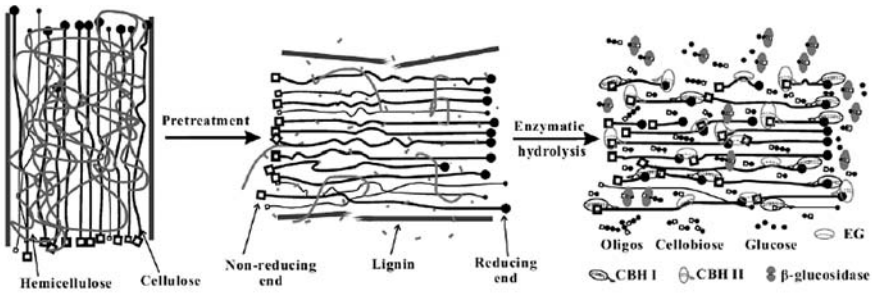

Fig. 5 Effects of pretreatment on different components in biomass and actions of non-complexed cellulases on celluloses [39, 42]

Factors impacting the activity of cellulases include enzyme source (e.g. organisms and producing conditions), concentration, and combinations. The normal enzyme dose for cellulose hydrolysis study is 10–60 FPU per gram of dry cellulose or glucan; glucanases to β-glucosidase ratio is approximately 1.75–2.0 IU of β-glucosidase for each FPU of glucanase used [29]. Most commercial glucanases are produced by *Trichoderma reseii* and the β-glucosidase is typically from *Aspergillus niger* [43].

Under research conditions, the reported digestibility or the conversion yield of cellulose from pretreated lignocellulose can be high (Table 4). However, actual glucose yield may vary greatly depending on the type of biomass, method/condition of pretreatment, cellulases (composition, source, and dose), solid to liquid ratio of the hydrolysis mixture, and other unspecified factors. The cellulose digestibility of corn stover and corn fiber can reach >90% following dilute acid or liquid hot water pretreatment [44], while the digestibility of rice hulls after similar pretreatment was about 50% [45]. Similar low digestibility results were obtained on dilute acid pretreated sorghum stubble in our lab (unpublished data). The variable digestibility of different biomass sources following dilute acid pretreatment may be an indication that this particular pretreatment is not universally effective. Currently, all the reported results for AFEX [44] and alkaline peroxide [44, 46] treated biomass sources showed consistently high cellulose recovery, and high digestibility, even at lower enzyme concentrations and shorter incubation time (48 h vs normal 96 h) [47] .

Digestibility, or glucose yield, is high when cellulose load is low (1–3% cellulose load) in the hydrolysis system. Glucose yield from pretreated biomass typically increases as enzyme load increases [47, 48, 49], while digestibility decreases as the cellulose load increases [48, 50]. We are unaware of any reports of >20% cellulose load with high digestibility. Starch-based ethanol production involves starch loadings of 20–25% or higher, that results in finished beers with ethanol concentration around 10–12% (w/v). Most lignocellulosic ethanol fermentation studies have used hydrolysates with 3–10% cellulose load, which resulted in a finished mash with ~3–5% (V/V) ethanol. Additional research is required to improve the

lignocellulose situation. Some non-cellulolytic enzymes (e.g. ferulic acid esterases and various xylanases) have been studied as pretreatment agents and showed promising results in increasing glucose yield from lignocellulose [51].

Since enzyme cost is a large contributor to the total production cost for lignocellulosic ethanol [30, 44], considerable research has been undertaken in attempts to increase the efficiency and reduce the cost of enzymes. Addition of protein (bovine albumen) and other additives (Tween 20 or 80, polyethylene glycerol, etc.) that reduce the affinity between cellulases and lignin all improve the efficiency of cellulose hydrolysis [27]. A recycling process using an ultrafiltration membrane to separate hydrolyzed glucose showed that cellulases could be re-used up to 3 times for pretreated low lignin biomass, or until ~50% of the cellulases were bound on accumulated lignin [48].

To help lower enzyme costs and possibly improve effectiveness, a research strategy has been developed to genetically-engineer biomass to express transgenic endocellulases. Microbial cellulose transgenes have been expressed in several crops: tobacco, potato, tomato, alfalfa, rice, maize, and barley [52–54]. Endoglucanase 1 (E1) concentration in some transgenic experiments has reached 1% (corn stover) [55] to 5% (rice straw) [54] of total soluble proteins. In some cases, both treated and non-treated E1 engineered biomass showed higher digestibility than biomass of their wild counterparts. Whether transgenic expression of appropriate enzymes is a viable long-term strategy when used for large-scale production remains under investigation.

6.4 Fermentation (Including SSF and C5 and C6)

For large-scale, economically viable use of lignocellulose there will be two input streams of sugars, one from hydrolysis of pretreated cellulose (C6 sugars such as glucose) and one from the hydrolysis of pretreated hemicellulose (C5 sugars such as xylose) since the common fermentation yeast (*Saccharomyces cerevisiae*) can only utilize C6 sugars, an additional technology is required for lignocellulose compared to starch or sucrose based ethanol production. The fermenting process for lignocellulosic ethanol production will include either two fermentation processes (*S. cerevisiae* for glucose and bacteria or other yeast for pentoses) or one C5 and C6 co-fermentation process (e.g. genetically-engineered microorganisms with specifically-designed metabolic pathways). To-date, several microbial species have been engineered to ferment both glucose and pentoses, including *E. coli*, *Zymomonas mobilis*, *Pichia stipitis*, *Thermoanaerobacterium saccharolyticum* and *S. cerevisiae* [56–58]. While these metabolically-engineered microbes show C6 and C5 fermentation, the ethanol yields have been too low for commercial applications [57]. In addition, many engineered organisms are susceptible to inhibitory compounds generated during pretreatment, and are not as tolerant to high ethanol concentration as the typical *S. cerevisiae* strains. Research continues to explore the possibilities for economic fermentation of both C6 and C5 sugars.

6.5 Butanol and Other Chemicals

Once hemicelluloses and celluloses in biomass feedstock have been hydrolyzed, the sugar "platform" can be utilized to generate a range of chemicals, including other fuels such as butanol [59]. Butanol has several advantages over ethanol as an alternative fuel (but not as an oxygenate) and may be a better choice for the large volume liquid transport fuel market. However, if other chemicals are produced in an ethanol plant, the final product separation process (distillation and dehydration) would be problematical. Separate down-stream production paths will be required in future biorefineries to accommodate the potential product flows, which may result in different designs and configurations [15].

6.6 Heat (Lignin)

The main component remaining in the solid residues following cellulose and hemicellulose hydrolysis to sugars is lignin (15–20% of the biomass feedstock) which has a heating value just slightly less than coal (~25 GJ/ton vs ~28 GJ/ton for coal). Therefore, lignin could be used as feedstock for co-firing, or gasification, in an integrated biorefinery to generate heat and electricity. Lignin, and associated phenolic compounds, can also be used as chemical intermediates, however, this market volume is probably limited. The main utilization will probably be for heat and electricity: both for internal use in the biorefinery and perhaps to generate surplus electricity that could be sold back to the grid, further capturing the economic benefit [60].

7 Current Outcome of Technological Implementation

7.1 Current Technology and Commercialization

For over 20 years, a considerable research effort has been made to overcome the technical and economic barriers that currently limit the use of lignocellulosic biomass. Most recently, the DOE has funded the development of several lignocellulosic biofuel facilities that will help further define the parameters for potential success. Some aspects of possible systems, such as concentrated acid hydrolysis, dilute acid and steam explosion pretreatment, are relatively well understood at the research level and will benefit from pilot-scale testing. Other aspects, such as fermentation inhibitors and fermentation of C5/C6 sugars, require further research to create sufficient improvements for commercial testing. Some technologies, such as biomass gasification, syngas conversion to biofuels by either fermentation or FT process, have been tested at a pilot scale and are ready for further scale-up and integration testing. This is a crucial period of time for lignocellulosic biofuel development: success with the current pilot scale operations will drive the required

investment for commercial scale, while poor results in the next 2–3 years may place a prohibitive restriction on future investment.

7.2 Major Industries and Technology Providers

Currently, over a dozen companies have demonstrated strong interest in exploring advanced R&D and/or pilot-scale facilities, with a view to building future commercial-scale plants. The following are a few examples, showing the range of locations, technologies, and feedstocks:

Abengoa Bioenergy, Inc. (http://www.abengoabioenergy.com) began to build the world's first commercial lignocellulosic ethanol plant in Babilafuente (Salamanca), Spain in 2005. With $76 million in funding from the DOE, the company is planning to build a lignocellulosic ethanol plant in Kansas by 2011, which will evaluate the use of corn stover, wheat straw, and other agricultural biomass.

BlueFire Ethanol, *Inc.* (http://www.bluefireethanol.com) recently received DOE funding of $80 million to build a 19 million gallons per year lignocellulosic ethanol plant in California. They plan to use urban trash (post-sorted MSW), rice straw, wood waste, and other agricultural residues as feedstock, combined with a concentrated acid process.

Coskata, Inc. (http://www.coskata.com) is exploring the integration of thermochemical and biochemical conversions: syngas is generated by gasification of lignocellulosic biomass and then converted into ethanol from the gas phase by anaerobic fermentation [61]. The company claims this technology can produce more than 100 gallons of ethanol per dry metric ton of feedstock with production cost of less than $1/gallon. There is no indication of when such numbers will be achieved in a practical large scale operation.

DuPont Danisco Cellulosic Ethanol LLC. (http://www.ddce.com) is a joint-venture between DuPont and Genencor (a subsidiary of Danisco). The company is cooperated with University of Tennessee to build a pilot lignocellulosic ethanol facility (PDU, 0.25 MG/y) in Tennessee by 2009. The plan is to combine DuPont's proprietary mild alkaline pretreatment and fermentation technologies with Genencor's enzymatic hydrolysis methods to convert corn stover and sugarcane bagasse into ethanol.

Etek Etanolteknik AB (http://www.sekab.com/) is located in Sweden and has set-up a pilot lignocellulosic ethanol plant with a capacity of about 400–500 L of ethanol/day (~2 ton dry substance/day). The plant has been functional since 2004, using the two-step dilute-acid hydrolysis process in combination with enzymatic hydrolysis. Feedstocks include cereal straws, organic waste, wood clippings, or forestry residues.

Iogen Co. (http://www.iogen.ca/) is located in Canada and has more than a decade of experience in ethanol production from lignocellulosic materials. The company currently runs a demonstration lignocellulosic ethanol plant using a modified steam-explosion pretreatment technology (dilute acid) and enzymatic hydrolysis,

with an annual capacity of 1 million gallons of ethanol. Feedstock includes wheat straw, barley straw, corn stover, and waste wood [62].

Mascoma Corporation (http://www.mascoma.com) is located in Massachusetts and was founded around the key technology of genetically-engineered bacteria that are capable of fermenting both hexoses and pentoses into ethanol. The company has recently raised $30 million and is building a 1.5–2.0 million gallon/year demonstration level lignocellulosic ethanol plant.

Poet (http://www.poetenergy.com) is one of the largest corn-based ethanol producers. With the help of an $80 million DOE grant, the company is expanding one of its plants in Iowa to produce 125 million gallons/year, of which 25 million gallons will be from lignocellulose (corn cobs and/or corn kernel fiber). Poet is currently researching possible methods for the collection and storage of corn cobs and the expanded facilities are expected to be operational by 2011.

Ranger Fuels (http://www.rangefuels.com/home) has began construction of a demonstration 20 million gallons/year lignocellulosic ethanol plant in Georgia (to be commissioned in 2009). The plant will use a thermochemical process (gasification and catalyst transformation) to turn wood, grasses, corn stover, and other available agricultural biomass into fuel ethanol.

Verenium (http://www.verenium.com/) was created by the merger of the former Celunol and Diversa companies. With DOE funding of $40 million, the company is in the process of building a 1.4 million gallon/year demonstration plant at Louisiana. The feedstock will include sugarcane bagasse, hard wood, rice hulls, and other agricultural residues.

ZeaChem, Inc. (http://www.zeachem.com/) has a technology that biologically transforms hemicellulose and cellulose into acetic acid. The acetic acid is then hydrogenated in a thermochemical process using hydrogen produced from gasification of lignin, to produce ethanol. Since no carbon dioxide is released during the biochemical conversion process, this process has a higher ethanol yield (up to 160 gallons/dry ton biomass) compared to the hydrolytic methods [63]. The plan is to build a 1.5 million gallon per year plant in Oregon with operational start-up in late 2009.

8 Summary

Global energy consumption will continue to increase, even as the reserves of easily available fossil fuels decline. Until alternative energy sources are developed for transportation, liquid fuels will remain in high demand. Crude oil production will be unable to meet future demands at affordable prices and fuels from renewable feedstocks will play a key role in contributing to the supply of liquid transport fuels.

Lignocellulose is a natural abundant material created by plants from sunlight, nutrients, and CO_2 capture. The potential volume of lignocellulose that can be theoretically produced and harvested is considerable and sufficient to make a major contribution to liquid transport fuel volume. In practice, there are several major challenges to lignocellulosic biomass production, collection, and storage that were not

addressed in this chapter but are the focus of research in many projects. Ultimately, the real cost of feedstock delivered to the conversion facility will be a major factor determining the magnitude of success for lignocellulosic biomass. Potential output products could include ethanol, butanol, biogasoline, FT liquids, and a range of chemical intermediates. Reaching this potential in an economically acceptable manner is a challenge, and requires an improved ability to convert the lignocellulosic feedstock to a useable fuel.

After more than two decades of intensive R&D, several technologies have been evaluated for biofuel production at the laboratory level. A few are now at the stage of advanced testing and pilot-scale evaluations. Presently, the challenges facing commercial conversion are such that no one technology has an absolute advantage over the others. The approach of thermochemical pretreatment and enzymatic hydrolysis followed by microbial fermentation has been the most extensively studied. The remaining challenges for this approach include further lowering pretreatment cost, improving hydrolysis efficiency and cost of cellulases (and hemicellulases), and improving the performance of fermentation organisms. The approach of thermochemical gasification combined with FT catalytic conversion has also been widely explored and may be promising under the appropriate conditions. The gasification approach would benefit from improved gasification efficiency, easier syngas cleanup, and better FT factors such as catalyst selectivity and longevity.

In some projects, various combinations (thermochemical front + biochemical, biochemical front + thermochemical) have been evaluated. For economic operation in an integrated biorefinery, it may be that such combinations of approaches will be required and that the combination utilized will depend on the feedstock, the location, the desired product stream, the degree of environmental impact, and the level of investment available. It is expected that the best technologies for specific challenges will be selected and implemented over the next 5–10 years and that the definitive answer on the size of the contribution from lignocellulosic biomass will become evident during that time.

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Environmentally Sustainable Biofuels – The Case for Biodiesel, Biobutanol and Cellulosic Ethanol

Palligarnai T. Vasudevan, Michael D. Gagnon, and Michael S. Briggs

Abstract Due to diminishing petroleum reserves and the deleterious environmental consequences of exhaust gases from fossil-based fuels, research on renewable and environmentally friendly fuels has received a lot of impetus in recent years. With oil at high prices, alternate renewable energy has become very attractive. Many of these technologies are eco-friendly. Besides ethanol, other alternatives are: biodiesel made from agricultural crops or waste cooking oil that is blended with diesel; biobutanol; gas-to-liquids (GTL) from the abundance of natural gas, coal, or biomass; oil trapped in the shale formations such as found in the western United States, and heavy oil lodged in Canadian tar sands. In this chapter, we examine advances made in environmentally friendly fuels such as biodiesel, biobutanol, and cellulosic ethanol in recent years.

Keywords Biodiesel · Cellulosic ethanol · Biobutanol · Lipase · Microalgae · Microbial · Enzymatic

1 Introduction

According to the Energy Information Administration [1], current estimates of worldwide recoverable reserves of petroleum and natural gas are estimated to be 1.33 trillion barrels and 6,186 trillion cubic feet, respectively. The world consumes a total of 85.4 million barrels per day of oil [2] and 261 billion cubic feet per day of natural gas [3]. The US consumes 24.6% of the world's petroleum (2), 26.7% of the world's natural gas (3), and 43% of the world's gasoline (1). At current consumption levels, worldwide reserves of oil will be exhausted in 40 years, and reserves of natural gas in 60 years.

P.T. Vasudevan (✉)
Department of Chemical Engineering, University of New Hampshire, Durham, NH 03824, USA
e-mail: vasu@unh.edu

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Along with diminishing petroleum reserves, the price of oil and natural gas has increased dramatically. A barrel of crude oil reached a record high price of $147.27 in July 2008, which is an increase of 1,190% over the $12.38 per barrel price in July 1998 [4]. Due to the rapid increase in the price of oil, the price per gallon of regular unleaded gasoline increased from $1.08 in July 1998 to $4.09 in July 2008 [5], representing an increase of 379%. As the price of petroleum increased, so did corporate profits. Exxon/Mobil reported a second-quarter profit of $11.68 billion in August 2008, when gas prices were the highest [6].

The concentrations of heat-trapping greenhouse gases in the atmosphere have significantly increased over the past century due to the burning of fossil fuels, such as oil and coal, combined with deforestation. As a result, the average temperature of the Earth's surface is increasing at an alarming rate [7]. The issue of climate change is one of the key challenges facing us and it is imperative that steps are taken to reduce greenhouse gas emission. The combination of diminishing petroleum reserves (it is generally believed that we reached a global "peak" oil or a global Hubbert's peak in 2006 [8]), and the deleterious environmental consequences of greenhouse gases has led to an urgent and critical need to develop alternative, renewable and environmentally friendly fuels. Examples include biodiesel, biobutanol, and cellulosic ethanol; the topics of this chapter.

Biodiesel is a renewable, non-toxic [9], biodegradable alternative fuel, which can be used in conjunction with or as a substitute for petroleum diesel fuel. Biodiesel is made entirely from vegetable oil or animal fats by the transesterification of triglycerides and alcohol in the presence of a catalyst. An advantage is that compression-ignition (diesel) engines, manufactured within the last 15 years, can operate with biodiesel/petroleum diesel at ratios of 2% (B2), 5% (B5), or 20% (B20), and even pure biodiesel (B100), without any engine modifications. Biodiesel contains no polycyclic aromatic hydrocarbons, and emits very little sulfur dioxide, carbon monoxide, carbon dioxide, and particulates, which greatly reduces health risks when compared to petroleum diesel.

Butanol is a four-carbon alcohol that can be produced from petroleum or biomass, and is currently used as an industrial chemical solvent. Biobutanol is an advanced biofuel that has an energy density, octane value, Reid vapor pressure (RVP), and other chemical properties similar to gasoline [10]. Without any engine modifications, it can either be blended at any ratio with standard grade petroleum gasoline or used directly as a fuel. Biobutanol can be produced from the fermentation of sugars from biomass or by the gasification of cellulosic biomass. Compared to gasoline, the combustion of butanol reduces the amount of hydrocarbons, carbon monoxide, and smog creating compounds that are emitted [11].

Cellulosic ethanol is ethyl alcohol, a two-carbon straight-chained alcohol, which is produced from wood, grass, or other cellulosic plant material, particularly the non-edible portions. Ethanol produced from renewable sources can be used as a high-octane biodegradable motor fuel, and is clean burning. It can be used in current automobile engines in blends up to 10% with gasoline (E10) without any engine modifications, and in higher percentages (E85 and E100) in Flex Fuel Vehicles (FFVs). Biomass consists of cellulose, hemicellulose, and lignin, which

requires pretreatment before processing. Enzymatic saccharification followed by fermentation and fermentation using cellulolytic microorganisms are the two main processing techniques used for the production of cellulosic ethanol.

In this chapter, we will examine the current state of the art in the production of biodiesel, biobutanol and cellulose ethanol, respectively.

2 Biodiesel

2.1 Background

Over the past decade, interest in biodiesel use has grown due to the increasing price of petroleum and the effect of carbon emissions on climate change. Biodiesel is a non-toxic and biodegradable alternative fuel, which can be used in conjunction with or as a substitute for petroleum diesel fuel. The first account for the production of biodiesel was in 1937 by the Belgian professor G. Chavanne of the University of Brussels, who applied for a patent (Belgian Patent 422,877) for the "Procedure for the transformation of vegetable oils for their uses as fuels" [12]. The chemical structure of biodiesel is that of a fatty acid alkyl ester, which is clean burning [13]. Biodiesel contains no polycyclic aromatic hydrocarbons, and emits very little sulfur dioxide, carbon monoxide, carbon dioxide, and particulates, which greatly reduces health risks when compared to petroleum diesel.

The first diesel engine was created in 1893 by a German mechanical engineer, Rudolph Diesel. The diesel engine is an internal compression-ignition engine that uses the compression of the fuel to cause ignition, instead of a spark plug for gasoline engines. As a result, a higher compression ratio is required for a diesel engine, which for the same power output (when compared to a gasoline engine), is more efficient and uses less fuel. The higher compression ratio requires the diesel engine to be built stronger so it can handle the higher pressure; consequently, the longevity of a diesel engine is generally higher than its gasoline equivalent. These vehicles therefore require less maintenance and repair overall, thus saving money [14]. In the European markets, over 40% of new car sales are diesel. This is due to a large influx of highly efficient diesel engines used in small cars.

An advantage of biodiesel is that current compression-ignition (diesel) engines, 15 years old or newer, can operate with pure biodiesel, or any blend, with no engine modifications. Older engine systems may require replacement of fuel lines and other rubber components in order to operate on biodiesel. The current infrastructure for petroleum diesel fuel can be utilized for biodiesel, thus reducing costs and widespread implementation criteria. The Environmental Protection Agency (EPA) in 2006 limited sulfur emission in diesel fuels to 15 ppm. New trucks and buses with diesel engines, from model year 2007, are now required to use only ultra low sulfur diesel (ULSD) with new emissions control equipment. The higher sulfur levels aided in diesel fuel lubrication; however, biodiesel is oxygenated and therefore is naturally a better lubricant and has similar material compatibility to ULSD. Many

countries are utilizing biodiesel's lubrication properties to blend with ULSD so that expensive lubricating additives are not needed [15].

The production of biodiesel is from the transesterification of triglycerides or by the esterification of fatty acids, which are both found in grease, vegetable oils, and animal fat. The transesterification of the triglycerides with a short chain alcohol (such as methanol, ethanol, propanol, or butanol) along with a catalyst, results in fatty acid esters (biodiesel) and glycerol as a by-product. The generalized transesterification reaction is given by the following stoichiometry

$$1[\text{triglyceride}] + 3[\text{alcohol}] \leftrightarrow 3[\text{fatty acid ester (biodiesel)}] + 1[\text{glycerol}]$$

The fatty acids are almost entirely straight chain, mono-carboxylic acids that typically contain 8–22 even number carbons. Fatty acids are obtained mainly from soybean, palm kernel, and coconut oils and from the hydrolysis of hard animal fats. The esterification of the fatty acids with a short chain alcohol along with a catalyst, results in a fatty acid ester (biodiesel) and water as a by-product. The generalized esterification reaction is given by the following stoichiometry

$$1[\text{fatty acid}] + 1[\text{alcohol}] \leftrightarrow 1[\text{fatty acid ester (biodiesel)}] + 1[\text{water}]$$

2.2 Feedstock

The large-scale production of a renewable and environmentally sustainable alternative fuel faces several technical challenges that need to be addressed to make biodiesel feasible and economical. The two main concerns with any renewable fuel are raw materials and the technologies used for processing. Advances in genetic modification and other biotechnologies are resulting in new or modified feedstocks that have significantly increased the yields of alternative fuels, such as genetically modified *Clostridium* to improve alcohol production [16]. Technological advancements are also being made to convert the feedstocks into fuels by improving techniques or developing completely new and environmentally friendly approaches to biofuel production.

There are many feedstocks for biodiesel production such as virgin oils, biomass, algae, and waste oils, to name a few. Feedstocks also vary with climate and location and what might be a great source in one place may not be a good source in another. A considerable amount of research has been done using edible sources of virgin oils from vegetables, like soybean, rapeseed, sunflower seed, and canola oils, to produce biodiesel. However, oil with water or high free fatty acid content can result in the formation of soap as a by-product. Therefore, additional steps must be taken to prevent soap formation, which requires the utilization of more resources.

The production of biodiesel has increased demand for soybean oil from 1.56 billion pounds in 2005–2006, to 2.8 billion pounds in 2006–2007 [17]. The increasing demand for virgin vegetable oil stocks has lead to an increase in price of these oils. The profitability of biodiesel relies heavily on the cost of its feedstock. The costs of

soybean oil can account for up to 75% of the final cost per gallon of biodiesel. This has resulted in crops being sold as fuel crops, reducing the food supply and leading to an increase in food prices around the world.

To help with this issue, many oil-bearing non-edible plants have been investigated for the production of biodiesel. These are mainly tree species that can grow in harsh environments, such as *Jatropha curcas, Pongamia pinnata, Castor, Mohva, Neem, Sal*, etc. *Jatropha curcas* has the most significant potential due to its characteristics and growth requirements [16, 18]. It requires very little fertilizer and water (as little as 25 cm a year), is pest resistant, and can survive in poor soil conditions such as stony, gravelly, sandy or saline soils. Most important, it is fast growing, and can bloom and produce fruit throughout the year with a high seed yield. Optimized production has been found to yield an average of more than 99% of Jatropha biodiesel [19], which has comparable fuel properties to that of diesel from petroleum. It is expected that some varieties of Jatropha can produce as much as 1,600 gal of diesel fuel per acre-year compared to the wild variety that produces about 200 gal/acre-year [20]. Jatropha trees can capture four tons of carbon dioxide per acre and the fuel emits negligible greenhouse gases.

There is a growing interest in using algae as a feedstock for biodiesel production within the United States. Algae have become an appealing feedstock due to their aquatic environment providing them an abundant supply of water, CO_2, and other nutrients. This results in a photosynthetic efficiency that is significantly higher than the average land based plants [21]. However, the power required to use artificial lighting to grow an aquatic species, such as microalgae, for the production of a biofuel would greatly reduce the overall efficiency of the process [22]. As the algae convert carbohydrates into triglycerides, the reproduction rate slows down so that the higher oil storing strains of algae reproduce at a much slower rate than lower oil storing strains [23]. This was shown by the Department of Energy's (DOE) Aquatic Species Program, which found the overall yield to decrease as the algae's oil storage increased.

Recently, Vasudevan and Briggs [21] summarized research on biodiesel production in a review article. According to them, a crude analysis of the quantum efficiency of photosynthesis can be done without getting into the details of the Calvin cycle; rather simply by looking at the photon energy required to carry out the overall reaction, and the energy of the products. In general, eight photons must be absorbed to split 1 CO_2 and 2 H_2O molecules, yielding one base carbohydrate (CH_2O), one O_2 molecule, and one H_2O (which, interestingly, is not made of the same atoms as either of the two input H_2O molecules.)

With the average energy of "Photosynthetically Available Radiation" (PAR) photons being roughly 217 kJ, and a single carbohydrate (CH_2O) having an energy content taken to be one-sixth that of glucose ($(CH_2O)_6$), or 467 kJ/mole, we can calculate a rough maximum efficiency of 26.9% for converting captured solar energy into stored chemical energy. With PAR accounting for 43% of incident sunlight on earth's surface [24], the quantum limit (based on eight photons captured per CH_2O produced) on photosynthetic efficiency works out to roughly 11.6%. In reality, most plants fall well below this theoretical limit, with global averages estimated

typically at between 1 and 2%. The reasons for such a difference generally revolve around rate limitations due to factors other than light (H_2O and nutrient availability, for example), photosaturation (some plants, or portions of plants receive more sunlight than they can process while others receive less than they could process), and photorespiration due to Rubisco (the protein that serves ultimately as a catalyst for photosynthesis) also accepting atmospheric O_2 (rather than CO_2), resulting in photorespiration.

In the US, the average daily incident solar energy (across the entire spectrum) reaching the earth's surface ranges from 12,000 to 22,000 kJ/m^2 (varying primarily with latitude). If the maximum photosynthetic efficiency is 11.6%, then the maximum conversion to chemical energy is around 1,400–2,550 kJ/m^2/day, or 3.8×10^{12} J/acre-year in the sunniest parts of the country. Assuming the heating value of biodiesel to be 0.137 GJ/gal, the maximum possible biodiesel production in the sunniest part of the US works out to be approximately 28,000 gal/acre-year, assuming 100% conversion of algae biomass to biodiesel, which is infeasible.

It is important to keep in mind that this is strictly a theoretical "upper limit" based on the quantum limits to photosynthetic efficiency, and does not account for factors that decrease efficiency and conversion. Based on this simple analysis though, it is clear that claims of algal biodiesel production yields in excess of 40,000 gal/acre-year or higher should be viewed with considerable skepticism. While such yields may be possible with artificial lighting, this approach would be very ill-advised, as at best only about 1% of the energy of the energy used to power the lights would ultimately be turned into a liquid fuel (clearly, one needs to look at the overall efficiency).

This upper limit also allows us to assess how truly inefficient many crops are when viewed strictly as biofuel producers. With soybeans yielding on average 60 gal of oil (and hence biodiesel) per acre-year, the actual fuel production is staggeringly small in comparison to the amount of solar energy available. This should further make it clear that using typical biofuels for the purpose of electricity generation (as opposed to the transportation sector) is an inefficient means of harnessing solar energy. Considering that photovoltaic panels currently on the market achieve net efficiencies (for solar energy to electrical energy) on the order of 15–20%, with multi-layer photovoltaics and solar thermal-electric systems achieving efficiencies of twice that in trial runs, biomass to electricity production falls far behind (considering typical plant photosynthetic efficiencies of 1–2%), with conversion of that biomass energy to electrical energy dropping the net efficiency to well under 1%.

Currently, the research for algae growth for fuel production is being done using photobioreactors. Unfortunately, current designs demand a high capital cost, which makes large-scale production uneconomical until a low cost design or new method of production is discovered. Storing energy as oil rather than as carbohydrates slows the reproduction rate of any algae, so higher oil strains generally grow slower than low oil strains. The result is that an open system (such as open raceway ponds) is readily taken over by lower oil strains, despite efforts to maintain a culture of higher oil algae. Attempts to grow higher oil extremophiles, which can survive in extreme conditions (such as high salinity or alkalinity) that most other strains cannot tolerate, have yielded poor results, in terms of the net productivity of the system. While an

extremophile may be able to survive in an extreme condition, that doesn't mean it can thrive in such conditions.

Many research groups have therefore turned to using enclosed photobioreactors of various designs as a means of preventing culture collapse or takeover by low oil strains, as well as decreasing the vulnerability to temperature fluctuations. The significant downside is the much higher capital cost of current photobioreactor designs. While such high costs are not prohibitive when growing algae for producing high value products (specialty food supplements, colorants, pharmaceutical products, etc.), it is a significant challenge when attempting to produce a low value product such as fuel. Therefore, substantial focus must be placed on designing much lower cost photobioreactors and tying algae oil production to other products (animal feed or fertilizer from the protein) and services (growing the algae on waste stream effluent to remove eutrophying nutrients, or growing nitrogen fixing algae on power plant emissions to remove NO_x emissions).

An additional challenge, when trying to maximize oil production with algae, is the unfortunate fact that higher oil concentrations are achieved only when the algae are stressed – in particular due to nutrient restrictions. Those nutrient restrictions also limit growth (thus limiting net photosynthetic efficiency, where maximizing that is a prime reason for using algae as a fuel feedstock). How to balance the desire for high growth and high oil production to the total amount of oil produced is no small task. One of the goals of DOE's well-known Aquatic Species Program was to maximize oil production through nutrient restriction; however their study showed that while the oil concentration went up, there was a proportionally greater drop in reproduction rate, resulting in a lower overall oil yield.

One approach to balancing these issues has been successfully tested on a small commercial scale (2 ha) by Huntley and Redalje [25], using a combination of photobioreactors and open ponds. The general approach involves using large photobioreactors for a "growth stage", in which an algal strain capable of high oil content (when nutrient restricted) is grown in an environment that promotes cell division (plentiful nutrients, etc.) – but which is enclosed to keep out other strains. After the growth stage, the algae enter an open raceway pond with nutrient limitations and other stressors, aimed at promoting biosynthesis of oil. The nutrient limitations discourage other strains from moving in and taking over (since they also require nutrients for cell division).

Waste oils, such as restaurant grease and spent frialator oil, can also be used in the production of biodiesel. This eliminates the "food or fuel" debate that affects virgin edible oil sources. These waste oils normally cost money for restaurants and other establishments to dispose off. This can have a negative feedstock cost which reduces the overall cost of production. However, like virgin oils, traditional processes of converting waste oils to biodiesel can result in soap formation due to the presence of water and free fatty acids. The waste oils usually contain particulates that require filtration or separation prior to processing. Demand for waste oil as a biodiesel feedstock has already resulted in companies now paying restaurants for their waste vegetable oil (WVO). Quantities of WVO are limited (it is estimated to be about 1.1 billion gallons per year in the US), but it is certainly a good option for producing biodiesel.

2.3 Comparison of Technologies

A conventional base-catalyzed reaction is used in the majority of transesterification processes to produce biodiesel. Sodium hydroxide is used as the catalyst when methanol is the acyl acceptor, and potassium hydroxide is used when ethanol is the acyl acceptor, due to solubility considerations [15]. The ethyl esters have a slightly higher energy value than the methyl esters due to the presence of the additional carbon atom, and ethanol can be more easily produced from renewable sources, such as corn. Typical reactions take place with a high molar ratio of alcohol to oil of about 6:1 with methanol, and 12:1 for ethanol [15]. The excess alcohol allows for complete conversion of the triglycerides to the fatty acid esters. An advantage of base-catalyzed transesterification is the relatively short reaction time to achieve conversion levels of 98% or greater, compared to other processes. The reaction is a direct process, needing no intermediate steps, and operates at a relatively low temperature and pressure of about 66°C and 1.4 atm, respectively. However, a major disadvantage of the base catalyzed process is the formation of soap when water or free fatty acids are present in the feedstock. Thus the feedstock should be anhydrous but the process still requires a large amount of base to be added to neutralize the fatty acids [15]. Soap formation results in additional downstream separation problems combined with a reduction in the fatty acid ester yield. The process also requires two steps and uses large amounts of chemicals as catalysts.

Acid-catalyzed transesterification is a viable alternative, in which sulfuric acid is typically used. One advantage over the base-catalyzed method [26] is that it is not as susceptible to soap formation. The resulting downstream product is easily separated and produces a relatively high quality glycerol byproduct. The process also requires only one step, compared to two steps in the base-catalyzed process. However, acid-catalysis reactions are slower and result in lower yields than base-catalysis, ranging from 56.8 to 96.4% depending on the feedstock [27]. A major disadvantage to either base or acid transesterification process is the disposal of the glycerol byproduct. Glycerin is already inexpensive, easily available, and is used in a wide array of pharmaceutical formulations. The major issue is with the purity of the glycerin; the byproduct glycerin from the production of biodiesel is 80–88% while industrial grade is 98% or higher [15]. The low market value of glycerin does not make purification economical. Many researchers are investigating innovative chemical and biological processes for the conversion of glycerin into value-added products including antifreeze agents, hydrogen, and ethanol [28].

A relatively new and promising development in the production of biodiesel is via enzymatic transesterification with lipase as the catalyst. Several microbial strains of lipases have been found to have transesterification activity; *Pseudomonas cepacia* [29], *Thermomyces lanuginosus* [30], and *Candida antarctica* [31] are a few that have been reported. The products of an enzyme-catalyzed reaction can easily be collected and separated. Unlike alkali-based reactions, enzymes can be recycled since they are not used up and require much less alcohol to perform the reaction. However, enzyme reactions take much longer to complete and can have lower yields due to inhibition of the enzyme caused by glycerol formation. Methanol, the acyl

acceptor, can also strip the essential water from the active site of the enzyme, resulting in deactivation of the enzyme. Enzymes are also expensive and require treatment such as immobilization, purification, pre-treatment, and modification [32].

New technologies are being developed to produce biodiesel that do not form glycerol as a byproduct. The hydrocracking process uses hydrocracking, hydrotreating, and hydrogenation reactions to convert a wide range of feedstocks to biodiesel with yields of 75–80% [15]. This process is currently being utilized in petroleum refineries and uses a conventional commercial refinery hydrotreating catalyst. However, the hydrocracking process requires hydrogen, which is primarily obtained from natural gas. To reduce the costs of hydrogen, the process could be easily integrated with a refinery.

The production of biodiesel has significantly increased over the past few years. The National Biodiesel Board reports an increase in production from 250 million gallons in 2006 to 450 million gallons in 2007, an increase of 55.6%. European countries produced 5.7 million tons of biodiesel in 2007 (∼1.5 billion gallons), which is an increase of 16.8% from 2006 according to the European Biodiesel Board. Germany is the World leader in biodiesel production and produced 2.9 million tons (∼790 million gallons) in 2007, which is over 50% of the European biodiesel market.

2.4 Summary

Biodiesel is a clean-burning fuel that is renewable and biodegradable. A recent United Nations report urges governments to beware of the human and environmental impacts of switching to energy derived from plants. There should a healthy debate about turning food crops or animal feed into fuel and the consequences of the switch to biofuels needs to be carefully thought out. The focus of biodiesel production needs to be on sources like waste oil and grease, animal fats, and non-edible sources. Current research has focused on these areas as well as on algae-based biofuels. Many technical challenges remain and these include development of better and cheaper catalysts, improvements in current technology for producing high quality biodiesel, use of solvents that are non-fossil based, conversion of the byproducts such as glycerol to useful products such as methanol and ethanol, and development of low cost photobioreactors.

3 Biobutanol

3.1 Background

Over the past few years, butanol made from biomass, popularly known as biobutanol, has gained a lot of attention as a biofuel. Butanol is an alcohol-based fuel that contains four carbons and has chemical properties similar to that of gasoline, thus making it an attractive substitute or additive. Biobutanol can be produced from the

fermentation of sugars from biomass or by the gasification of cellulosic biomass. It can be blended in any ratio with gasoline and be used in existing automobiles without any need for engine or fuel line modifications. It is an attractive substitute to gasoline because its BTU content is 110,000 BTU's per gallon, which is very close to the 115,000 BTU per gallon of gasoline, resulting in little change to fuel economy. The Reid vapor pressure (RVP) of butanol (0.33 psi) is low compared to ethanol (2 psi) or gasoline (4.5 psi), resulting in lower evaporative emissions. The octane values and energy density of butanol are also closer to gasoline than is ethanol. Ethanol is 100% soluble in water whereas the solubility of butanol is 9.1% at 25°C [10]; this results in less water absorbed and rust dissolved into the fuel from tanks and pipelines. An added benefit to the low solubility is reducing the spread into groundwater in case of a spill.

However, biobutanol is not a perfect fuel and has several disadvantages. Butanol is more toxic to humans and animals than lower carbon alcohols. The LD_{50} oral consumption for a rat for butanol is 790 mg/kg compared to 7,060 mg/kg for ethanol [13]. However, it is well known that gasoline contains chemicals such as benzene, which is toxic and carcinogenic. There have been no definitive tests as to whether butanol will degrade the materials in an automobile over time, but current evidence suggests that this is unlikely [10]. Environmental Energy, Inc. tested a 1992 Buick Park Avenue by driving it 10,000 miles on 100% butanol [33]. No modifications were done to the car and it passed all emission tests performed in 10 states with an average increase in gas mileage of 9%. Compared to gasoline, combustion of butanol reduces the amount of hydrocarbons, carbon monoxide, and smog-creating compounds that are emitted [33].

Butanol is used as an industrial solvent and the market demand is about 350 million gallons a year worldwide, with the United States accounting for 63%. The production of butanol via fermentation is the second oldest fermentation process, next only to ethanol. Since the 1950s however, production of butanol via fermentation has not been an economically viable alternative due to the historic low cost of petroleum. A new push for renewable alternative fuel sources has been fueled by the increasing cost of petroleum combined with the generation of more greenhouse gases. These two reasons and the development of new technologies form the underpinnings of the reemergence of the butanol fermentation process.

3.2 Comparison of Processes

The oldest method of butanol production is the acetone-butanol-ethanol (ABE) bacterial fermentation by *Clostridium acetobutylicum*, which dates back to Louis Pasteur in 1861 [13]. The bacterial microorganism, *C. acetobutylicum*, was first isolated by Weizmann [13]. In the ABE fermentation process, *C. acetobutylicum* produces acetic, butyric, and propionic acids from glucose that can be generated from various biomass sources. Potential feedstocks include corn, molasses, whey permeates, or glucose. An enzyme catalyzed reaction of acetoacetyl-CoA transfers

CoA to acetate forming acetyl-CoA. Through a series of metabolic reactions, butyryl-CoA is produced from acetyl-CoA, which is then converted to butanol in the solventogenic pathway [33]. Acetyl-CoA can also produce ethanol and acetone from acetoacetyl-CoA. A typical process produces acetone, butanol and ethanol in the ratio 3: 6:1.

The butanol yield from the ABE fermentation of glucose is relatively low, about 15–25 wt% typically [33]. This is due to the buildup of acetic, butyric, and propionic acids along with the products acetone, butanol, and ethanol, during the fermentation process. The solvents are toxic to *C. acetobutylicum*. The butanol destabilizes the cell membrane of the microorganisms ultimately resulting in cell death. Higher yields can be achieved by continuously removing the harmful solvents, mainly butanol, and/or by genetically modifying strains of microorganisms that can tolerate higher concentrations of butanol [33].

A butanol-tolerant mutant strain of *C. acetobutylicum* has been developed and designated as SA-1 [34]. This strain shows a 121% improvement in butanol tolerance over the typical strain used in ABE fermentation. The enhancement of the strain results in an overall increase in butanol production of 13.2%. Additional advantages of the mutated strain are an increase in growth rate, more pH resistance, more effective utilization of carbohydrates, and reduction in acetone concentration by 12.5–40% [34]. Other studies using genetic and metabolic engineering have modified strains, which have resulted in an increase of about 320% in the final butanol concentration [35]. The antisense RNA process helps down-regulate genes for butyrate formation by acidogenesis and increases the butanol yield through solventogenesis. The process has resulted in strains with butanol yields of 35% [36].

Tetravitae Bioscience has combined a patented mutant strain of *C. beijerinckii* and a continuous, integrated fermentation process that utilizes gas stripping. *C. beijerinckii* is a species of rod-shaped anaerobic bacteria that is known for the synthesis of organic solvents, and uses a broader substrate range and better pH range than *C. acetobutylicum*. The solvent genes of *C. beijerinckii* are located on the chromosome, which is more genetically stable than on the plasmid for *C. acetobutylicum*. The gas stripping process prevents the butanol concentrations from reaching toxic levels by sparging oxygen-free nitrogen or fermentation gases through the fermentation solution and the ABE captured in the gas are condensed [13]. The exhaust gas is then recycled back to the reactor to collect more ABE for removal. Advantages of this method are the low energy requirements, the fact that it does not remove important acid intermediates, and that it allows for efficient recovery of butanol [37].

Environmental Energy Inc. (EEI) and Ohio State University (OSU) have developed a two-step anaerobic fermentation process in a joint project to produce butanol from biomass. The first process converts the feedstock carbohydrates into butyric acid through acidogenesis using *C. tyrobutyricum*. The second step converts the butyric acid, using *C. acetobutylicum*, into butanol, which results in a significant improvement from conventional processes. The butanol solution requires purification from a recovery unit after the second step reactor. EEI's process uses a purification process that takes advantage of the azeotrope formed by butanol

(55%) and water (45%), which is used to minimize the energy required for distillation. These processes utilize OSU's proprietary fibrous-bed bioreactor (FBB) that has demonstrated improvements in long-term production with a scalable packing design. The packing consisted of a spiral-wound, fibrous matrix that allows for a high surface area with large enough voids to allow for a high cell density. Immobilizing the cells in the FBB minimizes the energy consumption required by the cells [33].

British Petroleum (BP) has partnered with DuPont to commercialize biobutanol using advanced metabolic pathways for 1-butanol. They have announced plans to produce 30,000 tons per year of biobutanol at the British Sugar facility in Wissington, UK. This will help meet the United Kingdom′s Renewable Fuels Obligation set for 2010. Along with 1-butanol, they plan on developing biocatalysts to produce higher octane isomers such as 2-butanol and iso-butanol, and to increase the interest and utility as a fuels additive or substitute [38]. BP and Dupont plan on initially marketing biobutanol to the current market as an industrial solvent and then implement a larger commercialization into fuel blending by 2010 [38].

A different approach to producing butanol utilizes a thermochemical route for the gasification of biomass by a syngas catalyst. W2 Energy Inc. is working to produce biobutanol from a Gliding Arc Tornado plasma reactor (GAT) for biomass gasification. The GAT is a non-thermal plasma system, which utilizes reverse vortex flow that allows for a larger gas residence time and ensures a more uniform gas treatment. An advantage to the GAT system is that because of the thermal insulation, it does not require high-temperature material, thus reducing costs [39]. The gasification of biomass is accomplished by the solid biomass undergoing a thermochemical reaction under sub-stoichiometric conditions with an oxidizing fuel. The biomass's energy is released in the form of CO, CH_4, H_2, and other combustible gases (syngas) [40]. The syngas consists of basic elementary components, which can be made into butanol using various petrochemical techniques. Other advances in gasification technology have been made by the National Renewable Energy Laboratory's (NREL) Battelle Labs.

3.3 Summary

Biobutanol is a renewable, biodegradable, alternative fuel, which can be used neat or blended with gasoline. Properties such as energy density, octane value, and Reid vapor pressure (RVP) are similar to gasoline; hence current vehicles can use biobutanol without any engine modifications. Biobutanol can be produced from biomass by the fermentation of sugars and starches or by thermochemical routes using gasification. The emergence of butanol as a fuel is growing with companies such as BP, DuPont, EEI, Tetravitae Bioscience, and W2 Energy Inc. investing in new technology as well as in manufacturing. Worldwide commercialization of biobutanol can replace or enhance blends of gasoline to reduce the dependence on petroleum as well as reduce greenhouse gas emissions.

4 Cellulosic Ethanol

4.1 Background

Henry Ford test drove his first prototype automobile called the Ford Quadracycle in July 1896 that ran on pure ethanol. He told the New York Times in 1925 that "The fuel of the future is going to come from fruit like that sumach out by the road, or from apples, weeds, sawdust — almost anything" [41]. Ethyl alcohol, or ethanol, is a two carbon, straight chain alcohol that is found in alcoholic beverages. Ethanol is a renewable, biodegradable, clean burning, alternative fuel that is usually produced by the fermentation of carbohydrates from sugar, corn, or fruits [13]. Ethanol has replaced methyl tert-butyl ether (MTBE) as an emissions reducing additive in gasoline due to concerns of MTBE ground water contamination that arose in late 2005. Ethanol can be used in current automobiles in blends up to 10% (E10) in gasoline without any engine modifications. Higher percentages of ethanol blends (E85 and E100) can be used in Flex Fuel Vehicles (FFVs).

Sugarcane-based ethanol edges out gasoline at an oil equivalent economic price of $40 per barrel [42]. In contrast, US corn-based ethanol has an edge over gasoline when oil price is $60 or higher. "Flex-fuel" vehicles are designed to run on ethanol, gasoline, or a mixture of the two. Ethanol is made through the fermentation of sugars, and sugar cane offers particular advantages. The energetic balance in ethanol production shows that for each unit of energy invested, sugar cane based ethanol yields eight times as much energy as corn [43]. Unlike corn-based fuels, sugarcane requires no fossil fuels to process. Cellulosic ethanol, derived from a range of crops, such as switchgrass and crop waste, is more economical than corn ethanol because it requires far less energy to produce. However, the economics of corn or cellulosic ethanol has been discussed widely in many articles. A central argument is that corn-based ethanol is literally a waste of energy. Detractors say that it takes more energy to grow the corn, process it, and convert it to ethanol than would be saved by using it. According to Pimentel and Pazek [44] "Ethanol production using corn grain required 29% more fossil energy than the ethanol fuel produced." Wang et al. dispute this and state that it takes 0.74 BTU of fossil fuel to create 1 BTU of ethanol fuel, compared with a ratio of 1.23 BTUs to 1 BTU for gasoline or 66% more than ethanol [45]. The conclusions of Wang et al. have largely been corroborated by Farell et al. [46]. According to them, "current corn ethanol technologies are much less petroleum-intensive than gasoline but have greenhouse gas emissions similar to those of gasoline." The authors however opined that cellulosic ethanol would be key to large-scale use of ethanol as a fuel. Hammerschlag compared data from ten different studies and used a parameter, r_E, defined as the total product energy divided by nonrenewable energy input to its manufacture [47]. Thus, $r_E > 1$ indicates that the ethanol has captured some renewable energy. The corn ethanol studies showed r_E in the range $0.84 \leq r_E \leq 1.65$, and three of the cellulosic ethanol studies indicated a range of $4.40 \leq r_E \leq 6.61$.

Because ethanol is made from crops that absorb carbon dioxide, it generally helps reduce greenhouse emissions. Although it is carbon neutral and renewable,

the GHG impact depends on farming practices, particularly the use of fertilizers. This is specifically true for ethanol made from corn. When ethanol is made from cellulosic sources there is considerable reduction in GHGs [48]. This is because producers of cellulosic ethanol burn lignin to heat the plant sugars whereas most producers of corn ethanol burn fossil fuels to provide the energy for fermentation. Cellulosic ethanol is a renewable, biodegradable, clean burning, alternative fuel. Cellulosic biomass typically contains 40–50% cellulose, 20–30% hemicellulose, and the remainder, 15–30%, is lignin and other components [49]. Cellulose consists of glucose monomers linked by a β-1,4 bond which forms a linear polymer [50]. Hemicellulose is a highly-branched complex polymer that is composed mainly of xylose and other five-carbon sugars [50]. Lignin is a phenyl propane polymer that acts as a binder, which cannot be converted into useful products. The hemicellulose is randomly acetylated and acts as an interface between the cellulose and lignin. The cellulose and hemicellulose can be broken down into simple sugars that are used to produce ethanol, while the lignin can be burned to produce heat, which helps to increase overall efficiency. What makes cellulosic ethanol promising is the diverse, abundant, low cost feedstock that is readily available. There are two main methods for the production of ethanol from biomass; enzymatic saccharification and fermentation, and fermentation by cellulolytic microorganisms.

However, cellulosic ethanol is not without its challenges and drawbacks. Commercial production of cellulosic ethanol currently requires high initial capital costs and involves risk. In 2002, a DOE study determined that for cellulosic ethanol to be competitive, the production cost would need to be $1.07 per gallon or less [51]. One of the most expensive steps in the production of cellulosic ethanol involves the pretreatment of biomass.

4.2 Comparison of Pretreatment and Manufacturing Processes

Pretreatment is required to alter the physical and chemical properties of the biomass to make it easier to process. The methods of pretreatment are similar for either enzymatic or microbial cellulosic ethanol processing. Removing or altering the lignin allows access to carbohydrates in the biomass. Higher lignin sources require chemical treatment to reduce the level to below 12% to enhance digestibility [50]. To gain access to the cellulose fiber, de-crystallization of the hemicellulose that is covalently bound with the lignin via hydrolysis is required [52]. The conversion of all the sugars derived from hemicellulose is highly desired to increase efficiency and minimize by-products. Pretreatment of the biomass is also required to increase the surface area and pore size, thus making it easier to digest. The increase in surface area is from the combination of hemicellulose solubilization, lignin solubilization, and lignin redistribution caused by various methods of pretreatment [53].

There are several methods by which pretreatment is performed: physical, chemical, and biological. Physical methods include ball and compression milling that shear or shed the biomass to de-crystallize the cellulose and increase the surface area and digestibility. However, these processes do very little to degrade hemicellulose

and lignin polymers. Milling also requires long processing times with high capital and operating costs, thus it is not economical and has not been pursued in scale-up operations [50, 54]. Radiation pretreatment utilizes gamma rays, electron beams, or microwaves to react to weaken and break the chemical bonds between hemicellulose and lignin through chemical reactions such as chain scission [55]. However, the high consumption of energy and capital costs makes this process economically unviable.

Dilute-acid pretreatment is a chemical process that increases the solubility of hemicellulose to 80–100%, extensively redistributes the lignin, and depolymerizes some of the cellulose [53]. The process soaks the biomass in a dilute solution of sulfuric, hydrochloric, or nitric acid and then raises the temperature by injecting steam to enhance the pretreatment method [50]. Autohydrolysis generates acids by the introduction of saturated steams into the biomass to breakdown the hemicellulose and lignin [50]. The pressure is rapidly released resulting in the breakup of the biomass due to the instant vaporization of the trapped water. This process is known as steam explosion pretreatment and results in 80–100% solubilization into a mixture of monomers and oligomers of hemicellulose. It also redistributes the lignin, and depolymerizes some of the cellulose [53]. Similar to steam explosion, ammonia fiber explosion pretreatment (AFEX) uses high temperature and pressure ammonia to de-crystallize cellulose, and increase the solubility of lignin by 10–20%, and of hemicellulose up to 60% while hydrolyzing about 90% to oligomers [53].

Other chemical pretreatment methods include "hydrothermal" processes using liquid hot water, supercritical carbon dioxide, "organosolv" processes that involve organic solvents in an aqueous medium, concentrated phosphoric or peracetic acid treatment, and strong alkali processes using sodium hydroxide or lime [50, 53]. A biological pretreatment process utilizes fungi, such as white rot, brown rot, and soft rot, to hydrolyze the cellulose component of biomass. Filamentous fungi, typically *Trichoderma* and *Penicillium* species, can be used directly for cellulose hydrolysis because of the greater capacity for extracellular protein production than that of cellulolytic bacteria [56]. However, it requires a three-fold reduction in cost for commercialization and the reaction rates for the hydrolysis of cellulose are relatively low in comparison to chemical pretreatment methods [56].

Enzymatic saccharification utilizes enzyme blends for recovering carbohydrates from the hydrolyzate generated after pretreatment [51]. Commonly, cellulase and hemicellulase enzymes are used as a "cocktail" with other enzymes to enhance yields and reduce enzyme costs. The products of enzymatic saccharification – the process of breaking a complex carbohydrate into its monosaccharide components – severely inhibit cellulases and hemicellulases [57]. To overcome this difficulty, Simultaneous Saccharification and Fermentation (SSF) of the pretreated hydrolyzate is preferred. Once the structure of the biomass is disrupted, the cellulose and hemicellulose is enzymatically converted to sugars by the saccharification process. During the fermentation process, yeasts such as *Saccharomyces cerevisiae,* convert the sugars to ethanol. The advantage of SSF over Separate Hydrolysis and Fermentation (SHF) is higher yields of ethanol but SSF requires more than double the fermentation time [58]. However, the hydrolyzate also contains acetic acid

and other toxic compounds. Together with increasing ethanol concentrations, this can inhibit the enzymes and fermentation organisms, thus lowering yields. New developments in enzymatic saccharification and fermentation have been developed by Iogen Energy Corporation and the NREL to develop effective "cocktails" of enzymes along with modified strains of yeast that can break down complex sugar molecules, which conventional fermentation yeast cannot.

Recently, Royal Dutch Shell (Shell) announced a partnership with Iogen Energy Corporation to advance cellulosic ethanol from agriculture residues such as cereal straw and corn cobs and stalks. And just recently, Iogen Energy shipped 100,000 L (26,417 gal) to Shell, which is the first installment of the initial order of 180,000 L (47,550 gal) of cellulosic ethanol. Iogen's demonstration facility located in Ottawa, which first began producing cellulosic ethanol in 2004, is being purchased by Shell for use in upcoming fuel applications [59].

Cellulolytic microorganisms, an alternative to yeast, utilize ethanol fermenting microbes that both hydrolyze and ferment the sugars into ethanol from a milder pretreatment process. Gram-negative bacteria, such as *Escherichia coli*, *Klebsiella oxytoca*, and *Zymomonas mobilis*, are being investigated as potential microorganisms for industrial production of ethanol [52]. Using genetic and metabolic engineering, NREL has developed a strain of *Z. mobilis* (Zymo) that can break down complex sugars like xylose, and tolerate higher concentrations of acetic acid [51]. Other studies have shown that the *Z. mobilis* strain can produce theoretical yields up to 95% and handle a wider range of feedstocks [52]. High technological costs have impeded the widespread production of cellulosic ethanol by microorganisms. Consolidated bio-processing or CBP has been developed to address this problem. This process utilizes cellulolytic microorganisms to perform the hydrolysis of biomass and the fermentation of sugars into ethanol within a single process, which is a large cost reducing strategy [53]. CBP is expected to reduce overall production costs by eight-fold compared to SSF under similar conditions.

Mascoma Corporation has dedicated their research team to focus on the commercialization of CBP, which is seen as the lowest cost configuration for cellulosic ethanol. Mascoma Corporation is in the process of developing a cellulosic fuel production facility that will use non-food biomass to convert woodchips into fuel. They are predicting that the new facility will produce 40 million gallons of ethanol and other valuable fuel products per year [60].

4.3 Summary

Cellulosic ethanol is ethyl alcohol produced from wood, grass, or the non-edible parts of plants, and is a sustainable and renewable biofuel that is biodegradable. The promising features of cellulosic ethanol are the diverse and abundant feedstock that can utilize existing waste by-products. Iogen Energy Corporation is currently producing cellulosic ethanol for Shell using enzymatic saccharification and fermentation in a small-scale commercial facility. Another approach to cellulosic ethanol is via the use of cellulolytic microorganisms. As commercialization of cellulosic

ethanol expands, it can be used to increase ethanol production without causing food shortages or demands, and will reduce greenhouse gas emissions and our dependence on fossil fuels.

5 Final Thoughts

Research on renewable and environmentally sustainable fuels has received a lot of impetus in recent years. With oil at high prices, alternative renewable energy has become very attractive. Many of these technologies are eco-friendly. Besides ethanol, other environmentally sustainable fuels include biodiesel and biobutanol.

A recent United Nations report urges governments to beware the human and environmental impacts of switching to energy derived from plants. There should a healthy debate about turning food crops or animal feed into fuel and the consequences of the switch to biofuels needs to be carefully thought out. Thus the focus of biofuel production needs to be on non-edible and waste sources. In the case of biodiesel, these include restaurant grease, non-edible sources like Jatropha as well as microalgae. Biobutanol is a renewable, biodegradable, alternative fuel, which can be used neat or blended with gasoline. Properties such as energy density, octane value, and Reid vapor pressure (RVP) are similar to gasoline; hence current vehicles can use biobutanol without any engine modifications. Biobutanol can be produced from biomass by the fermentation of sugars and starches or by thermochemical routes using gasification.

Ethanol is made through the fermentation of sugars, and sugar cane offers many advantages. Unlike corn-based fuels, sugarcane requires no fossil fuels to process. Cellulosic ethanol, derived from a range of crops, such as switchgrass and crop waste, is more economical than corn ethanol because it requires far less energy. While there is no single magic bullet that can completely replace our dependence of petroleum, the focus needs to shift on fuels that can not only alleviate our dependence on petroleum but are also renewable and environmentally sustainable.

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Biotechnological Applications of Hemicellulosic Derived Sugars: State-of-the-Art

Anuj K. Chandel, Om V. Singh, and L.Venkateswar Rao

Abstract Hemicellulose is the second most abundant polysaccharide in nature, after cellulose. As a substrate, it is readily available for the production of value-added products with industrial significance, such as ethanol, xylitol, and 2, 3-butanediol. Hemicellulose is a heterogeneous carbohydrate polymer with a xylose-linked backbone connecting to glucose, galactose, mannose, and sugar acids. In general, it represents about 35% of lignocellulosic biomass. It is estimated that the annual production of plant biomass in nature, of which over 90% is lignocellulose, amounts to about 200×10^9 tons per year, where about $8–20 \times 10^9$ tons of the primary biomass remains potentially accessible. Hemicellulose, which is generally 20–35% of lignocellulose amounts to nearly ~70×10^9 tons per year. Continuous efforts by researchers in the last two decades have led the way for the successful conversion of hemicellulose into fermentable constituents by developed candidate pretreatment technologies and engineered hemicellulase enzymes. A major challenge is the isolation of microbes with the ability to ferment a broad range of sugars and withstand fermentative inhibitors that are usually present in hemicellulosic sugar syrup. This chapter aims to explore and review the potential sources of hemicellulose and their degradation into fermentable sugars, as well as advocating their conversion into value-added products like ethanol, xylitol, and 2, 3-butanediol.

Keywords Hemicellulose · Ethanol · Xylitol · 2, 3-Butanediol · Hydrolysis · Fermentation

1 Introduction

Biomass in the form of cellulose, hemicellulose, and lignin provides a means of collecting and storing solar energy, and hence represents an important energy and material resource [1–3]. After cellulose, hemicellulose is the principal fraction of the

L.V. Rao (✉)
Department of Microbiology, Osmania University, Hyderabad-500 007 (A.P), India
e-mail: vrlinga@gmail.com

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plant cell wall that could serve as a potential substrate for the production of value-added products under optimized conditions [4]. In general, the secondary cell walls of plants contain cellulose (40–80%), hemicellulose (10–40%), and lignin (5–25%). The arrangement of these components allows cellulose microfibrils to be embedded in lignin, much as steel rods are embedded in concrete to form reinforced concrete [5]. The composition of hemicellulosic fractions from different natural sources is summarized in Table 1.

The carbohydrate fraction of the plant cell wall can be converted into fermentable monomeric sugars through acidic and enzymatic (hemicellulase/cellulase) reactions, which have been exploited to produce ethanol, xylitol, and 2, 3-butanediol via microbial fermentation processes [1, 4, 12]. In the hemicellulosic fraction of the plant cell wall, xylan is the major backbone, linking compounds like arabinose, glucose, mannose, and other sugars through an acetyl chain [4]. They can be characterized as galactomannans, arabinoglucuronoxylans, or glucomannans based on their linkage with the main xylan backbone [13].

Thermal, chemical, and enzyme-mediated processes and combinations thereof are being explored in order to obtain monomeric components of hemicellulose with maximum yield and purity. The depolymerization of hemicellulose by chemical or enzyme-mediated processes yields xylose as the major fraction and arabinose, mannose, galactose, and glucose in smaller fractions [12]. This sugar syrup can be converted into ethanol; xylitol; 2, 3-butanediol (2, 3-BD); and other compounds [4]. The use of hemicellulose sugar as a primary substrate for the production of multiple compounds of industrial significance is summarized in Fig. 1.

A wide variety of microorganisms are required for the production of metabolites from hemicellulosic-derived sugar syrup. The ability to ferment pentoses is not widespread among microorganisms and the process is not yet well-established in

Table 1 Cell wall composition among various lignocellulosic sources considered for biofuel (% of dry material)

	Cellulose	Hemicellulose*					
Lignocellulosic source	Glucan	Xylan	Arabinan	Mannan	Galactan	Lignin	References
Sugarcane bagasse	40.2	22.5	2.0	0.5	1.4	25.2	[6]
Wheat straw	32.1	19.5	2.8	0.6	1.1	20	[7]
Corn stover	37.5	21.7	2.7	0.6	1.6	18.9	[8]
Switch grass	34.2	22.8	3.1	0.3	1.4	19.1	[7]
Pine wood	44.8	6.0	2.0	11.4	1.4	29.5	[9]
Aspen wood	48.6	17.0	0.5	2.1	2.0	21.4	[9]
Spruce wood	41.9	6.1	1.2	14.3	1.0	27.1	[10]
	42.6	26.4	0.5	1.8	0.6	18.9	[9]
Birch wood	41.5	15.0	1.8	3.0	2.1	25.2	[9]
Douglas fir wood	46.1	3.9	1.1	14.0	2.7	27.3	[11]

*Total *hemicellulose* amount present in lignocellulosics on the basis of % of dry material- Sugarcane bagasse, 27.5; Switch grass, 30; Corn stover, 26.8; Wheat straw, 50; Pine, 26; Aspen, 29; Spruce, 26; Birch wood, 23; Salix wood, 21.7; Douglas fir wood, 20.3.

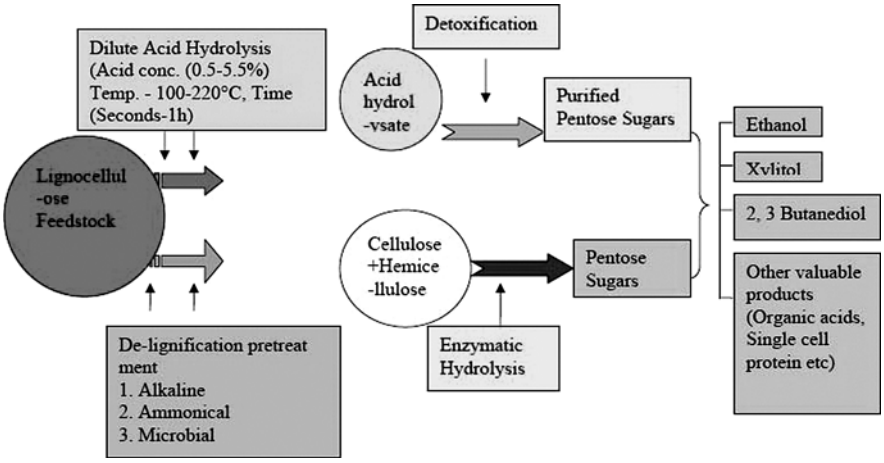

Fig. 1 Mechanistic steps involved in hemicellulose bioconversion into ethanol, xylitol and 2, 3-butanediol

industry. However, several yeast species have the basic ability to carry out these processes, i.e., *Candida shehatae, Pichia stipitis,* and *Pachysolen tannophilus* for ethanol production; *C. utilis*, *C. intermedia*, and *C. gulliermondii* for xylitol production; and *Klebsiella oxytoca* ATCC 8724, *Bacillus subtilis* (Ford strain), and *Aeromonas hydrophilia* for 2, 3-butanediol production [4]. This chapter presents significant advancements in hemicellulose biotechnology, with an emphasis on acidic and enzymatic hydrolysis and the conversion of hemicellulose hydrolysates into commercial products like ethanol, xylitol, and 2, 3-BD.

2 Background Research

To reduce the production of greenhouse gases and ensure sustainable global economic development, it is important to increase the use of renewable biomass resources [14]. There have been active movements accelerating the utilization of lignocellulose-derived products such as bioethanol, xylitol, microbial enzymes, and 2, 3-BD into alternative source of bioenergy [4, 15, 16]. Ethanol has drawn the most attention due to its rapid consumption and the global price fluctuations of crude petroleum [15, 17].

Due to developments in industrial biotechnology, the carbohydrate fraction of the cell wall can be converted into products of industrial significance. However, hemicellulose has been explored less extensively than cellulose due to several factors. The hemicelluloses in lignocellulosic materials are broken down into fermentable sugars by either chemical or enzymatic hydrolysis [18]. The latter is a promising method that breaks down hemicellulosic materials into fermentable sugars without

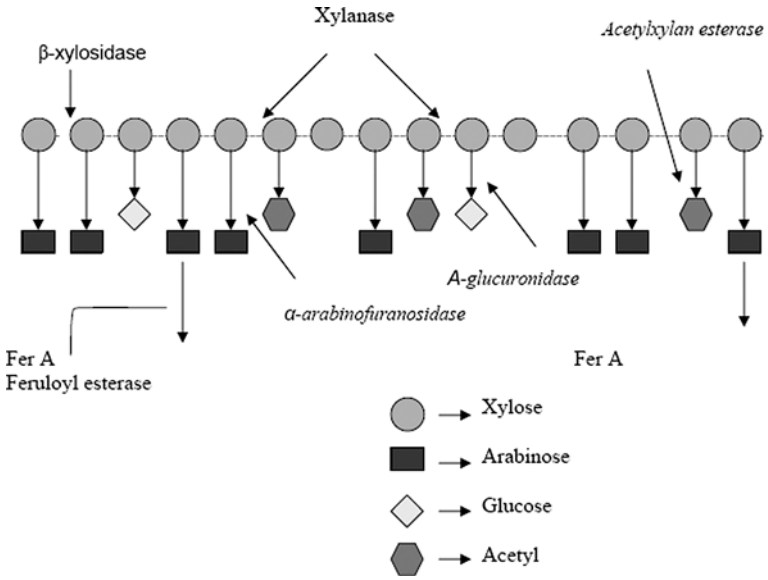

Fig. 2 Schematic presentation of coordinate action of hemicellulases on hemicellulose backbone into monomeric components

increasing the concentration of any inhibiting compounds in the hydrolysate, summarized in Fig. 2. These compounds are produced from hemicellulose hydrolysates by specialized microorganisms under a battery of cultivation techniques.

3 Technical Details – Materials and Methods

3.1 Hemicellulose Hydrolysis

In contrast to cellulose, which is crystalline, strong, and resistant to hydrolysis, hemicellulose has a random, amorphous structure with little strength. It is easily hydrolyzed by dilute acid or enzymatically using an arsenal of hemicellulase enzymes [19]. In addition, the lignocellulose can be mildly pretreated with chemicals prior to enzymatic hydrolysis for better saccharification into fermentable sugars. This reduces the crystallinity of the biomass and makes it more amenable to further coordinated enzymatic reactions [18, 20]. Various pretreatment strategies with dilute acid, alkali, ammonia fiber explosion, hydrogen peroxide, steam explosion, wet oxidation, liquid hot water, sodium sulfite, etc., have been discussed [3, 21].

3.1.1 Dilute Acidic Hydrolysis

Dilute sulfuric acid hydrolysis is a favorable method for pretreatment before enzymatic hydrolysis and also for the conversion of lignocellulose to sugars [22].

Compared to other pretreatment methods, it is especially useful for the conversion of hemicellulose into xylose, which can be fermented into ethanol by specialized microorganisms [3, 4]. Most dilute acid processes are limited to a sugar recovery efficiency of around 50%. It has been reported that the cell wall structure and components may be significantly different in different plants, which may influence the digestibility of the biomass [23]. A broad dilute acidic hydrolysis on a variety of lignocellulosic materials with respective ethanol production has been reviewed by Chandel et al. [3].

Formation of Inhibitors During Acid Hydrolysis

During acid hydrolysis of lignocellulosics, aliphatic acids (acetic, formic, and levulinic acid), furan derivatives, and phenolic compounds are formed in addition to the sugars. Furfural and 5-hydroxymethyl furfural (HMF) are the most important furans, formed by decomposition of pentoses and hexoses respectively [24]. Acetic acid has been reported in the hydrolysis of the acetyl groups into hemicellulose as a consequence of deacetylation of acetylated pentosan [25]. Multiple phenolic compounds are derived from lignin, including vanillin, vanillic acid, vanillyl alcohol, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, coumaric acid, syringaldehyde, syringic acid, cinnamaldehyde, dihydroconiferyl alcohol, hydroquinone, catechol, veratrole, acetoguaiacetone, homovanillic acid, and Hibbert's ketones [25]. HMF is converted at a lower rate than furfural, which may be due to lower membrane permeability and cause a longer lag-phase in the growth of microorganisms [26]. The phenolic compounds penetrate biological membranes and cause them to lose integrity, thereby affecting the membranes' ability to serve as selective barriers. The microbial growth was found to be inhibited in the presence of acetic acid (>3.5 g/l) in hemicellulosic hydrolysates, this phenomenon may occur due to the inflow of undissociated acid into cytosol [26].

Removal of Fermentation Inhibitors from the Hemicellulosic Hydrolysates

In order to enhance the efficiency of hydrolysate fermentation, several detoxification methods have been employed, including chemical, physical, and biological methods [25]. These methods include neutralization, overliming, use of ion exchange resins, adsorption onto activated charcoal or tin oxides, and treatments with enzymes such as peroxidase and laccase [3, 25]. Since detoxification increases the cost of the process, it is important to either overcome the need for detoxification steps or develop cheap and efficient detoxification methods. Overliming with CaO or $Ca(OH)_2$ is a classical chemical detoxification method. It efficiently removes furans and phenolics with marginal loss of sugars [24]. Organic solvents such as ether or ethyl acetate have also been applied to extract most of the inhibitors, such as phenolics, weak acids, and furans [25].

Activated charcoal treatment is an efficient and economical method of removing phenolic compounds, acetic acid, aromatic compounds, furfural, and HMF by adsorption [25]. Biological detoxification is another method that enhances the

fermentability of hydrolysates, substantially eliminating phenolic compounds. An enzymatic method using laccase was developed to eliminate the impurities of phenolic monomers and phenolic acids from hemicellulosic hydrolysates of sugarcane bagasse [24].

3.1.2 Enzymatic Hydrolysis

Hemicellulases, which catalyze the hydrolysis of plant cell polysaccharides, are multi-domain proteins generally containing structurally discrete catalytic and non-catalytic modules [27]. The most important non-catalytic modules consist of carbohydrate binding domains (CBD), which facilitate the targeting of the enzyme to the polysaccharide, interdomain linkers, and dockerin modules. The dockerin modules mediate the binding of the catalytic domain via cohesion-dockerin interactions, either to the microbial cell surface or to enzymatic complexes such as the cellulosome [27, 28].

The coordinated action of hemicellulases is necessary to obtain a satisfactory yield of pentose sugars from lignocellulosic as summarized in Fig. 2. Therefore, the development of low-cost and commercial hemicellulases is expected to be a limelight research area for cellulosic ethanol production. Table 2 shows the hemicellulase titers from different microorganisms and their mechanistic applications [29].

3.2 Hemicellulose Hydrolysates into Products of Industrial Significance

3.2.1 Ethanol

Bioethanol is a clean-burning (emits less CO_2 and other green house gasses due to availability of free O_2), non-petroleum liquid fuel that is considered to be a safe supplement to gasoline for transportation. The production and combustion of ethanol do not contribute to the total amount of carbon dioxide in the atmosphere [3, 21]. Ethanol can be mixed with gasoline in 10% (E10), 20% (E20), and 22% (E22) blends without engine modifications, but higher-level blends (such as 85% or 95%) require some engine modification. As a fuel additive, ethanol provides oxygen to the fuel, thus improving fuel combustion and reducing tailpipe emissions of carbon dioxide and unburned hydrocarbons.

Microorganisms

One of the main industrial uses of microorganisms has been alcoholic fermentation. The giant "microbial libraries" in current vogue can be studied for microbes that convert cheaper carbohydrates into value-added products, which can serve as raw materials for the fermentation of hemicellulosic-derived sugars into valuable commercial commodities [30]. The bioconversion process holds more promise of utilizing both hexose and pentose sugars from lignocellulosic materials. Microbial

Table 2 Hemicellulase titers from different microorganisms and their mechanistic applications (*Source*: Howard et al. [29].)

Microorganism	Enzyme	Substrate	Specific activity (μmol min^{-1} mg^{-1})	Mechanistic applications
Bacteria				
Fibrobacter succinogenes	Acetyl xylan esterase	Acetylxylan/alpha-naphthyl acetate	2,933	Hydrolyze the acetyl substitutions on xylose moieties
Thermoanaerobacter ethanolicus	Beta-1,4-xylosidase	o-nitrophenyl-beta-D-xylopyranoside	1,073	Hydrolyse xylobiose; release xylose
Bacillus polymyxa	Beta-Glucosidase	4-nitrophenyl-beta-D-glucopyranoside	2,417	Act upon Beta-Glucosidase to release glucose
Bacillus subtilis	Endo-alpha-1,5-arabinanase	1,5-alpha-L-arabinan	429	hydrolase activity, hydrolyzing O-glycosyl compounds
Escherichia coli	alpha-Galactosidase	Raffinose	27,350	Hydrolyzes the terminal alpha-galactosyl moieties from xylans
Clostridium stercorarium	Feruloyl esterase	Ethyl ferulate	88	Hydrolyze the ester bond between the arabinose substitutions and ferulic acid
Bacillus subtilis	Endo-galactanase	Arabinogalactan	1,790	Release of L-arabinose substituted D-galactooligosaccharides from arabinogalactan
Bacillus subtilis	Endo-beta-1,4-mannanase	Galactoglucomannan/ glucomannans/mannan	514	Acts upon interior side of beta-1,4-mannan to yield mannose
Fungi				
Phanerochaete chrysosporium	Alpha-Glucuronidase	4-O-methyl-glucuronosyl-xylotriose	4.5	Hydrolyses Alpha-1,2 Glycosidic bond the 4-O-methyl-D-glucuronic acid sidechain of xylans

Table 2 (continued)

Microorganism	Enzyme	Substrate	Specific activity (μmol min^{-1} mg^{-1})	Mechanistic applications
Aspergillus niger	Alpha-L-arabinofuranosidase	alkyl-alpha-arabinofuranoside/ aryl-alpha-arabinofuranoside/ Larabinogalactan/ L-arabinoxylan/ methylumbelliferyl-alpha-L-arabinofuranoside	396.6	Hydrolyzes arabinoxylan from terminal alpha-arabinofuranose
Aspergillus niger	Exo-beta-1,4-mannosidase	p-nitrophenyl-beta-D-galactoside	188	Acts upon outer side of beta-1,4-mannan
Trichoderma longibrachiatum	Endo-1,4-beta-xylanase	Beta-1,4-D-xylan	6,630	Cleaves interior Beta-1,4 linkage of xylan backbone

conversion of hexose sugars into chemicals is well established; however, the ability of these organisms to ferment pentose sugars is somewhat less so. The useful exploitation of lignocellulosics by fermentation can be enhanced by efficient utilization of the pentosanic fraction along with hexoses.

Yeasts that have been studied extensively for use in xylose fermentation include *Pachysolen tannophilus*, *Candida shehatae*, *Pichia stiptis,* and *Kluveromyces marxianus* [3]. The optimal performance of these microorganisms is usually controlled by the air supply. Other yeasts investigated for their xylose-fermenting ability include *Brettanomyces*, *Clavispora*, *Schizosaccharomyces*, several other species of *Candida* viz. *C. tenius*, *C. tropicalis*, *C. utilis*, *C. blankii*, *C. friedrichii*, *C. solani,* and *C. parapsilosis*, and species of *Debaromyces* viz. *D. nepalensis* and *D. polymorpha*. Maleszka and Schneider [31] screened 15 yeast strains for their ability to utilize D-xylose, D-xylulose, and xylitol for ethanol production under aerobic, microaerobic (low aeration), and anaerobic conditions using rich undefined or defined media. In almost all cases, ethanol production by *P. tannophilus* and species belonging to *Candida* and *Pichia* was better on rich media under microaerobic conditions [3, 4, 31].

Several pentose-utilizing fungal species like *Fusarium oxysporum*, *Rhizopus* sp., *Monilia* sp., *Neurospora crassa*, *Paecilomyces* sp., *Mucor* sp., *Neurospora crassa,* and *F. oxysporum* and bacterial species like *Bacillus macerans*, *B. polymyxa*, *Kiebsiella pneumoniae*, *Clostridium acetobutylicum*, *Aeromonas hydrophila*, *Aerobacter* sp., *Erwinia* sp., *Leuconostoc* sp., *Lactobacillus* sp., *Clostridium thermocellum*, *C. thermohydrsulfurium*, *C. thermosaccharolyticum*, and *C. thermosulfurogenes* utilizing pentose, hexose, and lignocellulose hydrolysates for ethanol production have been extensively reviewed [32].

Fermentation Methodologies

Researchers have performed all three fermentation processes (batch, fed-batch, and continuous) for biomass conversion into ethanol. The most suitable fermentation strategy depends upon the growth kinetics of the microorganism, the type of hydrolysate, and the economics of the process. For ethanol production from lignocellulosic biomass, batch fermentation has been extensively utilized in the past. The batch process is a multivessel approach that allows flexible operation and easy control in the bioconversion process [33]. In fed-batch fermentation, the microbial cells can be acclimatized at low substrate concentrations that later assist in accelerating the rate of ethanol formation during the entire course of the bioconversion process. Fed-batch fermentation processes are ideal to obtain a high cell density, which may help to achieve higher ethanol yields with greater productivity. Higher cell density also helps to reduce the toxicity of lignocellulose hydrolysates, particularly acid hydrolysates, to yeast cells. Continuous fermentation is another state-of-the-art technology in which microorganisms work at a lower substrate concentration, maintaining higher ethanol concentration during the entire course of the fermentation reaction [34]. Table 3 summarizes the fermentation profiles of different microorganisms utilizing a variety of lignocellulose hydrolysates.

Table 3 Fermentation of various hemicellulosic hydrolysates for ethanol production by different microorganisms

Lignocellulose material	Hydrolysis conditions	Released sugars (g/L)	Detoxification	Microbial strains	Ethanol yield (g/g)	References
Sugarcane bagasse	(2.5% (v/v) HCl, 140°C, 30 min), # Solid:liquid = 1:10	30.29	Overliming + ion exchanger	*C. shehatae* NCIM3501	0.48	[24]
Rice straw	Presoaking with 0.5% of H_2SO_4 for 18 h followed by steam heating at 15 bar pressure for 10 min, Solid:liquid = 600 g:4 L of 0.5% H_2SO_4	228 g sugar/Kg of substrate	Overliming	*Mucor indicus*	0.24	[35]
Wheat straw	(90°C, 1.85% (w/v) H_2SO_4, 18 h), Solid:liquid= 1:20	17.10	Overliming	*P. stipitis* NRRL Y-7124	0.41±0.01	[36]
Corn cob	(0.3 M H_2SO_4, 98°C, 1 h), Solid:liquid = 1:12	45.0	Overliming +ZSM-39 shaking	*P. stipitis*	0.44	[37]
Eicchornia crassipies	(1% (v/v) H_2SO_4, 7 h), Solid:liquid = 1:8	67.5	Overliming + sodium sulfite	*P. stipitis* NRRL Y-7124	0.35	[38]
Pine	Sulfur dioxide (30 min, 160°C + (225°C, 30 s, HCI equivalent to 1% of dry weight), Solid:liquid = 1:3	72	Overliming + sodium sulfite	*E. coli* K011	0.43	[39]

Table 3 (continued)

Lignocellulose material	Hydrolysis conditions	Released sugars (g/L)	Detoxification	Microbial strains	Ethanol yield (g/g)	References
Willow	Steam (1 bar pressure, soaked with gaseous SO, (1 g SO_2/100 g willow) 6 min, 206°C), Solid:gas = 100:1 g	9.0	Overliming + sodium sulfite	*E. coli* K011	0.51	[40]
Mixed wood	# # Acid hydrolysis	70.4	Electrodialysis + Sodium hydroxide	*C. shehatae* FPL-Y-049	0.48	[41]
Paja brava	Pre-steamed, impregnated with dilute sulfuric acid (0.5% or 1.0% by wt), + hydrolysis at temperatures between 170 and 230°C for a reaction time between 3 and 10 min. Solid:liquid = 1:10	22.2	No Detoxification	*P. stipitis* CBS 6054	0.20	[42]

Solid:liquid (Lignocellulose substrate: dilute acid solution).
Details are not available.

3.2.2 Xylitol

Xylitol is a naturally-occurring sugar with a wide spectrum of potential applications. It has a sweetening power matching that of sucrose (table sugar), and is used as a sugar substitute in the food processing industry [43]. Xylitol produces a perceived sensation of coolness in the mouth as it comes in contact with saliva because of its negative heat of solution [43]. Xylitol can be produced through microbial transformation reactions by yeast from D-xylose, or by both yeast and bacteria from D-glucose [44]; D-xylose can also be directly converted into xylitol by NADPH-dependent xylose reductase [45].

Microorganisms

Xylitol can be produced by bacteria and filamentous fungi [46], but often the best producers are yeasts, especially species of the genus *Candida,* such as *C. guilliermondii, C. pelliculosu, C. parapsilosis,* and *C. tropicalis* [47, 48]. Other yeast genera investigated for xylitol production from xylose include *Saccharomyces, Debaryomyces, Pichia, Hansenula, Torulopsis, Kloeckera, Trichosporon, Cryptococcus, Rhodotorula, Monilia, Kluyveromyces, Pachysolen, Ambrosiozyma,* and *Torula* [45]. Bacteria species such as *Enterobacter liqufaciens*, *Corynebacterium* sp., and *Mycobacterium smegmatis* [46] can also produce xylitol. The conversion of D-xylose to xylitol by microorganisms is important for industrial production and has been studied extensively in yeasts, as summarized in Table 4.

Fermentation Methodologies

Batch fermentation has been explored extensively for the production of xylitol *(47)*. Laboratory-based investigations in culture flasks did not show significant xylitol production. A higher substrate concentration is mandatory to obtain the genuine yield of xylitol in batch fermentation. Further studies will help to define the mechanism of xylitol fermentation under the desired set of fermentation reactions. The higher level of end products like ethanol, biomass and carbon dioxide in the media may also inhibit xylitol production [47].

In fed-batch operations, a constant substrate concentration can be maintained during the course of fermentation [48]. *C. boidinii* NRRL Y-17231 fermentations showed 75% theoretical xylitol yield in a fed-batch process, compared to 53% theoretical yield in a batch process [47]. Alternatively, continuous culture techniques have shown higher productivity with increased xylitol yields from several microorganisms. Feeding of nutrient media with an optimized dilution rate is a critical parameter in continuous cultures that helps achieve the higher rate of xylitol production. Table 4 lists a variety of microbial strains producing xylitol using different lignocellulosic sources.

Table 4 Fermentation of various hemicellulosic hydrolysates for xylitol production by different microorganisms

Lignocellulose material	Hydrolysis conditions	Sugars in hydrolysate (g/L)	Detoxification strategy	Microbial Strain	Xylitol Yield (g/g)	References
Sugarcane bagasse	1% (v/v) H_2SO_4, 120°C, 1 h, Solid:liquid = 1:5	30.0	Activated charcoal + Ion exchanger	*C. tropicalis*	0.65	[47]
Rice straw	126°C, 1% (v/v) H_2SO_4, 90 min, Solid:liquid = 1:10	20.7	Calcium hydroxide + Activated charcoal	*C. subtropicalis* WF79	0.73 g	[49]
Wheat straw	140°C, 30 min, Solid:liquid = 0.2:1	0.26 g sugar/g of wheat straw	Activated charcoal	*C. guilliermondii* FTI 20037	0.90	[50]
Brewer's spent grain	1.25% (w/v) H_2SO_4, 120°C, 17 min, Solid:liquid = 1:8	70	Alkali treatment	*C. guilliermondii*	0.78	[51]
Brewer's spent grain	2% (w/w) H_2SO_4, 121°C for 15 min, Solid:liquid = 1:8	26.3	Activated charcoal	*Debaryomyces hansenii* CCMI 941	0.50	[52]
Eucalyptus grandis	0·5% H_2SO_4, 140°C for 10 min, Total immersion time in acid solution 24 h and treated with CaO	54.7	Calcium hydroxide + NaOH	*C. guilliermondii* FTI 20037	0.54	[53]
Corn fiber	1% (v/v) H_2SO_4, 120°C, 1 h, Solid:liquid = 1:5	30.0	Activated charcoal + ion exchanger	*C. tropicalis*	0.58	[47]
Mixed wood	3.5% H_2SO_4, normal boiling temperature, 11 h	58–78	Activated charcoal	*D. hansenii* NRRL Y-7426	0.73	[54]

3.3 2, 3-Butanediol

2, 3-BD is the 2R, 3R isomer of 1, 4-butanediol, a potential bulk chemical that can be produced by a variety of microorganisms through microbial fermentation [55]. It has been utilized for the production of various chemical feedstocks and liquid fuels, including the formation of the liquid fuel additive methyl ethyl ketone by dehydration [56]. The esters of butanediol and suitable monobasic acids may find uses as effective plasticizers for thermoplastic polymers, such as cellulose nitrate and cellulose triacetates [55].

3.3.1 Microorganisms

Fermentation of xylose and glucose by *Klebsiella oxytoca* and *Aerobacter aerogenes* yields 2, 3-BD as the major product [55]. Other microorganisms capable of producing 2, 3-BD include *Bacillus subtilis* (Ford strain), *Aeromonas hydrophilia,* and several *Serratia* sp. [55]. *K. oxytoca* is able to yield high concentrations of 2, 3-BD as mixtures of stereoisomers from monosaccharides, but is unable to utilize polysaccharides. In comparison, *B. polymyxa* is able to ferment starch directly, yielding 2, 3-butanediol and ethanol in almost equal amounts [55].

3.3.2 Fermentation Methodologies

The efficiency of 2, 3-BD fermentation can be judged by the product yield from sugar, the final butanediol concentration, and the volumetric butanediol production rate. The theoretical yield of 2, 3-BD from glucose is 0.50 g/g. Higher levels of butanediol have been produced in fed-batch culture conditions that are maintained to minimize the effects of initial substrate inhibition and product inhibition. A higher production rate of 2, 3-BD was reported in continuous reactors [55]. However, product inhibition and incomplete substrate utilization remain challenging issues. Immobilization of live cells on a supporting material, i.e., matrix, has been attempted to increase the total yield of 2, 3-BD. In terms of overall performance, a two-stage continuous immobilized live cell reactor was found to be the most efficient for 2, 3-BD formation [55, 57].

The single greatest cost in most biomass conversion processes is the substrate cost [1, 2]. Hence, an inexpensive carbohydrate substrate is essential to develop an economical fermentation process for the production of 2, 3-BD. Different carbohydrate sources used by microorganisms producing 2, 3-BD under different culture conditions were reviewed [55]. pH is a crucial parameter during 2, 3-BD formation. A pH range from 5 to 6 was found to be optimal for accelerating the formation of 2, 3-BD by *K. oxytoca* [58]. In addition, a microbial growth temperature (i.e. 37°C) at which the sugar uptake can be managed by increasing the rate of 2, 3-BD formation is absolutely necessary [55]. Another important variable that affects the yield of 2, 3-BD and the productivity of the microorganisms is the rate of oxygen flow in the fermentation reaction [55]. These factors significantly contribute to 2, 3-BD

production, and they present the most challenges to maintaining a constant rate of 2, 3-BD formation during the entire course of the fermentation reaction.

3.4 Other Products

Besides ethanol, xylitol, and 2, 3-BD, other industrially significant products such as lactic acid, itaconic acid, and single cell protein (SCP) can be manufactured using hemicellulose sugars. These products have wide applications in the food, feed, pharmaceutical, and cosmetics industries. Garde et al. [59]. reported lactic acid production from wet-oxidized wheat straw by *Lactobacillus brevis* and *L. pentosus.* Sugar cane bagasse hemicellulosic hydrolysate was converted into lactic acid by thermotolerant acidophilic *Bacillus* sp. in a simultaneous saccharification and fermentation approach [60].

SCP production from hemicellulose is another cutting-edge area in hemicellulose biotechnology. Microorganism *Candida blankii* UOVS-64.2 was employed for SCP production from hemicellulose hydrolysates, and was increased by intraspecific protoplast fusion of auxotrophic mutants produced by UV irradiation followed by nystatin enrichment [61]. Pessoa et al. [62] showed microbial protein production from sugar cane bagasse hemicellulosic hydrolysate using *Candida tropicalis* IZ 1824 with a net cell mass of 11.8 g L^{-1} and a yield coefficient ($Y_{x/s}$) of 0.50 g g^{-1}.

4 Expert Commentary and Five-Year View

The current shortages and high prices of gasoline products are making it clear that a sustainable, economical, and environmentally benign process for producing fuel is needed. In the future, lignocellulosic-derived products are poised for sharp growth. According to a recent McKinsey report, the bio-based products market is expected to exceed $182.91 billion by 2015 [34]. Lignocellulosic-derived products may play a pivotal role to match this expectation and future markets seem very promising for ethanol, xylitol, organic acids, and 2, 3-BD. Mechanisms for higher yield and productivity of these value-added products can be developed by exploring the hemicellulose fraction of the cell wall in depth.

The fermentation of pentose sugars is not as easy as that of cellulosic-derived hexose sugars due to the unavailability of appropriate microorganisms and the lack of an established bioconversion process. In-depth studies of methods for hemicellulosic degradation are required. This will assist in limiting the role of fermentation inhibitors during hemicellulosic degradation. In the past five years, there has been substantial development in the area of hemicellulose hydrolysis using routine methodologies with known microorganisms. A newer approach to hydrolyzing technologies using a battery of hemicellulase titers needs to be developed to produce high yields of sugar monomers and eventually convert them into value-added products. Isolation and screening of potent hemicellulase-producing microorganisms and further development of mutants/cloned microorganisms may improve the

production yields of the desired titers on a commercial scale. Genetic engineering may also improve microbial efficiency for the overproduction of industrial products using cheaper sources of carbohydrates in fermentation media, the hallmark of commercial fermentation processes. The microbes will be more useful if they have characteristics such as thermotolerance, alkalotolerance, or tolerance of other extreme conditions.

Hemicellulose degradation into fermentable sugars is another area where the scope of research seems enormous. Efforts are underway at our laboratory for the production of ethanol and xylitol from lignocellulose feedstock. Multiple research projects are being sponsored by government agencies to improve the pretreatment process of lignocellulosics for their conversion into ethanol and xylitol [24, 63–69].

In the last five years, there has been comparatively less research into 2, 3-BD production than into ethanol and xylitol production worldwide. New research insights, such as the development of transgenic plants containing less lignin, may be helpful for the conversion of biomass into value-added products. Chen and Dixon [70] developed antisense-mediated down-regulation of lignin biosynthesis in alfalfa to reduce or eliminate the need for pretreatment. This may make the hemicellulosic fraction more accessible due to the reduced presence of lignin, which in turn will require a milder pretreatment and less enzymatic load to get the desired yield of fermentable sugars. Releasing genetically engineered plants may raise ethical issues among environmentalists; however, it can be assumed that the generation of new products from hemicellulose will strengthen the economy by saving foreign exchange reserves and promoting energy independence, which will benefit the environment.

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Tactical Garbage to Energy Refinery (TGER)

James J. Valdes and Jerry B. Warner

Abstract An emerging concept is the convergence of "green practices" such as systemic sustainability and renewable resources with military operational needs. One example is developmental tactical refineries. These systems leverage advanced biotechnology and thermochemical processes for energy production and provide sustainability to military forward operating bases for tactical purposes.

Tactical refineries are designed to address two significant problems in an overseas crisis deployment. The first problem is access to dependable energy. Recent military operations in Southwest Asia have shown that, despite advanced logistics and host nation resources, access to fuel, particularly during the early months of a crisis, can be difficult. Further, even temporary loss of access to energy during military operations can have unacceptable consequences. The second problem is the cost and operational difficulties for waste disposal of materials created by military operations. Delivery of food, supplies, equipment and material to forward positions creates huge volumes of waste, and its removal inflicts a costly and complex logistics and security overhead on US forces.

As a simultaneous solution to both problems, deployable tactical refineries are being designed to convert military field waste such as paper, plastic and food waste into immediately usable energy at forward operating bases, on the battlefield or in a crisis area. These systems are completely novel and are only becoming feasible by taking advantage of recent advances in biotechnology and thermo-chemical science. In addition to providing operational benefits to US Forces, these systems will provide significant cost savings by reducing the need for acquisition and distribution of liquid fuels via convoys which are vulnerable to attack. Tactical refineries would also serve a useful role in other military programs which support disaster relief or post-combat stabilization.

J.J. Valdes (✉)
Department of the Army, Research, Development and Engineering Command,
Aberdeen Proving Ground, MD 21010-5424, USA
e-mail: james.valdes@us.army.mil

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1 Introduction

The initial challenge was to mate the waste streams produced by small tactical units with technologies that were net energy positive at that scale. The TGER system was the result of a high level of optimization "from the trash up" and required a thorough scientific analysis and technology selection process with full consideration of the context within which it would be operating.

There are numerous waste to energy technologies, each with varying efficiencies and capabilities to digest complex waste streams [1]. Figure 1 breaks the problem set down to net power output (x axis) verses the type of waste (y axis), and shows the range of applications from landfill to onsite or tactical utilities. Incineration, for example, will handle all waste types including hazardous materials and metals, but has only 10% net power output at best and is most suited to large static operations such as landfills. By contrast, biocatalytic (i.e. enzymatic) approaches have much more limited ability to handle waste but are relatively efficient (~75%) in terms of net power output [2].

Biocatalytic approaches are therefore more suited to operations in which the waste stream is predominantly food waste and biomass. These two technologies occupy the extremes of this energy return spectrum.

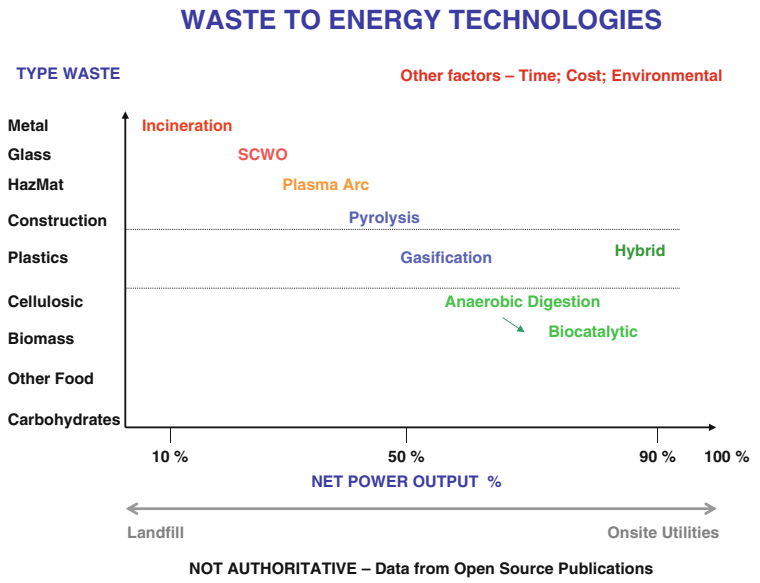

Fig. 1 Waste to energy technologies

The Tactical Garbage to Energy Refinery (TGER) design is a "hybrid" that utilizes both biocatalytic (fermentation) and thermochemical (gasification) subsystems in a complementary manner to optimize overall system performance and to address the broadest possible military waste stream. The hybrid design is based on detailed analysis of the waste stream combined with a modeling and simulation program unique to the TGER. Given the objective waste stream which includes both food and dry material wastes, a system which included a biocatalytic format for organic wastes such as food and juice materials, and a thermochemical format for solid wastes such as paper, plastic and Styrofoam, would have significant advantages over unitary approaches.

The Energy and Material Balance mathematical model showed that conversion of materials and kitchen wastes to syngas and ethanol would provide sufficient energy to drive a diesel engine and generate electricity. A downdraft gasifier was selected to produce syngas via thermal decomposition of solid wastes, and a bioreactor consisting of advanced fermentation and distillation was used to produce ethanol from liquid waste and the carbohydrates and starches found in food waste.

Both dry and wet field wastes (with the exception of metal and glass) are introduced into a single material reduction device which reduces both the wet and dry waste into a slurry. This slurry is then subjected to a "rapid pass" fermentation run which converts approximately 25% of the carbohydrates, sugars, starches and some cellulosic material into 85% hydrous ethanol. The remaining bioreactor mass is then processed into gasifier pellets which are then converted into producer gas, also known as "syngas". The hydrous ethanol and syngas are then blended and fumigated into the diesel engine, gradually displacing the diesel fuel to an estimated 2% pilot drip. The design process model is shown in Fig. 2.

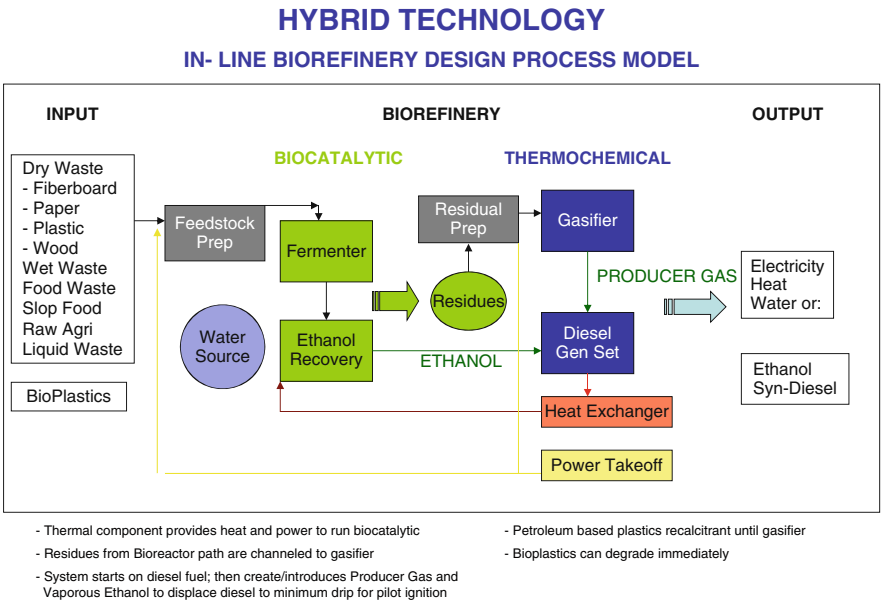

Fig. 2 In-line biorefinery design process model

Adding the advanced fermentation process to the design of the TGER added no significant energy costs, as heat generated by the engine's exhaust drives the distillation, which is carried out in an 8-foot-high column packed with material over which fractionation of ethanol and water occurs. The additions of a few small pumps used to transport the ethanol solution from the fermentation tank to the distillation column and finally to the ethanol storage tank, were the only additional power requirements. The combination of the two waste-to-energy technologies allowed for the remediation of a broader spectrum waste stream, both solid and liquid, the ability to extract much more energy from the waste, and operation of the generator at full power due to the anti-knock properties of the hydrous ethanol.

2 Background Research

There were two key bodies of knowledge that defined our research and technology transition plan. First, was the development of an understanding of the military context in which the tactical biorefinery was to serve and, second, was the search of the available "solution space", that is, the match of current and future technologies to requirements for energy generation and trash reduction.

Within the military context, a number of science and technology variables were considered. These included the type of input biomass, the type or types of biomass processing to be used, the output energy stream that results, and the kinds of military applications that would be served. A graphic depicting our "solution space" is shown in Table 1.

Table 2 illustrates the energy content of different fuels relative to diesel fuel [3–5]. The value of converting organic waste into ethanol is clearly shown. Ethanol

Table 1 Solution space for waste to energy

Waste	Technology	Energy product**	Military application served
Food waste (starch)	Bioprocessing	Ethanol (fluid.69)	Liquid fuel for burners/generators
Food waste (oil, grease)	*Starch	Methanol (fluid 0.51)	(primary or fuel additive)
Plastics	*Cellulosic	Bio-oil (fluid)	Gaseous fuel for modified generators
*Petroleum based	Pyrolysis to bio-oil	Biodiesel (fluid 0.6)	Fuel cells, PEMs generators
*Bio-based	Gasification to energy	Methane (gas 0.97)	Liquid fuel for advanced batteries
Paper (cellulosic)	Hybrid	Hydrogen (gas 0.2)	Direct electricity to power grid
Fiber board (cellulosic)	*Thermal		Hot water for troop use
Locally agriculture	*Bioprocess		

**Form and energy per unit volume, Gasoline = 1.0

Table 2 Relative energy content

Energy product	Energy index	Energy per unit volume*
Diesel	1.0	138,000 BTU 48 MJ/kg
Gasoline	0.98	125,000 BTU
Ethanol	0.69	84,600 BTU
Producer gas	0.2	10 MJ/kg

*Diesel = 1.0

has 69% of the energy content that the same volume of diesel fuel has, whereas synthetic gas has only 20% of the energy content of diesel fuel [6, 7]. Ignoring the potential energy contained within organic food and liquid wastes would result in a significant loss of energy and reduced diesel fuel savings.

3 Materials and Methods

The TGER prototypes were fabricated and commissioned at Purdue University and conformed to the following selection criterion:

a. Approach the problem as a "dual optimization" to develop a system which will simultaneously eliminate as much waste as possible while producing as much useful energy as possible.
b. Design of the TGER must be "tuned" to the operational context to ensure an easily available and reliable volume of military waste.
c. The TGER should be designed to be contiguous with both the input source of wastes and the end user for the output energy product, avoiding any reprocessing or transport costs.
d. The TGER must be operationally and tactically deployable via military airframe and able to be transported on the ground via standard military trailer.
e. The TGER should not need additional manpower or machinery costs for waste separation.
f. The process must minimize parasitic costs such as manpower, water, external energy, etc.
g. The refining process should have minimal residual waste.
h. Additional concerns of hazardous waste, safety, and troop use must be considered, and operation should be amenable to unskilled labor.

The selection of gasification and biocatalytic fermentation has strategic value in that both methods are well-demonstrated technologies supported by high levels of research by the Department of Energy and, in the long course, are very likely to improve as new advances are achieved.

Significant new advances in gasification include the introduction of integrated sensors and automated computerized control systems for the process. These recent

advances have resulted in gasification technologies with reliable and efficient conversion of waste to energy. Significant recent advances in biocatalytic fermentation include advances in genetically modified or modified via directed evolution enzymes and micro-organisms. Using methods developed at the Laboratory of Renewable Resources Energy at Purdue University, several commercial entities have broken new thresholds in domestic ethanol production techniques by applying new biocatalysts and processes, the result being the economically viable production of ethanol for fuel [8]. Current advances in enzymatic design and development bode well for further methods to reduce what would normally be considered unusable biomass waste (e.g. paper fines from shredded cardboard and other cellulosic wastes) into usable energy, allowing more energy to be harnessed from the same waste stream.

During the commissioning phase of the TGER, the system was able to deliver reliable power with very low parasitic costs required to operate the system internally. The core processes, gasification and fermentation for conversion of waste to energy, worked very well and the unique hybrid combination of thermochemical and biocatalytic technologies proved itself to be of considerable merit. These technologies could easily scale up to support military installations such as hospitals and major troop areas by converting waste into power, hot water, and usable fuel while eliminating costly waste removal expenses. Installation biorefineries could provide cost savings for US and overseas bases, reduce dependence on petroleum-based energy and support environmentally responsible initiatives, highlighting DoD's support of renewable energy resource technologies.

3.1 TGER Retrofits

The first TGER prototype (Fig. 3) was built as a part of a Phase II STTR (Small business Technology Transfer Research) program and demonstrated proof of principle,

Fig. 3 Original TGER prototype before retrofit

Fig. 4 TGER after retrofit

but was not rugged enough to deploy to an OCONUS (outside the continental United States) site for field testing and validation. The initial function of the follow-on effort was to upgrade the existing prototype with better, more advanced equipment that could withstand the stresses of a three month OCONUS deployment in an operationally harsh environment (Fig. 4).

Three of the key improvements identified during testing of the Phase II TGER and applied during the retrofit and fabrication are highlighted below.

(1) *First stage materials preparation (Industrial shredder and separations system)*. This component combines several key tasks which currently are done on the original prototype with separately acquired and integrated third-party components. Tasks include shredding, rinsing, auguring and compacting bioreactor residuals. The Industrial shredder performs these functions as a single component with half of the electrical power required by the original TGER. The new Industrial shredder was retrofitted onto the original prototype and included during fabrication of the second prototype.
(2) *Second stage pelletizer*. Testing demonstrated that the size and shape of the pellets were the most critical qualities of gasifier feed-stock, followed by pellet density and then proportions of waste content (plastic vs. cellulosic, other). Our original view of the feedstock had focused on the latter, i.e. waste content proportions, and had used a less expensive compaction channel for gasifier pellets. Subsequent off-line testing with pellets made with equipment demonstrated a marked improvement in gasifier performance and subsequent engine output. The pelletizer, shown in Fig. 5, was included in the second TGER design and was a retrofitted improvement to the original prototype.
(3) *Stainless steel commercial grade distilling column*. The stainless steel distilling column was upgraded from standard steel to stainless to prevent the introduction of rust into the distilling apparatus [9].

Fig. 5 Two high capacity laboratory pelletizers mounted on a single table with casters

3.2 Modifications of Second Prototype

Fabrication of the second TGER prototype began in early March 2008 and was completed in three weeks. During fabrication, additional modifications were applied to the second prototype that could not be applied to the first. These modifications are discussed in more detail below.

a. *Water circulation system*. The material rinsing water was routed away from the main system through an intermediate sump pump and into a 500 gallon tank (see Fig. 6), and then routed back into the wash tank on the system using a sump pump. There were several reasons for this modification. First, the intermediate sump pump broke up any large debris (e.g. food slop and paper material) that passed through the sieve. This ensured that the re-circulated liquids would not cause any clogging of the plumbing. Using the large 500 gallon tank at ground level also made it easier and more efficient for the operators to monitor the fermentation process and add the necessary biocatalysts.
b. *Rubber/flexible plumbing*. The plumbing on the first TGER prototype was fabricated using standard two inch PVC pipe. When operating in freezing temperatures, water would collect in the pipes after operation, freeze overnight and cause the pipes to burst, causing significant delays in operation due to the time required to repair the pipes. The second TGER prototype therefore used a flexible rubber hose with quick disconnect fittings instead of pipes, allowing the water to be drained from the hoses after operation in order to prevent the pipes from freezing. Flexible hosing also eliminated the possibility of pipes breaking

Fig. 6 Material rinsing water routed off the main system through an intermediate sump pump and into a 500 gal tank

due to excessive vibration of the TGER either while in operation or during transport.

c. *Chiller*. During testing of the first prototype, a chiller was needed to efficiently and quickly condense the distilled ethanol into a liquid state and collect it in the ethanol fuel tank. Due to design issues, the chiller could not be retrofitted on the first prototype but was included on the second. The chiller cooled a mixture of 50% water and 50% antifreeze and circulated it into a heat exchanger (condenser) where the ethanol vapor would condense into liquid ethanol, allowing the TGER to operate efficiently in hotter climates.
d. *Reflux valve*. The reflux valve is a programmable valve that automatically redirects condensed ethanol from the condenser to either the ethanol storage tank or back to the distillation column at a 5:2 time ratio. By redirecting condensed ethanol back into the distillation column at a 5:2 time ratio, the ethanol purity improved from 80% to 85%.
e. *Pellet auger/elevator*. An external pellet elevator was purchased in order to automate the process of supplying waste-derived pellet fuel into the downdraft gasifier (Fig. 7). On the original prototype, a technician was required to climb onto the top of the TGER in order to pour waste pellets from a bucket into the gasifier, a time consuming and unsafe process. The pellet elevator allowed the technician to dump the pellets into a large collection bin at ground level and the pellet elevator would automatically deliver the correct quantity of pellets into the gasifer based on data received from an infrared sensor suspended over the gasification chamber.
f. *Centrifuge pump and basket filter configuration*. On the original prototype, the centrifuge pump and basket filter had to be installed on their side. In order

Fig. 7 Pellet auger/elevator

to achieve optimal performance from the pump and filter it is necessary to install them upright. The frame on the second prototype was redesigned to accommodate an upright installation of both the pump and filter.

4 Current Outcome of Technical Implementation

Both TGER prototypes underwent a third party assessment conducted by the US Army Aberdeen Test Center. Three high risk and five medium risk hazards were identified on the TGERs. All risks were mitigated with minor hardware modifications, and sufficient safety devices and equipment were supplied as part of the basic issue items (BII). ***007-DT-ATC-REFXX-D5104***

Given that the mission of the Rapid Equipping Force is to quickly respond to field commanders' requests by accelerating new technologies, the two first stage TGER prototypes were deployed by intent at what was considered to be the minimum technical readiness level for field evaluation. TGER assessment during the 90 day deployment to Victory Base Camp, Iraq met its objectives by identifying the key engineering challenges needed to advance from a first stage scientific prototype to an acquisition candidate system (Fig. 8).

The Iraq deployment validated the utility of the TGER system as an efficient means to address a complex, mixed, wet and dry waste stream while producing power. The science and technology underlying the hybrid design of the TGER is unique and has considerable advantages over other unitary approaches. The engineering of the TGER system and, in particular, the difficulties which arose in having to modify third-party commercial off the shelf equipment to TGER purposes, were an expected and commensurate problem.

Overall, the TGER performed well as a system for the first month of deployment. During the second month, unanticipated problems with the downdraft gasifier arose

Fig. 8 Deployed TGER

which required considerable remedial attention by the technicians. With remote coordination with the manufacturer, many of these problems were quickly resolved, but the overall reliability and performance of the downdraft gasifier was in general decline over the three months, resulting in considerable down-time during the deployment.

Despite some initial tankage limitations (due to a delay in site prep by the Victory Base Camp DPW) and intermittent performance of the chiller system due to extremely high (120°F) ambient temperatures, the bioreactor performed well during the first month. The chiller was eventually upgraded with one of greater capacity, but during the final month the system encountered a compromised heat exchanger, some pumping problems, and apparent loss of biocatalyst efficacy due to heat exposure. The technicians were able to bypass the failed heat exchanger, modify pump elevations and add fresh biocatalysts to recover system performance.

About halfway through deployment, one of the two laboratory pelletizers became inoperative and could not be recovered. This resulted in a shift from a daily to an intermittent duty cycle (every other day) as the operators could not produce sufficient waste fuel pellets to keep the downdraft gasifier running continuously. The downdraft gasifier requires 60 lb/pellets/h and both pelletizers were needed to meet that throughput.

Alternatively, the biggest issues anticipated prior to deployment, i.e., the viability of the waste processing equipment involving the shredder, material transport/feeding and generator flex-fuel control performed reliably and were generally trouble free. Our pre-deployment effort on these critical system tasks ensured the system performed reasonably well during the first month, and allowed the other engineering issues to emerge from the background for proper identification and characterization for remedy.

Despite the mechanical issues, when the various elements of the TGER system were pulled together (routinely during the first month, then intermittently during the last two months) the system performed remarkably well. Field data demonstrated operations at or near 90% efficiency, with excellent throughput of both liquid and dry waste. The system generally conserved water at steady state and no environmental or safety problems emerged.

4.1 General TGER Parameters

Dimensions (L×W×H)	200”×88”×99”
Weight	10,000 lbs
Waste residuals per day (Ash):	
Emissions	EPA compliant
Consumable electric power produced	max 50 kW
Water supply	600 gal is required to initially charge the system
Manpower to operate	1–2 operators

4.1.1 Consumables:

Biological package, fuel, water, charcoal, and downdraft gasifier filter bags

Lactrol (Antibiotic): 1 g/day ($0.26/g)
Glucozyme (Enzyme): 50 g/day ($0.89/50 g)
Amylase (Enzyme): 50 g/day ($2.05/50 g)
Yeast: 200 g/day ($4.39/200 g)
Total cost for biological package: $7.59/day

Downdraft gasifier filter bags need to be replaced every 2 weeks 50 lbs of charcoal per month

4.1.2 Logistical Overhead:

Set-up/breakdown time: three days total to operate the system through one full cycle

4.1.3 Safety and health risk:

Received safety release from the Army Test and Evaluation Center for prototypes, certifying the prototypes safe for human use. TGER will require further safety evaluation to be cleared for soldier operation

4.1.4 Target MTBEFF:

TGER is composed of several subsystems, each with their own mean time between essential function failures (MTBEFF). The gasifier was the worst performer of the subsystems, with a MTBEFF of about 6 h. This has caused us to look at other gasification technologies to replace the current gasifier. The pelletizers in the material handling subsystem were the next worst performer. The pelletizers were undersized for the amount of throughput which caused some maintenance problems and breakdowns. The pelletizer MTBEFF was about 48 h. This problem should be resolved with pelletizers that have the right specifications. Applying the proper upgrades to the gasifier and replacing the pelletizers the target MTBEFF will be 1 month

4.2 Sub-system Specific Parameters Under Optimal Conditions Conus

Ethanol production and consumption	
Production	12 gal/day
Consumption	1 gal/h
Syngas production and consumption	
Production	65 m^3/h
Consumption	65 m^3/h
Pellet production and consumption	
Production	60 lbs/h
Consumption	60 lbs/h
Power efficiency	
Total power generated	54 kW
Parasitic power demand	14 kW
Total waste remediated per day	1,752 lbs
Solid	1,440 lbs
Liquid	312 lbs
Diesel fuel consumption per day	average 24 gal
Diesel fuel saved per day	average 86 gal

Although the TGER did not perform to its full potential during the 90 day assessment and validation, it did demonstrate its ability to convert waste to energy and reduce diesel fuel consumption in a harsh operating environment. Below is the system level parameters recorded during live testing in Iraq. Due to equipment problems, the TGER was not able to demonstrate its ethanol production capabilities and provide enough data to statistically evaluate the bioreactor performance. The harsher conditions in Iraq also required more maintenance time for the pelletizer, thus reducing their pellet production capabilities. These issues and others contributed to the reduced fuel efficiency of the TGER while in operation in Iraq.

Ethanol production and consumption	
Production	Insufficient data
Consumption	Insufficient data
Syngas production and consumption	
Production	65 Nm^3/h
Consumption	65 Nm^3/h
Pellet production and consumption	
Production	54 lbs/h
Consumption	60 lbs/h
Power efficiency	
Total power generated	54 kW
Parasitic power demand	14 kW
Diesel fuel consumption per day	average 48 gal
Diesel fuel saved per day	average 62.4 gal

Below are specific data taken from various days when the TGER was operating at its best in Iraq. Figure 9 illustrates the ability of the TGER to conserve diesel

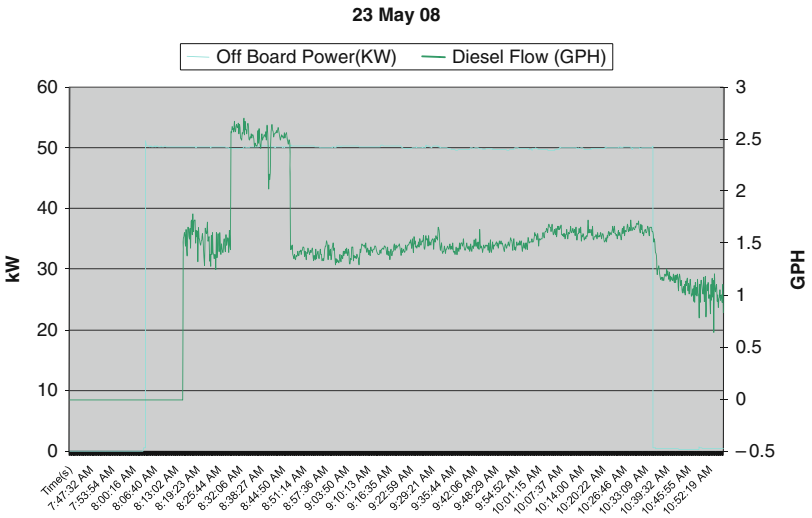

Fig. 9 Example test data (fuel/power over time)

fuel when running at high loads. The specifications for the Kohler 60 kW generator used on the TGER rates the engine's fuel consumption at 4.6 gallons per hour (gph) when less than 100% load. 100% load for the Kohler generator set using a 3-phase, 120/240 V 4P8 alternator at prime rating is 54 kW. The TGER maintained 50 kW of off board power (usable power) for approximately 2 h. During that same time the engine's diesel fuel consumption was on average 1.5 gph, a diesel fuel savings of 2.76 gph.

Figure 10 illustrates the power efficiency of the TGER. The yellow line represents all the power consumed by the TGER's subsystems and is referred to as parasitic power. All remaining power generated by the TGER (50 kW) is available for use by the customer, and is represented by the light blue line. To determine the TGER's power efficiency (pink line), we divided the power available to the customer (light blue line) by the total power generated (dark blue line). The TGER's average power efficiency was approximately 77.37% during the recorded timeframe.

Figure 11 illustrates the TGER's ability to continue to conserve diesel fuel in adverse environmental conditions. The generator exceeded the recommended load of 54 kW and generated 55.5 kW of off board power while consuming only 2.5 gph of diesel fuel. The most likely cause of the increase in fuel consumption from 1.5 to 2.5 gph was due to foreign debris (i.e. sand and dust) entering the system and causing the gasifier filters to clog, thereby reducing the amount of syngas supplied to the engine. This forced the engine to compensate by supplying more diesel fuel into the engine in order to maintain 55.5 kW of off board power. Even under these sub-optimal conditions, the TGER was able to conserve 2.23 gph of diesel fuel.

Table 3 shows data taken during field testing on 30 May 08 that was input into the TGER Energy Conversion Model. The model calculates the percent contribution

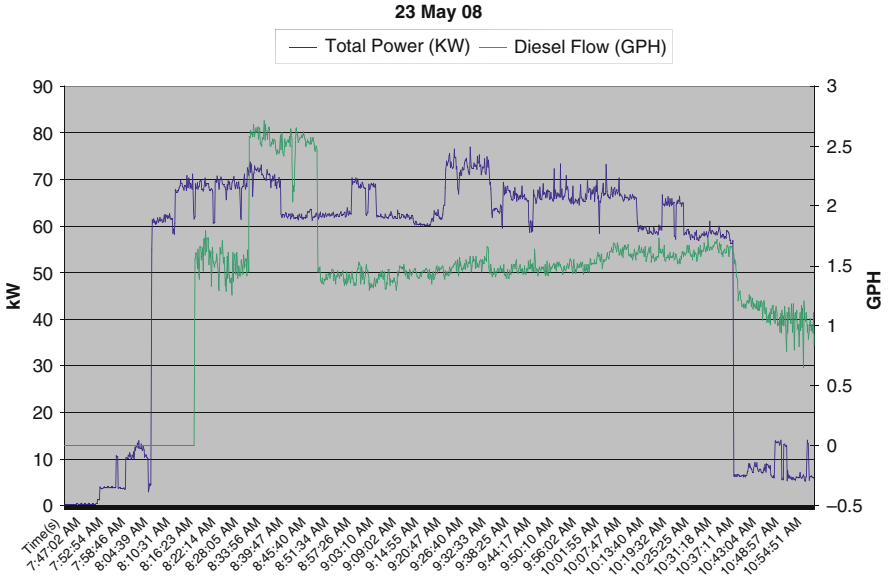

Fig. 10 Example test data (power components over tine)

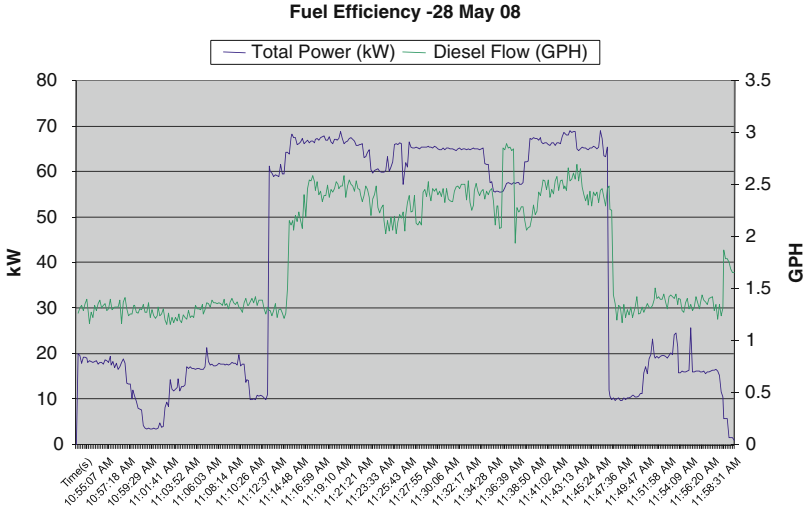

Fig. 11 Fuel efficiency and power (28 May 08)

that diesel fuel versus biofuels has to generating electrical energy. The model calculated that, of the total energy produced, the biofuels contributed 77.26% of the required energy and diesel fuel contributed 22.74%.

Figure 12 illustrates the effect of the introduction of ethanol on fuel consumption of the generator. Fuel consumption matches closely with the increase in power

Table 3 Data from TGER Energy Conversion Model

Feed materials (daily) -30 May 08	
Garbage (gallons)	70 20% paper, 50% cardboard, 30% plastic
Garbage (lbs)	399
Food (gallons)	40
Diesel (gallons)	9

Energy content of feed

Total (lb)	Component	Heats of combustion (btu/lb) LHV	Total energy (BTU)	Total energy (kWhr)
2.0	Carbohydrates	7200	14394.24	4.21871
279.3	Paper/cardboard	8000	2234400	654.8654
59.9	Plastic-polyethylene terephthalate	10250	613462.5	179.7956
59.9	Pastic-polystyrene	17800	1065330	312.2304
62.8	Diesel (DF2)	18397	1155700	338.7162
		Total	5083286	1489.826

Electrical energy production	
Total (kWh)	343
Offboard (kWh)	230

Total thermal-to-electrical energy conversion efficiency (% of energy content of feed)
23.0%

Offboard energy conversion efficiency (% of thermal energy content of feed)
15.4%

Diesel fuel savings (gallons)
33

Energy delivery efficiency (% of electrical energy for offboard use)
67.1%

	%Contribution to feed energy
Diesel	22.74%
Biofuels	77.26%

output until 1:30 pm, after which the fuel consumption drops off abruptly while the power output remains relatively steady. At 1:30 pm ethanol was introduced into the engine at rate of 0.5 gph causing the diesel fuel consumption rate to drop by more than 0.25 gph. Ethanol was supplied to the engine for approximately 30 min until mechanical difficulties with the ethanol pump began to occur and forced the operators to turn the pump off. When the ethanol pump is turned off the diesel fuel consumption gradually goes up while the power output remains relatively steady.

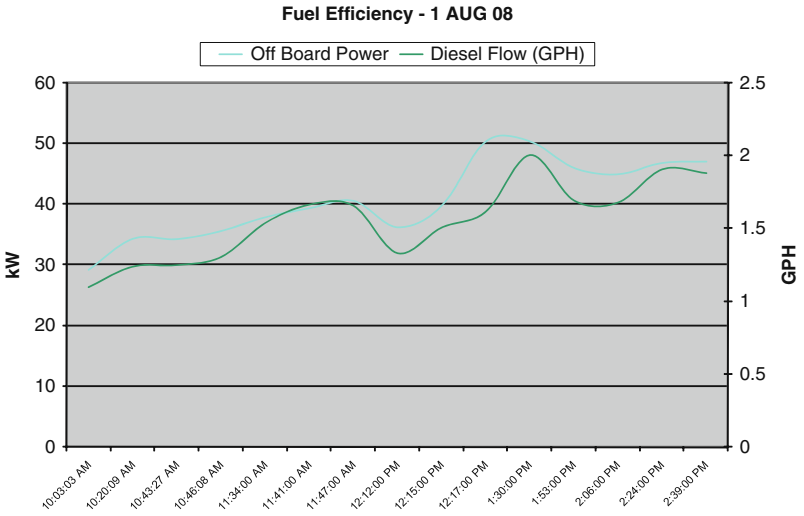

Fig. 12 Fuel efficiency and power (1 August 08)

Table 4 shows the use of the TGER Energy Conversion Model to analyze the performance of the TGER on 1 August 08. Biofuels contributed 92.92% of the required energy to generate electricity and diesel fuel contributed 7.08%. This shows that the TGER can run almost entirely on biofuels, although the increase in biofuel contribution did have a negative affect on the thermal to electrical conversion efficiency. The increase in the contribution of energy from biofuels lowered the thermal to electrical conversion efficiency from 23% on 30 May 08 to 16.8% on 1 August 08, which is attributable to the fact that the Kohler generator was specifically designed to run on diesel, rather than biofuels.

5 Expert Commentary and Five Year View

The Tactical Garbage to Energy Refinery (TGER) is a trailerable, skid-mounted device capable of converting waste products (paper, plastic, packaging and food waste) into electricity via a standard 60 kW diesel generator. Additionally, the system can utilize available local biomass as a feedstock. Waste materials are converted into bio-energetics which displaces the diesel fuel used to power the generator set. The system also co-produces excess thermal energy which can be further utilized via a "plug and play" heat exchanger to drive field sanitation, shower, laundry or cooling devices. With additional engineering, the TGER could include a small subsystem to recover water introduced with the wet waste and produce potable water to further reduce logistics overhead. The system requires a small "laundry packet" of enzymes, yeasts and industrial antibiotics to support the biocatalytic subsystem.

Table 4 Additional data from the TGER Energy Conversion Model

Feed materials (daily)-01 Aug 08	
Garbage (gallons)	90 20% paper, 50% cardboard, 30% plastic
Garbage (lbs)	513
Food (gallons)	58
Diesel (gallons)	3

Energy content of feed

Total (lb)	Component	Heats of comustion (btu/lb) LHV	Total energy (BTU)	Total energy (kWhr)
2.9	Carbohydrates	7200	20871.65	6.11713
359.1	Paper/cardboard	8000	2872800	841.9695
77.0	Plastic-polyethylene terephthalate	10250	788737.5	231.1657
77.0	Pastic-polystyrene	17800	1369710	410.439
20.9	Diesel (DF2)	18397	385233.2	112.9054
		Total	5437352	1593.597

Electrical energy production	
Total (kWh)	267.5
Offboard (kWh)	221.2

Total thermal-to-electrical energy conversion efficiency (% of energy content of feed)
16.8%

Offboard energy conversion efficiency (% of thermal energy content of feed)
13.9%

Diesel fuel savings (gallons)
27

Energy delivery efficiency (% of electrical energy for offboard use)
82.7%

	% Contribution to feed energy
Diesel	7.08%
Biofuels	92.92%

The residuals from waste conversion are environmentally benign including simple ash, which can be added to improve soil for agriculture, and carbon dioxide.

The TGER will deploy on a XM 1048 5-ton trailer and is designed to support a 550 man Force Provider Unit (FPU), which produces approximately 2,200 pounds of waste daily. On a daily operational basis, this would conserve approximately 100 gal of diesel. The capability for such conversion would provide

immediate and responsive energy requirements for expeditionary operations as well as yielding estimated cost savings of $2,905/day [10]. A projected fielding plan for the TGER involves identification of current Modified Table of Organization and Equipment (MTO&E) trailers associated with FPU kitchen support which would then be modified to include the waste conversion technology. This would avoid any changes to the MTO&E or prime mover designation. Estimations indicate that the additional tasks associated with maintenance support for the operator and mechanic would not exceed those standards for the assigned Military Occupational Specialty and Generator Mechanic. Higher order support may follow a Contractor Logistics Support or low density support plan similar to that for the reverse osmosis purification unit equipment.

Anticipated field employment of the system is such that the TGER would be pulled by the assigned 5-ton family of medium tactical vehicles assigned to accompany the FPU Containerized Kitchen. Upon occupation of the FPU site, the TGER would start up initially on diesel fuel alone. This would provide immediate power to the kitchen and begin to heat up/power the system components. As waste is developed from the kitchen, it will be introduced to the TGER and the two energetic materials (synthetic gas and ethanol) will begin to displace the diesel fuel. By six to twelve hours (depending on the waste stream), the TGER will run on 98% waste energetics and is capable of running for 12 h with a one hour maintenance shut-down intervening.

Improvements for future models revolve around three subsystems: the gasifier, bioreactor and materials handling. The current downdraft gasifier equipment is too complicated and unreliable under desert conditions. However, modifications to the current design could reduce the complexity of the system and, with a thorough inspection, repair and evaluation by the manufacturer, we believe a number of alterations to the downdraft gasifier would mitigate its reliability problems. Ultimately, it would be advantageous to consider alternative thermo-chemical approaches.

The issues with the bioreactor are much less complex and more easily addressed, as the system was custom built by Purdue University and several supporting subcontractors. Repairing and upgrading this system will primarily involve replacing and upgrading the two heat exchangers, modifying the system software to accommodate the changed thermo-dynamics and thermal management, and adjusting the "plumbing" of the ethanol collection and delivery system.

During the intervening 18 months since the TGER fabrication, the commercial field of biomass fuel processing has greatly expanded. There are a number of new options for third party equipment such as improved shredders, pelletizers and pellet drying systems which did not exist previously.

6 Conclusion

Throughout the course of the 15 month program the TGER underwent testing in a variety of conditions and environments. Performance characteristics of the TGER varied in each environment and provided valuable information as to how to improve

Table 5 Theoretical/optimal TGER performance data

Power output	Power efficiency	Diesel consumption rate	Ethanol consumption rate	Ethanol production rate	Solid waste processing rate (pellet production)	Liquid waste processing rate	Total waste processing rate	Diesel Savings
54 kW	90%	1 gph	1 gph	1 gph	60 lb/h	13 lb/h	1,752 lb/day	3.6 gph

Table 6 Power vs. Fuel Consumption Table Recorded at Purdue University

Power	Idle	25 kW	35 kW	45 kW	55 kW
Fuel					
Diesel	100%	1.3 gph	1.0 gph	1.2 gph	1.0 gph
Fuel gas	0 scmh	57 scmh	65 scmh	60 scmh	65 scmh
Ethanol	0 gph	0 gph	0 gph	0.5 gph	1 gph

Table 7 TGER performance data set recorded at VBC

Average TGER performance data at victory base camp						
Power efficiency	Diesel consumption	Pellet consumption	Solid waste processing (pellet production)	Liquid waste processing	Total waste processing	Diesel saved
~80%	2 gal/h**	60 lb/h	54 lb/h	13 lb/h	1,752 lb/day	2.6 lb/h

the overall design of the TGER in order to achieve what we believe to be the optimal theoretical performance characteristics shown in Table 5.

Prior to the deployment to Victory Base Camp, the TGER underwent testing in a controlled environment at Purdue University. The fuel consumption of all three fuels (syngas, ethanol and diesel) was measured at varying loads using digital flow rate sensors as seen in Table 6.

Although the TGER did not perform as well in Iraq as it had when in a controlled environment at Purdue University, it did demonstrate the ability to conserve fuel and remediate waste in a forward deployed operational environment. Table 7 shows the TGER's performance characteristics when it was running under optimal conditions at Victory Base Camp. With improved engineering and further development all of these performance characteristics can be improved, maximizing the TGER's potential as a viable portable power generation system.

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Production of Methane Biogas as Fuel Through Anaerobic Digestion

Zhongtang Yu and Floyd L. Schanbacher

Abstract Anaerobic digestion (AD) is a biotechnology by which biomass is converted by microbes to methane (CH_4) biogas, which can then be utilized as a renewable fuel to generate heat and electricity. A genetically and metabolically diverse community of microbes (mainly bacteria and methanogens) drives the AD process through a series of complex microbiological processes in the absence of oxygen. During AD, bacteria hydrolyze the polymeric components (e.g., polysaccharides, proteins, and lipids) present in the feedstock and further ferment the resulting hydrolysis products to short chain fatty acids (SCFA), H_2 and CO_2, which are ultimately converted to methane biogas (a mixture of CH_4 and CO_2) by archaeal methanogens. Various biomass wastes (e.g., livestock manure, crop residues, food wastes, food-processing wastes, municipal sludge, and municipal solid wastes) are especially suitable for AD. As one of the few technologies that can both cost-effectively generate bioenergy and reduce environmental pollution, AD has been increasingly implemented in different sectors to convert otherwise wasted biomass to bioenergy. AD technologies can be categorized in many different ways. Each AD technology has its own advantages and disadvantages that make it suitable for particular feedstocks or objectives (i.e., production of energy or stabilization and treatment of wastewaters). Both drivers and barriers exist for commercial implementation of AD projects, with the former stimulating, enabling, or facilitating AD implementation, while the latter function in opposite direction. This chapter will provide an overview of the microbiology underpinning the AD process, and discuss the characteristics of the biomass wastes suitable for AD and the AD technologies appropriate for each type of these feedstocks. The drivers and barriers for AD as well as the AD technology gaps and future research needs will also be discussed.

Z. Yu (✉)
Department of Animal Sciences and Environmental Science Graduate Program,
The Ohio Agricultural Research and Development Center, The Ohio State University,
Columbus, OH 43210, USA
e-mail: yu.226@osu.edu

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Abbreviations

AD	anaerobic digestion
BMP	biochemical methane potential
BOD	biological oxygen demand
CAFO	confined animal feeding operation
CMCR	completely mixed contact reactor
COD	chemical oxygen demand
CSTR	continuously stirred tank reactor
DRANCO	dry anaerobic combustion
EGSB	expanded granular sludge bed
HRT	hydraulic retention time
MPFLR	mixed plug-flow loop reactor
MSW	municipal solid wastes
OFMSW	organic fraction of municipal solid wastes
OLR	organic loading rate
RDP	ribosomal database project
SCFA	short chain fatty acids
SRT	solid retention time
SS	suspended solid
TPAD	temperature phased anaerobic digestion
TS	total solid
UASB	upflow anaerobic sludge blanket
VS	volatile solid

1 Introduction

Anaerobic digestion (AD) is underpinned by a series of bioconversion processes that transform organic compounds, especially biomass wastes, to methane biogas (a mixture of approx. 60% CH_4 and 40% CO_2). Although it has been used for more than a century in treatment of municipal sludge and high-strength organic wastewaters from industries, the main objectives have been to stabilize and sanitize the sludge and to remove the organic pollutants from the influents, with relatively little focus on biogas production. Recently, AD received tremendous renewed interest as the demand for and price of fuels continue to rise. AD is looked upon to be an important biotechnology to help build a sustainable society by simultaneously producing

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renewable bioenergy and protecting the environment. Indeed, a diverse range of feedstocks (e.g., municipal sludge, food-processing wastes and wastewaters, livestock manures, the organic fraction of municipal solid wastes (OFMSW), crop residues, and some energy crops) are being diverted to AD for increasing biogas production [4]. Although AD is a relatively slow process and its operation and performance are sometimes unstable, the methane biogas derived from biomass wastes has become competitive, in both efficiency and cost, with heat (via burning), steam, and ethanol production [31]. In this chapter, the microbiological underpinning of the AD process as well as the recent understanding of the microbial communities driving AD will be discussed from a biotechnological perspective. This chapter will also provide an overview of the common characteristics of feedstocks that have great biogas potentials and the AD technologies suitable for each of these types of feedstocks. The drivers and barriers for commercial AD implementation as well as the AD technology gaps and the research needs will also be discussed.

2 The Microbiology Underpinning Anaerobic Digestion

A very complex community of bacteria and archaeal methanogens drives the entire AD process [36, 65]. Fungi and protozoa are also found in anaerobic digesters [60] although their functions and contributions to the AD process are not known. The cell densities of microbes in anaerobic digesters are among the highest in managed environments, with bacteria being the most predominant (up to 10^{10} cells/mL of digester content) followed by methanogens. The entire AD process can be described as a synergistic process of four sequential phases: hydrolysis, acidogenesis, syntrophic acetogenesis, and methanogenesis (Fig. 1). Each phase is mediated by a distinct functional group, or guild, of microbes [36, 91]. During the first phase, some facultative or strictly anaerobic bacteria (e.g., *Clostridium* spp.) hydrolyze the biomass polymers (e.g., polysaccharides, proteins, and lipids) present in the feedstocks, giving rise to monomers or oligomers (e.g., glucose, cellobiose, amino acids, peptides, fatty acids, and glycerol). This hydrolysis step is catalyzed by the extracellular hydrolytic enzymes such as amylases, cellulases, xylanases, proteases, and lipases secreted by the hydrolytic bacteria. Kinetically, the hydrolysis step can proceed rapidly for soluble feedstocks such as starch. However, for insoluble lignocellulosic feedstocks that contain recalcitrant embedded lignin, the hydrolysis phase is rather slow and often becomes a major rate-limiting step of the entire AD process [2].

The resulting hydrolytic products are immediately fermented to short chain fatty acids (SCFA), CO_2, and H_2 during the subsequent fermentative acidogenesis by another guild of facultative or strictly anaerobic bacteria (e.g., *Bacteroides, Clostridium, Butyribacterium, Propionibacterium, Pseudomonas,* and *Ruminococcus*). The major SCFA formed include acetate, propionate, butyrate, formate, lactate, isobutyrate, and succinate, with acetate predominating. Small quantities of alcohols (e.g., ethanol and glycerol) are also produced. The fermentative acidogenesis typically proceeds rather rapidly [10]. In fact, when feedstocks

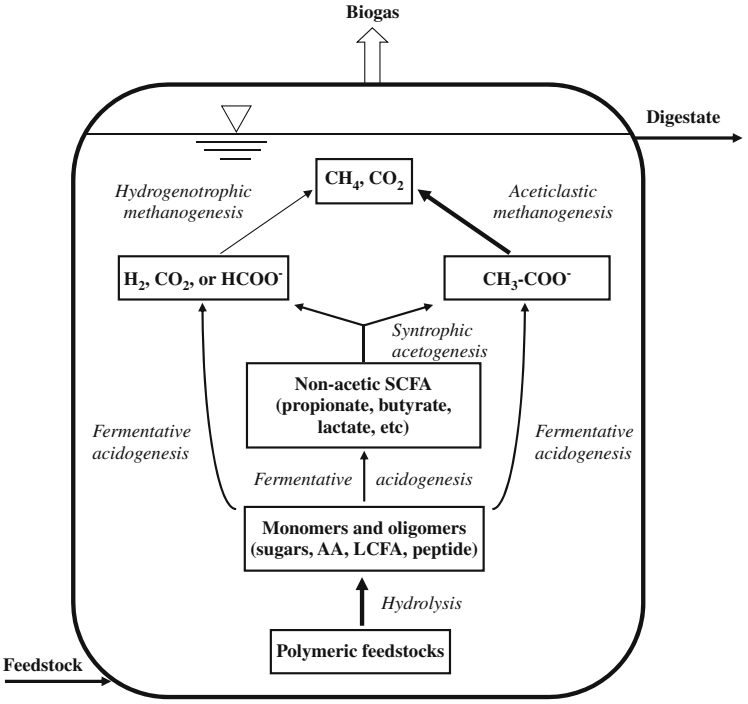

Fig. 1 The four phases of anaerobic digestion process

containing large amounts of readily fermentable carbohydrates (e.g., sugars and starch) are digested at high organic loading rates, the production of SCFA can exceed their consumption, leading to SCFA accumulation and consequential AD upset or even failure [10].

The final phase of AD involves methanogens of the *Archaea* domain. Methanogens are strict anaerobes and produce CH_4 as the major end-product of their catabolism. Most methanogens are fastidious microbes and only grow on a few substrates within a narrow spectrum of environmental conditions (neutral pH, $Eh < -300$ mV, etc.). Methanogens use a unique methanogenesis pathway to produce CH_4 [36]. Hydrogenotrophic methanogens produce CH_4 via the reduction of CO_2 by H_2 or by the conversion of other C_1 substrates (e.g., methanol and methylamines), while acetoclastic methanogens convert acetate to CH_4. It should be noted that the former accounts for approximately one third while the latter accounts for two thirds of the CH_4 produced in anaerobic digesters. This is because acetate is the major end product of the acidogenesis step in all anaerobic digesters [86]. In spite of this, only a few species of acetoclastic methanogens have been known and they are within genera *Methanosaeta* (formerly *Methanothrix*) and *Methanosarcina*. *Methanosaeta* spp. are obligate acetoclastic methanogens, while species of *Methanosarcina* also use C_1 substrates. Hydrogenotrophic methanogen species are found in genera *Methanobacterium, Methanospirillum, Methanobrevibactor, Methanococcus,*

Methanomicrobium, Methanoculleus, Methanogenium, and *Methanothermobacter*. All methanogens contain a unique cofactor, F_{420}, that is autofluorescent at a wavelength of 420 nm [38]. Some methanogens, especially hydrogenotrophic methanogens, contain so much of it that they appear blue when viewed under a microscope. Several trace elements, especially nickel and cobalt, are required by methanogens for methanogenesis and growth. For some feedstocks, supplementation with trace elements can significantly enhance methane biogas production and process stability [48]. Because of the low energy yield from the methanogenesis pathway, most methanogens grow slowly, especially acetoclastic methanogens (e.g., *Methanosaeta* spp. have a generation time of 3.5–9 days) [36]. However, methanogenesis is typically not a rate-limiting step of the entire AD process because the low-energy yield of the methanogenesis pathway forces it to run rather rapidly. Additionally, methanogens are susceptible to a host of factors (e.g., pH, ammonia, and metals) so they are often implicated in instability or sub-optimal performance of AD [17].

The small amounts of SCFA with three or more carbons (e.g., propionate, butyrate, isobutyrate, valerate) and the ethanol produced during the fermentative acidogenesis as well as the long chain fatty acids derived from lipid hydrolysis can not be used directly by any known methanogens. A unique guild of strictly anaerobic bacteria (referred to as syntrophic acetogens) can oxidize these intermediates to acetate, H_2, and CO_2 so that they can serve as the substrates of methanogenesis [75, 91]. However, the oxidation of these fatty acids and ethanol under fermentative conditions (referred to as syntrophic acetogenesis) is thermodynamically unfavorable; and hydrogenotrophic methanogens are needed to reside in close proximity to rapidly consume the H_2 produced by the syntrophic acetogens through interspecies hydrogen transfer [23]. *Syntrophomonas wolfei* and *Syntrophobacter wolinii* are thought to be important syntrophic acetogens in anaerobic digesters, with the former primarily oxidizing butyrate and the latter oxidizing propionate. With a generation time of greater than one week, syntrophic acetogens grow extremely slowly [24]. As a result, the solid retention time (SRT) in digesters has to be long (15 days or longer) to retain enough syntrophic acetogens. Hence, syntrophic acetogenesis can be a rate-limiting step during AD, and failure or suboptimal performance encountered during AD operation often involves this guild of bacteria, which is exemplified by AD failure when the organic loading rate was too high and the production of non-acetic SCFA exceeded that of their utilization [47]. Thus, syntrophic acetogens are important members of the microbial community of stable AD processes even though the carbon flux through them is relatively small, and it is critical to maintain a balanced production and consumption of these non-acetic SCFA by avoiding organic overloading. It should be noted that because they cannot be cultured as single cultures, syntrophic acetogens are not well studied. The recent advancement of genomics and metagenomics offers new opportunities to better understand this important guild of bacteria in anaerobic digesters (see [55] for a recent review).

Several features of feedstocks can have profound effects on AD, such as the content of readily fermentable carbohydrates, particle sizes of insoluble feedstocks (the hydrolysis step is especially affected by particle sizes), nutrient content and balance,

and presence and concentrations of inhibitory compounds. Feedstocks rich in starch and/or proteins are easier to digest than lignocellulosic feedstocks. Reduction of particle size of insoluble feedstocks can significantly speed up AD and increase CH_4 yields. Microbes need numerous nutrients to grow, with nitrogen and phosphorous being the most important. The optimal carbon (expressed as chemical oxidation demand, COD) to N to P ratios (COD:N:P) for efficient AD differ with different feedstocks and the AD technologies used. For most feedstocks, a C:N ratio of 25–32 is suitable for most AD processes [8].

3 Methane Biogas Production from Different Feedstocks

Any biomass can be used as feedstocks for AD. However, biomass wastes, especially those with a relatively high water content (>50%), are the most common feedstocks suitable for AD. In fact, methane biogas has been produced from millions of tons of biomass wastes arising from municipal, industrial and agricultural sources [91]. The characteristics of biomass wastes vary widely. The common feedstocks suitable for AD have been discussed with respect to features pertinent to AD and biogas potentials by Yu et al. [91]. The AD of several types of feedstocks has also been reviewed recently (e.g., [15, 66, 85]). Anaerobic digesters can be categorized in many different ways (see [80, 91] for an overview). No AD reactor is universally ideal or superior because each type of reactor has certain advantages and disadvantages that make it appropriate for particular type(s) of feedstocks. In this chapter, the features of individual feedstocks that have substantial methane biogas potentials and the AD technologies that are suitable for their AD will be discussed.

3.1 Anaerobic Digestion of Municipal Sludge (Biosolids)

Municipal sludge includes primary sludge and waste activated sludge derived from centralized wastewater treatment plants that employ biological treatment of sewage. It is probably the first type of feedstock subjected to AD. It has very high contents (95–99%) of water, low contents (15–20%) of volatile solid (VS, representing the biodegradable portion of total solid, TS), and low contents of readily fermentable carbohydrates [8, 94]. However, most municipal sludge has rich and balanced nutrients (nitrogen: 3–6%; phosphorus: 1.0–1.2%; of TS). The biochemical methane potential (BMP) of municipal sludge is relatively small, ranging from 85 to 390 m^3 CH_4/dry ton. Municipal sludge contains a high density of bacterial cells (mostly aerobic and facultative anaerobic bacteria), some of which may be pathogenic to humans and/or animals. Toxic compounds may also be present in some municipal sludge, especially those derived from large metropolitan areas. Approximately 6.2 million dry tons of municipal sludge are produced annually in the USA (based on 1999 data [39]), representing an annual potential of at least 6 billion m^3 of methane biogas. At present, however, only a portion of the municipal sludge is

digested and the methane biogas yields are relatively low. This is largely attributable to the relatively small net amounts of energy that can be produced. However, when municipal sludge is co-digested with carbohydrate-rich yet nitrogen-poor biomass wastes (e.g., OFMSW and food-processing wastes), the energy yields can increase substantially [4]. For example, in a full-scale two-staged AD system, a 25% increase in organic load rate (OLR) with OFMSW resulted in an increase in biogas yield by 80% and overall degradation efficiency by 10%, which resulted in an increase in electrical energy production by 130% and heat production by 55% [94]. Additionally, when co-digested with carbohydrate-rich yet nitrogen-poor biomass wastes, municipal sludge can stabilize the AD process of the former [46].

Municipal sludge is among the most studied feedstocks in AD. Numerous books and reviews have been published on AD of municipal sludge (e.g. [79]). In general, because of the presence of high levels of suspended solid (SS), most AD technologies are not suitable for the AD of municipal sludge. Continuously stirred tank reactors (CSTR) and completely mixed contact reactors (CMCR) are most commonly used in AD of municipal sludge [79]. For example, the CSTR with a total volume of 1,350 m^3 in Karlsruhe, Germany digests municipal sludge at 37°C and produces approximately 3,800 m^3 of biogas of 62–70% methane daily [33]. More recent research efforts have been directed at pretreatment to enhance degradation of the solid found in municipal sludge and production of methane biogas (see [28, 45] for reviews). Thermophilic AD, in single- or two-staged systems, is also being increasingly used to enhance biogas production and sanitation [92]. Additionally, because of the low solid contents (1–5%) and low BMP, large digesters are required for the conventional "wet" AD. Currently, "dry" AD technology is being evaluated to produce methane biogas from dewatered biosolids, which have significantly reduced water contents (70–85%) and thus reduced digester volumes [64]. Dewatered biosolids are also ideal feedstocks to be co-digested with other solid feedstocks, such as OFMSW and crop residues.

3.2 Anaerobic Digestion of Animal Manures

Animal manures represent a huge methane biogas potential. As estimated, 106 million dry tons of animal manures are produced each year in the USA, with approximately 87 million dry tons being available for methane biogas production [69]. Given a BMP of 200–400 m^3 CH_4/dry ton [8], the amount of animal manures available for AD provides a potential of 17–35 billion m^3 of CH_4 per year in the USA. The animal manures produced from confined animal feeding operations (CAFOs) offer one of the most abundant single feedstocks available for large-scale methane biogas productions. The composition and physical features (e.g., water contents) of animal manures vary widely from species to species and from operation to operation [58]. In general, animals manures have relatively high water contents, ranging from 75% (poultry manure) to 92% (beef cattle manure). Most of the animal manure is organic matter, with VS contents ranging from 72% (poultry manure) to 93% (beef cattle manure) of TS. Inorganic nutrients, including N,

P and K, are rich in animal manures, especially poultry manure. Because most of the readily degradable substances, especially carbohydrates, have been digested and absorbed by the animals, animal manures have very little readily fermentable substrates. Additionally, animal manures have high concentrations of amino nitrogen such as urea and ammonia and a large pH buffering capacity against acids. Thus, the fermentative acidogenesis during AD of animal manures typically does not result in significant pH decline, but high concentrations of ammonia can result, causing toxicity to methanogens, especially in thermophilic digesters where methanogens are very susceptible [43]. Furthermore, animal manures contain large amounts of microbial biomass, including bacteria and methanogens. Consequently, AD reactors digesting animal manures, especially livestock manures, can be started without the addition of external digested sludge as a start culture or inoculum.

Because of the relatively low contents of readily degradable substances, the methane biogas production from animal manures is generally slow. Thus, when digested alone a long retention time is needed. Co-digestion with nitrogen-poor yet carbohydrate-rich feedstocks, such as food-processing wastes and OFMSW, can substantially enhance CH_4 production and stabilize the AD process of animal manures [59, 94]. Some animal manures, especially dairy cattle manure, contain sand from the sand bedding [42], which settles in AD reactors and can cause operational problems if not dealt with properly. Due to the large differences in many physicochemical characteristics and degradability, different manures may require different AD technologies for efficient and cost-effective AD. Here the AD technologies suitable for beef manure, diary manure, swine manure, and poultry litter will be discussed.

3.2.1 Animal Manure Dung and Poultry Litter

The manures from beef cattle feedlots (or barns that do not use water to flush the animal manure) and poultry barns have relatively low water contents. They are often applied to farmland as fertilizer and thus have not been commonly subjected to AD. However, these two types of manures can be digested using dry AD processes [37] such as the dry anaerobic combustion (DRANCO) process, ECOCORP process, BEKON process, Kompogas process, and Linde process. These dry AD processes have several advantages over wet AD technologies and are described later in this chapter. Although not demonstrated on either type of manures [27], anaerobic leaching bed reactors may be suitable for the AD of these manures without any dilution.

Both types of manures can be diluted to slurry and digested in conventional wet AD reactors. For beef cattle manure, a slurry containing 12% TS can be digested, but for poultry litter, a higher dilution (TS <3%) is needed to minimize inhibition by ammonia [44]. Inevitably, such dilutions create the need for large reactor volumes and high capital and operating cost. Pretreatment may be needed to remove the uningested diet (e.g., hay in beef cattle manure) or course materials (e.g., bedding materials and feather in poultry litter) prior to AD. Because of the presence of high solid contents, only CSTR, CMCR, and mixed plug-flow loop reactor (MPFLR)

are suitable for AD of diluted beef cattle manure and poultry litter [20]. However, in a pilot study [9], upflow anaerobic sludge blanket (UASB) reactors were shown to be suitable and more efficient for the AD of diluted poultry feces. Co-digestion and thermophilic AD are also shown to improve digestion of poultry litter [16]. Thermophilic AD of poultry litter can be difficult due to the resultant high ammonia concentrations that render the AD process unstable [16]. The future will probably see more application of AD to both beef cattle manure and poultry litter, either alone or in co-digestion with other feedstocks, to both harvest bioenergy and produce fertilizer.

3.2.2 Dairy and Swine Manure Slurry

Some small dairy and swine farms do not use water to flush their barns so they produce manure with low water contents. These manures can be digested using dry AD [27]. Large dairy and swine farms, however, use water to flush the manure out of the barns and hog houses, respectively, generating manure slurries of moderate solid contents (>8%). Traditionally, both types of slurries are stored in waste lagoons built on the farms. By installing a flexible or floating gas-impermeable plastic cover, such lagoons can be easily converted to a unique type of digesters, covered lagoon digesters [68]. Covered lagoons typically have a long retention time (several months or longer) and high dilution rates [11]. Because of impracticality in temperature control, covered lagoons are left to operate at ambient temperatures and can produce biogas efficiently only in areas with moderate and elevated year round temperatures. Covered lagoons are simple and cheap to construct, operate, and maintain, which justifies their low AD efficiency. Another disadvantage is the slow but continuous accumulation of undigested solids at the bottom of the lagoons, which is costly to remove. One example of covered lagoons is located at Royal Farms in Tulare, California. It has three cells with a surface area of nearly 2,800 m^2. Supported by the US EPA AgSTAR Program (http://www.epa.gov/agstar/index.html), it was started in 1982 and has been in operation ever since. The biogas produced has been enough to fuel two Waukesha engine-generators to generate electricity to meet all of the farm's electricity needs with excess being sold to the local utility. The heat recovered from the generators is used as supplemental heat in the nursery barns, and the stabilized effluent is used as fertilizer. Barham Farm in North Carolina also operates a covered lagoon that has an effective volume of 24,500 m^3. It digests the manure slurry generated from 4,000 sows. Baumgartner Environics, Inc. and MPC Containment Systems, LLC are two providers and installers of anaerobic lagoon covers.

Another type of digester that has been successfully and commonly used in AD of dairy manure slurry is non-mixing plug-flow reactors [15], which can successfully digest manure slurries with high solid contents (up to 11–14%). With a HRT of 21 to 40 days, methane biogas containing more than 60% CH_4 can be produced at rates from 0.37 to 0.79 m^3/m^3 reactor volume/d. As estimated from the biogas yields of three such digesters, the daily biogas production ranged from 1.16 to 2.41 m^3 per cow per day [88]. Although non-mixing plug-flow reactors are

nearly maintenance-free, the gas production is rather slow due to poor mass transfer. Recently, MPFLR has been built at several dairy farms in the USA by GDH, Inc. Herrema Dairy located in Fair Oaks, Indiana operates a MPFLR, which receives more than 400 m^3 of manure slurry of 8% solids that is generated by 3,800 heads of cattle daily. Operated mesophilically with a HRT of 17 days, this reactor produces enough biogas to steadily fuel two Hess engine-generators of 375 kWh each. The separated solids from the effluent are dried and reused for bedding in the barns, while the heat recovered from the engine-generators is used to heat the digester, barns, and alleyways.

Both CSTR and CMCR have been used in AD of dairy manure slurry. The continuous mixing significantly enhances biogas production and reduces HRT (from months to 10–20 days) [11, 15]. Thus, implementation of CSTR and CMCR significantly reduces the digester volumes required to digest the manure derived from a given number of cows or hogs. Although these two types of digesters cost more to build and operate, the increased costs may be offset by the increased biogas production and TS reduction. Other types of reactors that have been tried on AD of manure slurries include hybrid reactors [26] and anaerobic filter reactors [87, 88]. However, to prevent clogging of the filter media of these two types of reactors, the SS has to be separated prior to feeding to these biofilm-based digesters, resulting in reduced biogas production [88]. The superiority of these digesters remains to be determined.

Recent studies have focused on improvement of VS degradation and concomitant increase in biogas production. Co-digestion with food wastes or crop residues was found to dramatically increase (by 2–3 folds) biogas production [51, 59]. This is attributed to the increased input of readily degradable substrate from these wastes. Temperature-phased AD (TPAD) also substantially improves AD [78], and TPAD of dairy manure slurry can be completed within a short HRT. The increased conversion rates at elevated temperature (55°C) are responsible for the improvement observed in TPAD systems [91].

3.3 Anaerobic Digestion of Solid Food and Food-Processing Wastes, Organic Fraction of Municipal Solid Wastes (OFMSW), and Crop Residues

These wastes are characterized by varying water contents, but high VS contents (>95%). However, these parameters vary considerably. Most food wastes have balanced nutrients and large amounts of readily fermentable carbohydrates and thus are among the most suitable feedstocks for AD. According to a recent study, 348 m^3 of CH_4 can be produced per dry ton of food wastes within only 10 days of AD [93]. Food wastes amount to approximately 43.6 million dry tons each year in the USA [81]. This represents a potential of 15.2 billion m^3 of CH_4 per year. During food processing, a significant portion of foodstuffs also ends up in wastes or wastewaters. For example, 20–40% of potatoes are discarded as wastes during processing. National data on the amount of food-processing wastes are not available.

The state of California generates more than 4 million dry tons of food-processing wastes each year [54], potentially producing 1,200 million m^3 of CH_4. This translates into an annual potential of several billions m^3 of CH_4 in the USA. Except for the wastes from animal meat processors, most food-processing streams are relatively poor in nitrogen, but rich in readily fermentable carbohydrates. As such, food-processing wastes can be co-digested with other nitrogen-rich feedstocks (e.g., municipal sludge or animal manures) to enhance AD system stability and CH_4 production [46].

Approximately 250 million dry tons of MSW are produced annually in the USA. The organic fraction, such as paper, yard trimmings, and food scraps, is biodegradable and can be converted to methane biogas. Although the composition of MSW varies dramatically depending on society, season, collection, and sorting, OFMSW accounts for more than 50% of the MSW in most societies. Most OFMSW has little moisture or readily fermentable carbohydrates and is relatively deficient in N or P, but has a relatively large BMP (300–550 m^3 CH_4/ton) if digested adequately [25]. The OFMSW generated annually in the USA has a CH_4 potential of 37.5 billion m^3.

Crop residues amount to an estimated 428 million dry tons each year in the USA. Although the majority of crop residues is typically left in the field, approximately 113 million dry tons are recoverable and available for conversion to methane biogas [69]. Crop residues typically have relatively low water contents, high VS contents, and variable contents of readily fermentable carbohydrates. Most crop residues are non-leguminous and are poor in available nitrogen. The BMP of crop residues varies from crop to crop (from 161 to 241 m^3 CH_4/ton) (124). If subjected to proper AD, at least 20 billion m^3 of CH_4 can be produced annually from the crop residues available for biogas production in the USA. Similarly for other nitrogen-poor biomass, co-digestion of crop residues with animal manures or municipal sludge substantially improves CH_4 yield [50]. In the EU, 1,500 million dry tons of biomass are available each year for biomethanation within the agricultural sector, with half of this being crops intended for bioenergy production [5]. It should be noted that production of bioethanol and biodiesel from energy crops only utilizes a fraction of the biomass, and implementation of AD by the bioethanol industry can generate substantially more energy (up to 30% of the total energy of the initial biomass) [3, 74]. This also holds true for many other biomass-based processes producing non-food products.

All these types of feedstocks likely contain bulky materials, such as peeling, papers, stems and leaves. Pretreatment, especially reduction of particle size by grinding or milling, is typically required to enhance AD [40]. Other pretreatments such as alkaline pretreatment [53] have also been evaluated to further enhance the hydrolysis step in laboratories, but few of them have been implemented in full-scale AD plants. As mentioned earlier for the AD of livestock manures, co-digestion with other nitrogen-rich biomass (e.g., municipal sludge or animal manure) can also substantially stabilize the AD process and increase CH_4 production [50, 94].

The above mentioned wastes have relatively low water contents. They can be digested using some wet AD processes (e.g., CSTR and CMCR) after dilution. The Lemvig Biogas plant in Denmark is one example of such wet AD. It is a centralized biogas plant consisting of three thermophilic CSTR with a total volume of 7,000 m^3

that digests various types of organic industrial wastes, source-sorted MSW, and manures [7]. The biogas produced is used to generate electricity and heat.

Apparently, dry AD is advantageous for these low-moisture feedstocks because it eliminates the need to dilute the feedstocks to a fluid state and produces a low-moisture digestate, which is easier to transport and disperse [90]. The DRANCO technology is a dry AD technology successfully used to convert low-moisture organic wastes (e.g., OFMSW and crop residues) to methane biogas [21]. The DRANCO technology requires the feedstock to be shredded and milled first to reduce particle sizes (<4.0 mm in diameter). A digested sludge or digestate is then mixed with the feedstock in a 6:1 to 8:1 ratio in a mixing compartment. The mixture is heated by steam (to 30–40°C for mesophilic AD or 50–55°C for thermophilic AD) and then pumped into the digester at the top. The feedstock descends by gravity while digestate is withdrawn at the bottom. The biogas rises and exits the digester through the roof of the digester. The retention time in a DRANCO digester averages 20 days with a pass-through time of 2–4 days. The DRANCO technology is marketed by the Organic Waste System (OWS) in Belgium (http://www.ows.be/index.php). According to OWS, the DRANCO technology has a number of advantages including high solid digestion, high loading rates (10–20 kg COD/m^3 of reactor/d), high biogas productivity (100–200 m^3 of biogas/dry ton of feedstock), small digester volumes, no maintenance or failures inside the digester, less energy consumption, well controlled external inoculation, and kill-off of pathogens and seeds. The largest DRANCO digester started operation in 2006 in Vitoria, Spain. This digester has an effective volume of 1,770 m^3 and a capacity of 120,750 tons/yr of primarily OFMSW. It produces 5,962 tons of biogas, which can generate 6,000 MWh of electricity, and 12,580 tons of compost per year. As of this writing, most of the DRANCO digesters in use are located in Europe, and the capacity of dry AD has exceeded that of wet AD of solid wastes [21]. The ECOCORP (www.ecocorp.com), BEKON (www.bekon-energy.de), Kompogas (www.kompogas.com), and Linde (http://www.anaerobic-digestion.com) processes are emerging dry AD technologies mostly used in Europe for dry AD of solid biomass wastes.

A new two-staged AD process was evaluated by Parawira et al. [67] in digesting solid potato wastes under mesophilic and thermophilic conditions. This process uses a solid leaching bed reactor for hydrolysis and acidification while an UASB reactor is used for methanogenesis. High loading rates (36 g COD/L/d), high methane yields (0.49 L/g COD removed), and stable operation were observed under mesophilic conditions. The utility of this new process remains to be validated for other types of feedstocks containing significant amounts of lignocellulose.

3.4 Anaerobic Treatment of Organic Wastewaters

Wastewaters generated from food- and beverage-processing industries often have little SS but high concentrations of soluble organic compounds (up to 50,000 mg/L of biological oxygen demand, BOD) such as starch, sugars, and proteins. Some common examples of these high-strength wastewaters come from cheese factories,

wineries, breweries, distilleries, slaughterhouses, potato processing, and ice cream factories. The organic compounds in these wastewaters can be readily degraded and converted to methane biogas, but the initial major objective of anaerobic treatment of such wastewaters was to degrade and reduce the organic pollutants in the wastewaters to satisfy governmental discharge requirements. With the push for bioenergy, the focus of anaerobic treatment of high-strength organic wastewaters has been shifted to methane biogas production. High-rate AD (HRT <24 h) has been commonly used to both reduce the organic strength of the wastewaters and recover the energy as methane biogas. UASB reactors [32] are among the most popular digesters used by many industries. A new variant of UASB reactors is the expanded granular sludge bed (EGSB) reactors. The advantages of EGSB over UASB, such as improved mass transfer and digestion rate, enhanced ability to handle high-strength influents, and high hydraulic loading rates (HRT <2 h), have been well recognized [82]. Therefore, during the last decade the number of EGSB reactors built exceeds that of new UASB reactors [32].

In addition to UASB and EGSB reactors, the following digesters have also been successfully used in the AD of these high-strength wastewater streams: CSTR (single staged, e.g. [74], or two-staged e.g. [72],), anaerobic contact filter reactor [83], anaerobic filter reactor [1], down-flow fluidized bed reactor [34], internal circulation (IC) reactors [29], and anaerobic hybrid reactors [14]. The sand-bed filter reactor manufactured by NewBio E Systems, Inc, [91] is another promising AD technology for such wastewater (unpublished data). Compared to the digesters used to digest feedstocks with high SS, most of these reactors have much higher loading rates. Thus, they have smaller footprints, but they need to be operated by well-trained digester operators. Detailed descriptions of each of these reactors and vendors is beyond the scope of this chapter, but interested readers should consult other recent books [79, 91] or reviews [10, 77]. Anaerobic treatment or digestion of specific high-strength wastewaters have also been extensively reviewed (e.g., see [56, 57, 66] for distillery wastewaters; and [18] for meat- and potato-processing and dairy wastewaters).

It should be noted that performance data from an existing AD digester can only be regarded as broadly indicative of how a similar AD technology may perform elsewhere, especially with respect to stability, efficiency of organic removal, biogas yield and quality. Only through studies using laboratory- and pilot-scale AD reactors on the feedstock of interest can the most suitable AD technology be identified for that feedstock.

4 Drivers and Barriers for Commercial Implementation of Anaerobic Digestion to Convert Biomass Wastes to Renewable Energy

The commercial installation of AD technologies is facilitated or obstructed by multiple interactive factors, respectively termed drivers or barriers. Drivers are factors that stimulate, enable, or facilitate implementation of a technology or project, whereas barriers are the factors that function in the opposite direction. Both drivers and

barriers may be technological, economic/financial, environmental, or sociopolitical, and may also include subjective psychological components such as uncertainty, perception, or fear. Despite their importance, the drivers and barriers for commercial implementation of AD by companies or farms are rarely disclosed or detailed in the literature because the information is typically related to business operations and confidential. However, experience from on-site and cooperative studies of AD for candidate factories or farms have shown that the satisfaction and resolution of multiple drivers and barriers, respectively, is crucial in the decision making to implement a specific AD project. Experience with pilot-scale AD studies conducted by anaerobic digester vendors has shown that both drivers and barriers are multi-faceted and interdependent and vary in importance depending on a host of factors associated with candidate factories and their biomass wastes. An AD project is unlikely to proceed unless the full range of drivers and barriers are considered and the drivers outweigh the barriers. The following section will discuss the drivers and barriers in general, and how the advancement of AD technologies can contribute to tipping the balance towards the drivers by mitigating many of the barriers, including those of economic and political nature.

4.1 Drivers for Commercial Implementation of AD

The drivers that stimulate commercial interest to implement AD include a complex set of economic, business, energy, environmental, and sociopolitical factors that are interactive and may be weighted differently for each AD implementation opportunity. The economic and business drivers relate to those that directly contribute to the profitability of an AD project through the rate of return on the investment. These drivers include (1) the revenues that can be realized by the production of biogas and other byproducts (e.g., fertilizer), (2) the cost savings derived from reduced waste disposal, (3) governmental credits (e.g., renewable energy credits, environmental credits, and carbon credits) that are earned by implementation of a AD project, and (4) potential business growth that results from overcoming the limitations posed by storage and disposal of the wastes generated from core business operations. Firstly, earned revenue from an AD project can be gained from sale of the biogas as fuel or energy produced therefrom. Additional revenue can be generated by receiving wastes from other factories or farms. A spillover benefit of such "service" is enhanced AD efficiency and process stability resulting from co-digestion of two or more types of biomass wastes. Secondly, AD is a proven technology to reduce pollution, and thus its implementation can reduce or eliminate the fees paid to the government for waste discharge or disposal. Depending on the nature and amounts of wastes, this saving can be substantial. Thirdly, methane biogas produced from AD of biomass wastes can replace fossil fuels, therefore implementation of AD should earn environmental and carbon credits as well as renewable energy credits that can be sold for additional revenues. Finally, for many factories or farms that produce large amounts of biomass wastes, the enterprise may be prevented from business

growth by the inability to dispose of the wastes. AD can help overcome such waste disposal limitation by reducing overall waste output.

Sociopolitical factors can also drive implementation of AD, but are situation dependent and variable in type and impact. Examples include reduced odor impact on surrounding communities from handling or disposing of biomass wastes (e.g., livestock and poultry manure disposal by land application), and better perception of and public opinion on the business operation. In some circumstances, sociopolitical factors may become a major driver to implement an AD project, superseding even the economic factors, especially in situations where the enterprise's ability to continue its operation is threatened by public opposition to its waste storage and disposal.

4.2 Barriers to Commercial Implementation of AD

A number of barriers may thwart the impetus of the drivers mentioned above. Barriers that can emerge during consideration of commercial AD projects include (1) uncertainty about the feasibility or reliability of the technology (both AD and energy production), (2) uncertainty about the economic and business outcome of the project, (3) uncertainty about public policy that might impact AD (e.g., incentive pricing or lack thereof for renewable energy, environmental rules and regulations and compliance therewith), (4) uncertainty about selling the renewable electricity to the main grid and standby fees, and (5) uncertainty about or fear of liability or penalties (from the complexity of compliance with environmental or safety regulations), and damage to corporate or product image from suboptimal or failed AD projects. The barriers to AD implementation are complex and vary in type and importance for each candidate site or enterprise. They can also be iterative whereby resolution of one concern may reveal a subsequent concern that also discourages implementation of an AD project (e.g., concern over disposal of digestate, or potential impact on material flow or heat recovery of the core operations of the business).

Among the major barriers is the concern over the capability and reliability of the AD system to digest the available feedstock(s) and the inability to validate the biogas yields predicted from the feedstock(s) because biogas and energy yield primarily dictates the economic viability of any commercial AD implementation. Although observation of a similar AD system operating elsewhere can alleviate this concern, concerns often exist over possible unpredictable digester failure that can potentially disrupt the core operation of the factory or farm. Such fear is a strong deterrent to AD implementation and can be difficult to overcome. Additionally, concerns can arise from the distraction from core business operations brought by the implementation of an "alien" technology (i.e., AD). Another barrier is the lack of supportive public policy that can provide assured markets for methane biogas and incentive pricing based on its fair value for not only the renewable energy, but also the environmental and social benefits. Uncertain long-term value of a feedstock or its value for alternative use (e.g., bioethanol production) can also further discourages commercial implementation of AD.

4.3 Tipping the Balance Between Drivers and Barriers

The balance between the drivers and the barriers for a potential commercial AD project is primarily centered on the financial economics of the AD system to be installed, which includes the capital and operating cost, the cost of the feedstock (e.g., the cost of acquisition, transportation, preparation, or alternative disposal), and the revenues and credits that can be realized from AD operations. To tip the balance towards the drivers, the capital and operating cost need to be minimized while the revenues and credits need to be maximized. More efficient, cost-effective, reliable, and versatile AD technologies are needed to strengthen key drivers and diminish many of the uncertainties related to AD technologies. However, a better understanding of the microbiological underpinning of AD processes is required to develop such AD technologies. Additionally, incentives from governments and public support are also important to encourage AD implementation.

5 Future Perspective

Methane biogas production is rather slow [89], and large digesters are often required to produce enough biogas to be recovered cost-effectively as energy. AD processes are also susceptible to a host of factors, which can render AD suboptimal or sometimes lead to unpredictable upset or total failure of AD [17]. The re-startup and recovery process after failure are often slow [17, 64, 84]. These limitations and other barriers can severely undermine the economic viability of AD processes and make many industries and farmers reluctant to implement this biotechnology. These issues and the research required to improve AD for bioenergy production are briefly discussed below.

5.1 Enhancing Biomass Conversion and Methane Production

From an economic, social and environmental perspective, lignocellulosic biomass wastes are good feedstocks for methane production through AD. Due to the slow hydrolysis of lignocellulose, however, methane production is slow, and a long retention time and large digester volumes are required to produce enough methane biogas for cost-effective recovery. In the case of livestock manure, 40–50% of the solid passes through mesophilic AD undigested [8]. Two-stage AD processes can improve solid reduction and stability by separating the more robust hydrolysis and acidogenesis from the less robust syntrophic acetogenesis and methanogenesis [13]. TPAD digesters are promising two-stage designs, with the hydrolysis being enhanced in the first digester operated at an elevated temperature (typically at 55°C) and syntrophic acetogenesis and methanogenesis being enhanced and stabilized in the second digester operated at a mesophilic temperature (typically 35°C) [46, 78]. Indeed, significant increases in hydrolysis, TS reduction, and methane production

resulted from the co-digestion of a primary sludge and OFMSW in a TPAD system [76]. Additionally, TPAD enhances sanitation of waste streams [73], reducing potential risks associated with certain types of feedstocks (e.g., municipal sludge and animal manures). Furthermore, TPAD processes eliminate the AD inhibition caused by the self-heating of mesophilic AD of high-energy feedstocks (e.g., energy crop and OFMSW) [49, 52]. The higher energy input required to operate TPAD is more than offset by the increased biogas and heat produced therefrom [22]. The TAPD technology will probably be applied more commonly in the near future when more lignocellulosic feedstocks (e.g., energy crops, animal manure, crop residues, and OFMSW) are subjected to AD.

Size reduction can dramatically enhance the AD of certain feedstocks, such as crop residues, OFMSW, and energy crops. Physical and chemical pretreatments can further enhance AD of these feedstocks [45, 53], but currently they may not be cost-effective, especially for those feedstocks that contain high water contents and for wastewaters. Low cost and efficient pretreatments need to be developed.

The entire AD process is often limited by three of the four steps of the AD process: hydrolysis, syntrophic acetogenesis, and methanogenesis. Hydrolysis of biomass polymers is typically the rate-limiting step of the entire AD process of lignocellulolytic feedstocks. Single or mixed cultures of lignocellulolytic microbes may be used to augment the capability of hydrolysis in digesters as exemplified by enhanced AD of cattle manure [62] and municipal sludge [30]. Methanogenesis can become the rate-limiting step when feedstocks containing large amounts of readily fermentable substrates (e.g., starch) are digested. In this scenario, acid-tolerant methanogen (e.g., *Methanobrevibacter acididurans*) cultures may be prepared and used to enhance the entire AD process or remediate upset AD operation. Bioaugmentation can also enhance the AD of feedstocks containing high concentrations of particular substances, such as lipids [19]. As more and more digesters are put into operation, there will be increasing needs for such specialty cultures to enhance existing digesters, start up new digesters, and prevent AD failures.

5.2 Optimizing AD Process Stability

AD process control on current digesters is still relying on input and output data: primarily biogas yield and composition, and pH. When the output data suggest any abnormality in performance, it is often too late to intervene, leading to severe disruption of normal operation. Thus, there is an urgent need for research and development of on-line systems that can monitor important parameters of the actual AD process. Some of the key parameters of AD and their modeling have been reported [35, 71], which can guide the research effort to develop online monitoring systems. Propionate was recently identified to be an important indicator of AD performance [12, 63], and online monitoring of this important SCFA using gas chromatography seems promising [12, 70]. Further understanding of the microbial communities involved in AD processes may also allow for the development of biosensors that

can achieve microbe-based continuous online monitoring. Such real-time monitoring can directly link to automated digester controls, such as loading and mixing. Advanced understanding of the microbial community structure, population dynamics, metabolic kinetics, and online monitoring in digesters will also improve the modeling of AD processes [6, 35].

5.3 Better Knowledge on the Microbial Communities in Digesters

The microbial community residing in digesters largely remains a black box [65]. This is largely attributed to the difficulties and inability to grow these microbes in laboratory media. The use of cultivation-independent DNA-based molecular biology and metagenomic techniques makes it possible to define the membership and functionality of this complex microbial community (e.g., reviewed in [41]). As indicated by the more than 5,265 bacterial and 839 archaeal 16S rRNA gene sequences of anaerobic digester origin archived in the Ribosomal Database Project (RDP) (as of this writing, unpublished data), our knowledge on this microbial community has expanded tremendously [61, 65]. These sequences represent approximately 2,500 species of bacteria (based on 97% 16S rRNA gene sequence identity) and 160 species of archaea (unpublished data). Our statistical prediction suggests that AD reactors can have at least 3,500 species of bacteria and 170 species of archaea. The continued studies using both molecular biology and metagenomic techniques should provide a better knowledge on the microbial community structure, population dynamics, adaptation, granulogenesis, and metabolic kinetics in digesters. Eventually, this knowledge will help develop more efficient and stable AD technologies.

5.4 Strengthening the Drivers and Eliminating the Barriers

Several barriers exist that make many industries and farmers reluctant to implement AD to convert their biomass wastes to methane biogas. Improvements in AD technologies with respect to efficiency, reliability, and cost-effectiveness will overcome some of the barriers related to the technologies. Other barriers can only be debased from policy and public supports. While it is broadly realized that the building of a sustainable society requires both renewable energy and protection of the environment, the valuation of such is incomplete. AD is one of the few technologies that help achieve both goals. Even the bioethanol and biodiesel industries generate biomass wastes, which need AD to extract otherwise wasted energy and to reduce environmental pollution. Therefore, AD should be regarded as a unique, indispensable, renewable energy-producing biotechnology that protects the environment. With continued improvement of AD technologies and supports from both the public and the government, AD will become more cost-effective, energy-efficient, reliable, and widely implemented. AD will evolve into one of the most environment-friendly biotechnologies that produce cost-effective bioenergy in the next five to ten years.

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Waste to Renewable Energy: A Sustainable and Green Approach Towards Production of Biohydrogen by Acidogenic Fermentation

S. Venkata Mohan

Abstract The global impact of increasing energy demands, depleting reserves of fossil fuels and increasing pollution loads on the environment due to the utilization of energy produced from fossil fuels have received considerable notice in recent years. Generation of energy from fossil fuels is generally convenient but the depleting reserves and associated global warming are major problems. One potential alternative is a shift from fossil fuel to a hydrogen (H_2) based economy. H_2 is considered to be a clean energy carrier with high-energy yield (142.35 kJ/g) and upon combustion it produces only water. H_2 can be produced by the biological routes of bio-photolysis, photo-fermentation and dark fermentation or by a combination of these processes. Dark fermentation offers the particular advantage of using wastewater as a substrate and mixed culture as catalyst. Wastewater contains high levels of biodegradable organic material with net positive energy. One way to reduce the cost of treatment is to generate bio-energy, such as H_2 gas by metabolically utilizing organic matter, at the same time accomplishing treatment. This chapter mainly focuses on the evaluation of fermentative H_2-generating processes utilizing wastewater as substrate and mixed culture as biocatalyst. A particular insight was also laid on to discuss the process based on important operating factors involved and to delineate some of the limitations. Various strategies such as multiple process integration, microbial electrolysis, polyhydroxyalkanoate (PHA) production, bioaugmentation, self-immobilization and metabolic engineering were discussed in overcoming some of the limitations in the direction of process enhancement.

Keywords Biohydrogen · Anaerobic · Dark fermentation · Wastewater treatment · Acidogenic · Pretreatment · Bioelectricity · Microbial fuel cell (MFC) · Microbial electrolysis · Bioaugmentation · Polyhydroxyalkanoates (PHA) · Mixed culture · Immobilization

S. Venkata Mohan (✉)
Bioengineering and Environmental Centre (BEEC),
Indian Institute of Chemical Technology (IICT), Hyderabad-500007, India
e-mail: vmohan_s@yahoo.com; svmohan@iict.res.in

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1 Introduction

Hydrogen (H_2) is a potentially sustainable energy carrier as because it produces only water and has a high energy yield of 122 kJ/g; 2.75 fold greater than that of hydrocarbon fuels and can be made from renewable resources, although at present nearly 90% of H_2 is produced from steam reformation of natural gas or light oil fractions at high pressure and high temperatures.

Biological H_2 production proceeds through two main pathways: photosynthesis and dark fermentation. Photosynthesis is a light-dependent process, comprised of direct biophotolysis, indirect biophotolysis and photo-fermentation, while anaerobic fermentation, also known as dark fermentation, is a light-independent catabolic process [1–4]. Photosynthetic microorganisms, such as algae, photosynthetic bacteria and cyanobacteria manifest H_2 production in photosynthetic processes [5–6] while, fermentative microorganisms generate H_2 during the acidogenic phase of the anaerobic digestion. Fermentative processes yield comparatively better H_2 production than the photosynthetic process and do not rely on the availability of light. They also utilize a variety of carbon sources such as organic compounds, wastes, wastewaters or insoluble cellulosic materials, require less energy, are technically much simpler, have lower operating costs and are more stable [3, 7–12]. Fermentative microorganisms also generally have rapid growth rates. Dark fermentation is a practically a more feasible process for the mass production of H_2. H_2 generation via biological routes is relatively pollutant free, requires low energy inputs and is therefore considered as a potential alternative to the conventional physical/chemical methods used for H_2 production. Most of the biological H_2 production processes are operated at ambient temperature and pressure, thus are less energy intensive. Research on photo-biological routes of H_2 production was initially reported with specific strains and defined medium. Subsequently, dark fermentation gained importance due to its feasibility of utilizing wastewater as a fermentative substrate and mixed cultures as biocatalysts. The process simplicity and efficiency are strong features.

2 Fermentative Process of H_2 Production

H_2 production by dark fermentation (acidogenic or acetogenesis) processes shares many common features with methanogenic-anaerobic digestion [7, 11, 12]. Anaerobic conversion requires four major steps and five physiologically distinct groups of microorganisms to convert hydrocarbons from complex to simple molecules through H_2 and acid as intermediates finally, to carbon dioxide (CO_2) and methane (CH_4) (Fig. 1a). Fermentative/hydrolytic microorganisms hydrolyze complex organic polymers to monomers, and then ferment those monomers to a mixture of low-molecular-weight organic acids and alcohols. Obligatory H_2 producing acetogenic bacteria (AB) oxidize fermentation products to acid intermediates and H_2,

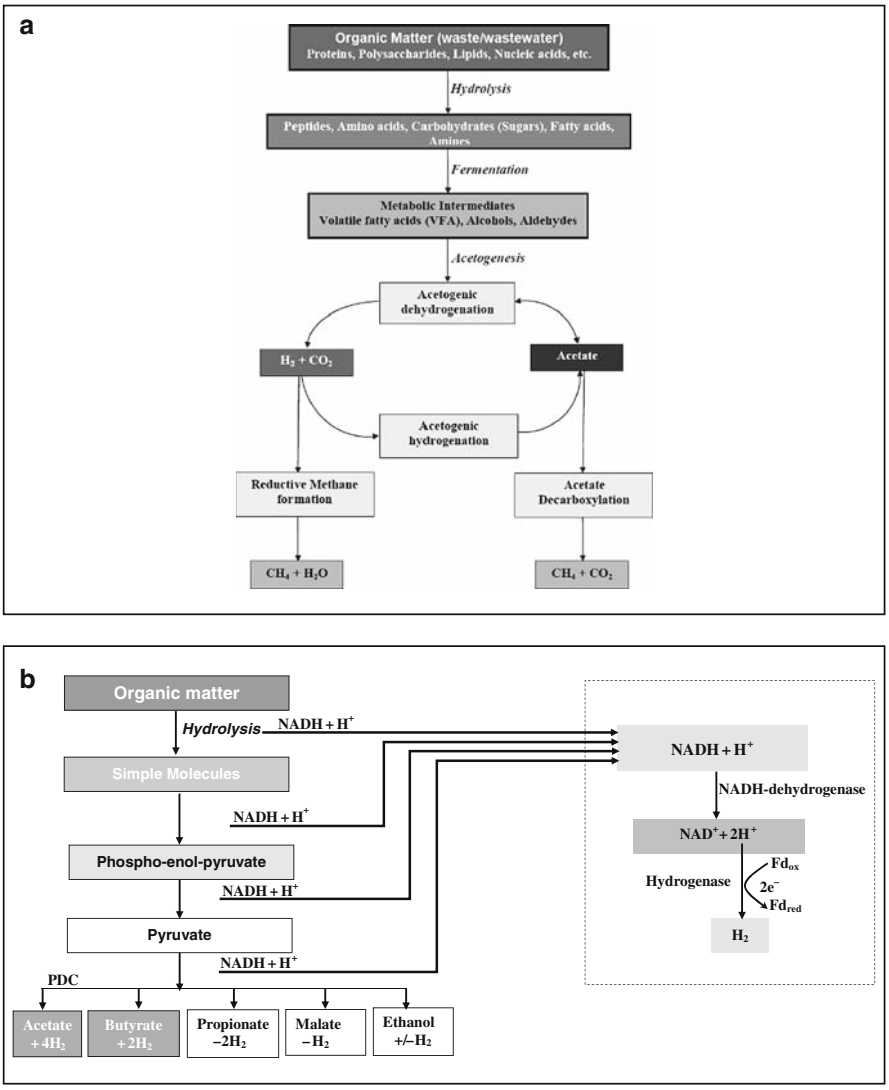

Fig. 1 (**a**) Anaerobic process depicting both acidogenic and methanogenic processes during conversion of complex organic molecules through biohydrogen to methane and carbon dioxide; (**b**) Metabolic pathways relating to possible routes of H_2 formation from proton (H^+) generated during substrate degradation

which also include acetate production from H_2 and CO_2 by acetogens and homoacetogens and finally acetoclastic methanogens convert organic acids to CH_4 and CO_2 [4, 11, 13]. H_2-producing AB grow in syntrophic association with hydrogenotrophic methanogens (H_2 consuming), resulting in low H_2 partial pressure thus allowing acetogenesis to become thermodynamically favorable by interspecies H_2 transfer [11].

2.1 Biochemistry

Fermentation is the process of deriving energy from the oxidation of organic compounds using an endogenous electron (e^-) acceptor, which is usually an organic compound [14]. This is in contrast to cellular respiration, where e^- are donated to an exogenous e^- acceptor, such as oxygen, via an electron transport chain (ETC). Considering glucose as substrate, fermentative H_2 production starts with the conversion of glucose to pyruvate through glycolysis by both obligate and facultative anaerobic bacteria. In facultative anaerobes, pyruvate is converted to acetyl-CoA and formate, which is catalysed by pyruvate formate lyase (PFL) [3] and H_2 is produced from formate by the formate hydrogen lyase (FHL) complex. In obligate anaerobes, pyruvate is converted to acetyl-CoA and CO_2 through pyruvate ferredoxin oxidoreductase (PFOR) and this oxidation requires reduction of ferredoxin (Fd) [3, 15]. The fate of pyruvate in the case of anaerobic operation depends on the operating pH. Under acidic condition pyruvate is converted into volatile fatty acids along with H_2 by acidogenic bacteria. Neutral operation leads to the formation of CH_4 and CO_2 by methanogenic bacteria. Under basic pH, anaerobic digestion leads to solventogenesis. At all the pH conditions, H^+ shuttling takes place between metabolic intermediates with the help of various redox mediators under anaerobic operation. The H^+ from the redox mediator is detached by a specific dehydrogenase (NADH-dehydrogenase) and combined with the e^- from oxidized ferredoxin to generate H_2 in presence of the hydrogenase enzyme (Fig. 1b). Hydrogenase activity is higher at acidic pH but with increase in pH, metabolic pathway might proceed to the next step of anaerobic digestion where H^+ get reduced to CH_4 (methanogenesis) or ethanol (solventogenesis).

Biodegradation of substrate is always accompanied by the release of protons (H^+) and electrons (e^-) associated with various redox reactions and enzymes. Dehydrogenase is one of the important enzymes involved in the inter-conversion of metabolites and the transfer of protons (H^+) between metabolic intermediates through redox reactions using several mediators (NAD^+, FAD^+, etc.). Redox mediators are capable of carrying H^+ and e^-, otherwise known as energy carriers as they are involved in biological energy (ATP) generation [16]. Generally, in the anaerobic microenvironment, inter-conversion of substrates takes place through degradation that increases the availability of H^+ in the cell. The protons associated with redox mediators are the main source of fermentative H_2 production. The protons from redox mediators are detached in presence of NADH-dehydrogenase and reduced to H_2 in presence of the hydrogenase enzyme with the help of e^- donated by oxidized ferredoxin (co-factor) [3]. Hydrogenases are complex metalloenzymes that can be classified into three groups based on the number and identity of the metals in their active sites: [NiFe]-, [FeFe]- and [Fe]-hydrogenases [17]. These enzymes are also responsible for the reversible conversion of molecular H_2 into two H^+ and two e^- [$H_2 \leftrightarrow 2H^+ + 2e^-$] [3]. The dehydrogenase activity is crucial along with the hydrogenase activity as it maintains H^+ equilibrium in the cell through redox reactions and inter-conversion of metabolic intermediates. Nitrogenase enzymes are also involved in H_2 production along with nitrogen-fixation. Nitrogenases irreversibly catalyze

the reduction of molecular nitrogen to ammonium by consuming reducing power (e^- mediated by ferredoxin, NAD^+ etc.) and ATP. Nitrogenase catalyzes H^+ reduction in the absence of nitrogen gas. Even in nitrogen atmosphere, H_2 production is catalyzed by nitrogenase as a side reaction at a rate of one-third to one-fourth that of nitrogen-fixation. Nitrogen-fixing cyanobacteria are potential candidates for H_2 production by nitrogenase but it is an energy-consuming process due to breakdown of many ATP molecules.

2.2 Soluble Metabolic Acid Intermediates

The soluble acid metabolites or volatile fatty acids (VFA) formed during the acidogenic process help in understanding the metabolic pathway [18]. The following equations show variable soluble acid metabolites generation during acidogenic fermentation.

$$C_6H_{12}O_6 + 2H_20 \rightarrow 2CH_3 \cdot COOH + 2CO_2 + 4H_2 \quad \text{[Acetic acid]}$$
$$C_6H_{12}O_6 \rightarrow CH_3 \cdot CH_2 \cdot CH_2 \cdot COOH + 2CO_2 + 2H_2 \quad \text{[Butyric acid]}$$
$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3 \cdot CH_2 \cdot COOH + 2H_2O \quad \text{[Propoinic acid]}$$
$$C_6H_{12}O_6 + 2H_2 \rightarrow COOH \cdot CH_2 \cdot CH_2O \cdot COOH + CO_2 \quad \text{[Malic acid]}$$
$$C_6H_{12}O_6 \rightarrow CH_3 \cdot CH_2OH + CO_2 \quad \text{[Ethanol]}$$

Depending on the pathway used by the microorganism and the corresponding end-products, H_2 yields are variable. Products formed from pyruvate such as acetate, butyrate, butanol, acetone, lactate or ethanol determine the theoretical yield of H_2 [3]. In obligate anaerobes, pyruvate is converted to H_2 from the reduced Fd by the action of hydrogenase resulting in maximum yield of 2 mol H_2/mole glucose. Two additional moles of H_2 can be produced from NADH produced during glycolysis, where NADH is oxidized by Fd reduction by NADH:ferredoxin oxidoreductase (NFOR) [3]. Further, H_2 can be produced from the reduced Fd by hydrogenase. The highest theoretical yield of 4 mol H_2/mole glucose can be obtained when acetate or acetone is the fermentation end-product. Two molecules of formate are produced from two pyruvate molecules where a theoretical maximum yield of 2 mol H_2/mole of glucose can be obtained. In the case of butyrate as the fermentation end-product, the maximum theoretical yield is 2 mol H_2/mole glucose. When alcohols are the end-products, lower yields of H_2 are obtained as alcohols contain additional H_2 atoms that have not been converted to H_2 gas [3]. The presence of higher concentrations of propionic acid or solventogenesis is generally not considered to be feasible for H_2 production.

3 Waste and Wastewater as Substrates for H_2 Production

One of the sustainable ways to reduce the cost of waste or wastewater treatment is to generate bio-energy, such as H_2 gas, from the organic matter present. Waste biomass contains enough energy to meet a significant fraction of the world's entire energy demand, if it could be efficiently converted to useful energy forms [19]. According

to one estimation, the energy value of all residual biomass in the United States is 0.2–0.3 TW [20] and conversion of this material to useful forms would meet approximately 7% of the USAs' total annual energy use (~3.3 TW) [19]. The fraction is much higher worldwide, perhaps 25% or more [19]. Major advantages of energy from wastes are the carbon neutrality, renewability, recovery of energy and simultaneous wastewater treatment. Simple sugars to complex industrial wastewaters have been evaluated to determine their potential as fermentative substrates for the production of H_2. Table 1 shows some of these studies using dark fermentation. Simple sugars such as glucose, sucrose and lactose are readily biodegradable substrates for H_2 production but are expensive. Various wastewaters generated from industrial or domestic activities function as good substrates for H_2 generation due to the presence of large fractions of degradable organics. Residue like agricultural crops and their waste products, wood and wood waste, food processing waste, aquatic plants, algae, and effluents produced in human habitats can all be used as fermentable substrates

Table 1 Various types of waste/wastewaters used as substrate for fermentative H_2 production

Nature of waste	Type of waste	References
Industrial Wastewater	Designed synthetic wastewater	[21–24]
	Chemical wastewater	[21, 22, 25, 26]
	Paper mill waste	[27]
	Dairy processing wastewater	[28–31]
	Cheese processing wastewater	[32, 33]
	Brewery wastewater	[34]
	Wine process wastewater	[35, 36]
	Molasses based wastewater	[37, 38]
	Palm oil mill effluent (POME)	[39–41]
	Citric acid wastewater	[42]
	Probiotic wastewater	[43]
	Slaughterhouse waste	[44]
	Starch based wastewater/starch effluent	[45, 46]
	Olive mill wastewater	[47]
	Food processing wastewater	[48]
Urban waste	Municipal solid waste	[44, 49, 48, 50, 51]
	Domestic sewage/wastewater	
	Activated sludge /sewage bio-solids	[52]
	Citrus peeling waste	[16]
Solid waste	Household solid waste	[53, 54]
	Vegetable based market waste	[55]
	Corn stalk	[56]
	Wheat starch/Wheat straw	[57–59]
	Fodder maize	[60]
	Chitinous waste	[61]
Agricultural waste	Cattle wastewater	[62]
	Mixed fruit peel waste	[63]
	Potato waste/Potato starch residue	[64, 65]
	Cellulose	[66–68]
	Hemicellulose-rich pine tree wood shavings	[69]

Table 2 Biohydrogen production and substrate degradation patterns observed during dark-fermentative treatment of various wastewater carried at BEEC, IICT

Type of wastewater	Organic loading range	Cumulative H_2 production (mmol/day)	Volumetric H_2 production (mmol H_2/m^3-day)	COD removal efficiency (%)	Specific H_2 yield (mol H_2/ kg COD_R)	References
Designed Synthetic	4.8 kg COD/m^3-day	27.89	6.97	32.4	14.35	[6, 23, 30]
Chemical	5.6–7.9 kg COD/m^3-day	10.26–23.81	2.56–5.95	17.21–26.7	6.06–13.44	[4, 21, 22, 25]
Distillery	9.6 kg COD/m^3-day	26.0	26.0	56.25	6.98	[71]
Dairy based	2.8–4.6 kg COD/m^3-day	39.5–40.8	21.94–22.96	62.6–67.8	13.4–15.05	[30, 31]
Vegetable based	4.4–57.6 kg COD/m^3	16.85–80.31	10.53–68.87	38.33–65.00	0.60–14.9	[55]
Citrus peeling waste	1.17–4.69 kg COD/m^3	1.05–10.07	32.81–271.25	36.69–88.62	1.05–3.59	[16]

[70]. Many agricultural and food industry wastes contain starch or cellulose, which are rich in terms of carbohydrate content and can also be used for H_2 production. The sludge generated in wastewater treatment plants contains large quantities of carbohydrates and proteins which can also be used for energy production. Table 2 shows data on fermentative H_2 production using various types of waste.

Unlike wastewater, cellulosic material or solid wastes typically require pretreatment to make the organic fraction soluble and bio-available to microorganisms for conversion to H_2. Due to its tightly packed, highly crystalline and water-insoluble nature, cellulose is recalcitrant to hydrolysis into its individual glucose subunits [70]. In the pretreatment step, a combination of chemical, mechanical, and enzymatic processes is typically used. Techniques viz., high temperature, high or low pH, hydrolytic enzymes, microwaves, ultrasound, radiation, and pulsed electric fields are being used for this purpose [19]. Some microorganisms can degrade cellulose effectively by using their cellulase enzymes resulting in monosaccharide products that can be converted into H_2 with dark fermentation [70].

4 Factors Influencing the Fermentative H_2 Production Process

4.1 Biocatalyst

Biocatalyst (inoculum) selection and its pre-treatment plays a vital role in selecting requisite microflora for efficient H_2 production [4, 7, 15, 30, 71, 72, 73]. Inoculum preparation affects both start up and the overall efficiency of H_2 production. Typical anaerobic mixed cultures cannot produce H_2 as it is rapidly consumed by H_2-consuming or CH_4-producing bacteria (MB) [74]. The most effective way to enhance H_2 production from anaerobic culture is to restrict or terminate methanogenesis by allowing H_2 to become a metabolic end product. Physiological differences between H_2-producing bacteria (AB) and H_2-consuming bacteria (MB) forms the main basis for the preparation of the inoculum to start up the acidogenic H_2-producing process [72]. Spore-forming H_2-producing bacteria can form spores which protect them when they are in an adverse environment (high temperature, extreme acidity and alkalinity), but methanogens have no such capability [72]. Some of the pretreatment methods normally used for selective enrichment of an H_2-producing inoculum are listed in Table 3. Methanogenesis could also be eliminated by maintaining short retention times (2–10 h) during reactor operation [75, 76] as H_2-producing bacteria grow faster than the methanogens [72]. Combining different pre-treatment methods also showed a positive effect on the H_2 production process [4, 21, 30, 38, 71]. In spite of good enhancement in H_2 production, marked reduction in substrate degradation efficiency was observed after applying pretreatment methods [4, 30, 71], which can be attributed to the inhibition of MB. The methanogenesis function is required to metabolize intermediates generated from the acidogenic process. Untreated anaerobic inocula showed low H_2 yield in spite of effective substrate removal leading to CH_4 formation due to the presence of MB.

Table 3 Pretreatment methods normally used to selectively enrich H_2 producing inoculum

Pretreatment method	Conditions	Function
Heat-shock	Enrichment under extreme temperature (>80°C)	Suppress non-spore forming bacteria
Acid	Enrichment in extreme acidic microenvironment (pH < 4)	Enrich spore-forming bacteria by specifically repressing the MB
Chemical	Enrichment in presence of specific chemicals such as	
	• 2-bromoethanesulfonic acid (BESA)	Selective inhibition of MB by suppressing the activity of co-enzyme, M reductase complex (chief component for methanogenesis)
	• Iodopropane	Prevents functioning of B12 co-enzyme (methyl group carrier)
	• Acetylene	Non-specific inhibition of MB
Alkaline	Enrichment in alkaline microenvironment (pH > 9)	Non-specifically inhibits MB
Load-shock	Direct enrichment in presence of higher substrate concentration	Leads to accumulation of high organic acids which prevents MB growth
Forced aeration (oxygen-shock)	Enrichment in presence of oxygen/air	Suppress the activity of MB

The influence of various pre-treatment methods applied individually and in combination on H_2 production and substrate degradation patterns from the treatment of diary based wastewater under acidic conditions is illustrated in Fig. 2

4.2 pH

Depending on organisms and growth conditions, changes in external pH can bring about subsequent alterations in several primary physiological parameters, including internal pH, concentration of other ions, membrane potential and proton-motive force [77]. pH also influences the efficiency of substrate metabolism, protein synthesis, synthesis of storage material and metabolic by-product release. This is especially important for fermentative H_2 production where the activity of acidogenic bacteria is considered to be crucial and rate limiting [24, 58]. The restricted nature of specific groups of bacteria at particular pH values helps to maintain the bioreactor in an acidogenic microenvironment. Maintaining pH in the acidic range (5.5–6.0) is ideal for effective H_2 production due to repression of MB, thus indirectly promoting H_2 producers within the system [21, 30, 72]. The activity of hydrogenase is observed to be inhibited by maintaining low or high pH in fermentation [58]. Most methanogens are limited to a narrow pH range (6.8–7.2), while most H_2-producing acidogenic bacteria can grow over a broader pH range. AB function well below pH 6, while for MB optimum range is between 6.0 and 7.5 [78, 79]. The pH range of

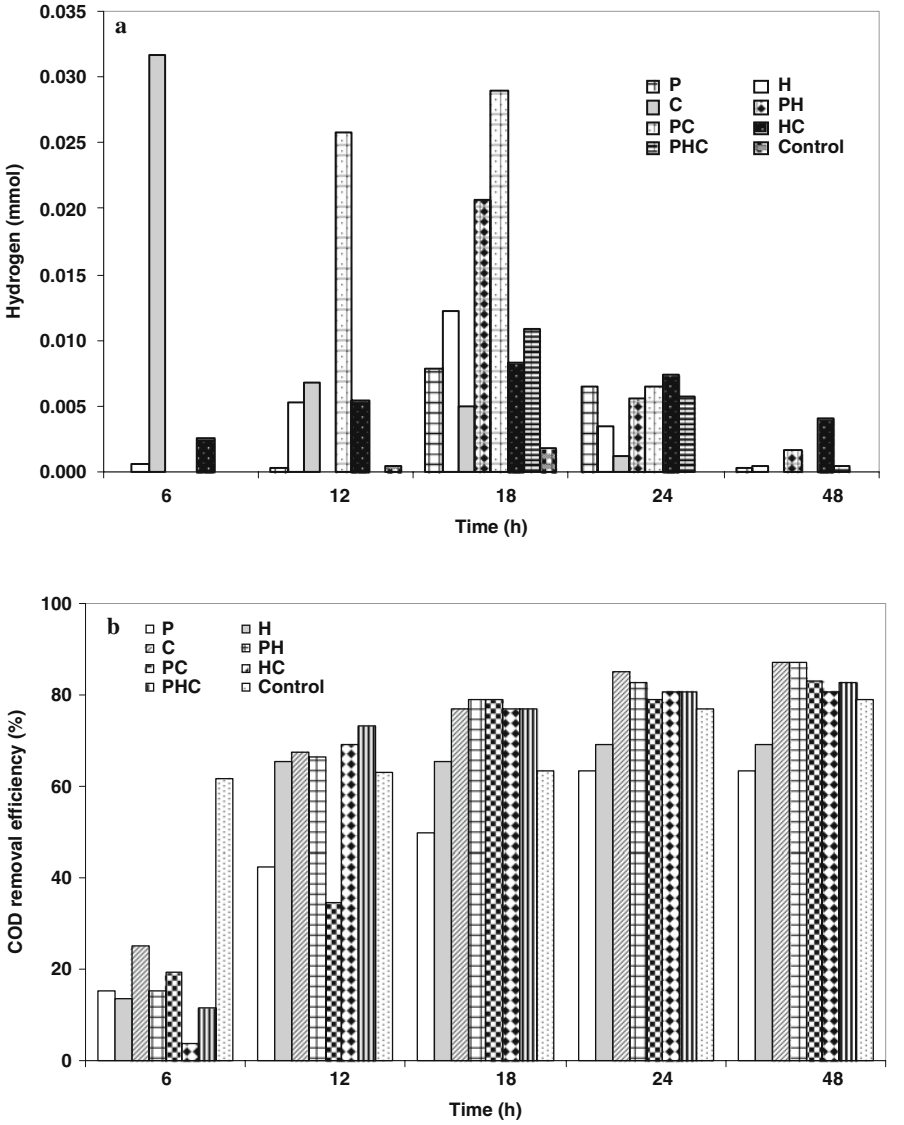

Fig. 2 Relative performance of various pretreatment methods application on anae [(**a**) H_2 production and (**b**) substrate (COD) removal efficiency [H, heat-shock; P, acid; C, chemical (BESA)]] [30]

5.5–6.0 is reported to be ideal to avoid both methanogenesis and solventogenesis [21, 79], which is important for good H_2 production. Effective H_2 production was observed by maintaining operating pH in and around 6 compared to near neutral pH [21, 75]. Increase in initial/feeding pH (from acidic to neutral) has resulted in suppressed H_2 production [21, 26, 31, 32]. However, highly acidic pH (<4.5) is detrimental to H_2 production as it inactivates H_2 producing bacteria [72, 80].

Cyclic voltammograms (CV) obtained at acidic and neutral pH conditions visualized well- defined redox pairs both in forward and reverse scans and the signal corresponded to intracellular electron carriers, NADH/NAD^+ (E_0, –0.32 V) [24] (Fig. 3). Shuttling of H^+ between metabolic intermediates can be correlated to the e^- discharge observed in CV. At acidic pH, the e^- discharge was almost similar at

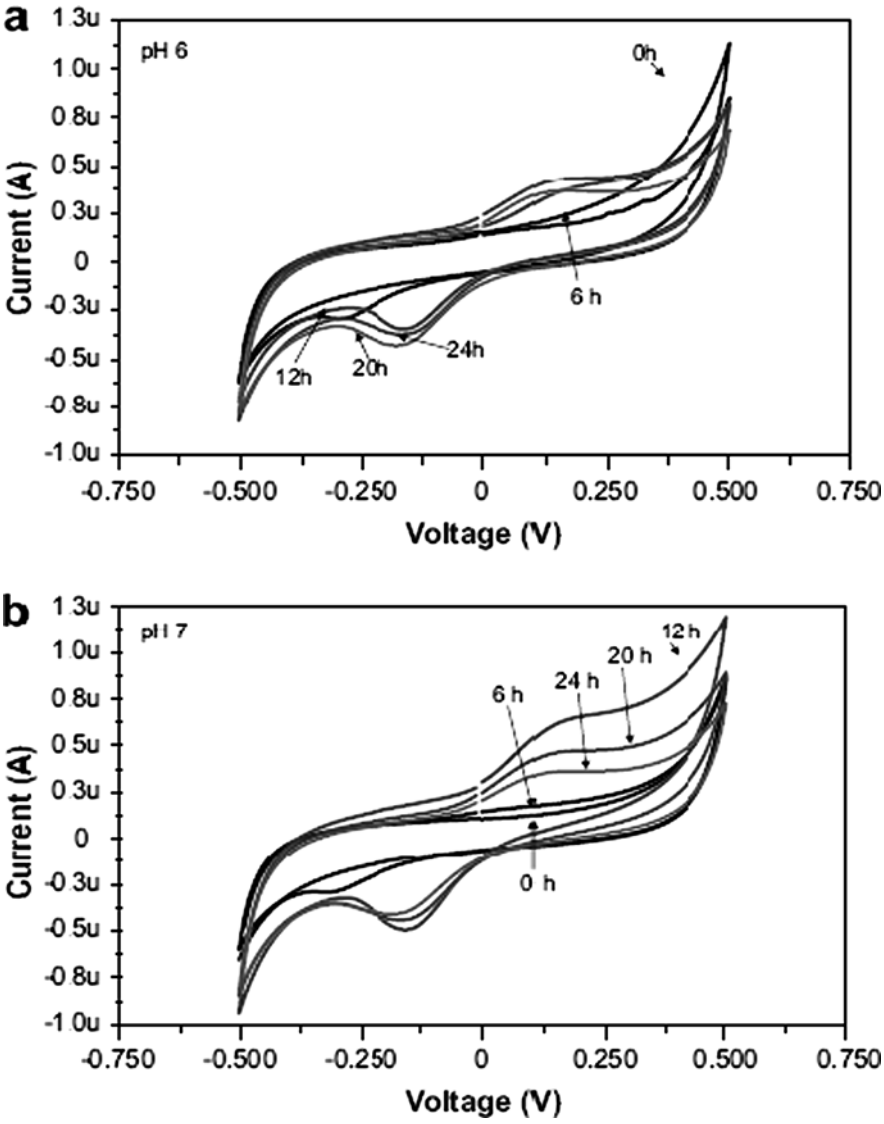

Fig. 3 Cyclic voltammograms (CV) of anaerobic mixed consortia (whole cell) with the function of feeding pH during fermentative H_2 production [(**a**) acidic and (**b**) neutral] [vs Ag/AgCl(S) (reference electrode); platinum rod (working electrode); graphite rod (counter electrode); wastewater (electrolyte); scan rate, 10 mV/s] [24]

12, 20 and 24 h suggesting the effective H^+ shuttling throughout the cycle operation. This helps to maintain the system under acidogenic conditions for longer periods leading to higher H_2 production. At neutral operation, the e^- discharge varied with time and approached maximum at 12 h prior to decrease suggesting the neutralization/reduction behaviour of H^+ by MB.

Acidic pH (below 6) showed less substrate degradation efficiency than the corresponding neutral operation due to reduced methanogenic activity [24]. Neutral pH illustrated effective substrate removal efficiency over the corresponding acidic operation. Maintenance of acidic conditions in association with pre-treatment has also been observed to be effective in H_2 production during treatment of various types of wastewater [30, 31, 38].

VFA (soluble acid metabolites generated from acidogenic fermentation) and pH are integral expressions of acid-base conditions of anaerobic microenvironments which provides information pertaining to the balance between two of the most important microbial groups (AB and MB). Production of acids gradually reduces the buffering capacity of system, which, in turn, results in a decline in the system pH due to accumulation of organic acids leading to process inhibition [23, 81]. If pH is not maintained in the optimum range, cessation of H_2 production will result along with a marked shift in microbial population [75]. Relatively higher levels of soluble metabolite production were observed under acidic operation over the corresponding neutral microenvironment, which corroborated well with H_2 production data [26, 31, 38, 82]. Therefore, pH can be considered as a manipulable variable for process control. Among the two process variables viz., influent pH and reactor pH, the later is more difficult to control. Bicarbonate-alkalinity is an important process parameter which indicates the system buffering capacity in association with pH microenvironment and VFA concentrations.

Sulfate, if present in wastes will be converted into hydrogen sulfide by sulfate-reducing bacteria (SRB) in the anaerobic microenvironment, resulting in toxicity to other anaerobes [83]. SRB are reported to have H_2 utilization hydrogenase and can readily use H_2 as the electron donor [84]. pH of the system microenvironment has a direct influence on the sulfate reduction linked to H_2 production. At acid pH, the SRB activity gets inhibited wherein H_2 production is unaffected. H_2 production has markedly recovered and increased when pH was reduced to 5.5, even in the presence of higher sulfate concentration (3 g SO_4^{2-} /l) [85].

4.3 Hydraulic Retention Time (HRT)

Hydraulic retention time (HRT) influences the H_2 generation process significantly. Reducing HRT from 18 to 12 h has improved H_2 yield without affecting substrate removal efficiency [57]. Maximum H_2 yield was reported between 0 and 14 h in all the experimental variations studied in batch mode during dairy, chemical and distillery wastewater treatment [22, 25, 31, 38]. Longer fermentation periods induce a metabolic shift from the acidogenic process to the methanogenic process which is unfavorable for H_2 production. Shorter HRT's have been shown to

dilute out slow growing MB [86]. However, in continuous operation mode, H_2 production was observed at long HRTs of 3 days (pH 6.4) without encountering problems with methanogenesis [87]. Optimal HRT mostly depends on the nature and composition of the substrate, function of biocatalyst, loading rate and fermentation pH employed. HRT can be considered as a readily manipulated variable for process control. Optimum HRTs from 8.0 to 14 h were reported for effective H_2 production [57].

4.4 Temperature

Temperature affects H_2 production, metabolite product distribution, substrate degradation and bacterial growth. Most studies on H_2 production have been conducted under ambient (15–27°C), mesophilic (30–45°C), and moderate thermophilic (50–60°C) temperatures, with a few studies of mixed cultures under extreme thermophilic conditions, over 60°C [88, 89]. The optimal temperature for H_2 production via dark fermentation varies widely based on the type of biocatalyst and the carbon substrate used. For pure cultures, the optimal temperatures are reported to be in the range of 37–45°C, whereas for mixed microflora diverse optimum temperatures were reported [62]. Both mesophilic and thermophilic temperatures were observed to be optimal for fermentative H_2 production processes. Thermophilic conditions were reportedly advantageous due to its thermodynamics [15, 62] which gives higher reaction rates with better process performance and decreased problems with contaminating H_2-consuming microorganisms. Although higher temperatures allow more favorable reaction kinetics, rapid changes in system pH may inhibit H_2 producing bacteria [90]. The changes in soluble metabolite composition were also observed with changes in operating temperature, resulting in metabolic pathway shifts correlated to bacterial functions dominant at that particular temperature [91]. Temperature control might not be a feasible option for process control.

4.5 Reactor Configuration and Operation

Various reactor configurations, viz., suspended growth, biofilm/packed-bed/fixed bed, fluidized bed, expanded bed, upflow anaerobic sludge blanket (UASB), granular sludge, membrane based systems, immobilized systems, etc., have been used successfully to produce H_2 by fermentation processes. Biofilm/attached-growth systems are generally robust to shock-loads compared to the corresponding suspended growth systems, with the biofilms acting as a buffer to reduce the effective concentration of toxic chemicals to which the organisms are exposed, protect the culture from predation, provide improved reaction potential due to the presence of high cell densities and provide resilience and resistance to change in the process parameters [26, 92–95]. Generally bacteria achieve maximum growth rates in biofilm resulting in improved reaction potential finally leading to stable and robust system which are

well suited for treating highly variable wastewater. Cell-immobilization approaches and granular processes also showed good H_2 production efficiency.

Various modes of reactor operation viz., batch, fed-batch, semi-batch/continuous, periodic discontinuous batch (sequencing batch operation) and continuous have been used to produce H_2. About a 25% improvement in H_2 production and substrate degradation efficiency was reported with batch mode operation compared to the corresponding continuous mode operation [92]. The efficacy observed in fed-batch mode operation might be attributed to the reduced accumulation of soluble metabolic intermediates formed during acidogenic fermentation due to fill-draw mode operation [24, 26, 31, 38, 92]. A fed-batch mode of operation with acidic pH showed highest H_2 production [92]. Poor biomass retention/cell washout encountered during continuous mode operation can be prevented to some extent with a batch mode operation [92, 96, 97]. Batch mode operation coupled with a biofilm configuration combines the operational advantages of both systems and helps to maintain stable and robust cultures suitable for treating highly variable wastewater [21–25, 98–100].

Morphologically similar bacteria were observed in the scanning electron microscopy (SEM) image [26] of the biofilm formed on the fixed-bed of bioreactor producing H_2 from the treatment of chemical wastewater (Fig. 4a). The biofilm reactor was inoculated with selectively enriched H_2-producing consortia and operated under an acidic microenvironment for more than 300 days. SEM imaging visualized slightly bent, scattered and short chain rods (predominant) along with a relatively low frequency of cocci shaped bacteria of approximate length of 10 μm. SEM images of isolated bacteria strains from a biofilm reactor (acidogenic mixed culture) (Fig. 4b, c) visualized slightly bent, rod shaped, thick fluorescent capsid bacteria with (~10 μm in length). Images of both the isolated strain and mixed consortia showed comparatively similar morphology demonstrating the presence of related groups of bacteria proliferated in the bioreactor producing H_2. Transmission electron microscopy (TEM) image showing sub-cellular structures of the isolated bacteria from an acidogenic mixed culture [26] (Fig. 4d). TEM image showing oval centralized spore formation with sub-terminal endospore development in rod shaped bacteria (1–7 μm in length). Terminal bulging with granulose accumulation was not observed. Flagellum attached subapically to the bacterium (two times length of the cell body) was observed. Vegetative cell surrounded by thick membrane (peptidoglycan layer) with two layers (inner and outer forming fibrillar capsule structure) on the cell surface was also visualized (Fig. 4e).

4.6 Substrate Loading Rate

The organic loading rate (OLR) of the wastewater also influences the H_2 production pattern, apart from other wastewater characteristics. H_2 yields were inversely proportional to the glucose feeding rate, while the highest H_2 yields were observed at lowest glucose loading rate [78]. Glucose concentrations exceeding 2 g/l (as co-substrate) showed suppression in H_2 production [21]. A marked reduction

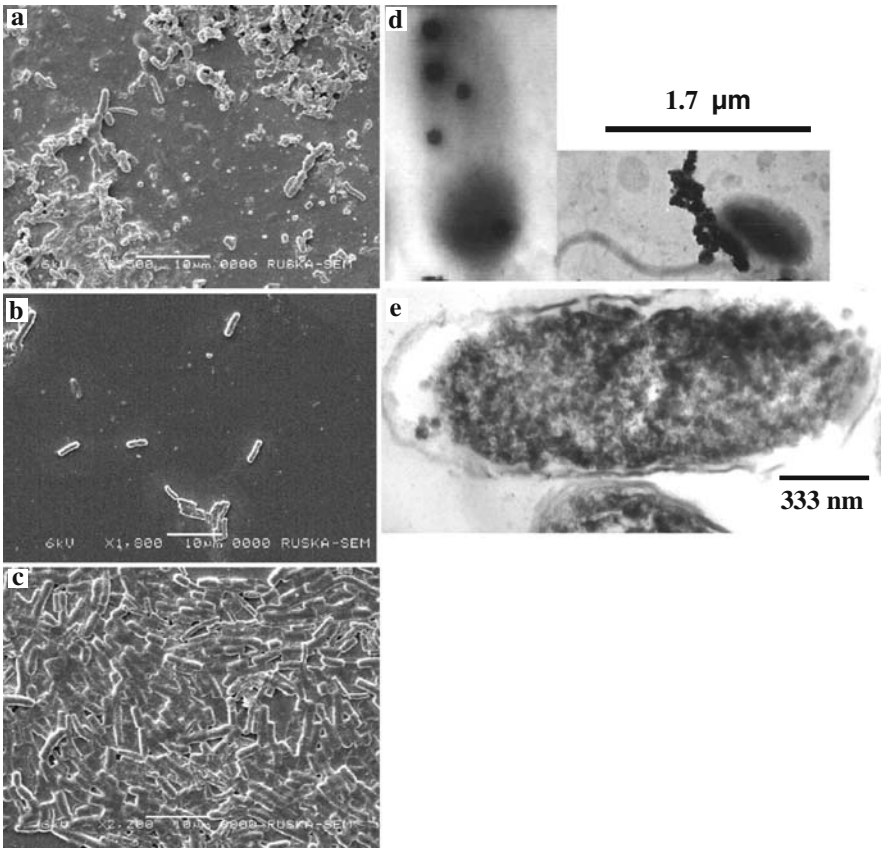

Fig. 4 SEM images of (**a**) acidophilic biofilm (X 2,500) (**b**) isolated strain (X 1,800) (**c**) isolated strain (X 2,200); (**d**) TEM images of isolate captured at different magnification (1.7 μm and 333 nm) [26]

in H_2 production rate was observed with an increase in OLR when chemical wastewater was used as the substrate [22]. H_2 production was also found to decrease with an increase in OLR when dairy wastewater was used as substrate [31]. Similar observations with substrate loading have been reported in the literature [101, 102]. Decreased H_2 production may also be due to end product inhibition by over-accumulated (supersaturated) soluble metabolites in the liquid phase at high OLRs [102]. However, each wastewater has its own threshold value, which relates to the system microenvironment and desired output [4, 22, 26, 31]. Feed consisting of only glucose as substrate showed a low H_2 yield, while feed with chemical wastewater admixed either with glucose or domestic sewage as co-substrates showed a positive influence on the H_2 generation rate [21, 71]. Domestic sewage addition showed a positive affect on the acidogenic fermentation process due to supplementation of

additional micronutrients, organic matter and microbial biomass in the direction of enhance the process efficiency.

4.7 Nitrogen and Phosphrous

Nitrogen is a necessary component in proteins, nucleic acids and enzymes and is second only to carbon as a requirement for bacterial growth [103, 104]. Nitrogen in an appropriate concentration range is beneficial to fermentative H_2 production, while at a much higher concentration can inhibit the process performance by affecting the intracellular pH of bacteria or inhibiting specific enzymes related to H_2 production [105–107]. Optimal nitrogen concentrations of 0.1 g N/l were reported for effective H_2 production [104]. Substrate degradation efficiency was also found to increase with increasing nitrogen concentration from 0 to 0.01 g N/l [104]. Appropriate ratios of C/N are fundamentally important, with the optimum being 47 [103]. Phosphate helps to maintain the system's buffering capacity during the H_2 fermentative process [91]. Using phosphate as an alternative to carbonate as a buffering supplement should increase the H_2 gas fraction [108]. An increase in the carbonate concentration increases the CO_2 fraction in the gas phase due to carbonate dissolution. Adding phosphate at a proper concentration is a useful strategy for optimal H_2 production [108]. Na_2HPO_4 affected the H_2 production in a concentration-dependent way with the optimal concentration being 0.6 g/l. Using a proper carbonate and phosphate concentration formulation, the H_2 production rate can be enhanced by 1.9 times which might be due to a shortening of the microflora lag-phase [108].

5 Combined Process Efficiency

When wastewater is used as a fermentative substrate for H_2 production, the extent of substrate degradation is important when process efficiency is considered [71]. There is a trade-off between technical efficiency based on H_2 production and substrate removal at different feeding pHs. Neutral pH is ideal for wastewater treatment while acidic pH is useful for effective H_2 production [21, 26]. Balanced conditions for effective combined performance and process optimization are especially important to sustain process economic viability. Process performance was evaluated using two diverse mathematical approaches [data enveloping analysis (DEA) and design of experimental (DOE) methodology] [71]. The role of some important factors such as type and origin of inoculum, pre-treatment procedure, inlet pH, co-substrate addition and feed composition were evaluated for combined process efficiency by the DEA methodology. DEA analysis showed that the untreated anaerobic inoculum under acidic conditions using simple wastewater as fermentative substrate showed combined process efficiency. Taguchi's DOE methodology was used to enumerate the role of selected factors on H_2 production and substrate degradation with the final

aim of optimizing the process [71]. This helped to identify the influence and contribution of individual selected factors on the process and to derive the relationship between variables and operational conditions. By adopting the derived optimum conditions, the performance with respect to H_2 production and substrate degradation could be improved significantly.

6 Limitations in Fermentative H_2 Production

In spite of striking advantages, the main challenge encountered with fermentative H_2 production processes are low substrate conversion efficiency and residual substrate present in acid-rich wastewater generated from the acidogenic process. Anaerobic bacteria have a theoretical maximum yield of 4 mol H_2/mole glucose [3]. In practice, yields are lower, as the NADH oxidation by NFOR is inhibited under standard conditions and only proceeds at very low partial pressures of H_2 [11]. Up to 4 moles of H_2 can theoretically be produced per mole of glucose through the known fermentative pathways [109]. However, various biological limitations such as H2-end-product inhibition and waste-acid and solvent accumulation limit the molar yield to around 2 moles per mole glucose consumed. Typical H_2 yields range from 1 to 2 mol H_2/mol glucose and result in 80–90% of the initial carbon remaining in the wastewater [7, 23, 25, 51, 109, 76, 110, 111]. Even under optimum conditions about 60–70% of the original organic matter remains as residue in the wastewater. Also a maximum yield of 4 mol H_2/mole glucose is still low for practical applications [3].

The generation and accumulation of soluble acid metabolites causes a sharp drop in the system pH and inhibits H_2 production. H_2 yield is lower when more reduced organic compounds, such as lactic acid, propionic acid, and ethanol, are produced as fermentation products, because these represent end products of metabolic pathways that bypass the major H_2-producing reaction [11]. The undissociated soluble metabolites can permeate the cell membrane of H_2-producing bacteria and then dissociate in the cell leading to physiological balance disruption [91]. Thus, some maintenance energy should be used to restore the physiological balance in the cell, which reduces the energy used for bacteria growth and inhibit the bacterial growth on the other hand. If the dissociated soluble metabolites is present in the system at a high concentration, the ionic strength will increase, which may result in cell lysis [91]. High concentrations of soluble metabolites can inhibit H_2-producing bacterial growth thereby reducing H_2 production [91, 78, 112]. The fermentation metabolic end-products and the resultant H_2 yields vary based on the environmental conditions even within the same bacterium [3, 86].

H_2 production is limited by the thermodynamics of the hydrogenase reaction, which involves the enzyme-catalyzed transfer of e^- from an intracellular electron carrier molecule to H^+ [11]. The partial pressure of H_2 is one of the important factors, as the pressure increases, H_2 production decreases [7]. H_2 production becomes thermodynamically unfavourable at H_2 partial pressures greater than 60 Pa [11].

Operating bioreactors at low H_2 partial pressure by stripping H_2 from the solution is as it is generated [57, 102], accomplishes both efforts simultaneously [11]. Conceptually, efforts are to be made in optimizing operational conditions to prevent consumption of H_2 by propionic acid-producing bacteria, ethanol-producing bacteria and homoacetogens and those that channel more reducing equivalents towards reduction of H^+ by hydrogenases to maximize H_2 production [11]. The physiological and physicochemical conditions under which the microorganisms give optimal H_2 yields is important and needs to be established. Optimization of process parameters is one of the vital steps as to enhance H_2 yield as well as to enhance substrate degradation efficiency and assumes significance prior to up-scaling the process.

7 Strategies to Enhance Process Efficiency

7.1 Process Integration Approach

Utilization of remaining carbon present in wastewater from acidogenic H_2 production (an organic acid rich effluent) for additional biogas (H_2 or CH_4) generation is one way to sustain the process. Integration of an acidogenic process with a terminal photo-fermentative process (for additional H_2 production) [6, 7, 110] or acidogenic process (for additional H_2 production) [86] or methanogenic process (for methane production) [23] were reported along with enhanced substrate degradation (Fig. 5). Soluble metabolites formed during methanogenic or from acidogenic processes could be utilized by photosynthetic bacteria [6, 7] or acidogenic cultures

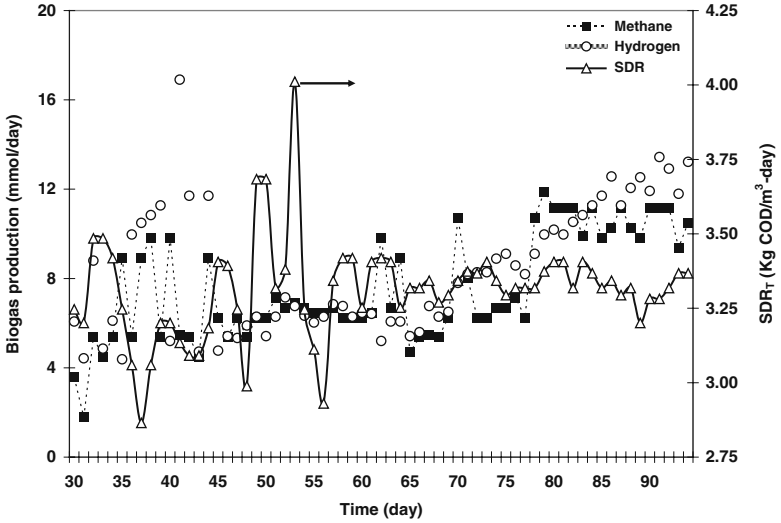

Fig. 5 Biogas generation and substrate degradation pattern during integration of acidogenic H_2 production (acidophilic) process with methaogenic (neutral) process [23]

[23] to produce additional H_2. Photosynthetic bacteria can produce H_2 by consuming organic acids which are abundant in the effluents generated from acidogenic H_2 fermentation processes [4, 6, 110]. Theoretically, the maximum H_2 yield may be obtained when glucose is converted to acetate as the terminal product through dark fermentation, then subsequently converted into H_2 through photo-fermentation [113]. Integrated systems showed higher H_2 yields compared to single-step fermentation [6, 13, 23, 73]. A two-stage process has been envisioned to obtain yields closer to the theoretical stoichiometric yield of 12 mol H_2/mole glucose [86, 113]. However, the efficiency of both H_2 production and substrate degradation were found to depend on the process used in the first stage along with the composition of the substrate [23]. The effluent from the first stage of operation generally contains ammonia, which inhibits the second stage process. This can be restricted by dilution and neutralization (to adjust the pH to 7) prior to feeding [10]. Integration of an acidogenic H_2 production process followed by a methanogenic anaerobic digestion for CH_4 production facilitated an enhanced energy yield along with higher substrate removal efficiency [23, 75, 114, 115]. Integration of the acidogenic process with a photo-fermentation process showed a more positive influence over the corresponding methanogenic process integration (Table 4). This might be due to the presence of a relatively higher concentration of VFA bound residual carbon corresponding to the methanogenic process. Multi-stage process was often used to maximize H_2 production. Initially, the process consisted of two stages, dark fermentation followed by photo fermentation [10] but three or even four stages have since been proposed in different configurations [109]. The acid-rich organic effluent generated from the initial process of dark fermentation was sent to photo-fermentative process followed by direct photolysis finally using microbial electrolysis cells to produce H_2 at fourth stage.

7.2 Microbial Electrolysis

Microbial aided electrolysis cells (MEC), also called bio-electrochemically assisted microbial reactor (BEAMR), use electro-hydrogenesis to directly convert biodegradable material into H_2 by applying external voltages in fuel cells in an anaerobic microenvironment [116, 117]. The supplemented voltage helps to decompose acetate spontaneously under standard conditions [116, 118]. Based on a thermodynamic analysis the addition of greater than 0.11 V to that generated by bacteria (–0.3 V) will yield H_2 gas at the cathode, but voltages of –0.2 V are needed because of electrode over-potentials [116]. This process, referred to as electro-hydrogenesis, provides a route for extending H_2 production past the endothermic barrier imposed by the microbial formation of fermentation end products, such as acetic acid [116]. Microbial electrolysis makes it possible to generate H_2 utilizing effluents generated from acidogenic fermentation and opens the possibility of using diluted organic matter varying in composition, such as wastewater, for H_2 production [119]. Membrane-less continuous flow microbial electrolysis cell (MEC) with a gas-phase cathode was also used to produce H_2 [119].

Table 4 Biogas (H_2 and CH_4) yield and substrate degradation pattern observed during two stages of process integration [acidogenic (pH 6)/methanogenic (pH 7) and photosynthetic (pH 7)] [6]

		H_2 yield (mol/kg COD_R)		CH_4 yield (mol/kg COD_R)	Substrate degradation rate (kg COD_R/m^3-day)	
Process Integration	Wastewater	Acidogenesis	Photo-fermentation	Methanogenesis	Acidogenesis (or) methanogenesis	Photo-fermentation
Acidogenesis + Photo-fermentation	Designed synthetic	18.64	15.16	–	0.525	1.05
Methanogenesis + Photo-fermentation	Designed synthetic	–	11.75	21.98	1.137	0.70
Acidogenesis + Photo-fermentation	Diary based	8.26	13.70	–	0.263	1.34
Methanogenesis + Photo-fermentation	Diary based	–	10.20	16.45	0.831	0.88
Acidogenesis + Photo-fermentation	Chemical	11.12	8.23	–	0.306	0.84
Methanogenesis + Photo-fermentation	Chemical	–	5.71	18.75	0.875	0.42

7.3 Polyhydroxyalkanoate (PHA) Generation Utilizing Acid-Rich Effluents

H_2 can also be viewed as an energy source and an intermediate towards the production of VFA which can be further transformed to polyhydoxyalkanoates (PHAs), or can be used for biohydrogenation of fatty acids into alcohols [120]. Polyhydroxyalkanoates (PHAs) are a group of biologically derived polyesters that represent a potentially sustainable replacement for fossil-fuel based conventional thermoplastics due to their biodegradability and capability of being produced from renewable resources. During growth-limiting conditions, bacteria produce PHAs as energy and carbon storage molecules. So far, many efforts have been made to produce PHAs from commercial grade VFAs using pure cultures. However, the PHAs produced in this manner are more expensive than polyethylene due to their high production costs [121, 122]. Almost 30% of total PHA production cost is attributed to the carbon source [123]. VFA bound acid-rich waste generated from acidogenic process of H_2 production can be used to produce PHAs using PHA accumulating organisms and is a promising approach to decrease the production cost. Production of PHA by mixed microbial cultures using wastes seems to have many advantages when compared to the existing well-known process where pure cultures and single defined substrates are used. Recently, the production of PHAs from the fermentation of syngas was also reported which is economically viable than that from sugar fermentation [124].

7.4 Bioaugmentation

Shifting the anaerobic reactor from methanogenesis (producing CH_4) to acidogenesis (to produce H_2) is important to make the process more feasible with wider application potential. Bioaugmentation is generally applied to improve the start up of a reactor, to enhance performance efficiency, to protect the existing microbial community against adverse effects or to compensate for organic or hydraulic overloading [99, 125]. In this way, the bioaugmentation strategy was applied to an operating anaerobic bioreactor (producing CH_4) to shift to acidogenic process so as to produce H_2 [25]. For this purpose selectively enriched H_2-producing mixed consortia (in immobilized form) was used as augmenting inoculum. After augmenting, the H_2 production rate almost doubled (Fig. 6). Bioaugmentation with co-cultures *Clostridium acetobutylicum* X_9 and *Ethanoigenens harbinense* B_{49} showed to improve cellulose hydrolysis and subsequent H_2 production rates from carboxymethyl cellulose [67].

7.5 Self-immobilization of Biocatalyst

The influence of self-immobilization of enriched acidogenic mixed consortia on fermentative H_2 production was studied on different supporting materials [SBA-15 (mesoporous) and activated carbon (granular; GAC and powder; PAC)] [126].

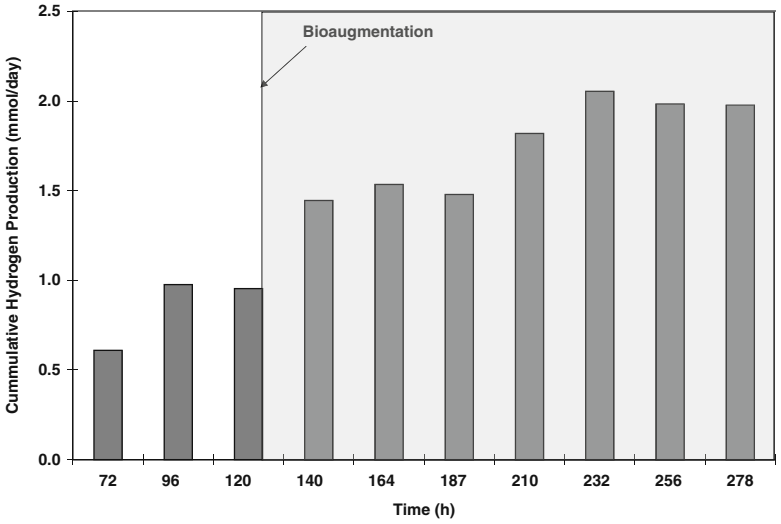

Fig. 6 H_2 production with the function of reactor operation (before and after augmentation with selective enriched H_2 producing culture) [25]

Suspended growth (SG-control) of cultures showed inhibition in terms of both H_2 production and substrate degradation, especially at higher loading rates. On the other hand, attached growth showed marked improvement in both H_2 yields and substrate degradation efficiency, particularly at higher loading rates. Self-immobilization on SBA-15 showed nine times higher H_2 production than the non-attached (SG) operation. Attached growth on GAC and PAC also showed marked improvement in the process performance at higher OLRs compared to SG operation. Immobilization of microflora on the support medium as biofilm results in high biomass hold up, which enabled the operation of the process at significantly higher liquid throughputs and OLRs. Immobilization protects the cells from environmental/chemical toxicity and from predation by other organisms and may enhance survival under extreme environments with relatively high survival rates even after prolonged storage [26, 92, 126]. Immobilized cells survive even at high temperatures.

7.6 Activators to Enhance H_2 Production

Some trace metals, organic compounds, nutrients and H^+ concentration generally have a stimulating effect on the enzymatic activity pertaining to biochemical processes and might enhance process efficiency if added at optmial concentrations (Table 5). Hydrogenases that are able to catalyze the oxidation of H_2 or the reduction of H^+ are classified into two major families: the [Ni–Fe] hydrogenases and the [Fe–Fe] hydrogenases, according to the metal content at their active site [130].

Table 5 Details of some of elements which may stimulate fermentative H_2 production process

Substance	Function	Reference
Mg^{+2}	Activates the α-subunit of catalytic site of E1 of pyruvate dehydrogenase complex (PDC) Activates the cytochromes for the efficient electron transfer	[127, 128]
Ni^{+2}	Important component for cell growth and [Ni-Fe] hydrogenase activity	[91, 129]
Fe^{+3}	Acts as micronutrient and mediates between hydrogenase and nicotinamide adenine dinucleotide (NADH)- ferredoxin reductase as electron carrier	[54]
Mn^{+}	Activates the Ni-hydrogenases and Ni-Fe hydrogenases	[127, 128]
Nitrogen	Helps in bacterial growth as nutrient and important constituent of all enzymes	[104]
Phosphorous	Has nutritious value and provides buffering capacity	[91]
Phosphoenol pyruvate	Activates the α-subunit of the catalytic site of E1 of PDC	[127, 128]
Adenosine mono phosphate	Activates the α-subunit of the catalytic site of E1 of PDC	[127, 128]
Acidic pH	Activates the NADH-dehydrogenase for the H^+ removal from the redox mediator (NAD^+) facilitaing H_2 formation	[127]

[Ni–Fe] hydrogenases have a higher substrate affinity [131]. During H_2 production process catalyzed by the [Ni–Fe] hydrogenases, electrons are transported through an intra-molecular electron transfer chain from the redox partner such as NADH or NADPH to the active site, meanwhile, H^+ are also transferred to the active site, and gets reduced by the e^- to produce H_2 [132, 133]. Since nickel is a fundamental component in [Ni–Fe] hydrogenases, it may influence the fermentative H_2 production by influencing the activity of [Ni–Fe] hydrogenases and thus plays an important role in fermentative H_2 production [91, 129]. A trace level of nickel is required for activation or function of [Ni–Fe] hydrogenases and thus is conducive to fermentative H_2 production [134]. Enhanced H_2 production potential was observed with increasing Ni^{2+} concentration from 0 to 0.1 mg/l [91]. Trace metals such as magnesium, sodium, zinc and iron showed considerable affect on the fermentative H_2 production with magnesium being the most significant one [134]. A nutrient formulation containing these four trace metals has shown a 66% enhanced H_2 production rate as compared to the control. Iron is an important element which helps to mediate between hydrogenase and nicotinamide adenine dinucleotide (NADH)-ferredoxin reductase [135, 136]. Low iron concentration limits hydrogenase activity to efficiently mediate a reversible reaction between H_2 and an electron donor such as reduced ferredoxin, thereby limiting H_2 production [2]. About a 1.59-fold increase

in H_2 production and six-fold increase in hydrogenase activity was observed by increasing the $FeSO_4$ concentration from 2.7 to 10.9 mg/l [137]. The role of metal ions (Mn^{+}, Mg^{+2}, Fe^{+3}, etc.) as well as primary and secondary metabolites (adenosine mono phosphate, phosphoenolpyruvate, etc.) which have stimulation effects on the enzymatic activity pertaining to fermentative H_2 production need to be studied to enumurate their specific function.

7.7 Molecular Engineering

Metabolic engineering is one of the promising areas which can be advantageously used to enhance H_2 production rate in dark fermentation processes. By the use of recombinant DNA technology one can try to restructure metabolic network to improve the production of H_2. Microbial metabolic manipulation by gene over expression, mutation and gene knocking out techniques were used for this purpose. H_2 molar yields can be increased significantly through metabolic engineering efforts [109]. Table 6 documents some of the work carried out in this area pertaining to fermentative H_2 production. By engineering the genetic expression of microorganisms the H_2 production rate can be influenced directly or indirectly.

8 Microbial Fuel Cell (MFC) – Bioelectricity Generation from Acidogenic Fermentation

Although H_2 produced from dark fermentation process is considered as a viable alternative fuel and energy carrier of the future, H_2 storage, purification, low production rates and the requirements of separate fuel cell systems for the generation of energy (electricity) are some of the inherent limitations. Alternatively, the microbial fuel cell (MFC) facilitates *in situ* conversion of energy in the form of bioelectricity from wastewater treatment by dark fermentation [111, 147–158]. MFC is a hybrid bio-electrochemical system, which converts the substrate directly into electricity by the oxidation of organic matter in the presence of bacteria (bio-catalyst) at ambient temperature/pressure [155, 156]. The potential developed between the bacterial metabolic activity [reduction reaction generating electrons (e^{-}) and protons (H^{+})] and electron acceptor conditions separated by a membrane manifests bioelectricity generation. In an acidogenic microenvironment, single and dual chambered MFC systems were evaluated for the production of bioelectricity using various types of wastewater viz., chemical wastewater, designed synthetic wastewater, domestic sewage and vegetable waste employing mixed cultures as anodic biocatalysts [147–158] (Table 7). The higher activity of intracellular e^{-} carriers which will help in the translocation of e^{-} from bacteria to the outside of the cell might be the reason for higher current generation observed under acidic pH operation [156]. Apart

Table 6 Details of some studied pertaining to metabolic engineering carried out to enhance fermentative H_2 production

Nature of genetic modification	Microorganism	Comments	References
Inactivating hycA gene and simultaneous overexpression of the formate hydrogen lyase activator fhlA gene	*Escherichia coli*	Improved H_2 production	[138]
Knocking out lactate dehydrogenase	*Escherichia coli*	35% improvement in H_2 production	[139]
Blocking the formation of alcohol and some organic acids using the proton suicide technique with NaBr and NaBrO3	*Enterobacter cloacae*	Improved H_2 production (3.4 mol H_2/mole of glucose)	[140]
Knocking out ackinase	*Clostridium tyrobutyricum* ATCC 25755	Improved H_2 production (2.61 mol H_2/mole of glucose)	[141]
Inactivation of *hycA* gene	*Escherichia coli* HD 701	14 fold increase in H_2 production at lower glucose concentration (100 mmol)	[142]
Overexpression of its own *hydA* gene encoding [Fe]-hydrogenase	*Clostridium paraputrificum* M121	1.7 fold improvement in H_2 production (2.4 mol H_2/mole n-acetyl glucosamine)	[143]
Inactivation of acetate kinase	*Clostridium tyrobutyricum*	1.5 fold improvement in H_2 production (2.2 mol H_2/mole glucose)	[144]
Double mutant	*Eneterobacter aerogens* strain AY2	2 fold improvement in H_2 production (1.2 mol H_2/mole glucose)	[145]
Knocking out formate hydrogen lyase (FHL) gene cluster hycABCDE in chromosomal DNA	*Enterobacter aerogenes* IAM1183	Improved H_2 production (from 18.3 to 45.2%) and purity (from 59 to 71%)	[146]
Altering expression of formate hydrogen lyase (FHL) by inactivating repressor gene and overexpressing the activator gene simultaneously	*Escherichia coli* K12	4 fold improvement in H_2 production	[147]

from power generation, the MFC also demonstrated an enhanced substrate degradation rate along with good color and total dissolved solid (TDS) removal efficiency compared to conventional anaerobic treatment [156]. MFCs can also utilize acid-rich carbon effluents generated from acidogenic processes as primary substrate for bioelectricity generation along with additional treatment efficiency.

Table 7 Studies on bioelectricity generation from the treatment of wastewater in microbial fuel cells (MFC) by acidogenic fermentation process carried at BEEC, IICT

Type of wastewater	Operation details	MFC configuration details	Maximum voltage (mV)	Maximum current (mA)	Power yield (W/kg COD_R)	COD removal efficiency (%)	References
Synthetic wastewater	pH 6.0*	Dual chamber; Nafion membrane; Plain graphite electrodes	586	2.37	0.63	72.20	[149]
	pH 7.0*	Single chamber; Nafion membrane; Plain graphite electrodes	348	2.44	0.21	48.68	[153]
Chemical wastewater	pH 5.5*	Dual chamber; Nafion membrane; Plain graphite electrodes	731	2.97	0.49	61.11	[151]
Domestic sewage	pH 6.0*	Single chamber; Nafion membrane; Plain graphite electrodes	478	2.72	4.64	66.67	[147]
Composite Vegetable waste	pH 7.0*	Single chamber; Nafion membrane; Plain graphite electrodes	266	1.51	0.231	62.86	[158]

*Anaerobic mixed culture as anodic biocatalyst without using mediators

9 Concluding Remarks

To establish an environmentally sustainable biohydrogen technology, multidisciplinary research approach is vital. Process engineering and optimization of operational factors govern the performance of any biological system and also have considerable influence on fermentative H_2 production. The persistence of an acidic microenvironment due to production of soluble acid metabolites as end-products inhibits the process leading to low substrate conversion efficiency to H_2. Apart from lower conversion efficiency, one of the important aspects to be paid significant attention is the non-utilized organic fraction that usually remains as a soluble fermentation product from acidogenic process. Various routes to utilize residual organic fraction of acidogenic process as substrate can be explored. Integration of multiple processes possible for additional revenue generation in the form of addition energy (H_2, bioelectricity, methane, etc.) and wastewater treatment utilizing acidogenic effluents are depicted in Fig 7. Application of genetic engineering aspects to stimulate conversion process efficiency is one potentially promising research area.

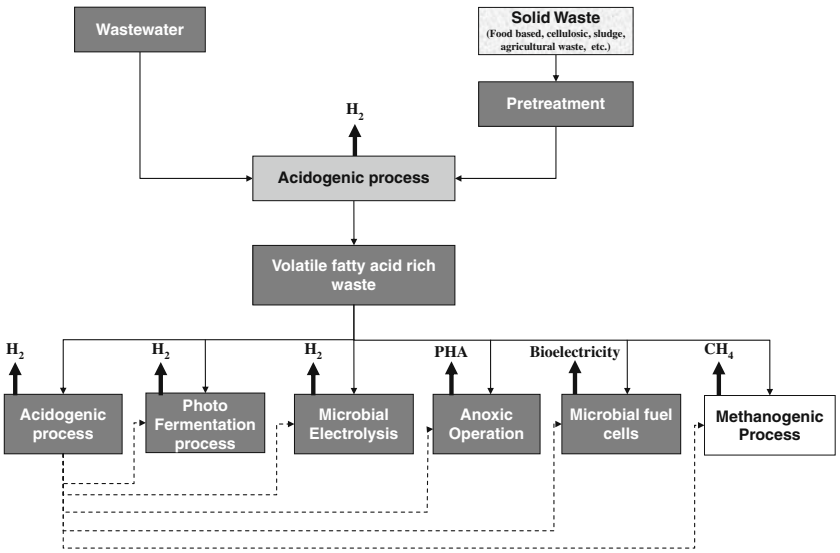

Fig. 7 Some of the possible process integration routes which can be used for generating renewable energy from waste and wastewater

Design and development of bioreactor systems for H_2 production is one of the areas where considerable focus is required. Scaling up of the process to pilot or large scale to generate baseline engineering data will sustain the technology with respect to commercialization. Interaction between the research community and industry from time to time to understand the requirements and design the technology accordingly holds the key to the successful commercialization of this process. Moreover, the process to convert existing/operating anaerobic reactors producing methane to

H_2 production will pave the way for large scale implementation of this technology and helps to achieve continuous H_2 production.

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Bacterial Communities in Various Conditions of the Composting Reactor Revealed by 16S rDNA Clone Analysis and DGGE

Keiko Watanabe, Norio Nagao, Tatsuki Toda, and Norio Kurosawa

Abstract Composting is an efficient and cost-effective process for organic waste treatment. In order to expand our knowledge regarding microorganisms in the composting reactor, bacterial community structures in a variety of composting processes were examined by 16S rRNA gene (rDNA) clone analysis including denaturing gradient gel electrophoresis (DGGE), as a case study. As previously reported, the dominant bacteria consist of members of the order *Bacillales* in a typical composting condition with woodchips as the bulking agent. However, these aerobic bacteria decreased to 14%, and anaerobes or facultative anaerobes arose when the decomposition rate of organic compounds dropped following aggregation of the contents. In the composting reactor operated with plastic bottle flakes as bulking agent, the order *Lactobacillales* co-dominated with the *Bacillales*, regardless of reactor size, accounting for about 70% of the detected organisms during first week of the operation, gradually decreasing to about 30% with maturation of the composting process. Most species detected by clone analysis have not been cultivated, and may be VBNC (viable but non-culturable) species, implying symbiotic interactions among the microorganisms. In addition, the 16S rDNA-clone and DGGE methods are also introduced in this chapter.

Keywords Aggregate · Bacterial community · Bulking agent · Clone analysis · Compost · Denaturing gradient gel electrophoresis (DGGE) · Large-scale fed-batch composting reactor · Plastic bottle · Polyethylene terephthalate · 16S rRNA gene (16S rDNA)

K. Watanabe (✉)
Faculty of Engineering, Department of Environmental Engineering for Symbiosis,
Soka University, 1–236, Hachioji, Tokyo, Japan
e-mail: kewatana@soka.ac.jp

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1 Introduction

Composting is one of the efficient and cost-effective biological processes to treat organic waste. However, some trouble may occur in a composting reactor, for example, aggregation of contents, decreasing pH and decreasing rate of decomposition. In composting processes, thermophilic and mesophilic microorganisms have important respective functions in terms of nutrient recycling and decomposition of complex organic substrates [1]. Therefore, an understanding of the microbial community and its succession is important to effectively manage the composting process. Thus, in this chapter, bacterial communities in the various composting reactors are revealed by using molecular biological methods, as a case study. These methods are similarly applicable to environmental samples, anaerobic digestion treatment reactors, and industrial reactors.

2 Background Research

Culture-based approaches have previously been used for this purpose, and various species of bacteria, e.g. members of the family *Bacillaceae*, *Clostridiaceae*, *Flavobacteriaceae*, *Neisseriaceae*, *Nocardiopsaceae*, *Staphylococcaceae*, etc., were isolated from composting reactors [2–7]. During the 1970s, a method which involved community DNA extraction from samples without cultivation of microorganisms was developed. Results of this type of analysis showed that the abundance of microorganisms which is able to be cultivated in the laboratory is less than 1%, and the remaining 99% are viable but non-culturable (VBNC) microorganisms [8, 9]. Dees and Ghiorse revealed microbial communities in compost by combining the cultivation method and 16S rDNA clone analysis. Furthermore, they detected some species which had not previously been detected from compost, and showed that the results of both methods were inconsistent, except for *Bacillus coagulans* [10]. Therefore, the cultivation method is not useful to reveal the "true (real)" microbial community.

All prokaryotes have 16S rDNA whose average length is about 1,500 bp. There are both conserved and variable regions (the V1–V9 regions), and sufficient information has been compiled with which to conduct for reliable phylogenetic analyses [8, 11]. Almost all the known sequences are registered on a DNA database, and it is possible to estimate the species on this basis. Mixed DNA extracted from environmental samples directly (without the cultivation of microorganisms) is called community DNA or environmental DNA. As mentioned above, by using 16S rDNA in this community DNA for analysis, it is possible to reveal the microbial community.

2.1 16S rRNA Gene (rDNA) Clone Analysis

Clone analysis is a method which uses recombinant DNA. PCR amplified-16S rDNA is cloned into a plasmid vector and a plasmid library is constructed. The microbial community can be estimated by analyzing this library. This method is

more cumbersome and expensive than other methods, i.e. DGGE analysis. However, it is suitable to detect minor species and to estimate species abundance. In addition, cell counting is helpful for clone analysis to estimate species abundance.

2.2 Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) is a gel electrophoresis method used to separate DNA fragments of the same length, but containing different base-pair sequences; it is used to determine the presence and abundance of different microbial species in a mixed population [12]. It is based on the principle that increasing the denaturant concentration will melt double-stranded DNA in distinct domains. When the melting temperature (T_m) of the lowest domain is reached, the DNA will partially melt, creating branched molecules with reduced mobility in a polyacrylamide gel [13]. DGGE analysis is able to compare many samples at the same time and to analyze them more easily than clone analysis and is thus suitable to reveal microbial community succession.

2.3 Case Study

2.3.1 Different Conditions of the Reactor

There are many types of fed-batch composting (FBC) reactors. However, FBC reactors cannot function continuously because the contents, which usually include waste and biomass-carriers such as wood chips, often aggregate after prolonged operation. Therefore, it is necessary to remove excess contents from the reactor regularly because of decreasing decomposition rate, and this requires secondary treatment of the aggregated contents. Since considerable amounts of partially degraded materials remain during the FBC process, secondary treatment of the products is necessary to obtain matured compost or complete degradation of waste to organic matter. Although the mechanism of aggregation in reactors is unknown, this process decreases the decomposition rate [14], and probably changes the microbial community. Therefore, this study was conducted to compare the optimal and aggregated conditions of a bacterial community in a FBC reactor using 16S rDNA clone analysis.

2.3.2 Types of Bulking Agent – Wood Chips or Polyethylene Terephthalate

In a long-term FBC reactor, a problem that is likely to occur and which needs to be monitored is the significant abrasion of the bulking agents. Nagao et al. noted that both plastic bottle flakes and wood chips were capable of maintaining a high rate of decomposition. In addition, the bacterial community was examined by 16S rDNA clone analysis and the difference in the community between the two bulking agents was compared [15].

2.3.3 Small-Scale and Large-Scale Reactor

As mentioned above, a variety of bacterial community structures in various composting reactors have been reported. Nevertheless, knowledge of the microbial community in large-scale, completely-mixed composting reactors is still lacking. Therefore, this study was conducted to clarify the bacterial community succession during the start-up period of a large-scale, completely-mixed composting reactor by using 16S rDNA clone and DGGE analysis, and to compare it with the bacterial community in a small-scale reactor.

3 Technical Details-Materials and Methods

3.1 Operation of the Reactors

Small- and large-scale, complete-mixing, composting reactors were used in this study. Three composting reactors for household use ("Namagomi-eater" TK400-H, Matsushita Electric Works, Japan) were used as the small-scale, FBC reactors. The working volume was 15 L. The biomass carrier (or bulking agent) comprised about 5 L of wood chips with a size range of 0.5–2.0 mm, or plastic bottle flakes with a size range of 2.0–10.0 mm. An artificial organic waste sample, made up of 500 g wet wt dog food (VITA-ONE, Nihon Pet Food, Japan) containing about 90% water, was loaded daily into each reactor. The contents in the reactors were gently mixed by automated paddles for 1 min each hour. Mechanical heating was used to maintain the temperature in all the reactors above 35°C to accelerate biodegradation. In the small-scale reactor, three experimental conditions (reactor A, B, and C) were used. In reactor A and C, a high decomposition rate of organic materials was maintained by "partial washing" [14, 16] as follows. Approximately 10% of the contents (0.75 L) were taken out every three days, mixed with 10 L of water, and then filtered on a 35 μm mesh filter. Upon filtration, the solid part retained on the mesh filter was dried in an oven at 60°C for 48 h, and then re-loaded into the reactor. This process prevents not only a decrease in decomposition rate but also aggregation of the contents in the FBC reactor [14]. In reactor B, there was no maintenance, except for moisture content, where spontaneous aggregation was allowed in the decomposition process. The moisture content in each reactor was kept at 40–50% by the addition of distilled water. Samples were obtained from each small-scale reactor after 60 days of operation.

To compare the difference of the scale of the reactor, large-scale composting reactor was also used (O-1, which we constructed). The working volume was 4 m^3. The bulking agent comprised about 2 m^3 of plastic bottle flakes with a size range of 2–10 mm. 600 kg wet weight food waste derived from a school cafeteria with 73% moisture content, was loaded into the reactor at the start of the experiment. This is termed batch operating. The contents of the reactor were gently mixed by automated paddles at 1.5 r.p.m. for 30 min once a day. Temperature was not regulated. Samples were obtained from the large-scale reactor, once a day, for 25 days. In the small-scale

reactor (reactors A, B, and C), 16S rDNA-clone analysis was performed to compare difference in the bacterial communities under each set of conditions, and in the large-scale reactor, both 16S rDNA-DGGE and clone analysis were performed to analyze how bacterial community succession changes day by day.

3.2 Extraction of Community DNA from Samples

Community DNA was obtained from samples by using a kit for extracting DNA from soil samples, e.g. UltraClean Soil DNA (Mo Bio Laboratories, USA), which includes different scales, and their use is recommend depending on the sample condition.

3.2.1 16S rDNA Clone Analysis

Construction of 16S rDNA Clone Library

Community DNA was used as the template DNA. Bacterial partial 16S rDNA (about 1,500 bp) was amplified by PCR with a forward primer B27F (5′-AGAGTTTGATCCTGGCTCAG, *Escherichia coli* position 9-27) and a reverse primer U1492RM (5′-GGYTACCTTGTTACGACTT, *E. coli* position 1512-1492) [17]. Amplification by PCR comprised 25 cycles of 30 s at 94°C, 30 s at 60°C, 1.5 min at 72°C, and a final extension of 5 min at 72°C using *Ex Taq* DNA polymerase (Takara Bio, Japan). PCR products were purified using a DNA purification kit (e.g. GFX PCR DNA and Gel Band Purification Kit, GE Healthcare, UK) and cloned into the plasmid vector (e.g. pT7Blue T-Vector, Novagen, Germany).

Transformation of *E. coli* and Sequencing of 16S rDNA Clone Library

E. coli strain DH5 alpha was transformed with this plasmid library. Plasmid DNAs were prepared from the cultures. The 16S rDNAs were sequenced using an appropriate DNA sequencer (e.g. DNA Analyzer 3730 *xl*, Applied Biosystems, USA).

Homology Search and Estimation of Phylogenetic Affiliations

A homology search was conducted using BLASTN database (BLAST, http://www.ncbi.nlm.nih.gov/BLAST/) [18]. Checks for chimeric sequences were conducted using the software Pintail [19] which is available from the Ribosomal Database Project, followed by NCBI BLASTN database [18].

Analysis of Homologous Coverage

Coverage of the clone library describes the extent to which the sequences in the library represent the total population. In order to calculate the coverage of a library, the criterion for what constitutes a unique sequence must first be decided. Various studies have used different criteria, generally based on sequence similarity (e.g. 97

or 99% similarity) or evolutionary distance (e.g. < 0.01). These values can then be used to plot a coverage curve (C vs D) that describes how well the library represents the total community given varying criteria of uniqueness. The homologous coverage (Cx) is calculated by the following formula Cx = 1–(Nx/n), where Nx is the number of unique sequences in the sample and n is the total number of sequences [20].

Construction of a Phylogenetic Tree

Sequences are aligned using the Clustal W program version 1.7 [21, 22], and all sites with gaps in any sequences and the regions of PCR primers are removed from the alignment. The phylogenetic trees are constructed by the neighbor-joining method [23] or the maximum-likelihood method [24] using the PHYLIP (Phylogeny Inference Package) program version 3.5c (http://evolution.genetics.washington.edu/phylip.html) [25]. The stability of relationships was assessed by performing bootstrap analyze of the neighbor-joining data based on 1,000 resamplings.

3.2.2 Denaturing Gradient Gel Electrophoresis (DGGE)

PCR Amplification of 16S rDNA

Community DNA was used as the template DNA for PCR. The bacterial variable V3 region of 16S rDNA was amplified by PCR with a forward primer, with a GC-clamp on the 5′ terminals, B341F-GC (5′-CGCCCGCCGCGCCCCGCGC CCGTCCCGCCGCCCCCACCCGCCTACGGGAGGCAGCA-3′, *E. coli* position 341-356) and reverse primer B515R (5′-TTACCGCGGCTGCTGGCAC-3′ *E. coli* position 533-515), which are modified Ellis et al. primer sequences [26]. Amplification of 16S rDNA by touch-down PCR using DNA polymerase comprised 3 min at 94°C, 10 cycles of 30 s at 94°C, 30 s at 65°C, 30 s at 72°C, and the annealing temperature was lowered for 1°C every 2 cycles from 65 to 60°C. Then, 30 cycles of 30 s at 94°C, 30 s at 60°C, 30 s at 72°C and a final extension of 3 min at 72°C was carried out. The PCR products were purified by using commercially available purification kit (i.e. GFX PCR DNA and Gel Band Purification Kit, GE Healthcare, UK).

Denaturing Gradient Gel Electrophoresis

Procedures for gel casting and electrophoresis were according to manufacturer's instructions. DGGE was performed on an 8% polyacrylamide gel with a 20–60% denaturant gradient, where 100% denaturant corresponds to 7 M urea and 40% formamide. Electrophoresis was run at a constant temperature of 58°C for 4 h at 200 V, using a DCode universal mutation detection system (Bio-Rad Laboratories, UK). PCR products separated on the gel were stained with 20 μL of ethidium bromide (10 mg/mL) in 200 mL of the buffer, which was used for electrophoresis, for 30 min and washed with the buffer for 30 min (3X 10 min washes) and then photographed with ultraviolet irradiation. A substitute for ethidium bromide,

SYBR Green I (Invitrogen, USA), can also be used for staining. In this case the concentration is 20 μL (undiluted solution) in 200 mL of distilled water.

DNA Recovery from DGGE Gel and Sequencing of 16S rDNA

Major DGGE bands were recovered from the gel by using the razor or nib of a Pasteur pipette, and washed with sterile purified water to use as template for re-amplification of PCR performed using the original protocols except for the forward primer, B341F (5′-CCTACGGGAGGCAGCAG-3′, *E. coli* position 341-356). The partial 16S rDNAs were sequenced by using an appropriate DNA sequencer, and a homology search was then conducted using the BLASTN database [18].

Cell Counting

About 0.2 g wet wt of samples from the reactors were each suspended in 5 mL of saline and gently shaken for 10 min at room temperature. Thereafter, 0.1 mL of the suspension was diluted with 0.9 mL of saline and subsequently filtered through a 0.02 μL-pore-size Anodisc filter (Whatman, UK). One drop of 0.25% of SYBR Gold (Invitrogen, USA) was placed on the Anodisc filter and kept in the dark for 15 min. The stained Anodisc filter was completely dried and mounted on a glass slide with a drop of antifade reagent (SlowFade Antifade Kit, Invitrogen, USA). The enumeration of cells was estimated by direct cell counting using epifluorescence microscopy [27]. The mean value was obtained from 20 independent experiments.

4 Current Outcome of Technological Implementation

With the progress of molecular biological techniques, microbial analysis methods have improved. 16S rDNA clone analysis and DGGE method have made possible to clarify the accurate microbial community structures including VBNC microorganisms that could not be detected by traditional culture dependent method.

As a case study, we compared the bacterial communities in (1) different conditions (decomposing rate) of the reactors, using (2) different types of bulking agent (wood chip or polyethylene terephthalate), and in (3) small-scale and large-scale reactor. Each result showed different communities and presence of the VBNC microorganisms (Figs. 1, 2, 3, and 4).

As previously reported, the dominant bacteria consisted of members of the order *Bacillales* in the typical condition of composting with woodchips as bulking agent [2–7, 10]. In reactor A, the order *Bacillales* was also dominant. However, in reactor B, they decreased to 14%, and anaerobes or facultative anaerobes increased when the decomposition rate of organic compound dropped following aggregation of the contents (Figs. 1 and 2). This might be correlated with accumulation of persistent substance like inorganic salt and decreasing of oxygen concentration [1]. The order *Lactobacillales* was not detected from the reactor A and B which using woodchips as bulking agents, however, the order *Lactobacillales* were detected from reactor

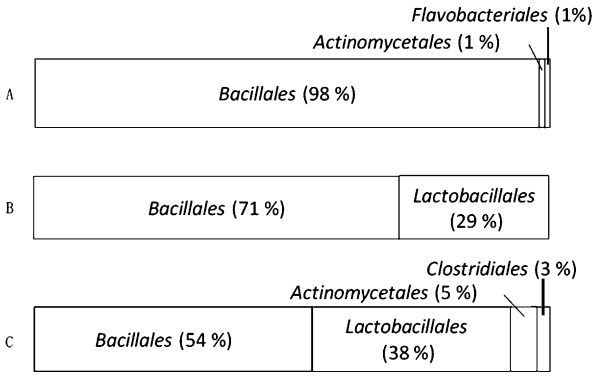

Fig. 1 Structure of bacterial community in small-scale reactors (reactor *A*, *B*, and *C*). Clones are classified by order level

C as dominant order (38%) which using plastic bottle flakes as bulking agents [15]. In general, the genus *Bacillus* is often detected from the reactor as the major species in the composting process [5, 6, 10, 28], and there are only a few reports in which the order *Lactobacillales* in the composting process is dominant [29, 30]. The dominance of the order *Lactobacillales* might be correlated with the type of bulking agent, because the bulking agent strongly affects the oxygen concentration or heat-retaining property in the reactor. There are three possible reasons for the dominance of the order *Lactobacillales*. Firstly, plastic bottle flakes do not have a water-absorbing property at the beginning of the run, therefore the moisture content of the reactor at the starting period was high and the inside of the reactor might be microaerophilic condition. This might be because the order *Lactobacillales*

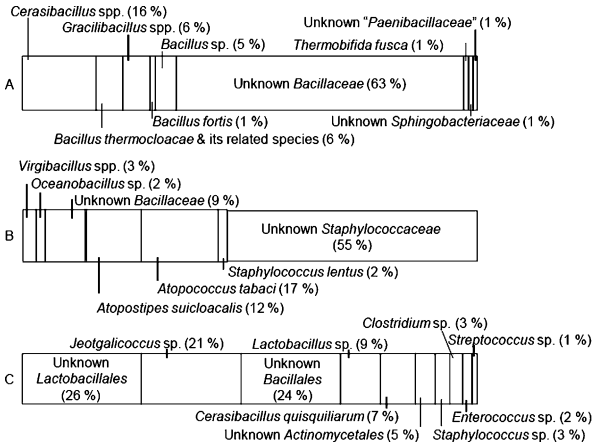

Fig. 2 Structure of bacterial community in the small-scale reactors (reactor *A*, *B*, and *C*). Clones are classified by species level

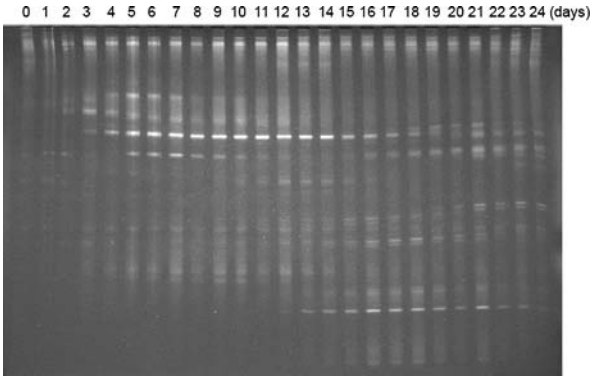

Fig. 3 DGGE profile of the sample from day 0 to 24 in the large-scale reactor

is dominant in an environment in which it can proliferate easily. Secondly, since decomposing waste attached to the surface of plastic bottle flakes is accompanied by decomposing organic waste, there is an anaerobic or microaerophilic condition between attached waste and plastic bottle flakes, i.e., aerobic, anaerobic, aerotolerant and microaerophilic microorganisms might coexistent in the reactor. Thirdly, the size of plastic bottle flakes is bigger than the woodchips. This may be inhomogeneous distribution, in which mixed aerobic and anaerobic conditions would occur partially in the reactor. Or because of density depression, heat insulation capacity of plastic bottle flakes was less than the woodchips [31].

In the small-scale reactor (reactor C) and in the large-scale reactor, operated with plastic bottle flakes as bulking agent, the order *Lactobacillales* also dominated with *Bacillales*, regardless of the reactor size (Figs. 1, 2, and 4). In the large-scale reactor, DGGE analysis showed that the bacterial community changed throughout the operation (Fig. 3). All of the bands before day 11 were of the order *Lactobacillales*

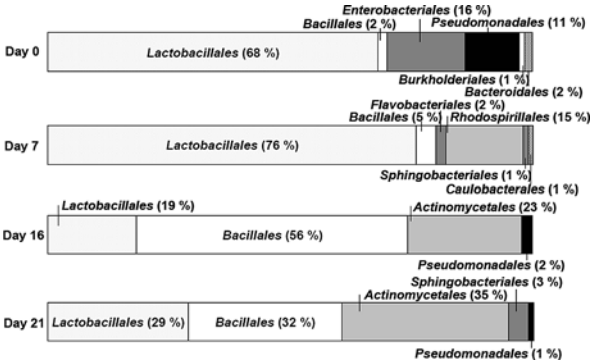

Fig. 4 Structure of bacterial community at day 0, 7, 16 and 21 in the large-scale reactor. Clones are classified by order level

(*Lactococcus* sp. and *Lactobacillus* sp.). They were dominant in the first 14 days, and these results showed a possibility that the dominant order of the *Lactobacillales* and decreasing pH may be correlated (data not shown), and this may cause rancidity depending on operating conditions [31]. After which order of the *Lactobacillales* decreased and other species, e.g. *Bacillus coagulans*, *Corynebacterium* sp., appeared. *B. coagulans* was often detected from various composting reactors [5, 10, 28, 29], even in different systems or wastes. Ishii et al. indicated how the acidic environment early in this phase is suitable for *B. coagulans*, which requires a slightly low pH value (6.0) for the initiation of growth [29]. The samples derived from day 0, 7, 16 and 21 were also analyzed by 16S rDNA clone analysis, which showed a bacterial community succession from the order *Lactobacillales* to the order *Bacillales* and *Actinomycetales* (Fig. 4). The large-scale reactor was batch operated. Therefore, this community succession, similar to field heaping compost, might be occurred.

In conclusion, 16S rDNA analysis showed that microbial community structures depended on the type or condition of the reactors, and some species can be used for indicators of reactor conditions. However, the family *Bacillaceae* is generally dominant or detected even though each composting reactor has independent microbial community structures. As we expected, unidentified genus were detected from each reactor. For example, 65% of the total clones might be derived from VBNC microorganisms in the reactor A (Fig. 2). It will be necessary to reveal the function of these VBNC microorganisms for totally understanding of biological reactions in the reactor.

5 Expert Commentary and 5 Year View

In recent years, microbial analysis by using molecular biological methods have been applied and developed to reveal its community or succession, to detect and determine quantity of them, Clone analysis and DGGE analysis, which were introduced in this manuscript, are able to apply ecological analysis in various environments including the artificial reactor. For example, to reveal the microbial community or succession in the soil, various aquatic environments, artificial reactor or processes such as anaerobic methane fermentation reactor, composting reactor, sewage disposal system, food and beverage industrial reactor (i.e. beer, juice) or ethanol fermentation reactor, and so on.

Clone analysis and DGGE analysis are useful to detect the contamination of microorganisms. Bacterial contamination is frequently a problem with yeast fermentations for the production of ethanol or food industrial reactors because of spoilage bacterium or fungus contamination. However, these methods are not able to give absolute quantity of microorganisms. Therefore, it is important to combine use of these methods and cell counting by using SYBR Gold (described in this manuscript) or Fluorescence in situ hybridization (FISH). FISH can detect not only absolute quantity but also biomass ratio, and it can detect microbial presence in which part of the samples.

In addition, some quantitative methods using PCR (termed quantitative PCR or Real-time PCR) have also been developed to detect and determine quantity specific microorganisms rapidly. Therefore, if you want to detect contamination, monitor or quantitative of some specific species, Real-Time PCR method is better than Clone analysis or DGGE analysis. Pedro et al. showed that the population changing of some bacteria by using Real-Time PCR [32]. One of the practical applications of the Real-Time PCR is to detect the harmful plankton which cause of red tide or shellfish toxin organism [33]. Moreover, these molecular biological methods can analyze not only prokaryote but also eukaryote by changing the PCR primer. Conventional methods for monitoring some process conditions like composting are texture or odor, which require experience. By using the molecular biological methods it is possible to analyze objectively and reproducibly and to prevent trouble in the reactor by optimizing operating conditions. As mentioned above, one can choose these methods in accordance with the intended use for general and applied microbiology.

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Perspectives on Bioenergy and Biofuels

Elinor L. Scott, A. Maarten J. Kootstra, and Johan P.M. Sanders

Abstract Alternative and renewable sources of energy have received much attention and there are a number of approaches: wind, hydro, solar nuclear and the use of biomass. Here discussion will focus on the use of biomass, however there are a number of alternative methods in applying this as a source of "energy". For example incineration (to generate heat and electricity), production of biodiesel and biomass to liquids (BTL) by chemical and thermal based processes respectively and the production of bioethanol and biogas using biotechnology. However the use of biomass for non-food applications raises a number of important issues which need to be considered for any conversion process such as: food production and price increases, destruction of the rainforest and greenhouse gases. Strategies which circumvent these should be explored and one potential route is the use of waste lignocellulose biomass (derived from primary agricultural practice) and its application for energy and fuels.

Keywords Lignocellulose · Bioethanol · Bioenergy

1 Introduction

Sustained global demand for fossil materials continues. In light of environmental concern, as well as unease over fluctuating prices due to supply and global events, the search for sustainable alternatives are being explored. Use of fossil resources for heat and electricity (industrial, commercial and residential), transportation fuels and chemical raw materials represent the major areas of use. Using European figures as a guideline, ca. 25% of all oil is used in the transportation sector [1]. Governmental and environmental bodies are currently setting targets for the reduction of CO_2 production and use of fossil raw materials, thus contributing to the replacement

E.L. Scott (✉)
Valorisation of Plant Production Chains, University of Wageningen, 6700 AA Wageningen, The Netherlands
e-mail: elinor.scott@wur.nl

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of petrochemical fuels with biofuels could make a significant contribution. This is exemplified by the European directive EC 3003/30/EC which deals with the promotion of the use of biofuels or other renewable fuels for transport [2]. Within this directive topics such as the objective of a 20% substitution of conventional fuels by alternative fuels in the road transport sector by the year 2020 as in The Commission Green Paper "Towards a European strategy for the security of energy supply" and that the promotion of biofuels in keeping with sustainable farming and forestry practices could create new opportunities for sustainable rural development.

Alternative and renewable sources of energy have received much attention and there are a number of approaches: wind, hydro, solar nuclear and the use of biomass. Here discussion will focus on the use of biomass however there are a number of alternative methods in applying this as a source of "energy". For example incineration (to generate heat and electricity), production of biodiesel and biomass to liquids (BTL) by chemical and thermal based processes respectively and the production of bioethanol and biogas using biotechnology. However the use of biomass for non-food applications raises a number of important issues which need to be considered for any conversion process.

1.1 Biomass for Non-food Applications and Possible Adverse Effects

Biomass is widely cultivated for non-food applications: cotton for clothes, timber for paper, building materials and furniture and tobacco for cigarettes. However the production of biofuels has initiated much discussion with regards to food versus fuel and land use.

1.2 Food Production and Price Increases

Recently the prices of foods such as rice, wheat and corn have seen a steep rise, which has led to protests in countries including Haiti, Egypt, Cameroon, Ethiopia, Yemen, the Philippines, Thailand, and Indonesia.

According to the report "World agriculture: towards 2015/2030" (launched by the UN Food and Agriculture Organization (FAO)), there will be enough food for a growing world population by 2030, but millions of people in developing countries will remain hungry and many of the environmental problems caused by agriculture will remain. This sentiment is echoed in the report "International Assessment of Agricultural Science and Technology for Development" published in April 2008 which discusses how new agricultural technology has brought increases in food production, but at a high environmental cost and has not really benefited the poor in developing countries and suggests more focus is required on the needs of small farms by improving income and providing a fair market for their products.

The World Bank and the International Monetary Fund, amongst others, have also commented on some of the many causes of rising food prices, which includes bad

weather, high oil prices for oil, increased demand for meat and dairy products in the some Asian countries and the push in the West to use biofuels derived from grain, especially maize, to reduce oil consumption. A knock on effect on the increase in the price of other crops (not for fuel application) may be envisaged by example in the production of first generation bioethanol, which uses carbohydrates such as starch and sugar from maize and sugarcane, which results in better prices for these crops. Thus farmers may switch production to these as opposed to the production of others crops, resulting in a price increase.

1.3 Destruction of the Rainforest

The production of biodiesel relies on the use of crops such as soya, rape, and palm which are pressed releasing oils (and rest presscake) which are transesterified yielding fatty acid mono alkyl esters (usually methyl) and glycerol and the topic of growth of these. For example the growth of soya in South America, and in particular in Brazil, has raised discussions about the destruction of the rainforest for soya production. The production of sugarcane in the Amazon is less suitable due to the wetter climate, however due to expansion of production of sugarcane, land used for soya production is therefore reduced leading to supplementary production of soya in the Amazon.

1.4 Greenhouse Gases

For the conversion of non-agricultural land such as grasslands to production of maize (for bioethanol) would first require removing the plants/grasses and converting it to agricultural land. This process leads to an overall increase in CO_2 emissions [3]. The effect of using fertilisers for cultivation should also not be ignored. Here the greenhouse gas N_2O is generated. For cultivation of forest areas the story is similar, as large amounts of CO_2 are formed as the organic material in soil (humus) is decomposed by microorganisms, taking many years of using the biofuel produced on that site to redress the CO_2 balance (paying back the carbon debt) [4]. However biofuels made from waste biomass, for example, has little or no carbon debt.

As well as the conversion of land use generating greenhouse gases, production of the biofuel itself should be considered. In the case of the distillation process of bioethanol, heating using coal is often used.

Therefore when considering the approach of converting biomass to fuel, smart strategies as to obtaining biomass that does not negatively interfere with food production and result in unfavourable land changes as well as having a good greenhouse gas balance with respect to obtaining the biomass and its conversion are of importance. As well as this one should not forget the potential for small scale farmers in developing countries to obtain benefits from the crops that they are cultivating.

1.5 Waste Biomass and Its Application for Energy and Fuels

The use of waste biomass is seen as a potential source of energy and fuel based on carbon debt. However for production, collection and transportation of this waste (often containing water) is required and at what economic cost? What types of conversion technologies are available at what scales and investment costs?

1.6 Biomass to Liquids (BTL)

BTL is a thermal based process to making biofuels from biomass including agricultural waste. Up till now the conversion of biomass to biofuels has generally focused on particular components of the biomass such as carbohydrates (for bioethanol) and fatty acids (for biodiesel) being used. In the BTL process the whole plant (if required) can be utilised in a multi-stage (thermal) process, which has been conventionally used in the fossil based industries. Initially the biomass undergoes a gasification process producing synthesis gas which is then converted via a Fischer-Tropsch (FT) process to hydrocarbons, which can be further processed using hydrocracking [5]. However such technology requires large amounts of the thermal exchange capacity. This in turn would require higher capital investments. Due to the types of investments required it could be expected that operation of such a facility may be operated in a more centralised location operating at larger scale, thus reducing the possibilities for opportunities for local communities.

1.7 Biogas

The production of biogas utilises a wide variety of raw materials with many using waste streams from other industries. A general description of the process is given in Fig. 1.

The raw materials can range from manures, waste from food production (and supermarkets) to rest streams from farming. The quality of the biogas should be improved to remove impurities such as water and hydrogen sulphide to allow it to be used as a methane source in traditional pipeline supplies and other installations. Biogas in this state may be used as traditional fossil methane (natural gas) that can be used as a source of energy (for heat and electricity) as well as transportation fuel. In general production is less dependant on specific crops for the process and thus biogas installations are being developed where organic waste is generated and offers the possibility for small scale production close to waste sites, so land use for its production is less controversial. A similar situation can be regarded for biomass incineration (not to be confused with incineration of municipal waste) which uses organic rest streams from agriculture (for example straws) and manures (such as chicken droppings). However, here only energy can be produced.

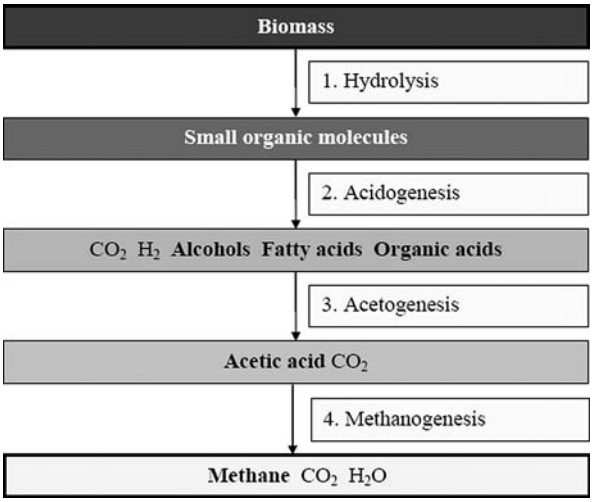

Fig. 1 The production of biogas from biomass: hydrolysis of biomass results in the formation of sugars, fatty acids and amino acids which are further converted to methane, carbon dioxide and water

1.8 Second Generation Bioethanol Production

There have been a wide number of reports on second generation bioethanol production as it utilises lignocellulosic rest streams (generated from primary agricultural production) such as straws from grain production, as a potentially abundant, inexpensive source of carbohydrates. A key technology in this approach is the use of bio-refinery where a particular crop may be separated into various fractions for food and other applications such as animal feed and raw materials for biofuels and materials. However there are some other technical challenges and in general this focuses on:

- The process(es) of obtaining the fermentable sugars from the cellulose (and hemi-cellulose).
- The conversion of other fermentable sugars e.g. xylose (together with glucose) to ethanol.

A schematic view of the general process is given in Fig. 2. Based on this general route there are a number of well documented processes.

- The BC International process uses an acidic hemicellulose hydrolysis which allows the resultant xylose containing liquid to be separated from the solid cellulose and lignin fraction. The cellulose is then hydrolysed to glucose (under acidic conditions) and two parallel fermentations of xylose (with a modified *E. coli*) and glucose to bioethanol takes place.

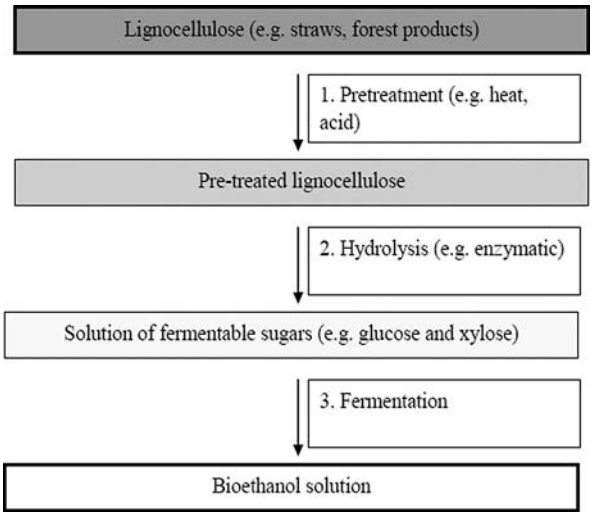

Fig. 2 The conversion of pretreated lignocellulose followed by hydrolysis and fermentation to bioethanol

- In the Iogen Corporation process the biomass undergoes as initial steam explosion and acidic hemicellulose hydrolysis to xylose, followed by a cellulose hydrolysis to glucose (under cellulase enzyme conditions). Subsequently, solid lignin is removed by separation and finally a simultaneous xylose-glucose fermentation to bioethanol takes place.
- The National Renewable Energy Laboratory (NREL) process uses steam pretreated biomass for an acid hydrolysis of the hemicellulose and the resultant mixture is fed to a reactor where simultaneous cellulose hydrolysis and xylose and glucose fermentation (using a genetically modified *Zymomonas mobilis*) takes place – simultaneous saccherification and co-fermentation (SSCF) [6].

1.9 Lignocellulose Pre-treatment for Bioethanol Production

Lignocellulose material consists of ordered (semi-crystalline) chains of cellulose, filled with lignin, hemi-cellulose and other organic compounds. There are a wide number of papers [7–9] that describe the role and potential mechanism of the pretreatment step. In order to make the carbohydrates contained in the lignocellulose more amenable to hydrolysis it needs to undergo a pretreatment step. Some have considered the use of lime [10], steam and the use of mineral acids such as sulphuric acid.

It is of importance in the (pre)treatment of lignocellulose with the use of acids (and in particular mineral acids) that process conditions are sufficient to allow pretreatment but should not lead to unacceptable amounts of degradation products of sugars such as furfural from pentoses and hydroxymethylfurfural (HMF)

from hexoses [11, 12]. This would not only lead to loss of fermentable sugars (lowering possible ethanol yields) but that these degradation compounds themselves may prove inhibitory in the fermentation process [13, 14] itself, again leading to lowering the efficiency of the process.

1.10 (Ligno)Cellulose Hydrolysis

Sulphuric acid can also be used for the hydrolysis conversion of the carbohydrates to free fermentable sugars, however in order to minimise acid costs as well as generating waste streams containing large amounts of inorganic waste recycling and reuse of the sulphuric acid. Such processes have attracted a lot of attention, with the Arkenol concentrated sulphuric acid process already being operated by NEDO/JGC in Izumi, Japan and BlueFire Ethanol Inc. Using their patented technology [15] all components of the biomass have found application and auxiliary reagents such as the sulphuric acid may be reused (via ion exchange) and residual acid converted to gypsum. Other methods using sulphuric acid are being explored [16]. The sulphuric acid recycling focuses on the use on anion selective membranes [17] and anaerobic treatment [18] – The Biosulfurol Process.

It is also possible to use enzymes (cellulases) in the hydrolysis process step. There has been a large amount of discussion as to the actual cost contribution of the cellulases per litre (or gallon) ethanol with early studies showing very high costs. Thus development in this area was required to improve enzyme production and efficiency to improve the costs of cellulase use. Strategies to help reduce cellulase production costs include strain improvement, using mutagenesis for example, or isolation of overproducing strains. The two major companies in the field, Novozymes and Genencor, have undertaken significant effort in cellulase development and this has resulted in cost reductions. In 2004 Novozymes announced a twelvefold reduction in the enzyme cost contribution in bioethanol production from >$5.00 to <$0.50 per gallon bioethanol [19].

1.11 Fermentation of Sugars

Traditional bakers yeast (*Saccharomyces cerevisiae*) allows ready conversion of glucose to ethanol. However in order maximise ethanol production, utilisation of pentoses (such as xylose and arabinose) that are released during pretreatment and hydrolysis should also be converted. Approaches include genetically engineered *Saccharomyces cerevisiae* yeasts that can ferment both xylose and glucose have seen development [20–22] and modified *Zymomonas, E. coli* and *Klebsiella* bacteria with improved pentose metabolism. Such progress in the field has culminated in announcements such as those from Danisco and DONG Energy to open a demonstration plant.

2 Technical Details and Status of Technological Implementation

Consider a biomass type (typical of a lignocellulosic material) with a composition of ca. 55% carbohydrates, ca. 15% lignin, ca. 10% protein and ca. 20% other organic components with a calorific value of 15–18 GJ of energy per tonne (dry weight) [23].

2.1 Q: Burn or Bioethanol?

- Assuming use for incineration for heat and electricity, comparisons with coal should be made. The biomass could be expected to generate 15–18 GJ of energy which could replace ca. 0.6 tonnes of coal.[1] Assuming coal has an average carbon content of 80%, then combustion of one tonne biomass could also save ca. 1.8 tonnes of CO_2 emissions [24][2]. Due to the lower cost of coal compared to other fossil materials, €3 per GJ[3] [25], the biomass has its lowest financial value. In this example it would have a potential value (based on coal) of ca. €50 per tonne.
- Should the same biomass be used for the production of ethanol then, assuming full conversion of all carbohydrates, 275 kg of ethanol could be produced (with 450 kg of unused organic material). The use of the ethanol[4] as a fuel could generate ca. 8.3 GJ of energy and combustion of the unused organic material could generate up to 8.1 GJ of heat energy. In terms of energy (providing no losses due to process) then this is comparable with the example above. However when the costs per GJ for the applications are considered this changes. Fuel sources such as petroleum have a value of ca. €8 per GJ. Thus the biomass could have a total value of ca. €90 per tonne for example (ca. €66 for fuel application and ca. €24 for heat application).

Thus one may conclude that the use of lignocellulosic material could generate more added value if used to produce fuel and more effectively if all components are used for an application providing the costs for isolation and/or transformation are favourable.

Is it possible to develop processes for the conversion of lignocellulose to bioethanol without (or reduce) the use of large quantities of corrosive sulphuric acid (for pretreatment and hydrolysis) circumventing the need for recycling of mineral acids and use of other chemicals such as lime?

One possible approach is a short, (low concentration organic) acid pretreatment followed by hydrolysis with an aqueous solution of cellulase enzymes. In such an approach there are several issues that need to be explored.

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[1] Calorific value of ca. 24 GJ per tonne

[2] 600 kg × 0.8 = 480 kg C, combustion of C results in 480 kg × 44/12 = 1,776 kg CO_2

[3] usually expressed as currency per British thermal unit (Btu), 2007 figures

[4] Calorific value of ca. 30 GJ per tonne

2.2 Pretreatment

- Can acid pretreatments only be carried out with sulphuric acid or is it also possible with other acids for example with (low levels) of less corrosive organic acids?
- What does the effect of the potential pretreatment conditions have on carbohydrate decomposition?
- What are the relative costs of traditional pretreatments (including capital costs) compared to potential alternatives?
- Are there possibilities to reduce costs?

2.3 Pretreatment Experiments

From preliminary studies it was found that pretreatment of various lignocellulose materials that a variety of acids, acid concentrations, temperatures and times could lead to interesting levels of glucose (after enzymatic hydrolysis) [26]. Based on this a more detailed study was performed where wheat straw was pre-treated with water (reference solution), sulphuric acid, or various organic acids (maleic and fumaric acid), at acid concentrations of 50 mM, over a temperature range of 130–170°C for a 30 min period. The effect of such conditions on furfural and HMF formation and fermentable sugar yields (after enzymatic hydrolysis) were studied [27].

After pretreatment only a small amount of glucose was formed, with most being formed, together with low levels of HMF, under higher temperature conditions using sulphuric acid. The production of xylose from hemicellulose (and degradation to furfural), was more pronounced under increasingly acidic conditions. It was found that the use of acid was in all cases superior to that of only water in increasing the susceptibility of lignocellulose for enzymatic hydrolysis to glucose. In general increasing temperature and use of acids, with increasing acid strength (Pk_a), led to higher levels of glucose being produced. Encouragingly the use of both sulphuric acid and maleic acid yielded near stochiometric amounts and the weakest (organic) acid used, fumaric acid, also resulted in very high levels (ca. 85%).

2.4 Pretreatment Costs and Acid Recovery

To determine if potential process costs could be reduced by decreasing the heating requirements by the use of smaller reactor volumes in the pretreatment stage, higher concentration of straw were pretreated, keeping the weight ratio of straw:acid constant. The results on glucose yield (after enzymatic hydrolysis) mirrored those previously observed using lower concentrations of straw, so such an approach could be a positive method of reducing costs.

However, compared to the reported traditional routes using sulphuric acid, the costs of organic acid per tonne ethanol still remain high as a method of pretreatment. It is estimated that the costs of the organic acid are ca. €300 per tonne ethanol

produced. In order to reduce costs it is thus imperative to recover the acid aqueous stream after pretreatment.

From investigations of the aqueous stream, after the solid pre-treated biomass had been separated, it was found that a significant percentage of the organic acid was lost from the aqueous phase (with respect to input), thus suggesting it is intimately attached to the straw. Indeed when the solid pre-treated straw was used in the subsequent aqueous enzymatic hydrolysis procedure, it was found that the remaining acid was detected in solution. To what extent the presence of organic acid in this media would have on the following fermentation process still remains to be investigated. However it does raise the issue, one acid solution recovery (directly after pretreatment) or two?

2.5 Enzymatic Hydrolysis

Within this study readily available cellulase from Genencor was utilised under the most optimal conditions that they recommend. No attempt to optimise these conditions were attempted, as the greatest contribution to improving this process step is the enzyme (and its production) itself and its price tag. This is an issue that producers such as Novozymes and Genencor are tackling.

2.6 Adding Value to Rest Streams

During the conversion process some organic material remains. Is it possible to use this for application as ruminant feed? Straw is not a significant source of dietary protein and its value as ruminant feed is dependant on the carbohydrate content. Very effective pretreatments of straw, as in the case of the use of sulphuric acid, would result in a rest stream low in carbohydrates and contain amounts of furfural and sulphate residues, making it unsuitable for animal feed. In the case of fumaric acid pre-treated straw some carbohydrates would still be present. The presence of non-recovered fumaric acid may also have some extra significance. In vitro studies have shown that methane emissions may be lowered [28, 29], thus fumarate in ruminant feed could prove a positive addition.

So to answer the original questions. . . pretreatments of lignocellulosic materials at elevated temperatures do not only need to be carried out using sulphuric acid. It is possible, under the same conditions, to achieve high glucose yields with other acids, for example with the use of less corrosive (organic) maleic and fumaric acid that also results in less degradation of any liberated free sugars. However the use of organic acids incurs more costs than processes operating on sulphuric acid, so recovery and re-use of acid rest streams requires further attention. However it can be mentioned that the price of sulphuric acid has risen significantly in the last year from $90 to $329 per tonne [30]. It has been suggested that such increases have been perpetuated by the rise in bioethanol production. However given the very large

production volumes of sulphuric acid (versus that for use in bioethanol production) the price, just like other chemicals, will have been substantially influenced by the oil price. No doubt this price rise will have an impact on the process/operating costs.

3 Commentary on Future Perspectives

Countries around the world are increasingly more committed to climate change and reducing emissions of CO_2. The UK became the first country in 2007 to introduce a Climate Change Bill which includes legally binding targets for CO_2 reduction [31, 32]. Other countries have since then have also introduced such Bills.

There are of course many ways to approach such a challenge, including the way we use our current resources by implementing more (energy) efficient, cleaner technologies in industry and transport or the use of sustainable sources to generate heat and electricity or the use of renewable resources to produce fuels and other materials. These (and more) approaches each play a role. Since the use of petroleum for transportation fuels represents such a substantial contribution to how we use our fossil resources (and to CO_2 emission) it is perhaps not surprising that so much attention is focused on this area and the promotion biofuels as indicated in European directive EC 3003/30/EC.

3.1 Tackling Adverse Effects of the Use of Biomass for Non-food Applications

This involves smart strategies in the production and application of biomass.

- Certification of biomass
  A biomass may be certified on sustainability criteria such as the influence of cultivation and harvesting on the environment (e.g. biodiversity and emissions) and social issues such as impact on food (production and market), working conditions on the plantations and the benefits for the local community should be considered. By only using certified biomass in production processes (for biofuel) some of the issues may be tackled.

  A Carbon Certification Project (E4Tech) and Sustainability Reporting Project (Ecofys) have been defined as part of The Department of Transports (UK) renewable transport fuel obligation (RTFO). Suppliers will be required to show documentation on the net greenhouse gas reduction and sustainability of the biofuels they supply and hence such methodologies are required.
- Use of non-food crops on arid land or areal which could not otherwise be cultivated for food purposes.
  Examples are the use of *Jatropha curcas* and algae cultivation. However stimulation by governments is required so farmers grow certain quotas of food crops and prevent some growers of turning agricultural land to cultivation of bio-fuel crops.

Biodiesel production involving the use of *Jatropha curcas* (shrub found in (sub)tropical areas) as a new (alternative) source of fatty acids for its production has merited attention in the last couple of years. *Jatropha curcas* is a non-food plant (all parts are toxic) that can grow in the wild in arid conditions traditional use has usually been to make soaps and mark land boundaries. The seeds of the plant can be pressed yielding the oil required for biodiesel production. Due to growing conditions and non-food properties, it addresses issues concerning "food-versus-fuel". While this area is fairly new, entrepreneurs have already established situations where intercropping of tender agricultural crops with *Jatropha curcas* (which protects them from harsh conditions) takes place. A successful venture in Tanzania has already been established offering labour to local farmers [33]. Another development is in the production using algae. The growth of algae is dependant on sun energy and CO_2 and due to its non-food qualities also helps tackle the "food-versus-fuel" issue. Due to the depth of penetration and intensity of sunlight required for biomass production, issues of surface area and location needed have arisen. Interest in the field is high with Sapphire Energy seeing (new) investments from Bill Gates (Cascades Investments LLC) [34] as well as Venrock (Rockefeller family venture capital facility), Arch Venture Partners and Wellcome Trust [35]. As well as this KLM announced in May 2008 that aims to use biodiesel derived from algae in some of its aircraft, thus further innovation, development and commercialisations in the area are reasonably expected.

- Use of (lignocellulosic) rest streams from primary agricultural production, as in the case of second generation bioethanol and biogas.
  It is reasonable to expect that improved biorefinery of agricultural materials will produce sufficient quality food and will lead to suitable biomass waste streams that can be used for biofuel production and other fractions that could be used for value added applications. As discussed elsewhere (Perspectives on chemicals from renewable resources), biorefinery is taking place for a number of renewable raw materials although it will require further development in order to obtain all (useful) fractions separated from biomass. However with such rest streams in hand technology is currently well placed in utilising them for bioethanol production.

  The (effective) pretreatment (and hydrolysis) of biomass often uses reagents such as mineral acids where most effort is involved on improving economics and reducing waste and focuses on the downstream processing of aqueous streams to isolate and reuse these reagents. For such materials this is often not trivial. Alternative strategies either using reagents that don't need to be removed (because of low cost and innocuous nature) or require less complex procedure for reuse e.g. direct feeding back into pretreatment should be considered. Where biotechnological techniques are implemented there are possibilities in obtaining fermentable sugars from pretreated biomass using cellulases and the conversion of glucose, xylose (and arabinose) to bioethanol using specially engineered yeasts and bacteria. Development of cellulases by Novozymes had already led to a twelvefold reduction in the enzyme cost contribution in bioethanol production to

<$0.50 per gallon bioethanol in 2004 and targets were set to reduce the costs to further for a viable technology. On the Cellulosic Roundtable [36] in November 2008 the Novozymes Global Director (Biofuels) Poul Ruben Andersen said: "The aim of Novozymes is to develop the first commercially relevant enzyme system needed for efficient and economical conversion of cellulosic materials into ethanol. This is the largest effort we have undertaken in our history and we plan to have this ready by 2010 for the first large scale plants expected to come on line in 2011."

Biogas technology utilises an organic and agricultural waste approach and continues to develop steadily with a growth in the number of installations that are appearing. While some use the biogas for local use (electricity generation), companies have been created that allows the upgrading of biogas to methane that is able to substitute natural gas (SNG) and be introduced into national gas supply pipelines. Some research has also been taking place in order to improve the digestibility of other lignocellulosic material with high lignin content, for instance bracken [37, 38]. Again the use of pretreatment technology is used as a means to improve the hydrolysis process.

3.2 Use of the Correct Raw Materials and Technology at the Right Scale

An article by Banholzer in 2008 [39], examined the use of various feedstocks to prepare olefins. Where carbohydrate containing feedstocks were used this involves ethanol production and integrated conversion to ethylene. Here capital and variable costs (feedstock costs) were plotted, with the diagonal lines showing the approximate equivalency of economic-cost-of-production. An adapted version, which includes an estimation for FT technology, is seen in Fig. 3. Interestingly the possible use of lignocellulosic biomass or starch or naphtha sit on the same economic-cost-of-production but illustrates the balance between the advantage of low biomass costs at the expense of (potential) high capital costs in order to build the facility.

4 Conclusion

In conclusion, biofuels can be readily produced from a number of biomass materials using a number of technologies. With the advent of second generation technology the production of bioethanol using lignocellulosic biomass (as an alternative to the use of sugar and starch) is being realised. Technologically, there are a number of solely chemical based methods (for pretreatment and hydrolysis) and some that make use of cellulase enzymes for the hydrolysis process. As well as this there are a number of options available to metabolise pentoses in the fermentation process to bioethanol (compared to traditional hexose metabolism of bakers yeast) therefore improving the over production potential of the biomass. It has taken many years of biotechnological development to achieve this but the technology

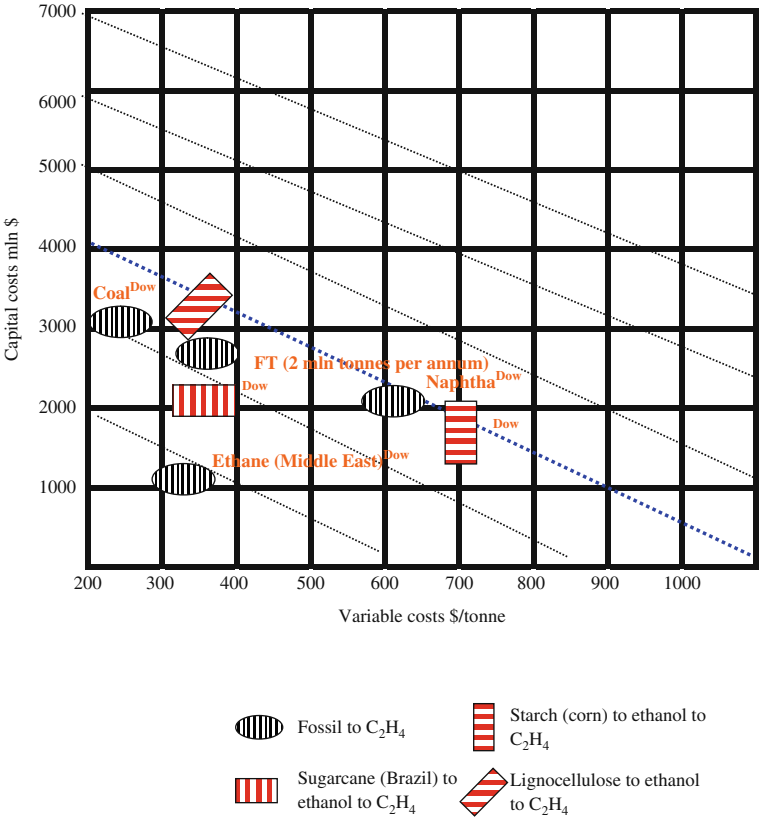

Fig. 3 Return on investment as a function of capital and variable costs (based on 1 million tonnes per annum production)

exists (to a large extent) and is on the brink for successful commercial application. Perhaps the most complex issue surrounding the production of biofuels lies with the renewable raw materials itself. Ethical, social and ecological issues surrounding the biomass with respect to competition with food production and prices, land conversion and carbon debt are complex and so strategies that address these sufficiently are perhaps the most rate determining step for acceptance and success in biofuel production.

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Perspectives on Chemicals from Renewable Resources

Elinor L. Scott, Johan P.M. Sanders, and Alexander Steinbüchel

Abstract Faced with increasing global demand for raw materials to meet the needs of transportation fuels, energy and chemical production coupled with depleting fossil reserves, fluctuating oil prices and ecological impact associated with CO_2 emissions, there has been heightened awareness for the need to utilise alternative and sustainable resources and production methods.

There have been many reports on the use of biomass for the production of chemical products. These have generally focused on the use of fatty acids, lignin and most significantly on the use of carbohydrates as raw materials. In the later case there have been a number of reports on the transformation of sugars by chemicals means but the majority of transformations involve fermentation and other biotechnological processes resulting in the formation of new and traditional chemicals used in industry. Less well explored is the use of proteins and amino acids in industry.

Here it is reported that protein and amino acid rest streams generated from processes such as the production of industrial starch, can be used as a potential raw material for the production of industrial products. With the aid of biotechnology, these complex rest streams may be refined to products with a more defined composition aiding the isolation of desired amino acids which can be used to produce an array of industrially significant products. More specifically it is described that Protamylasse®, generated from starch production, can be used as a media for the production of cyanophycin polymer which can be utilised as a source of arginine and aspartic acid for the production of compounds such as 1,4-butanediamine and succinic acid.

Keywords Amino acids · Decarboxylation · Cyanophycin · Rest streams

E.L. Scott (✉)
Valorisation of Plant Production Chains, University of Wageningen,
6700AA Wageningen, The Netherlands
e-mail: elinor.scott@wur.nl

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1 Introduction

Faced with increasing global demand for raw materials to meet the needs of transportation fuels, energy and chemical production coupled with depleting fossil reserves, fluctuating oil prices and ecological impact associated with CO_2 emissions, there has been heightened awareness for the need to utilise alternative and sustainable resources and production methods.

The production of chemicals and materials from renewable resources is not an area that is unfamiliar to industry. Prior to the advent of the petrochemical industry (as we know it today) a number of products have been developed, for example the production of soaps has been carried out for centuries by the saponification of fats as well as oils (such as olive oil) using sodium or potassium hydroxide.

1.1 Conversions of Fats and Oils

With the ever increasing use of the combustion engine and advent of the automotive industry there was a need to develop lubricants, and in some cases this involved the production of technical products from renewable raw materials. One such product was Castrol, the abbreviated name from the castor oil (derived from the castor plant) that was originally used in the early twentieth century. Chemical products from biological fats and oils (or oleochemicals) still continue to be produced and used: soybean oil is an additive in poly(vinyl chloride) (PVC) and fatty acid esters are used for biodiesel production.

Other components of renewable raw materials have also been utilised in industry. Carbohydrates can be used in fermentation processes as well as chemically transformed to produce chemicals. As well as this the use of lignin, and to a lesser extent amino acids, have been investigated.

1.2 Carbohydrate Conversions

The fermentation of glucose to ethanol has been widely described as a means to not only produce beverages but also produce ethanol for transportation fuels. Here first generation bioethanol uses expensive carbohydrate sources such as starch and sugar from corn and sugar crops while second generation is focusing developments on using inexpensive lignocellulose agricultural waste streams and lower investment costs. A third area is the use of ethanol produced from fermentation as a raw material for the chemical industry. Ethanol is dehydrated to produce ethylene, a major product for the production of a number of chemicals as well as being used as the monomer for the production of polyethylene (PE). Dow and Crystalsev have entered a joint venture in Brazil which will produce ethanol from sugarcane for conversion to ethylene and finally to PE. The integrated production is expected to start in 2011 with a capacity of 350 K tonnes per annum.

In the pharmaceutical industry the conversion of sorbitol to isosorbide has been carried out. Isosorbide is used as an intermediate for a number of products however this has been limited to applications on small scale with high product specification and cost. In recent years new markets and applications for isosorbide derivatives have been explored. Research, media publicity and public concern as to the health implications for the use of plasticisers in materials such as PVC have lead to investigation into safer alternatives. One such development is the formation of esters of isosorbide [1]. These types of compounds have shown suitable performance as a plasticiser while enjoying the benefits of reduced human toxicity, Fig. 1. Allied to this the isosorbide component of the plasticiser is bio-derived.

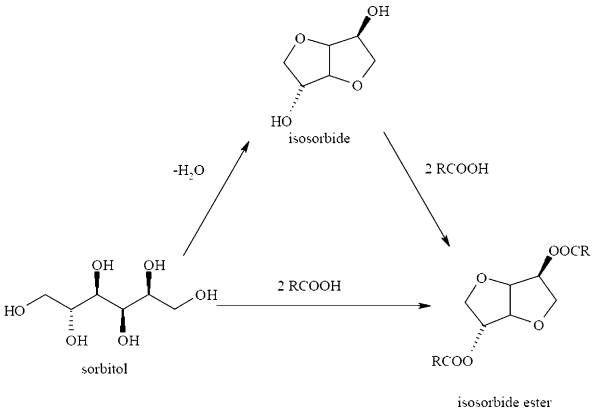

Fig. 1 Isosorbide ester synthesis from sorbitol by dehydration and esterification

Pentoses, from the hemicellulose component of plant material, has been widely studied and applied. Mainly they are obtained from agricultural waste streams generated from rice, corn and sugar production. Pentoses under the action of acid, usually sulphuric acid, results in dehydration and cyclisation resulting in the formation of furfural. Furfural itself has commercial application as a solvent but can also be converted by reduction to form furfuryl alcohol which can be used polymerised under acidic conditions to form polymeric resin materials used to form moulds in the metal (casting) industry. While the formation of hydroxymethyl furfural (HMF), formed by the acid catalysed dehydration and cyclisation of hexoses has also been described, its commercial production (to the best of our knowledge) has never been achieved despite its very rich chemistry. However recent years has seen a resurgence in interest in HMF and in particular its conversion towards furan ring containing di-functional monomers such as 2,5-furandicarboxylic acid (2,5-FDA). Here the emphasis has been the formation of compounds with similar chemical and structural properties to petrochemically derived monomers such as terephthalic acid thus offering the possibility to produce bio-derived monomers resulting in polymers with similar properties to conventional petrochemical materials.

Other strategies towards bio-derived monomers, such as 1,3-propanediol (1,3-PDO), have also been investigated. While the chemical synthesis of 1,3-PDO

has been known for some time, production was not without its problems. However, it was only with the advances in genetic engineering made by DuPont and Genencor that resulted in the successful commercial production of 1,3-PDO from glucose for use in the polymer poly(propylene terephthalate) (PPT), Sorona® [2]. The use of glycerol (from renewable raw materials) for 1,3-PDO synthesis has also been reported and a number of advanced studies in China have already been established to pilot scale (5,000 l) production using *Klebsiella pneumoniae* [3].

2 Conversions of Lignin

Lignin can be found in a wide variety of trees and lignocellulosic plant materials [4] and has the function of acting as a binder in the fibrous structure as well as providing anti-oxidative and anti-bacterial protection. Traditionally lignin is obtained during the pulping process in the paper industry and used as a fuel source and more recently as an additive in resins and cement. Cracking of lignin at elevated temperatures results in a complex mixture of (poly)hydroxylates and alkylated phenolic compounds [5–9]. Such aromatic compounds are of significance as potential precursors to current aromatic products, such as benzene, phenol and styrene. However, due to the complexity of the mixtures involved as well as the need to further "defunctionalise" the aromatic ring, the challenge remains in the further conversion and downstream processing of the fraction(s) obtained. Some attempts at simplifying the complex mixtures by extreme defunctionalisation have been attempted. This has been carried out under hydrodeoxygenation and hydrodealkylation conditions using hydrogen gas and catalysts at elevated temperatures and pressures [10].

2.1 Amino Acid Conversions

One area that has received less attention for the synthesis of chemicals is the use of amino acids. However lysine produced *via* fermentation from carbohydrates, has been described in the literature as a potential building block for the production of ε-caprolactam, the monomer used in nylon-6 production. The L-lysine hydrochloride salt was heated resulting in cyclisation to the lactam α-amino-ε-caprolactam which was subsequently deaminated with the use of hydroxylamine-*o*-sulfonic acid [11]. Another potential ε-caprolactam intermediate derived from lysine is 6-aminohex-2-enoic acid [12].

2.2 Other Biomass Conversions

The use of glycerol, a rest stream of biodiesel production, in industry has seen developments as companies invest to produce existing chemicals using this renewable feedstock. For example Solvay has already brought online the industrial production

of epichlorohydrin from glycerol using the Epicerol™ [13] process and Bio-Methanol Chemie uses glycerol to produce synthesis gas which is reformed to methanol [14]. Archer Daniels Midland also plan to use this feedstock to produce glycols [15], and others are investigating its use to produce (on pilot scale) 1,3-PDO using *Klebsiella pneumoniae* [3]. A review by van Haveren et al. in 2008 describes the potential to produce a wide number of chemicals from various biomass components including glycerol [16].

3 An Approach

If one considers the enthalpy changes involved in the petrochemical industry, the conversion of oil to other hydrocarbons are carried out with no major changes in the enthalpy of the raw materials and the products formed and also production is carried out with very efficient use of energy. However when hydrocarbons are used as the raw materials to produce chemicals functionalised with amine, carboxylic acid functionalities and the like, the resultant product has a lower calorific value than the original raw material. Allied with this large amounts of energy are expended in the production process itself and auxiliary energy is required for the production of co-reagents e.g. chlorine, ammonia, in order to introduce the required functionality. Renewable raw materials consist of a number of major components: oils, carbohydrates, lignin and protein, as well as a small amount of other compounds. Thus renewable raw materials contain functionality and indeed in some cases require the removal of some functionality in order to transform them to compounds similar to (or the same as) those currently produced in the chemical industry. This is opposed to the approach used with oil (or gas). It is conceivable to consider using biomass to make one common intermediate, for example conversion to synthesis gas (CO and hydrogen) and using this to prepare base chemicals which can be rebuilt to make the desired compounds. However this appears to be "using bonbons to make bars of chocolate" and does not take advantage of the functional groups present in the renewable raw material.

To achieve the best use of renewable raw materials (or biomass), and to be able to generate a respectable net income from a crop, it is desirable to utilise (all) components for application (in the broadest terms: food, heat, fuel, chemicals) by employing an efficient bio-refinery process [17]. In terms of raw material value per GJ of biomass produced by the farmer, if it is used only for its calorific value, then returns are low, too low to cover costs. However returns would increase if higher value applications such as fuels could be achieved and this should cover costs. For "high end value" applications such as functional chemicals, the returns would be high and the result very profitable. However it is unrealistic that all components could be used for such an application and that it is more likely different fractions (obtained from bio-refinery) for several applications could be achieved thus still giving rise to a good return. The fractions obtained should then be used for the most suitable application or transformation. For example, it would be more advantageous

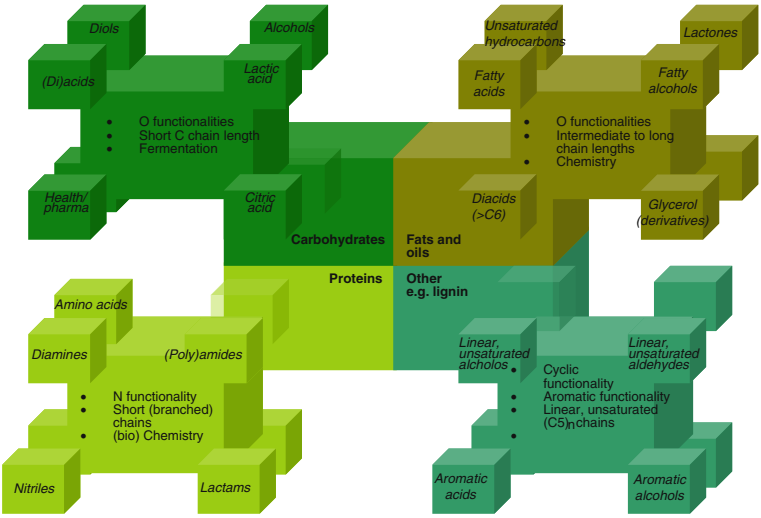

Fig. 2 The biorefinery toolbox

to utilise carbohydrates in fermentation processes or synthesis of oxygen containing products while amino acids (from proteins) could be used to synthesise small molecules containing amine (or other nitrogen) functionalities, Fig. 2.

The application of bio-refinery technology is currently implemented for example in the isolation of starch from potatoes and oils from soybean or rape seed for use in biodiesel production.

As various legislation and governmental aims come into place for the use of biofuels, production and use are set to increase. Assuming a 10% global substitution of transportation fuels, the use of wheat, corn, rape and palm amongst others for biofuel production will lead to large amounts of glycerol and residual protein materials. The use of glycerol has been previously described, however application of the large amount of protein waste has till now not been explored. The rest protein from such streams would amount to an estimated ca. 100 mln tonnes per annum. This volume is in excess of requirements for food and feed and so could provide a good source of raw material for upgrading. Indeed as we will describe in more detail later, amino acids contained in proteins have functionality that is conducive to preparing (bulk industrial) chemicals that are currently prepared by industry and so application of waste protein and hence amino acids as raw materials could find a place in the chemical industry. Rest streams containing proteins and/or amino acids are not contained to those generated from biofuels. Other rest streams from the use of renewable raw materials are also available. For example potatoes are cultivated and used for the preparation of starch for non-food purposes. During the process a rest "potato juice" stream, rich in amino acids is generated. In a concentrated form this is known as Protamylasse®.

While a number of routes to the production of chemicals from carbohydrates have been described, the use of protein/amino acids has to this far not been described in detail. The following discussion will focus on the use of amino acid containing waste streams in order to obtain and use amino acids suitable for the synthesis and production of industrial functionalised chemicals.

4 Technical Details and Status of Technological Implementation

The waste streams that contain proteins or amino acids are, more often than not, complex mixtures of amino acids (once they are hydrolysed) which require separating and isolating. A potential approach to obtaining the most (suitable) amino acids from these waste streams could be the use of bacteria that are selective in removing certain (desired) amino acids from the mixture by the formation of a molecule that is more readily separable from the mixture. For example cyanophycin, a poly(amino acid) consisting of a poly(α-L-aspartic acid) backbone with arginine side chains attached *via* the β-carboxylic acid of the aspartic acid, is produced by many cyanobacteria [18] as a (nitrogen) storage molecule during the transition from

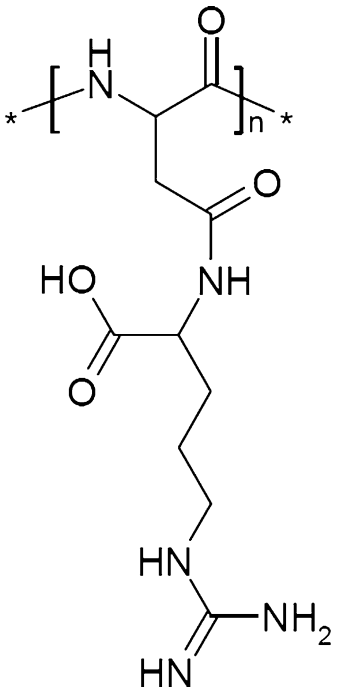

Fig. 3 Cyanophycin polymer (repeating unit in brackets)

exponential to stationary phase in the cell and is accumulated (at neutral pH) within the cytoplasm as insoluble cyanophycin granular peptide (CGP), Fig. 3.

The enzyme cyanophycin synthetase (CphA) [19] is responsible for the non-ribosomal biosynthesis, binding L-aspartic acid with L-arginine to form cyanophycin. However, slow growth of cyanobacteria and the low cyanophycin content means that efficient production of significant amounts are unattained. However *CphA* genes that have been cloned and expressed in bacteria, such as *Escherichia coli,* have lead to higher cell dry matter when cultivation was carried out in high quality (and thus expensive) media [20]. During the last years more interest has been directed towards cyanophycin as an interesting source for a number of applications. For example cyanophycin may be chemically treated in order to obtain a material with low (or reduced) arginine content [21]. This material has the properties of poly(aspartic acid) which could allow it to be used as a bio-derived and therefore biodegradable alternative to poly(acrylic acid). More recently it has been proposed that CGP could be used as a source of aspartic acid and arginine, which has a rich chemistry that could lead to the production of a number of commercially interesting compounds [22]. This will be dealt with in more detail later. Thus the production of cyanophycin could be used to accumulate, concentrate and isolate aspartic acid and arginine to obtain them for use as raw materials in chemical synthesis.

However production using CphA is constrained by the availability of the aspartic acid and in particular arginine. Thus for reasonable production these should be added to the growth media, consequently reducing the economic feasibility unless an inexpensive source of these amino acids is found. The potato juice concentrate, Protamylasse®, a rest stream obtained from the processing of potatoes to produce starch contains organic acids, sugars and minerals as well as peptides and amino acids including high levels of aspartic acid and arginine making it a particularly appealing and inexpensive production media or component [23].

Indeed it was shown that when using *E. coli* increasing the amount of protamylasse in the media increased not only the cell dry matter (CDM) but also the % of CGP in the CDM [24]. A maximum was reached at ca. 5% (vol/vol) Protamylasse®. These results were significantly better than those obtained when *Acinetobacter* and *P. putida* were used.

For the isolation from the fermentation media, of it has been described that CGP may be dissolved in acidic aqueous media. Using this approach allows separation from cell debris. Readjusting the pH to 7 allows precipitation and isolation of the CGP [20]. Such an acid extraction method of CGP was found to be more effective than previously reported methods [25] which were more cumbersome.

Once the CGP is obtained it may then be reacted to reduce (or remove) the arginine side chains or used as a source of aspartic acid and arginine. Both these amino acids due to their functionality and carbon chain length possibilities to be used as raw materials for products that are traditionally prepared in the petrochemical industry.

4.1 Possible Reactions of Amino Acids

Reactions, both chemical and enzymatic, involving amino acids have been extensively reported in the literature though the context of such studies have concentrated on the effects of amino acid conversion on nutrition, significance on physiological behaviour and medicine and origins of life. Such studies were not reported as a preparative method for the synthesis of those compounds. From these studies it is apparent that a number of the products that are (or can be) produced are also prepared industrially for various (technical) applications. For example in leucine degradation in the mevalonate pathway, dimethylallyl pyrophosphate (DMAPP), a pre-cursor to isoprene used in rubber manufacture, is formed. With the aid of the enzyme *isoprene synthase,* found in the leaves of a number of tree species [26–28], DMAPP may be converted to isoprene. Such observations are not limited to leucine and indeed for a large majority of amino acids show interesting product formation [22] including aspartic acid and arginine.

Acrylamide and acrylic acid are respectively synthesised by the hydrolysis reaction of acrylonitrile and the Reppe reaction using acetylene and are used as monomers for the production of the corresponding polymeric materials poly(acrylamide) and poly(acrylic acid) [29]. However, articles investigating the formation of acrylamide in food products have described that Maillard reactions, particularly in the presence of asparagine, had lead to the formation of acrylamide [30–33] while others report the formation of acrylic acid from an aspartic acid pathway [34]. Other reactions of aspartic acid are also known such as α-deamination under elevated temperatures in the presence of water resulting in the formation of fumaric and maleic acid [35]. Both these compounds have applications in the formation of unsaturated polyesters. The α-decarboxylation to β-alanine has also been reported [36]. Although β-alanine has somewhat limited use in its own right, it does make a potentially interesting precursor for the synthesis of a number of other compounds.

Diamines such as 1,6-hexanediamine (HMDA) and 1,4-butanediamine (BDA) are produced by the chemical industry. In the case of BDA production is carried out using reactions requiring propylene, ammonia and hydrocyanic acid. Diamines are used in the synthesis of nylon engineering polymers such as nylon-6,6 and nylon-4,6. Interestingly 1,4-butanediamine is the final product in arginine hydrolysis and decarboxylation (decomposition). Arginine is hydrolysed using the arginase enzyme [37, 38] to ornithine (and urea) which can then undergo decarboxylation resulting in formation of 1,4-butanediamine [39].

Based on the current knowledge as to possible reaction transformations of amino acids coupled with an overview of products and processes of the chemical industry one may envisage a synthetic scheme that leads to a number of interesting products, Fig. 4.

Synthesis of industrial products starting from either arginine or aspartic acid involves some hurdles to overcome. In the pathway A described above, it has

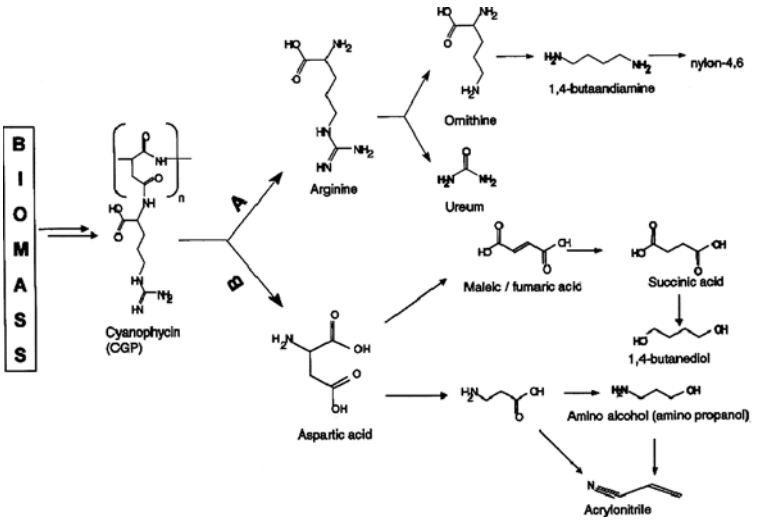

Fig. 4 Potential reaction products of cyanophycin from arginine (route A) and aspartic acid (route B)

already been described that the formation of ornithine and urea may be obtained using arginase enzyme, however hydrolysis of arginine may also be achieved by chemical hydrolysis [40–42]. The use of the enzyme offers the advantage of the use of ambient temperatures and no further decomposition of urea. However, issues of improving conversion rates and operational stability form an important role for successful large scale application. Alternatively, the use of chemical hydrolysis may be interesting due to potentially lower contact times and costs of reagents, however conditions can lead to an array of products (via cyclisation for example) and degradation of urea [40–42] which leads to losses in conversion and makes further downstream processing more complex (and costly).

In pathway B, the conversion of aspartic acid by α-deamination to fumaric acid has been described and leads to the formation of commercially interesting products such as fumaric acid, which in turn could be used as a precursor for other monomers for polymeric materials, such as 1,4-butanediol for poly(butylene terephthalate). Since this route results in the loss of ammonia, it is considered that the optimum use of the functionality is not achieved, for reasons that we described earlier. Thus research has focused on the conversion of aspartic acid to β-alanine. α-Decarboxylation of aspartic is not trivial. Only limited reports of (photo)chemical transformation are available [43]. The alternative to carry out this transformation is to use the enzyme, aspartic acid alpha-decarboxylase. This enzyme has not been widely studied with respects to its immobilisation, activity, stability and re-use although this forms the basis to determine the potential for efficient use at large scale for the synthesis of commercial products. A manuscript is has recently been submitted which describes these issues in more detail [44]. The conversion of β-alanine to a range of chemicals, including those given in Fig. 4 are now being studied.

5 Expert Commentary on Future Perspectives

While it has been described that (proteins and) amino acids could make good raw materials based on the functionality that they offer, allowing not only the possibility to reduce fossil raw materials for the carbon content in the product but also to circumvent (energy intensive) process steps, there are a number of challenges that are required.

5.1 Sourcing of Raw Materials

The rising interest and production in biofuels such as bioethanol and biodiesel from plant material looks set to continue. As previously discussed such developments would give rise to prolific amounts of rest protein. De-oiled cakes from soya or rape do currently have applications as animal feed. The (imported) cakes are used for example as compound (animal) feed or as raw materials to make hydrolysed vegetable products. In the case of animal feed, the digestion process only removes the required nutrients from the cake and the rest is expelled as manure. Thus the efficiency in the cakes use is not optimal and could be improved with relatively straightforward bio-refinery. Developments to refine press cakes to lignocellulose (for electricity), minerals (such as phosphates, P, and potassium containing compounds, K) for fertilisers and protein (for feed) are being carried out and could be implemented in the near future. This would in itself lead to more efficient and specific application of the renewable resources while generating extra value (from use as fertiliser and heat/electricity source) and leading to a contribution to the targets in generation and use of renewable energy, Fig. 5.

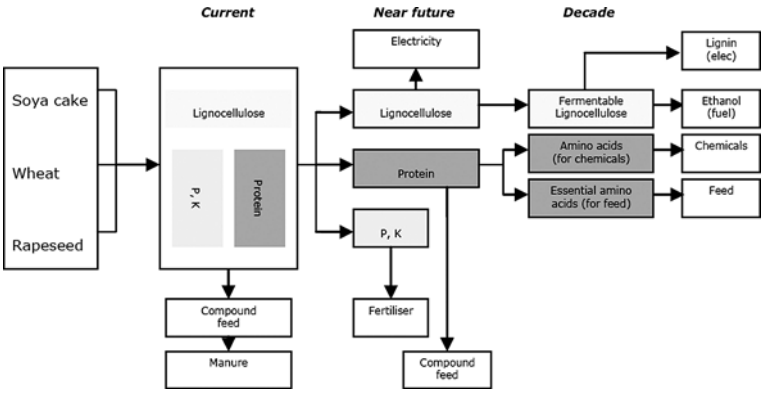

Fig. 5 Renewable raw materials and bio-refinery – possible developments for the co-production of fractions for mineral fertilisers (based on potassium, K and phosphorus, P), compound feed, heat fuel and chemicals

Further work is required to realise the potential for further refining which could lead to the lignocellulosic component being used as a raw material for transportation fuel and being able to resolve the protein into its constituent amino acids could lead to application in the chemicals industry (and satisfying the feed market). As importation of press cakes etc. already takes place at ports, it could be advantageous to implement the bio-refining there too. For large ports, with large integrated chemical facilities, such as the port of Rotterdam, this is particularly relevant.

For the use of other (aqueous) rest streams from other industries, such as Protamylasse®, it would be advantageous to have further conversions taking place as close to the original site to prevent the transportation of water. Integration of various industries (using renewable resources) have already been suggested, such as that between Dow and Crystalsev.

5.2 Protein Conversion to Amino Acids

In order to be able to utilise proteins as a source of amino acids a number of challenges need to be overcome. For example once the proteins are isolated they need to be hydrolysed, but how should this been done? Current practice (for making hydrolysed vegetable proteins) uses 6N hydrogen chloride (HCl) at elevated temperatures over a prolonged period of time. Amongst other things this leads to degradation of a number of (valuable) amino acids. As well as this, a copious amount of base is required to neutralise the solution. While the costs of HCl (and NaOH) are relatively low and production of technical grade HCl is often a result of the chloronation of hydrocarbons (such as in the synthesis of vinyl chloride in PVC production), the production of large amounts of inorganic salt is undesirable. They must be separated from the amino acid mixture as it may disrupt potential separation processes such as ion exchange (IE) or electrodialysis (ED), reducing the efficiency and increasing the costs of obtaining the desired (amino acid) end product. Thus alternative methods of hydrolysing the protein are required. One option to hydrolyse proteins and reduce salt (by-product) formation could be the (partial) use of enzymes such as proteases (although some salt may be produced to pH maintenance). Proteases are used in a number of applications in the food and beverages industry. The degree of hydrolysis, although not complete, can be quite high (up to 90%) [45] but does require high dosages and a cocktail of various proteases (with varying specificity) in order to achieve this. Due to this the costs are prohibitive for large scale, bulk chemical production applications.

5.3 Amino Acid Separation

The hydrolysed medium contains a mixture of amino acids which require separation in order that they can be used for their particular application. As previously described, the uses of IE and ED have been widely reported in the literature as

separation techniques. IE is effective in the separation of complex mixtures of amino acids, however, for (very) large scale applications costs are anticipated to be high due to elution times and the need for regeneration. ED shows potential of being an economically technique for the separation of charged species and has already been demonstrated at large scale for a number of applications [46]. ED separates ions in aqueous solution under the influence of a (electric) potential gradient therefore theoretically all amino acids could be separated on the basis of their isoelectric point (IP) and a number of studies have examined the isolation of amino acid containing materials using ED [47–49]. In practice, due to similarity in IP, only three different groups of species are isolated (acidic, basic and neutral amino acids). Thus strategies developing novel separation techniques need to be developed in order to effect greater separation.

5.4 Amino Acid Application and Modification

Once separated, what is the best application of a specific amino acid? For amino acids such as methionine, perhaps the most useful application is to use it for its nutritional value. The traditional method for the production of methionine is via chemical synthesis and the product cost is high. Some amino acids have similar chemical structures to chemical products currently on the market. For example serine if decarboxylated is converted to ethanolamine that is, amongst other things, an intermediate in ethylenediamine synthesis used in the production of chelating agents, some polymers, pharmaceuticals and agrichemicals. The question of which type of "transformation" technology should be used? Since an amino acid may be obtained in an aqueous environment, it would be useful to not need to remove the water and carry out (if possible) some of the synthetic procedure in the aqueous phase. For some transformations the use of enzymes are very useful. For example decarboxylation is often a valuable step and can be specifically carried out with the use of the correct enzyme at ambient conditions. In the above example serine can be decarboxylated to ethanolamine using serine decarboxylase. While enzymes offer this useful option they are prone to deactivation. Thus prolonged and/or repeated use can be troublesome. Some of these problems may be reduced with the aid of immobilisation of the enzyme. However, costs per kg product produced generally tend to be higher with enzyme technology compared to chemical transformation. In most cases the required enzyme is not commercially available and development is required to engineer them and allow production in microorganisms for application and also to reduce the costs. However, one should not be discouraged, as a number of viable processes have been developed for the production of industrial (bulk) chemicals using enzymes e.g. the production of acrylamide from acrylonitrile using nitrile hydratase.

Lysine, produced by fermentation, has been discussed as a raw material for ε-caprolactam [11]. Developments in plant breeding and genetic engineering has allowed the enhanced production of lysine *in planta*. The accumulation of lysine in potato has been well investigated [50–53]. Lysine biosynthesis was increased by

de-regulating the feedback mechanism specifically in potato tubers via genetic modification. This resulted in an increase of lysine content in tubers of 15-fold (up to 1% of FW). This opens the door to the question, could the production of chemical intermediates be produced in plants?

In conclusion there are many approaches using fermentation, chemistry and even possibly genetic engineering to produce chemicals from renewable raw materials. The functionality of renewable raw materials maybe used to advantage as a means to circumvent energy (and cost) intensive steps carried out in industry as well as also allowing the development of new (novel) materials with new functionality that would be too complex starting from petrochemicals. However, good choices of the component of the raw material coupled with the technology required together with the product types is required for efficient and sustainable product synthesis at competitive prices.

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Microbial Lactic Acid Production from Renewable Resources

Yebo Li and Fengjie Cui

Abstract Lactic acid is widely used in the food, chemical, textile, and pharmaceutical industries. New applications of lactic acid for the manufacture of biodegradable polymers have increased the demand for it. Lactic acid can be produced from cheese whey and starchy and lignocellulosic biomass via microbial fermentation with lactic acid bacteria (LAB) or fungi. Pure sugar and cheese whey can be directly fermented by lactic acid bacteria, while liquefaction and enzymatic saccharification of starchy biomass and pretreatment and enzymatic hydrolysis of lignocellulosic biomass are required for lactic acid production from biomass. Amylolytic lactic acid bacteria can direct convert starchy biomass to lactic acid. Lactic acid bacteria and methods used for lactic acid production from different feedstocks are summarized in this paper. Lactic acid productivity of 6.34 and 4.87 g/l·h and yields of 0.98 g/g lactose and 0.97 g/g glucose were obtained from cheese whey and wheat starch, respectively, using cell-recycle repeated batch fermentation by *Lactobacillus sp*. RKY2. Lactic acid bacteria such as *Lactobacillus pentosus*, *Lactobacillus brevis* and *Lactococcus lactis* can ferment glucose to lactic acid by homolactic fermentation and also effectively convert xylose or arabinose to lactic acid and acetic acid by heterolactic fermentation. The process for lactic acid production from lignocellulosic biomass needs to be improved to increase the lactic acid yield and productivity.

Keywords Lactic acid · Microbial fermentation · PLA · Lactic acid bacteria · Biomass · Starch · Cheese whey

1 Introduction

Lactic acid (2-hydroxypropanoic acid, $CH_3CHOHCOOH$) was discovered and isolated in sour milk in 1780 by the Swedish chemist Carl Wilhelm Scheele (1742–1786). It is the most widely occurring carboxylic acid in nature and has many

Y. Li (✉)
Department of Food, Agricultural and Biological Engineering, Ohio Agricultural Research and Development Center, Ohio State University, Wooster, OH 44691, USA
e-mail: li.851@osu.edu

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applications in food, chemical, textile, and pharmaceutical industries [1]. Recently, there is an increasing demand for lactic acid for the manufacture of biodegradable polymer, polylactic acid (PLA). The current worldwide demand for lactic acid is estimated roughly to be 130,000–150,000 tons per year [2, 3].

Lactic acid can be produced by chemical synthesis or microbial fermentation (Fig. 1). For the chemical synthesis, acetaldehyde and hydrogen cyanide are reacted in the presence of a base under high pressure to produce lactonitrile. Distillation is used to purify this crude lactonitrile. The purified lactonitrile is then hydrolyzed with sulfuric acid to produce lactic acid. A byproduct of ammonium salt is also produced [3, 4].

Feedstocks such as cheese whey and starchy and lignocellulosic biomass have been studied for microbial lactic acid production in the past decades. When cheese whey or simple sugars are used, lactose, glucose, or sucrose can be directly fermented to lactic acid (Fig. 1b, route 3). The starchy biomass can be hydrolyzed to glucose with enzymatic liquefaction and saccharification and then fermented to produce lactic acid (Fig. 1b, route 2). Starchy biomass can also be directly fermented to lactic acid by amylolytic lactic acid bacteria. When lignocellulosic biomass is used, a pretreatment process is required to break up the linkage among cellulose, hemicellulose, and lignin. The cellulose and hemicellulose are hydrolyzed by cellulase and hemicellulase to fermentable sugars. The obtained sugars can then be fermented with the appropriate microorganism to produce optically pure L (+) or D (–) lactic acid (Fig. 1b, route 1). Simultaneous saccharification and fermentation (SSF) can also be applied to allow the enzymatic hydrolysis and fermentation processes to be conducted in the same reactor. Separation and purification of lactic acid is required for microbial fermentation process.

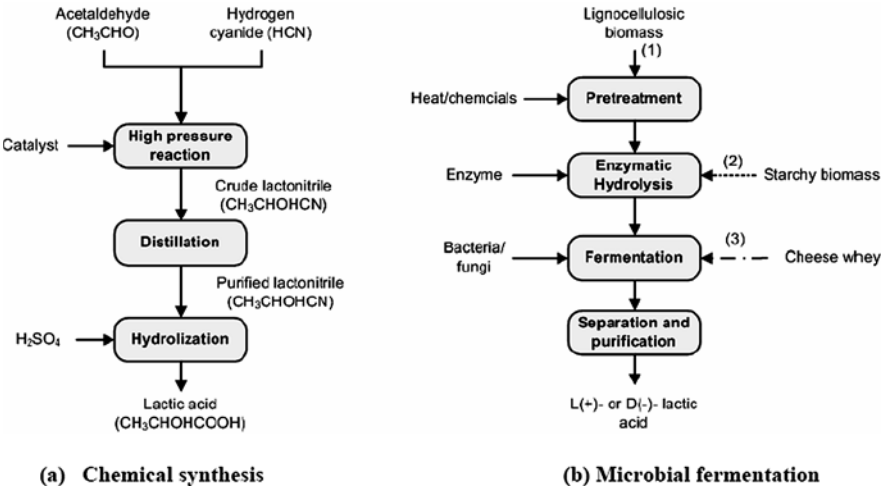

Fig. 1 Lactic acid production processes

There are two optical isomers of lactic acid: L (+) -lactic acid and D (–) -lactic acid. Pure L (+) and D (–) lactic acid can be obtained by microbial fermentation of renewable sources when an appropriate microorganism is selected. Racemic DL-lactic acid is always produced via chemical synthesis from petrochemical resources [3, 5]. While homopolymers form regular structures and develop a crystalline phase, copolymerization with D- or L- lactic acid leads to the interruption of the regular structures and the formation of amorphous materials. In order to achieve the desired polymer properties, high purity D- or L-lactic acid monomers are necessary [6].

2 Background Research

More than 100 different lactic acid bacteria (LAB) and filamentous fungi have been used for the microbial lactic acid production from renewable resources. However, *Lactobacillus (Lb.)* and *Lactococcus (Lc.)* species were most frequently studied in the past years for lactic acid production. LAB can be classified into two groups: (1) homofermentative and (2) heterofermentative. Homofermentative LAB convert sugars exclusively into lactic acid, while heterofermentative LAB produce other byproducts such as acetic acid, ethanol, and/or carbon dioxide along with lactic acid [5].

The two major pathways for assimilation of hexoses (glucose and galactose) and pentoses (xylose and arabinose) in the lactic acid bacteria are the Embden-Meyerhof-Parnas (EMP) pathway (Fig. 2, route c) and the pentose phosphoketolase (PK) pathway (Fig. 2, route b). Under conditions of excess glucose and limited oxygen, homofermentative LAB such as *Lc. lactis* [7], *Lb. delbruecki* [8], and *Lb. helveticus* [9] catabolize one mole of glucose in the EMP pathway to yield two moles of pyruvate. Intracellular redox balance is maintained through the oxidation of NADH, concomitant with pyruvate reduction to lactic acid. This process yields two moles lactic acid per mole of glucose consumed. Heterofermentation metabolizes sugars through the pentose phosphate pathway in some microoganisms. For example, *Lb. brevis* is a heterofermentative lactic acid bacterium, which produces lactic acid, carbon dioxide and ethanol from hexoses [10]. One mole glucose-6-phosphate is initially dehydrogenated to 6-phosphogluconate and subsequently decarboxylated to yield one mole of CO_2. The resulting ribulose-5-phosphate is cleaved to one mole glyceraldehyde phosphate and one mole of acetyl-phosphate. The glyceraldehyde phosphate is further metabolized to lactic acid, while the acetyl phosphate is reduced to ethanol via acetyl-CoA and acetaldehyde intermediates. *Lb. sunfruncisco* is another heterofermentative LAB which converts glucose to glyceraldehyde phosphate, then to lactic and acetic acid by catalyzing the acetyl- phosphate [11]. Lactic acid and other substances (typically acetic acid and/or ethanol) are the main end products and their compositions and ratios vary with the microorganisms and fermentation conditions [12].

Lactic acid bacteria also ferment other sugars such as lactose or galactose via different pathways [13, 14]. The catabolism of the disaccharide lactose involves the

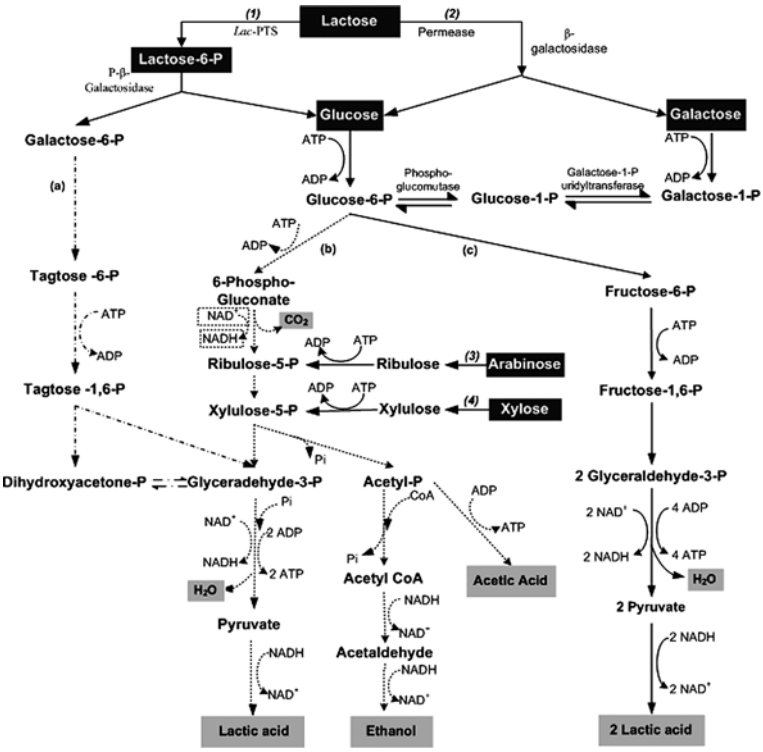

Fig. 2 Metabolic pathways of lactic acid bacteria

degradation of its two moieties, glucose and galactose. Normally, these LAB strains, including *Lactobacilli,* contain one or both of two systems for the transport of lactose and galactose into the cell: phosphoenolpyruvate-lactose phosphotransferase system (*Lac*-PTS) (Fig. 2, route 1) and lactose permease system (Fig. 2, route 2). When the *Lac*-PTS system is applied, lactose is translocated through the cell membrane to yield phosphorylation of the lactose molecule in the 6th position of the galactose moiety. The lactose phosphate is cleaved by phospho-D-galactosidase to yield glucose and galactose 6-phosphate. Glucose is further metabolized as described previously through the EMP or PK pathway, whereas galactose 6-phosphate is metabolized through the D-tagatose 6-phosphate pathway to form dihydroxyacetone phosphate and glyceraldehyde-3-phosphate, which are further metabolized by the enzymes of the glycolytic pathway (Fig. 2, route a). In the case of entry via a permease system, lactose is not modified, but broken down directly into glucose and galactose. Then glucose is converted to two moles lactic acid through the homofermentive pathway or to mixed lactic acid and acetic acid or ethanol through the heterofermentive pathway. Galactose is metabolized via the Leloir pathway. That is, first, galactose is phosphorylated from ATP at the C-1 position by galactokinase (Fig. 2). Galactose-1-phosphate is then converted into UDP-galactose

by galactose-1-phosphate uridylyltransferase with concurrent production of glucose-1-phosphate. The glucose-6-phosphate is formed from glucose-1-phosphate by phosphoglucomutase catalysis, and then enters the homofermentative or heterofermentative pathway to produce lactic acid, and ethanol and/or acetic acid.

Pentoses, such as xylose and arabinose, are mainly derived from the hemicellulose of lignocellulosic biomass. In xylose-utilizing strains, xylose is reduced and oxidized to xylulose by xylose reductase and xylitol dehydrogenase (Fig. 2, route 4) [15]. Xylulose is subsequently phosphorylated to xylulose-5-phosphate and metabolized through the pentose phosphate pathway. Similarly, arabinose is converted by arabinose-utilizing strains to the ribulose-5-phosphate and then enters the PK pathway for producing lactic acid and acetic acid or ethanol consequently (Fig. 2, route 3). LAB such as *Lb. pentosus, Lb. brevis* and *Lc. lactis* can ferment glucose to lactic acid by homolactic fermentation and can also effectively convert xylose to lactic acid and acetic acid by heterolactic fermentation [16, 17].

3 Materials and Methods

3.1 Pretreatment

When lignocellulosic biomass feedstock is used, a pretreatment process is required prior to enzymatic hydrolysis and fermentation (Fig. 1). Physical (grinding and irradiation), chemical (dilute acid and alkali), and physicochemical (steam pretreatment and autohydrolysis) processes have been performed to reduce the particle size and crystalinity, to increase solubilization of hemicelluloses and lignin, and to enhance the accessibility of cellulose to the enzyme in the following enzymatic hydrolysis step.

Dilute acid pretreatment is performed by adding dilute acid (<4% H_2SO_4) at elevated temperatures between 130 and 200°C for 2–80 min [18]. It is an inexpensive process with up to 90% hemicellulose and glucose yields during the enzymatic hydrolysis [19]. During acid hydrolysis of lignocellulosic biomass, it is important to select proper pretreatment conditions to maximize the solubilization of hemicellulose and minimize the formation of inhibitors such as furfural and hydroxymethylfurfural (HMF).

Alkaline pretreatment (NaOH or lime) can swell the pores of the cellulosic biomass at temperatures ranging from 25°C (ambient temperature) to 85°C [20, 21]. Alkali pretreatment can reduce the degree of polymerization and crystallinity, increase the surface area, and increase the solubilization of lignin and hemicelluloses [18]. The solid/liquid ratio of alkali treatment is about 10–20%. The pretreatment time ranges from 1 to 30 h depending on the pretreatment temperature and alkali loading. Alkali pretreatment is more effective on agricultural residues and herbaceous energy crops than on wood materials. Aqueous ammonia treatment has been successfully used as a pretreatment process of corn stover for lactic acid production [17]. Aqueous ammonia treatment is conducted at temperatures between 70 and 90°C and pressures of 15–20 atm for less than 5 minutes. The liquid-to-solid

ratio is generally between 10 and 30%. The alkali treated materials are washed with water until the pH value reaches around 7.0.

Steam pretreatment is performed with high-pressure saturated steam at a temperature between 160 and 240°C for a specific time ranging from several seconds to a few minutes. The severity of steam treatment can be described by R_o ($R_o = t$ exp $(T - 100)/14.75$) which is a function of treatment time (t, min) and temperature (T, °C) [22]. Previous work has shown that SO_2-catalyzed steam treatment is an effective pretreatment for lignocellulosic biomass [23]. Pretreatment using water at a controlled pH (5.0–7.0) and temperature (150–200°C) for 10–30 min has also been proven to be effective for corn stover [18].

3.2 Enzymatic Hydrolysis and Fermentation

Although pure sugars or disaccharides, including lactose, are good feedstocks for lactic acid production, feedstocks such as starchy and lignocellulosic biomass are cheaper and more abundant. Usually, starch-based materials and cellulosic biomass feedstocks used for fermentation have to be hydrolyzed to glucose before they can be fermented to lactic acid. Conventionally, gelatinization and liquefaction of starch is carried out enzymaticaly at high temperatures of 90–130°C for 10–30 min with α-amylase followed by enzymatic saccharification to glucose at 50–60°C for 2–4 h with glucoamylase [24]. The optimum pH values for the liquefaction and saccharification steps are 6.0 and 4.5, respectively [25].

Pretreatment of lignocellulosic biomass is required before enzymatic hydrolysis. When enzymatic hydrolysis and fermentation are performed sequentially, it is referred to as separate hydrolysis and fermentation (SHF). However, the cellubiose and glucose produced during lignocellulose saccharification strongly inhibit the cellulase activities. This two-step process involving consecutive enzymatic hydrolysis and microbial fermentation also prolongs the total processing time. Simultaneous saccharification and fermentation (SSF) is an effective process in which enzymatic hydrolysis and fermentation of lignocellulosic materials are conducted in the same reactor which can eliminate the substrate inhibition [26]. However, the different optimal temperatures and pH required for saccharification and fermentation are the main problems for SSF [27]. The optimal conditions for enzymatic hydrolysis are 50°C and a pH value below 5.0, while the optimum conditions for lactic acid fermentation are 37–42°C and a pH value of 5.5–6.5. Some compromise is needed for the SSF process in order to obtain high overall lactic acid yield and productivity.

Acid hydrolysis of starchy biomass, an alternative approach to enzymatic hydrolysis, is carried out with 1–5% H_2SO_4 with a liquid-to-solid ratio of approximately 8–10:1 (w/w) at temperatures ranging from 100 to 130°C for 1–3 h. After cooling to room temperature, the acid hydrolysate is neutralized to pH 6.5 with an alkali or $CaCO_3$ [16, 27].

Direct fermentation, which couples the enzymatic hydrolysis of carbohydrate substrates and microbial fermentation into a single step by utilizing the amylolytic lactic acid bacteria (ALAB), has received increasing interest [28]. A few amylolytic

LAB strains have been isolated, such as *Lb. amylophilus* JCM 1125 [29], *Lb. manihotivorans* LMG 18010T [30], *Lb. amylophilus* GV6 [31], and *Streptococcus bovis* 148 [32].

According to Hofvendahl and Hahn-Hagerdal [5], the optimum pH for lactic acid production by microorganisms varies between 5.0 and 7.0, depending on the microorganism species. Too-low or too-high pH levels would inhibit the strains' growth and lactic acid production. When fermentation experiments are carried out in orbital shakers, $CaCO_3$ (10–30 g/l) is generally added to neutralize the lactic acid produced and maintain the pH at the optimal range. A 4–10 M NaOH or NH_4OH solution is generally used to maintain the pH at the designated value in the fermentation reactor.

The influence of temperature on lactic acid production is related to the growth kinetic parameters of the organism, lactic acid production, and substrate consumption. Among the LAB, most lactic acid productivity studies have been conducted at a temperature of 35–39°C. Agitation influences the mixing and mass transfer rates. Agitation speed of 150–300 rpm is generally used for lactic acid production in fermentation reactors. The medium is generally supplemented with the desired salt or nutrient concentrations such as yeast extract 0.1–0.5%, K_2HPO_4 0.1%, and $MgSO_4 \cdot 7H_2O$, 0.5% etc.

Lactic acid production can be performed as a batch, fed-batch or continuous fermentation process [33–35]. For batch processes, the low level of nutrient that can be tolerated by the cells limits the final cell and product concentration. It is unable to attain and sustain high cell concentrations with the resources available in a typical growth medium. To overcome nutrient limitations, fed-batch processes have been practiced for lactic acid production with different substrates. For fed-batch fermentation, the microorganisms are grown in batch medium for 12–24 h, and then fresh media is continuously added into the reactor at a constant feeding rate, until the working volume of the reactor is reached. For repeated fed-batch fermentation, after 24 h of fermentation, half of the fermentation broth is withdrawn from the fermentor and an equal volume of fresh medium is added into the fermentor at a constant feeding rate [36].

Cell immobilization on a solid carrier is another method which has been widely studied for lactic acid production. A high cell density can be achieved in immobilization culturing which can be easily re-used for lactic acid production over a long period, especially for a fed-batch or continuous fermentation process [37].

3.3 Separation

In the traditional separation process, the fermentation broth is first neutralized by C_aCO_3 and then filtered to remove cells, carbon treated, evaporated and acidified with H_2SO_4 to get lactic acid and C_aSO_4. The insoluble C_aSO_4 is removed by filtration. Pure lactic acid is further obtained by hydrolysis, esterification, and distillation. This process yields calcium salt as a by-product, resulting in high chemical cost and waste generation.

Other alternative lactic acid separation processes such as adsorption [38], reactive distillation [39], electrodialysis [40], and nanofiltration [41, 42] have also been studied for lactic acid separation and purification. Advances in membrane technologies have improved their use in the fields of separation and purification. There has been a shift toward membrane separation processes because they are often more capital and energy efficient when compared with chemical separation processes. Membrane processes have advantages such as no energy-intensive phase changes or potentially expensive solvents or adsorbents as well as the potential for simultaneous separation and concentration of lactic acid.

The lactic acid fermentation broth contains lactate, lactose residues, cells, and other organic and inorganic fermentation residues. The cells and large molecular weight residues can be removed by microfiltration or ultrafiltration. Nanofitration membranes, with membrane molecular weight cut-off of 100–400, can retain 97–100% of lactose to obtain a permeate containing only lactic acid and water [41–43]. With reverse osmosis separation, lactic acid in the nanofiltration permeate can be further concentrated which can substantially reduce the energy cost of the subsequent evaporation process [44].

Electrodialysis is used to remove ions from the fermentation broth under the driving force of an electrical field generated by stacking cation- and anion-exchange membranes. For a two-stage electrodialysis, desalting electrodialysis is applied first to recover the lactate, and the water splitting eletrodialysis is applied for acidification of lactate using biopolar membranes which have both anion- and cation-exchange layers. One-stage water splitting eletrodialysis [45] and combined nanofitration and water splitting electrodialysis [46] have also been studied for the lactic acid separation and purification.

4 Results and Discussion

All lactic acid bacteria could ferment pure sugars such as lactose, glucose, xylose, and arabinose to lactic acid via EMP pathway and/or the pentose PK pathway. The performances of main strains with glucose as substrate are summarized in Table 1.

High lactic acid yield and productivity were obtained with most studied microorganisms when lactose and glucose were fermented through the EMP pathway [21]. Some LAB such as *Lb. Brevis* and *Lb. Pentosus* can ferment pentose (xylose, arabinose) using the PK pathway, but with a much lower lactic acid yield and productivity as acetic acid and/or ethanol is also produced along with lactic acid.

Currently, batch fermentation is still the most commonly used method in industrial lactic acid production. Lactic acid yield of 0.74 g/g glucose and productivity of 4.4 g/l·h were obtained with *Lb. casei* NRRL B-441 from glucose [52]. The lactic acid yield or productivity is limited by the inhibition of substrate (glucose) and product (lactic acid) in batch fermentation [61]. Coupled fermentation and separation with cell and sugar recycling have been practiced for lactic acid production to prevent nutrient depletion, prolong the growth phase, and increase the lactic acid

Table 1 Performance of microorganisms and processes on lactic acid production from pure sugars

Microorganisms	Substrate	Process	Yield (g/g sugar)	Productivity (g/l·h)	References
B. coagulans SIM-7 DSM 14043	Glucose	Batch/fed-batch		9.9	[47]
E. faecalis RKY1	Glucose	Batch/ repeated-batch	0.96	3.56–6.20	[48, 49]
Lb.casei subsp. *casei* CRL 686	Glucose	Continuous		9.72	[50]
Lb.casei NRRL B-441	Glucose	Batch	0.74–1	3.5–5.6	[51, 52]
Lb.casei LA-04-1	Glucose	Fed-batch	0.90	2.14	[53]
Lb.coryniformis sp. *Torquens* ATCC 25600	Glucose	Batch	0.98	2.6	[54]
Lb.delbrueckii ssp. *lactis* DSM 20073	Glucose	Batch and fed-batch		9.9	[47]
Lb.lactis BME5-18 M	Glucose	Fed-batch	0.97	2.2	[55]
Lb.rhamnosus IFO 3863	Glucose	Continuous	0.53–0.77	2.90–13.15	[56]
Lb.sp. RKY2	Glucose	Batch	0.91	6.21	[57]
Lc. Zeae ATCC 393	Glucose	Batch	0.98	5.0	[54]
Lc. lactis IO-1	Glucose	Immobilization		4.5	[58]
R. oryzae NRRL 395	Glucose	Batch, fed-batch/ immobilization	0.87–0.90	1.8–2.5	[59]
R. oryzae R1021	Glucose	Batch	0.77		[55]
R. sp. MK-96-1196	Glucose	Batch/fed-batch	0.93	1.80	[60]

productivity. Very high volumetric lactic acid productivity (9.72 g/l·h) was obtained from glucose with a two-stage immobilized packed-bed system by *Lb. casei* CRL 686 [50].

4.1 Cheese Whey

Whey is a waste stream of cheese production which contains lactose, protein, fat, and mineral salts. Deproteinized whey contains mainly lactose and recently has been extensively studied for lactic acid production. Theoretically, 4 moles of lactic acid could be obtained when lactose is broken down directly into glucose and galactose via a permease system, which are both converted to two moles lactic acid in the homofermentation pathway. The performance of different microorganisms for lactic acid production from cheese whey is shown in Table 2. Genus *Lactobacillus* is the main lactic acid producer which not only utilizes lactose with high conversion rates but also utilizes other nutrient such as protein present in whey [68]. The highest lactic acid productivity of 6.34 g/l·h and yield of 0.98 g/g lactose were obtained using the cell-recycle repeated batch production by *Lb.* sp. RKY2 [66]. High lactic acid yield (0.93 g/g lactose) and productivity (3.97 g/l·h) were also obtained with *Lb. casei* NRRL B-441 in a batch operated fermentation reactor [63].

Genus *Lactococcu*, like *Lc. lactis*, have been and are still being extensively studied due to their commercial potential for converting sugar to lactic acid. *Lc. lactis* subsp. *cremoris* 2487 could consume 99.1% lactose in the whey permeate and the highest lactic acid yield and productivity obtained with this strain were 0.88 g/g lactose and 4.6 g/l·h, respectively [67].

B. longum can also convert lactose into high-quality lactic acid although minimal research have been focused on this genus. Lactic acid yield of 0.81 g/g lactose was

Table 2 Performance of microorganisms and processes on lactic acid production from cheese whey

Microorganisms	Process	Yield (g/g sugar)	Productivity (g/l·h)	References
B. longum NCFB 2259	Batch, semi-continuous, or immobilization	0.51–0.82	0.3–0.7	[20, 62]
Lb. casei NRRL B-441	Batch	0.93	2.5–3.97	[63]
Lb. casei SU *No 22*	Fed-batch and immobilization	0.32–0.39	2.0	[36]
Lb. helveticus ATCC 15009	Batch	0.66	2.7	[64]
Lb. helveticus	Batch and immobilization		10.5	[65]
Lb. sp. RKY2	Batch and repeated batch cultures	0.98	6.34	[66]
Lc. Lactis sp.*Cremoris* 2487	Batch	0.88	4.6	[67]

obtained with *B. longum* NCFB 2259 in a batch fermentation reactor using cheese whey as the sole medium [62]. Lactic acid productivity was doubled during the 2nd and 3rd stages when a nanofiltration membrane was used to recycle lactose and cells in a semi-continuous fermentation mode [62].

4.2 Starchy Biomass

The performance of LAB and fungi for lactic acid production from starchy biomass is shown in Table 3. The maximum lactic acid volumetric productivity of 4.87 g/l·h and yield of 0.97 g/g glucose were obtained from wheat with *Enterococcus faecalis RKY1* after enzymatic liquefaction and saccharification [70]. A novel amylolytic lactic acid strain *E. faecium* No. 78 also demonstrated high lactic acid productivity of 3.04 g/l·h by a new continuous culture system with high cell density [28].

The filamentous fungus *Rhizopus* species has also attracted a great deal of attention not only because of its capacity to produce pure L (+) -lactic acid, but also due to some advantages, including amylolytic ability, low nutritional requirement, and easy downstream processing, with regard to the separation of the cell biomass from the fermentation broth [75]. Lactic acid yield of 1.0 g/g glucose and productivity of 1.65 g/l·h were obtained from corn starch with immobilized *R. oryzae* NRRL 395 [59]. Lactic acid yield of 0.86 g/g and productivity of 1.3–1.6 g/l·h were obtained by *R. arrhizus* DAR 36017 from waste potato starch [74].

Table 3 Performance of microorganisms and processes on lactic acid production from starch crops

Microorganisms	Substrate	Process	Yield (g/g sugar)	Productivity (g/l·h)	References
E. faecium No. 78	Sago	Continuous		3.04	[28]
E. faecalis RKY1	Corn, Wheat, Tapioca, Potato	Batch	0.93–1.04	0.5–4.8	[69, 70]
Lb. delbrueckii sp. *Delbrueckii* ATCC 9649	Wheat	Batch	0.82	1.6	[71]
Lb. delbrueckii sp. *bulgaricus* ATCC 11842	Wheat	Batch	0.11	0.56	[71]
Lc. Lactis sp. *Lactis* ATCC 19435	Wheat	Batch	0.76	3.0	[71]
Lc. Lactis sp. *Lactis* IFO 12007	Cassava	Recirculating, cell immobilization	0.76	0.6	[72]
Lc. Lactis sp. *Lactis* AS211	Wheat	Batch	0.77	1.7	[71]
R. oryzae NRRL 395	Corn	Fed-batch/Cell immobilization	≈1	1.65	[59]
R. oryzae RBU2-10	Rice	Batch/immobilized		1.84	[73]
R. arrhizus DAR 36017	Potato	stirred tank reactors		1.3–1.6	[74]

4.3 Lignocellulosic Biomass

The performance of LAB for lactic acid production from lignocellulosic biomass is shown in Table 4. Lactic acid conversion efficiency of 81% and yield of 0.27 g/g lime-treated straw were obtained by a fed-batch SSF process with GC-220 enzyme

Table 4 Performance of microorganisms and processes on lactic acid production from lignocellulosic biomass

Microorganisms	Substrate	Process	Yield (g/g sugar)	Productivity (g/l·h)	References
B. coagulans DSM 2314	Wheat straw	Fedbatch/SSF	0.27		[21]
B. sp.36D1	Sugar cane bagasse	Batch/SSF		0.60	[76]
E. faecalis RKY1	Wood	Batch		1.7	[35]
Lb. bifermentans DSM 20003	Wheat straw	Batch, immobilization	0.83	1.17	[77]
Lb. casei NCIMB 3254	Cassava bagasse	Batch/SSF		1.40	[78]
Lb. delbrueckii NCIM 2025	Cassava bagasse	Batch/SSF		1.36	[78]
Lb. coryniformis ssp torquens ATCC 25600	Waste cardboard	Batch/SSF	0.51	0.48	[79]
Lactobacillus pentosus CHCC 2355	Wheat straw	Batch	0.88		[16]
Lb. rhamnosus ATCC 9595 (CECT288)	Apple pomace, Cellulosic biosludge	Batch/SSF	0.36–0.88	0.82–5.41	[80, 81]
Lb. rhamnosus ATCC 7469	Paper sludge	Batch/SSF	0.97	2.9	[82]
Lb. delbrueckii ZU-S2	Corn cob residue	repeated batch fed-batch, immobilization	0.92	0.93–5.75	[83]
Lb. delbrueckii subsp. *delbrueckii* Mutant Uc-3	Sugarcane bagasse	Batch/SSF	0.83	0.93	[84]
Lb. delbrueckii UFV H2B20	Brewer's spent grain	Batch/flask	0.99	0.82	[85]
Lb. pentosus ATCC 8041	Vine-trimming wastes /Corn Stover	Batch/fed batch/SSF	0.65–0.77	0.1–0.9	[16, 17]
Lb. sp. RKY2	Rice and wheat bran	Batch		3.1	[86]
R. oryzae HZS6	Corncob	Batch	0.80	0.99	[87]
R. oryzae NRRL395	Corncob	Batch		0.31	[88]

and *Bacillus coagulans* DSM 2314 [21]. Moldes et al [86] investigated the lactic acid production by *Lb. delbruechii* NRRL-B445 from NaOH-pretreated wood with SSF and a 67% conversion of cellulose into lactic acid and productivity of 0.94 g/l·h were achieved in fed-batch operation supplying with fresh nutrient and enzymes.

Most research on lactic acid production focus on the conversion of the cellulose fraction of lignocellusic biomass. *Lb. pentosus, Lb. brevis* and *Lc. lactis* can ferment pentoses to lactic acid. Especially, *Lb. pentosus* can ferment hexose (glucose) through the EMP pathway under anaerobic conditions producing lactic acid as the sole product. This strain can also convert pentoses (xylose and arabinose) to equal moles of lactic acid and acetic acid through PK pathway [17]. *Lb. pentoses* ATCC-8041 has been used for converting glucose, xylose and arabinose in the hydrolytes of trimming vine shoots, barley bran husks, and corn cobs to lactic acid in SHF process. After 40-h fermentation, glucose, xylose and arabinose were almost completely utilized. The highest lactic acid yield of 0.76 g/g pentose was achieved by using hydrolysate from trimming vine shoots [16]. *L. pentoses* ATCC-8041 was also used for lactic acid production from aqueous-ammonia-treated corn stover by Zhu et al [17]. Lactic acid yield of 0.65 g/g and productivity of 0.7 g/l·h were achieved with fed-batch SSF process. *Lb. bifermentans* DSM 20003 has been used to convert glucose, arabinose and xylose from wheat bran hemicellulosic hydrolysate to lactic acid [77]. The maximum lactic acid yield, productivity and sugar utilization were 0.83 g/g consumed carbohydrate, 1.17 g/l·h, and 76%, respectively.

The filamentous fungus *R. oryzae* can also metabolize xylose as the sole carbon source to produce lactic acid. Ruengruglikit and Hang [88] obtained lactic acid yield of 0.3 g/g and productivity of 0.31 g/l·h from corncobs using *R. oryzae* after 48 h of fermentation. Lactic acid yield of 0.80 g/g and productivity of 0.99 g/l·h was obtained with *R. oryzae* HZS6 from corncob liquors obtained from acid hydrolysis [87].

Other raw materials, including sludges, have been used to produce lactic acid via SSF without any pretreatment due to the large amounts of short fiber cellulose and polysaccharide degradation products in the sludge. Lactic acid yield of 0.97 g/g carbohydrates and productivity of 2.9 g/l·h were obtained from biosludge with *Lb. rhamnosus* ATCC 7469 [82]. *Lb. rhamnosus* ATCC 9595 was another strain used to produce lactic acid from the Kraft pulp mill biosludge and lactic acid yield of 0.38 g/g biosludge and productivity of 0.87 g/l·h were obtained with SSF [81].

5 Expert Commentary and 5 Year View

In the United States, Archer Daniels Midland (ADM) and Natureworks LLC (Joint venture of Cargill and Teijin) are currently producing lactic acid from corn and other starchy biomass using microbial fermentation technology. The produced lactic acid at ADM is targeted for PLA and ethyl lactate (a solvent which dissolves many plastics and can soften the plastic for removal from a surface). Natureworks LLC is the first company to offer a family of commercially available biopolymers derived from

100% annually renewable resources with cost and performance that compete with petroleum-based packaging materials and fibers (http://www.natureworksllc.com). It has a capacity of 140,000 metric tons of polymer. Jiangxi Musashino Bio-chem Co., Ltd. (JMB) is a Sino-Japanese joint venture which has a capacity of 5,000 metric ton L-lactic acid from grain starch. With the increasing demand of lactic acid for biodegradable plastics production, processing technology for lactic acid production from abundant lignocellulosic biomass is expected to be substantially improved for conversion of multiple sugars derived from cellulose and hemicelluloses to lactic acid.

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Microbial Production of Potent Phenolic-Antioxidants Through Solid State Fermentation

Silvia Martins, Diego Mercado, Marco Mata-Gómez, Luis Rodriguez, Antonio Aguilera-Carbo, Raul Rodriguez, and Cristóbal N. Aguilar

Abstract The agroindustrial residues including plant tissues rich in polyphenols were explored for microbial production of potent phenolics under solid state fermentation processes. The fungal strains capable of hydrolyzing tannin-rich materials were isolated from Mexican semidesert zones. These microorganisms have been employed to release potent phenolic antioxidants during the solid state fermentation of different materials (pomegranate peels, pecan nut shells, creosote bush and tar bush). This chapter includes the critical parameters for antioxidants production from selective microbes. Technical aspects of the microbial fermentation of antioxidants have also been discussed.

Keywords Phenolic antioxidants · Solid-state fermentation · Gallic acid · Ellagic acid · Nordihydroguayaretic acid

1 Introduction

Antioxidants are bioactive compounds capable of slowing or preventing the oxidation of other molecules. This process is a chemical reaction that transfers electrons from a compound to an oxidizing agent. Free radicals are generally produced in the oxidation reactions, which start the chain reactions that damage cells. Chemically, antioxidants act modifying this chain of reaction removing free radical intermediates and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as polyphenols, which are natural compounds present in numerous plants, located in fruits, flowers, cases, ferns, crusts, wood, etc. The distribution of these compounds in plant cells varies depending on the vegetal [1]. Polyphenols, constitute an important kind of chemical substances,

C.N. Aguilar (✉)
Department of Food Science and Technology, School of Chemistry, Universidad Autonoma de Coahuila, Saltillo, Coahuila, México
e-mail: cag13761@mail.uadec.mx

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and are considered secondary metabolites of the plants with different chemical structures and activities, including more than 8,000 different compounds oligo or polymerized. The polyphenolic concept is used to define substances that have one or more hydroxyl groups together with an aromatic ring, these compounds are called polyphenols. Nevertheless, not all the hydroxyl groups are phenols, because they do not have phenol's properties [2]. An important property of phenolic hydroxyls is their acidic reactivity [3].

The use of natural antioxidant compounds, especially extracted from plants, as food preservatives is nowadays widely used. Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butyl hydroquinone (TBHQ), are used in food industry to depress rancidity of fats and oils. However, the toxicity of synthetic antioxidants as well as increasing consumer demand for natural products has reinforced the need for effective antioxidants from natural sources [4]. Several efforts are being carried out by the group of Food Bioprocesses of Universidad Autónoma de Coahuila to optimize the fungal production of certain phenolic antioxidants through solid state fermentation. Main target molecules are nordihydroguaiaretic acid (NDGA), gallic acid (GA) and ellagic acid (EA).

Diverse microbial bioprocesses were well developed during the first 60 years of last century; however, a bioprocess bad attended in that time was the solid state fermentation (SSF), an alternative method for cultivation of microbes, mainly, fungi. From the 1970s, western industries started to focus on the production of microbial metabolites and specific secondary metabolites by cultivation of fungi, yeasts and bacteria in SSF, in which microbes are grown on a moist solid substrate in the absence of free flowing water [5]. For specific applications, SSF offers improved yields and product spectra compared to the rest of fermentation systems. Currently, the main reason for the limited industrial application of SSF is the lack of engineering data and knowledge about the design and scale-up of solid state fermenters.

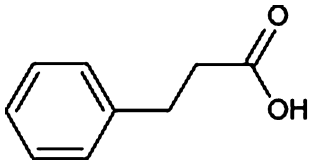
We have applied the SSF process to give an added value to agroindustrial residues, especially pomegranate husk, creosote bush, tar bush and pecan nut shells, tannin-rich materials and excellent sources of antioxidants derivated form biodegradation of lignans or tannins.

2 Background Research

2.1 Nordihydroguaiaretic Acid (NDGA)

Lignans are one of the most important groups of plant bioactive compounds structurally characterized by possession of a diphenolic ring, which contains a 2,3-dibenzylbutane structure formed from the oxidative dimerization of two cinnamic acid residues (Fig. 1). These compounds are widely distributed in numerous plant species and have been found at different levels of abundance in woody portions

Fig. 1 Cinnamic acid residue



of plants, roots, leaves, flowers, fruits and seeds [6]. Dimerization of the cinnamic acid residues, oxygen incorporation, and skeletal functionalization may occur in different ways giving a great diversity of structures. The important lignan skeletons are being listed in Fig. 2. The biological role of lignans in plants is related to the plant defence as well as to the regulation of its growth [7]. Due to a wide range of biological activities, including antifungal, antitumor, antiviral, hepatoprotective, and other properties, lignans are of considerable nutritional and pharmacological interest [8–10]. In vitro, animal, and epidemiological studies also suggest that these compounds may have cancer preventive properties through a variety of mechanisms, including anti-estrogenic, antiangiogenic, antioxidant, and pro-apoptotic properties [11, 12]. Lignans are one of three main groups of plant compounds classified as phytoestrogens, the other two being isoflavonoids and coumestans. All three groups are structurally similar to estrogens, which are known to modulate immune functions in humans [13, 14].

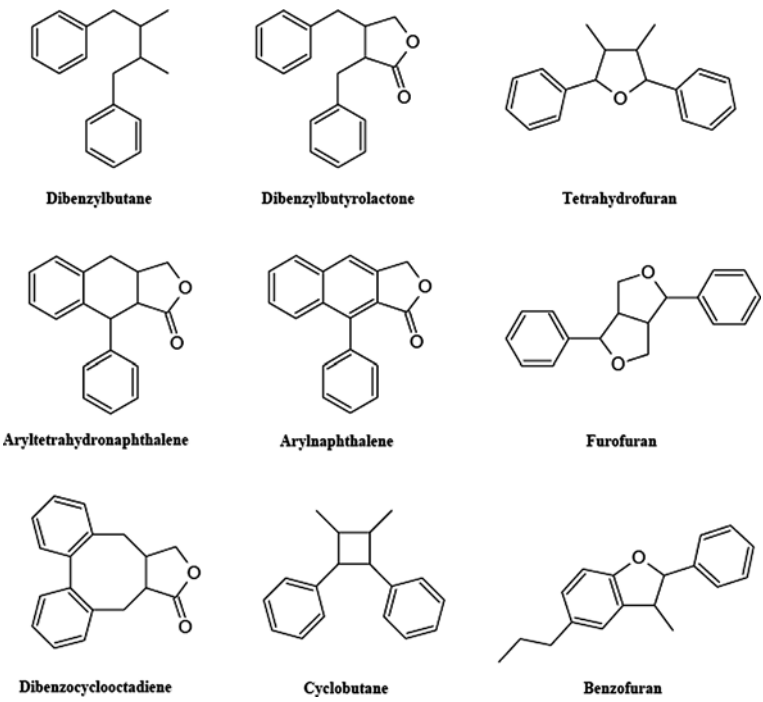


Fig. 2 Representative skeletons of lignans

The potential health effects of dietary phytoestrogens has attracted a great attention due to their protective action against several health disorders such as cancer, cardiovascular diseases, brain function disorders, menopausal symptoms and osteoporosis [12, 15].

Nevertheless, studies have suggested that in order to express their biological properties, plant lignans have to be converted into mammalian lignans by microbial action (i.e. demethylation and dehydroxylation) [16, 17]. Secoisolariciresinol diglycoside and matairesinol are two plant lignans that are not estrogenic by themselves, but are readily converted by the intestinal bacteria to the estrogenic mammalian lignans enterodiol and enterolactone, respectively [18]. The main phytoestrogen members and plant sources of each, as well as their major compound and corresponding human actives are summarised in Table 1 [14].

Another plant lignan with estrogenic activity, well known for its antioxidant properties, is nordihydroguaiaretic acid (NDGA) [19]. NDGA (Fig. 3) is originally from a semi-desert plant called *Larrea tridentata*, also known as creosote bush, which grows in the Southwestern United States and Northern Mexico. *Larrea tridentata* has been used for centuries by North American Indians as a remedy for several illnesses including inflammatory conditions as well as tumourous growths [20, 21]. Although NDGA is found in flowers, leaves, green stems and small woody

Table 1 Phytoestrogens classes: plant sources, major plant compounds, and corresponding human activities

Phytoestrogen class	Plant sources	Major plant compounds	Major corresponding (metabolic) active(s) in human
Lignan	Flaxseed	Secoisolariciresinol	Enterodiol/enterolactone
	Seed oils	Matairesinol	Enterolactone
	Grains	Pinoresinol	Enterodiol/enterolactone
	Berries	Lariciresinol	Enterodiol/enterolactone
	Green tea	Syringaresinol	Enterodiol/enterolactone
Isoflavone	Soy	Genistein/genistin	Genistein
	Red clover	Daidzein/daidzin	Daidzein/equol
	Lentils	Biochanin A/sissotrin	Genistein
	Legumes	Formononetin/ononin	Daidzein
		Glycitein/glycetin	Glycitein
		Prunetin	Genistein
Coumestan	Clovers	Coumesterol	Coumesterol
	Soy sprouts	4'-methoxycoumesterol	4'-methoxycoumesterol
	Alfalfa sprouts	4'-methoxycoumesterol	4'-methoxycoumesterol

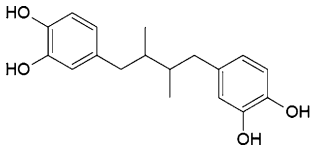


Fig. 3 Chemical structure of NDGA

stems, the highest concentrations of this lignan in *Larrea tridentata* are observed in leaves (38.3 mg/g) and green stems (32.5 mg/g) [22]. An amount of 10% of leaves dry weight, approximately, consists of NDGA, representing 80% of all phenolics in the resin.

Regarding the applications of NDGA in biological systems, several studies on its pharmacological activities have been conducted and are reported in Table 2. The earliest and best known property of NDGA is the capacity of inhibiting lipoxygenase [23, 24]. NDGA also inhibits the platelet derived growth factor receptor and the protein kinase C, both playing an important role in proliferation and survival of cancers [25]. Lee and co-workers [26] investigated the inhibitory effect of NDGA on the transforming growth factor-β (TGF-β) activity in osteoblastic cell lines. TGF-β

Table 2 Some of the NDGA pharmacological activities

NDGA pharmacological applications	Mechanisms proposed	References
Cancer chemopreventive activity		
Reactivation of methylation-silenced tumor suppressor gene p161NK4a	Cells arrest at the G1 phase and induction of cellular senescence in cancer cells	[103]
Suppression of breast cancer cells growth	Inhibition of the function or two receptor tyrosine kinases, IGF-1R and HER2/neu	[104]
Changes of DNA methylation during differentiation of human glioma cells	Inhibition of 5-cytosine DNA methyltransferase activity	[105]
Protection of DNA oxidation	Phenolic hydrogen atoms donation to quench hydroxyl radicals	[106]
Apoptosis of lypoxygenase-deficient FL5.12 cells	Depletion of glutathione and induction of a decrease in the mitochondrial membrane potential	[107]
Reduction of lung adenoma multiplicity of mice	Inhibition of 5-lypoxygenase	[108]
Antimicrobial activity		
Inhibition of *Entamoeba histolytica* in culture	Antiamoebic activity through the formation of the ortho-quinone form of NDGA	[109]
Inhibition of *Staphylococcus aureus*, *Bacillus subtilis* and *Streptococcus faecalis*	–	[110]
Fertility and reproductive effects		
Suppression of oxytocin-induced prostaglandin F2alpha	Delay of luteolysis	[111]
Ovulation inhibition, and leukotriene and prostaglandin reduction	Suppression of ovarian tissue PGE2 and PGF2alpha levels	[112]
Stimulation of pS2 expression	Binding to the estrogen receptor causing upregulation of the estrogen-responsive protein pS2	[113]

activity, which is a strong and multi-functional cytokine that affects various fundamental cellular and physiological events, was highly inhibited by NDGA. The mechanisms proposed to account for NDGA activity was the Smad2 translocation to the nucleus, as well as the strong inhibitory effect of NDGA on the phosphorylation of Smad2. In addition, several studies have demonstrated that NDGA can prevent tumour cell growth in vitro and in vivo [27–30]. A recent study evaluated the effect of NDGA on stomach carcinogenesis on specific pathogen-free Mongolian gerbil males infected with *Helicobacter pylori* [31]. A NDGA dietary level of 0.25% significantly decreased the incidence of gastric adenocarcinomas, which suggests that NDGA might be effective on gastric carcinogenesis prevention. The possible mechanisms appear to be related to inhibitory effects of NDGA on progression of gastritis and its antioxidant activity rather than direct antimicrobial influence. Moreover, NDGA induces apoptosis in certain cancer cells, including pancreatic and cervical cancer cells [32]. Different mechanisms mediating NDGA anti-carcinogenic effects have been suggested, such as glutathione depletion, peroxidation reactions, and mitochondrial stress [33, 34]. Some studies have also showed that NDGA may inhibit secretory and endocytic pathways which raise the hypothesis of other sites of action for this compound [35, 36].

Despite the fact that NDGA is likely to have several targets of action it is well tolerated in animals, which raises the interest in this compound for clinical studies. Nevertheless, due to NDGA's low solubility in water and the high concentrations required for efficacy, new analogues with improved solubility and functionality are needed [37–44]. Recently, Hwu and co-workers [45] synthesised a new series of NDGA analogues with appealing anti-HIV activity, also considering their water solubility and stability.

2.2 Gallic Acid

Gallic acid (3,4,5-trihydroxy benzoic acid) is a phenolic compound and the monomeric unit of the gallotannins and complex tannins, polyphenols considered to be the plant secondary metabolic products responsible of the prevention of attacks of microorganisms, birds, insects, etc. for this reason gallic acid and its structurally related compounds are found widely distributed in fruits and plants [46]. Gallic acid and its catechin derivatives are also present as one of the main phenolic components of both black and green tea. Esters of gallic acid have a diverse range of industrial uses, as antioxidants in food, in cosmetics and in the pharmaceutical industry [47, 48]. In the food industry, gallic acid finds application itself and as substrate of the synthesis of the propylgallate, additive used as antioxidant in fats and oils, as well as in beverages [49]. Studies utilizing gallic acid and related compounds have found them to possess many potential therapeutic properties including anti-cancer and antimicrobial properties [50].

Gallic acid and its esters can be obtained by the chemical hydrolysis of gallotannins. However, due to their commercial importance they can be produced by

biological way using fermentation [47, 51–55] and enzymatic synthesis [56–59]. However, these biological methods should be optimized to offer high productivity bioprocesses.

By fermentation, fungi, few bacteria and yeasts have been used to produce tannase, the gallic acid releasing enzyme, either by submerged (SmC) or solid state culture (SSC) [60–65]. Tannase (tannin acyl hidrolase EC: 3.1.1.20) is an inducible enzyme which catalyses the breakdown of hydrolysable tannins, gallic acid esters and diethyl diferulates [66, 67]. Tannase has been used as a clarifying agent in the beverage and brewing industries, coffee flavoured soft drinks, instant tea, grape wine and gallic acid manufacture [68]. Microbial production of gallic acid from myrobolan, tara, sumac, teri pods, creosotebush and chinese tannins as substrates has been published [1, 51, 69, 70]. Previous works have demonstrated that the xerophilic *Aspergillus niger* GH1 has the ability to growth at initial tannic acid concentrations higher than 20% [71, 72]. Figure 4 presents the structure of a gallic acid molecule.

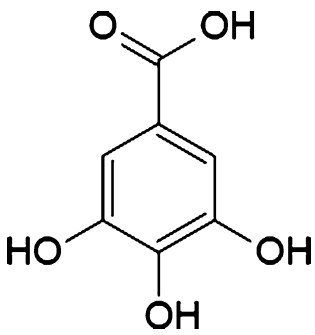


Fig. 4 Chemical structure of gallic acid

2.3 Ellagic Acid

Ellagic acid (EA) and ellagitannins (ET′s) are naturally occurring phenolic compounds widely distributed in plants. The importance of this compound is due its diverse properties reported as potent antioxidant, anti-inflammatory, anti-tumoral, anti-microbial, anti-viral and anti-proliferative capacities [73]. Major ellagitannins source are wood oak (*Quercus sp*), chesnut (*Castanea sp*) and myrobalan (*Terminalia chebula*) and some fruits like strawberry, raspberry, blueberry, cranberry, pecan and walnut [74]. Chemically, ellagitannins consist of glucose esterified with hexahydroxidiphenic acid, gallic acid and their derivates [75]. For industrial EA production from ET's, the acidic hydrolysis is the common method, however, it is an expensive and low-yield procedure [76]. Recently, several studies on biotechnological production of EA from several plant materials have been published [77–83].

We reported the first findings on fungal EA production through SSF [81, 82] demonstrating that the pomegranate husk residue is an excellent alternative for EA

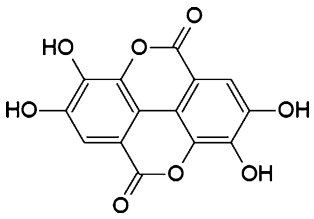


Fig. 5 Chemical structure of ellagic acid

production. Also, a biodegradation process of ET's for EA production has been proposed [73]. SSF is one of the most attractive alternatives to management of agro industrial by-products, in this case the residues of pomegranate husk contain an interesting profile of nutrients such as large amounts of insoluble carbohydrates, small amount of protein, minerals and some remaining juice and other soluble substances favoring a rapid microbial growth. These properties can be approached for the production of high value-added metabolites. Figure 5 depicts a typical structure of ellagic acid .

Extraction of bioactive compounds from plants is conventionally performed by heat-reflux extraction method. Nevertheless, different techniques including ultrasound-assisted extraction, microwave-assisted extraction, supercritical fluid extraction, and accelerated solvent extraction have been developed in order to decrease the bioactive compounds extraction time, as well as the solvent consumption, increase the extraction yield, and enhance the extracts quality [84–86]. Antioxidant phenolics extraction from *Larrea tridentata* is usually performed by conventional extraction by reflux. However, further research is needed in order to maximize the antioxidant's extraction lowering time and energy costs.

3 Technical Details

Figure 6 presents a scheme of work when phenolic antioxidants are produced by SSF. The process begins by activating the fungal strain on potato dextrose agar medium for sporulation (3–4 days at 30ºC), which were then collected in a suspension employing the detergent (0.01% Tween 80) and counted in a Neubauer chamber. Each fermentation batch inoculated at 60 millions of spores per gram of solid support. The dehydrated and pulverized plant material was 70% moisten with a minimal culture medium contained KH_2PO_4, $MgSO_4$ and NaCl. This wet material was referred as solid support and contains the rest of the nutrients for fungal growth. It is important to note that depending of the antioxidant required, the specified plant material was selected. We explored pomegranate husk for ellagic acid, and creosote bush for NDGA.

SSF is generally performed using several bioreactors, including aluminium trays, polystyrene plates, glass columns, steel columns or polyethylene bags. Inappropriate setup in bioprocesses may result into the contamination. A kinetic study of antioxidant production is always required to establish the culture conditions including

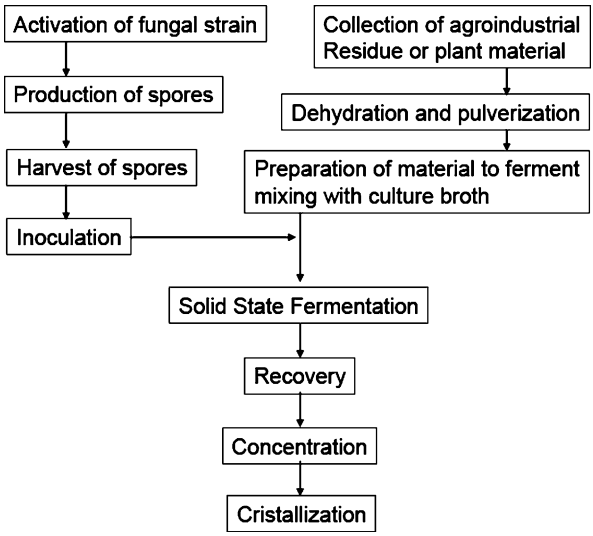


Fig. 6 Work scheme of SSF for the production of antioxidant phenolics

the time of incubation. After completion of fermentation, the SSF was stopped and the fermented solid support was mechanically removed by squeezing all the liquid contents. Fermented liquid contains the phenolic antioxidants released as result of enzymatic hydrolysis of natural polymers available in the plant material. Fungal strains were characterized by based content of specified enzymes.

The mechanical pressure did not found to be a good alternative; fermented solid support was re-suspended with the water and shacked in an immersion blender for two cycles of 30 s, the material was transferred at conic tubes and immersed in a vibrating sonic bath for 30 min. The material was centrifuged at 6,000 rpm by 30 min, decanted and the liquid fraction was recovered [87].

The biomass content was indirectly evaluated by spectrophotometry using the glucosamine content determination [88]. Substrate consumption (total polyphenols content) was evaluated as described by Makkar [89]. Each phenolic fraction recovered and quantified using HPLC method previously reported [90]. NDGA was also quantified by HPLC employing the method developed by Mercado Martinez and co-workers [91]. Gallic acid can be evaluated using HPLC [92] or employing a spectrophotometric method [93]. The separation of end product was carried out in Prodigy ODS column (5 μm; 250 × 4.6 mm, Phenomenex), maintaining temperature at 25°C. Different gradient profiles of mobile phases are used, acetonitrile, acetic acid, methanol and water are the solvents employed.

4 Current Outcome of Technological Implementation

Aspergillus niger PSH and GH1 are fungi of the DIA-UAdeC collection with a high capacity to release a great amount of antioxidant phenolics (NDGA, gallic acid

and ellagic acid). These fungal strains have demonstrated their capacity to degrade hydrolysable tannins and norlignans and to make possible the resultant accumulation of monomers which can be either consumed or accumulated during the process. The creosote and tar bush was determined to be the best sources of antioxidants. The highest consumption of total phenols in the samples collected at 48 h of SSF process was recorded. The initial concentration of total phenols in unfermented creosote bush extracts was 7.29 mg/g of plant; that was diminished up to 6.04 mg/g of plant after 48 h of fermentation. The total phenol in raw tar bush was 2.009 mg/g of plant that was diminished up to 1.001 mg/g of plant after 48 h of fermentation. Results revealed that *Aspergillus niger* PSH degrades the hydrolysable tannin polymers present in phenolic extract of both plants.

The monomers obtained by the hydrolysis of this kind of tannins were consumed by the fungus within 48 h of fermentation, and then the hydrolysis products were accumulated. However, it was observed that the monomers of condensed tannins were not consumed. The hydrolysable tannins present in the creosote bush extracts were utilized by 16% during the first 72 h of fermentation. At 96 h, the hydrolysable tannins were degraded and approximately 15% of monomers of phenolic acids were accumulated. In tar bush extracts the hydrolysable tannin consumption was approximately 40% at 48 h of fermentation.

The microbial biodegradation of condensed tannins and the respective catechin monomers accumulation were proportional to the culture time. The fungus strain recorded a similar behaviour in the fermentation kinetics of both substrates tested. The highest concentration of condensed tannins was reached at 96 h of fermentation process. An increase of condensed tannins from 42 and 83% were observed using creosote and tar bush extracts, respectively. The accumulation of gallic acid indicated the depolymerization of gallotannins and that this substance can be used as substrate. *A. niger* PSH consumed nearly 72% of free gallic acid in the extract, the minimum concentration reported was 0.14 mg/g of creosote bush at 48 h of the process. After this time, an accumulation of gallic acid was observed and this could be due to the reason that the rate of gallotannins hydrolysis was faster than the consumption rate of gallic acid. At 96 h, there was an increment in gallic acid by 152% with the concentration of 0.48 mg/g of creosote bush. In the fermentation of tar bush extracts, the gallotannis were depolymerised after 48 h followed by releasing of glucose and gallic acid. The highest level of gallic acid was reported at 96 h and was 0.08 mg/g of tar bush. Biodegradation of ellagitannins to ellagic acid and its accumulation was proportional to the fermentation time for both substrates. Initial ellagic acid concentration was 2.72 and 2.49 mg/g of tar and creosote bush respectively. After 96 h, the ellagic acid accumulated nearly by 92 and 177% in creosote and tar bush extracts respectively (Fig. 6). The highest consumption of total phenols, hydrolysable tannins in the phenolic extracts of both plants was recorded at 48 h of fermentation reaction. This could be due to the fact that the phenolic extracts of both plant materials have complex polysaccharides, and moreover the strain studied preferred to consume free monophenols and glycosides like gallic acid and glucose respectively present in the extracts before the production of hydrolytic enzymes to degrade tannins.

Some tannin-rich sources and several microorganisms [1, 51, 94, 95] have been used for gallic acid production and the hydrolytic enzyme responsible for its production is the tannase or tannins acyl hydrolase (EC, 3.1.1.20). It has also been reported earlier that tannase also can hydrolyse ellagitannins. However, the results of this study did not show this pattern and hence we consider that this enzyme is unable to degrade ellagitannins.

Results are in agreement by Shi et al. [75] on SSF of valonea tannins (79.2% at 168 h). This lower rate of hydrolysis could be due to low protein levels in the phenolics extracts of valonea. However, Belmares-Cerda et al. [96] reported better results with both substrates tested in this study using *A. niger*. This could be explained by fact that the earlier studies used leaves, a direct source of creosote and tar bush. This source contained high content of protein and tannins-protein complexes [97]. Several fungal species as *Penicillium, Chaetomium*, *Fusarium*, *Rhizoctonia*, *Cylindrocarpon*, and *Trichoderma* [98] were reported to use the monomers of gallic acid as substrate by the oxidative breakdown to simple oxidative acid, which then enter to citric acid cycle [99] and is converted to pyrogallol.

Important advances in EA production from ellagitannins have been reported in submerged co-cultures by Huang et al. [77–79] Recently, high EA yields (24%) were obtained after optimization of the co-culture of *A. oryzae* with *Trichoderma reesei* using acorn cups extract containing up to 62% ellagitannins as substrate [79]. However, in SSC the information is limited to those studies reported by Vattem and Shetty [100, 101], using cranberry pomace as support and source of ellagitannins with very low EA yields. Huang et al. [79]. suggested for the first time, the presence of ellagitannin acyl hydrolase as the enzyme responsible of the EA accumulation, which indicates that a new tannase is involved in the biodegradation ellagitannins. Also, they reported that such enzyme had a synergistic activity with other enzymes as xylanase and cellulase to enhance the EA accumulation. However, further studies are needed to define the catalytic role and properties of this new EHA or ellagitannin acyl hydrolase detected. Aguilera-Carbo et al. [82] reported that the SSF of *A. niger* GH1 using creosote bush ellagitannins impregnated in polyurethane foam could remarkably enhance EA accumulation.

The possibility to release NDGA from fermentation of *Larrea tridentata* was evidenced for the first time by Mercado Martinez [102], and high yields were reported. More than 75% of NDGA contained in the plant was recovered after fermentation process (Fig. 7). However, more efforts are needed to enhance NDGA accumulation and to improve the biotechnological process. Creosote bush is the best source of NDGA and gallic acid while pomegranate husk is the best source of ellagic acid.

5 Current Commentary and 5 Year View

The production of antioxidant phenolics by SSF is considered as an emerging technology in last five years. New information is being reported each year regarding new substrates, molecules, and procedures for recovery of the antioxidants. Also,

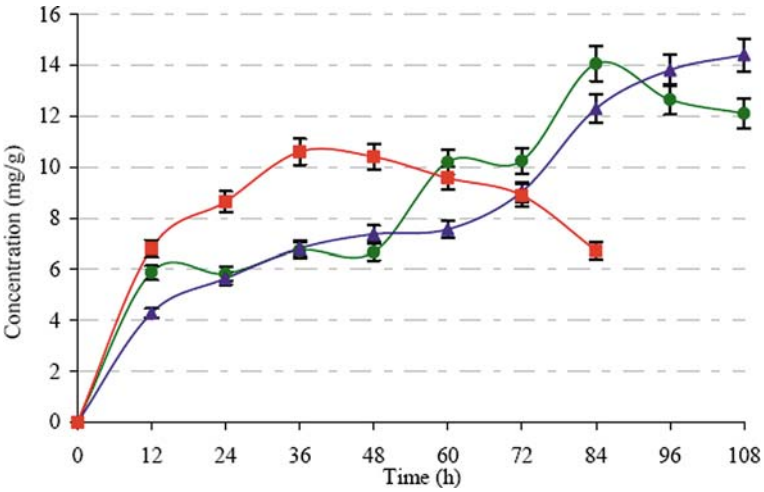


Fig. 7 Typical kinetic of NDGA production by *A. niger* GH1 (●), *A. niger* PSH (■) and *A. niger* Aa-20 (▲)

application studies are being reported. However, further studies are needed in order to know the microbial enzymes involved in the releasing of antioxidant from plant materials during the SSF, including their catalytic and physico-chemical properties. Scale-up research is also needed to allow the applicability of this technology in biotechnological industries.

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Photoautotrophic Production of Astaxanthin by the Microalga *Haematococcus pluvialis*

Esperanza Del Río, F. Gabriel Acién, and Miguel G. Guerrero

Abstract The global market for astaxanthin -the red carotenoid responsible for the color of salmon flesh, crustacean shells or flamingo feathers- rises markedly, with a preferential demand for the natural pigment from the microalga *Haematococcus pluvialis*. Current methodology for the production of astaxantin-rich cells follows a two-stage approach, first producing biomass under optimal growth conditions and then exposing the alga to adverse environmental conditions that promote encystment and accumulation of the carotenoid. An improved methodology involving a one-step-only continuous production strategy has been developed recently. Specific nitrate input and average irradiance are the most relevant parameters in determining the behavior of this continuous system that generates reddish vegetative cells, with astaxanthin representing more than 1% of the dry biomass. Feasibility of the method has been carefully analyzed indoors and verified outdoors in a tubular photobioreactor. Its singular capacity, besides a high quality of the reddish biomass product, made the system a real alternative to the two-stage option generating hard-walled red cysts.

Keywords Astaxanthin · Carotenoid · Continuous culture · *Haematococcus* · Irradiance · Nitrate input · Photobioreactor

1 Introduction

Microalgae combine properties typical of higher plants (efficient oxygenic photosynthesis and simplicity of nutritional requirements) with biotechnological attributes proper to microbial cells (fast growth in liquid culture and the ability to accumulate or secrete some metabolites). This particular combination supports

M.G. Guerrero (✉)
Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla-Consejo Superior de Investigaciones Científicas, 41092-Sevilla, Spain
e-mail: mgguerrero@us.es

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the use of these microorganisms for applied processes, and represents the basis of Microalgal Biotechnology. Microalgae are a major natural source for a vast array of valuable compounds, including a diversity of pigments, for which these photosynthetic microorganisms represent an almost exclusive renewable resource. Yellow, orange and red carotenoids have an industrial use in food products and cosmetics, as vitamin supplements and health food products, and as feed additives for poultry, livestock, fish and crustaceans. The growing worldwide market value of carotenoids is projected to reach over US $ 1,000 million by the end of the decade [1].

Astaxanthin is one of the most appreciated carotenoid pigments with applications in nutraceuticals, cosmetics, and food and feed industries. The major market for astaxanthin is as a pigmentation source in aquaculture, primarily salmon and trout [2–4]. The annual worldwide astaxanthin market is estimated to be about US$ 250 million [5, 6] at a price of about US$ 2,500 per kg. Most of this market is based on the synthetic carotenoid, the cost of this supplement representing around 10–15% of the salmon feed price [3]. The nutraceutical market for astaxanthin is increasing very fast on the basis of a growing variety of applications related to human health and nutrition [2, 7, 8]. The increase of consumers' demand for natural products provides an opportunity for natural astaxanthin although, despite technological progress in the current production process, natural astaxanthin cannot yet compete in price with the synthetic one [2].

Common sources of natural astaxanthin are the green algae *Haematococcus pluvialis* and the yeast *Phaffia rhodozyma*, as well as crustacean byproducts [7], but only that derived from *Haematococcus* or *Phaffia* might compete economically with synthetic astaxanthin. Currently, the yeast is manufactured by natural fermentation and marketed as a powder containing about 0.8% astaxanthin, utilized as an ingredient for salmonids feed [7]. However, the low yield of the production process has limited the further development of this pigment source [9].

The unicellular microalga *Haematococcus pluvialis* (Chlorophyceae) represents the richest source of natural astaxanthin, with its cysts or aplanospores accumulating over 3% astaxanthin, mainly in esterified form [10]. *H. pluvialis* is a natural inhabitant of temporary, small freshwater pools. It has a complex and not well defined life cycle, although a general consensus exists with regard to its main morphotypes. In its vegetative (growing and dividing) stage, both motile (flagellated) and non motile (palmelloid) cells (Fig. 1a, b) can be found. Green vegetative cells predominate

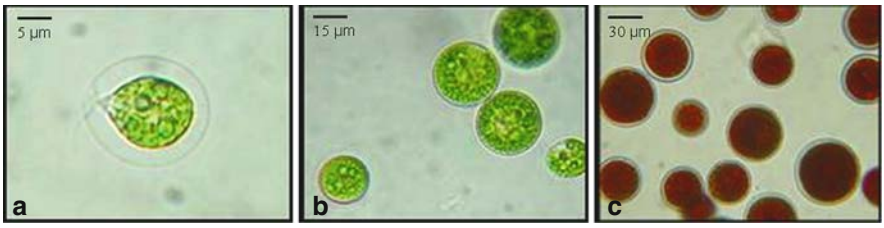

Fig. 1 *Haematococcus pluvialis* morphotypes: Flagellated (**a**); Palmelloid (**b**); Aplanospore (**c**)

under favorable growth conditions, whereas reddening and differentiation of vegetative cells to the resting form (cyst or aplanospore) is observed under suboptimal growth circumstances. Cysts are bigger in size than vegetative cells and are characterized by a thick cell wall and the massive accumulation of astaxanthin (Fig. 1c).

A relatively fast accumulation of astaxanthin occurs upon severe nutritional or environmental stress. A fair amount of information concerning factors influencing astaxanthin production and accumulation is available. High irradiance, nitrogen limitation, phosphate starvation or high temperature [11–14] leads to enhanced astaxanthin levels. Moreover, the presence of acetate [15–17], ferrous ion [13, 18], or high salt concentration [11, 19, 20] also seems to stimulate pigment accumulation. Nevertheless, the relative importance of individual factors is far from clear, since heterogeneity in culture conditions used by different authors (environmental conditions, length of experience, culture system) hampers the interpretation of results. This especially applies to conclusions drawn from experiences performed with batch cultures, an easy and widely used culture strategy.

Accumulation of astaxanthin under the effect of the stress factors mentioned above is generally assumed to be linked to cessation of growth and subsequent transformation of vegetative cells into cysts. This has led to the conclusion that cessation of cellular division is a pre-requisite for astaxanthin accumulation [11, 13, 19, 21–23]. Nevertheless, different authors have described astaxanthin accumulation in growing, flagellated and palmelloid, cells [24–29].

2 Current Methodology for the Production of *Haematococcus* astaxanthin: The Two-Stage Approach

The special characteristics of the physiology of *H. pluvialis* have determined the design of current production systems. An efficient production of astaxanthin by *Haematococcus* can be achieved in a two-stage process [5, 17, 21, 30, 31], first producing biomass under optimal growth conditions (“green” stage) and then exposing the alga to adverse environmental conditions as to induce the accumulation of astaxanthin (“red” stage).

In commercial systems, accumulation of astaxanthin is generally induced by a combination of nutrient deprivation (mainly nitrate and phosphate) and increase of irradiance and/or temperature [5]. Astaxanthin accumulation accompanies the development of red aplanospores, containing about 1.5–3.0% astaxanthin [32]. Harvesting of aplanospores is performed by settling and subsequent centrifugation. They are then dried and cracked afterwards, to ensure maximum availability of the astaxanthin [5].

Different systems exist for growth and handling of microalgae on a large scale, with sunlight as the energy source [33–39]. Within the open systems, the best choice seems to be the open shallow pond, made of leveled raceways 2–10 m wide and 15–30 cm deep, running as simple loops or as meandering systems. Each unit covers

an area of several hundred to a few thousands square meters. Turbulence is usually provided by rotating paddle-wheels which create a flow of the algal suspensions along the channels at a rate of 0.2–0.5 m s^{-1}. The adequate supply of carbon dioxide is very critical, and it is usually controlled through a pH-stat, thereby ensuring both provision of carbon and optimum pH of the culture, simultaneously. The open raceway pond reactor has some drawbacks that limit its use to strains that, by virtue of their weed-like behavior (e.g. *Chlorella*) or by their ability to withstand adverse growing conditions, as *Spirulina (Arthrospira)* or *Dunaliella*, can outcompete other microorganisms. The more recently developed and technologically advanced closed systems provide better options to grow virtually every microalgal strain, protecting the culture from invasion of contaminating organisms and allowing exhaustive control of operation conditions. These photobioreactors are either flat or tubular and can adopt a variety of designs and operation modes. In comparison to open systems, closed photobioreactors offer high productivity and better quality of the generated biomass (or product), although the latter are certainly more expensive to build and operate than the former systems. On the basis of the two-stage strategy, different approaches have been developed for the industrial production of astaxanthin, including even the use of artificial light and an organic carbon source for mixotrophic cultivation in closed photobioreactors [32, 40]. Usually, the systems utilized are based on photoautotrophic growth conditions and involve either a combination of a closed photobioreactor for the green phase and an open pond for the induction phase [41] or closed tubular photobioreactors for both stages (Algatechnologies, www.algatech.com).

A two-stage system operating under continuous illumination indoors yielded a product rich in astaxanthin (over 3% of dry biomass) and had a maximal reported productivity of 11.5 mg l^{-1} d^{-1} of carotenoids, with astaxanthin representing about 94% of the total [30]. Values for productivity of two-stage systems outdoors are rarely found in the literature [1]. Olaizola [41] reported a productivity of 2.2 mg astaxanthin l^{-1} d^{-1} for large scale commercial facilities, and Aflalo et al. [30] recently reported about 8–10 mg total carotenoids l^{-1} d^{-1} for the productivity outdoors of a combined experimental set-up composed of a flat vertical panel (green stage) and a horizontal tubular photobioreactor (red stage).

Currently, whereas commercial production of *Haematococcus*' astaxanthin is a reality, technological advances are required for a substantial reduction of costs. This would allow the competition of natural with synthetic astaxanthin so as to reach markets other than the nutraceutical one.

A reduction in production costs requires significant improvement in astaxanthin yields. A major factor influencing astaxanthin productivity is the yield at the growing phase, i.e., the "green stage" [17, 42]. However, most of the studies performed have been focused on the induction phase of pigment accumulation [10, 13]. Recently, García-Malea et al. set and modeled optimal conditions for enhancing growth in the "green stage" [43, 44]. Continuous culture with a simulated solar cycle has been performed in bubble-column reactors operated indoors. A significant productivity of green biomass (0.6–0.7 g l^{-1} d^{-1}) was obtained as a result of the combination of high irradiance and nitrate concentration.

Reactor design is another relevant issue that determines the process yield [35, 36, 45]. As mentioned above, for outdoor operation, closed tubular photobioreactors are most efficient, not only because of the high sensitivity of *Haematococcus* to contamination, but also because of the higher light availability inside this type of reactor in comparison to other systems [46].

3 The Alternative: The One-Step Strategy

The continuous culture system has recently been proposed as the approach of choice for unequivocal identification of factors influencing the accumulation of astaxanthin in *H. pluvialis* [25]. To this end, *H. pluvialis* cultures in photo-chemostats were maintained in continuous growth regime under steady state conditions. This approach allows to analyze the influence of a single variable while holding all other parameters constant, thus avoiding interferences in the interpretation of results. An in-depth analysis of factors determining astaxanthin accumulation in *Haematococcus* has been thus performed under continuous regime. The effectiveness of such a system, not only for analytical but also for production purposes, has led to the development of a one-step only strategy [25, 48].

The possibility of maintaining *Haematococcus pluvialis* cells under conditions promoting astaxanthin accumulation while maintaining full growth capacity had not received much attention until now. In the traditional two-step process, astaxanthin accumulation is induced through severe stress conditions that determine the transition of vegetative cells to cysts. The one-step system takes advantage of the capability of growing cells to accumulate astaxanthin. In continuously operated photoautotrophic cultures, nitrogen supply to the system is controlled to maintain a moderate nitrogen limitation that allows astaxanthin accumulation, without markedly affecting growth [25, 48]. This leads to high astaxanthin productivity, on the basis of maintaining a cell population that accumulates astaxanthin at a significant level, keeping besides a high growth rate (0.9 d^{-1}).

Performance of the alternative system has been validated for a variety of combinations of dilution rate, nitrate concentration in the feed medium, and incident irradiance (I_o). Dilution rate and nitrate concentration in the feed medium determine the nitrogen supply to the cells. Both factors, and especially the dilution rate, markedly affect population density. The most adequate parameter to quantify the nitrate supply to the cells is the "specific nitrate input": $SNI = [NO_3^-]^* D/C_b$, where $[NO_3^-]$ is the concentration of nitrate in the feed medium (mM); D, the dilution rate (d^{-1}); and C_b, the biomass concentration (g dry biomass l^{-1}). *SNI*, therefore, refers to the amount of nitrate (mmol) made available to cells (g dry biomass) per time unit (d).

Figure 2 shows, for constant incident irradiance, the influence of *SNI* on the yield of both biomass and astaxanthin in continuous culture of *H. pluvialis*. Lowering the *SNI* value up to a certain extent determines a slight reduction in biomass productivity, while a drastic increase in astaxanthin productivity is recorded under these

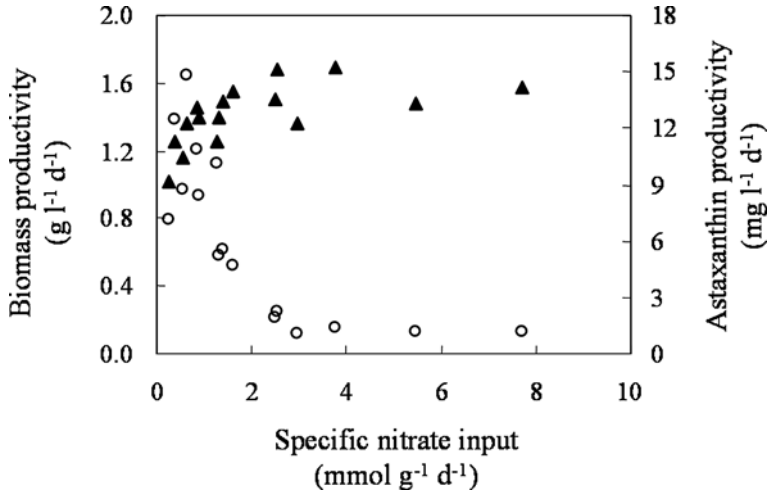

Fig. 2 Variation of biomass productivity (▲) and astaxanthin productivity (○) in continuous cultures of *H. pluvialis* as a function of specific nitrate input (I_o=1,000 μE m^{-2} s^{-1})

conditions. Optimal *SNI* value can be set in the range of 0.5–0.9 mmol NO_3^- (g dry biomass)$^{-1}$ d^{-1}, where the combination of biomass and astaxanthin productivities results more adequate.

For evaluating the influence of light availability on the behavior of the system, average irradiance, I_{av}, is the parameter of choice. I_{av} takes into account not only incident irradiance, but also biomass concentration and the geometry of the reactor [47]. Under conditions of nitrogen sufficiency, the growth rate is a hyperbolic function of average irradiance, and the cultures perform green, astaxanthin being present at basal levels only [48]. At *SNI* values lower than 2.7 mmol g^{-1} d^{-1} (nitrogen limitation), however, the cells accumulate astaxanthin, reaching 1.1% of the dry biomass at an *SNI* value of 0.8 mmol g^{-1} d^{-1}. For an optimal combination of *SNI* (0.8 mmol g^{-1} d^{-1}) and I_{av} (100 μE m^{-2} s^{-1}), a daily productivity close to 20 mg astaxanthin $l^{-1}d^{-1}$ is achieved. The increase in I_{av} results in enhancement of astaxanthin accumulation, but under nitrogen limiting conditions only. Astaxanthin does not accumulate at *SNI* values higher than 2.7 mmol g^{-1} d^{-1} (nitrogen sufficiency), even at high irradiance. Thus, once nitrogen limitation (the primary determining factor) triggers astaxanthin accumulation, the latter would behave as a linear function of light availability. Notwithstanding, contrary to the generalized belief [10, 13, 19, 21, 49], light itself does not suffice to promote astaxanthin accumulation when nitrogen and other nutrients are fully available. Nitrogen limitation is, therefore, the factor that triggers the accumulation of astaxanthin. Once this condition is established, pigment accumulation becomes a linear function of light availability.

Statistical analysis (ANOVA) of a very significant collection of data confirms that *SNI*, the specific nitrate input, and I_{av}, the average irradiance, are decisive parameters in determining astaxanthin content of the biomass, as well as the productivity

Table 1 Analysis of variance for astaxanthin content and productivity in continuous cultures of *H. pluvialis* as influenced by specific nitrate input and average irradiance ($\alpha = 0.05$; $n = 43$)

	Astaxanthin (mg g^{-1})		Astaxanthin productivity (mg l^{-1} d^{-1})	
Variable	*F*	*p-value*	*F*	*p-value*
SNI	49.74	0.0000	28.61	0.0000
Iav	5.29	0.0040	5.26	0.0041

of the system (Table 1). No doubt, these intensive variables control the behavior of the system. On these grounds, models have been developed that relate growth rate and astaxanthin accumulation with both average irradiance and specific nitrate input; these models accurately fit experimental data [48]. Efficient production of the carotenoid can be achieved through appropriate adjustment of the determining parameters. Thus, the developed model accurately reflects the behavior of the system, and allows estimation of productivity even for high average irradiance values (Fig. 3).

Productivity of astaxanthin by *H. pluvialis* in the one-step system compares favorably with any value obtained so far for the two-stage system operating under continuous illumination indoors. Notwithstanding, substantial effort is required for further development and scaling up prior to achieving steady operation of the one-step system at a large scale outdoors. In this context, the generated mathematical models represent powerful tools for both design and management of such systems [44, 48].

Although developments are obviously required, the one-step approach for astaxanthin production represents a serious alternative to the two-stage strategy. Besides

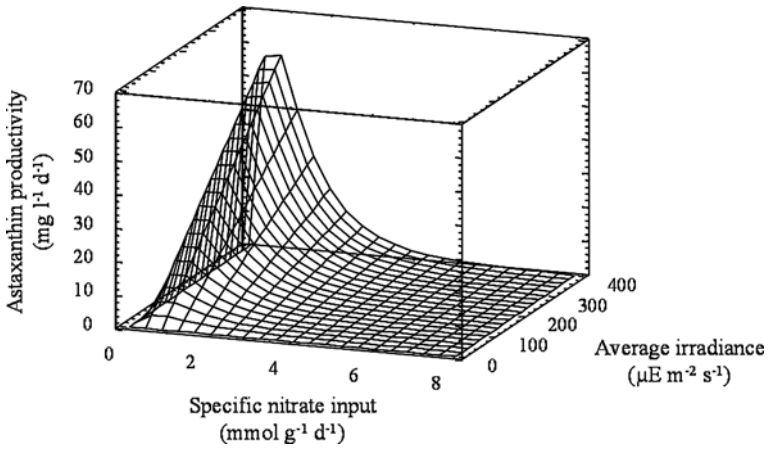

Fig. 3 Surface response curve of astaxanthin productivity by *H. pluvialis* in the one-step system as influenced by specific nitrate input and average irradiance

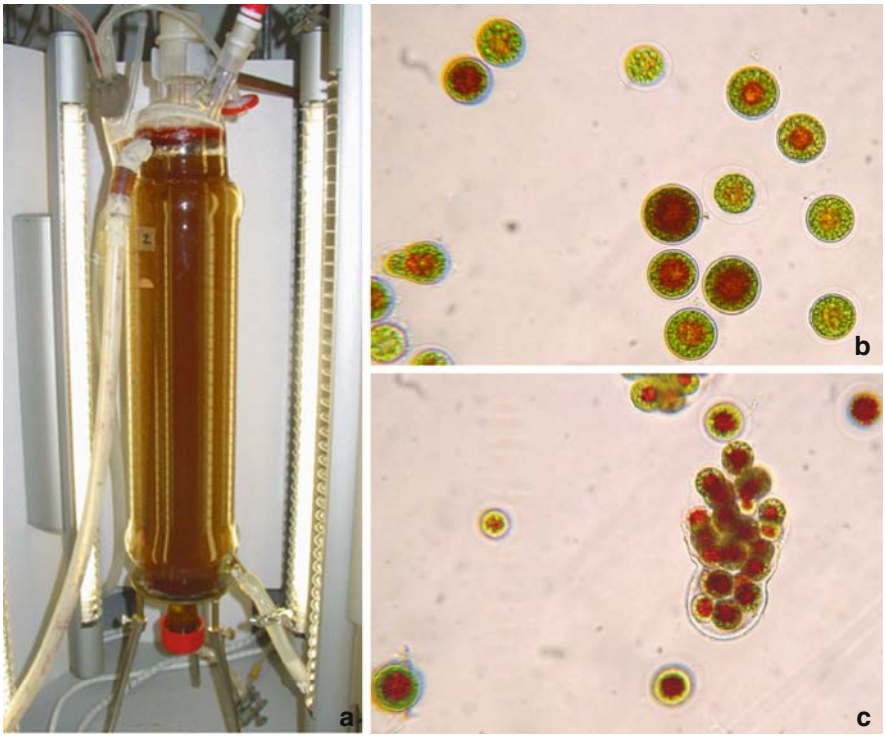

Fig. 4 Photochemostat operating under optimal conditions in the one-step mode for astaxanthin production (**a**), and microscopic view of the reddish *Haematococcus* population contained (**b**, **c**)

exhibiting superior productivity, the quality of the generated product also represents a plus. More than 95% of the cells composing the population generated in the one-step system are of the reddish vegetative type, either flagellated or palmelloid (Fig. 4), with only a small fraction of hard-walled aplanospores being present. Red aplanospore, on the other hand, is the morphotype of the accumulation stage in the two-step system.

Molecular composition of the reddish cells found in the one step system reflects a situation of moderate nitrate limitation, with a decreased protein content that, however, is higher than in cysts. Higher carbohydrate and lipid content is also found in reddish cells in comparison to the green stage (Table 2). Astaxanthin accumulation in *H. pluvialis* takes place in lipid vesicles in the cytoplasm, and a close relationship exists between fatty acid synthesis and astaxanthin accumulation [50].

Despite claimed advantages of the aplanospores as source of astaxanthin with regard to pigment content, storage stability and mechanical resistance [30, 51], the presence of the hard cell wall may hamper digestibility [27], reducing the direct applicability of cysts in aquaculture and animal nutrition. Therefore, alternatives are being sought to facilitate transfer of the pigment from *H. pluvialis* cells to the trophic chain. In this context, we have performed a study of the relative fragility of

Table 2 Molecular composition of different *H. pluvialis* populations (% of ash-free dry biomass)

Population	Protein	Carbohydrate	Lipid
Green*	30.1	35.6	9.3
Reddish*	13.8	48.7	18.7
Cysts**	8.8	51.1	25.5

*Green and reddish population generated in continuous one-step system.
**Cysts generated in two-step batch culture.

the reddish cells generated in the one-step system as compared with that of typical aplanospores (Table 3). Up to 95% of astaxanthin could be recovered from the reddish cells by extraction with methanol/chloroform (1:1), in comparison to a much lower pigment recovery when applied to cysts. Other chemical or physical treatments rendered always lower recovery from cysts as compared to reddish cells. It therefore follows that the reddish vegetative cells generated under continuous culture, lacking a hard cell wall and being amenable to cell disruption and carotenoid extraction with mild treatments, are an interesting astaxanthin source. The lesser stiffness of the cell envelopes in the biomass resulting from the continuous production process would favor bioavailability of the contained astaxanthin, for example when cells are used as an additive to fish feed [26, 27, 52]. Moreover, this reddish biomass is rich both in astaxanthin and fatty acids, exhibiting high antioxidant activity, analogous to that of cysts [53].

Thus, the demonstrated capabilities of the one-step production system, as well as its product quality, made it a viable alternative to the current two-stage systems for the production of astaxanthin-rich biomass. Performance and efficiency of outdoor operation needs verification at a convenient time and size scale. In this context, a recent viability test has been performed outdoors using a pilot scale tubular photobioreactor, consisting of a horizontal loop made of 0.025 m internal diameter tubes of a total length of 95 m, the culture volume in the reactor being 50 l [44]. The obtained results clearly support the validity of the developed models for different

Table 3 Relative fragility of astaxanthin-rich growing cells as compared with aplanospores

Treatment	One-step reddish cells astaxanthin recovery (%)	Two-step aplanospores* astaxanthin recovery (%)
Methanol/chloroform (1:1)	94.3	1.7
Methanol 70°C	62.2	24.6
French Press (20,000 psi)	64.2	14.9
Sonication (10 min, 40 W)	48.5	10.1

Astaxanthin was determined as described in Del Río et al. [25] 100% corresponds to 10 and 16 mg astaxanthin per g dry weight, for growing cells and aplanospores, respectively, disrupted with alumina in a mortar.
*Aplanospores generated following nitrate starvation in batch culture.

conditions and systems, as well as the robustness of the one-step system for the outdoor production of astaxanthin-rich *Haematococcus* cells.

In closed photobioreactors operated under continuous mode, according to the one-step strategy [44], the productivity values obtained are comparable to the maximum referenced values using the two-stage strategy outdoors [30]. Obviously, substantial effort is required for further development and scaling up prior to achieving steady operation of the one-step system at a large scale outdoors. In this context, the generated mathematical models represent powerful tools for both design and management of such systems. Improvements in design and operation of the photobioreactors, including predictive control tools, addressed to enhance performance and subsequent productivity, are expected to occur in the near future. The key factor to be acted upon in order to enhance productivity of the outdoor system is the availability of light to cells in the reactor, given that nitrate availability is easier to control. The controlled environment of the closed photobiorector certainly contributes to the prevention of contamination of *Haematococcus* cultures, a most crucial issue in outdoor handling of this valuable, but most delicate microalga.

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Enzymatic Synthesis of Heparin

Renpeng Liu and Jian Liu

Abstract Heparin and low-molecular-weight heparin have been used as anticoagulant drugs for decades. Pharmaceutical grade heparin is derived from mucosal tissues of slaughtered domestic animals. However, heparin can have severe side effects, has a potential risk of contamination and unstable resources. Thus, synthetic heparin that can be manufactured in a controlled environment is desirable. Although traditional chemical synthesis has successfully reduced the structural complexity of heparin, chemical synthesis of the heparin oligosaccharide is tedious and costly. In this review, we summarize recent progress toward the enzymatic synthesis of heparin/heparan sulfate. We will emphasize the efforts to develop novel enzymatic approaches for the synthesis of heparan sulfate mimics from *Escherichia coli* heparosan and to produce polysaccharide and oligosaccharide end products with high specificity for the biological target. These advancements provide the foundation for the development of heparan sulfate/heparin-based therapeutic agents.

Keywords Anticoagulant · Enzymatic synthesis · Heparan sulfate · Heparin

1 Introduction

Heparan sulfate (HS) is a highly sulfated polysaccharide that represents a unique class of natural products. Heparin is a special type of HS synthesized within mast cells. It was discovered in 1918 [1] and has been widely used as an anticoagulant drug for decades. HS/heparin consists of 50–100 disaccharide units carrying sulfo- groups. Pharmaceutical grade heparin is derived from slaughtered domestic animal mucosal tissues such as porcine intestine or bovine lung. Its complex structure causes many of the unwanted side effects of heparin, including hemorrhage, and

J. Liu (✉)
Division of Medicinal Chemistry and Natural Product, UNC Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599, USA
e-mail: jian_liu@unc.edu

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heparin-induced thrombocytopenia (HIT) [2]. Furthermore, since heparin is isolated from animal tissues, it could be contaminated by harmful substances. Most recently, the contaminated heparin drug made by SPL caused severe side effects including life-threatening anaphylactic reactions manifesting in abnormal low blood pressure, difficulty breathing and occasional vomiting [3]. Hundreds of patients worldwide have suffered the severe reactions linked to this contaminated heparin. This accident resulted in a major heparin recall in the USA, countries of the European Union and Japan. The "oversulfated chondroitin sulfate (OSCS)" was identified as a contaminant in heparin [3–5]. This tragic event suggested the heparin supply chain is vulnerable. Thus, a synthetic heparin that can be manufactured in a confined facility remains a high priority. Chemical synthesis is so far the most successful approach to the synthesis of structurally defined heparin mimetic. This effort has led to the total synthesis of the pentasaccharide drug, Arixtra, for the treatment of thrombotic diseases [6–10]. However, chemical synthesis of this pentasaccharide is complicated and costly. In this review, we will summarize the recent efforts of enzymatic and chemoenzymatic synthesis of heparin/HS.

2 Background Research

2.1 Structures and Biological Functions of HS

HS is widely expressed on the cell surface and in the extracellular matrix in the form of HS proteoglycans (HSPGs), which contain a core protein and polysaccharide side chains. HSPGs are involved in numerous biological processes, including blood coagulation, wound healing, embryonic development, regulating tumor growth, as well as the assisting of viral and bacterial infections [11–21]. HS polysaccharides play the essential role for the functions of HSPGs. The wide range of biological functions of HS attracts considerable interest in exploiting HS-based anticoagulant, antiviral and anticancer drugs. Heparin, an analog of HS, is a commonly used anticoagulant drug. HS/heparin belongs to the glycosaminoglycan family. Depending on the structures of the disaccharide repeating units, glycosaminoglycans include HS, chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS) and hyaluronic acid (HA). HS consists of highly sulfated repeating disaccharide units of 1→4-linked glucosamine (GlcN) and glucuronic (GlcUA)/iduronic acid (IdoUA). The majority of glucosamine residues are modified with either *N*-acetyl or *N*-sulfo groups (for detailed structure, refer to Fig. 1) [22–24]. However, up to 7% of glucosamine in HS is present in *N*-unsubstituted glucosamine ($GlcNH_2$) and may play an important biological role [25]. For example, it is known that the $GlcNH_2$ unit is involved in the binding of herpes simplex virus 1 (HSV-1) glycoprotein D (gD) [18, 26]. 6-*O*-sulfo glucosamine (GlcN6S) and 2-*O*-sulfo iduronic acid (IdoUA2S) are the most common sulfated monosaccharides, and these units play critical roles in binding to fibroblast growth factors (FGFs), fibroblast growth factor receptors (FGFRs) [27] and platelet factor 4 (PF4, a major cause for HIT) [28]. 3-*O*-sulfo

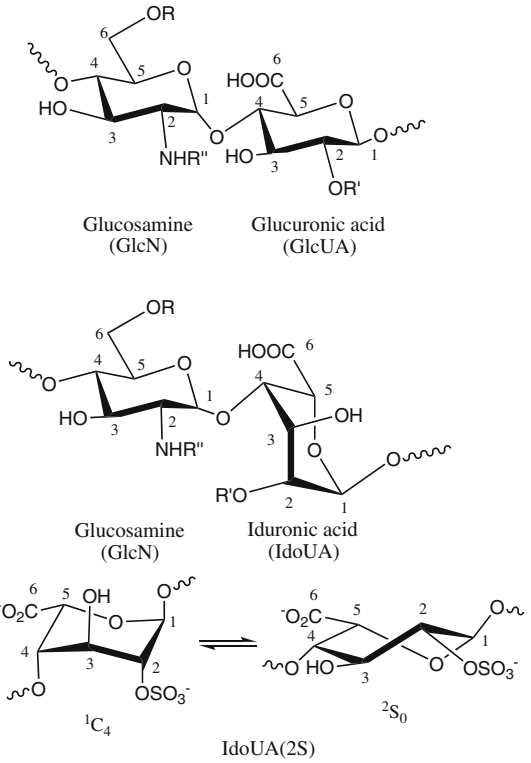

Fig. 1 Disaccharide repeating units of heparan sulfate and heparin. Sulfation (R= $-SO_3$) at Carbon 6 (known as 6-*O*-sulfated glucosamine, GlcN6S) of glucosamine is common. Sulfation (R′ = $-SO_3$) at Carbon-2 of iduronic acid (known as 2-*O*-sulfated iduronic acid, IdoUA2S) is common. Sulfation at Carbon-3 of glucosamine (known as 3-*O*-sulfated glucosamine, GlcN3S) is rare. Both *N*-acetylated (R″ = acetyl, GlcNAc) and *N*-sulfated (R″= $-SO_3$, GlcNS) are common. *N*-unsubstituted glucosamine (R″ = –H, $GlcNH_2$) is a low abundant component. IdoUA (2S) is presented in both $^{1}C_4$ and $^{2}S_0$ conformation. Both conformations are presented

glucosamine is a rare component of HS and plays an important role in binding to antithrombin (AT) [20] as well as binding to HSV-1 gD [29]. The distribution of different sulfo groups determines the biological function of HS. The structures of heparin and HS are similar; however, heparin has a higher content of IdoUA and more sulfo groups per disaccharide unit [20]. In fact, heparin has the most negative charge of all glycosaminoglycans.

2.2 Biosynthesis of HS

Heparin and HS share the same biosynthesis pathway. Understanding the biosynthetic mechanism of HS provides a tool to alter the synthesis of HS in the cells, and helps to delineate the contribution of HS in a specific biological process. Consequently, the results can be employed to improve the pharmacological drug properties of anticoagulant heparin and aid in the development of HS/heparin-based therapeutic agents with anticancer and antiviral activities. It should be noted that unlike proteins and nucleic acids, the synthesis of polysaccharides does not have a template; the specific saccharide sequences are governed by the expression level

of HS biosynthetic enzymes [15]. The biosynthesis of HS is accomplished by a complex pathway involving backbone elongation and multiple modification steps (Fig. 2). HS biosynthesis takes place in the lumen of the Golgi apparatus, although the core protein is biosynthesized in the endoplasmic reticulum (ER). The biosynthesis of HS is initiated as a copolymer of GlcUA and GlcNAc of which the polymer formation is catalyzed by copolymerases (EXT1 and EXT2) [14]. The backbone is then modified by a C_5-epimerase and different sulfotransferases. The first modification is *N*-deacetylation/*N*-sulfation to form the *N*-sulfo glucosamine unit (GlcNS) by *N*-deacetylase/*N*-sulfotransferase (NDST). NDST is a dual function enzyme that catalyzes the removal of the acetyl group from a GlcNAc residue and the transfer of a sulfo group to the amino group of the resulting $GlcNH_2$. NDST has four different isoforms which have different types of

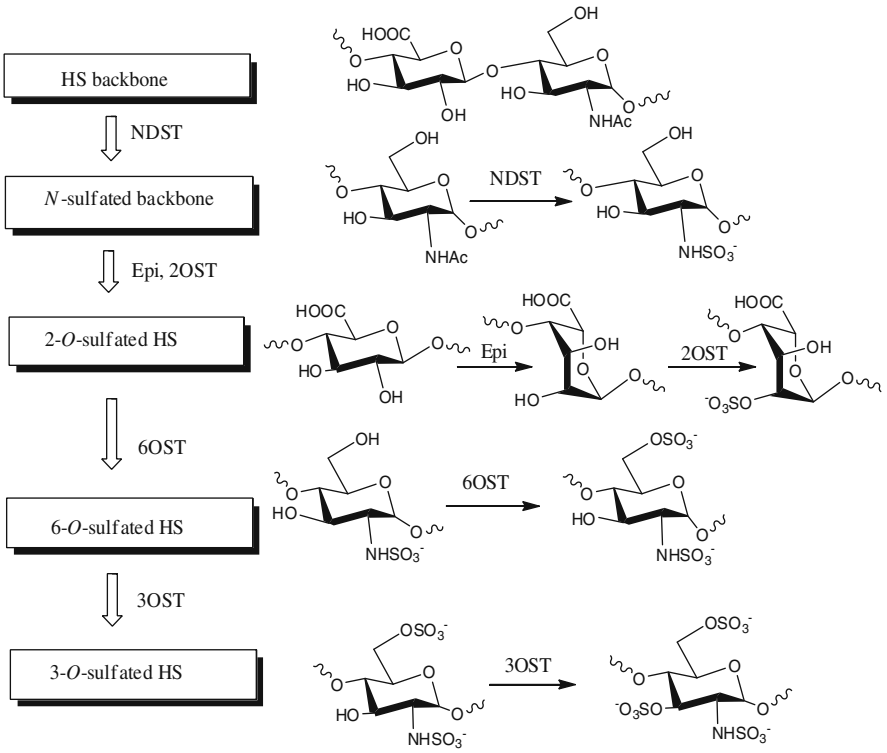

Fig. 2 HS modification pathway. Synthesis is initiated as a copolymer of GlcUA and GlcNAc by D-glucuronyl and *N*-acetyl-D-glucosaminyltransferase. The first modification is to form the *N*-sulfo glucosamine unit (GlcNS) by *N*-deacetylase/*N*-sulfotransferase (NDST). The C_5-epimerase then converts the neighboring GlcUA on the reducing side to an IdoUA unit. The chain modification proceeds with 2-*O*-sulfation at the iduronic acid (or to a lesser extent at a GlcUA), 6-*O*-sulfation at the glucosamine, and 3-*O*-sulfation at the glucosamine by different *O*- sulfotransferases. The reactions involved in polymer elongation steps are not shown

N-deacetlatylase/*N*-sulfotransferase activities [30–33]. After the *N*-sulfated backbone is generated, the C_5-epimerase converts the neighboring GlcUA unit on the reducing side to an IdoUA unit. The chain modification proceeds with 2-*O*-sulfation at the IdoUA/GlcUA (with a preference to IdoUA), 6-*O*-sulfation at the glucosamine, and 3-*O*-sulfation at the glucosamine by different *O*-sulfotransferases (OSTs [22]). C_5-epimerase and 2-*O*-sulfotransferase (2-OST) only have one isoform, while 6-*O*-sulfotransferase (6-OST) has three [34] and 3-*O*-sulfotransferase (3-OST) has seven isoforms [20].

2.3 The Role of HS/Heparin in Regulating the Blood Coagulation

Heparin has remained one of the main anticoagulant drugs since it was introduced in the 1930s [13]. The coagulation cascade consists of a series of proteases and their precursors. Factox Xa and factor IIa (or named as thrombin) are two key proteases in controlling the blood coagulation cascade. The advantages of heparin over other anticoagulant drugs include following: heparin is the only drug can inhibit both factor Xa and IIa activities, heparin has a fast anticoagulant response, and the excessive anticoagulant activity can be reversed by protamine [20]. Heparin is an exclusive product of mast cells, and is released during degranulation of mast cells. Therefore, HS, rather than heparin, is considered to be the "natural anticoagulant" in humans as HS is present on the surface of endothelial cells of blood vessels. The anticoagulant heparin and HS contain a structurally defined AT-binding pentasaccharide sequence with the structure of –GlcNS(or Ac)6S-GlcUA-GlcNS3S6S-IdoUA2S-GlcNS6S- (Fig. 3) [6–9]. The AT-binding site is the essential motif for the anticoagulant activity of heparin and HS. However, pharmaceutical grade heparin and low-molecular weight heparin (LMWH) also lead to untoward side effects include bleeding and HIT. HIT occurs in approximately 3% of patients receiving unfractionated heparin and about 0.2% in those patients receiving low molecular weight heparin [35].

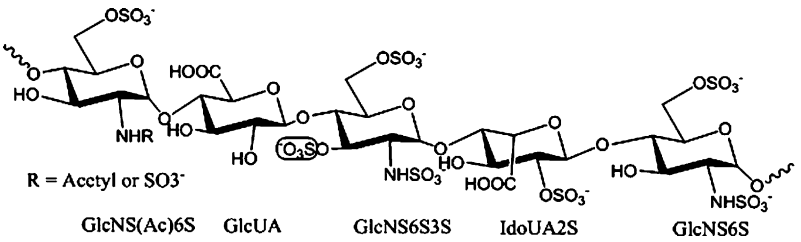

Fig. 3 The structure of AT-binding pentasaccharide. The 3-*O*-sulfation of glucosamine residue (3-*O*-sulfation is in red) is the critical modification to generate the AT binding site. GlcNS(Ac)6S, GlcUA, GlcNS3S6S, IdoUA2S and GlcNS6S represent the abbreviation of the individual monosaccharide residue

2.4 Chemical Synthesis of Heparin/HS

Chemical synthesis is a powerful approach to obtain structurally defined heparin/HS oligosaccharide. The most successful example is the total synthesis of an AT-binding pentasaccharide, which is currently a marketed drug with the trade name Arixtra used in clinics to prevent venous thromboembolic incidents during surgery. However, Arixtra only inhibits factor Xa activity and the synthesis of Arixtra is complicated and requires more than 60 steps with only a 0.5% yield [7–9]. Although the approval of Arixtra endorses the success of chemical synthesis of HS oligosaccharides, the high cost of Arixtra limits its application. In order to improve its pharmacological efficacy, a heparin mimetic with 16 saccharide units has been synthesized with both anti-Xa and anti-IIa activities. But this compound is a simplified hybrid molecule of HS oligosaccharides and highly sulfated glucose units that are not natural occurring heparan sulfate/heparin structure [6, 10]. The compound is effective in baboon [6]; however, it has not been marketed. Although many efforts continue to pursue the synthesis of heparin oligosaccharides, it has been difficult to generate authentic HS structures larger than a hexasaccharide solely utilizing chemical synthesis. HS biosynthetic enzymes offer a promising alternative approach for the synthesis of large heparin/HS oligosaccharides with the desired biological activities.

2.5 Enzymatic Synthesis of Heparin/HS

Several groups have reported the attempts to synthesize HS using biosynthetic enzymes to produce a product with anticoagulant activity [36–39]. For example, Rosenberg's group utilized HS biosynthetic enzymes to synthesize the HS containing AT binding sites with anticoagulant activity [36]. Although only microgram amounts of product were generated, this approach demonstrated the feasibility, and the yield (~1.1%) was higher than chemical synthesis. Lindahl and colleagues reported an alternative chemoenzymatic approach for the synthesis of anticoagulant heparin mimic in gram quantities from heparosan, an *Escherichia coli* (*E. coli*) capsular polysaccharide. The product was called neoparin [37].

Our lab has significantly improved on the enzyme-based synthesis of HS in three aspects. First, we enhanced the expression of enzymes and successfully coupled the synthesis with a 3′-phosphoadenosine-5′-phosphosulfate (PAPS) regeneration system, allowing us to prepare HS in milligrams quantities [40]. Second, we utilized this approach to identify a new anticoagulant HS, named Recomparin, which has a simplified structure [41]. Third, our lab prepared an octasaccharide that inhibited the infection of HSV-1 [42]. Here, we focus our attention on the progress.

3 Technical Details-Materials and Methods

3.1 Purification of Heparosan from E. coli

E. coli K5 strain was used to produce heparosan, a bacterial polysaccharide with repeating disaccharide *N*-acetylated glucosamine and glucuronic acid residues. Heparosan is structurally similar to unepimerized and unsulfated HS backbone. In this study, heparosan can be used to mimic HS backbone and can be modified by HS biosynthetic enzymes. Heparosan is harvested from *E. coli* cells in large quantities. Separation of heparosan from most proteins and other impurities was done using a diethylaminoethyl (DEAE) Sephacel column. To further remove any protein impurities, the eluted material was precipitated with ethanol followed by ammonium sulfate precipitation and phenol/chloroform treatment. Each liter of *E. coli* culture can yield up to 300 mg of heparosan.

Table 1 List of HS biosynthetic enzymes expressed in *E. coli*

Enzyme	Bacterial expression cells	Type of fusion protein	Yield (mg/L)	References
NDST-2	Rosetta-gami-B with GroEL	MBP-fusion protein	2	[46]
NDase-1[a]	Rosetta-gami-B with GroEL	Thioredoxin-fusion protein	5	Unpublished
NDase-2[a]	Rosetta-gami-B with GroEL	MBP-fusion protein	10	[46]
NDase-3[a]	Rosetta-gami-B with GroEL	Thioredoxin-fusion protein	5	Unpublished
NST[b]	BL21	GST-fusion protein	20	[47]
Epi	Rosetta-gami-B with GroEL	MBP-fusion protein	15	[52]
2-OST	Rosetta-gami-B	MBP-fusion protein	12	[40]
6-OST-1	Rosetta-gami-B with GroEL	MBP-fusion protein	14	[40]
6-OST-2	Rosetta-gami-B with GroEL	$(His)_6$-fusion protein	4	unpublished
6-OST-3	Rosetta-gami-B with GroEL	$(His)_6$-fusion protein	6	[41]
3-OST-1	BL21(DE3)RIL	$(His)_6$-fusion protein	8	[43]
3-OST-3	BL21(DE3)RIL	$(His)_6$-fusion protein	3	[44]
3-OST-5	Rosetta-gami-B with GroEL	$(His)_6$-fusion protein	8	[45]
KfiA	BL21 Star with GroEL	$(His)_6$-fusion protein	10	[49]
pmHS2	BL21 Star	$(His)_6$-fusion protein	20	unpublished

[a]NDase-1, -2, and -3 represents the *N*-deacetylase domain of NDST-1, NDST-2, and NDST-3, respectively.
[b]NST represents the *N*-sulfotransferase domain of NDST-1.

3.2 *Expression of HS Biosynthetic Enzymes in* E. coli

HS biosynthetic enzymes are traditionally expressed in mammalian or insect cells [36–39]. We have expressed these enzymes in bacteria that coexpresses a chaperone protein, which dramatically reduces the difficulty in obtaining the enzymes in large quantities [40–53]. We used a bacterial cell line that coexpresses chaperone GroEL/GroES to achieve high levels of expression by taking advantage of the fact that the chaperone helps the proteins fold correctly. These recombinant proteins have specific activities and substrate specificity comparable of those expressed in insect cells (Table 1).

3.3 *Coupling HS Sulfotransferase with a PAPS Regeneration System*

PAPS is the sulfo donor for sulfotransferases. The PAPS regeneration system, initially developed by Wong's lab [54], has been applied in HS/heparin enzymatic synthesis [40]. PAP is formed when the sulfo group is transferred to an acceptor. However, PAP inhibits the HS sulfotransferases activity, making milligram-scale synthesis difficult without continuously removing PAP. The PAPS regeneration

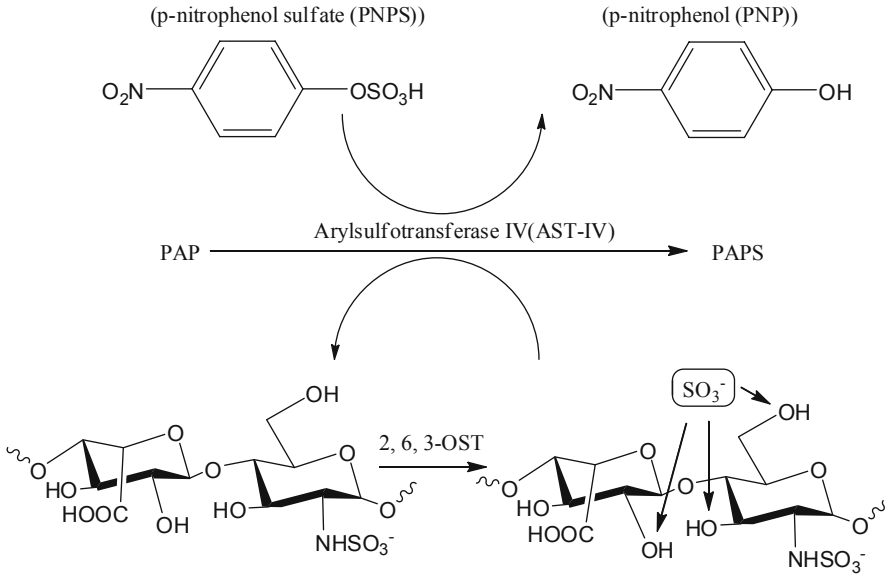

Fig. 4 PAPS regeneration system. A PAPS regeneration system converts PAP to PAPS through the action of recombinant arysulfotransferase-IV, which catalyzed the transfer of sulfo group from p-nitrophenyl sulfate (PNPS) to PAP. Thus, HS sulfotransferases use PNPS, instead of PAPS, as the sulfo donor

system converts PAP to PAPS through the action of arylsulfotransferase-IV, which catalyzes the transfer of a sulfo group from *p*-nitrophenyl sulfate (PNPS) to PAP. Thus, HS sulfotransferases use PNPS, instead of PAPS, as the sulfo donor (Fig. 4). The PAPS regeneration system offers two advantages for large–scale synthesis. First, it converts PAP to PAPS, eliminating the PAP inhibition effect. Second, the cost for PNPS is about 1,000 times lower than that for PAPS; therefore, its use significantly reduces the cost of sulfotransferase-catalyzed reactions.

4 Current Outcome of Technological Implementation

4.1 Enzymatic Synthesis of AT Binding Pentasaccharide

Rosenberg and colleagues achieved notable progress in the enzymatic synthesis of anticoagulant HS/heparin. For example, they developed an enzymatic route to synthesize a specific HS pentasaccharide that binds to AT (Fig. 5) [36]. The authors used heparosan as a starting material. Heparosan was treated with *N*-deacetylase/*N*-sulfotransferase 2 (NDST2) to prepare a partially *N*-sulfated polysaccharide, which was partially cleaved by heparin lyase I to generate a mixture of oligosaccharides of different sizes. A hexasaccharide fragment (hexasaccharide **1**) was separated by high-performance liquid chromatography (HPLC). This hexasaccharide was further treated with C_5-epimerase and 2-OST1 to generate an IdoUA2S residue at the reducing end. Next, selective 6-*O*-sulfation of two glucosamine units located at middle and non-reducing end was achieved by a 6-OST1 and 6OST2a mixture. After removal of the terminal uronic acid residue at non-reducing end by $\Delta^{4,5}$ glycuronidase, 3-*O*-sulfation of the middle glucosamine residue in the resulting pentasaccharide was accomplished by 3OST1, generating the AT-binding pentasaccharide. Either $PAP^{34}S$ or $PAP^{35}S$ was used in the 3OST1 modification step for structural characterization by electrospray ionization mass spectrometry (ESI-MS) or a gel mobility assay, respectively. Gel mobility assays confirmed that the synthetic pentasaccharide effectively binds to AT. This approach accomplished the synthesis of heparin pentasaccharide with fewer steps and a two-fold higher product yield as compared to traditional chemical synthesis. This demonstrated for the first time the feasibility of enzymatic synthetic strategies to synthesize structurally defined HS. However, only microgram amounts of product were generated, precluding further biological function studies.

4.2 Chemoenzymatic Synthesis of Anticoagulant HS from Heparosan

Lindahl and colleagues have reported an alternative chemoenzymatic approach for the synthesis of anticoagulant heparin mimic from heparosan [37]. The heparosan was initially *N*-deacetylated/*N*-sulfated and then GlcUA unit was converted to the

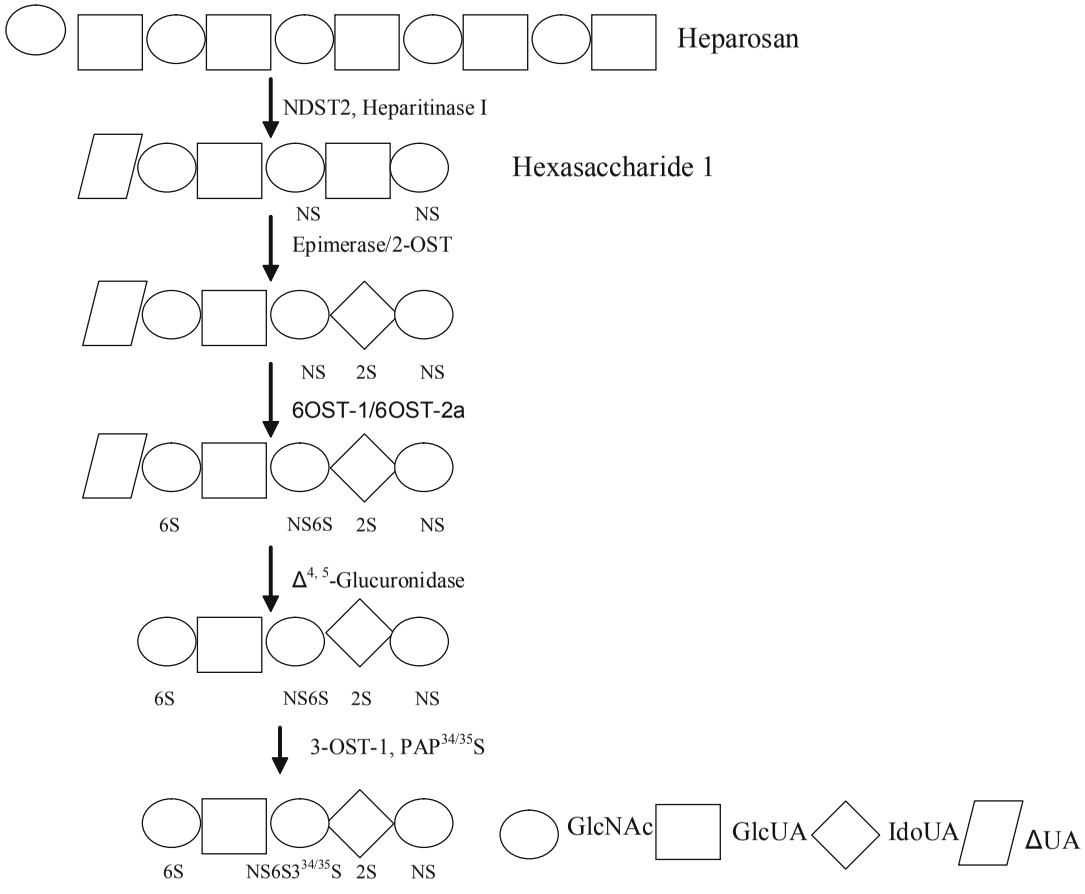

Fig. 5 Enzymatic synthesis of AT binding pentasaccharide. The synthesis was begun with heparosan. Six enzymatic steps were utilized to synthesize an AT binding pentasaccharide with anticoagulant activity. Either $PAP^{34}S$ or $PAP^{35}S$ was used in 3OST1 modification step for structural characterization by ESI-MS or gel mobility assay respectively

IdoUA unit by the C_5-Epimerase. After further chemical persulfation by pyridine sulfate and finally selective desulfation, a heparin mimic, known as neoparin, was generated. Although neoparin has levels of anti-Xa and anti-IIa activity similar to those of heparin, unwanted products, such as 3-*O*-sulfo GlcUA/IdoUA, were present in the polysaccharide, suggesting a limitation in the selectivity of chemical sulfation/desulfation in HS synthesis.

4.3 Enzymatic Redesign of HS

Our lab, in collaboration with the Linhardt group, has also developed an enzymatic approach to the synthesis of bioactive HS polysaccharides from HS backbone using the same strategy, starting from the chemically desulfated *N*-sulfated (CDSNS) heparin [40]. Only three enzymatic steps are required to synthesize anticoagulant HS (HS1 in Fig. 6) in milligrams. Immobilized enzymes were employed to permit reuse and to improve the stability of HS sulfotransferases. We further tested the activity of HS1 in inhibiting factor Xa and IIa. As expected, HS1 is a potent inhibitor of factor Xa and IIa via AT-mediated process. Its inhibition activity is very similar to that of

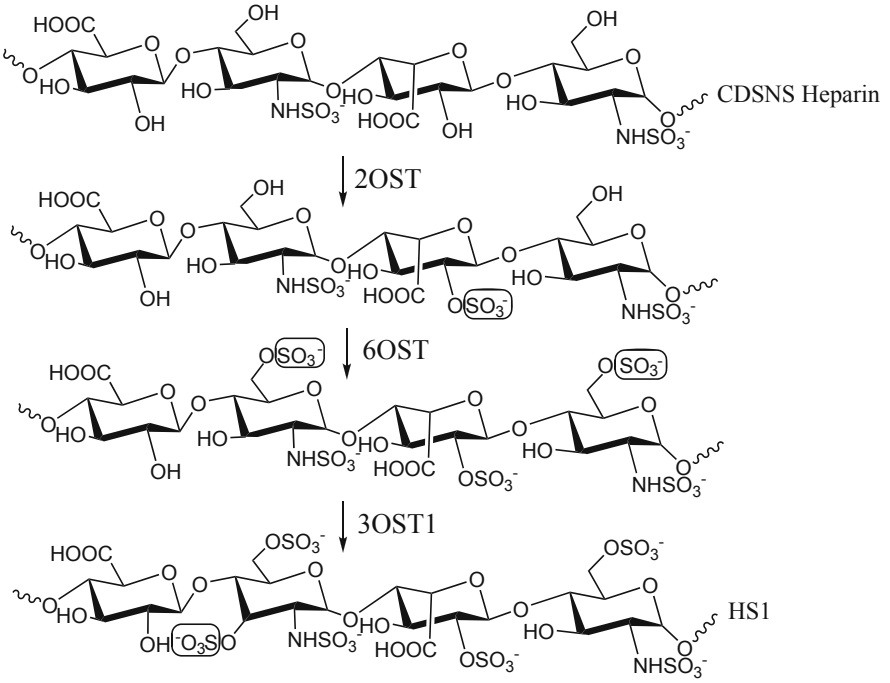

Fig. 6 Enzymatic synthesis of anticoagulant CDSNS heparin. The synthesis was begun with chemical de-sulfated/*N*-sulfated (CDSNS) heparin. CDSNS heparin was modified by 2-OST, 6-OST and 3-OST1 to generate anticoagulant polysaccharide HS1

heparin, suggesting that our enzyme-based modification system is indeed capable of converting HS backbones to anticoagulant polysaccharides. We also measured the binding affinity of AT to HS1 by surface plasmon resonance (SPR), which showed that the anticoagulant activities of HS1 correlated to its binding affinity to AT. We also generated other types of HS polysaccharides which bind to FGF2 or HSV-1 gD. Taken together, this demonstrates the feasibility of large-scale chemoenzymatic synthesis of heparin/HS with desired biological activities and could be used as a unique tool to explore the biosynthesis of heparin/HS.

4.4 Use of an Enzymatic Combinatorial Approach to Identify Novel Anticoagulant HS Structures

We applied this approach to the synthesis of a small HS library with different sulfation patterns [41]. In this study, *N*-sulfo heparosan had been chemoenzymatically prepared from heparosan starting material. A combination of recombinant HS biosynthetic enzymes was used to modify *N*-sulfo heparosan. Our lab discovered one polysaccharide, known as Recomparin (Fig. 7). Recomparin showed strong AT-mediated anticoagulant activity. Disaccharide analysis suggested that the Recomparin consists of a repeating tetrasaccharide (–GlcUA-GlcNS3S±6S-GlcUA-GlcNS6S-). It was somewhat surprising to discover Recomparin has strong anticoagulant activity despite the fact that Recomparin contains no IdoUA2S unit. Previous studies showed that the IdoUA2S unit was critical for a pentasaccharide bind to AT [7]. Furthermore, IdoUA adopts a skew boat ($^2S_0$) or chair ($^1C_4$) conformation, while GlcUA is mainly found in the chair conformation ($^4C_1$) (Fig. 1) [13]. The $^2S_0$ conformation was generally believed to be necessary for binding to AT [55]. Our results indicated that the structural flexibility of IdoUA unit is less important in polysaccharide-AT interaction. Indeed, further experimental data suggest that IdoUA unit is essential for binding to AT if the oligosaccharide is smaller than a hexasaccharide, while IdoUA unit is not essential when the oligosaccharide is larger than an octasaccharide [41]. Since IdoUA2S units are responsible for heparin binding to PF4 [28] and FGF [27], our results can help design novel heparin-based anticoagulant drugs with reduced chance of inducing HIT or stimulating cell growth. Indeed, we found that Recomparin, unlike heparin, had no activity in stimulating FGF/FGFR mediated cell proliferation, demonstrating that the anticoagulant activity and the activity in stimulating cell proliferation can be separated at the polysaccharide levels.

4.5 Preparation of 3-O-Sulfated Octasaccharide that Inhibits the Entry of HSV-1

We also utilized the enzyme-based approach to prepare a structure-defined octasaccharide with the purpose of developing novel anti-herpes drugs by targeting the viral

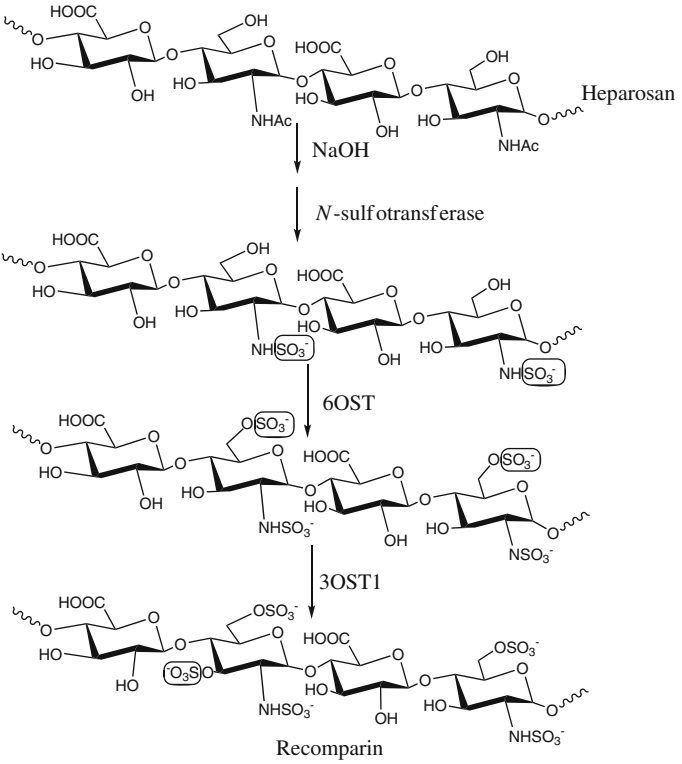

Fig. 7 Enzymatic synthesis of anticoagulant polysaccharide Recomparin. The synthesis was begun with heparosan. The acetyl group was removed by sodium hydroxide to yield the $GlcNH_2$ unit. The resultant $GlcNH_2$ unit was then *N*-sulfated by NST. After *N*-sulfated HS polysaccharide was generated, it was further modified by 6-OST and 3-OST1 to generate Recomparin

entry step [42]. It has been shown that 3-OST-3 modified HS is necessary for gD binding and that the octasaccharide is the minimum length for sufficient binding [26]. The octasaccharide exhibited the activity in inhibiting HSV-1 via saturating gD, a key envelope protein for promoting the entry of viral particles into the cells. HS that is modified by 3-OST isoforms, with the exception of 3-OST-1, binds to gD and serves as an entry receptor for HSV-1 [29, 53]. In this experiment, 3-OST isoform 3 was used. 3-OST isoform 3 transfers the sulfo group to the GlcN residue that is linked to an IdoUA2S unit at the nonreducing end [20].

The synthesis of 3-*O*-sulfated octasaccharide was completed by incubating purified 3-OST-3 enzyme and an octasaccharide substrate (Fig. 8). The octasaccharide substrate was purified from partially depolymerized heparin with heparin lyases. This 3-*O*-sulfated octasaccharide possesses a binding constant (K_d) of 19 μM as determined by affinity co-electrophoresis [29], which is comparable to the gD-binding 3-*O*-sulfated octasaccharide previously isolated from HS [26]. Further cell based assay [56, 57] demonstrated that this 3-*O*-sulfated

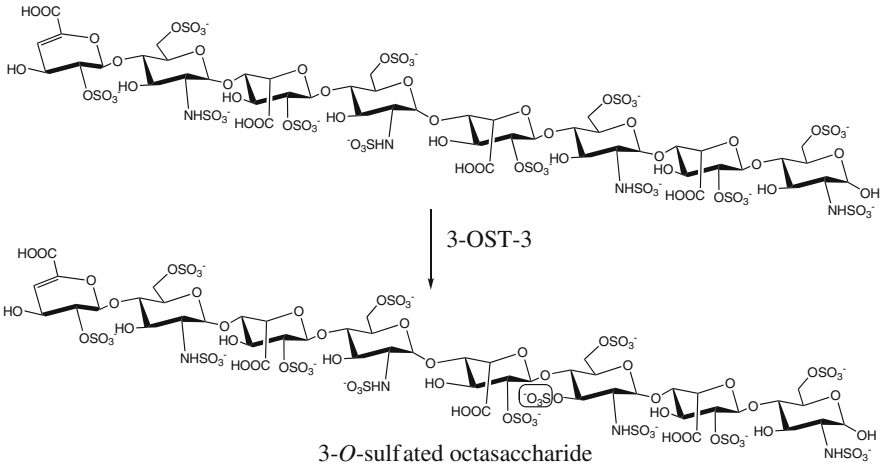

Fig. 8 Preparation of 3-*O*-sulfated octasaccharide that inhibits the entry of HSV-1. The substrate was purified from partially depolymerized heparin with heparin lyases. 3-OST-3 was utilized to transfer a sulfo group to the substrate to synthesize 3-*O*-sulfated octasaccharide

octasaccharide was indeed an inhibitor for HSV-1 infection. The structure of octasaccharide was characterized by ESI-MS and non-reducing and reducing end sequence analysis. The structure has been determined to be ΔUA2S-GlcNS6S-IdoUA2S-GlcNS6S-IdoUA2S-GlcNS6S3S-IdoUA2S-GlcNS6S (ΔUA is deoxy-α-L-threo-hex-enopyranosyluronic acid). These results demonstrate the application of enzymatic synthesis for a structurally defined HS oligosaccharide to inhibit HSV-1infection.

4.6 De Novo Synthesis of Heparin/HS Backbone

A major drawback of heparin/HS enzymatic synthesis from heparosan is that the heparosan is not a pure substrate because the size of the individual polysaccharide chain is not defined. Efforts have been made to synthesize size-defined HS backbones. One method is to use heparin lyase digested heparosan. Indeed, Rosenberg's lab has used this method to synthesize an AT-binding pentasaccharide. However, controlling the extent of digestion is difficult and HS oligosaccharides of other size and structures are difficult to achieve in large quantities. In contrast, *De novo* synthesis using HS backbone synthases provides a promising alternative approach. Bacterial glycosyl transferases offer the hope for the synthesis of HS oligosaccharide backbone. It's known that some bacteria such as *E. coli* K5 strain and *Pasteurella multocida* (*P. multocida*) can produce heparosan. Therefore, one can take advantage of the synthases involved in the biosynthesis of heparosan for HS backbone synthesis. Our lab and DeAngelis' lab have characterized some synthases involved in the biosynthesis of heparosan [48–51]. In *E. coli*, KfiA was originally

identified by Roberts' group to encode GlcNAc transferase activity, although the purified protein was not obtained [48]. Our lab developed an effective approach to express KfiA based on the published sequence [49]. The recombinant KfiA was harvested from bacterial culture at the yield of 10 mg/L. The substrate characterization study concluded that KfiA has high specificity for the UDP-GlcNAc substrate. Also, KfiA can efficiently transfer a GlcNAc group to an acceptor of various sizes, including disaccharides. DeAngelis' group successfully identified and cloned heparosan synthase pmHS1 [50] and pmHS2 [51] from *P. multocida*. Unlike KfiA, pmHS1 and pmHS2 have both GlcNAc and GlcUA transferase activities although the substrate specificities of pmHS1 and pmHS2 are believed to be distinct. The results from these studies could provide a new approach for the synthesis of heparin/HS backbone.

4.7 Alternative UDP Sugar Donor Substrates

UDP-sugar donors: UDP-*N*-acetylglucosamine (UDP-GlcNAc) and UDP-glucuronic acid (UDP-GlcUA) are widely used in the synthesis of heparin/HS oligosaccharide. The alternative UDP sugar donors can help us in the synthesis of unnatural products with novel biological or chemical properties. Several unnatural UDP-sugars have been synthesized and tested as substrates for GlcNAc or GlcUA transferase. For example, DeAngelis and colleagues reported the enzyme-catalyzed incorporation of unnatural UDP-sugar derivatives by pmHS1 and pmHS2 [58]. They found that pmHS1 required highly restricted donor structure, while pmHS2 was able to utilize several unnatural UDP-sugar analogs. For example, pmHS2 can accept UDP sugars with acetamido-containing uronic acid as GlcUA donors and it can tolerate glucosamine derivatives with longer acyl chain as GlcNAc donors. This flexible specificity of pmHS2 could be used to prepare heparin/HS analogs with novel structures. Recently, in order to study the specificity of different GlcUA transferases, Linhardt and colleagues have synthesized two UDP-GlcUA analogs: uridine 5′-diphosphoiduronic acid (UDP-IdoUA) and uridine 5′-diphosphohexenuronic acid (UDP-HexUA) [59]. In this study, pmHS1 and pmHS2 were utilized as GlcUA transferases. Unfortunately, their results demonstrated that UDP-HexUA failed to serve as a substrate for pmHS1 and pmHS2. When UDP-IdoUA was used as the substrate, sugar residues were transferred with low activity and only GlcUA was incorporated into the products formed. According to the authors, this is either due to the contamination of a small amount of UDP-GlcUA during the chemical synthesis of UDP-IdoUA or UDP-IdoUA might be isomerized to UDP-GlcUA by the GlcUA transferases via an unknown mechanism. These studies demonstrated the potential application of unnatural UDP-sugars in the chemoenzymatic preparation of synthetic HS/heparin.

In summary, our lab and other labs have demonstrated the potential of an enzyme-based approach for the synthesis of HS. This approach is clearly capable of preparing the HS with specific functions. The success of these efforts has improved

our understanding of HS biosynthesis. Most importantly, these efforts not only allow us to investigate novel synthesis of anticoagulant heparin, but also lead to a general method for preparation of structurally defined HS with various biological functions and to help develop novel heparin/HS based therapeutic agents.

5 Expert Commentary and 5 Year View

Heparin is a commonly used anticoagulant drug with annual sales close to $4 billion worldwide. Drawbacks of the drug include vulnerable supply of raw materials, severe side effects, and potential risk of contaminants [13, 60]. Over the past decade, many groups have achieved considerable progress in understanding heparin/HS biosynthesis, especially in the efforts to develop novel enzymatic approaches to synthesize HS from heparosan and to produce polysaccharide and oligosaccharide end products with high specificity for the biological targets [60]. It should be noted that heparosan can be prepared in a pharmaceutical manufacturing environment. Thus, synthetic heparin will eliminate the possibility of contamination and give the drug manufacturer a complete control over the safety and purity of the product. Optimizing the synthetic procedure will allow us to produce heparin with maximum pharmacological effects. The enzymatic synthesis will also provide an alternative approach to prepare structurally defined oligosaccharides. Although the synthesis of each different type of heparin/HS oligosaccharide remains a daunting task, the recent investigations reviewed above may accelerate our understanding of enzymatic/chemoenzymatic synthesis and help the future development of heparin/HS based drug from non-animal natural sources. Future direction includes the substrate specificity of biosynthetic enzymes, synthesis of structurally defined HS backbone, and utility of unnatural UDP-sugar donors as well as the molecular mechanism controlling the mode of heparin/HS action.

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Extremophiles: Sustainable Resource of Natural Compounds-Extremolytes

Raj Kumar, Dev Dutt Patel, Deen Dayal Bansal, Saurabh Mishra, Anis Mohammed, Rajesh Arora, Ashok Sharma, Rakesh Kumar Sharma, and Rajendra Prasad Tripathi

Abstract Microorganisms that thrive in extreme adverse environmental conditions are extremophiles. Examples of these conditions are temperature (>45°C; <10°C), pressure (>500 atmosphere), pH (>8.0; <5.0), oxygen tension, nutrient depletion, salinity (>1.0 M NaCl), high concentrations of calcitrant, heavy metals, high levels of radiation exposure etc. The discovery of extremophiles has enabled the biotechnology industry to innovate corresponding bioproducts, extremolytes, for people's benefit. The production of Taq DNA polymerase has revolutionized biotechnology research in many ways. Many thermostable enzymes including cellulase, lipase, amylase, and proteases have contributed significantly as industrial bioproducts. Extremophilic radioresistant bacteria and fungi can be used strategically for the development of radioprotective drugs to protect against radiation exposure. Further these extremophiles can be used to develop cryoprotectants. Categorically, the piezophilic microorganisms in the deep sea are a prominent source of specific bio-molecules that has ability to stabilize cell membrane blebbing by maintaining the membrane fluidity. Extremophiles are a sustainable resource for biotechnology industry, which needs to be explored. This chapter provides a comprehensive view of the extremophiles and their products with the possible implications in human interest.

Keywords Extremophiles · Radioresistant · Thermophiles · Piezophiles · Xerophiles · Radioprotectors

1 Introduction

The term extremophiles was coined by R D Macelroy in 1974 and organisms including microbes, plants and animals surviving in extreme environmental conditions are

R. Kumar (✉)
Radiation Biotechnology Laboratory, Division of Radiation Biosciences, Institute of Nuclear Medicine and Allied Sciences, Delhi-110054, India
e-mail: rajkumar790@yahoo.com

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named as such [44]. Radioresistant microorganisms are known to survive in ionizing and non ionizing radiation environments, which could otherwise, be lethal [74]. Certain evidence of past life like microfossils, stromatolites, isotopes of sedimentary carbon and sulphur indicates that microorganisms inhabiting earth during the Archaean period (the time before 2.5 Ga years) had developed metabolic functions similar to many present day living microbes. The evolutionary relationships of most of the extremophiles with present day extreme environments indicates novel genomic pools, biomolecules, and metabolic uniqueness of these microorganisms. Such extreme environment can be classified as geo-physical extreme (such as temperature, pressure, electromagnetic radiation i.e. ionizing and non ionizing radiation and cosmic radiation) and geo-chemical extreme including pH, salinity, desiccation, desert, gaseous toxicants such as reactive species of oxygen and nitrogen, redox potential, heavy metals etc.

Initially, major applications of extremophiles were identified in the production of temperature sensitive enzymes of industrial uses [12]. However, other biomolecules e.g. cryoprotectants, antifreeze proteins, membrane stabilizer lipids, antioxidants, anti-radiation agents and various other small molecules with therapeutic properties may be viewed from extremophilic origin. In a revolution of molecular biology, Taq DNA polymerase was extracted from a thermophilic bacterium *Thermus aquaticus* isolated from a hot spring in Yellowstone National Park USA. Other products of interest are thermostable polymerases and ligases including enzymes like "Antarctic phosphatase" isolated from psychrophilic (cold-loving) organisms [17]. Genencor^{TM} commercialized one of the first industrial extremozymes for use in textile detergents [76]. Therefore, in view of the tremendous industrial and therapeutic potential with extreme futuristic applications of the extremophiles, this chapter aimed to define the sustainable sources of distinguished extreme habitat and their habitant on the earth. In addition the applications of extremophiles and their products, extremolytes, with their possible implications in the human interest have been discussed.

2 Extremophilism

Life in any form requires not only an input of the energy but it must be able to control the flow of the energy. A balance in the redox states of the life chemistry is universal and need to be maintained unavoidably. An extremophile either lives within natural organic chemistry parameters, or guard against the outside world in order to maintain these parameters intracellularly [5]. Organisms that live in more than one extreme condition are called poly-extremophiles such as *Sulfolobus acidocaldarius*, an Archea that flourishes at pH 3.0 and temperature 80°C [61].

Other than living in extreme environmental conditions, the extremophiles have abilities tolerating other adverse environment. Among radioresistant *Deinococcus radiodurans* can tolerate 1,000 times more gamma radiation then the other normal microbial species [62]. Apart from vegetative microorganisms, the spores (i.e. *Bacillus* sp.), seeds and egg stages (i.e. shrimp) are more resistant to the environmental extremes than the vegetative forms. Tardigrades (water bears) in the

tun state can tolerate freezing to evaporating temperatures from –253 to 151°C, X-rays, vacuum and pressure up to 600 MPa, approximately, 6,000 times of atmospheric pressure at sea level [61]. A summary of some prominent extremophilic organisms and their habitat environmental extremes are being presented in Table 1.

Table 1 Extremophiles in different physical and geochemical environmental extremes

Environmental Parameters	Types	Definition	Examples	References
Temperature	Hyper-thermophiles	Growth> 80°C	*Pyrolobus fumarii*	[15]
			Aquifex	[26]
			Pyrococcus	[25]
			Pyrodictium	[25]
			Sulfolobus	[61]
			Thermococcus	[25]
			Thermoproteus	[25]
			Thermotoga	[25]
	Thermophiles	Growth 60–80°C	*Synechococcus lividis* *Methanobacterium* *Thermoplasma* *Thermus*	[54]
	Mesophiles	15–60°C	*Homo sapiens*	[61]
	Psychrophiles	<15°C	*Psychrobacter*, *Alteromonas*	[25]
Radiation	γ– radiation	>15 kGy	*Deinococcus radiodurans*	[9]
	γ– radiation	>15 kGy	*Rubrobacter*	[32]
			Dunaliella bardavil	[10]
Pressure	Psychro-piezophile	130 MPa	*Colwellia hadaliensis* BNL-1	[72]
		75–94 MPa	*Moritella japonica* DSK-1	[50]
		50 MPa	*Moritella yayanosii* DB21MT-5	[71]
		80 MPa	*Photobacterium profundum* SS9	[8, 21]
		28 MPa	*Photobacterium profundum* DSJ4	[90, 91]
		50–70 MPa	*Shewanella benthica*	[50, 80]
		30 MPa	*Shewanella violocea* DSS12	[70, 93]
	Thermophilic-pizophiles	51 MPa	*Methanococcus igneus*	[41]
		75 MPa	*Methanococcus jannaschii*	[41, 67]
		45 MPa	*Pyrococcus* sp. ES4	[88]
		20 MPa	*Pyrococccus* sp. GB-D	[45]
		40 MPa	*Thermococcus barophilus*	[65]

Table 1 (continued)

Environmental Parameters	Types	Definition	Examples	References
	Mesophilic-peizophiles	15 MPa	*Desulfovibrio profundus*	[4]
		60 MPa	*Pseudomonas* sp. Ms300	[51]
Desiccation	Xerophiles	Anhydrobiotic	*Artemia salina*; Nematodes	[84]
			Microbes, Fungi, Lichens	[79]
Salinity	Halophiles	Salt loving (2–5 M Nacl)	*Haloarcula, Halobacterium, Haloferax, Halorubrum, Dunaliella salina*	[61]
pH	Alkaliphiles	pH>9	*Natronobacterium, Natronococcus, Bacillus firmus* OF4, *Spirulina* sp. (all pH 10.5)	[61]
	Acidophiles	pH<4	*Ascidianus, Desulfurolobus, Sulfolobus, Thiobacillus, Cyanidium caldarium, Ferroplasma* sp.	[61]
O_2 Tensions	Anaerobes	Cannot tolerates O_2	*Methanococcus jannaschii*	[41]
	Micro-aerophiles	Tolerates some O_2	*Clostridium*	[67]
	Aerobe	Require O_2	*H. sapiens*	[61]
Chemical extremes	Heavy metals and gases	Can tolerate high concentation of metals and gases	*Cyanidium caldrium* (pure CO_2), *Feroplasma, Acidarmus*(Cu,As,Cd and Zn), *Ralstinia* sp. *CH34(Zn,Co,Cd,Hg,Pb)*	[61]

2.1 Factors Influencing Extremophilism

2.1.1 Temperature

Temperature creates a series of challenges to the living forms, from the structural destruction by formation of ice crystals at the freezing point to the denaturation of biomolecules at the higher temperature. The solubility of different gases in water is largely depending on the temperature, creating problems at high temperature for aquatic organisms requiring oxygen or carbon-dioxide for their metabolic processes. Increases in temperature up to 100°C, denatures bio-molecules including functional and structural proteins and nucleic acids. Natural thermal tolerance ranges (Fig. 1) from hyper-thermophilic (maximum growth >80°C) to psychrophilic (maximum growth <15°C) has been reported [61].

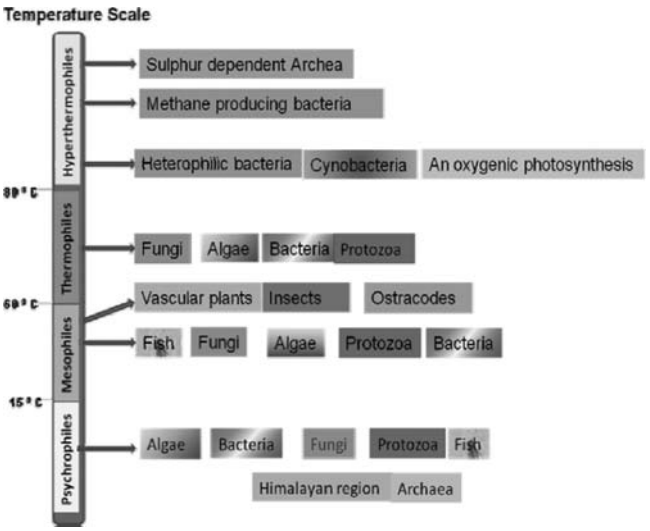

Fig. 1 Classification of microorganism based on the sustainable temperature [61]

The hyper-thermophilic microorganisms belonging to archaeabacteria include *Pyrolobus fumarii* (Crenarchaeota), a nitrate-reducing chemolithoautotrophic bacterium, grow at 113°C [15]. The enzymes derived from hyper-thermophilic bacteria have shown maximum catalytic activity at higher temperature such as the maximum amylopullulanase activity was reported at 142°C [83]. There are distinguished thermophiles among the phototrophic bacteria (cyanobacteria, purple and green bacteria), eubacteria (*Bacillus*, *Clostridium*, *Thiobacillus*, *Desulfotomaculum*, *Thermus*, lactic acid bacteria, Actinomycetes, Spirochetes etc.) and the archaea (*Pyrococcus*, *Thermococcus*, *Thermoplasma*, *Sulfolobus* and Methanogens). Invariably, the upper limit of temperature to sustained in an environment for eukaryotes including protozoa, algae and fungi is reported ~60°C. It is known that various microbes and animal cell lines are being preserved at –196°C (liquid nitrogen), whereas, lowest recorded temperature for an active microbial growth is –118°C. At low temperatures water freezes and ice crystal formed inside the cells. Ice crystals can rip cell membranes, and solution chemistry stops in the absence of liquid water. Freezing of intracellular water can be lethal to the living entities. The only exception to this rule reported so far, was a nematode *Panagrolaimus davidi.* This nematode was able to survive even after freezing of all body water [92].

2.1.2 Radiation

Radiation is energy in the form of either particles (i.e. neutrons, electrons, protons, alpha and beta particles or heavy ions) or electromagnetic waves (i.e. gamma rays, X-rays, ultraviolet (UV) radiation, visible light, infrared, microwaves or radio waves etc). Radiation background levels beyond the accepted limit are sufficient to qualify

for “extremophile” status of any organism. UV and ionizing radiation have importance in medicine, energy production, warfare and space programmes. However, the ionizing radiation causes oxidative damage among the vital biomolecules including proteins and nucleic acid. However, it is not unclear how the bacterium *D. radiodurans* can grow against supra-lethal ionizing radiation (up to 20 kGy of gamma radiation) and UV radiation (doses up to 1,000 J m) [9]. The extraordinary resistance of *D. radiodurans* is thought to be a by-product of its resistance against extreme desiccation. Other organisms that can stand high levels of radiation are *Rubrobacter* species [32] and the green alga *Dunaliella bardawil* [10].

In space research, planet ‘Mars’ atmosphere has a very thin ozone layer that changes with seasons and thus may be unable to protect potential mars micro biota if they exist [23, 57]. Endolithic cyanobacteria have been suggested as the ideal models for Martian biota by astrobiologists. These cynobacteria have the ability to protect themselves from UV rays by the producing photo-protective pigments. Desiccation- resistant strains of the cyanobacterium *Chroococcidiopsis* also exhibit resistance against ionizing radiation probably due to efficient DNA repair mechanisms [14]. Biosynthesis of radiation responsive pigments and DNA repair enzymes could be induced or activated by modern biotechnological techniques.

2.1.3 Desiccation

Water is an essential component of life under natural environment, however it generates extreme environments due to its insufficiency. Organisms that are able to tolerate extreme desiccation develop anhydrobiosis, a state characterized by little intracellular water with metabolically inactive life. Numerous organisms including bacteria, yeast, and fungi, are able to adopt anhydrobiotics, sustainable life [79]. In un-adapted situation, anhydrobiosis induced irreversible changes in the cell membrane’s lipids, proteins and nucleic acids lead to denaturation and structural breakage through Maillard reactions leading to the cell death. Anhydrobiosis also induce accumulation of reactive oxygen species during drying; especially the solar radiation is among major reasons of death in desiccated environment [28].

2.1.4 Pressure

Terrestrial life originated at an atmospheric pressure equal to 101 kPa (1 atmosphere $=$ 1.013 bar). Hydrostatic pressures are estimated to be increase at a rate of 10.5 kPa per metre depth, as compared with lithostatic pressure which increased at the rate of 22.6 kPa per metre. The pressure decreases with the increased altitude so that at 10 km above sea level, the atmospheric pressure is almost a one third of sea level. The change in pressure challenges life at the cellular level by compressing the packing of cell membrane lipids resulting in decreased membrane fluidity [7]. Many organisms have ability to survive at high pressure such as obligatory piezophilic species can grow at 70 to 80 MPa, though unable to sustain below 50 MPa [50]. Gravity is an integral component of the pressure. Organisms including human beings live on earth at $1g$. The effect of gravity and atmospheric pressure on

human health has been studied; however the effect of gravitational forces on microbial physiology including changes in biomass production, increase in conjugation, and changes in membrane permeability in *Escherichia coli* are among the major areas of space research.

2.1.5 Salinity

Physiology, biochemistry of an organism and molecular functioning of different biomolecules mostly depends on the salinity of the living environment. Osmotic aspects of life at high salt concentrations, particularly turgor pressure inside the cellular compartments, dehydration and desiccation of the cellular system are regulated by salinity of the surrounding environment. The osmotic regulation and life at high salt concentrations are two distinguished phenomenon of the same environmental event. Therefore, halophilic organisms can tolerate the osmotic stress. Halophiles especially archaea, cyanobacteria and the green alga *Dunaliella salina* including other marine microbial partners can survive for longer periods in a saturated salts environment [61] Table 1.

2.1.6 pH

pH is an inescapable factor to perform the chemical reactions in a biological environment. Most biological reactions are tends to occur at pH range 5.0–7.5 spectrum. However, some organisms are able to survive extreme low and high pH ranges. The best characterized acidophiles is *Cyanidium caldarium*, a red algae known to thrive at pH 0.5 [11]. The optimum growth of *Cyanidium caldarium* was observed at pH 2.0–3.0 [27]. The green alga *Dunaliella acidophila* and some fungi like *Acontium cylatium*, *Cephalosporium* sp. and *Trichosporon cerebriae* can survive at pH 1.0–2.0 [75]. A mysterious bacterium *Ferroplasma acidarmanus* has been reported growing at pH 0 in sulphuric acid mine drainage of an iron mountain in California [30]. Conversely, alkaliphiles prefer high pH, which is an equally challenging environment as with low pH. Most marine microorganisms grow at high pH range. Representatives of all domains and kingdoms of eukaryotes are able to tolerate pH as high as ~10 [47, 53] as represented in Table 1.

3 Extremophiles and Extremolytic Products

Extremophilic microorganisms have adopted a variety of imaginative strategies to sustain under extreme environmental conditions. The synthesis of extremolytes i.e. molecules that protect against extreme environments [35]. The extremolytes can protect the host organism from denaturation of biopolymers that usually occurs under intolerable conditions like salt concentrations >1 M and temperatures > 80°C.

Organisms exposed to osmolaritic environments can develop osmolyte strategies that have been referred to as extremolytes. Extremolytes provide protection to globular proteins, nucleic acids and whole cells. These protective effects may

partially be secondary effects of protein stabilization (e.g., stabilization of membrane proteins) in microorganisms. Extremolytes provide protection to this cells against drying environment probably by replacing of water molecules by hydroxyl group of Ectoine [59] and thus stabilize membrane fluidity. Hydroxyectoine (4S-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid) was originally discovered from an extremely halophilic phototrophic eubacterium *Halorhodospira halochloris*, isolated from Wadi Natrun, Egypt by Galinski et al. [33]. Later, hydroxyectoine substance was also found in a wide varieties of halophilic and halotolerant bacteria. The ability to accumulate ectoine was observed among the organisms such as *E. coli*, *B. subtilis*, *C. glutamicum*, and *S. melilotii* [46, 86].

The carbohydrate extremolytes such as mannosylglycerate (Firoin) and mannosylglyceramide (Firoin-A) are among top value-added extremolytes. Firoin accumulates in the cells in response to heat stress in thermophilic microbes. The chemically reactive end of the sugar of firoin forms a glycosidic bond with a hydroxyl group of glyceric acid or glyceramide. *Rhodothermus marinus* synthesizes both mannosylglycerate and mannosylglyceramide. Anionic mannosylglycerate is accumulated in the cells in response to heat stress. Whereas, uncharged mannosylglyceramide increase in the cells with elevated NaCl levels. Mannosylglycerate was also found in eukaryotic mesosphilic red algae [49].

Archaeabacteria are well known as extremophilic candidates among the microorganisms. Typical halotolerant and hyperthermophilic archeabacteria *Pyrococcus furiosus* and *Thermotoga maritima* accumulate negatively charged derivatives of inositol and glycerol at extreme temperature and high salt concentration [22, 82]. Di-myoinositol-1,1′-phosphate (DIP), a phosphodiester derivative of the uncharged osmolyte myoinositol found in eukaryotes, which provides tolerance against salinity. γ-Diglycerol phosphate (DGP) was identified as a new extremolyte in *Archaeoglobus fulgidus* and shown to be an effective protein stabilizer in vitro [56, 73]. DGP is also known to accumulate in response to elevated external NaCl concentrations, while temperature increases lead to enhanced DIP accumulation [55]. Similarly, cyclic 2,3-diphosphoglycerate (cDPG) and cyclic trianionic pyrophosphate were found to accumulate in archaea *Methanothermobacter thermoautotrophicus*. The primary role of 2,3-diphosphoglycerate (cDPG), in *Methanothermobacter* may be as a phosphate storage compound which may provide protection to glyceraldehyde-3-phosphate dehydrogenases at high temperature [42, 66]. Other novel extremolytes and their applications are being summarized in the Table 2.

Besides conventional means, non conventional sources such as marine macro and micro flora have been explored to isolate the novel drug candidates to inhibit or activate the vital signalling pathways lead to cure or prevent the particular diseases or disorder. Bryostatins, isolated from bryozoan, *Bugula neritina,* one of the novel protein kinase C (PKC) inhibitors is being evaluated for cancer cure. The marine microalgae, cyanobacteria, and heterotrophic bacteria, living in association with invertebrates (e.g. sponges, tunicates, and soft corals etc.) have been identified, as the original sources of many bioactive compounds (Kahalalide F, E7389, Curacin A, Salinosporamide A and Eleutherobin) and may be used as important

Table 2 Extremolytes and their specific applications

Compounds	Extremophilic organisms	Applications	References
Hydroxyectoine	*Streptomyces* strain	Protection of oxidative protein damage (LDH)	[1]
		Reduction of VLS in immunotoxin therapy	[6]
		Stabilization of retroviral vaccines	[24]
		Induction of thermotolerance in *E. Coli*	[63]
		Protection of *P. putida* against anhydrobiotic stress	[64]
Ectoine	*Halorhodospira halochloris*	Enzyme stabilization against heating, freezing, and drying	[59]
		Protection of LDH against heat and freeze-thawing	[36]
		Inhibition of insulin amyloid formation	[2]
		Stabilization of tobacco cells against hyperosmotic stress	[69]
		Block of UVA-induced ceramide release in human keratinocytes	[37]
		Protection of the skin barrier against water loss and drying out	[18]
		Protection of skin immune cells against UV radiation	[13]
		Reduction of UV-induced SBCs	[19]
		Prevention of UVA-induced photoaging	[18]
		Cytoprotection of keratinocytes	[20]
Mannosylglycerate	*Rhodothermus marinus*	Stabilization of enzymes against thermal stress and freeze drying	[16, 81]
		Stabilization of recombinant nuclease	[31]
DGP	*Archaeoglobus fulgidus*	Thermostabilization of proteins and rubredoxin	[55, 56]
Kahalalide F	*Elysia rufescens/ Bryopsis* sp. (mollusc/green alga)	Treatment of patients with severe psoriasis.	[40]
E7389 (halichondrin B derivative)	*Halichondria okadai* (sponge, synthetic)	Treatment for breast cancer	[3]
Curacin A	*Lyngbya majuscule* (cyanobacterium)	Potent inhibitor of cell growth and mitosis	[85]

drug candidate in human interest [3, 38, 40, 85]. The therapeutic agent Scytonemin isolated from an extremophilic marine cyanobacterium *Stigonema* sp. collected from Waldo Lake, Oregon and characterized as a protein serine/threonine kinase inhibitor [87]. Various other molecules have been screened in the hope of human interest to cure the cancer and related diseases. A battery of such compounds has been explored in the extremophilic marine organisms [29, 34, 43, 58, 60, 68, 77].

4 Future Implications of Extremolytes

Multiple irradiations such as X-rays, gamma rays, UV rays, and other electromagnetic radiations have shown tremendous application in human life. Also, various radioisotopes are being used in agriculture, medicine, diagnostic and therapeutic purposes. Apart from that, various nuclear installation sites or reactors are always prone to accidents. Besides the planed radiation exposure for human benefit, the unplanned radiation catastrophe cannot be ruled out. Under this scenario, development of an effective radioprotector is important. The extremophilic microbes and their niches are the best models having abilities to provide molecules of human interest for radioprotection. These sustainable resources of microorganisms have been explored in the past to find the lethal radiation environment [39, 48, 89]. We at Institute of Nuclear Medicine and Allied Sciences (INMAS) are involved in exploring the functional properties of radioresistant bacteria, isolated from anoxic rock samples, potentially leading to the development of an effective radioprotective biomolecule in future. Biomolecules extracted from the radioresistant bacteria has been tested for their antioxidant activities, in vitro DNA and protein protection properties and radioprotective efficacy in vitro and in vivo models. Studies performed at INMAS revealed potential support in lower animals, exposed to lethal doses of gamma radiation. The bio-molecule designed from radioresistant bacteria was also found to be capable enough to protect the radiosensitive organs (such hemopoitic, gastrointestinal and reproductive system) in mice model system. A future implication of mode of bio-molecule administration can be predicted to enhance the immunomodulatory activity with less post irradiation infection. The chances of survivability in irradiated mice, pre-treated with drug, are expected to increase compared to the control mice.

5 Expert Commentary and 5 Year View

In the past two decades thousands of molecules and drug candidates have been screened from different mesophilic microorganisms and evaluated for their possible therapeutic human applications. Many microbial bioproducts are being used as life saving drugs. However, comparatively insignificant efforts were focused on extremophilic microbes as the potential drug reservoirs of the future. In the recent years, much attention has given to the areas of astrobiology, oceanology, nuclear

energy, food production under extreme conditions. It is now being accepted that extremophiles have tremendous potential and can be sustainable resource for novel bioproducts. On futuristic approach, the radioresistant bacteria can be explored for innovation of radioprotective biomolecules that can be used in nuclear catastrophe, and may utilize to protect space travelers.

The isolation and maintenance of radioresistant bacteria remains a challenging issue due to their specific nutritional requirement, fear of pathogenesis, and unsecured genomic integrity, but the value-added bioproducts from extremophiles are of great potential utility. The antifreeze extremolytes are always in demand for people living at subzero temperature and many mountaineers facing frostbite disorder. Other than maintenance of extremophiles, the high throughput screening (HTS) and reference chemical libraries are limited that can screen a wider range of molecular specificity for strategic application of biomolecules. However, the mass spectrometry methods in analytical chemistry such as Electro Spray Ionization Ion Cyclotron Resonance Fourier Transform Mass (ESI-ICR FTMS), Fluorescence Activated Cell Sorter Multi SET^{TM} System (FACS-MS) and Nuclear magnetic resonance (NMR) methods such as Nuclear Magnetic Resonance-Structure Activity Relationship (NMR-SAR) and Saturation Transfer Difference- Nuclear Magnetic Resonance (STD-NMR) are being effectively utilized to screen chemical libraries. Not surprisingly, the sophisticated analytical instrumentation and logistic support may be a limiting factor until proven effective analysis of specified biomolecule [52]. Apart from exploring extremolytes, the evaluation of biological effectiveness, safety and toxicity of extremolyte is one of the major concerns routing drug development. The availability of animal models to generate effective data of specified biomolecule is still limited. The approaches and resources of extremophiles are broad, and have clear potential to generate value-added products for human society.

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