Clean Energy Production Technologies
Series Editors: Neha Srivastava · P. K. Mishra

Pankaj Chowdhary
Namita Khanna
Soumya Pandit
Rajesh Kumar *Editors*

Bio-Clean Energy Technologies: Volume 1

Springer

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Neha Srivastava, Department of Chemical Engineering and Technology, IIT (BHU) Varanasi, Varanasi, Uttar Pradesh, India

P. K. Mishra, Department of Chemical Engineering and Technology, IIT (BHU) Varanasi, Varanasi, Uttar Pradesh, India

The consumption of fossil fuels has been continuously increasing around the globe and simultaneously becoming the primary cause of global warming as well as environmental pollution. Due to limited life span of fossil fuels and limited alternate energy options, energy crises is important concern faced by the world. Amidst these complex environmental and economic scenarios, renewable energy alternates such as biodiesel, hydrogen, wind, solar and Bio-Clean Energy Technologies: Volume energy with zero carbon residue are emerging as excellent clean energy source. For maximizing the efficiency and productivity of clean fuels via green & renewable methods, it's crucial to understand the configuration, sustainability and techno-economic feasibility of these promising energy alternates. The book series presents a comprehensive coverage combining the domains of exploring clean sources of energy and ensuring its production in an economical as well as ecologically feasible fashion. Series involves renowned experts and academicians as volume-editors and authors, from all the regions of the world. Series brings forth latest research, approaches and perspectives on clean energy production from both developed and developing parts of world under one umbrella. It is curated and developed by authoritative institutions and experts to serves global readership on this theme.

Pankaj Chowdhary • Namita Khanna •
Soumya Pandit • Rajesh Kumar
Editors

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Editors
Pankaj Chowdhary
Environmental Microbiology Laboratory
CSIR-Indian Institute of Toxicology Research
Lucknow, Uttar Pradesh, India

Namita Khanna
Department of Biotechnology
Birla Institute of Technology and Science, Pilani, Dubai Campus
Dubai, United Arab Emirates

Soumya Pandit
Department of Life Sciences
School of Basic Sciences and Research
Sharda University
Greater Noida, India

Rajesh Kumar
Department of Microbiology
Babasaheb Bhimrao Ambedkar University
Lucknow, Uttar Pradesh, India

ISSN 2662-6861 ISSN 2662-687X (electronic)
Clean Energy Production Technologies
ISBN 978-981-16-8089-2 ISBN 978-981-16-8090-8 (eBook)
https://doi.org/10.1007/978-981-16-8090-8

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Preface

The editors of this book have had a long research career dedicated to studies on the development of biofuels and their need to be enforced in the current society. Apparently, we are no longer discussing "climate change," rather are on the way to face a "climate crisis." Our hydrocarbon-based economy that helped redefine technological feats achieved by civilizations has unfortunately led us to the brink of this climate crisis. To help prevent a temperature rise beyond 1.5°C, the foremost area of focus, besides changing societal attitudes and enforcing recycle and reuse, remains the development of alternative energy sources. This book is an exercise to discuss the latest advancements in the area of biofuel development. The contents of the book are broad, extensive, and informative regarding different aspects and types of biofuels. The contributors are experienced professors, academicians, and scientists associated with renowned laboratories and institutes in India and abroad.

Although there are several books in the market regarding biofuels, the need for this book stems from the fact that this is a rapidly evolving area of research, and this book content mainly discusses the recent advancements and the current status of the technology. This becomes imperative for the policymakers to decide the percentage of biofuel that can be generated and considered as a replacement for fossil fuel. This would also provide a road map of the various kinds of biofuels available for consideration, including both conventional and advanced algal-based biofuels, replete with the economic analysis of their production and implementation. Further studies on life cycle assessment of the biofuel under consideration assist to make a clear decision regarding the choice of biofuel and its adaptation to the local region. The fact that each country can generate their customized biofuel, in a decentralized manner, irrespective of its geographical location, political liaison, and wealth index, greatly adds to the appeal of implementing these renewable fuels into their economy.

The book is organized in two volumes to holistically cover the various aspects of biofuel production. The contents of Vol 1 are spread over 18 chapters. The introductory chapters of Volume 1 focus on introducing the readers to biofuel generations as well as the potential and challenges of renewable energy. Recent advancements in the development of genetic toolbox to manipulate both prokaryotic and eukaryotic

genomes have made genetic and metabolic engineering a key driver in the enhancement of biofuel yields. In view of this, four chapters in this volume are dedicated on briefing the readers on the latest tools and techniques to transform microbes into cellular factories for biofuel. Applications of nanotechnology in biofuel production is another rapidly evolving field. Therefore, six chapters of the book critically review the use and development of low-cost nano catalytic systems to develop next-generation biofuels. Further, a chapter is dedicated to the use of versatile algae in biofuel production. Opportunities and challenges preventing their large-scale commercialization are discussed in some details. Currently, feasible technologies include the production of biogas and biodiesel. Therefore, chapters have been dedicated to discussing biogas purification and anaerobic digestion. Lastly, the issue of "code red" to humanity, for climate change, in the latest IPCC report, has made it vital to evaluate the sustainability of every product released into the market as also the principles of recycling and reuse to prevent further environmental damage. Thus, chapters have been introduced to discuss the environmental impact of large-scale commercialization of biofuels and the techno-economic analysis of a biorefinery model for their production.

With so much literature available, it may be confounding to get a perspective on the latest trends in this technology. This book serves as a guide to inform the readers regarding the latest achievements in the field of biofuel. The proposed book also serves as an invaluable reference material to undergraduates, graduates, researchers, and policymakers working in the area of development and implementation of biofuels. Furthermore, this book also boosts up students, scientists, and researchers working in microbiology, biotechnology, environmental sciences with fundamental and advanced knowledge about the environmental challenges. In addition, readers can also get valuable information/awareness related to Bio-Clean Energy Technologies.

The editors would like to express their sincere thanks to the contributors for submitting their work in a timely and proper manner. The editors are also thankful to National and International reviewers for evaluation and valuable suggestions and comments to enhance book quality for readers. Dr. Chowdhary acknowledges the Council of Scientific and Industrial Research (CSIR), New Delhi, India, for Research Associate (RA) work. Besides, he acknowledges the support received from their family, especially Father (Mr. Ram Chandra), Mother (Mrs. Malti Devi). Further, editors also acknowledge the cooperation received from the Springer publishing team, for their guidance to finalize this book.

Lucknow, India — Pankaj Chowdhary
Dubai, UAE — Namita Khanna
Greater Noida, India — Soumya Pandit
Lucknow, India — Rajesh Kumar

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About the Editors

Pankaj Chowdhary is President, Society for Green Environment (SGE) at New Delhi. Currently, he is working as Postdoctoral Fellow in Indian Institute of Toxicology Research. He received his postgraduation degree (2011) in Biotechnology from Deen Dayal Upadhyaya Gorakhpur University, Uttar Pradesh (UP), India. Afterward, he obtained his PhD (2018) in the area of Microbiology from the Department of Environmental Microbiology at Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow, Uttar Pradesh, India. During PhD, his work has mainly focused on the role of ligninolytic enzyme producing bacterial strains in the decolorizing and degradation of coloring compounds from distillery wastewater. His main research areas are Microbial Biotechnology, Biodegradation and Bioremediation of Environmental Contaminants in Industrial Wastewaters, and Metagenomics. He has edited three international books *Emerging and Eco-friendly Approaches for Waste Management, Contaminants and Clean Technologies,* and *Microorganisms for Sustainable Environment and Health.* Besides, he has also authored two international books *New Technologies for Reclamation of Industrial Wastewater* and *Recent Advances in Distillery Waste Management for Environmental Safety.* He has published more than 70 research publications including research/review papers in national and international peer-reviewed journals of high-impact factor. He has also published many national and international book chapters and magazine articles on biodegradation and bioremediation of

industrial pollutants. He has presented many posters/papers relevant to his research areas in national and international conferences. He has also served as a potential reviewer for various national and international journals in his respective areas of the research. He is a life member of the Association of Microbiologists of India (AMI), the Indian Science Congress Association (ISCA) Kolkata, India, and the Biotech Research Society, India (BRSI).

Namita Khanna completed her graduate studies from the Indian Institute of Technology, Kharagpur, India, in 2013. Thereafter, she worked as Postdoctoral Fellow in Uppsala University, Sweden, till 2017. Currently, she works as an Assistant Professor in the Department of Biotechnology at Birla Institute of Technology and Sciences, Pilani, Dubai Campus. Her research is focused on enhancing biohydrogen production from wild type as well as from strains engineered with non-native synthetic hydrogen-producing circuits. Besides, her research interests include harnessing solar energy towards the production of synthetic biofuels and biodrugs. She has more than 27 publications to her credit in peer-reviewed journals and edited books. She has also co-authored one book on fundamentals of biohydrogen production technologies. Presently, she also serves as a Review and Guest Editor in Frontiers in Energy Research as well as a Guest Editor for Frontiers in Bioengineering and Biotechnology.

Soumya Pandit is currently working as senior assistant professor at Sharda University, Greater Noida, Delhi NCR, India. He pursued his doctoral studies from bioprocess engineering lab, Department of Biotechnology, Indian Institute of Technology, Kharagpur, in 2015 and completed his postdoctoral research work at the Department of Desalination & Water Treatment, the Zuckerberg Institute for Water Research (ZIWR), Ben-Gurion University of the Negev under Planning and Budgeting Committee (PBC) by Govt. of Israel. He is also a recipient of North-West University, South Arica Postdoctoral Fellowship. After completion of the PDF, he joined as assistant professor at Amity Institute

of Biotechnology, Amity University, Mumbai. He has published various papers at international conferences in the area of environmental biotechnology and bioenergy. He has authored 72 research and review papers in peer-reviewed journals such as (1) *Bioresource Technology*, (2) *International Journal of Hydrogen Energy*, (3) *ACS applied Materials and Interface*, (4) *Chemical Engineering Journal*, (5) *Biosensor and bioelectronics*, (6) ACS *Environmental Science and Technology*, (7) *Journal of Membrane Science*, and (8) *Journal of Cleaner Production*; he has authored 59 book chapters, 1 textbook, edited 2 books and published 9 Indian patents (2 granted) so far (H index—23). For the last 13 years, he has been working in the area of microbial electrochemical system for bioenergy harvesting, bacterial biofilm and biofouling study, biohythane production; microalgal biomass production for biofuel; nanomaterial synthesis and application in bioenergy harvesting and biofouling mitigation, etc. He has also presented his work at several national and international conferences. He is serving as an editorial board member in SCIREA *Journal of Biology* and *the Journal of Korean Society of Environmental Engineers* (JKSEE). He has attended more than 15 workshops, symposia, and faculty development programs (FDPs) both in India and abroad in his area of expertise. He has organized several FDPs and national webinars in the capacity of organizer and member of the organizing committee. Dr. Pandit is guiding his PhD students on the project related to Microbial Electrochemical System for Bioenergy Harvesting, and he has guided several MSc and MS students for their dissertation program. He has worked as a reviewer for several international journals. He has several international and national collaborations with scientists from CSIR Labs, IITs, NITs, the USA, South Korean, South African, and Israel Universities.

Rajesh Kumar postgraduate and doctorate in Microbiology, having 18 years of experience (excluding doctorate) in teaching and research of which initial 10 years at prestigious G. B. Pant University of Agriculture and Technology, Pantnagar. Presently, he is Professor of Microbiology and Head of the Department of Microbiology at Babasaheb Bhimrao Ambedkar University, (A Central University), Lucknow. He was awarded Senior Research Fellowship, INSA Visiting Fellowship at National Environmental Engineering Research Institute, Nagpur, and Innovator of the Year Award at Kasetsart University, Thailand, in the year 2016. He has guided more than a dozen of research students and published research papers in peer-reviewed journals of national and international repute with good impact factor. He has handled one Young Scientist Project of DST, New Delhi, two consultancy projects from Industry as Principal Investigator, Water Technology Initiative Project of DST as Co-PI, Uttarakhand Council of Science and Technology Project as Co-PI, and Organic Faring Project of Govt. of Uttarakhand as Co-PI worth more than 2.3 crores of rupees. Dr. Kumar hosted CV Raman International Fellow under DST-CV Raman International Fellowship for International Visiting Researchers in the year 2017–18. He is working on microbial secondary metabolites and their usage for remediation of hydrocarbons and heavy metal-contaminated sites. He has submitted around 93 DNA sequences to NCBI GenBank with Accession number, available in public domain. He has developed ELISA and QCM-based immunosensors for PGPR monitoring. Also developed vermicomposting unit for utilization of animal and agro-waste. He is the reviewer of many journals of repute such as Springer, and also reviewer for research projects at UCOST, UPCST, and Subject Expert in many panels for selection including MP Public Service Commission for various posts. He is Chairman of postgraduate studies, DRC, School Board member. He organized Industry-Academia workshops (2005–2010), conferences, trainings on water technology for CPCB, and handled different administrative positions in different capacities at various places.

Chapter 1
Brief Introduction to First, Second, and Third Generation of Biofuels

Pradyume Kumar, Bhoomika Singh, Sanchita Bipin Patwardhan, Smriti Dwivedi, Silpi Sarkar, Arpita Roy, and Soumya Pandit

Abstract Biofuels have drawn a great deal of consideration because of the expanding request on energy assets just raised worries about ozone-depleting substance discharges. Fluid biofuels can be great alternatives known as reasonable options in contrast to petroleum derivatives whose developing usage has effectively jeopardized both the climate and general well-being. First-generation biofuels have been broadly condemned for their negative effects on a reasonable stockpile of food and feed, while higher generations of biofuels have been at the focus point of consideration over the last few years. Second-generation biofuels were oil-oriented biodiesel waste alleviating environmental changes and greenhouse gas emissions. Since these sorts of biofuels require certain protocols and pretreatment, financially, their production is inefficient. Subsequently, research endeavors have been redirected toward the advancement of all the more financially feasible advances to defeat these difficulties. In this part, various ages of biofuels have been presented and reviewed subsequently.

Keywords Bioenergy · Renewable energy source · Starchy waste · Wastewater treatment · Biodiesel · Bio-hydrogen

P. Kumar · B. Singh · S. Pandit (✉)
Department of Life Sciences, School of Basic Sciences and Research, Sharda University, Greater Noida, India

S. B. Patwardhan
Amity Institute of Biotechnology, Amity University, Mumbai, Maharashtra, India

S. Dwivedi
Department of Applied Science (Chemistry), Galgotias College of Engineering and Technology, Greater Noida, Uttar Pradesh, India

S. Sarkar
Institution of Science and Technology, Vignan University, IOUT Pvt Ltd., Vadlamudi, Andhra Pradesh, India

A. Roy
Department of Biotechnology, School of Engineering, Sharda University, Greater Noida, India

P. Chowdhary et al. (eds.), *Bio-Clean Energy Technologies: Volume 1*, Clean Energy Production Technologies, https://doi.org/10.1007/978-981-16-8090-8_1

1.1 Introduction

With increase in the human population, the demand for food and energy has also increased. The main energy source for the manufacture of materials and transportation is oil. Currently, 84 million barrels (approximately) of oil are used daily for production and transport, with about 60% of this demand held by the transport sector. Greenhouse gases (methane, CO2, and nitrous oxide) are generated by the intensive use of fossil fuels and their derivatives, which can be minimized if we use plant-based biomass for bioenergy production. Biomass-generated bioenergy currently accounts for around 9% of the world's total energy supply and is an important replacement for fossil energy and has gained widespread worldwide attention (Popp et al. 2014). Biofuels produced using non-edible feedstock such as lignocellulosic biomass provide society with many benefits because they are sustainable, help fix carbon dioxide in the environment, promote growth and stimulation of the local economy, minimize air pollution by rotting biomass in fields, and also create high-tech employment for scientists and in fermentation fields.

Biofuels of the first, second, and third generations are categorized according to the raw material used, either from the origin of the biomass or identified as waste. It has always been difficult to identify biofuels, thereby restricting their use on a global scale. Biofuels of the first generation are obtained from power crops such as maize and sugarcane. The major fuels which have the ability to replace gasoline and diesel are ethanol and biodiesel. Biodiesel is made from raw vegetable oils derived from soybeans, sunflower, etc. and has recently become more desirable due to its environmental benefits as it is derived from renewable resources. Biodiesel's manufacturing process is very simple and offers great engine performance. Biofuels of the second generation are made from cellulose, hemicellulose, or lignin. The substantial content of lignocellulosic biomass in cellulose and hemicellulose serves as a critical source of sugar for bioethanol production. Biofuels of the third generation are derived from algal biomass. Algae consist of around 20–50 percent lipid content and high growth rates (Sun et al. 2018), but their high water content causes difficulties during lipid extraction, so centrifugation or filtration processes dewater them. By genetic engineering, cost-competitive algal fuels can be achieved to solve the problems of production, harvesting, and processing.

1.2 Biomass

Biomass is defined as a living organic matter. Biomass applies to all organic matter, whether of plant or animal origin, existing in the biosphere, as well as to certain materials obtained through natural or artificial transformation. It is a reliable source of energy, derived from trees, agricultural plants, and crops (Hakeem et al. 2015). Biomass can be terrestrial and aquatic. Terrestrial biomass consists of weeds, woods, leaves, grasses, fruit and vegetable solid waste, and organic municipal waste,

whereas aquatic biomass includes freshwater biomass and marine biomass (Takkellapati et al. 2018). Recently, weed is considered a potential biomass because of their ability to grow on soils which might not be suitable for the production of conventional crops. However, the production of biogas through weed biomass is also considered as a strategy for the management and control of weeds. Biogas from aquatic biomass involves the conversion of marine microalgae to a potential source of methane.

In comparison to fossil fuels, biomass is generally very bulky. When biomass is converted into liquid or gaseous biofuels, it becomes more energy-dense and less bulky. If biomass is used directly without conversion into liquid or gaseous biofuels, it will lead to some disadvantages such as (i) high moisture content making it biodegradable and difficult to store, which needs drying, and (ii) low efficiency because of substantial loss of energy on burning. The biorefinery is a multifunctional approach to processing of multiple energy products, which increases the economic value of feedstock and minimizes processing of waste streams. The International Energy Agency (IEA) has defined biorefinary as "sustainable processing of biomass into a range of marketable products (food, feed, materials and chemicals) and energy (fuel, power, heat)."

1.2.1 Forest Resources

The resource base for biomass consists of a broad range of forest and agricultural resources. The forest resource includes residues collected during the production of forest products, fuelwood harvested from forest lands, residues collected at processing plants for primary forest products, and forest resources that could be made available through fire threat reduction and forest health improvement initiatives (Table 1.1). The quantity of biomass extracted from forests is based on an evaluation of available resources and patterns in demand for forest products (Gonçalves et al. 2018). Residues from logging and other removals, fuel treatments, and urban wood residues are the three primary forest resources listed for this assessment.

1.2.2 Agricultural Resources

Grains and oilseeds are the main feedstocks for the processing of the majority of today's ethanol, bioproducts, and biodiesel consumed. Biomass resources derived from agriculture account for almost 25 percent of the current biomass consumption (Purohit and Chaturvedi 2018). Compared to currently available agricultural biomass resources, the quantity of biomass is limited, and the maximum capacity of small relative or agricultural resources is small (Table 1.2). By investing some land in the cultivation of perennial grass and woody crops, the quantity of sustainable

Table 1.1 Forest and agricultural biomass

Forest resources			Agricultural resource	
Primary resources	Secondary resources	Tertiary resources	Primary resources	Secondary resources
• Residues from traditional harvesting activities and residues from forest conservation and land cleaning activities are collected. • Extracted fuelwood from the forestland. • Excess biomass removal from woodland and other forest areas.	• Primary and secondary wood processing mill residues and pulping liquors.	• Residues of urban timber, constructive and demolition debris, tree timings, waste packaging, and consumer durables.	• Major crop residues such as corn, sugarcane, sunflower, small grain straw.	• Animal manures and food residues.

Table 1.2 Biogas composition

Component	Concentration (%)
Methane (CH_4)	55–60
Carbon dioxide (CO_2)	35–40
Hydrogen (H_2)	2–7
Hydrogen sulfide (H_2S)	2
Ammonia (NH_3)	0–0.5
Nitrogen (N)	0–2

biomass extracted from agricultural land could be further increased. Tertiary agricultural resources include municipal solid waste, post-consumer residues, and landfill gases (Mai-Moulin et al. 2019).

The composition of municipal solid waste is influenced by many factors, including geographical variations, climate variations, the degree to which recycling takes place, the extent of the collection, seasonal changes, and cultural traditions. It is anticipated that agriculture will continue to evolve and adapt to new technology and circumstances. By making genetically modified varieties of corn and soybeans viable, biotechnology is changing agriculture. Maize biotech hybrids now make up 40 percent of the total plant acreage (Ngoune Tandzi 2020). Biorefineries are distinguished based on the types of feedstocks used, the method of transforming biomass into bioenergy (including biochemical and thermochemical processes), and the types of intermediates produced. Phase I biorefinery only generates a single primary product by using only one feed material (Takkellapati et al. 2018), such as the production of biodiesel from vegetable oils and the production of ethanol from corn. Phase I and Phase II biorefineries are very similar, as they both utilize only one feedstock material, but phase II biorefineries are capable of producing multiple products (Takkellapati et al. 2018). For example, multiple carbohydrate derivatives

and bioethanol are produced from cereal grains. Phase III biorefinery consists of various groups which are whole grain, lignocellulose, green biorefinery, and two platform concept biorefinery (Takkellapati et al. 2018). These are the most advanced forms of biorefinery since they use different types of feedstock materials.

1.2.3 Lignocellulosic Biomass as Feedstock for Biofuel Production

Cellulose, hemicellulose, lignin, and other minor components (ash, protein, minerals, and pectin) constitute a complex matrix of lignocellulosic biomass. It is an effective alternative to sugarcane and maize, as it addresses the food and energy protection issues associated with the use of edible foods to produce biofuels (Figs. 1.1 and 1.2) .Corn Stover, rice husk, wheat straw, and sugarcane bagasse are the lignocellulosic feedstocks that earned the most coverage. Due to its recalcitrant nature, plant biomass decomposes slowly and can take several months to years for dead plants to be fully degraded. Whereas in biorefinery, the conversion of biomass to biofuels takes place in days. Hemicellulose-lignin complex crosslinks must be broken to improve the accessibility of cellulose and hemicellulose (Balan 2014). To accomplish this step, several pretreatment processes have been introduced.

Efficient pretreatments increase the rate of hydrolysis of the enzyme and greatly reduce the amount of enzymes required to turn biomass into sugars that microorganisms can use (Loow et al. 2016; Chowdhary et al. 2020). The efficiency of sugar conversion is determined by the amount of lignin present in pre-treated biomass; also, lignin is responsible for the unproductive binding of enzymes (Balan 2014). The reduction of lignin during enzyme reuses leads to substantial cost savings.

1.2.3.1 Chemical Pretreatment

For lignocellulose, acid hydrolysis is the most utilized method of conversion. The two fundamental forms of hydrolysis are dilute and concentrated acid hydrolysis. Both processes are conducted at high temperatures. There are mainly two forms of dilute acid pretreatment, namely, high temperature (more than 433 K) (Elliott et al. 2015), continuous flow process for low solid loading and low temperature (less than 433 K) batch process for high solid loading (Elliott et al. 2015). Pretreatment with dilute sulfuric acid is hemicellulose hydrolysis and allows cellulose to be more available for enzymatic hydrolysis (Elliott et al. 2015).

In alkaline pretreatment, alkaline catalysts such as calcium oxide (lime), ammonia, and sodium hydroxide are used for specific target groups of hemicellulose acetyl and lignin-carbohydrate ester linkages (da Costa et al. 2009). Alkaline catalysis is the saponification of intermolecular ester bonds that cross-link xylan hemicelluloses and

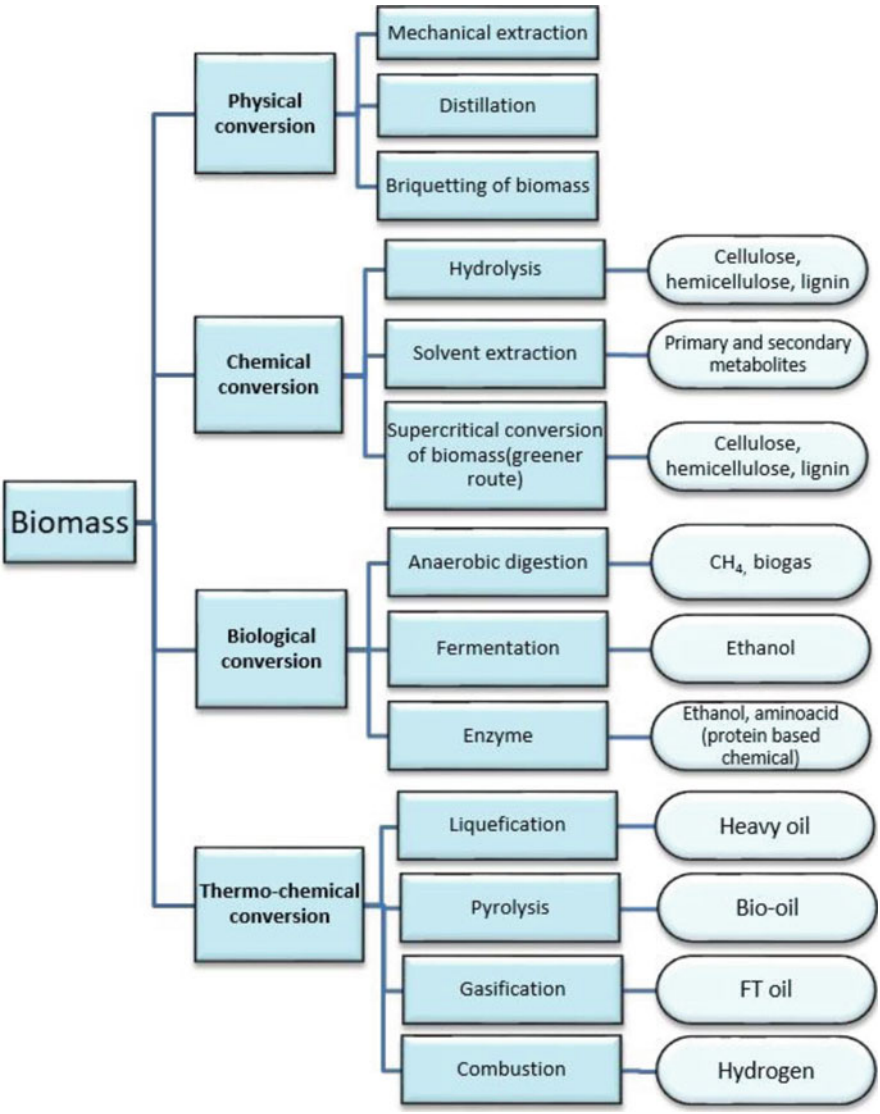


Fig. 1.1 Biomass conversion process

other components along with the elimination of crosslinks to increase the porosity of lignocellulose (Loow et al. 2016). Lignocellulose involves such diverse sources as grass switches, corn stalks, wood, herbaceous crops, waste paper, and paper products. Unlike starch-based biomass, lignocellulose materials are structurally complex.

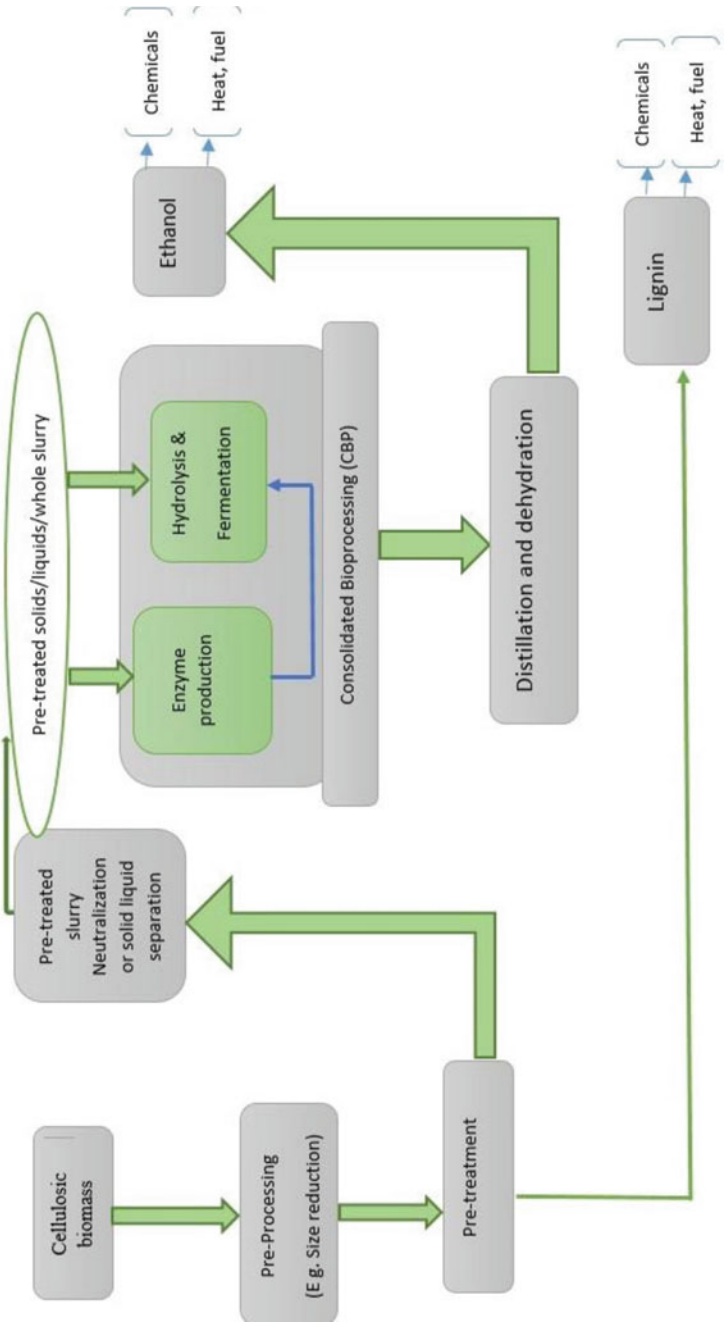


Fig. 1.2 Conversion methods of lignocellulosic biomass into various biofuels

1.2.3.2 Biological Pretreatment

The biological method for the pretreatment of cellulosic biomass utilizes microorganisms such as brown, white, and soft rot fungi (attacks cellulose and lignin). Such microorganisms secrete extracellular enzymes like lignin peroxidase and laccases. In contrast to costly reactor systems needed for chemical/physical pretreatment processes, biological pretreatments are run under mild conditions and require low capital costs (Balan 2014).

In contrast to costly reactor systems needed for chemical/physical pretreatment processes, biological pretreatments are run under mild conditions and require low capital costs. The biological process is relatively slower, requiring several days of biomass pretreatment. Besides, as compared to chemical pretreatment, sugar conversion after the microbial pretreatment method is lower. Biological pretreatment accompanied by chemical pretreatment is found to be successful in many situations and allows less extreme pretreatment conditions for the biomass to be hydrolyzed effectively (Balan 2014).

1.2.4 Microalgal Biomass

The world's eyes are recently focused on the production of microalgae fuel. To encourage this use of renewable energy, several countries in the Americas, Asia, and Europe have their own microalgae bioenergy research projects (Khan et al. 2018). Algae are the oldest freshwater, saline, and waste plants and are the feedstock for biofuels of the third generation. The lipid and protein found in the algae cells are greater than that of other terrestrial plants (Radakovits et al. 2010). Only by using sunlight, water, and carbon dioxide, it can generate biomass energy. Algae have a short period of growth, high photosynthesis ability, and does not occupy land. Microalgae are the main producers of marine environments and are small in size and only visible under a microscope. It is possible to use microalgae containing at least 30 percent lipids in the microalgae cell for biofuel conversion (Naik et al. 2010). Microalgae consist of chlorophyll and other photosynthetic organs that can photosynthesize to convert biofuel-produced organic compounds using sunlight (Fig. 1.3), water containing microalgae cells, and carbon dioxide from the air. Microalgae reproduction is generally split breeding form; the cell cycle is relatively short so that wide-scale breeding is easy. Microalgae can be grown in seawater, alkaline water, and even wastewater, so it is an important way of generating bioenergy in areas of freshwater scarcity and barren land areas.

Cultivation, depending on environmental conditions, water availability, the supply of carbon dioxide, and cultivation methods, is the first step in the development of bioenergy microalgae (Fig. 1.3). To obtain high-quality microalgae feedstocks, it is important to study cultivation techniques. As it involves microalgae processing, oil extraction, and energy conversion, this microalgae production technology is

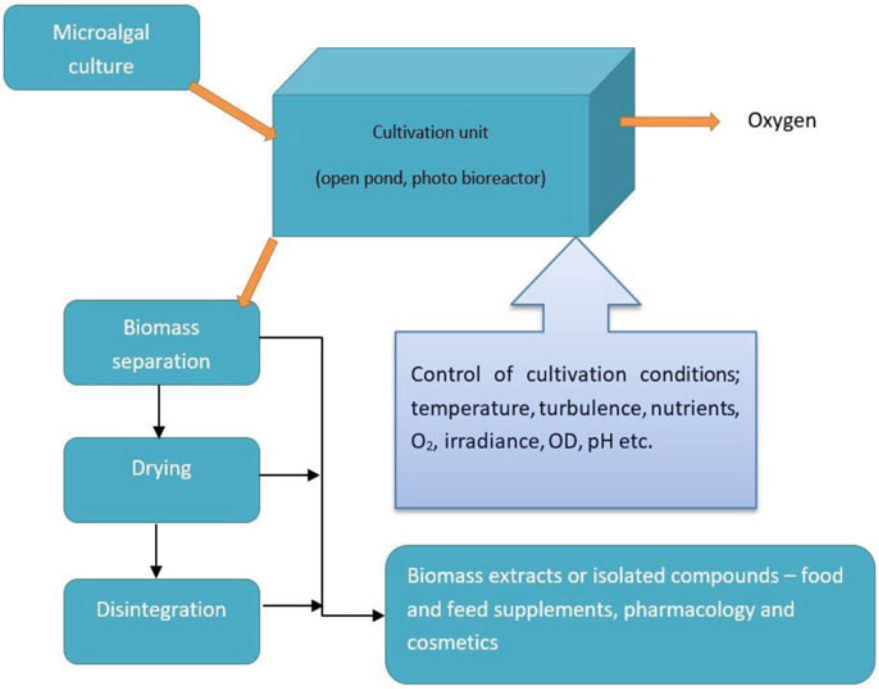


Fig. 1.3 Cultivation of microalgae for biofuel production

considered complex. Centrifugation and flocculation are the most widely used strategies for extracting microalgae (Zhu et al. 2018).

1.3 First-Generation Biofuels

First-generation biofuels are biofuels obtained from crops which accumulate sugar, starch, and oils (also used as human food or animal feed) (Balan 2014). Energy crops are effective consumers of solar energy for turning CO2 into biomass and can be used as energy sources. The biomass derived from these crops is made from wood (lignocellulose), sugar, starch, oil, and hydrocarbons.

The three main types of biofuels obtained in the first generation are biodiesel, bioethanol, and biogas, of which large quantities have been produced worldwide and for which the manufacturing process is considered to be technologically developed (Naik et al. 2010).

1.3.1 Biodiesel

Biodiesel is a form of environmentally friendly fuel that refers to a variety of fatty acid esters made from various types of vegetable oils, residual oils, and fats through a process known as transesterification. Biodiesel is commonly referred to as fatty acid methyl esters as "FAME." The conventional way to produce biodiesel industrially is illustrated in Fig. 1.4.

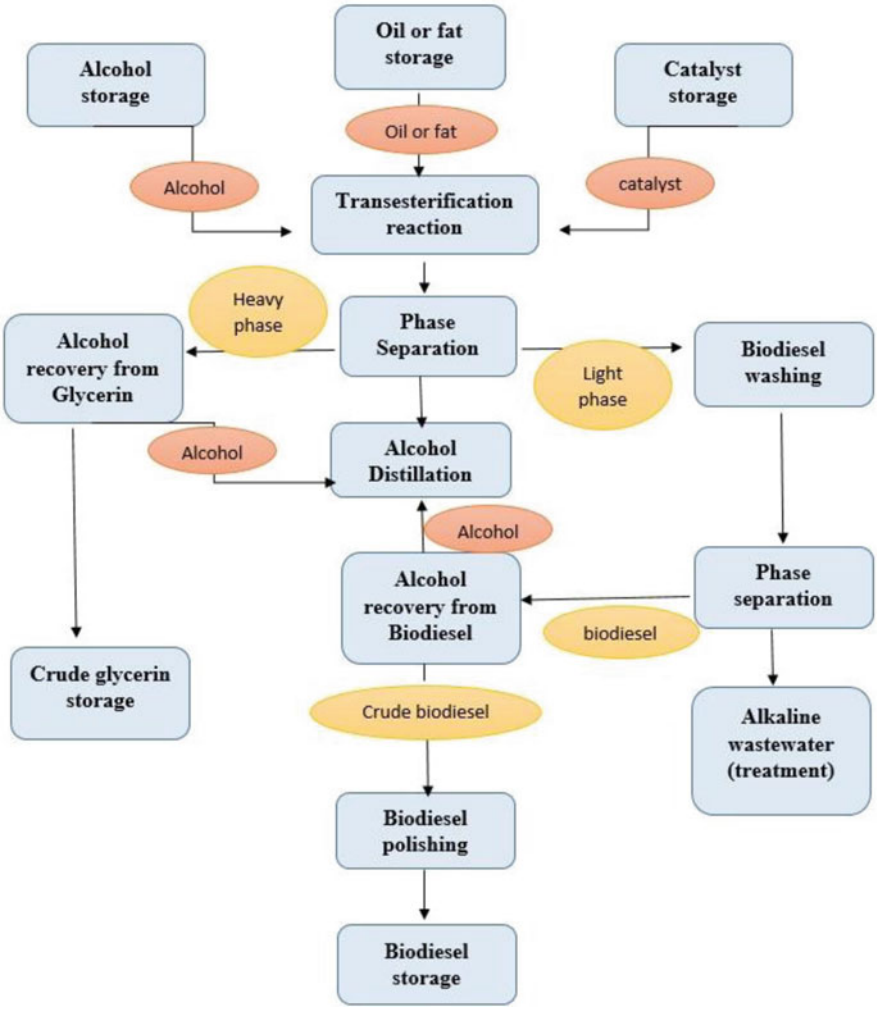
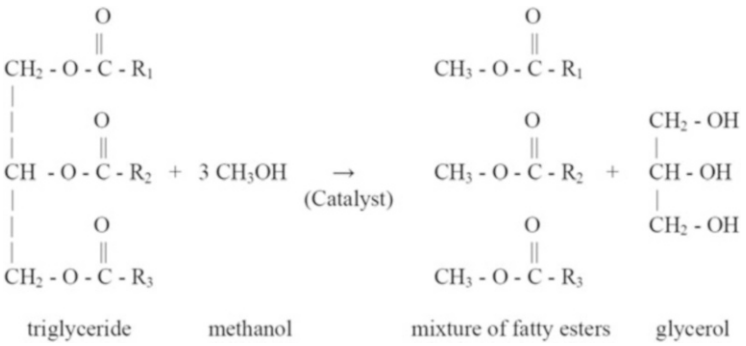


Fig. 1.4 Conventional pathway to produce biodiesel industrially

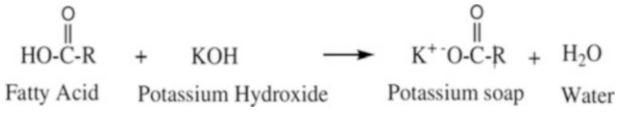
1.3.1.1 Transesterification

Transesterification is a process in which fats or oil reacts with alcohol to form ester and glycerol. This is a reversible reaction, and catalysts are used to increase the reaction rate. Triglycerides are first transformed into diglycerides, followed by their transformation into monoglycerides and then monoglycerides into glycerol.



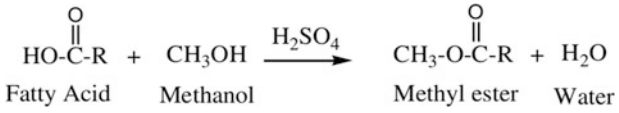
1.3.1.1.1 Transesterification Reaction

Selection of Catalyst: Acid catalyst, basic catalyst, and enzymatic catalysts can be used in this reaction. Basic catalysts are mostly used as they require a short time to complete the reaction even at room temperature. Examples of alkali-based catalysts are NaOH, KOH, carbonates, and alkoxides. Glycerides with relatively high fatty acid content and high water content are more suitable for acid-catalyzed transesterification.



But more methanol, high pressure (170–180 Kpa), and high-cost stainless steel equipment are required (Nasreen et al. 2018). When most popular sulfuric acid is used, there is low product yield. The acid catalyst transesterification reaction rate is also much slower than the alkali-based catalyst (Nasreen et al. 2018). Acid-catalyzed systems, however, are not of strong commercial interest.

Transesterification by Alkali Catalyst



1.3.1.2 Homogeneous Catalysis

Using homogeneous catalysts, this reaction can be mediated when the acid or alkaline compounds in the liquid formulations are destroyed. The most commonly used industrial-scale technique for biodiesel processing is alkaline transesterification. Some of the disadvantages of homogeneous alkaline transesterification are that high-purity raw materials are essential that are low moisture and free fatty acid content as during this process, saponification reaction can occur (Yadav et al. 2020).

Sulfuric, sulfonic, phosphoric, and hydrochloric acid are catalysts used in homogeneous acid catalysis. This method requires reaction equipment at high temperatures (50–150 °C) (Nasreen et al. 2018). These instruments are constructed of materials that accommodate such corrosion issues, as well as high-volume reactors until the response time of this route is low.

1.3.1.3 Heterogeneous Catalysis

Heterogeneous catalysts tend to be a fascinating alternative to biodiesel synthesis, making it simpler to isolate from the reaction mechanism and simplifying the phases of fatty acid alkyl esters purification. These provide a wider pore surface for greater interaction with the reagent and the oil supply. Heterogeneous acid catalysts can simultaneously catalyze esterification and transesterification reactions that become interesting when raw materials of low quality are used (Nasreen et al., 2018).

1.3.2 Bioethanol

Bioethanol can be derived from a broad variety of cellulose biomass, and these feedstocks are divided into the following types:

1. Sucrose containing feedstock (sugarcane and sugar beet).
2. Starchy compounds (rice, maize, wheat, barley).
3. Cellulosic biomass (wood, grass, and straw).

1.3.2.1 Bioethanol from Starch

Starch is a homopolysaccharide made of two glucose units, i.e., amylose and amylopectin, and is a high yield feeder for the production of ethanol. The manufacturing of starch bioethanol is a three-stage process: hydrolysis of higher sugars to monosaccharides, fermentation of glucose for the production of ethanol and carbon dioxide, and separation or purification of the product (Fig. 1.5).

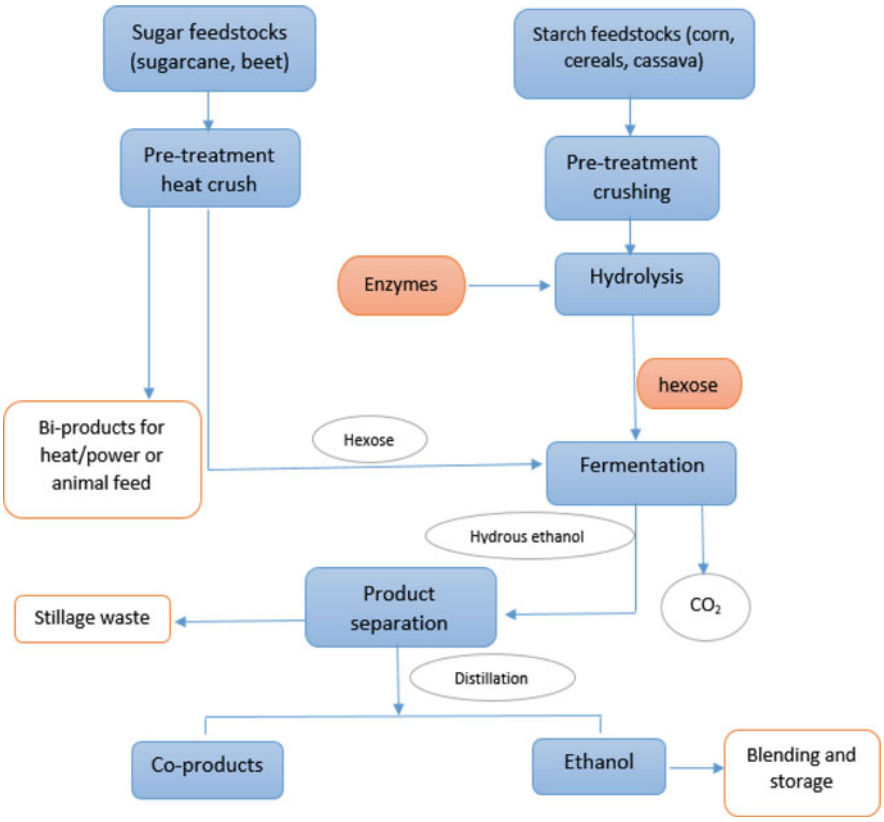


Fig. 1.5 Conversion of starch or sugar feedstocks to bioethanol

In hydrolysis, with the addition of a water molecule, a molecule is broken into two parts. Hydrolysis of starch can be done chemically by acids or enzymatically by amylase or cellulose. Starch feedstocks are grounded and combined with water in this process to create a mash that usually contains 20–30 percent starch (Naik et al. 2010). This mash is then boiled and eventually processed with two enzyme preparations at or near its boiling point. First, the enzyme amylase liberates maltodextrin oligosaccharides by the process called liquefaction. Enzyme pullulanase and glucoamylase further hydrolyze dextrin and oligosaccharide through the saccharification process. All dextrans are then converted into glucose, maltose, and isomaltose by saccharification. After the mash is cooled, yeast is added to begin the process of fermentation.

The fermentation process is the conversion of cellular biomass to ethanol using microbes such as yeast (Naik et al. 2010). The starch cannot be transformed directly into ethanol by the traditional fermentation method. The use of genetic engineering in microbes can increase the efficiency of ethanol fermentation of biomass-producing sugars that can ferment both hexose and pentose.

1.3.3 Biogas

Biogas is a colorless, flammable gas or a gas mixture produced by anaerobic bacterial fermentation of organic materials (Table 1.2). Biogas is known to be a low carbon fuel source providing rural communities the best ways to meet their energy demand. There are many prospects for the use of biogas such as:

- Improvement of agriculture in rural areas, which, through job growth, directly boosts the community economy.
- Reduction in waste by using organic agricultural waste and municipal solid waste to generate energy.
- Improving environmental quality by reducing the emission of carbon dioxide.
- Combining the disposal of organic waste with the production of useful methane from biogas energy.

The process of anaerobic degradation by organisms like nucleic acid and cellulose is required for the production of biogas. The substrate traditionally used for the processing of biogas is an industrial, agricultural, animal husbandry, or domestic waste product (Singh and Chandel 2018). These bacteria are hydrolytic, fermentative, and methanogenic bacteria, syntrophic H_2 producing bacteria, and acetogenic bacteria.

Anaerobic digestion in landfills is a potential source of methane emissions from solid waste. Anaerobic digestion of biodegradable components of municipal waste creates about the same amount of methane and carbon dioxide. These two main elements together with the traces of atmospheric nitrogen, oxygen, and organic compounds are known as landfill gases (Naik et al. 2010; Chowdhary et al. 2020). Similar to poor quality natural gas, landfill gas must remove volatile organic pollutants and carbon dioxide to achieve significant market value.

1.4 Second-Generation Biofuels

Second-generation biofuels are characterized as fuels created from a wide exhibit of feedstocks, particularly yet not restricted to non-edible lignocellulosic biomass. Biomass utilized for creation of second-generation biofuels is typically isolated in three principle classes: homogeneous, for example, white wood; quasihomogeneous, for example, agricultural and backwoods buildups; and non-homogeneous, including low-value feedstock (Hsu and Robinson 2019). The process of this biomass is complex and also depends on various technologies. What separates them from first-generation biofuels is the fact that feedstock used in producing second-generation biofuels is generally not food crops. Only the non-edible part of plants is used. The edible part of plant is used only after they have served their purpose. This helps farmers to not worry about the landmass which would be required to grow the biomass, as the waste produced after and during agriculture activities (only plant-

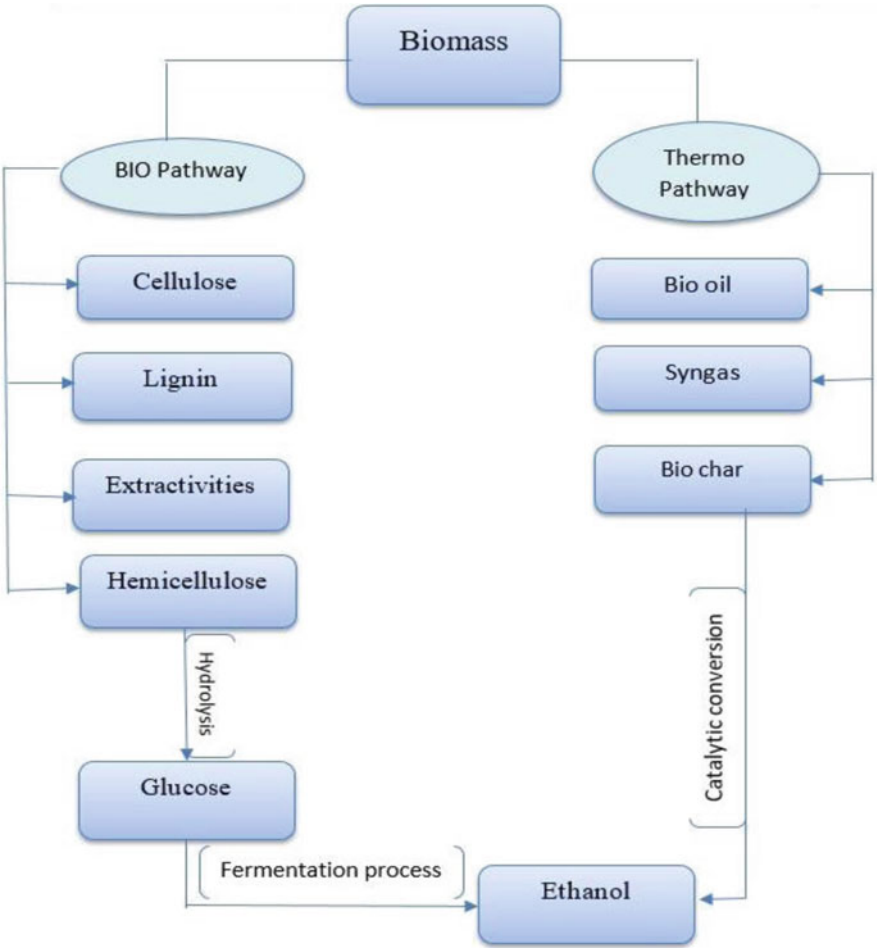


Fig. 1.6 Schematic representation of biochemical and thermochemical pathway

based waste from agricultural activities, not chemical wastes, e.g., wood chips, straws, etc.) is used for the production of biofuels (Table 1.2). By this process of conversion from non-edible plant parts, second-generation biofuel yield is higher than that of the first-generation biofuels. Common examples of second-generation biofuels are biodiesel, bio-alcohol, biogas, solid biofuels, vegetable oils, and syngas (Fig. 1.6) (Yadav et al. 2020). Biomass for second-generation biofuels include (i) plants that are specifically grown either for bioenergy production (bioenergy crops) on marginal lands, i.e., areas not suitable for food production, or on (ii) inedible parts of ordinary crops and forest trees that should be efficiently processed for bioenergy by improving the current technologies (Lee and Lavoie 2013). The only time food crops can act as second-generation biofuels is when they have already fulfilled their food purpose (non-edible part of food crop). For instance,

waste vegetable oil is a second-generation biofuel because it has already been used and is no longer fit for human consumption (Lee and Lavoie 2013). Virgin vegetable oil, however, would be a first-generation biofuel. Since second-generation biofuels are obtained from various feedstock, different innovations are frequently used to extract energy from them. This doesn't imply that second-generation biofuels can't be scorched straightforwardly as biomass. Indeed, a few second-generation biofuels, such as *switchgrass*, are developed explicitly to go about as immediate biomass (Haque and Bhat 2015).

Second-generation biofuels follow the conversion process of lignocellulosic biomass to biofuel in two different ways, namely, "thermochemical" and "biochemical" pathways (Naik et al. 2010). As the name suggests, the biochemical pathway consists of the use of microbes or microbial products for the conversion of biomass into useable biofuel, whereas in the thermochemical pathway, physical factors are considered while performing the conversion of biomass into biofuel. In the biochemical pathway, yeast is an example of microbe used (Naik et al. 2010). Yeast ferments lignocellulosic biomass into smaller constituents. As plant biomass mainly contains plant cell walls, which is made up of various sugars, after fermentation, the products will give high energy on consumption. Bioethanol is an example of biofuel which is obtained from the fermentation process (as mentioned above). The biomass obtained from the plant consists of various components: cellulose, lignin, extractives, hemicellulose, bio-oil, syngas, and biochar (Khan et al. 2020, 2021). Upon various processes like hydrolysis, pyrolysis, catalytic conversion, and fermentation, the final product is obtained in the form of bioethanol or biodiesel (depending on the conversion process used) (Fig. 1.6).

1.4.1 "Biochemical" Pathway

The "biochemical" pathway for the production of second-generation biofuels generally includes the separation of cellulose from the lignocellulosic biomass (Yadav et al. 2020) (Table 1.3).

Lignocellulosic biomass consists of cellulose (crystalline glucose polymer), hemicellulose (amorphous polymer of xylose and arabinose), and lignin (large polyaromatic compound) (Naik et al. 2010). In biochemical conversion, microbes play a vital role. They convert the sugars present in the plant-based lignocellulosic biomass into alcohol, which is a high-energy-yielding biofuel. This process includes the well-known and easy technique called pulping. To produce the highest quality of cellulose from the lignocellulosic biomass for the production of second-generation biomass biofuels, the pulping process has to be done precisely and efficiently (Singh et al. 2011).

There are various types of pulping process which can be used like classic pulping process, steam explosion, and organosolv process (Lee and Lavoie 2013). The isolation of cellulose from the biomass must be efficient in terms of energy and chemicals used. The production of these biofuels is directly related to the economic

Table 1.3 Types of biomass used in production of second-generation biofuels

Sr. no.	Types of biomass	Composition	Purpose in second-gen biofuel production	Example
1.	Energy crops	All plant-based biomass (lignocellulosic biomass) contains lignin, cellulose, hemicellulose	Grown specifically for biofuel production, non-edible parts	Switchgrass, sugarcane, wheat husks, mustard crops
2.	Agricultural waste			Straws, wood chips, bark, etc.
3.	Non-edible food waste			Any non-edible part of crop
4.	Industrial waste		Provides good quality of chemical and biological waste for biofuel production	Waste product after processing or production of food products from plants
5.	Forest waste and biomass		Ready-to-go biomass	Dead trees, non-edible plants and trees, fallen leaves and branches
6.	Municipal waste	Chemicals, plastic, paper, ashes, organic material, food waste		Excretory waste

expenditure and the effects on the environment. The next step is the saccharification of the purified cellulose, which is done with the help of enzymes or hydrolytic chemicals (acids). After the starch is obtained from enzymatic or hydrolytic process, the hydrolysis is then fermented with the help of yeasts. Upon fermentation, the sugar breaks down and produces alcohol. Sometimes, detoxification is required, because use of chemicals like acids may inhibit the fermentation process (Lee and Lavoie 2013).

1.4.2 "Thermo" Pathway

As the name suggests, thermochemical pathway includes the use of heat in the conversion of biomass into biofuels. Liquefaction, gasification, direct combustion, and pyrolysis are the processes which are included in the thermochemical pathway. This process of conversion is carried out in an oxygen-deficient environment (or minimal amount of oxygen is required). When biomass is heated in an oxygen-deficient environment, it produces three different types of products: solid (known as *biochar*), liquid (known as *bio-oil*), and gas (known as *syngas*). The synthesis of these different products depends on the temperature on which they are heated and the amount of oxygen provided. For example, if biomass is heated at a temperature of 250–350 °C in the absence of oxygen, the conversion product is biochar (solid) (Hsu and Robinson 2019). At a temperature range of 550–750 °C in the absence of oxygen, the conversion product is bio-oil (liquid). This is also known as *pyrolysis*,

which involves the change in the chemical composition and physical phase. At higher temperature range of 750–1200 °C, while providing the minimal amount of oxygen, the major conversion product is syngas (gas).

1. Liquefaction: In liquefaction, the biomass is processed in the presence of solutions of butanol, glycerin, propanol, alkalis, or direct liquefaction (Naik et al., 2010). High-viscosity oil products are obtained by liquefaction which eventually require solvents, as they are water-insoluble oil products. Catalyst are required during the conversion to overlook and fast pace the conversion process. Sodium carbonate (Na_2CO_3) and potassium carbonate (K_2CO_3) are used as catalysts in this process. CO and H_2 gases are also required during conversion alongside catalysts and biomass.
2. Gasification: In gasification, biomass is mixed with oxygen or steam to produce various gaseous products, CO, CO_2, CH_4, H_2, and N_2, which is known as syngas or producer gas, depending on the composition of the reaction mixture (Naik et al. 2010). This process does not require catalytic solvents to produce the syngas or producer gas. Syngas is then used to produce different types of fuels and chemicals.
3. Direct combustion: In the direct combustion method, biomass is directly heated or burned open (in the presence of air and oxygen). CO_2 and water are produced during direct combustion alongside the release of large amount of heat energy. Other harmful products like sulfur and CO are not produced (da Costa et al. 2009).
4. Pyrolysis: In pyrolysis, biomass is directly scorched in high temperature range (ranging from 300 °C to 1000 °C), in the absence of oxygen. The products obtained from pyrolysis are charcoal, bio-oil, and syngas (da Costa et al. 2009). Depending on the temperature and the size of the biomass, it is divided into three types:

Conventional pyrolysis—the temperature range of conventional pyrolysis lies between 300 °C and 650 °C. The rate of heating is around 0.1–1 K/s, which is rather slow than other methods (Lee and Lavoie 2013). Large hunks of wood are used in this method, making it a slow process. The time period for which biomass is treated under high temperature can be compared to biomass decomposition. Breakdown of simple and complex mechanism present in plant cell, like cell wall, water transport complex, bond dissociation, etc., occurs during this process.

Fast pyrolysis—the higher the temperature, the higher will be the rate of conversion of biomass in products which are used as fuels or intermediated for alternative fuel synthesis. The temperature range for this type of pyrolysis lies between 575 °C and 975 °C. The heating rate is much faster than conventional pyrolysis, about 10–200 K/s. The size of biomass is limited to <1 mm. After fast pyrolysis, it produces charcoal as main fuel product, along with the release of vapors (Lee and Lavoie 2013).

Flash pyrolysis—it is carried out at temperature range of 950 °C–1050 °C. The rate of conversion is very high, making biomass resting time in the reaction mixture very low. The heating rate is about 1000 K/s, which is very fast compared to the

other two methods. In this method, biomass size enters the nanometer world, ranging around 0.1–0.3 mm. The end product of this method is typically bio-oil, which can be converted into syngas under gasification conditions (Lee and Lavoie 2013).

1.5 Third-Generation Biofuels

Third-generation biofuels comprise of those fuel alternatives which are obtained from the process and culturing of algae and microbes. Apart from first-generation and second-generation biofuels, which solely depend on the quality of plant-based biomass like wood, chips, forest waste, etc., third-generation biofuels depend on the microbial aspect of biomass, for example, algae and microbes.

The main ingredient for the production of third-generation biofuels is microalgae. The use of microalgae for the production of biofuels as an alternative is currently more feasible than the production methodology of first-generation and second-generation biofuels. It is so because more than one type of biofuel can be obtained with this method (Fig. 1.5). Biodiesel and bio-hydrogen are some of the biofuels produced from the use of microalgae as the starter of biofuel production (Savla et al. 2020). The yield of biofuel from this process is much greater than the yield of first-generation and second-generation biofuel production. For comparison, a single unit of microalgae can produce up to 25–300 times than that of a current conventional method of first-generation biofuel can produce. Researchers have always focused on improving the yield and quality of biofuels obtained from microalgae. Distinction between the growth yield of biofuel produced from algal biomass is much more than the biofuels obtained from lignocellulosic biomass. A simple comparison between the yield difference is shown in Table 1.4.

Table 1.4 A yield comparison of fuels obtained from first-, second-, and third-generation biofuel production methodology

Biomass source	Liter/hectare/year	Barrels/hectare/year	References
Soybean (first gen biofuel)	400	2.5	Naik et al. (2010)
Palm oil (second gen biofuel)	6000	36	Naik et al. (2010)
Microalgae (third gen biofuel)	60,000–2,40,000	360–1500	("third generation biofuel from algae - ScienceDirect," n.d.)

1.6 Production of Microalgal Biomass

Microalgae are single-celled organism found in nature. They are found near waterbodies, like lake or pond. Microalgal production for biofuel purpose is expensive and complicated than normal plant agriculture. Sunlight, CO_2, water, and inorganic salts are required in the photosynthetic growth of microalgae. There is a need to strictly regulate the temperature regime. The temperature usually stays within 20 °C to 30 °C for most growth of microalgae (Khan et al. 2018). In order to minimize costs, the production of biofuels must rely on readily available sunlight, considering regular and seasonal fluctuations in the intensity of natural light. The inorganic elements that constitute the algal cell must be supplied by the growth medium. Nitrogen (N), iron (Fe), phosphorus (P), and silicon (Si) are the essential elements required for algal growth (Khan et al. 2018).

There are different ways microalgae can be cultivated. However, two widely used cultivation systems are (a) suspended cultures, including open ponds and closed reactors, and (b) immobilized cultures, including matrix-immobilized systems and biofilms (Alam et al. 2015). The most common large-scale production systems in practice are high-rate algal ponds or raceway ponds. Raceway ponds are open and shallow with paddle wheel to provide circulation of the algae and nutrients. Raceways are relatively inexpensive to build and operate but often suffer low productivity for various reasons (Hsu and Robinson 2019). Tubular photo-bioreactors are the only type of closed systems used at large-scale production of algae.

1.6.1 Microalgae-Harvesting Practices

After the production of microalgae, the collecting or harvesting of algae is the next important step. It can be carried out by various methods including chemical, biological, mechanical, and electrical methods (Fig. 1.6). The chemical method includes the flocculation of small-sized microalgae to increase its extraction efficiency (Branyikova et al. 2018). The mechanical methods comprise the use of centrifuge technique; it suspends and separate algae based on their weight, which can be later extracted. In electrical method, the net charge present on the microalgae is used for their separation. They have negative charge on their surface, which is used to separate them for biofuel production.

1.6.2 Biofuel Synthesis from Algae

Energy or biofuel production from microalgae can be carried out by multiple methods. Some of the commonly used conversion methods are (i) chemical conversion, (ii) biochemical conversion, (iii) thermochemical conversion, and (iv) direct

combustion, which clearly depends on the type of microalgae species used or some other desired end products along with the biofuel at the end. The conversion of microalgae into usable energy source or biofuel is shown below in Fig. 1.6 schematically.

1.7 Comparing the Different Generations of Biofuels

In modern-day times, majority of industries, research areas, commercial and residential areas, etc. consumes petroleum, coal, or natural gas. These fuel options are much cheaper and easily available. Upon consumption, petroleum and other currently used fuel options release some harmful compounds, e.g., CO, NO_X, CO_2, N_2O, SO_2, and hydrocarbons. These compounds are extremely harmful for the environment and many living organisms including human beings. Biofuels are a much cleaner fuel alternative which is used to overcome this problem. Depending on the biomass and where it is derived from, biofuels are categorically distinguished into different types: generally known as generation of biofuels (Figs. 1.7, 1.8, 1.9, 1.10, 1.11) and (Table 1.5).

First-generation biofuels are derived from plant-based biomass. First-generation biofuel's biomass comes from plants which have high sugar content and also accumulates high amount of starch and oils (vegetable oils). Sugar is a very good source of energy when processed correctly and efficiently. The production of first-generation biofuels is easy and can be carried out through hydrolysis, fermentation, and purification processes. As the main biomass for first-generation biofuel is sugar,

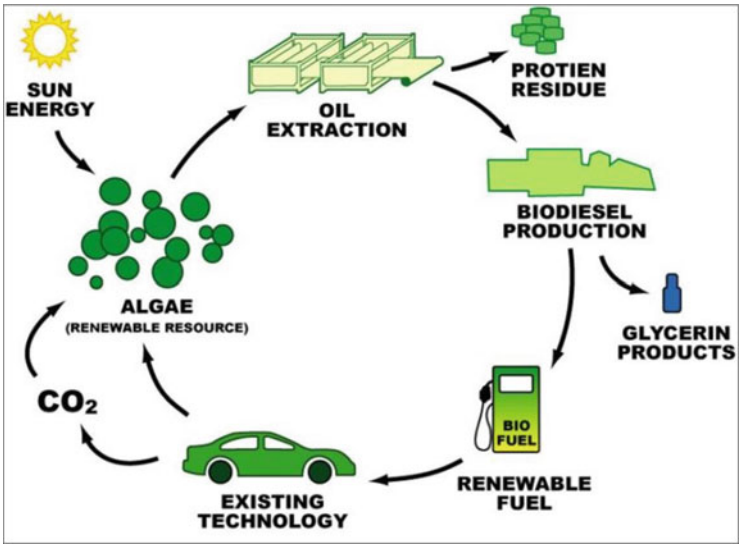


Fig. 1.7 Third-generation biofuels from microalgal biomass

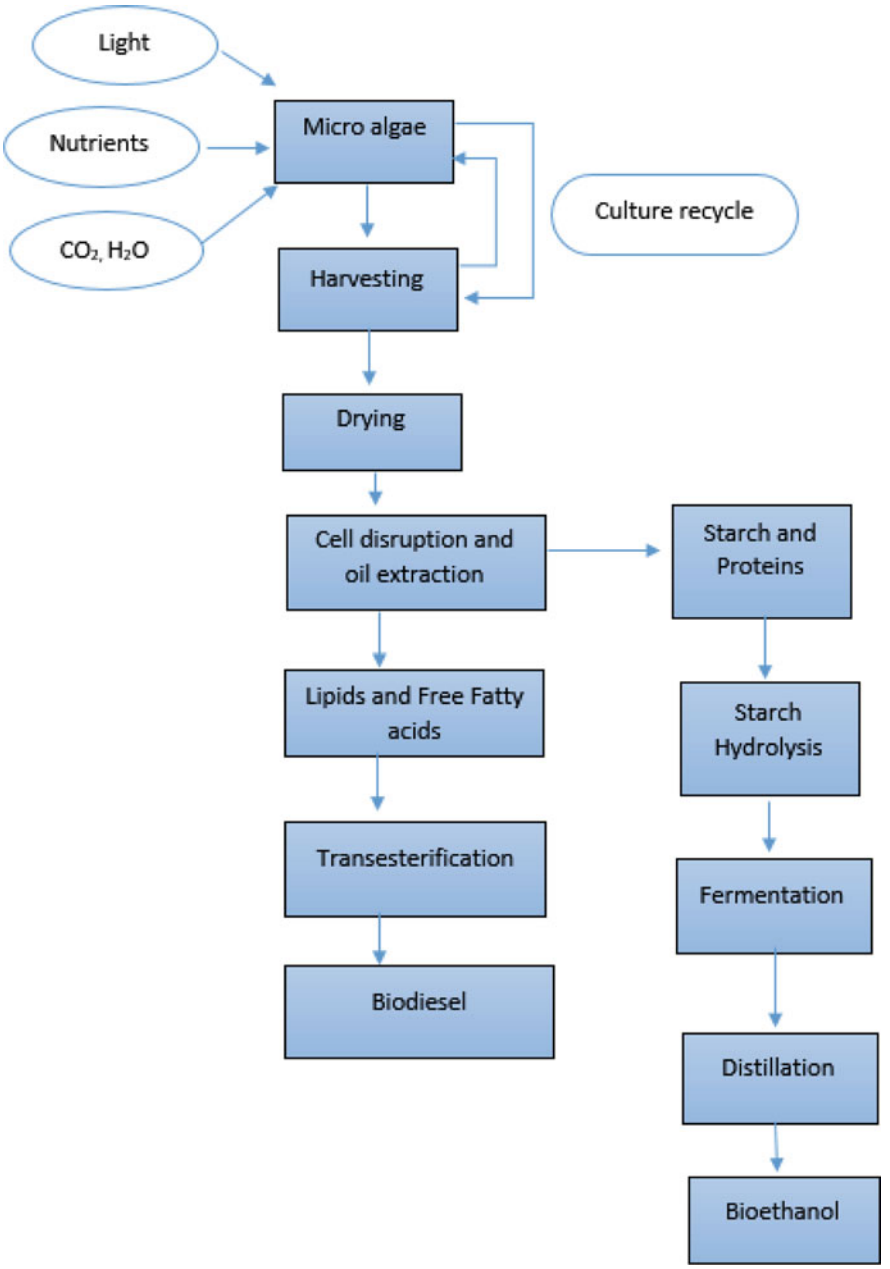


Fig. 1.8 Schematic representation of conversion of algae biomass into biodiesel and bioethanol through various steps

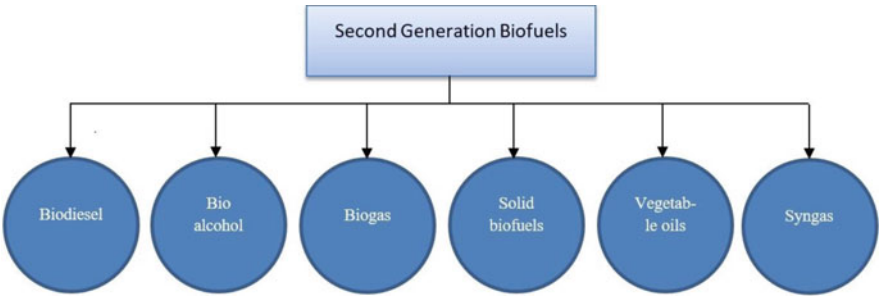


Fig. 1.9 Common examples of second-generation biofuels

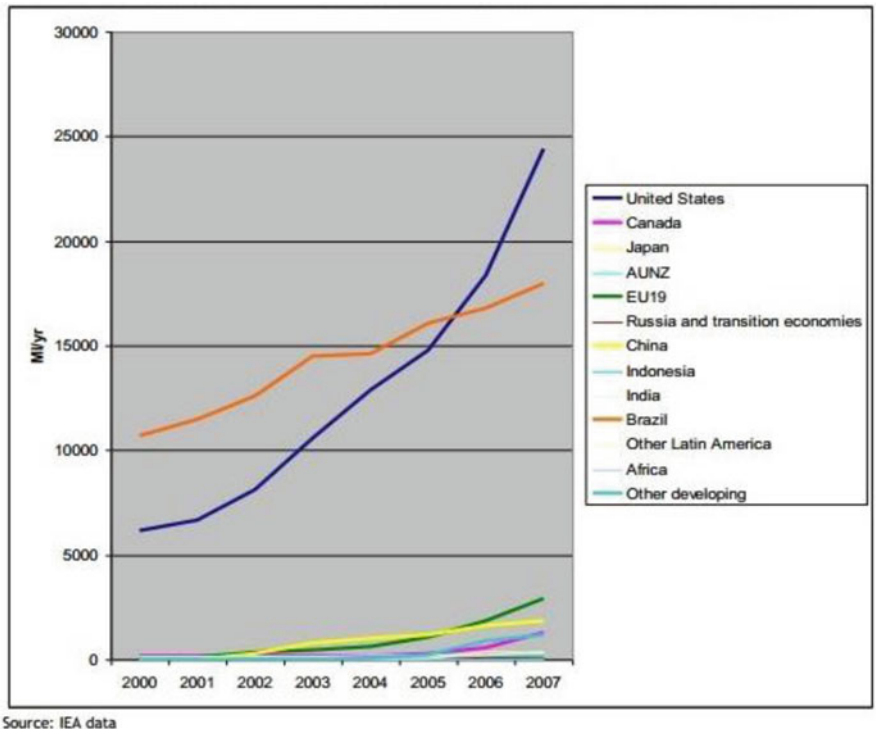


Fig. 1.10 Global ethanol production trends in major producing countries and region

starch, and oil-rich crops, food crops are most reliable sources for this. Using food crops as a biomass has its own demerits, like interrupting the food chain and also affecting the economical part of the world too. Consuming food crops for the production of biofuels results in increasing their market price. Also, first-generation biofuels require large area of landmass to grow their biomass or food crops. The yield-to-landmass ratio is less in case of first-generation biofuels. The highest yielding crop for first-generation biofuel is sugarcane, which gives about 650 gallons

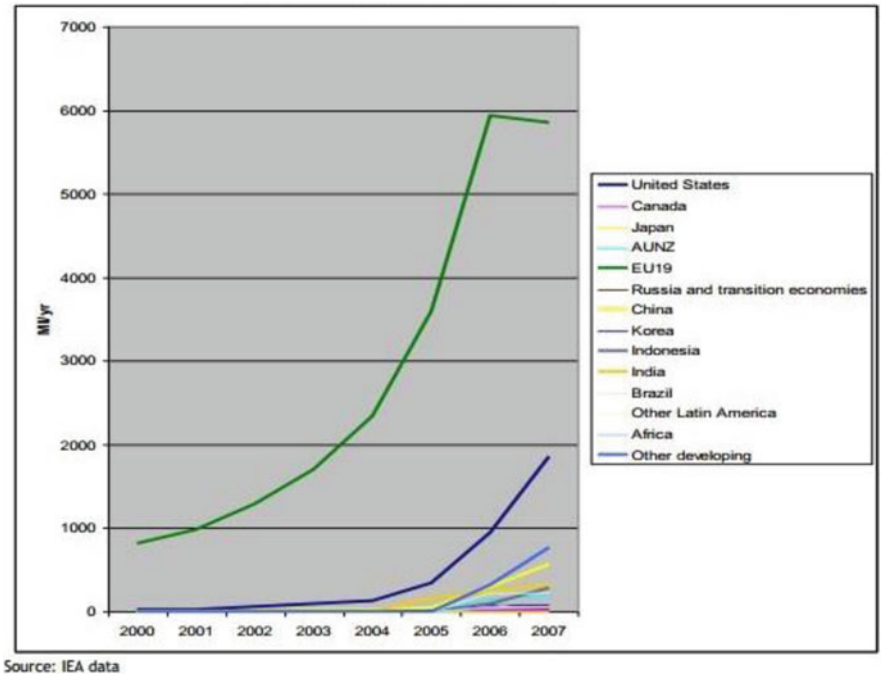


Fig. 1.11 Global biodiesel production trends in major producing countries and regions

of bioethanol per acre (Naqvi and Yan 2015), but it requires a lot of biomass for conversion process, which is very high compared to other types of biofuels.

Second generations of biofuels are derived from the lignocellulosic biomass. It contains high amount of lignin, cellulose, and hemicellulose. Breakdown of these compounds via biochemical or thermochemical pathways generates high amount of energy which can be used right away or can be stored in the form of biofuels. Bioethanol and biodiesel are two main examples of second-generation biofuels which are derived from biochemical conversion method. As the name suggests, the biochemical conversion methods exploit the ability of microbes to convert the sugars present in lignocellulosic biomass into energy, and the purification process is carried out by enzymes or chemicals. Another conversion method is used for second-generation biofuel production, i.e., thermochemical conversion method. Thermochemical conversion method involves the heating of biomass at various temperature ranges depending on what product we require. Typically, three types of products are obtained via thermochemical conversion, namely, biochar, biogas, and bio-oil. The temperature ranges from 200 °C to 1100 °C. The oxygen supply is also monitored during this process. Minimum to zero supply of oxygen is required. The yield of second-generation biofuel is relatively high than that of first-generation biofuel, because the biomass conversion efficiency of second-generation biofuel (230 liters of biofuel per ton of biomass) is higher than the conversion efficiency

Table 1.5 A complete comparison of different generation of biofuels

	First-generation biofuels	Second-generation biofuels	Third-generation biofuels
Biomass/ feedstock	Sugar, starch, and oil-rich crops	Non-edible part of crops, agricultural waste and other waste organic waste products, etc.	Microalgae and other microbes
Production methods	Hydrolysis, fermentation, transesterification, catalysis, and purification	Biochemical conversion and thermochemical conversion methods	Culturing techniques involving various species of microalgae and other microbes
Average yield	350 gallons of bioethanol/acre Feedstock—Corn 650 gallons of bioethanol/acre Feedstock— Sugarcane 70 gallons of biodiesel/ acre, Feedstock—Soybeans	506 gallons of bioethanol Feedstock—Lignocellulosic biomass, non-edible part of crops and other energy crops, and waste products	On average it is 9000 gallons/acre But it can go up to 20,000 gallons of biofuels/acre Feedstock—Algae
Conversion efficiency	Avg. 74.5 liters/ton	Avg. 230 liters/ton	7.4 g/L/d
Carbon footprint	Extremely less	Extremely less	Neutral
Products	Fatty acid methyl esters (FAME) Bioethanol Biogas Vegetable oils Biodiesel	Bioethanol Biomethanol BioDME Biodiesel	Biodiesel Butanol Gasoline Methane Ethanol Vegetable oil Jet fuel
Advantages	• Simple conversion of biomass to biofuel. • Whole crop can be used for biofuel production. • Infrastructure for harvesting and processing the crops is already in place. • Crops used are easy to grow and available in most regions. • Less carbon emission	• Biomass for second-gen biofuels can be used as a direct biomass. • Perennial crops are used, decreasing the need for regular plantation. • High energy yield in respect to the landmass used. • Less requirement of fertilizer. • It does not interrupt the natural food chain	• Extremely high yield, around 10X of traditional feedstocks can produce. • High diversity and quality. • Use of microorganism allows us to genetically modify it to according to our need. • Does not cause any engine damage. • Requires very less land mass for production.
Disadvantages	• High requirement of pesticides and fertilizer, can be harmful for soil. • Usage of edible food crops for biofuel	• It is not the most suitable way for producing bioethanol. • Conversion process is extensive.	• Chance of contamination during algae culture. • Selection of a particular species for a

(continued)

Table 1.5 (continued)

	First-generation biofuels	Second-generation biofuels	Third-generation biofuels
	production resulted in higher food prices worldwide. • Crops face various diseases and infestations from insects and pathogens. • Vegetable oil can cause engine damage if it is not refined properly	• Moist soil is required for their growth; they do not flourish in dry conditions. • Some second-gen biofuels, like vegetable oils or waste vegetable oil, can damage the machinery or reduce engine efficiency and life if not refined properly	particular biofuel is an extensive task. • Biofuel produced from algal biomass is less stable than the biofuels produced from other sources. • Requires large amount of nutrient media containing water, nitrogen, and phosphorus. • High cost of production than other methods as it requires large amount of fertilizer to grow

of first-generation biofuel (74.5 liters of biofuel per ton of biomass). As the crops used for second-generation biofuel production are specially cultivated for this purpose only, it does not require special care of separate landmass, and also their growth requirement is less than food crops, making it economically efficient too.

The use of microalgae as a biomass source is relatively new, and research is still ongoing on this very new field to improve and increase the quality and quantity of biofuel obtained from algae. This category of biofuel falls under the third generation of biofuels. The microalgae have the capability to store energy in the form of various biochemical molecules. For example starch, proteins, lipids, fatty acids, etc., these molecules on conversion with the help of methods like transesterification, fermentation, distillation produces different spectrum of biofuels. A typical algae species produces only ethanol, but with the help of genetic manipulation, the genome of the any algae species can be engineered in such a way that it will produce any biofuel that we need. Biodiesel, butanol, gasoline, methane, ethanol, vegetable oil, or jet fuel is some of the biofuels that can be produced from algae with the help of necessary genetic manipulation. A marginal concern about algae is that biofuel extracted from them seems to be less stable than biodiesel produced from other sources. This is because the oil present in algae appears to be relatively unsaturated. Unsaturated oils are more volatile and therefore more vulnerable to degradation, particularly at high temperatures. Apart from this issue, algae proved to be a great biomass for biofuel production due to its extremely high yield, about 9000 gallons of biofuel per acre. Some researchers have also reported a yield of up to 20,000 gallons of biofuel per acre using certain species of algae (Srivastava 2019).

1.8 Conclusion

Biofuels are much needed fuel alternatives which are dearly required considering present-time climate conditions. Fuels like petrol, diesel, coal, etc. are available in a very limited quantity for us to consume. It's even said that this resource will exhaust soon. Biofuels serve as a cleaner and efficient replacement for these fuels. Biofuels are obtained from naturally occurring plants and organisms, which on consumption release less carbon footprint. There are different types of biofuels depending on the biomass used for their production (Singh et al. 2011). First-generation biofuels are primary biofuels which are produced to this date. They are easy to produce, and their biomass is called lignocellulosic biomass consisting of lignin and cellulose. The lignocellulosic biomass used for the production of first-generation biofuel comes from those crops which are used on a daily basis, like sugarcane (Singh et al. 2011). Sugarcane is rich in sugar, which can be easily converted into energy source. Bioethanol, biodiesel, and biogas are the type of biofuels which are included in the first-generation biofuels. Second-generation biofuels are produced from lignocellulosic biomass. They rectify most of the limitation that comes with the first-generation biofuels. The biomass used for the production of second-generation biofuel is cultivated specifically for biofuel production. This decreases waste products like forest waste, agricultural waste, municipal waste, wood chips, and non-edible parts of a crop. All these waste products go into waste or thrown away, if not used for biofuel production. Second-generation biofuels use both biochemical and thermochemical means for their production. Biochemical pathway for production of second-generation biofuels implements all the practices which include the use of enzyme, chemicals, etc., for disrupting the bond, structure, and other biochemical factors to release energy. Third-generation biofuels are extremely easy and rapid to produce. These biofuels are mainly obtained from microalgae (different species of algae) and other microbes also. The main biomass for third-generation biofuels is algae. These are single-celled microscopic organisms which can be found in marine conditions. Their photosynthetic ability to convert CO_2 into energy attracted many researchers to work on more efficient and high-energy-yielding species of algae. It will drastically increase the product quantity without the need to increase the harvesting area. In economic terms, this will decrease the cost production while increasing the amount of energy produced simultaneously. There have been advancements in biofuel production, making its way to cleaner and more efficient biofuel step by step. The next generation of biofuels include the production of biofuels by exploiting the ability of algae and cyanobacteria to convert solar energy into energy. These types of biofuels are placed under the "fourth generation of biofuels." This area is still under development, but the promises are strong for future aspects. Compared to other generation of biofuels, fourth-generation biofuels are extracted from inexhaustible biomasses. This makes it the most efficient way to produce biofuels. In order to make production systems economically viable, the most revolutionary future scenarios include the production of biofuels in "synthetic factories" together with high-value chemicals.

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Chapter 2
Renewable Biofuel Sources as Bio-Clean Energy: Potential and Challenges

Dixita Chettri, Bhaskar Sharma, Shuchi Singh, Ashwani Kumar Verma, and Anil Kumar Verma

Abstract Growing population, urbanization, and industrialization have direct pressure-limited fossil fuels. As a result, these fossil fuels are on the verge of depletion, giving rise to great concern about the future source of fuel. Moreover, the environmental concerns of pollution generated by the use of these fuel sources are a major concern to be addressed by the nations of the world. In response to all these issues, research is being carried out into various forms of renewable energy to replace fossil fuels. One such alternative is biofuels derived from biomass. As it is the most abundant organic matter, it has enormous potential to become the next major source of energy. The chapter explores biomass as a beneficial alternative to fossil fuels in terms of sustainability and economic and environmental aspects.

Keywords Biofuel · Biomass · Lignocellulose · Pretreatment · Renewable energy

2.1 Introduction: Sustainable Development and the Concept of Renewable Energy

Sustainable development is generally defined as development that meets the needs of the present generation without compromising the ability of later generations to meet their needs (Future 1987). Among the various factors that have an impact on sustainable development, the continuous supply of energy from sustainable sources

D. Chettri · A. K. Verma · A. K. Verma (✉)
Department of Microbiology, Sikkim University, Gangtok, Sikkim, India
e-mail: akverma@cus.ac.in

B. Sharma
School of Life and Environmental Sciences, Faculty of Science, Engineering, and Built Environment, Deakin University, Geelong, VIC, Australia

TERI School of Advanced Studies, 10 Institutional Area, Vasant Kunj, Delhi, India

S. Singh
Department of Agricultural and Biological Engineering, University of Illinois at Urbana-Champaign, Urbana, IL, USA

P. Chowdhary et al. (eds.), *Bio-Clean Energy Technologies: Volume 1*, Clean Energy Production Technologies, https://doi.org/10.1007/978-981-16-8090-8_2

also plays a crucial role. The role that energy plays in the development of a society can be understood from the increasing demand for energy to improve and sustain the modern lifestyle. Energy in the form of electricity is used in households, offices, industrial equipment, as well as fuel for cars, generation of heat, etc. It has been estimated that 75% of the world's total energy supply is consumed by the limited 25% of the modern industrialized economy (Dunderdale 1990). The concept of sustainable development in terms of energy supply involves technological advancements and efficient energy production with renewable sources replacing fossil fuels while trying to reduce energy consumption (Lund 2007).

There are numerous drawbacks in the continuous use of fossil fuels as a source of energy such as global warming, air pollution leading to acid rain and water pollution, and depletion of the ozone layer which are the major environmental problems apart from limited supply and increased fuel prices which are the economic aspects of the problem (Chowdhary and Raj 2020). The use of renewable energy sources is seen as an alternative to solve these challenges. However, their implementation is not as simple as it is sometimes intensely advertised, leading to impractical claims being made (Dincer 2000). The main obstacle is the expansion of the amount of renewable energy generated. Although renewable energy has significant potential to be a major source of energy, its practical application is less than 15% of total primary energy consumption, with hydropower and fuel wood being the only renewable energy sources that contribute, while wind and solar energy contribute negligibly. For successful application of the concept of sustainability in energy supply, the integration of these renewable energy sources into the energy system to increase the amount of energy produced and the development of transportation for their distribution are the two major challenges that need to be addressed (Lund 2007). Other factors such as technological and technical realism, economic viability, reliability, applicability, and public acceptance must also be considered (Dincer 2000).

In the recent past, a growing interest and development in the use of biomass as a renewable energy source for biofuel production has been observed (Fig. 2.1).

Biomass-derived biofuels can be used for a variety of applications, from use as a transportation fuel to the generation of heat and electricity (Caspeta et al. 2013). The various sources and benefits of biofuels, as well as advances in technologies and policies for their successful large-scale application, are described below.

2.2 Sources for Biofuels

The fuel produced using the energy trapped in the form of biomass through the process of biological carbon fixation is called biofuel. Since the amount of carbon dioxide trapped during plant growth is equal to the amount released during combustion, biofuels are considered carbon neutral. Different biomass sources and forms, i.e., solid, liquid, or gas, can be used for biofuel production (Shalaby 2013; Khan et al. 2020, 2021).

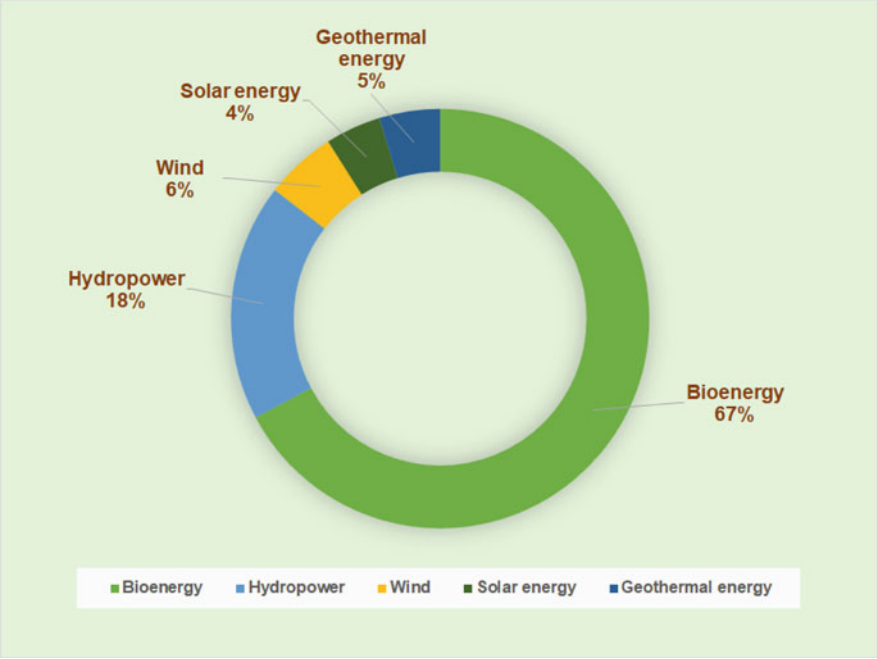

Fig. 2.1 Renewable energy distribution for the year 2018

Biofuels were classified based on the type and source of biomass used for their production. Based on the processing of biomass, biofuels were classified into primary and secondary biofuels (Demirbas 2008). Primary biofuels include traditionally used firewood, agricultural and forest residues, woodchips, etc. as biomass that does not require processing and is used directly for heat production. This type of biofuels is widely used in underdeveloped and developing countries for household applications such as cooking, firewood, etc. (Rodionova et al. 2017). Although this class of biofuels does not require high processing costs, its scope is very limited. The biofuels that are obtained by treating and processing primary biomass and have very high energy content with the ability to be used as an alternative to fossil fuels for commercial purposes are called secondary biofuels. Common secondary biofuels include bioethanol, biohydrogen, biodiesel, etc. (Rodionova et al. 2017; Savla et al. 2020).

Based on the source of biomass, secondary biofuels have been further classified into three generations, i.e., first, second, and third (Surriya et al. 2015). Table 2.1 compares these different generations of biofuels and biomass for their application with common biomass sources described for the production of these different generations of fuels (Fig. 2.2).

Table 2.1 Comparison of the three generations of biofuels based on biomass and energy production

First generation	Second generation	Third generation
Uses food crop as biofuel source	Uses lignocellulosic waste and non-food crops	Algae biomass are used
Requires arable land and water resource	Requires arable land	Can grow in any land or waterbody
Extensive use of fertilizers and pesticide	Does not require fertilizers and pesticide	Does not require fertilizers and pesticide
Less energy intense compared to third generation of biofuels	Less energy intense compared to third generation of biofuels	More energy intense per area of harvest
Raises food security issue since food crops are used as feedstock	Raises food security issue due to competition with agricultural resources	Does not raise any threat on food security
Examples are corn, sugarcane, vegetable oil	Examples are agricultural waste, non-food crops like alfalfa	Examples are *cyanobacteria*, *chlorella* sp.

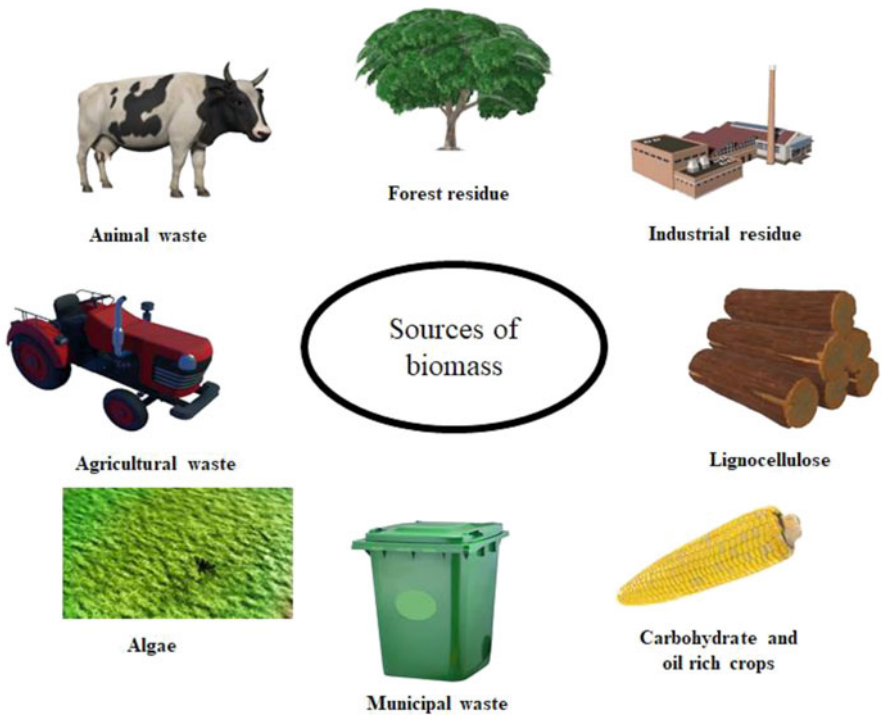

Fig. 2.2 Different potential sources of biomass for biofuel production

2.2.1 *Carbohydrate and Oil-Rich Crops (First-Generation Biofuel)*

Edible food crops, for instance, corn, wheat, barley, sugarcane, rice, rapeseed, soybean, sunflower seeds, etc., which are rich in carbohydrates and oils, are used as a biomass source for the production of first-generation biofuels (Hirani et al. 2018). Bioethanol is produced by fermentation or sequential hydrolysis and fermentation of starch and other polysaccharides present in these plants, with corn being the leading biomass for bioethanol production (60 billion liters), followed by sugarcane (20 billion liters), with the USA and Brazil being the principal supplier, respectively, for the year 2012 (Sawin et al. 2013). Oil plants are used to extract the oils, which are then esterified by mixing with alcohol to produce biodiesel. Palm oil with a value of 40% has a very high oil content and is a common oil crop used in Asian countries of Indonesia and Malaysia (Balat and Balat 2010). Rapeseed with an oil content of 35% is widely used in the European Union (EU) to produce biodiesel. Similarly, soybean with an oil content of 21% is a common feedstock in the USA (Ramos et al. 2009). In 2012, Germany was the largest producer of biodiesel, followed by the USA and Argentina (Sawin et al. 2013). These two main biofuels are commercially produced as substitutes for diesel and gasoline, respectively.

Although first-generation feedstocks have the advantage of easier processing and low cost for biofuel production, there are several drawbacks to using these feedstocks for long-term energy production (Voloshin et al. 2019). Competition with food raises the issue of food security and also leads to an increase in their prices, which has directly sparked the debate on the use of food as fuel (Gasparatos and Stromberg 2012). Since the production of these crops also requires arable land and water facilities, there is also competition for these basic resources (Fargione et al. 2008). Production and processing cost greatly increase the price of these fuels, making them unable to compete with fossil fuels (Doornbosch et al. 2007). The consistency of greenhouse gas (GHG) emission control and environmental impact of using these biofuels, when the total land-use change is considered, makes the use of first-generation biofuels not worthwhile (Paris 2008). Therefore, several factors need to be considered in the use of these edible biomass resources such as availability of limited resources like land, impact on water due to extensive use of agrochemicals to increase production, impact on food security, etc. (Singh et al. 2013). Therefore, the overall assessment of the impact of the use of these biofuels on the environment and socioeconomic conditions led to the search for alternative biomass sources for biofuel production (Ho et al. 2014).

2.2.2 *Lignocellulosic Material (Second-Generation Biofuel).*

Second-generation commodities include non-edible lignocellulosic biomass from agricultural, forestry, municipal, and industrial wastes. This biomass was classified

into three groups: (1) homogeneous biomass, which consists of wood chips from energy crops and has a value of US$100 to US$120 per ton; (2) quasi-homogeneous biomass, which consists of residues from forestry and agriculture and has a value of US$60 to US$80 per ton; and (3) non-homogeneous biomass, which includes municipal and household wastes with a value of US$0 to US$60 per ton (Lavoie et al. 2011). Perennial grasses such as switchgrass, reed canary grass, alfalfa, etc. are grown as energy crops on land with low soil quality, along with *Jatropha* and woody crops with short rotation. Agricultural and forestry residues such as bagasse and wood waste do not require land or other resources and are readily available to be used for biofuel production. Municipal and industrial wastes such as putrefaction products, vegetable and fruit peels, and paper industry wastes are also used under the concept of waste to energy (Ho et al. 2014).

Although these biofuel sources are expected to compete for land and water resources for food production, the total energy yield, in this case, is higher on a given land area, unlike first-generation biofuels. Further, nutrient-poor soils can also be used for their growth (Chakraborty et al. 2012). Moreover, the production cost of these biomasses is either zero or lower compared to first-generation biomasses like vegetable oil, sugarcane, etc. (Lee and Lavoie 2013). This is a major advantage of second-generation biofuels and has attracted a lot of attention for research to develop techniques to use these feedstocks to produce the most common forms of energy, i.e., heat and electricity. The whole process is considered to be carbon neutral or sometimes even negative for the combustion of the biofuel produced by these feedstocks (Alalwan et al. 2019). Moreover, some of the feedstocks can be used for the production of additional value-added products, which is the main concept of biorefineries.

However, the major drawback in using this type of biomass as feedstock is its complex nature which makes its treatment for biofuel production a challenging task (Nigam and Singh 2011). Since the success of any biofuel production technology lies in its economic aspect and cost friendliness, the success of second-generation biofuels is also directly related to the pre-treatment and production cost of lignocellulose, with the high cost of pre-treatment being the major obstacle (Lee and Lavoie 2013). Technical progress is expected to overcome this obstacle in the near future, as intensive research is already underway (Ho et al. 2014).

2.2.3 Algae (Third-Generation Biofuels)

Third-generation biomass consists of algal biomass, which has gained considerable attention in recent years for biofuel production. Algae are aquatic microorganisms with photosynthetic ability and rapid growth that live in various habitats such as wastewater, salt water, coastal seawater, or non-cultivable land (Chen et al. 2011; Chowdhary et al. 2020). They can photosynthesize carbon dioxide to produce large amounts of carbohydrates and other substances such as lipids, proteins, or pigments, which are stored as biomass. Algal biomass has great potential for use as a feedstock

for biofuel production and has been extensively researched. The algae *Botryococcus* and *Chlorella* sp. have a high lipid content of 50–80%, which makes them a suitable candidate for biodiesel production, while *Cyanothece* sp., *Spirulina platensis*, and *Chlamydomonas* sp. which produce large amounts of carbohydrates are used for bioethanol production. The algae are also used for biohydrogen and biomethane production (Costa and De Morais 2011).

The high growth rate of the algae, with the ability to double its biomass within 2–5 days, leads to a very high yield of dry biomass, which in the case of *Pleurochrysiscarterae* is about 60 tons/ha per year, from which an oil quantity of almost 20 tons is extracted. This is a major advantage of using algal biomass as the production rate is almost five times that of palm oil, which is the oil crop pant with the highest yield (Ho et al. 2014). The lower content of hemicellulose along with the absence of lignin provides the added advantage of high efficiency of hydrolysis, which has a direct positive effect on the fermentation rate, resulting in higher yield and making the process economical (Li et al. 2014).

The disadvantage in the successful application of algal biomass for biofuel production is the cultivation system, where the open pond cultivation requires low capital investment, but the biomass yield is also low, while the closed system requires high capital investment (Chen et al. 2011). The difference in growth rate, yield efficiency, and nutrient requirement of different algal species is another major problem (John et al. 2011). Downstream processing, photobioreactor design, and extraction strategies and techniques need to be developed to improve the commercial viability of using algae as biomass for biofuel production (Ho et al. 2014).

2.3 Technological Advances in Biofuel Production

This section focuses on technological developments in the biochemical routes of lignocellulosic biomass-based biofuel production (e.g., bioethanol, biobutanol, biogas, biohydrogen, etc.). This may involve modifications either in the process or in the biological components involved (microorganisms and plants). The conversion of lignocellulosic biomass into biofuels involves three main steps, namely, biomass pre-treatment, enzymatic/microbial hydrolysis of the pre-treated biomass to simple sugars, and fermentation for biofuel production. Enzymatic hydrolysis has been shown to be a rate-limiting step in overall biofuel production; thus, increasing the efficiency of the hydrolysis process is the most important part of biofuel synthesis. Efficient biomass pre-treatment also contributes to improved enzymatic hydrolysis. Therefore, researchers have focused more on improving these two steps in the past; however, direct intensification of the fermentation process has also been effectively attempted.

Lignocellulosic biomass, which is mainly available in the form of agricultural/forestry residues (Clauser et al. 2021), is a potential low-cost substrate for biofuel production. Lignocellulosic biomass is mainly composed of cellulose, hemicellulose, and lignin, of which the cellulose and hemicellulose can be hydrolyzed to

produce biofuels. By nature, the lignocellulosic biomass is highly crystalline, and the cellulosic fraction is densely entangled with hemicellulose and lignin, which hinders the enzymatic action on the cellulose to release fermentable sugar monomers (Kumar et al. 2009). This necessitates the pre-treatment of raw biomass prior to enzymatic hydrolysis for biofuel production. Therefore, the main objectives of pre-treatment are to reduce the biomass/cellulose crystallinity, increase the available surface area of the biomass for enzyme action, and remove the lignin. The various pre-treatment methods practiced for different lignocellulosic biomasses can be classified as physical, chemical, biological, and physicochemical pre-treatments.

2.3.1 Physical Pre-Treatment

Physical pre-treatments include comminution of biomass by using various physical methods such as grinding, milling, steam explosion, compressed hot water treatment, and energy radiation such as γ-radiation, microwave, etc. Mechanical comminution such as crushing, grinding, and milling involves comminution of raw biomass resulting in increased surface area and decreased crystallinity of biomass/cellulose. In one report, sequential chopping, grinding, and milling resulted in a material size of 10–30 mm after chopping and 0.2–2 mm after grinding or milling (Sun and Cheng 2002). Timothy and alfalfa grass were processed by mechanical crushing, hammer, and knife grinding (Alvo and Belkacemi 1997). Ball milling was applied to corn cobs, resulting in increased biohydrogen production (Zhang et al. 2019). Significantly higher glucose yield (~98%, g/g) was observed in enzymatic hydrolysis after compressed hot water and disk milling of oil palm mesocarp fibers (Zakaria et al. 2015). However, the energy required for mechanical comminution depends on the final particle size and biomass properties (Cadoche and López 1989) and incurs high energy and capital costs (Ghosh and Ghose 2003).

Another very suitable physical pre-treatment method for the production of liquid biofuels is steam explosion, in which the biomass is subjected to a sudden high pressure at a very high temperature (Ibrahim et al. 2011). Steam explosion of wild grass at 121 °C, 15 psi for 1 h, yielded a significant amount of sugar for ethanol fermentation (Das et al. 2013).

Liquid hot water pre-treatment is an emerging method where the treatment is carried out at elevated pressure to maintain the water in a liquid state (Yu et al. 2010), resulting in improved cellulose digestibility and dissolution of the hemicellulosic portion of the biomass. This method is advantageous in terms of the absence of fermentation inhibitors in the hydrolysate and the release of hemicellulosic pentose sugars ready for direct fermentation for bioalcohol production.

High-energy radiation such as γ-radiation (Su et al. 2020), electron beam (Fei et al. 2020), microwave heating (Ma et al. 2009), and ultrasound for biomass pre-treatment has also been extensively studied in the recent past. The mechanism behind the ultrasonic treatment of biomass has been extensively studied by some researchers to explore the physical and chemical effects (Singh et al. 2014a).

2.3.2 Chemical Pre-Treatment

Lignocellulosic biomass can be pre-treated by acids, alkalis, oxidizing agents, surfactants, and ionic liquids. The hemicellulosic portion of the biomass can be readily hydrolyzed with dilute acid and also dissolves the lignin to some extent. The resulting pentose sugars can be easily fermented for alcohol production (Bharadwaja et al. 2015). However, cellulose and lignin fractions, which are more crystalline than hemicellulose, can be treated with alkali to dissolve mainly lignin and leave a cellulose-rich residue (Singh et al. 2014a). Alkaline treatment also reduces the crystallinity of cellulose, resulting in improved digestibility during enzymatic hydrolysis (Ayeni et al. 2013).

Oxidants such as H_2O_2 have been used for biomass pre-treatment either alone or in combination with acid, alkali, or ammonia (Lee et al. 2021). Various studies show that hydrogen peroxide promotes lignin degradation and breaks lignin-carbohydrate bonds (Singh et al. 2014a, b, Lee et al. 2021). Biomass pre-treatment can also be carried out using surfactants (Gong et al. 2021). Surfactants are surface-active agents that improve mass transfer and increase the wettability of biomass by reducing the surface tension of water.

2.3.3 Biological Pre-Treatment

Biological pre-treatment mainly involves the use of wood-degrading microorganisms that lead to a change in the chemical composition and/or structure of the lignocellulosic biomass. Biologically pre-treated biomass is easier to degrade enzymatically, and this type of pre-treatment is also environment-friendly as it leaves no harmful by-products. Brown rot and soft rot fungi mainly affect cellulose with little effect on lignin, whereas white-rot fungi actively degrade lignin (Zheng et al. 2009). Biological pre-treatment of the field weed *Parthenium* sp. was attempted using the fungi *Trametes hirsuta* and *Marasmiellus palmivorus* PK-27 (Rana et al. 2013). For biogas production, biological pre-treatment of maize straw with mixed microbes accelerated biomass degradation rate and increased lignin degradation efficiency (Li et al. 2020).

2.3.4 Physicochemical Pre-Treatment

This category of pre-treatment can be a combination of the physical and chemical pre-treatments mentioned in the previous sections or can occur individually, such as the ammonia fiber expansion process (AFEX). The combined processes that are very commonly used are the chemical (CO_2, SO_2, acid or alkali) catalyzed steam explosion (De Bari et al. 2007), and the recently explored strategies are the

ultrasound-assisted chemical (acid/alkali) pre-treatments (Singh et al. 2014a, Suresh et al. 2014). In another very recent physicochemical pre-treatment, a two-step sequential approach using UV-catalyzed alkaline hydrogen peroxide (UHP) and ionic liquid treatment (IL) was applied to sisal waste. It resulted in a significant amount (69.2 g/100 g dry sisal waste) of sugar release after enzymatic hydrolysis. The analyses showed the main effects in terms of delignification and decrystallization of sisal waste biomass (Cao et al. 2021).

2.4 Advantages, Policies, and Challenges for Commercial Application

The use of biofuels has several advantages with long-term effects both in economic terms and for sustainable development. Biofuels are not only a potential substitute for fossil fuels but also have a significant impact on social, economic, and environmental aspects around the world. The environmental aspect of reducing greenhouse gas emissions and addressing climate change are important benefits of using biofuels as biofuels are carbon neutral (Gheewala et al. 2013). Another major advantage of using biofuels is the reduction of dependence on fossil fuels and the constant availability of an adequate amount of energy at a reasonable cost, thus ensuring energy security. The imbalance in the distribution of fossil fuels and the ever-fluctuating crude oil prices are the issues that are directly related to the economic and political influences, where the countries that have to import these fossil fuels are vulnerable. Renewable energy production can provide strategic benefits to these countries, especially developing countries, by giving them economic and political liberty in this context (Coelho et al. 2005). Renewable energy also provides social benefits in terms of improved health quality through the provision of cheap biofuels as alternatives, especially in rural households that rely on firewood as a common fuel source. Increased employment opportunities at the local level and rural development dependent on the bioenergy system is another positive aspect of using biofuels as renewable energy (Chum et al. 2011).

However, there are certain challenges in the production of bioenergy, the most important being the economic aspect. The high cost of enzymes used for both pre-treatment and fermentation processes for biofuel production is a major hurdle. Further, it is important to design the pre-treatment method in such a way that maximum benefits are obtained in an economical manner. Cost-effective pre-treatment requires the successful release of sugar polymers for enzymatic activity without the production of inhibitory compounds or residues, requires less energy and chemicals, and is a cost-friendly material for reactor design. The efficiency of fermentation also plays a crucial role, using several screening techniques along with genetic and protein engineering techniques to identify new as well as genetically modified organisms (GMOs) and enzymes with high production rate and stability under the industrial fermentation conditions (Gaurav et al. 2017).

Considering these advantages and limitations of biofuels, public policies for bioenergy production and utilization are being developed and implemented by the government worldwide. The Indian government formulated a national policy in December 2007 to promote bioenergy as a transportation fuel, where the biofuel was intended to use as a blend with gasoline at a level of 20% (Raman and Mohr 2014). To maintain the competitiveness of biofuels in terms of production, transportation, and distribution, the integration of the private sector in the regulatory framework is an important step. The Ministry of New and Renewable Energy (MNRE) has initiated several programs to promote bioenergy production, such as the National Biogas and Manure Management Program (NBMMP) and energy recovery from urban, agricultural, and industrial wastes (Gaurav et al. 2017). Nevertheless, research and development work are still required for the successful and economical implementation of biofuel policy.

2.5 Discussion and Future Prospective

As energy demand is a continuous process, the search for different energy sources will always exist. The biggest challenge of the coming generation is to find an energy source that provides sufficient energy, can be relied upon in the long run, and has little or no negative impact on the environment. Therefore, the future world will rely more on the various renewable energy sources to meet their needs. Among the numerous renewable energy sources, bioenergy, which is derived from abundant biomass, is the most reliable. The various biomass sources are already being used commercially, but further improvement of the technology supported by strong government policies to promote the use of renewable energy from biofuels is needed.

Acknowledgments The authors would like to thank the Department of Microbiology, Sikkim University, for providing the computational infrastructure and central library facilities for procuring references.

Conflict of Interest The authors declare that the study was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 3
Metabolic Rewiring and Cultivation Optimization for Photosynthetic Biofuel Production in Cyanobacteria

Hao Xie, Kateryna Kukil, Pia Lindberg, Rui Miao, and Peter Lindblad

Abstract The depletion and use of fossil resources together with global environmental concerns related to CO_2 emissions require a transition toward carbon-neutral, sustainable bioenergy. Photosynthetic organisms have great potential as cell factories for directly converting CO_2 into a broad range of value-added biochemicals, especially biofuels. Cyanobacteria are particularly attractive as platforms for bio-production, due to relatively fast growth rate, genetic tractability, and low nutrient requirements. Synthetic biology and metabolic engineering pave the way to develop cyanobacteria as green chassis for production of various biofuels, including, e.g., hydrogen, ethanol, butanol, and isoprene. However, engineered cyanobacteria show relatively low productivity compared with model heterotrophic microorganisms. Significant advances of biofuel production using cyanobacterial chassis are expected by implementing recently developed system biology tools as well as optimized cultivation systems with newly developed photobioreactors.

Keywords Biofuel · Cyanobacteria · Metabolic engineering · Microbial consortia · Photobioreactor · Synthetic biology · System biology

3.1 Introduction

Cyanobacteria are the most widely spread autotrophic organisms which can be found in all types of environments, from ocean to freshwater, from hot springs to deserts and glaciers. They play an important role in CO_2 fixation and O_2 evolution and are considered as main producers for the significant level of O_2 in the Earth's atmosphere (Brocks et al. 1999). Cyanobacteria are present in several morphology

H. Xie · K. Kukil · P. Lindberg · P. Lindblad (✉)
Department of Chemistry-Ångström, Uppsala University, Uppsala, Sweden
e-mail: peter.lindblad@kemi.uu.se

R. Miao
Science for Life Laboratory, KTH - Royal Institute of Technology, Stockholm, Sweden

Department of Protein Science, KTH - Royal Institute of Technology, Stockholm, Sweden

P. Chowdhary et al. (eds.), *Bio-Clean Energy Technologies: Volume 1*, Clean Energy Production Technologies, https://doi.org/10.1007/978-981-16-8090-8_3

variants, such as unicellular, filamentous, or colonial. They possess a bilayered cell wall of a gram-negative subclass, but with a thicker layer of peptidoglycan (Stanier and Cohen-Bazire 1977). Moreover, cyanobacterial peptidoglycan is complexed with similar type of polysaccharides found in gram-positive bacteria (Castenholz 2015).

In cyanobacteria, both oxygenic photosynthesis and respiration are performed in the thylakoid membrane, where several electron transport apparatuses for these two reactions are shared (Vermaas 2001). Moreover, cyanobacteria have a higher photosynthetic efficiency, compared to microalgae and plants (Branco Dos Santos et al. 2014; Mur et al. 1977). It is believed that the photosynthetic capacity of algae and plants evolved from cyanobacterial endosymbionts (Tomioka and Sugiura 1983).

The transformation of energy from light to biofuel by photosynthesis shows the potential to alleviate environmental concerns related to greenhouse gas emission and addresses the global demand for renewable energy and chemicals (Woo 2017). As photosynthetic microorganisms, cyanobacteria have superior properties, such as fast growth rate, simple nutrient requirement, and ease of genic manipulation, and growing them does not require arable land. Moreover, some cyanobacterial strains even can be cultivated in wastewater (Markou and Georgakakis 2011; Oliver et al. 2016; Chowdhary et al. 2020). Due to the rapid development of genetic engineering techniques, cyanobacteria have been metabolically engineered to produce commercially important chemicals, e.g., biofuels (Nozzi et al. 2013), nutraceuticals (Gademann 2011), pharmaceuticals (Vijayakumar and Menakha 2015), and secondary plant metabolites (Angermayr et al. 2015). Different metabolic engineering approaches, ranging from conventional synthetic biology tools to systems biology tools, have been applied to cyanobacteria for various compound production, such as ethanol (Deng and Coleman 1999), higher alcohols (Atsumi et al. 2009; Liu et al. 2019), and hydrogen (Bolatkhan et al. 2019). When compared with heterotrophic microorganisms, e.g., *Escherichia coli* and *Saccharomyces cerevisiae*, a comprehensive study of the biology of photosynthetic microorganisms is far behind. As a consequence, there are limited tools developed and available for genome manipulation and metabolic engineering in cyanobacterial biotechnology (Sun et al. 2018). Nevertheless, significant efforts and progress have been made in the past few years (Lindblad et al. 2019; Wichmann et al. 2021), making it possible to use cyanobacteria as a platform to produce biofuel.

This book chapter focuses on recent advances in developing cyanobacteria as chassis for biofuel production. We discuss and highlight the current challenges as well as opportunities in applying different tools and strategies to further assist cyanobacterial engineering toward carbon-neutral clean energy production.

3.2 Various Cyanobacterial Strains as Biofuel-Producing Chassis

The usage of certain cyanobacterial chassis is usually determined by several characteristics that need to be taken into consideration, such as optimal growth temperature, growth rate, availability of tools for genetic modifications, and salinity

requirements. The most extensively studied cyanobacterial strains are listed in Table 3.1. Note that all indicated doubling times are strongly dependent on cultivation conditions and should be used as an orientation.

Among various species, fundamental and applied research has been primarily concentrated on unicellular non-nitrogen fixing cyanobacteria. *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*), *Synechococcus elongatus* PCC 7942, and *Synechococcus* sp. PCC 7002, known as "model organisms" nowadays, have been comprehensibly studied for decades. For instance, the discovery and study of the circadian clock in cyanobacteria were elucidated in *S. elongatus* PCC 7942 (Ouyang et al. 1998), which is now used as a model strain to study molecular oscillations during diurnal growth (Welkie et al. 2018). On the other hand, filamentous cyanobacterial strains are extensively used for photosynthetic hydrogen production, due to their ability to form a specialized type of cell called heterocyst with a specific micro-oxygenic environment for nitrogen fixation (Avilan et al. 2018; Dutta et al. 2005; Hansel and Lindblad 1998; Wegelius et al. 2018b). Major bottlenecks for industrial application of model cyanobacterial strains for biofuel production, such as relatively slow growth rate and low product titer compared to heterotrophs, force the scientific community to search for new fast-growing strains and to enhance the growth and productivity of available strains via metabolic engineering approaches.

Several fast-growing cyanobacterial strains, *Synechococcus* sp. PCC 11901, *Synechococcus elongatus* UTEX 2973, *Synechococcus elongatus* PCC 11801, and *Synechococcus elongatus* PCC 11802, have been discovered and described recently, all with growth rates close to yeast (Jaiswal et al. 2020; Jaiswal et al. 2018; Wlodarczyk et al. 2020; Yu et al. 2015). Because of more efficient photoautotrophic growth and higher robustness in various living environments, the newly discovered strains significantly expand the range of cyanobacterial strains that can be used as promising biofuel-producing chassis (Knoot et al. 2018; Song et al. 2016). Freshwater strain *S. elongatus* UTEX 2973 is attributed as the fastest-growing cyanobacterial strain whose genomic sequence shares 99.8% similarity to that of *S. elongatus* PCC 7942. This high similarity makes it more promising to transfer and apply the existing genetic tools previously developed for *S. elongatus* PCC 7942 in *S. elongatus* UTEX 2973. Furthermore, remarkably high sequence similarity to a slower-growing strain makes *S. elongatus* UTEX 2973 a new model organism to reveal the genetic features of fast photoautotrophic growth (Abernathy et al. 2017; Ungerer et al. 2018; Yu et al. 2015). Recent reports on genome-scale model and flux balance analysis suggest that the high growth rate of *S. elongatus* UTEX 2973 is linked to the nearly complete incorporation of fixed carbon into biomass formation due to an efficient reincorporation of photorespiratory lost carbon (Hendry et al. 2019).

Table 3.1 Extensively studied cyanobacterial strains for metabolic engineering

Strain	Genome sequence available	Genome size (Mbp)	Doubling time (h)	Transformation methods	Notes
Synechocystis sp. PCC 6803	1997 (Kaneko and Tabata 1997) GenBank: BA000022.2	3.6	6–12 Williams (1988)	Conjugation Natural transformation	Extensively studied model strain Freshwater Unicellular
Anabaena sp. PCC 7120	2001 (Kaneko et al. 2001) GenBank: BA000019.2	6.4	15.1 Zhao et al. (2007)	Electroporation Conjugation	Model filamentous strain Nitrogen-fixing
Nostoc punctiforme ATCC 29133	2008 GeneBank: GCA_000020025.1	8.2	16 Summers et al. (1995)	Electroporation; conjugation	Model filamentous strain Nitrogen-fixing
Synechococcus elongatus PCC 7942	2005 GeneBank: CP000100.1	2.7	4.9 Yu et al. (2015)	Conjugation Natural transformation	Model strain for circadian clock studies Freshwater Unicellular
Synechococcus sp. PCC 7002	2008 GeneBank: CP000951.1	3	4.1 Yu et al. (2015)	Conjugation Natural transformation	Model marine strain Unicellular Euraline, cobalamin auxotroph
Synechococcus elongatus UTEX 2973	2015 (Yu et al. 2015) GeneBank: CP006471.1	2.7	2.1 Yu et al. (2015)	Conjugation Natural transformation	Freshwater Unicellular Newly described fast-growing strain
Synechococcus elongatus PCC 11801	2018 (Jaiswal et al. 2018) GeneBank: CP030139.1	2.7	2.3 Jaiswal et al. (2018)	Natural transformation	Freshwater Unicellular Newly isolated fast-growing strain Closely related to *S. elongatus* PCC7942 and *S. elongatus* UTEX 2973

Synechococcus elongatus PCC 11802	2020 (Jaiswal et al. 2020) GeneBank: CP034671.1	2.7	2.8 Jaiswal et al. (2020)	Natural transformation	Freshwater Unicellular Newly isolated fast-growing strain Closely related to *S. elongatus* PCC 11801
Synechococcus sp. PCC 11901	2020 (Wlodarczyk et al. 2020) GeneBank: CP040360.1	3	2.14 Wlodarczyk et al. (2020)	Natural transformation	Marine Unicellular Cobalamin auxotroph Newly isolated fast-growing strain Closely related to *S. elongatus* PCC 7002

3.3 Generate, Analyze, and Improve Biofuel-Producing Strains Via Various Approaches

Ever since the full genomic sequence of *Synechocystis* became available in 1997 (Kaneko and Tabata 1997), research on cyanobacteria accelerated extensively. Already in 1999, the cyanobacterial strain was metabolically engineered for ethanol production (Deng and Coleman 1999). Nowadays, cyanobacteria are engineered as cell factories for various chemicals (Fig. 3.1). However, titers and productivity for most target products remain lower in cyanobacteria than those reported in heterotrophic organisms. In contrast, heterotrophic growth relies on the addition of carbon sources usually in a form of sugars to the growth media, whereas phototrophic cyanobacteria allow the completely sustainable production of chemicals and biofuels by assimilating CO_2 as a carbon source (Knoot et al. 2018).

Biofuels and chemicals produced by cyanobacteria are usually derived from a few central metabolites. Consequently, a basal approach of metabolic engineering for improving product titer is to increase the availability of the corresponding central precursors. Directing fixed CO_2 from the CBB cycle into the pathways for biosynthesis of desired products requires modifications of the metabolic network, by removal (deletion of competing reactions for the same precursors) or plug-in of components (boosting the formation of precursors) via genetic modifications.

Metabolic modifications often have a detrimental effect on growth, since part of the carbon is channeled away from anabolic reactions of biomass formation. Thus, it is beneficial to develop synthetic circuits with balanced biomass/product formation. However, in some cases, a strong carbon sink was shown to promote photosynthetic capacity (Abramson et al. 2016), since the cells compensate for the lost carbon. Another approach consists of fine-tuned regulation of switch between anabolic and product formation pathways via induction systems.

3.3.1 Synthetic Biology

Synthetic biology, a multidisciplinary research subject, aims to design and generate biological systems with given properties and functions for useful purposes (Benner 2003). Generally, the synthetic biology workflow represents repetitive cycles of design and combining of standardized parts, testing assembled genetic constructs, and, finally, evaluation of results for new design cycles. First implemented in model heterotrophic organisms, e.g., *E. coli* and yeast, synthetic biology has allowed a better understanding of biological mechanisms. Currently, about 80 cyanobacterial full genomic sequences are available and published (https://gold.jgi.doe.gov/), making synthetic biology a fundamental tool in cyanobacterial research.

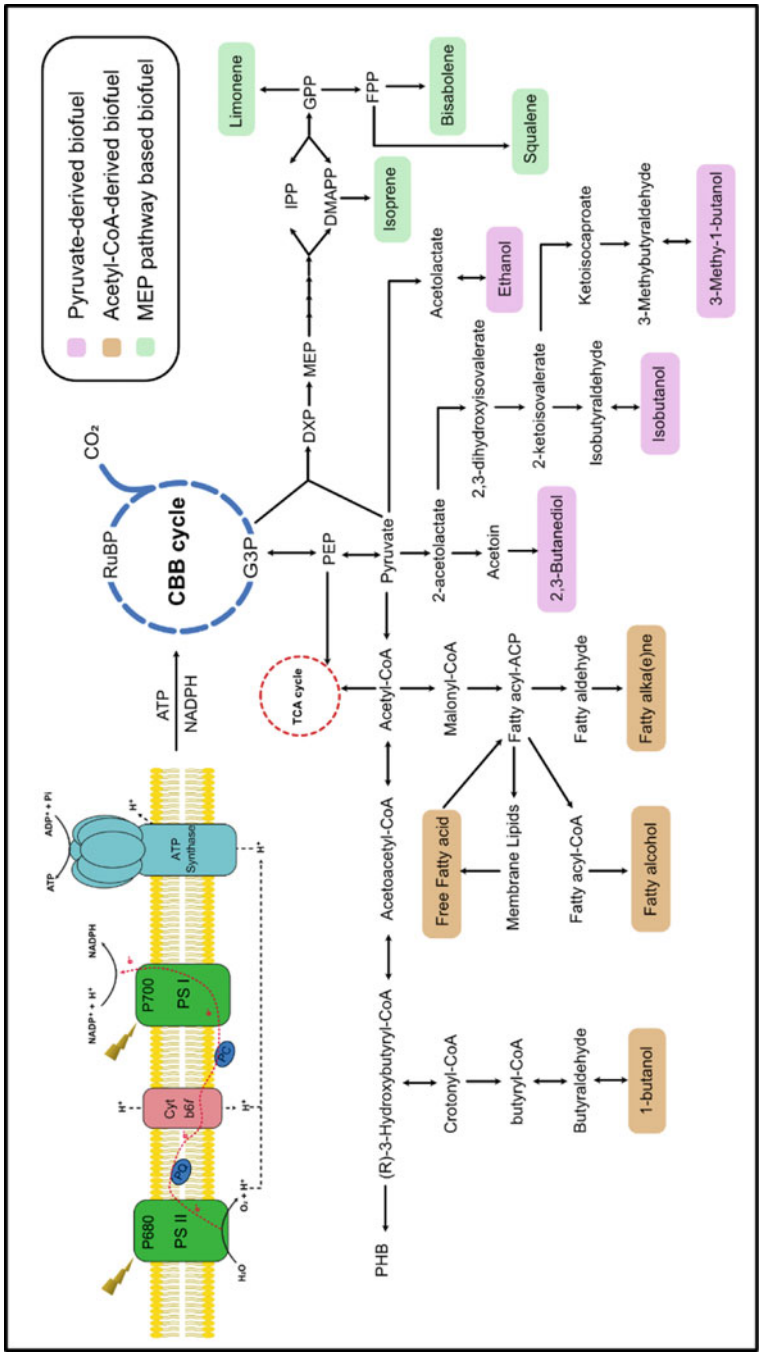

Fig. 3.1 Overview of photosynthetic metabolism and central carbon metabolism in cyanobacteria. ATP and NADPH support CO_2 fixation via Calvin-Benson-Bassham (CBB) cycle. Target biofuel production was achieved in cyanobacteria through native pathways and/or engineered pathways. Pink boxes, products derived from pyruvate; brown boxes, products derived from acetyl-CoA; green boxes, terpenes and terpenoids; PS I, photosystem I; PS II, photosystem II; PQ, plastoquinone; b_6f, cytochrome-b6/f; PC, plastocyanin; RUBP, ribulose 1,5-biphosphate; G3P, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2-C-methylerythritol 4-phosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; PHB, polyhydroxybutyrate

3.3.1.1 Genetic Tools for Gene Expression and Regulation

For successful genetic manipulation that allows controlled gene expression and metabolic reconstruction, a large variety of genetic tools are required. Promoters, the traditional regulatory elements for gene expression, have been mostly evaluated in model cyanobacterial strains. For more details on promoters and other regulatory genetic elements implemented in cyanobacteria, refer to earlier published reviews (Berla et al. 2013; Sun et al. 2018; Till et al. 2020). Briefly, promoters can be distinguished according to the origin: native, heterologous, or synthetic. Additionally, they can be classified depending on their regulatory mode: constitutive or inducible. Most of the strong constitutive promoters used in cyanobacteria originate from native essential genes related to CO_2 fixation (P*rbc*) and photosynthesis (P*cpc*, P*psbA*), whereas native metal-responsive promotors, such as nickel-inducible P*nrsB* and iron deficiency-dependent P*isiAB*, are derived from metal homeostasis genes.

It is important to mention that well-characterized promoter systems from *E.coli* are often not compatible with cyanobacteria, due to the different holopolymerase (Heidorn et al. 2011). For instance, commonly used in *E.coli*, lactose-induced P_{lac} and tetracycline-induced P_{tet} promotors were shown to function poorly in *Synechocystis* (Huang et al. 2010). This has forced researchers to work on synthetic elements (Ferreira et al. 2018). Recently, many inducible synthetic and semisynthetic promotors have been designed and ready for application in cyanobacteria, such as anhydrotetracycline (aTc)-induced P_{tet}, rhamnose-induced P*rhaBAD*, vanillate-induced P*vanCC*, and arabinose-induced P*BAD* promoters. However, leakiness and low expression strength are still not completely solved. In addition, external chemical inducible promoters are not optimal in large-scale cultivation, due to the requirement of a large number of chemicals and the high economical cost of the chemicals. Moreover, some inducers, such as aTc, are highly sensitive to light, which makes them difficult to keep high induction levels in large-scale cultures. Autoinducing or inducer-free tunable promotor systems driven by environmental signals, such as light or CO_2 level, seem to be highly attractive for industrial-scale cultivation of cyanobacteria and microalgae (Sengupta et al. 2020; Sun et al. 2018). As an example, Immethum et al. developed a dark inducible system based on transmembrane Cph8 chimera of *Synechocystis* native photoreceptor Cph1 and histidine kinase EnvZ of *E. coli* that activates the transcription from *E. coli* P_{ompC} promotor in darkness. A dark-inducible system can be advantageous when expressing heterologous enzymes which are sensitive to photosynthetic oxygen, such as hydrogenase, or when decoupling production from light-driven cellular processes is advantageous for target product formation (Immethun et al. 2017).

Experimental evidence indicates that the portability of promoters across even closely related strains is quite limited (Markley et al. 2015). Therefore, promoter elements need to be systematically evaluated to create reliable libraries for various cyanobacterial strains. A better understanding of cellular machinery would help not only expand the cyanobacterial toolbox by adapting tools from heterotrophic model

organisms but also use described promoter elements in newly isolated fast-growing strains.

Ribosome binding sites (RBSs) are important elements for synthetic biology to initiate the translation of downstream genes. It was found that the same RBS gives different gene expression efficiency depending on the surrounding nucleotides, possibly due to the formation of secondary structures at the 5'UTR (Englund et al. 2016; Thiel et al. 2018). While several RBS calculators have been established for *E. coli* to predict in silico specific ribosome-mRNA interaction based on statistical thermodynamic modeling ("RBS calculator," "UTR designer," "RBS designer"), the performance of these tools in cyanobacteria needs further experimental validation, as several recent studies found a relatively low correlation and predictability (Thiel et al. 2018; Wang et al. 2018). An approach to overcome the issue of surrounding genetic context is the use of "bicistronic design" (BCD) (Mutalik et al. 2013), which was successfully applied in cyanobacteria to give a reliable translation initiation (Englund et al. 2018; Liu et al. 2019; Wegelius et al. 2018b). The BCD consists of a short nucleotide sequence called leader sequence, which is placed upstream and partially overlaps with the coding sequence of the gene of interest. This leader sequence is translated firstly into the leader peptide, thus enabling the ribosome to melt any secondary structure of the transcript and facilitate the translation initiation of the downstream gene of interest (Mutalik et al. 2013).

In synthetic biology, transcriptional terminators are used for terminating transcription and to isolate the transcriptional units from one to another. To date, little scientific work was devoted to the characterization of termination efficiencies in cyanobacteria. Widely used terminator elements in cyanobacterial studies are the native Rubisco terminator T*rbcS*, *E. coli rrnB* transcription terminator (Geerts et al. 1995), and terminator of bacteriophage T7 (Haselkorn 1991). Recently, a library of native transcription terminators in *Synechocystis* was established (Liu and Pakrasi 2018).

Regulatory RNAs, a powerful tool for fine-tuning gene expression, act independently from promoters. Regulatory RNA elements can be riboswitches, which are *cis*-regulatory elements, or *trans*-activating riboregulators. Riboswitches are non-coding RNA elements that form a stem-loop structure in the 5' UTR of mRNA including RBS; binding of a specific ligand or signal molecule induces conformational changes of riboswitch, resulting in ON/OFF gene expression. In the case of riboregulators, *cis*-element on mRNA undergoes structural changes after binding of *trans*-activating RNA. In the synthetic biology toolbox for cyanobacteria, only a few riboswitches are considered to be suitable, one of which is theophylline-dependent riboswitch (Ma et al. 2014; Ohbayashi et al. 2016). Other described systems are not applicable due to a central role of the ligands and their toxic impact on the cell upon addition. More comprehensive information on regulatory RNAs in cyanobacteria was summarized in earlier reviews (Sun et al. 2018; Till et al. 2020).

3.3.1.2 Genetic Modifications and Genome Editing

In the synthetic biology of cyanobacteria, heterologous genes can be expressed either extra- chromosomally via plasmid addition or through chromosome editing via homologous recombination. Both approaches are commonly used while each has certain limitations and advantages. Foreign DNA elements can be delivered inside the cyanobacterial cell via natural uptake, conjugation, or electroporation. Many unicellular cyanobacterial strains are naturally transformable, which considerably simplifies the delivery of DNA into host cells**.**

Genetic modification through gene expression on self-replicating plasmids is a routinely used procedure, where standardized parts are assembled and swapped in standard plasmids. Self-replicating plasmids are maintained in the host cell with selective marker genes (usually antibiotic resistance). A homologous recombination is a conventional approach for genome editing in cyanobacteria. Heterologous DNA sequences can be introduced into the chromosome or endogenous plasmids to create a deletion, insertion, or replacement. For model strains, several *loci* of neutral sites are identified and used, where heterologous DNA insertion seems not to affect metabolism.

Importantly, many cyanobacterial strains are polyploid, which requires more effort to reach full segregation after mutagenesis. When full segregation is achieved, these mutants are stable over time without the application of selective pressure (Bentley et al. 2014). Nonetheless, even after full segregation, the loss of heterologous genes appears to happen in some cases (Jacobsen and Frigaard 2014; Takahama et al. 2003). Mechanisms related to genetic instability, as part of the adaptive evolution to stresses, have not yet been fully elucidated in cyanobacteria (Jones 2014).

The small number of available selectable markers limits the possibility of multiple gene deletions or insertions for extensive strain engineering. Therefore, marker-less genetic modification of chromosomes has been developed in cyanobacteria. The marker-less modification is based on the principle of counter-selection, where the second round of transformation is required to remove the selective marker gene. *SacB* counter-selection marker (Lagarde et al. 2000) and FLP/FRT-based recombination (Tan et al. 2013) are two widely used approaches. These methods allow multiple reuses of selection markers. However, due to the need for several rounds of transformation and a low success rate, the process becomes highly time demanding.

As the most recent state-of-art in genome engineering technique, CRISPR technology allows one-step precise and efficient modification of DNA sequences. It can be used for marker-less specific point mutations, knock-ins, and knock-outs in a wide variety of organisms (Bortesi and Fischer 2015; Jiang et al. 2015; Sander and Joung 2014). One such system originates from the bacterial CRISPR-Cas9 antiviral defense system. As an editing tool, it requires two components, Cas9 nuclease protein and single-guide RNA (sgRNA). A complex of sgRNA and Cas9 DNA nuclease can initiate double-stranded breaks. The apparent toxicity of Cas9 nuclease in cyanobacteria restrains the usage of this system (Wendt et al. 2016). As an

alternative, Cpf1, from *Francisella novicida*, was successfully applied in *Synechocystis*, *Anabaena* sp. PCC 7120, and fast-growing strain *S. elongatus* UTEX 2973 (Ungerer and Pakrasi 2016). Another tool adapted from the CRISPR-Cas9 system, termed CRISPR interference (CRISPRi), was employed for multiple knockdowns of gene expression in several cyanobacterial strains (Gordon et al. 2016; Yao et al. 2016). In this case, the system is based on inactive Cas9 (dCas9). The dCas9, coupled with sgRNA, can bind target DNA without creating a double-stranded break. CRISPRi can be used for both repression and activation of gene expression (CRISPRa) (Bikard et al. 2013).

3.3.2 Advanced Systems Biology Approaches Applied in Cyanobacteria

Using photosynthetic organisms as platforms for biofuel production is a promising strategy to alleviate both the high consumption of fossil fuels and the high rate of climate change (Ducat et al. 2011; Fargione et al. 2008). Unicellular cyanobacteria are promising chassis for biofuel production, with the ability to store energy from light in valuable products. However, limited advancements have been achieved with traditional biology tools. Therefore, with rapid development in genome sequencing and systems biology, it is possible to develop and apply various systems biology tools for a comprehensive understanding of the genome-scale metabolic network of cyanobacteria, which can further guide the optimization of biofuel production in cyanobacteria.

3.3.2.1 Genome-Scale Metabolic Models (GSMMs)

A genome-scale metabolic model (GSMM) is a large-scale stoichiometric model that represents all the metabolic pathways theoretically and/or experimentally characterized (Kim et al. 2008). It offers insights into cellular function and organization and further serves as a powerful framework to rationally modify existing pathways and create novel pathways to produce desired end-products (Zhang and Hua 2015). The construction of a GSMM contains four stages: (1) construction of initial draft metabolic networks; (2) manual curation and refining of the established draft metabolic networks; (3) conversion to a mathematical format known as the stoichiometric matrix; and (4) model evaluation and analysis (Gudmundsson et al. 2017).

With many computational methods developed, GSMM is currently an indispensable tool to discover new biological knowledge and to guide metabolic engineering (Likic et al. 2010). It has rapidly been widely applied for different aims, such as biochemical production and drug development. Starting with GSMMs of model organisms, including *E. coli* (Edwards and Palsson 2000) and *S. cerevisiae* (Lu et al.

2019), GSMMs of various organisms have been generated and optimized, including cyanobacteria.

A number of GSMMs of cyanobacteria have been constructed (Table 3.2). The first GSMM of cyanobacteria was developed for *Synechocystis* (Fu 2009). Since genome databases, bioinformatics tools, and literatures are updated continuously, GSMMs are accordingly expanded and updated based on the newly curated genes and biochemical knowledge (Klanchui et al. 2018). This is exemplified by the GSMMs of *Synechocystis*: *i*Syn811 (Montagud et al. 2011), *i*Syn731 (Saha et al. 2012), and *i*HK677 (Knoop et al. 2013). As an updated and comprehensive GSMM of *Synechocystis*, *i*SynCJ816 contains 816 genes, 1045 reactions, and 925 metabolites (Joshi et al. 2017). Different from the previously published models, molecular mechanisms of the photosynthetic network around the thylakoid membrane are included in this newly updated model to get a comprehensive understanding of the interactions between respiration and photosynthesis within the cells. Furthermore, another significant improvement of this model is the inclusion of thermodynamic analysis of more than 500 reactions (Joshi et al. 2017).

With GSMMs established, Flux Balance Analysis (FBA) is used to study biochemical networks, by identifying flux distribution that maximizes an objective, such as specific chemical production or cell growth. It can not only be used to calculate the maximum theoretical yield of end-products but also further be used to identify target pathways for optimization to attain this yield (Orth et al. 2010). To study the metabolic capacities of biofuel production in cyanobacteria, GSMM, coupled with other computational algorithms, could be a useful tool to suggest strategies for enhanced biofuel production. Different algorithms were successfully developed and applied for improved biochemical productivity via identifying gene deletion targets, such as OptKnock (Burgard et al. 2003), OptGene (Patil et al. 2005), OptORF (Kim and Reed 2010), minimization of metabolic adjustment (MOMA) (Segre et al. 2002), Computational Approach for Strain Optimization aiming at high Productivity (CASOP) (Hadicke and Klamt 2010), and Constrained Minimal Cut Sets (Hadicke and Klamt 2011). Based on the original GSMM of *Synechocystis* published by Knoop et al. (Knoop et al. 2013), Erdrich et al. (Erdrich et al. 2014) implemented two computational methods, CASOP and Constrained Minimal Cut Sets, to explore possible intervention strategies for enforcing coupled biomass and high yield biofuel production. A suitable knockout gene set was identified for improved synthesis of ethanol and isobutanol in *Synechocystis*, which is further needed to be verified experimentally. In another study, with the combined use of OptGene and MOMA, different knockout sites in the *Synechocystis* genome have been identified for improved production of biofuels (Shabestary and Hudson 2016). Besides, Shabestary et al. used a third algorithm, OptKnock, to identify knockouts that result in coupling biofuel and growth (Shabestary and Hudson 2016). As a similar algorithm to OptKnock, OptORF was applied in *Synechococcus* sp. PCC 7002 to explore new strategies for improved fuel productivity (Vu et al. 2013). On the other hand, Flux Variability Analysis (FVA) is another useful approach to predict the minimum and maximum flux of different metabolic

Table 3.2 List of GSMMs developed in different strains of cyanobacteria

Strain	Model name	Number of genes	Number of reactions	Number of metabolites	Sub-cellular compartments	References
Arthrospira platensis PCC 8005	n/a	n/a	121	134	1	Cogne et al. (2003)
Arthrospira platensis C1	*i*AK692	692	875	837	2	(Klanchui et al. (2012)
	*i*AK888	888	1096	994	6	Klanchui et al. (2018)
Arthrospira platensis NIES-39	n/a	620	746	673	2	Yoshikawa et al. (2015)
Synechocystis sp. PCC 6803	n/a	633	831	704	1	Fu (2009)
	*i*Syn669	669	882	790	2	Montagud et al. (2010)
	*i*Syn811	811	956	911	2	Montagud et al. (2011)
	n/a	393	493	465	2	Yoshikawa et al. (2011)
	*i*JN678	678	863	795	4	Nogales et al. (2012)
	*i*Syn731	731	1156	996	4	Saha et al. (2012)
	n/a	677	759	601	6	Knoop et al. (2013)
	*i*SynCJ816	816	1060	925	7	Joshi et al. (2017)
	*i*mSyn61	n/a	729	679		Gopalakrishnan et al. (2018)
Cyanothece sp. ATCC 51142	*i*Cce806	806	667	587	1	Vu et al. (2012)
	*i*Cyt773	773	946	811	4	Saha et al. (2012)
Synechococcus sp. PCC 7002	*i*Syp611	611	552	542	2	Hamilton and Reed (2012)
	*i*Syp708	708	602	581	2	Vu et al. (2013)
	*i*Syp706	706	649	542	2	Song et al. (2015)
	n/a	728	742	696	7	Hendry et al. (2016)
	*i*Syp821	821	744	777	n/a	Qian et al. (2017)
Cyanothece sp. PCC 7424	*i*Cyc792	792	1242	1107	4	Mueller et al. (2013)
Cyanothece sp. PCC 7425	*i*Cyn731	731	1306	1160	4	Mueller et al. (2013)
Cyanothece sp. PCC 7822	*i*Cyj826	826	1258	1110	4	Mueller et al. (2013)

(continued)

Table 3.2 (continued)

Strain	Model name	Number of genes	Number of reactions	Number of metabolites	Sub-cellular compartments	References
Cyanothece sp. PCC 8801	*i*Cyp752	752	1172	994	4	Mueller et al. (2013)
Cyanothece sp. PCC 8802	*i*Cyh755	755	1161	973	4	Mueller et al. (2013)
Synechococcus elongatus PCC 7942	iSyf715	715	851	838	2	Triana et al. (2014)
	*i*JB785	785	850	768	7	Broddrick et al. (2016)
Nostoc sp. PCC 7120	n/a	n/a	804	777	6	Malatinszky et al. (2017)
Synechococcus elongatus UTEX 2973	*i*Syu683	687	1178	1028	n/a	Mueller et al. (2017)
Anabaena variabilis ATCC 29413	*i*AM957	957	960	912	4	Malek Shahkouhi and Motamedian (2020)
Synechococcus sp. BDU 130192	*i*Syn706	706	908	900	4	Ahmad et al. (2020)

reactions, which has been successfully applied in *Synechocystis* to find non-intuitive overexpression targets for improved isoprene production (Englund et al. 2018).

Even with proof-of-concept studies in using cyanobacterial GSMMs for predicting metabolism changes, model-driven strain design for optimizing biofuel production in cyanobacteria is still in the early infancy stage. Nevertheless, the advances in GSMMs and corresponding algorithms to redesign cyanobacterial strain's metabolism, together with the genetic tools developed for cyanobacteria, allow the further implementation of system metabolic engineering tools in the field of cyanobacterial biofuel production.

3.3.2.2 Application of Metabolomics and Fluxomics in Rational Design of Cyanobacteria as Cell Factories

Metabolomics and fluxomics are two techniques developed to investigate and analyze the metabolism of cyanobacteria via monitoring the biochemical reactions within cells (Young et al. 2011) by measuring the abundance of metabolites and reaction rates between different metabolites.

3.3.2.2.1 Metabolomics

Metabolomics is the comprehensive profiling of the metabolome of a cell. In general, two different approaches have been applied for metabolomics analysis: targeted and nontargeted methods. Targeted metabolomics is able to identify and quantify specific metabolites, which are predefined based on the specific scientific question (Babele and Young 2020). On the contrary, nontargeted methods aim to measure a large pool of metabolites, and relative amounts of different metabolites are obtained, which is able to offer a comprehensive relationship between interconnected metabolites from various pathways (Johnson et al. 2016).

Metabolomics and analysis are divided into three steps: (1) quenching, metabolic extraction, and sample preparation; (2) metabolic abundances measurement; and (3) data analysis of metabolome. Although the techniques used are transferable between different organisms, there are still many alternatives available for selection based on specific organisms and specific scientific questions. As a crucial step to identify metabolites via advanced analytical instruments, there are different options available with different experimental aims. Mass spectrometry (MS) coupled with chromatography is widely used in metabolomics studies and helps obtain large-scale omics data, while NMR spectroscopy is suitable for structural analysis of target metabolites. By combined use of liquid chromatography-mass spectrometry (LC-MS) and NMR, previously unknown metabolites with low abundance were elucidated from the cyanobacterial sample (Lin et al. 2008).

Metabolomics has been successfully applied in strain improvement for enhanced biofuel production. By performing targeted metabolomics on *Synechococcus elongatus* BUOHSE, the reductive reaction of butanoyl-CoA to butanal was

identified as a bottleneck in the coenzyme A (CoA)-a dependent pathway for 1-butanol synthesis (Noguchi et al. 2016), which was further confirmed by widely targeted metabolic profiling (Fathima et al. 2018). The results were used to guide further engineering, improving the activity of CoA-acylating propionaldehyde dehydrogenase (PduP). The resulting strain was able to produce 33% more 1-butanol (Fathima et al. 2018).

3.3.2.2.2 Fluxomics

Unlike metabolomics, which presents snapshots of metabolite levels, fluxomics is a method to uncover metabolic pathway activities and assess the gene, protein, and metabolite interactions within metabolic networks (Sauer 2006). These cannot be measured directly. Instead, they must be inferred from measurable quantities through computer model-based interpretation.

^{13}C metabolic flux analysis (^{13}C MFA) is developed to determine intracellular flux by isotopic labeling of substrates (Jazmin et al. 2017). ^{13}C MFA is applied to identify rate-limiting steps in a selected pathway that limits maximum production (Koffas and Stephanopoulos 2005) by analyzing how fluxes change after a new biosynthetic pathway is introduced, which guides metabolic engineering strategies to remove the bottlenecks for further enhanced productivity. Unlike heterotrophs, cyanobacteria use CO_2 as the carbon source for cell metabolism. Conventional steady-state isotopic ^{13}C-MFA is incapable to provide flux distribution maps for photoautotrophs assimilating carbon solely from CO_2 since the final labeling steady state of isotopomers is attained independent of the flux distribution (Shastri and Morgan 2007). However, the rate of isotopomers to reach a steady state depends on the flux distribution. Therefore, a new approach, named isotopically nonstationary ^{13}C metabolic flux analysis (INST-MFA) (Wiechert and Noh 2005), was developed to monitor flux changes within photoautotrophic systems, including cyanobacteria. It is worth noting that an underlying assumption behind this approach is that the metabolic fluxes and the pool size are the same throughout the labeling experiments and are not influenced by the introduction of ^{13}C tracer (Young et al. 2011). This approach was first applied to the model cyanobacterium *Synechocystis* cultivated in bioreactors (Young et al. 2011). Below are some examples of how the INST-MFA approach has been applied for enhanced biofuel production in cyanobacteria.

After performing INST-MFA on wild-type *S. elongatus* PCC 7942 and the IBA-producing strain SA590, the conversion of phosphoenolpyruvate (PEP) to pyruvate, catalyzed by pyruvate kinase (PK) reaction, was identified as the hidden bottleneck for isobutyraldehyde (IBA) production (Jazmin et al. 2017), which is the direct precursor for biofuel candidate isobutanol. The bottleneck was successfully removed by overexpressing PK, and a significant improvement in IBA specific productivity was observed (Jazmin et al. 2017). In addition, after comparing the central carbon fluxes on the strains (SA590 and SA590 with PK overexpressed) mentioned in (Jazmin et al. 2017), it was found that the aldehyde flux was positively correlated with flux through PK and acetolactate synthase (ALS), and negatively

correlated with the flux through pyruvate dehydrogenase (PDH) and PEP carboxylase (PEPc) (Cheah et al. 2020). As expected, it was experimentally verified that downregulation of PEPc flux or PDH expression level results in an improved IBA production (Cheah et al. 2020).

Apart from providing a new direction for metabolic engineering strategies, INST-MFA is also a powerful systems biology tool to elucidate metabolic network regulation in the whole biological system. Specifically, when a non-native pathway for biofuel production is introduced into cyanobacterial cells, INST-MFA could be adapted to analyze the interactions between native pathways and non-native pathways, further providing insights into the nature of phototrophic metabolism (Xiong et al. 2015). Sustainable production of ethylene has been achieved in *Synechocystis* by expressing *efe* encoding an ethylene-forming enzyme from *Pseudomonas syringae pv. phaseolicola* (*Ungerer* et al. 2012). After applying INST-MFA to wild-type *Synechocystis* strain and an ethylene-producing strain, it was shown that significantly enhanced flux flowed through the modified tricarboxylic acid (TCA) cycle, resulting in efficient ethylene production (Xiong et al. 2015). These results confirm the high plasticity of cyanobacterial metabolism and prove the possibility to reprogram carbon flux through the TCA cycle for high biofuel production, even though the TCA cycle is generally considered to have limited carbon flux (You et al. 2014; Young et al. 2011).

3.3.3 *Phototrophic Microbial Consortia for Increased Biofuel Production*

Cyanobacteria have been explored as platforms for biofuels production, as well as other commodity chemicals, by the introduction of heterologous pathways which give the engineered cells the capacity for specific biofuel production. However, the resulting strains only produce biofuel with low productivity, which is not sufficient for commercialization, when compared with the model heterotrophic microorganisms, e.g., *E. coli* and *S. cerevisiae*. One of the barriers of productivity is the metabolic burden, which was difficult to resolve. Additionally, heterologous chemical production and the accumulated intermediates can lead to physiological stress to the cells (Glick 1995). To alleviate those challenges, a strategy of Division of Labor (DoL) using microbial consortia with cyanobacterium as energy provider could be applied (Dong et al. 2014) for completely carbon-neutral biofuel production.

Previously, artificial consortia have been designed to co-cultivate heterotrophic microorganisms for various applications, such as waste treatment (Fedeson et al. 2020) and production of valuable compounds (Castro et al. 2019; Weiss et al. 2017). With cyanobacteria proposed as promising candidates to produce carbohydrate feedstock to support fermentative bio-industrial processes (Hays and Ducat 2015), a novel approach for carbon-neutral biofuel production may be achieved by establishing a phototrophic microbial consortium consisting of two bio-modules:

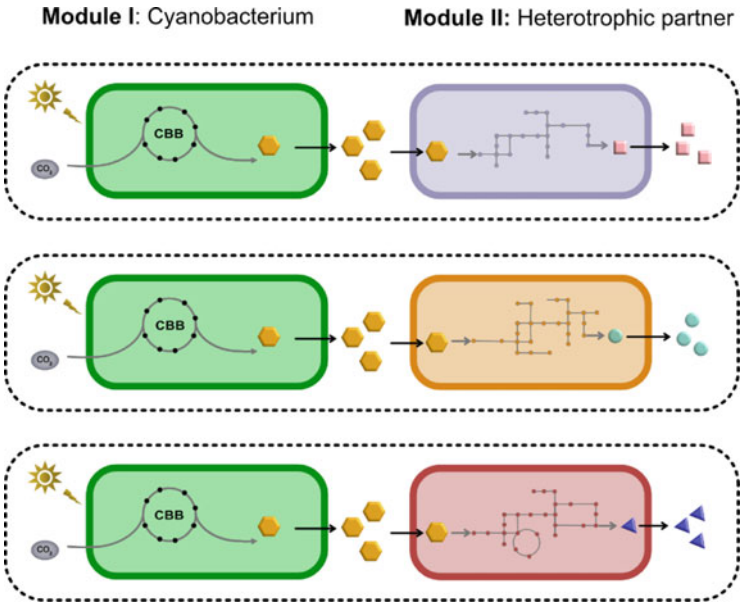

Fig. 3.2 Schematic illustration of phototrophic microbial consortia for biofuel production from CO_2 and light. CO_2 is fixed via the Calvin-Benson-Bassham (CBB) cycle to make an organic carbon source (Hexagon, Module I), which in turns secretes into the surrounding medium and is metabolized by Module II for biofuel synthesis. The swapping of Module II allows for production of various biofuels (square, circle, and triangle)

bio-module I, in which the cyanobacterial strain is responsible to perform CO_2 and light energy capture via photosynthesis and provide organic carbon, and bio-module II, where the organic carbon is used to support the co-cultured partner for biomass accumulation as well as chemical production (Fig. 3.2).

S. elongatus PCC 7942, a unicellular cyanobacterial model strain, has been engineered to synthesize and secrete sucrose by expressing the *E. coli* H+/sucrose symporter CscB (hereafter, *S. elongatus* CscB) under osmotic stress (Ducat et al. 2012). *S. elongatus* CscB was successfully co-cultivated with distinct model microorganisms (*Bacillus subtilis*, *S. cerevisiae*, and *E. coli*) and the resulting consortia can be stabilized over time and withstand perturbation (Hays et al. 2017). Furthermore, through co-cultivating *S. elongatus* CscB and *Rhodotorula glutinis*, the final biomass, and overall lipid content, was up to 40–60% higher than in a mono-culture of *S. elongatus* CscB (Li et al. 2017). The produced lipid can be further converted into, e.g., biodiesel (Wahlen et al. 2011). On the other hand, *Chlamydomonas reinhardtii*, a microalga, is able to achieve optimal lipid production under photomixotrophic conditions in the presence of acetate. Instead of adding acetate into mono-culture of *C. reinhardtii* for lipid production, co-cultivated *C. reinhardtii* and acetate-producing cyanobacterium *Synechococcus* sp. PCC 7002 could be

adopted to achieve optimal carbon-neutral lipid/biofuel production (Therien et al. 2014).

To date, there is limited literature on different kinds of biofuels successfully produced via a synthetic consortium using a cyanobacterium as the bio-module to capture CO_2 as a carbon source. Instead, most reported synthetic consortia still rely on organic carbon as an energy source (He et al. 2011; Jiang et al. 2020; Minty et al. 2013) to achieve biofuel production. Two main limiting factors are restricting the feasibility of phototrophic microbial consortia for light-driven biofuel production: low productivity and co-culture instability. Multiple approaches are promising to cope with those challenges. First, instead of co-culturing two microbial partners freely in the medium, the cyanobacterial strain may be encapsulated within alginate hydrogels (Lee and Mooney 2012). This approach was demonstrated to improve the productivity as well as stability for long-term production of PHB by a synthetic consortium of *Halomonas boliviensis* and alginate-encapsulated *S. elongatus* CscB. Second, fast-growing cyanobacterial strains were isolated and identified (Song et al. 2016), which may be employed in phototropic microbial consortia as a partner with increased photosynthetic efficiency. Conversely, heterotrophic partners could be bioengineered for better growth in the co-culture conditions (Weiss et al. 2017). Last, but not least, considering the high flexibility of synthetic consortia, the co-cultures could be programmed for new target biosynthesis pathways for various biofuel production by swapping the downstream module with high production performance (Fig. 3.2).

3.4 Biofuel Production Using Cyanobacteria

3.4.1 Hydrogen Production in Cyanobacteria

Hydrogen is an efficient energy carrier that has distinct advantages over carbon-based energy carriers. H_2 has around 3 times the energy density per mass (145 MJ kg^{-1}) as methane (55 MJ kg^{-1}) or gasoline (46 MJ kg^{-1}), and its combustion, or use in a fuel cell, yields only water. Hydrogen production via a photobiological process is a strategy that provides a possibility to generate clean energy through a carbon-neutral or even carbon-negative process. Many cyanobacterial strains have the ability to produce hydrogen naturally (Dutta et al. 2005; Savla et al. 2020). Nitrogenase and bidirectional hydrogenase (Hox-hydrogenase) are the two enzymes carrying out H_2 production in different cyanobacteria. Nitrogenase produces H_2 as a by-product during N_2 fixation (Fig. 3.3). The bidirectional Hox-hydrogenase catalyzes both H_2 production and H_2 consumption in many of both N_2-fixing and non-N_2-fixing strains. There is the second hydrogenase called uptake hydrogenase (Hup-hydrogenase) that exists in nitrogen-fixing cyanobacterial strains to recycle the H_2 produced from nitrogenase (Tamagnini et al. 2007) (Fig. 3.3).

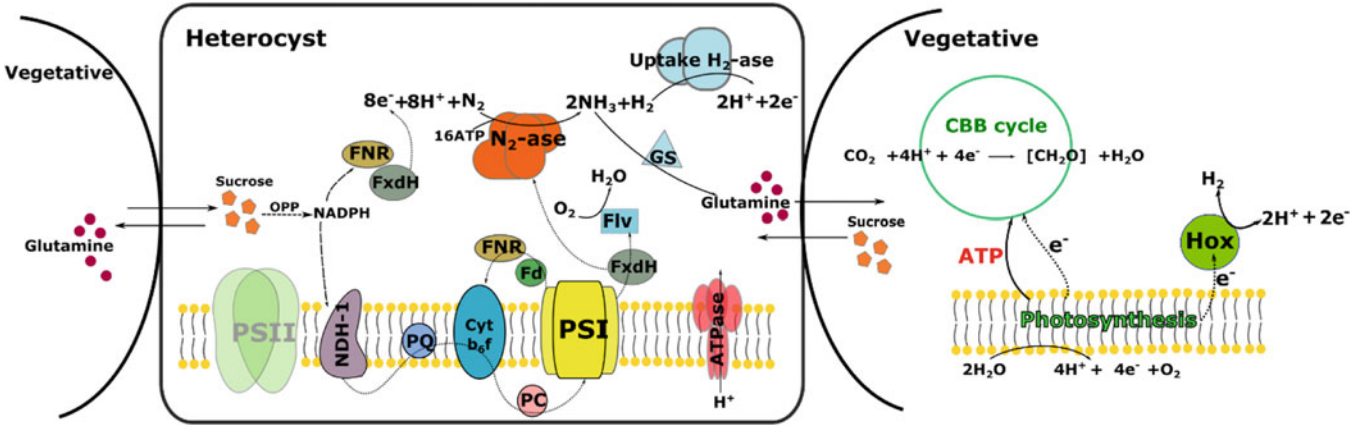

Fig. 3.3 Scheme of biochemistry and energy transport in heterocysts and vegetative cells of the filamentous cyanobacterium *Anabaena* PCC 7120. Some of the electron transport routes are proposed by different studies. Parts of the biochemistry in vegetative cells are also illustrated. Enzyme positioning in the figure does not represent their real location in heterocysts. The yellow double-layer structure is thylakoid membrane where photosynthesis and respiration apparatus are located. N_2-ase, nitrogenase; GS, glutamine synthetase; FNR, ferredoxin-$NADP^+$ reductase; FxdH, heterocyst-specific ferredoxin; Fd, ferredoxin; NDH-1, NADH dehydrogenase; PQ, plastoquinone; Cyt-b_6f, cytochrome-b6/f; PC, plastocyanin; ATPase, ATP synthase; PSI, photosystem I; PSII, photosystem II; OPP, oxidative pentose phosphate pathway

Native H_2 productivity of cyanobacteria is low, as there is no evolutionary advantage to the cell of releasing hydrogen. Limited light utilization efficiency is also an obstacle for high H_2 production in photosynthetic microorganisms since H_2 production in cyanobacteria is heavily dependent on the energy produced by the light reaction during photosynthesis. In recent decades, various strategies have been applied to enhance photosynthetic H_2 production from different cyanobacterial strains. Here we introduce some examples representing selected strategies.

Modifying Hydrogenase: The uptake hydrogenase, HupSL, is one of the significant obstacles for enhanced hydrogen production in N_2-fixing cyanobacteria since it directly recycles the H_2 produced by nitrogenase. Thus, efforts have been directed to inactivate the uptake hydrogenase for enhanced H_2 production via various approaches. Inactivating the genes encoding HupS and HupL, separately or together, in different cyanobacterial strains has resulted in a higher H_2 production rate compared to wild-type cells (Happe et al. 2000; Masukawa et al. 2002). More recently, a protein engineering approach was employed to generate a HupS mutant in *Nostoc punctiforme*, where the modification was made on one of the [FeS] clusters in HupS. The engineered HupS contributed to a light-driven H_2 production under N_2-fixing conditions (Raleiras et al. 2016). Furthermore, [FeFe]-hydrogenase from *C. reinhardtii* (CrHydA1) was heterogeneously expressed in *Synechocystis* and activated in vivo by a synthetic [2Fe] subcluster mimic complex. This novel semisynthetic activated enzyme bypassed biological maturation and regulation challenges and resulted in successful H_2 production both in light and in darkness (Wegelius et al. 2018a). In a more recent study, *Synechocystis* NiFe-hydrogenase HoxYH was fused to the 4Fe4S cluster F_B in the PsaD subunit of photosystem I (Appel et al. 2020). This fusion efficiently directs electrons from PSI to Hox and resulted in high photoautotrophic H_2 production.

Optimize Conditions for Nitrogenase Performance: Nitrogenase evolves hydrogen concomitantly with the reduction of nitrogen to ammonia. As an enzyme complex, it consumes a large number of electrons and energy in the form of ATP. A minimum of 25% of the electrons are used for hydrogen production. In addition, it is extremely sensitive to oxygen, which is one of the biggest difficulties for photosynthetic H_2 production in cyanobacteria. To overcome this obstacle, approaches aiming to remove O_2 around nitrogenase have been examined in different cyanobacterial strains. Three major approaches have been applied: (1) Remove O_2 from growth media; (2) reduce photosynthetic O_2 production; and (3) increase O_2 uptake. Sparging with an Ar-N_2-CO_2 gas mixture allows a higher hydrogen production yield (Sveshnikov et al. 1997). Oxygen generation can be reduced when photosynthesis is inhibited biologically or chemically. In addition, the inhibition on photosystem or photosynthetic electron flow can redirect more electrons to nitrogenase for H_2 production (Khetkorn et al. 2012). More recently, the heterocyst-specific flavodiiron protein Flv3B was overexpressed in *Anabaena* PCC7120 for increased O_2 reduction. In cyanobacteria, Flv proteins reduce oxygen into water using NADPH as electron donor, with Flv3B shown to be responsible for light-induced O_2 reduction in heterocysts (Ermakova et al. 2014). Overexpression of Flv3B resulted in

significantly higher H_2 production under aerobic conditions compared to wild-type cells (Roumezi et al. 2020).

Cell Immobilization: Artificial biofilm generated by entrapping cyanobacterial cells into polymer matrices is a novel technology and has been shown as an effective method to arrest cell growth to enhance energy flux toward the production of desired end products and to increase cell fitness (Kannaiyan et al. 1994; Kosourov et al. 2017; Leino et al. 2012; Vajravel et al. 2020). For instance, Ca^{2+}-alginate immobilized wild-type *Synechocystis* cells showed a significantly prolonged viability lasting for more than 30 days (Touloupakis et al. 2016), but retained a higher H_2 producing rate compared to suspended cells. An N_2-fixing filamentous strain, *Fischerella muscicola*, isolated from soils in Thailand, was immobilized by agar via entrapment method (Taikhao et al. 2013). The maximum H_2 producing yield from the agar-immobilized cells was 23-fold higher than that from cell suspension (Wutthithien et al. 2019). In another study, three different support materials were applied to immobilize a unicellular halotolerant cyanobacterial strain *Aphanothece halophytica* for H_2 production. Agar-immobilized cells showed the highest H_2 accumulation (around 160 $\mu molH_2\ g^{-1}$ DCW) after 48 hours in N_2-deprivation condition. Besides whole filamentous cultures, it has been demonstrated that isolated heterocysts also can be used to develop biofilm by using extracellular sucrose as an energy resource. Immobilized heterocysts showed at least 3 times longer nitrogenase activity compared to suspended heterocysts under the same condition (Volgusheva et al. 2019).

3.4.2 Carbon-Based Biofuel Production in Cyanobacteria

Cyanobacteria have been utilized as chassis to produce various carbon-based compounds, including ethanol, butanol, isoprene, alka(e)ne, and fatty acid. Most of the investigations toward carbon-based biofuel production in cyanobacteria focus on two model cyanobacterial strains: *Synechocystis* and *S. elongatus* PCC 7942. Below, we discuss major achievements of carbon-based biofuel production in cyanobacteria, consisting of three main categories: (1) pyruvate-derived biofuels; (2) acetyl-CoA-derived biofuels; and (3) methylerythritol 4-phosphate (MEP) pathway-based biofuels.

3.4.2.1 Pyruvate-Derived Biofuels

Pyruvate is closely positioned to the carbon fixation reactions of the Calvin-Benson-Bassham cycle and is generated from glyceraldehyde 3-phosphate (GAP). Ethanol, being used as engine fuel for decades, was first produced in *S. elongatus* PCC 7942 (Deng and Coleman 1999). Recently, systems-level analysis has been performed for further improvement of ethanol production (Kopka et al. 2017). In addition, as better biofuel candidates than ethanol, higher alcohols were successfully synthesized from

pyruvate in cyanobacteria through introducing heterologous pathways/genes, including isobutanol (Atsumi et al. 2009), 2-methyl-1-butanol (Shen and Liao 2012), 3-methyl-1-butanol (Miao et al. 2017), and 2,3-butanediol (Kanno et al. 2017). These alcohols are generated from branched-chain amino acid (BCAA) intermediates by decarboxylation and reduction.

3.4.2.2 Acetyl-CoA-Derived Biofuels

Acetyl-CoA, synthesized from pyruvate via pyruvate dehydrogenase complex (PDC) and pyruvate/ferredoxin oxidoreductase (PFOR), is used as starting metabolite to produce various biochemicals in cyanobacteria. By implementing the CoA-dependent pathway for 1-butanol synthesis, originating from *Clostridium* species, into *S. elongatus* PCC 7942, 1-butanol production was first manifested in a cyanobacterial strain (Lan and Liao 2011). With further enzymes screening, 404 mg L^{-1} of 1-butanol was produced in *S. elongatus* PCC 7942 (Lan et al. 2013). In a recent study, a 1-butanol titer of 4.8 g L^{-1} with a rate of 302 mg L^{-1} day^{-1} was achieved in *Synechocystis* by applying modular pathway engineering strategies, which is a new record of photosynthetic 1-butanol titer (Liu et al. 2019). Further analysis of the engineered 1-butanol producing strain revealed that up to 60% of fixed carbon is allocated for 1-butanol synthesis (Wichmann et al. 2021).

Fatty acids and their derivatives have the potential to be used as alternative fuels and chemicals. In nature, cyanobacteria synthesize alka(e)ne from fatty acyl-ACPs. Alka(e)ne overproduction has been explored by rationally overexpressing enzymes involved in the native pathway (Wang et al. 2013). Besides, further efforts have been invested in generating engineered cyanobacterial strains with the capability to produce fatty acyl-ACP-derived products, including free fatty acids (FFAs) (Ruffing 2014), fatty alcohols (Tan et al. 2011), and fatty acid methyl ester (Yunus et al. 2020).

3.4.2.3 MEP Pathway-Based Biofuels

Cyanobacteria are capable to synthesize terpenoids via the native MEP pathway, initiated from pyruvate and GAP. Terpenoids produced via MEP pathway are vital in maintaining the cell physiology of cyanobacteria. By introducing terpene synthases from plants, different terpenoid compounds have been produced using cyanobacteria as cell factories, including isoprene (Gao et al. 2016; Lindberg et al. 2010), squalene (Englund et al. 2014), limonene (Lin et al. 2017), and bisabolene (Davies et al. 2014; Rodrigues and Lindberg 2021), all promising substitutes of fuels due to their chemical similarity with traditional petroleum-derived fuels. It is worth noting that 1.26 g L^{-1} of isoprene was produced from CO_2 in engineered *S. elongatus* PCC 7942 (Gao et al. 2016).

3.5 Photobioreactors for Photosynthetic Production of Biofuels

Photobioreactors (PBRs) are designed for fine-tuned illuminated cultivation of phototrophic microorganisms for biomass accumulation or valuable chemical production. The developments of PBRs in various scales have been used for both fundamental research and industrial application purposes.

Large-Scale Open Pond System: Opinions differ on whether open ponds can be called PBRs. Some argue that bioreactors are cultivators where the cultivation environment is isolated from the outside environment. On the other hand, open ponds do have control on many cultivation parameters; thus, it may be counted as a PBR. The outdoor open pond cultivation system is one of the major large-scale phototrophic biomass production facilities because of its lower economical cost and simpler design compared to many closed PBR systems.

However, open ponds have many drawbacks which limit their utilization possibilities. (1) Contamination is a serious obstacle during cultivation since it is not possible to perform complete sterilization in open ponds. Using cultivation conditions, for example, temperature or pH, to kill contaminants is not always efficient, and the feasibility heavily depends on the tolerance of cultivated strains. (2) From the products' point of view, many of the new biofuel compounds are not suitable to be produced in open ponds due to their volatility. Open ponds are usually used for producing chemicals that do not excrete out of the cells, e.g., lipids and polysaccharides. (3) Due to the direct gas exchange with the surrounding environment, open pond cultivation may demand a large amount of energy input to maintain a suitable temperature for optimal cell growth. Furthermore, more water is required in the open ponds due to the significant evaporation level. Cultures need to be diluted frequently for maintaining correct nutrients concentration and for minimizing self-shading in thick cultures. (4) Open ponds can only be placed horizontally which occupies much land and cause considerable self-shading in the culture. No matter what kind of materials are used to construct the open ponds, the major part of incident light comes from the top, which may cause significantly increased non-photochemical quenching and photoinhibition in the cells. At the same time, cells close to the bottom of the ponds are in the shadow of the cells above them and thus do not get enough light to support optimal growth. (5) In addition, cultivating cyanobacteria, especially engineered cyanobacterial strains, in open ponds is not always allowed due to the potential contamination threat to the surrounding environment.

Large-Scale Closed PBR System: To overcome most of the obstacles of open pond cultivation, strong interest in the design and construction of closed PBRs has been shown from both research and industry in recent decades. Different types of closed PBRs have been developed, e.g., flat-panel PBRs, bubble column PBRs, vertical tubular PBRs, and helical PBRs. Each type of PBR shows specific characteristics and advantages, but several core principles are shared no matter which type of closed PBR is designed (Pruvost et al. 2016).

Among all the cultivation parameters, light supply in large-scale PBRs is the most important and the hardest factor to be fine controlled. A well-designed PBR should

provide suitable light intensity for each cell to support efficient growth and product formation. Materials used to construct PBRs, e.g., glass, PVC, polyethylene, or plexiglass, are generally with a transmission rate between 70% and 99% for the light within the photosynthetically active radiation (PAR) range (Johnson et al. 2018). The shape of the PBRs also matters for the light supply, for instance, flat-panel and helical PBRs have high surface-to-volume ratio which contributes to higher energy transfer rate and productivity (Marsullo et al. 2015). Moreover, the positioning of PBRs can significantly affect photosynthetic efficiency (Posten 2009). For example, a vertically positioned flat-panel PBR showed 1.5-fold biomass production per mol of PAR photons supplied compared to a horizontal positioned PBR since less light energy was wasted in the form of heat or non-photochemical quenching in the vertical panels (Cuaresma et al. 2011). Due to the low occupation of land per PBR, vertical positioning can also result in higher areal productivity. Nevertheless, more optimization of size, distance, and orientation of PBRs is needed based on the cultivation location and the cultivated strain (Slegers et al. 2011).

In large-scale PBRs, a significant amount of freshwater is used, which may put more pressure on the already strained clean water utilization plan. In addition, the supplementation of various nutrients indirectly results in pollution and extra energy consumption (Chowdhary and Raj 2020). Large consumption of both water and nutrient also causes the high economic cost to operate PBRs. Therefore, efforts are being carried out to utilize different water sources, such as wastewater or industrial water, to cultivate photosynthetic microorganisms in PBRs (Arias et al. 2017). It is important but difficult to select cyanobacterial strains with high tolerance to the wastewater environment. Not all the nutrients and heavy metals are needed, or needed in such a high concentration, for the growth of cyanobacteria. Thus, some sort of filtration or dilution system may be attached to PBRs for medium optimization. Recently, a novel attached growth PBR was designed for lipid production from the filamentous cyanobacterial strain *Oscillatoria* grown in synthetic wastewater (Economou et al. 2015). This is a good example of a cost-effective system to achieve biofuel production and wastewater treatment at the same time.

Laboratory-Scale PBRs: Different from large-scale outdoor PBRs, the general aim of utilizing lab-scale fined-tuned LED illuminated PBRs is to carry out fundamental research and strain development. Among the four different cultivation methods, batch, fed-batch, chemostat, and turbidstate, the two continuous cultivation techniques have been preferred in cyanobacterial physiology and metabolism studies (Melnicki et al. 2013). Similar principles are shared with large-scale PBRs design, and light supply is still the most important factor. However, the light source for lab-scale PBRs is generally LED tubes or panels, which can be fined-tuned from the light spectrum to light intensity. This advantage provides more opportunities to cultivate photosynthetic microorganisms in specific precise light conditions (Nedbal et al. 2008). Continuous cultivation is an essential cultivating process for deep investigation of growth and biofuel productivity of engineered cyanobacterial strains. It overcomes the obstacles on light transmission efficiency, evaporation, and self-shading during batch cultivation. It also can provide detailed online information about the correlation among photosynthesis, growth, and product formation.

All the knowledge can guide further metabolic engineering design for enhanced productivity. However, benchtop PBRs have limitations in throughput, and the cleaning, assembling, and sterilizing processes are time-consuming. A novel technique, called microfluidic photobioreactor technology, has been developed for high-throughput screening and evaluation. This technology provides unprecedented single-cell experimental resolution and economically friendly high-throughput workflow; details were well-reviewed (Yang and Wang 2016). Therefore, it will be a handy tool to assist future metabolism modeling and metabolic engineering work.

3.6 Conclusion

Significant progress has been made in the metabolic engineering of cyanobacteria as biocatalysts for biochemical production, including biofuels. With the rapid development of synthetic biology, an increasing number of genetic tools are available for application in designing cyanobacterial cell factories through optimizing native pathways and introducing heterologous pathways. Applying systems biology approaches, including GSMM, metabolomics, and fluxomics, it is possible to get a comprehensive understanding of cyanobacterial metabolism and monitor the whole metabolic network within a cell, offering more insights on further improvement of biofuel productivity in cyanobacteria. However, with many proof-of-concept studies performed, there is very limited success in commercializing cyanobacteria cell factories on an industrial scale. The biggest factor impeding commercialization lies in the low titers and low productivities. To attain industrial-level biofuel productivity in cyanobacterial chassis, further improvements are needed: enhancement of photosynthetic efficiency, optimization of large-scale cultivation, and increased cellular resistance toward target products. As more and more fast-growing cyanobacterial strains are being identified and characterized, and with further development of the available tools for metabolic engineering, biofuel production from cyanobacteria will gain more attention in the near future and have greater potential in addressing the global energy and climate change issues.

Acknowledgments This work was supported by the Swedish Energy Agency (project numbers P46607-1 and 38334-3), the NordForskNCoE program "NordAqua" (project number 82845), and the Svenska Forskningsrådet Formas (project number 2016-01325).

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Chapter 4
Role of Enzymes in Biofuel Production: Recent Developments and Challenges

Arvind Bangaru, Kamasani Aarya Sree, Chandana Kruthiventi, Meenakshi Banala, Vadapalli Shreya, Y. Vineetha, A. Shalini, Bishwambhar Mishra, Rajasri Yadavalli, K. Chandrasekhar, and C. Nagendranatha Reddy

Abstract The increasing interest in the production of renewable and clean fuel has led to various cost-effective and efficient strategies with minimal impact on the environment. One such strategy of producing biofuels using enzyme-mediated catalysis has gained much attention globally. This chapter aims at improving the overall yield in a less energy-intensive and more environmentally friendly way compared to its production by conventional processes. The production of various clean fuels, various enzymes used so far for biohydrogen and biodiesel production, the significance of immobilization and improving the biofuel efficiency by identifying novel enzymes through metagenomic approach and enhancing the enzyme/metabolite production, and various obstacles faced and future perspectives have been elaborated in this chapter.

Keywords Enzymes · Biofuel · Novel enzymes · Biohydrogen · Biodiesel · Immobilization

Arvind Bangaru, Kamasani Aarya Sree and Chandana Kruthiventi contributed equally with all other contributors.

A. Bangaru · K. A. Sree · C. Kruthiventi · M. Banala · V. Shreya · Y. Vineetha · A. Shalini · B. Mishra · R. Yadavalli · C. N. Reddy (✉)
Department of Biotechnology, Chaitanya Bharathi Institute of Technology, Hyderabad, Telangana, India
e-mail: nagendranath_biotech@cbit.ac.in

K. Chandrasekhar
Green Processing, Bioremediation and Alternative Energies Research Group, Faculty of Environment and Labour Safety, Ton Duc Thang University, Ho Chi Minh City, Vietnam

P. Chowdhary et al. (eds.), *Bio-Clean Energy Technologies: Volume 1*, Clean Energy Production Technologies, https://doi.org/10.1007/978-981-16-8090-8_4

4.1 Introduction

Biofuels are considered renewable and preferred fuels of the future as they are derived from biomass. Agriculture, plants, animal wastes, and microorganisms act as a source of biological materials which undergo anaerobic digestion and help in the formation of biofuels. Organic components which act as a substitute source of energy are known as biomass and are of three classes, namely, solid, liquid, and gaseous. Due to the formation of the tremendous amount of energy, large prosperity, and low price of solid and liquid biofuels, they are widely used for energy inception (Datta et al. 2019). Biofuels that are produced from biomass, viz., bioethanol, biohydrogen, biomethane, biodiesel, bioelectricity, etc., are carbon neutral and sustainable. Biofuels are categorized into first, second, third, and fourth generation biofuels. The third and fourth generation biofuels are considered advantageous due to their inherent characteristics of higher biomass production with higher lipid content, high manufacture rate, more capability to capture CO_2, less space and time requirement, etc. when compared to other generations.

Biofuel production can be performed through different methods, viz., thermochemical, mechanical, physical, enzymatic, and microemulsion processes. Of these processes, the enzymatic method is considered to be a safe, clean, easy, cost-effective, and environment-friendly technique. The enzymatic methods are gaining universal acceptance and are aimed at overcoming the limitations associated with conventional chemical catalysts, improving the yield and efficiency, reducing negative environmental impacts, and enhancing the quality of the produced biofuels; repeated use of the enzyme is essential from the economic point of view. For instance, the utility cost of enzymatic hydrolysis is much lower compared to the alternative methods of acidic hydrolysis because it is carried out in mild conditions and does not require subsequent treatment steps. Various types of enzymes are used for the production of different types of biofuels, but we will mainly focus on biodiesel and biohydrogen as they are gaining global acceptance and are mostly used in most industries. These enzymatic systems allow continuous separation of products and prevent enzyme inhibition during the process. Research attention is also focused on genetic engineering in enzyme production. Recently, genes of various enzymes have successfully been cloned, and more genes are promised to be cloned rapidly in the coming years. The use of recombinant DNA technology to produce large quantities of recombinant enzymes will help lower enzyme costs. In addition, protein engineering will help create novel enzyme proteins that are more resistant and highly thermostable. The introduction of a new generation of cheap enzymes, with enhanced activities and resilience, should change the economic balance in favour of enzyme use.

4.2 Role of Enzymes in Biofuel Production

4.2.1 Biodiesel

Biodiesel is a biodegradable, non-toxic, and renewable fuel which is recognized as a potential alternative to current renewable sources of fuel. Biodiesel is the mono-alkyl esters of long-chain fatty acids produced using animal fat, plant-derived or vegetable oil, microbial oil, and other sources of oil.

Vegetable oil can be blended with conventional diesel and can be used for a shorter period of usage. But these ester blends are impractical in long-term use and are unsatisfactory because of their high viscosity, gum formation due to free fatty acid oxidation. Hence, the vegetable oils must be processed before use in automobiles as a fuel source. Biodiesel can be produced by different processing methods such as pyrolysis, using microemulsions, and transesterification (Maria Manuela Camino Feltes et al. 2011). Transesterification is the most popular method for biodiesel production. Transesterification results in the conversion of triglyceride esters into a mixture of glycerol and mono-alkyl esters. The mono-alkyl ester is biodiesel. The catalyst required in the transesterification process can be an acid catalyst, alkali catalyst, alcohol in a supercritical state, or a biocatalyst. Using a biocatalyst for biodiesel production gives purity and minimal downstream operations (Ranganathan et al. 2008). The biodiesel production using lipase was patented by Haas (1997).

4.2.1.1 Lipase

The triglyceride transesterification from waste oil by lipases is considered to be the most efficient process so far. They catalyse the breakdown of carboxylic ester linkages in triacylglycerol to form monoglycerides, diglycerides, glycerol, and free fatty acids. Lipases can be intracellular or extracellular. They can be immobilized and used to catalyse the transesterification reaction for better efficiency. The lipases commercially used are mostly of microbial origin. Lipases from different sources are known to exhibit different properties along with the difference in catalytic efficacy. Lipases from *Candida antarctica* B and *Pseudomonas* sp. are the most researched enzyme in biodiesel production (Nevena et al. 2011). Upon immobilization, lipase can be recovered and reutilized for biodiesel production. Several alcohols such as butanol, ethanol, and methanol are used along with the catalyst as acyl acceptors. Higher conversion rates were obtained by using methanol as an acyl acceptor by minimizing its inhibitory effects by using an observed by using common solvent for both oil and method. Many researchers found this effective because insoluble methanol is the cause of inhibition. The highest rate of conversion was obtained by adding methanol stepwise to oil which ensures that the concentration of methanol in the medium is always less thereby eliminating enzyme deactivation (Ranganathan et al. 2008). *Rhizopus oryzae*, *Aspergillus niger*, *Streptomyces* sp., *Candida*

antarctica, *Chromobacterium viscosum*, *Candida rugosa*, *Mucor miehei*, *Pseudomonas* sp., *Photobacterium lipolyticum*, and *Thermomyces lanuginosus* are the most used organisms for lipase in biodiesel production (Sevil et al. 2012).

4.2.2 Biohydrogen

Biohydrogen, a clean fuel high in energy, is also a potential alternative to the non-renewable source of fuels. Conventionally, electrolysis of water or methane reformation is used for hydrogen production. One of the most promising approaches for the generation of renewable energy is the culture of photosynthetic microorganisms for producing molecular hydrogen. It is less polluting and is carbon dioxide neutral. The most known methods of producing biohydrogen from renewable resources are direct or indirect biophotolysis and photo- and dark fermentation. Many facultative and obligate anaerobic bacteria are used for dark fermentative hydrogen production. In direct biophotolysis, microalgae are grown in anaerobic conditions. During the photosynthetic phase, carbon dioxide is fixed in hydrogen-rich substrates due to the light-mediated generation of molecular hydrogen. The second phase involves the incubation of microalgae in sulphur-free media (Levin 2004; Savla et al. 2020). Photofermentation is anaerobic photosynthesis carried out by non-sulphur purple bacteria. In biohydrogen production, the light is absorbed, and electrons are transferred to enzymes – hydrogenases and nitrogenases. For biohydrogen production, potential feedstock includes agricultural waste, biomass, lignocellulosic biomass, sewage sludge, livestock effluents, waste from food processing, etc. (Show et al. 2012; Khan et al. 2020, 2021).

Enzymes hydrogenase and nitrogenase play an important role in hydrogen production. Nitrogenase is involved in the production of hydrogen under nitrogen-deficient conditions, while hydrogenase oxidizes hydrogen to recycle protons, electrons, and ATP for the metabolism of energy.

4.2.2.1 Hydrogenase

Enzyme hydrogenase is used in biohydrogen production. It is inhibited in the presence of oxygen. The enzyme bidirectional hydrogenase catalyses the hydrogen production reaction (Rodionova et al. 2016). Based on their metal content, hydrogenase are classified into three different categories – (Fe) hydrogenases, (Ni-Fe) hydrogenases, and (FeS) cluster-free hydrogenase which are metal-free hydrogenases (Bhari and Singh 2017).

In photolysis, oxygen is considered a serious problem that inhibits hydrogenase. The best-known approach so far is to isolate oxygen-resistant hydrogenase from nature and re-engineer it. Though several studies have been conducted, the expression of hydrogenase in microalgae is still a significant challenge due to their complex structure (Oh et al. 2011).

4.3 Enzymes in Biofuel Production

Production processes of biofuel can be broadly categorized as a chemical process or a biochemical process. While the chemical process uses chemical catalysts and heat, a biochemical process involves the usage of biological catalysts (enzymes) to convert raw material into fuel. The usage of enzymes can overcome the disadvantages associated with the usage of conventional chemical catalysts of being energy-intensive, environmentally friendly, etc. (Sulaiman et al. 2011).

4.3.1 Amylases

The first generation of biofuel production involved the treatment with α-amylases for hydrolysis of starch molecules at α-1,4 linkages to produce the short chains consisting of dextrins. These short chains are further hydrolysed to produce maltose, glucose, and isomaltose by glucoamylases which can then be easily fermented to butanol or ethanol (Lee et al. 2007; Visioli et al. 2014). Most widely used bacterial species for amylase production is *Bacillus*, while fungal source includes *Penicillium* and *Aspergillus* (Ajita et al. 2014).

4.3.2 Lignocellulosic Biomass

The second generation of biofuel production involved lignocellulosic biomass as the raw material. It is a high potential raw material for biomass production. It consists of lignin, hemicellulose, and cellulose in the ratio 3:3:4. These are initially pre-treated and then broken down by enzymatic hydrolysis by saccharification enzymes to simpler sugars which are then converted to the desired chemical product such as bioethanol by fermentation (Linde et al. 2007).

4.3.2.1 Cellulase

Cellulose is a linear polysaccharide consisting of repeating units of glucose bound together by β-1,4-glycosidic linkages. Around 15–45 repeating units of glucan make up the cellulose. The glucan units are arranged into fibrils which form microfibrils that give cellulose an alternate crystalline and amorphous structure. Due to this structure, the surface area is increased thereby protecting it from degradation (Juturu and Wu 2014).

Cellulase is a widely used industrial enzyme. Cellulase breaks down complex sugar molecules of cellulose to simpler sugars of glucose. Several species of fungi are known to produce cellulase. Commercially microorganisms such as *Aspergillus*

niger and *Trichoderma reesei* are used for the production of cellulase (Srivastava et al. 2015a, 2020a). The cellulase enzyme system consists of three enzymes (Jahirul et al. 2012):

(a) endoglucanases
(b) exoglucanases,
(c) β-glucosidases

Cellulase has low enzyme stability and is difficult to reuse. Various approaches such as protein engineering and immobilization are being employed to improve enzyme stability (Khoshnevisana et al. 2017). The conversion of cellulose to sugar is an important cost-determining step in biofuel production (Garvey et al. 2013).

4.3.2.1.1 Endoglucanase

Endoglucanase also known as carboxymethyl cellulase (CMCase) breaks the internal bonds and thereby randomly introducing a nick in amorphous chains of cellulose which leads to the conversion of lignocelluloses into monomeric sugars (Narra et al. 2014). Due to their lower energy intake and higher specificity, they are considered inexpensive (Daniel et al. 2012). Commercial endoglucanase is produced using fungal strains such as *Aspergillus* and *Trichoderma* along with other bacterial strains such as *Streptomyces* sp. and *Bacillus* sp. They belong to the glycosyl hydrolases family. Endoglucanases are glucoside hydrolases and have been separated into families of GH5, GH6, GH7, GH9, GH12, GH44, GH45, GH48, GH51, GH74, and GH124. Endoglucanases have two mechanisms of action for hydrolysis of cellulose – retention and inversion.

4.3.2.1.2 Cellobiohydrolase

Cellobiohydrolase (CBH) also known as exoglucanase acts synergistically with endoglucanase and β-glucosidase to hydrolyse cellulose. Cellobiohydrolase is an exocellulase enzyme. It degrades the cellulose molecules by breaking down 1,4-β-D-glycosidic bonds from the terminals of cellulose. There are two types of cellobiohydrolases – CBH1and CBH2. The CBH1 moves along the reducing end of cellulose, while CBH2 moves along the non-reducing end of cellulose, thereby cleaving the cellobiose (Wang et al. 2012a). Cellulose hydrolysis into cellobiose is considered to limit the overall rate of cellulosic degradation. This involves the cooperation between endoglucanase and cellobiohydrolase (exo-endo synergism) along with the cooperation between CBH1 and CBH2 (exo-exo synergism). This facilitates the removal of the residue of cellobiosyl molecules from both the non-reducing and reducing ends from cellulose (Liu et al. 2011a, b). Commercially CBH is produced using fungal species such as *Trichoderma reesei*, *Penicillium* sp., and *Polyporus* sp. (Fang and Xia 2013) and bacterial strains including *Flavobacterium* sp. and *Paenibacillus* sp. (Islam and Roy 2019). CBH increases

the substrate proximity to glycosyl hydrolase proximity. This enhances the accessibility while modifying the cellulose surface crystals (Sánchez et al. 2003).

4.3.2.1.3 β-Glucosidases

While the endoglucanase is responsible for exposing the non-reducing and reducing ends of cellulose polysaccharide chains by introducing nicks, the exoglucanase acts on these exposed chains and liberates the cellobiose and glucose. The most significant and final step in the saccharification of cellulose to simpler sugars is played by β-glucosidases (BGL). BGL completes the process by converting cellobiose produced by exoglucanase into final products, namely, glucose (Horn et al. 2012). It maintains the overall hydrolysis rate of cellulase as the cellobiose causes the inhibition of exoglucanase and endoglucanase. The feedback inhibition of BGL by glucose is making it a rate-limiting enzyme. Hence, an efficient BGL that is tolerant to the high concentrations of glucose is needed for producing commercially viable processes for the production of biofuel (De Andrades et al. 2019). Well-documented microorganisms for the production of BGL are *Aspergillus* sp. and *Penicillium* sp. and some bacterial species of *Bacillus*. Though BGL is produced by bacteria, fungi, animals, and plants, at the commercial level, bacteria are preferred due to their rapid growth, ease of handling, and engineering (Amore et al. 2013). Due to the lower production and higher cost, a greater level of production of BGL is needed for the biofuel industry. Immobilization of BGL increases the hydrolysis rate (Muhammad et al. 2016). This approach is expected to cut the cost while also saving time on biofuel production.

4.3.2.2 Xylanases

Hemicellulose is a branched heteropolymer containing hexose and pentose sugar molecules. Xylan is the most abundant type of hemicellulose containing β-D-xylopyranosyl residues with β-1,4-glycosidic linkages (Beg et al. 2001). Cellulose, xylan, and lignin are bonded together by noncovalent and covalent bonds and form the cell wall. It is present at the interface of cellulose and lignin, a position significant for maintaining cell wall integrity. The enzymes that break down xylan – xylanase – belong to the glycoside hydrolase (GH) family. Several xylanases act synergistically on xylan to completely hydrolyse the xylan backbone. To completely hydrolyse the xylan backbone, the side chains must be cleaved through the glycosidic bonds between xylosyl units that are not necessarily cleaved (Table 4.1).

Xylanases increase the saccharification of lignocellulosic wastes including hemicelluloses (xylan), which results in more sugar available for fermentation, therefore, enhancing the productivity of biofuel (Sakthiselvan et al. 2015). For commercial use, bacterial as well as fungal species are used, such as *Trichoderma* sp., *Aspergillus* sp., *Bacillus* sp., and *Streptomyces* sp.; *Aspergillus terreus* is a potential organism for

Table 4.1 Several xylanases and their action

Enzyme	Action on xylan backbone
β-1,4- endoxylanase (xylanase)	Hydrolyzesglycosidic bonds
arabinofuranosidase	Hydrolyzes arabinose side chains
xylan esterase	Removes of acetate group
α-glucuronidase	Removes the glucuronic acid side chains of xylosyl units
β-xylosidase	Hydrolyzexylobiose to xylose

Srivastava et al. (2020a, b, c, d, e, f)

production of xylanases. Currently, *Humicola insolens* is being used by various companies for the production of xylanases (Srivastava et al. 2020a, b, c, d, e, f).

4.3.2.3 Laccase

After cellulose, lignin is the second most abundant naturally existing polymer. It is the only renewable aromatic feedstock. It contributes significantly to provide structure to plant cell walls (Jouanin and Lapierre 2012). It is a structurally diverse heteropolymer. It is a complex aromatic polymer with phenylpropanoid aryl-C3 units bound together by CC and CO bonds (Bugg et al. 2011). In the production of biofuel, the removal of lignin from lignocellulosic biomass is a great challenge due to the chemically sturdy structure. Hence, pre-treating the lignin is necessary.

The enzymes required for the complete degradation of lignin include ligninase which contains three different enzymes – manganese peroxidase, laccase, and lignin peroxidase (Wang et al. 2017a). Laccase is well studied for the degradation of lignin of all three enzymes. It belongs to the family of copper-containing oxidases called multicopper oxidase (MCO). During the natural course of evolution, the white-rot fungi have come to express laccase abundantly due to their improved expression system of the laccase gene family which is involved in lignin metabolism (Villalba et al. 2010). Many genres of fungus such as basidiomycetes, deuteromycetes, and ascomycetes produce laccase. Some of the laccase-producing bacteria are *Marinomonas mediterranea*, *Streptomyces lavendulae*, *Bacillus subtilis*, *Geobacillus thermocatenulatus*, and *Aquisalibacillus elongatus* (Srivastava et al. 2020a, b, c, d, e, f).

For biofuel production from lignocellulosic biomass, the polysaccharides must be completely hydrolysed. This can be achieved through the pre-treatment of biomass. Laccase is used as a potential agent for the removal of lignin.

4.3.2.4 Pectinases

Pectin is a polymer containing rhamnogalacturonan molecules in the backbone chain which is connected to other polymers and carbohydrates. It is a linear polymer of D-α-(1,4) anhydro-galacturonic acid (Munarin et al. 2013). Pectinases hydrolyse

pectin. Polygalacturonases (PG) hydrolyse the glycosidic bands beside the free carboxyl group. Pectinase lyase (PL) hydrolyses using the process of β elimination. Both enzymes randomly divide the pectin chain. Fungal strain *Aspergillus* sp. and *Penicillium* sp. produce PG (Patil and Chaudhari 2010). *Bacillus* sp. are known to produce pectinase. In biofuel production, pectinases play a role in the alteration of plant cell walls to bioethanol. In cases where lignin is at lower levels, pectinases help in the degradation of enzymes (Srivastava et al. 2020a, b, c, d, e, f). Novel extraction techniques are required to bring the cost of pectinases which helps in the economical production of biofuel.

4.3.2.5 Proteases

The enzyme used to break down the protein present in the biomass used for biofuel production is protease by proteolysis. They can be exopeptidases that separate the amino acids from carbon and nitrogen terminus or endopeptidases that break the internal bonds in polypeptide chains (Domsalla and Melzig 2008). Proteases are produced by a wide range of microorganisms of fungi, yeast, and bacteria, such as *Aspergillus oryzae*, *Bacillus subtilis*, and *Penicillium roqueforti*, to name a few (Kasana 2010). In biofuel production, proteases enhance the microorganism efficiency of converting sugars to biofuels by making nitrogen available to them (Paritosh et al. 2017) and higher alcohols liberated from branched-chain 2-keto acid for biofuel conversion (Huo et al. 2011) (Table 4.2).

4.4 Applications of Enzymes in Biodiesel and Biohydrogen Production

Due to the advancement in technologies, economic growth, and the increase in the world's population, the world is facing serious energy problems. Regular sources of energy are scarce and sometimes will be unavailable, and many harmful gases are also generated by the atmosphere, so an alternative that is less environmentally destructive and sustainable is required. Therefore, the progression usage of less harmful biofuel substitutes like biogas, biodiesel, biohydrogen, and bioethanol is required to lessen the effects. Microorganisms are being used for different applications, and enzymes produced by them are now studied for biodiesel and biohydrogen production. As the production of biodiesel from enzyme catalysts is a green approach, many researchers are attracted towards its study and development. Abundantly used commercial enzymes for the production of biofuel are cellulases, lipases, and proteases (Neha et al. 2018; Chowdhary et al. 2020; Chowdhary and Raj 2020). The biomass is converted to biofuel which involves various biochemical steps and reactions. Pre-treatment of biomass is done in the presence of enzymes before the production process. Enzymatic hydrolysis is economical and gives more yield than

Table 4.2 Enzymes in biofuel production

Name of enzyme	Commercial function	Source	References
Amylases	Hydrolysis of starch molecules to fermentable sugars	*Bacillus*, *Penicillium*, *aspergillus*	Ajita et al. (2014)
Cellulase	Breaks down complex molecules of lignocellulose to simple sugars	*Aspergillus Niger*, *Trichoderma reesei*	Sadhu and Maiti (2013), Agrawal Shweta (2014)
Endoglucanases	Lignocellulose to monomer sugars	*Aspergillus Niger*, *Trichoderma reesei Streptomyces*, *bacillus*	Srivastava et al. (2020a, b, c, d, e, f), Ariffin et al. (2008)
Exoglucanases	Breaks down cellulose molecules by degrading 1,4-β-D-glycosidic bonds resulting in cellobiose	*Trichoderma reesei*, *Penicillium*, *Flavobacterium*	Srivastava et al. (2020a, b, c, d, e, f)
β-Glucosidases	Converts cellobiose to glucose	*Aspergillus*, *Penicillium*, *bacillus*	Zhao et al. (2018)
Xylanases	Hydrolysis of hemicellulose into simple sugar xylose and oligoxylosaccharides	*Trichoderma*, *aspergillus*, *bacillus*, *Streptomyces*, *Humicola insolens*	Sagarika Garg (2016)
Laccase	Degradation of lignin	*Marinomonas mediterranea*, *Geobacillus thermocatenulatus*, *Aquisalibacillus elongatus*, *Streptomyces* sp., *bacillus* sp.,	Srivastava et al. (2020a, b, c, d, e, f)
Pectinases	Hydrolyses pectin	*Aspergillus* sp., *Penicillium* sp., *bacillus* sp.	Patil and Chaudhari (2010)
Proteases	Breaks down the protein present in biomass	*Aspergillus oryzae*, *Bacillus subtilis*, *Penicillium roqueforti*	Kasana (2010)
Lipases	Transesterification of triglycerides to produce biodiesel	*Candida antarctica* B, *pseudomonas* sp.,	Nevena et al. (2011)
Hydrogenases	Biohydrogen production; light is absorbed, and electrons are transferred to hydrogenase to produce biohydrogen	*Escherichia coli Clostridium*, *Desulfovibrio*, *Thermotoga*	Satenik et al. (2017), Morra et al. (2017)

acid hydrolysis. It is performed by a set of enzymes called cellulases; it is further divided as endoglucanase, which affects low crystallinity regions in cellulose, creating free chain ends to break polymer chains, exoglucanases that hydrolysate disaccharide glycosidic bonds, and β-glucosidase (Neha et al. 2020a, b). Microbial lipolytic enzymes have received attention for their ability to catalyse ester bond-containing compound biotransformation reactions such as waste conversion into

high-energy products, such as biofuel and other value-added products using energy-efficient pathways. The overall biofuel processing method can be made more versatile by thermophilic or thermostable enzymes derived from thermophilic or thermotolerant fungi. (Neha et al. 2020a, b).

4.4.1 Suspended Vs. Immobilized

The biological production of hydrogen (H_2) is an eco-friendly process. Photosynthetic or dark-fermentative pathways have been widely studied to produce H_2. In one of the case studies, yields up to 3.8 mol H_2/mol glucose have been noticed to be produced under the photo-fermentative route by bacteria like *Rhodopseudomonas* species and dark-fermentative conditions by *Caldicellulosiruptor*, *Enterobacter*, and *Thermotoga* species (Patel and Kalia 2012). This combination resulted in a twofold increase in H_2 yield. The efficiency of the whole process has to be increased to yield 12 mol H_2/mol glucose (Patel and Kalia 2012). To increase the efficiency of the production process, the metabolic activities of bacteria have been complimented for biowaste as feed. Further usage of intermediates for abovementioned two processes polyhydroxyalkanoate and methane production is likely to increase the feasibility of meeting the ever-increasing energy demand (Patel and Kalia 2012).

Enzymes supported on nanomaterials have led to a new path to the research of biofuel production. Lipase *Rhizopus oryzae* with immobilization has exhibited high stability for industrial application. The immobilization technique improves acidic alkaline, and that affects reusability and conditions stability (Song et al. 2012). Calcium phosphate mineralized chitosan beads' anti-swelling characteristics have been significantly enhanced and have improved storage capacity and heat stability (Han et al). The chitosan-based and agarose beads' structures to immobilize β-galactosidase promoted batch catalysis of the hydrolysis of whole milk lactose (Vieira et al. 2013). Immobilized β-galactosidase was highly efficient for hydrolysis of lactose exceeding 94 percent. *Saccharomyces cerevisiae* cells on a magnetite chitosan complex and nanomaterials for biohydrogen processing have been immobilized. Cellulose-coated magnetic nanoparticles are used to enhance ethanol production and found that the kinetics of ethanol synthesis relied on each type of carrier (Ivanova et al. 2011). Researchers tested a green lipase immobilizing support material (*Pseudomonas cepacia*) that was based on chitosan and polyvinyl alcohol in the carrier. They discovered that their new support material had dramatically improved lipase catalytic efficiency and could also be readily processed in future industry sectors where biological processes for their biokinetics could be enhanced (Badgujar and Bhanage 2014). To research the enzymatic efficiency of immobilized lipase, the researchers have examined Na-alginate-chitosan and Ca-alginate-chitosan and then concluded on heterogeneity in enzymatic behaviour about synthetic conditions for the preparation of enzyme carriers (Liu et al. 2010). In recent studies, chitosan nanoparticles with magnetic core shell have been prepared to immobilize lipase and have also observed a significant improvement in lipase stability and

overall successful enzymatic behaviour when immobilized when doing so (Ghadi et al. 2015). When immobilized on a chitosan-based carrier attached to polyvinyl chloride beads and additional bead carriers made from a combination of chitosan and polyvinylpyrrolidone, the team demonstrated an improvement in thermal stability of the fenugreek β-amylase enzyme. They demonstrated the green aspects of novel immobilization service products under which they are not harmful, reusable, and readily adaptable to complex immobilized matrices (Srivastava et al. 2015c). There are several other advantages and benefits of using immobilized enzymes.

4.4.2 Nanomaterial Immobilized Enzymes

Important advances in the field of accelerated nanotechnology have expanded their future uses for enhancing biological processes. The availability of nano-structured materials, nanoparticles, which have sufficient chemical and physical properties, has especially helped this (Patel and Kalia 2012). The rapid improvement of nanomaterials has created a new research path for enhancing the efficiency of the biohydrogen process by stimulating the bioactivity of microorganisms because of their unique physio-chemical properties (Lin et al. 2016). There are several advantages of nanomaterial such as their excellent biodegradability; customizing nature; ease of separation; low cytotoxicity to biomass cell; ease of synthesis; maintaining stability after mechanical, chemical, and physical modifications; and ability to bind multiple targets, to name a few (Lamberti et al. 2014). Overall, the nanoparticles studied for bioenergy processing, the magnetic nanoparticles, are most commonly used due to their fast recoverability. Enzymes used in the generation of biodiesel or bioethanol can be immobilized as a carrier using magnetic nanoparticles. During the methanogenesis process, high coercivity and great paramagnetic properties of magnetic nanoparticles also make them useful for biogas processing. The very first approach to biohydrogen development would be to convert the condition of the "powdered nanoparticles" into a "tubular, concentrated, mixed or sheeted arrangement of incorporated inert support materials" using nanoparticles. Briefly, these nanoparticles might be stimulating biohydrogen production by their surface and quantum size effect. The thermal stability of the enzymes can be significantly enhanced by the immobilization of cellulase into nano-matrix (Sivagurunathan et al. 2018).

There are several technological advancements for biohydrogen production processes, like biophotolysis, photo-fermentation (PF), biophotolysis, dark fermentation (DF), microbial electrolysis (MEC) based on H_2 production processes, and photo dark coupled fermentation. The major problem of the low yield of the product was due to the insufficient conversion of substrates which was addressed by nanotechnology (Azwar et al. 2014). Due to the nanoparticles and nanomaterials in biofuel production, there have been many benefits in important parameters such as conversion or energy efficiency, biohydrogen yield, capital cost, and HPR (Bunker and Smith 2011). Dehydrogenase and cellulases are the two prominent enzymes that

have been regularly used for biohydrogen production. The biohydrogen production processes when carried out separately have major advantages as they can be performed at their own optimum conditions to produce maximum yield. However, due to the wettability of the sample, the downside of the dispersion of certain nanoparticles is the difficulty of dispersing in the aqueous solution where the hydronium ions become ineffective in the solution. The findings revealed that cellobiose conversion was up to 96.0 percent higher than traditional conversion (32.8%) without catalysts for acid-functionalized magnetic nanoparticles with 6 percent of the sulphur content (Peña et al. 2014). Researches have progressed towards the immobilization of enzymes particularly chitosan-based to promote the enzymatic action of dehydrogenase and cellulase moieties in biofuel production which can increase the bioavailability of cellulosic biomass and their derivatives of improved hydrolysis (Sivagurunathan et al. 2018). Compared to the current one from coal, the biodiesel fuel from vegetable oil does not contain sulphur oxide and minimizes the soot particulate by 1/3 times. And biohydrogen production from green sources has the scope of applications for many purposes. Because of these environmental benefits, it is possible to foresee biodiesel fuel to replace traditional diesel fuel.

There are disadvantages of nanotechnology in the production process. In metallic nanoparticles, there can be the high cost of synthesis material, limitations in scale-up production processes, poor dispersion abilities, and mobility dependent on environment compatibilities. The major disadvantage of cellulosic biomasses is the crystallinity of cellulose, lignin, and surface area, and hemicellulose over coating cellulose; these all decrease the yield of biofuels at the commercial level. As they contribute to agglomeration, oxidative stress, and unreliable nutrient availability, NPs are detrimental to microalgae. Therefore, screening studies of nanoparticles with an effect on microbial and enzymatic behaviour are required to investigate their wide range of concentrations. The process involving nanoparticles and proteins in the production should be analysed at the molecular level. The characteristics and properties of materials including electric, magnetic, and optical behaviours can be more crucial due to the quantum effects (Khoo et al. 2020) (Bogani and Wernsdorfer 2008).

4.4.2.1 Challenges

For poor and underdeveloped countries, the use of biofuels may be difficult because they are too expensive as it needs wide trade, technical, and social policies. These are important for the development of the biofuel industry without weakening the food security of undeveloped countries (Neha et al. 2020a, b). Pre-treatment, enzymatic hydrolysis, and cultivation of biomass during initial processes are challenges for industries before conversion to bioenergy. The enzymes for biofuel production are selected based on their efficiency and commercial advantages. Isolation, Identification, and growth of enzymes from bacteria, yeast, or fungus for obtaining desired enzymes have been major challenges to produce biofuels at the commercial level. However, when exposed to the oxidation process, metals such as cobalt and nickel

that are introduced into the synthesis show toxic and susceptible compounds, so further scientific studies are needed to solve these problems.

Though cellulase enzymes have configurable industrial uses, performance enhancement, cost reduction, and energy usage are often selective criteria for the bioconversion of cellulose biomass into biofuels (Neha et al. 2018). Studies on the development processes of microbial lipases and the function of lipid substances used as inducers in the production of lipases are scarce, considering their significance. Lipases are a particularly versatile category of extracellular bacterial enzymes capable of conducting several major reactions, providing a promising area for potential study (Neha et al. 2020a, b). Structure-function relationship understanding would enable researchers to customize new lipases for biotechnological applications.

Apart from the purification of enzyme and end-product isolation, minimal understanding of protein engineering and its application often add a barrier to the overall bioconversion process. Researchers should be able to develop more viable and efficient enzymes and commercially economic processes to replace existing technologies.

The major drawback to prevent nano-catalysts is nanoparticle sintering. At extreme heat in the reactive conditions of many catalytic processes, metal atoms are mobile to the point where they cause significant changes in size and form of metal nanoparticles. Those conformational changes lead to undesirable effects such as loss or reversion in selectivity and inhomogeneity (Zuliani et al. 2018).

Research should concentrate on applied genomics and proteomics technology for the discovery of novel lignocellulolytic enzymes and engineering enzymes with enhanced industrial-scale applicability activities. The considerable demand opportunity and the critical role that cellulases and xylanases play in the developing industries of bioenergy and bio-based goods provide incentives to create improved enzyme preparations for the hydrolysis of plant cell walls. These developed cellulases and xylanases must also have the required functionality for biorefineries, such as higher catalytic efficiency on insoluble cellulosic substrates (Prajapathi et al. 2018).

The integration with hydrogen purification and storage are the common challenges that are experienced by all renewable hydrogen production. The two key obstacles to biohydrogen development are the comparatively low yield of hydrogen and the cost of production. To enhance metabolic pathways, it could be possible to improve hydrogen yield by using adequate process change, successful bioreactor architecture, bacterial strain, and even genetic and molecular engineering techniques. There are now some integrated strategies being developed: two-step hybrid fermentation process, multistage bioreactors, or the use of modified microbial fuel cells (Show et al. 2011, and Ueno et al. 2007). The synthetic cascade enzyme has shown an increase in the yield of biohydrogen close to the maximum theoretical value. But the low stability and high cost of purified enzymes in the cascade system are a drawback for the commercial level of production.

4.4.2.2 Recent Trends

An extensive search for new microorganisms and their enzymes will lead to novel yet simple techniques for commercial procedures leading to different ways to address environmental problems. Collaboration, exchange of knowledge, experience between biochemists, crystallographers, enzyme kineticists, geneticists, and chemical, biochemical, and food engineers will be seen. In order to explore the feasibility of integrating nanoparticles on large-scale production of bioenergy basis, pilot research is essential. In addition, future research is not limited to bioenergy sources and development, where nanoscience could overcome the technological shortcomings of science and engineering by contributing to the areas of transition, transport, energy conservation, and the use of end product as well as storage of bioenergy (Nizami and Rehan 2018). Safety tests are being carried out because, with increasing use in biofuel applications, nanoparticles have shown apparent human and environmental toxicity consequences. The toxicity of nanoparticles has been studied using many methods, most of which include in vitro nanotoxicity investigation. Now, innovative and durable methods for enzyme encapsulation from nanosheets, nanoparticles, lipid vesicles, and nanotubes are provided by nanotechnological instruments (Cacicedo et al. 2019).

Currently, about 38Mt of hydrogen is produced all over the world every year. The demand for biohydrogen is expected to increase annually. The increase in H_2 output is related to the concentration of nanoparticles and their characteristics. Cu nanoparticles, on the other hand, demonstrated a detrimental impact on the H_2 output yield at a lower 2.5 mg/L concentration, using mixed and pure culture from both bio-wastes and sugars as a feed; the variable effects of NPs or their mixtures on the biohydrogen production were observed. These nanoparticles mainly increase the output yield of H_2 by their major positive effects on the development of the organism, the quality of feed degradation, and the profile of intermediate metabolites. The intermediate metabolites are mainly transferred towards a higher ratio of acetate to butyrate and inhibition of ethanol and propionate production in the presence of nanoparticles (Mohanraj et al. 2016).

Extensive screening of new microorganisms and their lipolytic enzymes will open up new, easy routes for synthetic processes and hence new ways of addressing environmental problems (Neha et al. 2020a, b). There has been a steady growth in interest worldwide in the quantum of research efforts, findings, and the resulting number of reviews and research publications published in the last few decades on the use of the potential of nanoscience (Sivagurunathan et al. 2018). Because of the environmental benefits, it is possible to foresee biodiesel fuel to replace traditional diesel fuel.

4.5 Improving Biofuel Efficiency

Sustainable sources of energy are the need of the hour in a world with an ever-increasing demand for energy. Presently, fossil fuels account for a major supply of energy, but the depletion of fossil fuels and the negative environmental health impact of the use of such sources of energy call for the development of safer and sustainable sources available at affordable costs. Biofuels offer an alternative; they are sustainably produced and help with confronting climate change, greenhouse gas emissions, and health risks associated with the use of fossil fuel. Progress has been made in biofuels, but the cost of producing biofuels is still high, and it is not yet viable to replace fossil fuels with biofuels.

4.5.1 Novel Enzymes Involved

Enzymes play an important role in the production of biofuels. Common processes in biofuel production like the breakdown of lignocellulosic biomass into ethanol for bioethanol production, transesterification of lipids into biodiesel, photobiological water splitting for biohydrogen production, etc. are dependent on and limited by enzymes. Therefore, enzymes with increased activity and a wider range of substrates are required to minimize costs in the development of biofuels. A variety of basic properties including catalytic activity, broad substrate specificity, and tolerance to extreme temperatures, broad levels of pH, aqueous and non-aqueous solvents, and high salt concentrations should be improved to increase the efficiency of biofuel production. Production of biofuels relies on raw materials high in lignocellulosic biomass which comprises cellulose, hemicellulose, and lignin. Lignin, in particular, is difficult to digest and decreases the accessibility of cellulose and hemicellulose to enzymes. This increases the recalcitrance of biomass and makes its conversion inefficient and costly. It is known that highly efficient lignocellulose biomass degradation is carried out by specialized plant-parasitic fungi, decomposers, and microbial communities from termite gut and cattle rumen (Bhari and Singh 2017). Strategies for identifying novel enzymes from these communities as well as some novel enzymes discovered using such strategies are discussed in this chapter.

Some strategies have used metabolic engineering to engineer biosystems to better utilize these enzymes for biofuel production. They use genetic engineering to modify the metabolic pathways in organisms to increase the synthesis of desired compounds like hydrogen, lipids, etc. Such strategies are also discussed in this chapter.

4.5.1.1 Metagenomic Approach for Identification of Novel Enzymes for Biofuel Production

Microbial enzymes are extremely important for the breakdown of biomass for the production of biofuel. Microorganisms, especially prokaryotes, are the most diverse and proliferate group of organisms on earth. They populate almost all environments, thriving even in extreme conditions like salt springs, hot springs, and extremes of temperature. They remain a large pool of unexplored biological and genetic diversity that can be explored to find novel enzymes with high activities and other beneficial properties. Conventional methods for culturing bacteria limited this exploration as most of the bacteria from environmental samples are unculturable (Jünemann et al. 2017). Metagenomics helps overcome this problem by being culture-independent. In metagenomics, genomic DNA is isolated directly from environmental samples and explored for novel genetic resources (Simon and Daniel 2009). This library of metagenomic DNA can be explored in two ways: sequence-driven and function-driven screening. Sequence-driven screening approaches include the sequencing of this metagenomic DNA and using bioinformatics tools to predict the functions of genes discovered. These methods rely on the accuracy of the sequencing techniques and prediction algorithms, thus may not correlate well with reality in most cases due to the low accuracy of prediction algorithms. Therefore, function-driven screening is more widely used to discover novel genes from metagenomic libraries as they employ assay-based techniques (Singh et al. 2009). Some recent assay-based techniques used in function-driven screening are biological function-based screening, compound configuration screening, DNA sequence-based screening, and substrate-induced gene expression (SIGEX) (Wang et al. 2019). Enzymes like cellulases, xylanase, laccase, pectinases, proteases, and feruloyl esterase synergistically carry out the degradation of lignocellulosic biomass (Fig. 4.1 and Table 4.3). Function-driven screening has helped identify novel enzymes of such classes from metagenomic libraries of specialized microbiomes. Some such enzymes are discussed below.

4.5.1.1.1 Cellulase

Cel7482 is a novel cellulase identified from metagenomic sequences of DNA isolated from anaerobic beer lees. Cel7482 showed maximum glycoside hydrolase activity at 5.5 pH and 60–70 °C. Cel7482 is highly halotolerant (up to 2 M NaCl) and tolerant to ionic liquids which makes it suitable for industrial application (Yang et al. 2016). Active cel7482 can be easily expressed extracellularly using the twin-arginine translocation (Tat) pathway of *Bacillus subtilis* 168 making it even more suitable for industrial applications due to ease of downstream processing and overexpressing of the enzyme. Another cellulase, MeBglD2, which showed β-galactosidase and β-fucosidase activities along with β-glucosidase activity, was identified from a soil metagenomic library. MeBglD2 was not inhibited by the

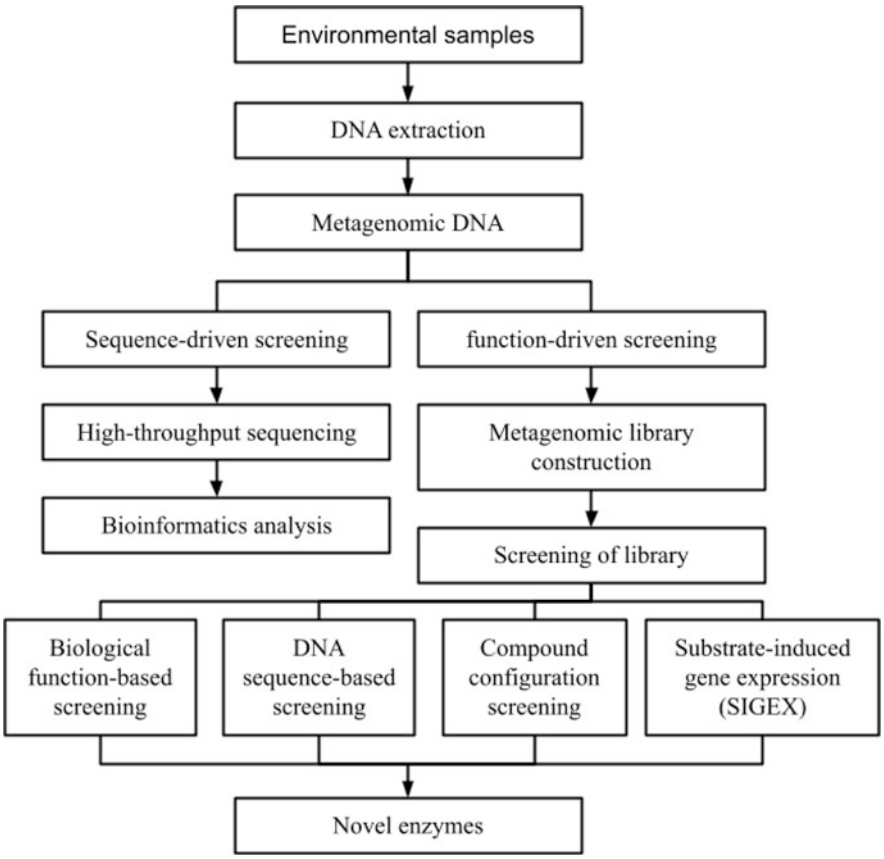

Fig. 4.1 Metagenomic approach for the identification of novel genetic resources (enzymes)

products (monosaccharides and disaccharides) of cellulose breakdown like other cellulases. Thus, it improved the saccharification yield when used along with other cellulases (Matsuzawa and Yaoi 2017).

4.5.1.1.2 Amylase

Amylases hydrolyse starch to produce glucose which can later be used for biofuel production. A novel α-amylase gene called amyPL was identified from a faecal metagenomic library of the pygmy loris which showed optimal activity at 50 °C and 5.6 pH (Xu et al. 2014). Another α-amylase, AmyI3C6, was identified using a functional metagenomic approach from ikaite column samples which showed optimum activity at 10–15 °C, 8–9 pH, and was very well suited for low-temperature applications (retained 70% activity at 1 °C) (Vester et al. 2015).

Table 4.3 Characteristics and abilities of novel enzymes for biofuel production

Source	Enzyme type	Enzyme name	Optimal temperature (°C)	Optimal pH	Special properties	References
Anaerobic beer lees	Cellulase	Cel7482	60–70	5.5	Halotolerant (up to 2 M NaCl) and tolerant to ionic liquids	Yang et al. (2016)
Soil microbiome	Cellulase	MeBglD2	60	6	Lowered feedback inhibition of cellulose degradation	Matsuzawa and Yaoi (2017)
Pygmy Loris faecal matter	Amylase	amyPL	50	5.6	First α-amylase Isolated from a gastrointestinal metagenomic library	Xu et al. (2014)
Ikaite column	Amylase	AmyI3C6	10–15	8–9	Suited for alkaline and low-temperature applications	Vester et al. (2015)
Saline-alkaline soil	Xylanase	Xyn22	65	7	Thermostable and halotolerant	Li et al. (2019b)
Chicken cecum	Xylanase	xynAMG1	45	6	Tolerant to organic solvents and salts	AL-Darkazali et al. (2017)
Acidic bog soil	Laccase	LacM	50	4	Tolerant to organic solvents and salts	Ausec et al. (2017)
Soil microbiome	Feruloyl esterase	FAE-Xuan	30	5	Stable over a broad pH range (3.0 to 10.0)	Li et al. (2019a)
Mangrove soil	Lipase	Lip906	74	7.8	Resistant to industrial detergents and low temperatures	Tang et al. (2017)
Solar saltern	Esterase	EstSP	40	8	Resistant to non-aqueous solvents and halotolerant	Jayanath et al. (2018)

4.5.1.1.3 Xylanase and Laccase

Xylanase degrades xylan which is a major component of lignin. Numerous efforts have been made to find novel xylanases with increased efficiency and tolerance. Xyn22, a novel thermostable and halotolerant GH11 xylanase, was identified from a saline-alkaline soil metagenomic library which retained 58% relative activity in 5 M NaCl. Its thermostability was further improved by introducing aromatic interactions between the amino acid residues to make Xyn22T48Y (Li et al. 2019b). Another xylanase, xynAMG1, which is salt tolerant and resistant to common organic solvents, was identified from the chicken cecum metagenomic library (AL-Darkazali et al. 2017). Laccases also can degrade xylan. Laccase LacM was identified from acidic bog soil metagenomic libraries. It was found to be tolerant to organic solvents and salts and had the ability of azo and triphenylmethane dye decolonization (Ausec et al. 2017).

4.5.1.1.4 Feruloyl Esterases

Hydroxycinnamic acid (HCA) units, in particular, ferulic acid (FA), form cross-links in the lignocellulosic mass derived from plants. This hinders their accessibility to enzymatic digestion. Feruloyl esterases (FAEs) are enzymes that cut these linkages and improve the accessibility of other enzymes (Underlin et al. 2020). FAE-Xuan is a novel Feruloyl esterase found in a soil metagenomic library which showed high activity towards methyl ferulate (40.0 U/mg) at 30 °C and 5.0 pH and was quite stable over a broad pH range from 3.0 to 10.0 (Li et al. 2019a)

4.5.1.1.5 Lipolytic Enzymes

Lipases and esterases are lipolytic enzymes that hydrolyse acylglycerols with carbon chain length > 10 and carbon chain length ≤ 10, respectively (Ramnath et al. 2017). They are important in biodiesel production from lipids. A novel lipase Lip906 was identified from a metagenomic library constructed from mangrove soil. Lip906 showed maximum activity at 74 °C and pH 7.8 and was resistant to industrial detergents and low temperature (up to 4 °C) (Tang et al. 2017).

A novel esterase, EstSP, was identified from a solar saltern metagenomic library which showed high stability in the presence of industrial solvents and halotolerant up to 5 M NaCl retaining 60% of activity. Due to these remarkable properties, EstSP holds a high potential for industrial applications in non-aqueous solvents.

4.5.1.1.6 Multifunctional Enzymes

A multifunctional enzyme R_09–02 from glycosyl hydrolases family 43 (GHF43) was identified from fiber-adherent microbiome from cow rumen using metagenomic

analysis. R_09–02 showed activity on xylose, arabinose, glucose, and galactose containing oligosaccharides (Ferrer et al. 2012). Cow rumen microbiomes are known for their rapid digestion of plant biomass, and GHF43 makes up 7% of the GH genes found in the cow rumen. This along with their multifunctional activity towards xylose and arabinose-containing substrates suggests that GHF43 must be studied more for its role in lignocellulosic biomass digestion.

4.5.2 *Improving the Efficiency of Fungal Enzyme/Metabolite Production for Biofuel Synthesis*

Fungi are used for the production of about 60% of commercially available enzymes (Østergaard and Olsen 2011). Single-celled fungi like *Saccharomyces cerevisiae* and *Candida albicans* are very well studied and often used as model organisms and as hosts for recombinant protein production. Filamentous fungi like *Aspergillus* sp. and *Trichoderma reesei* have been used for their high protein production potential. Still, there is a need to improve their efficiency for producing biofuels in a commercially viable manner.

Fungi are used to produce enzymes for the breakdown of biomass, and several strategies are used to improve their production like random mutagenesis, cloning, heterologous expression, metabolic engineering, co-cultivation, etc. Heterologous expression of two xylanase genes (GH10 and GH11) from *Malbranchea cinnamomea* was carried out in *Pichia pastoris* X33. These xylanases improved the hydrolysis of acid−/alkali-treated rice straw by 1.5 times when used along with commercial cellulases when compared to only using commercial cellulases (Basotra et al. 2018). Metabolic engineering was used to improve the saccharification efficiency of *Trichoderma reesei* strain RUT-C30. This was achieved by introducing six modifications using CRISPR/Cas9 gene editing which include the introduction of mutated cellulase regulator XYR1, extracellular proteases SLP1 and PEP1, β-glucosidase CEL3A from *Talaromyces emersonii*, invertase SUC1 from *Aspergillus niger*, and the deletion of cellulase repressor ACE1. This resulted in very high extracellular protein production (80.6 g/L, highest recorded in *T. reesei*), a 72-fold increase in β-glucosidase activity, and a 42-fold increase in xylanase activity (Fonseca et al. 2020).

Fungi have also been used for biodiesel production in two ways. Firstly, they have been used for producing biomass with high lipid concentrations (like single cell oils). Genetic engineering has helped increase the lipid content by overexpression of lipid biosynthesis. The overexpression of acetyl-CoA carboxylase (ACC1), fatty acid synthase 1 (FAS1), and fatty acid synthase 2 (FAS2), in *Saccharomyces cerevisiae*, has led to lipid accumulation of more than 17% of dry biomass, a fourfold increase compared to control (Runguphan and Keasling 2014). Secondly, they have been used to produce enzymes that help in the transesterification process during the synthesis of biodiesel from lipids. Genetic engineering has been applied for

Table 4.4 Modifications in fungal systems for biofuel production

Strain	Alteration	Modification observed	References
Saccharomyces cerevisiae	Overexpression of acetyl-CoA carboxylase (ACC1), fatty acid synthase 1 (FAS1), and fatty acid synthase 2 (FAS2)	Lipid accumulation of more than 17% of dry biomass	Runguphan and Keasling (2014)
Trichoderma reesei	Heterologous expression of lipase B of *Candida antarctica* (CalB)	Production of highly pure extracellular recombinant CalB of up to 4 grams per litre	Rantasalo et al. (2019)
Pichia pastoris X33	Heterologous expression of xylanase (GH10 and GH11) from *Malbranchea cinnamomea*	Up to 1.5 times increase in the hydrolysis of lignocellulose substrate when used in conjunction with cellulases	Basotra et al. (2018)
Trichoderma reesei strain RUT-C30	Metabolic engineering Introduction of: • Mutated cellulase regulator XYR1. • β-Glucosidase CEL3A, • Invertase SUC1. • Proteases SLP1 and PEP1. Deletion of cellulase repressor ACE1	Extracellular protein concentration of 80.6 g/L and a 72-fold increase in β-glucosidase activity and a 42-fold increase in xylanase activity	Fonseca et al. (2020)

increasing the production of recombinant enzymes for transesterification. *Trichoderma reesei* has been engineered using CRISPR/Cas9-enabled multiplex genome editing to work with synthetic expression system (SES) which enabled the production of recombinant CalB (lipase B of *Candida antarctica*) enzyme. It also enabled repression of other enzymes using glucose medium to produce highly pure recombinant CalB of up to 4 grams per litre (Rantasalo et al. 2019) (Table 4.4).

4.5.3 *Tuning Algal Genomes Using Genetic Engineering Approaches for Improved Metabolite Production*

Third-generation biofuels make use of oil-rich microorganisms for biodiesel production. Algae is the most promising candidate for biodiesel production due to its high lipid accumulation, good growth characteristics, low land area requirements, and potential to grow on treated wastewater (Leong et al. 2018). They can also be used for commercially important secondary metabolite production. However, high initial capital and insufficient biomass production have limited their commercial application.

Similar to fungi, algae have been engineered to produce higher contents of lipid accumulation in their biomass using metabolic engineering specifically by overexpression/suppression of genes. Starch and lipids both can act as storage

molecules in algae and have common carbon precursors. Environmental conditions decide whether starch or lipids get accumulated. By hindering the pathway of starch synthesis, the entire carbon flux can be dedicated to the synthesis of lipids. The enzyme acyl-CoA:diacylglycerol acyltransferase (DGAT) catalyses the esterification of diacylglycerol and acyl-CoA, a final committed reaction in triacylglycerol (lipid) synthesis, and has a central role in cytosolic lipid accumulation. Based on this, *Phaeodactylum tricornutum* was engineered to overexpress its endogenous type 2 acyl-CoA:diacylglycerol acyltransferase (DGAT2B) and an Δ5-elongase (OtElo5) from *Ostreococcus tauri* which led to doubling of lipid accumulation in biomass when cultivated under nitrogen depleted conditions (Haslam et al. 2020).

Phosphoenolpyruvate carboxylase (PEPC) catalyses the formation of oxaloacetate from phosphoenolpyruvate and is an important enzyme in carbon fixation as carbohydrates during photosynthesis. Its downregulation has been found to increase the carbon flux towards fatty acid synthesis. The downregulation of PEPC in *Chlamydomonas reinhardtii* using artificial miRNA (amiRNA) inhibition has led to a 48% increase in fatty acid content (Wang et al. 2017b).

Some microalgae also can produce H_2 in the absence of oxygen. Biohydrogen production in algae is catalysed by [FeFe]-hydrogenase which is very sensitive to the presence of oxygen and limits hydrogen production. This incompatibility has lagged the development of green alga as an H_2 production system (H. Li et al. 2015). The actual efficiency of hydrogen production is ten times lower than that predicted theoretically, so the potential for improvement in biohydrogen production is very high. To overcome this limitation, the native [FeFe]-hydrogenases in *Chlamydomonas reinhardtii* strain D66ΔHYD were replaced with O_2-tolerant clostridial [FeFe]-hydrogenase CaI which leads to sustained hydrogen gas production even at high levels of oxygen (Noone et al. 2017).

Another approach for increasing H2 production is by suppressing the photosystem II (PS II) of photosynthesis in algae. It was found that the temporary inactivation of *C. reinhardtii* PS II by sulphur depreciation led to a prolonged H2 production. Based on this phenomenon, Li et al. engineered *C. reinhardtii* by knocking out the Oxygen Evolving Enhancer (OEE2) gene, an important protein in PS II using amiRNA technology which led to a twofold increase in H_2 production (Li et al. 2015) (Table 4.5).

4.5.4 *Termite Microbiome for Novel Enzymes for Lignocellulose Degradation*

Termites are eusocial insects which mostly feed on dead plant material. They can digest 74–99% of cellulose and 65–87% of hemicellulose in the lignocellulosic matter they ingest (Ohkuma 2003). They do this chiefly with the help of symbiotic microorganisms which reside in their gut as well as with their enzymatic secretions (endogenous enzymes). These termite gut microbiomes have been explored for

Table 4.5 Modifications in algal systems for biofuel production

S. no	Strain	Alteration	Modification observed	References
1	*Phaeodactylum tricornutum*	Overexpression of an endogenous type 2 acyl-CoA:Diacylglycerol acyltransferase (DGAT)	Higher lipid yield and enhanced levels of eicosapentaenoic acid and docosahexaenoic acid in triacylglycerols	Haslam et al. (2020)
2	*Chlamydomonas reinhardtii*	Artificial miRNA inhibition of phosphoenolpyruvate carboxylase	Improvement of fatty acid from 0.086 g g^{-1} to 0.105 g g^{-1}	Wang et al. (2017b)
3	*Chlamydomonas reinhardtii* strain D66ΔHYD (hydA1 − hydA2 −)	Replacement of [FeFe]-hydrogenases with more O2-tolerant clostridial [FeFe]-hydrogenase CaI	Sustained H_2 production even at high O_2 levels and prolonged illumination	Noone et al. (2017)
4	*Chlamydomonas reinhardtii*	Artificial miRNA silencing of OEE2 gene	A twofold increase in H_2 production	Li et al. (2015)

novel enzymes which can help in more efficient cellulose and lignin degradation. Some novel endoglucanases (EG) and β-glucosidases (BG) have been identified from termites that have been cloned and overexpressed in heterologous hosts for easy production. Novel xylanases have also been identified from termite gut microbiomes using metagenomic approaches which can help in lignin digestion. Novel endoglucanases CfEG5 and CfEG3a identified in *Coptotermes formosanus* have been cloned and overexpressed in *E.coli*, showing specific activities of 328 and 325 units/mg, respectively. C-terminal His-tagged version tCfEG was also prepared to facilitate easy purification of the enzyme (Zhang et al. 2011). Two EGs, RsEG and NtEG, identified from *Reticulitermes speratus* and *Nasutitermes takasagoensis*, respectively, have been successfully produced in the *Aspergillus oryzae* host, which is more suitable than *E.coli* for industrial production (Hirayama et al. 2010). NkBG is an endogenous BG identified in salivary glands of *Neotermes koshunensis* which was first expressed in heterologous host *E.coli* and then more efficiently mass-produced in *A. oryzae* (Ni et al. 2007). A more thermostable BG, bgl-gs1, was identified from the gut symbiotic microbiome of *Globitermes sulphureus* which showed optimal activity at 90 °C (Wang et al. 2012b).

Novel xylanases mXylB8 and Xyl6E7 have been identified using functional metagenomic screening of symbiont microbiota of *Reticulitermes santoensis* and *Macrotermes annandalei*, respectively. These were then cloned in heterologous host *E.coli* and showed xylanase activity on beechwood xylan (Mattéotti et al. 2012; Liu et al. 2011a, b). It is also demonstrated that microbiota from termite gut can be cultivated in bioreactors using lignocellulose as a substrate and be used to produce carboxylates. In particular, the *Nasutitermes ephratae* gut microbiome showed the highest levels of lignocellulose degradation along with high levels of xylanase and cellulase activity (Auer et al. 2017) (Table 4.6).

Table 4.6 Novel enzymes identified from termites and their symbiont microbiomes

Source organism	Enzyme type	Specific activity (U/mg)	Optimal temperature (°C)	Optimal pH	Expression host	References
Coptotermes formosanus CfEG3a CfEG5	EG EG	328 325	37 43	5 5.6	*E.coli*	Zhang et al. (2011)
Reticulitermes speratus RsEG						
Nasutitermes takasagoensis NtEG	EG	1392	65	6	*A. oryzae*	Hirayama et al. (2010)
Neotermes koshunensis NkBG	BG	156	45	5	*E.coli*	Ni et al. (2007)
Symbiont of *Globitermes sulphureus* Bgl-gs1						
Symbiont of *Reticulitermes santoensis* mXylB8	Xylanases	1837	55	5	*E.coli*	Mattéotti et al. (2012)
Gut symbiont of *Macrotermes annandalei* Xyl6E7	Xylanases	733	50	7.5	*E.coli*	Liu et al. (2011a, b)

One unit (U) is defined as the amount of enzyme that releases 1 μmol of reducing sugar per minute

4.6 Conclusions and Future Perspectives

Biofuels have gathered attention as they are a sustainable and environment-friendly alternative to the rapidly depleting fossil fuels. However, at present, biofuels are not economically viable. Their chemical synthesis uses a lot of harsh chemicals and is energy inefficient, whereas biological synthesis suffers from the low effective activity and other inefficiencies. Enzymatic digestion of lignocellulosic biomass remains a challenge. Metagenomics-based approaches allow the identification of novel enzymes from previously unexplored environments. Although many enzymes have been screened and identified, further research is necessary for the standardization and scale-up of the process. Understanding the degradation mechanisms of lignin by various enzymes derived from fungal and termite biomes opens more avenues for biofuel production in the future. There is also a need to develop a greater range of alternative hosts with good expression of foreign genes of metagenomic origins. Different techniques like metabolic engineering using genetic engineering for pathway modification and optimization of production of desired metabolites have

been tried and successfully implemented at a laboratory scale. But such studies have been limited to a few organisms. Lack of knowledge of metabolic pathways is a major bottleneck for metabolic design, but the sequencing of more genomes, development of better bioinformatics tools, and study of metabolic pathways will help design better systems for efficient production of biofuels. More research is needed to bring biofuel production to commercially viable levels.

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Chapter 5
Engineered Strains in the Development of the Biofuel Industry

Poojhaa Shanmugam, Sanchita Bipin Patwardhan, and Elvis Fosso-Kankeu

Abstract The maintenance of consistent electric power production remains a challenge. This could be achieved if the intermittent part of the system is effectively controlled. The activities of microorganisms and the type of nutrients available determine to a large extent the amount and continuous generation of electricity. The ideal microorganism or communities of microorganisms are expected to tolerate conditions in the system, degrade nutrients through a pathway that will lead to maximum production of electrons and protons, and ensure proper transfer of electrons to the anode electrode. Very few microorganisms may be able to exhibit all these properties, and the need for symbiotic interaction among microorganisms is required. Numerous microorganisms have been identified as able to contribute for the electricity generation. Although wild strains have also been used successfully, biomolecular transformation or physical pretreatment has been required to improve the performance of some strains in other instances. This chapter will highlight the performance of a number of microorganisms that have the potential to play a major role for electricity generation as well as the different techniques that have been used to enhance their performance.

Keywords Biofuel production · Electricity generation · Symbiotic interactions · Engineered strains · Enhanced performance

P. Shanmugam · S. B. Patwardhan
Amity Institute of Biotechnology, Amity University, Mumbai, India

Pune Expy, Panvel, India

E. Fosso-Kankeu (✉)
Department of Mining Engineering, College of Science Engineering and Technology, University of South Africa, Florida, South Africa

P. Chowdhary et al. (eds.), *Bio-Clean Energy Technologies: Volume 1*, Clean Energy Production Technologies, https://doi.org/10.1007/978-981-16-8090-8_5

5.1 Introduction

At the current stage of development, microbial fuel cells cannot seem to match the capacity of conventional fuel systems, but remain attractive for several reasons: (1) The current is extracted from renewable biomass; (2) the bioelectrochemical processes involved in the conversion of biomass to electricity are considered to be carbon neutral; and (3) the overall process is sustainable and cheap (Lovley 2008; Osman et al. 2010). The important achievements in this field have mainly been in the design and development of the electrodes and the configuration of the system for producing biofuel. This has certainly brought about a significant increase of the power densities. Some progress has also been made toward the understanding of the interactions among fuel components which include substrates and biocatalysts. Wang et al. (2009) came to realize that the substrates being fed to the system is of utmost importance in determining the start-up time. The substrates are often readily available in abundance in the environment; a number of sources have been listed by Pant et al. (2010), including, among others, brewery wastewater, starch processing wastewater, meat processing wastewater, and wastewater from canning of fruits and vegetables. It is equally important to ensure that the right microorganisms are present in the system to effectively convert these substrates into electricity. Whether the microorganisms possess the enzymes capable of using these substrates through an adequate pathway to produce more electrons and less toxic byproducts is one thing; moreover, the electrons generated must reach the anode electrode. The transfer of electrons to the anode electrode can be attained by using artificial mediators which may be costly and/or toxic for microorganisms; some bacteria called exoelectrogens can transfer electrons through a direct or indirect mechanism, the second being carried out by mediators produced by the microorganisms; *Pseudomonas aeruginosa* and *Clostridium butyricum* are some of the species that produce their own mediators capable of mediating electron transfer and ensuring increased power output (Park et al. 2001; Rabaey et al. 2005). Contrarily, there are species such as *Geobacter sulfurreducens* and *Shewanella oneidensis* with the ability to establish a direct communication with the electrode through the enzymes at their outer membranes. Biofilm formation often enhances direct contact of microorganisms with the electrode, providing also the possibility of electron transfer by mobile mediators present in the biofilm; however, only biofilm-forming species can develop such conditions (Rabaey and Verstraete 2005).

It is therefore imperative that the microbial species present in the anodic matrix possess individually or as a combination the potential to oxidize available substrates for maximum emission of electrons and eventually transmit them to the anode electrode. Combining more than one species holds promise for the occurrence of bioelectrochemical reactions or pathways required for effective electricity generation; several researchers (Kim et al. 2007) have suggested the usage of mixed cultures for better processing of mixed fuel in practical applications such as wastewater treatment; however, carefully selected pure species with desired potentials could match the performance of mixed cultures (Ringeisen et al. 2007; Xing et al.

2008). The intensive research carried prior to this review has allowed the identification of a number of microorganisms which have demonstrated some important attributes for power generation; these include *Geobacter sulfurreducens*, *Shewanella*, *Clostridium*, *Pseudomonas*, *Desulfovibrio*, and *Enterobacter* which could successfully be used in combination for the biofuel synthesis. This review will mainly focus on the potential of these microbes to effectively oxidize a number of substrates, their capacity to transfer electrons, and the mechanism used. In some cases, the potential of these microorganisms has been improved through molecular engineering; modification of the respiration rates, electricity shuttles pathway, control of the number of pili, and cofactor manipulation have been carried out to improve electron transfer, while other changes focused on biofilm dispersion and utilization of specific substrates.

5.2 Microbial Use of Substrates

The resultant power of the biofuel production system derives mainly from the biochemical activities taking place in the anode compartment of the system; these activities are correlated to the diversity of the species and the type of substrates available (Grüning et al. 2014; Lee et al. 2008; Liu et al. 2009). The composition of the anodic microbes concerning the metabolic function determines the effective use of substrates and conversion to bioelectricity. An increment in the rate of hydrolysis-acidification and the overall efficacy of anaerobic digestion could be achieved if there is a proper knowledge and understanding of the microbial community. This section will mainly discuss the ability of predominant microorganisms to use substrates through the pathways suitable for electron and proton generation.

Substrate type mainly affects electricity generation from the perspective of its accessibility, the analysis of the microbe's morphology and characteristics, and the metabolic path undertaken during anaerobic digestion; fermentable sugars are used by several microbes which have greater growth yields than the anode-respiring bacteria; however, factors such as increased biomass, H_2, and CH_4 during fermentation act as electron sinks in the anode therefore inhibiting bioelectricity generation. Although organic acids may not be effectively used as substrate by some anodophiles and exoelectrogens, anaerobic digestion of the organic acids such as acetate produces less gases and soluble organic matters that can hinder electron transfer to the anode (Freguia et al. 2007; Torres et al. 2007). Biopolymer biomasses are abundantly available and renewable, but very few microorganisms are able to hydrolyze them and effectively use the monosaccharide derived from their breakdown (Pant et al. 2010; Rezaei et al. 2009).

5.2.1 Clostridium *species*

The biocatalysts *Clostridium butyricum* and *Clostridium beijerinckii* are capable of metabolizing a great variety of substrates to generate electricity; they have the ability to digest several substrates including complex carbohydrates, monosaccharides, and even low-molecular-weight compounds such as lactate, converting them into electricity (Niessen et al. 2004). *Clostridia* make use of branched fermentation pathways to catabolize carbon sources into a variety of end products which could be the energy generated or reducing equivalents, reduced, and oxidized products of various amounts (Abbad-Andaloussi et al. 1996). Some *Clostridia* species are solventogenic, fermenting carbohydrates through two distinctive phases: during the first phase also known as acid-producing phase, growth is accelerated, and acetate, butyrate, H_2, and CO_2 are generated (Gheshlaghi et al. 2009); in the second stage or solventogenesis, the organic acids are transformed into butanol, acetone, ethanol, and carbon dioxide (Patakova et al. 2013). By studying the metabolic changes in *C. acetobutylicum*, Finch et al. (2011) observed that the biofuel system generated dual voltage peaks over a week-long period, which was correlated to the glucose metabolism. As a result, they hypothesized that the first voltage peak corresponded to acidogenic metabolism, while the second peak corresponded to solventogenic metabolism. The capacity of *C. acetobutylicum* ATCC824 to uptake and metabolize hexose, pentose, disaccharide, and starch has been investigated through transcriptional analysis of the genes involved; it was suggested that for the transport of hexoses and disaccharides, this strain uses a phosphoenolpyruvate-dependent phosphotransferase system (PTS) and gluconate, H^+ transporters, while symporters and ATP-binding cassette transporters ensure the uptake of pentoses. Pyruvate is a prime intermediate in clostridial metabolism; hexose and pentose are broken down through the Embden-Meyerhof-Parnas (EMP) pathway and the pentose phosphate pathway, respectively (Ounine et al. 1983); the pentose sugars are first converted to fructose-6-phosphate and then glyceraldehyde-3-phosphate before entering the EMP pathway. A complete breakdown of pentose to pyruvate will yield 5 mol ATP and 5 mol NADH (Rogers 1986). Glucose and glycerol behave differently when used as carbon source by *Clostridia* species; the excess of NADH produced during glucose catabolism is relatively higher compared to the glycerol metabolism; the same study reported the simultaneous glucose-glycerol consumption by *C. butyricum* when grown on glucose-glycerol mixture. However, Zhang et al. (2011) have described a disappearance of *Clostridium* species from the biofuel production system after the substitution of glucose by acetate. Natural carbohydrates, such as herbal biomasses, are abundant and make good substrates; they are waste materials from agriculture and industrial activities and are easily available in the environment. However, they mostly consist of cellulose and starch, which are complex carbohydrates that can only be exploited by a few microbes with the appropriate enzyme system. Cellulose is a linear polysaccharide linked by −1,4-linkages and organized in a variety of crystallinity patterns. The β-glycosidic bonds of the structural carbohydrate cellulose are highly resistant against starch. Niessen

Table 5.1 Microbial strains that are metabolically engineered leading to the generation of biofuel

Microbial strains (engineered)	Substratum for growth	Metabolic pathway followed	Yield generated	Production efficiency	References
Clostridium acetobutylicum, *Saccharomyces cerevisiae*	Glucose, corn, starch	Clostridial acetoacetyl-CoA-derived pathway	*n*-Butanol	30 g/L	Das et al. (2020), Luo et al. (2017)
Clostridium autoethanogenum	Synthetic media	Ferredoxin oxidoreductase	Ethanol	10.3 mM	Liew et al. (2017)
Clostridium Tyrobutyricum	Glucose, xylose	Xylose metabolic pathway	*n*-Butanol	12 g/L	Yu et al. (2015)
Escherichia coli	Synthetic media	Fatty acyl-ACP reductase-dependent	Fatty alcohol	0.75 g/L	Liu et al. (2014)
Saccharomyces cerevisiae	Glucose, galactose	Butanediol biosynthetic	2,3-Butanediol	100 g/L	Lian et al. (2014)
S. cerevisiae strain XUSAE57	Xylose, glucose	Xylose-isomerase pathway	Ethanol	0.49 g/L	Ko et al. (2020)
Enterobacter cloacae	Lignocellulose	Pentose phosphate	2,3-Butanediol	119.4 g/L	Li et al. (2015)
Klebsiella pneumoniae	Glucose	Meso-2,3-butanediol synthesis	2-Butanol	1030 mg/L	Chen et al. (2015)
Methylobacterium extorquens	Ethylamine	Ethyl malonyl-CoA	1-Butanol	13.6 mg/L	Hu and Lidstrom (2014)
Synechocystis sp.	Glucose	Ehrlich	Isobutanol	450 mg/L	Varman et al. (2013)

et al. (2004) have reported current densities between 1 and 1.3 $mAcm^{-2}$ generated from the digestion of starch by *C. butyricum* and *C. beijerinckii*. Bioelectricity production by *C. hydrolysis*, while the association of cellulose to lignin makes it a recalcitrant substrate for enzymatic and microbial hydrolysis. Amylose and amylopectin are two types of polysaccharides found in starch. Amylose is made up entirely of D-glucose residues with -(1,4) linkages, while amylopectin contains 5% -(16) branch interactions; to utilize starch, bacteria must break down the complex structure using extracellular enzymes α-amylase and glucoamylase (Singh 2008). *Clostridia* species are capable of digesting several species including complex carbohydrates such as cellulose and *C. acetobutylicum* and *C. thermohydrosulfuricum* utilizing cellulosic waste from the paper industry as reported by Singh (2008). Using rumen microorganisms dominated by *Clostridium* spp., Rismani-Yazdi et al. (2007) achieved a maximum energy density of 55 mW/m^2 with cellulose as substrate. Metabolism of electron in *Clostridium thermocellum* was engineered to boost the bioethanol synthesis (Lo et al. 2017). Table 5.1 displays the various strains of microorganisms that have been metabolically engineered to generate biofuel.

5.2.2 Desulfovibrio

Microbes of the genus *Desulfovibrio* are the most examined sulfate-reducing organisms. Sulfate-reducing prokaryotes are a heterogeneous group of microbes consisting of bacteria and archaea which are capable of using different carbon sources as electron donors and sulfate as TEA. Sulfate-reducing bacteria (SRB) utilize easily degradable compounds such as fermentation products and intermediate breakdown products; according to Hansen (1994), the available pure cultures of sulfate reducers are capable of oxidizing only over 125 compounds. *Desulfovibrio* species are well-known to preferably use low-molecular-mass compounds such as fumarate, lactate, pyruvate, malate, ethanol, and glycerol or molecular hydrogen as substrates for sulfate reduction (Fareleira et al. 1997; Matias et al. 2005; Savla et al. 2020). The breakdown of these compounds results in the formation of acetate and CO_2; although the genome annotation of *D. vulgaris* indicates the absence of pyruvate dehydrogenase, *D. vulgaris* is reported to change pyruvate to acetyl-CoA and CO_2 via an oxidoreductase (Tang et al. 2007). The capability of *Desulfovibrio* to employ sugars and the possible pathway remain uncertain. Although the annotated genome sequence of *D. vulgaris* has genes coding for the enzymes in glycolysis, Tang et al. (2007) have shown that it is not capable of developing on glucose or fructose. However, *Desulfovibrio fructivorans* has been reported to develop on fructose as the only source of carbon (Ollivier et al. 1988). Very few SRBs have the potential to degrade natural biopolymers such as glycogen, starch, and proteins or lipids therefore depending on the activities of other organisms for providing them with fermentation and degradation products. Early attempts to use SRB for producing biofuel included the investigation of the potential use of *Desulfovibrio desulfuricans* Essex 6 that recuperates electrons emitted by the reduction of sulfate to hydrogen sulfide with lactate as carbon source (Cooney et al. 1996). Chou et al. (2013) operated five double-chamber biofuel-producing reactors with a mixed culture of SRB supplemented with artificial contaminated water consisting of lactate and sulfate.

5.2.3 Enterobacter

Enterobacter are bacteria belonging to the *Enterobacteriaceae* family, which includes *Klebsiella pneumoniae*, *Klebsiella oxytoca*, and *Serratia marcescens*. They grow quickly in a simple medium and metabolize glucose and xylose to form 2,3-butanediol (Ji et al. 2011; Li et al. 2015). They preferably use glucose as substrate and inhibit the pathway of other carbon sources when they are present with glucose, and they will be used once glucose is depleted. The strain SDM of *Enterobacteriaceae cloacae* has been reported to efficiently use xylose and pentose sugars and arabinose from lignocellulose and other cheap biomass such as cassava for 2,3-BD production (Peters 2007). The breakdown of these monosaccharides

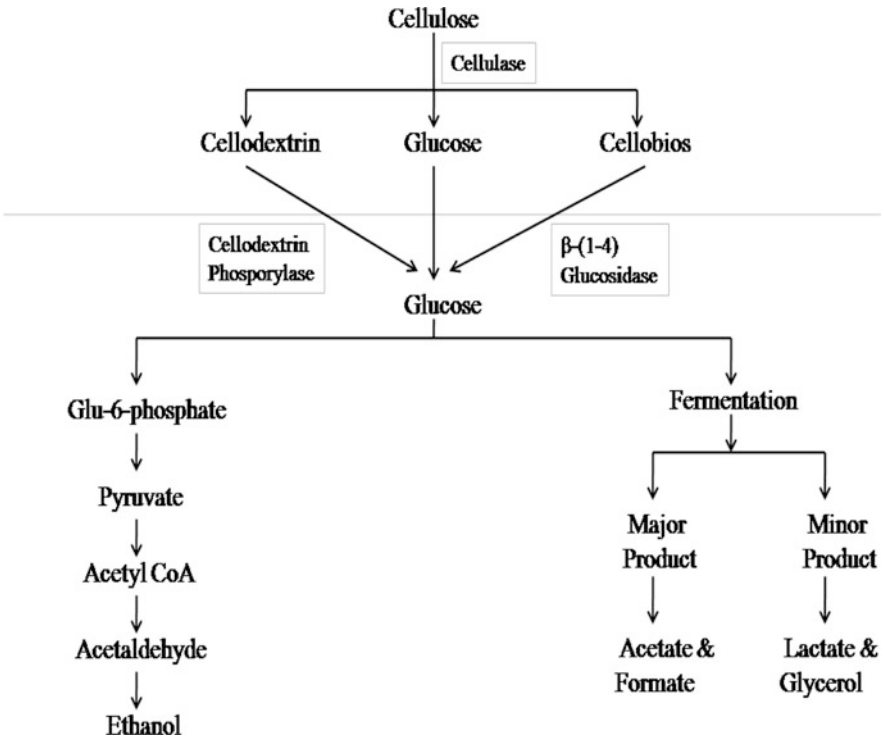

Fig. 5.1 Cellulose breakdown mechanism

starts by the conversion into pyruvate in a comparatively simple manner via the Embden-Meyerhof pathway. The pyruvate so produced is channeled into lactate, formate, acetate, succinate, acetoin, and ethanol through a mixed of acid-2,3-BD fermentation pathway (Magee and Kosaric 1987; Song et al. 2019). It has been assumed that *Enterobacter* could degrade cellulose as they possess endo-1,4-β-D-glucanase activity (Sami et al. 2008). This was further confirmed as *E. cloacae* was used as the source of microbes to accomplish both cellulose degeneration (Fig.5.1) and power production (Rezaei et al. 2009). It was however found that the strain ATCC 13047 could not utilize common fermentation end products; hence, the deposition of different volatile fatty acids and acetate was observed when a pure culture was fed on cellulose; the addition of other microbial strains will therefore be required for total utilization of the breakdown products to generate biofuel.

5.2.4 Pseudomonas

Pseudomonas species have a great oxidizing potential for various monoterpene hydrocarbons, along with the monoterpene aldehydes and alcohols (Schrader and

Berger 2001). Geranic acid was formed when geraniol was oxidized within the cells of *P. putida* DSM 12264 grown in E2 medium (Mi et al. 2014). Due to the greater oxidation capability of *P. putida* DSM 12264, the production rate of geranic acid was around 0.43 mM/h. Yet another strain of *Pseudomonas* species, *P. putida* KT2440, was engineered which gave a geranic acid concentration of 5 mM. The geraniol synthase (GES) gene was taken from *O. basilicum* for de novo geraniol synthesis in *P. putida* (Iijima et al. 2004). This was then inserted into *P. putida* using a plasmid vector pMiS1, to obtain a new strain. As a result, geraniol was generated at around 0.6 μM concentration, whereas geranic acid contributed to about 8 μM. There was a zero manufacturing of geraniol and geranic acid in the *P. putida* DSM 12264 which has no vector incorporated into it (Speelmans et al. 1998).

Various phenazine compounds including phenazine-1-carboxylic acid (PCA) (Thomashow et al. 1990) and 2-hydroxy-phenazine (2-OH-PHZ) (Liu et al. 2016) are formed by *P. aeruginosa* and *P. chlororaphis*, respectively. An antimicrobial compound, phenazine-1-carboxamide (PCN), was extracted and purified by HPLC and was investigated for the phenazine production under wild-type strain of *Pseudomonas*. However, the effects of an engineered strain, *P. chlororaphis* HT66, displayed a higher production of phenazine of about 424.87 mg/L. Single mutants like HT66Δ*psrA* and HT66Δ*rpeA* and double mutants like HT66Δ*psrA*Δ*rpeA* were examined for the impact of the omission of *psrA* and *rpeA* genes on phenazine bio-production in *P. chlororaphis* HT66. It was conjectured that the *psrA* controls the phenazine production via quorum sensing autonomous regulators *rpeA*/*rpeB* in *P. chlororaphis* HT66 (Peng et al. 2018).

5.2.5 Thermophilic bacteria

Thermophiles may break down cellulose and hemicellulose, as well as ferment pentose and hexose sugars synthesized during polysaccharide hydrolysis (Arora et al. 2015). The addition of thermophilic bacteria to the fermentation process improves ethanol production. *Clostridria*, *Thermoanaerobacter*, and *Geobacillus* are the most common thermophilic bacteria with ethanologenic characteristics (Taylor et al. 2009). *T. ethanolicus* and other *Thermoanaerobacter* species ferment D-glucose and D-xylose from composite biomass (Scully and Orlygsson 2015). On employing beet molasses for the substratum, the ethanol is produced at about 6.65 g/L. *Thermoanaerobacter* BG1L1 consumes raw corn and wheat hydrolysates along with 42% of xylose intake. Hence, ethanol is produced by the corn stover at about 63.3 g/L, whereas wheat hydrolysate yields an amount of 50.9 g/L (Di Donato et al. 2019).

Thermoanaerobacter sp. X514 was engineered to convert crystalline cellulose to obtain a five times higher generation of ethanol (Williams-Rhaesa et al. 2018). *Thermoanaerobacterium saccharolyticum* is transformed by the deletion of genes to enhance the production of second-generation bioethanol. *Thermoanaerobacterium* is a genus for few anaerobic bacteria that are genetically

manipulated by subduing the formation of residues and by excessive expression of dehydrogenase enzymes. Eliminating the gene *ldh* in *T. mathranii* BG1 showed an increment in the production of ethanol by 35%. Excessive expression of a bifunctional alcohol and aldehyde dehydrogenase in *T. mathranii* BG1L1 removes the *ldh* and yields ethanol for about 95% (Yao and Mikkelsen 2010). On the other hand, engineering of the *T. saccharolyticum* TD1 gave a 98% yield of ethanol (Bala and Singh 2019). On elimination of the genes *ldh* and *pfl* (pyruvate-formatelyase) in *Geobacillus thermoglucosidasius*, the yield of ethanol increased twice the previous amount. The regulation of expression of *pdh* gene in this strain produced 15 g/L of ethanol (Cripps et al. 2009; Van Zyl et al. 2014).

5.2.6 Zymomonas

Commercial generation of ethanol is carried out by employing *Zymomonas mobilis* which converts glucose, a hexose sugar, to bioethanol. However, it is unable to act on pentose sugar to convert it, unlike *Saccharomyces cerevisiae*. This becomes a hindrance in the production of biofuel from lignocellulose which is enriched in pentose sugars (Zhang et al. 1995). *Z. mobilis* was engineered in such a way that it utilizes pentose sugar for synthesizing bioethanol. Genes coding for xylose isomerase, xylulokinase, transaldolase, and transketolase enzymes were inserted into *Z. mobilis* and were allowed to express. This leads to the formation of a metabolic pathway that can easily convert xylose to glyceraldehyde-3-phosphate and fructose-6-phosphate of the EMP pathway resulting in generation of ethanol (Fig.5.2). Current research designed an improvised strain, *Z. mobilis* TMY-HFPX, which produced 90% of ethanol by the consumption of xylose (Wang et al. 2016).

5.2.7 Bacillus

A strain of *Bacillus subtilis* is produced when the gene encoding lactate dehydrogenase is blocked by the insertion of a chromosome containing the genes coding for pyruvate decarboxylase and alcohol dehydrogenase II from *Z. mobilis*, which is administered by the ldh native promoter. This strain is tagged as BS35 (Majidian et al. 2018). In comparison to the wild species of the bacterium, the new strain decreased the cell growth and glucose intake by 60–70%. However, it generated enough ethanol and butanediol. For yielding a higher concentration of ethanol of about 89%, further alteration of the BS35 to BS36 (BS35 *ΔalsS*) is necessary. The incorporation of a chromosome of transhydrogenase gene from *E. coli* inactivates the *alsS*, producing a new strain BS37 (BS35 *ΔalsSudhA*+) which has the potential to generate 8.9 gL^{-1} of ethanol (Romero et al. 2007).

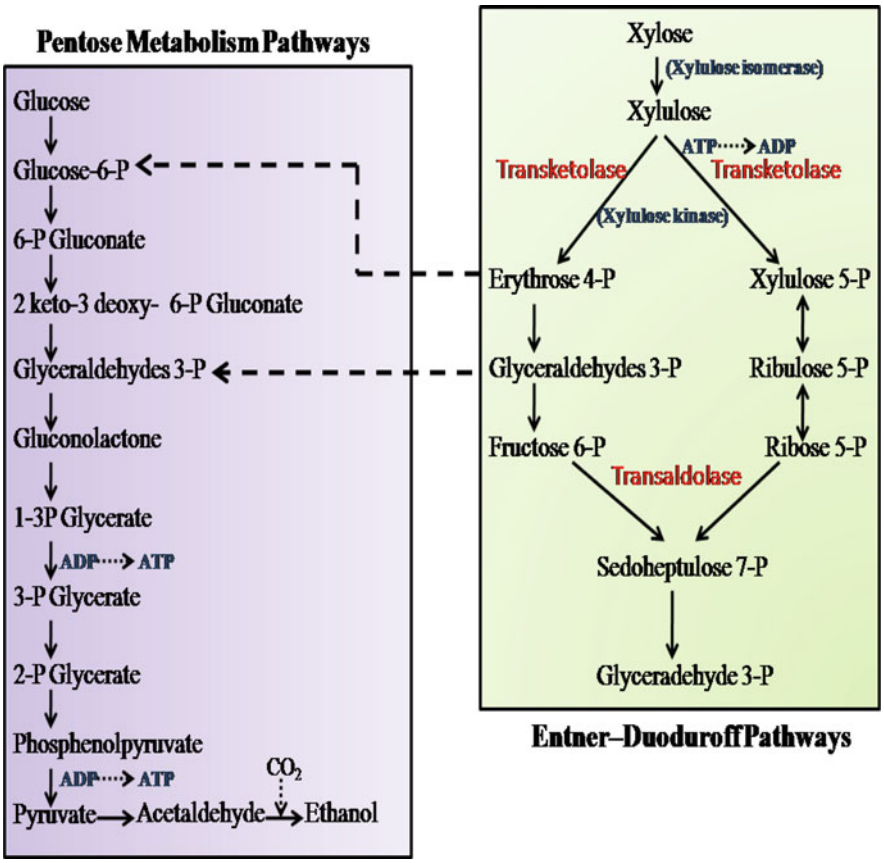

Fig. 5.2 Pentose metabolism pathway and Entner-Doudoroff pathway *by Zymomonas mobilis*

5.2.8 Klebsiella

Chen et al. (2015) altered a strain, *Klebsiella pneumoniae* HR526, to synthesize 2-butanol. The researchers added diol dehydratases and alcohol dehydrogenases to the bacterium's 2,3-butanediol production pathway. This led to the synthesis of 1030 mg/L of 2-butanol. *K. pneumoniae* M5al fermented glycerol to synthesize ethanol with a maximum concentration of 18 g/L when exposed to nitrogen (Cheng et al. 2007; Oh et al. 2011). When lactate synthesis was lowered by omitting the gene producing lactate dehydrogenase, a strain of *Klebsiella oxytoca* M5al with a glycerol synthesis of 19.5 g/L was engineered, which produced more ethanol (Yang et al. 2007). A γ-irradiated mutant strain of *K. pneumoniae* was discovered to produce significant amounts of ethanol from glycerol. The genes for pyruvate decarboxylase and alcohol dehydrogenase from *Z. mobilis* were expressed in *K. oxytoca* M5A1 to

synthesize ethanol (Ohta et al. 1991). The optimal recombinant strain M5A1 (pLOI555) produced 40 g/L of ethanol.

5.2.9 Escherichia coli

Gonzalez et al. (2008) demonstrated that *Escherichia coli* can anaerobically ferment glycerol. High expression of aldehyde dehydrogenase and excision of lactate dehydrogenase resulted in a new *E. coli* strain, and a maximum concentration of ethanol of 20.7 g/L was achieved (Durnin et al. 2009). *Z. mobilis* genes for pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adhII*) were introduced into the chromosome of *E. coli* B at the pyruvate formate lyase (*pfl*) site to develop *E. coli* strain KO11.The resulting KO11 strain was capable of producing large volumes of ethanol. Another mutant of *E. coli* KO11 (*E. coli* SE2378) has been produced that can efficiently ferment both hexose and xylose to ethanol using just natural enzymes (Xu and Koffas 2010).

5.3 Other Strains

5.3.1 Microalgae

Biodiesel can be made from a variety of microalgae species. Biodiesel made from microalgae has a significant advantage and is referred to as a third-generation biofuel. Microalgae grow quite quickly compared to other crops, and many of them are extremely oily. Microalgae increase biomass content to twice the original amount within 24 h. Similarly, exponential growth increases biomass to four times in 3.5 h. Microalgae can have an oil content of up to 80% of dry biomass, and oil contents of 20–50% are normal (Spolaore et al. 2006).

Most significantly, autotrophic algae do not compete with beginning plant materials for biofuel generation because of their photosynthesis. Algae, on the other hand, fix CO_2 and thereby reduce its concentration in the atmosphere, a gas that contributes to global warming. In reality, a few start-up businesses are presently experimenting with the idea of capturing carbon dioxide streams released by coal plants for autotrophic, photosynthetic microalgae development (Tollefson 2008).

Furthermore, research is also being performed into the use of heterotrophic algae for the generation of biodiesel employing sugars as a substrate. When compared to phototrophic algae, heterotrophic algae can achieve substantially higher growth densities (and thus biodiesel concentrations). In addition, unlike phototrophic algae, heterotrophic algae's dark development provides no engineering challenges. However, the method necessitates the use of beginning plant materials as substrates, and the technique's overall economic sustainability is now being investigated (Metting 1996).

Despite the present rapid rate of fossil fuel depletion, many nations are substantially investing in algae-based biofuel production, including genetic modification (GM) research to improve baseline biofuel properties. The latter is particularly crucial because no strong, commercially viable strain has emerged from non-GM algae research in the recent decade. As a result, GM algae strains are being studied in order to make algal biofuel more financially viable than fossil fuels. However, the commercial mass growth of GM algae in open ponds, with the potential for environmental contamination, is a major cause of concern (Chowdhary and Raj 2020).

Although the green algae *Chlamydomonas reinhardtii* has been genetically manipulated to exhibit several critical biofuel properties, most of the model strains have poor biomass production rates and lipid content, preventing them from being industrially relevant (Ahmad et al. 2015). For instance, the phenotypic variation observed in different wild-type isolates of the green algae *Chlamydomonas reinhardtii* may now be easily connected with the genomic sequence of these isolates, allowing researchers to link alterations in genomic DNA sequence to desirable phenotypes (Flowers et al. 2015). In the other study, Radakovits et al. mapped the genome of *Nannochloropsis gaditana* and devised transformation methods for modifying the lipid synthesis pathways, allowing significant strain improvements utilizing genetic engineering (GE) technology (Radakovits et al. 2012).

5.3.2 Saccharomyces cerevisiae

Saccharomyces cerevisiae lacks the ability to metabolize xylose. However, two pathways have been examined to incorporate this ability into the yeast cells (Cai et al. 2012; Kim et al. 2013). The oxidative-reductive route, catalyzed by the enzymes xylose reductase (XR) and xylitol dehydrogenase (XDH), is one of them. The xylose isomerase (*xylA*) pathway, on the other hand, is more efficient. This pathway follows a one-stage transformation of xylose to xylulose. The total amount of ethanol which is generated is enhanced (Li et al. 2016). PE-2 is a strain that ferments xylose into ethanol efficiently.

5.4 Co-Cultivation Systems for Biofuel Synthesis

Synergism, commensalism, competition, and mutualism are all used by artificial microbial consortia to build a co-cultivation system (Ding et al. 2016). In the synthesis of biofuel, these systems have proven to be effective. Few of the examples are discussed as follows (Jiang et al. 2019).

Argyros et al. (2011) developed a *C. thermocellum-T. saccharolyticum* co-cultivation framework in which organic acid generating pathways in both strains were abolished, yielding 38 g/L of ethanol. For cellulosic synthesis of ethanol, a

Bacillus/yeast framework was created. Eight cellulosomal genes derived from *C. thermocellum* are inserted in the recombinant *B. subtilis*. The gene encoding glucosidase (NpaBGS) from a rumen fungus is harbored by the yeast ally, *Kluyveromyces marxianus* KY3-NpaBGS. As a result, 9.5 g/L of ethanol is synthesized (Ho et al. 2012). A co-cultivation system was constructed in which *C. phytofermentans* and *S. cerevisiae* performed cellulose hydrolysis and ethanol synthesis, yielding 22 g/L of ethanol (Zuroff et al. 2013).

5.5 Future Prospects and Conclusion

Concerns about global warming, combined with the depletion of fossil fuels, have promoted increased interest in alternative commercial fuels. To produce a better fermentation product, the model strain for biofuel generation must be capable of using a large amount of substrate, transmitting sugar via rapid and regulatory routes, and withstanding inhibitory substances and end products and have higher metabolic fluxes. By exploiting metabolic pathways, engineering microorganisms could be an excellent method for producing biofuel from lignocellulosic biomass at a low cost (Chowdhary et al. 2020). Metabolic engineering is a cutting-edge approach for creating highly efficient microbial cell factories, and it's an important part of the next-generation bioeconomy. Microbial metabolic engineering is a difficult process, particularly when it comes to identifying efficient strains and that it is critical for the biofuel industry's progress. The key metabolic pathways must be fully comprehended, as well as the enzymes involved. Metabolic engineers and synthetic biologists are employing several methodologies to identify the limitations of pathways. The metabolic engineering approach for improving recombinant protein expression continues to develop and become more sophisticated. Industrial microorganisms have been altered or created to increase the output of recombinant metabolites despite saving time and resources.

CRISPR/Cas9, a modern technology that allows for rapid and effective genome editing, is being used to speed up microbial genetic engineering. The Cas9-sgRNA attaches to the specific DNA sequence and uses the Cas9 protein endonuclease to cleave it. This facilitates protein metabolic engineering and gene editing, which could improve inhibitor resistance or increase the use of alternative biofuel substrates.

In conclusion, microorganisms play a significant role in biofuel production. However, the yield of natural strains is insufficient, necessitating the development and improvement of new strains using gene modification techniques. Current research has emphasized on using gene alteration to maximize high output and energy value at a lower expense of manufacturing model strains. There is a good chance that database mining will uncover more distinct metabolic pathways for biofuel synthesis in the near future. Using modern technologies such as omic technologies and the CRISPR/Cas9 system, metabolic engineers can develop new strains of microbes for the efficient synthesis of biofuel.

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Chapter 6
Genetic Engineering: An Optimism for Sustainable Biofuel Production

Hiren K. Patel, Jaydeep B. Dobariya, and Priyank S. Patel

Abstract Fossil fuel depletion, rising demand, and global climate change problems have motivated researchers to find alternative sources of renewable fuel. Biofuels offer a possible road to the avoidance of global market instability and environmental concerns resulting from traditional fossil fuels. Most biofuels are presently derived from starch or other carbohydrate from different sources, such as microbes, plants, algae, etc., in the form of ethanol. The quantity and sample preparation procedures involved, however, are quite costly. As an alternative genetic engineering organism, large quantities of raw products are produced, and the cost of sample preparation is also minimized by metabolic process alteration. Genomics methods encourage genetic selection approaches to give significant genetic improvement of organisms as biomass sources for biofuel generation. Genetic alteration of organisms offers a wide range of possibilities for improving biomass composition and helping generate biofuels and ensures a safe and environmentally friendly energy source. While there are considerable barriers to making biofuel generation cost-effective and on a large size to replace a large proportion of transporting petroleum, as well as potential environmental issues, they have been examined extensively.

Keywords Biofuel · Genetic engineering · Genetically modified crops (GMO) · CRISPR/Cas9

6.1 Introduction

Petroleum is the essential wellspring of energy utilized in cooking, transportation, fabricating, and so forth. Nonetheless, our fuel assets are running out because of an impractical utilization by an always expanding total populace. Indeed, the explosive growth in fuel use, specifically in fast-growing agricultural countries, has been

H. K. Patel (✉) · J. B. Dobariya · P. S. Patel
School of Science, P. P. Savani University, Surat, India

P. S. Patel
School of Science, P. P. Savani University, Surat, India

P. Chowdhary et al. (eds.), *Bio-Clean Energy Technologies: Volume 1*, Clean Energy Production Technologies, https://doi.org/10.1007/978-981-16-8090-8_6

expected to result in a half growth in petroleum demand by 2025 (Paudel and Menze 2014). Not only has indiscriminate exploitation and use of minor petroleum reserves contributed to the depletion of energy sources, but it has also harmed the ecosystem. Exhaustion of petroleum derivative stores and the expansion in worldwide temperature because of expanded degree of climatic carbon dioxide (CO_2) are inescapable twin issues of consuming nonrenewable energy sources. Enormous endeavors looking for elective and manageable wellsprings of energy to substitute or possibly to enhance petroleum product are being made everywhere on the world. Today's rise in fuel demand is still driven by oil production, but the fact that oil is a scarce resource and that its availability may be jeopardized in the future are important motivators for this massive global investigation into alternative energy sources such as biofuels. Biofuel is defined as solid, liquid, or gaseous fuels derived from biorenewable materials, according to (Demirbas 2011).

Ethanol is a biofuel that is expected to be most commonly used worldwide, as it can be obtained from abundant biomass supplies from every life form and plant-based components, including animal waste, starch, sugar, and oil plants that have been successfully used for food and energy. Furthermore, ethanol is nonpoisonous, degrades quickly, and produces fewer air pollutants than fossil fuels. Greenhouse gas levels are also decreased by the growth of raw material crops for bioethanol processing, largely due to the use of greenhouse gases in the atmosphere in photosynthetic activity. Because the converting biomass into ethanol and the burning of ethanol produce pollution, a considerable reduction in greenhouse emissions compared to fossil fuels may have a net effect, implying that using bioethanol somehow doesn't result in an increase in net atmospheric carbon dioxide emissions (Bothast and Schlicher 2005).

Furthermore, biofuel, mainly biodiesel derived from plant oils, brings enormous long-term possible outcomes due to its sustainability, higher efficiency of exhaust emissions, biodegradability, photorespiration origin of carbon in the air, and non-toxicity and is essentially free of sulfur and alkanes. Biodiesel is not only a limitless renewable source for diesel engines, but it also has the potential to reduce pollution and carcinogenic (Singh and Singh 2010; Chowdhary et al. 2020; Chowdhary and Raj 2020).

There are two significant biorenewable fuel fills worldwide, bioethanol and biodiesel. Biodiesel pulled in numerous specialists and governments for its utilization as option inexhaustible diesel fuel. It is nontoxic and biodegradable and has lower emanation of greenhouse gas when consumed. Biofuels can be classified into four categories: first generation biofuels (FGBs), second generation biofuels (SGBs), third generation biofuels (TGBs), and fourth generation biofuels (FGB).

6.1.1 First-Generation Biofuel

First-generation biodiesel can be acquired utilizing consumable oil like soybean and sunflower oil. Trans-esterification of this oils or just breaking of it can yield

biodiesel. Utilizing yeast fermentation of the harvest plants yields bioethanol. Sugars from wheat, grain, and potato are appropriate for creation of bioethanol. Original biofuels are extremely savvy; its lower creation cost makes it more available to non-industrial nations. Albeit, higher creation of it can make issues for farming businesses (Rizwan et al. 2019).

6.1.2 Second-Generation Biofuel

Second-era biofuel come from lignocellulosic biomass from agrarian waste and non-eatable oil of *Jatropha curcas* L. feedstock. Notwithstanding second-era bioethanol and biodiesel from starch and greasy harvests, biobutanol and syndiesel are additionally created from lignocellulosic biomass. Second-era biofuel creation is more possible because of economical crude materials and more effective utilization of land space. Additionally, it utilizes non-palatable oils; thus, harm to agrarian ventures is low. Albeit, the expense of the proteins utilized in the process is a lot higher which makes the creation of second-era biofuels all the more exorbitant (Rizwan et al. 2019).

6.1.3 Third-Generation Biofuel

Third-era biofuel can be obtained from macroalgae and microalgae. Among these feedstocks, microalgae are the most productive ones. Here, we convert sun-oriented energy into fluid fuel utilizing photosynthetic microorganism. Microalgae are single-celled or multilayered photosynthetic microorganisms that can be prokaryotic or eukaryotic. Prokaryotic microbes are represented as *Chlorophyta*, and diatoms are *Cyanophyceae* and eukaryotic microalgae, respectively (*Bacillariophyta*) (Mata et al. 2010).

There are numerous benefits to utilizing these microalgae or cyanobacteria for biofuel creation, for example, they have sequenced genomes, and furthermore, hereditary apparatuses are accessible for control, higher developing rate, exceptionally proficient photosynthesis, and high lipid content. Microalgae can absorb CO_2 from the air while also capturing solar energy at a higher rate than earthbound plants. Microalgae is also used in the production of bioethanol. They grow at a rate that is several times faster than earthbound plants, and they can double their biomass in a single day. Their ability to fill in harsh circumstances with a limited supply of supplements allows them to fill in zones that are otherwise unsuitable for cultivation (Lam and Lee 2012).

6.1.4 *Fourth-Generation Biofuel*

Fourth-generation biofuels (FGB) rely on genetically engineered (GE) algae to improve bioenergy. Even though genetically engineered algal biofuels are a well-known alternative to fossil fuel sources, human health hazards remain a major concern. An assessment of these issues and, as a result, the development of effective mitigation measures to address them are critical to the commercialization of FGB. While much study has been done on genetic engineering and other developments aimed at increasing the production of algae strains, only a few of them address the legal restrictions on harvesting and processing genetically modified algae (Fig. 6.1).

6.2 Genetic Engineering

Basically, it is a process to manipulate genomes of an organism to introduce desirable traits. Genetic engineering also provides information about many metabolic pathways and enzymes associated with it. It can also be used to find cis-acting and trans-acting elements, as well as other regulating components. It is the process of adding or removing genes to alter its metabolic functions in a way such as production of a certain metabolites increases. Many environmental and nutritional elements affect control of metabolic pathways (ACCase) (Jarvis and Dais 1996).

In any organism at a time, thousands of metabolic reactions are ongoing involving large set of organic molecules. By metabolic pathway engineering, concentration

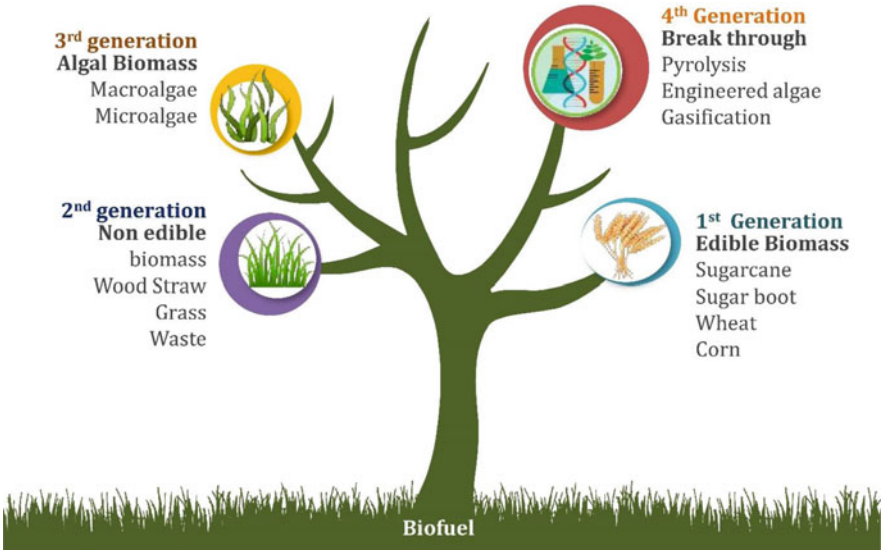

Fig. 6.1 Different generations of biofuel

of certain metabolites can be increased or decreased. There are many different approaches to it. One of them is "knockout." Genes that are involved in synthesis of unwanted biomolecules can be knocked out. It can be achieved by RNA interference (RNAi) technology, which reduces concentration of mRNA of proteins involved. Another approach is overexpression of the corresponding gene. Concentration can be enhanced by altering the gene transcription involved in the production of desired macromolecules (Larkum et al. 2012).

6.3 Role of Plant Genetic Engineering to Enhance Biofuel Production

Developments of new varieties of plants with higher concentration of oil and enhanced quality of it have been made by using some conventional breeding strategies. To achieve these genetic traits, germplasm collection, somaclonal variation, inter-species hybridization, mutation, and genetic engineering have been used, although these techniques are very time-consuming. Recent advances in sequencing techniques such as NGS and other metagenomics, metabolomics, and other novel approaches in genetic engineering can be extremely beneficial tools for generating desired oil crops.

Plant cells are low in starch, with cellulose, hemicellulose, and lignin constituting the cell wall. In addition to these components, arabinose, xylose, galactose, and other polysaccharides are also available. Using yeast, these plant cell constituents can be fermented into ethanol. However, fermentation of cellulose and hemicellulose is a simple process, but presence of lignin hinders it. Plants having lower content of lignin proved to be more easily processed using enzymes, because this biomolecule can bind to cellulose degrading enzymes irreversibly, and it reduces the efficiency of these enzymes.

Mixture of different polysaccharides with cellulose makes the process more difficult for biochemical conversion of cellulose to simple sugar for biofuel production. Therefore, genetic modifications leading to increased cellulose content are a primary way to increase efficiency of biofuel production. Cellulose synthase enzymes are involved in cellulose synthases, and the desired objective can be achieved by altering these enzymes. CES3A is a mutated gene found in tobacco plants that leads to increased cellulose synthesis (Furtado et al. 2015).

6.3.1 Production of Hydrolysis Enzymes in Plants

Fundamentally, it is an interaction to control genomes of an organic entity to present attractive qualities. Hereditary designing additionally gives data about numerous metabolic pathways and proteins related with it. It is additionally useful for the

recognizable proof of cis-acting components, executing components, and other administrative variables. It is the way toward adding or eliminating qualities to modify its metabolic capacities in a manner, for example, creation of a specific metabolites increment. Numerous natural and dietary components influence control of metabolic pathways (ACCase) (Sticklen 2008).

When cell wall degradation enzymes are concentrated in cellular components, they are preferred over cytosol precipitation in plants. When targeted for deposition in cellular components, these enzymes are more likely to show appropriate packing and activity, glycosylation, less fragmentation, and higher stability than when targeted for deposition in the cytosol (Sticklen 2006). The recombinant enzymes can be isolated as part of the plant total soluble protein (TSP) from fresh or dry recombinant crop biomass and then added to pretreatment crop biomass for processing into fermentable sugars. TSP can be collected quickly and easily from fresh or dry material, and it might be employed in ethanol production facilities. However, more research is needed to assess the biological capacity of isolated plant-produced hydrolysis enzymes in TSP when treated for different lengths of time before being used in degradation at cold temperatures.

Subcellular targeting has already resulted in the production of several microbial hydrolysis enzymes in plants. The majority of studies have been done on tobacco and alfalfa, which are not commonly used as biomass fuels. As mentioned above, because of possible interaction with metabolic processes, the cytosol may not be an optimal place for the deposition of heterologous substances. In many situations, the apoplast has been chosen because it is the most voluminous division and hence capable of storing enormous amounts of foreign molecules (Ziegler et al. 2000). This compartment, on the other hand, is best used for the storage of thermophilic hydrolysis enzymes (biologically active at higher temperatures). If the recombinant enzymes remain active at in situ temperatures, they can destroy plant cell walls prior to lignification. The targeting of the same enzyme to several compartments in the same plant may enhance the efficiency of enzyme activity, as shown for xylanase regulating either chloroplasts or peroxisomes independently compared to its synthesis in both divisions in the same plant (Hyunjong et al. 2006).

6.3.2 Increasing Cell Wall Polysaccharide Content

The identification of characteristics involved in both cellulose and hemicellulose production has been aided by functional genomics and mutant research. Despite the fact that cellulose production has been studied for a long period of time, the majority of the steps in this process are still unknown ((Andersson-Gunneråsa et al. 2006); (Haigler et al. 2009); (Abramson et al. 2013)). Future research will focus on improving our understanding of the biosynthesis of these plant cell-divider polysaccharides, as well as their genetic control to produce polysaccharides for better cellulosic biofuel production.

6.3.3 Increasing the Overall Biomass

Genetically altering feedstock plants could also result in increased and enormous fodder biomass. This could entail tinkering with plant growth regulators. Transgenic mixed poplar with increased gibberellin production, for example, demonstrated enhanced growth and increased biomass (Eriksson et al. 2000), Gibberellin's effects on plant height are most able to convict. There are a few different possibilities for increasing plant biomass in particular (Sticklen 2007). A new study including genetic manipulation has yielded encouraging results in the goal of increasing biomass in a high-yielding plant. ADP-glucose pyrophosphorylase (AGP), a key enzyme in endosperm starch production, was expressed at higher levels in rice using an endosperm-explicit promoter. This resulted in a stunning 20% increase in plant biomass (Smidansky et al. 2003).

6.3.4 Lignin Modification

Downregulation of lignin biosynthesis pathway chemicals to alter the substance structures of lignin components and reduce plant lignin content is a promising strategy for lowering pretreatment costs in bioethanol production from cellulosic biomass (Ragauskas et al. 2006). Lignin is made up of three precursors that are made in separate but linked mechanisms: paracoumaryl, coniferyl, and sinapyl alcohols. Antisense oligonucleotides were initially used to downregulate lignin production catalysts; however, RNA interference (RNAi) technology has also been used.

Downregulation of 4-coumarate 3-hydroxylase (C_3H) in horse feed (*Medicago sativa*) brought about a sensational move in the lignin profile and ensuing adjusted lignin structure, causing improved absorbability of C_3H-insufficient hay lines in ruminants (Ralph et al. 2006). Downregulation of another lignin production pathway enzyme, cinnamyl alcohol dehydrogenase (CAD), in alfalfa led to changes in lignin residue composition and enhanced in situ digestibility, according to another study. However, the downregulation of CAD in alfalfa did not contribute to a decrease in lignin levels in the crops. Another potential method is to shift plant carbon resources away from the synthesis of lignin, which has the potential to improve the strength properties of biofuels. For example, in aspen (*P. tremuloides*), energy shifts from lignin biosynthesis to polysaccharide production have been observed. Down guideline of 4-coumarate CoA ligase (4CL) brought about a 45% lessening in lignin content and an accompanying 15% increment in cellulose content. These figures were additionally expanded to a 52% decrease in lignin content and a 30% expansion in cellulose content when coniferaldehyde 5-hydroxylase (CAld5H) was likewise downregulated (Li et al. 2007). Finally, in transgenic tobacco, downregulation of cinnamoyl CoA reductase (CCR) resulted in a decrease in lignin concentration and an increase in xylose and glucose connected to the cell wall (Chabannes et al. 2001).

Significantly, genetic regulation of lignin biosynthesis pathway catalysts has been shown to reduce the need for pretreatment steps in the production of ethanol production. In a recent solid evidence study, downregulation of six different lignin biosynthesis pathway enzymes in alfalfa was demonstrated to reduce or eliminate the need for chemical pretreatment in the digestion of bioethanol ((Chen and Dixon 2007); (Chapple et al. 2007)).

6.3.5 Modifying Features of Cellulose

Increasing cellulose permeability can increase saccharification, providing another option for reducing pretreatment requirements. For instance, in green growth, exopolysaccharides, for example, acetan, hyaluronan, alginate, levan, and chitosan, are water dissolvable. Levan sucrose transgenic expression from the bacterium *Erwinia amylovora* (which facilitates the development of sucrose-based water-soluble fructan) increases the absorption of algal cell walls. Furthermore, cellulose production was boosted in transgenic algae that expressed exogenous hyaluronan and chitin synthase in the extracellular matrix (Kawasaki et al. 2002). These studies could be significant since algae could be exploited as a source of biofuel (Fig. 6.2).

6.4 Examples

6.4.1 Genetic Engineering for Improving Seed Size in Jatropha

Seed size is a key feature of improving oil yield in *Jatropha*, so increasing seed size is a potential to enhance the potential for oil storage. Sun (Sun et al. 2017) auxin response factor 19 (JcARF19) was shown to be a critical aspect in growing the size of seeds in *J. curcas*. They claimed that the overexpression of 19 (JcARF19) in two plants, i.e., *A. thaliana* and *J. curcas*, led to improved seed size. In proportion to the increase in the oil content of seeds and an increase in the majority of female flowers and, subsequently, in the number of fruits in Jatropha, an increase in the oil yield of this bioenergy plant was considered as a remedy. For instance, (Pan et al. 2016) thidiazuron may increase the fruit number by treating inflorescence meristems of *Jatropha*.

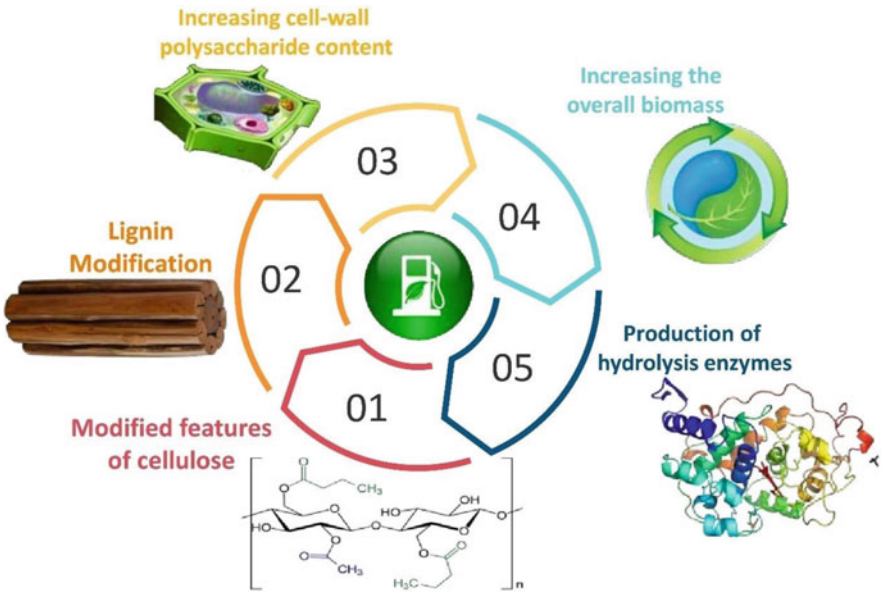

Fig. 6.2 Factors determining the biofuel production

6.4.2 Genetic Engineering for Improving Oil Composition in Jatropha

Designing the qualities ascribed to yield boundaries (leafy foods size, oil substance, leaf size and numbers, early blossoming, quickly developing, and so forth) is considered as the main technique to improve oil creation in *Jatropha*. The qualities encoding proteins engaged with oil biosynthesis or digestion just as those engaged with transcriptional control of these cycles are significant instruments to unfurl biotechnological upgrade of oil substance and quality in *Jatropha* by direct hereditary designing (Moniruzzaman et al. 2016). Three key proteins, i.e., DGAT, LPAT, and GPD, assume significant part in lipid biosynthesis pathway. In any case, different compounds, for example, carboxyl transferase of the ACCase b subunit, the biotin carboxyl transporter protein of ACCase, acyl ACP thioesterase A, acyl-CoA restricting protein, malonyl-CoA:ACPtransacylase, betaketo acyl ACP synthase II, 3-keto acyl ACP reductase, beta-keto acyl ACP synthase I, acyl transporter protein ω-3-unsaturated fat desaturase, ω-6-unsaturated fat desaturase, and long chain acyl-CoA synthetase, are likewise associated with lipid biosynthesis pathways in *Jatropha* and could be promising contender for oil yield and oil composition designing ((Natarajan et al. 2010); (Moniruzzaman et al. 2016)).

In plants, FAD2 is a crucial enzyme in the production of linoleic acid. In the genome of *Jatropha*, three potential fatty acid desaturase genes have been found. When the JcFAD2–1 gene was suppressed by RNAi technology, it resulted in substantial increases in oleic acid content and a decrease in polyunsaturated fatty

acid content in *Jatropha* plants. The acyl-acyl carrier protein (ACP) thioesterase called JcFATB1 is yet another gene involved in determining the oil composition in *Jatropha*. This gene enhances the concentration of saturated fatty acids and, in particular, palmitate in plant oils. So suppression of this gene would enhance the production of unsaturated fatty acids such as oleic acid (Qu et al. 2012).

6.4.3 *Genetic Engineering for Reduction of Toxic Metabolites in Jatropha*

The existence of certain toxic metabolites such as phorbol esters and curcins (a type 1 ribosome inactivating protein) in seeds is among the most significant disadvantages of *Jatropha* for the development of biofuel.

Skin-irritating and cancer-promoting compounds reduce the nutritional and health value of this species' seeds and oil (Gu et al. 2015). As a result, lowering these toxins will improve *Jatropha*'s oil content greatly. RNAi technology has been utilized successfully in some research trials to reduce *Jatropha* curcinum. The curcinum could be reduced by up to 98 percent if this gene is suppressed ((Patade et al. 2014; Gu et al. 2015). *J. curcas* leaves were used to acquire cDNA for the gene encoding curcin, i.e., curcin2. *J. curcas* MD44 has three Curcin genes that have been isolated and purified (Wu et al. 2009). Furthermore, RNAi was used to downregulate a phorbol ester gene (JcCASA: casbene synthase gene). This suppression strategy could effectively reduce the gene expression and resulted in a final 85% reduction of phorbol esters level in seeds (Li et al. 2014).

6.4.4 *Enhancing Biotic Stresses Tolerance in* Jatropha

Jatropha isn't impervious to most nuisances, and microorganisms produce a lot of a few antibacterial and poisonous alkaloids taking all things together its tissues; however, it has been shown that this plant could be assaulted by different organisms, infections, bugs, and so forth that cause critical monetary harm (Anitha and Varaprasad 2012). Consequently, it is additionally of importance to attempt *Jatropha* reproducing programs for nuisance and infectious prevention. The Archipsmicaceanus (Walker), a moth of the family Tortricidae, is known as perhaps the main irritations of *Jatropha*. In 2014, a half breed of cry1Ab and cry1Ac qualities encoding δ-endotoxin proteins from the bacterium *B. thuringiensis* which gives protection from lepidopteran bugs was communicated in *Jatropha* utilizing *Agrobacterium*-intervened change and a synthetically managed, Cre/loxP-interceded DNA recombination framework (Gu et al. 2015). It was reported that the insecticidal bioassays of the GM line of *Jatropha* resulted in 80–100% mortality of larvae of *A. micaceanus* 4 days after infestation.

6.4.5 *Enhancing Abiotic Stresses Tolerance in* Jatropha

Despite its reputation as a drought-tolerant plant that grows in semiarid soils unsuitable for food crops, *Jatropha*'s oil yield is now uneconomical under such stress circumstances. Therefore, increasing drought tolerance is critical for the commercialization of this plant as a bioenergy plant and could lead to improving *Jatropha*'s oil profitability even in semiarid regions. Three kinds of drought tolerance transgenic *Jatropha* plants were developed by Tsuchimoto during an investigation (Tsuchimoto et al. 2012). Saltiness is another pressure antagonistically influencing the development and yield of *Jatropha*. Salt resilience in plants fundamentally relies upon various physiological, biochemical, and sub-atomic transformations. It is notable that antiporters Na+/H+ are significant variables engaged with plant salt resistance. They assist plants with keeping up Na + homeostasis in the cytosol. Ion homeostasis is typically performed either by vacuolar Na+/H+ antiporters (NHX1) sequestration of excess sodium into the vacuoles or by active exclusion by Na+/H+ antiporters (SOS1) situated on the plasma membrane (Shi et al. 2000).

6.5 Genetically Improving the Seed Size and Oil Content in *Camelina*

Camelina has been subjected to a variety of genetic modification studies in order to increase oil production, oil arrangement, and abiotic stress tolerance. The low oil content of *Camelina* plants is one of the disadvantages of using it as a biodiesel feedstock; therefore, increasing seed oil yields could be one of the main targets of GM systems. The compound encoded by the quality DGAT1 has been portrayed as one of the most important variables in plant oil biosynthesis pathways. Three DGAT1 qualities (CsDGAT1A, CsDGAT1B, and CsDGAT1C) were identified and recreated during the advancement of *Camelina* seeds (Kim et al. 2014). CsDGAT1B overexpression in *Camelina* reflected in a 24% rise in total seed oils and larger embryonic cells and a higher number of cells relative to the wild type. Recent times, under the guidance of seed-specific promoters, DGAT1 and yeast cytosolic GPD1 genes have been co-expressed in *Camelina*. This co-expression resulted in up to 13% higher seed oil content and up to 52% increase in seed mass compared with the wild-type plants. For the GM lines, the oil harvest index has been almost 2 times greater than the wild type (Chhikara et al. 2018). Another research used an *Agrobacterium*-mediated transformation with a vacuum infiltration technique to transfer the castor fatty acid hydroxylase gene to *Camelina* to increase hydroxyl fatty acid synthesis in the fruit. The genetically engineered seeds showed the synthesis of a new castor fatty acid hydroxylase (Krohn and Fripp 2012).

6.5.1 *Genetically Improving Oil Composition in* Camelina *Seeds*

The degree of immersion and length of the FAs chain in oils have been shown to have a significant impact on fuel properties such as thickness, burning efficiency, start latency, and oxidative dependability. As a result, improving *Camelina*'s oil synthesis for better biodiesel is critical. Since higher oleic acid content improves biodiesel efficiency by increasing oxidative stability, lowering cetane number, and improving cold-flow properties, high oleic content *Camelina* lines are of great importance to the biofuel industry (Durrett et al. 2008). *Camelina* has benefited from new gene silencing methods such as RNAi and CRISPR/Cas9 genome editing. The repression of FAD2 and FAE1 genes by RNAi resulted in a reduction in linoleic, linolenic and eicosenoic acid levels, while oleic acid concentrated at rates as high as 66% (Krohn and Fripp 2012). CRISPR/Cas9 gene editing was recently used in *Camelina* to achieve selective mutagenesis for three related 12desaturase (FAD2) genes. Both mutations resulted in a decrease in polyunsaturated fatty acid levels and an increase in oleic acid accumulation in the crude. The heritability of single, double, and triple mutations in the three isological CsFAD2 genes was shown by genetic analysis of mutants over four generations. Furthermore, for the triple FAD2 locus, the combinatorial association of distinct alleles raised the accumulation of oleic acid in the oil of the mutant *Camelina* lines from 10% to 62 percent (Morineau et al. 2017). In another investigation, the CRISPR/Cas9 was utilized to focus on the FAD2 quality in *A. thaliana* and *C. sativa* to upgrade seed oil creation. Thus, transgenic *Camelina* seeds showed a critical increment of oleic corrosive substance (16-half of the unsaturated fat structure) and huge abatements in less attractive polyunsaturated unsaturated fats content, i.e., linoleic corrosive (a reduction from ~16% to <4%) and linolenic corrosive (a lessening from ~35% to <10%). These attributes were steady up to T3 and T4 age *Camelina* seeds (Jiang et al. 2017).

The subsequent procedure to streamline oil arrangement would be the statement of qualities engaged with the biosynthesis of medium-chain FAs (MCFAs, 8:0–14:0). These sorts of FAs are ordinarily utilized as Stream A fuel (Kallio et al. 2014). In an investigation, three FatB qualities (i.e., CpuFatB3, CvFatB1, and CpuFatB4) associated with high amassing of MCFAs in *Cuphea* seeds were recognized. The statement of the FatB qualities and coconut LPAT in *Camelina* in single or co-articulation structure prompted gathering of capric corrosive (10:0), 16:0, myristic corrosive (14:0), lauric corrosive (12:0), or 14:0 in *Camelina* seeds. Also, RNAi approach has been utilized for seed explicit concealment of *Camelina* KASII which brought about the decrease of 12:0 yet improved palmitic corrosive (16:0) and MCFA substance in seeds to copy Stream A fuel arrangement. In another investigation, concurrent articulation of a 12:0-acyl-transporter thioesterase quality (UcFATB1) from California sound (*Umbellularia californica* Nutt.) and concealment of the *Camelina* KASII qualities by RNAi develops prompted upgraded gathering of three medium-chain immersed FAs, including laurate (C12:0), myristate (C14:0), and palmitate (C16:0) in *Camelina* seed oils (Salehi Jouzani et al. 2018).

6.5.2 *Improving Biotic and Abiotic Tolerance in* Camelina

Camelina is usually resistant to pests and bacteria, although a few parasitic nematodes, such as *Plasmodiophora brassicae*, *Albugo candida*, *Erwinia carotovora*, *Fusarium sporotrichioides*, *Alternaria brassicae*, and *Candidatus phytoplasma asteris*, as well as some creepy crawly bothers (aphids) (Sainger et al. 2017). As a result, GM *Camelina* varieties encoding antimicrobial proteins such as cecropin P1 or defensin and pathogenesis-related genes (PR1 and PR3) have been successfully established to improve tolerance to *E. carotovora*, *F. sporotrichioides*, and *A. brassicae* (Chamil et al. 2014).

Camelina is often referred to as a drought adaptive plant, and this characteristic may lead to decreases in its yield productivity during abiotic stresses. Lee et al. (2014) moved a record factor quality MYB96 to *Camelina*. The outcomes affirmed that the outflow of this quality improved the declaration of a few *Camelina* cuticular wax biosynthetic qualities by two- to sevenfold and subsequently expanded collection of cuticular wax (Lee et al. 2014). The 1-aminocyclopropane-1-carboxylate deaminase (acdS: ACC deaminase) encoding gene that improves abiotic tolerance in plants by reducing ethylene stress has been transmitted to *Camelina*. The GM *Camelina* lines communicating acdS showed higher root length and weight, and seed creation, just as seed quality and seed oil content under salt pressure contrasted and the wild kind (Heydarian et al. 2016).

Isoprene can stifle receptive oxygen species created in plants during various abiotic stresses; accordingly, improving the convergence of isoprene in plants particularly during abiotic stresses could be promising in actuating resistance. It has been very much recorded that plants with high isoprene outflows are normally lenient to some abiotic stresses, like high temperatures, high light forces, and high O3 fixations. To improve such capacities in the plants lacking isoprene emanation, like *Camelina*, it is conceivable to move isoprene synthase quality (PcISPS) to the plant genome (Rossi et al. 2014). Also, the citrus limonene synthase quality (ls), geranyl diphosphate synthase (Gross domestic product), monoterpene synthase (LS), farnesyl diphosphate synthase (FDS), and sesquiterpene synthase (CDNS) have been moved to the *Camelina* genome in the single or in co-articulation shapes and brought about more transformation to abiotic stresses in *Camelina* ((Augustin et al. 2015); (Borghi and Xie 2016)). It has been likewise shown that the overexpression of γ-glutamylcyclotransferase quality (GGCT2) in *Camelina* could improve oxidative pressure resistance in this plant. This protein is a piece of γ-glutamyl cycle associated with the amalgamation and debasement of glutathione and reusing of amino acids (Ablordeppey 2014).

6.6 Genetic Engineering Applications in Microalgae

Previously, several researchers used foundational engineering to include a high centralization of biomass for modern uses, as well as altered metabolic pathways to produce more valuable products. Shift in microalgae can be accomplished using a few techniques.

6.6.1 Trans-Conjugation

It's the transfer of DNA from a cyanobacterial cell to a bacterial cell. It is generally accomplished through direct cell-to-cell contact or through the use of a scaffold, such as the connection of two cells. It is an adaptable technique that can be used for hereditary control of marine and freshwater green growth. This technique is exhibited first to transformation of five strains of marine cyanobacteria *Synechococcus*, *Synechocystis*, and *Pseudanabaena* (Sode k. 1992). Conjugation was done using transposon and vector pKT230(IncQ). This research confirmed wide application of conjugation in microalgae. By interspecific conjugation with *E. coli*, a plasmid containing green fluorescent protein (GFP) was introduced into the *Prochlorococcus* strain, and protein expression was observed by Western blotting and cellular fluorescence (Tolonen et al. 2006).

6.6.2 Natural Transformation and Induced Transformation

This strategy permits engrossing extracellular DNA straightforwardly in normally capable cells or misleadingly instigated capable cells. In marine green growth, common change has been accounted for just *Synechococcus* sp. PCC7002, and others are for the most part freshwater strains of cyanobacteria. It utilizes treatment with ethidium bromide for the strain (Qin et al. 2012). The system of fitness is practically comparative in the two sorts of cyanobacteria; however, the change proficiency of marine *Synechococcus* was much lower than freshwater *Synechococcus* strain. It happens fundamentally because of the presence of certain polysaccharides which thwarts the take-up of DNA. These days, fast and straightforward change techniques are accessible for marine strains like electroporation.

6.6.3 Electroporation

The electroporation strategy is utilized for change in bacterial cells for quite a while. It is a straightforward and profoundly proficient strategy for a limited quantity of

DNA (Neumann et al. 1982). By electroporation method, outward DNA can be moved autonomously from the cell's capacity. Electroporation was first acted in marine *Cyanobacterium Synechococcus* sp. (Matsunaga et al. 1990). The strength of an electric field needed for marine cyanobacteria was than that of freshwater strains. This effectiveness can be expanded by pretreatment with $CaCl_2$. Electroporation interceded change was additionally accomplished in eukaryotic *Chlamydomonas reinhardtii* strains utilizing 14Kb plasmid (Brown et al. 1991).The electroporation technique is substantially more effective than the glass globules strategy to move exogenous DNA. Recently, change convention has been set up for oil-creating green growth *Nannochloropsis* sp. by electroporation strategy, and a few qualities were taken out utilizing homologous recombination technique (Kilian et al. 2011).

6.6.4 Biolistic Transformation

The miniature molecule siege strategy has been the most proficient technique for direct quality exchange. There were conventions set up for the change of numerous atomic and chloroplast articulation frameworks of microalgae.

Advantages of the biolistic method:

- Including plants, creatures, microorganisms – different cells and tissues can be presented by exogenous DNA by this strategy, by biolistic technique chloroplasts, mitochondria, and different organelles.
- Due to restricted data on genomes of most green growth, it is hard to plan endogenous vectors. Vectors from *E.coli* were generally useful in algal biolistic change.
- Gene gun usually used for particle bombardment; but it can be controlled, and mostly all physical and chemical parameter can be adjusted.

Marine cyanobacterium *Synechococcus* was changed utilizing molecule assault with bacterial attractive particles. A phospholipid coating coats the molecule, allowing it to bind larger amounts of DNA (Matsunaga et al. 1990). Devices accessible for hereditary control of diatoms are restricted. Biolistic change is the most effective device for change in diatoms. Change techniques with molecule barrage have been set up for some types of diatoms, *Thalassiosira pseudonana*, *Thalassiosira weissflogii*, and *C. cryptica* (Qin et al. 2012).

6.6.5 Glass Beads

With the assistance of glass dabs, unfamiliar DNA can be productively acquainted with microalgae. *C. reinhardtii*, a novel water alga, was discovered for the first time. Glass dots are a less expensive and more targeted alternative to biolistic change. Glass dabs were used to build up the hereditary change framework in Dunaliella

salina more efficiently than biolistic approach and electroporation strategy (Feng et al. 2009). Using glass beads, red seaweed Porphyra haitanensis also transformed, which have very thin walls (Wang et al. 2010). The primary downside of this technique is that it can't be useful for thick cell-walled green growth. Thick cell divider should pretreated with proteins that digest the dividers. In any case, in some microalgae, cell perceivability is diminished when cell dividers are eliminated and furthermore separation of callus prevented.

6.6.6 Silicon Carbon Whiskers Method

Glass beads cannot be useful for thick cell-walled algae for efficient transformation. But when *C. reinhardtii* cells agitate with silicon carbon (SiC) whiskers for some time, it can give transformants a very good efficiency. It gets over the cell wall's barrier to foreign DNA insertion. There was protocol established for the transformation of marine dinoflagellates using SiC.

6.6.7 Microinjection

It is an immediate actual technique to infiltrate the cell divider. It doesn't need a protoplast recovery framework. Microinjection is useful to present substances in an extremely controlled way and at explicit targets. Because of trouble in immobilization of algal cells, microinjection technique is infrequently utilized in marine green growth change (Neuhaus and Spangenberg 1990). A high yield transformation was established for marine green algae *Acetabularia mediterranea* by microinjection (Neuhaus et al. 1986). However, the dynamic and delicate mechanism can be considered to be an extremely efficient and cost-effective method of transformation for marine algae.

6.6.7.1 Artificial Transposon Method

Transposons are mobile DNA elements discovered in maize, which is used as a genetic tool for genetic manipulation. Artificial transposon can be separated and transformed from the natural one. It has been created for in vitro mutagenesis and hereditary change. Artificial transposons with a high frequency of interpretation can coordinate unknown DNA into the receptor cell's genome, avoiding the random mixing of genes (Wu et al. 2011). Using a common Tn5 translation and a cation liposome complexed with electroporation to boost the change efficiency (Reznikoff 2008).

6.6.8 Agrobacterium tumefaciens-*Mediated Genetic Transformation*

Agrobacterium tumefaciens-mediated transformation changes plants by moving and incorporating Ti plasmid with huge DNA parts to plant genome with the assistance of Vir proteins for T-DNA move. In marine ocean growth *Porphyra yezoensis*, hereditary change by *A. tumefaciens* previously revealed. The change recurrence of quality exchange to new water alga *C. reinhardtii* by *A. tumefaciens* new water strains is a lot higher than that of the glass globule change (Kumar et al. 2004). Transformation by this technique is influenced by numerous variables, for example, strains utilized or plasmid vectors. Because of low duplicate number of Ti plasmid, plasmid disengagement and control become more troublesome, and thus, change becomes more troublesome.

6.7 CRISPR/Cas9-Mediated Genome Engineering

As other regular strategies, it doesn't include disconnection and move of hereditary material. It is REM (RNA-guided endonuclease-interceded) approach. The CRISPR/Cas9 framework is a collection of regularly interspaced short palindromic repeats that bacteria use to protect themselves against viral assault. They are little dull successions flanked by viral or phage hereditary material. At whatever point, bacterium experiences bacteriophage or infection, and some portion of its hereditary material has been coordinated in its own genome. Whenever new experiences occur, the framework recognises them with the help of RNA groups that are interpreted. Cas catalyst severs DNA at specific base location with the help of RNA guidance. It utilizes CRISPR RNA (crRNA) trancrRNA to manage cas9 nuclease protein to make breaks in genomic DNA. Microbial cells can be changed for a more effective biofuel yield utilizing CRISPR/Cas9 intervened genome designing.

6.8 Conclusion

Plants have been genetically engineered to have less lignin to grow biofuel with a high return on investment. The most efficient structure of plant cell divider can be created using various molecular biology approaches. Higher oil content and consistency, as well as decreased tolerance to environmental conditions, have been linked to some GM crops. In addition to their abundant material pool and programmable cells for plan and production, cyanobacteria and eukaryotic algal growth have enormous potential. Significant strides have been made in the field of molecular biology in the last decade. With the help of current technology like NGS and CRISPR/Cas9, we may reinvent metabolic pathways and networks, as well as living

organisms themselves. It is now critical to plan for methodologies to amass more techniques and devices for marine algal cells for adjustable modern-scale frameworks.

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Chapter 7
Algal-Based Biofuel Production: Opportunities, Challenges, and Prospects

Prathmesh Anerao, Hemant Kumar, Roshan Kaware, Komal Prasad, Manish Kumar, and Lal Singh

Abstract The consumption of energy around the globe is increasing continuously, which is mostly fulfilled by fossil fuel which is a nonrenewable source of energy. To meet the demand and supply, researchers need to identify and promote a renewable source of energy. Among various source of renewable energy, microalgae would be considered as an emerging and reliable feedstock which would be able to replace the fossil fuel-based source. Due to its high lipid contents (≈30% of dry cell mass) and high growth rate, microalgae would be able to produce higher amount of bioenergy in comparison to various renewable energy sources. Still, the key challenges associated with algal biodiesel are recovery of lipids from algal biomass and their conversion to fatty acid methyl esters (FAME). Therefore, the current chapter enlightens the whole process of biodiesel production including selection of feedstock, harvesting of algal biomass, extraction and purification of lipids, and finally production of biodiesel and value-added products via various routes.

Keywords Biodiesel · Microalgal biomass · Photobioreactor · Bioflocculation · Downstream processing · Microalgal harvesting

7.1 Introduction

In the current scenario, the rapid growth of the population leads to an increase in high energy consumption as a result of modernization, urbanization, industrialization, and transportation (Yin et al. 2020; Kumar et al. 2021). Overexploitation of fossil fuel as an energy source generates enormous environmental pollution resulting in climate change, ecological imbalance, and health problems (Okoro et al. 2019; Zhu et al. 2019; Kumar et al. 2020; Chowdhary and Raj 2020). Presently, the level of carbon dioxide in the atmosphere is 394.5 ppm (parts per million) which is estimated to reach 500 ppm by 2050. To reduce the effect and emission of carbon dioxide and

P. Anerao · H. Kumar · R. Kaware · K. Prasad · M. Kumar · L. Singh (✉)
CSIR-National Environmental Engineering Research Institute (CSIR-NEERI), Nagpur, Maharashtra, India
e-mail: lalsingh@neeri.res.in

P. Chowdhary et al. (eds.), *Bio-Clean Energy Technologies: Volume 1*, Clean Energy Production Technologies, https://doi.org/10.1007/978-981-16-8090-8_7

other harmful gases, the utilization of nonrenewable sources of energy like fossil fuels should be reduced, and an alternate source of energy (mostly renewable source) must be prioritized (Mathimani and Mallick 2018; Mahlia et al. 2020; Kumar et al. 2020).

To overcome these environmental issues, many nations had stepped forward to produce and use bioenergy (Kumar et al. 2021), with the USA, Germany, and Brazil contributing around 50% of the world's total biofuel production. Apart from this, Africa has less than 1% share in it. A renewable source of energy from biomass resources like animal wastes, food waste (Kumar et al. 2021), municipal waste (Kumar et al. 2018), plant biomass (Prabha et al. 2021; Kumar et al. 2020), and algae biomass (Nie et al. 2020) can be proved as a source of a clean and sustainable source of bioenergy. Biodiesel showed properties and characteristics of being a biodegradable, renewable source of bioenergy (Venturini et al. 2019; Sanchez et al. 2019). Biodiesel contributes approximately to 82% of total biofuel, and its demands are increasing exponentially every year, to fulfill the economic and environmental objectives of the nation (Mata et al. 2010). In a report, the European Union expected that 3% of their energy demands will be fulfilled from renewable sources by 2050.

India stands in fifth position for energy consumption, consuming 4% of total global energy after the USA, Russia, China, and Japan. India imports 80% of crude oil out of which petrol and diesel have 40% share. Due to the high dependency on imports, and rising crude oil price globally, India started thinking about alternate sources of bioenergy (Kumar et al. 2018, 2020). In 2009, a National Policy on biofuel was proposed to meet the high demand for fuels and also to provide energy security. The major objectives were to utilize nonedible feedstock for the production and cultivation of biofuel on wasteland followed by R&D support for its commercialization. In India, various government and private organizations are working in the area of biofuel production and its distribution. Here, majorly, the Biodiesel Association of India (BDAI) and the Indian Renewable Energy Development Agency Limited (IREDA) are the agencies which provide the supporting infrastructure for feedstock cultivation and processing and biodiesel production, storage, and distribution (Marousek et al. 2015). Here, *Jatropha curcas*, *Azadirachta indica*, *Manilkara zapota*, *Madhuca longifolia* (mahua), etc. are being used as feedstock for biofuel production.

More recently, it has been reported that microalgae have been a potential nonedible source of biodiesel due to their high oil yield, high productivity, and less area requirement in comparison to other feedstock (Kumar et al. 2020; Nie et al. 2020; Sharma and Singh 2017). Algae can be cultivated on degraded land or in wastewater water with only sunlight as a basic requirement. This can produce a relatively high amount of biomass with rapid growth and high lipid content (Dewangan et al. 2018). Furthermore, harvested algal biomass can be utilized in production of biofuels and range of materials via biorefinery approach (Fig. 7.1).

Therefore, this current chapter provides a comprehensive discussion on the production of biodiesel from algal biomass. This chapter also critically evaluated the various technologies/methodologies applied so far in harvesting algal biomass

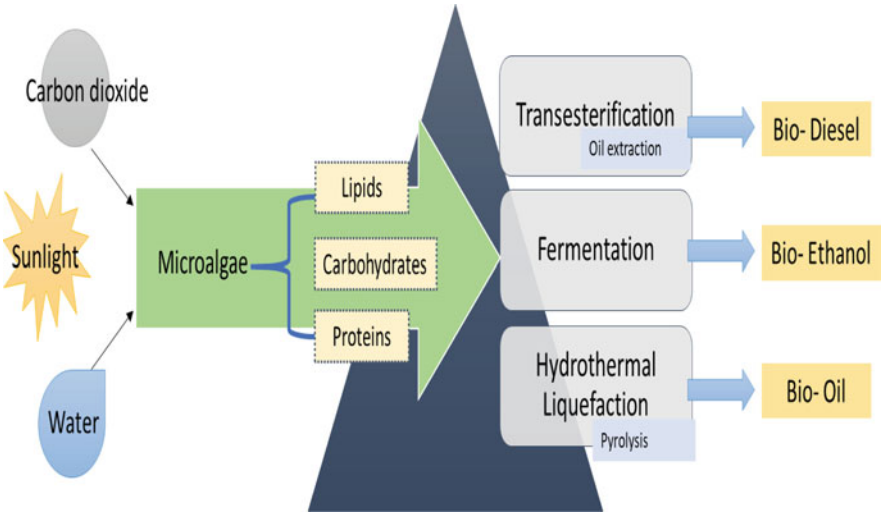

Fig. 7.1 Schematic representation of biorefinery model of algal biomass

and algal lipids and their conversion into biodiesel via process of transesterification. Moreover, this chapter also provides a glimpse of side products obtained during bio-valorizations of algal biomass.

7.2 Classification of Biofuels

The classification of biofuels is based on the origin of feedstock, i.e., first-, second-, and third-generation feedstock. Biofuel's properties such as quality, efficiency, and purity are mainly governed by their feedstock's types and properties.

7.2.1 First-Generation Biofuel

The first-generation biofuel is generally produced from edible feedstocks like sugarcane, grains, or edible oils. From the initial era of biodiesel production, the use of edible feedstock was commonly practiced as first-generation feedstock where vegetable oil is being extracted from the edible source and mixed with methanol in presence of catalysts, resulting in the production of biodiesel and glycerol as end products (Bhatia et al. 2017; Morya et al. 2018). Likewise, fermentation of the feedstock (starch, sugar, or sugarcane) produces bioethanol by the utilization of microbes. This bioethanol can be utilized in alcoholic beverage industries, or after blending, it could be utilized as biofuel (Velazquez Lucio et al. 2018). Utilization of edible feedstock for the production of biodiesel would not be feasible as its leads to

food security as well as increasing the cost of edible feedstocks, which are the major stumbling block in development of first-generation biofuel (Mishra et al. 2020).

7.2.2 Second-Generation Biofuel

To overcome the problem of first-generation biofuel, biodiesel production is carried out by the use of nonedible feedstocks named as second-generation biofuel. Species like *Jatropha curcas*, *Calophyllum inophyllum*, *Madhuca longifolia*, neem, *Karanja*, rubber seed, etc. are extensively used in production of second-generation biofuel. The greatest advantage of these feedstock is that they can be cultivated on the degraded or wastelands which made them more eco-friendly and more beneficial for the environment (Mohamed Shameer et al. 2017). Mostly, the production of biodiesel from second-generation feedstock is carried out by two different processes, i.e., biological process and thermochemical process. In the biological process, enzymatic hydrolysis of feedstock is carried out which results in the production of biofuel, whereas in the thermochemical course, the feedstock is being pyrolyzed in the absence of oxygen, or the gasification of feedstock can be done in a similar condition. But the major problem with these feedstocks is that the yield and efficiency of biofuel are low (Mahdavi et al. 2015).

7.2.3 Third-Generation Biofuel/Biodiesel

Given the limitations of both first- and second-generation biofuel, the researchers tend to search a feedstock which imposes lower food security, least investment, and high yielding and can also contribute to mitigate climate change (Verma et al. 2016). These competencies have been found in the biodiesel produced by animal fats or fish oil, waste cooking oil, and microalgae. These are categorized as third-generation biofuel. Algae are aquatic organisms that differ in size ranging from micro to macro and can be easily cultivated in all variety of water and have the ability to fix atmospheric CO_2 (Saladini et al. 2016). Algae possess some unique features like high lipid productivity, high carbon sequestration rate, the capability of growing in fresh as well as brackish water, less generation time, and can be cultivated in open or closed vessels (Najafi et al. 2011). Different species of algae with various chemical compositions and their presence in diverse habitats may lead to production of a range of biofuels such as biodiesel, biohydrogen, bioethanol, syngas, etc. (Janaun and Ellis 2010). The replication and mass cultivation can be done to microalgae in 24 h or in less time easily with the help of appropriate genetic engineering tools.

7.3 Algal-Based Biodiesel Production

The production of algal biodiesel comprised various processes like selection and cultivation of algal species and treatment or pre-treatment process followed by lipid extraction which leads to the main process of transesterification of algal oil. After this process, the product can be purified and separated into two forms: One is crude biodiesel, and the other is crude glycerol which could be further purified to obtain pure biodiesel or glycerol (Gong and You 2015) (Fig. 7.2).

7.3.1 Selection and Cultivation of Microalgae

The selection of microalgae is done based on their efficiency to utilize environmental pollutants and production of biomass. Microalgae are unicellular auto-heterotrophic photosynthetic organisms which utilize carbon dioxide (CO_2), sunlight, and water as their carbon and energy source and for the synthesis of lipids, phospholipids, proteins, and nucleic acid (Enamala et al. 2018). Some potential algal species which could be used for the production of biodiesel are *Nannochloropsis occulta*,

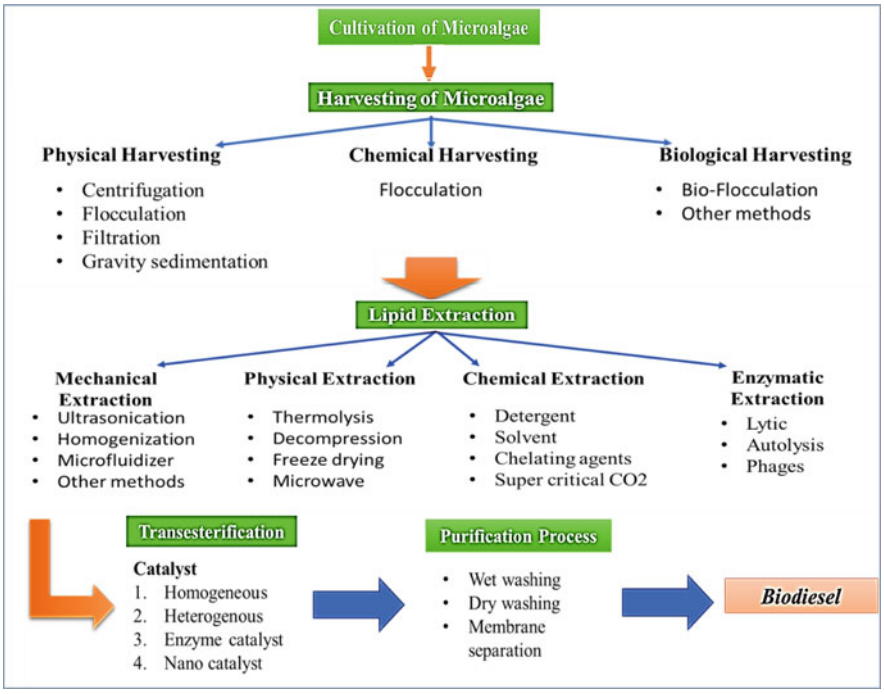

Fig. 7.2 Schematic representation of algal biodiesel production process

Botryococcus braunii, and *Chlorella vulgaris* (Mohamadzadeh et al. 2017). The cultivation of algae is carried out in open or closed pond systems. Generally, atmospheric CO_2 and sunlight are utilized for the production of algal biomass in the pond system. The circulation and mixing of external nutrients are carried out by the paddle system in the open pond. This system provides a higher yield of biomass with low capital investment and maintenance, but the probability of contamination in an open pond is more; therefore, maintaining the sterilized condition in an open pond system is tedious (Lam et al. 2017). To overcome this problem, a closed photobioreactor system is designed with plastics, glass, and polythene bags for algal cultivation which has sunlight penetration up to 6–11 cm. The system is designed for proper liquid circulation and also enhances the gas exchange process which plays a major role in supplying CO_2 to the photobioreactor. The key advantage of microalgae is that they can be cultivated in a small area and even in wastewater enriched with organic waste, phosphate, and nitrates which helps in the enhancement of lipid production by algae (Nugroho and Zhu 2019). The optimization of operational parameters like pH, light, and temperature during cultivation is key to improving lipid accumulation by algae (Lowrey et al. 2015).

Microalgae can absorb light of a wavelength ranging from 400 to 700 nm which helps initiate photosynthesis and in the translation of inorganic matter (Sajjadi et al. 2018). The distribution of light and its intensity varies in different cells of microalgae when grown in the photobioreactor. Many species of microalgae can be cultivated in both dark and light atmospheres periodically like *Spirogyra*, *Picochlorum*, *B. braunii*, and *Chlorella* (Naderi et al. 2017). Species like *Nostoc commune*, *Chroococcus turgidus*, *Chlorella* sp., and *Lyngbya confervoides* are best known for their growth under natural pH, whereas *Nannochloropsis salina* could resist pH between 8 and 9 in range (Qiu et al. 2017). Therefore, for high-quality algal biomass and better lipid accumulation, these factors must get under consideration.

7.3.2 Harvesting of Microalgae

The most important step after the cultivation of the microalgae is harvesting and dewatering for the production of biodiesel. The harvesting process is difficult and expensive due to smaller cell size of microalgae, i.e., (2–20 μm), which involves solid-liquid separation consuming 20–30% energy of total biomass produces and also the most expensive step (Barros et al. 2015). To reduce this cost and energy consumption, the harvesting and dewatering could be carried out in bulk quantity. There are several methods for harvesting microalgae like physical, chemical, and biological. Moreover, the combination of these methods was also carried out to obtaining high biomass and proper separation (Mathimani and Mallick 2018).

7.3.2.1 Physical Methods of Harvesting

The physical method comprises various processes including centrifugation, flocculation, filtration, and gravity sedimentation.

7.3.2.1.1 Centrifugation

The finest and most appropriate harvesting of microalgal cells is carried out via the process of centrifugation, where the harvesting is carried out by the use of centripetal force which avoids the use of harsh solvents and chemicals which could cause contamination in separation process (Laamanen et al. 2016). By regulating the biomass concentration and centrifuge type, the efficiency rate could be increased. Research investigated that the efficient method for separating algae at a density rate of 0.02–0.05% using the disc stalk centrifuge method increases algal separation by 95%. Apart from these benefits, this process has the drawback of being too costly, having high energy expenditure and heavy maintenance, and also potentially causing damage to microalgal cells due to its high centrifugal force and the physical stress applied in this process (Mathimani and Mallick 2018).

7.3.2.1.2 Flotation and Flocculation

Flotation is a process that works on the inverted sedimentation where air or bubbles lift the solid particle carrying toward the liquid surface. These air bubbles carry microalgal particles of lower density and lift them through their lifting force which results in the separation of microalgae (Laamanen et al. 2016). There are two types of flotation, i.e., dissolved flotation and dispersed flotation, used for harvesting microalgae, generating microbubbles of diameter ranges from 10 to 100 μm for dissolved and 700 to 1500 μm for dispersed types of flotation. This process of attachment between air bubbles and algae depends upon two major factors, one is the instability of particles and the aqueous contact angle between microalgae and air bubbles. This method is efficient to be used in small spaces and short duration of time for harvesting microalgae. However, the efficiency of this method widely depends upon the algal biomass, bubble size, and pH of the media used (Barros et al. 2015).

7.3.2.1.3 Filtration

Filtration is a process for separation of solids from liquids via a selectively permeable membrane through which only liquid could pass either physically or mechanically (Enamala et al. 2018). This is majorly known as the dewatering process where algal suspension culture is passed through the filter which results in the flow of water

and slurry of algae left behind. The filtration is carried out using different sizes of membrane filters for the separation of algae of diverse size. This process shows a 70–80% of efficiency rate because it does not disturb the cell structure of algae. The majorly used process is ultrafiltration and microfiltration which separate low-density culture easily due to its tangential flow. The drawback of this process is the slow filtration rate which leads to logging, increase in insufficient cell separation, high maintenance cost, and filters requiring extensive washing with screening (Fuad et al. 2018).

7.3.2.1.4 Gravity Sedimentation

Predominantly, the separation of solid and liquid is being executed by gravity sedimentation. In this process, separation of the algal biomass from water is performed under the force of gravity at a particular velocity based on cell size and density (Nie et al. 2018). This method is very cost-effective and requires less energy compared with other methods, but the settling rate is very low and in the range of 0.1–2.6 cm/h; therefore, this method is not preferable at the commercial scale (Kumar et al. 2017).

7.3.2.2 Chemical Harvesting Process

7.3.2.2.1 Flocculation

The effective and most conventional method for microalgae harvesting is flocculation. This is a key process that must be followed before floatation, filtration, or gravity sedimentation. In the process of flocculation and coagulation, the small microalgae cells collide and adhere to each other and form a large flocs which tends to easy separation and sedimentation (Zhu et al. 2018). This process can be carried out with the help of chemical and biological agents. The mechanism of flocculation involves charge neutralism, patching, sweeping, and adsorption bridging. In this method, a large quantity of inorganic flocculants is required to initiate the separation of solid liquid, but it can also generate a large amount of sludge.

7.3.2.3 Biological Harvesting

7.3.2.3.1 Bio-flocculation

The predominant and emerging technology in the current scenario is bio-flocculation where a variety of microorganisms with their biopolymers like EPS (extracellular polymeric substance) (Kumar et al. 2019) or some acid like glutamic acid helps in harvesting microalgae (Chen et al. 2018). Bio-flocculation can be carried out by various micro-based methods through bacteria, fungi, and actinomycetes. Mostly,

the use of co-cultured microorganisms results in higher lipid production, but it also leads to contamination which could be caused by the interaction of microalgae with other organisms which affects the downstream process of biodiesel production (Chen et al. 2018; Chowdhary et al. 2020). The bio-flocculation process can be made more energy-efficient, cost-effective, and feasible with the combination of other processes like sedimentation or centrifugation.

7.3.2.3.2 Other Methods

Techniques like electric-assisted and magnetic methods can be used for harvesting microalgae. The magnetic method separates the particles by applying a magnetic field which is easy to operate, time-saving, and a reusable process (Markeb et al. 2019). These days, for effective harvesting of microalgae, electric fields are also being used due to its efficiency in comparison to flotation and flocculation methods. As the microalgae are negatively charged, it gets concentrated toward anode when exposed to an electric field (Barros et al. 2015). Overall, all the above discussed algal biomass harvesting methods have their advantages and disadvantages, so, any lone method has not that much efficacy in harvesting microalgae from the culture medium; therefore, harvesting should be carried out in the integration of various methods to improve efficiency, reduce cost, and improve productivity.

7.4 Lipid Extraction or Pre-Treatment Process

The core process for the production of biodiesel is microalgae harvesting and lipid extraction, which could be performed simultaneously reducing duration and improving efficiency. The prominent aim of this technique is the disruption of the cell wall which allows the accesses of solvent through it and uses intracellular lipids improving the lipid extraction process (Sierra et al. 2017). This extraction can be carried out in the physical, chemical, mechanical, and enzymatic processes.

7.4.1 Mechanical and Physical Extraction

This mechanical lipid extraction involves the disintegration of microalgae through various forces (electric, waves, or shear) before the introduction of the solvent. This involves ultrasonication, microwave, homogenization, and electric pulse field. In ultrasonication, the microalgae cell disruption is carried out by using ultrasonic wave energy of frequency ranges 10 KHz (kilohertz) to 20 KHz. The use of shock waves in this technique for propagating fluctuation causes a mechano-acoustic effect resulting in rupturing of the cell wall (Naveena et al. 2015). The emission of shock wave collapses due to the reduced pressure and microbubble which tends to

cytoplasmic disruption of cells. The shape, culture cell size, age of cell culture, process exposure time, and frequencies are the parameters which highly governed the efficiency of disruption of algal cells. The enrichment in the lipid process with reduced extraction time in this method is up to 50–500% (Lari et al. 2019). Ultrasonication method is more effective against species possessing rigid cell wall like *Chlorella* sp. of algae because flexible cell wall algae would retain the lipid due to its coiling. The major drawbacks of this process are denaturation and high energy loss due to the high temperature produced during the process (Dong et al. 2016).

The most efficient conventional method for lipid extraction is microwave. This method was developed in the 1980s uses electromagnetic radiation for cell disruption whose frequency ranges from 0.3 to 300 gigahertz (GHz) which is much higher as compared with the general range of algal cell disruption, i.e., 2450 megahertz (MHz) (D'Hondt et al. 2017). This process provides rapid, secure, and cost-effective lipid extraction without the requirement of dewatering. In this method, the heat is generated through intra- and intercellular movements produced by an oscillating electric field which forms the water vapors which open and rupture the cell membrane leading to release of intracellular lipid. This microwave-assisted treatment on algae species *Botryococcus braunii* results in the increase in yield by 18–38% where the temperature provided was 45 °C for the period of 15 min (Rokicka et al. 2018). This microwave-assisted technique provides a high disruption rate in a short duration of time where it can recover lipids from the reaction mixture quickly within 15–20 min.

Similarly, a clean, promising, cheap, and less complicated process of lipid extraction from microalgal cells is pulse electric field (PEF). In this process, the cell membrane is made permeable using electroporation which is utilized for the extraction of oil from microalgae (Silve et al. 2018). This is a continuous system designed where specific short electric pulses and high voltage are being utilized for the disruption of cells. This method made the downstream process quite easier because this extraction does not produce cell debris (Carullo et al. 2018). At an industrial scale, PEF could be combined with solvent for lipid extraction from microalgae which prevents loss of solvent less than 1% (Raso et al. 2016).

Some other techniques like depressurization and the bead mill method are commonly used for cell disruption to extract intracellular compounds. In depressurization, a diffusible gas like CO_2 is made to pass through the cell membrane which results in expansion of the cell wall until its saturation. The pressure generated by gas has high disruption efficiency which leads to the rupture of the cell membrane (Uquiche et al. 2016). This process is more advantageous due to less extraction time and a toxic-free environment during the process. Research on *Chlorococcum* sp. of algae where the use of supercritical fluid for lipid extraction was carried out shows reduced time up to 5.6 times compared with soxhlet extraction method. Likewise, the bead mill method is the most conventional and easy method used for extraction where the agitation caused between the beads brings algae cell disruption (Onumaegbu et al. 2018). Eventually, these mechanical methods for lipid extraction are successful and can show more efficiency when used in combination with non-mechanical extraction methods.

7.4.2 Chemical Methods

The chemical or non-mechanical methods for lipid extraction are solvent extraction, alkali/acid extraction, and ionic method. These methods can be scalable as per the extraction requirement. In the process of solvent extraction, polar and nonpolar solvents are responsible for microalgae lipid extraction proficiency. The most conventional solvent used methods for lipid extraction are the Folch procedure and Biligh and Dyer method where methanol and chloroform solvents were utilized in the ratio of 1:2 and 2:1 v/v (Guo et al. 2019). Major solvents like ethanol, chloroform, methanol, and hexane are prominently used on the basis of class of algae in the lipid extraction process where polar solvents are attached with the nonpolar solvents for total extraction of lipids from algae. Some solvents are excluded at large-scale lipid extraction from algal biomass because of their toxic and adverse effect on the environment like hexane and chloroform (Mubarak et al. 2015). To achieve efficient extraction in a short duration, the solvent extraction method could be combined with other methods like heat, pressure, or any mechanical methods.

Like the solvent extraction method, for disruption of cell biomass, strong acids and alkalis are being used known as the acid/alkali method. In alkali and acid, the chemicals used for intracellular lipid extraction are sodium hydroxide and sulfuric acid respectively (Xia et a1. 2015). Here, alkali saponifies lipids and acid hydrolyses polymer of cell wall which helps in cell disruption by the disintegration of cell structure. Some drawbacks of this method like the use of chemicals causing corrosion in the reaction chamber and extreme pH lead to the denaturation of valuable components of cells like proteins, etc.; this method is not attractive (Dixon and Wilken 2018).

Some green solvents are used prominently in the form of ionic liquids like 1-ethyl-3-methylimidazolium diethyl phosphate, 1-ethyl-3-methylimidazolium acetate, and tetrafluoroborate with cations and anions which dissolve microalgal biomass by extracting low-density lipids in ionic liquids (Olkiewicz et al. 2015). These ionic liquids show good yields of extraction because of their solubility, thermal stability, and high polarity in organic and inorganic solvents which helps them reduce the drying process cost by generating good extraction yield. It can be combined with microwave-assisted extraction for a large-scale yield up to 10–15 times (Pan et al. 2016).

7.4.3 Biological Methods

This method of lipid extraction from algal biomass comprises use of enzymes which hydrolyze the cell wall and make it permeable by binding to the molecules of the cell wall. Using biological catalysts like pectinase, lysozymes, or chitinase which are responsible for the degradation of the cell membrane may depend on the diversity of algal biomass (Zabed et a1. 2019). This is the simplest method because it does not

utilize any sophisticated equipment and harmful chemicals. In research on *Chlamydomonas reinhardtii*, the use of the enzyme autolysin achieved a 30% high lipid extraction yield. By managing the pH of biomass and media, temperature, and enzyme selection, the efficiency and yield of extracted lipid can be increased (Sierra et al. 2017) (Table 7.1).

7.5 Transesterification Process

Lipids extracted from algal biomass cannot be used directly as biodiesel. It needs to be converted into fuel by different methods like dilution, micro-emulsification, and transesterification process. Biodiesel produced by the transesterification process has equal commercial value and similar properties when compared with other processes because it can be carried out in dry as well as wet form (Mahmudul et al. 2017). It is also known as alcoholization because of the reaction of triglycerides with alcohol to produce three-part of ester (FAME) by the reaction of three parts of alcohol one part of triglycerides in the presence of catalyst (Chozhavendhan et al. 2020). This process is a reversible process that increases the separation rate and maintains the forward flow of reaction utilizing excess alcohol. The quality of biodiesel produced here depends on the types of catalyst ratio of alcohol and oil, water content, reaction time, and temperature.

7.5.1 Catalyst

To fasten the rate of transesterification reaction and to obtain the yield in a minimum duration of time, the catalyst is being used. Catalyst is basically of two types, homogeneous and heterogeneous (Carvalho et al. 2017). These are found in alkali or acid form, and their choice depends on the fatty acid content of the oil. Some detailed study of catalyst is discussed below.

7.5.1.1 Homogeneous Catalyst

The homogeneous catalyst (acid and alkali) is being used in the transesterification process generally. Acid catalyzed such as sulfuric acid and hydrochloric acid are used in case there is high possibility of free fatty acid in the feedstock (Kumar and Thakur 2018; Kumar et al. 2016). Generally, engagement of such catalyst is not prominently done in biodiesel production because of its slow reaction rate (Vyas et al. 2010).

As a base catalyst, potassium and sodium hydroxide are commonly used, wherein the presence of potassium hydroxide catalyst oil and methanol is being stirred for 3 h at 60 °C; after completion, it is remained undisturbed for at least 18 h which leads to

Table 7.1 Selected references for pre-treatment method used for various algal biomass modified

Pre-treatment method	Conditions	Algal biomass	Extracted compounds	Advantages	Limitation	References
Enzymatic	Endogalactouronase 800 U/g, esterase 3600 U/g, protease 90 U/g, pH 6, 50 °C, 24 h	*Scenedesmus obliquus* *Chlorella pyrenoidosa*	Carbohydrates Lipids	Cell disruption With minimal damage High lipid recovery	Strongly dependent on pH; extraction efficiency is also dependent on incubation time. Enzymes are expensive	Ometto et al. (2014)
Pulsed electric field	17.9–71.7 kWh/m^3, 36–54 °C	*Synechocystis* sp.	Cell disruption	No additional chemicals required Less energy requirement Easy scalability Less time-consuming Less operating cost	No lipid is detected in extracellular medium Formation of oil content due to the existence of C–C double Bonds of unsaturated fatty acids	Sheng et al. (2011)
Microwave	Acetone, 50 W, 56 °C, 5 min	*Dunaliella tertiolecta*	Pigments	High heat and mass transfer More economical Usage of minimal solvent Improved extraction yield	Maintenance cost is higher An additional process is required to remove solid residue The scale-up process is difficult	Pasquet et al. (2011)
Pulsed electric field and solvents	1 cm electrode distance, 45 kV/cm ethyl acetate/methanol/water	*Ankistrodesmus falcatus*	Lipids	No additional chemicals required Less energy requirement Easy scalability Less time-consuming Less operating cost	No lipid is detected in extracellular medium Formation of oil content due to the existence of C–C double bonds of unsaturated fatty acids	Zbinden et al. (2013)
Ionic liquid and solvent	Ionic liquid 1 h, ambient temperature Adding hexane mixture 30 s 15 min	*Chlorella vulgaris*	Cell disruption and lipids	Stable green solvent Non-flammable and less hazardous	Water immiscible ionic liquids have lower extraction Scale-up process is difficult; expensive approach	Orr et al. (2015)

(continued)

Table 7.1 (continued)

Pre-treatment method	Conditions	Algal biomass	Extracted compounds	Advantages	Limitation	References
Ultrasound	65–130 W, 40 kHz, 25 min	*Chlorococcum* sp.	Carbohydrates	Increases the extraction rate No solvent required No effects on fatty acid profile Eco-extraction process with higher penetration	High heat generation Produces free radicals High power consumption Scale-up process is difficult	Halim et al. (2012)
Pulsed electric field	15–25 kV/cm, 60–150 μs, 10–40 °C	*Arthrospira platensis*	Pigments	No additional chemicals required Less energy requirement Easy scalability Less time-consuming Less operating cost	No lipid is detected in extra-cellular medium Formation of oil content due to the existence of C—C double bonds of unsaturated fatty acids	Martínez et al. (2017)
Alkaline-peroxide	H_2O_2 1–7.5% (w/w), 50 °C, 1 h	*Scenedesmus obliquus*, *Scenedesmus quadricauda*, *Nitzschia* sp. *Aphanothece* sp. *Desmodesmus spinosus* *Nitzschia palea*	Carbohydrates and by-products			Juárez et al. (2016)
Hydrothermal water	1:13 (w/v), 147 °C, 40 min	*Scenedesmus* sp.	Glucose			Yuan et al. (2016)
Acid hydrolysis	H_2SO_4 2 N, 120 °C, 30 min	*Scenedesmus obliquus*	Carbohydrates			Miranda et al. (2012)
High pressure homogenization	500–850 bar, 15 min	*Chlorococcum* sp	Carbohydrates			Halim et al. (2012)

Bead milling	200 µL glass beads, 10 min cycles, 30 s vortexing, 30 s cooling on ice,	*Synechocystis* sp.	Proteins			Zhou et al. (2014)
Freezing/ thawing	3 cycles, 10 min freezing −80 °C, 5 min thawing 37 °C	*Synechocystis* sp.	Proteins			Zhou et al. (2014)
High pressure homogenization	10 mL compression chamber, 50–270 MPa, 3 °C	*Nannochloropsis oculata Porphyridium cruentum*	Cell disruption			Montalescot et al. (2015)
Acid hydrolysis	Acid hydrolysis H_2SO_4 1 M, 80–90 °C, 120 min	*Mix of microalgae (Scenedesmus*, *chlorella*, *Ankistrodesmus Micromonas*, *Chlamydomonas*)	Carbohydrates			Castro et al. (2015)

phase separation process (Onay et al. 2014). This reaction is faster compared with the acid-catalyzed process because of the short reaction time (Thakur et al. 2018). The reusability in this process is not possible due to saponification caused by a high fatty acid content (Avhad and Marchetti 2015). Microalgae *Scenedesmus* sp. catalyzed by sodium hydroxide which yields 55% of biodiesel per gram of lipid in 2 h are more efficient compared with acid-catalyzed (10 h). These homogeneous catalysts are mostly used in industrial production due to their higher efficiency and lower consumption in a single run (Alhassan et al. 2015).

7.5.1.2 Heterogeneous Catalyst

To overcome problems like reusability, purification of biodiesel, and recovery of glycerol using a homogeneous catalyst, the heterogeneous catalyst is applied (Olkiewicz et al. 2016; Kumar et al. 2018). It comes in both acid and base form where heterogeneous acid catalyst helps extract pure form of glycerol increasing purification rate up to 80%. This heterogeneous acid catalyst can yield 99% of methyl ester when used with sulfonated carbon and polymeric membranes. Similar to the homogenous acid catalyst, heterogeneous base catalyst serves disadvantages of more energy consumption and lower activity (Lee and Wilson 2015). The base heterogeneous catalyst shows high chemical steadiness and high activity rate at encompassing temperature which is great compared with heterogeneous acid catalyst (Yang et al. 2018).

7.5.1.3 Enzyme Catalyst

Serving various advantages like low energy consumption, high selectivity, low reaction temperature, and reusability, the enzyme catalyst overcomes the problems of chemical catalysts (Aguieiras et al. 2015). Mostly lipids obtained from microalgae species like *Chlorella*, *Botryococcus*, and *Aurantiochytrium* sp. use fungal and bacterial lipase and Novozymes for enzyme-catalyzed reactions. 90% of biodiesel conversion can be achieved using high-purity enzymes at an optimum temperature of 30–50 °C for 8–90 h. The enzyme-associated catalyst is not feasible for large-scale industrial production because of its low reaction rate and its non-cost-effectiveness.

7.5.1.4 Nano-catalyst

Similar to enzyme-associated catalyst, nanoparticles use a nano-catalyst to overcome the disadvantages of a homogeneous and heterogeneous catalyst. The yield of the transesterification process for biodiesel extraction from microalgae is 99% using nano-catalyst like nano-calcium methoxide (Ca $(OCH_3)_2$) at a reaction time of 3 h for 80 °C (Siow et al. 2016). Likewise, zinc oxide (ZnO) nanoparticles also show decent

catalytic performance compared with conventional methods. Recently, nanoporous carbon, biochar, and some of its derivatives showed their application in transesterification of lipids as catalyst/catalyst support. Major parameters like catalyst size, surface composition, functionality, and toxicity play a key role in the selection of nano-catalyst (Kumar et al. 2020; Ashu and Anshul 2017).

7.5.2 Alcohol to Oil Ratio

In the transesterification process generally, the yield of three parts of FAME and glycerol is carried out by the reaction of one part of triglycerides and three parts of alcohol where an excess amount of alcohol is consumed for forwarding and completion of the reaction (Hailegiorgis et al. 2015). This excess alcohol ratio of 6:1 molar is being catalyzed in reaction, and unused alcohol settles down with glycerol during the separation process, leading to difficulty in purification. The microalgae species *Chlorella vulgaris* shows conversion efficiency of 90% of fatty acid to FAME at 4:1 molar alcohol ratio within 4 h. The purity of biodiesel depends upon the alcohol-to-oil ratio in any reaction where it should not be more than 6:1 after which it affects the yield of biodiesel as well as complicates the purification process and would affect the expenditure and efficiency of production (Kumar et al. 2017, 2017).

7.5.3 Composition of Free Fatty Acid (FFA) and Water

In biodiesel production process, adverse effect has been shown by the water content of the feedstock. The hydrolysis of triglycerides present in oil leads to the formation of FFA resulting in the enhancement in the production of biodiesel (FAME) carried out in the presence of solid acid catalyst (Suwannakarn et al. 2009). Excess in water can lead to energy-intensive frying biomass, which also initiates saponification and formation of soap and make downstreaming process tedious. Excess amount of water used in transesterification reaction would decrease the translation of oil to methyl ester which affects the process and cause an adverse effect on the yield of the reaction (Demirbas 2009).

7.5.4 Reaction Time and Temperature

The factors which importantly determine the investment cost for the production of biodiesel and its yield capacity are reaction time and temperature. The reaction time and temperature are highly influenced by the solvent used for extraction, lipids present in microalgae, and catalyst (Jazzar et al. 2015). As temperature increases,

the conversion rate also increases up to 95%, and the reaction time is also reduced to 30 min to 10 min. In dry biomass, the reaction time is 5 times faster in alkali catalysts compared to acid catalysts. The biodiesel yield increases in temperature, ranging from 200 to 400 °C, due to an increase in the breakdown of the cell wall and helps in methanol-lipid interactions (Cao et al. 2013).

7.5.5 Purification Process

Impurities like water, acid, glycerol, metal ions, and excess alcohol must be removed to obtain the purified form of biodiesel for better emission characteristics and fuel efficiency (Shirazi et al. 2013). During purification, glycerol can be separated from biodiesel through gravitational settling. There are few downstream techniques which are frequently used for purification like wet washing, dry washing, membrane separation, etc. (Banga et al. 2014).

7.5.5.1 Wet Washing

The most conventional and traditional method used for the removal of impurities from biodiesel is wet washing, where the removal of residual impurities like glycerol, catalyst, and methanol soap takes place. This process is carried out in two phases: the first phase uses 5%HCl followed by saturated CO_2, water wash, and the second phase of washing uses neutral water where the remaining mixture is shaken well and is kept to settle down for some time; afterward, it is centrifuged to obtain the purified biodiesel. The acid wash neutralizes the base catalyst and decomposes the soap produce during reaction, which is further washed off along with remaining impurities (Berrios et al. 2011). This method achieves 99% of pure biodiesel by the removal of glycerol, excess alcohol, and soap. The major drawback of this method is the requirement of the excess quantity of water used for centrifugation and washing; also, this requires a large surface area for setting up the tank, centrifugation chamber, and washing area which is non-economical.

7.5.5.2 Dry Washing

To control the excess flow of water in wet washing, dry washing is developed which removes the contaminants using an adsorption process from crude biodiesel. In this method, ion exchangeable absorbents like Amberlite, Magnesol, etc. are frequently used (Mansir et al. 2018). Some resins both positively and negatively charged are used for absorbing their opposite charge to purify glycerol and remove soap; therefore, in this method, no wastewater is generated. The drawbacks of these methods are resin-associated absorbents are not reusable and methanol cannot be

separated by this method, so other separation processes are required for proper purification (Veljkovic et al. 2015).

7.5.5.3 Membrane Separation

To overcome the drawbacks of wet and dry washing techniques, the membrane-based separation technique is being utilized in biodiesel purification. In this method, the polymeric, microporous, nanofiltration, ceramic membranes which are solvent resistant and prepared by phase inversion process are applied (Torres et al. 2017). The average pore size of the membrane is between 12qm and 400qm which can tolerate adverse conditions like pH 12 and temperature up to 60 °C. This method also serves some drawbacks like the membrane can be damaged by soap content produced during the production of biodiesel which led to the replacement of these expensive membranes which is not cost-effective.

7.6 Application of by-Products

The majority of challenges have been faced by the feedstock which is being used for microalgal biodiesel production like cell harvesting, low productivity, extraction of lipids, separation of biodiesel, and wholesome cost of investment. A variety of valuable by-products obtained from used algal biomass like proteins, vitamins, pigments, and antioxidants can be a source of additional profit (Chew et al. 2017). After removal of lipids, the spent algal biomass can be applied for extraction of proteins by using ethanol. After solvent recovery, the remaining biomass can be utilized as good protein source for animal feed, and the rest of the microalgal biomass can be utilized in combustion (generation of energy) or biochar production or as fertilizer (Kumar et al. 2020; Nie et al. 2020; Khan et al. 2020, 2021).

Another major by-product obtained from biodiesel post transesterification process is crude glycerol. As per estimation, 4 kg of glycerol is produced as a by-product for each 40 kg of biodiesel with some impurities like water, salt, methanol, etc. Glycerol is a versatile compound used in cosmetics, food, and pharmaceutical industries producing high-value products. Currently, crude glycerol is being sold at a lower cost as compared to animal feed and also used as co-combustion fuel in burning or boiling chambers due to its high energy value (Chozhavendhan et al. 2017). Crude oil is a rich source of carbon that is utilized by microorganisms as an energy source and produces important chemicals like biohydrogen, succinic acid, polyhydroxyalkanoates (PHAs), and ethanol (Morya et al. 2018; Kumar and Thakur 2018; Savla et al. 2020). This crude glycerol could be used as a potential feedstock for biopolymers and fuel additive chemical production (Pradima and Kulkarni 2017). Zero discharge of waste from biodiesel industries by the utilization of microalgae, protein, lipids, pigments, and crude glycerol for converting waste into wealth is completely waste on the circular economy model or zero-waste generation model.

7.7 Conclusions

To meet the global demand for biodiesel, the first- and second-generation biodiesel fail, but the use of microalgae as a potential source for biodiesel production leads to a sustainable and renewable solution to get rid of energy deficiency around the world. Despite various drawbacks in the commercialization of microalgal biodiesel, various research has been successfully carried out to produce fast-growing oleaginous microalgae in the further production of biodiesel. To enhance the production efficiency of algal biodiesel, various parameters need to be considered such as microalgal growth rate, harvesting, extraction of lipids, and purification. The major portion of the capital cost investment is contributed by the downstream process, but utilization of integrated concepts would lead to overcoming this problem and helps in the production of high yielding biodiesel in a short duration of time and making the process cost-effective. This would encourage and promote the economic viability of biodiesel industries.

Acknowledgments The authors are thankful to the Director of the CSIR-National Environmental Engineering Research Institute for providing necessary facilities for this work.

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Chapter 8
SAPO-34 Zeolite and Membranes for Biogas Purification

A. Hernández-Palomares, Y. Reyes-Vidal, and F. Espejel-Ayala

Abstract Biogas is a renewable fuel that can be used to produce heat and electricity. It is the result of the decomposition of organic matter from microorganisms under anaerobic conditions. Biogas has a varying composition according to the different conditions in which it is produced (substrate, digester, temperature, etc.). Biogas is a mixture of methane (CH_4 50–75%) and carbon dioxide (CO_2 25–45%) with traces of other gases (H_2S, N_2, H_2, and water steam). To increase calorific power, the removal of CO_2 and other gases is necessary employing absorption or separation processes. Nevertheless, although the gas sweetening process using liquid solution of amines is used, this technology has serious disadvantages. Then, in this work, zeolites and membranes are studied as advantageous technologies for biogas purification. Specifically, zeolite and SAPO-34 membranes are shown with emphasis for obtaining biogas enriched in methane. In addition, SAPO-34 outstanding characteristics are shown: thermal, mechanical, and chemical stability, good resistance to erosion, and stability at high pressures, which allows the material to be used for a long time. Moreover, the influence of the synthesis parameters on the performance of the SAPO-34 is also shown to understand its performance in the purification of biogas.

Keywords Ceramic membrane · Biogas · Zeolite · Hydrothermal synthesis · CO_2 separation

8.1 Introduction

Biogas has an average calorific power of 6.56 kWh/m^3 which means 0.6–0.65 oil (Dobre et al. 2014). Calorific power depends on the methane content in biogas being a variable from the organic matter employed to generate biogas, reactor type, and operational conditions. From these principal parameters, the composition of biogas can be expected (Table 8.1).

A. Hernández-Palomares · Y. Reyes-Vidal · F. Espejel-Ayala (✉)
Center of Research and Technological Development in Electrochemistry, Parque Tecnológico Querétaro, Querétaro, Mexico
e-mail: ahernandez@cideteq.mx; mreyes@cideteq.mx; fespejel@cideteq.mx

P. Chowdhary et al. (eds.), *Bio-Clean Energy Technologies: Volume 1*, Clean Energy Production Technologies, https://doi.org/10.1007/978-981-16-8090-8_8

Table 8.1 Typical composition of biogas considering different organic matter (Fantozzi and Buratti 2009)

Component	Organic matter					
	Fowl	Cattle manure	Pig manure	WWTP sludge	Domestic residues	Crop residue
CH_4 (%vol)	54.5	29.7	37.8	60.0	60.0	68.0
CO_2 (%vol)	37.2	35.3	21.3	33.0	33.0	26.0
O_2 (%vol)	0.0	0.0	0.0	0.5	0.0	0.0
H_2S (ppm)	–	285.0	410.0	717.4	645.7	286.9
CO (ppm)	330.7	394.7	432.5	–	–	–

Biogas can contain the following components: CH_4, CO_2, H_2S, water vapor, hydrogen, nitrogen, ammonia, siloxanes, and particulate matter. CO_2 is the principal component, after CH_4, which can decrease the calorific power. Another undesirable gas is H_2S that generates corrosion in the energy system that burns biogas. Therefore, the removal of these gases is desirable to reach a calorific power of 11 kWh/m^3. Purification techniques of biogas principally are applied to eliminate CO_2 and H_2S. Physical and chemical absorption cryogenic methods and membranes are the technologies used at the industrial level (Angelidaki et al. 2018). However, the selection of technology must consider the technical and economical issues due to energetic penalties in the applied process: the spent energy to purify biogas must be less than the energy supplied from biogas. With this consideration, the implemented technology of purification is sustainable. Technologies to purify biogas are described as follows.

8.1.1 Sorption

This process is very much used in biogas treatment and other gases. Physical adsorption implies the sorption of gas into a solid adsorbent. Activated carbon, zeolites, silica gel, or other molecular sieves can form the fixed bed in a column. Adsorption of gases occurs from electrostatic forces between the solid adsorbent surface and molecules of gases. Moisture, pressure, temperature, and the presence of particles can affect the volume of gas adsorbed as well as the selectivity. Commonly, physical adsorption can be described by models that consider the monolayer adsorption in porous media. Langmuir model is employed to describe the maximal capacity of adsorption although a multi-component form of the equation is considered.

In contrast, the chemical absorption process uses aqueous solutions of NaOH or ammines, principally. Approximately, at 48 ° C, CO_2 and H_2S react with the aqueous solutions generating carbonates, bicarbonates, or carbamates. Although the process is used at the industrial level, the issue is the generation of waste due to deterioration of ammines and NaOH solutions in the regeneration of solutions by thermal

treatment (Kamopas and Kiatsiriroat 2019). In this process, biogas is compressed and passed into a column at counter-current about solutions.

8.1.2 Pressure Swing Adsorption (PSA)

PSA is a technology used at the industrial level to separate gases like CO_2 from biogas. Separation occurs from high pressure in the system. Gases are attracted in the surface solid adsorbent as activated carbon or zeolites. The solid adsorbent is chosen depending on the type of gas for separating. The solid adsorbent is regenerated by a decrease of gradual pressure, and the sorbed gas is liberated for loading again the column. The process has a principal disadvantage: the adsorption capacity of the system depends on the amount of solid adsorbent; then, a great plant can be installed into two or eight columns in parallel (Canevesi et al. 2019).

8.1.3 Cryogenic Methods

This technology is based on the volatility of gases at very low temperatures to separate gases by the different boiling points. Companies use this process to separate and concentrate gases from the air. Considering CO_2 and CH_4 boiling points are -78.46 and -161.5 °C, respectively. Then, CO_2 is first condensed making the separation relatively simple. However, to reach these temperatures, energetic penalties must be considered due to high energy consumption.

8.1.4 Membranes

A selective barrier is used to permit that one gas permeates and another not. Using membranes, molecules, ions, and small particles, separation is carried out. However, to obtain a high-purity permeate, sequential membrane systems are necessary. Separation occurs by three principal mechanisms shown in Fig. 8.1 (Koros and Fleming 1993).

Knudsen diffusion is carried out when the pore diameter in the membrane is less to mean the free path of molecules. That is, molecules hit with the wall in the pores with high frequency and diffuse into pores. Equation (8.1) defines the Knudsen diffusion:

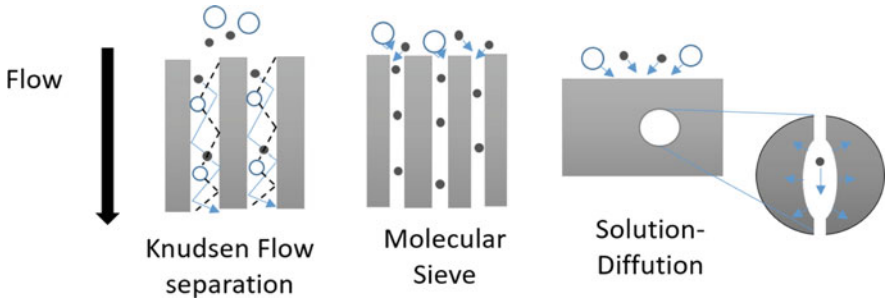

Fig. 8.1 Mechanisms of mass transport in a membrane (Adapted by Koros and Fleming 1993)

$$D = \frac{1}{\sqrt{\frac{M_A}{M_B}}} \tag{8.1}$$

where D is the diffusion and M_A and M_B are the molecular weight of A and B, respectively. Separation occurs by the difference or similitude of molecule and pore sizes. If the molecular diameter is higher pore size the molecule gas does not cross the membrane. When the difference in the diameter of the molecule is great, the separation will be simpler. Then, in the case of oxygen and nitrogen, their separation is complicated by the Knudsen diffusion mechanism. The molecular sieve mechanism occurs when the pore size is low than molecular size. Filtration is similar to molecular sieve, but, in this case, gaseous adsorption can be considered in the general mechanism. In polymeric membranes, molecules diffuse due to the diffusion capacity of molecules in the polymer. Diffusion in polymeric membranes depends on the density of polymer and molecules size (Javaid 2005).

Membranes separates biogas according to different molecular size in the mixture. Membrane pores must retain methane and allow smaller molecules such as CO_2, H_2S, and N_2 to pass through the membrane. Selectivity and permeability membrane are key parameters for gas separation. Selectivity is inversely proportional to gas permeability; the relationship between selectivity logarithm and highest permeability logarithm is limited to achieve the desired result of high selectivity combined with high permeability (Robeson 1991).

Polymeric membranes show a greater ability in the gas separation market for separating CO_2/CH_4 mixtures, but CO_2 in high concentration can plasticize them and decrease their separation capacity. Research is still being carried out to increase resistance, selectivity, and permeability. Table 8.2 shows polymeric materials examples used for membranes manufacture and their efficiency in CO_2/CH_4 separation.

Table 8.2 Polymeric materials for membranes manufacture and their efficiency in CO_2/CH_4 separation

Polymeric material	Permeability CO_2 (Barrer)	Permeability CH_4 (Barrer)	Selectivity CO_2/CH_4	Surface area (m^2/g)	References
TR-α-PBO	4045	73	55.4	535	Park et al. (2007)
TR-α-PBI	1624	35	46.4	447	Han et al. (2010)
TR-α-PBO-co-PI	269.5	–	8.3	389	Jo et al. (2015)
PIM basic	4646	–	–	550	Rogan et al. (2014)
PIM with substituted groups	2130	177	12	410	Carta et al. (2014)
PIM-PI	218	6.7	32.5	250	Zhuang et al. (2016)
PIM-TR-PBO	8.8	0.5	16.6	5	Li et al. (2013)

PMP Poly(4-methyl-2-pentyne), *TR* thermally rearranged polymers, *PBO* polybenzoxazole, *PBI* polybenzimidazole, *PI* polyimidazole, *PIM* polymers of intrinsic microporosity

8.1.5 Zeolites

Inorganic membranes potentially have significant advantages over polymeric membranes due to high thermal, mechanical, and chemical stability and good erosion resistance (Sahota et al. 2018). Zeolitic materials, natural or synthetic, have uniform pores and channels, and these have varied dimensions and arrangements depending on zeolite type. According to their structure, they can be used in catalysis, adsorption, and separation processes.

Zeolites have a three-dimensional structure created from rigid tetrahedral composed of a cation and four oxygen atoms, which, when bonded with adjacent tetrahedral, share oxygen atoms (tetrahedron is represented as TO_4). Mainly the cation is silicon (Si) and aluminum (Al), but they can be different elements like Ti, P, Be, Zn, Mg, Co, and B, or any other tetrahedrally coordinated cation. The zeolite unit cell is described by Eq. (8.2):

$$M\frac{y}{m}\left[(SiO_2)_x(AlO_2)_y\right]\cdot wH_2O \tag{8.2}$$

where M is valence cation; m is generally alkali or alkaline earth metals, although organic cations can be used to counteract the negative charges generated by aluminum ions; w is water contained in the cell; and, finally, x and y are tetrahedral numbers per unit cell.

Zeolites are porous materials with pore aperture dimensions (3–13 Å) varying due to structure type. The pore size is mainly defined by the number of tetrahedrally coordinated elements that form rings when linked, in addition to the influence of cation charge compensating. These factors define a large number of different geometries, such as large internal cavities or extensive uniform channels throughout the entire crystal. There are about 40 types of natural zeolites and about 200 synthetic zeolites. These characteristics have made the separation of various gas mixtures from different arrangements of zeolite channels of different types. In recent years, different types of membranes have been studied, among which are the membranes based on SSZ-13 (Falconer et al. 2015), DDR (Van den Bergh et al. 2008), silicalite-1 (Liu et al. 2016), ZSM-5 (Amedi and Aghajani 2016), SAPO-34 (Falconer et al. 2016), and AlPO-18 (Wang et al. 2015).

8.1.6 Silicoaluminophosphates

Aluminophosphates are molecular sieves with structures composed of aluminum, phosphorus, and oxygen. They have some hydrophilicity due to the difference in electronegativity between aluminum and phosphorus (Lohse et al. 1986).

In the 1960s, several attempts were made to synthesize materials with the isomorphic replacement of silicon by phosphorus. The results were several aluminosilicates and phosphates with similar properties or even showing no beneficial properties such as molecular sieve (Barrer and Marshall 1965). At the beginning of the 1980s, a patent was presented with the description of the synthesis process of a new class of silicon substituted aluminophosphates or silicoaluminophosphates (SAPO), which at the same time were both crystalline and microporous (E. U. A Patent no US4440871A 1982).

This class of materials are made up of PO_2^+, AlO_2^- y SiO_2 tetrahedra, and the synthesis refers to reaction mixtures free of alkali metals; therefore, its empirical formula in a molar ratio of oxides is represented as:

$$aR_2O : \left(Si_xAl_yP_z\right)O_2 : bH_2O$$

where R is an organic molecule that acts as a structure-directing agent (SDA) and a represents the moles of R per mol of$(Si_xAl_yP_z)O_2$; its minimum value is 0.3, and maximum value depends on the molecular dimensions of the SDA used for each type of silicoaluminophosphate. b has a preferable value of 2 to 30. The mole fractions of silicon, aluminum, and phosphorus are represented by x, y, and z, respectively. These mole fractions are within the pentagonal compositional area defined by points ABCD and E of the ternary diagram (E. U. A Patent no US4440871A 1982). The points ABCD and E represent the values shown in Table 8.3 for x, y, and z.

Table 8.3 Molar fraction for points ABCD and E (E. U. A Patent no US4440871A 1982)

Point	Molar fraction		
	x	*y*	*z*
A	0.01	0.47	0.52
B	0.94	0.01	0.05
C	0.098	0.01	0.01
D	0.39	0.60	0.01
E	0.01	0.60	0.39

Table 8.4 Types of bonding of Si and Al atoms

Element	Bond
Al	Al(4Si)
Si	Si (0Al, 4Si), Si (1Al, 3Si), Si (2Al, 2Si), Si (3Al, 1Si), y Si (4Al, 0Si)

8.1.6.1 Structural Composition of Silicoluminophosphates

There are several criteria to determine the distribution of the Al and Si atoms in the SiO_4 and AlO_4 tetrahedra that form the structure of the aluminosilicates. Löwenstein's rule states that bonds between two Al tetrahedra cannot be carried out (Flanigen et al. 1986). Al atom can only bond with four adjacent Si atoms, and Si atoms can bond in different ways in the zeolite structure. Table 8.4 shows the types of bonds that Si and Al atoms can form.

The substitution of the Si atom by P atoms in aluminosilicates gives rise to the family of aluminophosphates that are formed by AlO_4 and PO_4 tetrahedra. However, Al and P atoms can be isomorphically replaced by Si, Li, Be, B, Ge, As, Si, Mg, Cr, Mn, Fe, and Co atoms (Bennett et al. 1986). Different isomorphic substitution mechanisms (SM) of Al and P atoms were established to understand the metal cations (Me) and for Si atoms in the $AlPO_4$ structure. Metal cations can replace Al atoms (SM I); these can be monovalent, divalent, and tetravalent elements of Al atoms (SM Ia, SM Ib, and SM Ic, respectively); this results in a Me-O-P bond. P atoms (SM II) can be substituted by tetravalent and pentavalent elements of P atoms (SM IIa and SMIIb, respectively), and this results in a Me-O-Al bond. Replacing the atoms Al and P adjacent metal cations form a Me-O-Me being this link very stable. On the other hand, Si is the only one that can be substituted by Al and P atoms at the same time (SM III) (Martens et al. 1990).

SM III substitution can form silicoaluminophosphate (SAPO) structures. Structure SAPO is formed by the combination of the substitution mechanisms SM IIa and SM III. Equations (8.3) and (8.4) show the species present in the final structures (Sastre et al. 1997):

$$\underset{\text{AlPO}_4\text{ Structure}}{\text{SM IIa} : [\text{AlPO}_4] + \text{Si}^{4+} + \text{H}^{+}} \rightarrow \underset{\text{SAPO Structure}}{[\text{Si, H}]_{\text{P}} + \text{P}^{5+}} \tag{8.3}$$

$$\underset{\text{AlPO}_4\text{ Structure}}{\text{SM III} : [\text{AlPO}_4]} + 2\text{Si}^{4+} \rightarrow \underset{\text{SAPO Structure}}{[2\text{Si}]_{\text{Al,P}}} + \text{P}^{5+} + \text{Al}^{3+} \tag{8.4}$$

where species are in subscripts and final structures are in brackets. At the beginning of the reaction, Si atoms are incorporated by the SM IIa mechanism. Replacement of a pair of adjacent Al and P atoms with two Si atoms (SM III ho) generates a Si-O-P bond, but this bond is unstable. From a critical concentration of Si, which can be below the stoichiometric level of substitution SM IIa, substitution SM III begins to occur simultaneously and extends until individual regions of the crystal become siliceous, forming a heterogeneous substitution (SM III silicon patches). Substitution SM III has resulted in a neutral electron structure comprising layers of $AlPO_4$ and layers of SiO_2. At the boundary of two crystal domains, Si (3Si, 1Al) and Si (1Si, 3Al) are present. Negative charges are generated in structure when Si atoms are replaced by Al atoms in the SM II substitution. Si substitution mechanisms are sensitive to different synthesis parameters, for example, Si content (Martens et al. 1990), nature of SDA (Wang et al. 1991), SDA/Al_2O_3 ratio, and P_2O_5/Al_2O_3 in the reaction mixture (Jahn et al. 1990), pH (Mertens 1989), and crystallization temperature (Young and Davis 1991).

8.1.6.2 Alkali Metal Content in Silicoaluminophosphate Synthesis Gel

The chemical composition of the SAPO-34 structure includes Si, Al, P, and SDA atoms, but it doesn't include alkali metals or other cations that may be present in the reaction mixture. When these cation species are present, they function as charge balance ions for AlO_2^- tetrahedra that aren't associated with a PO_2^+ or an organic ion derived from SDA (E. U. A. Patent no US4310440A 1980). A high content of alkali metals in synthesis gel increases the pH and forms aluminophosphates with dense structures without the presence of pores (E. U. A Patent no US4440871A 1982). Alkali cations appear in the SAPO structure when they are unintentionally introduced into the reaction mixture as impurities. The cations can be observed forming foreign compounds or as indicated above, as structural cations that balance the net negative charges of some crystal lattice sites. Different commercial sources of silicon, aluminum, phosphorus, and SDA contain alkali metal concentration as impurities. For example, TEAOH has been observed to contain potassium 1.6% by weight and sodium 0.05% by weight, while phosphoric acid frequently contains more than 0.01% of alkali metals (E. U. A. Patent no US7528201B2 2005). In the synthesis of SAPO CHA/AEI, with an alkali metal content of 0.1–0.5 g/mol of Al_2O_3 the obtained average particle size was 2.2 μm. Also in the alkali metal content of 1–2.5 g per mole of Al_2O_3, the result was a material with a higher percentage of AEI type structure (E. U. A. Patent no US7528201B2 2005).

8.2 SAPO-34 and Its Applications

SAPO-34 is a silicoaluminophosphate material with Si, Al, P and O atoms in their structure type chabazite (CHA) forming channels and eight ring pore of 0.38 nm. This material was first synthesized by Union Carbide Corporation (E. U. A Patent no US4440871A 1982). This material is used in the petrochemical industry as a catalyst in the conversion of methanol to olefins (MTO) (Askari et al. 2016). With pore size equal to the kinetic diameter of methane, SAPO-34 membranes have shown high efficiency in separating the CO_2/CH_4mixture (Xue et al. 2013) and hydrogen purification (Falconer et al. 2008). SAPO-34 in coordination with transition metals obtained a material used for catalysis of nitrogen oxides from combustion gases (Schneider et al. 2016).

8.2.1 SAPO-34 Membranes

Inorganic membranes have advantages over polymeric membranes due to their chemical and thermal stability. The SAPO-34 pore size of 0.38 nm gives the membranes the capacity for selective separation. Membranes with an excellent separation capacity have been synthesized by molecular sieving of different gas mixtures.

8.2.1.1 Membranes Preparation

Zeolite membranes are synthesized on porous support by hydrothermal process. It is difficult to control zeolite nucleation and membrane growth; therefore, it is important to control the synthesis parameters to obtain a membrane without defects. Different methods allow the growth of defect-free zeolite membranes, for example, in situ growth and secondary growth.

8.2.1.2 Secondary Growth

Seed growth is a method of creating flawless thin-layered molecular sieves supported on different substrates. In this method, ex situ crystallization is performed first. This method consists, first of all, of ex situ crystallization of the seed. The SAPO-34 seed is synthesized by different methods that will produce crystals with different characteristics each. Seed characteristics will affect the membrane properties. The seed is deposited on the substrate by rubbing or dipping and will form a thin film in the secondary growth process.

8.2.1.3 Seed Synthesis

The characteristics of the seed will depend on the synthesis conditions; likewise, the seed layer that covers the surface of the support ensures that the core of the support is dense and uniform; therefore, the possibility of defect formation is considerably reduced. The seed size is an important parameter. At a smaller size, the seed can enter the support pores and reduce the permeation flow. The crystal size should be around 30–120 nm; if the seed size is too large, membrane cross-linking will decrease, and membrane thickness will increase (Jiang et al. 2004).

8.2.1.4 Hydrothermal Synthesis

The most used SAPO-34 synthesis method is the hydrothermal method. This method is based on the crystal growth in superheated mineral solutions in water at temperatures between 180 and 200 °C in presence of the structure-directing agent. This process is carried out in sealed autoclaves, heated in an oven at a specific temperature, and time and autogenous pressure is generated.

8.2.1.5 Crystallization Time

This is a very important factor in the hydrothermal synthesis of zeolites. It is the time that the precursor solution is under hydrothermal treatment. The zeolite crystalline phase and crystal size depend on the crystallization time. In the SAPO-34 hydrothermal synthesis, after 14 h of crystallization, the amorphous phase disappears completely (Askari et al. 2012), and the pure phase and the surface area increase (Wang et al. 2011). Crystal growth with increasing time is attributed to the Ostwald maturation phenomenon, which describes that, with increasing crystallization time, the smaller crystals dissolve and settle into larger particles (Pavlov et al. 2012). However, at minimum crystallization time, a crystalline phase cannot be obtained, and an amorphous material will be obtained. In zeolites, at a longer crystallization time, more stable crystalline phases appear, and the less stable ones disappear (Pavlov et al. 2012). In Table 8.5 is shown the effect of the SAPO-34 crystallization time and its effect on gas separation.

The table shows that the crystallization time is proportional to the membrane thickness. Chen et al. (2017) report that the minimum time for adequate crystallization of SAPO-34 membrane is 8 h, and at a shorter time, the capacity to separate gases decreases (Chen et al. 2017).

Gas permeation units (GPU) don't contain the thickness of the membrane. To analyze the effect of crystallization time with membrane thickness, it is necessary to convert the units of permeability from GPU to Barrer. Equation (8.5) shows the equivalence of 1 GPU:

Table 8.5 Effect of crystallization time in SAPO-34 for gas separation

Composition Al_2O_3:P_2O_5: SiO_2: SDA: H_2O	Time (h)	Temperature (°C)	Membrane thickness (μm)	Permeability (Barrer)	References
1: 1: 0.3: 1 TEAOH: 1.6 DPA: 150	2	220	4	217.4	Liu et al. (2020a, b)
	4	220	7	491.3	
	6	220	7.5	387.5	
	8	220	7	351.2	
1: 1: 0.45: 1,2 TEAOH: 1,6 DPA: 100: 6.5% PDMS	18	180	8	191.1	Rehman et al. (2020)
1: 1: 0.45: 1,2 TEAOH: 1,6 DPA: 100: 7.5% PDMS			9	225.8	
1: 1: 0.45: 1.2 TEAOH: 1.6 DPA: 100: 8.5% PDMS			12	290.3	
1:1:0.3:1.0 TEAOH:1.6 DPA:150	6	210	6	430.1	Mu et al. (2019)
	12	210	6	448.02	
	22	210	7	501.7	
1: 1: 0.6: 1.2 TEAOH: 55	6	180	2	0	Chen et al. (2017)
	6	180	3	0	
	12	180	5	273.2	
	12	180	7	286.4	
	18	180	12	344.08	
	18	180	15	362.9	
	24	180	9	352.1	
	24	180	11	446.8	
	30	180	13	528.07	
	30	180	11	335.1	

SDA Structure director agent, *TEAOH* tetramethylammonium hydroxide, *PDMS* α, ω-dihydroxypolydimethylsiloxane, *DPA* dipropilamine

$$1\ \text{GPU} : 3.35 \times 10^{-10} \frac{\text{mol}}{\text{m}^2 \cdot \text{s} \cdot \text{Pa}} \tag{8.5}$$

To convert from GPU to Barrer, multiply the value of the membrane thickness by the equivalence of 1 Barrer.

$$1\ \text{Barrer} : 3.35 \times 10^{-16} \frac{\text{mol} \cdot \text{m}}{\text{m}^2 \cdot \text{s} \cdot \text{Pa}} \tag{8.6}$$

From the data observed in Table 8.5, the crystallization time vs thickness of the membranes was plotted. The graph shows that the crystallization time has a proportional effect on membrane thickness. The reports also mention that at a long

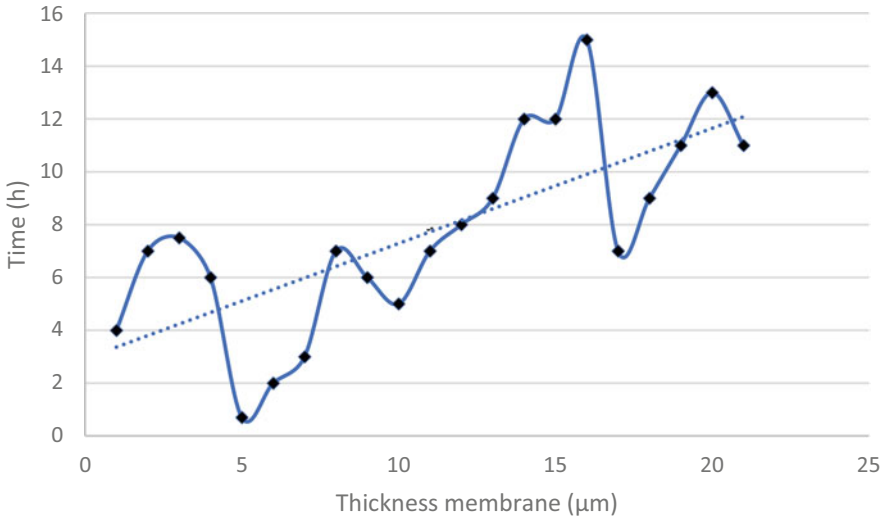

Fig. 8.2 Effect of synthesis time on membrane thickness

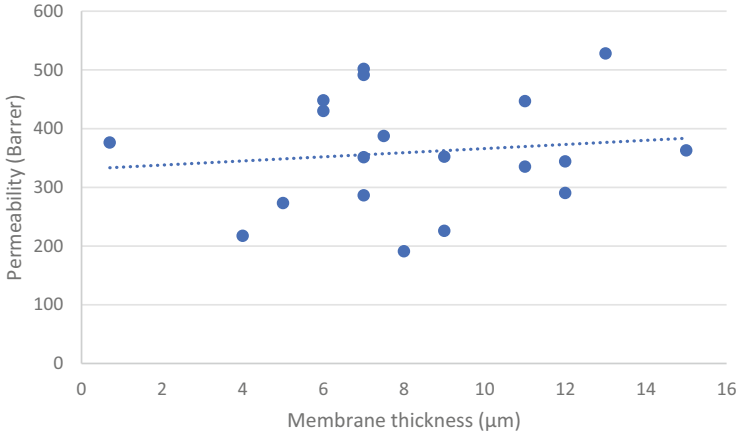

Fig. 8.3 Effect of membrane thickness on CO_2 permeability

crystallization time, the thickness of the membrane increases (Chen et al. 2017) (Fig. 8.2).

The influence of membrane thickness on the membrane gas separation capacity was evaluated. It's important to assess whether the thickness of the membrane has a significant effect on the gas separation capacity of the membrane. Figure 8.3 shows the graph of membrane thickness vs permeability with the data in Table 8.5 plotted, and a slightly upward almost linear trend is observed. It was observed that the membrane thickness doesn't affect CO_2 permeability. Therefore, the permeability

of the SAPO-34 membrane is measured in GPU where it's not necessary to consider the membrane thickness (Fig. 8.3).

8.2.2 *Nature and Concentration of the SAPO-34 Seed Synthesis Precursors*

The origin of the aluminum, silicon, and phosphorus sources used in the SAPO-34 synthesis influences the crystallinity, crystal size, and morphology of the final product. In the case of silicon, silica sol or fumed silica is preferably used (E. U. A Patent no US4440871A 1982). These silicon sources are used for SAPO-34 membrane synthesis for gas separation (Australia Patent no AU2007249452B2 2007). Other sources of silica are used, for example, precipitated amorphous silica (Ali et al. 2019), silica gel (Makertihartha et al. 2020), silicon alkoxides (Varzaneh et al. 2020), and other sources such as silicic acid and sodium silicate (Lu et al. 2019). Table 8.6 shows that the most widely used source of silica is colloidal silica (Ludox AS-40) and fumed silica. Some reports using silica gel or silica alkoxides were also observed.

The reports show that decreasing the silicon concentration increases the CO_2 separation capacity. At medium-low silicon concentration, the SAPO-34 formation increases without presenting the combination of other crystalline structures. The particle diameter of the silicon source is important for solubility in an aqueous medium. The silica particle will dissolve from the outside in, so that, with smaller particle diameter, it dissolves to obtain a greater quantity of silicon species in reaction (Iler 1979). Aluminum alkoxides such as aluminum isopropoxide and pseudo-boehmite are preferred for SAPO-34 formation. Moreover, crystalline or amorphous aluminophosphates can be used as a source of aluminum and phosphorus. Table 8.7 shows the most used aluminum sources for the SAPO-34 synthesis.

The most widely used source is aluminum isopropoxide due to its greater ability to dissolve; this favors the formation of nuclei and crystallinity. Defect-free membranes are formed with these aluminum sources. Aluminum sources such as $AlCl_3$, $NaAl_2$, and Al_2SO_4 aren't mentioned because they alter the pH of the gel or contain cations that prevent silicoaluminophosphate formation (E. U. A Patent no US4440871A 1982). The use of pseudoboehmite aluminum isopropoxide, hydrargalite, α-Al_2O_3, and aluminum chloride for the synthesis of SAPO was reported. With Argilita and aluminum chloride, dense phases like cristobalite were obtained, without the presence of SAPO structures. When using α-Al_2O_3, the aluminum did not react due to the low solubility. Using pseudoboehmite and aluminum isopropoxide, it was possible to obtain a SAPO material without an amorphous material present (Weyda and Lechert 1990). The use of $Al(OH)_3$ was reported for SAPO-34 membrane synthesis, and membranes with clogged pores were obtained, hindering the permeability of CO_2 and He (Funke et al. 2012). In other reports, the membranes synthesized with $Al(OH)_3$ showed greater efficiency in

Table 8.6 Effect of silicon source on gas separation

Silicon sourses	Composition Al_2O_3: P_2O_5: SiO_2: SDA: H_2O	Ratio Si/Al	SDA	Permeability $CO_2 \times 10^{-8}$ mol/m^2 s Pa	References
Silica gel	1: 1: 0.4: 1.2 TEAOH: 47	0.2	TEAOH	4.8	Siamak et al. (2010)
Silica gel	1: 1: 0.6: 1.2 TEAOH: 47	0.3	TEAOH	3.6	
Coloidal silica (Ludox AS-40)	1: 1: 0.6: 1.0 TEAOH:1.6 DPA: 150	0.78	DPA	2.5	Mu et al. (2019)
Coloidal silica (Ludox AS-40)	1: 2: 0.6: 1.0 TEAOH: 1.6 DPA: 75	–	TEAOH/ DPA	2.7	Mirfendereski (2019)
Fumed silica	1: 1: 0.8: 2 TEAOH: 55	0.49	TEAOH	2.01	Makertihartha et al. (2020)
Coloidal silica (Ludox AS-40)	1: 2: 0.6: 4.0 TEAOH: 75	–	TEAOH	2.6	Bai et al. (2017)
Coloidal silica (Ludox AS-40)	1: 2: 0.6: 4.0 TEAOH: 75	2.3	TEAOH	1.82	Mei et al. (2018)
Coloidal silica (Ludox AS-40)	1: 1: 0.6: 1.2 TEAOH: 55	2.3	TEAOH	1.83	Liu et al. (2020a, b)
Coloidal silica (Ludox AS-40)	1: 1: 0.6: 1.2 TEAOH: 55	–	TEAOH	60.6	Rehman et al. (2020)
Coloidal silica (Ludox AS-40)	1: 2: 0.6: 4.0 TEAOH: 75	–	TEAOH	4.1	Zhang et al. (2019)
Sílice coloidal (Ludox AS-40)	1: 2: 0.6: 1.0 TEAOH: 1.6 DPA: 75	0.1554	TEAOH	32	Mirfendereski (2019)
Coloidal silica (Ludox AS-40)	1: 0.6: 1: 2 TEAOH: 60	–	TEAOH	4.02	Huang et al. (2015)
Coloidal silica (Ludox AS-40)	1: 1: 0.3: 1.2TEAOH: 80	–	TEAOH	18	Chew et al. (2011)

CO_2/CH_4 mixture separation compared to the membranes synthesized with aluminum isopropoxide (Zhou et al. 2013). Good results were also achieved by using Al $(OH)_3$ in membrane synthesis on a larger scale, increasing the size from 5 to 25 cm (Li et al. 2010). In the phosphorous source case, phosphoric acid (H_3PO_4) is most used. Variation on concentration influences the physicochemical characteristics in

Table 8.7 Effect of aluminum source on gas separation

Aluminum source	Composition Al_2O_3:P_2O_5: SiO_2: SDA: H_2O	Ratio Si/Al	SDA	Permeability $CO_2 \times 10^{-6}$ mol/m^2 s Pa	References
Pseudoboehmite	1: 1: 0.8: 2 TEAOH: 55	0.49	TEAOH	2.01	Makertihartha et al. (2020)
$Al(OH)_3$	1: 2: 0.6: 4.0 TEAOH: 75	–	TEAOH	2.6	Bai et al. (2017)
$Al(OH)_3$	1: 2: 0.6: 4 TEAOH: 75	–	TEAOH	1.08	Zhou et al. (2013)
Isopropóxido de aluminio	1: 2: 0.6: 1.0 TEAOH: 1.6 DPA: 75	–	TEAOH/ DPA	2.7	Mirfendereski (2019)
$Al(OH)_3$	1: 2: 0.6: 4 TEAOH: 75	–	TEAOH	0.8	Chang et al. (2018)
$Al(OH)_3$	1: 2: 0.32: 1 TEAOH: 0.8DPA: 75	0.16	TEAOH/ DPA	0.44	Li et al. (2010)
Bohemite	1: 0.3: 1: xMorfolina: 66	0.402	Morfolina	1.2	Das et al. (2012)
Bohemite	1: 0.3: 1: xMorfolina: 66	0.402	Morfolina	2.75	Das et al. (2012)
Aluminum isopropoxide	1: 1: 0.6: 1.2 TEAOH: 55	–	TEAOH	60.06	Rehman et al. (2020)
Aluminum isopropoxide	1: 2: 0.6: 4.0 TEAOH: 75	–	TEAOH	4.1	Zhang et al. (2019)
Aluminum isopropoxide	1: 2: 0.6: 1.0 TEAOH: 1.6 DPA: 75	0.1554	TEAOH/ DPA	32	Mirfendereski (2019)
Aluminum isopropoxide	1: 0.6: 1: 2 TEAOH: 60	–	TEAOH	4.02	Huang et al. (2015)
Aluminum isopropoxide	1: 1: 0.3: 1.2TEAOH: 80	–	TEAOH	18	Chew et al. (2011)

TEAOH Tetramethylammonium hydroxide

the zeolite; at high concentration of phosphorus a combination of phases is obtained decreasing the surface area in the total material (Sedighi et al. 2014).

8.2.2.1 Water Content

In the hydrothermal process, the water amount is important to know the components of zeolite dissolve. Water amount influences pH and ion amount in the gel and affects the crystallinity of the final product (Table 8.8).

Water amount is important in SAPO-34 synthesis. An increasing of ions in the gel of synthesis of SAPO-34 occurs with the variation of water in the system that benefits the formation of the zeolite. At the same time, when the water content in

Table 8.8 Effect of water content on SAPO-34 synthesis

Phase	Crystal size (μm)	Surface area (m^2/g)	Composition Al_2O_3:P_2O_5: SiO_2: SDA: H_2O	References
SAPO-34	6	592	1: 0.4: 1: 0.5: 1.5MOR: 60	Sedighi et al. (2014).
SAPO-34	3	462	1: 0.4: 1: 0.5: 1.5MOR: 75	
Indefinite phase	1	330	1: 0.4: 1: 0.5: 1.5MOR: 100	

MOR Morpholine

the synthesis gel increases, the ion concentration decreases, causing a change in pH, and alkalinity medium decreases. Altered pH affects zeolite nucleation (Sedighi et al. 2014). In membrane synthesis, the water amount could influence the defects number. For example, when using a greater water amount, the membrane continuity could be affected, creating spaces without a membrane in the support, and this would affect the gases separate ability of the membrane (Sedighi et al. 2014).

8.2.2.2 Effect of Structure Director Agent Type

The SDA type is important for SAPO-34 synthesis because of its role in nucleation and crystallization. In addition, it can affect the solution alkalinity and precursor interaction (Yang et al. 2013). The synthesis of SAPO-34 with different SDAs was reported (Carreon et al. 2008). SDAs contain nitrogen, phosphorus, arsenic, and antimony with at least one alkyl or aryl group having one to eight carbon atoms. Amines are the main nitrogenous SDAs and quaternary ammonium compounds. Polymeric quaternary ammonium salts $[(C_{14}H_{32}N_2)\ (OH)_2]_x$, where "$x$" has a value of at least 2, is thoroughly. The mono-, di-, and tri-amines are used alone or in combination with a quaternary ammonium compound or another template compound. SDA mixtures can produce silicoaluminophosphate mixture. This zeolite has been shown to be synthesized with various templates such as tetraethylammonium hydroxide (TEAOH), dipropylamine (DPA), diethylamine (DEA), triethylamine (TEA), morpholine, piperidine, and others. Membranes synthesized with different SDAs have different properties for gas separation (Carreon et al. 2008). Table 8.9 shows the SDA effect on SAPO-34 synthesis.

TEAOH, morpholine, and DPA showed better results in the synthesis of SAPO-34 membranes for gas separation (Carreon et al. 2008). High concentration of morpholine benefits the synthesis of SAPO-34 zeolites while the SAPO-5 is obtained at low concentrations. TEAOH in synthesis gel creates smaller crystals which are favorable when SAPO-34 is used for the MTO reaction. Table 8.9 shows that SAPO-34 with morpholine as SDA yields larger crystal sizes compared to TEAOH, DPA, and TEA. The surface area of morpholine/SAPO-34 doesn't exceed 500 m^2/g; on the other hand, TEA/DEA/SAPO-34 almost reaches 800 m^2/g. No relationship was observed between membrane surface and separate gases ability. The surface area of the membrane is not reported in papers. The seed crystals surface

Table 8.9 Effect in the synthesis parameters for formation of SAPO-34

Crystalline phase	Molar composition Al_2O_3: P_2O_5:SiO_2:SDA:H_2°	Time (h)	Temperature (°C)	Si, Al, and P sources	SDA	Surface area (m^2/g)	Crystal size (μ)	Note	References
SAPO-34	1.0:0.56:1.0:1.72:66.5	12	200		Morpholine		0.6	Seed-assisted synthesis for crystal size reduction	Ali et al. (2019)
Cu-SAPO-34	0.26:0.1:0.12:0.09:7.2:0.09	48	200	Fumed silica Pseudoboehmite Phosphoric acid	Morpholine	346		Cu/SAPO-34 catalyst	Cheng et al. (2019)
SAPO-34	x:1.0:0.6:3:80: (0.02–2000) PEG (x = 1.0, 1.5 y 2.0)	48	200	Pseudoboehmite TEOS Phosphoric acid	Morpholine	528	2–4	PEG and high Al ratio were used to obtain a petal morphology	Zhang et al. (2019)
SAPO-34	1:1:0.6:3:50	48	200	Pseudoboehmite/ aluminumisopropoxide Silica gel, phosphoric acid	Morpholine / TEA	598	0.2–0.5	Morpholine treatment to reduce SAPO-34 from micro to nanoparticles	Wang (2020)
SAPO-34	1.0:1.0:(0.3–1):1:50	6, 12, 24, 36 y 48	150, 170, 190 y 200	Aluminum isopropoxide, colloidal silica, TEOS, sodium silicate, phosphoric acid	Morpholine	609	0.3–0.8	The optimal conditions were temperatures of 200 ° C, time greater than 12 hours, and the best was colloidal silica	Lu et al. (2019)
SAPO-34	1.0:1.0:0.4:4.7:70	24	180	Fumed silica Pseudoboehmite Phosphoric acid	Triethylamine (TEA)	569	10	TEA etching to create larger secondary pores	Pan et al. (2019)

(continued)

Table 8.9 (continued)

Crystalline phase	Molar composition Al_2O_3: P_2O_5:SiO_2:SDA:H_2O	Time (h)	Temperature (°C)	Si, Al, and P sources	SDA	Surface area (m^2/g)	Crystal size (μ)	Note	References
SAPO-34 hierarchicalpores	1.0:1.0:0.6:3:60	48	200	Silica sol Pseudoboehmite Phosphoric acid	TEA	628	2	Creation of larger secondary pores was done with oxalic acid and heat treatment	Liu et al. (2019)
SAPO-34	1:0.3:1: 3.9:70: X DEA·HCl (x = 0.2,0.4 y 0.6)	24	200	Pseudoboehmite Silica sol phosphoric acid	TEA/DEA-HCl	799	1–2	Combination of TEA/DEA-HCl templates produced hollow SAPO-34 particles	Yang et al. (2019)
SAPO-18/34	1.0:1:0.3: 7.0:15.7	48	200	Aluminum isopropoxide Coloidal silica phosphoric acid	TEA	604	1	The synthesis of two SAPO phases was carried out under the hydrothermal method with stirring	Sun et al. (2020)
Cu-SAPO-34	6.7:6.2:1:7.7:161.3	24	200	Fumed silica Aluminum isopropoxide Phosphoric acid	TEAOH		–	Mix SDA with copper precursor before synthesis to increase catalytic activity	Pétaud et al. (2019)

SAPO-34	1.0:2.0:0.6:4.0:75	7	180	Aluminum isopropoxide Colloidal silica Phosphoric acid	TEAOH	501	–	Microwave-assisted hydrothermal synthesis was performed in addition to assisted ozone for template removal	Rehman et al. (2019)
Nanosheets-SAPO-34	1:2: 0.6:2:105:0.4 TPOAC	96	180	Colloidal silica Aluminum isopropoxide Phosphoric acid	TEAOH	578	0.1	Nano sheets of SAPO-34 were synthesized from an organosilicone surfactant	Chen et al. (2019)
SAPO-34	1.0: 2.0: 0.6: 1.0/1.6: 75	24	190	Aluminum triisopropylate Coloidal silica Phosphoric acid	TEAOH/DPA		0.5–5	Different treatments were used to obtain membranes with uniform growth	China Patent no US20190169035A1 (2016)

PEG Polyethyleneglycol

area for membrane synthesis does influence its uniformity. Most surface area favors the growth uniformity of membrane (Askari et al. 2016).

8.2.2.3 Seeding Method

The seeding method is the fixation of seed crystals (preformed SAPO-34) on the substrate. The most used techniques for the synthesis of SAPO-34 membranes are described below.

8.2.2.4 Rubbing Method

In this method, the support is rubbed with SAPO-34 powder on the surface of the support where the membrane will grow when subjected to the hydrothermal process. To promote the interaction between the substrate and seed, a positively charged colloidal suspension was used, and the substrate surface was negatively charged to fix the seed on the substrate by electrostatic forces (Boudreau et al. 1999). The seed size is important; the smallest seeds will coat the surface evenly, and the membrane will grow homogeneously. The use of different-sized crystals was reported. The seed size was modified by mechanical milling, and seed sizes of 800, 290, and 300 nm were used, obtaining a CO_2/CH_4 selectivity in the synthesized membranes of 38–43, 59–64, and 118–166 AU, respectively (Chen et al. 2017). Table 8.10 shows the synthesis reports of SAPO-34 membranes using the rubbing method as seeding.

Table 8.10 Synthesis of SAPO-34 membranes from rubbing seeding

Gel composition	Substrate	Crystal size (nm)	Membrane thickness (μm)	Permeability de $CO_2 \times 10^{-6}$ mol/m^2 s Pa	References
$1Al_2O_3$:$1P_2O_5$: $0.6SiO_2$: 1TEAOH: 55 H_2O.	6 channel tube α-Al_2O_3	800	6	1.67	Chen et al. (2017)
		290	7	1.86	
		300	10	1.38	
$1Al_2O_3$:$1P_2O_5$: $0.8SiO_2$: 2TEAOH: 55 H_2O.	Silica tube	200–400	1.7	2.1	Makertihartha et al. (2020)
$1Al_2O_3$: $1P_2O_5$: $0.3SiO_2$: 1TEAOH: 1.6DPA: 150H_2O· Al$(OH)_3$	Al_2O_3 tube	1–0.3 μm	0.8	2.6	Bai et al. (2017)
$1Al_2O_3$: $1P_2O_5$: $0.45SiO_2$: 1.2TEAOH: 1.6 DPA: 100H_2O	4-channel tube α-Al_2O_3	300	7	1.68	Rehman et al. (2020)

8.2.2.5 Dip Coating Method

In this method, the support is used to immerse it in a suspension with SAPO-34 crystals. The crystals settle on the surface of the substrate where the membrane will grow when the support is introduced into the synthesis gel and is subjected to the hydrothermal process. (Lin et al. 2002). The implementation of a layer of polyvinylpyrrolidone (PVP) in the substrate has been reported before applying the SAPO-34 seeding. Subsequently, a 3% solution of SAPO-34 was prepared, and the particles were dispersed with ultrasonic treatment for 2 hours. A mullite tube was used as a substrate. From the implementation of the PVP coating, it was possible to increase the H_2/CO_2 selectivity from 2.67 to 4.2 (Das et al. 2012). Table 8.11 shows the synthesis reports of SAPO-34 membranes using the dip-coating method as seeding.

8.2.2.6 In Situ Crystallization

In this method, the support is introduced directly to the synthesis gel to later induce the growth of the membrane in a hydrothermal process (Meng-Dong et al. 1994). Membrane formation under hydrothermal conditions involves the formation of the supersaturation region adjacent to the substrate surface, nucleation, aggregation, crystallization, and crystal growth. The driving force for the deposition of particles is the gravity when the substrate is at the bottom of the autoclave; the particles are attracted by electrostatic forces between the particle and the substrate surface. The membrane thickness resulting from in situ crystallization averages 10 μm. In this method, there is no relationship between local supersaturation and synthesis conditions (Lin et al. 2002). The first in situ synthesis was reported as SAPO-34 membrane on ceramic support of α-Al_2O_3 in the shape of the disc of 5 mm in diameter and 3 to 5 mm in thickness and a diameter pore of 0.4 0.3 μm. At the end of the synthesis,

Table 8.11 Synthesis of SAPO-34 membranes from dip coating seeding

Gel composition	Substrate	Seed weight concentration	Membrane thickness (μm)	Permeability $CO_2 \times 10^{-6}$ mol/m^2 s Pa	References
1Al_2O_3: 0.3SiO_2: 1P_2O_5: 66H_2O	Tube α-Al_2O_3	34.3%	–	2.75	Das et al. (2012)
1Al_2O_3: 2P_2O_5: 0.6SiO_2: 4TEAOH: 75 H_2O.	Stainless steel tube	0.042%	3–5	2.8	Zhou et al. (2013)
1Al_2O_3: 0.5SiO_2: 1P_2O_5: 1.5TEAOH: 100H_2O	Tube α-Al_2O_3	1%	1.6	0.6	Jabbari et al. (2014)
1Al_2O_3: 1P_2O_5: 0.32SiO2: 1TEAOH: 0.8 DPA: 52 H_2O,	Stainless steel tube	1%	1.6	1.12	Kim et al. (2016)

Table 8.12 Synthesis of SAPO-34 membranes from in situ crystallization

Gel composition	Substrate	Substrate pore size	Membrane thickness (μm)	Permeability $CO_2 \times 10^{-6}$ mol/m^2 s Pa	References
1Al_2O_3: 1P_2O5: 0.3 SiO_2: 1TEAOH: 1.6 DPA: 150 H_2O.	α-Al_2O_3	200 nm	6–15	1.82	Liu et al. (2020)
1Al_2O_3: 1P_2O5: 0.3 SiO_2: 1TEAOH: 1.6 DPA: 150H_2O	α-Al_2O_3	100 nm	6–7	1.0	Mu et al. (2019)
1Al_2O_3: 1P_2O5: 0.3SiO_2: 1TEAOH: 1.6DPA: 75H_2O	α-Al_2O_3	100 nm	0.7	0.94–4.02	Huang et al. (2015)
1Al_2O_3: 1P_2O5: 0.3SiO_2: 1.2TEAOH: 80H_2O	α-Al_2O_3	–	4	1.8	Chew et al. (2011)
1Al_2O_3: 1P_2O5: 0.3SiO_2: 1.2TEAOH: 80H_2O	Disco de α-Al_2O_3	–	4	17.5	Chew et al. (2019)

no change was observed with the naked eye, but when performing the gas permeate tests, significant changes were observed. During the first synthesis, defects were observed in the membrane; after the second synthesis, the substrate surface is completely covered by cross-linked crystals (Meng-Dong et al. 1994). The synthesis of the membranes was also carried out in tubular supports of α- Al_2O_3 with a diameter of 0.7 cm, a length of 0.7 cm, and a pore diameter of 200 nm. The same synthesis method was used for the wafer substrate. The results obtained were notably improved with a permeability (mol/(m^2·s·Pa)) de N_2 de $<7 \times 10^{-11}$. With this, it was observed that arrangement in tubular membranes gave better results than membranes in 2D (Poshusta et al. 1998). Table 8.12 shows of in situ crystallization of SAPO-34 membranes and their effect on the separation of CO_2/CH_4.

8.2.3 Membrane SAPO-34 Post-Synthesis Modification

Post-synthesis treatments are required to correct membrane errors and increase selectivity. Modifications can also increase selectivity toward a specific molecule or promote the greater mechanical property of the membrane. The use of vacuum-assisted deposition (VAD) was reported to cure defective membranes supported on α-Al_2O_3 with a bis (triethoxysilyl) ethane or (BTESE)-organosilica coating. This coating led to a significant increase in CO_2/CH_4 selectivity by a factor of 2.5 (Mu et al. 2019). Also, a post-treatment was carried out to increase the resistance of SAPO-34 membranes in humid conditions. N-dodecyltrimethoxysilane (DTMS) was used to coat the outer surface of the α-Al_2O_3 hollow fiber support. The selectivity in the CO_2/CH_4 mixture for the membranes before and after modifying

was 0.9 and 65, respectively, which shows a significant increase (Rehman et al. 2019). SAPO-34 membranes were functionalized with various organic aminocations, such as ethylenediamine, hexylamine, and octylamine. With this, it was possible to obtain a selectivity in CO_2/CH_4 mixtures of up to 245 (Venna and Carreon 2011).

8.2.4 Environmentally Friendly Synthesis

The hydrothermal synthesis process of SAPO-34 has a negative environmental impact. The hydrothermal process requires large amounts of water that later becomes waste with high ion concentrations. Also heating the reactor up to 200 ° C for long periods is high energy consumption. Finally, to remove the SDA to obtain a material with free pores, it is necessary to heat up to 550 ° C, and toxic vapors are released. Different studies have shown that the environmental impact of hydrothermal synthesis of SAPO-34 can be decreased.

8.2.5 Mother Liquid Recycling and SDA Use Reduction

Zeolites synthesized without SDA have been reported (Hosseinpour et al. 2020), but this hasn't been reported for SAPO-34. SSZ-13 is a zeolite with a CHA-type structure, the same as SAPO-34; this zeolite was synthesized without SDA. N,N, N-trimethyl-1-adamantammonium ($TMAda^+$) was substituted for ammonium fluoride (NH_4F), and a 100% efficient crystalline material was obtained for the MTO reaction. This is an important advance for structure-directing agent-free zeolite synthesis. This could indicate the possibility of synthesizing SAPO-34 without SDA. In zeolite synthesis, the material is recovered by decantation. The residual liquor has a high content of organic and inorganic species. The possibility of reusing the mother liquor resulting from the synthesis of SAPO-34 has been reported. Reuse of SAPO-34 mother liquor was reported up to four times. The synthesis mother liquor of Cu-SAPO-34 has also been reused, and an efficient material in NOx catalysis was obtained. Also, from the mechanical mixing of SAPO-34 precursors and thermal treatment application, solvent-free synthesis was reported (Jin et al. 2013).

8.2.6 Fast SAPO-34 Synthesis

Hydrothermal synthesis of SAPO-34, temperatures around 200 ° C, and several days are used for its crystallization (E. U. A Patent no US4440871A 1982); with other methods, it is possible to reduce the crystallization time to only a few minutes using

heating by microwave (Ding et al. 2018), oil bath (Sun et al. 2015), or ultrasonic-assisted synthesis (Azarhoosh et al. 2019). The synthesis of SAPO-34 in 10 minutes was reported with the help of the combination of the seed-assisted method and rapid heating in an oil bath (Sun et al. 2015). This was accomplished by combining the seed-assisted method and rapid heating in an oil bath. Chemical methods have also been improved, whereby by adding chelating agents to the synthesis gel, it has been possible to reduce the hydrothermal treatment time to 40 min (Liu et al. 2020a, b).

8.3 Conclusion

Biogas is a promising renewable energy option and a good replacement of natural gas. However, its purification is necessary to increase the calorific power; moreover, corrosion in installation can be avoided with the biogas enriched in biomethane. The technology to reach this objective must be sustainable, clean, and economical. Adsorption or separation processes are considered as a promising options for purification of biogas and other gases. Zeolites are considered as excellent candidates to separate gases as carbon dioxide and methane. Specifically, zeolite and SAPO-34 membranes show a good performance when they are prepared taking account the different parameters in the synthesis process of zeolites. Then, to obtain a SAPO-34 zeolite with high performance in the purification of biogas, the following parameters are important: sources of Si, Al, and P (colloidal silica, aluminum isopropoxide, and phosphoric acid, respectively) and type of SDAs although the TEAOH is preferable when the SAPO-34 zeolite is used for gas separation. SAPO-34 zeolite adsorption and separation processes to purify biogas are still in development before reaching an industrial scale; currently, pilot testing is carried out. However, the proposal technology showed herein promises to promote the sustainability using a removable energetic as biogas.

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Chapter 9
Nanotechnology-Based Biofuel Production

Bhaskar Sharma, Ashwani Kumar Verma, Dixita Chettri, Shuchi Singh, and Anil Kumar Verma

Abstract Immoderate employment of traditional fossil fuel culminated in environmental pollution, rapid fuel stock depletion, and a rise in fuel prices. For the last few decades, many countries are shifting their energy dependence from conventional fuels to renewable energy resources. Biofuels are emerging as an excellent renewable energy source and alternative to fossil fuels, but their full potential could not be realized due to several barriers such as low yield, high production cost, and lack of technologies to manage the efficient utilization of substrate. Recently, nanotechnology has manifested extraordinary applications in the energy sector. Nanoscale materials exhibit unique properties that can be utilized to improve biofuel production to achieve a higher yield at a feasible cost. This chapter will shed light on the potential use of nanomaterials for the advancements in the most popular biofuels, i.e., biogas, biodiesel, bioethanol, and biohydrogen production. We have discussed various technical limitations associated with biofuel production and their solution through the application of nanomaterials. Our review summarizes the potential of nanotechnology in biofuel production and anticipates a lot of scope for future developments in the biofuel industry through nano-sciences.

Keywords Nanotechnology · Biohydrogen · Biodiesel · Bioethanol · Biogas

B. Sharma
School of Life and Environmental Sciences, Faculty of Science, Engineering, and Built Environment, Deakin University, Geelong, VIC, Australia

TERI School of Advanced Studies, New Delhi, India

A. K. Verma · D. Chettri · A. K. Verma (✉)
Department of Microbiology, Sikkim University, Gangtok, India
e-mail: akverma@cus.ac.in

S. Singh
Department of Agricultural and Biological Engineering, University of Illinois at Urbana-Champaign, Urbana, IL, USA

P. Chowdhary et al. (eds.), *Bio-Clean Energy Technologies: Volume 1*, Clean Energy Production Technologies, https://doi.org/10.1007/978-981-16-8090-8_9

9.1 Introduction

Traditional fossil fuels such as oil, gas, and coal are the major source of human energy consumption on the planet. Unfortunately, the limited availability of fossil fuels and several environmental consequences such as climate change and greenhouse emission are associated with the consumption of fossil fuels which led to the search for new alternatives sources of energy. The combustion of fossil fuels containing carbon and hydrogen leads to the incorporation of oxygen and the production of carbon dioxide, methane, nitrous oxide, and fluorinated gases along with energy production. Carbon dioxide is a major gas produced on earth that maintains the atmospheric temperature. The increased consumption of fossil fuels majorly liberates the carbon dioxide gas which contributes to the escalation of earth temperature and thereby effectuates the greenhouse effect and adverse consequences on the living organisms on earth (Höök and Tang 2013). On the other hand, nitrous oxide, fluorinated gases, carbon monoxides, and sulfur oxide produced from the combustion of fossil fuels pollute the air and degrade the ozone layer. The ozone layer on the outer surface of the earth protects living organisms from harmful ultraviolet radiation originating from the sun. The damage to the ozone layer allows ultraviolet radiations to enter the earth's environment and gives rise to health risks to all living organisms (Hoel and Kverndokk 1996). The compounds released from fossil fuel combustion frequently pollute the air, water, and soil reservoirs and pose greater health risks to biodiversity (Withagen 1994). Moreover, the cost of extraction, purification, and consumption is too high, thus making it lesser feasible for the world population. Therefore, we need an alternative solution for energy production from a continuously available renewable source with minimum or no environmental pollution at a low cost to fulfill the demand of the ever-increasing world population.

Fuels originating from biological sources or biomass are termed "biofuels." Biomass accounts for about 80% of the energy production of global renewable energy. Biomass can be used to produce heat, fuel, and electricity (Strzalka et al. 2017). It can also be termed as "bioenergy" which are gas, solid, and liquid fuels that originated from a biological source. Biofuels can be categorized into primary and secondary biofuels. Primary biofuels are produced directly from forests, animal waste, plants, crop residues, etc., while secondary biofuels are obtained from biomass feedstocks and microorganisms (Enagi et al. 2018; Khan et al. 2020, 2021). Secondary biofuels are categorized into three generations, i.e., first-, second-, and third-generation biofuels. The first-generation biofuels are generated from edible crops such as wheat, barley, sugarcane, corn, sunflower, sorghum, etc. (Naik et al. 2010). The second-generation biofuels are produced from biomass residues such as jatropha, corn, wood chips, cassava, wheat straw, waste cooking oil, grass, etc. (Zhang et al. 2018). The second-generation biofuels sources are the most feasible choices as residues biomass or nonfood crops are widely available and do not compete with edible sources and pose no environmental implication (Aditiya et al. 2016; Naik et al. 2010). The third-generation biofuels are produced from algae microorganisms. The microalgae can survive and grow vigorously under various

environmental conditions and are capable of mass-producing different variety of biofuels such as biodiesel, biohydrogen, and biogas (Leong et al. 2018).

Nowadays, biofuels such as bioethanol, biohydrogen, biodiesel, and biogas are attracting scientists and industries due to their cheaper availability, production from nonfood biological sources, and eco-friendly properties (Ghimire et al. 2017). Biogas has been widely accepted and a sustainable biofuel which is commonly produced from organic agricultural and animal waste. Biogas production involves the biochemical process of anaerobic digestion facilitated by archaeal and bacterial species found in wastewater, landfills, field composts, poultry farms, etc. (Christy et al. 2014). The biogas production process is operated at various operational parameters such as temperature, substrate concentration, pH, inoculum size, and hydraulic retention time to produce a balanced concentration of methane (50–75%), carbon dioxide (25–45%), and traces of other gases such as hydrogen sulfide (Adekunle and Okolie 2015; Kirtley et al. 2015). The biogas production plants are installed in many countries such as the USA, China, Germany, the Netherlands, and Brazil. The world's most biogas anaerobic digesters are installed at farm sites in Europe to treat agricultural and animal waste (Bond and Templeton 2011). Europe has increased biogas production to 14.9 million tons in 2014 where Germany is leading with 7850 biogas facilities installed by 2014 (Hijazi et al. 2016; Scarlat et al. 2018). Biohydrogen is attracting researchers due to its features such as diverse feedstock utilization, a wide range of bacteria utilization, high energy content, carbon sequestration capabilities, and production at ambient temperature. However, the production is exclusively dependent on the operational parameters such as temperature, pH, substrate concentration, and hydraulic retention time; therefore, production optimization is necessary for maximum yield (Das et al. 2008). The biohydrogen can be used in the biorefinery for improving energy recovery through dark fermentative biohydrogen production which produces volatile compounds used in secondary fermentation for high energy recovery in the form of biomethane, bioelectricity, and photo-fermentation. The process allows soluble products to be converted into various alcohols (Sekoai et al. 2018).

Bioethanol is a major alternative fuel component to petroleum and is majorly produced by sugar fermentation process utilizing wheat, maize, willow, reed canary grass, and sorghum plants (Abbaszaadeh et al. 2012). The global production of bioethanol was estimated at around 100 billion liters in 2017 (Aditiya et al. 2016). Biodiesel is another form of biofuel which is a suitable and environmentally friendly alternative to petroleum diesel, produced from non-edible oils. Biodiesel, bioethanol, and biomethane are foreseen as an alternative to fossil fuels to meet the current energy demands and environmental concerns. The production of second-generation biofuels has not been established, yet while the development of first-generation biofuels has reached an advanced stage. There are still numerous challenges associated with the production and usage of first-generation biofuels which suggest searching for new methods for biofuel production (Shafiei et al. 2014; Gaurav et al. 2017). Irrespective of the modern technological intervention and discoveries, biofuel production still faces technological limitations such as production cost, lack of infrastructure, establishment and optimization of mass

production strategies, and tedious and sensitive production methodologies (Patumsawad 2011). The fermentation inhibitors such as phenolics, furan derivatives, aliphatic compounds, homoacetogens, sulfate, and nitrate-reducing bacteria are formed during the fermentation process which turns down biofuel yield and quality (Jönsson et al. 2013; Show et al. 2012). The lignocellulose-based biomass requires tedious and costly chemical, physical, or radiation pretreatments to release fermentable sugar before the biofuel production process which is a major challenge for optimization and production of lignocellulose biomass-based biofuels (Zheng et al. 2014). The third-generation biofuel production from microalgae is encountering numerous hurdles such as cost of production, large-scale cultures maintenance difficulties, efficacy, and lack of established conversion methodologies for new species (Hallenbeck et al. 2016). Such challenges are crucial and require effective solutions to be accepted as a strong alternative to conventional fuels. In this chapter, we have reviewed the most critical challenges for biofuel production and their solution through the intervention of nanotechnology-based products. The biofuel industry performance needs to be improved to establish a sustainable and robust mechanism for energy production.

9.2 Nanomaterial Characteristics

Nanotechnology has emerged as the most diverse field of science covering wide interdisciplinary research areas and applications. Nanotechnology deals with nanoscale particles, retaining a size range of 1–100 nm. The major characteristics of the nanomaterials are large surface area-to-volume ratio, increased number of active sites, faster interaction, diverse morphologies at different dimensions, easy passage, high adsorption capacity, better catalytic activity, unique optical properties, etc. The nanomaterials can be synthesized using either a top-down approach where bulk particles are broken down to nanoscale dimension or a bottom-up approach in which smaller-sized particles such as ions combine to attain nanoscale dimension. The synthesis methodologies of the nanomaterials include self-assembly, phase separation, co-precipitation, laser ablation, electrochemical deposition, etc. depending on the type of application. Nanoparticles with different shapes can be synthesized using an arc discharge method for carbon nanotubes, an electrospinning method for nanofibers, and thermal exfoliation for nanosheet production. After synthesis of the desired size and morphology of the nanomaterials, the surface is functionalized with a suitable ligand or functional group to enhance the properties of the synthesized nanomaterials. The surface functionalization of the nanomaterials can be performed to stabilize the nanoparticles, or enhance or inhibit the reactivity, and solubilization of the material in the solvent. The functionalization compounds can be synthetic polymers, proteins, enzymes, polysaccharides, lipids, polypeptides, etc. depending on the application. Nanostructures are categorized into four categories based on the dimension: (1) zero dimension (0-D), (2) one dimension (1-D), (3) two dimension (2-D), and (4) three dimension (3-D). The ultrafine grain size less than

50 nm can be used to characterize the dimensions of the nanomaterials. Thus, nanomaterials exhibit different physical, chemical, and optical properties than normal-sized materials.

The wide range of nanomaterials can be metallic, polymer, ceramic, carbon-based, lipid-based, semiconductors, etc. A wide variety of size, shape, surface reactivity, and adsorption capabilities allow their direct and indirect application in the biofuel production processes. The metabolic reaction can be enhanced by using metallic nanoparticles, nanofibers, and nanotubes. Nanocrystals, nanodroplets, and nanomagnets are used as nano-additives to enhance the blending efficiency of the fossil fuels petrol and diesel. Nanomaterials can be utilized as catalytic agents (Table 9.1) to improve the activity of the anaerobic consortia, modulate the electron transfer, and reduce inhibitory compounds in the biochemical processes during fermentation.

9.3 Nanotechnology for Biofuel Production

9.3.1 Biogas Production

Biogas production involves four steps, i.e., hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Firstly, the larger molecules such as carbohydrates, lipids, and proteins are transformed into fermentative simple molecular forms such as amino acids and soluble sugars through physical or chemical pretreatments (Mao et al. 2015; Kirtley et al. 2015). The acidogenic bacteria metabolize the monomers into carbon dioxide, hydrogen, and alcohol via various biochemical reactions in the second step of biogas production. Later, acetic acid, hydrogen, and carbon dioxide are produced from soluble intermediates by acetogenic bacteria which can grow along with acidogenic bacteria under low partial pressure (Angelidaki et al. 2018). In the fourth stage of biogas production, acetoclastic and hydrogenotrophic methanogenic bacteria utilize acetic acid, carbon dioxide, and hydrogen to give rise to methane under low hydrogen pressure conditions (Buitrón et al. 2014; Mao et al. 2015; Khan et al. 2017).

Impurities such as carbon monoxide, hydrogen sulfide, and ammonia reduce the calorific value and density of the methane gas that prevents methane use in natural gas grids (Sahota et al. 2018). These impurities are corrosive to the equipment used in biogas production and storage. Zero-valent iron nanoparticles are a core-shell structure where the core comprises metallic iron nanoparticles and the oxide shell contains iron oxides and hydroxide groups (Karri et al. 2005). The outer shell provides a larger surface area for chemical reactions and various interactions, while the core region acts as an electron donor to the molecules. Higher stability, water solubility, and electron donor capabilities make ZVI a suitable candidate for contaminant removal from biochemical reactions through chemical adsorption (Sun et al. 2006). The zero-valent iron nanoparticles obtain a positive charge at lower or acidic pH and attain a negative charge at higher or alkaline pH. ZVI nanoparticles

Table 9.1 Major developments in biofuel production through nanomaterial use

S. no.	Type of biofuel	Nanomaterial used for enhancement	Improvements/ developments	References
1	Biogas	Zero-valent iron nanoparticles	Contaminant removal during fermentation through adsorption	Karri et al. (2005)
		ZVI and iron oxide nanoparticles	Methane production during anaerobic digestion	Yang et al. (2013)
2	Bioethanol	Iron oxide	Beta-glucosidase immobilization, catalytic and binding efficiency of enzyme	Verma et al. (2013)
		Silicon oxide nanoparticles	Co-immobilized culture of *Saccharomyces cerevisiae* and *Kluyveromyces marxianus*, rapid hydrolysis of the substrate	Beniwal et al. (2018)
		MnO_2 nanoparticles	Immobilization of cellulase enzyme, catalytic and binding efficiency of enzyme	Cherian et al. (2015)
		Calcium alginate and chitosan	Microbial immobilization, catalytic and binding efficiency of enzyme	Ivanova et al. (2011), Duarte et al. (2013)
		Silica nanoparticles and methyl functionalized cobalt-ferrite-silica nanoparticles	Enhanced gas-liquid mass transfer for high bioethanol yield	Kim et al. (2011)
		Carbon nanotubes	Immobilization of the cells and enzymes	Pan et al. (2007)
		Graphene oxide sheets with copper nanoparticles, ZnO nanosheets	Detection of compounds such as reducing sugars and ethanol	Lin et al. (2016), Santos et al. (2016)
3	Biodiesel	Sulfamic and sulfonic silica-coated crystalline magnetic nanoparticles	Catalytic activity and transesterification	Wang et al. (2015)
		Iron oxide and silica core-shell nanoparticles	Catalytic activity and transesterification	Chiang et al. (2015)
		Calcite, dolomite, eggshell, CaO, and	Nanocatalyst	Hebbar et al. (2018), Bet-Moushoul et al. (2016)

(continued)

Table 9.1 (continued)

S. no.	Type of biofuel	Nanomaterial used for enhancement	Improvements/ developments	References
		calcite-Au nanocatalysts		
		ZnO, MgO, and CaO nanocatalysts	Improved enzyme activity, nanocatalyst	Baskar et al. (2018), Bet-Moushoul et al. (2016)
4	Biohydrogen production (dark fermentation)	Silver and gold nanoparticle	Substrate utilization efficiency, electron transport, ferredoxin protein, and [Ni-Fe]- and [Fe-Fe]-hydrogenase enzyme activities	Zhang and Shen (2007), Zhao et al. (2013)
		Ag, Pb, Cu, and FeO nanoparticles	Immobilization and encapsulation of microorganisms improved the biohydrogen production rate	Beckers et al. (2013)
		ZVI, Ni, SiO_2, $FeSO_4$, and TiO_2 nanoparticles	Microbial processes, and catalytic efficiencies and significantly enhanced the biohydrogen yield	Mohan et al. (2008), Vi et al. (2017), Nath et al. (2015), Hsieh et al. (2016), Lin et al. (2016)
	Biohydrogen production (photo-fermentation and photocatalysis)	Silver, Iron gold, ZVI, and TiO_2 nanoparticles	Nitrogen metabolism, photosynthetic activities, pigment (carotenoids and chlorophyll) production in microalgal species, carbohydrate production, and metabolic enzymes such as glutamine synthase, nitrate reductase, and glutamate dehydrogenase	Pandey et al. (2015), Eroglu et al. (2013)
		Silica nanoparticles	Chlorophyll concentration and photo-fermentative efficiencies algal growth	Giannelli and Torzillo (2012)
		Cadmium sulfide nanofibers	Surface electron transfer and photocatalysis	Hernández-Gordillo et al. (2015)
		TiO_2 nanoparticles	Photocatalysis	Hakamizadeh et al. (2014)

were found to be effective in removing the contaminants produced in biogas production and boosting biogas recovery (Karri et al. 2005; Sun et al. 2006). The 0.1% dose of ZVI in biogas production enhanced the methane gas production by at least 5% and ameliorated the biogas production by approximately 30% (Su et al.

2013). The use of ZVI nanoparticles in biogas production could reduce the sulfate impurities in the final product while enhancing methane production significantly (Su et al. 2013). The ZVI nanoparticles can be used to remove the sulfide, aromatic hydrocarbons, inorganic ions, and complex organic compounds formed during the biochemical reaction as products, by-products, and intermediates due to their high reactivity, remarkable adsorption capacity, and high surface area-to-volume ratio (Pikaar et al. 2015; Su et al. 2013).

Furthermore, the low concentration of ZVI and iron oxide nanoparticles could enhance the activity and population of the methanogens in the anaerobic digestion process which boosted methane gas production by 120% and 117%, respectively (Yang et al. 2013; Zhang and Lu 2016). Nanoparticles increase the production of the intermediate compound in biogas production which ultimately enhances methane production. The optimized concentration of the ZVI nanoparticles enhances the production of hydrogen which is utilized by the hydrogenotrophic methanogens. Nanoparticles promote electron transfer in the bacterial-archaeal syntrophic interactions and induce the production of methane in the reactor (Park et al. 2018). Nanoparticles such as copper oxide and zinc oxide significantly inhibit biogas production because they exhibit a negative impact on the growth of the methanogenic bacteria (Luna-delRisco et al. 2011).

9.3.2 Bioethanol Production

Bioethanol has emerged as the best suitable alternative to traditional fossil fuels due to the minimum or no environmental risk associated with production and utilization. Bioethanol production has been increasing over the past decade and reached 108 billion liters in the year 2018 (Sydney et al. 2019). Due to severe environmental consequences and high cost associated with the use of traditional fuels, renewable fuel alternatives especially bioethanol are seen as a major preference in the travel and energy sector (Limayem and Ricke 2012). Bioethanol consists of high evaporation enthalpy, high octane number, and a broad range of flammable properties (Waqas et al. 2016). Bioethanol production is predicted to continuously rise considering the huge demand for blending with conventional hydrocarbon fuels obtained from crude oil (Limayem and Ricke 2012). Bioethanol produced from edible crops such as wheat, rice, corn, barley, sorghum, etc. are termed first-generation biofuels, and China, the USA, and Brazil are among the top producers of bioethanol (Balan 2014). However, the use of edible crops for bioethanol production will not be feasible as the world is already running short of food production to fulfill the demand. Currently, efforts are being made to increase bioethanol yield from second-generation biofuels, i.e., non-edible crops and crop residues, and third-generation fuels, i.e., algal sources (Limayem and Ricke 2012; Leong et al. 2018).

Nowadays, lignocellulose feedstock is drawing the attention of researchers for bioethanol production due to its availability at a cheaper rate, abundance, and environmentally safe degradation properties. The lignocellulosic production of

bioethanol suffers from several technical limitations such as the presence of plant-based contaminants which interrupt the production of ethanol by inhibiting the metabolism of *Saccharomyces cerevisiae* and other microorganisms that are vital for the fermentation process (Kim et al. 2011). Another major drawback of the lignocellulose substrate is the structural complexity which needs pretreatments to break down the lignocellulose into cellulose, hemicellulose, and lignin before fermentation because microorganisms used for fermentation are either not efficient to degrade lignocellulose or require cost- and time-intensive methodologies (Balan 2014; Chowdhary et al. 2020). Moreover, the intermediates formed during the pretreatment process such as phenolic compounds and furan compounds are inhibitors of the fermentation organism *Saccharomyces cerevisiae* (Ximenes et al. 2010). To address these issues, immobilization of the enzymes or microorganisms was found to help increase bioethanol production and reduce contaminants' interference. The enzyme beta-glucosidase immobilization in iron oxide nanoparticles enhanced bioethanol production with 50% catalytic activity retention and 93% binding efficiency after 16 fermentation cycles (Verma et al. 2013). Moreover, it was observed that polymer magnetic nanofibers could show stable immobilization with prolonged reusability (Lee et al. 2010). Similarly, beta-glucosidase could be immobilized in silicon oxide nanoparticles for hydrolyzing the whey substrate and co-immobilized culture of *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* at the same time (Beniwal et al. 2018). MnO_2 nanoparticles offer a large surface area and are therefore used for bioethanol production from sugarcane leaves through immobilization of cellulase enzyme that drives faster hydrolysis at a broad range of pH and temperature (Cherian et al. 2015). The cellulase immobilized on the nanoparticles resulted in higher production of the bioethanol with 60% catalytic efficiency and 75% binding efficiency. Moreover, microbial cell immobilization in the matrix of calcium alginate and chitosan increased the bioethanol production without any contamination, and the immobilized cells could be reused multiple times (Ivanova et al. 2011; Duarte et al. 2013).

The silica nanoparticles and methyl functionalized cobalt-ferrite-silica nanoparticles with higher surface area and higher reactivity enhanced the gas-liquid mass transfer for increased bioethanol production (Kim et al. 2014). Carbon nanotubes due to their lighter weight and unique thermal and mechanical properties can be used for the immobilization of cells and enzymes. For instance, carbon nanotubes loaded with rhodium were used as a reactor for the production of bioethanol from the reaction of carbon monoxide and hydrogen gas (Pan et al. 2007). Nanoparticles can be utilized to detect the compounds during the fermentation process. Graphene oxide sheets with copper nanoparticles were used for the detection of reducing sugar for better efficiency of the fermentation system (Santos et al. 2016). Similarly, ZnO nanosheets containing silver nanoparticles could be used for the detection of ethanol in the fermentation mixture (Lin et al. 2016). These nano-detectors exhibit higher sensitivity and reusability. Nanomaterials are very useful tools for the production of bioethanol. The numerous applications of nanomaterials indicate the potential to become an integral part of bioethanol production.

9.3.3 Biodiesel Production

Biodiesel is produced through various methods such as pyrolysis, microemulsion, and transesterification (Yusuf et al. 2011; Meher et al. 2006; French and Czernik 2010). The most adopted method for biodiesel production is transesterification which utilizes oils or fats and methanol in the presence of homogeneous or heterogeneous catalysts. Biodiesel is a biodegradable compound that does not pollute the environment and is a renewable source of fuel (Atabani et al. 2012; Chowdhary and Raj 2020). Biodiesel offers a high cetane number, better combustion efficiency, faster degradation, and lesser emission of carbon monoxide, carbon dioxide, particulate matter, and hydrocarbons (Shameer et al. 2017; Sangle et al. 2017; Rounce et al. 2010). Biodiesel consists of comparatively smaller quantities of sulfur and aromatic compounds than fossil fuels (Meher et al. 2006; Yusuf et al. 2011). Moreover, biodiesel can be produced using a wide range of edible and non-edible oils which provides an option to produce fuel from abundant and waste feedstocks. Biodiesel can offer sustainable solutions to the growing fuel demand by establishing the local source of fuel production and minimize the dependence on imports (Lapuerta et al. 2008). The major advantage of biodiesel is that it can be blended with conventional petroleum diesel fuel which facilitates flexibility in the gradual adaptation of the fuel for future use. However, large-scale production and utilization of biodiesel are limited due to technical challenges such as high viscosity, higher flash and cloud point, low engine speed, high copper strip erosion, higher nitrogen oxide compound emission after combustion, lower energy value, and higher cost of production (Rounce et al. 2010; Lapuerta et al. 2008).

Nanotechnology is an interesting tool for improving biodiesel production. It has been observed that higher surface area, small particle size, and flexible surface functionalization capabilities allow their use to improve catalytic efficiency of the biodiesel production in the transesterification method (de Araújo et al. 2013). For instance, sulfamic and sulfonic silica-coated crystalline magnetic nanoparticles were used for transesterification of glyceryl trioleate which resulted in 95% of biodiesel conversion and an improved catalytic activity (Wang et al. 2015). Likewise, functionalized iron oxide and silica core-shell nanoparticles enhanced biodiesel production to 97.1% when used with an algal oil source (Chiang et al. 2015). Similarly, the addition of 1.5% CaO nanocatalyst with *Bombax ceiba* oil during the transesterification process could increase biodiesel yield to 96.2% (Hebbar et al. 2018). Calcite, dolomite, eggshell, and calcite-Au nanoparticles were used as nanocatalysts for improving biodiesel production from a variety of oil sources. The simple combination of MgO and CaO nanoparticles maximized the biodiesel production to 98.95% from waste cooking oil within 5 hours at methanol to oil ratio of 7:1 (Bet-Moushoul et al. 2016). Similarly, the ZnO nanoparticles improved biodiesel production to 95.20% from castor oil transesterification within an hour's time. Moreover, the nanocatalyst could be used numerous times without compromising the activity (Baskar et al. 2018). Therefore, nanoparticles present attractive

solutions to biodiesel productivity with enhanced recovery and reusability making the transesterification economically feasible for large-scale production.

Furthermore, the new methods for biodiesel production include lipid catalyzed transesterification due to its reusability, low energy requirements, and use of various feedstock. Nanomaterials can be used to immobilize lipase enzymes for efficient substrate utilization with minimum loss. The immobilized lipase efficiency increases due to the presence of a high surface area which provides maximum interaction and incubation at the surface (Tian et al. 2017). The immobilized enzymes on nanoparticles can enhance biodiesel production. The use of nanoparticles in biodiesel production can be a revolutionary step toward providing cutting-edge, economical, environment-friendly, and quick solutions to major production and compatibility issues. The addition of the functionalized nanomaterials to the transesterification process as a catalyst has been beneficial to biodiesel production. More applications of nanomaterials need to be identified for improving biodiesel production and extension to the large-scale setup at the lowest possible cost. Nanotechnology has the potential to provide a platform for the development of second and third-generation biofuels on large scale.

9.3.4 Biohydrogen Production

Hydrogen is a high-energy value candidate which, unlike carbon-based fuels, does not harm the environment. Biohydrogen production can effectively replace hydrogen production dependence on fossil fuels. The biohydrogen production is mainly performed through (1) dark fermentation (light-independent reaction) and (2) light-dependent reaction (photo-fermentation and photocatalysis) (Sivagurunathan et al. 2017; Savla et al. 2020). Anaerobic bacteria accelerate the production of molecular hydrogen under the controlled environment of temperature, pH, hydraulic retention period, and substrate concentration. The higher yield of the molecular hydrogen depends on the optimum microorganism growth condition, type of feedstock or substrate, and metabolic capacity of the microorganism used for fermentation (Łukajtis et al. 2018; Das et al. 2008).

9.3.4.1 Biohydrogen Production in Dark Fermentation

In the dark fermentation method, hydrogen and carbon dioxide gases are produced by anaerobic microorganisms from biomass under a carbon-neutral process. The dark fermentation method provides higher hydrogen evolution rates compared to the light-dependent method. However, carbon dioxide is a major carbon additive generated that requires separation before frequent usage. The lower biohydrogen production through weaker catalytic efficiency and neutral microbial processes are major limitations in the dark fermentation method (Rittmann and Herwig 2012). The nanoparticles could be used as an additive for enhancing molecular hydrogen

production in microorganisms through higher substrate utilization and promoting the activities of biohydrogen-producing enzymes. The optimum concentration of nano-additives such as silver and gold nanoparticles can enhance biohydrogen production. The 20 nmol/L concentration of the silver nanoparticles increased glucose conversion by 62% which boosted the biohydrogen production (Zhao et al. 2013). Silver nanoparticles help accelerate the acetic reaction and acidogenic phase throughout the fermentation process. Similarly, gold nanoparticles provide a large surface area for anaerobic bacteria to bind and access the substrate which resulted in higher substrate utilization efficiency (56%) and an increment in biohydrogen production by 46% (Zhang and Shen 2007). Gold nanoparticles enhance the activity of biohydrogen-producing enzymes that involves electron transporters, ferredoxin protein, and [Ni-Fe]- and [Fe-Fe]-hydrogenase in the microorganism that influences the rate of biohydrogen production. Metallic nanoparticles such as Ag, Pb, and Cu were used along with FeO nanoparticles as an additive in the fermentation from *Clostridium butyricum*. The metallic nanoparticles were immobilized on silica which enhanced electron transfer in the microbial cells coated on the surface of the nanoparticles (Beckers et al. 2013). The immobilization and encapsulation of microorganisms improved the biohydrogen production rate by 58% in comparison to nanoparticles coated with *Clostridium butyricum* on nanoparticles without FeO nanoparticles (Beckers et al. 2013). Similarly, zero-valent Fe, Ni, Si, $FeSO_4$, and TiO_2 nanoparticles, Ni-graphene, and iron oxide-silicon oxide nanocomposites have been supplemented in the dark fermentation process which stimulated the microbial processes and catalytic efficiencies and significantly enhanced biohydrogen yield (Mohan et al. 2008; Vi et al. 2017; Nath et al. 2015; Hsieh et al. 2016; Lin et al. 2016). The properties of nanoparticles such as high surface area, small size, electron transfer efficiency, and rich surface functionalization capabilities allow improved interaction and metabolic efficiency of microorganisms for biohydrogen production. On the other hand, few nanoparticles such as copper and $CuSO_4$ were found to be reducing biohydrogen production through interfering with volatile fatty acids, acetate, and butyrate production (Mohanraj et al. 2016).

9.3.4.2 Biohydrogen Production in Photo-Fermentation and Photocatalysis

Biohydrogen-producing microalgae utilizing photosynthetic activity are a vital source for biohydrogen production. Photo-fermentation can achieve better biohydrogen yield than dark fermentation, but the major constraint in photo-fermentation is the slow growth rate of anoxygenic phototrophic bacteria. Due to this reason, the biohydrogen yield achieved is two times lower than dark fermentation. Photo-fermentation through lignocellulosic sources can be feasible, but the high cost of pretreatment and co-product contamination limit its widespread usage (Zhang and Zhang 2018). The use of nanoparticles enhanced photosynthetic properties, biomass growth, nitrogen metabolism, protein, and other metabolic pathways such as lipid metabolism and photosynthetic pigments. The nanoparticles were

found to be promoting algal growth through carbohydrate production and metabolic enzymes such as glutamine synthase, nitrate reductase, and glutamate dehydrogenase. For instance, the optimum concentration of silver, gold, ZVI Fe, and TiO_2 nanoparticles significantly enhanced biohydrogen production through improving photosynthetic activities and pigment (carotenoids and chlorophyll) production in microalgal species such as *Chlorella vulgaris* and *Rhodobacter spheroids* (Pandey et al. 2015; Eroglu et al. 2013). The biohydrogen production rate was increased as much as 4.2 mol hydrogen per mol sucrose medium with the addition of the ZVI concentration of 8–16 gram per liter and 1990 mL hydrogen per liter with TiO_2 nanoparticle addition to the fermentation medium (Pandey et al. 2015; Eroglu et al. 2013). Similarly, the silica nanoparticles increased the growth and production of *Chlamydomonas reinhardtii i*CC124 through significant enhancement of the chlorophyll concentration and photo-fermentative efficiencies (Giannelli and Torzillo 2012). The silica nanoparticles could scatter light in the fermentation reactor which in turn promotes algal growth.

Photocatalysis involves the splitting of the water molecule into oxygen and hydrogen using an illuminating source as a photocatalyst (Tentu and Basu 2017). Numerous methods are available for biohydrogen production from either algal cultures or isolated components. The photosynthetic bacterial and algal cultures are best known for their photocatalysis properties and biohydrogen production. The photocatalysis method for biohydrogen production is commonly limited by high cost and low photosynthetic efficiency. Titanium oxide nanoparticles have been recognized as an excellent photocatalytic agent with low-cost availability, chemical stability, and nontoxic properties. Mesoporous titanium oxide-activated carbon nanoparticles could enhance hydrogen production through photocatalysis up to 75 times (7490 micromoles per hour per gram photocatalyst) compared with convention photocatalyst P25 (Hakamizadeh et al. 2014). Similarly, the photocatalytic potential of cadmium sulfide nanofibers integrated with ethylenediamine, butanol, and tetrahydrofuran was explored in the presence of blue light. The cadmium ions surface-loaded with ethylenediamine create a surface state to store the electrons generated from blue light and later transferred to protons to produce hydrogen molecules (Hernández-Gordillo et al. 2015). Nanomaterials are excellent tools to assist in influencing chemical and biological properties to stimulate hydrogen production.

9.4 Conclusion and Future Prospects

Biofuels are an excellent alternative renewable energy source to conventional fossil fuels. Sufficient, efficient, and economical biofuel production confronts several technical restrictions and practical challenges. The properties of nanoscale particles could put forward pivotal applications for improvement of the biofuel production. This chapter reviews various aspects of nanomaterials for boosting biofuel production through supplying solution to technical obstructions related to efficient

utilization of a range of substrates, catalytic properties, microbial metabolic and biochemical capabilities, and enzyme activities. However, there is a lot of scope for new advancements in research related to biofuel production. The inception of the second- and third-generation biofuels has given new hope for a revolutionary transformation in the energy sector. The interventions from nanotechnology should focus on cost-effective, nontoxic, and environmentally safe methodologies for the biofuel industry. There is a need to establish a library of screened nanomaterials for improving energy production from a variety of sources supported by solid evidence. Future studies should focus on exploring new dimensions of nanomaterials for third-generation biofuels and impact assessment.

Acknowledgments The authors would like to thank the Department of Microbiology, Sikkim University, TERI School of Advanced Studies New Delhi, and School of Life and Environmental Sciences, Deakin University, Geelong, Australia, for providing the computational infrastructure and central library facilities for procuring references and plagiarism analysis (Turnitin: Plagiarism Detection Software).

Conflict of Interest The authors declare that the study was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 10
Advancement of Nanoparticles in Gaseous Biofuel Production

Pritam Kumar Dikshit, Divya, Neetika Jamnal, Shruti Singh, Jatin Kumar, Amit K. Das, and Soumya Pandit

Abstract Biofuels are considered as the most important alternatives to fossil-derived fuels in order to mitigate the global fossil fuel depletion and environmental hazards associated with the use of petroleum-based fuels. Among several biofuels (bioethanol, butanol, and biodiesel), biohydrogen and biogas have received special attention due to their unique properties. However, high production cost and low yield are the major limitations to the technical development and successful commercialization of this process. Of late, nanotechnology is being applied in various fields including agriculture, food, cosmetic, electronic, and pharmaceutical industries due to its distinct properties. Nanoparticles are also being used in several biological processes for the improvement of microorganisms/enzyme activity leading to higher product throughput. This chapter summarizes various literature reports on the application of nanoparticles over biohydrogen and biomethane production. In addition, the present chapter also highlights the types of nanoparticles, viz., metallic, nanofiber, nanotubes, etc., and their influence over biohydrogen and biomethane production.

Keywords Nanoparticles · Microorganism · Anaerobic digestion · Methane · Biohydrogen

10.1 Introduction

The depletion of fossil fuel reserves vis-a-vis continuous population growth has caused serious threats to the environment in terms of deforestation, global warming, decrease in biodiversity, and energy resources. Fossil fuels contribute around 88% of the total energy production worldwide. Additionally, these fuels are nonrenewable,

P. K. Dikshit (✉)
Department of Biotechnology, Koneru Lakshmaiah Education Foundation, Guntur, India

Divya · N. Jamnal · S. Singh · J. Kumar · A. K. Das · S. Pandit
Department of Life Sciences, School of Basic Sciences and Research, Sharda University, Greater Noida, India

P. Chowdhary et al. (eds.), *Bio-Clean Energy Technologies: Volume 1*, Clean Energy Production Technologies, https://doi.org/10.1007/978-981-16-8090-8_10

and their burning causes extensive emission of greenhouse gases resulting into numerous environmental and ecological problems (Ajay et al. 2020). In view of this, production, process development, and utilization of various renewable energy sources such as wind, solar, and biomass have received significant research interests in the past few years. Among these renewable energy sources, biofuels (ethanol, butanol, methanol, hydrogen, methane, algal biofuel, etc.) derived from biological carbon fixation are becoming extremely important. These biofuels are mainly categorized into two groups: (i) primary biofuels, and (ii) secondary biofuels. The primary biofuels are produced directly from plants, forest waste, animal wastes, crop residues, etc., whereas a combination of biomass feedstocks and microorganisms are used for the production of secondary biofuels. These secondary biofuels are further subdivided into first-generation, second-generation, and third-generation biofuels (Ho et al. 2014; Naik et al. 2010).

Among these biofuels, biohydrogen has attained special importance due to its unique properties such as production from various feedstocks, use of diverse group of microorganisms, high-energy content, and simplest production processes. Optimization of various process parameters, such as pH, temperature, substrate concentration, and hydraulic retention time (HRT), enhances the biohydrogen production. Biological hydrogen production can be achieved by adopting several methods which include photofermentation, biophotolysis, microbial electrolysis, and dark fermentation. Among these biological processes, dark fermentation and photofermentation processes are the two most widely used methods in the production of biohydrogen. The H_2 production through dark fermentation process holds dominance over the photofermentation process in terms of high productivity and non-requirement of light to accomplish the process.

Alternatively, biogas has contributed a crucial role in the development of the biofuel sector due to its widespread acceptance and sustainability. The production of biogas is carried out using anaerobic digestion (AD) process involving several groups of archaea and bacterial species. In AD process, diverse groups of microorganisms act on waste biomass under anaerobic condition to convert it into energy-rich biogas. Methane (CH_4) is the major component of biogas contributing 50–75%, followed by carbon dioxide (CO_2) putting up 25–45% with a trace amount of other gases such as hydrogen sulfide (H_2S) (Sekoai et al. 2019). Various operating conditions such as temperature, pH, carbon to nitrogen ratio (C/N), hydraulic retention time, etc. influence the performance of AD process. The biomethanation process is mainly reliant on the interspecies electron transfer between the syntrophic microorganisms. Several parameters such as choice of substrate, pretreatment of substrate, operating conditions, reactor configuration, and addition of organic/inorganic additives are continuously improvised to enhance the biogas production.

Significant developments of nanotechnology over the past few decades have diversified its applications in several sectors including agriculture, food, cosmetic, electronic, and pharmaceutical industries. In addition, the technology is also being used in various biological processes for improving the production rate and yield. Diverse applications of nanotechnology are attained due to the unique, and beneficial physical and chemical features of nanoparticles (NPs) which include nanoscale size

(1–100 nm), greater reactivity, and high surface-to-volume ratio. Additionally, several other characteristics of NPs such as high crystallinity, catalytic activity, chemical stability, and high adsorption capacity aid in their functionality. In view of these excellent properties, NPs are being used in biofuel production for improvement of electron transfer, reduction of inhibitory compound formations, and improvement of catalytic activity of anaerobic consortia.

The present study recapitulates applications of nanotechnology in gaseous biofuel production especially on biogas and biohydrogen. The effects of various nanostructure materials such as zerovalent metal NPs (Ag, Cu, Au, Ni, Fe, Pd, etc.), metal oxide (Fe_2O_3, Fe_3O_4, NiO, SiO_2, TiO_2, ZnO, CuO, CeO_2, etc.), multi-compound NPs, and carbonous nanomaterials over biogas and biohydrogen production are discussed briefly.

10.2 Biohydrogen Production

Biohydrogen is considered as the cleanest and cost-effective biofuel with energy content of 122 kJ/g. Nowadays, most of the hydrogen is produced by using conventional processes (coal gasification and electrolysis of water) which have several drawbacks like high operating conditions (840 °C) and environmentally unsafe processes. Therefore, production of hydrogen using a biological route is the most viable option as compared to other processes. Biological hydrogen production can be achieved by adopting four different methods, viz., (i) photofermentation, (ii) dark fermentation, (iii) biophotolysis, and (iv) microbial electrolysis.

Among these methods, dark fermentation and photofermentation methods are widely adopted for the production of biohydrogen. Various physiological factors such as pH, temperature, type of inoculum, substrate composition, etc. impact the microbial hydrogen production. Diverse groups of microorganisms belonging to the genus of *Clostridium*, *Bacillus*, *Klebsiella*, *Escherichia*, *Rhodobacter*, *Enterobacter*, and *Citrobacter* produce biohydrogen under mesophilic/thermophilic conditions by using pure sugars as substrates. To make this process sustainable with an aim to reduce the production cost and overcome environmental hazards, several waste biomasses are also explored for the production of biohydrogen (Kahyaoğlu et al. 2012).

10.2.1 Application of NPs Over Biohydrogen Production

Addition of NPs during biohydrogen fermentation can significantly increase the microbial activity by enhancing the electron transfer, which further leads to an increase in H_2 yield. Several previous studies reported positive impact of different NPs (Au, Ag, Pd, Cu, Ni, Fe, Ti, SiO_2, and carbon nanotubes) over biohydrogen

Table 10.1 Summary of the effect of various NPs on biohydrogen production

NPs	Concentration	Organism	Substrate type	Effect on anaerobic digestion	References
Zerovalent metal NPs					
Ag	20 nmol/L	Mixed culture	Glucose	• Exhibited much higher H_2 yields (2.48 mol/mol glucose). • Reduction in lag phase.	Zhao et al. (2013)
Au	1.0 mM	Anaerobic sludge	Acetate	• Maximum hydrogen production rate achieved ~105 mL/L day.	Khan et al. (2013)
	10 nM	Anaerobic culture	Wastewater	• Gold particles of 5 nm size performed better than other sizes (10 nm and 20 nm). • 50% yields of hydrogen production for 5 nm NP.	Zhang and Shen (2007)
Cu	2.5 mg/L	*Enterobacter cloacae* 811,101 and *Clostridium acetobutylicum* NCIM 2337	Glucose	• Higher inhibitory effect on microorganisms observed with the addition of Cu NPs in fermentative process.	Mohanraj et al. (2016)
Fe	5.0 mg/L	Anaerobic sludge	Glucose	• 37% enhancement in hydrogen yield.	Taherdanak et al. (2016)
	100 mg/L	*Enterobacter cloacae* DH–89	Glucose	• 100% increase in hydrogen production. • Higher cell growth was observed compared to control.	Nath et al. (2015)
	400 mg/L	Mixed bacterial consortium	Glucose	• 38.2% higher hydrogen yield	Zhang et al. (2015)
	250 mg/L	Mixed culture and *Clostridium butyricum* TISTR	Water hyacinth	• Maximum hydrogen yield of 57 mL/g of dry weight-based plant biomass.	Zada et al. (2013)

(continued)

Table 10.1 (continued)

NPs	Concentration	Organism	Substrate type	Effect on anaerobic digestion	References
Ni	5.0 mg/L	Anaerobic sludge	Glucose	• No significant effect was observed.	Taherdanak et al. (2016)
	5.7 mg/L	Anaerobic sludge	Glucose	• 22.71% increase in hydrogen production. • At optimum conditions, the hydrogen production reached 2.54 mol of H_2/mol of glucose.	Mullai et al. (2013)
	60 mg/L	Anaerobic sludge	Wastewater	• 23% improvement in hydrogen production.	Elreedy et al. (2017)
Pd	5 mg/L	*E. cloacae* 811,101 and mixed culture	Glucose	• The maximum hydrogen yields reached 1.48 and 2.48 mol H_2/mol glucose using *E. cloacae* and mixed culture as inoculum, respectively.	Mohanraj et al. (2014a, 2014b)
Metal oxide NPs					
Fe_2O_3	175 mg/L	*C. acetobutylicum* NCIM2337	Glucose	• The hydrogen production increased by 33% compared to control experiment. • Enhancement in hydrogen production rate and content was observed. • Addition of FeNPs led enhancement of ferredoxin activity leading to higher hydrogen production.	Mohanraj et al. (2014a, 2014b)
	200 mg/L	*Enterobacter aerogenes* ATCC13408	Glucose	• Increase in hydrogen yield noticed from 164.5 to	Lin et al. (2016)

(continued)

Table 10.1 (continued)

NPs	Concentration	Organism	Substrate type	Effect on anaerobic digestion	References
				192.4 mL/g with increase in concentration of NPs from 0 to 200 mg/L.	
	125 mg/L	*E. cloacae* 811,101	Glucose	• Addition of 125 mg/L and 200 mg/L NPs improved the H_2 yield reaching a maximum value of 2.07 mol H_2/mol glucose and 5.44 mol H_2/mol, respectively.	Mohanraj et al. (2014a, 2014b)
	50 mg/L	Anaerobic sludge	Glucose	• Biohydrogen production increased by 53%. • The metal NPs are not consumed by the microbes, and only act as an enhancer.	Engliman et al. (2017)
Fe_3O_4	400 mg/L	Anaerobic sludge	Glucose	• 26% higher cumulative hydrogen yield was observed in comparison to control.	Zhao et al. (2011)
	50 mg/L	Mixed culture	Wastewater	• Specific hydrogen yield obtained as 44.28 mL H_2/g COD at a rate of 80.7 mL/h.	Malik et al. (2014)
	200 mg/L	Anaerobic sludge	Sugarcane bagasse	• 69.6% enhanced hydrogen yield.	Reddy et al. (2017)
NiO	200 mg/L	Anaerobic sludge	Glucose	• 5.47% increase in hydrogen yield in comparison to control. • Metal NPs were not consumed by the microbes.	Engliman et al. (2017)

(continued)

Table 10.1 (continued)

NPs	Concentration	Organism	Substrate type	Effect on anaerobic digestion	References
SiO_2	40 mg/L	*Chlamydomonas reinhardtii* CC124	Air:CO_2 (97:3)	• Higher culture growth rate was observed for cultures grown on NPs.	Giannelli and Torzillo (2012)
TiO_2	50 mg/L	*C. pasteurianum* CH5	Glucose	• No substantial increase in biohydrogen yield. • Increase in hydrogen production rate from 5.0 L–H_2/L–d to 5.6 L–H_2/L–d was observed.	Hsieh et al. (2016)
	100 mg/L	*Rhodopseudomonas palustris*	Waste sludge	• 46.1% increase in hydrogen production. • Degradation of protein and polysaccharide to smaller organic molecules increased with the addition of NPs. • Increase in nitrogenase activity and growth of photosynthetic bacteria with reduced H_2 uptake hydrogenase activity was observed.	Zhao and Chen (2011)
	60 mg/L	*R. sphaeroides* NMBL–02	Malate	• 1.54-fold increase in rate and 1.88-fold increase in duration in the presence of NPs. • 1900 mL/L maximum hydrogen production observed with 63.27% malate conversion.	Pandey et al. (2015)

production. A summary of literature reports on the influence of various NPs over biohydrogen production by different microorganisms is given in Table 10.1.

Zhang and Shen (2007) reported the effect of Au NPs size over substrate uptake and biohydrogen production. For this purpose, three different sized NPs (5, 10, and 20 nm) at a concentration of 10 nM were added to the fermentation mixture containing anaerobic mixed culture and artificial wastewater. The results showed 56% improvement in substrate utilization with 46% increase in hydrogen yield. Higher surface area to volume ratio of Au NPs provides better binding of microorganisms to the active site of the molecules that further stimulates the activity of biohydrogen-producing enzymes (ferredoxins and [Fe–Fe] and [Ni–Fe] hydrogenases), leading to enhancement in hydrogen yield. The optimum concentration of NPs is crucial for maximizing productivity as the higher NP concentrations inhibit growth of microorganisms. Ag NP (20 nmol/L) addition into anaerobic batch reactor dominated *Clostridium butyricum* strains improving the glucose conversion by 62% and a highest biohydrogen yield of 2.48 mol of H_2/mol glucose (Zhao et al. 2013). As per the report, the addition of Ag NPs during anaerobic digestion not only augmented the hydrogen yield but also assist in reduction of the lag phase of fermentation.

Numerous studies have explored the influence of Fe, Ni, Cu, and Pd NPs over biohydrogen production (Elreedy et al. 2017; Mohanraj et al. 2014a, 2014b; Mohanraj et al. 2016; Mullai et al. 2013; Nath et al. 2015; Taherdanak et al. 2016; Zada et al. 2013; Zhang et al. 2015). Taherdanak et al. (2016) studied the effect of Fe^0 and Ni^0 NPs versus Fe^{2+} and Ni^{2+} ions over hydrogen production using anaerobic sludge as the inoculum and glucose as the carbon source. The results of this study indicated significant effect of Fe NPs with 37% enhancement in hydrogen yield, while no change in hydrogen production was observed with the addition of Ni NPs during anaerobic digestion. In contrast, biologically synthesized Cu NPs at a concentration range of 2.5–12.5 mg/L showed a negative effect on *Clostridium acetobutylicum* NCIM 2337 and *Enterobacter cloacae* 811101H2, further reducing the H_2 production from glucose (Mohanraj et al. 2016).

Other than zerovalent metal NPs, several metal oxide NPs like Fe_2O_3, Fe_3O_4, NiO, SiO_2, and TiO_2 are being assessed for the biohydrogen production (Table 10.1). The addition of Fe_2O_3 NPs at concentrations of 125, 175, and 200 mg/L into pure cultures of *E. cloacae* 811101, *C. acetobutylicum* NCIM2337, and *E. aerogenes* ATCC13408 showed enhancement in hydrogen production by 21.8, 33.9, and 17.0%, respectively (Lin et al. 2016; Mohanraj et al. 2014a). Mohanraj et al. (2014b) also ascertained that the biohydrogen production by *E. cloacae* 811101 got influenced by the choice of carbon source in the fermentation medium in addition to Fe_2O_3 NPs. Glucose was found to be a more ideal substrate for *E. cloacae* 811101 as compared to sucrose, and an increase in hydrogen production was observed with increase in NP concentration from 25 to 125 mg/L. Similarly, Engliman et al. (2017) used two different metal oxide NPs, Fe_2O_3 and NiO, in batch processes using glucose-fed anaerobic mixed bacteria. The results showed 34.38% and 5.47% higher hydrogen yield as compared to control for Fe_2O_3 and NiO NPs, respectively. The analysis of fermentation metabolites further

corroborated that the hydrogen production followed the acetic acid pathway. These results also suggested that iron oxide NPs were not consumed by the microbes and act only as an enhancer for improvement in hydrogen production.

NPs are also used in photofermentative and photocatalytic biohydrogen production for improving the H_2 production. A detailed discussion on these processes has been given in earlier studies (Sekoai et al. 2019). In photofermentative biohydrogen production process using microalgae, NPs have been used to enhance the biomass growth, nitrogen metabolism, photosynthetic activity, and protein content (Eroglu et al. 2013). Furthermore, the addition of NPs improves the activity of major enzymes, viz., glutamine synthase, glutamate dehydrogenase, and glutamate-pyruvate transaminase involved in the algal metabolism.

10.3 Methane

Methane is the simplest alkane with chemical formula of CH_4. It is a part of natural gas and produced by anaerobic decomposition. Methane, also called as biogas, is primarily produced by anaerobic digestion process in the presence of animal dung, mostly by cow dung. The process of methane production is called as biomethanation and is carried out in anaerobic conditions. Methanogens (also called methane-oxidizing bacteria or *Methanobacterium*) are mainly responsible for the production of methane while utilizing various substrates in the process. Cow dung, sewage, rumen of sheep, cattle, and mud are some of the good sources of methanogens.

10.3.1 Conventional Methane Production

Anaerobic digestion (AD) is one of the attractive, eco-friendly, and economical biological processes that utilizes the organic content of complex biomass and waste for the production of high energy containing biogas. The biogas is produced under anaerobic condition (i.e., in the absence of oxygen) in which series of reactions take place with the involvement of various microorganisms for the degradation of complex organic matters (Aryal et al. 2018; Romero-Güiza et al. 2016; Chowdhary and Raj 2020; Khan et al. 2020, 2021). The primary components of produced biogas are methane (CH_4) and carbon dioxide (CO_2) along with trace amount of hydrogen sulfide, nitrogen, hydrogen, and carbon monoxide. In AD process, several groups of bacteria and methanogenic archaea acts sequentially on the organic waste for its conversion to biogas. The conversion of substrates into various products in AD process occurs in four different stages as mentioned below:

1. Hydrolysis
2. Acidogenesis

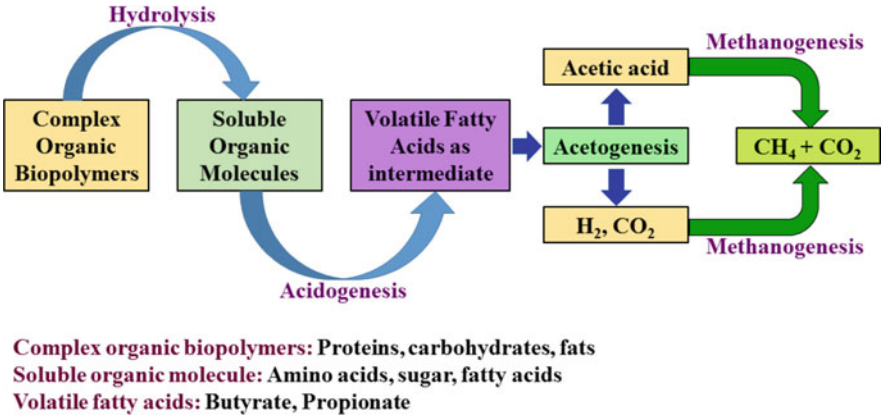

Fig. 10.1 Schematic representation of various processes and products of anaerobic digestion process

3. Acetogenesis
4. Methanogenesis

The process of digestion starts with the action of hydrolytic bacteria which break down complex organic molecules into simple monomers and oligomers, to make those available for the other microorganisms. Hydrolysis of complex substrates is considered as the most crucial as well as rate-limiting step of the anaerobic digestion process as this leads to the generation of non-desirable volatile fatty acids or toxic by-products (Buffière et al. 2018; Wang et al. 2018; Yap et al. 2018). Subsequently, the acidogenic bacteria convert these monomeric sugars, fatty acids, and amino acids into organic acids, CO_2, H_2, and NH_3. These organic acids released in the acidogenesis process are further converted into acetic acid and hydrogen by acetogenic bacteria. In the end process, i.e., methanogenesis, the acetic acids are finally converted to methane and carbon dioxide. A schematic representation of these processes is given in Fig. 10.1.

10.3.1.1 Hydrolysis

Hydrolysis is the initial stage of the anaerobic digestion process. During this, the complex organic polymeric substrates containing carbohydrates, proteins, and lipids are converted into simple soluble monomers in the form of sugars, amino acids, and long-chain fatty acids, respectively. The hydrolysis is mainly carried out by hydrolytic enzymes of anaerobic hydrolytic bacteria which are usually found in soil, AD sludge, animal rumen, and compost (Castellano-Hinojosa et al. 2018). Degradation of these complex polymers or substrates begins with the formation of a multienzyme complex (cellulosome) by the hydrolytic bacteria in which different hydrolytic enzymes (glucanases, chitinases, hemicellulases, and liganases) are released for the

degradation of polymeric substances. The degradability of various polymers by the anaerobic bacteria majorly depends on the composition, type, and complexity of polymers, and this further controls the duration of hydrolysis from hours to many days. Five different phyla of hydrolytic bacteria such as *Bacteroidetes*, *Firmicutes*, *Fibrobacter*, *Thermotogae*, and *Spirochaetes* are mainly responsible for degradation. Among these, bacteria belong to the phyla *Bacteroidetes*, and *Firmicutes* are abundantly found during this process (Yap et al. 2018). Nevertheless, several operational conditions such as temperature, type of inoculum, composition of substrates, and cell retention time affect the relative abundance of hydrolytic bacteria (Wang et al. 2018). The optimum temperature and pH for hydrolysis are 30–50 °C and 5–7, respectively (Meegoda et al. 2018).

10.3.1.2 Acidogenesis

In acidogenesis, the end products of hydrolysis (carbohydrates, amino acids, and fatty acids) are utilized by acidogenic bacteria for the production of short-chain (C1–C5) volatile fatty acids (VFAs). VFAs like acetic acid, butyric acid, propionic acid, and acetate are formed in this process along with some amount of alcohol, carbon dioxide, and hydrogen. The intermediately formed hydrogen ion concentration directly influences the end product type, i.e., increase in partial pressure of hydrogen leads to the formation of fewer reduced compounds (acetate) and vice versa (Oh et al. 2018; Westerholm et al. 2018; Zhou et al. 2018). In general, acidogenesis steps are divided into two stages: the first stage is accomplished by facultative anaerobic acidogens, and on the contrary, obligate anaerobic acidogens are more active in the second stage. These acidogenic bacteria belong to the phyla *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Firmicutes*. The population and abundance of acidogenic bacteria in the AD process are regulated by varying the operating parameters such as temperature, digester design, cell retention time, and substrates types.

10.3.1.3 Acetogenesis

Acetogenesis is the third phase of AD, which involves the conversion of produced VFAs (propionic, butyric, and pentanoic acid) and other intermediates in the previous acidogenesis phase into acetate along with hydrogen (Hansen and Cheong 2013). Acetate formation takes place by the involvement of two different groups of acetogenic bacteria with a different mechanism. The first groups, i.e., homoacetogenic bacteria, are known for the constant conversion of hydrogen and carbon dioxide to acetate. These homoacetogens belong to the genera of *Acetobacterium*, *Acetogenium*, *Butyribacterium*, *Eubacterium*, *Clostridium*, etc. The accumulation of hydrogen (with increase in higher hydrogen partial pressure) in the system inhibits the acetate-forming bacteria leading to a decrease in acetate concentration. However, the presence of hydrogenotrophic methanogens decreases

the concentration of hydrogen by converting it into methane and obliterating the inhibitory effect of hydrogen on homoacetogens. Therefore, the activity of hydrogenotrophic methanogens is enormously important for maintaining low hydrogen partial pressure and typical activity of homoacetogens in the AD process.

10.3.1.4 Methanogenesis

Methanogenesis is the end stage of AD process in which the accessible intermediates are consumed by methanogenic archaea for the production of methane (Ferry 2010). In addition to methane production, hydrogenoxidation, carbon dioxide reduction, and its utilization are some of the additional features of methanogens. Methanogens represent slow-growing obligate anaerobic archaea that are very sensitive to minor presence of oxygen. Until now, 65 different species of methanogens are reported, which are further categorized in five different orders (*Methanococcales*, *Methanobacteriales*, *Methanomicrobiales*, *Methanopyrales*, and *Methanosarcinales*) (Laiq Ur Rehman et al. 2019). Based on substrate specificity, these methanogens are further classified into three different groups, i.e., (i) methylotrophic methanogens (utilize single carbon compound and methyl), (ii) hydrogenotrophic methanogens (utilize CO_2 and H_2 for the production of methane), and (iii) acetoclastic methanogens (convert acetate to methane). In comparison to other methanogens, acetoclastic methanogens account for the majority of methane production.

Proper functioning and prolonged stability of AD process with optimum biogas production depend on various operational parameters (pH, temperature, C/N ratio, retention time, substrate composition, organic loading rate, trace elements, etc.) and the presence of different microbial communities with their activity at various stages of the AD process.

10.3.2 Application of NPs in Anaerobic Digestion Process

Enhancement in the AD process for improving biogas production is essential for the successful commercialization of the process. In view of this, several strategies such as co-digestion with similar substrate to maintain required C/N ratio (Siddique and Wahid 2018; Luo and Angelidaki 2013), prior pretreatment of substrates for breaking down the complex structure and release of simpler organic matter to improve microbial action (Dahunsi et al. 2017), modification of reactor design (Li et al. 2017), maintaining optimal process conditions (Leitão et al. 2006), and usage of organic/inorganic additives (Nzila 2017) to accelerate microbial action have been implemented in the AD process. These additives can be further classified into organic (enzymes, microbial cultures, and green biomass) or inorganic (macronutrients/micronutrients and carbonous materials) additives. The macronutrients (phosphorous, nitrogen, sulfur, etc.) added into the AD process help in maintaining the

buffer capacity, while the micronutrients (nickel, cobalt, iron, molybdenum, etc.) are essential components for cofactors and enzymes involved in these processes. These micronutrients in the form of pure metals, metal oxides, salts, and nanostructured materials are added into the AD process to enhance the productivity. Significant research works are conducted on the supplementation of nanostructured materials to the AD process. Addition of these materials enhances the methane production rate by acting as an electron conduit that further aids in the interspecies electron transfer between syntrophic microorganisms (Baniamerian et al. 2019).

These nanostructured materials are added into the AD process in four different forms, viz., zerovalent metal ions, metal oxide-based NPs, multi-compound NPs, and carbonous nanomaterials. A detailed discussion on the effect of above mentioned nanomaterials over the anaerobic digestion process is given in the following sections.

Nanoscale zerovalent metals (NZVMs) hold several unique properties in terms of high efficiency, large specific surface area, and great chemical reducibility in comparison to zerovalent metals (ZVMs) for various environmental applications (Li et al. 2016, 2016). With decreasing particle size, the fraction of atoms positioned on the surface increases due to increment in surface-to-volume ratio. This in turn increases the particle tendency for various reaction processes (Crane and Scott 2012). As the AD process is performed by diverse groups of microorganisms (fermentative bacteria and methanogen), interspecies electron transfer (IET) between these species plays a significant role in improving the performance. The IET occurs mainly in three different modes, i.e., (i) interspecies hydrogen transfer, (ii) interspecies formate transfer, and (iii) direct interspecies electron transfer (DIET). Several zerovalent metal nanoparticles like Ni, Co, Fe, Cu, Ag, Au, etc. have been successfully studied for improving the AD process. A summary on application of various NPs in AD process and its effect on biogas/methane production is given in Table 10.2. Among these, nanozerovalent iron (nZVI) is one the most largely used NPs in the field of AD process for the production of biogas. The treatment of sludge with nZVI improves the hydrolysis and methanogenesis activity. Suanon et al. (2017) reported the effect of nanoscale zerovalent iron (nZVI) and commercial iron powder (IP) on the anaerobic digestion of sewage sludge. The results of this study substantiated that the addition of 100 mg/L of nZVI and 1600 mg/L of IP into batch biodigester increased the methane yield by 25.2% and 40.8%, respectively. Furthermore, compared to the control experiment (with COD removal −44.6%), 54.4% and 66.2% enhancement in chemical oxygen demand (COD) removal efficiency was observed with the addition of nZVI and IP. In another study, Su et al. (2013) reported 30.4% and 40.4% increase in biogas and methane production with the addition of 10 mg/L nZVI in the AD process, respectively. Besides biogas yield, the addition of NPs also significantly reduced the H_2S concentration by 98% in the biogas. Amen et al. (2017) reported the effect of four different NP additives, i.e., nZVI, zeolite, mixture of nZVI and zeolite, and nZVI coated zeolite on the anaerobic digestion performance of domestic sludge. The reactor was operated for 14 days, and various parameters such as methane content, biogas production, pH, and COD were monitored on daily basis. The cumulative

Table 10.2 Summary of the effect of NPs over anaerobic digestion

NPs	Concentration of NPs	Substrate type	Effect on AD process	References
Zerovalent metal NP				
Nickel (Ni)	2 mg/L	Manure slurry	• 1.74 times increase in biogas volume in comparison to control. • 2.01 times increase in methane volume in comparison to control.	Abdelsalam et al. (2017a)
	5–10 mg/KgVS	Sewage sludge	• Ni in the presence of Nitrilotriacetic acid increased 10% methane yield.	Tsapekos et al. (2018)
Cobalt (Co)	1 mg/L	Manure slurry	• 1.64 times increase in biogas volume in comparison to control. • Methane volume increased by 1.86 times compared to control.	Abdelsalam et al. (2017a)
Nanozerovalent iron (nZVI)	10 mg/g TSS	Waste activated sludge	• Methane production increased by 120%.	Wang et al. (2016)
	100 mg/L	Sewage sludge	• Methane production increased by 25.2%.	Suanon et al. (2017)
	20 mg/L	Raw manure	• 1.59 times increase in methane volume. • 1.45 times increase in biogas volume.	Abdelsalam et al. (2017b)
	10 mg/L	Sewage sludge	• Biogas production increased by 30.4%. • 40.4% increase in methane production.	Su et al. (2013)
	1000 mg/L	Domestic sludge	• 105.46% increase in cumulative biogas production.	Amen et al. (2017)
	56, 560, and 1680 mg/L	Digested sludge	• 20% decrease in methane production.	Yang et al. (2013)
Copper (Cu)	10–1500 mg/L	Granular sludge	• IC_{50} values of acetoclastic and hydrogenotrophic methanogens were 62 and 68 mg/L.	Gonzalez-Estrella et al. (2013)
Silver (Ag)	40 mg/L	Digested sludge	• NPs at moderate concentrations (40 mg/L) showed negligible effect on methanogenic assemblages and anaerobic production.	Yang et al. (2012)
	130 mg/L	Cellulose	• 33% inhibition on ordinary heterotrophic organisms.	García et al. (2012)
	5, 1.5, and 100 mg/L	Biosolids from wastewater treatment plant	• Low concentration didn't impact on anaerobic degradation. • At high concentration, the cationic NP demonstrates toxicity.	Gitipour et al. (2016)

(continued)

Table 10.2 (continued)

NPs	Concentration of NPs	Substrate type	Effect on AD process	References
	5–1000 mg/gTS	Municipal waste activated sludge	• 12.1% decrease in methane production.	Ünşar et al. (2016)
	1500 mg/L	Granular sludge	• No toxic effects on the methanogenic activity	Gonzalez-Estrella et al. (2013)
Gold (Au)	75 mg/L	Cellulose	• Zero or slight toxicity effect on ordinary heterotrophic organisms, ammonia-oxidizing bacteria, and anaerobic bacteria.	García et al. (2012)
Metal oxide NPs				
ZnO	10–1500 mg/L	Granular sludge	• IC_{50} values of acetoclastic and hydrogenotrophic methanogens were 87 and 250 mg/L.	Gonzalez-Estrella et al. (2013)
	10, 300, 1500 mg/L	Waste activated sludge	• 75.1% inhibition of methane production.	Mu et al. (2011)
	5–500 mg/L	Waste activated Sludge	• Biogas production reduced by 25%. • Reduction on methane production by 50%.	Zhang et al. (2017)
	30 or 150 mg/g–TSS	Mixed primary and excess sludge	• The methanogenic archaea population decreased. • Methane production reduced.	Zheng et al. (2015)
	100, 500, 1000 mg/L	Sludge	• 65.3% decrease in biogas production.	Nguyen et al. (2015)
	7.5–480 mg/L	Cattle manure	• 74% inhibition in biogas production.	Luna-del Risco et al. (2011)
CuO	5–1000 mg/gTS	Municipal waste activated sludge	• 5.8–84.0% increase in anaerobic digestion. • EC50 values for short inhibition −224.2 mgCuO per gTS and for long-term inhibition −215.1 mgCuO per gTS.	Ünşar et al. (2016)
	7.5–480 mg/L	Cattle manure	• Up to 96% biogas production inhibition.	Luna-del Risco et al. (2011)
	10–1500 mg/L	Granular sludge	• IC_{50} for acetoclastic methanogens was 223 mg/L.	Gonzalez-Estrella et al. (2013)

(continued)

Table 10.2 (continued)

NPs	Concentration of NPs	Substrate type	Effect on AD process	References
Fe_2O_3	100 mg/g TSS	Waste activated sludge	• Increase in methane production by 117%.	Wang et al. (2016)
	750 mg/L	Granular sludge	• Increase in methane production by 38%.	Ambuchi et al. (2016)
	1500 mg/L	Granular sludge	• No toxicity was observed on methanogenic activity.	Gonzalez-Estrella et al. (2013)
Fe_3O_4	20 mg/L	Raw manure	• 1.96 times increase in methane volume. • 1.66 times increase in biogas volume.	Abdelsalam et al. (2017b)
	100 mg/L	Crystalline cellulose	• 180% increase in biogas production. • 8% increase in methane production.	Casals et al. (2014)
	50–125 mg/L	Municipal solid waste	• Methane production increased by 117%.	Ali et al. (2017)
CeO_2	5–1000 mg/gTS	Municipal waste activated sludge	• Methane production increased by 9.2%.	Ünşar et al. (2016)
	640 mg/L	Cellulose	• 100% decrease in biogas production. • Toxicity effect.	García et al. (2012)
	100, 500, 1000 mg/L	Sludge	• Decrease in biogas production by 35%.	Nguyen et al. (2015)
	1500 mg/L	Granular sludge	• No toxicity observed on methanogenic activity.	Gonzalez-Estrella et al. (2013)
Mn_2O_3	1500 mg/L	Granular sludge	• No toxicity observed on methanogenic activity.	Gonzalez-Estrella et al. (2013)
Al_2O_3	1500 mg/L	Granular sludge	• No toxicity observed on methanogenic activity.	Gonzalez-Estrella et al. (2013)
MgO	500 mg/g TSS	Waste activated sludge	• 99% decrease in methane production.	Casals et al. (2014)
SiO_2	1500 mg/L	Granular sludge	• No toxicity observed on methanogenic activity.	Gonzalez-Estrella et al. (2013)
TiO_2	150 mg/g TSS	Mixed primary and excess sludge	• No significant effect on methane production.	Zheng et al. (2015)
	840 mg/L	Cellulose	• No effect.	García et al. (2012)
	1500 mg/L	Granular sludge	• No toxicity observed on methanogenic activity.	Gonzalez-Estrella et al. (2013)

IC_{50} – half maximal inhibitory concentration

biogas production was increased up to 105.46% with addition of 1 g/L of nZVI, and the increment was 286.75% for nZVI coated zeolite. Zeolite acts as porous support for maximum immobilization of NPs on the surface as well as in the pores (Bhowmick et al. 2014). In contrast, Yang et al. (2013) reported 20% decrease in methane production due to disruption of cell integrity while using nZVI with an average size and concentration of 55 nm and 56 mg/L, respectively. Some of the possible reasons for enhancement in methane content in biogas with the addition of nZVI could be due to the following: (i) the electrons formed upon oxidation of iron aid in AD process and improve the growth as well as the metabolism of microorganisms, (ii) the presence of iron additives enhances the conversion of carbon dioxide to methane by electron transfer, and (iii) it improves the production of hydrogen through Fe^0 which further leads to increasing methane production. Abdelsalam et al. (2016) verified the influence of various NPs, viz., Co (1 mg/L), Ni (2 mg/L), and nZVI (20 mg/L), on AD of raw manure, and the results showed 50%, 70%, and 80% increase in biogas yield as compared to control condition, whereas significant increase in methane which yields 67%, 117%, and 100% was noticed compared to the control while adding Co, Ni, and nZVI NPs, respectively. The cobalt (Co) and nickel (Ni) are predominantly used as trace minerals by the methanogenic bacteria for improving the growth rate. In view of this, the influence of these nanoparticles at various concentrations level on biogas production from livestock manure has been reported by Abdelsalam et al. (2017a). As per the study, increasing cobalt concentration (up to 1 mg/L) enhanced the biogas and methane volume by 1.64 and 1.86 times, respectively, and a further increase in concentration to 2 mg/L caused a negative impact on methane production. In contrast, the biogas and methane production increased with the addition of nickel NPs up to a concentration of 2 g/L. Other zerovalent metal NPs such as copper (Gonzalez-Estrella et al. 2013), silver (García et al. 2012; Gitipour et al. 2016; Gonzalez-Estrella et al. 2013; Yang et al. 2012; Ünşar et al. 2016), and gold (García et al. 2012) at different concentration were used in AD process to enhance the biogas/methane yield. García et al. (2012) reported negative influence of Ag NPs over the biogas production with a reduction in biogas yield by 33–50%, whereas the Au NPs exhibited zero or minor toxicity.

In addition to zerovalent metals, several metal oxide NPs such as CuO, TiO_2, ZnO, NiO, MgO, Fe_2O_3, Fe_3O_4, etc. are evaluated to improve the performance of AD process. Wang et al. (2016) investigated the effect of two different metal oxide NPs (MgO and Fe_2O_3) over methane production during AD of waste activated sludge. These results reported that supplementation of 100 mg/g TSS Fe_2O_3 and 500 mg/g TSS MgO NPs into the AD process increased the methane production rate by 117% and 98.92%, respectively. Further, the results confirmed that the low concentration of Fe_2O_3 NPs promoted growth and enzymatic activity, while the higher concentration of MgO caused inhibition. Other studies also reported enhancement in the activity of methanogens while using Fe_3O_4 NPs which further lead to higher methane production (Abdelsalam et al. 2017a, 2017b; Ali et al. 2017). Studies also reported the use of TiO_2 and CeO_2 NPs in the wastewater treatment process to assay the activity of some specific microorganisms such as ammonia-oxidizing

bacteria, heterotrophic organisms, and thermophilic and mesophilic anaerobic bacteria (García et al. 2012; Chowdhary et al. 2020). The results of this study demonstrated that CeO_2 at 640 mg/L caused the highest inhibition, i.e., nearly 100%, while the TiO_2 NPs caused negligible inhibition. The results of this study was in close agreement with earlier report by Zheng et al. (2015) which showed no significant impact of TiO_2 NPs on any of the steps (hydrolysis, acidification, and methane production) of AD process. To ascertain the inhibitory effect of various inorganic oxide NPs on acetoclastic and hydrogenotrophic microorganisms, Gonzalez-Estrella et al. (2013) used various NPs (CeO_2, Al_2O_3, CuO, Mn_2O_3, Fe_2O_3, SiO_2, ZnO, and TiO_2) in the AD of granular sludge. The results of this study corroborated higher inhibition effect of ZnO and CuO NPs toward acetoclastic and hydrogenotrophic methanogens, while in contrast no significant inhibition of the activity of methanogens was not inhibited at higher concentration (1500 mg/L) of other NPs.

In addition to zerovalent metal and metal oxide NPs, several carbon-based nanostructures such as graphene (Lin et al. 2017; Tian et al. 2017), fullerene (Nyberg et al. 2008), multiwalled carbon nanotubes (MWCNT) (Ambuchi et al. 2016), and short-walled carbon nanotubes (SWCNTs) (Li et al. 2015; Yan et al. 2017) are successfully used in AD process for enhancement of methane production.

10.4 Conclusion and Future Perspective

It is manifested that the addition of nanoparticles enhances the gaseous biofuel production owing to its physicochemical properties like large surface-area-to-volume ratio, high reactivity, good dispersibility, high specificity, etc. Four different types of nanoparticles, namely, zerovalent metal NPs, metal oxide NPs, carbonous nanomaterials, and multi-compound NPs, are successfully used in the production of biomethane and biohydrogen. Significant improvement in biofuel production is observed for most of the NPs, while a few exhibited negative/insignificant effects over the gaseous biofuel production. However, successful commercialization of this process is obstructed by many technical barriers such as toxicity of NPs, high cost of NPs, stringent operating conditions, etc. which need to be addressed in advance. These barriers can be curtailed by the synthesis of nontoxic NPs to minimize the inhibitory effect over microorganisms, the use of less expensive NPs, and adopting the biological synthesis method as a substitute to the chemical synthesis method to avoid harsh operating conditions.

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Chapter 11
Nanocatalyzed Transesterification of Thumba Oil for Biodiesel Production Using Hydrodynamic Cavitation

Abhijeet D. Patil, Saroj S. Baral, and Prashant B. Dhanke

Abstract Available fossil fuels are decreasing day by day. So the use of renewable sources like alkyl esters (biodiesel) is increasing in CI engines. Biofuel or fatty acid alkyl ester (C_{13}–C_{23}) is derived from short-chain alcohols. Various processes are reported to formulate from animal fats and vegetable oils. Alcoholysis is a commonly employed biodiesel preparation method where oil is mixed with suitable CH_3OH or C_2H_5OH and a suitable catalyst. Alcoholysis mainly reduces the viscosity of oils as well as lowers the Sox and NOx emissions from the oil. Various techniques are available to carry out alcoholysis reactions, viz., mechanical stirring, probe type ultrasonic cavitation, pyrolysis, and hydrodynamic cavitation. Hydrodynamic cavitation (HC) coupled with nanocatalyst is one of the energy-efficient processes for alkyl ester (biofuel) synthesis in which cavities are created by the reduction in the pressure caused by allowing the liquid to flow through constriction. This technique gives a high yield and it is a time-saving one. The different operating variables, viz., cavitation number, inlet pressure, and system geometry of hydrodynamic cavitation, influence the percent conversion of triglycerides and the yield of alkyl ester. A variety of successful applications of the HC technique has been investigated, and research is still going on. This paper broadly covers the basics of the HC technique and its use for the production of biodiesel through the transesterification of thumba oil. The effects of operating parameters such as oil-to-alcohol molar ratio, TiO_2 concentration, the inlet pressure of cavitation, and cavitation number have been briefly discussed.

Keywords Biodiesel · Fatty acid methyl ester (FAME) · Hydrodynamic cavitation (HC) · Free fatty acid (FFA) · Alcoholysis

A. D. Patil · P. B. Dhanke
Chemical Engineering Department, PVPIT, Sangli, MH, India

S. S. Baral (✉)
Department of Chemical Engineering, BITS Pilani K K Birla, Goa Campus, Goa, India

P. Chowdhary et al. (eds.), *Bio-Clean Energy Technologies: Volume 1*, Clean Energy Production Technologies, https://doi.org/10.1007/978-981-16-8090-8_11

11.1 Introduction

Energy is a primary need of each country for its economic growth. Every sector of each country is mostly dependent on energy, viz., domestic, agriculture, industry, transport, and commercial (Pal et al. 2010). The increasing demand for energy gives rise to a more substantial dependency on natural gas, coal, and oil. But these sources are also finite. The more significant dependence on these fossil fuels has increased local environmental issues, which are the primary cause of global warming. Presently, the problem of uncertainty of fuel availability shortage leads to finding alternative fuel sources that can abundantly available in the future, which can be considered infinite sources.

An alternative fuel source may a biodiesel or biofuel. Biodiesel is termed as fatty acid alkyl ester. It is the fuel that is derived from biological sources. Animal fats and vegetable oils are the primary biological sources. Biofuel or biodiesel is known as mono-alkyl esters of long-chain fatty acids obtained from a primary biological energy source. Alkyl esters fall in chain length of fatty acid ranging from C_{13} to C_{23} derived from short-chain alcohols. Alkyl esters can be quickly produced and readily available with environmental acceptability (Ghayal et al. 2013). Biodiesel can offer specific merits such as reducing greenhouse emissions, no engine modification, and low cost compared to diesel, high cetane number, and energy-efficient. It is the only fuel that is nearly comparable with diesel. Biodiesel is characterized by fewer exhaust emissions (Ghayal et al. 2013). The synthesis of higher-order ethyl or methyl esters from the various edible or nonedible oils is a better option than others as it has biodegradability.

In the last 40 years, innovation in the biodiesel field has intimated to extract fuel from various plants (Ghayal et al. 2013). Under the Indian economic and environmental situation, plants producing nonedible oils, shrubs, and herbs are available. Thumba plant is one of them. Biodiesel can be prepared by various methods such as blending, cracking, pyrolysis, micro-emulsification, and transesterification. Transesterification (alcoholysis) is the commonly employed process for biofuel generation. The emission of aromatic and toxic compounds from the resources is strictly prohibited by transesterification (Pal et al. 2010). Several studies reported that biodiesel prepared from the transesterification process possesses a high viscosity index and O_2 content compared to conventional diesel, which increases NOx and SOx emissions (Meher et al. 2006). In blending, vegetable oil is directly mixed with fuel with minor modifications in the engine system. But here, fuel viscosity is not reduced. In micro-emulsion, oil is emulsified with solvents like butanol and octanol, which form a colloidal equilibrium suspension. In cracking, vegetable oils are pyrolyzed using heats in the absence of air to create biofuel. Tung oil was initially saponified and pyrolyzed to yield a crude oil.

On the other hand, nanomaterial use, such as titanium dioxide, has attracted interest across various chemical and allied industries. It has excellent dielectric property and catalytic activity, which finds its application in the pigment industry and as a photocatalyst in wastewater treatment (Mahshid 2007). TiO_2 nanoparticles

are widely used as photocatalyst because it possesses wide bandgap, better selectivity, and reusability. It is chemically stable and highly reactive (Gardy et al. 2016; Byrne et al. 2007). It mainly exists in brookite, rutile, and anatase phases (Gardy et al. 2016). TiO_2 nanoparticles can store energy within them, which increases their reactivity for a more extended period, and can be effectively used as a catalyst in biodiesel synthesis (Gardy et al. 2016). Such a characteristic is not observed in another catalyst like metal oxides and Amberlyst-15 (Salinas et al. 2016). It is reported that the use of TiO_2 in base fuel of mustard oil methyl ester resulted in a reduction in HC, CO, and smoke emissions and improved oxidation capability as well as enhanced thermal conductivity and catalytic effect (Yuvarajan et al. 2018). TiO_2 nanoparticles are synthesized by several processes such as sol-gel, mechanical alloying solvothermal, and hydrothermal methods. One commonly employed method in the synthesis of TiO_2 nanoparticles is sol-gel (Gardy et al. 2016; Salinas et al. 2016; Wang et al. 2007). The concentration of precursors is one of the most crucial parameters in TiO_2 crystallization behavior, as well as the characteristics of nanoparticles prepared. Solvent-to-precursor ratio strongly affects the crystalline size and morphology of the produced TiO_2 nanoparticles. A variety of synthetic routes are available to prepare TiO_2 nanoparticles and their composites (Ambati and Gogate 2018).

Various precursors are available under the sol-gel method for TiO_2 synthesis. Many researchers reported sol-gel method integrated with ultrasound power as an efficient process for the synthesis of TiO_2 nanoparticles as well as its composites (Ambati and Gogate 2018; Alam and Rahman 2013). The aging period and the hydrogen concentration in the solution affect the morphology, properties, and crystalline size of prepared TiO_2 nanoparticles (Ambati and Gogate 2018). Compression and rare fraction cycles in the sonication method lead to the formation of cavitation bubbles in the solvent-precursor mixture, which in turn collapses without any heat loss. This phenomenon occurs at higher operating temperatures and pressure and affects the morphology of TiO_2 nanoparticles. The effects of operating variables such as ultrasound power, temperature, reactor size, ultrasonication time, and solvent-to-precursor ratio affect the mesoporous TiO_2 nanocatalyst properties. Increasing the ultrasonic irradiation time was favorable for the fragmentation of the TiO_2 particles (Wang et al. 2007). High ultrasonic power density supplied higher energy to the reactor system, causing cavitation bubbles at higher temperatures and increased crystallinity.

Several studies revealed that the direct use of nanoparticles or nanofluid reduces these emissions and improves oxidation capacity, however affecting the thermal resistivity (Alam and Rahman 2013). Nanoparticles promote secondary atomization, which lowers emissions and increases the evaporation rate (Macedo et al. 2006; Sharma and Singh 2010). TiO_2 nanoparticles may act as a catalyst during a combustion process. It holds energy within it, which in turn improves the reactivity and combustion characteristics due to its large specific surface area.

Various techniques are available to carry out vegetable oils' alcoholysis using suitable catalyst, viz., mechanical stirring, microwave heating, supercritical methanol, and ultrasonic cavitation. In a mechanical stirring method, a reaction occurs in a

three-necked flask equipped with a stirrer so that mixing will take at a particular speed. Several studies reported the transesterification kinetics of Camelina sativa oil using various metal oxides, i.e., magnesium oxide, calcium oxide, and barium oxide, catalysts under mechanical stirring as microwave heating method (Wang et al. 2007). This study revealed that solid catalysts and heating methodology have an essential role in determining the kinetics of this reaction (Patil et al. 2018). Power ultrasound cavitation is also a useful method for improving diffusion transfer between two immiscible liquid mixtures. The power ultrasound irradiation generates cavities with required energy in both alcohol and oil, resulting in microbubble formation. The collapse of bubbles is asymmetric and affects phase boundary, and liquid creates the microjets and creates effective emulsification.

Many researchers have recently reported hydrodynamic cavitation as an energy-efficient technique for biodiesel production (Patil et al. 2019; Dhanke et al. 2020). HC is one of the novel methods for biofuel generation and wastewater treatment (Dhanke et al. 2020; Pal et al. 2010). Cavitation generated by passing the fluid through restriction followed by expansion and bursting of cavities is called hydrodynamic cavitation. The process intensified approach of the HC method can result in a higher conversion of triglycerides up to 90% within 20 minutes of the reaction period. The parameters such as the geometry of HC, number of holes, and reactor configuration are significant in gaining a higher conversion of triglycerides. Several studies performed biodiesel synthesis from frying oil with CH_3OH using KOH as a catalyst in the hydrodynamic cavitation reactor (Ghayal et al. 2013). Almost 95% conversion was obtained within the first 20 minutes because many pulses were generated during cavity oscillation. The magnitude of the pulses was minimal. Micro-level turbulence overcomes somewhat mass transfer resistances, which indicated that HC has a good potential of obtaining higher conversions.

So far, there are no reports on biodiesel production using various nanoparticles as heterogeneous catalysts under hydrodynamic cavitation (Maddikeri et al. 2014; Dhanke et al. 2020). So this review work reveals the sources, methods of biodiesel production, and how HC is better and energy effective in producing biofuel from thumba oil (Maddikeri et al. 2014; Kolhe et al. 2017; Malade and Deshannavar 2018; Bargole et al. 2019).

11.2 Renewable Sources of Alkyl Esters (Biodiesel)

A variety of renewable sources are available to synthesize biodiesel. These sources are broadly classified into two major categories, viz., edible or nonedible vegetable oils and animal fats. All these sources are insoluble in water and hence considered hydrophobic substances. Vegetable oil and animal fats consist of some saturated and specific unsaturated fatty acids. The high proportion of saturated fatty acids in any oil indicates a possible biodiesel source. Vegetable oils are the primary sources of biodiesel and are considered renewable sources. They are more utilized in biodiesel

synthesis as it is an inexhaustible source of energy. It has high-energy content as compared to conventional available fuel.

Commonly used vegetable oils include palm, rapeseed, soybean, canola, corn, soybean, groundnut, maize, cottonseed, sesame, and coconut (copra). Some of the oils are derived from nuts and shells, which include walnut, macadamia, and almond (Pal et al. 2010). Edible oils include grape seed, amaranth, artichoke, avocado, hemp, kapok seed, meadowfoam seed, mustard, pine nut, apricot, argan, Lallemantia, lemon seed, macauba fruit, and wheat germ. Nonedible oils include copaiba, babassu tree, Karanja, honge, Jatropha, mahua, and algae. Oils like thumba (Citrullus colocynthis), castor, and radish oil are considered underutilized oils.

All these oils are effectively used in biodiesel production in various countries based on their respective country availability. Jatropha oil and Karanja (Pongamia pinnata) are most widely used in multiple Asian countries. Oil extracted from soya seeds is mainly employed in biodiesel production in the UK as well as in the USA. Rapeseed oil is the primary source in many south and north European countries for biodiesel production. Palm oils and coconut oil are underutilized in Sri Lanka, Indonesia, Bali, Singapore, and Malaysia. Countries like Thailand, Malaysia, Argentina, and Indonesia majorly export vegetable oils, while significant countries like China and the UK import them. Countries like Singapore, the USA, Germany, and Bali export and import vegetable oils (Demirbas and Demirbas 2007).

Poultry oil extracted from cock, booster, beef, tallow yard are the alternative sources of biofuels. Animal fats are mainly wasted in adipose tissue cells, while the rest are to be found in the intermuscular connecting tissue, bones, and around nervous tissue, kidneys, and other organs. Biolipid is another new source of biodiesel. It includes virgin vegetable oils, viz., mustard, hemp, palm oil, and animal fats, including yellow grease, tallow, and lard. Many researchers have reported that algae are another alternative feedstock for biofuel synthesis. Algae is composed of 40–50% of fatty acids, which can be significantly converted to biodiesel via transesterification. Some algae can synthesize biodiesel up to 60%. The extraction of oil from algae and its simultaneous conversion into alkyl ester on a commercial basis have not been reported yet.

11.3 Alcoholysis

Alcoholysis is the significant process of converting triglycerides from oil into alkyl esters. In this process, one alcohol from a product displaces another alcohol. It is also called transesterification. A catalyst is required to accelerate the reaction. Alcoholysis is a reversible process by which different triglycerides in oils combine with CH_3OH or C_2H_5OH to yield alkyl esters and glycerol in the presence of suitable catalysts at a particular temperature. The catalyst may be acidic, basic, enzymatic, and lipase type. The optimized oil-to-alcohol molar ratios are chosen, and excess

alcohol is more preferred. Generally, the molar ratio of 1:6 or 1:4.5 is taken (Demirbas 2008; Patil et al. 2018; Pal et al. 2010).

Triacylglycerols are mixed with glycerol in the alcoholysis mechanism, and formed acids are converted into alkyl esters under the influence of catalyst. The principal operating parameters are temperature, acid value, water content, alcohol moles-to-moles of oil ratio, and inlet pressure. The product yield and TG conversion increase with temperature but decrease slightly beyond the boiling point of alcohol (Demirbas and Demirbas 2007).

$$(\text{TriglyceridesG}) + CH_3OH \rightarrow \text{diglycerides} + R_1COOCH_3$$

$$(\text{DiglyceridesG}) + CH_3OH \rightarrow \text{monoglycerides} + R_2COOCH_3$$

$$(\text{Monoglycerides}) + CH_3OH \rightarrow \text{glycerin} + R_3COOCH_3$$

Alcoholysis is one of the widely used processes to lower the primary source's viscosity, i.e., oil. It yields by-product, namely, glycerin, which is used as cosmetics at a commercial scale. Many researchers have reported that fatty acid alkyl esters produced via transesterification possess similar physical characteristics as conventional diesel (Demirbas 2009; Wang et al. 2007; Parmar and Gautam 2017). The factors which affect the mechanism are reactant purity, moles of alcohol-to-moles of oil ratio, inside temperature, and reaction period. Each parameter has its significance based on the application (Demirbas 2009; Patil et al. 2018).

11.4 Cavitation Methods of Biodiesel Synthesis

So far, two cavitation methods are reported for the synthesis of alkyl ester. The choice of the technique depends upon the application and operating condition. Each method has its own merits and demerits. Some of the ways are summarized below.

11.4.1 Hydrodynamic Cavitation (HC)

HC's working principle is somewhat resembled acoustic cavitation, as cavitation is a fundamental phenomenon in both cases. Figure 11.1 depicts a diagrammatic representation of HC. It consists of a stainless steel (SS-316) tank/reactor with a specific liter capacity, pressure gauges, valves, and a centrifugal pump of power rating maximum up to 2000 W. The thumba oil-methanol mixture's temperature is maintained as per requirement by passing the coolant through the jacket that surrounds the reactor. The discharge line of the pump is a bisected cavitating zone line and bypass line. All these lines carry the reaction back into the reactor. Both these lines are provided with ball valves and pressure gauges to adjust the upstream pressure in the cavitation zone, employing orifice plates/venturi tubes. Samples are

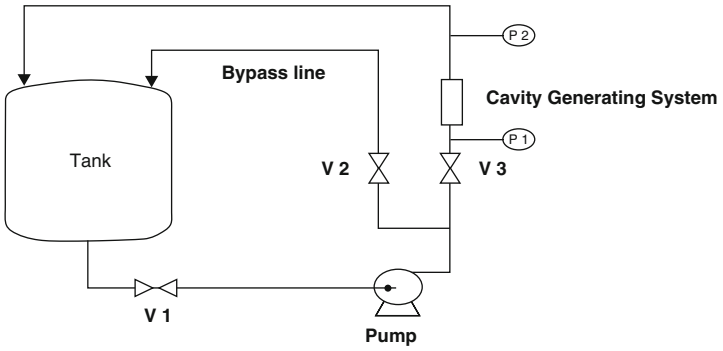

Fig. 11.1 Setup of hydrodynamic cavitation

continuously withdrawn from both lines through collection ports. Hydrodynamic cavitation of different intensities is generated by incorporating different geometrical orifice plates in the cavitating zone line.

When the reaction mixture passes through the constriction of the cavitating zone, its velocity increases due to a decrease in flow area and, in turn, decreases the pressure. When the pressure at a point in the cavitating zone is less than the reaction mixture's vapor pressure, vapor clouds are created in the reaction mixture. The cavities created get suddenly collapsed due to the regaining of pressure, and it releases a large quantity of heat. This increases the point pressure as well as temperature and favors the reaction. Thus, microfine bubbles are formed, which collapse and affect the interface of alcohol and oil. This results in supersonic alcohol jets (Patil et al. 2019). Because of this, the rate of reaction becomes faster. The mixture is circulated through orifice holes until it is converted to biodiesel.

Many reporters have considered HC as one of the novel, energy-efficient, and economical function methods for alkyl ester synthesis (Patil et al. 2019). Frying oil was alcoholized with CH_3OH using KOH in HC. Almost 95% conversion of triglycerides was achieved within the first 10 min because of a large number of pulses. It was also reported that HC's geometry plays a significant role in its process intensification (Ghayal et al. 2013). Thumba oil was transesterified with methanol in the hydrodynamic cavitation reactor with NaOH as a catalyst. Almost 80% yield was obtained within the first 30 minutes, and the yield increased with the number of orifice holes (Ghayal et al. 2013). So hydrodynamic cavitation is a time-saving method and viable at an industrial scale.

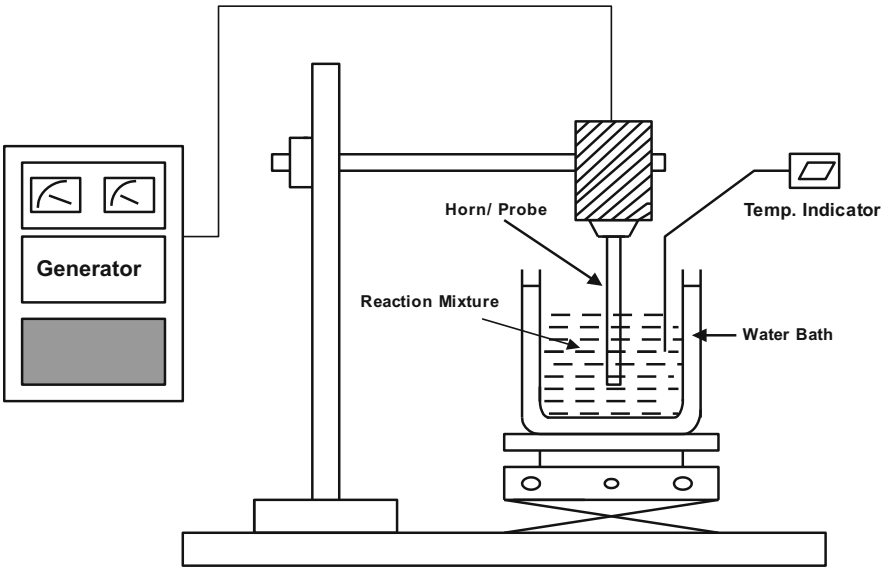

Fig. 11.2 Probe type of ultrasonicator

11.4.2 Ultrasonication

This method works on the principle of compression-rarefaction cycles. Figure 11.2 depicts ultrasonicator. The ultrasound cavitation system consists of a horn connected to the transducer, which produces ultrasound irradiation in the mixture. It is supported on a supporting tray for sliding motion with a beaker containing a reaction mixture. In this process, cavities are created using power ultrasound with some energy in the reaction mixture. Thus, fine bubbles of micro sizes are formed, which collapse and affect the interface of alcohol and oil. This results in the formation of supersonic alcohol jets (Ghayal et al. 2013). This results in the emulsification of the mixture (Theerayut et al. 2004).

Thumba methyl ester was synthesized under ultrasonic cavitation using Amberlyst-15 as a catalyst, and almost 90% yield of thumba methyl ester was obtained within 60 min. The ultrasonic cavitation method offers an advantage as one of the rapid, effective, and economically functional methods for preparing alkyl esters (Meher et al. 2006). The problem of adequate mixing could overcome using ultrasonication. Higher yields of FAME are obtained within a short reaction period compared to the conventional method (Patil et al. 2019; Dhanke et al. 2018a, b, c, d; Ghayal et al. 2013). A probe is subjected to damage. Table 11.1 summarizes catalysts used in biodiesel synthesis.

Table 11.1 Summary of catalysts used in biodiesel production

Sr. no.	Type of catalyst	Examples	Features of catalyst	Ref.
1.	Homogeneous base catalyst	KOH, NaOH, CH_3ONa, CH_3OK	Fast and high conversion, the catalyst cannot be recovered, must be neutralized leading to waste chemical production, limited use of continuous, methodology sensitive, high cost	Casio et al. (2006)
2.	Homogeneous acid catalyst	H_2SO_4, HCl, $AlCl_3$, 1-butyl-3-methylimidazolium hydrogen sulfate (BMIM HSO_4)	Fast and high conversion, but subjected to soap formation, high cost	Yusuke et al. (2009)
3.	Heterogeneous basic catalyst	SrO, MgO, t-MgO, CaO, $CaCO_3$, activated carbon, BaO, ZnO nanoparticles (K/BC-Fe_2O_3)	Moderate conversion, continuous fix bed operation possible, no emulsion, soap formation, reuse possible, cheaper	Jianbing et al. (2006)
4.	Heterogeneous acid catalyst		Higher conversion, no emulsion, and soap formation, reuse possible, cheaper, requires higher temperatures	Patil et al. (2011)
	1. Sulfated metal oxide	Sulfated zirconia, sulfated titania, TiO_2		
	2. Sulfonic ion exchange resin	Amberlyst-15, EBD, Nafion		
	3. Zeolites	Natrolite, zeolite X, zeolite Y		
	4. Hetropolyacids	H_3PWO_{40}, $H_3P_2W_{18}O_{62}$,		
	5. Carbon-based	Sulfonated carbon, asphalt-based, glycerol-based		

Table 11.1 summarizes the catalysts used so far in biodiesel production. Ghayal et al. (2013) reported biodiesel production of used frying oil with CH_3OH using KOH as a homogeneous catalyst in HC. KOH was added into the HC tank by mixing it with methanol. Almost 97% conversion was achieved within 20 min using KOH as a catalyst. Bargole et al. (2019) examined catalytic activity of NaOH for the alcoholysis waste oil in HC, where he achieved the intensification approach by increasing the ratio of throat diameter to the cross-sectional area and reported a maximum 99% yield of alkyl ester under at a temperature of 65 °C and NaOH concentration of 1 wt % of oil within 5 min. Kolhe et al. (2017) reported alcoholysis of frying oil using 0.55% (w/w) of oil concentration of NaOH at a molar ratio of 1: 4.5. Almost 93.5% conversion was observed by Kolhe et al. (2017) in 60 min.

Patil et al. (2019) examined the use of TiO_2-Cu_2O nanocomposites first time in HC in the preparation of alkyl ester from thumba seed oil. This is the first study reported so far where a hydrodynamic cavitation reactor was integrated with heterogeneous catalyst TiO_2-Cu_2O and obtained the conversion of 64% in 1 h at 1.2 wt%

of TiO_2-Cu_2O. Patil et al. (2011a, b) studied the transesterification reaction rates and the fatty acid methyl ester (FAME) conversion rates using heterogeneous metal oxide catalysts, i.e., BaO, CaO, MgO, and SrO, and two different heating methods. This study concluded that BaO and SrO catalyst generated higher FAME yields than the CaO and MgO catalysts and added that the selection of solid catalysts plays a vital role in improving the reaction kinetics and optimizing the reactors (Dhanke and Wagh 2020).

11.5 Hydrodynamic Cavitation in Biodiesel Synthesis

The most successful application of the HC method is in wastewater treatment as well as in the synthesis of fatty acid alkyl esters by alcoholysis of alcohol and oil. The conventional stirring method was complicated and time-consuming because additional steps were required for the separation, but HC is free from such additional steps. Ghayal et al. (2013) have reported biodiesel production of used frying oil with CH_3OH using KOH as a homogeneous catalyst in HC. The setup was made so that a 10-L reservoir was equipped with a centrifugal pump in a closed loop. He concluded that the geometry of HC plays a significant role in its process intensification. Pal et al. (2010) examined the alcoholysis of thumba oil with CH_3OH using NaOH as catalyst (1% by weight of oil) through hydrodynamic cavitation. In his experimentation, the mixture of CH_3OH and NaOH was mixed with this oil, and finally, the entire mixture was charged to feed the tank. He reported a yield of up to 80% within the first 30 min. The yield was increased with orifice holes, and afterward, it was nearly constant for all other types of orifice plates employed in the experimentation. The setup included a tank, centrifugal pump (2.2 kW), mainline, and bypass lines with ball valves and gauges to accommodate the orifice plate. Alcoholysis was done with methanol at different molar ratios. The reaction was carried at optimized process conditions (temperature, 60 °C; reaction period, 60–90 min; and KOH concentration of 1.5% (w/w) of oil). A rapid increase in the yield took place within 60 min. The results indicated that the rate of reaction was high in the early stages of reaction. Few results were attributed to the high concentration of methyl ester in the reaction vessel to promote the reverse reaction.

Patil et al. (2019) examined the synthesis of TiO_2-Cu_2O nanocomposites and their use in the preparation of alkyl ester from thumba seed oil. Biodiesel was prepared under hydrodynamic cavitation. Bargole et al. (2019) examined process intensification of alkyl ester synthesis from waste oil in HC, where he achieved the intensification approach by increasing the ratio of throat diameter to the cross-sectional area under optimized conditions (temperature, 65 °C; reaction period, 20 min; and NaOH concentration of 1.% (w/w) of oil). The maximum 99% yield of alkyl ester was obtained in 5 min in HC. Kolhe et al. (2017) described the alcoholysis of frying oil using an HC reactor with purification technology. The alcoholysis was reported at optimized process conditions (temperature, 60 °C; reaction period, 1 h; and KOH

Table 11.2 Orifice plate and venturi structures

Orifice plate		Venturi tube	
Parameter	Dimensions	Parameter	Dimensions
Hole diameter (mm)	3	Throat diameter (mm)	3
No. of holes	1	Diverging angle	6.4°
Total flow area (m^2)	7.07×10^{-6}	Converging angle	22.6°
Total perimeter (m)	9.42×10^{-3}	Total flow area (m^2)	7.07×10^{-6}
Pipe diameter (mm)	19	Pipe diameter (mm)	19
Pipe perimeter (m)	59.7×10^{-3}	Breadth of diverging section (mm)	25.5
Cross-section area (m^2)	283.85×10^{-6}	Total length from converging to end of the diverging section (m)	0.133
β	0.158	β	0.08

concentration of 0.55% (w/w) of oil) and the molar ratio of 1:4.5. Almost 93.5% conversion was observed by Kolhe et al. (2017) in 1 h.

Here, we consider thumba methyl ester synthesis from thumba oil in HC. Ghayal et al. (2013) reported geometry of HC plays a significant role in its process intensification. Patil et al. (2011a, b) examined the alcoholysis of thumba oil with CH_3OH using NaOH as catalyst (1% by weight of oil) through hydrodynamic cavitation. In his experimentation, the mixture of CH_3OH and NaOH was mixed with this oil, and finally, the entire mixture was charged to feed the tank. He reported a yield of up to 80% within the first 30 min. The yield was increased with orifice holes, and afterward, it was nearly constant for all other types of orifice plates employed in the experimentation. Patil et al. (2019) examined the synthesis of TiO_2-Cu_2O nanocomposites and its use in the preparation of alkyl ester from thumba seed oil. Biodiesel was prepared under hydrodynamic cavitation. He reported that few results indicated the high concentration of the biodiesel in the reactor and favored reverse reaction.

Thumba oil was transesterified with methanol in the HC reactor using TiO_2 as a heterogeneous catalyst. The cavitation of different intensities was generated by incorporating the orifice plate and venturi tubes. The details of the geometry of the venturi tube and orifice plates are mentioned in Table 11.2. The upstream pressure was varied from 3 bar to 5 bar with an orifice plate and venturi tube geometry. Biodiesel was synthesized as per the general process shown in the block diagram, viz., Fig. 11.3. The performance of the hydrodynamic cavitation method in biodiesel synthesis was evaluated in terms of the alcohol-to-oil ratio, catalyst concentration, and inlet pressure and cavitation number. All these parameters strongly affect the conversion of thumba oil to biodiesel.

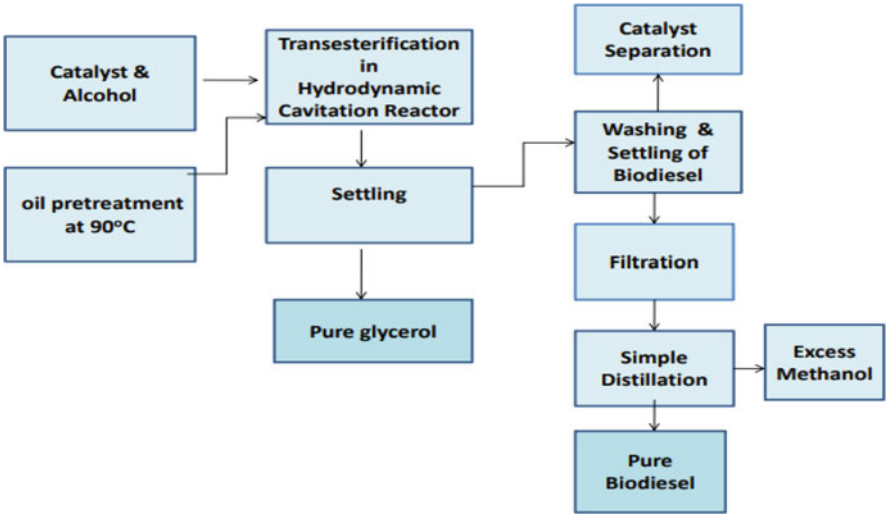

Fig. 11.3 Generalized process of biodiesel production

11.5.1 Variation in Oil-to-Alcohol Ratio

Conversion of triglyceride was increased with the change in molar ratio up to a certain value, but beyond this value, the conversion was decreased. The conversion was reduced due to inadequate separation from glycerol (Patil et al. 2019; Dhanke et al. 2018a, b, c, d; Ghayal et al. 2013). Methanol was considered as an excess reactant to achieve the equilibrium within a short reaction period. Demirbas (2009) reported 1:6 as an optimum molar ratio for transesterification using acid catalysts and 1:9 using alkaline catalysts. Meher et al. (2006) has reported 1:6 as suitable oil-to-alcohol molar ratio for the transesterification using an acid catalyst. Ghayal et al. (2013) has reported six as suitable alcohol-to-oil ratio for biodiesel production of used frying oil.

Patil et al. (2011a, b) have examined the alcoholysis of thumba oil with CH_3OH in an optimum molar ratio of 1:4.5 using NaOH as a catalyst. Patil et al. (2018) have examined the alcoholysis of thumba oil with CH_3OH in the ultrasonic cavitation reactor. He reported six as a suitable molar feed ratio for biofuel synthesis from thumba oil using Amberlyst-15. The vegetable oils are transesterified in the range of oil-alcohol molar ratios 1:6–1:40 in the presence of catalysts. Figure 11.4 depicts the variation in molar ratio and its effect on the conversion of triglycerides in HC using TiO_2 as a catalyst. The highest conversion, up to 75%, was achieved with an orifice plate at a molar ratio of 1:6. Lower molar ratios have not shown good results for both these geometries because of the dominancy of esterification reaction in earlier stages of reaction. Maddikeri et al. (2014) observed similar effects and reported an optimum value of 1:12 with a yield of 89% in the HC reactor. Gole et al. (2013) studied

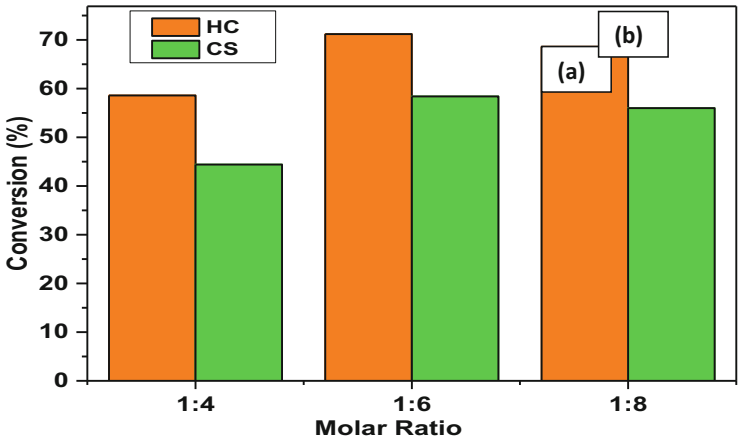

Fig. 11.4 (**a**) Effect of molar ratio on conversion of triglyceride in HC (temperature, 65 °C; inlet pressure, 2 bar; TiO_2 concentration, 1.2% of oil; time, 1 h); (**b**) comparison of performance of HC with conventional method

similar results in the HC reactor based on the constriction through orifice or venturi tubes

11.5.2 Variation in TiO_2 Catalyst Concentration

The main application of TiO_2 is to provide initial active ions to enhance the rate of alcohol reaction and achieve complete conversion of feedstock oil into methyl ester. This mechanism depends upon the concentration of catalyst. To study the effects of variation in TiO_2 catalyst concentration on the conversion of TG, experiments were performed in the range of 1–1.4 (weight % of oil), and results are depicted in Fig. 11.5. The effect of TiO_2 concentration was observed with a molar ratio of 1:6 in 1 h under HC. As TiO_2 concentration increased, the conversion of triglycerides was also increased up to optimum value. Percent conversion increased from 55.8% to 71%, with an increase in TiO_2 concentration from 1 and 1.2 wt. % of oil in 1 h. The lower TiO_2 concentration of 1.0 wt. % resulted in the lower conversion of TG due to a lack of availability of active sites at the initial stages, which were necessary for the reaction's progress (Wagh et al. 2017).

It was observed that the increase in TiO_2 catalyst concentration provided more active ions for alcoholysis reaction turning into the higher conversion of TG to TME. A further rise in TiO_2 concentration from 1.2 to 1.4 wt.% of oil slightly decreased the conversion of TG into biodiesel. It was found that 1.2% TiO_2 concentration was the

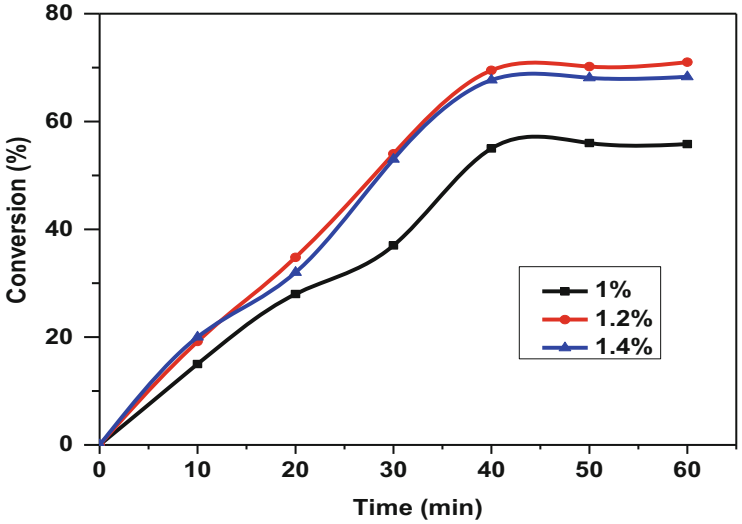

Fig. 11.5 (**a**) Effect of TiO_2 concentration on conversion of triglyceride in HC (temperature, 65 °C; inlet pressure, 5 bar; TiO_2 time, 1 h)

optimum one. Beyond the TiO_2 concentration of 1.2 weight % of oil, some emulsified products were formed.

11.5.3 Variation in Inlet Pressure of Cavitation

An increase in cavitation pressure is a favorable condition to achieve a higher conversion of triglycerides to biodiesel. Ghayal et al. (2013) observed that an increase in pressure at the inlet (0.5–3 bar) improved the liquid's velocity to pass through the constriction, which in turn reduced the reaction period. Many researchers have reported that the increase in inlet pressure beyond 5 bar results in choked cavitation, which slightly decreases the conversion (Patil et al. 2019; Dhanke et al. 2018a; Ghayal et al. 2013). Figure 11.6 depicts the variation in TG conversion with inlet pressure for both the geometries. The conversion increased up to 5-bar pressure for all the geometries of hydrodynamic cavitation. The highest conversion, up to 73%, was achieved with a venturi tube at an inlet pressure of 5 bar. Here also, smaller diameter venturi tubes have shown promising results as compared to orifice plate for all pressure.

The rate of this reaction was increased from 3 to 5 bar with orifice plate B. No further change was observed at a pressure of more than 5 bar. This may be due to the presence of chocked cavitation and the complete absence of mass transfer resistance at a pressure greater than 5 bar.

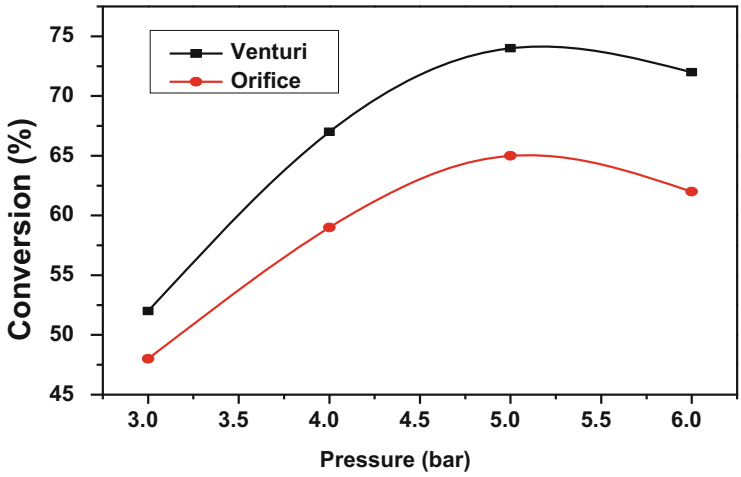

Fig. 11.6 Effect of inlet pressure on conversion of triglyceride (molar ratio, 1:6; temperature, 65 °C; TiO_2 concentration, 1.2% of oil; time, 1 h)

It was also observed that a few numbers of active sites were available to enhance the selectivity toward TME products (Patil et al. 2019; Dhanke et al. 2018a; Ghayal et al. 2013). Any enhancement in triglycerides' conversion using TiO_2 nanoparticles can be achieved by a proper synergy of reaction time, molar ratio of thumba oil to CH_3OH, and TiO_2 loading (Banerjee and Sahani et al. 2019).

In case of the venturi tube, a maximum of 57.8% conversion was achieved at 5 bar within 1 h, whereas 72% conversion was obtained at 5 bar using orifice plate in 1 h. The smooth convergent angle of venturi resulted in the reaction mixture's laminar flow across the throat and reduced the intensity of turbulence. But the reverse mechanism was observed with all the orifice plates. The sudden decrease in a cross-sectional area across orifice holes led to the turbulent flow and high shear and reduced the mass transfer resistance between thumba oil and alcohol. Mukherjee et al. (2020) also reported that significant numbers of cavities are formed at low Cv values at varying pressures. These cavities are carried away with the reaction mixture. Similar observations were also reported by many researchers (Dhanke et al. 2020).

11.5.4 Variation in Cavitation Number (Cv)

The hydraulic characteristics of the hydrodynamic cavitation reactor were determined based on the cavitation number (Cv). The change in the Cv was observed by

varying inlet pressure/operating pressure (2 bar to 6 bar) for both devices. The generation of cavities will be more when the Cv is less than one. The flow rate and velocity of the reaction mixture increased with inlet pressure and reduced the value of Cv. Hence, the flow rate of the reaction mixture and inlet pressure changed the cavitation number (C_V). In the study of hydraulic characteristics of the cavitation system, the flow rate was measured at the different inlet pressure (2 bar to 6 bar) to determine the velocity of the reaction mixture, and Cv was calculated as per the following equation:

1.2 % of oil, time = 1 h

$$Cv = \frac{P_2 - P_V}{0.5\left(\rho \times V_o^2\right)} \tag{11.1}$$

where P_2 is pressure across the downstream side, P_V is the vapor pressure of the reaction mixture depending upon the individual moles of alcohol and oil, V_0 is the velocity of the reaction mixture, and ρ is the liquid density of the reaction mixture depending upon the individual moles of alcohol and oil.

Decreasing Cv produces more cavities and simultaneously collapses, which results in more cavitation effects with the high shear area and more turbulence for the treatment (Patil et al. 2020). Cavitation is favorable when $Cv < 1$ because its intensity increases as Cv decreases (Bargole et al. 2019). Reaction mixture velocity increased with the inlet pressure, and it reduced the Cv value in the HC reactor. Table 11.3 summarizes flow rate, the velocity of the liquid, and cavitation number for selected geometries at different pressure obtained in our study.

Table 11.3 Calculation of flow rate and velocity of liquid for different plates at different pressure

	Orifice			Venturi		
Inlet pressure (bar)	Q (l/m)	V (m/s)	C_v	Q (l/m)	V (m/s)	C_v
2	4.2	9.9	1.96	5.1	12.2	1.3
3	5.25	11.3	1.49	6.4	15.2	0.83
4	6.2	14.6	0.9	7.6	18	0.59
5	7.4	17.4	0.63	9.6	22.6	0.37
6	10.5	24.8	0.31	10.7	25.2	0.30

11.6 Conclusions

The concept of using alternative renewable fuels in the future is gaining significant importance due to the depletion of available fuel sources and global warming. Biodiesel can be synthesized from a wide range of available nonedible oil, animal fats, and edible oils. At present, biofuel is mostly synthesized from various edible oils such as palm, sunflower, soybean, and rapeseed in European and Asian countries. The nonedible oils that are currently utilized are Jatropha, neem, P. pinnata, rubber seed, mahua, etc. Alcoholysis is a widely used process for the synthesis of alkyl ester, which is a chemical reaction carried with or without a catalyst. Nanocatalyst has its own merit as well as a demerit. HC is the most successful and promising technique for obtaining higher yields of biodiesel and reductions in Sox and NOx emissions. Inlet pressure of 5 bar is optimal to achieve maximum conversion of triglycerides. HC can be efficiently used in the synthesis of alkyl esters (biofuels) from thumba oil by optimizing inlet pressure and oil-to-alcohol ratio. The kinetics of the alcoholysis reaction depends upon concentrations of all reactants as well as the catalyst.

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Chapter 12
Application of Nanotechnology in Production of Biofuel

Ritika Luthra, Shreeja Datta, Arpita Roy, Suresh Ghotekar, and Muhammad Bilal

Abstract With a continuous increase in demand for energy, there is a considerable rise in the utilization of fossil fuels. However, these fossil fuels undoubtedly are the primary source of environmental pollution. This highlights the need for alternative sources of energy possessing enormous potential. Biofuels are regarded as one of the most promising substitutes due to numerous benefits exhibited by them. The commercially produced biofuels suffer from higher costing and technical obstructions. A novel and emerging field of nanotechnology in the production and process stages can assist in improving and overcoming the economical, technical, as well as environmental problems. It provides engrossing approaches like using nanoparticles which can act as nanocatalysts for immobilizing enzymes in biogas, bioethanol, and biodiesel production. It is beneficial for recovering and reusing the catalysts. Furthermore, nanomaterials turn out to be advantageous in gasification reactions for biofuel production from biomass as well as enhance the efficiency of lipid extraction. Therefore, this chapter aims to focus on the significant applications of nanotechnological methods for biofuel production which show promising prospects. The concerns regarding the use of this technology along with future prospects will also be discussed.

Keywords Biofuels · Bioenergy · Nanotechnology · Nanomaterials

R. Luthra · S. Datta
Delhi Technological University, Delhi, India

A. Roy (✉)
Department of Biotechnology, School of Engineering & Technology, Sharda University, Greater Noida, India

S. Ghotekar
Department of Chemistry, Smt. Devkiba Mohansinhji Chauhan College of Commerce and Science, University of Mumbai, Dadra and Nagar Haveli, India

M. Bilal
School of Life Science and Food Engineering, Huaiyin Institute of Technology, Huai'an, China

P. Chowdhary et al. (eds.), *Bio-Clean Energy Technologies: Volume 1*, Clean Energy Production Technologies, https://doi.org/10.1007/978-981-16-8090-8_12

12.1 Introduction

The energy demand is growing with a rise in the populace globally. The use of fossil fuels has risen all over the world in a manner that the utilization per capita is 105 times higher in comparison to its production through nature (Satyanarayana et al. 2011). It has been projected that almost all existing reserves for fossil fuels would be fully depleted by 2050 (Demirbas 2009). It is well acknowledged that the shortage of fossil energy is indeed a global concern. Besides that, climate changes attributed rise in greenhouse gas emissions and economic problems which are some other serious challenges related to usage of fossil fuels (Zhang et al. 2010).

Issues about the economy, energy, and environmental safety arising from undue reliance on petroleum and its low supply drive nations around the globe to seek alternatives such as biofuels, in particular bioethanol and biodiesel (Cherubini 2010; Silva and Chandel 2014; Lee et al. 2014). They have received significant prominence in current times because of their low greenhouse gas emissions and minimized carbon footprints, most significantly owing to their self-sustaining characteristics (Hussein 2015). In order to overcome these problems, alternative fuels are required to fulfill the current demands.

Biodiesel, which is long-chain fatty acid esters, is yet another essential renewable source of energy being utilized in diesel engines driven by technical, ecological, and operational benefits (Enweremadu et al. 2011). Furthermore, biodiesel has become a highly sophisticated diesel fuel procured from petroleum which needs practically no improvement within the existing fuel distribution network. Other benefits of biodiesel in comparison with petroleum diesel incorporate the lessening of many other vehicle emissions, good biocompatibility, greater viscosity, and intrinsic lubricity which enhances the mechanical system of fuels and increases the viability of engine components as well as possibility of them possessing a domestic nature (Antunes et al. 2014). Biodiesel as well as bioethanol generation using raw materials is presently in operation which is termed as first-generation biofuels (Zabed et al. 2014). These approaches require huge quantities of feed materials, primarily animal fats and vegetable oils for producing biodiesel and sugarcane sucrose or cornstarch for producing bioethanol (Silva and Chandel 2014). However, there are some drawbacks in the processing of these fuels due to the numerous applications of these essential feed materials like corn, vegetable oils, and animal fats used for biofuel production. Technological advances for the generation of biofuels employing nonedible feed materials, such as wood wastes as well as crop residue, are thus being used (Eggert and Greaker 2014). Biofuels generated using these biomass resources are referred to as second-generation biofuels (Bradley et al. 2009). Further prospect for producing these fuels by utilization of microbial populations as primary matter is termed as third-generation of biofuels (Ho et al. 2014; Milledge et al. 2014). Microbial algae recently appeared as a good candidate for biofuel feed material owing to its rapid growth as well as lipid productivity. In contrast to various other crops for oil production, algae possess the capacity to thrive in salt and wastewater and therefore don't contest with other agricultural foods for

farmland. In addition, certain control parameters for algae could be modified due to increased biomass and lipid composition, which otherwise is not seen in other oilseed crops (Mandotra et al. 2016; Upadhyay et al. 2016). While these current groups of biofuels have several benefits, manufacturing processes still raise several drawbacks, including production costs which remain high in comparison with the first generation of biofuels. Further, the current network does not seem to be adequate for the development process. There are many technical challenges in the manufacturing process, and thus further work is required to improve approaches pertaining to enzymes, pretreatment, as well as fermentation processes for implementing them further in an economical and productive manner (Patumsawad 2011).

In view of the requirement for scientific studies to test newer technologies, nanotechnology can provide practical solutions by modifying the attributes of feedstock and biocatalysts utilized during biofuel generation. It is a promising field which has received notable recognition recently. A possible benefit of nano-biotechnology for renewable biofuel generation as well as biosensors has motivated many researchers in recent times to examine new nano-scaffolds for constructing functional nano-biocatalytic technological tools (Verma et al. 2013a, b, c). Nanoparticles possess several special properties in comparison with conventional substances as they are tiny enough to accommodate their atoms and create quantum mechanics. Studies on the utilization of nanomaterials for production of bioenergy and biofuels are currently experiencing rigorous research. Hence, this chapter aims to provide a comprehensive review on the role of nanotechnology in the production of biofuels. The use of nanomaterials and nanocatalysts during the process and the concerns anticipated regarding the use of nanotechnological methods and its future scope have also been addressed.

12.2 Conventional Methods for Biofuel Production

Thermochemical and biochemical methods are commonly utilized for converting different feedstocks into biofuels. Thermochemical processing is perceived to be a viable approach for producing biodiesel, bioethanol, pyrolysis oil, biosynthetic gas, as well as biohydrogen. Nevertheless, biochemical or microbial transformation is primarily utilized in the manufacturing of fuels in liquid or gas forms employing diverse biological agents by fermentation and anaerobic respiration, among others (Mitrovi et al. 2012). Biofuel generation using lignocellulosic material can be classified into two phases. During the first phase, the entire feedstock is broken down to provide expandable liquid or gaseous frameworks. This stage is generally carried out by thermochemical decomposition for producing syngas via gasification or bio-oils via pyrolysis or liquefaction process. Hydrolysis of the lignocellulose material is also performed for producing sugar monomers, which are then processed to form biofuels such as bioethanol via microbial transformation (Akia et al. 2014).

As previously discussed, thermochemical transition techniques are typically accomplished via gasification or by direct liquefaction. The gasification of biomass has the ability to transform numerous widely dispersed and lower-value lignocellulose to syngas that can be subsequently utilized for producing energy, liquid fuels, heat, chemical compounds, as well as hydrogen (H_2) (Ozaki et al. 2012). The gasification method appears to be of great significance as a wide range of lignocelluloses may be deemed suitable for the method (Luo and Zhou 2012). Gasification process can be performed into two specific mechanisms, namely, low-temperature gasification and high-temperature gasification. Nevertheless, application of particular gasification methods varies depending on the formation of a kind of biofuel (Ozaki et al. 2012).

Direct liquefaction along with variations in several physical and chemical processes can lead to conversion of biomass into liquidized substances. This method uses various biomass molecules which are disintegrated to form smaller molecules through heat treatment often aided by a catalyst. In addition, direct liquefaction is typically accomplished by liquefaction as well as pyrolysis techniques. The operating temperature for liquefaction and pyrolysis approaches usually ranges between 250–325 °C and 377–527 °C, respectively (Akia et al. 2014). Additionally, numerous biological and biochemical techniques for biofuel generation have been established. Diverse bioagents, including plants and microbes, have been widely utilized explicitly or implicitly for their effective manufacturing. In microbes, photoautotrophs, like cyanobacteria and algae, have also drawn immense focus for the development of third-generation biofuels. However, using photoautotrophs has some drawbacks, including reduced cell rate of growth, and therefore there is lower yield of metabolites (Sheehan 2009; Sarkar and Shimizu 2015).

Accordingly, certain microbes such as *Zymomonas mobilis* (Galbe and Zacchi 2007), *Saccharomyces cerevisiae*, *Hanseniaspora uvarum*, *Starmerella bacillaris* (Wang et al. 2014; Ingle et al. 2017), *Pichia stipitis*, *Kluyveromyces marxianus* (Sheikh et al. 2016), *Aspergillus niger*, as well as *Mucor mucedo* (Oyeleke and Okansanmi 2008; Li et al. 2009) are most widely employed for fermentation process of ethanol using different plant-based raw resources in industrial applications. Amidst plants, edible and nonedible oilseed foods such as soybean, *Camelina*, rapeseeds, mustard, canola, safflower, sesame, olives, castor bean, sunflower, and many more (Ahmad et al. 2011; Ardebili et al. 2011; Chang et al. 2017) are widely employed for producing biofuels. Nevertheless, these edible and nonedible fuels are not commercially feasible, and productivity is also a key problem. It is therefore important to establish commercially feasible, secure, and more efficacious strategies. In the light of novel nanotechnology advances in the area of biofuels and bioenergy processing, research is being carried out globally on using different nanoparticles for quick and accurate biofuel production.

12.2.1 Magnetic Nanoparticles

Magnetic nanoparticles possess numerous applications in the fields of biomedical, environment and material science, and biotechnology, among others. There are multiple kinds of magnetic nanostructured materials that have been produced which are used as a catalyst. Numerous substances, including alloy of nickel, platinum, cobalt, or iron, as well as transition metals, could be made in use for building molecules as catalysts (Laurent et al. 2008). The key objective of the analysis is the development of various forms of magnetic nanoparticles (Abraham et al. 2014). The attributes of these particles incorporate their greater ratios of surface/volume, quantum phenomena, as well as their capability of holding other substances, like medicines, as a result of their smaller size. Another benefit of magnetic nanoparticles is their ability to be used as extremely effective catalysts, rendering immobile materials removed effortlessly by applying sufficient magnetic fields in the absence of toxic effects (Baig and Varma 2013; Nicolas et al. 2014). They have a promising implementation throughout the sectors of biofuels as well as bioenergy. For instance, they assist mostly in the development of sugar and bioethanol using lignocellulose by immobilizing enzymes such as cellulase as well as hemicellulase. Such immobilized enzymes can be retrieved by magnetic means and then further recycled for reused (Alftren 2013). The magnetic nanoparticles are distributed in a similar way like various nanomaterials when there is no magnetic field. Apart from being useful in assisting the immobilization of enzymes, they could also be covered or utilized for attaching other catalytic nanoparticles, thus allowing them to be suitable nanocatalysts across a variety of purposes. Such nanocatalysts appear to be of great potential and can be employed for processes like photooxidation, hydrogenation, as well as induction heating, among others, via applying large-frequency magnetic fields (Lu et al. 2007; Govan and Gunko 2014). These nanoparticles are also being regarded as carriers of enzymes that would be easy to manage under magnetic fields. Furthermore, studies conducted by several research groups such as Cherian et al. (2015) showed that immobilized enzymes possess greater thermal stability in comparison with the mobilized enzyme and were observed to be constant at a temperature of 70 °C. Raitaa et al. (2015) utilized iron oxide magnetic nanoparticles for immobilizing lipases along with their application in generation of biodiesel using palm oils. This same biocatalyst demonstrated greater durability as well as catalytic performance because of immobilization. In addition, immobilized enzymes can be isolated while introducing magnetic flux for utilization in five consecutive groups having greater than 80% output.

12.2.2 Carbon Nanotubes

Carbon nanotubes are manufactured using a range of approaches, including laser ablation, chemical vapor deposition, as well as arc discharge (Feng and Ji 2011).

Such particles incorporate sheets of graphite folded in a cylindrical form having nanoscopic diameter which is highly biocompatible, thus allowing them to be popular to immobilize enzyme (Feng and Ji 2011). Carbon nanotubes are essential for catalysis due to various deployments in electrochemical cells and several more equipment employing catalysis of electrochemical reactions. Conversely, among several nanoparticles, these nanotubes exhibit distinctive structural, thermal, and mechanical qualities as well as good biocompatibility. A great deal of interest is observed among scholars because of the possible applications in biotechnology, especially in the formation of biosensors and biofuel generation using enzyme-conjugated carbon nanotubes (Shi et al. 2007). In addition, they have a wide surface area, which allows greater loading ability along with lesser tolerance to diffuse. Latest researches have shown the improved reactivity when enzymes are combined with these nanotubes (Lee et al. 2009). The performance of these nanoparticles could be increased by modifying the surfaces (Johnson et al. 2011). The surface modification of carbon nanotubes greatly affects catalytic performance of an immobilized enzyme, for instance, immobilizing lipases on the biocompatible multiwalled nanotube support systems with the help of cross-linking agent gluteral. The lipases, which are available and bound by multiwalled nanotubes, have been assessed biochemically for hydrolysing esters in aqueous solutions (Pavlidis et al. 2010; Verma et al. 2013a, b, c) and contrasted to certain components such as polymeric materials that influence reactivity by cognitive decline or modifications in their attributes with time, eventually resulting in enhanced levels of aldehydes, carboxyl groups, and peroxides (Goh et al. 2012). They also possess capability of attaching at the enzymes' embedded binding sites for transferring electrons directly. The application of carbon nanotubes in generation of biofuels has also encouraged researchers. This is due to their 3D electroactive regions that raise the abundance of enzymes as well as different redox substances on the substrate. In addition, certain unique characteristics of these nanotubes, including permeability and conductivity, have rendered them as highly valuable substances for immobilizing biomolecules for biofuel and biosensor purposes (Holzinger et al. 2012).

12.2.3 Other Nanoparticles for Heterogeneous Catalysis

Heterogeneous catalysis is utilized for processing of biofuels. This method possesses several benefits like simpler isolation along with no contamination of the products procured with the catalytic particles that are usually nontoxic, eco-friendly, and exhibiting good specificity and longer life span. Certain researches show employment of heterogeneous catalysts, which possess promising outcomes in converting lignocellulosic materials for generating biodiesel as well as bioethanol. Among them, few scientists identified metallic nanoparticles into permeable substances or nanostructured catalysts which are basic in nature for deconstructing cellulose or for producing methyl esters of fatty acids (Feyzi et al. 2013; Kuo et al. 2013). The features of metallic nanomaterials rendered for heterogeneous catalysis are important

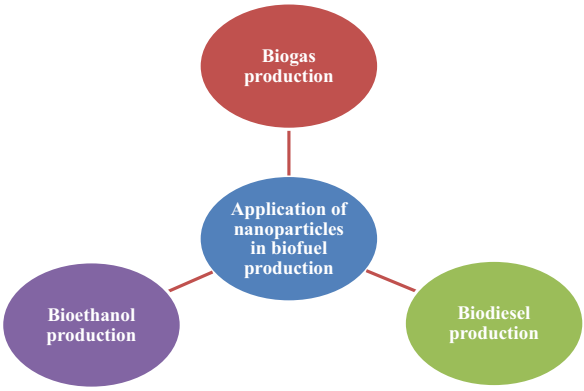

Fig. 12.1 Nanotechnology in biofuel production

for them to be used, including surface area, pore size, acid concentration, as well as strength (Kondo et al. 2005). In such scenarios, development of nanoporous nanomaterials has been performed using a number of methods allowing a proper movement of ultimate structure, homogeneity, size, and dispersion of particle, culminating in the refinement of particle characteristics as required (Wu et al. 2013). A study explained several other possible outcomes including oxide of transition metals, H-forms of zeolites, and cation-exchange polymers and endorsed solid-state acid, complex substances, and carbon-based solid acid (Guo et al. 2012). In other research, integrated utilization of nanomaterials using ionic liquid in solid-state heterogeneous catalyst method was done. Ionic liquids were employed for preparing as well as stabilizing nanomaterials that are necessary given the widely found thermodynamically unstable metallic nanomaterials, which need to be balanced in a solution to prevent accumulation (Julis et al. 2010) (Fig. 12.1).

12.3 Nanotechnology in Biogas Production

Biogas is derived by anaerobically digesting biodegradable wastes, including plants, animals, as well as human refuse, along with agricultural wastes. These waste materials are an abundant source of carbon and nitrogen, and the carbon/nitrogen content mainly determines the amount of energy released via anaerobic digestion (Feng et al. 2014). It has been found that adding some specific metal ions in less quantities improves functioning of methanogens. Therefore, they can serve as catalysts for increasing energy generation. Studies show that nanoparticles are advantageous over other atomic or dense components as methanogens need limited quantities of nickel, iron, or cobalt for biogas production (Feng et al. 2010). In the biomethanation phase, magnetic beads may be utilized as they possess good paramagnetic properties as well as better coercive nature (Yang et al. 2015). Research demonstrated the use of nickel or cobalt nanomaterials, which improved generation

of methane gas. Further, on comparing the performance of iron and iron oxide nanomaterials, it was found that iron oxide nanomaterials showed better performance (Abdelsalam et al. 2015). Recently, another research indicated the impact of several nanomaterials like nickel, iron, cobalt, as well as magnetic iron oxide nanoparticles, which produced maximum biogas along with generation of methane gas by anaerobically processing the cow manure (Abdelsalam et al. 2016). Similarly, another study demonstrated that by applying magnetic iron oxide nanoparticles on biodegradable wastes while anaerobically digesting them, there was an improvement in the action of breakdown along with more generation of biogas and methane (Casals et al. 2014).

12.4 Nanotechnology in Biodiesel Production

Biodiesel constitutes long-chain esters of fatty acids that are predominantly generated from plants and animals via transesterification process. Plant oil or animal fats having short-chain alcohols like ethanol, which fulfill particular requirements for being utilized as transportation fuels, are mainly incorporated in biodiesels. In contrast with fossil fuels, biodiesel offers numerous benefits, which include their environment-friendly nature and preferable lubricating characteristics along with their possible generation from renewable sources without production of any particulate pollution (Feyzi and Norouzi 2016). Nanocatalysts offer numerous possible applications in this field. Various functional nanoparticles have shown successful implementation in biodiesel generation. Wang and his colleagues (Wang et al. 2015) produced magnetic nanomaterials functionalized by acids and subsequently illustrated their utilization as heterogeneous catalysts in producing biodiesel. Here, crystal-type iron/iron oxide base or shells coated with silica sulfamic and sulfonic acid magnetic nanomaterials were incorporated for producing biodiesel by transesterifying glyceryl trioleate. It was found that both of these heterogeneous nanocatalysts exhibited notable catalytic functions. Still, nanocatalyst synthesis from sulfamic acid was better in terms of functioning in comparison to the nanocatalyst synthesis from sulfonic acid. Some researchers also studied the production of biodiesel using cooking oil. Here, they utilized calcium oxide and magnesium oxide nanomaterials which were manufactured using sol-gel and sol-gel autoignition processes, respectively. It was observed that calcium oxide nanomaterials increased the production of biodiesel significantly in comparison with magnesium oxide nanomaterials (Tahvildari et al. 2015). Another engrossing technique involves magnetic nanocatalyst which can be effortlessly retrieved and recycled, thus making the method economical. Based on this, a study suggested an unchallenging and quick method based on nanotechnology for biodiesel generation using soybean oil. Here, researchers employed a combination of iron oxide and cadmium or tin oxide nanoparticles possessing magnetic properties, which was constructed by coprecipitating as nanocatalyst for producing biodiesel. Among these nanocatalysts, iron and tin oxide nanoparticles were most efficient and generated a yield of 84%.

Noteworthy prospects toward processes like hydrolysis, transesterification, as well as esterification of soybean oil and fatty acids obtained from them have been bestowed by these nanocatalysts (Alves et al. 2014). Comparably, another research illustrated zirconium dioxide nanocatalysts which are loaded with potassium bitartrate with dimensions ranging from 10 nm to 40 nm. Furthermore, they examined biodiesel generation aided by nanocatalysts that were synthesized for transesterifying soybean oil as well as methanol in a distinct molar ratio along with more variables such as amount of nanocatalyst used, temperature, and time interval. It was observed in the reacting mixture with a molar ratio of 16:1 of methanol/oil incorporating 6% of nanocatalysts at 60 °C for a period of 2 h led to highest biodiesel production of around 98.03% (Qiu et al. 2011). Many more researches have also been outlined that utilized nanocatalysts for generation of biofuels. For instance, a study used KF/CaO nanocatalysts for producing biodiesel using Chinese tallow seed oils. These nanocatalysts were formed via facile impregnation technique and had size ranging from 30 nm to 100 nm. Around 96.8% of biodiesel was produced, which exhibited the prospects of these kinds of nanocatalysts in this field (Wen et al. 2010). Contrastingly, a group of researchers used particles acquired from hydrotalcite along with magnesium or aluminum oxides as nanocatalyst. These were formed via coprecipitation technique utilizing precipitating factor as urea. They procured 95.2% of biodiesel (Deng et al. 2011). Another study revealed a novel nanocatalyst named Cs/Al/Fe_3O_4, which was assessed with respect to the catalytic efficiency it had for generation of biodiesel. Consequences of various molar ratios of Cs/Al and Cs/Fe along with their calcinating setup on the catalytic activity were taken into account. It was observed that the nanocatalysts having Cs/Al and Cs/Fe in 2.5:1 and 4:1 molar ratio manifested great prospects for generating biodiesel. It resulted in a yield of 94.8% when kept at 58 °C and stirred constantly for 2 h (Feyzi et al. 2013). Magnesium oxide nanocatalysts have also been synthesized in the form of nanosheets using aerogel technique. These have been suggested to be employed for producing biodiesel from sunflower oil as well as rapeseeds and confirming up to 98% of product (Verziu et al. 2008). In recent times, research made use of Ca/Fe3O4@SiO2 nanocatalyst for biodiesel generation. This nanocatalyst exhibited magnetic effects and procured by combining two separate methods, namely, sol-gel and dry impregnation technique. These nanocatalysts appeared quite efficient at ideal conditions and showed the highest yields of around 97%. Their magnetic characteristic assisted them to be reused numerous times without any deprivation in the catalytic performance (Feyzi and Norouzi 2016). Another study synthesized new calcium oxide nanocatalyst from seashells, namely, *Polymesoda erosa*, via several stages including calcination, followed by hydrating and dehydrating processes. The efficiency of this catalyst for producing biodiesel using inedible crude oil such as *Jatropha* was also observed. It was revealed that the highest of 98.54% yield was found when the molar ratio of methanol/oil was 5.15:1 for a time span of 133.1 minutes at 0.02:1 (w/w) ratio of nanocatalyst (Reddy et al. 2016) (Table 12.1).

Table 12.1 Nanocatalysts and feed materials for producing biodiesel generation

Feed material	Nanocatalyst used	Biodiesel yield	References
Jatropha oil	Calcium oxide	98.54%	Reddy et al. (2016)
Sunflower oil	$Ca/Fe_3O_4@SiO_2$	97%	Feyzi et al. (2013)
Glyceryl trioleate	Crystal-type iron/iron oxide base or shells coated with silica sulfamic and sulfonic acid magnetic nanomaterial	95%	Wang et al. (2015)
Soybean oil	Iron/cadmium and iron/tin oxide nanoparticle	84%	Alves et al. (2014)
Sunflower oil	Cs/Al/Fe3O4	94.8%	Feyzi et al. (2013)
Jatropha oils	Particles procured from hydrotalcite with magnesium or aluminum oxide	95.2%	Deng et al. (2011)
Soybean oil	ZrO_2 loaded with $C_4H_4O_6HK$	98.03%	Qiu et al. (2011)
Chinese tallow seed oil	KF/CaO	96.8%	Wen et al. (2010)
Sunflower as well as rapeseed oil	Magnesium oxide	98%	Verziu et al. (2008)

12.5 Nanotechnology in Bioethanol Production

Bioethanol, also called ethyl alcohols, are commonly synthesized from carbon sources of grains, sugarcane juices, etc. It can also be produced by making use of fermentable sugars that are liberated from vegetable biomass like lignocellulosic materials. Such materials mainly consist of cellulosic as well as hemicellulosic components that are carbohydrate polymeric structures and lignin which is a polymer-like molecule consisting of phenolics (Antunes et al. 2014). Some preliminary treatment is required for using sugars present in cellulose and hemicellulose in order to disintegrate recalcitrance of biomass and break down the polymer units into fermentable monomeric compounds. Generally, after the initial preliminary treatment, cellulosic fractions of these materials undergo hydrolysis in the presence of enzymes (Rai et al. 2016). This technique can be used for producing monomers of glucose under moderate reaction conditions such as lesser temperature, with low-pressure demand, in comparison with the chemical methods. It also avoids the formation of unwanted fermentation by inhibiting substances during the process. For instance, application of cellulase enzymes in hydrolysing lignocellulose feedstock accounts for about 18% of the net cost concerned with this technique for producing bioethanol. Thus, newer approaches that can enable the easy recuperation as well as reuse of enzymes can possibly lower the costing. Taking this into consideration,

nanotechnology enables immobilizing a variety of enzymes like cellulase as well as hemicellulase that are employed on various nanoparticles for producing bioethanol. As, for instance, magnetic nanoparticles immobilized by enzymes are a very propitious approach based on the application of magnetic fields that allows restoration of enzymes easily as well as reusing them for multiple turns (Alftren 2013; Rai et al. 2016). Magnetic nanomaterials showed the enzyme immobilizations that are used for producing bioethanol. Usually, enzymes immobilized on nanomaterials are accomplished via covalently binding or physically adsorbing them. Nonetheless, binding covalently is considered more favorable as it decreases desorption of proteins as covalent bonds are formed between nanomaterials and enzymes (Abraham et al. 2014). In order to ensure steady immobilization of enzymes on nanoparticles, such substances must be altered or covered using polymeric compounds which are chemically active and can give functional groups required for linking enzymes. A study showed binding of enzyme β-glucosidase on polymeric magnetic nanofiber by entrapping them for generating cellulosic ethanol. β-Glucosidase is accountable for formation of glucose by transforming cellobiose that can be metabolized using microbes for synthesizing bioethanol. Moreover, entrapping β-glucosidase on magnetic nanofibers offers enzyme stability as well as allows them to be used multiple times by segregating them using magnetic fields (Lee et al. 2010). Likewise, another research assessed immobilization of β-glucosidase, which was isolated from fungus, on magnetic nanomaterials and utilized it as a nano-biocatalyst for producing bioethanol. It also showed 93% binding efficacy of enzymes, exhibiting around 50% of its preliminary performance in the 16th turn (Verma et al. 2013a, b, c). Another group of researchers examined the enzyme recycle while hydrolysing the cellulose microcrystals using carbodiimide as the connecting polymer for immobilization on iron oxide nanomaterials. By the virtue of the magnetic nature possessed by these nanoparticles, the enzyme was recuperated with ease and reused in six cycles (Jordan et al. 2011). Another study verified that the enzymes required for producing bioethanol undergo immobilization in single-wall carbon nanotubes that were previously included by iron oxide nanomaterials for displaying magnetic attributes. Here, modifying the amounts of iron oxide nanomaterials in nanotubes may control the activity of enzymes that had undergone immobilization. Hence, for prolonged storing of immobilized enzymes, they could be stored in an acetate buffer at 4 °C (Goh et al. 2012). Various studies have been conducted for immobilization of enzymes on different nanomaterials. One of them stated the immobilizing lipases on magnetic chitosan microspheres, which are formulated by chemically coprecipitating them. Glutaraldehyde was employed for linking enzymes and magnetic chitosan microspheres via covalently binding them (Xie and Wang 2012). Furthermore, enzyme immobilization on TiO_2 nanomaterial aided by adsorption techniques was also effectively employed for hydrolysing lignocelluloses, thus directing toward bioethanol generation (Ahmad and Sardar 2014). Cherian and his colleagues revealed that cellulase enzymes procured from *Aspergillus fumigatus* were immobilized on nanomaterials of manganese dioxide via covalent linkage. They also stated that enzyme immobilization exhibited more promising results in enhancing the thermostable properties by bestowing stability up to 70 °C in

comparison with free enzymes. Cellulases, which underwent immobilization, moderated the hydrolysis process along with subsequent utilization of synthesized yeast for producing 21.96 g/L bioethanol from agricultural waste. The immobilized enzyme displayed about 60% of its performance after continuous use for around five cycles (Cherian et al. 2015). In addition to magnetic nanomaterials, other particles like silica and titanium oxide, as well as carbonic particles like graphite, fullerene, carbon nanotube, and polymer nanoparticles, may also be utilized in nanotechnological processes. All these substances have been used for immobilizing various enzymes employed during bioethanol generation (Huang et al. 2011; Cho et al. 2012; Pavlidis et al. 2012; Verma et al. 2013a, b, c). A study reported that applying carbon nanotube, which was trapped with Rh components, showed improved catalytic function for ethanol generation. Indeed, free-standing pores present on the carbon nanotube have proved to promote the inclusion of substances of various regards (Pan et al. 2007). Another study investigated cellulase enzyme immobilization on silicon dioxide nanomaterials, indicating the efficiency of immobilized as well as free enzymes while hydrolysing cellulose to form glucose. It has been found that this immobilized cellulase led to higher production of glucose in comparison with the free enzymes, thus indicating that immobilized enzymes may be utilized simultaneously in fermentation as well as saccharification processes (Lupoi and Smith 2011). Cells of different microbes may undergo immobilization on nanomaterials and subsequently utilized for fermentation during ethanol generation. For instance, researchers designed a technique for immobilizing cells of brewer's yeast on magnetic nanomaterials. Moreover, they also showed that fermenting continuously for bioethanol generation, immobilized brewer's yeast cells displayed higher ability of producing ethanol. Thus, investigations carried out on the immobilization of enzymes or the cells of microbes on various nanoparticles have given enough proof that these kinds of techniques would be appropriate for safe and low-cost bioethanol synthesis by utilizing cheap lignocelluloses (Ivanova et al. 2011).

12.6 Future Scope

There is a need for developing an economical method for producing bioenergy for which a technical evaluation needs to be performed along with considering cost for synthesizing nanomaterials that can affect net cost of production. It also puts an emphasis on the formation of nanomaterials which are cost-effective in order to build the entire process achievable to be commercialized. Small-scale preliminary studies are needed for examining the feasibility of application of nanomaterials for producing bioenergy on a larger scale. Moreover, future investigations should not be exclusive only to source and generation of biofuels where techno-economic constraints can be addressed by nanotechnological methods by being utilized for transportation, modifications, efficient generation and storage of fuels, and also utilization of end products (Nizami et al. 2018). Furthermore, till date there is very

little research on the application of nanoparticles as fuel additives. Different techniques for overcoming accumulation of nanoparticles, their erosion, and clearing down are still needed. Along with this, there are not many tangible outcomes as well as comprehension of methods of heat transferring for commercializing the nanoparticles as fuel additives. Additionally, safety evaluations should be performed as nanomaterials exhibit evident effect on exposing to living beings as well as the environment. Currently, toxic nature of these nanoparticles has been carried out via various methods where in vitro studies are mostly performed (Malorni et al. 2017). Yet, in vivo studies also need to be performed with huge focus on nanomaterials specifically employed for producing biofuels (Antunes et al. 2017). This includes the safe and suitable microbes which are used as nanoparticles also need to be generated. Research of nanomaterials need to be studied for identifying the wide spectrum of contents and their effect on performance of microbes and enzymes. Molecular-level studies are required for examining the approach using nanoparticles during the production. Correspondingly, appropriate operation conditions for generation of biofuels can be established.

12.7 Conclusion

The less availability of conventionally used fossil fuels and their effect on the environment along with extensive demand of energy is a global issue. This has motivated researchers to search for newer alternatives like biofuels. Several nanocatalysts like metallic or magnetic nanoparticles, carbon nanotubes, etc. offer an eco-friendly approach for generation of biofuels that allow them to conduct greater catalytic conversions having more selective nature and requiring moderate conditions for operation with longer shelf life. They also overcome the limitations of biofuel production via conventional methods like technical barriers and high production cost. Thus, they are being utilized for producing bioenergy as well as biofuels like biodiesel, bioethanol, and algal biofuels. However, there are certain concerns regarding the use of these nanoparticles for commercialization due to some of their effects on environmental as well as human health. These issues need to be investigated in the future for applying nanotechnology on a commercial scale.

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Chapter 13
Application of Nanotechnology in Biofuel Production

Jahanvee Mitra, G. K. P. Srilekha, Nilesh Wagh, and Jaya Lakkakula

Abstract One of the greatest challenges the world is facing is the indiscriminate consumption of energy resources due to population explosion. Thus, alternative resources to produce renewable and biodegradable biofuel were necessary to meet the crisis of fossil fuels. Nanotechnology has attracted a greater deal of attention from many researchers because of nanomaterials possessing characteristics like small size, large surface area, and the most important excellent catalytic activity that give them great potential in the production of biofuels. Several nanomaterials act as nanocatalysts and play a vital role in catalytic degradation for bioethanol production and catalyze the transesterification and esterification reaction for extraction of biodiesel from edible and nonedible oils. Nanomaterials associated with microbial enzymes and immobilized onto various forms of lipases have been explored for improved biodiesel production. Nanoadditives have been explored to enhance biogas production. Hence, this chapter aims to review the innovative and outstanding applications of nanomaterials in the field of biofuel production.

Keywords Nanotechnology · Biofuel · Nanocomposites · Nanoparticles · Nanocatalyst · Nanotubes

13.1 Introduction

Fossil fuels, like oil, coal, and natural gas, are the primary sources of energy in various industries. Since fossil fuels are widely consumed by many countries around the world, the demand for petroleum and other fossil fuels increased exponentially (Chow et al. 2003). This led to volatility in the prices of petroleum and environmental concerns along with the rapid depletion of nonrenewable resources (Sagar and Kartha 2007). To overcome these issues, significant numbers of studies were conducted to produce biofuels in a sustainable, efficient, and economically viable way. Biofuels, including biodiesel and bioethanol, are produced using organic

J. Mitra · G. K. P. Srilekha · N. Wagh · J. Lakkakula (✉)
Amity Institute of Biotechnology, Amity University, Mumbai, Maharashtra, India

P. Chowdhary et al. (eds.), *Bio-Clean Energy Technologies: Volume 1*, Clean Energy Production Technologies, https://doi.org/10.1007/978-981-16-8090-8_13

matter, also known as biomass, such as soybean, rapeseed, sugarcane, cottonseed oil, etc., algal biomass, and even organisms (Luque et al. 2008; Chowdhary et al. 2020; Khan et al. 2020). Over the past few decades, uses of biofuels, with low carbon footprint, have immensely altered the environmental and financial conditions of various industries such as transport, agriculture, cottage industry, and household. The potential substitution of biofuels with depleting fossil fuels seems to be reliable because of its cost-effective and environmentally safe nature (Gowen 1989).

The conventional downstream production of biofuels from feedstock was popular; however, because of its high-energy input and production costs, it had many drawbacks. To overcome these limitations, a widely emerging technology was introduced, which is known as nanotechnology (Mandotra et al. 2018). Nanotechnology deals with particles at atomic scale which are known as "nanoparticles" that lie between the range of 1 and 100 nm and are made of different types of materials (Navya et al. 2019). Though these particles are small, their properties are much more advance than particles that are larger in size, due to their high surface-to-volume ratio, stability, and feasibility. Nanotechnology has been applicable in various fields, including biomedical, automobile, agriculture, architecture, etc., and is slowly emerging in the field of industrial biotechnology (Averback 2004).

Over the past few years, various nanomaterials, such as nanotubes, nanocatalysts, nanocomposites, nanoparticles, nanofibers, etc., are being used to convert crop oils or algal biomass into fatty acid methyl ester (FAME) or biodiesel. Being smaller in size and having a high surface-to-volume ratio, they immobilize enzymes easily and generate high catalytic effect, which boosts the conversion of biomass to biofuels (Yiu and Keane 2012; Ren et al. 2011). Transesterification is the process in which chemical reactions lead to the conversion of crop or algal oils into FAME, also known as biodiesel. Oils are extracted from crops, algae, animals, etc. which react with alcohol under the influence of heterogeneous catalysts (Zhang et al. 2013). This process has several specifications like reaction time, reaction temperature, optimal molar ratio of oil and alcohol, concentration of moisture and free fatty acids, and catalytic dosage (Sarno and Iuliano 2019). Usually, the enzymes used during the process of transesterification become inactive due to the presence of substrates or formation of by-products (Kumari et al. 2009). However, with the help of different nanomaterials, the enzyme system can be stabilized also, due to their high thermostability and efficiency, and low-cost input nanoparticles can be replaced with traditional methodologies used in biodiesel production (Verma et al. 2013).

Wen et al. performed an experiment in which biodiesel was produced from Chinese tallow seed oil using KF/CaO nanocatalyst. KF/CaO nanocatalyst was synthesized (impregnation method) and characterized using TEM, BET, and XRD. It was observed that the size of the pores of the catalyst was between 30 and 100 nm (TEM), with a surface area of 109 $m^2\ g^{-1}$ and an average per size of 97 nm (BET), as well as the formation of a new crystal $KCaF_2$ in the catalyst (XRD). Consecutively, Chinese tallow seed oil was transesterified using KF/CaO under optimal conditions like reaction temperature of 65 °C and an alcohol-oil molar ratio of 12:1. Under optimal conditions, the catalyst usage was between 1% and 5% w/w of oil; however, at 4% specifically, the biodiesel yield was found to be maximum. The production of

biodiesel was 96.8% due to high catalytic activity and stability, because of the large pore size of the KF/CaO catalyst (Wen et al. 2010). Similarly, biodiesel was produced using date palm seed oil with the help of a CaO-Fe_3O_4 nanomagnetic catalyst. Ali et al. prepared CaO-Fe_3O_4 nanomagnetic catalyst using chemical precipitation method and characterized it. Further, it was reported that calcination of CaO-Fe_3O_4 leads to the formation of calcium ferrite and the presence of iron components of calcium ferrite. To produce high amounts of biodiesel, a batch reactor was utilized for transesterification of date palm seeds oil under optimal conditions. These optimal conditions included 300-min reaction time, 65 °C reaction temperature, 10 wt% of CaO-Fe_3O_4 catalyst, and 20:1 molar ratio of methanol/oil. Under optimal conditions, biodiesel yield obtained from date palm seeds oil with the help of CaO-Fe_3O_4 nanomagnetic catalyst was around 69.7% (Ali et al. 2017).

The main objective of the chapter is to give a broad and thorough understanding of the studies conducted by various researchers to produce biofuels using nanotechnology. It primarily discusses the use of nanotubes, nanocatalysts, and nanocomposites for the conversion of biomass or waste feedstock into fatty acid methyl ester (FAME) or biodiesel. Furthermore, it gives a detailed outline of the transesterification processes yielding varying results with the help of different nanomaterials. The nanotechnological application to produce enhanced and efficient biodiesel or FAME yield, low-cost input, and environment-friendly biofuel production has been critically reviewed.

13.2 Application of Nanotechnology in Biofuel Production

13.2.1 Nanotubes

Nanotubes are one of the most widely used nanostructures in the field of industrial biotechnology due to its low toxicity, high efficiency, and feasibility. For the production of biofuels, nanotubes are functionalized with different substances to enhance their yielding capacity. This makes nanotubes a highly promising and potential nanostructure for the green synthesis of biofuels.

The depletion of petroleum has increased the demand for other sources of fuel especially green sources such as vegetable oil or animal fats. Biodiesel is the most economical, nontoxic, and biodegradable fuel. An experiment was conducted in which sodium titrate nanotubes acted as activators in the transesterification reaction of processed cooking oils to produce biodiesel. The morphology and microstructures were observed using BET and TEM, whereas the chemical bonds were studied by FT-IR spectroscopy. It was revealed that sodium titrate nanotubes of average diameter 5.37 nm and length varying from 50 to 80 nm (TEM) showed the presence of water molecules on titanate structures (FT-IR) and surface area of 120 $m^2\ g^{-1}$ (BET). The catalyst activity was observed at different conditions for 2 h, and it was found that 95.9% of biodiesel was yielded at 80 °C. Zaki et al. (2019) reported that with an increase in sodium titrate nanotubes (catalysts) and the amount of cooking

oil, the biodiesel production increased. Hence, the utilization of cooking oil for biodiesel production was very cost-effective, and the output (biodiesel) was found to be 1.2 times greater than the input (cooking oil) in which energy means highly productive (Zaki et al. 2019).

In recent years, significant consideration has been given to the production of biodiesel using various green sources like animal fats or vegetable oil. Also, the use of functionalized multiwalled carbon nanotubes (MWCNTs) has a huge contribution to the production of biodiesel. A comparative study was made in which a carboxylated MWCNT (MWCNT- COOH) and butylamine MWCNT (MWCNT- BA) were used for immobilization of *Candida antarctica* lipase B (CALB) for the enzymatic production of biodiesel using rapeseed oil. The morphological characteristics of MWCNTs were observed, which revealed that the diameter of functionalized MWCNTs was less than 20 nm (TEM), and the presence of carbon, nitrogen, and oxygen was confirmed (XPS) which led to the increased of the absorptivity of nanotubes. It was observed that a maximum yield of 92% was obtained when MWCNT-COOH was used, whereas the yield decreased to 86% when MWCNT-BA was used. Rastian et al. (2016) reported that immobilization of CALB stabilized the reaction and increased the output of biodiesel (Rastian et al. 2016). Lately, the production of biodiesel via transesterification reaction using refined vegetable oil, as the primal matter, has been known to be the most common approach. To examine the efficiency of sulfuric acid-regenerated MWCNTs as an activator to produce biodiesel using palm fatty acid distillate (PFAD), an experiment was conducted. The MWCNTs were 40–60 nm in diameter and 1–2 μm in length which was measured using SEM and FT-IR spectroscopy. It was observed that the as-synthesized MWCNTs produced less biodiesel as compared to the acid-regenerated MWCNTs. This was because the regenerated MWCNTs were cut into shorter tubes which enhanced their ability for esterification reaction. It was stated that acid regeneration of MWCNTs increased their catalytic activity, thus leading to a yield of 93.5% under certain conditions. Hence, acid-regenerated MWCNTs were found to be a very efficient catalyst to produce biodiesel using PFAD (Shuit and Tan 2015).

Considering the global environment, it is necessary to center the renewable energy sources and the production of biofuels. Transesterification reaction of oil feedstock using lipase as the catalyst is the most favorable method for the production of biodiesel. In another experiment, superparamagnetic multiwalled carbon nanotubes (MWCNTs) loaded with iron oxide and functionalized with polyamidoamine (PAMAM) were immobilized on *Burkholderia cepacia* lipase (BCL) and further used for biodiesel production. TEM revealed the dimension of around 40 nm of modified MWCNTs, whereas FT-IR spectroscopy was used to observe the lipase immobilization on MWCNTs. It was observed that biodiesel yield was maximum in the presence of water (2%), the temperature was in the range of 25–35 °C, and the ratio of oil to methanol increased from 1:2 to 1:5. Fan et al. (2016) reported that BCL-immobilized m-MWCNTs-PAMAM were the most effective catalyst to produce biodiesel using transesterification. Thus, magnetized BCL-

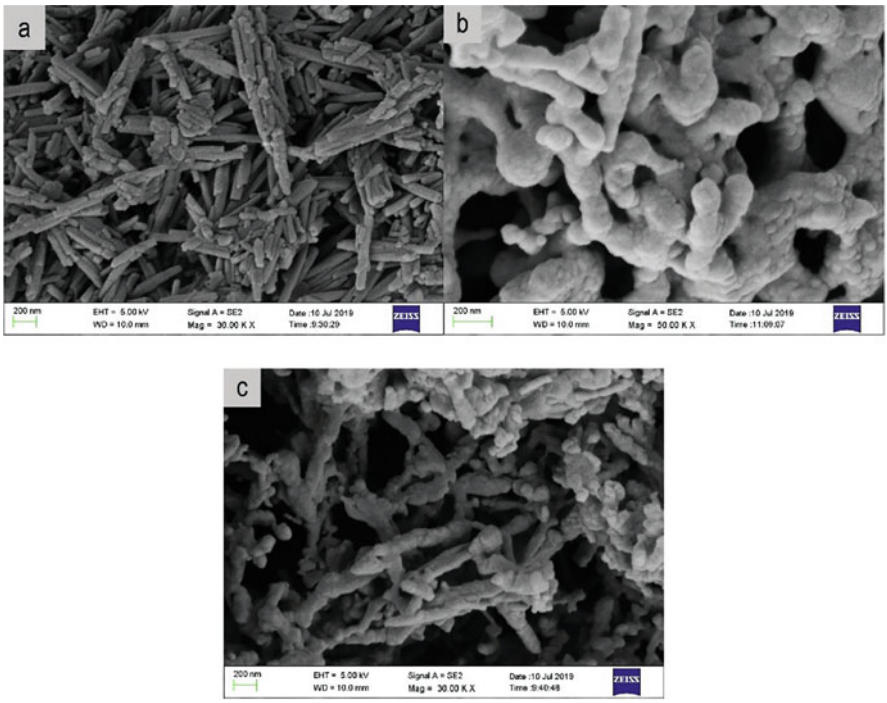

Fig. 13.1 SEM images of different samples: (**a**) HNTs, (**b**) HNTs-Ca, (**c**) HNTs La-Ca

mMWCNTs-PAMAM are proved to be the most promising nanobiocatalysts for increased production of biodiesel (Fan et al. 2016).

Halloysite is known to be a magnificent nanomaterial and has a nanotube-like structure with a layer of aluminosilicate. It has been strongly used in water purification, energy storage, and separation of gas. The nanocavity structure resists the mass transfer and the very effective catalytic activity of the halloysite. In this experiment, functionalized halloysite nanotubes (HNTs) with La-Ca (lanthanum and calcium) bimetallic oxide were utilized for the catalysis of transesterification reaction producing biodiesel. The catalyst HNTs/La-Ca of diameter 18.35 nm and crystalline phase structure was characterized using SEM (Fig. 13.1) and XRD, respectively. It was observed that along with La_2O_3 and CaO, HNTs-La/Ca catalysts were highly efficient and produced a yield of 97.5%. Lin et al. (2020) noted that the halloysite nanotubes when functionalized gave the highest productivity with 88.7% yield at the fifth reused cycle. Thus, a promising and favorable stimulus for the transesterification reaction for effective biodiesel production was identified (Lin et al. 2020).

Immobilization of various forms of lipases has been very helpful for biodiesel production; lipases can be immobilized both covalently and non-covalently, but the covalent immobilization was proved to be more stable and efficient. An experiment

was conducted in which *Candida antarctica* lipase B (CaL-B) was immobilized covalently on carboxylated SWCNTs and used to produce biodiesel. SWCNTs of 0.8–1.6 nm (inner diameter) and 1–2 nm (outer diameter) and length of 5.33 μm (observed under TEM) were utilized for this experiment. It was observed that the biocatalyst used could convert 83.4% of oil in 4 h at 35 °C retaining more than 90% after ten reusable cycles. Bencze et al. (2016) noted that covalent immobilization of enzyme was very favorable and stable for the biocatalyst since it generated conformational changes in the protein. Thus, a very active and useful catalyst to produce biodiesel was advanced (Bencze et al. 2016).

Extensive use of nanoparticles especially magnetic carbon nanotubes (m-CNTs) has been observed for the immobilization activity due to their attributes like immense surface area, chemical stability, porosity, and elevated heat conductivity. In this experiment, *Rhizomucor miehei* lipase (RML) is immobilized onto magnetic MWCNTs modified with polyamidoamine (PAMAM) dendrimer and used for the transesterification reaction of waste vegetable oil. The synthesized catalyst was studied using TEM and FT-IR which revealed the diameter of MWCNTs to be around 40 nm, and the presence of PAMAM and immobilization of RML was confirmed. After immobilization of the enzyme, the recovery activity reached 2808%, and the reaction activity went up 27 folds when compared with free enzymes. Fan et al. (2017) noted that 94% of the waste oil could get converted into biodiesel when PAMAM-m-MWCNTs immobilized with RML were used as catalysts in the transesterification reaction. A very efficient, stable, and reusable catalyst was developed which produced high-quality biodiesel (Fan et al. 2017).

Recently, the use of a solid acid catalyst containing ion-exchange resins, sulfated oxides, mobile composite material number 41, and Nafion has been reported. But they have a disadvantage that the acid side of the catalyst easily gets reduced by hydration of acidic hydroxyl groups (OH). The sulfonation and MWCNTs were further characterized, and it was noted that MWCNTs have poor dispersibility in methanol (UV-vis spectra) and average poor diameter (12.3 nm) and surface area (92.37 $m^2\ g^{-1}$) (BET). An effective protonic acid catalyst was prepared by grafting MWCNTs with SO_3H (sulfonation), then treating it with sulfuric acid, and further incorporating it with ultrasonication. The catalytic activity was observed to produce biodiesel. It was examined from the experiment that the optimum condition for synthesis was 10 wt% $(NH_4)_2SO_4$ solution with 10 min of ultrasonication treatment at a temperature of 235 °C. Shuit et al. (2015) reported that this method of sulfonation for catalyst production was very feasible, less toxic, and economical. Also, the produced catalysts had good thermal stability and dispersibility. In conclusion, s-MWCNTs prepared from $(NH_4)_2SO_4$ solution was advanced technology for production of biodiesel (Shuit et al. 2015).

Nanotubes and nanorods are the most promising and advanced one-dimensional nanostructures which are widely used as catalysts for different applications. One of the types of nanotubes is kaolinite nanotubes which possess a very large surface area and porous structure. An experiment was conducted in which kaolinite nanotubes were synthesized and doped with potassium ions (K+/KNTs) and further used for catalysis in the transesterification reaction. The doped nanotubes were characterized

using XRD and BET techniques. It was observed the results after experimenting 4 h using 6 wt% of K^+/KNTs catalyst that 99.4% of biodiesel was extracted from the sunflower oil. A novel catalyst was developed with 14.5 nm pore diameter and 7.43 mmol OH/g basicity which could produce biodiesel with high quality and properties (Abukhadra et al. 2020).

The most frequently used feedstock for biodiesel production is food-grade crops, yet there are some issues related to it like seed oil containing high free fatty acid (FFA) that cannot be converted into biodiesel in the presence of catalyst since saponification can occur. Thus, whenever a high FFA content oil is used as feedstock, biodiesel was produced using an acid catalyst. In this experiment, MWCNTs functionalized with sulfonated organosilane were used to fabricate biodiesel from high FFA seed oil. With a rise in the temperature, the mass of MWCNTs decreased (TGA), and the functionalization of acidic groups on MWCNTs added stability to it. Macawile et al. (2020) reported that conversion of high FFA accommodating oil into biodiesel was a two-step process and was very feasible in the presence of SO_3H-MWCNTs acid catalyst. Thus, a very efficient acid catalyst was successfully developed (Macawile et al. 2020).

Nanotubes are widely used in the production of biofuels; one of the most common types of nanotubes used is carbon nanotubes. Goh et al. (2012) filled single-walled carbon nanotubes (SWCNT) with iron oxide to obtain magnetic single-walled carbon nanotubes (mSWCNT). The enzyme amyloglucosidase (AMG) was immobilized on these mSWCNT (through physical adsorption and covalent immobilization) to hydrolyze starch, a biomass prototype used in this study. The carbon nanotubes were characterized using TEM and SEM. It was confirmed that SWCNTs of size 20–50 nm (in a bundle) (SEM) were loaded and covered with Fe_2O_3 nanoparticles (TEM). Also, SEM images showed adsorbed AMG over mSWCNT. It was determined that mSWCNT showed decreased enzymatic activity, which helps in less alteration in the structural conformation (CD) of AMG, eventually making it possible to recycle AMG for further use. It was also proved that AMG-immobilized mSWCNT increases the formation of AMG and other enzyme complexes, hence increasing the production of biofuel (Goh et al. 2012).

13.2.2 Nanocatalysts

13.2.2.1 Metal Oxides

With an increasing amount of research on biodiesel production using nanocatalyst, Gurusamy et al. (2019) conducted a study on *Ulva lactuca* seaweed to elevate the production of biodiesel in the presence of TiO_2-ZnO nanocomposite catalyst. The nanocatalyst was prepared using the coprecipitation technique and consecutively characterized using TEM which revealed that the size of nanocatalyst of around 12 nm and XRD confirmed its amorphous nature. The conversion of hydroxydecanoic acid to fatty acid methyl ester (FAME) was performed under

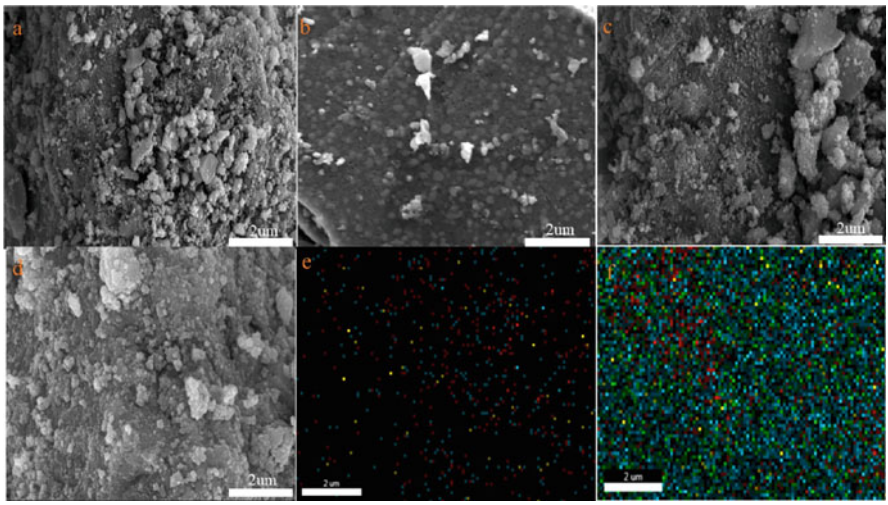

Fig. 13.2 (**a**) The SEM images of ZnO-TiO2–500 °C, (**b**) the SEM images of La3+/ZnO-TiO2–500 °C, (**c**) the SEM images of La3+/ZnO-TiO2–600 °C, (**d**) the SEM images

optimum and mild conditions, i.e., 4 wt% catalyst, 60–80 °C reaction temperature, and 4 h of reaction time. It was noted that a biodiesel yield of 82.8% was attained upon conversion from hydroxydecanoic acid to FAME. Furthermore, authors used the leftover algal biomass to synthesize silver nanoparticles (AgNPs) to study its antimicrobial activity (Gurusamy et al. 2019). Another study was conducted using waste cooking oil as biomass to produce biodiesel with the help of La^{3+}/ZnO-TiO_2 photocatalyst. Guo et al. (2021) prepared La^{3+}/ZnO-TiO_2 photocatalyst through the sol-gel method and characterized it. It was found that TiO_2-ZnO nanocomposite of size 8–11.5 nm (TEM), calcination temperature of 400–600 °C (TG), and crystal structures were observed on the surface using SEM-EDS (Fig. 13.2). Initially, the experiment was conducted under optimal conditions like reaction temperature of around 35 °C, 4 wt% of catalyst dosages, an ethanol-oil ratio of 12:1, UV irradiation, and reaction time of 3 h which resulted in 96.14% conversion of waste cooking oil to ethanol through photocatalytic esterification. Consecutively, transesterification of waste cooking oil was accomplished with the help of NaOH as a catalyst. Although after five cycles of this experiment it yielded 87% biofuel, this method was proved to be more stable and sustainable (Guo et al. 2021).

Like the previous experiments, Lin et al. (2020) performed an experiment using functional CaO/Au nanocatalyst for the production of biodiesel from soybean oil. However, in this experiment, Au (III)-polluted waste eggshell was used to synthesize CaO/Au nanocatalyst and further use it for biodiesel production. To achieve this goal, the eggshell powder was prepared and used for the synthesis of CaO/Au nanocatalyst; furthermore, the catalyst was characterized. It was observed that a thick layer of Au nanoparticles was found over the surface of CaO (XRD) making the particle size up to 2–5 nm (TEM); also, EDS confirmed even distribution of Au

nanoparticles on CaO. The transesterification process was performed with the help of methanol and soybean oil in a molar ratio of 12:1, 1 wt% catalyst concerning soybean oil, 70 °C reaction temperature, and 3 h of reaction time. The results obtained reported a total biodiesel yield of 88.9% and reusability up to five times without degradation in its efficiency (Liu et al. 2021).

The transformation of biomass into biofuels can be achieved using magnetic nanoparticles. Mapossa et al. (2020) conducted a study using $NiFe_2O_4$ and $Ni_{0.3}Zn_{0.7}Fe_2O_4$ magnetic nanoparticles to convert soybean oil into biodiesel. $NiFe_2O_4$ and $Ni_{0.3}Zn_{0.7}Fe_2O_4$ were synthesized by combustion reaction with the help of a conical reactor. The crystallinity of $NiFe_2O_4$ and $Ni_{0.3}Zn_{0.7}Fe_2O_4$ nanoparticles was found to be 55% and 72%, respectively (XRD), and the size of the nanoparticles was confirmed to be in the range of 13–20 nm. The experiment was performed under controlled optimal conditions, i.e., 2 wt% catalyst dosage, reaction temperature of 180 °C, a reaction time of 1 h, and oil-to-alcohol molar ratio of 1:12. The biodiesel yield was found out to be 94%; this procedure was conducted due to its efficiency, stability, and high activity, and hence this method can be helpful in the immediate future (Mapossa et al. 2020). Similarly, another experiment was conducted by Guo et al. (2012) in which a magnetic solid base catalyst made up of Na_2SiO_3 and Fe_3O_4 nanoparticles were used for converting cottonseed oil to biodiesel or FAME. Firstly, the synthesis of catalysts was carried out by depositing Na_2SiO_3 and Fe_3O_4 nanoparticles; consecutively, catalyst showing high catalytic activity was obtained at 350 °C calcination temperature, 2 h of aging time, Si/Fe molar ratio of 2.5, and calcination time of 2.5 h. The nanocatalyst was characterized by VSM and TEM to determine its magnetic strength and morphology, respectively. It was found out that Fe_3O_4 nanoparticles were spherical ball-like structures of size 20 nm (TEM) with high magnetic strength (VSM). Under optimal conditions like 60 °C reaction temperature, 100 min reaction time, 1:7 oil-to-methanol molar ratio, 5 wt% catalyst concentration, and 400 rpm stirring speed, the biodiesel yield was analyzed. A yield of 99.6% was obtained with a very good recovery rate of Na_2SiO_3/Fe_3O_4 nanocatalyst (Guo et al. 2012).

Recently, Santha et al. (2019) produced an experiment in which sustainable biofuel was produced, using CuO nanoparticle-based heterogeneous nanocatalyst, with the help of biogenic waste. Transesterification was carried out on waste cooking mustard oil using CuO nanoparticles as a catalyst which were fabricated by the coprecipitation method. The formation of CuO nanoparticles of size 13 nm and morphology resembling facets broken flower was confirmed using XRD and FESEM, respectively. EDAX was used to check the presence of impurities in the CuO catalyst, after which the transesterification process was carried out. During this process, certain optimal conditions were maintained (temperature 70 °C, time 2.5 h, and centrifuged at 4200 rpm for 15 min) to separate biofuels from by-products. A FAME yield of 88% was achieved through the ultrasonic reactor method, and the presence of methyl and ester groups was confirmed using FT-IR (Santha et al. 2019). Another experiment, in which potassium fluoride (KF) was infused on CaO/NiO, was performed to study the conversion of waste cottonseed oil to biodiesel. 20 wt% KF was impregnated on CaO/NiO through the wet impregnation method and

consecutively characterized by TEM, SEM, XRD, and BET. Based on the characterization, it was revealed that rhombus-shaped cluster particles of the catalyst of size 150 nm were formed (TEM and SEM); also the surface area of CaO/NiO decreased with an increase in KF impregnation (BET). Now, the transesterification process was carried out at 65 °C, for 4 h, with 5 wt% catalysts and methanol-to-waste cottonseed oil molar ratio of 15:1 to convert waste cottonseed oil to FAME. Kaur and Ali (2014) reported biodiesel yield of more than 96.5% and reusability up to four times without any degradation in its efficiency (Kaur and Ali 2014). The production of renewable energy has captivated many researchers due to the diminishing fossil fuel reserves. Biodiesel is the most environmentally safe fuels because of its properties like clean, nontoxic, and aroma-free nature. In this experiment, the CaO nanocatalyst was synthesized through the sol-gel method to churn out biodiesel. It was observed that the surface methodology using CaO nanocatalyst was very efficient and 97.61% yield was obtained. Further, it was reported that the conversion of soybean oil to biodiesel was observed to be 60 °C for 2 h with 3.675 wt% of the catalyst. Thus, CaO as nanocatalyst showed the highest rate of conversion in the transesterification as compared to the other nanocatalyst (Bharti et al. 2019).

In the following study, a palm kernel shell was used for the synthesis of highly mesoporous activated carbon by hydrothermal-assisted carbonization (HTC) for an enhanced catalytic activity. Waste cooking oil was used as a biomass prototype, to which the activated carbon acts as a degrading agent. Furthermore, the HTC-based activated carbon was infused with K_2CO_3 and CuO (through wet impregnation) to carry out esterification and transesterification processes. Using different characterization methods, the presence of K_2CO_3 and CuO nanoparticles was determined (FESEM) also, and the formation of a broad band in the range of 1366 cm^{-1} and 1346 cm^{-1} was determined (FT-IR). Abdullah et al. (2021) reported an increased production of biodiesel when 4 wt% of the catalyst was added, while 6 wt% of catalyst did not show any changes; however, it disturbed the mixing process between oil, methanol, and catalyst. The catalyst helped in increasing the contact between the active sites on the catalyst and reactant for esterification and transesterification processes (Abdullah et al. 2021).

Pollution is the main problem all over the world, and one of the main causes of pollution is excess use of fossil fuels (Chowdhary and Raj 2020). New techniques are being introduced to produce nonpolluting fossil fuels that would be safe for the environment and available on a large scale. One such technique is the use of seed oils like sunflower, soybean, date seed, etc. An experiment was conducted in which recycled cooking oil was used to produce biodiesel via transesterification reaction with nano-CaO and nano-MgO as catalysts. The nanocatalysts were synthesized by the sol-gel method. It was observed that nano-CaO was more efficient as compared to nano-MgO due to the high contact area of CaO. Tahvildari et al. (2015) stated that biodiesel yield was enhanced when both the catalysts were used together. Nano-MgO was not a very efficient catalyst when used individually due to its basic affinity, but when combined with CaO, it gave highly promising results (Tahvildari et al. 2015). The fuel prices have gone unreasonably high due to an increase in demand and decrease in amount. To overcome this issue, alternative fuels that are easily

producible and renewable are being synthesized using green sources like vegetable oils. Waste cooking oil is one of the feedstocks used for biodiesel production since it cannot be used for human consumption and has low cost and high availability. MgO nanocatalysts were produced using the coprecipitation method and used in the synthesis of biodiesel from waste cooking oil. The nanostructured phase and characteristics were observed using UV-vis and FT-IR spectroscopy. The MgO nanoparticle size and energy bandgap (Mg-O) were observed to be 7.86 nm and 5.84 eV (FT-IR and UV-vis). Ashok et al. (2018) investigated the biodiesel yield of 93.3% with 2 wt% of MgO catalyst at a temperature of 65 °C. Based on the experiment, it was inferred that MgO nanocatalyst was very effective in producing high-quality biodiesel (Ashok et al. 2018).

Characteristics like magnetically controlled drug delivery, sensors, memory storage devices, and catalysis make magnetic nanoparticles highly promising in the field of biofuel production. Also, they can be recovered and reused which makes them cost-effective too. In this experiment, the Fe_3O_4 nanoparticles were modified with tetraethyl orthosilicate (TEOS) and 3-chloropropyl trimethoxysilane (CPTMS) and then immobilized with different amines for transesterification of soybean oil for biodiesel production. Characterization of the modified nanoparticles was done using FTIR, SEM, and TEM spectroscopy. The particle sizes were revealed to be 45 and 65 nm (SEM and TEM), and the immobilization of amines was confirmed through FT-IR spectroscopy. A yield of 96% was observed at 160 °C within 3 h with 6% of the modified nanoparticles used as catalysts. Farzaneh et al. (2018) reported that the nanoparticle Fe_3O_4@SiO_2@CPTMS@amine (nanocatalyst) was highly stable and reusable because of which they were very efficient in the production of biodiesel. Thus, functionalization and modification of the nanoparticles increased their efficiency and activity rate (Farzaneh et al. 2018).

The use of renewable sources of energy due to the excessive use of petroleum and depletion of fossil fuels has become indispensable. Biofuels are produced using various bioresources and thus do not possess any kind of harm to the environment. An experiment was conducted in which microalgae *Chlorella vulgaris* was used to produce fatty acid methyl esters (FAMEs) type of biodiesel. Superparamagnetic few-layer graphene oxide and Fe_3O_4 (MGO) immobilized with lipase (ROL) were used as nanobiocatalysts in the transesterification reaction. The characterization of graphene oxide and its functionalization with 3-aminopropyl triethyoxysilane (AP) and glutaraldehyde (GA) was studied using SEM, XRD, and FT-IR spectroscopy. The graphene oxide layers were characterized by AFM. It was observed that the highest amount of biodiesel conversion occurred when ROL/MGO-AP-GA was used as a nanocatalyst. Tahir et al. (2020) reported that ROL/MGO-AP-GA was the most efficient catalyst even after reusability and converted microalgae bio-oil into biodiesel effectively. Thus, the functionalization of MGO improved their characteristics like loading capacity, thermal stability, and storage stability and successfully produced a large amount of biodiesel from microalgae (Nematian et al. 2020).

In the following experiment, biodiesel was produced by electrolysis from two microalgae strains, namely, *Chlorella vulgaris* and *Spirulina platensis*, using CaO/KOH-Fe_3O_4 and KF/KOH-Fe_3O_4 as magnetic nanocatalysts. SEM and XRD

were used to characterize the nanocatalysts, and the sizes were measured to be 55.91 nm and 42 nm, respectively. Electrolysis technique was conducted using two graphite electrodes, and the production of biodiesel at various conditions like weight percentage of catalyst, reaction time, and the ratio of alcohol was observed. It was depicted that the optimum condition for biodiesel production was at 25 °C using 1.5 wt% of catalyst at a molar ratio of 1:6 (methanol/oil). Farrokheh et al. (2020) submitted a report stating that *Chlorella vulgaris* showed better results in the yield as compared to the *Spirulina platensis* microalgae and that the KF/KOH-Fe_3O_4 were highly efficient nanocatalyst since they took less processing time and saved raw material. Thus, the method of electrolysis was highly potential; it reduced the transesterification reaction time and increased the efficiency of biodiesel (Farrokheh et al. 2020). Production of biodiesel through the transesterification reaction of vegetable oils and fats with methanol is the most acquainted and efficient technique. Among the vegetable oil, castor oil is the highly produced oil in India and is known for its high viscosity and density. In this experiment, castor oil was used to produce biodiesel in the presence of a heterogeneous Ni-doped ZnO nanocatalyst. The structural and functional characterization of Ni-doped ZnO nanocatalyst was done using AFM and XRD spectroscopy. It was observed that the Ni-doped ZnO increased the catalytic activity due to the surface area, and a maximum yield of 95.20% was noted. Baskar et al. (2018) reported that the optimum conditions for the biodiesel yield through castor oil were 55 °C for 60 min, 11 wt% of catalyst, and 1: 8 ratio of oil/methanol. Consequently, the nanocatalyst was highly promising to produce biodiesel from castor oil which is a low-cost feedstock (Baskar et al. 2018).

The nanocatalytic technology has played a vital role in the synthesis of biodiesel through a transesterification reaction. They are well known for increasing the efficacy of biodiesel production from various vegetable oils. The nanocatalysts modify the surface morphology for efficient production, whereas other catalysts such as carbonates, alkalis, zeolites, etc. do not actively modify the surface at the atomic scale. An experiment was conducted in which magnesium oxide nanocatalysts were used for biodiesel production through surface structural modifications. The prepared nanocrystalline-MgO particles underwent in situ aberration-corrected TEM, and the XRD technique was used to study other structural properties. Following the in situ AC-TEM results, it was observed that vacancies were devised at oxygen sites in the plane connecting Mg atoms. Thus, Gai et al. (2009) submitted a report stating that the vacancies between adjacent Mg-Mg atoms were the active sites and responsible for high biodiesel synthesis (Gai et al. 2009).

The destructive effect of pollution and high use of nonrenewable and toxic fossil fuels has laid a foundation for an alternative energy production method that is less toxic and detrimental to the environment. Presently many new techniques are being identified to produce biofuels through various green sources, one of which is the transesterification reaction of vegetable oils with the application of heterogeneous catalysts. A study was made to examine the efficiency of two different nanostructured oxides and their activity for biodiesel production. The nanoparticles synthesized were characterized using BET and SEM analysis. The size of nanoparticles was examined to be 15–55 nm (SEM). Parameters like acidity/basicity and surface

area (BET) were analyzed, and it was observed that the highest activity was related to surface basicity, not surface area. Further, it was reported that heterogeneous catalysts were dependent on temperature and only CaO was active at 70 °C converting 98% into biodiesel, whereas all other oxides were active at 150 °C. Thus, it was detected that in catalysis the effect of surface basicity was superior to that of particle size, and it was successful in advancing the transesterification reaction (Do Nascimento et al. 2012).

During the past decades, the production of methane-rich biogas through the method of anaerobic digestion (AD) has been established greatly. Scientists have recognized microalgae as a highly promising feedstock to produce biogas. Also, recently, the application of nanotechnology for improved biofuel production on a large scale is being appreciated. In this experiment, the potential of *Chlorella pyrenoidosa* (microalgae) in the presence of α-Fe2O3 nanoparticles (IONPs) for biogas production was studied. The IONPs were characterized using TEM and SEM spectroscopy. It was observed that there was an increase in the growth of microalgae in the presence of IONPs and biomass composition was enhanced. Rana et al. (2020) examined from the biochemical methane potential test that biogas productivity was improved with IONP supplementation. A rise of 25.14% with 22.4% enhanced methane content was reported. Consequently, microglial biogas production was a total success (Rana et al. 2020).

13.2.2.2 Metals

In recent years, waste materials are being used to produce biofuels to maintain sustainability and efficiency of biofuels at the same time. Ganesan et al. (2020) produced biofuel from *Jatropha* oil with the help of silver waste generated from discarded X-ray films. Silver nanoparticles (AgNPs) of size 60–70 nm (FESEM) were synthesized by green synthesis using oak gall's extract and *Camellia sinensis* extract. Using various characterization techniques, the absence of impurities in AgNPs (EDX) and crystallite size of AgNPs (25 nm) was confirmed (XRD). Moreover, the catalytic cracking process was initiated by maintaining catalytic cracking conditions, i.e., the temperature range was kept between 400 and 500 °C at a fixed WHSV (= hourly mass feed flow rate/*catalyst* mass). The oil was then passed through a condenser (0 °C), because of which gaseous products, as well as liquid products, were formed and collected. The obtained products were analyzed using the GC analysis technique, based on which it was revealed that 90% *Jatropha* oil was converted to 85% biofuel and 79% gaseous product, i.e., gasoline (Ganesan et al. 2020).

Biomass is known to be the premier green source of renewable energy and supplies 14% of the total energy demand. This is because of its abundant nature in all the areas like agriculture, forest residues, and unwanted plants (weed). An experiment was conducted in which two weed plants *Cannabis sativa* and *Parthenium hysterophorus L.*as biomass for biodiesel production were used in the presence of nanocatalysts Co and Ni. The structural details of the nanocatalyst were

measured using SEM and XRD spectroscopy. Gasification of *Cannabis sativa* led to the extraction of 12% gas, 53% oil, and 34% biochar, whereas *Parthenium hysterophorus L.* extracted 17% of gas, 44% oil, and 38.36% biochar. Tahir et al. (2020) reported that *Cannabis sativa* was more efficient and produced 53% more biodiesel as compared to *Parthenium hysterophorus L.* Hence, the production of biodiesel from unwanted plants like weeds had a high potential to avail the energy demands (Tahir et al. 2020). Unwanted weed biomass from cultivated fields was found to be potential biomasses in the biofuel production. This experiment was performed to check the activity of nickel and cobalt nanoparticle-based nanocatalyst on the lignocellulosic part of weed biomass. Ali et al. (2020) used different weed plants like *Carthamus oxyacantha*, *Asphodelus tenuifolius*, and *Chenopodium album* to produce biodiesel, biochar, and biogas using nanocatalyst. Initially, nickel and cobalt nanoparticles were produced using hydrothermal method and characterized TEM, XRD, and SEM. It was found that nickel and cobalt nanoparticles of size 2–90 nm (SEM), when used for the gasification of weed biomass, resulted in the yield of 18% biochar, 23.75% biogas, and 57.5% bio-oil which was further analyzed for transesterification. GC-MS results revealed that the biodiesel sample accommodated 65.47% esters, which demonstrates the improved quality of biodiesel (Ali et al. 2020).

Recent works have proved magnetic nanoparticles to be highly proficient in catalysis. They have emerged on a large scale due to their properties like magnetic separation from the reaction medium and surface area. The aim of this experiment was an extraction of biodiesel from soybean oil using magnetic mixed iron/cadmium (ICdO) and iron/tin oxide (ISnO) nanoparticles as catalyst. No significant difference in the catalytic activity of both the catalysts was detected, which led to an equal yield of biodiesel. Alves et al. (2014) reported that 90% of fatty acid could be converted into biodiesel using ISnO and ICdO as catalysts and that these catalysts could be magnetically recovered and reused. Hence, they were recognized as catalysts showing high potential in the generation of biodiesel and were also cost-effective (Alves et al. 2014). Researchers have recognized bimetallic nanoparticles as highly promising metal nanoparticles for the production of biodiesel. They have certain characteristics and better reactivity which make them a suitable catalyst for the transesterification reaction. This study is about the synthesis of bimetallic gold-silver core-shell nanoparticles (Au@AgNPs) and their application in biodiesel synthesis from sunflower oil. The characterization of the catalyst Au@AgNPs was done using TEM, energy dispersive X-ray (EDX) analysis. It was observed that when silver particles were deposited on the gold nanoparticles, it showed high catalytic activity in the transesterification reaction of sunflower oil. Banerjee et al. (2014) reported that the required optimum conditions for biodiesel extraction from sunflower oil are 65 °C for 2 h with catalyst concentration of 5%. The results depicted that Au@Ag core-shell nanoparticles were of high potential as a catalyst for transesterification of sunflower oil (Banerjee et al. 2014).

The demand for biodiesel is seen to be increasing worldwide because of the increase in vehicle population and industrialization. To subdue this problem, biofuels like biodiesel and bioethanol were prepared as an alternative for fossil

fuels. An experiment was directed in which a magnetically separable zinc ferrite nanocatalyst was used to produce biodiesel from waste cooking oil. The structural features of nanocatalyst were studied using XRD, FT-IR, and SEM. The average crystalline size was revealed to be around 74 nm (XRD). The results of FT-IR spectra showed the presence of H-O-H vibrations. A maximum yield of 98.6% of biodiesel in transesterification reaction using $ZnFe_2O_4$ nanocatalyst was observed at 60 °C temperatures for waste cooking oil. Ashok et al. (2019) reported that zinc ferrite nanocatalyst was successful in producing biodiesel efficiently through the transesterification reaction of waste cooking oil and is a non-spontaneous reaction (Ashok and Kennedy 2019). In developing countries like India, it has become very necessary to evolve some alternative sources for fuels. Biodiesel and bioethanol production has reached great heights due to their renewable and nontoxic nature. An experiment was conducted in which esterification of palmy fatty acid for biodiesel production was done in the presence of immobilized lipase on magnetic nanoparticles. The structural and morphological characteristics were studied using FT-IR and TEM spectroscopy. Gupta and Rathod (2020) reported 82.47% conversion due to esterification of palm fatty acid into biodiesel with lipase-immobilized magnetic nanoparticles. The nanoparticles were reusable up to five cycles giving a yield of 80.19%. Immobilizing the nanoparticles with lipase increased its efficiency to 85%. Hence, these particles were having great potential as catalysts for producing high-quality, cost-effective, and pollutant-free biodiesel from soybean oil (Gupta and Rathod 2020).

The most suitable biological resource for biodiesel production is microalgae. They possess certain characteristics such as photosynthetic efficiency by harvesting solar radiations, allowing for rapid growth rate and the production of biomass in large quantity. In this study, the impact of carbon/nitrogen (C/N) on microalgal growth and application of nanoparticles for biodiesel extraction. Three types of microalgae species *Chlorococcum* sp., *Scenedesmus* sp., and *Euglena* sp. were isolated, and a comparative study was made in the presence of different carbon sources and titanium (Ti) nanoparticles. The morphological characterization of Ti NPs was done using SEM analysis. Among all the carbon sources used, it was identified that sucrose was the most suitable microalgal biomass enhancement. An increase in biomass from 3.5 g/L to 5.02 g/L and lipid content from 58 to 60% after the addition of Ti nanoparticles was observed. Khanra et al. (2020) reported that the optimum concentration of Ti nanoparticles was 15 ppm with the harvesting potential of 82.46% and the most effective algae for biodiesel production was determined to be *Chlorococcum* sp. Hence, according to the results, the Ti nanoparticles were highly promising and most viable to produce biodiesel from microalgae feedstock (Khanra et al. 2020). The current advancement in processes to produce cost-effective and biodegradable fuel energy with the use of magnetic nanocatalyst has been accepted worldwide. In this experiment, a magnetic nano-sized solid catalyst was produced with the help of *Citrus sinensis* peel ash (CSPA)@Fe_3O_4 and used to produce biodiesel from waste cooking oil. The structural and morphological characteristics were examined using TEM, XPS, XRD, and FT-IR spectroscopy. The average particle size was 12–15 nm (TEM). The chemical composition of the

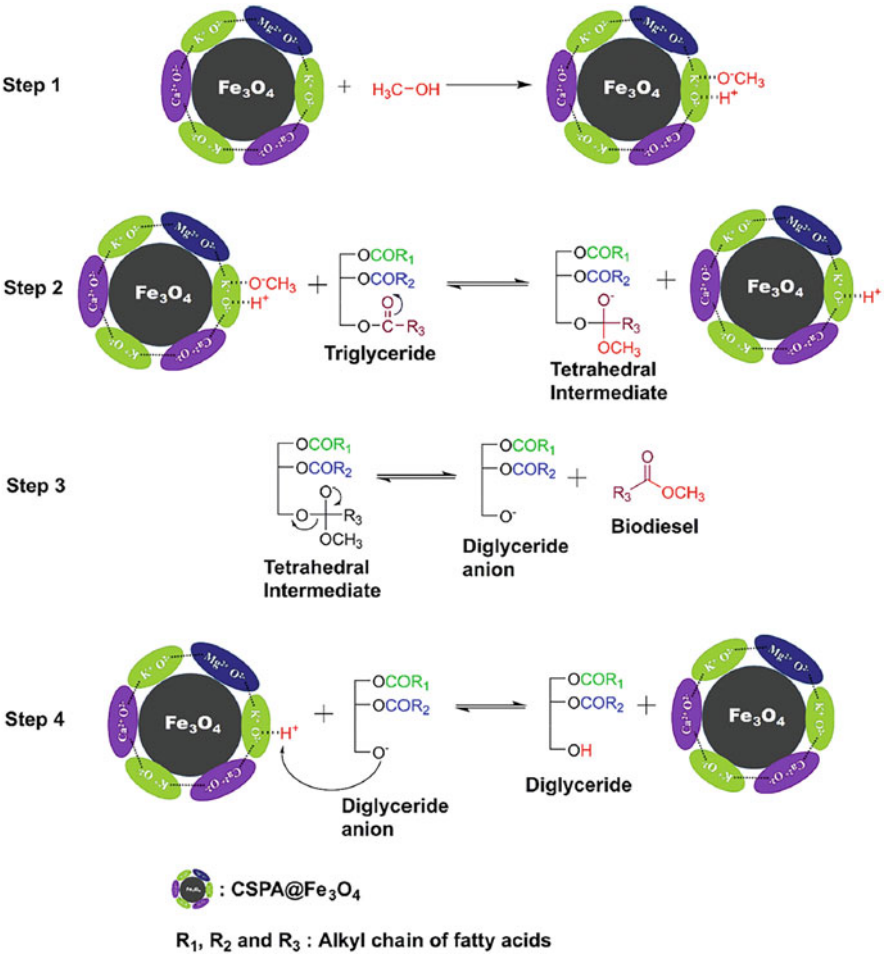

Fig. 13.3 Probable mechanism of CSPA@Fe_3O_4-catalyzed transesterification of WCO

catalyst was observed to be Ca, K, C, and O(XPS). Also, the presence of O-H group was revealed in the catalyst (FT-IR). It was observed that the size, shape, and surface properties of the catalyst were enhanced due to its core-shell structure. Changmai et al. (2021) noted that the synthesized catalyst could give a yield of 98% in the transesterification reaction (waste cooking oil) (Fig. 13.3) and also that the magnetic catalyst was easily recoverable. Therefore, this method using iron oxide nanoparticles was a great success and showed excellent activity in biodiesel production (Changmai et al. 2021).

Microalgae are considered as best source for biodiesel production since it has a high value of triglycerides and polysaccharides and shows increased growth rate in any environmental conditions. A study was made in which microalgal biomass was

produced in which lignocellulosic biomass (LCB) was the carbon source and then further biodiesel was extracted from it using iron nanomaterials. The size of iron nanoparticles was observed to be 3.347 nm (SEM). Two types of cultures *Dictyococcus* sp. and *Coelastrella* sp. each 44% and 52% were grown from which FAME was extracted. Sakthi Vignesh et al. (2020) reported that the two microalgae each produced a yield of 90 and 28%, respectively. The lipid content was enhanced by nitrogen starvation for better results. Thus, the product obtained through bio-iron nanoparticles was evaluated for its activity, and quality was found to be highly efficient (Sakthi Vignesh et al. 2020).

Similarly, another study by Ashok et al. (2021) demonstrated the use of waste cooking oil to produce biodiesel with the help of magnetically recoverable magnesium-substituted zinc ferrite nanocatalysts. The characterization techniques revealed that the nanocatalyst of size 8–10 nm (HR-SEM) had a high magnetic moment and saturation magnetization property which is necessary for the separation of the catalyst from the reaction medium (VSM). Furthermore, it was revealed that at standard conditions such as 3 wt% of nanocatalyst dosage, 18:1 methanol-oil ratio, a reaction time of 30 min, and 65 °C reaction temperature, the biodiesel yield increased to 99.9%. Also, the magnetically separated nanocatalyst could be recovered up to ten cycles with 94% yield of biodiesel (Ashok et al. 2021).

13.2.3 Nanocomposites

Nanobiotechnology is a branch of biotechnology that has promising applications in all aspects of science and technology. Enzymes are the biological catalysts that enhance the speed of any reaction, but recent studies have evolved their function by immobilizing it with any type of nanomaterials. This study is about how immobilization of different nanomaterials like nanoparticles, nanocomposites, and nanofibers on enzymes can be considerate for the stabilization and production of biofuels. Different nanomaterials along with enzyme immobilization were characterized using BET, AFM, XRD, and, FT-IR spectroscopy analysis. Verma et al. (2013) noted that enzyme activity was increased after immobilization because nanomaterials have a large surface area and thus load a large quantity of enzymes on them. Also, because of their high tensile strength, they cannot break easily. Hence, this research leads to an establishment of a novel nanobiocatalytic technique that was very suitable to produce biodiesel (Verma et al. 2013).

Another way of producing biodiesel from biomass is through an electrocatalytic process which involves the use of electrodes made from nanoparticles. This experiment was performed to transform waste palm oil to biodiesel with the help of Pt-Ni/NiO/Ni_2O_3 nanocatalyst-based electrodes. Pt-Ni/NiO/Ni_2O_3 nanocatalyst was synthesized and characterized using TEM, XRD, DTG, TG, FT-IR, and Raman analysis. It was found that nanocatalyst of size 50 nm with quasi-spherical morphology consisted of a bridge between Pt and Ni/NiO/Ni_2O_3 nanoparticles (TEM), and the presence of nickel oxides was confirmed by FT-IR, whereas Raman analysis helped

in the confirmation of the formation of Pt and Ni-Ni oxide nanoparticles. Sarno et al. (2020) performed transesterification of palm oil into biodiesel using low voltage and temperature, i.e., 10 V and 40 °C, respectively, in a two-phase system with less amount of nanocatalyst. The palm oil was treated at 240 °C for 7 days with a reaction time of 3 h, and a high yield of biodiesel was observed. A biodiesel yield of 96.2% was obtained, proving it as a substitute in the production of biodiesel in a sustainable way (Sarno et al. 2020). Likewise, a heterogeneous base nanocatalyst was used to produce biodiesel from vegetable oil. In this experiment, Se-doped ZnO nanocatalyst was used to carry out a transesterification process to convert vegetable oil to FAME and eventually into biodiesel. Se-doped ZnO nanorods were prepared by mechanothermal method and characterized using XRD, XPS, HR-TEM, and FE-SEM. XRD confirmed the presence of doped Se in ZnO crystal, while the rod-shaped structure of Se-doped ZnO nanoparticles of an average diameter of 50 nm was determined using FE-SEM and HR-TEM, respectively. Rao et al. (2021) performed a transesterification process under optimal conditions such as 65 °C temperature, 5 wt% catalyst concentration, 1:20 oil-to-methanol volume ratio, and 3 h of reaction time. A total biodiesel yield of 94.7% was obtained, along with which it was found that the catalyst was recyclable up to five cycles without any degradation in the FAME yield (Rao et al. 2021).

With the arrival of new technologies, various methods are used to convert fatty acids into biofuels or fuel additives. One of these technologies is the use of nanocomposites as heterogeneous catalysts for sustainable production of biodiesel and fuel additives. Peixoto et al. (2021) prepared organo-sulfonic aryl-silica nanoparticles (by post-grafting methods) as a highly active heterogeneous catalyst for the esterification of free fatty acids (FFA), levulinic acid (LA), and aldol condensation of furfural. Firstly, characterization of nanoparticles was studied using XPS, TEM, SEM, and FT-IR (average size of 10–100 nm), the structure (uniform spheres) and presence of oxygen, carbon, and silica on the surface of nanoparticles were confirmed. Furthermore, in the case of esterification reactions of FFA and LA, the temperature was kept at 120 °C for 2 h with 10 wt% of catalyst dosage and reused for 6 and 10 cycles, respectively. On the other hand, for the aldol condensation reaction, the temperature was maintained around 65 °C for 2 h. Based on the results, it was revealed that esterification of LA showed 100% biodiesel yield with 83.2% yield even after ten cycles. Moreover, the aldol condensation reaction of furfural with 3-methyl furan produced a high yield within 10 min of reaction proving its high catalytic effect (Peixoto et al. 2021).

The following study was conducted on cottonseed oil to produce biodiesel using nanocrystalline lithium impregnated calcium oxide as a nanocatalyst. Li^+ ions were impregnated over CaO by the wet impregnation method; additionally, their structural and chemical characteristics were studied using XRD and TEM. These techniques helped confirm the size (50 nm) and nanostructure of the catalyst. Furthermore, the transesterification process was carried out under optimal conditions, i.e., reaction temperature of around 35 to 65 °C, time of 0.5–8 h, catalyst dosage of 5 wt%, and oil-to-methanol molar ratio of 1:12. Along with these, moisture content of up to 15 wt% and free fatty acid content up to 6 wt% were maintained. Kumar and Ali

(2010) reported that when the moisture content and free fatty acid content were kept high, the production of biodiesel from cheap feedstock was found to be more, making it possible to reduce the production cost of biofuels (Kumar and Ali 2010).

Fatty acid alkyl ester (FAME) is not only renewable but also biodegradable and less hazardous. Lipases are recognized to be an efficient catalyst especially lipase deriving out of yeast. In this work, *Magnusiomyces capitatus* yeast was used as a source of lipase. The *M. capitatus* A4C extracellular lipase was an extraordinary biocatalyst that could catalyze both the esterification and transesterification reactions. $CuSO_4.5H_2O$ hybridized with ECL was used for biodiesel production, and its structural analysis was done with the help of SEM and FT-IR. The morphological changes that occurred after the hybridization of $CuSO_4.5H_2O$ were observed (SEM). The results observed were 89.7% conversion of triglycerides into FAME while 98.7% of oleic acid into FAME in an equal time. Baloch et al. (2021) reported that the ECL-$CuSO_4$ hybrid was preferable for the esterification reaction as compared to the transesterification. So, the biocatalyst produced from *M. capitatus* yeast had a very promising effect on biodiesel production as an alternative for depleting fossil fuels (Baloch et al. 2021).

As a result of increased population and industrialization, it has been observed that the need for energy sources is also increasing. To meet this increasing demand, new techniques and sources are being established to produce renewable, nontoxic energy. Transesterification is one of the techniques utilized to generate fatty acid methyl esters (FAME) used as biodiesel. This reaction is usually catalyzed by nanomaterials and is the most suitable catalyst to produce biodiesel. In this experiment, transesterification of soybean oil was achieved with the influence of CaAlSi mixed oxide nanoparticles. The structural and morphological characteristics of oxide nanoparticles were examined using XRD, FT-IR, and SEM spectroscopy. It was observed that CaAlSi nanoparticles were effective in catalyzing the transesterification reaction of soybean oil. Farzaneh et al. (2017) submitted a report which described the most suitable condition for biodiesel production through soybean oil was 60 °C in the presence of 6% catalyst (oxide nanoparticles). It was a successful attempt that resulted in a 95% yield of biodiesel within 8 h. Also, it was analyzed that the catalysts were heterogeneous and reusable (Farzaneh et al. 2017). Fatty acid methyl ester (FAME) is one biodegradable and nontoxic biofuel prepared by the researchers to meet the crisis arising due to the lack of petroleum and other fossil fuels. Techniques like transesterification and esterification in the presence of nanocatalysts especially oxides have reached a high success in biodiesel production. In this study, the zeolite imidazolate framework (ZIF-8) was hybridized with potassium (KNa/ZIF-8) that further catalyzed the transesterification reaction of soybean oil for biodiesel production. The KNa/ZIF-8 catalyst was characterized using FT-IR, XRD, and SEM spectroscopy. It was observed that after the addition of potassium to the Na/ZIF-8, the basicity was increased and so the activity of the catalyst. Saeedi et al. (2016) reported that KNa/ZIF-8 was the finest catalyst to fabricate biodiesel due to its high basic nature and surface area. The optimum conditions identified for a yield of greater than 98% were 10:1 ratio for methanol/oil for 3.5 h. Hence, the catalyst was highly stable and active showing excellent

results for the production of biodiesel through soybean oil (Saeedi et al. 2016). Waste frying oil (WFO) is a new feedstock found by scientists that have a high capability for biodiesel production when treated in the presence of certain nanocomposites as the catalyst. This study is about the synthesis of CaO-KOH-Al_2O_3 nanocomposites and examines their role in biodiesel production from canola oil. Different methods like coprecipitation, impregnation, sol-gel, and MW-assisted solution combustion synthesis (M-SCS) were used for the synthesis of nanocomposites. The structure and function of synthesized nanocomposites were examined using SEM, XRD, BET, and FT-IR analysis. It was observed that the nanocomposites formed by the M-SCS method showed greater basicity and thus activity. 86% yield of biodiesel was noted when this nanocomposite was used as the catalyst. Nayebzadeh et al. (2019) reported that the nanocomposites synthesized by M-SCS were more simple, economical, and active as compared to others. They increased porosity, surface area, and stability and thus were very effective in the production of biodiesel (Nayebzadeh et al. 2019).

Traditionally used heating systems had many disadvantages like the need for high temperatures, energy loss, and cost. To overcome these scientists found a technique known as microwave irradiation; it gained high success due to its proficiency in completion of chemical reaction in a short period. An experiment was conducted in which KOH/$Ca_{12}Al_{14}O_{33}$ nanocatalyst was prepared using microwave irradiation for biodiesel production. Interestingly, yield of biodiesel increased from 30.2% to 93.4% with an increase in the amount of catalyst and reaction time from 20 to 60 mins. Heydari et al. (2018) reported that KOH/$Ca_{12}Al_{14}O_{33}$ nanocatalyst successfully converted many triglycerides into biodiesel since they had a large pore size and surface area. Therefore, calcium aluminate was proved to be a highly promising nanocatalyst to produce biodiesel, especially through the microwave irradiation method (Heydari et al. 2018). Biodiesel is a type of renewable energy with low sulfur and aromatic content and is used widely as a green fuel. It can be derived from edible as well as nonedible vegetable oils and animal fats. *Capparis spinosa* is one of the vegetable oils which can be comprehensively generated and is available in all climates and areas. In this experiment, biodiesel was fabricated from *Capparis spinosa* oil in the presence of a heterogeneous catalyst nanozeolite NaX. The structural characteristics of the NaOH/NaX catalyst were examined, and yield of 90.81% was observed at optimal conditions. Helmi et al. (2021) reported that *Capparis spinosa* seeds contain 30–50% convertible oil in them and the optimum temperature for transesterification is 60 °C with methanol-to-oil ratio of 1:6.7 and the weight of catalyst to be 2.3 wt%. Therefore, *Capparis spinosa* oil is the most effective feedstock producing magnificent quality of biodiesel, and NaOH/NaX catalyst is a nonheterogeneous catalyst of high potential and activity (Helmi et al. 2021).

Lately, the Earth's natural resources are seen to be diminishing with increasing demand and industrialization. This is the fundamental cause of developing new approaches for the production of biodiesel. The efforts of researchers have recognized and developed several techniques that can use pyrolysis, microemulsions, and vegetable oils for biodiesel synthesis. An experiment was conducted in which they

used five different types of calcium oxide-based catalysts along with gold nanoparticles (AuNPs) as nanocatalysts for the production of biodiesel through transesterification. SEM and XRD analyses were used for the characterization of CaO-based/Au nanoparticles. The gas chromatography results revealed that the biodiesel produced with AuNP-supported CaO catalyst was better than that produced by conventional CaO catalyst. It was further reported that oil conversion of about 90–97% was observed at an optimum temperature of 65 °C with 3 wt% of catalyst and also that the reusability of the catalyst was ten times which makes it the most efficient and economical (Bet-Moushoul et al. 2016).

Metal nanoparticles are extensively used nanoparticles in different sectors of industries. Nowadays, metal nanoparticles are used for the production of biofuels from biomass. Recently, in 2019, Laskar et al. (2020) experimented using ZnO-supported silver nanoparticles (AgNPs) to produce biodiesel from palm oil. Initially, ZnO@AgNPs were synthesized by the homogenous precipitation method, wherein polyethylene glycol acted as a surfactant and reducing agent and examined different aspects using TEM, NMR, BET, and XRD. Based on the results obtained by the characterization of ZnO@AgNPs, the deposition of Ag on ZnO nanoparticles (XRD), the size of ZnO (85 nm) and AgNPs (23 nm), and the surface area of 28.13 m^2/g (BET) were confirmed. The transesterification process was carried forward at 60 °C, for 60 min, with oil-to-methanol molar ratio of 10:1 and 10 wt% of catalyst. A FAME yield of 97% was reported; moreover, the catalyst recyclability tests confirmed the reusability of ZnO@AgNPs up to five cycles without any loss in its efficiency (Laskar et al. 2020).

13.3 Conclusion

Most of the experiments mentioned in the above chapter focused on the production of biodiesel using various types of biomasses. Currently, researchers have observed that nanoparticles are seen to be replacing the traditional heterogeneous catalysts. A great deal of attention was toward the application of nanotechnology for the efficient production of biodiesel. Several nanomaterials such as nanotubes, nanoadditives, and nanocomposites were proven to be the most effective nanocatalyst for the catalysis of transesterification and esterification reaction of biodiesel production. A higher amount of yield and an excellent quality of biodiesel were extracted using nanomaterials as seen in Table 13.1. Certain characteristics of the nanocatalysts like small size, large surface area, high rate of activity, stability, and reusability make them suitable catalysts for the reactions. Therefore, the sustainable and cost-effective synthesis of biodiesel was only possible due to the use of nanocatalysts of various forms catalyzing the reactions of various green sources of biomass like vegetable oils and microalgae.

Table 13.1 The amount of yield obtained using different biomass prototypes for the production of biodiesel using various nanomaterials

Type of nanomaterial	FAME yield (%)	Biomass	Size	Characterization	References
Carbon nanotubes (MWCNTs)	85–98	Soybean oil	D = 40–60 nm	XPS, FT-IR, TEM	Fan et al. (2016)
Sulfonated MWCNTs			D = 40–60 nm, L = 1–2 μm	FT-IR, Raman spectra, BET	Shuit et al. (2015)
CaO nanocatalyst			8 nm	SEM, TEM, XRD, FT-IR, BET	Bharti et al. (2019)
Fe_3O_4 nanoparticles			45–65 nm	FT-IR, XRD, VSM, SEM, TEM	Farzaneh et al. (2018)
NiFe2O4 and Ni0.3Zn0.7Fe2O4 magnetic nanoparticles			13–20 nm	XRD, FT-IR, GC, SEM, TEM	Mapossa et al. (2020)
Magnetic nanocatalysts			Cadmium (228.80 nm) Tin (189.99 nm) iron (259.95 nm)	XRD, Raman spectroscopy, UV-vis	Alves et al. (2014)
CaO/au nanocatalyst			2–5 nm	XRD, EDS, FESEM, TEM, XPS	Liu et al. (2021)
CaAlSi mixed oxide nanoparticles			48–54 nm	XRD, SEM, FT-IR	Farzaneh et al. (2017)
Nanostructured sodium-zeolite imidazolate framework with potassium (ZIF-8/K)			D = 1–1.5 nm	FT-IR, XRD, TGA, SEM	Saeedi et al. (2016)
Mg-substituted zinc ferrite nanocatalyst	82–100	Waste cooking oil	8–10 nm	EDX, DRS, VSM, XRD, FT-IR, HR-SEM	Sakthi Vignesh et al. (2020)
Zinc ferrite nanocatalyst			8.55–13.04 nm	XRD, FT-IR, DRS, SEM	Ashok and Kennedy (2019)
Sodium titanate nanotubes			D = 5.37 nm, L = 50–80 nm	XRD, TEM, BET, FT-IR	Zaki et al. (2019)
La^{3+}/ZnO-TiO2 photocatalyst			8–11.5 nm	TG, XRD, SEM-EDS, HRTEM, BET, UV-vis, Raman spec	Guo et al. (2021)

CaO and MgO heterogenic nanocatalyst			65–70 nm	XRD, SEM	Tahvildari et al. (2015)
Magnesium oxide nanocatalyst			7.86 nm	XRD, UV-vis, FT-IR,	Ashok et al. (2018)
Magnetic nanocatalyst			12–13 nm	TEM, XRD, FT-IR, XPS	Changmai et al. (2021)
K2CO3-CuO nanocatalyst			2–17 nm	XRD, FESEM, EDX, FT-IR, TCD, BET	Abdullah et al. (2021)
Sulfonated MWCNT	82–98	Palm oil	D = 40–60 nm, L = 1–2 μm	SEM, FT-IR	Shuit and Tan (2015)
Halloysite nanotubes			D = 17.47 nm	XRD, SEM, XPS	Lin et al. (2020)
Magnetic nanoparticles			10–20 nm	FT-IR, BET, TEM, UV-vis	Gupta and Rathod (2020)
Zinc oxide-supported silver nanoparticles			23–85 nm	XRD, TEM, BET, HNMR, GC	Laskar et al. (2020)
Pt-Ni/NiO/Ni2O3 nanoparticles			2–3 nm	XRD, TG, DTG, Raman spec, FT-IR, TEM	Sarno et al. (2020)
Oxide nanoparticles	85–98	Other oils	15–55 nm	XRD, BET, zeta potential, SEM	Do Nascimento et al. (2012)
Plasma-functionalized MWCNT			D = 10–20 nm, L = 10–30 μm	XPS, TEM, BET	Rastian et al. (2016)
Ni-doped ZnO nanocatalyst			35.1 nm	AFM, XRD, VSM	Baskar et al. (2018)
Silver nanoparticles			60–70 nm	XRD, UV spec, EDX, FESEM	Ganesan et al. (2020)
SO3H-MWCNTs			D = 20–30 nm, L = 10–30 μm	SEM, FT-IR, TGA, BET	Macawile et al. (2020)
NaOH/NaX nanoheterogeneous catalyst			70.44 nm	XRD, SEM, BET	Helmi et al. (2021)

(continued)

Table 13.1 (continued)

Type of nanomaterial	FAME yield (%)	Biomass	Size	Characterization	References
Graphene oxide magnetic nanobiocatalyst	82–99	Algae	20–30 nm	AFM, FT-IR, XRD, BET, zeta potential	Nematian et al. (2020)
Titanium nanoparticles			15 ppm Ti nps	SEM	Khanra et al. (2020)
CaO/KOH, Fe3O4 magnetic nanocatalysts			42–55.91 nm	SEM, BET, FT-IR	Farrokheh et al. (2020)
TiO2-ZnO nanocomposite catalyst			~ 12 nm	XRD, FT-IR, TEM, EDAX	Gurusamy et al. (2019)
Iron nanoparticles			3.347 nm	SEM, BET, FT-IR	Ashok et al. (2021)
Iron oxide nanoparticles			<50 nm	UV-vis, XRD, TEM	Rana et al. (2020)
Gold-silver core-shell nanoparticle	83–100	Sunflower oil	12 nm	UV-vis, TEM, XRD, FT-IR	Banerjee et al. (2014)
Carbon nanotubes (SWCNTs)			D = 1–2 nm, L = 5–33 μm	TEM, HNMR spectra	Bencze et al. (2016)
Kaolinite nanotubes			D = 14.5 nm	XRD, BET, TEM	Abukhadra et al. (2020)
CaO-based/au nanoparticles				XRD, SEM	Bet-Moushoul et al. (2016)
Magnetic MWCNTs	80–95	Vegetable oil	40 nm	TEM, XRD, FT-IR	Fan et al. (2016)
Se-doped ZnO nanocatalyst (nanorods)			50 nm	XRD, XPS, HRTEM, FE-SEM	Rao et al. (2021)
Magnesium oxide nanocatalysts			15–19 nm	SEM, TEM, XRD, XPS	Gai et al. (2009)
CaO/NiO Nanocatalyst	96–100	Cottonseed oil	1.8 μm	XRD, TEM, SEM	Kaur and Ali (2014)
Na2SiO3 and Fe3O4 nanoparticles				VSM, TEM	Guo et al. (2012)

Nanocrystalline lithium-ion impregnated calcium oxide			50 nm	XRD, TEM, BET, FESEM	Kumar and Ali (2010)
Nanocatalysts (co, Ni)	50–60	Unwanted weed	20–50 nm	SEM, XRD, FT-IR	Tahir et al. (2020)
Nanocatalyst nickel and cobalt			2–90 nm	XRD, TEM, SEM	Ali et al. (2020)
KOH-CaO-Al_2O_3 nanocomposites	86–93	Canola oil		XRD, FT-IR, SEM	Nayebzadeh et al. (2019)
KOH/calcium aluminate nanocatalyst			18–30 nm	XRD, BET, FT-IR, SEM	Heydari et al. (2018)
Nano-entrapped lipase (ECL)	98–100	Acids		FT-IR, TGA, SEM, XRD	Baloch et al. (2021)
Organ sulfonic aryl-silica nanoparticles			10–100 nm	XPS, FT-IR, TEM, SEM	Peixoto et al. (2021)
Magnetic carbon nanotubes	–	Starch	20–50 nm (bundle)	FT-IR, TEM, SEM, Raman analysis, ICP-OES	Goh et al. (2012)
CuO nanoparticles	88	Biogenic waste	13 nm	XRD, EDAX, FESEM, FT-IR	Santha et al. (2019)
Nanoparticles, nanofibers, nanosheets, nanopores	–	–	Pore size 4–40 nm	BET, AFM, XRD, TEM, FT-IR	Verma et al. (2013)

SEM scanning electron microscope, *TEM* transmission electron microscope, *XRD* X-ray diffraction, *XPS* X-ray photoelectron spectroscopy, *FT-IR* Fourier transform infrared spectroscopy, *BET* Brunauer-Emmett-Teller, *UV-vis* UV visible spectroscopy, *EDX/EDAX/EDS* energy-dispersive X-ray spectroscopy, *AFM* atomic force microscopy, *ICP-OES* inductively coupled plasma atomic emission spectroscopy, *H-NMR* proton nuclear magnetic resonance, *TGA* thermogravimetric analysis, *VSM* vibrating sample magnetometer, *GC* gas chromatography.

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Chapter 14
Application of Nanotechnology in Biofuel Production

Sougata Ghosh

Abstract Reduction in carbon footprint in biodiesel has emerged as a potential alternative considering the rapid exhaustion of available natural fossil fuel reserves. In order to overcome the challenges of future energy deficiency, nanotechnology has been employed to develop robust, stable, and cost-effective nanobiocatalytic systems for production of biofuel. This chapter presents an elaborate account on recent advances in nanotechnological application for nanostructured oxides of calcium, strontium, titanium, and iron for catalytic transesterification of edible oil, nonedible oil, and waste oil resulting in generation of biofuel. Also nonmetallic nanostructures such as graphene oxide, carbon nanofibers, carbon nanotubes, and biochar are employed for efficient catalytic conversion of oil to biofuels. Further, microbial lipases from *Pseudomonas cepacia*, *Mucor janaicus*, and *Candida antarctica* are also conjugated with nanoparticulate zirconia, silica, polystyrene, chitosan, and polylactic acid for efficient biodiesel production. Hereby, the role of various nanocatalysts for biofuel production is of utmost significance, and careful optimization of process parameters would enable scaling up of the same up to industrial level.

Keywords Biofuel · Nanoparticles · Feedstock · Transesterification · Lipase immobilization

14.1 Introduction

The rapid rate of urbanization, flourishing industrialization, and increasing populations has led to an upsurge in the energy demands, most of which is obtained from nonrenewable sources. However, overexploitation of the natural resources has led to detrimental effects on the climate and the environment. In the past decades, biofuel has emerged as a promising alternative with the potential role to check climate change, environmental pollution, and replenish the renewable energy resources (Abdullah et al. 2019; Chowdhary and Raj 2020). Some of the important

S. Ghosh (✉)
Department of Microbiology, School of Science, RK University, Rajkot, Gujarat, India

P. Chowdhary et al. (eds.), *Bio-Clean Energy Technologies: Volume 1*, Clean Energy Production Technologies, https://doi.org/10.1007/978-981-16-8090-8_14

parameters in biofuel production include the biomass generation, bioreactor design, optimization of reaction parameters, and selection of appropriate catalytic agents. More recently, nanotechnology gas gained wise attention toward biofuel production because using nanomaterials can significantly reduce the production cost and enhance the efficiency of the whole process (Nizami and Rehan 2018; Chowdhary et al. 2020; Khan et al. 2020).

Nanotechnology has emerged as a potential field where smaller particles with dimensions 1 to 100 nm are used for various applications like sensors, solar cells, pharmaceuticals, cosmetics, food, and agriculture (Robkhob et al. 2020; Karmakar et al. 2020; Kitture and Ghosh 2019; Ghosh et al. 2019). Nanoparticles are synthesized either by physical, chemical, or biological routes (Joshi et al. 2019; Jamdade et al. 2019; Ghosh 2018; Rokade et al. 2018; Ghosh et al. 2016a, b). Silver, gold, platinum, palladium, and bimetallic nanoparticles have tremendous potential for catalysis, free radical scavenging, and therapy (Ghosh et al. 2015a, b, c; Shende et al. 2017; Adersh et al. 2015; Ghosh 2016c, d).

Hereby, nanoparticles with large surface area, exotic shape and size, high catalytic activity, stability, and crystallinity are being fabricated for successful use in processes related to biofuel generation. Thus, metal, metal oxide, and functionalized and hybrid nanoparticles are used in several processes like pyrolysis, transesterification, hydrogenation, anaerobic digestion, and gasification for generation of biogas, biodiesel, and other renewable hydrocarbons.

In this chapter, we discuss various nanoscale materials for production of biodiesel that include oxides of calcium, magnesium, strontium, and titanium. Further, mesoporous and magnetic nanomaterials are discussed which are employed for recovery and reuse in biofuel production. Similarly, the catalytic role of carbon-based nanostructures like graphene oxide, carbon nanofibers, carbon nanotubes, and biochar for biodiesel production is also elaborated as seen in Table 14.1. Finally, strategies like nanoencapsulation and nanoentrapment mediated immobilization enzymes to ensure stability and reusability of the enzymes that are essential for biofuel production are furnished in this chapter.

14.2 Nanostructures for Biofuel Production

14.2.1 Calcium-Based Nanostructures

Transesterification of edible oil like soybean oil to biodiesel (fatty acid methyl ester (FAME)) is brought about by acids, bases, and enzymes. Liu et al. (2008) used CaO as a solid base catalyst for production of low-cost biodiesel. The process has several advantages that include long catalytic lifetime, enhanced activity, and no use of hazardous reaction conditions. It was observed that a 12:1 molar ratio of methanol to oil was more suitable for the transesterification. Moreover, 8% CaO as catalyst and a reaction temperature of 65 °C facilitated the reaction. Further, water content in methanol up to 2.03% was more effective resulting in more than 95% yield at 3 h.

Table 14.1 Role of nanoparticles in biofuel production

Sr. no.	Nanocatalyst	Feedstock	Yield	References
1	CaO	Soybean oil	95	Liu et al. (2008)
2	MgO (nanosheet, conventional nanoparticles, and aerogel)	Sunflower oil and rapeseed oil	98	Verziu et al. (2008)
3	SrO	Soybean oil	100	Liu et al. (2007)
4	$TiO_2/PrSO_3H$	Used cooking oil	98.3	Gardy et al. (2017)
5	Mesoporous calcium titanate	Waste cooking oil	80	Yahya et al. (2016)
6	SO_4/Fe-Al-TiO_2	Waste cooking oil	96	Gardy et al. (2018)
6	Improved and modified graphene oxide	Oleic acid	97.6	Mahto et al. (2016)
7	Carbon nanofibers	Triolein	72	Stellwagen et al. (2013)
8	Sulfonated multiwalled carbon nanotube	Trilaurin	97.8	Guan et al. (2017)
9	Biochar	Canola oil and waste vegetable oil	89%	Dehkhoda et al. (2010)

Compared to calcined K_2CO_3/γ-Al_2O_3 and KF/γ-Al_2O_3 catalysts, CaO had a longer lifetime with no loss in activity even after repeatable use for 20 cycles. No significant reduction in the yield of the biodiesel at 1.5 h indicates the reusability and recyclability of the CaO as excellent catalyst for transesterification.

Initially, extraction of H^+ from H_2O was initiated as CaO solid base catalysts provided basic site that later formed surface OH^- which further extracted H^+ from methanol resulting in the formation of methoxide anion and H_2O. This highly basic methoxide anion effectively catalyzed transesterification reactions. Additionally, extraction of H^+ from the hydroxyl group of methanol by O^{2-} also formed surface methoxide anions that initiated transesterification by attaching itself to the carbonyl carbon atom of the triglyceride molecule resulting to a tetrahedral intermediate. This tetrahedral intermediate, reacted with methanol to form methoxide anion, further picks up an H^+ atom from the surface of CaO leading to the final rearrangement resulting in the synthesis of glycerol and biodiesel.

14.2.2 Magnesium-Based Nanostructures

In another study, Verziu et al. (2008) reported transesterification of sunflower and rapeseed oil by three morphologically distinct catalytic MgO for biofuel formation. A green synthesis approach was followed for the generation of the nanocrystalline

MgO(111) nanosheets (MgO (I)), conventionally prepared MgO (MgO (II)), and aerogel prepared MgO (MgO (III)). MgO (I) was synthesized by employing a sol-gel process involving 4-methoxy-benzyl alcohol template subjected to supercritical drying and calcination in air at 773 K. These nanosheets exhibited lattice fringes with a distance of 0.24–0.25 nm parallel to its main surface. On the other hand, MgO (II) was prepared by boiling commercial MgO in water which was eventually dried at 393 K and dehydrated at 773 K under vacuum. Interestingly, MgO (II) showed interconnected MgO domains about 2 nm with parallel (110) planes with lattice spacing of 1.48 Å. MgO (III) was synthesized by hydrolysing $Mg(OCH_3)_2$ in a methanol-toluene mixture which was then subjected to supercritical solvent removal with simultaneous formation of a $Mg(OH)_2$ aerogel that was dehydrated under vacuum at 773 K. MgO (III) were 1 and 3 nm in size with lattice spacing of 2.1 Å corresponding to the (100) plane. Temperature played a significant role in the transesterification process. At 493 K, conventional microcrystalline MgO catalyst transesterified 80% of sunflower oil resulting in 55% methyl ester. On reducing the reaction temperature to 373 K, the conversion of oil and the methyl ester yield also reduced to 25% and 32%, respectively. The highest catalytic activity for MgO (II) and (III) was observed at 583 K. Microwave and ultrasound facilitated the transesterification significantly.

14.2.3 Strontium-Based Nanostructures

Liu et al. (2007) reported SrO-based solid-based catalyst for transesterification of soybean oil to biodiesel. Calcinations of $SrCO_3$ in a muffle furnace at 1200 °C for 5 h resulted in the formation of SrO. Initially the nanocatalyst was dispersed in methanol followed by addition of 50 g soybean oil that was continuously stirred at 1000 rpm under heating. After 30 min of transesterification, SrO was separated by centrifugation, excess methanol was distilled off under vacuum, glycerol was removed, and biodiesel was recovered. Over 95% biodiesel was produced with SrO as a catalyst within 30 min when temperature was kept below 70 °C. Temperature played a significant role in the biofuel production. At low temperature (55 °C), a slow reaction rate was observed that resulted in only 30% yield after 30 min. However, 100% yield was observed when the temperature was increased to 65 °C. Although the main advantage of high temperature was shorter reaction time, it is to be noted that at temperature beyond the boiling point of methanol, reaction rate decreases due to evaporation of methanol. Another important parameter influencing the yield of biodiesel was the molar ratio of methanol to triglyceride. The yield of biodiesel increased from 1% to 59% as the molar ratio increased from 6:1 to 18:1. Also, the biodiesel yield improved with increasing SrO addition, and the maximum biodiesel yield was increased up to 92% on addition of 3% SrO at 5 min. It is significant to note that SrO catalyst had a prolong activity even after ten cycles indicating long catalytic lifetime and reusability. In view of the background, transesterification of

soybean oil to biodiesel using SrO as a catalyst can have promising commercial viability.

Among various nanomaterials, titanium dioxide nanoparticles (TiO_2NPs) have gained high popularity due to their attractive features that include high chemical and mechanical stability, reusability, wide band gap, acidic nature, high surface area, lower cost, low toxicity, superior redox selectivity, and better availability. Hence, these nanomaterials have got wide range of applications in cosmetics, photocatalysts, solar cells, gas sensors, antimicrobial surfaces, and catalysis.

14.2.4 Titanium-Based Nanostructures

Recently, Gardy et al. (2017) reported a novel, efficient, and recyclable mesoporous $TiO_2/PrSO_3H$ solid acid nanocatalyst for the synthesis of FAME via simultaneous esterification and transesterification reactions from used cooking oil (UCO). Initially, the TiO_2NPs were treated with ammonium hydroxide solution for 30 min that significantly reduced agglomeration and simultaneously enhanced the receptor sites. The treated TiO_2NPs were then washed and dried at 80 °C for 4 h in an oven. Finally, 1 g of the dried TiO_2NPs was reacted with 20 mL solution with 0.1 molar 1,3-propane sulfone in dry toluene under reflux for 72 h at 120 °C. The $TiO_2/PrSO_3H$ was recovered by centrifugation which were washed and dried overnight at 80 °C. Agglomerated particles formed larger clusters as depicted in Fig. 14.1. Such structures were attributed to the interaction between the head groups of $PrSO_3H$ on the surface of TiO_2-NPs. Surface grafting resulted in the enlarged particle sizes of $TiO_2/PrSO_3H$ as indicated by the red arrows. Various shapes of the $TiO_2/PrSO_3H$ nanocatalyst particles included tetragonal, cubic, and hexagonal morphologies with superior crystallinity and size distribution ranging between 8.2 and 42 nm with an average particle size of 23.1 nm. However, the average particle size of initial TiO_2NPs was 22.34 nm.

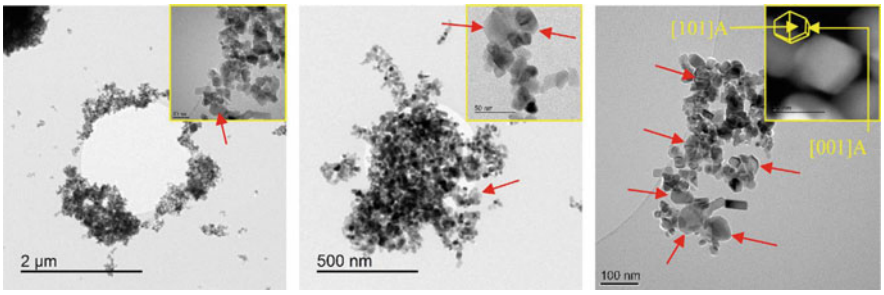

Fig. 14.1 TEM images at different magnifications of synthesized $TiO_2/PrSO_3H$ nanocatalyst. Reprinted with permission from Gardy, J., Hassanpour, A., Lai, X., Ahmed, M.H., Rehan, M. (2017) Biodiesel production from used cooking oil using a novel surface functionalised TiO_2 nanocatalyst. Applied Catalysis B: Environmental 207:297–310. Copyright © 2017 Elsevier B.V

Significantly, 98.3% of FAME was obtained at 60 °C reaction temperature with 1:15 molar ratio of oil to methanol and 4.5 wt% catalyst loading after 9 h. Notably, this one-pot post-surface functionalization strategy with hydrophilic functional groups (–SO_3H) increased the acid strengths of the nanocatalyst significantly resulting in enhancement of acid sites in the reactants. This further improved the accessibility of methanol to the triglycerides (TG)/free fatty acids (FFAs) by increasing the pore volumes/sizes of the nanocatalyst. The TiO_2/$PrSO_3H$ nanocatalyst exhibited high reusability and recyclability.

Mesoporous calcium titanate (MCT) catalyst was reported as superior catalyst for biodiesel production from waste cooking oil (WCO). The surface area, stability, and catalytic performance were strategically enhanced due to the nanostructure with calcium supported on titanate. The synthesis of the nanocomposite was achieved by reacting glacial acetic acid with ethanol followed by drop-wise addition of titanium tetrabutoxide. The resulting white gel was then added to calcium oxide solution that was subjected to autoclaving for 24 h at 150 °C. The resulting material was ovendried for 12 h at 80 °C followed by calcination at 550 °C for 3 h resulting in the formation of the final MCT catalyst which appeared as aggregated flakes with a porous interior. WCO was filtered and mixed with methanol and catalysts and transesterified under varying reaction conditions and ratios of the reactants. The solid nanocatalyst was separated by filtration on completion of the reaction and reused. Around 80.0% biodiesel production was achieved when methanol was reacted with WCO with molar ratio 3:1 in the presence of 0.2 wt % of MCT catalyst for 1 h at 65 °C. The catalyst could be reused without significant reduction of its catalytic potential for five successive runs (Yahya et al. 2016).

14.2.5 Magnetic Nanostructures

In another such study, Gardy et al. (2018) reported the use of magnetic SO_4/Fe-Al-TiO_2 solid acid catalyst for biodiesel production from waste cooking oil (WCO). The catalytic nanocomposite was synthesized by stepwise deposition of alumina and iron oxides onto commercial TiO_2NPs as depicted in Fig. 14.2. In brief, commercial rutile/anatase mixed phase TiO_2NPs were sequentially functionalized with alumina as a buffer layer followed by hematite to impart magnetic character. Further, Brønsted acidity was introduced by sulfation with chlorosulfonic acid.

The size of agglomerated majorly spherical aluminum functionalized titania was approximately 50 nm. Interestingly, exotic shapes with tetragonal, hexagonal, and even core-shell-like structures were also seen. Also anatase truncated octahedron crystallites were observed with a mean particle size of 15–25 nm. The amorphous alumina shell was 3 nm in thickness that encapsulated the titania core. It is important to note that after functionalization of iron oxide, Al-TiO_2 formed denser aggregates with irregular shape and 50 nm diameter which was attributed to the magnetic property of the hematite. Transmission electron microscopic (TEM) images of the sulfonated Fe-Al-TiO_2 that exhibited similar irregular aggregates with 50 nm size

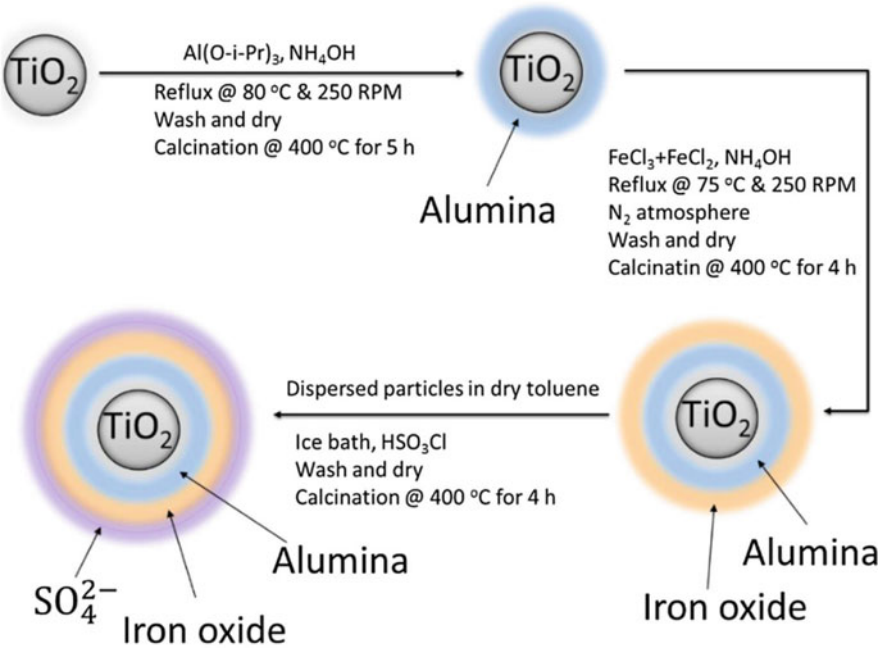

Fig. 14.2 Schematic of SO_4/Fe-Al-TiO_2 catalyst synthesis. Reprinted with permission from Gardy, J., Osatiashtiani, A., Céspedes, O., Hassanpour, A., Lai, X., Lee, A.F., Wilson, K., Rehan, M. (2018) A magnetically separable SO_4/Fe-Al-TiO_2 solid acid catalyst for biodiesel production from waste cooking oil. Applied Catalysis B: Environmental 234: 268–278. Copyright © 2018 Elsevier B.V

and lattice fringes of 0.356 nm which was attributed to the rhombohedral millosevichite form of $Al_2(SO_4)_3$ as seen in Fig. 14.3a. The [110] planes of anatase (Fig. 14.3b), [110] and [012] planes of rutile TiO_2 (Fig. 14.3c) and rhombohedral hematite (Fig. 14.3e) were clearly noticed. Further, Fig. 14.3c, d also confirmed that the core-shell structure of the parent Al-TiO_2 nanoparticles are retained following Fe and S modification.

Biodiesel production was achieved by charging the pretreated WCO with specific amounts of SO_4/Fe-Al-TiO_2 catalyst and methanol in a reactor at room temperature. After 2.5 h, 96% fatty acid methyl ester (FAME) was obtained using 3 wt % of the magnetic catalyst and a methanol/oil molar ratio of 10:1 at 90 °C. The transesterification potential was significantly stable even after ten recycles confirming its reusability and recyclability.

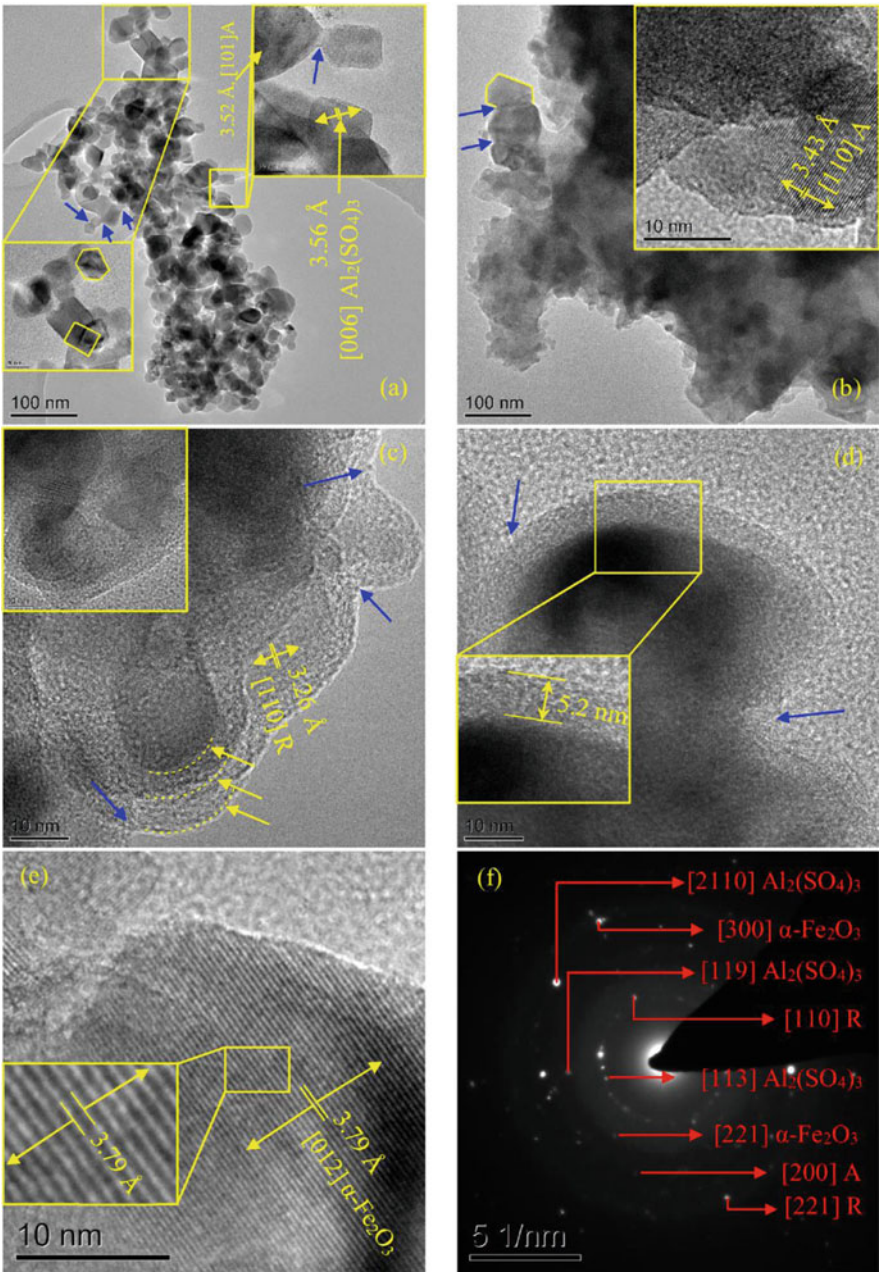

Fig. 14.3 (**a**–**e**) TEM images and (**f**) SAED of SO_4/Fe-Al-TiO_2. Reprinted with permission from Gardy, J., Osatiashtiani, A., Céspedes, O., Hassanpour, A., Lai, X., Lee, A.F., Wilson, K., Rehan, M. (2018) A magnetically separable SO_4/Fe-Al-TiO_2 solid acid catalyst for biodiesel production from waste cooking oil. Applied Catalysis B: Environmental 234: 268–278. Copyright © 2018 Elsevier B.V

14.2.6 Carbon-Based Nanostructures

In recent years, carbon-based acid catalysts referred to as carbocatalyst have drawn significant attention toward biodiesel production. Particularly, sulfonic acid functionalized carbocatalyst which is an active protonic catalyst is promising for biodiesel production. Among various forms of carbon, graphene oxide with attractive physicochemical and optoelectronic properties shows diverse oxygen-containing functionalities such as SO_3H, hydroxyl, epoxy, carboxyl, and carbonyl groups at the edges and its basal plane.

14.2.6.1 Graphene Oxide

Mahto et al. (2016) reported two different graphene oxides as IGO (improved graphene oxide) and MGO (modified graphene oxide) synthesized by modified Hummers' method for esterification of oleic acid with methanol. IGO was synthesized by adding expandable graphite flake into concentrated sulfuric acid followed by mixing for half an hour. Then potassium permanganate was added, and the reaction was allowed at 50 °C for 2 h followed by addition of 70 mL water and final reaction for 15 min at 98 °C for 15 min. The reaction was eventually terminated by adding water and hydrogen peroxide (30%). The resulting solid product was recovered by centrifugation, washed, and dried resulting in final IGO. Similarly, MGO was prepared by slight modification of the method. The nanocomposites were composed of thin-layered structures of IGO with 2–3 nm thickness with specific arrangements forming cavities as seen in Fig. 14.4. It was speculated that the SO3H groups in graphene oxide mediated the esterification that significantly varied with reaction time, temperature, methanol-to-oleic acid ratio, catalyst amount, and catalyst recyclability. Remarkable improvement in the esterification potential was

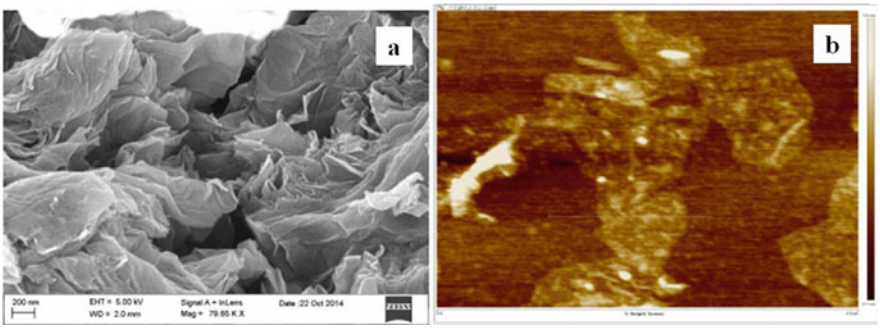

Fig. 14.4 Morphology of IGO: (**a**) FESEM image of IGO, (**b**) AFM image IGO. Reprinted with permission from Mahto, T.K., Jain, R., Chandra, S., Roy, D., Mahto, V., Sahu, S.K. (2016) Single step synthesis of sulfonic group bearing graphene oxide: A promising carbo-nano material for biodiesel production. Journal of Environmental Chemical Engineering 4:2933–2940. Copyright © 2016 Elsevier Ltd

observed with the increase in temperature which was indicated by 97.4% conversion at 100 °C. The highest conversion of 97.6% was obtained at methanol volume of 0.1972 mol, while 89.3 to 97.6% esterification was achieved when the amount of IGO increased from 2.5 to 5 wt%. Transesterification followed a pseudo-first-order kinetics with maximum esterification at 4 h and 90 °C when 8 mL methanol was reacted with 2 g of oleic acid. The catalytic property decreased with the successive reuse. The yield in the first cycle was 96.7% which reduced to 87% and 74% at the second and third cycle, respectively.

14.2.6.2 Carbon Nanofibers

Stellwagen et al. (2013) reported functionalized carbon nanofibers (CNFs) for biofuel formation. Initially, homogeneous deposition precipitation strategy was used for fabrication of a Ni/SiO_2 growth catalyst. Using this prereduced growth catalyst, fishbone-type CNFs were synthesized flowing a $CO/H_2/N_2$ mixture at about 3 bar and 550 °C for 24 h. The resulting product was refluxed, purified, and acid treated to obtain CNFs. CNFs with mesoporous structure served as a better support material that were further functionalized with aryl sulfonic acid groups by employing in situ diazonium coupling. The functionalization was initiated by reacting the CNFs initially with sulfanilic at 70 °C followed by reaction with isoamyl nitrite for 16 h. The final product was then recovered by centrifugation, washed, and dried overnight at 120 °C. This strategy enhanced the accessibility of the active sites to the triglyceride reactants which was attributed to strong acidic sulfonic acid groups. This carbocatalyst composite was used for catalytic transesterification of triolein and methanol. CNF-based catalysts resulted in 72% yield of methyl oleate (MO) after 4 h of reaction.

14.2.6.3 Carbon Nanotubes

In another research, Guan et al. (2017) reported biodiesel production using sulfonated multiwalled carbon nanotube (S-MWCNTs). The high acidity of this solid catalyst was attributed to the polycyclic textural matrix. MWCNTs were reacted for 5 h in a mixture of concentrated H_2SO_4 and HNO_3 (1:1) in the presence of ultrasonication. The resulting nanostructures were washed and dried which were further subjected to sulfonation using H_2SO_4 solution till 2 h leading to the formation of the S-MWCNTs. The MWCNTs with a surface area of 228.1 $m^2\ g^{-1}$ exhibited a pore size ranging from 20 to 45 nm. On the other hand, the S-MWCNTs exhibited a surface area of 198.9 $m^2\ g^{-1}$ and pore sizes ranging from 5 to 35 nm, majority being 10–15 nm. Transesterification of trilaurin in ethanol using S-MWCNTs resulted in a biodiesel yield up to 78% when the reaction was carried out at 150 °C for 30 min. The yield increased to 97.8% upon increasing the reaction time till 1 h. Interestingly, the overall conversion of 90% was observed when the reaction took place for 20 min at 170 °C with a 3.7 wt% catalyst loading and a 1:20 ethanol-to-trilaurin mass ratio.

14.2.6.4 Biochar

More recently, biochar has come up as promising catalytic agents for production of biofuels (Khan et al. 2020, 2021). Dehkhoda et al. (2010) synthesized three biochar-based sulfonated catalysts reacting to concentrated sulfuric acid at 150 °C for 24 h. The resulting slurry of biochar was washed and dried at 70 °C for 1 h. The biochars generated by fast pyrolysis of hardwood (RTI), hardwoods and softwoods, and wood waste, white wood, bark, and shavings (DynaMotive) were named as Cat A1, Cat A2, and Cat A3, respectively. The biochar-based catalysts exhibited a highly irregular, convoluted fibrous surface structure and less regular texturing with discrete pores as illustrated in Fig. 14.5. The biochars were used for transesterification of canola oil and waste vegetable oil in the presence of ethanol. Cat A1, Cat A2, and Cat A3 resulted in 89%, 77%, and 88% conversion, respectively, when transesterification of canola oil was carried out at 60 °C for 3 h with 28:1 alcohol-to-oil (A:O) molar ratio and 5 wt.% catalyst loading.

14.2.7 Lipase Immobilized Nanoparticles

Lipases from numerous sources were reported to be immobilized on nanocarriers that effectively enhanced the activity, stability, and reusability of the enzyme (Kim et al. 2018). Nonmagnetic nanoscale zirconia, silica, polystyrene, chitosan, and polylactic acid were used along with their magnetic counterparts for the immobilization. Among various carboxylic acids, stearic acid exhibited superior activity and enantioselectivity when grafted on zirconia nanoparticles immobilized with lipase from *Pseudomonas cepacia*. Likewise, attachment of silica nanoparticles to ethylenediamine (EDA) was achieved using a coupling agent (glutaraldehyde (GA) or 1,4-phenylene diisothicyanate (NCS)) which were further used for

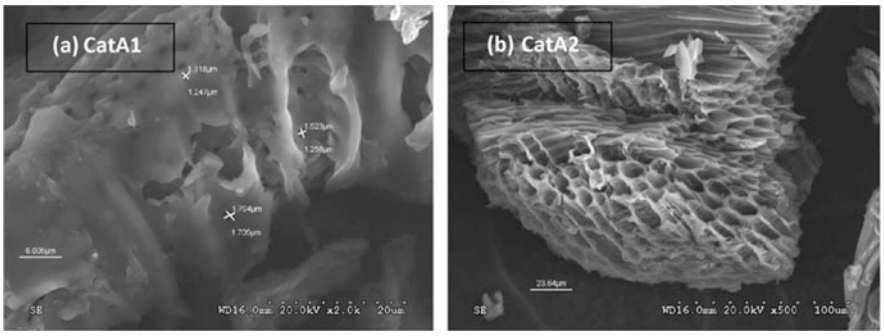

Fig. 14.5 SEM images of biochar-based catalysts. (**a**) Cat A1 indicating pore sizes and (**b**) Cat A2 emphasizing fibrous channels and pore network. Reprinted with permission from Dehkhoda, A.M., West, A.H., Ellis, N. (2010) Biochar based solid acid catalyst for biodiesel production. Applied Catalysis A: General 382: 197–204. Copyright © 2010 Elsevier B.V

immobilization of lipase from *Mucor janaicus*. This immobilization strategy rendered superior pH tolerance and high thermal stability to the enzyme that could benefit during biofuel production. The relative activities of immobilized enzyme were 115% and 107% for EDA-GA and EDA-NCS, respectively. Polystyrene nanoparticles were also used for adsorption of *Candida antarctica* lipase B (CAL-B) which enhanced the stability of the enzyme under varying pH. Immobilized enzyme exhibited 1.81-fold higher activity compared to free enzyme. Various magnetic nanoparticles, particularly γ-Fe_2O_3, after activation with acetyl or amine groups were used for immobilizing lipase from *Candida rugosa* (CRL). The interaction took place with the amine groups of lipases. In some cases poly(glycidyl methacrylate) (GMA) was grafted onto the surface of Fe_3O_4/SiO_x by radical polymerization for further enzyme immobilization for biofuel production.

14.3 Conclusions and Future Perspectives

Nanomaterials have recently gained much attention as alternative catalysts for biofuel production. Both metallic and nonmetallic nanocatalysts have tremendous scope to develop industrial process for generation of biofuel from the renewable feedstock. In this respect, various edible and nonedible oils have been explored for biofuel production. However, the high cost and the concern for using food for fuel are the major obstacles toward the acceptability of biofuel production. Hereby, tracing alternative sources for oil like waste cooking oil and microalgae-derived oil can be exploited for biodiesel production. In the presence of a catalyst, the short-chain alcohols act as an acyl acceptor during the transesterification process. However, high free fatty acid content hinders the alkaline catalysis. Thus, alternative strategy using lipases from bacteria, fungi, and plants can be beneficial when nonedible oil and waste cooking oil are used for biofuel production. Such processes are environmentally benign, cost-effective, low in energy consumption, and broader in feedstock specificity. Further, optimization of the process parameters like oil-to-alcohol ratio, time, temperature, concentration of the catalyst, agitation, etc. should be carefully optimized to enhance the productivity. Efficient scale-up and low-cost posttreatment strategy will enable the implementation of these technologies on an industrial level.

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Chapter 15
Production of Biodiesel from the Bacterial Lipid of Sewage Sludge: Versatile Future of Bioenergy in Developing Countries

Rohit Kumar, Kalpana Sharma, Shaily Chauhan, Ankit Kumar, Piyush Kumar Gupta, Soumya Pandit, Sanchita Bipin Patwardhan, and Srijoni Banerjee

Abstract Production of biodiesel from unconventional methods or renewable resources needs to be exploited for the economic incentives of the future. Biodiesel made from lipids is a green and sustainable form of bioenergy that may be used to replace petroleum-based fuels. Sewage sludge, hospital waste, and dump yards contain a huge number of bacteria, which are a significant source of raw material for biodiesel manufacturing. Lipid extracts from microbes such as bacteria and algae are adequate forerunners for renewable biodiesel productions. However, commercialized scale extraction of lipids from cell biomass required intensive research and understanding of lipid accessibility mechanism and mass transfer approaches. Lipids are obtained from a mixed bacterial population during the treatment of waste from hospitals and wastewater treatment plants (WWTP). The yield cost of biodiesel from bacterial lipid could be higher due to lipid exaction techniques from wet cell biomass. Instead of these conventional lipid extraction approaches, cell disruption and lipid mass transfer are favored because it removes the need of expensive drying approaches. Among lots of lipid-producing microorganisms, the use of oleaginous microbes is preferably significant due to simple cultivation and more production

R. Kumar · S. Chauhan · A. Kumar · P. K. Gupta · S. Pandit
Department of Life Sciences, School of Basic Sciences and Research, Sharda University, Greater Noida, India

K. Sharma
Department of Life Sciences, School of Basic Sciences and Research, Sharda University, Greater Noida, India

S. B. Patwardhan
Amity Institute of Biotechnology, Amity University, Mumbai Campus, Panvel, Maharashtra, India

S. Banerjee (✉)
Advanced Technology Development Center, Indian Institute of Technology, Kharagpur, West Bengal, India

Department of Biotechnology, School of Life Science and Biotechnology, Adamas University, Kolkata, West Bengal, India

P. Chowdhary et al. (eds.), *Bio-Clean Energy Technologies: Volume 1*, Clean Energy Production Technologies, https://doi.org/10.1007/978-981-16-8090-8_15

efficiency. Conventional approaches for lipid extractions are costly, due to which production of biodiesel does not reach the commercialized scale till the twenty-first century. Recent advances in phage cell lysis methods for the secretion of lipids have been highlighted due to intensive researches on bacteriophages. This chapter discusses the challenges and future research needs for expensive lipid extraction techniques and biodiesel generation from extracted lipids.

Keyword Biodiesel · Extracellular lipids · Transesterification process · Bioenergy · Bacterial lipid

15.1 Introduction

Lipid-based biodiesel is the ideal alternative to petroleum-based fuel since it is renewable and harmless. Bacterial lipids can be used as a precursor in the production of lipid-based biodiesel. Lipid-based biodiesel is regarded as one of the safest and most beneficial biofuel forms since lipids may be generated utilizing environmentally friendly and sustainable techniques without the use of hazardous toxins (Singhvi et al. 2014). The demand for the production of renewable nontoxic fuels is becoming enlarged due to the exhaustion of conventional resources of fuels such as petroleum-based fuels and increasing atmospheric challenges because of pollution and CO_2 levels. The biological extraction of lipids using bacteria, fungi, and microalgae has been widely used (Chen et al. 2015a, b; Meng et al. 2009) because the cultivation of these microorganisms is a promising approach for biofuel production. Wax esters (WEs), triacylglycerols (TAGs), and polyhydroxyalkanoates are all lipids that can be used to make bacterial-based biofuels (PHAs). Tags have been utilised as the major raw material in the production of tags.

Transesterification converts biodiesel into fatty acid methyl esters (FAMEs) or fatty acid ethyl esters (FAEEs) (Hwangbo and Chu 2020; Reham et al. 2015). Wax esters are produced by long-chain alcohols and long-chain fatty acids. Many microorganisms such as algae, microalgae, and bacterial species can produce various kinds of lipids (Qadeer et al. 2018; Chowdhary et al. 2020). Many bacterial species have been reported to produce TAGs and WEs (Qadeer et al. 2018; Sharma et al. 2018). Bacterial lipids can be an attractive source for manufacturing low-cost lipid-based biodiesel due to their ease of cultivation and rapid growth rate. The cost of production of lipid-based biodiesel is high due to the use of expensive techniques such as downstream processes. The elicitation of lipids from bacterial cell biomass, which accounts for half of the total energy use in biodiesel synthesis, is one of the most challenging aspects of the process (Dassey et al. 2014; Hwangbo and Chu 2020). The yield cost of biodiesel from bacterial lipid is higher due to lipid exaction techniques from wet cell biomass. The cost of extracting lipids from bacterial cell biomass has decreased as a result of this drop, making it an essential and difficult stage in the manufacture of industrial-based biolipid-based biodiesel. Traditional

extraction methods have several limitations for commercialized scale production of biodiesel such as the following: (1) Separating the solvent from the lipids necessitates a significant amount of energy (Shi et al. 2017). (2) The used solvent is disposed of as a secondary waste stream (Qadeer et al. 2018).

Some bacteria produce a variety of alkanes that can be developed to create biodiesel after being sequestered. Lipids are produced by using fatty acids as precursors in the exponential development of different bacteria. TAGs, which may be isolated from these bacteria and utilized as biodiesel precursors, can also be obtained (Bharti et al. 2014). Biodiesel produced from the lipid extraction of bacteria is nontoxic, and due to low flammability, its uses are nonhazardous. The use of biodiesel is environmentally friendly and does not cause any development to global warming. It overcomes the problem like sulfur and unburned hydrocarbon ejaculation as compared to petroleum-based biofuel (Bharti et al. 2014; Demirbas 2008; Chowdhary and Raj 2020). The increasing demand for biofuel is accomplished by the conventional sources of biofuel such as petroleum, diesel, and kerosene oil. But the major problems associated with the use of these conventional sources of biofuel are limited availability and the increasing verge of peak pollution. The use of biodiesel which is an alternative and nonconventional source of biofuel is the only way to meet this continuous demand for energy (Fernando et al. 2006). It is well known that the transport and the feasibility of machines and engines are dependent on fossil fuel for working. These conventional sources of fuels are CNG (compressed natural gas), diesel, gasoline, and LPG. But emission of sulfur and unburned hydrocarbon from these fossils causes serious hazards to the environment and climate. An alternative fuel to these petroleum-based fossils must be economically competitive, environmentally friendly, and technically feasible. Biodiesel is one of the suitable fuels which have all these characteristics of ideal fuel. Biodiesel is gaining popularity as a blending component across the world, and it has a lot of potential as a replacement fuel for petroleum-based diesel fuel (Demirbas 2017). The cost of feedstock, which accounts for approximately 75% of the total operating costs, is a key reason for low biodiesel output. The high extraction cost of bacterial lipid utilized in biodiesel synthesis raises the cost of production (Fadhil et al. 2015). Some significant and virtuous research is needed for costly lipid extraction methods, and the production of biodiesel from extracted lipids is an important aim. The production cost of biodiesel varies upon many factors such as the geographic location of bacterial cell biomass, labor cost, and lipid extraction cost; hence, low-cost cell biomass and cheap extraction techniques are needed for the production of economically competitive biodiesel. Biodiesel is the best unconventional and nontoxic energy method as compared to fossil petroleum-based fuels, but, currently, biodiesel costs three times more than fossil fuels (Demirbas 2007). When compared to other microorganisms, the major benefit of using bacterial species for biodiesel production is their rapid growth rates. The bacteria *Rhodococcus* has a doubling time of 3–4 h (Young et al. 2012). *Nannochloropsis* has a 2–3-day doubling period when compared to microalgae (Srinophakun et al. 2017). When compared to other microbial species such as microalgae, the cell biomass density of bacteria utilized for lipid extraction for biodiesel generation is quite high (Srinophakun et al. 2017). Due to all

these advantages such as fast growth rate, easy cultivation techniques, and high cell biomass density, bacterial lipid extraction is an ideal source for the production of biodiesel. Sludge from wastewater treatment facilities is a significant source of bacterial lipids; however, the enormous amount of liquid makes it difficult to separate biolipids from other sludge elements using different lipid extraction techniques. Microbial processing, lipid adsorption on sludge particles, and bioreactor design are the most difficult elements, bioreactor design, and regulatory concerns in the production of biodiesel (Demirbas 2017). Sewage sludge contains lots of bacterial species and heterotrophic bacteria that consume the organic waste of the wastewater. Instead of useful substance, sludge of wastewater treatment plants also contains toxic and hazardous substances such as dioxins, furans, pesticides, chlorine derivatives, hydrocarbons, phenolic compounds, and antibiotics (Zhu et al. 2012). Separation and extraction techniques of lipids from these compounds make the production of the biodiesel process costly. Sludge extracted from wastewater treatment plants is rich in bacterial species and organic matter. The first is a two-step procedure that begins with organic solvent extraction and ends with acid-catalyzed esterification or transesterification of the extracted oil fraction. The second is a one-step direct transformation that involves extracting and converting the lipid component from sewage sludge in one step. A heterogeneous acid Zr-SBA-15 catalyst is utilized in both options (Melero et al. 2015). Production of biodiesel through transesterification of TGAs, diglycerides, and phospholipids contained oils and fat. Instead of this, cell membrane of bacterium present in sludge is mostly consisted of phospholipids. Before the extraction of lipids, the treatment of sewage sludge is processed because sewage sludge consists of many organic matter such as vegetables, textiles, woods, etc. After the treatment of primary sludge, wastewater treatment plant sludge is most often free from organic matters and nutritive substances (Demirbas 2017). TAGs/WEs are often synthesized by bacteria when they are stressed, such as when there is a nitrogen deficit combined with an excess of carbon. TAGs may be made from a variety of carbon sources, including sugars, organic acids, and alcohols, as well as lignocellulosic biomass (Hwangbo and Chu 2020). It has been noted that lipid extracted from the bacterium and other microorganisms that are used for feedstock for biodiesel production has many privileges over the use of animal lipids and plant oils for feedstock. By 2016, the worldwide biodiesel industry is expected to reach 37 billion gallons, with a 42 percent annual growth rate. For the next decade or so, Europe will remain the largest biodiesel market, closely followed by the United States (Sims et al. 2010). This chapter is covering the related methods and techniques of extracting lipids with cost efficiency and the production of biodiesel for commercialized scale (Fig. 15.1).

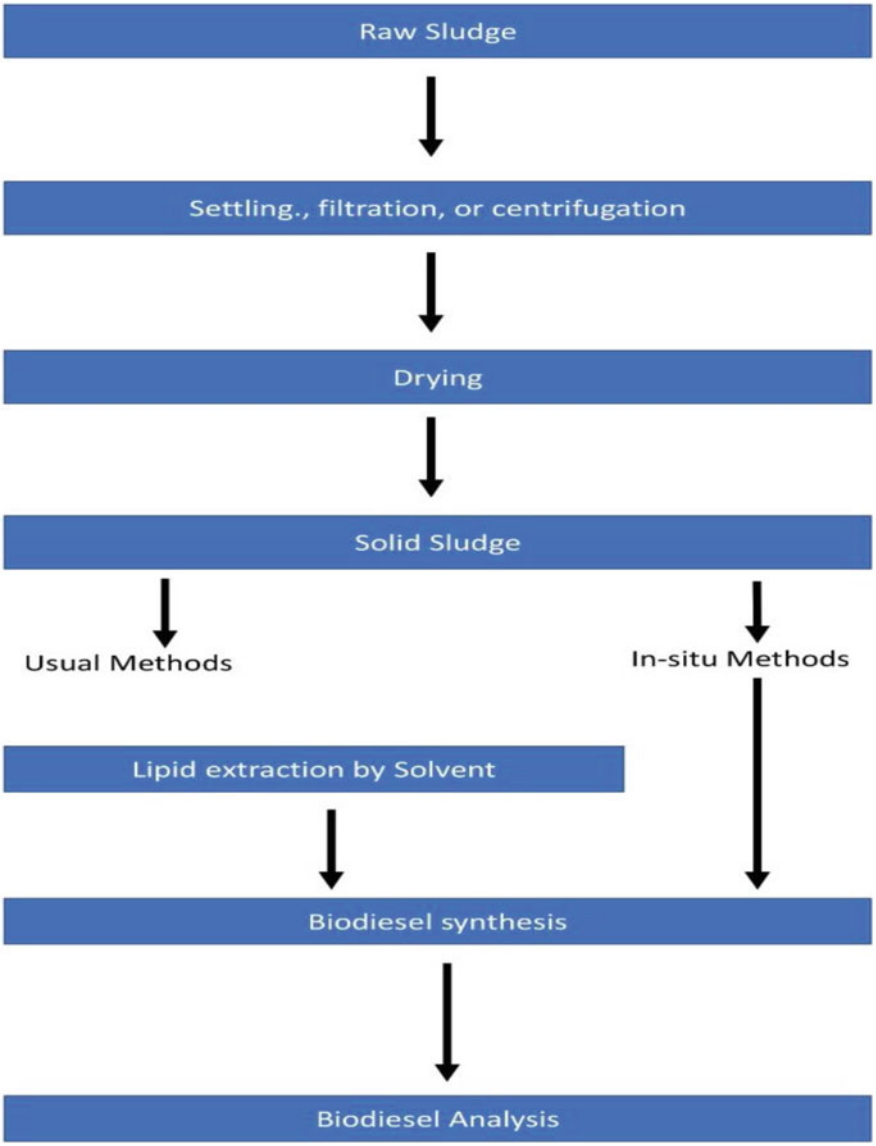

Fig. 15.1 Schematic representation of biodiesel production pathway

15.2 Bacterium Species for Lipid Extraction and Production of Biodiesel

Several bacteria species in our environment catalyze a variety of beneficial processes, including esterification. We need bacterial species with high lipid content to make biodiesel. Because of its high lipid content, *Serratia* sp. ISTD04, a carbon dioxide sequestering bacterium, has been described as a biodiesel feedstock (Kumar et al. 2017). Several immobilization techniques are used in the extraction of lipids, but these techniques are costly and not able to retain the feasibility of the production of biodiesel. Physical adsorption is a very useful method to overcome the problems of several immobilization methods such as expensive, difficult to use, toxicant, and inconvenient. *Serratia* sp. ISTD04 was chosen because of its high lipid content of 64.7%, greater saturated fatty acid content, and palmitic acid as the main components (Bharti et al. 2014). The following transesterification method was utilized to extract lipid content from bacteria: methanol-molar ratio (1:6), catalyst immobilized lipase (100 mg)/immobilized liquid lipase/NaOH (1%), and shaking at 300 rpm for 3 h. The samples were allowed overnight to allow the various layers to settle (Khosla et al. 2017). Another species of gram-positive, aerobic bacteria known as *Corynebacterium rubrum* nov. sp. is capable of producing a high amount of lipids and is highly preferable for the production of biodiesel (Crowle 1962). If activated sludge has a growth yield efficiency of 0.5–1 mg dry weight per milligram of biological oxygen demand (BOD), 1 kg of BOD removed will produce 0.5–1 kg of dry surplus sludge, depending on sludge age. For the extraction of lipid contents from these bacterial species, transesterification and esterification are the chosen methods accordingly. The extracted lipid content depends on the ability of the solvent to permeate biomass of lipid content and solubility of fatty acids (Frkova et al. 2020). *Rhodococcus* sp., like these bacteria, is known to digest refractory organic debris as well as lipid buildup within the cell. In comparison to some fungal and algae taxa, the lipid content of bacteria has a high carbon content (Goswami et al. 2017). The first important step in the production of biodiesel is to remove the water content of the sludge of wastewater treatment plants. Various drying techniques are used to dewater the sludge. Vacuum drying at 60 °C is used to minimize the water content of sludge (Frkova et al. 2020) (Fig. 15.2).

15.3 Extraction of Lipid from Bacteria

Various extraction techniques such as solvent extraction method, cell disruption-assisted method, and biological methods are used for lipid extraction from bacteria. All of these approaches, which range from conventional to innovative, have been identified as a key challenge in achieving commercial-scale production of lipid-based biodiesel (Hwangbo and Chu 2020).

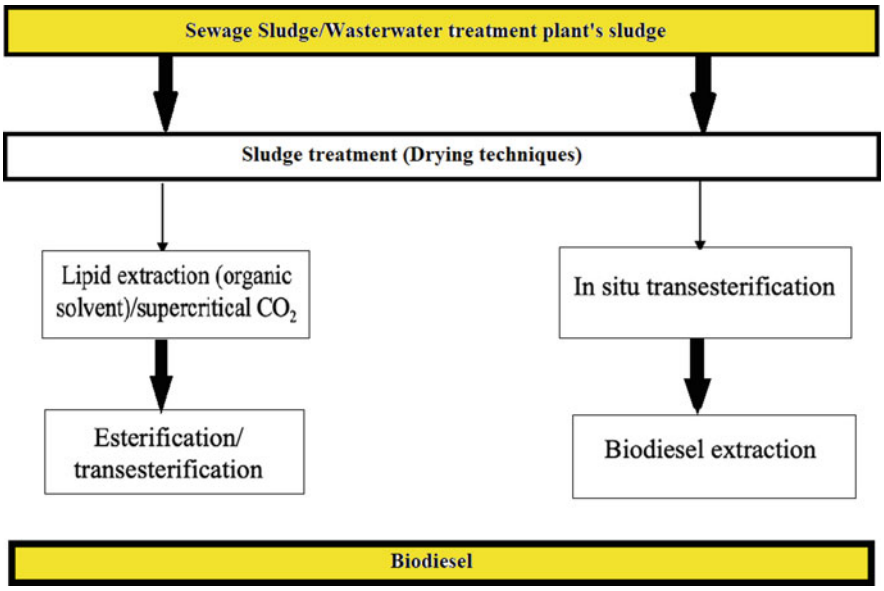

Fig. 15.2 Routes of biodiesel production from sewage sludge and wastewater treatment plants (Frkova et al. 2020)

15.3.1 The Solvent Extraction Method of Lipid Extraction

Biolipids are isolated from the whole bacterial cell using an organic solvent combination with polar solvent. To differentiate neutral lipids from non-neutral lipids, a mixture of polar and nonpolar solvents is necessary. Because the polar lipids of the bacteria's cell wall have such a strong hydrogen bond, low polarity solvents are difficult to extract their neutral lipids alone (Harris et al. 2018). Polar solvents such as methanol and acetone are used to access the strong hydrogen bond present in the cell wall. This polar solvent easily breaks the structure of neutral lipids. Hence, to access the neutral lipids, a mixture of polar solvent and low polarity solvent must be aimed to disrupt the neutral polar lipid complexes (Harris et al. 2018; Kourilova et al. 2020).

15.3.2 Biological Methods of Lipid Extraction

Recent novel biological approaches to release lipids are successfully implemented to increase the extraction efficiency recombinant. *E. coli* MG 1655 strain containing phbCAB genes from *Alcaligenes eutrophus* can thrive on a 2× LB medium with 21% glucose and ampicillin, generating up to 80.2% PHB after autolysis, and has

potential extraction tactics without the need of traditional lipid extraction methods (Hwangbo and Chu 2020).

Only the host bacterial cell may be infected by bacteriophage. In the lytic cycle of bacteriophage infection, the phage takes over the bacteria's cell reproduction machinery and creates its offspring. The capacity of bacteriophages to destroy bacterial host cells, resulting in internal component release and rapid replication, has sparked interest in environmental applications (Correia et al. 2016).

Peptidase, lysozyme, and glycosidase are some of the enzymes that may be employed to break down the bacterial cell wall (Vermassen et al. 2019). Treatment of bacterial cells with enzymatic activity is very effective for extracting the lipid content from bacteria. Lysozyme can hydrolyze the peptidoglycan of the cell wall, so it is a common enzyme for degrading the cell wall of gram-positive bacteria (Salazar and Asenjo 2007). Glycosidase breaks the amide linkage in the peptidoglycan chain of bacterial cell walls, and peptidase cleaves the peptide bonds.

15.3.3 Cell Disruption-Assisted Method for Lipid Extraction

Cell walls and cell membranes are the outer covering of bacteria. For extraction of lipid from bacteria, both of these structures are needed to disrupt by solvents. Gram-positive bacteria have a thin peptidoglycan coating on their cell walls, while gram-negative bacteria have a thick peptidoglycan layer. Lots of chemical solvent treatments and mechanical processes such as ultrasonication and bead methods are used to disrupt the cell wall of bacteria. In bead-beating method, it generates solid compressive stresses on the cell wall components of microorganisms such as bacteria (Patel et al. 2018). The efficiency of bacterial cell disruption can be influenced by a variety of parameters such as temperature, bead size, and bead load. Ultrasonication, in which cavitation and shock wave propagation are employed to break the cell wall, is another technique (Lee et al. 2017). Cavitation and shock wave propagation create pressure on cells during ultrasonication, and cells are ruptured by the sheer force produced by pressure (Lee et al. 2017). Cell disruption of *Rhodococcus* species is a tough process because of the complex cell wall structure.

Cell disruption is also done by chemical-assisted methods such as adding base and solvents. Sulfuric acid (H_2SO_4) is commonly used in methods of lipid extraction due to minimum cost efficiency (Lee et al. 2017). Solvents like dimethyl sulfoxide and methanol are used to remove the lipid components from the cell wall, and these solvents are also very effective for cell lysis (Harris et al. 2018). Various enzymes such as peptidase, glycosidase, lysozyme, and protease are also used for the lysis of bacterium cell walls for lipid extraction (Hwangbo and Chu 2020). The peptidoglycan of the cell wall is easily degraded by lysozyme; thus, it is preferably used to degrade the bacterium cell wall. The amide linkage and glycosidic linkage are degraded by peptidase and glycosidase, respectively. Lipids are a viable source of biodiesel feedstock. However, numerous obstacles such as expensive lipid extraction

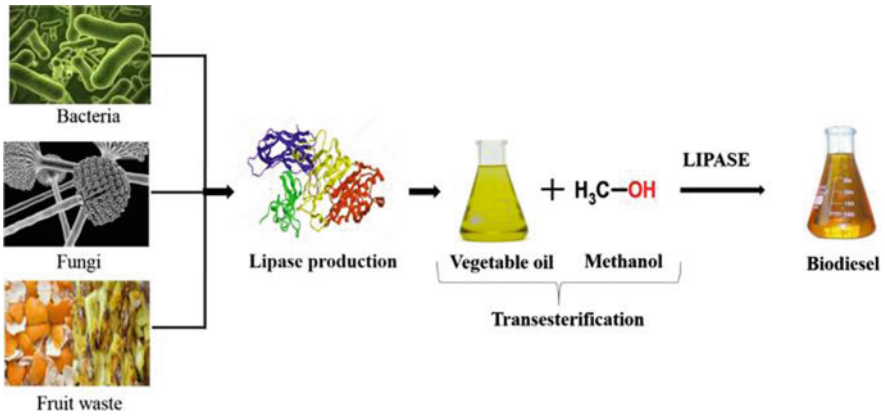

Fig. 15.3 Biodiesel production using fruit waste and microorganisms using transesterification process

techniques, optimal biodiesel synthesis, and bioreactor design are hindering industrial biodiesel manufacturing. All the above lipid extraction methods from bacterium are costly to commercialize scale production (Fig. 15.3).

15.4 Dewatering and Drying of Extracted Lipid

Extracted lipid from bacteria is needed for drying or removal of water before further processing. Cost-effective methods of dewatering and drying for extracted lipid affect the energy consumption and production of biodiesel. Various techniques of drying lipids such as freeze drying, convective drying, and solar drying are usually needed, depending on required products. For dewatering the lipid content, techniques like filtration and flotation are used. Solar drying is a very cost-effective method of drying, but it has the drawback of being time-consuming and having large surface area (Chen et al. 2015a, b). Closed solar drying devices raise the atmospheric temperature from 35 °C to 60 °C and remove the moisture content up to 90% in the end product. Convective drying or hot air drying such as oven-drying methods is also an effective method of drying, although the quality of drying end product from this method may not be stable.

15.4.1 Filtration for Water Removal

The filtration process is used to dewater the lipid biomass obtained from the sludge system. The filter process under pressure seen with a chamber filter press or rotary

drum can recover a relatively large content of lipid from bacterium biomass. The factors which affect the operational process of drying and dewatering are pore size, cross flow, and transmembrane pressure (Chen et al. 2015a, b).

15.5 Bioreactor Design for Maximum Production of Microbial Biomass

Bioreactors are specially designed to increase the production efficiencies of microbial biomass. Hence, the two bioreactors were designed, viz., bubble columns or plate photobioreactors. They can be used in the optimization of the effect of light dilution. Bubble column bioreactor is versatile to use and has economic advantage, i.e., low cost due to simple structure and also low production cost (Schügerl et al. 1977). All of these advantages and characteristics of bubble column reactor are valid for application in the maximum production of biomass. To prevent the effect of sedimentation of microbial cells, mixing is necessary to carry the process of distribution of O_2 and CO_2 (Tanaka et al. 2007). The two important factors for increasing microbial biomass transfer efficiency are shaking and stirring because the shaking process enhances the contact of bacterial cells with air and substrates (Tanaka et al. 2007). Moreover, suitable industrial studies may vary extremely on the applications of several precise parameters. For instance, including improved efficiencies based on optimum bioreactor depth, or integrating bulk nutrient cost economies of scale, has a significant impact on the plant's measured profitability. Simply extending the life of a plant from 5 to 10 years increases productivity while incurring limited additional capital costs. As a result, the conservative conclusion is that biodiesel production systems from bacterium lipid might already be economically feasible using current low-tech approaches (Tanaka et al. 2007). The main challenges are the collection of sludge and bioreactor design for the economic production of biodiesel. The main concern about boosting the production of biodiesel depends on reactor design and type of reactor (Fig. 15.4).

15.6 Effect of Temperature and pH on Biodiesel Production

The optimal pH and optimal temperature lead to the maximum microbial production which is the ultimate result of maximizing biodiesel production. Under the initial high glucose intake, a pH of 7.5 boosted biomass production and lipid synthesis (Sun et al. 2015). The role of pH did not make any significant difference in microbial accumulation in the end product, but optimal pH is necessary for excess biodiesel production. Bacterium species present in wastewater sludge adapts to the optimal pH of (approximately 7.5). It's still unclear if activated sludge grown at an optimum pH

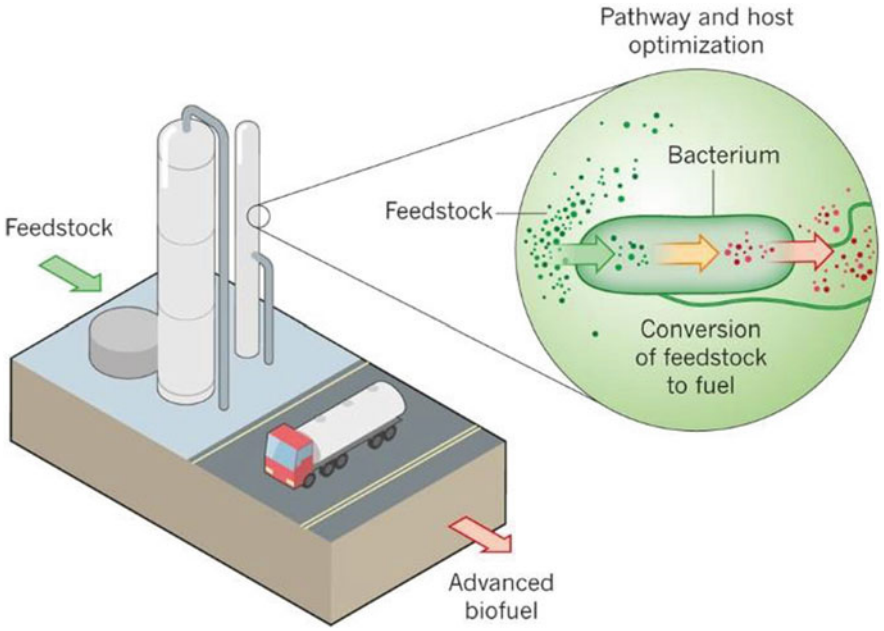

Fig. 15.4 Fermentative production of biofuel from a novel combination of feedstocks and various pathways

or with an unregulated pH would have distinct microbe growth and lipid properties (Sun et al. 2015). Maximum lipid concentration is obtained during the cultivation under controlled pH. It is due to maximum enzymatic activity at optimum pH, and the speed of nutrition uptake on controlled pH also increases. The high lipid yielding was achieved at the temperature of 70 degrees to 120 degrees attain at the 50%–75% of sample dryness (Islam et al. 2014).

15.7 Technological Challenges for the Production of Biodiesel

Production of biodiesel from sewage sludge as per the requirement of commercialized scale poses lots of challenges to overcome. These difficulties include (1) sludge collection, (2) optimal biodiesel production, (3) product quality maintenance, (4) soap generation and chemical separation, (5) bioreactor design, (6) pharmacological ingredients in sludge, (7) regulatory issues, and (8) biodiesel production cost (Kargbo 2010). Techniques, such as transesterification and extraction of lipids and drying for dewatering the sludge, for biodiesel production from sludge have an estimated cost of $3.11 per gallon (Kargbo 2010). Biodiesel produced from extracted lipid is directly used in regular vehicles, but in older vehicles, there is a

Table 15.1 Main challenging elements for biodiesel production

Challenging elements	Causes
Accumulation of sludge	Accumulation of sludge for lipid production can affect the production's cost
Dissociation of lipid content	Centrifugation and filtration
Dewatering and drying	Freeze drying, convective drying, and solar drying are costly processes
Maintaining product's attributes	The increase in production of primary sludge and improvement in the solubility of lipid content
Bioreactor design	Boosting the production of biodiesel depends on reactor design and type of reactor
Extraction techniques (maintenance)	Influence the overall cost of production

need of replacing the fuel pipes and rubber parts to use biodiesel. Instead of all these, several other factors are making the production of biodiesel costly: first, the high cost of extraction of lipid content and purification of lipid. The lipid is purified and characterized using a sequential process that includes ammonium sulfate precipitation, dialysis, and DEAE-cellulose ion-exchange chromatography. These all techniques and biochemical characterization of lipid are very sensitive to organic solvents and detergents and must be performed with precautions. Transesterification with methanol in the presence of catalysts such as acid, alkalis, and lipase is required for biodiesel synthesis (Khosla et al. 2017). Recuperation of these catalysts and biocatalysts for the production of biodiesel poses lots of challenges and economical drawbacks (Table 15.1).

15.8 Scale Up the Production of Biodiesel

Computational tools were used for the modeling of the process scale-up and the different configurations of lipid extraction to optimize this step, as it is the most expensive. The operational variables with a major influence in the cost were the extraction time and the amount of solvent. Sludge produced during wastewater treatment, on the other hand, requires special handling before disposal and is a significant cost in the operation of a wastewater treatment plant (WWTP). Bacterial lipids derived from sludge may be thought of as a low-cost, abundant, nonedible feedstock that can help make biodiesel manufacturing viable. The price of feedstock, which accounts for roughly 80% of the overall operational cost in the production of biodiesel, is perhaps the most important economic element to consider. The high cost of edible oil used in biodiesel manufacturing increased the expense of production. Other significant expenditures are labor and methanol and catalyst pricing. Biodiesel fuel costs vary based on the base stock, geographic location, overall agricultural

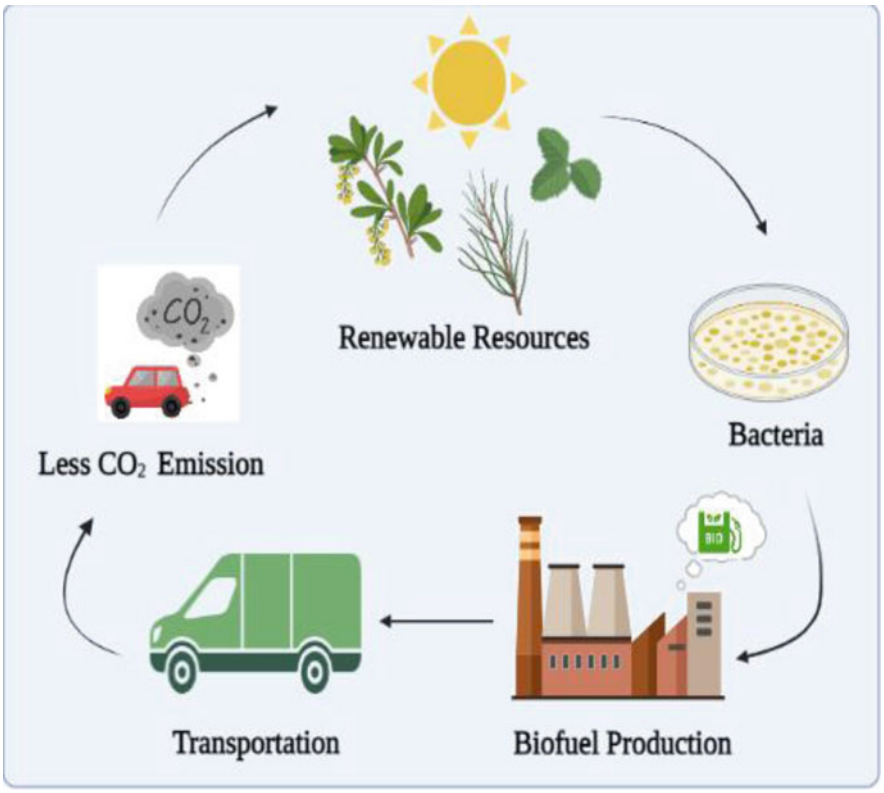

Fig. 15.5 Biofuel production cycle, burning of fuels, renewable sources of raw substances, bacterial growth for lipid extraction

variability from season to season, crude petroleum price, and other factors. Biodiesel made from microorganisms such as bacterial lipids, algae, and fruit waste is not cost-competitive with petroleum-based diesel fuel; therefore, low-cost feedstocks are required. Bacterial lipids, fruit peels, yellow grease, and algal oil are all inexpensive feedstocks for biodiesel production. The algae might be used to make biodiesel and other by-products like animal feed and bacterial lipids. If economic prospects are to be fulfilled, biodiesel manufacturing from sewage sludge faces enormous hurdles. Due to the vast volume of liquid, lipid adsorption onto sludge particles, and the fact that the optimum temperature and other lipid extraction parameters change for the primary and secondary sludge fractions, extracting lipids from sludge poses a difficulty. Collecting sludge, separating lipids, microbial processing, optimal biodiesel production and product separation, soap generation in basic catalytic transesterification, preserving product quality, bioreactor design, biodiesel production economics, and regulatory issues are the key obstacles (Fig. 15.5).

15.9 Toward an Economical Approach

Biodiesel is the most leading source of energy, but its cost and economic approach made its growth uncompetitive to petroleum-based fuels. Alternative ways to the production of biodiesel such as lipid feedstock and sludge waste are compatible and significant to facilitate its growth. This makes the inherence of cost-efficient and reliable feedstock of biodiesel for large-scale production. Many obstacles, such as sludge collection, optimal biodiesel production, product quality maintenance, and bioreactor design, make biodiesel manufacturing a highly significant topic. Since the organization of these operations is essential and needs cost-effective and environmentally acceptable procedures, significant ways for bacterial biomass recovery and lipid extraction have been developed. Advanced techniques such as flocculation methods, optimum bioreactors designing and maintains product quality by affecting bacterial biomass separation and cellular disruption for successful lipid removal from bacteria, intending to produce a long-lasting lipid-based biofuel to boost the bio-economy strategy. Flocculation has become a reliable and cost-effective method of harvesting bacterium biomass. The use of wet condensed bacterial biomass as an oil extraction feedstock is especially appealing because it reduces the need for additional concentration and/or drying techniques. This reduces and removes at least two time-consuming procedures in the lipid-based oil refining process. Disruption of bacterial cells with proper disruption method is very necessary to increase the efficiency of lipid extraction. Pretreatment using chemicals is used on the bacterium biomass to break the cell walls which ease the process of lipid extraction (Patel et al. 2018). The use of thermal or osmotic shock pretreatments, which might result in the release of lipid bodies in the bulk fluid based on the cell wall attributes of the bacteria, may be the most cost-effective and ecologically beneficial alternative. When comparing various extraction methods, prices, scalability, protection, and environmental considerations should all be taken into account. To make bacterial lipid extraction a more commercially feasible alternative that can compete with traditional oil industries, techniques that are not only productive but also safe to function and safe to use must be introduced and developed.

15.10 Conclusion

Sewage sludge can be used as a source of high-quality, low-cost microbial lipids for biodiesel processing, resulting in a more cost-effective operation. The demand for the production of renewable nontoxic fuels is drastically rising due to the exhaustion of conventional sources of fuels such as petroleum-based fuels and increasing atmospheric challenges because of pollution and CO_2 levels. Various extraction techniques such as solvent extraction method, cell disruption-assisted method, and biological methods are utilized for lipid extraction. The yield of lipids and the saponifiable fraction is reduced by popular sludge drying methods. Furthermore,

they necessitate a large amount of energy. Freeze drying, convective drying, and solar drying are some of the suggested alternate lipid drying strategies that are often used to lower the final biodiesel price. These techniques can lessen the negative effects on the environment and lower the costs of traditional lipid extraction methods/techniques. Prokaryotic biolipids have many benefits, since the researcher's analysis centered on developing the more efficient and accurate prokaryotic biolipid extraction method. Future studies should focus on improving lipid extraction performance from these new biological methods for lipid-assembling prokaryotes.

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Chapter 16
Environmental Impact and Economic Benefits of Biofuel Production

Gyanaranjan Sahoo, Afaq Majid Wani, Singam Laxmana Swamy, and Amita Sharma

Abstract Energy crisis and environmental degradation are two major concerns disrupting the growth and economic development of many countries. Within the current context, the search for alternate fuels to fossil fuels that build harmonious connection with sustainable growth, conservation of resources, power, and protection of the environment has become imperative. The quest for biofuels for renewable transport has been stimulated by negative environmental effects of fossil fuels, nonrenewable nature, and looming market prices of crude oil. A biofuel should deliver a net energy benefit, have environmental benefits, be economically efficient, and be manufactured in large amounts to be a viable substitute without impacting the chain of food supplies. Several organisations are promoting biofuels for a number of perceived advantages, targeted to stabilize domestic energy, decrease greenhouse gas (GHG) emissions, balance fossil fuel equivalents, improve economic growth, and alleviate poverty in rural settings. Biofuels if made domestically may result in decrease in fuel imports. In the face of rising biofuel production and use, we are reducing our reliance on imported fossil fuels. If biofuel incentives were expanded, economic disruptions may be avoided. Reducing our demand for fossil fuel may conjointly minimize its value, generating economic advantages for customers, however doubtlessly increasing petroleum consumption, and thereby increase the risk of environmental degradation and also potential danger of rapidly exhausting fossil fuel reserves. Biofuels are green fuels, which are environmentally friendly and economically better fuels for meeting the energy needs on sustainable basis. This article is an analysis of the implications of environmental and economic advantages

G. Sahoo (✉)
KrishiVigyan Kendra, OUAT, Angul, Odisha, India

A. M. Wani
Department of Forest Biology and Tree Improvement, College of Forestry, SHUATS, Prayagraj, India

S. L. Swamy
TCB College of Agriculture and Research Station, Sarkanda, Bilaspur, Chhattisgarh, India

A. Sharma
Krishi Vigyan Kendra, RVSKVV, Gwalior, Madhya Pradesh, India

P. Chowdhary et al. (eds.), *Bio-Clean Energy Technologies: Volume 1*, Clean Energy Production Technologies, https://doi.org/10.1007/978-981-16-8090-8_16

of biofuels over fossil fuels with relevant examples from developing and developed countries in the world.

Keywords Biofuel · Economics · Environmental impact · Greenhouse gas · Pollution

16.1 Introduction

A biofuel is energy produced from biomass by existing processes, instead of a fuel generated by the precise measured environmental progressions convoluted in the establishment of fossil fuels, like oil. Since biomass is hypothetically utilized unequivocally as fuel (e.g., wood logs), the words biomass and biofuel are employed conversely by specific people (Chowdhary and Raj 2020). In any case, for the most part, the word biomass just indicates the living regular asset from which the fuel is delivered or some kind of strong finished result that is thermally/artificially adjusted, for example, frozen pellets or briquettes. Frequently, the term biofuel is utilized for liquescent or fumy energies that are utilized for transport. In the event that the biomass associated with biofuel creation is produced rapidly, the fuel is for the most part considered as a sort of sustainable power (SCOPE 2009; Gasparatos et al. 2011).

Biofuels will deliver replies to present global drive and fiscal crises, each in the form of a sustainable supply of energy and by promoting economic growth, especially in developing countries' rural areas. Dependence on nonrenewable fossil fuels likewise as environmental issues associated with pollution and greenhouse emission effects causative to global warming and climate change has aroused interests of policy-makers and trade to push bioenergy with respect to energy security and environment conversion extenuation methods. However, the growth of the feedstock production for biofuels has been debatable due to possible differing aspect possessions on systematic environments and also the services they supply (Fischer et al. 2009). Ecosystem services are the advantages that individuals derive from ecosystems (Mace et al. 2012) and propose a useful way to measure effects related to biodiversity and energy use and its implications. There's dearth of covenant on the grade to that biofuels each offer affirmative bionetwork facilities (e.g., energy, environmental parameter) and conciliation alternative ecosystem services (e.g., biodiversity, food). Some oppose that biofuel will be carbon-fair in view of all biomass crops accrete carbon (C) to a cautious degree—in a general sense all harvests move CO_2 from over-the-ground course to underground limit in the roots and moreover the incorporating soil.

Though it is assumed that biofuels are more climate-friendly than petro fuels, several arguments are against and support biofuels. In general, biofuels are believed to be C-neutral and fossil-free. However, the literature poses important questions concerning the carbon benefits of existing biofuels (Giampietro et al. 1997; Lal 2004). In true sense, biofuel production also ingests a substantial quantity of energy derivative from remnant gases. Production inputs, including plowing, composts,

insect killers, irrigation, harvesting and transport machinery systems, steam and construction energy, etc., entirely represent remnant drive, resulting in a substantial remaining atmospheric C gained with the time that the biofuel is inevitably consumed (Pimentel and Patzek 2005; Farrell et al. 2006). Consequently, the production of biofuels has non-atmospheric natural impacts such as soil disintegration owing to ploughing, eutrophication due to excessive fertiliser usage, and pesticide effects that harm the ecosystem and biodiversity (Chowdhary and Raj 2020).

The contrary deviations happening within the Cerrado region of Brazil or the rainforests in Indonesian Borneo, which are biodiversity regions, are related to growth of soybean and oil palm plantations, severally (NRC 2011). Indeed, the EU's biofuel directive has already wreaked havoc on forest lands, as Peter Mandelson stated, "Europeans will not pay a premium for biofuels if the ethyl alcohol in their car is produced in an unsustainable manner by repeatedly burning fields once harvests, or if it comes at the expense of rainforests". We won't enable the switch to biofuels to become associate degree environmentally unsustainable stampede in the developing world." In view of such limitations, the actual environmental impact of biofuels is blurred and taxed (Cotula et al. 2008). Biofuels are financially motivated by the fact that they are a cost-effective, low-effort, locally produced oil replacement and require minimal effort, making it constantly becoming more expensive and weird from politically unpredictable regions (Robertson et al. 2014). The inflated interest for agro-industry from biofuels additionally can address the overall issue of declining farmstead returns. Nevertheless negative impacts on food and accordingly the climate are taking steps to counterbalance the beneficial outcomes on government assistance as a fuel source. This, nonetheless, shouldn't be alluring. Seeing anyway biofuels can affect asset obligation, energy and food costs, innovation selection, pay assumption, and so forth and is significant at this frightfully beginning phase of advancement (Dufey 2006).

Biodiesel is trans-esterified from the extraction of lubricants or flabs, and it is the most popular biofuel in Europe. It may remain recycled even by way of pure form (B100) vehicle fuel but is often recycled as a diesel stabilizer to minimize diesel-powered vehicle, dust, carbon monoxide, and hydrocarbons. Consequently, 152 billion litres (40 billion gallons) of biofuel were produced in 2018, up 7% from 2017, and these provided three-dimensional fills for street transportation. The International Energy Agency needs biofuels to satisfy a significant sector of realm of interest for transference fills by 2050, conducive to decrease reliance on oil. Be that as it may, the creation and utilization of biofuels aren't destined for success to satisfy the IEA's practical improvement situation. From 2020 to 2030, global biofuel yield should increment by 100% yearly to prevail in IEA's objective (Chowdhary and Raj 2020).

16.1.1 Biofuel Directives

The Union Renewable Energy Directive 2009 mandated the making of biofuels for countries belonging to it. This has led to agricultural expansion and crop substitution, causing environmental and economic impacts in turn. Environmental impacts are focused on the change in land use, while financial benefits have managed to intensify world crop costs switching these recently established biofuel croplands (Gasparatos et al. 2013).

16.1.2 Arguments Concerning the Invention and Practice of Biofuel

There are numerous communal, cost-effective, conservational, and practical problems with biofuel invention and practice that are mentioned within the prevalent mass media and methodical magazines (Gasparatos et al. 2013). These comprise the delayed consequence of coordinating oil costs, "food versus fuel" battle, food costs, destitution decline potential, energy quantitative association, energy needs, petroleum derivative side effect levels, sensible biofuel creation, removing forests and soil degradation, negative impact on biodiversity and groundwater, attainable changes critical to move the engine on biofuel, and further impact on energy use and its efficiency (Faaij 2006). The International Resource Panel, which gives consistent self-ruling assessments and master proposition on a bargain of source-related items, overviewed the issues about biofuel use and in its preliminary report toward attainable creation and utilization of resources: assessing biofuels. "It was specifically outlined that the more comprehensive and interrelated elements that should be considered when choosing the comparable assets of following one biofuel over another should be considered whenever picking the similar assets of following one biofuel over another should be considered" (Fearnside 2002; Curran et al. 2004). It is everywhere on that not all biofuels perform similarly as to consequence on atmosphere, energy security, and biologic systems and directed that ecological and social effects should be evaluated all through the total life cycle.

The other problem in utilization and development of biofuels is that the USA modified its mandates over consequently production taking longer than anticipated. The Renewable Fuel Standard (RFS) laid down by Congress for 2010 was moved with a target to providing 100 million gallons of pure ethanol at best in 2012 (not emulsified with a fossil fuel).

The environmental benefits of crop rotation include improved weed, insect, and disease management, increased nutrient availability, soil carbon accumulation, and better yields (Gao et al. 2011). In conjunction with higher yields, these edges lead to reducing agrochemical contribution and GHG releases. In addition, the amplified variety of produces engrained in the field (each directly or throughout the year) can reduce the number of chemical applications needed. For instance, combinations that

incorporate grasses and nitrogen-fixing vegetables may limit pesticide application requirements (Gardiner et al. 2010). Giampietro et al. (1997) thought about previous corn, switchgrass, and blended field crops in Michigan and observed that switchgrass and blended grasses upheld superior plenitude of arthropod generalist regular adversaries of yield bugs. Indeed, the standardized harvest change between maize and soybean would allow managers to bother and minimize the insect killer's work by disregarding the example of monoculture irritations and diseases that may be present. The board is likely to add coordinated annoyance to a decline in pesticide production (Fornara and Tilman 2008).

16.2 Environmental Influences

The effects of growing crops for use as biofuels on the environment are complex and widespread. The level of impurities and ecological depletion caused directly by biofuel manufacturing is frighteningly significant, particularly when considering the fact that when promoting how "eco-friendly" biofuels really are, few people take these issues into account (Gasparatos et al. 2013).

16.2.1 Habitat Devastation

If the manufacturing of biofuels harms indigenous species and disrupts ecosystem structure and function, these might be the broad shared elements determining biofuels' environmental consequences. In terms of ethanol generation, in contrast, sugarcane is more efficient; if carbon-rich tropical rainforests are removed to produce sugarcane crops, it results in significant rise of greenhouse gas emissions; thus, the overall efficiency of sugarcane plantations is rapidly decreasing.

If the full environmental advantages of tropical forests are included, e.g., for the conservation of biodiversity, hydrological functioning, and preservation of the soil, such comparisons are even more lopsided.

16.2.2 Trace GHG Emanations from Enrichers

Another typically forgotten product generated from crops that need the use of fertilizers in development, especially N. The most effective biofuel crops, such as maize and canola, require a lot of fertilisers (Hertel et al. 2010). When these penetrate into soil and enter the groundwater, they ultimately reach streams and rivers, the second exposed to the in-flight; these will be converted into a big spring of nitrous oxide, a crucial fume that rescinds stratospheric ozone. This can be called a trace gas emission. A trace gas emission may be called this. Again, when the real

residual greenhouse gases have been merged, measured GHG emissions used in development (Fargione et al. 2008), such crops could be worse than fossil fuels for global warming, not to mention the number of health and environmental threats resulting directly from the use of fertilizers along with loam nutrient diminution and poisonous contamination of confined imbibing liquid (Melillo et al. 2009).

16.2.3 Aquatic Insufficiency

On each continent of the globe, aquatic inadequacy marks one in three people. As water needs rise along with inhabitant development and urbanization and domestic and trade habits increase, the situation is getting worse. At the meantime, the use of water for agricultural production, in particular, is causing the greatest demand for water over the world. This downside may become much worse by supporting a drastic increase in biofuel production (Nelson and Robertson 2008).

16.2.4 Outsized Balance Farming

In order to have a cost-effective supply of fuel, the cultivation of biofuel crops should typically take place on relatively large-scale, trendy plantation farms. The ecosystem is implausibly degraded by this form of agriculture (Rosegrant et al. 2008). The heavy machinery required to collect such large annual crops should not be fuelled by fossil fuels. The techniques of agricultural growing leave the soil implausibly exhausted, resulting in continuous deforestation as additional and additional land is required to produce a similar amount of crop (Royal Society 2008).

16.2.5 Vigor Possessions

Biodiesel is more secure for people to relax. Inspection directed inside the USA shows biodiesel outflows have debilitated degrees of all objective polycyclic sweet-smelling hydrocarbons (PAH) and nitrite PAH mixes, when contrasted with oil diesel fumes. PAH and nPAH mixes are known as potential malignant growth incurring mixes (Gasparatos et al. 2013). Directed PAH mixes were decreased by 75 to 85%, except for benzo(a)anthracene, which was diminished by about 50%. Target nPAH mixes were moreover decreased significantly with biodiesel fuel, with 2-nitrofluorene and 1-nitropyrene diminished by 90%, additionally, the remainder of the nPAH exacerbates to solely follow levels. Those decreases are consequently established truth; the biodiesel fuel contains no sweet-smelling mixes (Shi and Goto 2013).

16.2.5.1 Can Biofuels Simplify Moderate Climate Conversion?

Once the C fuels recycled as an element in its construction are also taken into account, the contribution of various biofuels to sinking fossil fuel depletion differs extensively. The fossil vigor balance of a biofuel attributed to feedstock characteristics, geographic location, farming methods, and therefore the availability of drive cast off to the adaptation system (Gasparatos et al. 2013). Moreover, various biofuels behave differently with respect to their mitigation potential of GHG emissions. Biofuels are only alone among range of alternatives to minimize GHG emissions (Schott 2009). Alternative options may prove less costly, as well as entirely different forms of renewable energy, extreme energy consumption, and exaggerated clean energy, in view of policy goals and restoration, and decreased emissions from clear felling and soil degradation (Turner et al. 2007). Apart from the impact of exaggerated biofuel innovations on GHG emissions, soil, water, and other natural resources differ widely across nations; biofuels, feedstocks and raw materials, harmonious pathways to life-cycle research, greenhouse emission balances, and sustainability requirements are crucial. For all feedstocks, carbon emission balances aren't positive. Investment should be geared into crops with the best positive energy and greenhouse balances at ever low environmental and social prices for climate change functions (UNCTD 2009).

Although the making of biofuels remains scarce in connection with overall energy demand, the current level of agricultural production is significant. It is important to consider the possible environmental and social consequences of its continued development. Diminished carbon issues, for instance, are midst the obvious signs of a few vital drives to support the assembly of biofuels (Rosegrant et al. 2008). Errant negative impacts on terrestrial, wildlife habitat are often one of the results of agricultural invention; however, they're of specific distress regarding biofuels. Moreover, the degree of such disruptions is based on the composition and processing of biofuel feedstocks, the development dimensions, and, in particular, their effect on land-use adjustment, entrenchment, and economic growth (Zhang et al. 2013).

Notwithstanding these probable benefits, in any case, scientific considerations have unconcealed that totally extraordinary biofuels fluctuate wide in their nursery emanation offsets when put next with petroleum. Considering the approaches that will be used to produce the feedstock and the fuel technique, a few harvests will even produce extra ozone-harming substances than do petroleum products. For example, N_2O, a gas outflow with an overall reheating probable around much more greater than that of CO_2, is liberated from N fertilizers (Shi 2012). Similarly, ozone-depleting compounds are transferred at will throughout the development of bioenergy crops and biofuels, including in the formation of composts.

Ozone-harming substances can even be delivered by straight or auxiliary land-use alterations provoked by intensified biofuel creation, for example, once C accumulated in lumbers or meadows, it is emitted from the topsoil all through land change to collect harvests. In fact, maize grown for ethanol will consistently yield 1.8 Mg CO_2

ha^{-1} of GHG investment funds, while switchgrass, a prospective second-age crop, will consistently produce 8.6 Mg ha^{-1} of GHG investment funds; the variation of biome to supply those harvests will release 300 Mg ha^{-1}, and transformation of forest land will release 600–1000 $Mgha^{-1}$ (Cotula et al. 2008). Doornbosch and Steenblik (2007) surveyed the fossil fuel by-products dodged by fluctuated ethanol and biodiesel raw materials created on prevailing farmland (e.g., sugar stick, maize, wheat, and sugar beet for ethanol and oilseed and forested biomass for diesel). By converting agricultural land to forest, a metric ton of carbon would be reclaimed over a 30-year period. It was challenged that on the off chance that the objective of biofuel upholds arrangements is to lessen warming, by then, forest management and refurbishment will be more effective options for the executives and restoration.

Apart from the decisions for tumbling conservatory air transmissions that are shortly being referenced, biofuels are one essential unique—anyway in a few cases rising energy effectiveness and preservation (Zhang et al. 2013), expanding C storage through reafforestation or shifts in rural practices, or abuse elective sorts of environmentally friendly power might be more affordable. In fact, in the United States, increasing average car fuel efficiency by one mile per gallon may reduce ozone-harming chemical emissions by as much as all current US ethanol output from maize combined (Fargione et al. 2008). According to Righelato and Spracklen (2007), the cost of reducing ozone-harming substance discharges through biofuels above US$500 could be as high as US$4520 (sugar beet and maize ethanol) in terms of nursery emanation appropriations per unit of weight in the United States (maize-based ethanol), and thus the cost inside the EU-higher than the market estimation of CO_2 (Zhang et al. 2013).

Enkvist et al. (2007) opined that nearly simple procedures to reduce energy consumption, as higher fortification of late edifices or intensified capability of heating and freezing structures, have lower energy emanation costs of however €40 per Mg. Both the scientific and strategy measurements of practical bioenergy advancement are developing rapidly (practically consistently). To ensure that bioenergy crops have a synergistic and meaningful influence on climate security efforts, a thorough understanding of the key concerns, including land-use change and proper assessment of GHG emission adjustments, is required. The intricacy of things about land-use adjustment has intersection rectifier to its exclusion from most bioenergy life-cycle examinations anyway as it continues to be a significant bit of knowledge that administrations should mull over in planning public bioenergy strategy (Zhang et al. 2013).

16.2.5.2 What Variations to Farming Property Would Biofuel Production Necessitate?

In the newest modes of the production and method of energy crops, economic effects are made, but processes associated with land-use change and intensification appear to predominate (Chowdhary and Raj 2020). In the subsequent decade, a fast, strategic increase in want for biofuels is likely to gear up the transformation of

nonfarm land into biofuel land. It might arise invariably in the growth of biofuel feedstock, as well as by chance in the case of replacement products emigrating from standing crops (Zhang et al. 2013).

Yield will rise, and cautious employments of data sources shall be fundamental components in mollifying land-use pressure from each diet and essential produces (Robertson et al. 2014). Devoted examination, resource in apparatus, and solid foundations and framework are needed. Possible outcomes vary widely between feedstocks, production methods, and geographic locations, and are largely dependent on how land-use change is implemented. Converting perennial feedstocks (such as oil palm, *Jatropha*, or perennial grasses) for annual yields would improve soil C adjustments (Gasparatos et al. 2013).

In the last five decades, much of the growth in income of foreign agricultural trade products (about 80%) was derived from increased yields, with the remaining augmented by crop acreage and cropping intensity (Fischer et al. 2008). The speed of growth in want of biofuels has so far exceeded historical growth rates in market for commodities in agriculture and crop yields over the past few years. This means that alteration of land use, and thus the related environmental effects, could become a major problem for first- and second-generation technologies (Zhang et al. 2013). This demand could also increase in the short run, by growing the area covered by biofuel crops, whereas within the intermediate and extended run the event of enhanced biofuel harvest diversities and vagaries in science live out and novel skills (conversion of cellulose) could initiate to dictate (Robertson et al. 2014). Increased yield opportunities and innovative developments could be fundamental for the economical making of biofuel feedstocks to diminish quick land-use alteration in zones effectively under development and consequently the variation of land, not consequently in produce harvestings, similar to field or forest land. Of the general territory of place where there is 13.5 billion ha in the nation, generally 8.3 billion ha are well under prairie or forest, and 1.6 billion ha are in other landscapes (Hazell and Wood 2008).

A large part of the land in green area, ground or elective uses, offers significant ecological types of assistance, along with carbon confiscation, rainwater filtration, and diversity protection; thus, development of harvest conception in these territories may be prejudicious to the climate. After removing wooded areas, endangered zones, and assets required to meet increased demand for food and domesticated animal products, estimates vary from 250 to 800 million hectares of land that will be available for prolonged agricultural harvest, with the majority of this land located in Latin America or Africa (Cassman et al. 2005).

A portion of this land may be utilized unswervingly for biofuel feedstock formation; however, expanded biofuel creation on existing cropland may conjointly produce movement in the creation of non-biofuel harvests somewhere else (Robertson et al. 2014). Increased maize conception for ethyl alcohol in the central United States, as one, has uprooted soybean on some present agricultural fields, which could actuate enlarged soybean creation and change of plot or forest land somewhere else. Accordingly, each immediate and aberrant land-use change

brought about by extended biofuel creation should be contemplated for handling expected ecological effects (Hertel et al. 2010).

16.2.6 Amplification

Though zone development for biofuel feedstock creation is probably going to assume a promising job in fulfilling expanded interest for biofuels over following not many years, the rigorous practice of land use has made enhanced advancements, and the executive practices can be constrained to supplement this determination, especially if creation is to be supported inside the since quite a while ago run. Grain yields have typically been higher in densely populated Asia than in Sub-Saharan Africa and Latin America, and paddy and wheat yields have been higher than maize yields. A broad range of public and individual interest in research on increasing hereditary materials, information and water usage, and genomic resources has fought a functional part presently aiming at these income successes (Fischer et al. 2008).

Similarly as enlarged solicitation for biofuels triggers quick and indirect changes in land use, yield changes, each straightforwardly inside the production of biofuel feedstocks and by suggestion inside the making of elective yields, can likewise be foreseen to inspire varieties, given that adequate ventures are made to create framework, innovation, and admittance to data, evidence, and arcades (Robertson et al. 2014). A few insightful examinations are getting down to survey the adjustments in land use normal from expanded biofuel request; however there is practically no experimental evidence on that to put together projections on how yields will be changed—either directly or indirectly—or how quickly yields would be influenced. In one model, ethyl liquor advisors in Brazil accept that, even while not hereditary advancements in sugar stick, produce will increase inside the shift of 20% may be accomplished over following 10 years only over improved administration in the development succession (Nelson and Robertson 2008).

16.2.7 How Will Biofuel Invention Distress Water Properties?

The rapid expansion of agricultural assembly frameworks for biofuel feedstocks, as well as the resulting modification in existing agriculture lands, can have ecological consequences, including effects on ozone-depleting material emissions. The kind and extent of those impacts are dependent on criteria such as the size of the creation, the type of feedstock, the development and land-the-board performance, the region, and the downstream cycle courses (Robertson et al. 2014). The remaining sections of the sign are restricted to impacts that are specifically linked to manufacturing; however, the vast majority of the issues resemble those generally identified with farming creation—H2O weariness and pollution, loam deprivation, supplement

diminution, and therefore the deficiency of untamed and agrarian diverseness (Chowdhary and Raj 2020).

Water, rather than property, deficiency could convincingly be the basic restricting issue for biofuel feedstock creation in a few prospective. Approximately 70% of the fresh material removed is used for farming drives. As a result of increased competition with local or mechanical jobs, water resources for agribusiness have become increasingly scarce in a few countries. In addition, the expected consequences of temperature fluctuation on reduced precipitation and overflow in several major producer provinces (including the Near East, North Africa, and South Asia) might effectively place additional strain on limited resources.

Enduring plants like *Jatropha* and *Pongamia*, which can be filled in semi-parched regions on minimal or debased terrains, can require some water system during sweltering and dry summers. Moreover, huge amounts of water, mostly for cleaning plants and seeds and for cooling, will be utilized to transform feedstocks into biofuels (Robertson et al. 2014). Be that as it may, the flooded yield of these essential biofuel feedstocks would have the best impact on the equilibrium of nearby supplies of water.

Water quality, as well as quantity, can be affected by delivering a huge amount of biofuel production. Transforming the fields or forests into maize fields could probably crop up the fuel issues like dissolving, sedimentation and excess supply of N and P that spill over into surface waters, and infiltration into soil water from increased fertilizer application. The oxygen-starved "no man's land" inside the Gulf of Mexico, where a few types of marine life can't survive, might be attributed to an abundance of gas in the stream system. Runge and Senauer (2007) contend that as maize-soybean revolutions are uprooted by maize, edited ceaselessly for plant item creation in the USA, significant increment in gas compost use and overflow can intensify aforesaid issues.

Bioethanol and alcohol making prompts naturally the untreated wastewater released, which may increase eutrophication of water bodies. Notwithstanding, current wastewater treatment advancements will manage natural contaminations and squanders (Melillo et al. 2009). Aging frameworks will decrease the organic oxygen interest of sewer water thru quite 90%, all together that liquid is reused for cycle, and methane arrangement is caught inside the treatment framework and utilized for power age. As respects the dissemination and capacity periods of the cycle, therefore ethanol and biodiesel are transitory; when petroleum derivatives are used quickly, the likelihood of unwanted properties on soil and rainfall from drainage and spills is reduced (Robertson et al. 2014).

16.2.8 How Will Biofuel Creation Distress Dirt?

Mutually property reclamation modification and augmentation of farming creation on standing terrains will have notable antagonistic effects on earth; anyhow these effects—even concerning any yield—depend basically on farming practices.

Unsatisfactory development practices will decrease dirt living material and increment destroying by eliminating lasting loam refuge. The expulsion of plant buildups will lessen soil supplement substance and increment gas discharges through misfortunes of soil carbon (Robertson et al. 2014).

Yet, at that point, protection culturing, crop pivots, and diverse improved administration practices will, under the right circumstances, diminish opposite impacts or maybe advance natural quality related to extended biofuel feedstock creation (Cotula et al. 2008). Developing perennials, for example, palms, short-pivot vegetation, sugar stick, or switchgrass, rather than yearly yields would boost topsoil class by expanding soil protection and natural carbon intensities. Idealistic impacts on biodiversity are accomplished related to nonlaying and diminished fertilizer and pesticide inputs (Erixon 2012).

Different feedstocks have different soil effects, augment demands, and, as a result, the land area they need. Soil class is supported by the use of supplements from sugar plant and mechanical plant wastes; however, mistreatment of extra pulp by way of drive contribution to ethyl liquor creation would diminish its use. Inside and out creation frameworks need reutilization of deposits to reuse supplements and keep up soil ripeness; generally, exclusively 25–33% of accessible harvest deposits from grasses or maize is gathered reasonably (Righelato and Spracklen 2007). By making a commercial center for rural deposits, broadened interest for energy may, if not appropriately accomplished, redirect deposits to the muster of biofuels, with presumably biased consequences for topsoil superiority, especially on soil natural issue (Doornbosch and Steenblik 2007).

Hill et al. (2006) initiated that the assembly of soybean for biodiesel inside the USA needs a great deal of less manure and insect killer per part of vigor created than ensures maize. They contend, notwithstanding, that every feedstock requires more significant levels of information and preferred land quality over second-age feedstocks, for example, switchgrass, woody plants, or various combinations of grassland swards and forbs. Lasting lignocellulose harvests, e.g., eucalyptus, poplar, willow, or grass, require less serious upkeep and less contributions of energy and can even be totally developed on degraded land, while soil C and quality will in general increment over the long haul (IEA 2011).

16.2.9 How Will Biofuel Production Distracts Biodiversity?

Biofuel development will have a positive effect on wild and agricultural biodiversity in some respects, but many of its impacts will be negative, such as through the regeneration of degraded lands; as an example, once usual ecosystems are turned into energy crop homesteads or vegetable, mulch plots are sapped (CBD 2008). Consequently, unoccupied biodiversity is threatened by the loss of its environment as the world below yield creation expands, although rural diverseness is susceptible within the event of extensive mono-cropping that relies on a slim pool of genetic material and might conjointly cause abridged practise of outmoded diversities.

The first pathway for diversity misfortune is living space misfortune following area transformation for crop creation, as an illustration from timberland or meadow. According to CBD (2008) synopses, a few current crops of biofuel are very much coordinated for tropical regions. This would increase the financial incentives for countries with biofuel production on the horizon to convert natural habitats into feedstock farms (such as oil palm), resulting in a loss of wild biodiversity in these areas. Whereas oil palm homesteads don't want a lot of fertilizers or pesticides, even on poor soils, their enlargement will cause damage of tropical forest. While the damage to natural ecosystems has been calculated in various countries, acreage adaptation for the growth of biofuel feedstock has not (CBD 2008), there is still a lack of knowledge and analysis needed to determine its reach and implications. Nelson and Robertson (2008) observed however rising good costs instigated by enlarged biofuel demand might persuade farmland modification and augmentation in Brazil and located that agrarian enlargement focused by advanced costs might imperil ranges made in bird class variety.

The second significant pathway is the loss of agro-biodiversity, initiated by boosting on pastures, inside the kind of harvest hereditary consistency. Most biofuel feedstock ranches upheld one species. There additionally are contemplations concerning low degrees of hereditary variety in grasses utilized as feedstocks, similar to sugar stick (The Royal Society 2008), that will build the status of those yields to new nuisances and infections (FAO 2012). On the other hand, the opposite is valid for a harvest like class *Jatropha* that has an especially serious level of hereditary variety, the majority of that is unchanged, prompting an expansive change of hereditary qualities that subvert its business cost.

A number of the supported groups are listed as invasive species with respect to second-generation feedstocks, creating new considerations on a way to handle them and prevent unmotivated consequences. In addition, some of the carbohydrase required aimed at its transformation are inherently engineered in order to improve their efficacy and can be fastidiously accomplished via locked trade fabrication procedures (Tilman et al. 2006).

Optimistic possessions on diversity are renowned in sullied or minimal regions wherever novel perpetual diverse classes are familiarized to revive environment effectively and increase diverseness (Soyka et al. 2007). Empirical information from check plots on despoiled and relinquished soils indicate that cost-effective high-variety combinations of local meadow perennials—which supply a spread of environment administrations, just as natural life territory, water aeration, and energy storage—jointly generate higher net energy gains (estimated as burning energy released), lower discharge of ozone-damaging substances, and less crop cultivation pollution than maize ethanol or soybean biodiesel, which will increase with the total number of organisms.

16.3 How Could a Biologically Justifiable Biofuel Invention be Certified?

Institutional approaches toward principles and verification may not be the first or best option for certifying widespread and equitable participation in biofuel production. Living things that combine best practise and capability erection may produce better short-term results and provide the tractability needed to adapt to changing environments. Expenses aimed at conservational amenities can additionally signify a tool on behalf of inspiring obedience by justifiable production strategies:

- It is important to treat biofuel feedstocks and various diet and agrarian crops equally. Conservational concerns surrounding the production of biofuel feedstock are constant with regard to the effects of improved pastoral invention as a whole; steps to confirm imperishable should therefore be routinely extended to any or entire produce.
- Effective agronomic systems, such as farming systems, will minimize the C footprint and also the negative environmental effects of the production of biofuels, as these will usually be for in-depth farmed invention. The perennial trees and grasses enhance diversity of production base and enable minimal or sullied soils to be enriched.
- Internal administration strategy should teach us about the global outcomes of biofuel improvement. Practical and conceivable biofuel commitments and targets will be encouraged by worldwide discourse, usually through existing components.

16.4 Certifying Environmentally Defensible Biofuel Invention

16.4.1 Decent Observes

Moral reviews are expected to utilize open data to deal with the manageability measurements of on-ranch biofuel staple creation, harvest, and cycle. In addition to the life-cycle examination used for gas outflows, this argument relates to everyday capital the board concerns like land, soil, water, and variety, and determines if a certain biofuel is more friendly than a fuel environmental transition. Soil, water, and crop insurance in fair positions, the executives' energy and water, the board's supplement and agrochemical, variety and scene protection, and selection, cycle, and appropriation all consider some genius place practices that are required to deal with manageable improvement of bioenergy as a real part of the territories.

Protected agriculture is one example of how unfazed circles have created a viable and profitable agroindustry for agrarians and rural people by employing little soil disturbance, continuous natural soil cover, and diversified crop pivots. Concerning

present focus on carbon stockpiling and on advances that decrease energy force, it looks especially satisfactory. Also, the strategy ends up being conscious to things anyplace where work is scant, and there is a requirement for them to save soil wetness and ripeness. Interferences such as automated topsoil plowing are minimally simplified, and interventions including agrichemicals and mineral or organic supplements are applied at an optimum level.

16.4.2 Regular Sustainability Measures and Amenability

Despite the fact that the various ecological effects of bioenergy advancement don't differ meaningfully from those of various kinds of cultivation, the inquiry survives from anyway they will best be surveyed and reflected in field exercises. Existing natural effect appraisal procedures and vital ecological measures give a respectable starting line to dissecting the biophysical factors (Sahoo and Wani 2020). There furthermore exists an abundance of specialized data drawn from rural improvement all through the previous 60 years. New bioenergy pledges incorporate logical structures for both bioenergy and food security; work on the blend ecological effects, along with soil acidification, unreasonable plant food use, and variety of misfortune, contamination, and compound noxiousness (Doornbosch and Steenblik 2007); and work on community and natural property standards, along with limits on deforestation, rivalry with food creation, and unfriendly effects on various dissolving and supplement actions (Faaij 2007).

The biofuel segment is characterized through a large variety of participants with numerous benefits. This, shared by the speedy growth of the world, has resulted in a creation of enterprises to confirm viable bioenergy improvement. Among several personal and civic teams, morals, principles, and needs are taken into account alongside acquiescence devices to assess performance and guide the world's development (Zhang et al. 2013). The Roundtable on Sustainable Biofuels include a range of public, personal, and nonprofit initiatives (UNCTAD 2008). Such miscellany proposes that a method for harmonizing the assorted approaches could also be required, particularly within the lightweight of strategy directives and goals that assist to inspire additional energy invention.

The vast majority of the groups are as of now being created in industrialized nations and are intended for verifying that biofuels are made, conveyed, and used in an ecologically supportable way before they're recorded in worldwide business sectors (Shi and Goto 2013). The EU Commission, for example, has just arranged models that allow to be viable with the World Trade Organization rules (European Commission 2008). Nonetheless, to this point, none have however been tried, especially related to government uphold plans like endowments or once chose for invaluable treatment beneath worldwide economic deals (Zah et al. 2007).

The expression "guidelines" infers thorough frameworks for action boundaries against defined measures, during which inability to obey would thwart a nation from sending out its item (Mosnier et al. 2013). Such globally joint frameworks exist

currently for a spread of sanitation, compound, and human well-being subjects. Is the biofuel area sufficiently created for evolving such a framework, and are the dangers sufficiently pleasant that its nonappearance would present huge, irreversible dangers to human well-being or the climate? Should biofuels be dealt with extra carefully than various agrarian products?

Also, for example, just if most normal impacts of biofuels are vague from those of extended farming origination overall, it might be battled that identical standards ought to be applied in all cases. Likewise, limiting territory use modification may confiscate open entryways for nonmechanical countries to benefit from extended interest for country items (Chalmers et al. 2011). On the other hand, there are sturdy conflicts that agrarian producers and procedure makers should acquire from earlier mistakes and sidestep the deleterious standard influences that have went with country land change and were heightening already.

In the 2008 release of *The State of Food and Agriculture*, sections for natural administrations were personally talked about. This agreement might compensate farmers for specific services that misuse the generation in more sensible ways. Chapters can be coupled to consistence with norms and certification plans joined at the worldwide level. Payment plans for natural administrations, however troublesome and modern to execute, might speak to an additional apparatus to affirm that biofuel territory unit was made in a really maintainable way.

16.4.3 Probable Ecological Properties of Accumulative Biofuel Fabrication

It gives a blueprint of the life-cycle appraisal procedure for the most part used to survey natural aftereffects of creating and utilizing biofuels:

- It analyzes this condition of information with respect to key ecological impacts. In terms of processing feedstocks, converting them to fills, and burning them, all environmental outcomes are listed, if appropriate. Effect estimation techniques and even the expected outcomes or determined results conveyed in the disclosed literature are conferred. Gaps in information handiness and shortages in winning demonstrating stages, everything about adds to vulnerability in surveying natural impacts, additionally are recognized inside the accompanying regions:
  - GHG emanations
  - Air superiority
  - Water superiority
  - Water amount and immoderate use
  - Soil
  - Biodiversity
  - Environment administrations

- For instance, local conservational valuations of biofuel creation are utilized on the grounds that the impacts of biofuel creation are explicit and the discoveries are taken from local ecological appraisals that appear differently in relation to the wide running state assessment of added substance impacts.

16.5 Environmental Aids and Costs

16.5.1 Prospective of Sinking GHG Emanation

The obvious plausible GHG decrease of biofuels was tested by the phenomenally high GHG emanations once high C stock grounds like tropical woodlands or peatlands are recovered to farming terrains. Numerous examinations taking into account the change of such grounds to oil palm manors in Southeast Asia have indicated that the "carbon obligation" made by the underlying release of GHG outflows all through land clearing will require many years or possibly hundreds of years to repay. However, the quick conversion of high C stock grounds to rural terrains for the production of biofuels raw materials is required (alluded to as immediate changed nature), as well as the improvement of farmland from, for example, the production of food crops to the growth of biofuel feedstock, will allow high C terrains to be dislocated by food yields to pay for the reduced production of food crops (Erixon 2012). This has been a topic of a great deal of conversation, especially versus the count method and vulnerabilities worried inside the assessment. On the other hand, considering land and by item/buildup/squander the platform, as well as avoiding aggravation of high carbon stock grounds, would significantly reduce GHG emissions.

In this way, it's critical to examine life cycle implications alongside land use changes, each immediate and actuated, to guarantee that the points of interest, e.g., decrease GHG emissions, are in a real sense accomplished. Be that as it may, measuring the capacity of diminishing GHG, in genuine circumstances, could well be tricky (Gheewala 2011). A new report has demonstrated that techniques and stock information regularly control the estimation of GHG emanations. Another important consideration includes perhaps, even if GHG reductions are always accomplished, what is the significance of such reductions and whether there are other options that are less expensive. Some ethanol studies in Asian countries, for example, showed that once ethanol blends with hydrocarbons, only nominal GHG reductions were achieved. However, the values of the decreases weren't positive by way of association with several different choices like cogeneration and energy potency (Fargione et al. 2008). In general, using biomass for heating and generating electricity might increase value. Moreover, aside from producing biofuels for the automobile sector, land productive ways to reduce GHG emissions; particularly if coal is the fuel substituted.

16.5.2 General Environmental Effect

Creation and utilization of biofuels are frequently helpful to some natural characteristics and asset base and have unfriendly belongings for other people. Consequently, the natural impacts of biofuels can't be fixated on a couple of environmental limitations (for instance, GHG emanations). A valuation of generally speaking natural outcomes needs a framework procedure that considers fluctuated ecological impacts at the same time utilizing a setup of pointers. Such evaluation would get to be led across spatial measures as consequences of certain impacts are restricted though others are territorial or worldwide (Gheewala 2011). A framework appraisal of natural impacts would add to building up a biofuel industry that adjusts commutations and diminishes undesirable effects.

Despite the fact that using biofuels has the potential to provide net environmental benefits as compared to using oil-based energy sources, the ecological result of biofuel creation can't be secured, while no scene besides biorhythm image of any place and the way the bioenergy feedstocks are completely developed satisfies the RFS2 (Renewable Fuel Standard) utilization command. Such scene and life-cycle idea would limit the capability of adverse immediate and circuitous land-use and ground vegetation variations, empowering the situation of plastic, advancement of raw material in territories that may improve the nature of the dirt or energize the decrease of overflow of agrarian supplements, expectation and decrease of the limit with regard to trade of groundwater bills, and upgrade of living environments. A fitful solidarity to expanding the biofuel business doesn't basically consider anyway bioenergy feedstocks that may be best incorporated into a farming scene to streamline natural favorable circumstances (Huang et al. 2013).

16.5.3 Encouragement and Strengthening of Rural Participation

Biofuels are similarly supported by the governments of Japan and India to frame commitment in addition to success in provincial territories. Current lessons demonstrate that bioenergy incorporates a bigger optimistic effect on employment conception in country zones than elective fuel sources. Biofuel firms can use a large number of workers per unit of power generated more often than the fuel company. An ADB amendment has uncovered that biofuel creation would drastically build the expense of feedstock, land, and work, increasing the income of farmers and rural communities (ADB 2009). Similarly, several laws in the region have put in place developing biofuel arrangements with the goal of creating biofuel and biofuel feedstock send out companies to improve local professions and financial processes.

For instance, the overall vision of the Philippine biofuel strategy assessment is that the nation can turn into a main net exporter of biofuels by 2030. A few nations need at this point made expanded occupation through biofuel exchanges. Indonesia,

which utilizes about 1.7–three million people inside the oil palm area and traded 1225 million liters of biodiesel in 2011, may create an extra 2.5 million positions through amplification of palm biodiesel creation. However, the type of bioenergy framework determines whether the jobs created represent a net increase to country employment. Because bioenergy is derived from naturally generated biomass, the use preferences that emerge from the bioenergy framework are dependent on the relative labour force of the feedstock crop, which was previously grown on nearly comparable land (Condon et al. 2013). For example, if the bioenergy feedstock requires less effort than the previous harvest or land use system, the bioenergy framework may result in a net savings at the farm level. Furthermore, there are contemplations concerning harmful effects onto land residency and ownership identified with the multiplication of biofuels. In many cases, handles that are named "desolate" or "wastelands" truly uphold underestimated segments of the general public for fuelwood, feedstuff, and so forth; proof of such acreage appointment has been rumored for genus *Jatropha* development in India as well as in China.

16.6 A Profitable Accounting of Biofuels

16.6.1 Practicality and Reasonableness of the Biofuel Industry

Biofuels could not be typically viable without government intervention. Biofuels including biodiesel are typically more expensive than fossil fuels on an energy equivalent basis. The usual costs of biodiesel from edible fat and waste grease are USD 0.54–0.62 and USD 0.34–0.42 per metric capacity unit, respectively; in distinction, value of pre-tax diesel is about USD 0.18 per metric capacity unit in the USA and USD 0.20–0.24 per metric capacity unit in some EU countries. In China, biodiesel from *Jatropha* was assessed at varying prices of 4–11.5 RMB/L (US$0.51–1.47/L), whereas retail cost for diesel in northern China was about 4.55–4.92 RMB/L (US$0.58–0.63/L) at the same time. The substantial variance is attributed to no viable *Jatropha* oil or *Jatropha* biodiesel production existing in China. Resource, value involvement, and professional obstacles perform vital roles within the development of biofuels (FAO 2011). If government support the biofuels, it will be much cheaper than fossil fuels; as an example, in Thailand, retail costs for E85 were 30–40% cheaper than premium gasoline in 2008, whereas state was promoting to subsidize biofuels if their costs are beyond fossil fuels. Despite the federal support, the cost of biofuels is extremely unstable and fluctuates with market price feedstock and oil. For example, the value of cassava, a usual feedstock for ethanol production in Asia, became doubled from 2006 to 2008. Besides, the seasonal fluxes in supply of feedstocks may cause issues for biofuels as feedstock costs typically go up in non-harvest slack seasons. The long vegetative period between planting and maturity of biodiesel crops (4–5 years for *Jatropha* and

6–7 years for *Pongamia*) could cause further increased risks of biodiesel than bioethanol producers. The affordability of biofuels is also dependent on geographic location (Gerasimchuk et al. 2012). Due to its crop-like characteristics, biofuels made in some regions could also be extra competitive than others. As an example, several Southeast Asian nations are encouraged to sell biofuels to China, wherever land isn't abounding and climate is favorably smaller. Thus, oil, primarily oil-based biodiesel, made in Southeast Asia became highly competitive compared to EU market.

China fixes bioethanol value at 90% of the ex-factory value of gasoline so it encourages to stimulate the utilization of biofuels. Whereas mandates can encourage the investment needed to increase the money viability of biofuel, necessary mixing could encourage biofuel production to an extent that can't be met on a sustainable method (Huang et al. 2013). Such policy interventions could prevent unsuccessful market due to aspects like external forces, public goods, upcoming industry, and uncertainties. In contrary to fossil energy, biofuels also face considerable dissipative obstacles like weak setup to beat in arcades and regulative and managerial footraces. The biggest hurdle to biofuels in Asia Pacific is government defrayal on grants controlling the value of fossil fuels for consumers.

16.6.2 The Expense of Government Uphold

Biofuel production, particularly in developed countries, is not taken seriously, a few Asian countries including China, Malaysia, and Thailand are amalgamating a blend of orders, appropriations, fuel extract attributes, and expense occasions to advance biofuels in a big way. India, in its arrangement to advance the expansion of a *Jatropha* biofuel exchange, gives various motivations and appropriations like local area improvement programs and least help cost for *Jatropha* seed. In 2008, the Government of the Philippines forced a few expensive impetuses for environmentally friendly power improvement, and those incorporate assessment occasions, obligation-free imports of apparatus and materials, and duty liberated from C credits created from environmentally friendly power.

16.7 Financial Influences of Biofuels

16.7.1 Biofuels and Nutrition Safety

Food safety remains a chronic problem faced by many countries in Asia. As per the FAO, 563 million people in this continent accounting 14% of the total inhabitants are subjected to food insecurity, receiving less than recommended levels of dietary energy supply (FAO 2012). Malnutrition of women and children is common in the region. In such conditions, the majority of biofuels produced for transportation fuel

markets employ assets that may be better used in the food system, raising serious concerns about growing biofuel crops at the expense of food crops.

16.7.2 Biofuels' Impact on Food Prices and Poor Households

Biofuels have undoubtedly had an effect on food security through resource competition mainly land, water, labor, and food crops used in food production systems (FAO 2012). This competition will lead to augment food costs and accessibility, which ultimately affect the poor. While there is only a smattering of consensus on the magnitude of the impact fifty, biofuels manufactured from farmed crops are acknowledged to be one among a number of factors driving up food prices in recent years (Sahoo and Wani 2020). Despite the fact that the entire use of growing crops for biofuel production is negligible, the division's new utilization of just barely any essential feedstocks (e.g., maize and palm oil) has raised the realization that world market expenses of those items are finished if biofuels weren't made. This, for example, may likewise sometime affect item subsidiaries not utilized as biofuel feedstock as they will be subbed to conciliate order in utilization or swapped thus the resistance for property and elective information sources.

Higher food costs will not only affect the source of living and food access of poor people but also increase their major expenditures in the form of food purchases. As an example, the FAO has assessed that proportion of the domestic budget spent on food is over 60% in Vietnam and over 70% in Pakistan. In developing countries of Asia, the ADB projected that a 10% escalation in domestic food costs could lead to a surplus of 64.4 million deprived societies or a 17.9-point increase in the proportion of poor within the country. In the same study, it had been observed that nutrition worth upsurges additionally lower the living standards of these people already living below the poverty line. The increase in the nutritional requirement of net food consumers will have an effect on food items by decreasing demand for higher-value nourishments and increasing demand for staples across a wide range of styles and food preferences (ADB 2009).

The studies on biofuel systems indicate that there is significant competition over more or less the tools recycled in food production, with various possible final effects on dietary values. Biofuels derived from farmed crops and deposits are most closely related to agricultural markets and thus have the utmost probable to have an effect on the cost and production of food. Interestingly, the cutting-edge biofuels got from dialect cellulosic biomass and photosynthetic green growth may have less immediate connections to food creation frameworks; creation of those feedstocks can in any case contend with the rural business somewhat for sharing assets like land, water, and manure and furthermore for work (Huang et al. 2012). The effect of upper food costs is moreover not basically uniform and will end in edges for elective gatherings. An examination by the World Bank uncovered that the effect of changes in food costs on net food buyers and merchants in nine nations demonstrated that helpless net food retailers will take advantage if food value will increase.

16.7.3 Reducing Necessity on Fossil Fuels

The production of biofuels decreases the reliance on conventional fossil fuels and increases the availability of diverse fuels. Sustainable biofuels are an attractive option to fossil fuels because their production is not limited to areas where it is possible to drill fossil fuels, allowing for a more flexible geographical supply.

16.7.4 Communal and Profitable Doles and Expenses

Discussion on regional social and financial effects of biofuels is likewise questionable and highlights the vital problems: the capacity of biofuels to fill in as a synergy to provincial economies, property asset exploitation, and food safety. A few arguments in favour of and against biofuels should be expressed as much as the strategy for biofuel feedstock development is viable, then financial points of interest will most likely be clearly formed by the method for generation. Despite the typical variety within each, modern scale farms, farmlands developing autonomously for market, and grouped designs under which organisations contract smallholders to deliver feedstock for the good of them will all have their own unique game plan of impacts.

16.7.5 Biofuels as an Upgrade to Provincial Economies

Under the precise conditions, as an example, oil palm will yield positive socioeconomic edges to rural communities ensuring employment and infrastructure enhancements and will increase the land worth and net financial gain from biofuel crop production (World Bank 2010). Soybean production has brought an important financial gain to landowners as it made economic multipliers within the downstream food trade (Peters and Thielmann 2008), though anecdotal proof suggests that edges are focused merely on larger land holdings.

One of the foremost advantages ventured to go with enormous scope estates is increment in profession (Andrade and Miccolis 2010). In Indonesia and Malaysia, the oil palm sector employs anywhere between 0.08 and 0.5 people per hectare, with smallholder-based production having the highest business rates, followed by smaller-scale projects and learning (World Bank 2010). Firms show an inclination toward employees with foundations in inactive agribusiness in their recruiting reviews and inside the strategy keep native networks from getting forthcoming points of interest (Colchester 2010).

In Brazil, sugarcane and ethanol creations have created precarious influence on business, and the amount of representatives used in sugarcane making has diminished by 62% as a result of computerization (Peters and Thielmann 2008).

Considerable influence may be found in the soy region, where mechanised cultivation employs just 0.05 to 0.06 units per ha^{-1} on average (Cotula et al. 2008), still this happens in regions of rather low populace concentration any place off-ranch pay acquire assists with fortifying country vocations. Notwithstanding the confined work power of certain feedstocks, farmstead occupation will in general be untalented and amazingly shaky because of between time obligations; edges to financial condition assuaging are, hence, usually restricted (Ortiz and Rodrigues 2006).

In spite of the fact that the net indigenous budgetary gains from plantation farming stay a subject of discussion, evidence from smallholder-based feedstock creation is relatively encouraging. In specific cases, feedstocks like oil palm have given bigger net incomes to property and work to smallholders in contrast with various cash crops (Rist 2010). In any case, such restitutions depend on the addition of the harvest relative to various decisions and on market access. Feintrenie et al. (2010) found that smallholder cooperatives are basic to the conviction of such advantages once operational in light of a legitimate concern for individuals, regarding their parts in allotting improved expenses and in considering firms capable to compose arrangements. Experiences from countries such as India and Africa demonstrate that given the resources and expert assistance, anything is possible (Practical Action 2009).

16.7.6 Effects of Biofuels on Property Possession and Control

Probably the key issue concerning the expanding biofuel exchange, joining the exceptional job of mechanical scale manors, is its consequences for neighborhood land rights. An amalgamation by Cotula et al. (2008) focuses on developing evidence for the negative impacts of enormous scope business biofuel creation because of diminished land and water entry and mandatory land seizures. In Indonesia, the NGO Sawit Watch has realized 630 land questions between oil partnerships and neighborhood networks, though the public land department realized 3500 debates associated with oil palm within the nation (Watch 2010).

The large number of disputes emerges from land and related revenue, absence of clarity land rights, lack of accountability, lack of free consent, and inequitable sharing of benefit among stakeholders (Mueller et al. 2011; Borras et al. 2010). Indigenous people with traditional claims to land are particularly underprivileged by the extension of oil palm, as special status of their concerns is restricted. Corporate obligations on land in Latin America are imposed through entirely distinct mechanisms, with far-reaching repercussions for indigenous rights to land, while in Bolivia the high soybean amplification has removed lands occupied by indigenous populations, so distressing their livelihoods (Ludewigs et al. 2009). However, the voluntary market transactions sell their land to larger operators in the short run as a spectacular opportunity for smallholders. The latter contributed to the consolidation of land holdings both in key farming expenses and in reform settlements (Cronkleton et al. 2009).

16.7.7 Influences of Biofuels on Food Security

The development of biofuels will have two essential impacts on food security. From one side, the direct impacts could happen from side to side to this propensity for mechanical balance feedstock creation to dislodge usual employments. Then again, the repercussions of those land-use changes and employments of multi-reason feedstock on food costs will subvert food access of poor people. According to FAO (2008b), the development of biofuel production might jeopardise every family's food security and public-sector food independence by increasing food costs.

Because the impoverished spend a disproportionately large amount of their family's income on food, the price of food is likely to rise (FAO 2008a). Numerous reports estimated that the growing plea for biofuels will have a negative effect on world food prices over time (Fischer et al. 2009). However, the additional applicable consequences of this particular topic are those affected by changes in tenure, profession, and usage. For the current dialogue, the challenge of fringe lands is as important as they are open for contentious environmental impact argument. The questionable "marginal" lands are usually expected to be uninhibited or infertile, so exterminating several undesirable possessions on native family food security.

16.7.8 Prospective Profitable Paybacks of Biofuel Production

Substituting petroleum products with substitute biofuels has likely to concoct assortment of extra returns. In qualification to nonrenewable energy sources, which are non-inexhaustible assets, biofuels are sustainable feedstocks. Accordingly, their creation and use may, in idea, be consistent indeterminately. While the collect of biofuels winds up in GHG discharges at numerous phases of the technique, EPA's (2010) examination of the Renewable Fuel Standard (RFS) extended that numerous types of biofuels may yield lower life-cycle GHG outflows than gas throughout a long-term time skyline.

The examinations identified with exploitation of alternative budgetary models have discovered that biofuels will bring about decreases in life cycle of GHG outflows compared with customary energies (Huang et al. 2012, 2013). Biofuels from the second and third generations might significantly reduce GHG emissions as compared to older technologies since feedstocks will be manufactured of marginal property. Furthermore, in the case of waste biomass, no further rural development is necessary, and market-interceded GHG emissions will be insignificant if the wastes do not have any other economic usage. Biofuels will be produced locally, resulting in a reduction in fuel imports (Huang et al. 2013). If the development and use of biofuels reduces our demand on foreign petroleum substitutes, we may be less vulnerable to the negative consequences of supply disruptions (US Environmental Protection Agency 2010).

The decline in the demand for fossil fuels could also decrease its value, creating social consequences for US consumers, but it is also likely to increase fossil oil consumption abroad (Mosnier et al. 2013). In part, the use of bioethanol could minimize emissions, particularly ethanol, which is almost combustible, reducing carbon monoxide emissions (US Environmental Protection Agency 2010). Nevertheless, the production and use of biofuels would not only, in and of itself, decrease emissions of GHGs or traditional waste materials but also reduce the imports of fossil oil or relieve the strain on exhaustible resources. In order to accrue these benefits, biofuel production and usage should synchronize with reduction in the production and use of fossil fuels.

16.8 Disbenefits

Feedstock for biofuels contains some crops that could preferably be recycled as feed for animals. Promoting biofuels for these crops could lead to a lot of expansion being redirected to agriculture, increased use of polluting inputs, and improved food prices. For resources (land, water, fertilizer, etc.) that would ideally be devoted to food production, polymeric feedstocks can also compete. Some opponents indicate that the generation of energy may lead to several unfavorable innovations. By reducing terrestrial carbon stocks in the atmosphere, changes in land-use patterns may increase GHG emissions (Searchinger et al. 2008).

Biofuel feedstock produced from land unoccupied from tropical timberlands, similar to soybeans inside the Amazon and feather palm in geological location, creates remarkably high GHG emanations (Fargione et al. 2008). Indeed, even utilization of plastic feedstocks will awaken higher harvest costs that support the development of farming into lacking area expanding GHG emanations and biodiversity adversities (Danielsen et al. 2009). GHGs can likewise be unharnessed by biofuel preparation and measure practices. About non-GHG natural effects, a few investigations announced that creation of biofuel feedstocks, mostly food crops like corn and soy, may prompt increment contamination from supplements, insect killers, and residues (NRC 2011; Chowdhary et al. 2020; Khan et al. 2020, 2021). Unreasonable water system and grain ethanol cleaning may dry springs (NRC 2011). Air quality may get crumbled in certain areas if the effect of biofuels on tailpipe emanations and the additional discharges produced at biorefineries increase air contamination (NRC 2011).

16.9 Recommendations

In order for the use of biofuels to be a lucrative benefit, community and environmentally friendly care must be taken of the following:

1. Biofuel policy: The success of biofuels depends on their compulsory use, tax services, subventions provided by the state, evaluation to shoppers, the popularity of the rights of employees, and therefore the thousand and one ways that develop from the agricultural societies and operative use of their land.
2. Grants: The creation of biofuels in the world is profitably attributable to grants and enticements for renewable energy; however, they should make sure that these subsidies are allotted to the foremost susceptible.
3. Soil use: The issue of land use denotes the average and long-term ecological demands that can only be met with resources derived from biofuel production.
4. Second-generation biofuels: They ought to flip the eye to second-generation biofuels; the benefits are that they'll be acquired from biomass that's not seized for food providers or contend with them and protect this soil use.
5. Exploration and growth: Each advanced and emerging state should concentrate to the returns related to analysis and development and adopt new technologies, leading to improved eco-friendly tradition and getting economic advantages within the development of biofuels.
6. Profit vs. environmental aids: The motivations for the revenues ought not to exceed the advantages of environmental preservation. In reference to environmental protection, any effective path resulting in a reduced consumption of nonrenewable energy collides with identical difficulty: the decrease of the gain or unusual incomes.

The growth of the bioethanol market would gear up the labor requirements. Thus, the typical income of the frugality increases compared to the standard, which could raise the cost of output and have a negative effect on sectors with value complex demand. The decline in the value of additional goods in these sectors offsets a number of increases in the production of bioethanol, changing the progressive effect on actual GDP across all situations.

Regions that changed activities, in any case, issue resources into the budget to be utilized somewhere else. This incorporated land that, through circumstances, is used fundamentally for maize creation could be redirected to other productive farming crops like pulses, cassava, sorghum, and groundnut. These returns account for about half of the food crop insects less carbohydrates produced by rural and low-income metropolitan family units. The endogenous move in land use consequently limits the unfavorable impact on mechanical crop establishment. The bioethanol feedstock creation decreases the amount of land available for conventional farming use, bringing about a decrease in volume of agriculture food crops made and available for their utilization.

16.10 Conclusion

Biofuels are being praised by several emerging country policymakers as a viable means of strengthening regional economies, strengthening energy retreat or acquiring unfamiliar trade, and tumbling GHG outflows and by enterprises as a way of encouraging proficient prospects. Life cycle, money-saving advantage, and framework investigations, nonetheless, showed that the growth of biofuels will have some brunt on contemporary land use, food creation frameworks, and asset provision and fuel costs. The conceptual provides various particular examples of how biofuel production may result in positive, neutral, or negative environmental outcomes depending on the individual ecological consequences of interest, the harvest utilized, the land acquainted with develop the yield and its past use, the administration practices utilized, and different factors also as conservational impacts from market-intervened farmland and vegetation changes. In contrast, while biofuels' capacities in tumbling GHG outflows are generally featured, land-use alteration (immediate and aberrant) may refute such ecological aids. The paper primarily highlights the implications for biofuel production to have positive, neutral, or negative ecological consequences in terms of the produce recycled, the land acclimated to produce the yield and its previous use, the administrative procedures followed, and a variety of other factors such as natural impacts from market-interceded land-use and vegetation changes. Biofuels are commonly not frugally inexpensive compared with petroleum products. While government approval is necessary for the biofuels industry to be viable, the protocols in which that of the help ought to be thoroughly curtained and a heap of strategy tools ought to be ratified. The sustainability of biofuels development in Asia will be improved by a variety of techniques, including appropriate strategic planning, growth, reasonable rural observes, territorial norms, and the development of next-generation biofuels. The social, economic, and environmental dimensions should be judiciously addressed in policies for manageable advancement of biofuels.

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Chapter 17
Advanced Anaerobic Processing of Bioresources for Production of Clean and Sustainable Gaseous Biofuels

K. B. Ramachandran, Praveen V. Vadlani, and Sai Praneeth Thota

Abstract Anaerobic processing of organic bioresources for gaseous fuel production is an established technology with global recognition. While these processes have been well adopted to treat diverse waste streams from industry with adequate production of gaseous biofuels as an end product, the need for high solid residence time, large process footprint, and undesirable variability in their operation has been a serious drawback toward wider acceptability. Recently, developments of high-throughput anaerobic reactors with high hydraulic and organic rates with consistent methanogenic activity have played a major role toward sustainable and clean gaseous biofuel production. Particularly, upflow anaerobic sludge blanket (UASB) technology has become a trailblazer in biological waste treatment due to its unique ability to generate mixed microbial granules as a consequence of an effective startup strategy, thereby effectively delinking hydraulic and solid residence times, which enables processing of high organic loading rates with enhanced methane productivity. Further, the high-performance UASB processing follows a modular design enabling a minimum footprint and flexibility in plant layouts and functioning, which enables the incorporation of process intensification schemes to meet circularity and sustainability goals in industrial/domestic waste processing.

Keywords Bioresources · Gaseous biofuels · Anaerobic digestion · UASB reactor · Sustainability

K. B. Ramachandran (✉)
Department of Biotechnology, Indian Institute of Technology Madras, Chennai, Tamil Nadu, India
e-mail: kbram@iitm.ac.in

P. V. Vadlani
VisvAum LLC, Puttaparthi, Andhra Pradesh, India

S. P. Thota
Department of Chemistry, School of Basic Sciences, SRM University Sikkim, Gangtok, Sikkim, India

P. Chowdhary et al. (eds.), *Bio-Clean Energy Technologies: Volume 1*, Clean Energy Production Technologies, https://doi.org/10.1007/978-981-16-8090-8_17

17.1 Introduction

Over the last two centuries, the world witnessed unprecedented global growth by following a linear economic model. The natural resources of the Earth were effectively converted to value-added products and services, which created a vibrant standard of living among the global citizenry. While the material and economic benefits were enormous, the linear model also generated vast quantities of industrial and domestic wastes; it is projected the total wastes will reach about 20 billion tons by 2025, thereby severely stressing the existing waste treatment facilities and the environment in general. The global community must switch gears and inculcate circularity and sustainability criteria in the utilization of natural resources, adopt process intensification and waste minimization methods during industrial processing, and stringently follow the rules to safeguard global climate change (Charpentier 2007; Vadlani 2020).

Anaerobic digestion (AD) is a fascinating nature-based technology to clean up the seasonal leftovers of forestry and agricultural residues to generate a clean gaseous biofuel comprising methane and carbon dioxide. The AD process has been successfully extended to treat industrial and domestic wastes with effective removal of biochemical oxygen demand (BOD)/chemical oxygen demand (COD) (Xia et al. 2016; Sikora et al. 2017). In addition, gaseous biofuels derived from dark fermentation of lignocellulosic biomass resources will be a dominant factor to service the current and future renewable energy needs in transportation and energy industries (Kucharska et al. 2018; Guragain and Vadlani 2021). Compared to liquid biofuels, gaseous biofuels have demonstrated better energy balances, significant greenhouse gas emission reductions, and a minimum waste footprint (Kucharska et al. 2018). Depending on the waste characteristics and volatile fatty acid content, the composition of the biogas varies between 65 and 75% methane, the rest being mostly carbon dioxide (Vadlani and Ramachandran 2008a). Recently, innovative carbon dioxide capture technologies have been developed (Abdelkareem et al. 2021) that can reduce the CO2 level in the biogas stream to less than 3%, which enables the enriched methane to be used as compressed gas in the transportation industry (Xia et al. 2016). The entire waste treatment facility should be successfully incorporated in the value chain of the industrial/domestic processing industry, and sustainable financing and valuation methods should be adopted to usher in the new circular bioeconomy (Vadlani 2020). Biofuel could be either in liquid or in gaseous form, produced from biomasses such as trees, grasses, crops, animal materials, and other bioresources. These biological materials can be used as a fuel; however, they can also be converted by the AD process to liquid or gaseous form to conveniently transport them to the usage site for the generation of electric power and heat and conversion to useful chemicals or for use as an automobile fuel. Industrial effluents containing carbohydrates and proteins can also be converted to biofuels by anaerobic processing (Sikora et al. 2017; Guragain and Vadlani 2021). Biofuels have several benefits over traditional fuels. They are produced from renewable bioresources. Source materials can be grown and later energy harvested as fuel at regular intervals.

This provides economic security to nations by reducing dependence on imported oil. With increasing oil prices, it is even becoming cost-effective. They are relatively less flammable compared to fossil fuels and are environmentally friendly by minimizing the emission of greenhouse gases (Ghiat et al. 2021). Some examples of liquid biofuels are ethanol produced from sugarcane, corn, and lignocellulosic materials (Guragain and Vadlani 2021); biodiesel derived from vegetable oils, animal fats, oleaginous microbes, and other plant sources; and algal fuels (Vadlani 2020). Examples of gaseous biofuel are biogas and hydrogen. Biogas is primarily methane derived from animal manure and other digestible organic materials or industrial effluents by anaerobic processing (Ali Shah et al. 2014; Van et al. 2020).

17.2 Gaseous Biofuels

Production of gaseous biofuels instead of liquid biofuels from agricultural and waste materials has several advantages. They can be produced from a variety of agricultural and waste materials which do not require extensive pretreatment before bioconversion to gaseous fuels. Gaseous fuels, after production, do not require an extensive separation process to bring them to a useable form. The gas generated by anaerobic digestion mainly consists of methane and carbon dioxide. Carbon dioxide can be separated easily from the gas mixture, and the remaining gas rich in methane can be used as a fuel or converted to other useful products (Kucharska et al. 2018).

Gaseous biofuels have several advantages over other forms of fuels. It is safer and easier to store when compared to other fossil fuels. They can also be transported to required sites by means of pipes. However, there are some disadvantages with them. They require very large tanks for their storage and are relatively costly compared to other fuels. They are also highly inflammable, and hence, greater care is needed for their storage (Sinharoy et al. 2020).

17.3 Anaerobic Microbiology and Biochemistry

Anaerobic digestion of complex organic material is a multiphase biological process affected by the integral action of a heterogeneous population of microorganisms (Sikora et al. 2017). In this process, microorganisms break down the biodegradable material in the absence of oxygen. First, the digestion process begins with bacterial hydrolysis of the input materials. Insoluble organic polymers, such as proteins and carbohydrates, are broken down to soluble derivatives, amino acids, and sugars, respectively, which become available for other bacteria, namely, acidogenic bacteria. Acidogenic bacteria then convert the amino acids and sugars into carbon dioxide, hydrogen, ammonia, and organic acids. In acetogenesis, bacteria convert these resulting organic acids into acetic acid, along with additional ammonia, hydrogen,

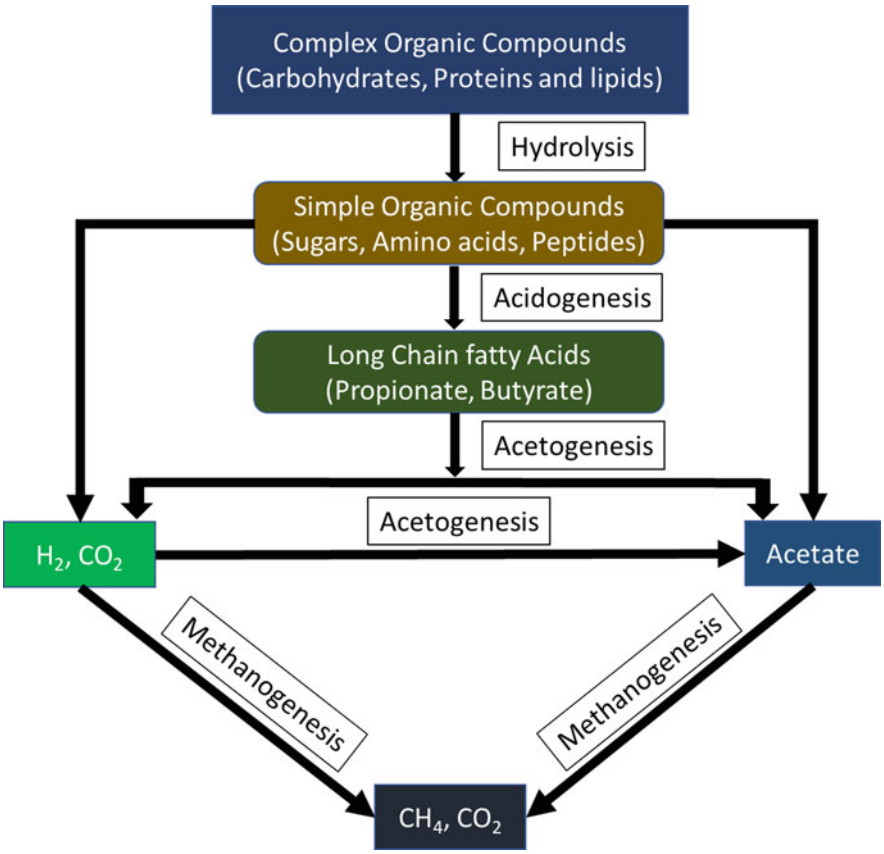

Fig. 17.1 Metabolic steps and microorganisms involved in anaerobic digestion (Sikora et al. 2017)

and carbon dioxide among other compounds. Finally, methanogens convert these products to methane and carbon dioxide. The methanogenic archaea populations play an indispensable role in anaerobic processing. In the digestion process, optimal proportions of all these types of bacteria are required for efficient bioconversion of the biodegradable material to gaseous biofuels (Sikora et al. 2017). The interaction of various types of bacteria in the anaerobic process is illustrated in Fig. 17.1.

Anaerobic processing is influenced by several environmental factors. Among these are temperature, pH, and toxic substances. It takes place efficiently between temperatures 25 °C and 40 °C since the majority of anaerobic bacteria responsible for the process have temperature optimum in this range. At temperatures between 55 °C and 60 °C, anaerobic digestion is brought about by thermophilic bacteria. Most methanogenic bacteria have optimum pH between 7 and 8, whereas VFA-degrading bacteria mostly have lower pH optima. The optimal pH for a mesophilic biogas reactor is between 6.7 and 7.5, and its performance is strongly

inhibited at pH below 6 or above 8 (Vadlani and Ramachandran 2008a, b). The tolerance of the digester for toxic compounds depends on their type and concentration in the influent stream and those produced within the system. Many organic and inorganic materials may exhibit toxic or inhibitory effects on the anaerobic process. A high-throughput anaerobic system, hence, should maintain high levels of these heterogeneous populations at optimum ratio and also provide optimal physical and chemical environmental conditions for its efficient operation.

17.4 Anaerobic Reactors and Process Technology

Anaerobic digestion process technology has gained increasing attention since the 1970s (Anna Sikora et al. 2013). Due to stricter environmental regulations, the demand for more cost-effective and sustainable treatment systems was required for the expanding manufacturing industries and also due to the worldwide oil crisis at that time. Fervent research had greatly improved the knowledge of the microbiology and biochemistry of anaerobic microorganisms and consequently the digestion system reactors. Anaerobic treatment of agricultural and liquid wastes is preferred over aerobic treatment because of its merits. The main advantage is the production of gaseous fuel, biogas, and its ability to treat high COD loads. The anaerobic process can also withstand fluctuation in the influent and provide good stabilization of solids.

The design and operation of anaerobic reactors have come a long way from the earlier ones (Chong et al. 2012; van Lier et al. 2015; Xia et al. 2016). Initially, a simple septic tank was developed and used for the treatment of sewage and the generation of biogas. These early digesters were not mechanically stirred, and their efficiency was reduced by the formation of a thick scum layer. To prevent scum formation and to ensure better contact between bacteria and waste material, various types of mixing devices were introduced, ranging from recirculation to propeller action draft tube. These digesters, though could meet the limited requirement, had several operational limitations for application in large. This led to the development of modern high-rate anaerobic bioreactors.

Much of the performance in an anaerobic digester depends on the conversion of acetic acid into methane by methanogenic bacteria, which grows very slowly with a doubling time of 8 to 80 days, compared to acetogenic bacteria, which doubles in 4–8 h. This necessitated a very high hydraulic retention time to maintain the desired level of methanogenic bacteria in the reactor to have a reasonable rate of digestion. The modern high-rate bioreactor design and operation succeeded in extending the retention time of microbes in the reactor while at the same time reducing hydraulic retention time to few hours. Simultaneously, improvements in reactor design and operation allowed increased concentration of the bacteria in the system by many folds, thereby resulting in an increased rate of conversion of organic material to methane. This new breed of reactors, referred to as "biomass retained reactors," effectively delinked the solid retention time from hydraulic retention time, thereby allowing large throughput of wastes to be treated (van Lier et al. 2015). All modern

Table 17.1 Immobilization principles applied in high-rate anaerobic reactor

	Immobilization principle	Treatment system
I	Bacterial sludge attachment	
A	To stationary packing materials (attached film)	Upflow anaerobic filter (AF) Downflow anaerobic filter (AF)
B	To particulate carrier materials (attached film)	Fluidized bed/expanded bed reactor (FBR/EBR) anaerobic attached film expanded bed (AAFEB) reactor
II	Bacterial sludge aggregation, granulation, floc formation	Upflow anaerobic sludge blanket (UASB) reactor Anaerobic baffled reactor (ABR)

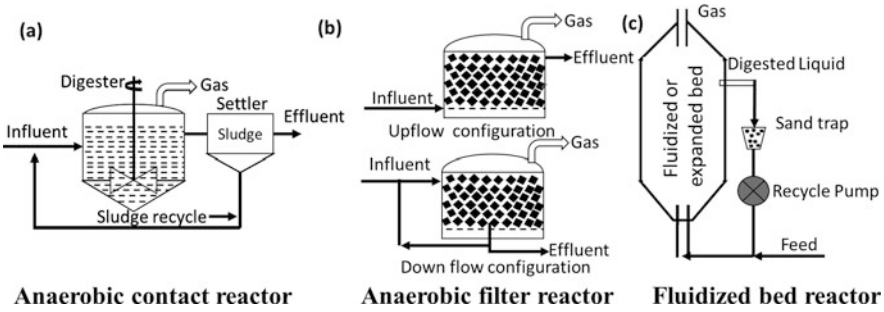

Fig. 17.2 Various types of anaerobic reactors

high-rate anaerobic treatment system is based on some kind of sludge retention or immobilization principle to retain as many viable cells as possible in the reactor (Table 17.1).

17.5 High-Rate Anaerobic Reactors

Examples of high-rate digesters are anaerobic contact reactors, anaerobic filters, fluidized or expanded bed reactors, and upflow anaerobic sludge blanket (UASB) reactors. Anaerobic contact reactor consists of a high-rate digester coupled to an external settler (Fig. 17.2a). The microbial cells in the effluent are separated, and the settled biomass is recirculated back into the reactor, thereby increasing its concentration to 5–10 kg VSS/m^3. The major operational difficulty encountered in the operation of the contact process is the required separation and concentration of biomass in the settler prior to its return to the digester. Large hydraulic retention time in the settler reduced considerably the throughput through the reactor.

Anaerobic filter (AF) reactor contains solid support packing material on which the microorganisms are attached (Fig. 17.2b). Biomass densities up to 10–25 kg VSS/m^3 could be obtained using packing material with a porosity of 0.8 to 0.9. High-strength soluble wastes with a high loading rate of 10–25 kg COD/m^3-day have been

treated in this type of bioreactors, achieving 70% COD conversion and good process stability (van Lier et al. 2015; Jaibiba et al. 2020; Sinharoy et al. 2020). The main limitation of this reactor is the accumulation of solids in the packing material which tends to plug the reactor (Young and Dahab 1983; Narayanan and Narayan 2019). The plugging may be caused by suspended solids from the wastewater or by material precipitated from the waste (e.g., calcium carbonate). This problem can be obviated by operating the reactor in downflow mode, usually referred to as a downflow stationary fixed film reactor. The influent stream entrains the flow of methanogenic bacteria, removes any clogging, and induces better mixing conditions at the top of the reactor. In this reactor, loading rates are limited by the amount of active biomass that can be retained in the reactor; however, very dilute wastes can be treated by this reactor (Ali Shah et al. 2014; van Lier et al. 2015; Ribera-Pi et al. 2020; Van et al. 2020).

In a fluidized or expanded bed reactor (FBR/EBR), the bacteria attach to the small particles of the carrier, which may be nonporous such as sand, sepiolite, carbon, and different types of plastics or porous foam such as polyurethane (Fig. 17.2c). The particles in the FBR can be small (0.2–1 mm), providing a high specific surface area (2000–5000 m^2/m^3) for bacterial biomass attachment. By choosing the particle diameter and density, the settling rate of the biolayer-covered particles can be up to 50 m/h, and biomass concentration of 30–40 kg VSS/m^3 can be maintained in this reactor. Very high loading rates of 20–30 kg COD/m^3-day have been sustained with high-strength soluble waste (Sreekrishnan et al. 1991). However, this reactor requires high pumping cost and process control is difficult. The anaerobic attached film expanded bed (AAFEB) reactor is conceptually similar to the anaerobic FBR but uses much smaller support particles of 20–30-micron (μm) diameter and much lower recycle rates. When the bed expansion is up to 30%, the reactor is called EBR, and if the expansion is far more than 30%, it is called FBR.

17.6 Upflow Anaerobic Sludge Blanket Reactor

The most widely used, reliable, and effective system for anaerobic treatment is the upflow anaerobic sludge blanket (UASB) reactor. UASB reactor as an alternative process for anaerobic wastewater treatment was pioneered by Lettinga and his co-workers in the late 1970s (Lettinga and Hulshoff Pol 1991). Attracted by its favorable prospects, CSM N.V. (now Corbion N.V.), a food and biochemical company in the Netherlands, participated in the study concerning the scaling up of the UASB design for full-scale application. From 1973 to 1977, pilot plant studies in 6 m^3, 30 m^3, and 200 m^3 volume capacity treatment units were undertaken (Souza 1986). The process in the last few decades has gained wide acceptance in the treatment of industrial and domestic effluents in both developed and developing countries (Kosaric and Blaszczyk 1990; Vadlani and Ramachandran 2008b; van Lier et al. 2015).

In UASB reactors, biomass is retained in the form of granules within the reactor. The propensity of bacteria, especially methanogens, to form self-immobilized granular structures is exploited in this reactor. The process is performed in one reactor, and no supporting medium is required for attachment of the biomass, which decreases the capital cost and minimizes the possibility of plugging. The energy requirement is also small because there is no mechanical mixing within the reactor, no recirculation of sludge, and no high recirculation of the effluent (Tiwari et al. 2006). The reactor is simple to construct, and granular sludge formed can withstand high loading rates and can tolerate fluctuations in influent feed concentration and composition, temperature, and pH (Singhal et al. 1998; Vadlani and Ramachandran 2008b; Mainardis et al. 2020).

17.7 Fundamentals of UASB Technology

The basic principles underlying the UASB process are (1) formation of stable anaerobic sludge, preferably granular one, from loose dispersed biomass, which is affected by a good startup strategy; (2) an effective separation of biogas, treated effluent, and sludge by the internal gas-solid separator (GSS) device; and (3) even distribution of influent waste at the bottom of the reactor where concentrated sludge bed is present (Souza 1986; Lettinga and Hulshoff Pol 1991). This is more required at lower loading rates and low wastewater strength. The schematic diagram of the UASB reactor is shown in Fig. 17.3a.

The installation of a GSS at the upper part of the reactor is essential for the proper performance of the system, irrespective of the nature of the sludge (Souza 1986; Lettinga and Hulshoff Pol 1991). For proper operation of GSS, the following

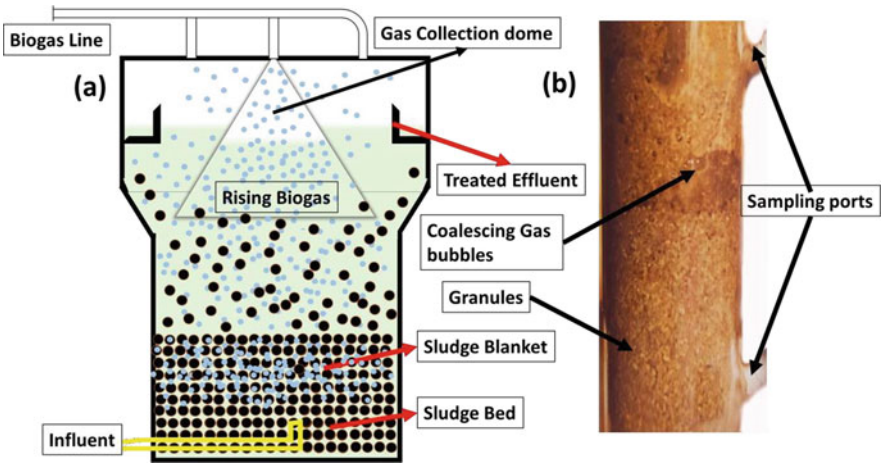

Fig. 17.3 (**a**) Schematic of an UASB bioreactor, (**b**) granular bed inside a lab UASB reactor

conditions should be fulfilled: (1) The walls of the settler should be inclined at an angle of approximately 50°. (2) The surface load of the settler should be kept below 0.7 m/h, and the average flow through the aperture between the gas collectors should be below 2 m/h. (3) Sludge of good settleability should be formed in the reactor. (4) The separated sludge should slide back into the reactor. (5) Excessive foaming in the gas collector should be prevented. The presence of a sludge blanket, even if it extends up to the settler chamber, is beneficial. It serves as a lock for extensive sludge bed expansion into the settler chamber (Liu et al. 2003); it also acts as a buffer spring, absorbing the initial turbulence of upflow gas bubbles, thereby alleviating the load on the settler chamber, and it also gives a further reduction in COD (Lettinga and Hulshoff Pol 1991). The importance of the feed inlet distribution system is felt more while treating dilute waste or when operating at low temperatures. Proper inlet distribution is necessary when the sludge has a remarkably high settleability because, in that case, a dense layer of sludge bed will be easily formed leading to dead zones. Improper distribution creates channeling, leading to reduced COD removal. For a granular sludge, one inlet nozzle/m^2 of reactor area is recommended, whereas 5 nozzles/m^2 is required for thin flocculant sludge (Souza 1986; Lettinga and Hulshoff Pol 1991).

The rising gas bubbles through the reactor usually coalesce and form a larger bubble. When the buoyancy of these large bubbles balances the granular bed above them, as shown in Fig. 17.3b, then a distinct gas zone is formed within the reactor. The upward movement of this gas zone subsequently outbalances the bed above it and erupts toward the settler. UASB reactor is a fascinating multiphase operation involving movement of liquid influent through a solid granular bed with the biogas formation as a product; the proper study of the hydrodynamics and gas separation along the length of the granular bed is essential (Singhal et al. 1998). The most used construction material for UASB reactors has been either steel or concrete. The use of steel generally implies easier construction and installation for shapes with circular cross-sections, while for concrete the rectangular section is easier (Souza 1986).

17.8 Effective Startup Strategy for UASB Reactor

After the UASB system has been designed and constructed to meet a particular waste processing requirement, effective startup of the reactor is the deciding factor to make the reactor fully operational at the earliest. Typically, a startup in advanced anaerobic reactors is defined as the duration it takes to charge the reactor with seed sludge (inoculum) and starting the input feed to the point when microbial sludge gets stabilized, and the process meets the design specification in biogas production and COD removal. In the case of the UASB process, additionally, the initial inoculum gets transformed into specialized self-immobilized granular entities in the startup phase. Proper startup strategy is essential for rapid granulation of the cells and efficient operation of the reactor (Vadlani and Ramachandran 2008a, b). After a

successful startup, the reactor is typically exposed to high organic loading rates at high superficial velocities while meeting the designed COD removal efficiency (Liu et al. 2003; Zhou et al. 2007). Effective startup involves a crucial interplay of seed sludge quality (initial microbial consortia and the accompanying suspended solids), hydraulic and organic loading regime during multiphase reactor operation, the influent waste stream characteristics, and environmental factors. Each of these requirements is explained in more details as follows (Vadlani and Ramachandran 2008a).

17.8.1 Seed Sludge

- The source of seed sludge is critical. Preferably, it should be taken from an active UASB/anaerobic reactor that is processing the same waste that the UASB system is designed for. Further, the sludge should have adequate suspended microparticles to enable microbial aggregation.
- The amount of sludge should be between 10 and 20 kg VSS/m^3 of reactor volume. At this stage, the specific methanogenic activity of the seed sludge is not of much importance.
- The addition of disintegrated granular sludge from a working UASB reactor (preferably processing the same waste) will accelerate the granulation process and as a consequence reduce the startup duration.

17.8.2 Process Operation

- After charging the reactor with seed sludge, the reactor operation is initiated with an organic loading rate between 2 and 4 kg COD/m^3-day. Essentially, the low flow through the reactor enables loosening of the seed sludge, acclimatization of the microbial flora to the waste as substrate, sequential utilization of biomolecules with biogas production as the final step, and separation and removal of dispersed and non-active sludge from the reactor. No recycle of the effluent stream is entertained at this juncture.
- Typically, during startup, the influent media concentration is kept constant, but the organic loading rate is varied by increasing the upflow velocity. After about 80% reduction in biodegradable COD is attained, the organic loading rate is increased in steps, and this process is continued until the active component is retained within the reactor and the non-active sludge is washed out from the reactor (Vadlani and Ramachandran 2008a).
- If it is feasible, samples are collected over the length of the reactor and analyzed for sugars and volatile fatty acid reduction. Further, the pH measurement along the length and the inception and growth of granules by microscopic analysis of the samples should be monitored.

17.8.3 Environmental Factors

- The temperature within the reactor, particularly at the bottom one-third of the reactor, should be between 37 and 39 °C for mesophilic and 45 and 55 °C for thermophilic operation, respectively.
- pH should be above 6.2 to prevent inhibition of methane-forming bacteria in the reactor; the desired pH range should be maintained between 6.6 and 7.6. In the certain influent waste stream, the sugars and VFA components can be adjusted so that when they are utilized the desired pH is maintained along the length of the reactor (Vadlani and Ramachandran 2008b).
- The influent waste stream should be analyzed for the availability of divalent/trivalent trace elements and growth factors. If these are not present, it is advisable to augment the influent stream with these in available form (Tiwari et al. 2006).
- Toxic/inhibitory compounds that are detrimental to microbial growth should be removed from the influent waste stream.

17.8.4 Granular Formation

A good startup strategy results in the formation of granular sludge from dispersed microbial cultures. As the influent flow of the waste stream is initiated, the various groups of microbial cultures, including acidogens, acetogens, and methanogens, start growing after getting suitably acclimatized to the waste ingredients (Kosaric and Blaszczyk 1990; Liu et al. 2003). With the increase in influent flow rate, the hydraulic loading rate and the gas formation separate the loose and fluffy biomass from seed sludge and wash them out of the reactor. This separation of the biomass into an active component, which is growing and getting aggregated, and a non-active component is a consequence of a well-designed loading rate regime followed during startup. The reactor height-to-diameter ratio establishes the appropriate superficial liquid and gas upflow velocities in the multiphase reactor. The environmental factors enumerated for a startup are conducive for the incipience of granular formation. To further aid in granulation, the following guidelines are suggested during the startup phase of the reactor:

- The initial specific loading rate should be between 0.05 and 0.1 kg COD/Kg VSS-day.
- Samples should be collected along the length of the reactor, and specifically the VSS concentration and pH should be monitored. The decrease (due to washing out of the non-active component) and subsequent increase in VSS indicate the startup regime is in the right direction (Vadlani and Ramachandran 2008a, b).
- Prolonged periods of substrate overloading or under-loading the reactor is not conducive for granulation.

The seed sludge usually has suspended solids that act as micro-carriers to enable microbial linkages. Granular formation results from a complex interplay between the physical forces (influent upflow and gas velocities) and chemical interacting forces comprising the carrier-microbial linkages and the bacterial cell-to-cell aggregation (Kosaric and Blaszczyk 1990; Liu et al. 2003). The adhesion between cells is facilitated by the formation of an external microbial layer of the glycocalyx, which is composed of polymers and is also called extracellular polymeric substances (EPS) (Zhou et al. 2007). Depending on the prevailing reactor hydrodynamics and the influent substrate characteristics, about 1–2% of EPS has been reported in bacterial aggregates (Fernández et al. 2008; Tsagkari and Sloan 2018; Feng et al. 2021). Further, the surface properties of the micro-carriers, such as surface charge and hydrophobicity, can induce linkages with the microbial surface.

The presence of metal ions, particularly divalent ions, either in the seed sludge or suitably supplemented in the influent stream, acts as a bridge between the negatively charged surfaces of the microbial cells. Of these ions, the presence/addition of calcium salts in the feed has proven to be efficacious for granular formation (Kosaric and Blaszczyk 1990; Vadlani and Ramachandran 2008a). Other metal ions such as Fe, Al, Ba, Mg, and trace metals also induce aggregation, which was deduced when these ions were found to be incorporated in microbial granules (Vadlani and Ramachandran 2008a). Ba promoted rapid aggregation, whereas Al (trivalent ion) promoted rapid settling (Kosaric and Blaszczyk 1990; Liu et al. 2003; Tiwari et al. 2006). It is evident that the chemical bond strength of the metal ions affects the granular formation of microbes; higher atomic weight ions have a pronounced effect on the floc density, thereby increasing the sedimentation rate (Liu et al. 2003). Further, the addition of disrupted granular biomass from an established UASB process, preferably treating similar waste characteristics, and addition of treated and functionalized nano-polymers can aid in the acceleration of granular formation.

The use of low selection pressure, high solid retention time, and allowing the non-active component of the seed sludge to get washed out leads to the rapid development of granular pellets consisting predominantly of the rod-shaped Methanosaeta sp (formerly Methanothrix soehgenii). With increasing loading rates in steps, this rod-shaped microbe grows and entraps other unattached bacteria leading to dense and compact aggregates. It is also suggested that the Methanosaeta sp. colonizes the central cavities of Methanosarcina clumps formed at initial high acetate concentration in the influent stream (Tiwari et al. 2006). In our studies performed in a laboratory reactor that used synthetically mixed acid waste, incipient granules measuring between 100 and 200 μm in diameter were formed after 50 days of startup phase (Vadlani and Ramachandran 2008a). Figures 17.4a, b show the nascent granule and the larger magnification of the surface characteristics and gas/substrate transport channels of the granule, respectively. It is evident that the Sarcina-type microorganisms were predominant on the surface since the influent feed had a high acetate concentration of 4.8 g/l during startup. After the startup period, the UASB reactor was operated at a steady loading rate of 20.2 kg COD/m^3-day. Figures 17.4c, d show the SEM photo of a matured granule and

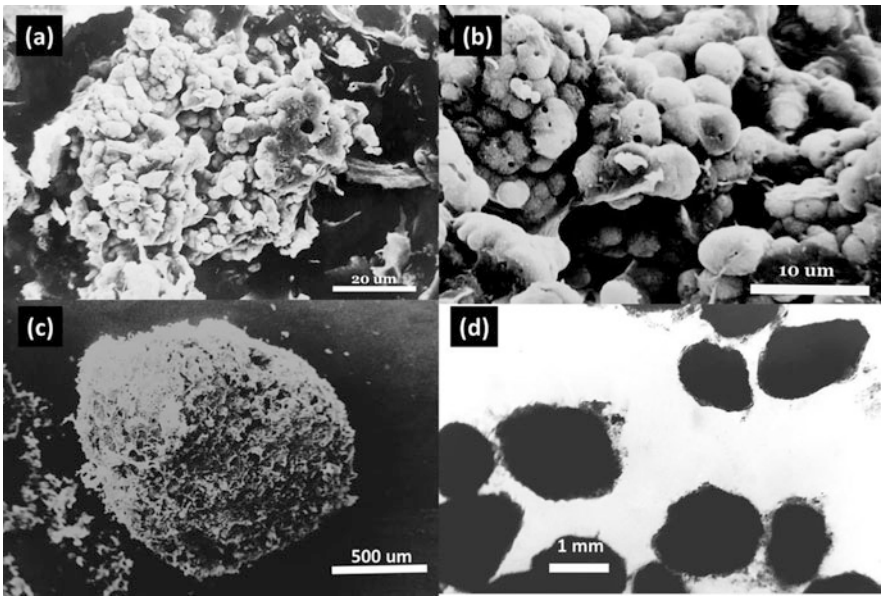

Fig. 17.4 (**a**) Incipient granule, (**b**) gas/substrate channels in granules, (**c**) matured granule (100th day of reactor operation), (**d**) photomicrograph of granules

photomicrograph of granules taken from port 1 on the 100th day of reactor operation, respectively. Again, the surface characteristics of the granules did not change much since startup and had predominantly Sarcina-type microbes. When these granules were disrupted, rod-shaped Methanosaeta sp. were observed, thereby confirming their presence in the inner core of the structure. The Sarcina-type microbes then grew on this inner core along with other classes of microbes; together the consortia matured into a fully developed granular structure of 1–2 mm in diameter.

17.8.5 UASB Reactor Performance

The onset of granulation and the development of a stable granular bed and blanket constitute an effective startup phase; the duration varies between 30 and 60 days depending on the waste characteristics, seed sludge source, startup strategy, and reactor design (Chong et al. 2012; Mainardis et al. 2020). In our studies, during this phase, the organic loading rate was increased in steps from 3 to 30 kg COD/m^3-day, and the sludge loading rate increased from 0.12 to 1.2 kg COD/kg VSS-day. Collecting samples along the length of the reactor helps in monitoring the COD uptake rate, VSS concentration, and environmental conditions. Further, considering the region between the two sample ports to be well mixed, an analytical expression was developed to calculate the specific uptake rates of individual VFA and COD,

which provided a powerful tool to monitor the reactor performance during the startup (Vadlani and Ramachandran 2008a). During this period, the specific methanogenic activity (SMA) of the initial seed sludge taken from a domestic treatment plant increased from 0.075 to 0.75 kg COD-CH4/kg VSS-day, and the methane yield (methane expressed as the equivalent COD per kg of COD removed) stayed promising between 0.83 and 0.86 kg COD-CH4/kg COD removed. Better reactor performance was observed with a seed sludge taken from a distillery waste treatment plant (Vadlani and Ramachandran 2008a).

After a successful startup period, an efficient UASB reactor operates at the design loading rate, which typically is between 20 and 25 kg COD/m^3-day at a hydraulic loading rate (HRT) between 8 and 12 h. The effective delinking of HRT from solid retention time (SRT), which is usually between 30 and 50 days, is a significant feature of advanced anaerobic reactors, including UASB reactor, which enables reactor operation at a high throughput of influent waste with consistently high SMA and methane yield (Vadlani and Ramachandran 2008a). For agricultural and domestic wastes, the loading rate is between 10 and 15 kg COD/m^3-day (Álvarez et al. 2006; Chong et al. 2012). The methane content of the ensuing biogas is between 72 and 75%; interestingly, when the influent media was changed to only VFA during steady-state operation, the methane content increased to 83–85% of biogas (results unpublished). Further, the presence of stable granular sludge with high methane productivity allows the UASB system to operate for a longer duration and could sustain shock high loadings and periodic interruptions in the influent stream (Mainardis et al. 2020).

The UASB process has been used to treat industrial and domestic wastes in different capacities and at different places in the world (van Lier et al. 2015). The industrial wastes include food, beverages, distilleries, feed, paper, meat, and leather processing; domestic waste is predominantly from municipals and large urban areas (Álvarez et al. 2006; Chong et al. 2012; Mainardis et al. 2020). In addition, the UASB system has been operated in a two-stage mode for better stability and higher methane yield, for bio-hydrogen and VFA production by inhibiting the methanogenic activity, in combination with activated sludge process/AF/ABR for higher BOD/COD removal efficiency, and as anammox process for the abatement of ammonium and nitrite in waste streams (Chong et al. 2012; Mainardis et al. 2020). The advance in GSS design with additional support material/filter at the reactor top has enabled the modular design of the UASB reactor with reduced footprint and flexibility in the mode of operation (van Lier et al. 2015).

Figure 17.5 shows multiple streams of by-products from different units, which have great potential to be further processed into industrial chemicals, bio-fertilizers, and road/building materials. The biogas generated from the modular UASB system is processed to capture CO2, and if H_2S is present, then it can be removed by passing the biogas stream through an activated carbon column. The resulting enriched methane will be a clean gaseous biofuel, which can be used as compressed gas for the transportation industry (Xia et al. 2016) or as a benign feedstock for the methane/

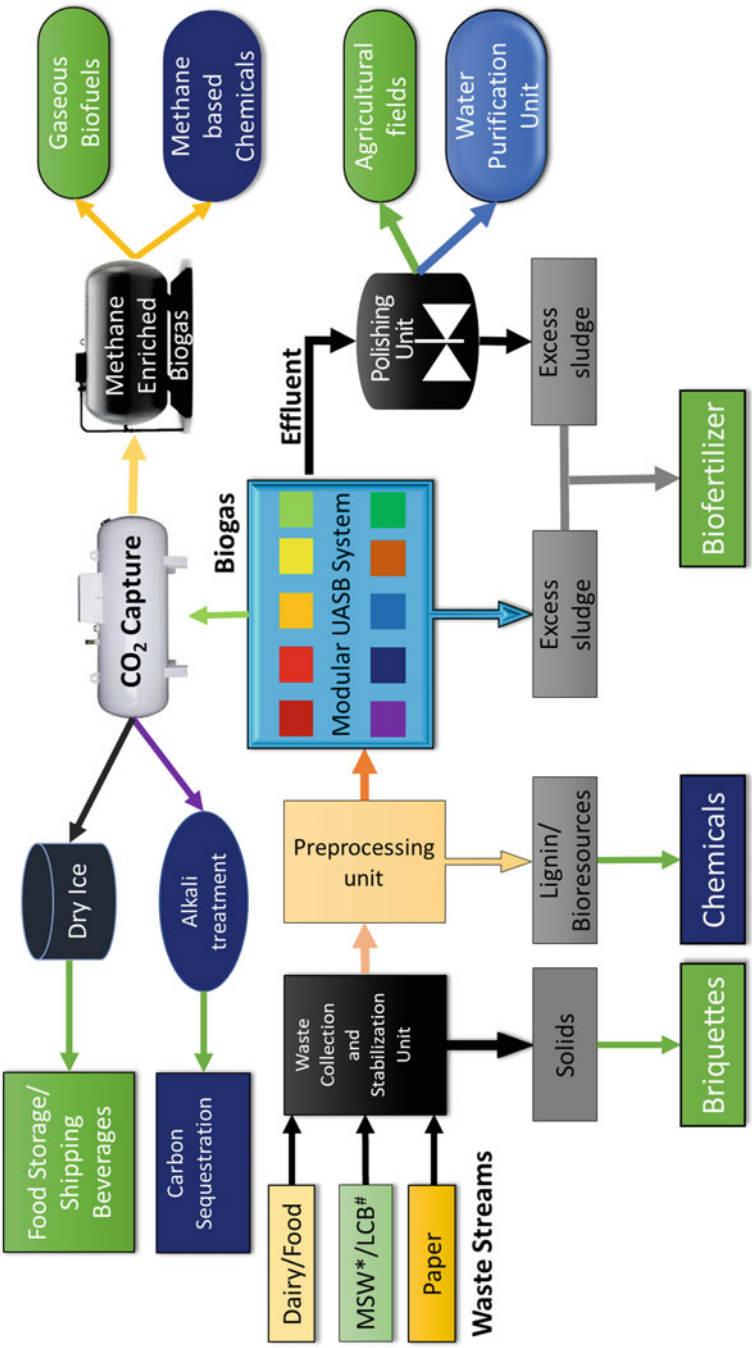

Fig. 17.5 Integrated anaerobic processing—ease of operation and flexibility in plant layout. * municipal solid waste, # lignocellulosic biomass

methanol-based platform chemical industry. The captured CO_2 can be sequestered or used in the beverages and food storage/shipping industries.

17.9 Production of Clean Gaseous Biofuels and Value-Added Products

Recent advances in advanced anaerobic processing have enabled the industrial and domestic wastewater treatment industry to meet the stipulated pollution prevention requirements. The whole approach was geared toward BOD/COD removal and not on gaseous biofuels as a source of revenue (Chowdhary and Raj 2020). Further, to compete with natural gas markets and to emerge as a feasible alternative for the transportation and energy industry, gaseous biofuels must be produced at the economy of scale with clear product consistency. The existing industrial/domestic treatment plants are not designed and operated for this purpose. Figure 17.5 presents a schematic of an integrated anaerobic processing complex to generate clean gaseous biofuel comprising of highly enriched methane gas, along with a slew of products generated from various processes included in the complex. Waste streams from food and other allied industries can be pumped/shipped to a central waste collection point, wherein the collective waste is stabilized and recalcitrant solids/materials are separated. The environmental factors and other media components are adjusted to provide a waste stream of designed influent characteristics.

Lignocellulosic biomass residues and municipal domestic waste will require a pretreatment step to enable deconstruction of complex polymers to simple sugars, oligopeptides, and VFA (Kucharska et al. 2018; Guragain and Vadlani 2021; Khan et al. 2020, 2021). These two units (waste stabilization and preprocessing units) will deliver a consistent influent stream of desired suspended solids and BOD/COD strength to the anaerobic processing unit. With the recent advances in UASB system modular design, an array of reactors in series and parallel can be envisaged to efficiently process the influent stream with a minimum footprint in layout. The compactness of the modular design will allow appropriate usage of the reactor capacity. For example, if the influent flow is reduced due to variations in industrial outputs or due to maintenance needs, then some of the reactors can be shuttered while enabling the continuous processing of the waste stream. The additional polishing unit, which can be an/a activated sludge/anaerobic filter/membrane reactor, is a required posttreatment unit to smoothen the effluent characteristics with additional BOD/COD and other pollutants removal to meet the Environmental Protection Agency (EPA) regulations. Water from the polishing unit can be used in agricultural fields or processed to meet drinking water specifications.

17.9.1 Carbon-Negative Biofuels with Carbon Capture

Reduction of greenhouse gases emissions is a primary requirement, and Kyoto-based approaches have been promulgated to effect this change. However, these methods are rudimentary at best without any tangible outcome. Sequestration of atmospheric carbon is a feasible approach to attain this reduction. The atmospheric carbon dioxide concentrations have risen steeply in recent years; in May 2021, atmospheric CO_2 reached 416 ppm, a level higher than any reached in the last 800,000 years (Mazzoldi et al. 2008). Enabling carbon sinks is possible with promising technologies such as carbon capture and storage (CCS), which is an important solution for the mitigation of the current global greenhouse gas emissions. The visible correlation between rising CO_2 emissions and the global warming phenomenon demands a concrete development of cost-effective and efficient CCS technologies; widespread development of them will lead to a significant reduction in cost and improved techniques to monitor the stored CO_2 (Abdelkareem et al. 2021). Further, to meet the sustainability criteria, cost-effective biodegradable materials, such as those derived from cellulose, cellulosic derivatives, and nanocellulose, have been developed as CO_2 selective adsorbents/membranes (Đào and Leo 2021) and multiscale carbon superparticles synthesized by soft-templating lignin nanocellulose and microbead cellulose nanofibril combination for CCS technology (Zhao et al. 2021). Different fuel cells, including molten carbonate fuel cells, solid oxide fuel cells, and algae-based fuel cells, are also used to capture CO_2 (Abdelkareem et al. 2021).

The development of sustainable CO_2 capture technologies is critical to address issues associated with global warming (Xia et al. 2016). Plants have the capability of withdrawing and storing atmospheric CO_2, and therefore sustainable biomass harvesting enables new growth and subsequent carbon withdrawing from the atmosphere. In addition, CO_2 released during biomass conversion can be captured and stored permanently in underground caves, thereby creating a net carbon sink based on life-cycle analysis (Abdelkareem et al. 2021). Using the CCS technologies that are established, CO_2 present in anaerobic processing-derived biogas can be sequestered, transported/stored, or used for a wider gamut of applications ranging from dry ice production used for beverages to food storage/shipping industries (Fig. 17.5). Synthesis of dry ice from the captured CO_2 for use in cold storage has many advantages apart from safe transfer options (Zhao et al. 2021). Further, CO_2 can be catalytically converted to useful chemicals and fuels (David Bem 2019). CO_2 sequestration/utilization is an attractive way forward to tangibly achieve COP26 targets to tackle global warming. Typically, small-scale operation of biogas production will not sustain the capital-intensive operation of combined heat and power production (CHP) and utilization of CO_2. Large integrated anaerobic processing of multiple wastes, as proposed in Fig. 17.5, will provide the cash flow liquidity and financial stability derived from numerous products to meet the sustainability criteria (Vadlani 2020).

17.9.2 Bio-Hydrogen Production from Anaerobic Processing

Anaerobic processing of bioresources results in about 0.33–0.35 m^3 of biogas/kg COD removed, and the biogas composition is typically about 65–75% methane, about 25–35% CO_2, and about 1% hydrogen (Sikora et al. 2017). If the metabolic steps leading to acetate from simple organic compounds and long-chain fatty acids are blocked, methanogenic steps are inhibited (Fig. 17.1), and formation of hydrogen and CO_2 gets enhanced. Alternatively, the anaerobic reactor can be operated at high HRT (less than 4 h) to effectively wash out the methanogens, thereby leading to bio-hydrogen production (Chang and Lin 2004). Under this regime, bio-hydrogen production between 0.1 and 0.2 m^3/kg COD removed are reported, the yield depending on the waste being processed, with sugar utilization leading to higher yield. The composition is about 35% hydrogen and 65% CO_2 (Antonopoulou et al. 2008). This clearly shows that bio-hydrogen production through anaerobic processing is a promising venture provided the necessary operating parameters and usage of high hydrogenic microbial consortium are followed.

Hydrogen is environmentally friendly and is a clean fuel; it produces water as the end product after combustion. Besides, electricity can be produced directly from hydrogen using fuel cells (Show et al. 2010; Savla et al. 2020). A high production rate and capacity to convert large organic wastes into a valuable energy source are possible through the dark fermentation method (Kucharska et al. 2018; Xia et al. 2016). Bio-hydrogen production is a viable alternative to fossil fuels that can facilitate decarbonization and provide a carbon-free energy carrier (Xia et al. 2016). Renewable bioresource-derived bio-hydrogen has garnered attention and is a promising alternative with a high yield of energy of 122 kJ/g. Various approaches are in progress to commercialize bio-hydrogen technologies; however, to attain sustainable production, utilization of bioresources as a feedstock is essential (Kim et al. 2021).

High treatment efficiency and short hydraulic retention time can be achieved with a UASB process (Chang and Lin 2004). Studies showed that a column-shaped reactor with granular sludge is the preferred choice for organic wastewater conversion to hydrogen (Show et al. 2010). Further, a two-stage UASB processing is conceivable; the first stage is operated at high HRT and different pH, leading to high hydrogen productivity, and the second stage is optimized for methane productivity. The two gaseous streams can be subjected to effective CO2 removal in the CCS unit (Sect. 4.1). The resulting combination of the enriched hydrogen and methane streams can potentially lead to an exciting gaseous biofuel with high-energy content and act as a feedstock for value-added chemical processing.

17.9.3 Process Intensification and Sustainable Engineering in Anaerobic Processing

The integrated and central processing of large-scale combined industrial/domestic waste stream ensures circularity and a sustainable approach of waste treatment with tangible multiple revenues (Fig. 17.5). The recent advances in process intensification include hybridization/consolidation of unit operations, vessels with multifunctionality in modes of production, and microengineering and microtechnology (Charpentier 2007). Some of the promising research initiatives for the process intensification in anaerobic processing include waste stream integration on a qualitative and quantitative basis, design of vessels to perform multiple unit operations, production of designer granules/biofilms/biofilters that constitute the appropriate microbial consortia, and heat minimization and integration. The integrated facility should be designed to handle lignocellulosic biomass feedstocks for optimum output of gaseous biofuels. In addition, sustainable anaerobic processing should synchronously pursue economic growth, environmental protection, and societal needs, using closed-loop material flow by embracing the 6Rs principle – reduce, reuse, recycle, recover, redesign, and remanufacture (Clark et al. 2016). Since anaerobic processing involves the removal/degradation of waste to useful gaseous biofuel via a microbial consortium activity, it is a clean technology that closely aligns with sustainable engineering principles.

17.10 Summary

Advanced anaerobic processing seamlessly integrates with the required circular global economy. Integrated operation of multiple waste streams will generate gaseous biofuels, such as enriched methane and bio-hydrogen, at an economy of scale to compete with and complement the existing natural gas industry. Process innovation and integration of new knowledge such as data analytics, AI, and robotics with the anaerobic system will provide a high-throughput sustainable platform to generate multiple products from industrial/domestic waste at a price point to compete/replace the existing paradigm of natural gas-based chemicals and fuel production. Identification of microbial flora involved in the sequential utilization of waste components at the molecular level and rapid advances in gene editing/splicing techniques should lead to the development of designer granules/biofilters/biofilms for more efficient BOD/COD removal leading to clean biofuel production and to treat refractory and complex wastes from industries such as pharmachemicals and specialty chemicals.

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Chapter 18
Techno-economic Analysis of Lignocellulosic-Based Biofuels: An Approach Toward a Practical Biorefinery

Piyush Parkhey

Abstract Lignocellulosic (LC)-based biofuels have attracted considerable interest in recent times as an exciting alternative to fossil fuels. However, higher costs of conversion of LC biomass to biofuels make them noncompetitive with fossil fuel, which severely impacts their commercial prospects. Therefore, in order to achieve a cost-effective and commercially viable LC biomass-based biofuel, technology and economically feasible approach needs to be developed in place. Of the numerous strategies to develop such a technology, techno-economic analysis (TEA) and life-cycle assessment have been identified as key tools. Techno-economic analyses have essentially been highly beneficial in identifying the technical and economic aspects of an LC-based biorefinery. It not only allows to assess the practicability of the bioprocessing plant but also helps in identifying the key economic drivers of the process. A well-established techno-economic study can help in developing sustainable, practical, and marketable technology for biomass-based biofuels. This chapter describes the basics of techno-economic analysis and discusses some recent progress in the domain toward achieving a practical solution.

Keywords Techno-economic analysis · Biorefinery · Lignocellulose · Biofuel · Biomass

18.1 Introduction

The world has seen a rapid increase in the development of new biofuel technologies in recent times. This increment can be attributed to the current day energy scenario which is affected by the incessant population rise, exhausting oil-based reserves, apprehensions of climate change due to the ever-increasing release of GHGs, and obligation of developing an alternative energy source on a commercial platform.

P. Parkhey (✉)
Amity Institute of Biotechnology, Amity University Chhattisgarh, Raipur, Chhattisgarh, India
e-mail: pparkhey@rpr.amity.edu

P. Chowdhary et al. (eds.), *Bio-Clean Energy Technologies: Volume 1*, Clean Energy Production Technologies, https://doi.org/10.1007/978-981-16-8090-8_18

Biofuels developed from lignocellulosic biomass have been at the center of interest of the scientific community owing to their obvious advantages over fossil fuels.

Lignocellulosic biomass is a renewable and indigenous fuel resource that has been recognized to reduce not only the dependency on fossil fuels but also the impact of energy on the environment and climate (Zucaro et al. 2018). A recent report suggests that energy derived from lignocellulosic substrates contributes to one-tenth of the total world's energy production, wherein by the end of 2019 the biofuel production increased by 6% from 87MT and is further predicted to increase annually by 3% till 2025 (Röder et al. 2017; Saini et al. 2020).

However, this increase has also seen the emergence of few serious concerns related to their sustainability at a large scale, efficiency, and emissions. The process economics of biofuels, especially from second-generation feedstocks such as lignocellulosic agro-waste, have come under the scanner of cost-effectiveness and scale-up. Also, owing to the issues related to biomass complexity, pretreatment type, capital costs involved, and energy required for fractionation and fermentation, the commercialization of LC biomass-based biofuels has been severely affected (Saini et al. 2020). If lignocellulosic fuels are to replace fossil fuels anytime in the near future, they need to become price competitive with fossil fuels. The preexisting technologies, though, have demonstrated successful conversion of lignocellulosic biomass into fuels and high-value chemicals, but the viability of the process has still not been fully realized, that is to say, that the current technologies need to be modified or replaced with efficient ones to obtain higher yields at an economic price, with higher conversion rates and minimum GHG emissions. This can be achieved with a thorough analysis of the technical aspect of biofuel production along with the evaluation of the economics associated with it. This brings the concept of techno-economic analysis into the forefront which can help in understanding the stages which can be optimized for reduction of energy usage and cost of the process and therefore play a significant role in realizing the possibility of developing commercially profitable and viable biofuel technology (Singh et al. 2021).

Although several techno-economic analyses have been conducted in the past decade, many of them present a rather intricate survey that is difficult to acknowledge. Most of the conclusions are in terms of complex economic terminologies which are challenging for an early career researcher to comprehend. Therefore, the authors felt there is a need to discuss and summarize the techno-economic studies conducted into terms that are easier to understand and put to use for execution by all.

In the present book chapter, the authors have first discussed the concept and importance of techno-economic analysis in realizing economically feasible biorefineries. This is followed by a brief description of the terminologies that define the techno-economic assessment. Finally, few major studies that have been undertaken by various researchers in the past decade related to this domain have been summarized. The aim is to present a comprehensible scenario related to the technical and economic feasibility of the commercial aspect of lignocellulosic-based biofuels.

18.2 Techno-economic Analysis

The sustainability of any bioprocess is reliant on the technical viability and the economics of the process. In other words, the commercial practicability of the process and its products thereof is decided upon by evaluating the technical feasibility using economical aspects such as cost of equipment, labor, consumables, and other variables. This whole assessment is called techno-economic analysis and is useful in drawing out the cost estimates of a project or an active biofuel plant. The analysis not only helps in improving the research planning and its management but also aids in the prediction of plant performance in terms of efficiency, emission of off-gases, and investment recovery time with due consideration of all the economic entities such as inflation, depreciation, and interest rates (Singh et al. 2021). Therefore, a well-detailed and thorough techno-economic analysis leads to the identification of the main economic drivers of the bioconversion process that can be of huge assistance in developing a sustainable and commercially feasible biorefinery.

18.3 Software Commonly Used for TEA

Aspen Plus® and SuperPro® are the two most commonly used software for techno-economic analyses of biorefinery processes.

Aspen Plus is a process simulation software tool developed by AspenTech, Virginia. It allows users to build a process model and then simulate it using complex calculations and also assists in developing new processes in addition to improving the existing ones. The Aspen Process Economic Analyzer allows users to map, size, and estimate the costs for equipment directly from process simulators, while Aspen Capital Cost Estimator helps the users in conceptualizing and detailing the estimates of process model costs. In addition to these, the users can optimize the small capital and draw out maintenance plans for existing facilities (Aspen Economic Evaluation, Reduce Process Costs, AspenTech n.d.).

SuperPro® is a valuable tool developed by Intelligen, Inc. which enables modeling, evaluation, and optimization of the integrated batch as well as various continuous processes. The single tool consists of models that allow for simultaneous designing and optimizing manufacturing processes, economic evaluation, resource tracking, as well as environmental impact assessment (Intelligen Inc. n.d.). The simulation software also allows for the comparison of various pretreatment processes and substrate efficiency for evaluation of economics and sustainability of the biorefinery (van Rijn et al. 2018).

18.4 Economic Performance Indicators

In this section, we have tried to simplify few terms which are commonly used in the description of techno-economic assessment of biorefineries. These have also been called economic performance indicators by some researchers. Preliminary knowledge of these terms would help the readers in a better understanding of the assessment reported in the literature.

18.4.1 Total Capital Investment (TCI)

As the term suggests, TCI corresponds to the overall invested capital in the biorefinery plant. It is the sum of *fixed capital investment* (FCI), which corresponds to the capital needed for manufacturing and plant facilities, equipment and installation, etc., and *working capital* (WC), which is the capital necessary for running the plant. Working capital is also referred to as the operating costs of the plant. While few investigators propose a 10–20% of TCI as initial WC, few prefer using 10–35% of yearly operating costs as a more pertinent measure (Chovau et al. 2013).

18.4.2 Variable and Fixed Operating Costs

Operating costs refer to the costs incurred in running the biorefinery plant. It includes raw material costs, labor costs, laboratory QA/QC costs, utilities, maintenance, waste disposal, and other miscellaneous costs. The most significant component of the operating costs of a lignocellulosic biorefinery is the enzyme cost. The availability, ambiguity in price, and difference in estimation methods make the analysis difficult (Kuo and Yu 2020), and therefore, considerable efforts must be put in to correctly estimate the enzyme costs incurred in a biorefinery to accurately analyze its economic feasibility. While maintenance costs are generally taken to be approximately 10% of the equipment costs, the miscellaneous costs include components such as insurance, taxes, and factory expenses (van Rijn et al. 2018). Operating costs also take into consideration depreciation costs, which are calculated throughout the useful life of the facility.

18.4.3 Discounted Cash Flow Analysis

Discounted cash flow analysis is an assessment technique to ascertain the value of an investment depending upon the future expected cash flow. It thus evaluates the investment based on the time value of money or in simpler terms tries to define

the current value of an investment based on projections of how much money would be generated in the future. The discounted cash flow is calculated by using a discount rate which gives the present value of expected future cash flows. Positive returns are expected if the discounted cash flow is more than the current cost of investment.

18.4.4 Net Present Value

NPV is a major economic performance indicator used extensively in techno-economic studies. It explains an investment's profitability with respect to the total value of future net cash flows over the entire duration of the project (Kuo and Yu 2020). An economically viable project is indicated by a positive NPV, while a negative value suggests an economically non-feasible project, thus signifying that higher NPV correlates to higher profit returns.

18.4.5 Sensitivity Analysis

In a financial model, sensitivity analysis regulates how the final variable is affected by the change in variable factors of the project. In a typical LC biorefinery, sensitivity analysis is performed to identify the variables that affect the MSP of the end product and estimate the extent to which they affect it. Parameters such as raw material cost, utility cost, labor costs of waste disposals, fixed capital costs, and discounted rates are frequently assessed for their effect on biorefineries.

18.5 Lignocellulosic Biomass-Based Biorefinery

The prime bottleneck in utilizing any lignocellulosic biomass for commercial-scale biofuel production is its complexity. This requires a pretreatment procedure which breaks down the recalcitrant structure and exposes the polymeric sugar chains for hydrolysis into monomeric forms which can be subsequently or concomitantly fermented into biofuel. The pretreatment requirement is irrespective of the LC biomass, though the severity of the process may vary depending upon the type of substrate and its composition. In addition to that, lignin contained in LC biomass often gets degraded into compounds such as furfurals and ferulic/glucuronic acids which act as inhibitors and decrease microbial growth and activity by interfering with their metabolism (Jönsson and Martín 2016). The pretreatment technique employed for biomass fractionation not only influences the subsequent hydrolysis efficiency, bioconversion, and product purification but also significantly affects the overall process economics. Therefore, biomass pretreatment constitutes an important step of LC biorefinery and has a major impact on the economics as well as the

technical and environmental sustainability of the bioprocess. Considering the primary importance of pretreatment in any biorefinery and to identify the best pretreatment conditions for different biomass substrates, the techno-economic assessment and life-cycle assessment of six pretreatment technologies and their comparison with NREL State of Technology (SOT) were described by Tao et al. (2013). Of the six methods compared, the MSSP of the glucose and xylose released upon pretreatment was minimum with a dilute acid treatment and SO_2-impregnated steam explosion method after NREL SOT, making them the most economic and technically viable biomass fractionation processes. Nevertheless, an ideal pretreatment method depends on many factors including the type of feedstock, chemicals required, electricity credits, and other fixed costs. Therefore, it is important to take into consideration all these factors before the most appropriate method for biomass fractionation can be decided upon. For instance, a comparison of the organosolv method of pretreatment with dilute acid for ethanol production using softwood biomass was reported by Gurgel et al. (2018). The study revealed that while organosolv pretreatment consumed almost twice as much energy as dilute acid pretreatment, it, nevertheless, also resulted in twice the amount of ethanol. The total costs of dilute acid pretreatment were considerably less, but the organosolv method consumes less water and offers higher savings in terms of equipment costs and biomass usage. Therefore, it is predicted that further optimizations in solvent recovery can further decrease the cost and increase ethanol concentration, thereby decreasing the MSP of ethanol and making organosolv treatment more commercially competitive than other methods.

18.6 Biofuel Biorefineries

Cellulosic ethanol is the most promising biofuel that is proposed to replace petroleum-based energy sources. Techno-economic analysis of bioethanol production from cellulosic sources could help in understanding the key parameters that can substantially reduce the MESP of ethanol and make it comparable to the market price of gasoline. Primarily, the key factors that regulate the MESP of cellulosic ethanol are the cost of the feedstock, the size of the biorefinery, and the enzyme load for the saccharification and fermentation. A TEA did use a pilot-scale biorefinery for bioethanol production from sweet sorghum and sugarcane bagasse and confirms that developing better high-yield feedstocks, improved microbial strains, and enzyme systems can significantly improve the process economics (van Rijn et al. 2018). Another study described the techno-economic assessment of cellulosic ethanol from *Miscanthus* (Boakye-Boaten et al. 2017) and also concluded that the MESP of cellulosic ethanol is largely governed by the feedstock cost along with the internal rate of return and corporate taxes. Improving enzyme production, pretreatment, and feedstock processing can make the technology of cellulosic ethanol from *Miscanthus* commercially viable.

Because feedstock accounts for a considerable fraction of the production cost of any biorefinery (Stephen et al. 2014), identification of a substrate that is economic, virtually inexhaustible, and easily available and has good biofuel production potential has been strongly advocated. One such cost-effective feedstock could be forestry residues. Frankó et al. (2016) used a variety of such residues as sawdust, fuel logs, early thinning, tops, and branches and determined the economic and technical feasibility of ethanol, biogas, and electricity production. While they do represent an abundant and sustainable source of biomass for biofuels and other commodity products, some types of residues such as softwood are recalcitrant to hydrolysis and therefore require severe pretreatment steps. This, other than adding to the technical complexity of the process, also adds onto to the input costs of chemicals and reagents which further have a negative environmental impact. The viability of biofuel production (and MESP), therefore, depends on the bark content of the forest residue, and hence, sawdust and shavings have the highest ethanol potential and yield, while hog fuel, debarked pulpwood, tops, and branches with a negative NPV are not prescribed for biofuel production until significant cost improvements are achieved with enhanced sugar conversion.

Along similar lines, another study conducted by Quintero et al. (2013) compared the techno-economic viability of ethanol production from four lignocellulosic substrates which were sugarcane bagasse, coffee cut stems, rice husk, and empty fruit bunches. Of the four substrates, the production cost of ethanol was minimum, and the yield was maximum from empty fruit bunches which confirms that holocellulose content, input costs, and energy consumption play a key role in deciding the feasibility of the LC biomass biorefinery. Another interesting low-cost feedstock for bioethanol production is bamboo, which in the area of abundance can prove to be an economic substrate due to its perennial nature, rapid growth, and easier management (Littlewood et al. 2013). Bioethanol production from bamboo has been shown to be technically and economically feasible; however, further cost reductions are still required to make the process competitive with the cost of petrol worldwide without tax exemptions and subsidies.

Given the high costs of cellulase enzymes and their impact on the economics of LC-based ethanol production, a different approach to make the process economically viable could be integrating cellulase production and fermentation of sugars into ethanol. One such analysis concluded that such an approach can significantly improve the process economics if higher yield and productivity of cellulose can be achieved. In addition, while the energy efficiency did not show much difference, the GHG emissions from ethanol produced from the integrated approach was much less as compared to the one produced from the purchased enzyme (Olofsson et al. 2017).

Another fuel of commercial importance that can be derived from lignocellulosic biorefineries is butanol. Jang and Choi (2018) carried out a techno-economic analysis of a pilot-scale study for concentrated acid pretreatment and continuous fermentation of LC biomass for biobutanol production. They described variable operating expenses and fixed operating investment as the major governing factors of the butanol MSP and therefore suggested that reducing these costs can make the technology commercially feasible. While the variable operating costs (except

feedstock cost) can be reduced by revenue from the by-products, the feedstock costs can be decreased by a well-developed location strategy. The pretreatment and hydrolysis process that contribute majorly to the fixed capital investment also can be optimized by using low-priced material and increasing the equipment capacity, thus improving the process economics and making it viable.

Lignocellulosic feedstocks have not only been explored for on-road vehicles but marine biofuels as well. Marine fuels need to have separate characteristics of viscosity, flash point, and water content for their usage in ship engines and off-shore applications. One of its TEA analyses explored the feasibility of few thermochemical technologies for lignocellulosic marine biofuel blendstock in terms of their technical, environmental, and economic performance. While the marine fuels from LC biomass have at least three to four times higher MSP than fossil-based fuels, the GHG emissions from agro-based feedstocks were significantly less (Tanzer et al. 2019). These findings suggest that there is still much scope of research before marine biofuels can be developed at a commercial level from LC biorefinery.

18.7 Integrated Strategy

It has been well understood that the lignocellulosic biorefinery for the production of only a single fuel is economically not viable and can't compete with the traditional petroleum refineries (Zang et al. 2020). Albeit such high prospects of lignocellulosic biofuel, a report suggests that much of the renewable fuel produced worldwide is conventional biofuel (corn-based), while only about 2% is from cellulosic biomass (EPA finalizes Renewable Fuel Standard for 2019, reflecting cellulosic biofuel shortfalls—Today in Energy—U.S. Energy Information Administration (EIA 2018)). Nevertheless, cellulosic biofuel is still very much in contention of becoming the next-generation biofuel, and substantial research is still being done to optimize the biorefinery and make it economically viable (Golecha and Gan 2016).

The major bottleneck in the effective utilization of LC biomass and making the biofuel thereof to be economically competitive in comparison to the traditional biofuel is the presence of lignin. The heterogeneous polyaromatic polymer makes the biomass recalcitrant toward depolymerization and subsequent conversion of fuel or other value-added products (Ahmad and Pant 2018).

This, however, can be overcome if an approach is developed for efficient biomass utilization with minimum energy consumption and waste generation along with cogeneration of high-value end products (Fernand et al. 2017; Mukherjee et al. 2017). An integrated biorefinery is one such strategy that can be put to use to overcome the bottlenecks. Though there are issues of high technical and market risks associated with it, it can still be made commercially viable by minimizing the complexity by a thorough techno-economic analysis (Alonso et al. 2017; Sun et al. 2017).

One such approach was proposed by Zang et al. (2020), who described integration of switchgrass biomass fractionation and xylan conversion in a single stage for the production of digestible pulp, lignin, and furfural. They also compared the techno-economic analysis of the process with industrial furfural production via chemical means. The study revealed that biomass fractionation required maximum energy input and as the leftover solid waste residues are minimum, the associated electricity production is very low which therefore required drawing additional electrical energy from the grid. In terms of yield, the mass balance study confirmed the conversion of 49% carbon into furfural, lignin, and ethanol. The feedstock cost contributed the maximum to the operating costs (~40.6%), while furfural and ethanol yielded maximum contribution to the revenues (34.7 and 47.2%, respectively). The sensitivity analysis showed that technical parameters such as temperature and solid loading and not the economic parameters maximally affected the MFSP which shared a negative linear relationship with lignin and ethanol selling price. The complete TEA established that even though 24.3 MW of electricity was additionally required, the co-production process owing to revenue generation through varied value-added products and feedstock utilization is still economically viable.

In addition to furfural, another product of commercial importance that can be co-produced with ethanol from the C5 sugars of lignocellulosic biomass is xylitol. A comparative study of producing only ethanol against co-producing ethanol with furfural or xylitol revealed that despite the higher cost of investment for the co-production of compounds and even further reduced revenue generation due to lesser electricity production, the strategy for integrating xylitol production with ethanol is profitable from an economic viewpoint (Giuliano et al. 2018). The reason is the much higher market price of xylitol, as compared to ethanol alone or ethanol and furfural together, which makes the process economically viable and sustainable.

Another important industrial product that can be derived from the C5 sugars of the hemicellulosic fractions of LC biomass is 1,5-pentanediol (PDO), an efficient plasticizer and a component of polyester and polyurethane. A novel co-production biorefinery strategy was demonstrated to improve the overall process economics which could produce ethanol at par technical and economic assumptions of NREL cellulosic ethanol. The main cost regulators of the process were biomass fractionation, enzyme loading, hydrolysis, and fermentation yield of PDO from C5 sugars. The approach requires supplementary steps which adds to the complexity, capital, and operational costs of the process; nevertheless, the PDO co-production can compensate for extra costs by credit generation (Huang et al. 2018).

Further to the earlier reported works on process economics of C5 utilization, Kim et al. (2020) analyzed the techno-economic of an integrated process for co-production of an eco-friendly bioplastic monomer 2,5-furandicarboxylic acid (FDCA) along with 1,5-pentanediol (1,5-PDO) from the insoluble cellulosic and soluble hemicellulosic portions of LC biomass, respectively. The TEA revealed that conversion of C5 sugars to 1,5-PDO and FDCA was a much economic process, despite the high capital and operating costs arising due to multiple conversion steps. This could be attributed to the revenue generated from the by-products. Furthermore, the MSP of FDCA, which was majorly governed by total capital investment and

1,5-PDO price as determined by sensitivity analysis, was much lower than petroleum-based TPA which shows that FDCA can effectively replace TPA. Their assessment also took into account the environmental impact of the process and showed that electricity consumption was the main contributor to climate change and fossil fuel depletion. The overall conclusion of the study was that the biomass-based FDCA can be made more eco-friendly if renewable resources can be used for covering the electricity requirement.

Lignin is another component of LC biomass other than cellulosic and hemicellulosic fractions, which can be utilized for the production of various commodity products, the most important being phenolics. An integrated approach that can simultaneously utilize lignin of LC biomass for value-added products with other applications could make the whole bioprocess refinery economically viable. In a separate study (Fernández-Rodríguez et al. 2020), an integrated biorefinery was designed for utilizing the waste streams of LC biomass fractionation for the production of phenolic compounds. They compared the production of phenolic compounds from depolymerization of lignin from solid pretreated biomass as well as black liquor obtained during precipitation. During the lignin extraction stage, a higher yield was obtained with soda extraction as compared to organosolv but at a higher solvent consumption. In addition to this, organosolv (OS) method also consumed large volumes of water. However, since OS allowed for the recovery of solvents, the fixed capital investment (FCI) and the manufacturing costs (COM) were low. Therefore, regardless of depolymerization of lignin, directly either from liquor or from an earlier precipitation stage into monomeric phenolics, organosolv was evaluated to be the most viable process for pretreatment and subsequent production of phenolics.

A novel integrated strategy for lignocellulosic biorefinery was demonstrated in a recent report (Naderi Nasrabadi et al. 2020). They discussed integrating an electrochemical reactor for converting biorefinery lignin into value-added chemicals along with hydrogen. In their study, they described the electrochemical oxidation of lignin in the anodic compartment of an electrochemical reactor for the production of hydrogen. The economic viability of the process was analyzed by integrating the reactor to a lignin biorefinery, whose 50% lignin stream is directed to the reactor, with the other 50% going to a boiler to generate or recover process energy. The integration imparted additional manufacturing costs to the process for electrochemical conversion due to significant electricity requirements. The additional costs can only be compensated, and the process can become feasible if the lignin oxidation products have commercial value. The TEA revealed that the electrochemical oxidation with 88% conversion of lignin into high economic value products and hydrogen cogeneration can counterbalance the capital and energy costs, along with other costs such as depreciation, taxes, and return on investment, thus improving the process economics.

In any integrated biorefinery approach, the most important factor is the added operating costs that emerge due to additional processing steps, chemicals, and the requirement of instruments. If the infrastructure and utilities of the biorefinery can be shared, it will not only lead to an efficient treatment system for the effluent from the

main unit but also decrease the capital costs required for additional steps. One such study was proposed by Geng et al. (2020) where they compared the production of hemicellulose from various LC biomass co-localized with a pulp mill. Due to sharing of the utilities and infrastructure, the costs of capital investments and manufacturing were much lower, thereby suggesting that co-location can yield better and reasonable process economics for commercial purposes. This was further established in a study (Özüdoğru et al. 2019) which investigated the cost-effectiveness of an integrated biorefinery co-producing electricity with other products such as xylitol, citric acid, and glutamic acid from sugarcane biomass while annexed to a sugar mill. While the co-production of citric acid electricity was not economically viable, the other two approaches, i.e., co-production of electricity and xylitol or glutamic acid, demonstrated considerable feasibility as compared to the base case scenario where biomass was exclusively burned for electricity production. While this study confirms that integrated approaches could make the LC biorefineries economically attractive, their efficacy to a great extent depends upon the type of commodity being produced. Co-production of higher-value chemicals, like xylitol and glutamic acid, in this case, can ensure the economic competitiveness of the biorefinery. An ideal biorefinery should therefore concentrate on complete and total utilization of all the fractions of the biomass for bioproduct generation, thus leading to a sustainable and resource-efficient valorization of biomass, a concept which has been recently called as circular bioeconomy (Table 18.1).

18.8 TEA of Sugar Production

A key parameter in designing an efficient and economic lignocellulosic biorefinery is to maximize the yield of sugars, both hexoses and pentoses from the biomass. Techno-economic analysis of sugar production from LC biomass would therefore be highly supportive information for assessing the commercial viability of the biomass-based products.

One such analysis and simulation to determine the production costs of sugars from biomass has been recently reported (Kuo and Yu 2020). They analyzed the process viability for the production of C5 and C6 sugars and the influence of a combined heat and power (CHP) system along with the utilization of residual biomass for energy recovery. The operating costs for sugar production are mainly affected by raw material and biomass costs. The major contributor to raw material is the cost of enzymes for biomass hydrolysis. Another contributing factor to the operating costs of the process is the utilities required for the process such as steam, cooling water, and electricity. The utility costs can be substantially decreased by integrating a combined heat and power system to sugar production where residual biomass is burnt for recovering energy and production of electricity. An interesting finding, however, is that if the biomass solids are not burned but returned to the soil

Table 18.1 A comparative analysis of techno-economic assessments of various integrated biorefinery approaches from LC biomass

Biorefinery description	Products	Simulation software	Techno-economic assessment	Special remarks	References
One pot biomass fractionation using a biphasic solvent comprising aqueous choline chloride (ChCl) and methyl isobutyl ketone (MIBK)	Furfural integrated into ethanol production	Aspen Plus	49% feedstock carbon conversion into products (17.9% to furfural, 16.0% to lignin, and 15.1% to ethanol) MSP of furfural was 37% less than market price	Technical parameters such as reaction temperature and solid loading impact more on the MFSP than the economic parameters (material and installation cost)	Zang et al. (2020)
Sugarcane lignocellulosic biorefinery annexed to a sugar mill	Electricity, co-produced with xylitol, citric acid, or glutamic acid	Aspen Plus	Electricity co-production with either xylitol or glutamic acid was economically feasible (IRR of 12.3% and 31.5%, respectively)	The economic feasibility of integrated biorefineries was compared to a combined heat and power (CHP) plant baseline scenario that solely combusts biomass for electricity co-production	Özüdoğru et al. (2019)
An integrated approach for catalytic biomass conversion to C_6 and C_5 sugars for subsequent product formation	2,5-Furandicarboxylic (FDCA) from C_5 sugars via furfural co-produced with 1,5-pentanediol from cellulose via HMF	Aspen Plus	Conversion of C_5 sugar to 1.5-PDO had a positive impact on the process economics as compared to C_5 sugar to furfural The MSP of FDCA was 29% lower than the market price of TPA	Heat integration can counterbalance the energy requirement of the process LCA showed biomass-derived FDCA had a lower impact on fossil depletion but higher impact of climate change as compared to petroleum-derived TPA	Kim et al. (2020)
Organosolv fractionation of lignocellulosic biomass followed by catalytic conversion of hemicellulose and enzymatic hydrolysis and fermentation of cellulose	Co-production of C_5 sugars to 1,5-pentanediol and C_6 sugars to ethanol	Aspen Plus	Co-production of ethanol and 1,5-PDO based on NREL prescribed process parameters and economic assumptions for cellulosic ethanol led to an MESP of $1.35/gal 1,5-PDO SP at $1600/ton, which is the	Co-production of ethanol with 1,5-PDO results in sufficient revenue to offset the additional process steps, complexity, and operational and capital costs Biomass fractionation,	Huang et al. (2018)

			lowest reported price of all C4–C6 α,ω-diols	enzyme loading and overall yield of C_5 and 1,5-PDO regulate the overall process economics	
Multiproduct biorefinery from corn stover utilizing C_5 sugars compared with ethanol base case	Co-production of xylitol or furfural from C5 sugars obtained from biomass hydrolysis	Aspen Plus	While the operating costs for ethanol-only production were almost equivalent to co-production with xylitol or furfural, the total investment cost for co-production strategies were significantly lower	Xylitol coproduction with ethanol was commercially profitable due to its higher market price as compared to furfural co-production or ethanol-only approach	Giuliano et al. (2018)
Lignocellulosic biorefinery annexed to a sugar mill utilizing bagasse and trash as feedstock	Ethanol or 1,3-butadiene production	Aspen Plus	The MSP of biobased butadiene and ethanol were higher than fossil-based products, which was taken as baseline	The greenhouse emissions were significantly lower for biomass-based butadiene as revealed from LCA, thus confirming a lesser environmental impact	Farzad et al. (2017)
An electrochemical reactor integrated to a lignocellulosic biorefinery	Biorefinery lignin converted to higher-value industrial chemicals with cogeneration of hydrogen	–	At low level of lignin conversion, the break-even cost of biofuel production from the lignin oxidation stream would be higher in integrated approach This is because of significant input costs in the form of electricity due to electrochemical integration	The suggested biorefinery approach could only be economically feasible if it generates a lignin oxidation product that has inherent industrial value The biorefinery product from electrochemical process should be of a sufficiently high value as a feedstock for industrial production	Naderi Nasrabadi et al. (2020)

for carbon sequestration, it still did not increase the MSP of sugars, but rather may as well reduce it by a reduction in the carbon tax credit.

Utilization of the by-products generated after sugar extraction from lignocellulosic biomass for the production of value-added products can also counterbalance the sugar production costs in a biorefinery. The hemicellulosic and the lignin components can be transformed into commercial products which can considerably reduce the minimum sugar selling price. One such work was reported recently (Geng et al. 2020) wherein the hemicellulosic fraction was converted to xylitol and lignin was liquefied to polyols. The production of xylitol and polyols increases the operating costs of the biorefinery due to an increase in raw materials, expensive chemicals, and the requirement of extra energy. However, combustion of unreacted lignin for energy production and higher market value of polyols and xylitol can offset the increased operational costs by about 23%, thereby adding substantial credits to the lignocellulosic biorefinery. Improving the technical aspects of the process such as chemical recycling and increasing the xylitol and polyol yield can further result in the viability of the biorefinery.

18.9 Other Products

Although lignocellulosic biomass has been extensively used for the production of biofuels and other chemicals, however, they are also an attractive substrate for other value-added substances such as amino acids and other compounds of nutritive value. The C5 and C6 sugars released after LC fractionation can be channelized effectively into the production of diversified products via different bioprocesses, making it a multifaceted substrate. Techno-economic analysis of these bioprocesses would allow for the possible exploration of a new dimension toward commercializing lignocellulosic biorefineries.

One such study (Chen et al. 2019) described the TEA of L-lysine production from corn stover using *Corynebacterium glutamicum*. The obtained yield was much less than that of starch fermentation pathways but multifold greater than that from other feedstocks. The TEA analysis revealed that the minimum lysine selling price was higher than the market price, but futuristic advancements in the technical details of the bioprocess which increase the lysine yield may significantly bring down the cost, thereby making the process commercially viable.

Lactic acid is another product of commercial importance that can be produced along with ethanol from biomass feedstock. Another study (Manandhar and Shah 2020) has analyzed the techno-economic feasibility of lactic acid production from corn grain and compared the yields from yeast, bacteria, and fungi. While the lowest production costs were obtained with yeast fermentation, further improvements in process efficiency, costs related to feedstocks, chemicals, and equipment may additionally improve the techno-economic viability and make the process comparable with petroleum-based lactic acid production. This supplements the notion of the development of a sustainable bioeconomy wherein the existing infrastructure for

corn-based ethanol can be utilized for producing a high-value chemical with commercial applications.

The economic viability and environmental assessment of the co-production of lactic acid from sugars of sugarcane bagasse and leaves have also been assessed. While the cellulosic fractions of substrate resulted in a higher rate of lactic acid production, it also affected the total capital investment, operating costs, as well as revenue generation. The approaches where gypsum isn't co-produced required lesser chemicals and therefore have a lower impact on the environment, improved life cycle, and better economic performance. Even though using acid-tolerant bacteria for LA production led to a lesser cost of operation and total capital investment, the rate of production was less, thereby making the approach economically unattractive (Gezae Daful and Görgens 2017). Nevertheless, the study highlights that industrially important chemicals and reagents can also be attractive by-products from LC biorefinery.

LC biorefineries also put forward an exciting and sustainable alternative for the production of industrially important compounds while relieving environmental loads of their conventional production processes. One such compound is 1,3-butadiene (BD), a key monomeric unit used in rubber and polymer products which is industrially produced from thermal cracking of naphtha, an environmentally and energetically non-friendly process. An economically feasible approach for the production of BD from lignocellulosic substrates can therefore have a major impact on the rubber industry. A life-cycle analysis and techno-economic assessment of BD production attached to a sugar mill, with co-production of electricity from sugarcane residues, were therefore undertaken (Farzad et al. 2017), and it was observed that integrating BD production with ethanol led to lower capital costs and energy consumption. The economic sensitivity analysis showed the MSP to be dependent upon the total capital investment, the desired internal rate of return, and the yearly operational period. The simulations and multi-criteria analysis demonstrated that even though the BD production from LC biomass was not economically profitable, as the MSP was about three times that of the current market price, there was a significant decrease in GHG emission by 85%. This advocates that biorefinery-based commodity production and achieving a sustainable and green economy still have a long way to go and require considerable basic research.

18.10 Future Prospects

An ideal biorefinery should therefore concentrate on complete and total utilization of all the fractions of the biomass for bioproduct generation, thus leading to a sustainable and resource-efficient valorization of biomass, a concept which has been recently called as circular bioeconomy. With this proposition, Valdez-Vazquez and Sanchez (2018) have described the development of a biorefinery using mixed cultures that can serially defragment the fractions of lignocellulosic biomass into biofuels, namely, hydrogen, methane, and butanol. As this approach mimics the

natural processes, it effectively skips the requirements of harsh chemicals and reagents, costly reactor setups, and other expensive arrangements for biomass fractionation and product formation. The techno-economic analyses further strengthened the notion that such biorefineries have a lower environmental effect and are less energy-intensive, thereby suggesting that a profitable bioprocessing strategy can be developed using mixed culture fermentation.

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