Clean Energy Production Technologies

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Vijai Kumar Gupta *Editors*

# Microbial Strategies for Techno-economic Biofuel Production

Springer

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**Series Editors**

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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 bioenergy sources, which can produce 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.

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# Foreword

Replacement of fossil fuels with renewable energy is important to save the environment from its hazardous effect as well as fulfilling the needs of society. Renewable energy production is a unique sustainable replacement option of fossil fuels in the form of biofuels production, which is cheap, renewable, and environment friendly. The cost of technology involved in this green fuel production process is the main limitation of its practical and commercial implementation. Production process such as enzymes used in biofuels production, processing of waste biomass for enzymatic hydrolysis, imbalance, the cost economy of this process, and so required attention for commercial utility and environmental protection. Various approaches like microbial and bioprocess improvement may be able to resolve this issue to some extent.

The publication of the book entitled *Microbial Strategies for Techno-economic Biofuel Production* is a commendable step in the proposed area. I am writing this message with satisfaction as a researcher in the same area. This book essentially contains ten chapters addressing various issues on practical ground level to improving the economy of the system. The book presents details of various microbial and microbes-related parameters used in different biofuels production technologies including biogas, biobutanol, bioethanol, biohydrogen, and biodiesel. In my view, the book will prove itself as an asset to those working and interested in this field, which includes scientists, researchers, teachers, students, and industries.

I appreciate the efforts of Dr. Neha Srivastava [IIT (BHU), Varanasi], Dr. Manish Srivastava [IIT (BHU), Varanasi], Prof. (Dr.) P.K. Mishra [IIT (BHU), Varanasi], and Dr. Vijai Kumar Gupta [TTU, Estonia] for bringing out the book entitled *Microbial Strategies for Techno-economic Biofuel Production*. The effort taken to complete this book will surely cover the whole and demand of industries, scientists, teachers, researchers, and students. I congratulate the editors for their hard work and bringing a final shape to this book.

Department of Science, Galway-Mayo Institute of Technology, Galway, Ireland

Anthonia O'Donovan

# Acknowledgments

The editors are thankful to all the academicians and scientists whose contributions have enriched this volume. We also express our deep sense of gratitude to our parents whose blessings have always prompted us to pursue academic activities deeply. It is quite possible that in a work of this nature, some mistakes might have crept into the text inadvertently and for these we owe undiluted responsibility. We are grateful to all the authors for their contribution to this book. We are also thankful to Springer Nature for giving this opportunity to editors and the Department of Chemical Engineering & Technology, IIT (BHU) Varanasi, U.P., India, for all technical support. We thank them from the bottom of our heart.

# Contents

# About the Editors

**Neha Srivastava** is an expert in the field of biofuel production, microbial bioprocessing and enzyme technologies. She is currently working as a post-doctorate fellow at the Department of Chemical Engineering and Technology, IIT (BHU) Varanasi, India. She has published 25 research articles in peer-reviewed journals and holds 3 patents and 1 technology transfer. She completed her PhD in Bioenergy at the Department of Molecular and Cellular Engineering, SHIATS, India. She has received 6 Young Scientist Awards. Her current research focuses on biofuel production (cellulase enzymes; production and enhancement, biohydrogen production from waste biomass, bioethanol production).

**Manish Srivastava** is an expert in the field of nanomaterial synthesis and the application of nanomaterials as catalysts for the development of electrode materials for energy storage, biosensors and biofuel production. He is currently working as a member of the DST INSPIRE faculty at the Department of Physics and Astrophysics, University of Delhi, India. He has published 45 research articles in peer-reviewed journals, authored several book chapters and filed 1 patent. He worked as a post-doctorate fellow at the Department of BIN Fusion Technology, Chonbuk National University. He received his Ph.D. in Physics from the Motilal Nehru National Institute of Technology, Allahabad, India. His areas of interest include synthesis of nanostructured materials and their applications as catalysts for development of electrode materials in energy storage, biosensors and biofuels production.

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# Chapter 1
# An Introduction to Algal Biofuels


**Manisha Verma and Vishal Mishra**

**Abstract** Desires of living a higher standard of life bring regular consumption of non-renewable energy resources. This conventional fuel consumption causes emission of $CO_2$, particulate matter, and greenhouse gases to the atmosphere; in such conditions, energy crisis brings ignited focus on algaculture for producing biodiesel and other liquid biofuels. Algal oil or algal biofuels are third-generation biofuels, emerged as a renewable alternative to conventional liquid fuels. These algal oils are also a replacement for conventional biofuels, which are obtained from agricultural sources like corn, sugarcane, oilseed plants, and some animal fats. Unlike oilseed crops, they do not need vast farmland and hence keep agrarian lands available for food crops. Algaculture is possible in freshwater, saline water, and wastewater from various sources with minimal impact. Algaculture has a significant effect on environmental pollution as it assimilates nitrate and phosphate present in wastewater while continuously contributing to $CO_2$ sequestration. Algal oils are biodegradable and comparatively less harmful to the surrounding if spilled. Open outdoor cultures are used for algae cultivation for their low cost, but generally, they are profoundly affected by environmental disturbances like light availability and temperature swings. Ongoing research in algal biofuels is focused on the rapid growth of biomass, high lipid production, and thermal tolerance.

**Keywords** Renewable energy · Algal biofuel · Algaculture · Wastewater · $CO_2$ sequestration · Third-generation biofuel

## Abbreviations

| | |
|---|---|
| ADP | Adenosine diphosphate |
| AMP | Adenosine monophosphate |
| ASTM | American Society for Testing and Materials |

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| | |
|---|---|
| ATP | Adenosine triphosphate |
| C | Carbon |
| Ca | Calcium |
| CNRL | Canadian Natural Resources Limited |
| $CO_2$ | Carbon dioxide |
| Cu | Copper |
| DNA | Deoxyribonucleic acid |
| Fe | Iron |
| GHG | Greenhouse gas |
| HAMGM | Highly assimilable minimal growth medium |
| HDRD | Hydrogen-derived renewable diesel |
| K | Potassium |
| $K_2CO_3$ | Potassium carbonate |
| Mg | Magnesium |
| Mn | Manganese |
| Mtoe | Million tons of oil equivalent |
| N | Nitrogen |
| $NO_X$ | Nitrogen oxides |
| P | Phosphorus |
| PBR | Photobioreactor |
| S | Sulfur |
| TAGs | Triacylglycerides |
| TPES | Total primary energy supply |
| Zn | Zinc |

## 1.1 Introduction

Petroleum reservoirs are depleted due to unlimited consumption of petroleum as an energy source and cause an increased demand for fuel in developed and developing countries worldwide. Fossil fuel combustion causes serious climate issues such as global warming by emitting greenhouse gases (GHG) (Deng et al. 2020). Biofuels are emerged as a budding hope to reduce the reliance on crude oils with some limitations. Lipid-based fuels from algal sources are well-known renewable sources for many years. Algae are easy to grow and harvested at outdoor cultivations in freshwater, saline, or wastewater. Extracted algal oils contain various forms of lipids or triacylglycerides (TAGs) that can be processed further for the production of fatty acids, methyl ester (biodiesel), synthetic jet fuel, and hydrogen-derived renewable diesel (HDRD) which can be employed as fossil fuel alternatives (Lam et al. 2019). Photosynthetic microorganisms like microalgae can fix atmospheric carbon dioxide more rapidly than terrestrial plants or trees (Paul et al. 2020). Microalgae could be either prokaryotic or eukaryotic and have a reasonable growth rate in harsh conditions (Li et al. 2008a; Richmond 2008); it could be served as a basic raw material for

the production of biodiesel, ethyl alcohol, methane, and bio-hydrogen (Tsukahara and Sawayama 2005). Microalgae cultivation for biodiesel serves several other purposes, such as elimination of ammonia, nitrogen oxides, and phosphates from domestic or industrial effluents (Wang et al. 2008) and $CO_2$ sequestration during photosynthesis (Wang et al. 2008). Very less greenhouse gases are emitted during the production process of biodiesel, and the residual biomass left after oil extraction can be utilized as bio-fertilizer for agricultural lands (Directive 2003). It was also noticed that high lipid content was obtained when microalgae was cultivated under nutrient-limited condition (Rehman and Anal 2019). Algae generate more lipid content as compared to conventional oilseed crops (Moradi-kheibari et al. 2019). Recent trends of hike in the price of crude oil along with GHG and pollutant emission drive a new focus on microalgae biodiesel production. Several companies started a suitable market by selling either the complete process unit or some parts of it (photobioreactors and cell disruption units) for algaculture and biodiesel production (Barclay and Martek Biosciences Corp 2005; Behrens et al. 2007; Kanel et al. 1999). Algae cost more per unit biomass production as compared to other lignocellulosic non-food crops because of their expansive processing and operation (Carriquiry et al. 2011). To ensure availability and reducing the production cost of algal biofuels commercially, several industries and governments provide funds (Omidvarborna and Kim 2019).

## 1.2 Algal Species Involved in Biofuel Production

Table 1.1 represents different freshwater and marine algal species and the range of dry weight lipid content in percent (Chisti 2007; Meng et al. 2009; Li et al. 2008b; Terme et al. 2017).

**Table 1.1** List of various algal species and their dry weight lipid content%

| Algae | Type of algae | Lipid content% dry-weight | References |
|---|---|---|---|
| *Botryococcus braunii* | Colonial-green microalgae | 25–75 | Chisti (2007), Meng et al. (2009) |
| *Chlorella* sp. | Single-celled green algae | 22–63 | Li et al. (2008b) |
| *Dunaliella primolecta* | Single-celled, photosynthetic marine-green alga | 23 | Chisti (2007) |
| *Crypthecodinium cohnii* | Dinoflagellate microalgae | 20 | Meng et al. (2009) |
| *Isochrysis* sp. | Haptophytes | 25–33 | Chisti (2007) |
| *Nannochloris* sp. | Green algae | 20–35 | Chisti (2007) |
| *Nitzschia* sp. | Marine diatom | 45 | Chisti (2007), Meng et al. (2009) |
| *Sargassum* | Brown (class Phaeophyceae) macroalgae (seaweed) | 0.13–2.96 | Terme et al. (2017) |

Different microalgae species accumulate different amounts of lipid. In order to enhance the yield of lipid from microalgae cultivation, algal cells can be cultivated under some favorable environmental conditions (Sheehan et al. 1998). In certain controlling circumstances, lipid content could arise up to 90% of the dry weight of microalgae, but in general, the average content of lipid ranges between 1 and 70% of dry weight biomass (Li et al. 2008a, c; Chisti 2007; Spolaore et al. 2006). Screening of seaweed, diatoms, and other algae species for biofuel production relies on growing efficiency, lipid content, oil yield, and ability of microalgae to assimilate available nutrients in certain environmental conditions. As represented in Table 1.1, widely used algal species are *Botryococcus braunii, Chlorella* sp., *Cylindrotheca, Crypthecodinium* sp., *Dunaliella* sp., *Isochrysis* sp., *Nitzschia* sp., and *Nannochloris* sp. These species contain up to 20–60% dry weight lipid content. Different algal species accumulate different compositions of fatty acids in their lipid content, which have an enormous impact on biodiesel characteristics. Usually, saturated and unsaturated fatty acids containing 12–22 carbon atoms are found in freshwater microalgae species (Thomas et al. 1984). Fatty acid composition could be affected by the growth phase of cells, and under certain nutritional limitations, environmental and cultivation conditions such as salt stress and nitrogen depletion in culture medium induce C18:1 accumulation (Thomas et al. 1984).

## 1.3 Types of Biofuels Produced from Microalgae

In the field of renewable energy fuels, microalgae part a huge contribution by being a producer of multiple biomass-generated energy sources. Efforts of producing biofuels for replacing fossil fuels are not novel; instead, now it is taken seriously by seeing the scarcity and escalating hike in the price of fossil fuels. Figure 1.1 represents renewable fuels generated from different processes by microalgae biomass. Anaerobic digestion of microalgae biomass produces methane (Serna-García et al. 2020). Algal lipids are used for biodiesel production by transesterification (Duran 2020). Direct or indirect photolysis process of algae yields bio-hydrogen (Jiménez-Llanos et al. 2020). Microalgae have also been reported as a promising feedstock for the alcohol fermentation because of their sufficient starch accumulation inside the cell and cell wall (Harun et al. 2010), which conclude that microalgae could have great potential to generate biodiesel as well as ethanol-based biofuels.

### *1.3.1 Biodiesel*

Nowadays, biodiesel is a proven energy fuel produced from plants, animals, and microalgae oils. Biodiesel from soybeans is available in the United States. Jatropha, corn, palm, and canola have been used for agro-based diesel generation (Blackshaw et al. 2011). Under some specific growth conditions, microalgae produce a large

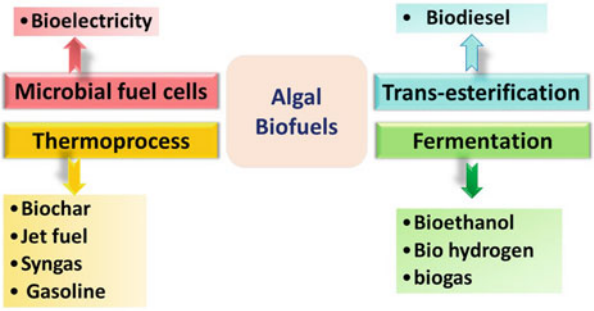


**Fig. 1.1** Representation of different fuel alternatives produced from microalgae

amount of lipid in their biomass (Sharma et al. 2012a). Microalgae have more potential to deliver higher biodiesel content than cotton and palm oil (Singh et al. 2011). Algal oils are quite abundant in double bonds of polyunsaturated fatty acids as compared to other vegetable/seed oils (Belarbi et al. 2000). Biodiesel of microalgae oil origin is required to match with some standards. According to the European Union, two different grades are applied for biodiesel: (1) standard EN 14214 in automobile engines and (2) standard EN 14213 as heating or burning fuel, while in the United States, ASTM standard D 6751 biodiesel is used (Knothe 2006). Figure 1.2 represents the chemical reaction of lipid transesterification into methyl esters.

Algal oil consists of triacylglycerides, which have three moles of fatty acids esterified by a glyceride molecule. For obtaining biodiesel, triacylglycerides have to undergo a reaction known as alcoholysis or transesterification (acids, alkali, and lipase as catalysts) with 3 moles of methanol, which yield 3 moles of methyl esters of fatty acid (which is biodiesel) with 1 mole of glycerol (Fukuda et al. 2001). The transesterification process could be optimized for reducing the production cost of biodiesel, such as direct transesterification. Direct transesterification includes chemical extraction of complex algae cell walls and transesterification of these extracted algal oils in the same step (Li et al. 2011; Johnson and Wen 2009). Figure 1.3 represents microalgae processing steps for biodiesel production. Harvesting microalgae cells is the primary procedure performed by gravity sedimentation, dewatering, filtration, centrifugation, and flocculation. The selection of suitable harvesting techniques depends on the quality of the desired product (Richmond 2008). Processing is a significant aspect to optimize for the low-cost production of any commodity since it should be highly specific for the desired product. In microalgae processing, drying of algal biomass is the next step to extend the storage

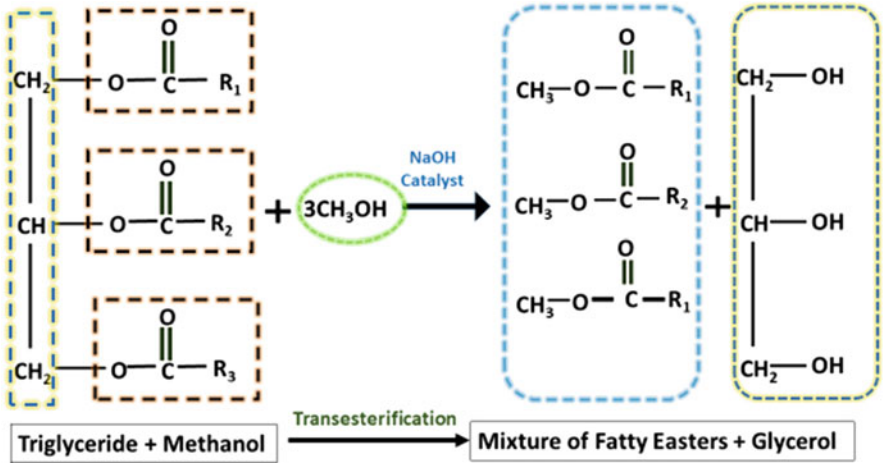


**Fig. 1.2** Transesterification of lipids into methyl esters

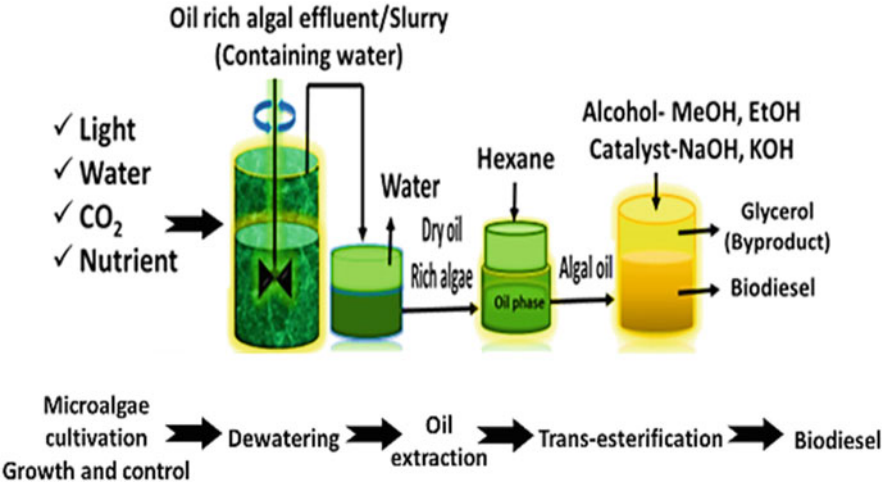


**Fig. 1.3** Microalgae processing steps for biodiesel production

life of raw algal biomass, and spray dryer, freeze dryer, drum dryer, and sun drying are the main processes involved in this step (Richmond 2008). Cell disruption is the next step to obtain algal oil. Cell disruption has been done by mechanical (homogenization, ultrasonication) or any other chemical (acid–alkali treatment) or enzymatic methods. For lipid production, extraction of algal oils is the next process involving solvent extraction by using solvents such as ethyl alcohol, hexane, or hexane–ethyl alcohol mixture (Richmond 2008; Grima et al. 2003; Cravotto et al. 2008).

### 1.3.2 Biobutanol

Escalating price and scarcity of petroleum and other fossil fuels demand sustainable fuel at an economical cost. Up to 85% of biobutanol can be smoothly blended with gasoline; it is used as a substitute for energy in Japan, USA, and Europe, which include 8%, 33%, and 19% of their total fuel energy supplement, respectively. Up to 15% of biobutanol blend is allowed in Europe while a 16% blend is permitted in the United States. Butanol correctly fixes into the standards of replacing ethanol as a gasoline additive as it has very high viscosity with lower heat of vaporization and lowers volatility as compared to ethanol (Qureshi et al. 2001). Microalgae biomass contain carbohydrates, lipid, protein, and many biomolecules, so microalgae that consist of the majority of storage as glucose, starch, and cellulose serve as feedstock for fermentation media (Chen et al. 2013). Storage polysaccharides of microalgal cells are released by some biological (enzymatic), chemical, mechanical, and thermal pretreatment. Cellulose and lignin need to be hydrolyzed by some mild treatments. However, polysaccharide accumulating strains could be improved by genetic engineering for efficient biobutanol production (Passos et al. 2014). After algal lipid extraction, the leftover green cellular waste could be used as polysaccharide feedstock for butanol production. Fermentation could be carried out by some bacteria of genus *Clostridia* (Potts et al. 2012).

### 1.3.3 Biogasoline

Gasoline consists of C4–C12 n-alkanes and iso-alkanes with a mixture of various arenes, cycloalkanes, and oxygenates. The antiknock index is the main characteristic of gasoline and is measured by octane rating (Gibbs et al. 2009). Higher the octane number tends to raise the compression ratio, which generates more energy and enhances the functioning of the motor engine. After the three-step conversion of biomass-derived levulinic acid into angelica lactone dimer, gasoline-like branched C7–C10 hydrocarbon is obtained. Figure 1.4 shows the chemical conversion reactions of levulinic acid into alpha-angelica lactone dimer.

This levulinic acid derived from algal biomass undergoes intramolecular dehydration by using K10, i.e., montmorillonite clay and yields >90% angelica lactone. The next step is the dimerization of angelica lactone in the presence of anhydrous $K_2CO_3$, which yields >94% alpha angelica dimer; this dimer is used as feedstock in hydro-deoxygenation reaction, in the presence of some noble catalysts, and yields C7–C10 hydrocarbons of gasoline volatility range (Mascal et al. 2014).

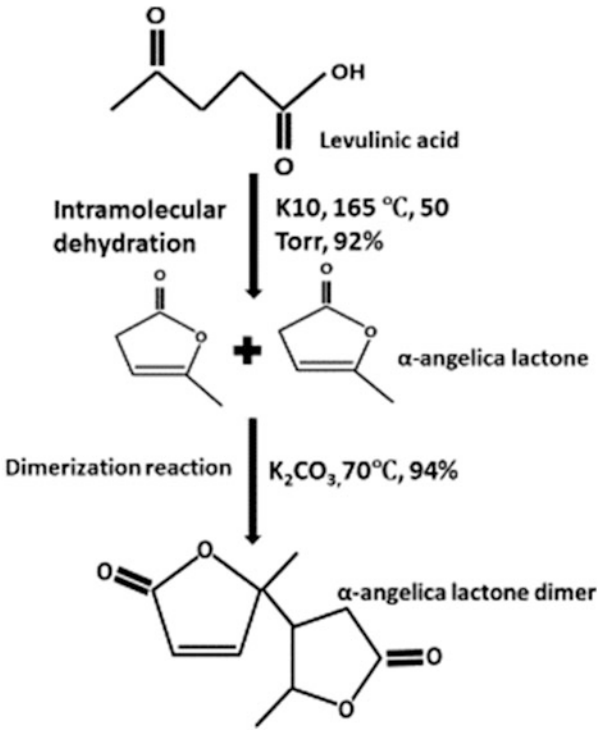


**Fig. 1.4** Formation of alpha-angelica lactone dimer for biogasoline production

### *1.3.4 Methane*

Microalgae have substantial nutritional value. They contain proteins, carbohydrates, and lipids in abundant quantities. For anaerobic digestion and methane production, *Chlamydomonas reinhardtii, Dunaliella salina, Arthrospira platensis, Euglena gracilis*, and *Scenedesmus obliquus* are some microalgae species that have been utilized (Mussgnug et al. 2010). Processed algae leftovers (after oil extraction) are the finest raw material for methane generation by anaerobic digestion as compared to unprocessed microalgae (microalgae that do not undergo the process of lipid extraction). Unprocessed microalgae have lipid content which generates volatile fatty acids that inhibit anaerobic digestion (Zhao et al. 2014).

### *1.3.5 Ethanol*

Microalgae and algal species have cell walls derived from various monosaccharides and complex polysaccharides, the majority of which are cellulosic contents; also algae have starch accumulation as a storage substrate (Goh and Lee 2010). Up to 70% of polysaccharide content is found in some marine microalgae are cellulose,

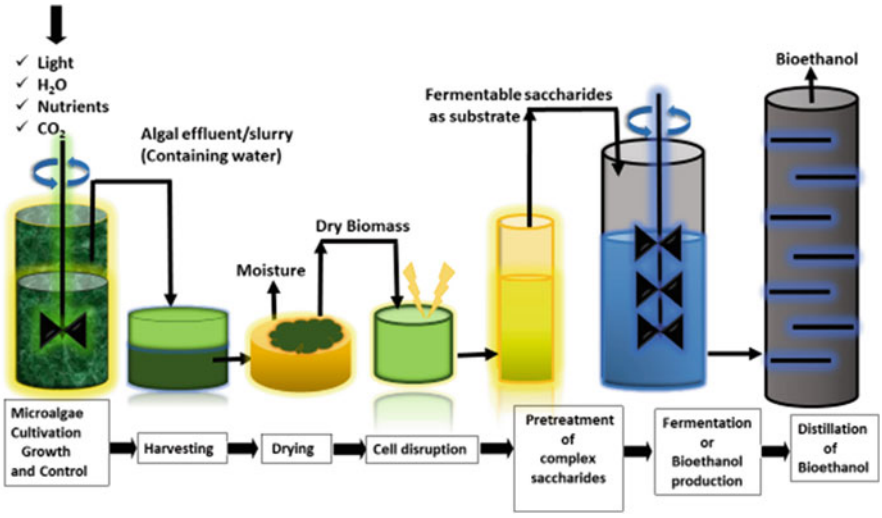


**Fig. 1.5** Schematic representation of bioethanol production from microalgae feedstocks

hemicellulose, mannon, and xylan present as cell wall components. Agar, algin, and carrageenan present as intracellular saccharides, while floridean starch, laminarin starch, and amylopectin are present as food storage of cells (Okuda et al. 2008). All these polysaccharides are the finest feedstock and need some pretreatments to convert complex polysaccharides into fermentable saccharides for ethyl alcohol fermentation. Algal feedstock has various advantages over other crops to obtain saccharide or starch as fermentable sugar, which include utilization of different domestic/industrial effluents or saline/blackish wastewater, carbon dioxide recycling, high productivity in terms of area, and there is no need of traditional agricultural land; hence, algae are the most valuable nonedible feedstock for biofuel production (Wijffels and Barbosa 2010). *Laminaria hyperborean* is a brown seaweed used to extract mannitol, which is utilized as a raw substrate for bioethanol fermentation by the bacteria *Zymobacter palmae* (Horn et al. 2000). Up to 15% of bioethanol blends are used in transportation fuel and hence make 99% of overall biofuel utilization in the USA. However, ethanol has less energy efficiency as compared to gasoline, but it has many advantages, such as fewer pollutant emissions, and is produced from waste biomass (Rao and Bantilan 2007). Algae are a nonfood feedstock, and having high photosynthetic efficiency could be considered as the most promising feedstock for ethanol fermentation (Hossain et al. 2008; Wijffels et al. 2010). Figure 1.5 represents microalgae pretreatment and processing for the bioethanol fermentation.

Microalgae cell is the natural source to obtain fermentable saccharides. Utilizing microalgae for bioethanol fermentation is based on three consecutive processes: (1) preprocessing of microalgae biomass, (2) saccharification, and (3) bacterial or yeast fermentation of saccharides. Microalgae pretreatment is a necessary process for improving the production efficiency, which focuses on accessing all intracellular

saccharides as well as saccharides present in cell walls. Fermentable sugars can be extracted from algal biomass by utilizing some biological, chemical, mechanical and thermal methods of cell diruption and extraction. Cell disruption usually involved mechanical methods (high-pressure homogenization, ultrasonication, ultrasound, pulsed electric field) (Miranda et al. 2012; Zhao et al. 2013a) or non-mechanical methods like enzymatic hydrolysis of cell wall components by amylases, cellulases, and amyloglucosidase (Günerken et al. 2015). Obtained saccharides are further processed under the fermenter in the presence of some alcohol-producing yeast such as *Saccharomyces cerevisiae* or other *Saccharomyces* sp. (Eshaq et al. 2011*;* Abate et al. 1996) or bacteria like *Zymobacter palmae* (Horn et al. 2000) and *Zymomonas mobilis* (Abate et al. 1996).

## 1.4 Nutrients and Growth Inputs for Algal Growth

Microalgae are found either independently or in symbiosis with other living beings on both terrestrial and aquatic ecosystems; microalgae could be able to get efficient biomass and high growth rate by utilizing light energy, $CO_2$, and water via photosynthesis. Microalgae cultivation gains massive concerns for some therapeutic proteins and the sustainable biofuel generation (Abdeshahian et al. 2010). Large-scale cultivation of microalgae is affected by various aspects such as nutrient, light availability/intensity, color, and temperature conditions (Xenopoulos et al. 2002). Studies show that chemical elements like N, K, Ca, Cu, Fe, Mg, Mn, P, S, and Zn are crucial for microalgae growth in the form of salts; however, the amount of these micro- and macronutrients varies for every algal growth medium (Kaplan et al. 1986; Oh-Hama and Miyachi 1988). The selection of growth medium is a significant aspect, and it depends on the chemical composition of media that affects the biomass growth (Borowitzka 2005). Bold's basal (BB) medium, acidified Bold's basal medium, Chu10 medium, BG (Blue-green) 11 medium, and modified Hoagland's medium are mostly used for culturing microalgae (Ilavarasi et al. 2011).

### *1.4.1 Bold's Basal Medium (BBM)*

One liter of Bold's basal medium is prepared by adding 10 mL of each stock solution from Table 1.2, items 1–6, and 1 mL of items 7–10 in a volumetric flask with 1 L distilled water.

**Table 1.2** Composition of Bold's basal medium (Ilavarasi et al. 2011)

| S No. | Stocks of chemicals | g/L |
|---|---|---|
| 1. | $NaNO_3$ | 25.00 |
| 2. | $KH_2PO_4$ | 17.50 |
| 3. | $MgSO_4 \cdot 7H_2O$ | 7.50 |
| 4. | $K_2HPO_4$ | 7.50 |
| 5. | NaCl | 2.50 |
| 6. | $CaCl_2 \cdot 2H_2O$ | 2.50 |
| 7. | **Trace elements**: | |
| | • $ZnSO_4 \cdot 7H_2O$ | 4.42 |
| | • $CuSO_4 \cdot 5H_2O$ | 1.57 |
| | • $MnCl_2 \cdot 4H_2O$ | 1.44 |
| | • Co $(NO_3)_2 \cdot 6H_2O$ | 0.49 |
| | • $MoO_3$ | 0.71 |
| 8. | $H_3BO_3$ | 11.40 |
| 9. | **EDTA and KOH solution**: | |
| | • EDTA $Na_2$ | 50.00 |
| | • KOH | 31.00 |
| 10. | $FeSO_4 \cdot 7\ H_2O$ with 1.0 mL concentrated $H_2SO_4$ | 4.98 |

### *1.4.2 Acidified Bold's Basal Medium*

Add 250 mg of $(NH_4)_2SO_4$ in 800 mL of deionized water, dissolve it completely, and add each of the stock solutions from Table 1.3, items 1–6, and then 6.0 mL from trace element solution. Add vitamin $B_1$ and $B_{12}$. Make up to 1 L with deionized water and bring the pH to 3.0 with HCl.

### *1.4.3 BG11 (Blue-Green Medium)*

Make up to 1 L with deionized water and then adjust it to pH 7.1 with 1 M NaOH.

### *1.4.4 Chu10 Medium*

Take 2.5 mL from each stock solution, and prepare the media by completing the volume to 1 L by adding deionized water.

Microalgae growth highly depends on the composition of media, and nutrient-rich water promotes more algal blooms (Castellanos 2013; Schenk et al. 2008). The presence of all essential micro- and macronutrients is necessary for media to produce adequate algal biomass (Dauta et al. 1990). Carbon is a significant ingredient for algal growth, obtained from various inorganic and organic substrates such as carbon

**Table 1.3** Composition of acidified Bold's basal medium (Ilavarasi et al. 2011)

| S. No. | Chemical formula of each salt | Distilled water, g/1000 mL | Stock solutions in 1 L final medium |
|---|---|---|---|
| | **Stock solution** | | |
| 1. | $NaNO_3$ | 25.0 g | 30.0 mL |
| 2. | $KH_2PO_4$ | 17.5 g | 10.0 mL |
| 3. | $K_2HPO_4 \cdot 3H_2O$ | 7.5 g | 10.0 mL |
| 4. | $MgSO_4 \cdot 7H_2O$ | 7.5 g | 10.0 mL |
| 5. | $CaCl_2 \cdot 2H_2O$ | 2.5 g | 10.0 mL |
| 6. | NaCl | 2.5 g | 10.0 mL |
| 7. | **Trace element composition** | **mg/1000 mL** | **6.0 mL** |
| | • $MnCl_2 \cdot 4H_2O$. | 41.0 mg | |
| | • $FeCl_3 \cdot 6H_2O$ . | 97.0 mg | |
| | • $ZnCl_2 \cdot 6H_2O$ | 5.0 mg | |
| | • $Na_2MoO_4 \cdot 2H_2O$ | 4.0 mg | |
| | • $CoCl_2 \cdot 6H_2O$ | 2.0 mg | |
| | • $Na_2$ EDTA | 0.75 g | |
| 8. | **Vitamin $B_1$** | **0.12 g/100 mL** | 1.0 mL |
| 9. | **Vitamin $B_{12}$** | **0.1 g/100 mL** | 1.0 mL |

dioxide, acetic acid, and peptone (Cysewski and Lorenz 2004). Nitrogen is another macronutrient that builds more than 10% of the total biomass content in the form of amino acids or proteins. Nitrogen is added to the culture medium in the form of nitrogen oxides such as nitrate (Cysewski and Lorenz 2004). It has been reported that nitrogen-deprived media in culture lead to decreased chlorophyll content and increased amounts of carotenoids and lipids (triacylglycerides) (Cysewski and Lorenz 2004). Phosphorus is the third essential element mostly available in the form of orthophosphate in the medium, and phosphorus is involved in cellular processes in the form of ATP/ADP/AMP for energy transmission and also a constituent of DNA (Cysewski and Lorenz 2004). Calcium, iron, magnesium, potassium, selenium, and sodium are other micronutrients. Boron, copper, manganese, molybdenum, and zinc are trace elements that are usually involved in the catalytic activity of enzyme reactions (Cysewski and Lorenz 2004) (Tables 1.4 and 1.5).

### *1.4.5 Wastewater as a Source of Nitrogen and Phosphate*

Microalgae need inorganic nutrients, sunlight, and $CO_2$ for their growth. Utilizing wastewater for algaculture reduces the cultivation cost of adding culture media and minimizes the consumption of fresh water. Three significant origins for wastewater are agricultural, domestic, and industrial effluents. These effluents are rich in various organic and inorganic ingredients and nitrogen and phosphorus components.

**Table 1.4** Composition of blue-green media BG11 (Ilavarasi et al. 2011)

| S. No. | Chemical composition | | Stock solutions (in mL) per 1 L final medium |
|---|---|---|---|
| | **Stock solution** | **Per 500 mL** | |
| 1. | $NaNO_3$ | 75.0 g | 10.0 |
| 2. | $MgSO_4 \cdot 7H_2O$ | 3.75 g | 10.0 |
| 3. | $K_2HPO_4$ | 2.0 g | 10.0 |
| 4. | $CaCl_2 \cdot 2H_2O$ | 1.80 g | 10.0 |
| 5. | Ammonium ferric citrate | 0.30 g | 10.0 |
| 6. | Citric acid | 0.30 g | 10.0 |
| 7. | $Na_2CO_3$ | 1.00 g | 10.0 |
| 8. | EDTA $Na_2$ | 0.05 g | 10.0 |
| 9. | **Trace metal solution** | **Per 1000 mL** | **1.0** |
| | • $ZnSO_4 \cdot 7H_2O$ | 0.22 g | |
| | • $MnCl_2 \cdot 4H_2O$ | 1.81 g | |
| | • $H_3BO_3$ | 2.86 g | |
| | • $Na_2MoO_4 \cdot 2H_2O$ | 0.39 g | |
| | • $Co(NO_3)_2 \cdot 6H_2O$ | 0.05 g | |
| | • $CuSO_4 \cdot 5H_2O$ | 0.08 g | |

**Table 1.5** Composition of Chu10 medium (Ilavarasi et al. 2011)

| S. No | Chemical composition | Concentration in g/L | Stock solutions (in mL) per 1 L final medium |
|---|---|---|---|
| 1. | $K_2HPO_4$ | 4.0 | 2.5 |
| 2. | $MgSO_4$ | 10 | 2.5 |
| 3. | $CaCl_2$ | 16 | 2.5 |
| 4. | $NaNO_3$ | 8.0 | 2.5 |
| 5. | $FeCl_3$ | 0.32 | 2.5 |
| 6. | $Na_2CO_3$ | 8.0 | 2.5 |
| 7. | NaCl | 30 | 2.5 |
| 8. | EDTA Na | 4.0 | 2.5 |
| 9. | **Trace elements** | | |
| | • $MnCl_2 \cdot 4H_2O$ | 0.02 | 2.5 |
| | • $(NH_4)\ 6Mo_7O_{24} \cdot 4H_2O$ | 0.028 | 2.5 |
| | • $H_3BO_3$ | 0.288 | 2.5 |
| | • $CuSO_4 \cdot 5H_2O$ | 0.08 | 2.5 |
| | • $ZnSO_4 \cdot 7H_2O$ | 0.224 | 2.5 |
| | • $COCl_2 \cdot 6H_2O$ | 0.004 | 2.5 |
| 10. | $Na_2SiO_3$ | 5.7 | 2.5 |

Ammonium, nitrates, and nitrites are sources of nitrogen, and phosphates are used as the source of phosphorous available in various wastewater effluents which could be utilized by microalgae cultivation. Some studies reveal assimilation of heavy metals, nitrogen, and phosphorus by microalgal cells, and hence, these are used for bioremediation of wastewater (Cho et al. 2013; Cabanelas et al. 2013). Microalgae can bio-mitigate the effects of industrial and municipal effluents by consuming carbon, nitrogen, and phosphorus compounds and simultaneously overcome the problem of eutrophication for maintaining the aquatic ecosystem (Cai et al. 2013). By utilizing microalgae for tertiary treatment of wastewater and combining biofuel production along with that, a zero-waste concept is implemented, so wastewater algaculture is considered as sustainable cultivation for biodiesel industries (Rawat et al. 2013).

### *1.4.6 Impact of Growth Conditions on Microalgal Biomass*

Table 1.6 represents optimization of various growth conditions and their outcomes in terms of biomass, lipid, and protein content.

Optimization of several growth aspects such as the chemical composition of nutrient media, light availability, temperature, and some other factors has a significant impact on biomass, lipid, and protein accumulation. It has been reported that the pattern of lipid accumulation in microalgae is reflected from its usual pattern of lipid accumulation under several diverse conditions (Sato et al. 2000; Thompson Jr 1996; Guschina and Harwood 2006). Nitrogen starvation approach is the most studied technique to increase triacylglyceride production (Widjaja et al. 2009). Light availability could be optimized at different growth levels, and light–dark photoperiod cycles have a significant impact on microalgal lipid accumulation. Microalgae culture in stationary phase under 12:12 h continuous intense light conditions gained greater storage of triacylglycerides with monounsaturated and saturated fatty acids compared to a culture grown under the lower intensity of light (Juneja et al. 2013).

## 1.5 Different Microalgae Cultivation Methods

For ideal large volume growth of microalgae, two major approaches have been taken: the indoor or outdoor photobioreactor cultivation method with electric lights for photosynthesis and the outdoor pond cultivation method with natural sunlight. In artificial photobioreactors, species-specific optimization of growth conditions is convenient (Liu et al. 2015; Xu et al. 2009). The light source, reactor setup, the materials used for the vessel, nutrient medium, temperature, circulation system, and $CO_2$ supply are the basic condition to be optimized in photobioreactor (Liu et al. 2015; Xu et al. 2009). It is divided into closed or open systems and open cultivation directly exposed to sunlight and air, while the closed system is indoor away from

**Table 1.6** Representation of various experimental growth conditions and their effects on biomass/lipid production

| Growth condition/ composition | Experimental methods | Effects observed | References |
|---|---|---|---|
| By limiting nitrogen source ($NH_4$ and $NO_3$) | Optimization of $NaNO_3$, $KH_2PO_4$, $NH_4HCO_3$, $MgSO_4 \cdot 7H_2O$, $K_2HPO_4$, $(NH_4)_2HPO_4$ based on HAMGM and BBM | • Raised concentration of algal biomass: 40% (0.73 g/L) by BBM<br>• Elevated lipid concentration: 85% (281 mg/L) by HAMGM | Widjaja et al. (2009) |
| Nitrogen sources: $CO(NH_2)_2$, $KNO_3$, $NaNO_3$, and $NH_4NO_3$ | *Chlorella sorokiniana* Optimization the concentration of $CO(NH_2)_2$: 0–10 g/L | 1.50 g/L $CO(NH_2)_2$ gives<br>• More significant biomass generation (0.220 g/L)<br>• Lipid content: 61.50% | Ramírez-López et al. (2016) |
| Optimization of nitrogen and phosphorus concentration | Nitrogen concentration optimized: 0–56 mg/L<br>Phosphorus optimization: 0–19 mg/L | • Nitrogen/phosphorus ratio: 10.0<br>• Biomass concentration: 1.58 g/L | Sharma et al. (2015) |
| N and other trace elements | Optimize Ca, Mn, N | • Approximately threefold lipid accumulation | Alketife et al. (2017) |
| $NO_3$ | Optimizing nitrate utilization and accumulation of protein | • Protein accumulation raised to 44.30% | Morschett et al. (2017) |
| Photoperiod and light intensity | By optimizing light condition | • Appropriate light intensity: 2000 lux<br>• Optimum light–dark period: 12:12 h | Xie et al. (2017) |
| Elevation of light intensity | 400 µmol photon/ms | • Yellow color develops in microalgae due to high xanthophyll production (molecular sunglasses)<br>• Xanthophyll antioxidants protect algal cells from radiation | Lu et al. (2013) |
| Variation in illumination methods | • Continuous light<br>• Periodic light–dark durations<br>• No light<br>• Continuous dark with flashing light | Flash lighting elevates the total fatty acid accumulation and the growth rate significantly in *Chlorella vulgaris* | Grudzinski et al. (2016) |
| Optimization in red light intensity | Intensities at 800–2000 µmol/$m^2$/s | • Optimal light for best biomass growth: Red wavelength<br>• The optimal light concentration: 1200–1600 µmol/$m^2$/s | Choi et al. (2017) |
| Nitrogen starvation and light limitation | Outer shading and dimming the lights | • Reduction in algal growth | Zhao et al. (2013b) |

(continued)

**Table 1.6** (continued)

| Growth condition/ composition | Experimental methods | Effects observed | References |
|---|---|---|---|
| Optimization of carbon sources: Glucose, acetate, and glycerol | Autotrophic, heterotrophic, and mixotrophic modes | • The maximum biomass produced at heterotrophic mode: 8.90 g/L<br>• Lipid content is highest in a heterotrophic way: 36.19% in culture medium | Schreiber et al. (2017) |
| Optimization for $CO_2$ concentration light intensity N deficiency P uptake | • $CO_2$: 0.03–12%<br>• Intensity of light: 40–200 µmol photons/m/s<br>• Nitrogen starvation<br>• Phosphorus (optimized) | Best results obtained at<br>• $CO_2$ concentration: 4%<br>• Light intensity: 200 µmol photons/m/s<br>• Polyphosphate maximum uptake rate: 2.08 mg/L/day<br>• Observe better performance in N-deficient condition than N-rich condition | Morowvat and Ghasemi (2016) |
| Temperature | • Ranged between 20 and 30 °C | • Optimum temperature for lipid productivity: 27 °C<br>• Optimum N concentration:1.50 g/L<br>• Optimum cell density: 50% | Chu et al. (2014) |

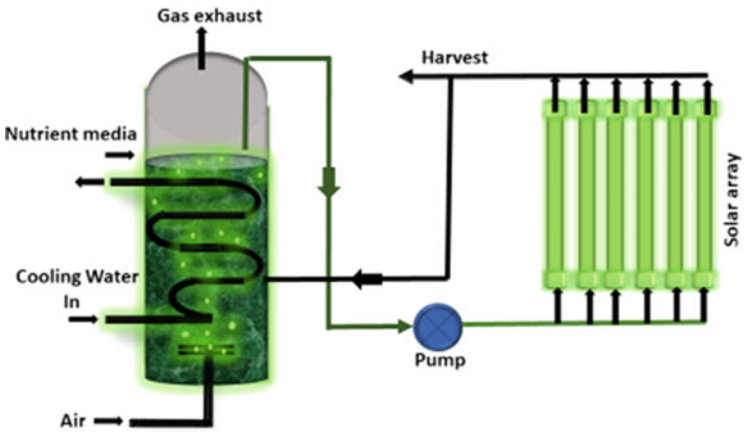


**Fig. 1.6** Schematic representation of a photobioreactor assembly for microalgae cultivation

sunlight (Juneja et al. 2013). Figure 1.6 represents the schematic assembly of a photobioreactor for microalgae cultivation.

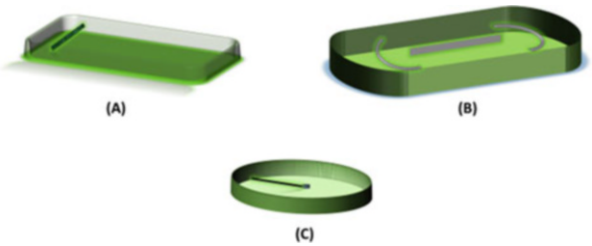


**Fig. 1.7** A diagrammatic representation of open cultivation by using ponds. (**a**) Unstirred pond. (**b**) Raceway pond. (**c**) Circular pond

### *1.5.1 Open System*

In open systems, pond's depth control light availability, stirring optimity, temperature, and evaporation. A shallow pond is better for light availability to the algae cells while a minimum depth is required to control the evaporation rate and proper mixing; shallow lakes and ponds provide extensive surface evaporation, which results in an ionic imbalance in the growth medium (Tredici 2004). Open cultivation systems are easy to build in the form of ponds and lakes. Pond designs include variety such as open, covered, raceway, circular, inclined, and big shallow ponds (Borowitzka 1999; Mata et al. 2010). However, the risk of bacterial, fungal, microalgal, and protozoal contamination is high in an open pond system. Figure 1.7 represents open systems (unstirred pond, raceway pond, and circular pond) for the cultivation of microalgae.

### *1.5.2 Closed Systems or Indoor Photobioreactors (PBRs)*

An indoor photobioreactor is made up of any transparent material which provides optimization for several growth conditions such as aeration rate, $CO_2$ supply, cell density, pH, temperature, light, and water. For PBR designing, the surface to volume ratio is the main parameter regarding light availability for photosynthetic efficiency (Singh and Gu 2010). The most widely used PBR architecture is tubular and flat plate photobioreactor in continuous mode (Singh and Gu 2010), which provides a high surface to volume ratio (Ilavarasi et al. 2011). In the flat plate reactor, narrow panels are arranged in a vertical or horizontal manner and provide adequate exposure to light and air, generally used to get high biomass (Singh and Gu 2010), whereas the tubular photobioreactors consist of transparent tubes of approximately 1 dm in diameter arranged in helical, horizontal, or vertical parallel loops. These closed tubes have an advantage over external contaminants (Carvalho et al. 2006; Morweiser et al. 2010). Figure 1.8 represents various configurations of photobioreactors designs which have a general surface to volume ratio of 80–100 $m^2/m^3$ (Posten 2009).

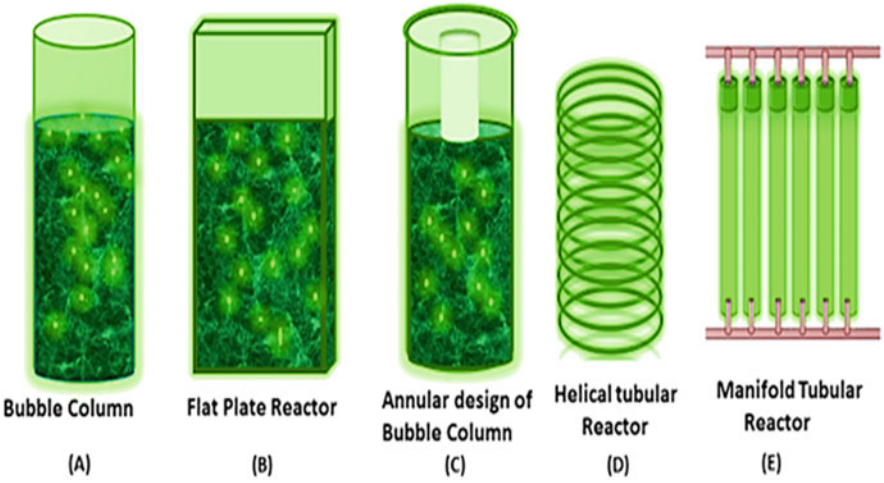


**Fig. 1.8** Common geometries of closed photobioreactors. (**a**) Bubble column. (**b**) Flat plate reactor. (**c**) Annular design of bubble column. (**d**) Helical tubular reactor. (**e**) Manifold tubular reactor

Table 1.7 provides a comparative study on the open and closed system of microalgae cultivation (Carvalho et al. 2006; Chen 1996; Del Campo et al. 2007; Canela et al. 2002; Piccolo 2010).

## 1.6 Concept of Biorefineries

Microalgae is a rich source of carbohydrate, lipid, and protein; they uptake carbon dioxide and light from the atmosphere, just like plants, so it could be considered as a bio-based crop. Figure 1.9 demonstrates how phototropic microalgae biomass is useful for various commodities such as biofuel, animal feed application, protein supplements, and carbohydrate feedstocks (Williams and Laurens 2010). Microalgal biomass needs a complete fractionation and valorization similar to any petrochemical or oil refineries, which bring the concept of biorefineries for microalgae. Biorefinery facilities include harvesting of microalgae cells, cell disruption, and then product extraction and fractionation (Wijffels et al. 2010; Vanthoor-Koopmans et al. 2013).

### *1.6.1 Evaluation of the Biorefinery Processes*

Nowadays, techno-economic advancements in large-scale cultivation of microalgae and its downstream processing are the basis on which a biorefinery should be evaluated. Figure 1.10 demonstrates a simplified view of biorefinery processes.

**Table 1.7** Comparison of microalgae growth conditions in the closed and open system

| Culture conditions | Photobioreactor | Ponds |
|---|---|---|
| Space requirement | Depends on productivity | Same as PBR |
| Light efficiency | Highly efficient utilization of light | Less-efficient utilization |
| Sterility | Sterilizable | Could not be sterilize |
| Contamination control | Convenient | Difficult to prevent contaminants |
| Contamination risk | Less | High risk of contamination |
| Area/volume | High | Low |
| Cell density | High | Low |
| Operation mode | Batch and semicontinuous | Batch and semicontinuous |
| Mixing and aeration | Uniform throughout PBR | Nonuniform |
| Evaporation rate (culture media) | Less evaporation | A high rate of evaporation |
| Temperature control | Uniform temperature throughout the system | Difficult to maintain the same temperature at various depths |
| Operating cost | High | 3–10 times lesser than photobioreactors |
| Biomass yield | High, 3–5 times more in PBR | Less yield than PBR |
| Processing and control mechanism | Convenient | Difficult |
| Investment | High investment | Needs less investment than PBRs |
| Monitoring and control of gas transfer | Highly efficient | Less efficient |
| Scale-up of system | Demanding/complex | Demanding/complex |

A complete biorefinery should have total revenues greater than total reproduction and economically feasible (Ruiz et al. 2016; Grima et al. 2003). Table 1.8 summarizes all the practices involved in a biorefinery approach to get multiple commodities along with biofuel production from microalgae biomass.

Biomass recovery is a significant criterion which contributes up to 25–30% of net algal biomass generation costs (Grima et al. 2003). Before applying harvesting techniques, an additional step of flocculation, flotation, or a combining approach of both is used on algal cells for their aggregation. Microalgal cell aggregation causes ease in biomass harvesting due to large effective particle size (Grima et al. 2003), then various recovery practices such as centrifugation, filtration, sedimentation, and ultra-filtration are applied to recover microalgal biomass (Grima et al. 2003). Recovered biomass is further processed by dewatering or dehydration step to increase the storage life of raw feedstock (biomass) and most commonly include drum dryer, freeze dryer, conventional sun drying technique, and spray dryer (Richmond 2008). Cell disruption is the next process to release of desired metabolites (carbohydrate, lipid, protein, pigments, and some other high-value compounds) from dry algal biomass. The selection of cell disruption techniques for microalgae usually relies on the composition of the cell wall/cell membrane and the nature of the

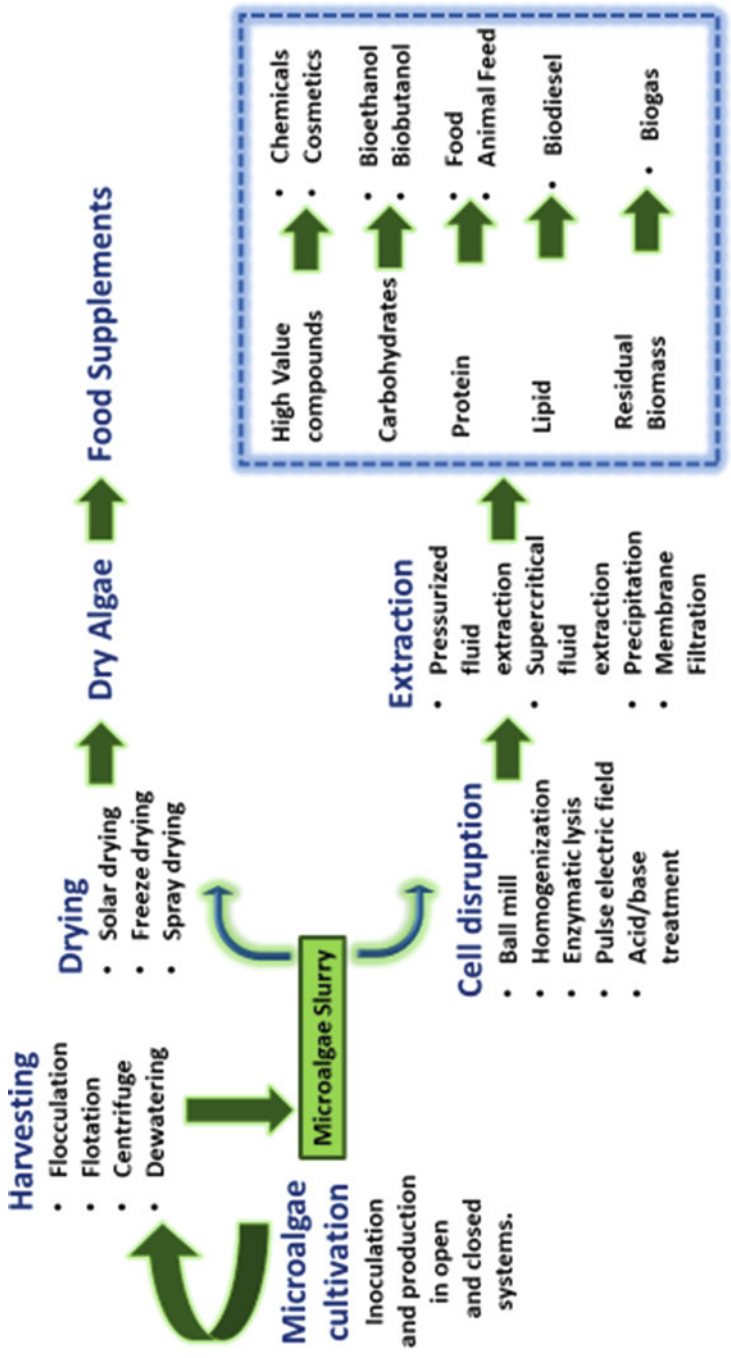


**Fig. 1.9** Representation of the biorefinery approach to obtain useful commodities from algae

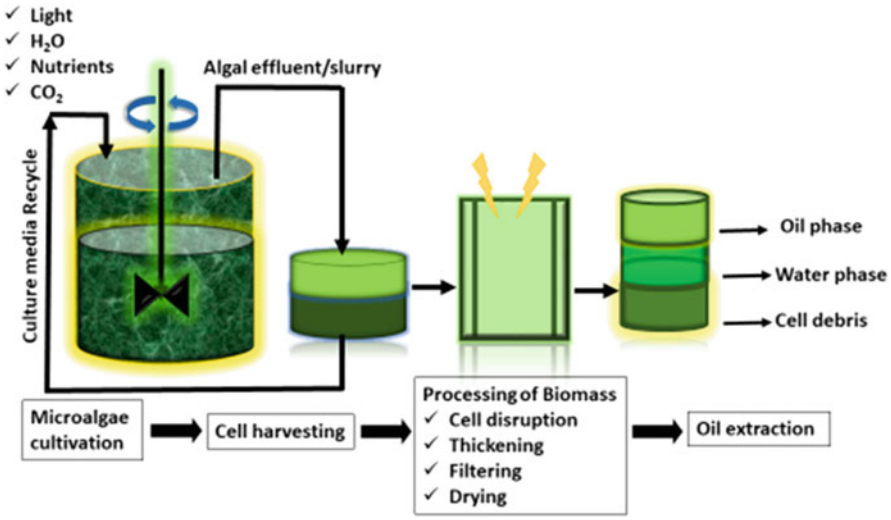


**Fig. 1.10** Diagrammatic representation of biorefinery approach

**Table 1.8** Techniques involved in microalgae biorefinery processes for biofuel production

| Process | The technique involved in DSP | References |
|---|---|---|
| Biomass harvesting | Flocculation, filtration, flotation, sedimentation, centrifugation | Coons et al. (2014), Gerardo et al. (2015), Vandamme et al. (2013), Roselet et al. (2015) |
| Cell disruption | • Mechanical: High-pressure homogenization, bead mill<br>• Biochemical: Enzymatic treatment, acid–base treatment<br>• Physical: Ultrasonication, microwave, pulsed electric field | Günerken et al. (2015) |
| Product extraction | Alkali extraction, aqueous two-phase extraction, organic solvent extraction, supercritical extraction | Eppink et al. (2017), Passos et al. (2016), Sari et al. (2015), Du et al. (2015), Orr and Rehmann (2016) |

desired metabolite. All the practices involved in cell disruption can be broadly categorized as mechanical and nonmechanical techniques of cell disruption. Mechanical actions include conventional practices like autoclave, bead milling, homogenization, and some other new methods such as ultrasound, multimode microwave, and sonication. Nonmechanical actions for cell disruption involve some biological and chemical treatments such as acid–alkali treatment, enzymatic hydrolysis of cell wall and cell membrane by amyloglucosidase, cellulases, and pectinases (Cravotto et al. 2008). After cell disruption obtained metabolites further undergo the extraction process where biofuel-based industries are focused on maximum extraction of lipids and fatty acids, solvent extraction is efficient and quick extraction practice is applied conventionally for lipids. In solvent extraction of

lipids, solvents such as ethyl alcohol (96%) or hexane has been used. Sometimes a mixture of 96% ethyl alcohol and hexane has been used to extract pure fatty acids (98% quantitatively) (Richmond 2008). Ethyl alcohol acts as an excellent solvent for solvent extraction besides lipid extraction. It could also extract some other cellular metabolites like saccharides, salts, proteins, and pigments, which is desirable for the biorefinery approach but undesirable for pure lipid extraction. Microalgal metabolites have a wide range of utilities. Instead of starch-containing food crops, biofuel industries utilize microalgae-derived carbohydrates as fermentable feedstock for bioethanol production, whereas high lipid accumulation drives interest in biodiesel production. Some other saturated and unsaturated fatty acids, proteins, and pigments of microalgal origin have their pharmaceutical and nutraceutical applications. For the economic feasibility and commercial viability of microalgal metabolites and fuels, the biorefinery process needs further evaluation.

## 1.7 Advantage and Disadvantage of Biofuels

Microalgae, diatoms, and seaweeds are the ideal resources for biofuel production as they have excessive biomass yield and are able to grow in saline or wastewater. Utilizing wastewater as a source of nutrients saves consumption of freshwater and reduces the cost of additional nutrient media (National Research Council 2013a). Third-generation biofuels have one more advantage over second-generation oilseed crops to keep agricultural lands available for food crops to assist the food crisis. Algal biofuels have various social and environmental benefits, but the production process of algal diesel also have some detrimental effects which are associated with almost all kinds of energy/fuel production (Georgianna and Mayfield 2012). The perspective of sustainable green fuel get its way by reducing particulate matter emissions and GHG emissions, but biodiesel attracts moisture and less suitable for low temperature regions, which is not a good sign in terms of engine durability. Hence, these potential fuels are still inferior to conventional fossil fuels. Table 1.9 presents a quick view of all possible aspects regarding the potential and limitations of biofuels (Haines-Young and Potschin 2010; Sharma et al. 2012b; Xue et al. 2011; Tesfa et al. 2014).

## 1.8 Policies Regarding Algal Biofuels Worldwide

In the modern lifestyle, fuel is an essential requirement and the most precious source of energy demanded by worldwide consumers and industries. Fossil fuels have direct command on the economy and growth of any country (Cleveland et al. 2000); the proportional relationship of the fuel stockpile shows the growth of the country. The higher the storage, much better is the nation's growth. Energy and per capita gross national product keep a correspondence correlation while the nation consumes more energy per head with a higher gross national product (Stern 2010). Exponentially

**Table 1.9** Potential and limitations of biofuels

| | Criteria | Potentials of biofuels | Limitations |
|---|---|---|---|
| A. | Environmental pollution | Comparatively less air pollution | Industrial pollution |
| | | Carbon sequestration, significantly less harmful carbon emission compared to conventional diesel | Cause increase in nitrogen oxide ($NO_X$) emissions |
| | | Emission of particulate matter is reduced | More likely to attract moisture as compared to conventional diesel |
| | | Greenhouse gas reduction | Reduce fuel economy |
| | | Biodegradable | |
| B. | Energy availability | Unlimited source of energy, produced from a vast range of biomass | Require some more investment in biorefinery and equipment |
| | | Viability of third-generation biofuel production | High production cost |
| | | Fuel varieties are available from a single source, such as bioethanol, biobutanol, methane, and biodiesel | High water use |
| | | Renewable source of energy | |
| | | Less dependency on petroleum import | Unique management is needed for storage and transportation |
| C. | Utility of biofuels | Less flammable compared to conventional fuels | Not applicable for low temperatures |
| | | Have better lubrication property | Biodiesel can only be used in diesel-powered engines |
| | | High combustion efficiency | Less engine durability |

escalating energy demands led to massive combustion of coal with hydrocarbons in the nineteenth century. In contrast, the twentieth century consumes oil more than coal, and in the twenty-first century, demands are raised for natural gas (Hall 2016; IEA 2016; Sayre 2010). The worldwide demand for energy could be analyzed by a report of total primary energy supply (TPES), which states that during 1973 energy consumption is 6101 Mtoe (million tons of oil equivalent), which almost raised to approximately 13,699 Mtoe by 124% in 2016 (Birol 2017).

### *1.8.1 Indian National Policy of Biofuel 2008*

Detrimental impacts of conventional fossil fuels on the atmosphere cause stress on the government to set up an effective policy for the efficient use of renewable energies. India is a region rich in biodiversities and has sufficient ability to explore renewable energy resources. In international conventions, such as the United Nations Framework Convention on Climate Change 1992 and the Kyoto Protocol 1997, India makes legal commitments for supporting sustainable energy sources and

reducing greenhouse gas emissions. The preamble of biofuel policy has a feature that food crops are not being involved in biofuel production for the purpose of ending the debate on fuel production versus food crisis (Murali et al. 2016). This policy recommends India to target blending up to 5% biofuel by 2012, up to 10% blending by 2017, and after 2017 blending is increased up to 20% (Chandel et al. 2017). This policy permits 100% equity to attract foreign investors for direct investment and also draws a roadmap for the development of biofuel industries and technology (Chandel et al. 2017). The prime minister constitutes a committee named the National Biofuel Coordination Committee, which plays a key role in coordination and provides policy guidance to achieve recommended biofuel targets. The ambition of microalgae biofuel production requires efforts at grassroot level in biofuel industries, and its technology is still in infancy; hence, India needs vast marketing of microalgal biofuel from both private and government sectors. Research on microalgae fuels and other renewable resources of energy is at its primitive level in India. In contrast, engineers, scientists, industries, and policymakers need to take a step in building a robust framework for biofuel policies.

### *1.8.2 Biofuel Policies in the United States*

At G20 Pittsburgh, several measures were discussed with the leaders, and they were agreed on a commitment that demands investments in renewable and clean energy resources to deal with the detrimental impacts of climate change; it also demands to reduce the subsidies for conventional fuels which results in wasteful exhaustion of energy security. So, according to the policy, the US state and federal governments reduce the subsidies provided to the oil industries. The reduced amount of subsidies is approximately $2.84 billion, which is decided to be invested in biofuel industries to create a fairer algal biofuel market. The legislation is passed in the year 2010 by the US House of Representatives, demanding the equal parity in federal tax credit programs for algal-based third-generation biofuels as the cellulosic/lignocellulosic biofuels. An act HR 4168 was implemented to promote microalgae-based renewable biofuels that permit 50% bonus depreciation for biofuel industrial setup and its property while giving access to $1.01 per gal production tax credit for biofuel projects. In 2011, domestic biofuels began to introduce to enhance the National Security Act (Bracht 2011).

### *1.8.3 Biofuel Policies in Canada*

After 1975 oil crisis, the United States, Europe, and Canada make several policies for the promotion of renewable biofuels; implementing these policies, Canada exempted all excise taxes on natural gas, and these exemption of excise taxes are then further extended in 1992 for methanol and bioethanol. In 2006 Canadian federal government opens a strategy on renewable fuels with the following four aspects: to

support the expansion of renewable fuel production in Canada, increasing biofuel availability, accelerating the opportunities in biofuel industries, and commercialization of the new biofuel technologies (O'Connor 2011). In 2006, the Canadian federal government showed its attentiveness by introducing the Alternative Fuel Act 2006 to encourage biofuel technology and industries and make a commitment to increase its purchase power (Gao et al. 2012). In 2009, Saskatchewan implemented a requirement to introduce 2% biodiesel. In January 2010, Canadian provinces in BC introduced the requirement of blending 5% ethanol and 5% biodiesel. In April 2011, Alberta implemented an obligation to add 5% ethanol and 2% biodiesel (O'Connor 2011). National Research Council of Canada introduces some flagship programs and research on algae carbon conversion. In May 2013, for this flagship program, the national research council of Canada made a tie-up to the CNRL and pond biofuels to build an industrial-scale biorefinery setup nearby Bonnyville, Alberta (National Research Council 2013b, c).

## 1.9 Companies Involved in Algal Biofuel Production

In biofuels industries, regular new attempts have been tried to explore the potential of microalgae, and prices of conventional fossil fuels are not much costly to acquire microalgal fuels as an alternative, but the hike in crude oil prices touches to $100 for a barrel, whereas developing countries such as China and India continuously struggle to escalate the costs and demands of crude oil. The crisis of fuel all over the world brings interest to alternatives of crude oil (Stephens et al. 2010). In April 2006, Solix Biofuels, a startup in Fort Collins, Colorado, develops a microalgae reactor that runs along with power stations to run the $CO_2$ in closed cycles (Sears and SunSource Ind 2007). In June 2006, PetroSun, an oil company, owned a subsidiary known as algal biofuels to operate in Australia and the US and investigate the production of ethanol, biodiesel, hydrogen, methane, and methanol from algal biomass. Later, the company was renamed as PetroSun Biofuels, and committed an agreement with a company BioAlternatives, PetroSun supplies half of its algal production to BioAlternatives as a feed for biofuel production (Gross 2008). Table 1.10 represents some top leading companies and their working areas in the field of biofuel technology.

## 1.10 Conclusion

The current scenario of climate change is the result of the extreme consumption of conventional fuels, although the desires of a better lifestyle increase the demand of energy between people and industries in day-to-day life. The concept of algal biofuels has been introduced to us since the 1950s, and initially emerged as a ray of hope as an alternative green energy source. Still, the concept of biofuels is not economically and commercially viable. Funding in the sector of algal fuels is fluctuating. However, its beneficial impact on the atmosphere still makes it a prior

**Table 1.10** Leading companies involved in biofuel production and innovations

| Company | About the company | Work/production/patent | References |
|---|---|---|---|
| Algenol biofuels | • Industrial biotechnology company<br>• In partnership with the US Department of Energy's bioenergy technologies Southwest Florida | • Patented technologies for biodiesel, ethanol, gasoline, jet fuel production using algae | Algenol (2014), Energy (2015) |
| Blue marble biomaterials (blue marble energy corporation) | • Seattle, Washington-based company, utilize waste biomass as raw material to produce bioenergy and biochemical products | • Focuses on the recovery of microalgae blooms from infested water sources, cleans up the aquatic domains along with bioenergy generation<br>• Utilizes algae for various productions such as biofertilizer, flavor enhancers for food, an anti-inflammatory drug, and other high-value commodities | Sims (2012) |
| Solazyme | • They are supported by oil companies such as Chevron<br>• Sponsored by imperium renewables, the Roda group, and blue crest capital finance | • Developed a process to utilize 80% of dry microalgae as oil. The process requires the algae cultivation in a dark chamber and supplies the growth media. This cultivation practice is said to generate maximum oil from algae than those cultivated in light<br>• Oil industries utilize this algal oil for their conversion into biodiesel | Nathan (2013), Solazyme Integrated Biorefinery (2012), Solzayme (2012) |
| Sapphire energy | • Algal biofuel industry, New Mexico, USA<br>• It is sponsored by bill gates' Cascade investment, Monsanto, and Wellcome Trust | • This company keeps producing "green crude" from algae. Open raceway ponds are used to cultivate biomass<br>• Built the first commercial algal fuel facility to produce biofuels, in New Mexico | The Sapphire Story (2014), Herndon (2013) |
| Diversified technologies Inc. | The United States of America | • Created a patent on the algal extraction method, named pulsed electric field (PEF) technology. PEF put a high voltage electric pulse continuously to the microalgal slurry | Diversified Technology Inc (2013), Kempkes (2016), Environmental Protection Agency (2013) |

(continued)

**Table 1.10** (continued)

| Company | About the company | Work/production/patent | References |
|---|---|---|---|
| Origin oils Inc. | Los Angeles, Southern California city | • Researching the helix bioreactor, helical patterns help to utilize lower energy lights, which ensure to provide the required amount of light intensity to each microalgal cell | Piccolo (2013), Mantai and Bishop (1967) |
| Proviron | Belgian microalgae company | • Proviron is known for its plasticizers, de-icing solutions, livestock algae, and some other products. | Home-Proviron (2019) |
| Genifuels | A US-based water technology firm | • Licensed for the fuel extraction process, involves hydrothermal liquefaction and gasification in a vessel running at extreme high temperature (662 °F) and pressure (3000 PSI). It builds a pilot-scale oil extraction facility and utilizes this process to make biofuel in massive quantities | PNNL (2013) |
| Qeshm microalgae biorefinery Co. (QMAB) | Iran-based biofuel company, first and largest microalgae biotechnology company in the Middle East | The farm mainly focuses on the following areas:<br>• Production of various range of nutraceutical products of microalgal origin<br>• Food additive production<br>• Production of green crude oil from microalgal biomass<br>• QMAB introduces BAYA biofuels, deriving algal oils from the *Nannochloropsis* sp. | Home (2014) |

choice as fuel sources. Utilizing microalgae in tertiary treatment of wastewater mitigates the impact of nitrogen and phosphorus compounds and assimilates some organic and inorganic pollutants as a source of the nutrient. $CO_2$ sequestration and less GHG and particulate matter emissions raise the demand for production and commercialization of algal fuels as an alternative to fossil fuel. The current scenario of biofuel production from microalgae is at its infancy and needs huge investment and efforts from algaculture farms, oil industries investors, and the automobile sector.

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# Chapter 2
# Paper Mill Sludge as a Potential Feedstock for Microbial Ethanol Production


**Subramaniapillai Niju and Vidhya Vijayan**

**Abstract** Health and environmental impacts of fossil fuel utilization have urged the need to find alternative bioresources that could act as renewable source of energy called as biofuels. Bioethanol is currently the most produced biofuel. First-generation feedstocks result in food–fuel competition; hence, second-generation feedstocks are mostly preferred, which consist of lignocellulosic biomass, but they have a costly and difficult pre-treatment techniques that will add up to the total cost of the production of bioethanol. Pulp and paper industry is one among the fast-growing industries which simultaneously generates large amount of residues which are disposed in landfills. These residues are wastes from paper making that is rich in monosaccharides or even polysaccharides besides lignin and hence can be used as a proper feedstock for bioethanol production which requires lesser pre-treatment. It acts as zero or negative feedstock cost and has the potential for the production of cellulosic ethanol. The aim is to cover the recent developments and key challenges for the successful introduction of paper mill sludge in the bioethanol production industry. The covered subjects are sustainability of biofuel, paper-making process, Indian scenario of paper mills, and environmental impacts of paper mill sludge along with the pre-treatments employed on paper mill sludge for bioethanol production.

**Keywords** Paper mill · Bagasse · Sludge · Pre-treatment · Fermentation · Bioethanol

## 2.1 Introduction

The depletion of petrochemical fossil fuels and the environmental pollution caused due to the combustion of fossil fuels have brought in interest for developing alternative energy resources that are renewable, sustainable, and environment friendly. Scientific developments have presented mankind with different ways to

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utilize resources to progress with the quality of life. A development is said to be 'sustainable' if it 'meets the needs of the present without compromising the ability of the future generations to satisfy their own needs' (Brundtland et al. 1987). Preference given to unsustainable alternatives, along with the ever-rising world population, has resulted in the depletion of available resources. The world population reached 7.3 billion in 2015 and is said to increase by 33% to reach 9.7 billion in 2050 (Melorose et al. 2015). To meet the energy demand of such a growing population has been one of the major challenges faced by the humanity (Smalley 2003). Combustion of fossil fuels, specifically, oil, coal and natural gas is the source of most of the global energy. In 2015, these fuels accounted for 86% of the total energy consumed (World Energy Council 2016). Fossil fuels are non-renewable energy sources, and their global supplies are unlikely to last for more than 120 years (International Energy Agency 2016) if consumed at the current rate of consumption. Further, burning of fossil fuels is one of the major sources of greenhouse gas (GHG) emissions, whose adverse effects are on climate change and global warming.

To reduce GHG emissions and to supplement fossil fuels, low-carbon and clean energy alternatives are being developed. The growing energy demand along with insecurity of fossil fuel supply remains the key drivers in the diversification of energy sources and gradually balancing the renewable energy. These include energy from solar, nuclear, hydrothermal, wind, geothermal and biomass, which have grown unexpectedly over the last 15 years (World Energy Council 2016) and continue to facilitate rapid advancements in terms of technology and infrastructure. The depletion of fossil fuels and the inability to replenish the non-renewable resources like crude oil, coal and petroleum, along with the growing global demand for fuel efficiency, environmental quality and energy security, have elicited the need to replace fossil fuels with liquid bio-based fuels such as bioethanol and biodiesel.

Bioethanol has long been considered as a suitable alternative to the depleting fossil fuels either as a petrol substitute in road transport vehicles or as an additive in fuel blends which requires no engine modification when mixed up to 30%. Bioethanol being the most dominant biofuel has shown a rising trend in its global production over the last 25 years (Talebnia et al. 2010). Commonly employed feedstocks across the world are sugar-based, starch-based and lignocellulosic-based which are being used for bioethanol production. Sugar or sucrose-based feedstocks include sugarcane, sweet sorghum and sugar beet which are rich in fermentable sugars. Starch-based feedstocks include corn, wheat and rice which consists of polysaccharide comprising of long chain homopolymer of D-glucose, which is hydrolysed to obtain glucose syrup suitable for ethanol production (Balat et al. 2008). Lignocellulosic feedstocks include those materials which contain complex mixtures of cellulose, hemicellulose and/or other polysaccharides like lignin. It can be produced from biodegradable fractions obtained from agricultural wastes, industrial wastes and municipal wastes which are processed through fermentation resulting in cleaner emissions (steam, carbon dioxide and heat) during combustion. Plants take up the carbon dioxide produced and process it through photosynthesis.

Lignocellulosic feedstock has gained importance for the production of bioethanol in the last few years due to major factors such as increased costs of petroleum fuel, environmental pollution caused by the petroleum fuels and energy security (Sukumaran and Pandey 2009), and also the agricultural crop residues serve as low-cost feedstock. They contain lignin components which do not allow the enzymes to act on the cellulose and hemicellulose and hence pre-treatment is necessary.

Pre-treatment helps to alter the biomass structure along with the chemical composition making hydrolysis of carbohydrate to monomeric sugars more rapid (Sindhu et al. 2011). The pre-treatment method adopted to be cost effective is a major challenge of cellulose to ethanol production technology research and development. The available pre-treatment techniques that have been employed in sugar cane bagasse, sugar cane tops, rice straw etc. include acid pre-treatment, alkali pre-treatment, ultrasound-assisted pre-treatment and microwave-assisted pre-treatment. These pre-treatments require harsh conditions due to the lignin removal being difficult. The feedstocks which has lesser amount of lignin are preferred, thus decreasing the extent of pre-treatment required for the production of bioethanol.

Paper mill sludge, a solid by-product of the paper industry, is considered as a promising feedstock for bioethanol production, which helps in replacing fossil fuels and mitigating greenhouse gases. India is one among the fastest growing paper and pulp market in the world with over 10% growth rate in a year in per capita consumption, which is expected to grow in future. The paper industry has an output of more than six million tonnes annually making it among the top 15 global players today (Kumar and Marimuthu 2012). Among the lignocellulosic biomasses that act as the feedstock, agricultural residues are widely used but pre-treatment is required due to the presence of lignin which further increases the total cost of the process.

An alternative approach to reduce the pre-treatment and optimization challenges is to choose feedstock which contains lower lignin content such as industry wastes and by-products. Paper mill sludge being a by-product of the paper mill industry has lower amount of lignin due to the chemical processing done during the paper-making process known as the chemical bleaching. Hence, the pre-treatments opted for the paper mill sludge would require less harsh conditions. In this chapter, the discussion reviews about the different pre-treatments employed in paper mill sludge for bioethanol production which includes acid-based pre-treatment, alkali-based pre-treatment and solvent-based pre-treatment. Pre-treatments chosen might lead to toxic substances in the process which further inhibits the activity of the organism on the paper mill sludge for fermentation. Hence there is a need for the appropriate pre-treatments to be chosen in terms of higher yield and lower amount of toxic inhibitors.

## 2.2 Bioethanol: A Sustainable Renewable Biofuel

Biofuel made from plant biomass is recently gaining attention as it acts as a countermeasure to global warming and as an alternative to petrol. Bioethanol is practically one of the important liquid bio-fuels and can be produced using variety of cheap substrates that does not compete with food resources. According to an estimate, it helps to reduce greenhouse gas emissions by approximately 30–85% compared to the available gasoline, depending on the feedstock used for its production (Fulton et al. 2004). Commonly employed feedstocks across the world are sugar-based, starch-based and lignocellulosic-based which are being used for bioethanol production. Sugar or sucrose-based feedstocks include sugarcane, sweet sorghum and sugar beet which are rich in fermentable sugars. Starch-based feedstocks include corn, wheat and rice which consists of polysaccharide comprising of long-chain homopolymer of D-glucose, which is hydrolysed to obtain glucose syrup suitable for ethanol production (Balat et al. 2008).

## 2.3 Common Feedstocks Used for Bioethanol Production

Lignocellulosic feedstocks include those materials which contain complex mixtures of cellulose, hemicelluloses and/or other polysaccharides like lignin. Lignocellulosic biomasses are considered to be efficient feedstocks for bioethanol production, among which agricultural residues are more significant. According to an estimation of 114.5 billion tons of biomass being generated through plant photosynthesis every year, lignocellulosic materials form the majority (Wang et al. 2011). They include biomasses such as woody materials, agricultural residues like wheat straw, sugarcane bagasse, rice straw and sugarcane tops, and other waste plant materials obtained from the field, which serve as sustainable alternative feedstocks that help in producing bioethanol.

### *2.3.1 Rice Straw*

Rice straw is a by-product of rice production, and various pre-treatments like treating with different chemicals (acid, alkali) and physical conditions (subcritical water, ultrasound) are being employed for lignocellulosic conversion to sugar. Alkali treatment using sodium hydroxide, acid pre-treatment using sulphuric acid and acid-assisted ultrasound pre-treatment were carried out, and the final pre-treated sample was fermented using *Saccharomyces cerevisiae* yeast. Among this, acid-assisted ultrasound pre-treated sample had the highest sugar yield up to 44% w/w along with ethanol concentration also being the highest when compared to other pre-treatment methods (Yoswathana et al. 2010).

### 2.3.2 Sugarcane Bagasse

Sugarcane bagasse is a solid residue left over after the extraction of juice from sugarcane and is considered as a promising feedstock for the generation of bioethanol (Anwar et al. 2014). Calcium hydroxide and alkaline hydrogen peroxide pre-treatments are some of the techniques used to remove the lignin component present in the biomass and solubilize the hemicelluloses. The solid fraction obtained after the pre-treatment was subjected to enzymatic hydrolysis and subsequently fermentation. The calcium hydroxide pre-treated sample was found to preserve most of the cellulose when compared to alkaline hydrogen peroxide pre-treatment (Rabelo et al. 2011). Other studies have shown that pre-treatment with alkaline hydrogen peroxide minimizes the formation of inhibitors, and hence, the energy spent is also reduced decreasing the overall cost of the pre-treatment along with high yields of sugar (Niju and Swathika 2019).

### 2.3.3 Sugarcane Tops

Sugarcane tops include the leaves that contain sugar in the form of cellulose. Due to its rich cellulosic matter and large biomass availability, extraction of ethanol can be done, and this will not in turn affect food supply. Screened SCT were pre-treated by ultrasonication along with alkaline hydrogen peroxide (AHP) followed by enzymatic hydrolysis and fermentation using *Saccharomyces cerevisiae*. The results showed 59% lignin removal and 50% cellulose recovery under the optimum condition of 45 min of ultrasonication and 3%(v/v) of AHP concentration with pH 11.5 and 5% (w/v) of biomass loading at 40 °C and 8.9% of ethanol produced during the fermentation (Niju et al. 2020).

### 2.3.4 Waste Paper

Waste paper as a feedstock in the bioethanol production serves as advantageous when compared to other lignocellulosic biomass since the cellulose, hemicellulose and lignin present is less complicated due to the pulping process during the manufacturing process. Papers produced in the world during the chemical pulping removes almost all the lignin present, and as a result, the papers are either of pure cellulose or with very much lignin (Bajpai 2012). Waste papers are usually recycled and used again, but at a certain stage, recycling becomes difficult due to the loss of the fibre quality and thus ends up in the landfill. Bioethanol production can offer environmental benefits when compared to the incineration of the landfills since it can lead to groundwater contamination and greenhouse gas emissions (Al-Azkawi et al. 2019).

Pre-treatment is considered to be necessary before enzymatic hydrolysis in order to increase the glucan and xylan accessibility towards the enzyme activity. Dilute acid pre-treatment is a favourable method chosen by the industries and has been used in the pre-treatment of wide range of lignocellulosic biomasses. The waste paper cut is mixed with 1% v/v of sulphuric acid solution incubated in rotary incubator at 50 °C and 150 rpm for 3 h along with the addition of surfactant, Tween 80 (3% v/v), and showed that the cellulose conversion was higher by 70% when compared to dilute acid pre-treated waste office paper without any addition of surfactant (Alencar et al. 2017). Alkali pre-treatment helps in the breakage of the linkages like benzyl ether, glycosidic ether and benzyl ester present in lignin–cellulose complexes. Sodium hydroxide is the mostly used alkali by the industries due to the high rate of delignification and rapid dissolution of cellulose and hemicellulose (Zhu et al. 2006). Different concentrations of NaOH (1.0–5.0%) were used for different time periods (24 and 48 h) for lignin degradation, and the maximum amount of lignin was removed in 4.0% of alkali-treated sample for 24 h (Bilal et al. 2017).

### *2.3.5 Paper Mill Sludge*

The main waste streams produced from pulp and paper mills are the primary clarifier stream which comprises short fibres, ash and trace amount of heavy metals. The paper-making process produces large amounts of waste water. Conventional primary–secondary water treatment system has been adopted to treat these waste water generated (Monte et al. 2009). The removal of the suspended solids from the effluent via sedimentation is the first stage of waste water treatment, where the solids are pressed to form primary sludge (Mendes et al. 2014). Primary sludge (on a dry basis) is said to be about 4% of the total paper product in virgin fibre mills and 15–30% in processed recycled fibre mills (Chen et al. 2014). Secondary sludge originates from the secondary treatment unit clarifier in the biological units of the waste water treatment plant (Monte et al. 2009). The secondary clarifier obtained from the aerobic activated sludge section generates smaller volumes of sludge since most of the heavy fibres have been removed initially from the primary clarifier (Coimbra et al. 2015). Secondary sludge is more difficult to process due to the presence of high microbial content; hence, primary sludge is considered for further processing (Gottumukkala et al. 2016).

## 2.4 Pulp and Paper Mill Industry

Pulp and paper mills are a type of industry that makes paper from wood pulp and other residues accomplished through a variety of special machines like tree chipper, a digester and a Fourdrinier machine. Pulping processes in the paper making helps in the separation of lignin from plant products. There are around 400 million tons of

paper and paperboard production in the world annually and is said to reach up to 550 million tons by 2050.

## 2.5 Paper-Making Process

The paper industry uses raw materials which is either cellulosic being derived from forest, agricultural residues, and waste paper or non-cellulosic including sodium hydroxide, sodium sulphide and coal. Availability of wood-based raw materials being limited tends India to use extensively the non-wood raw materials like bagasse, cereal straw, bamboo, jute and flax (Bajpai 2012). It undergoes many processes to produce paper as the final product (shown in Fig. 2.1).

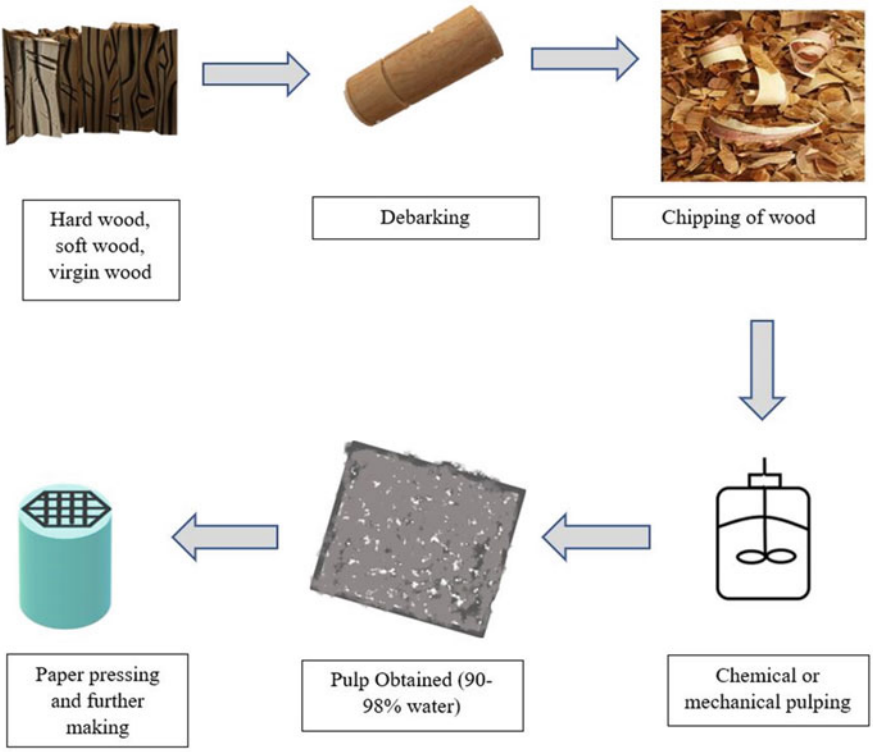


**Fig. 2.1** Steps involved in paper-making process

## 2.6 Preparation of Raw Materials and Processing

The bark has to be removed from the wood, and the logs are chipped into small pieces, so that they can be easily cooked for pulp production. Dust along with oversized chips and knots has to be removed by screening.

### *2.6.1 Pulping*

Pulping process is done to separate and clean the fibres wherein the bonds within the wood structure are ruptured. The choice of pulping process to be chosen, either chemical or mechanical, depends on the final product requirement. Mechanical pulping involves crushing of raw materials to separate the fibres leading to process being economical with higher yield and quality, but the quality of product is low having lower resistance to ageing leading to discoloration. Hence, this method is opted in the production of newsprint papers. The most widely used method for the production of papers is chemical pulping due to the high quality of the paper. The wood chips are fed into the digester where they are cooked in an acidic or basic solution to dissolve the lignin and separate the plant fibres. The obtained cellulose is further processed to produce paper.

### *2.6.2 Pulp Washing and Chemical Recovery*

The pulps that are treated with chemicals are washed thoroughly, and the chemicals are recovered. Washing helps to improve chemical recovery and reduce the chemicals responsible for bleaching since high cooking liquor would aggregate with the chemicals and results in increased chemicals used. Chemical recovery further helps in reducing the pollution load in wastewater.

### *2.6.3 Bleaching*

Bleaching is the last step of the process, which aims to whiten and brighten the pulp by removing the lignin residues as well as dust and foreign particles by the addition of alkali and other chemicals which include oxygen, hydrogen peroxide, peracetic acid, sodium hypochlorite, ozone, chloride oxide and chlorine. As a result of this chemical processing, lignin will be in soluble form. Bleaching can be further divided into two categories: (1) total chlorine free which uses oxygen and (2) elemental chlorine free which uses chloride dioxide. The use of elemental chlorine is not

recommended since there are chances of chlorine to degrade the cellulose content along with harmful AOX emissions in the aquatic environment.

### *2.6.4 Paper Pressing and Paper Making*

Here the pulp gets squeezed through large cloth-wrapped cylinders which help to remove more amount of water. This process is also called the wet end process. This is the point at which the product starts to get its smoothness and thickness. Fillers and sizing agents are added to improve the properties like optical, penetration control of liquids which will further improve the printing properties. The final stage is where the paper enters the dryer section and is dewatered.

### *2.6.5 Paper Mill Sludge as a By-Product*

After the paper-making process, the paper mill sludge is generated as the main organic residue from the wastewater treatment section. The presence of short cellulosic fibres of the recycled papers makes it difficult to be processed again in the machinery, and hence, it comes along with the sludge.

## 2.7 Indian Scenario of Paper Mills

Developing countries like India find it difficult to meet the increasing energy demands with the existing fossil fuel deposits. Coal remains to be the primary energy resource which amounts to 58% of the total energy that is being produced Power sector at a glance all India (2017). Import of coal has been a recent stage due to the poor quality and challenges in domestic production. Further, import of coal and crude oil has considerably affected nation's economy widening the scope for alternative renewable energy sources which are sustainable and eco-friendly (Chandel et al. 2016). Waste generated from the industrial and domestic sector is a depository of chemical energy, and development of technology for efficient energy generation remains a challenge.

Paper industries are one of the fast-growing sectors in India, contributing to 2.6% of the total global paper production (2013) with per capita paper consumption being 13 kg. Around ten million tons of paper is produced every year and is the sixth largest producer of pollutants after oil, cement, leather, textile and steel industries. Small-scale and large-scale units are prominent in India, and they utilize wood, agro residues and residual paper as raw materials (Balabanič et al. 2017). Nearly 750–800 paper mills are located in India including small-, medium- and large-scale units, thus

providing employment to 0.46 million people. Large-scale units have the capability to produce ≥20,000 tons of paper per annum.

## 2.8 Paper Mill Sludge

Sludge is the final solid waste obtained from the wastewater treatment process in pulp and paper mills. The world supply of PMS is high and is expected to increase due to the policies brought about by the government which helps to support and encourage paper recycling and create public awareness of environmental issues. It is said that for every 1 ton of paper produced, around 300 kg of sludge was produced, and the global paper production in 2014 was 407 million metric tonnes compared to 399 and 403 million tonnes in 2012 and 2013. Thus, along with the paper production, the PMS is also expected to increase annually in the future due to increase in the demand (Bajpai 2015). Considering the increasing requirement and that the amount of ethanol that could be produced from PMS theoretically due to its high cellulose and hemicellulose composition, PMS could be a promising substrate. Further it has less amount of lignin, thus contributing less to the pre-treatment cost (Al-Azkawi et al. 2019).

### *2.8.1 Paper Mill Sludge Composition*

Paper sludge (PS) produced from the paper industry has mainly cellulosic fibres, with calcium carbonate and some ink generated during the pulping mechanism or from the recycling process. These fibres generated during the recycling processes cannot be used for making new paper. Higher the paper is recycled, the shorter cellulose fibres are produced. Paper sludge obtained from recycling usually contains about 50% cellulose, 10% hemicellulose, and smaller amounts of materials like calcium carbonate and ink. When compared to other cellulosic biomasses available for bioethanol production, PMS serves as more efficient since there is negligible amount of lignin present and hence pre-treatment costs can be reduced (Al-Azkawi et al. 2019). Sludge discharged from paper mills is divided into four categories:

1. Primary sludge (PS), which comes from the production of virgin wood fibre
2. De-inking paper sludge (DPS), which comes from the process of removing inks from recycled paper
3. Secondary sludge (SS; activated sludge), which comes from the secondary wastewater treatment system
4. Combined primary and secondary sludge

### 2.8.2 Environmental Impacts of Paper Mill Sludge

Paper and pulp industry is considered as one of the most pollution-causing industry in the world (Thompson et al. 2001; Sumathi and Hung 2006). Whole processes that this industry undertakes are very energy- and water-intensive processes (Pokhrel and Viraraghavan 2004). The wastewater that is being generated from this process includes high concentration of chemicals such as sodium hydroxide, sodium carbonate, sodium sulphide, bisulphites, elemental chlorine or chlorine dioxide, calcium oxide and hydrochloric acid which are accumulated during the step-wise processes (Sumathi and Hung 2006). The variation in the components also causes the variation in the type of paper produced (Pecar and Gorsek 2015).

Environmental concerns of pulp and paper industry include wastewater generation, solid wastes including sludge generating from wastewater treatment plants and air emissions; hence, effective disposal and treatment approaches are essential. The solid wastes such as lime mud, lime slaker grits, green liquor dregs, boiler and furnace ash, scrubber sludges, wood processing residuals and wastewater treatment sludges are generated from different mills. Disposal of these solid wastes causes severe environmental problems because of high organic content, partitioning of chlorinated organics, pathogens, ash and trace amount of heavy metal content (Monte et al. 2009). Most of the paper mill sludge produced is currently being disposed in landfills or being burned which further pollutes the environment and thus wastes the potentially valuable bioenergy resource (Peng and Chen 2011) causing economic problems. The amount and type of materials that can be deposited in landfills are restricted by legislative trend in certain countries, because landfills are running out of storage space and bring about potential ground water contamination (Prasetyo et al. 2010). This could be overcome by making use of the PMS as an attractive biomass feedstock for the production of fermentable sugars and further for the production of bioethanol (Lin et al. 2012) being beneficial from economic and environmental perspective (Prasetyo et al. 2010).

### 2.8.3 Industrial Uses of Paper Mill Sludge

The paper mill sludge being produced as a by-product has found to be applicable in many industries as depicted in Fig. 2.2.

#### 2.8.3.1 Brick Manufacture

The manufacturing of eco-friendly light weighted bricks by the binary mixing of paper mill sludge and soil. The soil used can be of two types: laterite and alluvial. The mix of 10% PMS with both the soils at a firing temperature of 9000 °C is found to be the optimum condition for brick production. The mixing of PMS helps to

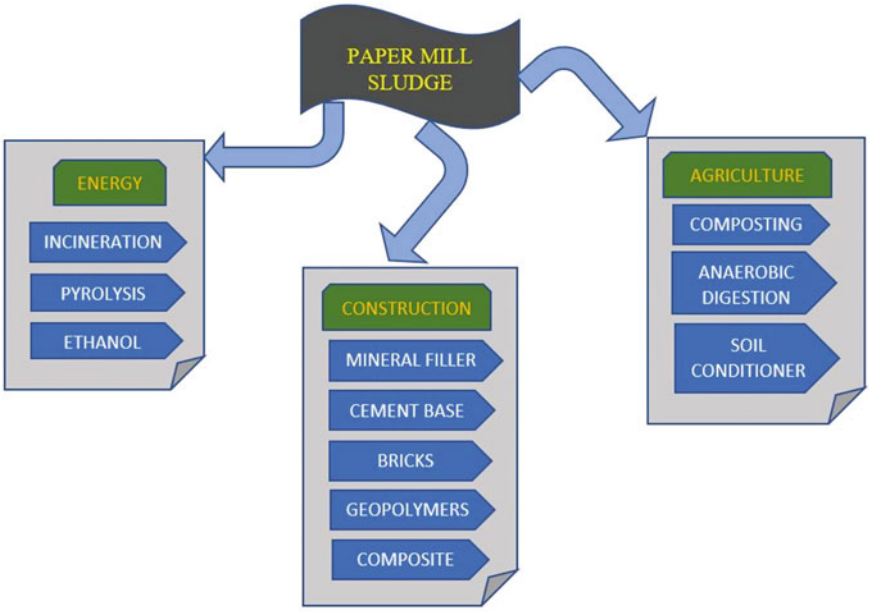


**Fig. 2.2** Uses of paper mill sludge

enhance the porosity, thereby leading to weight reduction (Goel and Kalamdhad 2017).

#### 2.8.3.2 Anaerobic Digestion

It acts as a waste treatment technique in which diverse groups of organisms naturally present biodegrades and helps in producing renewable energy from the discarded organic materials, thus simultaneously acting as a solution in resource management (Rittmann and McCarty 2012). Paper industry containing anaerobic digestor have the ability to treat 100 tonnes of PMS per day, further yielding biogas up to 0.25 $m^3$/kg of volatile solids as feed (Veluchamy and Kalamdhad 2017).

#### 2.8.3.3 Cement Base

The black and grey sludge which upon addition in wood cement boards showed difference in physical and mechanical properties. The properties have shown more improvement in black sludge when compared to the grey sludge. Based on the sludge type, 20% wt of total composite weight can be used for the commercial production of wood-cement boards (Cavdar et al. 2017).

#### 2.8.3.4 Soil Conditioner

Bioremediation of paper mill sludge by mixing it with cattle dung in various proportions and further subjecting to aerobic co-composting and vermicomposting (*Eisenia fetida*). Vermicomposting showed higher decline in organic carbon with increase in the content of nitrogen and phosphorous with lower electrical conductivity and higher pH, thus indicating that *E. fetida* helps in quick conversion of toxic paper mill sludge in about 100 days (Kaur et al. 2010).

#### 2.8.3.5 Bioethanol

Paper mill sludge serves as a source of lignocellulosic biomass that can be used for bioethanol production and is classified as second-generation biofuel. Different factors such as fermentation process, the cellulose and hemicellulose content and the type of paper mill sludge govern the conversion into bioethanol process.

## 2.9 Paper Mill Sludge Resource for Bioethanol Production

Paper mill sludge is produced in large quantities during the paper-making process which is being deposited as landfill. The paper and pulp industries are one among the largest industries in the world and the major consumer of woody biomass that generates high amount of waste residues rich in monosaccharides, or polysaccharides besides lignin, which can be utilized as a proper feedstock for second-generation bioethanol production (Branco et al. 2019). It is a waste-to-waste energy conversion process with transportation and harvesting costs being reduced when compared to the lignocellulosic biomass to ethanol conversion process (Robus et al. 2016).

Pre-treatment is considered to be the crucial step in conversion of the feedstock as it directly impacts the efficiency of bioethanol production as well as cost-effective production (Table 2.1). The enzymatic activity within the cellulosic fibres can be inhibited due to the presence of impurities and fillers, hence making the overall process challenging for bioethanol production (Gurram et al. 2015). Also, the cellulose structure being crystalline represents a higher resistance to hydrolysis and the presence of lignin decrease the accessibility of the hydrolysing agent by absorbing the enzymes (Branco et al. 2019). Pre-treatment processes include physical, chemical, physicochemical, biological or a combination of these methods. Even though different pre-treatments are available, development of a cost-effective process that produces less inhibitor with maximum efficiency is a challenge in bioethanol production (Rabelo et al. 2011).

**Table 2.1** List of pre-treatments employed for paper mill sludge (PMS)

| S. No. | Pre-treatment technique | Optimized process condition | Percentage removal of lignin/ hemicellulose | Yield | Reference |
|---|---|---|---|---|---|
| 1. | Acid pre-treatment | 10% w/v of PMS with 1 M HCl<br>Overnight stirring: 500 rpm | Ash removal: 65.8–76.7% | 75.6% yield of glucose per gram of cellulose | Gurram et al. (2015) |
| | Mechanical pre-treatment | 10 g of PMS in 500 mL of DW in standard disintegrator for 5 min, 11,500 rpm | Ash removal: 70.1–88.9%<br>Lignin removal: 64% | | |
| | Accelerants: c-PAM | Two c-PAM accelerants, high MW (30%) and low MW(100%) in 0.1% (g polymer/g dry PMS) | – | | |
| | c-PAM and hydrogen peroxide | PMS with 1% $H_2O_2$ (35% concentration Solution)<br>Temperature: 30 °C<br>Incubation: overnight solids loading 2.5, 5, 10, and 20% (w/v) | – | | |
| 2. | Thermal | Temperature:170 °C<br>Time: 1 h | – | Carbohydrates and glucose<br>6.5 g/L; 4.1 g/L | Gogoi et al. (2018) |
| | Mechanical (ultrasonication) | 20 kHz<br>Intensity: 1 kW/L<br>Time: 30 min<br>Temperature: <55 °C | | 4.7 g/L; 1.85 g/L | |
| | Alkaline | NaOH pellets<br>pH: 12<br>Temperature: 140 °C<br>Time: 1 h | | 7.23 g/L; 5.3 g/L | |
| | | 15% concentration of alkali pre-treated PMS | | 0.559 g/L of ABE | |

| | | | | | |
|---|---|---|---|---|---|
| 3. | Thermal | Temperature: 121 °C<br>Time: 15 min | – | Ethanol<br>SSF (glucan loading)<br>3%: 13.6 g/L<br>6%: 25.5 g/L<br>SSCF<br>42 g/L: single feed run<br>45 g/L: fed batch | Kang et al. (2010) |
| 4. | Thermal | Temperature: 121 °C<br>Pressure: 1 atm<br>Time: 20 min | – | Ethanol<br>$SHF_s$: 73.3 kg/ton<br>$SHF_{ns}$: 64.8 kg/ton<br>$SSF_{ns}$: 88.3 kg/ton | Schroeder et al. (2017) |
| | Chemical | 0.1 mL of a 2% sodium azide solution to each 0.1 g of the RPS | | | |
| 5. | Thermal | Temperature: 121 °C<br>Time: 20 min | – | – | Zhu et al. (2019) |
| 6. | Acid pre-treatment | [Cho][OAc] IL/PMS ratio of 10% (w/w)<br>Time: 1.0 h (oil bath)<br>Temperature: 120 °C | Hemicellulose-36.38 ± 4.51%, lignin removal: 17.42 ± 1.19%, cellulose recovery: 82.17 ± 4.28% | **Ethanol**<br>11.21 g/L | Farghaly et al. (2017) |
| 7. | Ammonia | 17 wt% aqueous ammonia solution<br>Temperature: 150–220 °C | 30%: lignin<br>50%: hemicellulose | – | Kim et al. (2000) |
| | Ammonia and hydrogen peroxide | 17 wt% aqueous ammonia solution<br>Temperature: 150–220 °C<br>5.0 wt% $HO_2$ | | | |

## 2.10 Steps Involved in Bioethanol Production

The process of conversion of lignocellulosic biomass into bioethanol involves degradation of recalcitrant cell wall structure of lignocellulose into fractions of lignin, hemicellulose and cellulose done by various pre-treatment techniques followed by enzymatic or acid hydrolysis and subsequent biological fermentation, where the sugars are fermented into ethanol and finally followed by purification via distillation (Chandel et al. 2007; Mu et al. 2010) depicted in Fig. 2.3.

Pre-treatment helps to decompose the polymeric components of the lignocellulose and form monomeric sugars, thereby altering the biomass size and structure along with its sub-microscopic chemical composition leading to higher rate of hydrolysis and greater yields of bioethanol along with increased surface area for the enzymes to act (Singh et al. 2014). Cellulose and hemicellulose released are hydrolysed into free monomer molecules that help in fermentation to produce bioethanol. Hydrolysis are of two types that involve either acid or enzyme (Limayem and Ricke 2012).

1. Pre-treatment step: to make the raw material suitable to hydrolysis condition
2. Hydrolysis step: to break down the cellulose present in solid fraction into monomeric sugars
3. Collection of liquid hydrolyzate and detection of inhibitory compounds
4. Fermentation of the five and six carbon containing sugar solution using suitable micro-organisms
5. Distillation and dehydration step as the final purification step to produce absolute ethanol

## 2.11 Pre-treatment Techniques Employed in Paper Mill Sludge

### *2.11.1 Acid Pre-treatment*

The main challenge in the ethanol production is in the process of pre-treatment of lignocellulosic biomass wherein the pre-treatment should improve the formation of sugars like glucose and xylose and avoid the formation of inhibitors for hydrolysis and fermentation process. Paper sludge pre-treated by using phosphoric acid followed by enzymatic hydrolysis showed that conversion of cellulose to glucose was more than 83% and that fermentation efficiency of glucose to bioethanol was 98% (Pecar and Gorsek 2015). Chemical or mechanical pre-treatments can also be employed to remove ash and to improve the cellulose recovery of the paper mill sludge. 10% w/v of PMS is mixed with 1 M HCl and stirred overnight at 500 rpm. It helps to remove calcium carbonate that acts as fillers in paper mill sludge (Gurram et al. 2015).

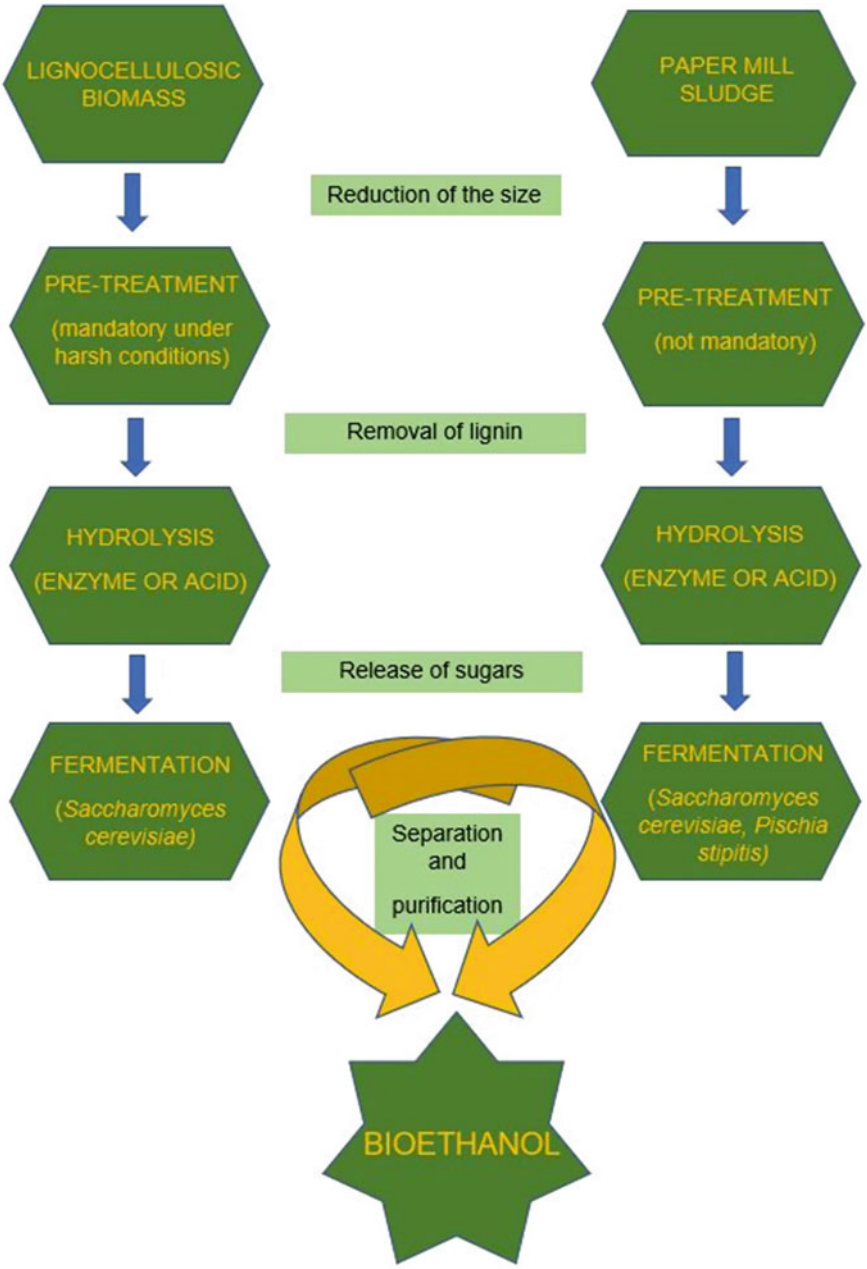


**Fig. 2.3** Steps in bioethanol production

### 2.11.2 Alkaline Pre-treatment

Alkali-based pre-treatments help to remove lignin and increase the digestibility of the cellulose (Sindhu et al. 2014). Sodium hydroxide is the most commonly used and is widely used in industries while few other alkalis like ammonia can also be used. It comprises a delignification process in which part of the lignin is removed and cellulose and hemicellulose produced is further hydrolysed (Xu and Huang 2014). The reaction involved is the saponification reaction of the ester bonds that cross links the hemicelluloses and lignin which further causes cleavage of bonds, improving the contact between cellulose, hemicellulose and enzymes for hydrolysis to take place. In this process, the formation of inhibitory compounds is very less when compared to acid pre-treatments. The sludge sample is subjected to varying concentrations of NaOH pellets followed by the heating of the sample to 140 °C for 1 h. The characterization of alkali pretreated samples revealed that they were distorted and the surface area was increased, indicating the disruption of the complex cellulose structure and making the reducing sugar and carbohydrates available for the break-down and utilization by microorganisms during fermentation process (Gogoi et al. 2018).

### 2.11.3 Pulping-Based Pre-treatment

Pulping forms a part of the paper-making process which serves as a pre-treatment technique, and it results in the formation of aliphatic acids. Chemical pulping is implemented on lignocellulosic biomass to degrade lignin. Two types of pulping processes that are used generally are kraft and sulphite pulping. Kraft pulping is based upon treating the wood chips with sodium hydroxide and sodium sulphide which helps to break the bonds between the components. In sulphite pulping, various salts of sulphurous acids, either aqueous mixture of bisulphite ($HSO_3$) or sulphite, are being used depending on the pH. In both the processes, lignin and some hemicelluloses are degraded in black liquor which can further be removed (Jönsson and Martín 2016).

### 2.11.4 Ultrasound Pre-treatment

Ultrasound pre-treatment (UP) can be used for the fractionation of lignocelluloses, and it can complement the prevailing lignocellulosic biomass pre-treatment approaches such as alkali treatment, resulting in enhanced performance and efficiency. UP is based on the application of ultrasound wave which results in the cavitation of microsize. These cavities produce high temperature and pressure gradients for few microseconds which create an enormous effect on biomass.

These high energy densities bring alterations in the surface morphology of the biomass, further disrupting structures for the effective production of fermentable sugars (Subhedar and Gogate 2016). Ultrasound pre-treatment does not produce any by-products and also helps to improve the overall yield of sugars. Ultrasound-assisted approaches result in the reduction in treatment time and also reduce the chemical/enzyme requirement. Overall, the ultrasound-assisted approach is considered to be a novel and environmentally friendly green technique, giving a significant degree of intensification.

### *2.11.5 Solvent-Based Pre-treatment*

Ionic liquids are groups of organic salts, eco-friendly solvents due to its characteristics like negligible vapour pressure, non-flammability, high thermal and chemical stability, and it can be recoverable (An et al. 2015). It has melting point below 100 °C. Choline acetate ionic liquid in combination with dilute HCl concentration gradually increases the glucose yield and saccharification ratio of $33.2 \pm 0.3$ g/L and $99.0 \pm 0.9\%$, respectively, with HCl being 1% for 1.0 h (Farghaly et al. 2017).

## 2.12 Challenges Faced in Available Pre-treatment Techniques

### *2.12.1 Acid-Based Pre-treatment*

Even though this technique is one of the efficient techniques that are being used in the industries due to its less cost and its ease of operation, it has some drawbacks which include the formation of inhibitors mainly furans and phenylic compounds and gypsum formation during neutralization after pre-treatment. Moreover, the lignin after pre-treatment may remain on the surface of crystalline cellulose which further blocks the enzyme accessibility (Farghaly et al. 2017).

### *2.12.2 Alkali-Based Pre-treatment*

Alkali-based technique being more efficient when compared to acid-based pre-treatment has a major drawback of being high cost. They also generate acetic acid, hydroxyl acids and minor amounts of furan aldehydes as by-products (Jönsson and Martín 2016).

### *2.12.3 Solvent-Based Pre-treatment*

The main drawback behind this pre-treatment is that the pre-treated biomass will contain small ionic liquids and solvents which pose as toxic to the micro-organisms and the enzymes (Jönsson and Martín 2016).

## 2.13 Conclusions and Future Prospects

The biorefinery concept is becoming more attractive, taking into account the present economic model based on non-renewable fossil resources for energy production. The cost of bioethanol is primarily affected by the cost of feedstock used, secondarily by the process employed. From the overall study, it is clear that most of the pre-treatments resulted in maximum lignin removal from PMS. Though many pre-treatment techniques are being employed, it cannot be used for commercial production due to factors like expensive technique, time-consuming and formation of inhibitors due to chemical pre-treatment which requires further separation and purification processes that lead to increase in process cost. Hence, hybrid pre-treatments are necessary to cut down the process cost and give results favourable with less inhibitors and maximum cellulose recovery.

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# Chapter 3
# Application of Hydrolytic Enzymes in Biorefinery and Its Future Prospects


**Bikash Kumar and Pradeep Verma**

**Abstract** Biomass is an alternative to the depleting fossil fuels which are primary source of the biofuel and biochemicals. It majorly consists of lignin, cellulose, hemicelluloses along with small fractions of proteins and extractives. Some of the biomass is also rich in pectin and lipids. The two major steps involved in biomass to biofuel conversion is pretreatment and enzymatic hydrolysis. The groups of hydrolytic enzymes that act on these constituents are cellulase, laccases, peroxidases, lytic polysaccharide monooxygenases (LPMOs), hemicellulases, pectinases, amylases, lipases, proteases, etc. The enzymes are obtained from a wide variety of living organisms such as microbes, plants, and animals. The specificity and activity of enzyme differ for different microorganisms. Therefore, search of novel isolates or design of microbes with desired property is essential to attain high yield in biomass-based biorefinery. The chapter gives an insight into source and function application of hydrolytic enzymes along with its techno-economic evaluation. This chapter also discusses the recent advances in the area of enzyme technology to further strengthen the concept of integrated biorefinery.

**Keywords** Biomass · Biorefinery · Hydrolytic enzymes · Enzymatic hydrolysis · Lytic polysaccharide monooxygenases

## 3.1 Introduction

The energy and most of chemical requirements are met by non-renewable fossil fuels. The depletion of fossil fuels has led to investigate for alternative energy sources (Kumari and Singh 2018). Biomass is available abundantly in nature which can be exploited for the generation of biofuel and value-added biochemicals.

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In addition, biomass is also an excellent feedstock for enzyme production (Ben-Iwo et al. 2016). The enzymes play a vital role in enzymatic hydrolysis of the biomass and generation of different precursor chemicals (Singh et al. 2019a). The biofuel can be divided into generations based on the type of biomass used, i.e., in the production of first-generation biofuel, food crops rich in sugar and starch are used, whereas in the second- and third-generation biofuels production, lignocellulosic biomass, and algal biomass are used (Kumar et al. 2020) respectively. The biomass consists of several constituents such as lignocellulosic biomass that are rich in celluloses, hemicelluloses, lignin, pectin, amylase, some oils, and proteins, whereas algal biomass mostly constitutes lipids and proteins with cell wall consisting of polysaccharides such as hemicelluloses and celluloses. The enzymes are highly selective that acts on specific constituents of biomass; therefore, different groups of hydrolytic enzymes are required for the breakdown of complex biomass into their monomer units. The group of enzymes that can facilitate the hydrolysis of these components are cellulases, hemicellulases, laccases, peroxidases, lytic polysaccharide monooxygeneases (LPMOs), pectinases, lipases, and proteases (Escamilla-Alvarado et al. 2017). The enzymes are required by several living organisms for their specific requirements; thus, these enzymes can be obtained from several sources such as microbes, plants, and animals. However, micro-organisms such as bacteria and fungus are preferred source due to its ability to grow fast, easy regulation, and manipulation for the desired yield of enzymes (Gurung et al. 2013).

The enzyme can act individually or in combination for efficient removal of lignin (ligninolytic enzymes), hydrolysis of cellulose, starch, hemicelluloses, pectin for the generation of fermentable sugars (Manisha and Yadav 2017). The lipases and proteases are used during transformation of the oils, lipids, and proteins into biofuels (Escamilla-Alvarado et al. 2017). Based on the application of steps at which enzymes during conversion of biomass to biofuel, reaction types are classified as separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), and recently developed consolidated bioprocessing (CP). During SHF, biomass is separately hydrolyzed followed by fermentation with ethanologenic microbes (Gupta et al. 2009). The SSF involves hydrolysis and fermentation together using hydrolyzing enzymes producing microbes or directly enzymes with the ethanologenic microbes (Escamilla-Alvarado et al. 2017; Bhardwaj et al. 2019b). In consolidated bioprocessing, pretreatment (ligninolytic enzymes), enzyme production (cellulases, xylanases, and pectinases), hydrolysis, and fermentation are conducted in a single pot/vessel (Tanimura et al. 2015).

The challenge associated with enzymatic hydrolysis is the development of robust and highly efficient enzymes for cheap conversion of variety of biomass (Bhardwaj et al. 2019c). The understanding of enzyme structure, kinetics, and metabolic pathways allows designing microbes or enzymes with robust applicability (Pröschel et al. 2015). Several private stakeholders such as Novozymes and DuPont have been in the field for long and are not developing and setting benchmark in the area of enzymatic hydrolysis (Brooks and Tchelet 2014). National renewable energy laboratory, USA, is also working exhaustively in the area of developing efficient pretreatment process, robust enzymes, and designing process to obtain bioethanol

and platform chemicals using biomass as starting/precursor material (Gao et al. 2014). Several initiatives by the Indian government is also leading to the development of biobased biorefineries plant in India (TOI 2018). This chapter gives an insight into structure, function, source, application of enzymes in breaking down different constituents of biomass, along with its techno-economic evaluation. This chapter also focuses on the approaches used for improving the yield and efficiency of the enzymes and its roles in the development of consolidated and integrated biorefineries.

## 3.2 Biomass Structure

The organic material obtained from different living organisms can be collectively defined as biomass, e.g., grass, vegetable, agricultural and forest residues, microbial biomass, and organic wastes generated from meat, fish, food processing industries, and municipal wastes. These biomasses are abundantly available and can be readily converted to bioethanol and bio-oils using microbial enzymatic systems. The biomass is composed of lignin, cellulose, hemicelluloses, pectin, starch, chitin, fats, and oils, with minor quantities of proteins, inorganic materials, and extractives (Mussatto and Dragone 2016).

The lignocellulosic biomass mostly consists of celluloses (30–50% of dry weight) and is located in the secondary cell wall of plant cells. Cellulose is a homopolysaccharide structure made up of β-D-glucopyranose moieties with ~10,000–15,000° of polymerization and linked via β-(1,4)-glucosidic bonds (Crocker and Andrews 2010). Followed by cellulose, the second abundant component in nature is hemicellulose, which is a highly branched hetero-polymeric structure, made up of a wide variety of moieties. Pentoses (xylose and arabinose), hexoses (mannose, glucose, galactose, and rhamnose), and sugar acids (acetic acid, D-glucuronic acid, 4-*O*-methyl glucuronic acid, and D-galacturonic acid) are the major moieties present in the hemicelluloses (Xu 2010). The amount of hemicelluloses can range from 20 to 40% of the dry biomass weight in lignocellulosic biomass (Saha 2003).

Lignin is a heteropolymeric constituent which imparts recalcitrance to lignocellulosic biomass. It is an amorphous structure built up by phenyl propanoid units. The major phenyl propanoid units are coniferyl alcohol, p-coumaryl alcohol, and sinapyl alcohol that are interlocked by highly resistant C-C and *β-O-4*-aryl ether bonds. The aromatic constituents of the different alcohols in lignin are identified as syringyl (S), p-hydroxyphenyl (H) and guaiacyl (G) (Isikgor and Remzi Becer 2015). These components are arranged in a reticulate manner to give lignin a complex structure and is insoluble in most solvents (Isikgor and Remzi Becer 2015). Therefore, for depolymerization of polyphenolic lignin, pretreatment (physical, chemical, biological, physicochemical) steps are required prior to hydrolysis of polysaccharide constituents (cellulose and hemicelluloses) into respective monomers or fermentable sugars (Balan et al. 2009; Adsul et al. 2011). In general, normal hardwood contains

20–28% lignin, whereas normal softwood contains 26–32% lignin herbaceous biomass which usually has a lower lignin content (10–25%) compared to woody biomass.

Several extractives such as resins, waxes, gums, fatty acids, chlorophyll, a variety of phenolic substances, and terpenoids are also present in the biomass (Milagres et al. 2011). Lignocellulosic biomass also consists of extractable minerals, i.e., ash. Inorganic elements such as K, Ca, Si, and Mg are also present in minute quantity as part of ash in LCB (Xu 2010). The ash content of hardwood and softwood is 0.5% and 0.4%, respectively (Demirbas 2009; Mussatto and Dragone 2016).

Pectin is usually part of middle lamella in the cell wall of young plant. Pectin is an acidic heteropolysaccharide made up of galacturonic acid, i.e., galactose-based sugar acids. It constitutes approximately 35% and 5–10% in dicots/monocots and grasses in primary cell wall (Vogel 2008). In lignocellulosic biomass, pectin constitutes only 5–10% of total dry weight; however, agricultural and food waste residues such as fruits and vegetable peels are rich in pectin. Lipids and protein are the constituents of living organisms in varying percentage. Several proteinaceous food crops (jatropha seed, olive seed, etc.), waste stream from oil industries, and oil industry wastes along with microalgae can be used as a rich source of lipid and proteins.

## 3.3 Application of Hydrolytic Enzymes in Generation of Bioethanol from Biomass

The biomass to biorefinery conversion involves numerous steps such as (1) collection of biomass, (2) biomass preparation, (3) pretreatment (physical, chemical, biological, and physicochemical), (4) hydrolysis/saccharification, (5) fermentation, and (6) downstream processing (distillation). The process of pretreatment, i.e., delignification, usually involves the role of ligninolytic and LPMOs; the hydrolytic enzymes such as cellulases, hemicellulases, LPMO, amylase, and pectinase play important role in the conversion of polysaccharide carbohydrate component to respective monomeric fermentable sugars during saccharification or hydrolysis (Fig. 3.1). The lipases and proteases are important enzymes for the utilization of lipid and protein-rich substrates such as algal biomass and food/oil wastes. Therefore, each hydrolyzing enzymatic families along with their structure, function, source, and application with respect to biorefinery has been discussed below.

### *3.3.1 Cellulase*

The cellulase family constitutes a group of enzymes which act synergistically in order to depolymerizing celluloses (Gupta et al. 2009). As discussed above, cellulose is a homopolymer of glucose with high degree of polymerization. This polymer is

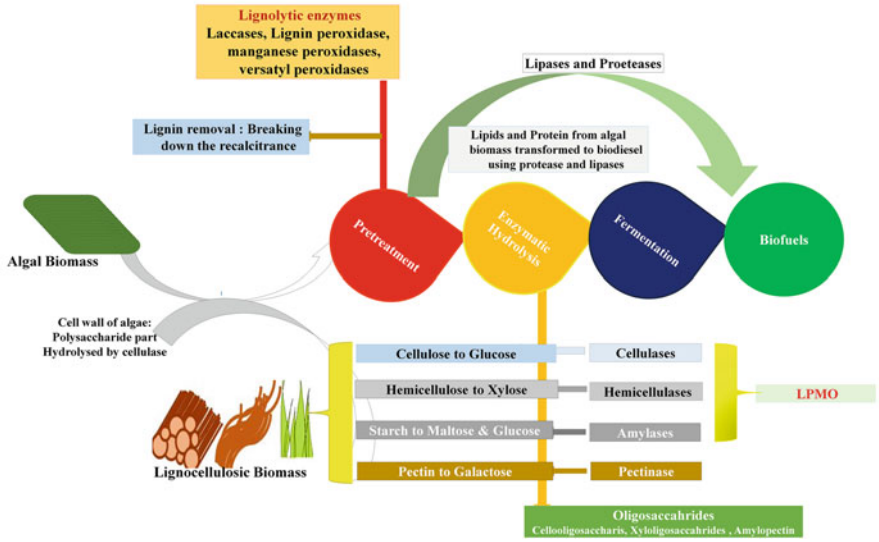


**Fig. 3.1** Schematic representation of the role of enzymes: biomass to bioethanol generation

broken down by the systematic action of the constituting enzymes, i.e., endoglucanase and exoglucanase (cellobiohydrolases and β-glucosidase) (Knowles et al. 1987; Lynd et al. 2002; Zhang et al. 2006). First, endoglucanases attack the amorphous regions and randomly cleave at β-1,4 linkages of glucan chain resulting in the generation of cellooligosaccharides. The cellobiohydrolases (1,4-β-D-glucan-cellobiohydrolases) act on reducing and non-reducing ends of cellooligosaccharide chain and result in the formation of cellobiose (Fig. 3.2). Lastly, β-glucosidases (β-D-glucoside glucanohydrolase, EC 3.2.1.21) mediates the conversion of cellobioses to the glucose monomers (Behera et al. 2017). The β-glucosidases also play a vital role in the release of glucose from non-reducing end of cellooligosaccharides (Jørgensen et al. 2007).

Cellulases are produced on industrial scale mostly by fungi and bacteria. The fungus isolated from soil, compost, and wood rot are rich sources of cellulases. Several mesophilic fungal strains such as *A. nidulans*, *A. niger*, *A. oryzae.*, and mesophilic aerobic bacteria such as *Cellulomonas fimi, Cellvibrio japonicus*, and *Pseudomonas fluorescens* isolated from soil and compost, *Bacillus brevis*, isolated from animal gut are source of cellulases (Obeng et al. 2017).

#### 3.3.1.1 Cellulases: Application in Biorefinery

During the process of lignocellulosic biomass to biofuel conversion, the biomass is first pretreated with the lignin-degrading enzymes. The delignified biomass is then hydrolyzed to the fermentable sugars through synergistic action of cellulase hemicellulases, pectinases, LMPO, etc. The fermentable sugars are then converted

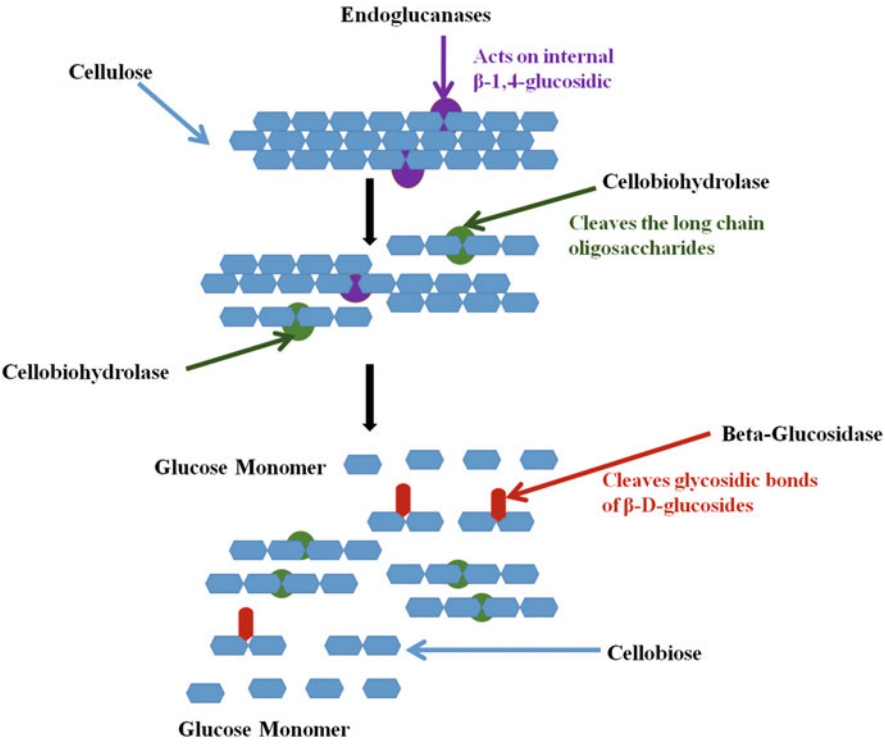


**Fig. 3.2** Mode of action of cellulolytic enzyme system

to ethanol by using ethanologenic microbes. Kumar et al. (2019) reported the generation of 7% ethanol by simultaneous saccharification and fermentation of microwave-pretreated rice straw using cellulase enzyme *from S. commune* NAIMC C-F-03379 along with *S. cerevisiae* MTCC 173. Similarly, NaOH-pretreated rice straw was hydrolyzed using *T. harzianum* cellulase followed by its fermentation by *Clostridium acetobutylicum* ATCC 824 resulted in biobutanol yield of 0.16 g/g of glucose generated (Rahnama et al. 2014). Cellooligosaccharides are generated by the action of endoglucanases and CBH (Basholli-Salihu et al. 2013). Thus, simultaneous production of low-cost product like ethanol and high-value chemical such as cellooligosaccharides can help in regulating the overall cost of lignocellulosic biomass-based biorefinery.

### *3.3.2 Hemicellulases*

Hemicellulose is heteropolymer having xylan as its major constituents. The xylan can be completely hydrolyzed into its constituent components through synergistic action of different xylanolytic enzymes. The *endo*-1,4-β-d-xylanases randomly

cleaves the backbone of xylan to generate longer xylooligosaccharides chains which are further broken down to xylose in the presence of β-d-xylosidases. The action of acetylxylan esterase, α-glucuronidase, and α-l-arabinofuranosidases helps in the removal of acetyl and phenolic side chains. The *p*-coumaric esterase and ferulic acid esterase assist in cleaving the ester bond. Thus, these enzyme complex collectively play a vital role in the generation of pentose sugar and high value compounds such as xylooligosaccharides from xylan (Walia et al. 2017; Bhardwaj et al. 2019c).

Several strains of bacteria (*Arthrobacter*, *Bacillus,* and *Clostridium*), fungus (*Aspergillus, Chaetomium,* and *Trichoderma*), and algae (Jensen et al. 2018) are potent source of hemicellulases. Being ubiquitous in nature, xylanase is also found in several protozoa, crustaceans, insects, plants, and seeds (Bhardwaj et al. 2019c). On industrial scale, bacteria such as *Bacillus*, *Actinomycetes*, and *Clostridiales* (Kulkarni et al. 1999; Rabemanolontsoa and Saka 2016) have been widely used. Several filamentous fungi such as *A. niger*, *Humicola insolens*, *A. oryzae*, *Trichoderma reesei, Trichoderma longibrachiatum, Thermomonospora fusca*, and *Trichoderma koningii* (Howard et al. 2003) have been used for the commercial production of xylanase.

#### 3.3.2.1 Hemicellulases: Application in Biorefinery

The action of xylanase enzyme causes fiber swelling that improves the biomass porosity. This results in the enhanced accessibility of cellulase to biomass causing efficient hydrolysis. Sanjivkumar et al. (2017) performed the production and purification of xylanase from *Streptomyces olivaceus* (MSU3) using Sephadex-G-75 column chromatography resulted in enhanced xylanase-specific activity of 153.11 ± 2.11 IU/mg. The purified xylanase enzyme resulted in reducing the sugar yield of 52.19% with 4.19 g/L bioethanol yield. The commercial substrates for xylanase production are such as beech wood xylan, birch wood xylan, and oat spelt xylan. These substrates are very costly, thus there is an increase in the overall production cost of the xylanase enzymes. Bhardwaj et al. (2017) demonstrated xylanase production by *Aspergillus oryzae* LC1 using different agro-residues such as rice straw, rice husk, wheat straw, wheat bran, and sugarcane baggase. They observed maximum xylanase yield with rice straw as substrate. They also demonstrated the saccharification of different lignocellulosic biomasses, i.e., rice husk, rice straw, wheat bran, wheat straw, sugarcane bagasse, groundnut shell, barley husk, and pearl millet husk with the saccharification yield of 7.8%, 34.5%, 16.7%, 8.9%, 15.6%, 28.1%, 19.3%, and 26.2%, respectively. Later, the same group has purified *Aspergillus oryzae* LC1 xylanase using single-step aqueous two-phase system which helped in enhanced substrate specificity during hydrolysis. The application of partially purified xylanase resulted in the generation of wide range of xylooligosaccharides from different lignocellulosic biomass mentioned above (Bhardwaj et al. 2019a).

### 3.3.3 *Ligninolytic Enzymes*

The ligninolytic enzyme family comprises a group of enzymes, i.e., laccases, lignin peroxidases (LiP), manganese peroxidases (MnP), and versatile peroxidases (VP) which play a key role in the biodegradation of lignocellulosic biomass and several organic wastes. These enzymes act on phenylpropanoid aryl-C3 unit linkage of several complex organic compounds such as lignin, dyes, and recalcitrance compounds via oxidation with $H_2O_2$ as the first electron acceptor. The peroxidases contain iron containing hemo group as co-factor (Robles-González et al. 2012).

Laccases (E.C. 1.10.3.2) belong to multicopper consisting oxido reductase class and are often called as p-diphenol oxidase or benzenediol. Laccase is an inducible enzyme whose production is induced by the existence of copper, dyes, or recalcitrance molecules (Minussi et al. 2007). Laccase has low substrate specificity and uses oxygen as co-factor and oxidizing agent. The lignin peroxidase (LiP) (EC 1.11.1.14) and manganese peroxidase (MnP) (EC 1.11.1.13) are collectively known as heme-peroxidases due to the presence of iron in the prosthetic group (protoporphyrin IX). LiP mediates the decomposition of numerous aromatic compounds such as methoxy benzenes and 3,4-dimethoxybenzyl (veratryl alcohol). The catalytic action of MnP results in the formation of $Mn^{3+}$ that helps in oxidization of the aromatic compounds (Hofrichter 2002). The $Mn^{+3}$ can easily pass through the lignified cell wall due to its small size and high redox potential. Therefore, it is key to biomass delignification and penetration of other enzymes to biomass (Martínez 2002; Hammel and Cullen 2008). Versatile peroxidase (EC 1.11.1.16) is also a hemoprotein with a broad range of specificity for phenolic as well as non-phenolic substrates and can oxidize them even in the absence of manganese (Polak and Wilkolazka 2012).

Ligninolytic enzymes are found in microorganisms such as bacteria and fungi. The ligninolytic enzyme can be obtained from wood decaying bacterial strains belonging to α-proteobacteria and γ-proteobacteria and actinomycetes groups. Lacasses have been found to be produced by several plants such as tobacco and Zea mays and insects as well (Plácido and Capareda 2015) (Table 3.1).

#### 3.3.3.1 Ligninolytic Enzymes: Application in the Biorefinery

The ability of laccase to degrade the polyphenolic aromatic recalcitrance compound is used in the removal of lignin from lignocellulosic biomass also referred as biological delignification/pretreatment prior to biofuel production (Moreno et al. 2012). The ligninolytic enzyme also plays a key role in simultaneous waste decomposition and electricity generation in microbial fuel cells with wastewater or industrial effluents as substrate collectively known as biofuel cell. The concept of biological delignification has been described below.

**Table 3.1** Hydrolytic enzymes used in biorefinery: its EC number, CAZy family classification, used substrate, and resulting products

| Enzyme systems | Enzymes | Substrates/sites for acting of enzyme | Products | References |
|---|---|---|---|---|
| Cellulases | Endo-1,4-β-glucanase | Amorphous cellulose | Cellooligosaccharides | Lynd et al. (2002), Kumar et al. (2018), Benedetti et al. (2019) |
| | Cellobiohydrolase | Reducing and non-reducing ends of cellulose | Cellobiose | |
| | β-Glucosidase | Cellobiose | Glucose | |
| Hemicellulase | *Endo*-1,4-β-d-xylanases | Xylans | Xylooligosaccharides | Alalouf et al. (2011), Bhardwaj et al. (2019b) |
| | β-d-xylosidases | Xylooligosaccharides | Xylose | |
| | Acetyl xylan esterase | Acetyl xylan esters residues | Removal of acetyl groups from xylans | |
| | p-Coumaric esterase or ferulic acid esterase | Side group of ferulic acid and p-coumaric acid | Removal of side chains such as ferulic acid and p-coumaric acid | |
| Ligninolytic | Laccases, lignin peroxidase, manganese peroxidase, versatile peroxidase | Lignin, aromatics, veratryl alcohol, methoxybenzenes | Oxidized products, guaiacol, protocatechuic acid and vanillic acid | Rabemanolontsoa and Saka (2016), Agrawal et al. (2018) |
| Lytic polysaccharide monooxygenase (LPMOs) | Lytic polysaccharides monooxygenase | Cellulose and chitin | Oligosaccharides | Payne et al. (2015), Chylenski et al. (2019) |
| Amylases | α-amylase | Starch at random site | Short oligosaccharides such as maltotriose and glucose | El-Enshasy et al. (2013), Escamilla-Alvarado et al. (2017) |
| | β-Amylase | Non-reducing end of the starch molecule | β-Maltose | |
| | Glucoamylase or γ-amylase | Amylose, amylopectin | Glucose | |
| | Pullulanase | Amylose, amylopectin | Glucose | |

(continued)

**Table 3.1** (continued)

| Enzyme systems | Enzymes | Substrates/sites for acting of enzyme | Products | References |
|---|---|---|---|---|
| Pectinases | Pectin acetyl esterase<br>Pectin methyl esterase<br>Polymethylgalacturonase | Pectin | Galacturonic acids and galactose | Kiran et al. (2014), Garg et al. (2016) |
| | Rhamnogalacturonan endolyase (RGL | Rhamnogalacturonan | | |
| Lipases | Lipases | Triglycerides | Monoacylglycerols, diacylglycerols, glycerol | Bajaj et al. (2010) |

Biological Delignification

The existence of complex and highly recalcitrance lignin makes it difficult for the application of lignocellulosic biomass in biomass-based biorefinery. Therefore, the ligninolytic enzyme-producing microbes basically fungus or obtained ligninolytic enzymes can been used for biomass delignification. Based on the type of applications, Plácido and Capareda (2015) classified the fungal delignification into four broad categories. Considering the role of all the types of microorganisms and its ligninolytic enzymes, we can further modify those classification as (a) microbial delignification (MD), (b) enzymatic delignification (ED), (c) laccase-mediator system (LMS), and (d) integrated microbial fermentation (IMF).

(a) **Microbial Delignification**: This process involves direct application of the ligninolytic microorganisms subjecting the biomass in submerged (Lu et al. 2010; Martin-Sampedro et al. 2011) or solid state fermentation (Wan and Li 2010; Salvachúa et al. 2011). The microorganisms selectively degrade lignin and sometime hemicelluloses as well, while it is expected that cellulose remains intact (Moreno et al. 2015).
(b) **Enzyme Delignification**: The long duration of 13–50 days required for microbial delignification can be reduced to 1–4 days by the direct application of enzymes (Asgher et al. 2013). During enzymatic delignification, in-house crude extracts, partially purified, purified. or commercially available ligninolytic enzymes are used (Mattinen et al. 2011). Agrawal et al. (2019) used partially purified laccase from *Myrothecium verrucaria* ITCC-8447 for lignin removal from wheat straw, and a delignification of 64.7% was achieved.
(c) **Laccase Mediator System**: Laccase has often reported to have low redox potential thus often not able to break recalcitrance molecules on its own, thus delignification efficiency is limited. The other factors which also affect laccase ability to degrade the biomass are its size, complexity, and composition of the substrate (Agrawal et al. 2018). Therefore, the researchers have come up with the addition of compounds having high redox potential which act as a bridge between enzyme and substrate for electron transfer. These mediator compounds are either chemicals such as 1-hydroxybenzotriazole (HBT), and 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) or exist naturally such as vanillin and syringaldehyde (Fillat and Roncero 2009).
(d) **Integrated Microbial Fermentation (IMF)/Consolidated Processing**: The multistep process of biomass to biofuel conversion is not only tedious but also costly. Therefore, there is a requirement of a single-step process to convert biomass to biofuel directly. The application of a microorganism or a group of microorganisms for direct bioethanol generation from different biomass in a single vessel, the process is also known as consolidated bioprocessing (Lynd et al. 2005). Several reports suggest that thermophillic Gram-positive (+ve) Firmicutes belonging to *Thermoanaerobiales and Clostridiales* order can be used potentially in consolidated bioprocessings.

### 3.3.4 Lytic Polysaccharide Monooxygenases (LPMOs)

Lytic polysaccharide monooxygenases (LPMOs) are also referred as PMO, and structurally LPMO are multi-copper enzyme with immunoglobulin- or fibronectin-type III-like structure. LPMOs consist of β-sandwich made up of 8–10 β-strand (Quinlan et al. 2011). LPMOs have a characteristic property, i.e., presence of "histidine brace" that binds to the copper (Quinlan et al. 2011; Ciano et al. 2018). LPMOs have high affinity toward copper and have ubiquitous presence of copper (Quinlan et al. 2011; Aachmann et al. 2012). Earlier studies showed that catalytic action of LPMO requires one reductant, two electrons, and one molecular oxygen and reaction (Beeson et al. 2015; Walton and Davies 2016; Meier et al. 2017) and is represented in Scheme 3.1.

**Scheme 3.1** $RH + O_2 + 2e^- + 2H^+ \rightarrow R\text{-}OH + H_2O$ (Vaaje-Kolstad et al. 2010)

Bissaro et al. (2017) used mass spectrometry, enzyme assays, and experiments with labeled oxygen atom to establish the fact that $H_2O_2$ is a more preferred co-substrate rather than $O_2$ for the LPMOs. The reaction is represented in Scheme 3.2.

**Scheme 3.2** $R\text{-}H + H_2O_2 \rightarrow R\text{-}OH + H_2O$ (Bissaro et al. 2017)

LMPOs are naturally produced by several bacteria (*Bacillus licheniformis, Serratia marcescens*), fungi (*Myceliophthora thermophila, Phanerochaete chrysosporium, T. reesei, and Aspergillus oryzae*), and insect (*Thermobia domestica*) (Hemsworth et al. 2015; Sabbadin et al. 2018). However, most of the characterized LPMOs are recombinant in origin, such as *E. coli* for bacterial and *Pichia pastori*s and fungal hosts for fungal LPMOs (Eijsink et al. 2019).

#### 3.3.4.1 LPMOs: Application in Biorefinery

The monooxygenases or LPMOs show auxiliary activities (AA) that act synergistically along with several other hydrolytic enzymes (GH) such as cellulase, hemicellulase, chitinase, and amylases thus able in better hydrolysis of cellulose, hemicelluloses, chitin, and starch as compared with hydrolyzing enzymes. The efficient cellulose degradation requires collegial action of endo-1,4-β-glucanases (random cleavage), cellobiohydrolases (generation of cellobiose), and β-glucosidases (glucose from cellobiose). Several commercial cocktails have blending of LPMOs which increase the overall hydrolytic efficiency. Hu et al. (2014, 2015) and Müller et al. (2015) suggested that spiking of Celluclast and Novozyme 188 mixture with TaAA9A LPMO can result in improvement in cellulose conversion efficiency by 18–63% from a wide range of pretreated lignocellulosic biomass (i.e., corn stover, poplar, and loblolly pine) and commercial substrate Avicel. Several studies suggest that the LPMO can play a vital role in biomass processing, fermentative valorization of biomass. However, to utilize the potential of LPMO at

industrial scale requires changes in the process design, e.g. the regulation of the addition of co-substrates (oxygen or hydrogen peroxide) need to be done when LPMO is used with the ligno-hemicellulosic cocktail (Müller et al. 2018).

### *3.3.5 Amylases*

The amylase family consists of four enzymes, i.e., α-amylase (EC 3.2.1.1), glucoamylase or γ-amylase (EC 3.2.1.3), β-amylase (EC 3.2.1.2), and pullulanase (EC 3.2.1.41). α-Amylases are metalloenzymes (require metal ions $Ca^{+2}$ to initiate reaction) that cleave randomly on α-1,4-glycosidic bonds of the linear chain of starch molecule breaking it into shorter oligosaccharides. β-Amylase or 1,4-α-d-glucan maltohydrolase is an exoenzyme that acts on non-reducing end of linear starch molecule breaking the α-1,4-glycosidic linkage and generates β-maltose as major and β-limit dextrin as minor end products. Glucoamylase or γ-amylase or glucan α-1,4-glucosidase is an exoamylase that acts on non-reducing end of amylase and amylopectin at last α-1,4-glycosidic linkages and generates glucose. Pullulanases acts on the α-1,6 linkages of the partially hydrolyzed amylopectin. Pullulanases are usually used in combination with the glucoamylases and α-glucosidases to improve the saccharification yield in starch-rich substrates (El-Enshasy et al. 2013).

Microorganisms (bacteria, fungi, and Actinomycetes), plants, and animals are source of amylase enzyme. It plays a vital role in human digestion and is found predominantly in the saliva and pancreatic fluids. Several bacterial strains from *Bacillus* species have a desired character needed for its industrial application (Kathiresan and Manivannan 2006). Several hyper-thermophilic bacteria and Archaea such as *Rhodothermus marinus* and *Clostridium thermosulfurogenes* are also capable of producing amylases specifically pullulanases (Gomes et al. 2003; Gangadharan and Sivaramakrishnan 2009).

Several fungal strains from genus *Aspergillus* (*A. flavus*, *A. oryzae*) and *Penicillium* (*P. chrysogenum*) and several other fungal strains such as *Cryptococcus flavus*, *Pycnoporus sanguineus*, and *Mucor* sp. have shown amylase production potential (Sundarram and Murthy 2014).

#### 3.3.5.1 Amylases: Application in Biorefinery

The first-generation biofuels are obtained from the conversion of mainly edible parts of food crops rich in starch, sugars, or oils (Kumar et al. 2020). The role of amylase is of great importance in the conversion of the starch-rich substrate to bioethanol and bio-butanol. Through a series of steps, the starchy biomass can be transformed to ethanol. The bio-conversion of carbohydrate (starch) to fermentable reducing sugar requires the role of these amylases (Lewis and Van Hulzen 2013). The starchy materials are first grounded to pulp and are then subjected to liquefaction by the action of amylases. The liquefaction process involves the action of α-amylase

conversing pulp to water soluble maltodextrin oligosaccharides which are subsequently hydrolyzed to fermentable sugars such as glucose, maltose, and iso-maltose and dextrins via the action of pullulanase and glucoamylase. The fermentable sugars thus generated are subsequently converted to ethanol via action of the ethanologenic microbes (Lewis and Van Hulzen 2013).

### 3.3.6 Pectinases

The pectic substrate is degraded by the de-esterification and de-polymerization reaction carried out by esterases and hydrolases/lyases, respectively (Singh et al. 2019b). Basically, the pectinases are grouped based on its mode of action on pectin. Pectin acetyl esterase (E.C. 3.1.1.6) and pectin methyl esterase (E.C. 3.1.1.11) are two esterases that act on pectin. The depolymerization reaction of pectin is catalyzed by depolymerases (Kiran et al. 2014; Garg et al. 2016).

Naturally, pectinases help in natural ripening of the fruits, thus it is produced by plants, but commercially, the pectinases are produced by the microbes usually bacteria, fungi, and yeasts. Strains from genus *Aspergillus* (*A. niger* and *A. fumigates*) and *Penicillium* (*P. notatum* and *Penicillium occitanis*) and other fungal strains such as several bacterial strains such *B. subtilis, Sclerotium rolfsii*, *Chryseobacterium indologenes*, and *Pectobacterium carotovorum* are potent pectinases producing bacterium (Singh et al. 2019c). Till date very few report on the application of pectinases in the biorefinery. However, the bio-waste generated from the fruits, vegetables, and food processing industries can be used for the further biofuel and value-added compound generation. The food, fruit, and vegetable industry wastes can be valorized by the action of pectinase enzyme.

### 3.3.7 Lipases

Lipases (EC 3.1.1.3) also known as triaglycerol acyl hydrolases can efficiently hydrolyze the fats (lipids) such as triacylglycerols, fatty acids, oils, and glycerol. Unlike conventional lipases, phospholipases and sphingomyelinases exist in nature which catalyzes hydrolysis of glycerophospholipids (attack on ester and phosphodiester bond) (Murakami and Kudo 2002) and sphingomyelin. The lipases catalyze different reaction by the acidolysis, alcoholysis, aminolysis, and transesterification (Saxena et al. 2003).

The lipases are produced by microbes as well. Bacterial strains from genus *Bacillus* (*B. subtilis*), *Pseudomonas* (*Pseudomonas aeruginosa*), *Streptomyces* (*Streptomyces aureus*, *Streptomyces hyicus*, *Streptomyces epidermidis*, *Streptomyces* sp.), and other strains such as *Aeromonas hydrophila* and *Xenorhabdus luminescens* reported to be as potent lipase producer. Fungal strains from genus *Penicillium* (*Penicillium aurantiogriseum*), *Rhizopus* (*Rhizopus rhizopodiformis*),

*Aspergillus* (*Aspergillus carneu*), and *Candida* (*Candida cylindracea*) have shown good lipase production potential using oil-rich plant components as substrates such as olive oil, olive bagasse, olive mill wastewater, wheat bran, soybean oil, and oleic acid (Singh and Mukhopadhyay 2012).

#### 3.3.7.1 Lipases: Application in Biorefinery

The biotechnological application of lipases is food, dairy, laundry, organic synthesis, medicine, health, cosmetics, etc. With respect to the biorefineries, the lipases can be used for the conversion of crude/waste oils, lipids, and fats to the biodiesel (Bajaj et al. 2010; Singh and Mukhopadhyay 2012; Kiran et al. 2014). The first- and third-generation (1G, 3G) fuels are based on transesterification of vegetable oils (crude or waste) and third-generation fuel based on lipids (algal biomass) to biodiesel through trans-esterification. Therefore, the lipases are important for 1G and 3G biofuels.

### *3.3.8 Proteases*

Proteases are the enzymes that catalyze the proteolysis, i.e., breaking down protein into smaller fragments of polypeptides or even to single amino acids. The proteases are ubiquitous in nature existing in all types of the living organisms from prokaryotic to eukaryotic or single cell to complex organisms. Microbial proteases are classified into three different types based on the activity at the respective pH, i.e., alkaline proteases (9–11), acidic proteases (3.8–5.6), and neutral proteases (5–8) (Pushpam et al. 2011; Vadlamani and Parcha 2011; Razzaq et al. 2019). Different strains of bacterial genus *Bacillus* such as *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus* sp., and *B. lentus* have been reported to be used in commercial production of the proteases (Razzaq et al. 2019). Fungal strains from genus *Aspergillus*, *Penicillium*, and *Rhizopus* have been used as microbial proteases (Kiran et al. 2014).

#### 3.3.8.1 Proteases: Application in Biorefinery

The proteases find application in leather, detergent, dairy, food, and pharmacy due to its ability to break down proteins which are intermediate or starting material for the product being generated in these industries. Algae or microalgae are primary substrates for third-generation biofuel, as they are rich in the protein/lipids which need to be hydrolyzed for being converted to biofuel or biochemicals (López-Otín and Bond 2008; Sari et al. 2016; Li et al. 2018; Tavano et al. 2018; Razzaq et al. 2019). Several chemical and enzymatic methods have been used, and proteases and lipases can be used for enzymatic hydrolysis of algal protein for its conversion to biofuels. The global need of petrochemical is decreasing due to the depletion of petroleum

reserve. The proteases can play an important role to accelerate bio-based chemicals in order to meet the global need arising from chemical requirement (Li et al. 2018).

## 3.4 Strategies Employed for Improving the Hydrolytic Enzyme Yield and Efficiency

### *3.4.1 Immobilization of Enzyme*

The use of enzyme in biorefinery is limited due to the high cost of enzyme and lack of efficient method to reuse the enzymes. The immobilization of enzymes enables the enzymes to be more stable and enables its reusability without losing much of the activity (Manisha and Yadav 2017). Different methods for immobilization of enzyme on a durable and reusable matrix are suggested such as covalent binding (support such as polyaniline via glutaraldehyde, HP-20 (styrene-divynilbenzene adsorbent resin) with glutaraldehyde) (Kapoor and Kuhad 2007; Madakbacs et al. 2013), ionic binding (using Q-sepharose, Dowex-50W) (Kapoor and Kuhad 2007; Hu et al. 2018), physical absorption, crosslinking, entrapment, encapsulation (Reis et al. 2019), magnetic nanoparticles, and carbon nanotubes (Long et al. 2017).

These encapsulation methods help to reuse the immobilized enzyme upto 5–15 cycles of hydrolytic process (Hu et al. 2018). Hu et al. (2018) performed immobilization of xylanase into alginate beads using glutaraldehyde. They suggested that lowering the glutaraldehyde loading can improve enzyme immobilization and xylo-oligomer conversion efficiency (~65%). The immobilization leads to improvement in the β-xylosidase and endoxylanase activities by 40% and 80%, respectively. The efficiency of the immobilized enzyme remains almost similar even after five cycles of application.

### *3.4.2 Screening of New and Robust Isolates from Extreme Habitats*

The industrial application of enzymes requires characteristics property such as high specificity (specific to substrate), activity, and stability. The industrially potent enzymes need to endure a wide range of temperature and pH, metal ions and solvent concentrations, highly alkaline, and halophilic. Usually the enzymatic hydrolysis during conversion of biomass to biofuel requires high temperature; therefore, there is a need of thermophilic enzymes (Bhardwaj et al. 2019c). These thermophilic enzymes are key to the development of simultaneous saccharification and co-fermentation (SSCF) strategy and consolidated biorefinery (CP) system. The thermophillic enzymes may be obtained from the microbes from the thermophillic environments such as hot spring. Zarafeta et al. (2016) isolated a bacterial strain

belonging to *Thermoanaerobacterium* sp. from Icelandic hot springs. The isolate has been characterized to have a GH5 cellulase gene (*CelDZ1*) which is thermotolerance and halotolerance. An extremely halophilic bacterial isolates from genera *Salinicoccus* were able to produce amylase and proteases extracellularly (Jayachandra et al. 2012). Bhardwaj et al. (2017, 2019a) isolated a fungal strain of *A. oryzae* from leaf litter samples collected from the forests of Assam. The xylanase obtained from the isolated strain is found to be alkali and thermo-tolerant and efficient in the saccharification of biomass to sugars and xylooligosaccharides. Similarly, Kumar et al. (2018) demonstrated production and purification of thermo-alkali stable cellulase from *S. commune* NAIMCC-F-03379. The purified cellulase showed significant potential to be in biomass hydrolysis.

### *3.4.3 Genetic Engineering*

Several molecular approaches such as recombinant DNA technology and genetic engineering that can be used for the enhanced enzyme yield with high specific activity (Gopinath et al. 2017). Genetic engineering or protein engineering as in case of enzymes can be broadly divided into three approaches: (1) rational approaches, (2) semi-rational, and (3) directed evolution. Rational approaches utilize the available structural information in the enzyme sequence to be applied for site direct mutation leading to specific sequence modification (Plácido and Capareda 2015). Deletion of transcription factor encoding gene amyR of *A. niger* CICC2462 resulted in the enhanced amylase production specifically with no or low background protein secretion (Zhang et al. 2016). During semi-rational approaches, a selected amino acid in the hotspot region of enzyme is replaced with other amino acid (Mate and Alcalde 2015). This approach has even helped to improve the enzyme efficiency by three- to eight-folds (Andberg et al. 2009). In the absence of the structural information, the directed molecular evolution is applied by utilizing random mutation followed by recombination and selection of efficient mutant. This approach has resulted in the improvement of the solvent tolerance to catalytic activity (Mate and Alcalde 2015).

Recombinant DNA technology involves several steps first being selection of suitable gene and second the gene is inserted in a suitable vector system. The third step of RDT involves transformation to an efficient expression system such as *E. coli* and yeast, and the fourth step is the selection of the recombinant followed by downstream processing and characterization of the protein thus produced by the recombinant. Bhardwaj et al. (2020) showed that xylanase gene (*XynF1*) of *A. oryzae* LC1was transferred to *E. coli* BL21(DE3) (a prokaryotic system), for the production of recombinant xylanase having very high titer (1037.3 U/mg) of specific activity that is higher than that of the native strain by 9.3-fold.

### 3.4.4 *Metagenomics Approach for the Identification of the Potential Hydrolytic Enzyme*

All the microbes are cultivable under laboratory condition; therefore, in order to explore this microbiome, metagenomics approach is used. Metagenomics is a technology of directly isolating DNA from the natural habitat such as soil, water, and composed leaves. The open reading frame of the isolated DNA is screened in order to identify the novel genes for different enzymes. Several lipases from forest topsoil, activated sludge, cold-sea sediment, and fat contaminated soil have been identified using metagenomic approach (Lee et al. 2004; Roh and Villatte 2008; Jeon et al. 2009; Glogauer et al. 2011; Manisha and Yadav 2017).

The beginning of highly advanced next-generation sequencing (NGS) technology has arrived as a boon to the field of metagenomics. The advancements have enabled the discovery of potential source of hydrolytic enzymes. The application of multi-substrate-derived microbial consortium approach has taken a leap due to the combination of NGS-assisted metagenomics. By using this approach, 25 GH families have been identified from anaerobic beer under high-temperature condition (Yang et al. 2016).

## 3.5 Integrated Biorefineries: Future of Biomass-Based Biorefinery

Previously, the bio-refineries usually used the commercial enzymes for the generation of bioethanol which resulted in the high cost of the overall process. Therefore, it was suggested to produce the enzymes in-house, and also the waste generated in the overall process is further used up for the generation of high-value chemicals, making the overall process more feasible economically. Several studies such as one by Bozell and Petersen (2010) at the US Department of energy suggested that chemicals such as aspartic acid, fumaric acid, glutamic acid, glycerol, itaconic acid, malic acid, levulinic acid, sorbitol, succinic acid, and xylitol can be generated along with the bioethanol/biofuel in the biomass-based biorefinery. Further, the integrated biorefinery can be attributing to the generation of essential precursor molecules such as benzene, ethylene, propylene, toluene, and xylene as well. Thus, the process of integrating the process from collection to complete valorization can be defined as integrated biorefineries.

Robles-González et al. (2012) demonstrated an integrated biorefinery during the conversion of agave to mescal. The overall process involves first extraction of juice, and the bagasse generated can be used for in-house enzyme production along with the production of compost. The juice is fermented to produce mescal, and the microbial biomass generated during the process was suggested to be used for the generation of enzymes such as laccase, peroxidases, biofuel such as methane and other value-added compounds such as antioxidants. The four-stage H-M-Z-S

model-based integrated biorefineries are suggested to use the in-house generated hydrolytic enzymes for the conversion of municipal solid waste to bio-hydrogen, for hydrogen fuel cells. Also, methane produced during the process is used to meet the energy need of the biorefineries (Escamilla-Alvarado et al. 2014). The role of enzyme is important for success of the integrated biorefinery concept and may even help in fulfilling the dream of biomass-based circular bio-economy.

## 3.6 Summary

Enzyme is the key player in the biomass-based biorefinery with (1) polysaccharide hydrolysis mediated by cellulase, amylase, xylanase, and pectinase, (2) removal of lignin and generation of platform chemical by ligninolytic enzyme, and (3) transformation of oils, lipids, and protein-rich biomass by lipases and proteases. The microbial sources are needed to be further exploited to attain high yield of these enzymes at lower cost by approaches such as genetic engineering and process optimization. The conversion of biomass to bioethanol is a multistep process which eventually leads to higher cost. Thus, there is also need to minimize the number of steps by using approaches such as consolidated bioprocessing which may be developed using advance technologies such as metagenomics and cell surface engineering. There is a need to have better understanding of the biochemical and kinetic properties of different hydrolytic enzymes. This improved understanding coupled with advance technologies such as metagenomics, next-generation sequencing, and cell surface engineering can help to develop microbes that can be used in developing integrated biorefineries.

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# Chapter 4
# Cultivation of Microalgae: Effects of Nutrient Focus on Biofuels


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**Abstract** Several microalgae have potential to produce biofuels, carotenoids, polyunsaturated fatty acids, peptides, and phytosterols. Microalgae are capable of producing biofuels competently as another potential alternate as feedstock and may help to generate extra revenue, when its cultivation is handled scientifically at large-scale. The growth medium components, which is a major part of their cultivation, play a key role to improve its cellular components and mass accumulation. The medium components are varied according to the nature of microalgae, i.e., heterotrophic, autotrophic, and their nature of availability. In this chapter, nutritional factors, suitable compositions of media used for various microalgae cultivation, photosynthesis process, micronutrients requirements, and bioreactors for microalgae are discussed. For enhanced production of biofuels and bioactive compounds, optimized environmental conditions and nutritional factors for effective cultivation of microalgae have been revealed.

**Keywords** Microalgae · Photosynthesis · Lipid production · Cultivation

## Nomenclature

| | |
|---|---|
| ATP | Adenosine triphosphate |
| BBM | Bold basal medium |
| BG 11 | Blue-green medium |
| E | Energy |

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| | |
|---|---|
| EDTA | Ethylenediamine tetraacetic acid |
| ESM | Enriched sea water medium |
| h | Plank's constant |
| K medium | Keller medium |
| $k_L a$ | Mass transfer coefficient |
| MBM | Modified Bristol Medium |
| MDM | Modified Detmer's Medium |
| $NADPH_2$ | Nicotinamide adenine dinucleotide |
| PBR | Photobioreactor |
| PU | Poly unsaturated fatty acids |
| TAP | Tris–acetate–phosphate medium |
| TG-FA | Triglyceride fatty acids |
| $\nu$ | Frequency |

## 4.1 Introduction

Microalgae are photosynthesizing microorganisms; they may be prokaryotic or eukaryotic in nature, present in aquatic areas like ponds, lakes, streams, oceans, deep sea waters, soil, and on earth. They are characterized by rapid growth even at high pressures (deep seas), in acid environments, and in extreme conditions. The microalgae play a critical role in $CO_2$ sequestration, bioactive compounds, and biofuel production, involving in wastewater treatment, and needed a potential research for the enhancement of the end products (Guzzon et al. 2008; Kumar et al. 2009). Therefore, a critical research is needed to achieve full-scale and continuous process including (1) assess nutrient uptake, (2) improved algae growth, (3) wastewater utilization, (4) $CO_2$ sequestration efficiency, (5) improved algal harvesting, (6) enhanced extraction and purification, and (7) economic considerations (Pulz 2001; Carvalho and Malcata 2001).

## 4.2 Types of Microalgae

Microalgae are observed as single cells to colonies (a group of cells) and also as filamentous eukaryotic structures (chain like multiple cells), from prokaryotes to eukaryotes, and are classified according to biological evolution upon the time from cyanoprokaryotes to eukaryotes. Cyanoprokaryotes are with no distinct nucleus and chloroplasts (primitive of life), whereas eukaryotes are having well-defined cell wall, nucleus, and other cellular organelles. Further in algae vascular tissues, embryo and sex organs are absent like higher plants. Eukaryotic algae are classified into several divisions, i.e. *Chrysophyceae*, *Chlorophyceae*, *Euglenaphyceae*, *Cyanophyceae*, *Rhodophyceae*, *Pyrrophyceae*, *Phaeophyceae*, and *Xanthophyceae*. Further, general

**Table 4.1** Major differences in characteristics present in various divisions of algae

| Division | Cell wall | Pigment type | Storage type | Habitat | Type of algae |
|---|---|---|---|---|---|
| Chlorophyceae | Cellulose/pectose | Chlorophyll A and B | Starch | Marine and fresh water | Micro and macroalgae |
| Chrysophyceae | Cellulose with silicate | Chlorophyll A and C | Leucosin with oil drop | Marine and fresh water | Microalgae |
| Cyanophyceae | Mucopeptide with carbohydrates | Chlorophyll A and D | Starch and protein | Marine and fresh water | Microalgae |
| Euglenophyceae | No cell wall, cover proteinacious pellicle | Chlorophyll A and B | Paramylon | Fresh water | Microalgae |
| Phaeophyceae | Cellulose | Chlorophyll A and C | Laminarin | Marine water | Macroalgae |
| Pyrrophyceae | Stiff cellulose plates on outer wall | Chlorophyll A and C | Starch | Marine and fresh water | Microalgae |
| Rhodophyceae | Cellulose/ mucilagenous | Chlorophyll A and D | Floridean | Marine and fresh water | Micro and macroalgae |
| Xanthophyceae | Cellulose and hemicelluloses | Chlorophyll A | Leucosin | Marine and fresh water | Microalgae |

classification depends on the pigments present, such as green (chlorophyll), blue (phycocyanin), yellow (xanthophylls), orange (carotene), red (phycoerythrin), and brown (fucoxanthin) algae (Frac et al. 2010). The major differences in characteristics and the classification of algae are summarized in Table 4.1.

## 4.3 Components Present in Algae

The major components of microalgae are very rich in lipids, carbohydrates, proteins, and also vitamins, pigments, etc. in various percentages of their dry weight, and these percentages may vary depending upon the variety of microalgae. Among them largest fractions of lipids and proteins are present, lipids are used for production of biofuels and edible oils (Delrue et al. 2012). In case of proteins and carbohydrates, they are used for the protein supplements and polymers manufacturing, respectively (Tables 4.2 and 4.3).

**Table 4.2** Various components present in microalgae as percentages of dry weight

| Name of the algae | Lipids | Proteins | Carbohydrates | References |
|---|---|---|---|---|
| *Phaeodactylum tricornutum* | 16.1 | 34.8 | 16.8 | Tibbetts et al. (2015) |
| *Scenedesmus obliquus* | 12–14 | 48–56 | 10–17 | González López et al. (2010) |
| *Dunaliella* sp. | 14.36 | 34.17 | 14.57 | Kent et al. (2015) |
| *Chaetoceros calcitrans* | 23 | 40 | 37 | Velasco et al. (2016) |
| *Chaetoceros muelleri* | 31 | 59 | 10 | Velasco et al. (2016) |
| *Spirulina maxima* | 6–7 | 60–71 | 13–16 | Becker (2007) |
| *Nannochloropsis granulate* | 22 | 30 | 10 | Kent et al. (2015) |
| *Porphyridium cruentum* | 9–14 | 28–39 | 40–57 | Brown (1991) |
| *Tetraselmis chui* | 12 | 31–46 | 25 | Tibbetts et al. (2015) |
| *Tetraselmis* sp. | 15 | 36 | 24 | Schwenzfeier et al. (2011) |
| *Isochrysis galbana* | 11 | 27 | 34 | da Silva Gorgônio et al. (2013) |
| *Prymnesium* sp. | 22–38 | 28–45 | 25–33 | Ricketts (1966) |
| *Schizochytrium* sp. | 50–77 | – | – | Chisti (2007) |
| *Botryococcus braunii* | 25–34 | 39–40 | 19–31 | Tibbetts et al. (2015) |
| *Chlorella pyrenoidosa* | 02 | 57 | 26 | Chisti (2007) |
| *Aphanizomenon flos-aquae* | 03 | 62 | 23 | FAO (2018) |
| *Chaetoceros calcitrans* | 15 | 36 | 25 | Milledge (2011) |
| *Chlorella vulgaris* | 14–22 | 51–58 | 12–17 | Wolkers et al. (2011) |
| *Diacronema vlkianum* | 06 | 57 | 32 | Bleakley and Hayes (2017) |
| *Dunaliela bioculata* | 08 | 49 | 04 | van Krimpen et al. (2013) |
| *Euglena gracilis* | 22–38 | 39–61 | 14–18 | van Krimpen et al. (2013) |
| *Haematococcus pluvialis* | 15 | 48 | 27 | Bleakley and Hayes (2017) |
| *Prymnesium parvum* | 22–38 | 28–45 | 25–33 | van Krimpen et al. (2013) |
| *Scenedesmus dimorphus* | 16–40 | 8–18 | 21–52 | van Krimpen et al. (2013) |
| *Scenedesmus quadricauda* | 1.9 | 47 | 21–52 | van Krimpen et al. (2013) |
| *Spirogyra* sp. | 11–21 | 6–20 | 33–64 | van Krimpen et al. (2013) |
| *Arthrospira platensis* | 4–9 | 46–63 | 8–14 | Milledge (2011) |
| *Arthrospira maxima* | 6–7 | 60–71 | 13–16 | Milledge (2011) |
| *Synechococcus* sp. | 11 | 63 | 15 | Christaki et al. (2011) |
| *Tetraselmis maculate* | 03 | 52 | 15 | van Krimpen et al. (2013) |
| *A. Platensis* F&M-C256 | 10.7 | 63.9 | 12.8 | Niccolai et al. (2019) |
| *Klamath* | 6.1 | 62.4 | 18.8 | Niccolai et al. (2019) |
| *N. spheaeroides* F&M-C117 | 15.1 | 50.8 | 14.5 | Niccolai et al. (2019) |
| *C. sorokiniana* F&M-M49 | 22.7 | 51.3 | 15.5 | Niccolai et al. (2019) |
| *C. sorokiniana* IAM C-212 | 27.9 | 39.9 | 10.7 | Niccolai et al. (2019) |
| *C. vulgaris* Allma | 16.9 | 56.8 | 5.9 | Niccolai et al. (2019) |
| *T. suecica* F&M-M33 (S) | 22.4 | 18.3 | 36.8 | Niccolai et al. (2019) |
| *P. purpureum* F&M-M46 | 13.1 | 34.2 | 17.0 | Niccolai et al. (2019) |
| *P. tricornutum* F&M-M40 | 20.5 | 38.8 | 11 | Niccolai et al. (2019) |

(continued)

**Table 4.2** (continued)

| Name of the algae | Lipids | Proteins | Carbohydrates | References |
|---|---|---|---|---|
| *Tisochrysis lutea* F&M-M46 | 27.9 | 42.9 | 8.6 | Niccolai et al. (2019) |
| *N. oceanic* F&M-M24 | 28.2 | 43.1 | 14.3 | Niccolai et al. (2019) |

## 4.4 Cultivation of Microalgae

Microalgae are generally unicellular autotrophs/heterotrophs which convert inorganic compounds in the presence of light energy into organic compounds. The earliest photoautotroph organisms (anoxygenic photosynthetic process) utilize light source to extract the protons and electrons from donors ($Fe^{2+}$, $H_2S$, etc.), and reduction of $CO_2$ ultimately produces organic compounds. Blue-green algae (cyanobacteria) are prokaryotic cells containing DNA in nucleoplasm and peripheral region (Chromoplast) covered with photosynthetic sheets in parallel and closed to cell surface. Eukaryotic algae (microalgae) have special organelle chloroplasts with alternative layers of thylakoids and stroma which is involved in photosynthetic process. Microalgae have the potential for $CO_2$ fixation, biofuel, and bioactive compounds production due their capability of photosynthesis which transforms $CO_2$ in to biomass and into several value-added products. So, microalgae cultivation is an option to reduce $CO_2$ release from the fossil fuel combustion.

During photosynthesis, light energy–controlled oxidoreduction reaction yielded by chlorophyll in which $CO_2$ and $H_2O$ are transformed into oxygen and sugars in two stages, i.e., light and dark reactions. In thylakoid membranes, the light power is transformed into chemical force, facilitating a biochemical reductant $NADPH_2$ and ATP. In stroma, $NADPH_2$ and ATP are utilized for consecutive biochemical reduction of $CO_2$ to sugars (Fig. 4.1). Light energy is available as photons (quanta) and as a product of plank's constant and its frequency ($E = h\nu$). The light energy incident on the surface was measured as radian flux energy; the units are power per area (W $m^{-2}$ or $Jm^{-2}$ $s^{-1}$). In the process of photosynthesis, photon flux density is considered as the quantity of photons (quanta) that are accrued on the surface per unit time which is measured in μmol quanta $m^{-2}$ $s^{-1}$ or $\mu Em^{-2}$ $s^{-1}$.

## 4.5 Nutritional Requirements of Algae Growth

Algae are categorized as autotrophs and heterotrophs and, depending upon energy and carbon sources, are categorized as photoautotrophs, chemoauthotrophs, chemoheterotrophs, phagocytosis. Further due to mixed characteristics, they are again subdivided into photoheterotrophs, auxotrophs, and mixotrophs. The autotrophic algae depend on light for the reduction of $CO_2$ and water by the release of $O_2$. Photo-autotrophs need only inorganic minerals for their growth, whereas some organic combinations are also required for growth (e.g., vitamins). Vonshak

**Table 4.3** Various components present in microalgae as percentages of dry weight

| Name of the algae | Lipids | Proteins | Carbohydrates | References |
|---|---|---|---|---|
| *A. cylindrical* | 4–7 | 43–56 | 25–30 | Becker (2007) |
| *C. vulgaris* | 14–22 | 51–58 | 12–17 | Becker (2007) |
| *D. salina* | 6 | 57 | 32 | Becker (2007) |
| *E. gracilis* | 14–20 | 39–61 | 14–18 | Becker (2007) |
| *Arthrospira maxima* | 6–7 | 60–71 | 13–16 | Becker (2007) |
| *Scenedesmus obliquus* | 12–14 | 50–56 | 10–17 | Becker (2007) |
| *Poryphyridium cruentum* | 9–14 | 28–39 | 40–57 | Becker (2007) |
| *Spirogyra* sp. | 11–21 | 6–20 | 33–64 | Becker (2007) |
| *Spirulina platensis* | 4–9 | 46–63 | 8–14 | Becker (2007) |
| *Synechococcus* sp. | 11 | 63 | 15 | Becker (2007) |
| *Chlamydomonas reinhardtii* | 21 | 48 | 62 | Becker (2007) |
| *Chaetoceros calcitrans* | 16 | 34 | 6 | Brown (1991) |
| *Chaetoceros gracilis* | 7.2 | 12 | 4.7 | Brown (1991) |
| *Nitzschia closterium* | 13 | 26 | 9.8 | Brown (1991) |
| *P. tricornutum* | 14 | 30 | 8.4 | Brown (1991) |
| *Skeletonema costatum* | 10 | 25 | 4.6 | Brown (1991) |
| *Thalassiosira pseudonana* | 19 | 34 | 8.8 | Brown (1991) |
| *Dunaliella tertiolecta* | 15 | 20 | 12.2 | Brown (1991) |
| *N. atomus* | 21 | 30 | 23 | Brown (1991) |
| *Chlorella vulgaris* | 13 | 48 | 8 | Brown (1991) |
| *Chlorella pyrenoidosa* | 2 | 57 | 26 | Brown (1991) |
| *Chroomonas salina* | 12 | 29 | 9.1 | Brown (1991) |
| *Nannochloropsis oculata* | 18 | 36 | 7.8 | Brown (1991) |
| *Tetraselmis chui* | 17 | 31 | 12.1 | Brown (1991) |
| *Tetraselmis suecica* | 10 | 31 | 12 | Brown (1991) |
| *Isochrysis galbana* | 23 | 29 | 12.9 | Brown (1991) |
| *Pavlova lutheri* | 23 | 29 | 12.9 | Brown (1991) |
| *Pavlova salina* | 12 | 26 | 7.4 | Brown (1991) |
| *Arthrospira platensis* | 7 | 64 | 25 | Brown (1991) |
| *Arthrospira maxima* | 2 | 65 | 20 | Brown (1991) |
| *Chlamydomonas rheinhardtii* | 21 | 17 | 48 | Hossain et al. (2019) |
| *Chlorella* sp. | 19 | 22 | 56 | Hossain et al. (2019) |
| *Spirogyra* sp. | 16 | 55 | 20 | Hossain et al. (2019) |
| *Porphyridium cruentum* | 11 | 50 | 35 | Hossain et al. (2019) |
| *Spirulina platensis* | 8 | 12 | 60 | Hossain et al. (2019) |
| *Dunaliella salina* | 6 | 57 | 32 | Hossain et al. (2019) |
| *Bellerochea* sp. | 15 | 24 | 03 | Hossain et al. (2019) |
| *Chaetoceros* sp. | 18 | 18 | 02 | Hossain et al. (2019) |
| *Rhodomonas* sp. | 15 | 74 | 09 | Hossain et al. (2019) |
| *Scenedesmus* sp. | 12 | 56 | 18 | Hossain et al. (2019) |

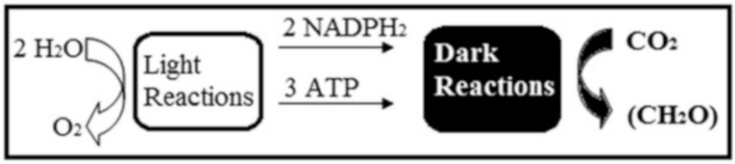


**Fig. 4.1** Photosynthesis in microalgae with both light and dark reactions

(1986) summarized the nutritional necessities for the fabrication of algal recipes, i.e., (a) the total salt contents according to the habitat from where the algae originate, (b) the main ionic contents such as $K^+$, $Mg^{2+}$, $Na^+$, $Ca^{2+}$, $SO_4^{=}$, and $Cl^-$, (c) the nitrogen source, (d) carbon source either $CO_2$ or $HCO_3^-$, (e) pH, (f) minor elements and chelating agents, and (g) vitamins. Carbon (C), nitrogen (N), and phosphorus (P) are the three main essential nutrients for autotroph growth, and their supply is essential for the effective microalgae growth. The nutrient-rich supply could effectively impacted on the algal cultivation process for optimal biomass production.

### *4.5.1 Carbon*

Carbon is an essential element as sole feedstock to form the carbon skeleton in the microalgae biomass growth and must available in the form of inorganic or organic source enriched medium. For high growth rate of microalgae, $CO_2$ or $HCO_3^-$ are very important in the medium as it is maintained the pH like as buffer system in the liquid medium that is optimal for mass cultivation. The buffering system chemically expressed as $CO_2$ – $H_2CO_3$ –$HCO_3^-$– $CO_3^{2-}$ is having greater importance in maintenance of the pH in cultivation of microalgae. On the other hand, glycylglycine and Tris(2-amino-2-[hydroxymethyl]-1–3-propanediol) are frequently preferred as buffering system. Chemically the buffering system involving photosynthesis process to produce $CO_2$ is as following:

$$2HCO_3^- \leftrightarrow CO_3^{2-} + H_2O + CO_2 \tag{4.1}$$

$$HCO_3^- \leftrightarrow CO_2 + OH^- \tag{4.2}$$

$$CO_3^{2-} + H_2O \leftrightarrow CO_2 + 2OH^- \tag{4.3}$$

The above reactions shows that $OH^-$ accumulation leads to rise in pH during photosynthetic $CO_2$ fixation. This is natural phenomenon to rise in pH upto "11" and increased density of algal growth without additional supply of $CO_2$. One can control the pH rise to "11" by additionally sparging $CO_2$ into the growth medium; it ultimately leads to highly dense algal growth. Further, as discussed above, the carbon sources provide organic acids, alcohols, and sugars for mixotrophic algal cultivation.

### 4.5.2 Nitrogen

Nitrogen is an important element and occupies >10% of dry weight of microalgae and helps in biomass production. Nitrogen could be supplied as ammonia ($NH_4^+$), urea, and nitrate ($NO_3^-$) to the growth medium in mass cultures. Ammonia is useful in pH maintenance in case of additional supply of $CO_2$, but here the mechanism is releasing of protons ($H^+$). To achieve higher yields of microalgae biomass production, adequate supply of nitrogen is needed. Few cynobacterial species are capable of reducing the atmospheric elemental $N_2$ to $NH_4^+$ by nitrogenase enzyme. In the efficient production of biofuels by microalgae, in the microalgae medium ingredients few nutrients are rationalized or limited in quantity. In this regard, nitrogen is limited supply material (nitrogen starvation) in the medium leads to accumulation of lipids reaching to higher levels in microalgal biomass. In nitrogen starvation the microalgae synthesize the TG (triglyceride) and utilized to storage of energy and carbon. These stored TG-FA have undergone transesterification process and release methyl and ethyl esters. Nitrogen scarcity can lead to the accumulation of lipids, but it compromises the cell growth (Chen et al. 2017). To avoid it in the first stage of growth, rich supply of nitrogen is needed; in the later stage, nitrogen scarcity helps to build up lipids in biomass for biofuel production as good feedstock.

### 4.5.3 Phosphorus

This is a critical element for algae growth, energy transfer, and also in the biosynthesis of nucleic acids, adenosine triphosphate, phospholipids, etc. Algal biomass contains >1% of phosphorus, and it is easily bound with other ions (iron, $CO_3^{2-}$) which leads to precipitation, resulting in unavailability of phosphorus to algal uptake. In the growth medium, the preferred form of phosphorus is orthophosphate, and the uptake is energy dependent. Adenosine triphosphate is the preliminary product of photosynthesis of algae, so phosphorus is essential for achieving higher yields of microalgae production. Phosphorus limitation or scarcity has no superior effects on microalgae growth as nitrogen scarcity (Kamalanathan et al. 2015). Therefore, phosphorus supply is sufficiently needed (Chu et al. 2013) to attain effective density of microalgae for the biofuel production at the first stage, and then nitrogen scarcity is required at the second stage.

### 4.5.4 Macro- and Micronutrients

Essential nutrients required except phosphorus, nitrogen, and carbon are 30 elements in the form of minerals of high quantity (macronutrients) and in trace amounts (micronutrients) for microalgae growth. Macronutrients are Ca, Mg, S, K, Na, Fe,

etc. and micronutrients are enlisted as Cu, Mn, B, Zn, Co, Mo, V, Se, etc. Calcium is an important element in cell wall and membrane structures and performs as intracellular messenger that harmonizes response toward environmental changes. Magnesium as part of enzymes is directly related to photosynthesis and $CO_2$ fixation. Magnesium supplementation in the culture media could increase the photosynthesis activation, thereby increases the accumulation of lipids in biomass. Esakkimuthu et al. (2016) explained in their work on the starvation of calcium and phosphorus that decrease in lipid content in microalgae and they suggested as lower amount of calcium must be provided than the starvation. In modern cultivation of algae primarily provided all nutrients richly and later stages starvation of nitrogen, little amount of calcium could be preferred to achieve the higher yields of biomass and optimized production of biofuels. Salinity of the growth medium could be maintained by the addition of salts, i.e., NaCl, KCl, $MgCl_2$, $CaCl_2$, NaCl, and KCl are used as salinity modifiers to increase the external osmotic pressure of the cell which may affect the fluidity and permeability of the membrane. $MgCl_2$ and $CaCl_2$ are not modifying the salinity of the medium efficiently than the above salts but involving in other regulations. Sajjadi et al. (2018) revealed that the when high salinity in the medium, the osmo-regulation mechanism is started in the cell to equilibrate external salinity condition. This equilibration and osmoregulation could induce fatty acid metabolism which leads to accumulation of lipids. Silicon is also an important constituent which is involved in the formation of cell wall especially in diatoms. Cobalt is essential for the synthesis of vitamin $B_{12}$, and other minor nutrients are very important as they are cofactors with the enzymes and involved in the metabolism. Metal chelators (EDTA) are required to avoid phosphorus precipitation in the medium and avoid the unavailability of phosphorus which is essential for cellular structures and division of microalgal cells. So, the macro and micro inorganic nutrients are most necessary for microalgae-augmented biomass harvest and biofuel production. Several microalgae media available (55 types) in the literature are depicted in the Tables 4.4, 4.5, 4.6, 4.7, 4.8, and 4.9 along with their ingredients, quantities and compared with conventional medium. However, here the components, quantities, and suitability of the medium for microalgae cultivation are considered by diversity of algae and target of the product to be achieved.

### *4.5.5 Other Considerations*

Along with the above conditions, many other parameters will influence the effective cultivation of microalgae. They are (1) temperature, (2) light, (3) pH, (4) $CO_2$, (5) salinity, (6) $O_2$, (7) mineral bioavailability, (8) carbon bioavailability and cellular characteristics, (9) growth inhibition, (10) cell density, and (11) cell fragility. Reactor characteristics affecting the microalgae growth are (1) rate of mixing, (2) gas bubble size, (3) gas bubble distribution, (4) fluid dynamics, (5) gas exchange capacity, (6) mass transfer, (7) dilution rate, (8) light intensity, (9) culture depth, and (10) maintenance of dark spots. Further, the cultural growth characteristics also

**Table 4.4** Various culture media and their compositions (g $L^{-1}$ or otherwise stated)

| Name of the component | Name of the culture medium | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Zarrouck's | BG 11 | AW (g/kg) | MN | ASN III | Modified Allen's | Bristol's | Modified Zarrouck's | Bold's Basal BBM |
| $NaNO_3$ | 2.5 | 1.5 | 1.667 | 0.75 | 0.75 | 1.5 | 0.125 | – | 0.25 |
| $KNO_3$ | – | – | – | – | – | – | – | 2.5 | – |
| $K_2HPO_4 \cdot 3H_2O$ | 0.5 | 0.04 | – | 0.02 | 0.02 | 0.039 | 0.0375 | 0.5 | 0.075 |
| $KH_2PO_4$ | – | – | – | – | – | – | 0.0875 | – | 0.175 |
| $MgSO_4 \cdot 7H_2O$ | 0.2 | 0.075 | – | 0.038 | 3.5 | 0.075 | 0.0375 | 0.2 | 0.075 |
| $MgCl_2 \cdot 4H_2O$ | – | – | 9.395 | – | 2.0 | – | – | – | – |
| $CaCl_2 \cdot 2H_2O$ | 0.04 | 0.036 | 1.316 | 0.018 | 0.5 | 0.025 | 0.0125 | 0.04 | 0.084 |
| $Ca(NO_3)_2 \cdot 4H_2O$ | – | – | – | – | – | 0.02 | – | – | – |
| $SrCl_2 \cdot 6H_2O$ | – | – | 0.0214 | – | – | – | – | – | – |
| $Na_2SiO_3 \cdot 9H_2O$ | – | – | 3.000 | – | – | 0.02 | – | – | – |
| Citric acid | – | 0.006 | – | 0.003 | 0.003 | 0.006 | – | – | – |
| Fe-ammonium citrate | – | 0.006 | – | 0.003 | 0.003 | – | – | – | – |
| $FeCl_3$ | – | – | 0.016 | – | – | 0.002 | 0.001 | – | – |
| $FeSO_4 \cdot 7H_2O$ | 0.01 | – | – | – | – | – | – | 0.01 | 0.00498 |
| EDTA, 2Na–Mg salt | 0.08 | 0.001 | – | 0.0005 | 0.0005 | 0.001 | 0.025 | 0.08 | 0.05 |
| $NaHCO_3$ | 16.8 | – | 0.170 | – | – | – | – | 16.8 | – |
| $Na_2CO_3$ | – | 0.02 | – | 0.01 | 0.02 | 0.02 | – | – | – |
| NaCl | 1.0 | – | 20.758 | – | 25.0 | – | 0.0125 | 1.0 | 0.025 |
| KCl | – | – | 0.587 | – | 0.5 | – | – | – | – |
| $K_2SO_4$ | 1.0 | – | – | – | – | – | – | 1.0 | – |
| $Na_2SO_4$ | – | – | 3.477 | – | – | – | – | – | – |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| NaI | – | – | 0.0027 | – | – | – | – | – | – |
| KOH | – | – | – | – | – | – | 0.0155 | – | 0.031 |
| KBr | – | – | 0.0845 | – | – | – | – | – | – |
| $H_3BO_4$ (μg $L^{-1}$) | 2.86 | 2.84 | 0.0225 | 2.86 | 2.86 | 2.86 | 0.00571 | – | 11.42 |
| $MnCl_2 \cdot 4H_2O$ (μg $L^{-1}$) | 1.81 | 1.81 | – | 1.81 | 1.81 | 1.81 | 0.00072 | – | 1.44 |
| $MnSO_4 \cdot 4H_2O$ | 0.222 | – | 0.054 | – | – | 0.222 | – | – | 8.82 |
| $ZnSO_4 \cdot 7H_2O$ (μg $L^{-1}$) | – | 0.222 | 0.0073 | 0.222 | 0.222 | – | 0.00442 | – | – |
| $Na_2MoO_4 \cdot 2H_2O$ (μg$L^{-1}$) | – | 0.391 | – | 0.390 | 0.390 | 0.391 | – | – | – |
| Vitamins | – | – | Thiamin, Vit. $B_{12}$ | – | – | – | – | – | – |
| Sodium glyceryl $PO_4$ | – | 0.667 | – | – | – | – | – | – | – |
| $CuSO_4 \cdot 5H_2O$ (μg $L^{-1}$) | 0.08 | 0.079 | – | 0.079 | 0.079 | 0.079 | 0.000079 | – | 1.57 |
| $Co(NO_3)_2 \cdot 6H_2O$ (μg$L^{-1}$) | – | 0.0494 | – | 0.0494 | 0.0494 | 0.0494 | 0.000025 | – | 0.49 |
| $CoSO_4 \cdot 7H_2O$ | – | – | 0.0016 | – | – | – | – | – | – |
| $MoO_3$ (μg $L^{-1}$) | 0.01 | – | – | – | – | – | 0.000036 | – | 0.71 |
| $H_2SO_4$ | – | – | – | – | – | – | 0.001 | – | – |
| Adjust final pH | – | 7.4 | – | 8.3 | 7.5 | 7.8 | – | 6.5 | – |
| References | Grobbelaar (2004) | Grobbelaar (2004) | Paul et al. (1980) | Rippka et al. (1979) | Rippka et al. (1979) | Grobbelaar (2004) | Bold (1942) | Pai et al. (2008) | Grobbelaar (2004) |

**Table 4.5** Various culture media and their compositions (g $L^{-1}$ or otherwise stated)

| Name of the component | Name of the culture medium | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Sorokin/ Krauss | George's | Amara and Steinbüchel | Wu's medium | Modified Chu's (μg/L) | Kessler and Cygan | MBM | M 11 | Ben-Amotz and Avron |
| $NaNO_3$ | – | – | 1.5 | – | – | – | – | 0.1 | – |
| $KNO_3$ | 1.25 | 0.20 | – | – | 400 | 81 | – | – | 0.505 |
| $K_2HPO_4 \cdot 3H_2O$ | – | 0.02 | 0.5 | 0.3 | 80 | 47 | – | 0.01 | 0.014 |
| $KH_2PO_4$ | 1.25 | – | – | 0.7 | – | 36 | 1.25 | – | – |
| $MgSO_4 \cdot 7H_2O$ | 1.0 | 0.02 | 0.2 | 0.3 | 200 | 25 | 1 | 0.75 | 1.2 |
| $MgCl_2 \cdot 4H_2O$ | – | – | – | – | – | – | – | – | 0.1 |
| $CaCl_2 \cdot 2H_2O$ | 0.04 | – | 0.012 | – | 107 | 0.15 | 0.111 | 0.40 | 0.033 |
| Citric acid | – | – | – | – | 100 | – | – | – | – |
| Fe-ammonium citrate | – | 0.035 | 0.018 | – | 20 | – | – | – | – |
| $FeSO_4 \cdot 2H_2O$ | 0.05 | – | 0.01 | 0.003 | – | 0.06 | 0.0498 | 0.01 | – |
| EDTA, 2Na–Mg salt | 0.5 | – | 0.08 | – | – | 0.08 | 0.5 | 0.01 | – |
| $NaHCO_3$ | – | – | 9.214 | – | – | – | – | – | 1.7 |
| $Na_2CO_3$ | – | – | 7.143 | – | – | – | – | 0.30 | – |
| NaCl | – | – | 1 | – | – | 47 | – | – | 117 |
| $K_2SO_4$ | – | – | 0.571 | – | – | – | – | – | – |
| Tris–HCl | – | – | – | – | – | – | – | – | 6 |
| $H_3BO_3$ (μg $L^{-1}$) | 114 | – | – | – | 5.72 | 0.005 | 0.1142 | – | 6 |
| $MnCl_2 \cdot 4H_2O$ (μg $L^{-1}$) | 14 | – | – | – | 3.62 | 0.005 | 0.0142 | – | – |
| $MnSO_4 \cdot H_2O$ (μg $L^{-1}$) | – | – | – | – | – | – | – | – | – |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| $ZnSO_4 \cdot 7H_2O$ (μg $L^{-1}$) | 88 | – | – | – | 0.44 | 0.002 | 0.0882 | – | – |
| $ZnCl_2$ | – | – | – | – | – | – | – | – | 14 |
| $Na_2MoO_4 \cdot 2H_2O$ (μg $L^{-1}$) | – | – | – | – | 0.084 | 0.002 | – | – | – |
| $CuSO_4 \cdot 5H_2O$ (μg $L^{-1}$1) | 16 | – | – | – | 0.16 | – | 0.0157 | – | – |
| $Co(NO_3)_2 \cdot 6H_2O$ (μg $L^{-1}$) | 5 | – | – | – | – | – | 0.0049 | – | – |
| $CoCl_2 \cdot 6H_2O$ (μg $L^{-1}$) | – | – | – | – | 0.02 | – | – | – | 4.8 |
| Vitamin $B_1$ | | – | – | 0.01 | – | – | – | – | – |
| $MoO_3$ (μg $L^{-1}$) | 7 | – | – | – | – | – | – | – | – |
| Peptone | – | 1 | 0.1 | – | – | – | – | – | – |
| Glycine | | – | – | 0.1 | – | – | – | – | – |
| Yeast extract | – | – | 0.01 | 4 | – | – | 6 | – | – |
| Glucose (mg $L^{-1}$) | – | – | – | – | – | – | 10 | – | – |
| Glycerol | – | – | – | 10 | – | – | – | – | – |
| Adjust final pH | 6.8 | – | – | – | 7.5 | 6.5 | – | 8.0 | 7.5 |
| References | Grobbelaar (2004) | Atlas and Parks (1997) | Amro and Steinbüchel (2013) | Xiong et al. (2008) | Yamaguchi et al. (1987) | Kessler and Czygan (1970) | Xiong et al. (2008) | Yagi et al. (1979) | Grobbelaar (2004) |

**Table 4.6** Various culture media and their compositions (g $L^{-1}$ or otherwise stated)

| Name of the component | Name of the culture medium | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Walne | Guillard | "F" medium | Fresh water WC | Half strength Chu's (μg/L) | TAP | Algal Assay Procedure (AAP) (mg/L) | Diatom medium (mg/L) | SOT |
| $NaNO_3$ | 100 | 84.2 | 75 | 0.08501 | – | – | 25.5 | – | 2.5 |
| $K_2HPO_4 \cdot 3H_2O$ | – | – | – | 0.00871 | 0.005 | 0.108 | 1.04 | – | 0.5 |
| $Na_2HPO_4 \cdot 3H_2O$ | 20 | 10 | 5 | – | – | – | – | – | – |
| $KH_2PO_4$ | – | – | – | – | – | 0.056 | – | 12.4 | – |
| $MgSO_4 \cdot 7H_2O$ | – | – | – | 0.03697 | 0.07 | 0.1 | 14.7 | 25 | 0.2 |
| $MgCl_2 \cdot 4H_2O$ | – | – | – | – | – | 0.05 | 12.6 | – | – |
| $CaCl_2 \cdot 2H_2O$ | – | – | – | 0.03676 | – | 0.05 | 4.41 | – | 0.004 |
| $Ca(NO_3)_2 \cdot 4H_2O$ | – | – | – | – | – | – | – | 20 | – |
| $Na_2SiO_3 \cdot 9H_2O$ | 30 | 50 | 30 | 0.02842 | – | – | – | 57 | – |
| $FeCl_3 \cdot 6H_2O$ | 0.8 | 3.5 | 3.15 | 0.00315 | 0.001 | – | 1.6 | – | – |
| $FeSO_4 \cdot 2H_2O$ | – | 2.9 | – | – | – | 4.99 | – | 2.25 | 0.001 |
| EDTA, 2Na–Mg salt | 45 | – | 4.36 | 0.00436 | – | 50 | 3 | 2.25 | 0.008 |
| $NaHCO_3$ | – | – | – | 0.0126 | – | – | – | 15.9 | 16.8 |
| NaCl | – | – | – | – | 0.04 | – | – | – | 1 |
| KOH | – | – | – | – | – | 16 | – | – | – |
| $K_2SiO_3$ | – | – | – | – | 0.02 | – | – | – | – |
| $NH_4Cl$ | – | – | – | – | – | 0.4 | – | – | – |
| $(NH_4)_2SO_4$ | – | – | – | – | 0.025 | – | – | – | – |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Tris–HCl | – | – | – | 0.0005 | – | 2.42 | – | – | – |
| $H_3BO_3$ ($\mu g\ L^{-1}$) | 33.6 | – | – | 0.001 | 2.48 | 11.4 | 1.9 | 2.48 | 0.286 |
| $MnCl_2 \cdot 4H_2O$ ($\mu g\ L^{-1}$) | 0.4 | 0.36 | 0.180 | 0.00018 | – | 5.06 | 42 | 1.39 | 0.250 |
| $MnSO_4 \cdot H_2O$ ($\mu g\ L^{-1}$) | – | – | – | – | 1.47 | – | – | – | – |
| $ZnSO_4 \cdot 7H_2O$ ($\mu g\ L^{-1}$) | – | – | 0.022 | 0.000022 | 0.23 | 22 | – | – | 0.022 |
| $ZnCl_2$ | 2.1 | – | – | – | – | – | 0.327 | – | – |
| $Na_2MoO_4 \cdot 2H_2O$ ($\mu g\ L^{-1}$) | – | – | 0.0063 | 0.000006 | – | – | 0.726 | – | 0.0021 |
| $(NH_4)_6 MoO_7O_{24}$ ($\mu g\ L^{-1}$) | 0.9 | 1.26 | – | – | 0.07 | 1.10 | – | 1.0 | – |
| $CuSO_4 \cdot 5H_2O$ ($\mu g\ L^{-1}1$) | 2 | 1.96 | 0.0098 | 0.00001 | 0.1 | 1.57 | – | – | 0.0079 |
| $Co(NO_3)_2 \cdot 6H_2O$ ($\mu g\ L^{-1}$) | – | – | – | – | 0.14 | – | – | – | – |
| $CoCl_2 \cdot 6H_2O$ ($\mu g\ L^{-1}$) | 2 | 2 | 0.010 | 0.00001 | – | 1.61 | 0.143 | – | – |
| Hcl (mL) | 10 | – | – | – | – | – | – | – | – |
| Glacial acetic acid (mL) | – | – | – | – | – | 1 | – | – | – |
| Thiamine HCl | 20 | 0.2 | 0.002 | 0.0001 | 50 | – | – | 0.04 | – |
| Biotin | 0.1 | 0.01 | 0.01 | 0.0005 | 2.5 | – | – | 0.04 | – |
| Vitamin $B_{12}$ | 0.1 | 0.01 | 0.01 | 0.0005 | 2.5 | – | – | 0.04 | – |

(continued)

**Table 4.6** (continued)

| Name of the component | Name of the culture medium | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Walne | Guillard | "F" medium | Fresh water WC | Half strength Chu's (μg/L) | TAP | Algal Assay Procedure (AAP) (mg/L) | Diatom medium (mg/L) | SOT |
| Vitamin $B_1$ | 0.2 | – | – | – | – | – | – | – | – |
| Glycylglycine | – | – | – | 0.0005 | – | – | – | – | – |
| Glucose (mg $L^{-1}$) | – | – | – | – | 50 | – | – | – | – |
| Adjust final pH | – | – | – | 7–8 | – | – | – | 6.9 | – |
| References | Vivi et al. (2012) | Vivi et al. (2012) | Guillard and Ryther (1962) | Guillard and Lorenzen 1972 | Chu (1942) | Harris (1989) | ASTM (2012) | Barsanti and Gualtieri (2006) | Ogawa and Terui (1970) |

**Table 4.7** Various culture media and their compositions (g $L^{-1}$ or otherwise stated)

| Name of the component | Name of the culture medium | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | M4 (mg/L) | M7 (mg/L) | Combo (mg/L) | MNK (μg/L) | "K" medium (μg/L) | ESM medium (μg/L) | LO medium | MDM (mg/L) | Carefoot (mg/L) | F/2 (mg/L) |
| $NaNO_3$ | 0.0274 | 0.274 | 17.0 | 20 | 75 | 120 | 20 | 1000 | 247 | 75 |
| $K_2HPO_4 \cdot 3H_2O$ | 0.0184 | 0.184 | 1.742 | 1 | – | 5 | 1 | – | 90 | 60 |
| $Na_2HPO_4 \cdot 3H_2O$ | – | – | – | 0.28 | – | – | 0.28 | – | – | – |
| $KH_2PO_4$ | 0.0143 | 0.143 | – | – | – | – | – | 250 | 23 | – |
| $MgSO_4 \cdot 7H_2O$ | 123.3 | 123.3 | 36.97 | – | – | – | – | 250 | – | – |
| $CaCl_2 \cdot 2H_2O$ | 293.8 | 293.8 | 36.76 | – | – | – | – | 100 | 11 | – |
| $Na_2SiO_3 \cdot 9H_2O$ | 1 | 1 | 10 | – | – | – | – | – | – | 10 |
| $Na_2VO_4$ | – | – | 0.0018 | – | – | – | – | – | – | – |
| $Na_2Se\ O_3$ | 0.0022 | 0.0022 | – | 0.03 | 1.6 | – | 0.05 | – | – | – |
| $H_2\ Se\ O_3$ | 0.0016 | – | – | – | – | – | – | – | – | – |
| $FeCl_3 \cdot 6H_2O$ | 1 | 1 | 1 | – | – | – | – | – | 196 | 3.16 |
| $FeSO_4 \cdot 7H_2O$ | 0.996 | 0.249 | – | – | – | – | – | 200 | – | – |
| EDTA, 2Na–Mg salt | 2.5 | 0.625 | 4.36 | 3.7 | 37,224 | – | 2.5 | – | 1000 | 4.4 |
| Fe-EDTA | – | – | – | 26 | 4927 | 259 | 30 | – | – | – |
| Mn-EDTA | – | – | – | 33 | – | 332 | 15 | – | – | – |
| $NaHCO_3$ | 64.8 | 64.8 | 64.8 | – | – | – | – | – | – | – |
| NaBr | 0.016 | 0.004 | – | – | – | – | – | – | – | – |
| KCl | 5.8 | 5.8 | 5.96 | – | – | – | – | 100 | 15 | – |
| LiCl | 0.31 | 0.077 | 0.31 | – | – | – | – | – | – | – |
| RbCl | 0.07 | 0.018 | 0.07 | – | – | – | – | – | – | – |
| $SrCl_2 \cdot 6H_2O$ | 0.15 | 0.038 | 0.15 | – | – | – | – | – | – | – |
| KI | 0.0033 | 0.0033 | 0.0033 | – | – | – | – | – | – | – |

(continued)

**Table 4.7** (continued)

| Name of the component | Name of the culture medium | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | M4 (mg/L) | M7 (mg/L) | Combo (mg/L) | MNK (µg/L) | "K" medium (µg/L) | ESM medium (µg/L) | LO medium | MDM (mg/L) | Carefoot (mg/L) | F/2 (mg/L) |
| $H_3BO_3$ (µg $L^{-1}$) | 2.86 | 0.715 | 24 | – | – | – | – | 286 | – | – |
| $MnCl_2 \cdot 4H_2O$ (µg $L^{-1}$) | – | 0.090 | 0.18 | 9 | 178 | – | 15 | – | 36 | 0.18 |
| $MnSO_4 \cdot H_2O$ (µg $L^{-1}$) | – | – | – | – | – | – | – | 250 | 47 | – |
| $ZnSO_4 \cdot 7H_2O$ (µg $L^{-1}$) | – | – | 0.022 | 2.4 | 23 | – | 4 | 22.2 | 22 | 0.21 |
| $ZnCl_2$ | 0.013 | 0.013 | – | – | – | – | – | – | – | – |
| $Na_2MoO_4 \cdot 2H_2O$ (µg $L^{-1}$) | – | 0.016 | 0.022 | 0.7 | 7.3 | – | 1.5 | 2.1 | 2.5 | 0.07 |
| $Na_2$ Glycerophosphate | – | – | – | – | 2.2 | – | – | – | – | – |
| $(NH_4)VO_3$ | 0.0006 | 0.0006 | – | – | – | – | – | – | – | – |
| $CuSO_4 \cdot 5H_2O$ (µg $L^{-1}$) | – | – | 0.001 | 0.06 | 2.5 | – | 0.06 | 7.9 | – | 0.07 |
| Cu $Cl_2 \cdot 2H_2O$ | 0.0165 | 0.0042 | – | – | – | – | – | – | – | – |
| $CoSO_4 \cdot 7H_2O$ | – | – | – | 1.2 | 14 | – | 2 | – | – | 0.112 |
| $CoCl_2 \cdot 6H_2O$ (µg $L^{-1}$) | 0.010 | 0.010 | 0.010 | – | – | – | – | – | 4 | – |
| Thiamine HCl | 0.075 | 0.075 | 0.1 | 20 | 101 | 100 | 40 | – | 20 (µg) | 100 (µg) |
| Biotin | 0.0008 | 0.0008 | 0.0005 | 0.15 | 0.5 | 1 | 0.3 | – | 0.2 (µg) | 5 (µg) |
| Vitamin $B_{12}$ | 0.001 | 0.001 | 0.00055 | 0.15 | 0.5 | 1 | 0.3 | – | 0.2 (µg) | 5 (µg) |
| Adjust final pH | – | – | – | – | – | – | – | 8.0 | 7.5 | – |
| References | Samel et al. (1999) | Samel et al. (1999) | Samel et al. (1999) | Noël et al. (2004) | Keller et al. (1987) | Watanabe et al. (2000) | Noël et al. (2004) | Watanabe et al. (2000) | Carefoot (1968) | Guillard and Ryther (1962) |

**Table 4.8** Various culture media and their compositions (g $L^{-1}$ or otherwise stated)

| Name of the component | Name of the culture medium | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | AF-6 (mg/L) | CA (mg/L | C (mg/L) | HUT (mg/L) | D (mg/L) | MA (mg/L) | MW (mg/L) | P35 (mg/L) | URO (mg/L) | VT (mg/L) |
| $NaNO_3$ | 140 | 100 | – | – | 689 | 50 | 1.7 | – | – | – |
| Urea | – | – | – | – | – | – | 8.5 | – | – | – |
| $KNO_3$ | – | – | 100 | – | 103 | 100 | 10 | – | – | – |
| $KHCO_3$ | – | – | – | – | – | – | 9 | – | – | – |
| $NH_4NO_3$ | 22 | 50 | – | – | – | – | – | 100 | 5 | – |
| $NH_4Cl$ | – | – | – | – | – | – | 0.42 | – | – | – |
| $K_2HPO_4 \cdot 3H_2O$ | 5 | – | – | – | – | – | – | – | – | – |
| $KH_2PO_4$ | 10 | – | – | 20 | – | – | – | – | – | – |
| $Na_2HPO_4 \cdot 3H_2O$ | – | – | – | – | 111 | – | – | – | – | – |
| $MgSO_4 \cdot 7H_2O$ | 30 | – | – | 25 | 100 | – | – | 40 | 10 | 40 |
| $MgCl_2 \cdot 6H_2O$ | – | – | – | – | – | 50 | – | – | – | – |
| $CaCl_2 \cdot 2H_2O$ | 10 | – | – | – | – | – | 14 | 74 | 10 | |
| $CaCO_3$ | 10 | – | – | – | – | – | – | – | – | – |
| $Ca(NO_3)_2 \cdot 4H_2O$ | – | 20 | 150 | – | – | 50 | 100 | – | – | 117.8 |
| $CaSO_4$ | – | – | – | – | 60 | – | – | – | – | – |
| $Na_2\ SO_4$ | – | – | – | – | – | 40 | – | – | – | – |
| $FeCl_3 \cdot 6H_2O$ | – | 196 | 196 | – | 0.5 | 0.5 | 196 | 196 | 196 | 196 |
| Ferric citrate | 2 | – | – | – | – | – | – | – | – | – |
| Potassium citrate | – | – | – | 40 | – | – | – | – | – | – |
| $Na_2$ Acetate | – | – | – | 400 | – | – | – | 1000 | – | – |
| Citric acid | 2 | – | – | – | – | – | – | | – | – |
| EDTA, 2Na–Mg salt | – | 1000 | 1000 | – | – | 5 | 1000 | 1000 | 1000 | 1000 |
| Fe-EDTA | – | 1 | – | – | – | – | – | – | 0.5 | – |

(continued)

**Table 4.8** (continued)

| Name of the component | Name of the culture medium | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | AF-6 (mg/L) | CA (mg/L | C (mg/L) | HUT (mg/L) | D (mg/L) | MA (mg/L) | MW (mg/L) | P35 (mg/L) | URO (mg/L) | VT (mg/L) |
| HEPES | – | 400 | – | – | – | – | – | – | – | – |
| NaCl | – | – | – | – | 8 | – | – | – | 1 | – |
| KCl | – | – | – | – | – | – | – | 50 | – | 50 |
| NTA | – | – | – | – | 100 | – | – | – | – | – |
| KI | – | – | – | – | – | 20 | – | – | – | – |
| $H_3BO_3$ (mg $L^{-1}$) | 2860 | – | – | – | 500 | – | – | – | – | – |
| $MnCl_2 \cdot 4H_2O$ (μg $L^{-1}$) | – | 36 | 36 | – | – | 5 | 36 | 36 | 36 | 36 |
| $MnSO_4 \cdot H_2O$ (μg $L^{-1}$) | 2500 | 20 | 40 | – | 2280 | 15 | – | – | – | – |
| $ZnSO_4 \cdot 7H_2O$ (μg $L^{-1}$) | 222 | 22 | 22 | – | 500 | | 22 | 22 | 22 | 22 |
| $ZnCl_2$ | – | – | – | – | – | 0.5 | – | – | – | – |
| $Na_2MoO_4 \cdot 2H_2O$ (μg $L^{-1}$) | 21 | 2.5 | 2.5 | – | 25 | 0.8 | 2.5 | 2.5 | 2.5 | 2.5 |
| β-$Na_2$ glycerophosphate | – | 30 | 50 | – | 100 | 20 | – | 50 | 4 | 50 |
| $CuSO_4 \cdot 5H_2O$ (μg $L^{-1}$) | 79 | – | – | – | 25 | – | – | – | – | – |
| Cu $Cl_2 \cdot 2H_2O$ | – | – | – | – | 45 | – | – | – | – | – |
| $CoCl_2 \cdot 6H_2O$ (μg $L^{-1}$) | – | 4 | 4 | – | – | 5 | 4 | 4 | 4 | 4 |
| Polypeptone | – | – | – | 600 | – | – | – | – | – | – |
| Yeast extract | – | – | – | 400 | – | – | – | – | – | – |
| Glycylglycine | – | – | – | – | – | – | 100 | – | – | 500 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Bicine | – | – | – | – | – | 500 | – | – | – | – |
| Thiamine HCl ($\mu g\ L^{-1}$) | 10 | 10 | 10 | 0.4 | – | – | 20 | 10 | 10 | 10 |
| Biotin ($\mu g\ L^{-1}$) | 2 | 0.1 | 0.1 | – | – | – | 0.2 | 0.1 | 0.1 | 0.1 |
| Vitamin $B_{12}$ ($\mu g\ L^{-1}$) | 1 | 0.1 | 0.1 | 0.5 | – | – | 0.2 | 0.1 | 0.1 | 0.1 |
| Vitamin $B_6$ ($\mu g\ L^{-1}$) | 1 | – | – | – | – | – | – | – | – | – |
| Tris (aminomethane) (mg) | – | – | 500 | – | – | – | – | 500 | – | – |
| Adjust final pH | 6.6 | – | 7.5 | 6.4 | – | 8.6 | 7.2 | 8.0 | 7.5 | 7.5 |
| References | Kato 1982 | Ichimura and Watanabe (1974) | Ichimura (1979) | Hutner et al. (1966) | Castenholz 1969 | Ichimura (1979) | Sako et al. (1984) | Ichimura (1979) | Kimura and Ishida (1985) | Robertson et al. (2001) |

**Table 4.9** Various culture media and their compositions (g $L^{-1}$ or otherwise stated)

| Name of the component | Name of the culture medium | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | W (mg/L) | M-ASP7 (mg/L) | STP (mg/L) | ASN (g/L) | PCR-S11 (mg/L) | Johnson's (g/L) | ESAW (g/L) | Allen (mg/L) | DY-III (mg/L) |
| $NaNO_3$ | – | 50 | 200 | 0.75 | – | – | 46.7 mg | – | 20 |
| Urea | 17 | – | – | – | – | – | – | – | – |
| $KNO_3$ | 10 | – | – | – | – | 1 | – | – | – |
| $NaHCO_3$ | – | – | – | – | – | 0.043 | 0.174 | – | – |
| $NH_4SO_4$ | – | – | – | – | 2.68 | – | – | 132 | |
| $NH_4Cl$ | – | – | – | – | – | – | – | – | 2.68 |
| $K_2HPO_4 \cdot 3H_2O$ | – | – | 10 | 0.02 | – | – | – | – | – |
| $KH_2PO_4$ | – | – | – | – | – | 0.035 | – | 27.2 | – |
| $NaH_2PO_4 \cdot 2H_2O$ | – | 20 | – | – | – | – | 3.09 mg | – | – |
| $Na_2HPO_4 \cdot 3H_2O$ | – | – | – | – | 14 | – | – | – | – |
| $MgSO_4 \cdot 7H_2O$ | 15 | 9000 | – | 3.5 | – | 0.5 | – | 24.6 | 50 |
| $MgCl_2 \cdot 6H_2O$ | – | – | – | 2 | – | 1.5 | 9.592 | | |
| $CaCl_2 \cdot 2H_2O$ | – | 300 | – | 0.5 | – | 0.2 | 1.344 | 7.4 | 75 |
| $Ca(NO_3)_2 \cdot 4H_2O$ | 100 | – | – | – | – | – | – | – | – |
| $Na_2SO_4$ | – | – | – | – | – | – | 3.55 | – | – |
| $VSO_5 \cdot 5H_2O$ | – | – | – | – | 0.098 | – | – | – | – |
| $Na_2WO_4 \cdot 2H_2O$ | – | – | – | – | 0.198 | – | – | – | 2 |
| $Na_2CO_3$ | – | – | – | 0.02 | – | – | – | – | – |
| $Na_2SiO_3 \cdot 9H_2O$ | – | – | – | – | – | – | 30 mg | – | 14 |
| $Na_2SeO_3$ | – | – | – | – | – | – | 0.173 | – | 2 |
| $FeCl_3 \cdot 6H_2O$ | 196 | 63 | – | – | – | 2.44 | 1.77 | – | 1 |
| $NH_4$ ferric citrate | – | – | – | 0.003 | – | – | – | – | – |
| $NH_4VO_3$ | – | – | – | | – | – | – | 23 | – |
| Citric acid | – | – | – | 0.003 | – | – | – | – | – |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| EDTA, 2Na–Mg salt | 1000 | 1000 | – | 0.0005 | 8 | 1.89 | 3.09 | | 8 |
| Fe-EDTA | – | – | – | – | – | – | – | 30.16 g | – |
| HEPES | – | – | – | – | 200 | – | – | – | – |
| NaCl | – | 25 | – | 25 | – | – | 21.19 | – | – |
| KCl | – | 700 | – | 0.5 | – | 0.2 | 0.599 | – | 3 |
| NTA | – | 70 | – | – | – | – | – | – | – |
| KBr | – | – | – | – | 0.714 | – | 0.0863 | – | – |
| NaF | – | – | – | – | – | – | 0.0028 | – | – |
| KI | – | – | – | – | 0.498 | – | – | – | – |
| $SrCl_2$ | – | – | – | – | – | – | 0.0218 | – | – |
| $H_3BO_3$ (mg $L^{-1}$) | – | 1130 | – | 2.860 | 18.549 | 0.61 | 0.023 | 2.86 g | 0.8 |
| $MnCl_2 \cdot 4H_2O$ (μg $L^{-1}$) | 36 | 32 | – | – | – | 0.41 | – | 1.79 g | 200 |
| $MnSO_4 \cdot H_2O$ (μg $L^{-1}$) | – | – | – | 1.81 | 10.140 | – | 0.54 | – | – |
| $ZnSO_4 \cdot 7H_2O$ (μg $L^{-1}$) | – | 46.6 | – | 0.222 | 1.725 | | 0.073 | 220 | 40 |
| $ZnCl_2$ | – | – | – | – | – | 0.041 | – | – | – |
| $Na_2MoO_4 \cdot 2H_2O$ (μg $L^{-1}$) | 2.5 | – | – | 0.390 | – | – | 1.48 mg | – | 20 |
| $(NH_4)_2Mo_7O_{24} \cdot 4H_2O$ | – | – | – | – | 0.494 | 0.38 | – | 130 | – |
| β-$Na_2$ glycerophosphate | 20 | – | – | – | – | – | – | – | – |
| $CuSO_4 \cdot 5H_2O$ (μg $L^{-1}$) | – | – | – | 0.079 | 0.749 | – | – | 79 | – |
| $Cu\ Cl_2 \cdot 2H_2O$ | – | – | – | – | – | 0.041 | – | – | – |
| $CoSO_4 \cdot 7H_2O$ | – | 0.93 | – | – | – | – | 0.016 | – | – |

(continued)

**Table 4.9** (continued)

| Name of the component | Name of the culture medium | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | W (mg/L) | M-ASP7 (mg/L) | STP (mg/L) | ASN (g/L) | PCR-S11 (mg/L) | Johnson's (g/L) | ESAW (g/L) | Allen (mg/L) | DY-III (mg/L) |
| $CoCl_2 \cdot 6H_2O$ ($\mu g\ L^{-1}$) | 4 | – | – | – | – | 0.015 | – | | 8 |
| $Co(NO_3)_2 \cdot 6H_2O$ | – | – | – | 0.0494 | 0.873 | – | – | – | – |
| $Cd(NO_3)_2 \cdot 6H_2O$ | – | – | – | – | 0.925 | – | – | – | – |
| $NiCl_2 \cdot 6H_2O$ | – | – | – | – | 0.713 | – | 1.49 mg | – | – |
| $Cr(NO_3)_3 \cdot 9H_2O$ | – | – | – | – | 0.240 | – | – | – | – |
| $SeO_2$ | – | – | – | – | 0.333 | – | – | – | – |
| $VOCl_2$ | – | – | – | – | | 0.041 | – | – | – |
| $KAl(SO_4)_2 \cdot 12H_2O$ | – | – | – | – | 2.846 | – | – | – | – |
| Sodium glutamate | – | – | 500 | – | – | – | – | – | – |
| Glucose | – | – | 200 | – | – | – | – | – | – |
| Glycine | – | – | 100 | – | – | – | – | – | – |
| D,L-Alanine | – | – | 100 | – | – | – | – | – | – |
| Tryptase | – | – | 200 | – | – | – | – | – | – |
| Yeast autolysate | – | – | 200 | – | – | – | – | – | – |
| Sucrose | – | – | 1000 | – | – | – | – | – | – |
| Soil extract | – | – | 50 | – | – | – | – | – | – |
| Glycylglycine | 100 | – | – | – | – | – | – | – | – |
| Thiamine $B_1$ ($\mu g\ L^{-1}$) | 2 | 50 | 50 | – | – | – | 100 mg | – | 100 mg |
| Biotin $B_7$ ($\mu g\ L^{-1}$) | 0.2 | 0.1 | 0.1 | – | – | – | 1 mg | – | 0.5 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Vitamin $B_{12}$ ($\mu g\ L^{-1}$) | 0.2 | 1 | 1 | – | 10 | – | 2 mg | – | 0.5 |
| Nicotinic acid | – | 10 | 10 | – | – | – | – | – | – |
| Calcium pantothenate | – | 10 | 10 | – | – | – | – | – | – |
| *p*-Aminobenzoic acid | – | 1 | 1 | – | – | – | – | – | – |
| Inocitol | – | 500 | 500 | – | – | – | – | – | – |
| Folic acid | – | 0.2 | 0.2 | – | – | – | – | – | – |
| Thymine | – | 300 | 300 | – | – | – | – | – | – |
| Tris (aminomethane) (mg) | – | 1000 | | – | – | 2.45 | – | – | – |
| Adjust final pH | 7.5 | 8.0 | 7.5 | 7.5 | 7.5 | 7.5 | 8.2 | | 6.5 |
| References | Watanabe (1983) | Watanabe (1981) | Provasoli et al. (1957) | Laura and Paolo (2014) | Laura and Paolo (2014) | Laura and Paolo (2014) | Laura and Paolo (2014) | Allen (1959) | Laura and Paolo (2014) |

influence the growth of microalgae, i.e., (1) toxic chemicals to microalgae, (2) pathogens to microalgae, (3) competitive growth of other algae, (4) management of photo inhibition, (5) management of photorespiration, and (6) yield frequency of microalgae.

In the designing of the microalgae medium, the nutritional ingredients and their quantities are consider for the enhanced yields of specific metabolites. In few instances, it is essential to alter the quantities and amounts of one or more nourishments (Shin et al. 2018). So, one must know the definite features of each and every group of algae, their physiology, nourishment prerequisites, growth circumstances, and specific considerations to alter the composition of medium (Milano et al. 2016). Cuellar García et al. (2019) evaluated various culture mediums on two microalgae *S. obliquus* and *C. vulgaris* for biodiesel productivity. They used Bristol, Bold, and AAP (modified algal assay procedure) culture conditions and studied about which one is the best in favor of biomass growth for lipid accretion. The results have been revealed that the usage of Bold medium and AAP medium for *S. obliquus* and *C. vulgaris* respectively, showed higher production of biomass containing more lipids when compared with other media. BG 11 medium is suggested for freshwater and soil Cyanophyceae, diatom medium is recommended for freshwater *Bacillariophyceae* algae, and DY-III medium is the best for freshwater *Chrysophyceae* group of algae. Bold Basal Medium (BBM) is used broadly for the freshwater *Chlorophyceae, Xanthophyceae, Chrysophyceae*, *and Cyanophyceae*. AF-6 medium is useful for algae cultivation where slight acidic condition is required, i.e., Euglenophyceae, xanthophytes, dinoflagellate, green ciliate, volvocalean algae, and many cryptophytes. AK medium will be used for the cultivation of almost all varieties of marine algae. Both "F" medium and "G" medium are useful for marine algae cultivation in salt as well as brackish waters. Both "K" medium and "LO" medium are useful for marine microalgae, especially oligotrophic salt and brackish waters. Combo medium is used broadly for the cultivation of cryptophytes, cyanobacteria, diatoms, and green algae, whereas MNK medium is the best suitable for especially marine microalgae, *coccolithophores*.

Sueoka's medium is especially used for the cultivation of *Chlorophyceae* family algae. In the similar manner, MES and Jaworski's media are used broadly for pure water microalgae cultivation. Walne's culture medium is broadly used for marine microalgae principally set in commercial culturing. ASN-III, PCR-S11, and Chu-II media are most preferably recommended for the Cyanobacterial strain cultivation. *Botryococcus braunii* is a colonial microalgae cultivated in modified Chu 13 medium for the production of lipids (Yamaguchi et al. 1987). F/2 medium and ESAW medium are used for the broad spectrum for oligotrophic marine algae and coastal open ocean algae cultivation. CY-II, Zarrouk's, and Johnson's media were used to cultivate *Cyanophora paradoxa*, *Arthrospira* sp., and *Dunaliella* sp. (halophilic algae), respectively. *Chlorella vulgaris* is studied with Chu medium, and it is found that hydrocarbons (HC), free fatty acids, sterol esters (SE), acetone mobile polar lipids, aliphatic alcohols (ALC), and phospholipids (PL) yield are more. Debjani et al. (2012) experimented on different media, i.e., dry-grind ethanol thin stillage (TS), soy whey (SW), and modified basal medium (MBM) as media for the

cultivation of *C. vulgaris*. After 4 days of incubation, the biomass yield was 9.8, 6.3, and 8.0 g $L^{-1}$ and the oil content was 43, 11, and 27% (w/w) in the media TS, SW, and MBM, respectively. The quality of fatty acid profile is also tested as linoleic and linolenic forms, the oil is richer and good quality when grown in TS and MBM media. Xiong et al. (2008) reported on the cultivation of *C. protothecoides* to achieve more dense biomass in the production of biodiesel using Wu's culture medium successfully.

As reported by Richmond and Becker (1986), the higher yields of algal biomass could be achieved when (a) providing nutrition-rich medium, (b) positive environmental parameters, and (c) turbulent flow mixing. The turbulent flow mixing enhances the diffusion rate of $CO_2$, exchange rate of nutrients in betwixt the algal cells, and fluid medium; further augmented light/dark periods lead to efficient photosynthesis and productivity (Grobbelaar 1994). Microalgae is small in size and available in liquid medium as suspension offers direct contact with nutrients present in medium. Upon the sparging of air due to higher surface area available on algae, $CO_2$ fixation and also solar energy transmission rate are high. The main advantage of microalgae cultivation is that the maximum annual biomass productivity is more, due to nutrients, solar light, adsorption of $CO_2$, and high surface area availability on algae. High surface area allows the easy exchange of gases, nutrients, and solar energy which leads to faster growth than other sources of biofuels.

## 4.6 Bioreactors for Microalgae Cultivation

Since microalgae could grow well at immense biodiversity, possibility to use waste water as nutritional medium, can sequester $CO_2$; producce $O_2$ and can produce high cell density are great features so that leads to much attention by scientists and considered as microalgae as feedstock for biofuels production. Accurate fabrication, design of reactors, and optimization of process by biotechnology of microalgae facilitate the achievement of augmented cell density mass cultures. The overall knowledge is required to scale up the operational parameters, i.e. (1) light energy, (2) mass transfer, (3) shear forces, and (4) rate of mixing. The above-mentioned variables are strongly interconnected and decide the yields and effectiveness of a particular reactor. Further, the augmentation in algae yield entails better microalgae culture and amplified $CO_2$ capture. In the scale-up process to industrialization, maintenance of controlled conditions in open ponds is the major issue (Fig. 4.2). To maintain the controlled conditions, closed photobioreactors are considered as alternative system against open ponds. Photobioreactor system has several advantages than open pond system except capital and operation costs.

Capital cost may be required for the manufacturing, installation, and supporting accessory systems. The operational cost due to the $CO_2$ addition, $O_2$ removal, maintenance and cleaning, etc. will increase the costs. The harvesting cost is low when compared with the open ponds, which compensates the initial costs. The other important factors, i.e., biomass quality, biomass concentration, production

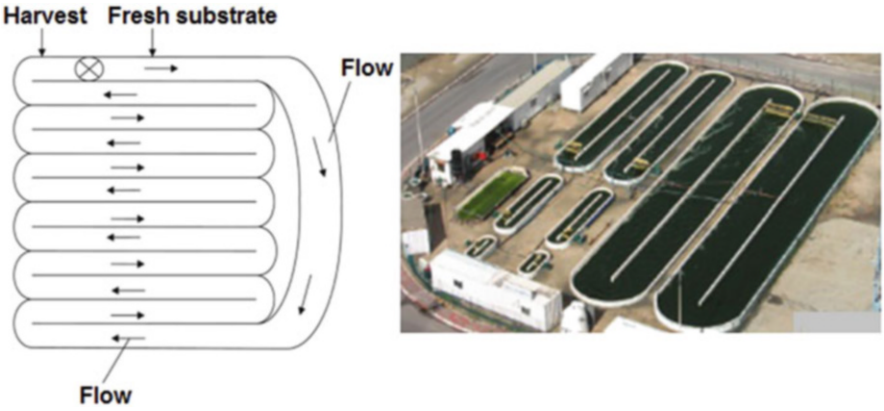


**Fig. 4.2** Schematic diagram of raceway pond for the cultivation of microalgae. (Adapted from Seambiotic 2009)

flexibility, process controls, and reproducibility are more preferred in the PBRs than the open ponds. In case of open ponds, the requirement is huge space, water, and $CO_2$ loss and the contamination risk is also more.

### *4.6.1 Closed Reactor Designing for Microalgae Cultivation*

Generally, the reactor is in cylindrical shape and fits with several accessories for proving specific temperature, pH, and mixing devices. Algae cultivation needs light source as energy which can be provided by a light source or sunlight for photosynthesis. Since algae cultivation is through a light source, these reactors are calling as photobioreactors (PBR). PBRs offer a protection (Fig. 4.3) from the contaminated species (bacteria, fungi, etc.) and make available closed environments to maintain desirable temperature, pH, mixing, etc. and ensure the specific algal strain cultivation. The below-mentioned variables are essential features that must be considered in the design of closed reactor system: (1) light source and orientation, (2) nutrition by culture medium, (3) water, (4) algae circulation (mixing), (5) pH, (6) temperature control, (7) $CO_2$ feed, (8) $O_2$ removal, (9) materials of construction, and (10) reactor maintenance. The parameters remains the same but there may be changes in their intensities as per the array of higher and lesser points for specific strain cultivation management.

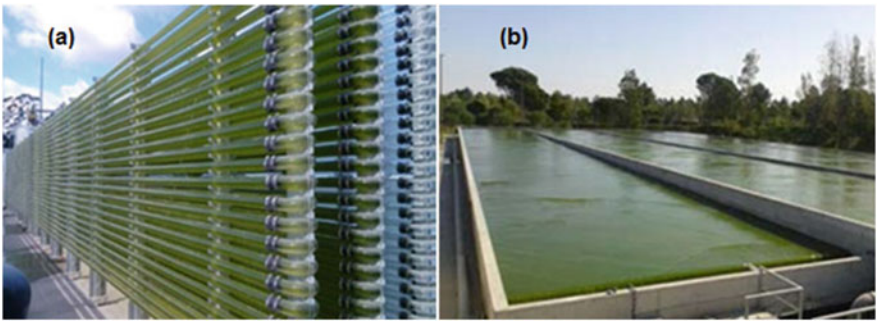


**Fig. 4.3** Tubular photobioreactor (**a**), open pond (**b**) for microalgae cultivation. (Adapted from Li et al. 2008)

### *4.6.2 Classification of Photobioreactors (PBRs)*

Photobioreactors are classified into various types depending upon the design of reactors, i.e., (a) tubular or flat, (b) horizontal, (c) inclined, (d) vertical or spiral, (e) manifold or serpentine, (f) hybrid, (g) floating, and (h) biofilm reactors.

Depending on the mode of operation, photobioreactors are categorized as (1) single-phase reactors and (2) air-mixed reactors. In single-phase reactors, gas exchange takes place, whereas in air-mixed reactors, both liquid and gas mass transfer occurs.

Based on the construction materials, again PBRs are classified into (a) glass or plastic and (b) rigid or flexible photobioreactors. Other than these, axenic PBRs are used for specific cultures and intended for sterilization before the operation of reactor.

#### 4.6.2.1 Light

Light is an essential energy for photosynthesis; light must be radiated inside the reactor to avoid the dark zones (less-intensity light at some places in the reactor) or illuminate the fluorescent lights close to the outer surface of the reactor. When the light penetrates with high intensity and reaches to the cells with good frequency in a reactor then it is considered as an optimal reactor. Spatial dilution of illumination is a significant feature which reduces the mutual shading of cells in the culture medium. This may lead to higher growth rate, but the product component inside the cell is less. One can consider how to exploit the volume to surface area ratio and geometry of reactor for efficient light distribution in the designing process of PBR (Fig. 4.4).

The light gradient directly influences the productivity of cultured algae and is determined by the reactor dimensions and algal cell density. The cell density formation is a microalgae strain specific property, and it is essential to manage optimal conditions for the light penetration and light intensity to reach each and every cell in the reactor. The critical cell density is an operating parameter, which is

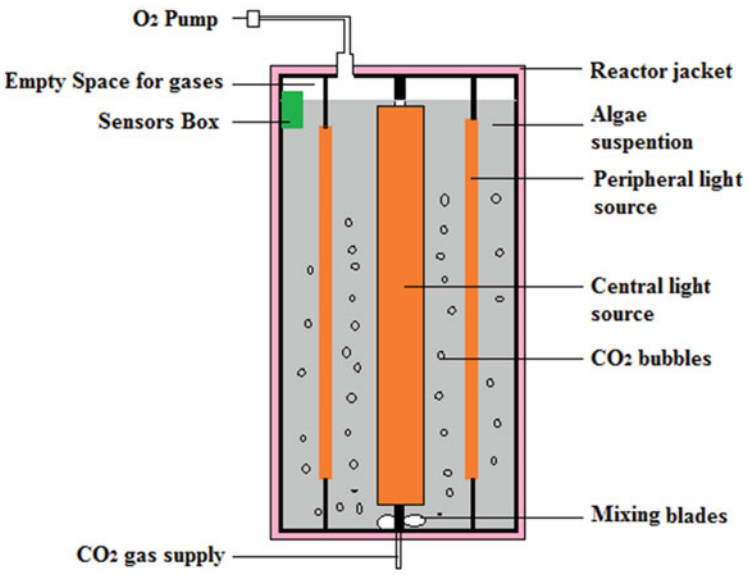


**Fig. 4.4** The typical photobioreactor for microalgae production

nothing but the greatest cell mass without reciprocated shadows and one can take into consideration this point while designing the PBR. Naturally light/dark periods also influence the efficacy of photosynthesis of the microalgae. Janssen et al. (2001) explained the impact of illumination on photosynthetic effectiveness of *D. tertiolecta* and on short irradiance or dimness compared with continuous light. Photon flux of the illumination tenure and time of liquid duration in various radiance regions are two key parameters to attain the optimal dark period. The light regime of PBR is influenced by the extent of light/dark periods and illumination strength, and the light regime directly responsible for physiological responses of cells is called as photoacclimation. Zu and Richmond (2000) reported that the sudden flash of light is fatal for various algal species. Based on the photosynthetic efficiency and other data, the theoretical maximum fuel yield per area can be determined. LEDs are more prominently used as light source in algal cultivation, due to low cost, and can also use it as flash light to pulse the light whenever it is required to improve the productivity.

#### 4.6.2.2 Mixing

Mixing is the critical parameter which enhances the rate of recurrence of the illumination and murk cycles and leads to improve the productivity and cellular contents. It provides the effective mass transfer from nutrients to the cell in the presence of light. In the designing of the bioreactor, effective mixing will be considered for effective availability of nutrients and light to the cell by avoiding

the diffusion barriers. Ugwu et al. (2008) scale out tubular reactors by setting up of static mixers with augmented tube diameter and confirmed that the enhanced light exploitation and microalgae mass improved yields. For optimal cell growth, the reactor maintains the capability to sustain the mass transfer coefficient ($k_L$a), it build upon the category of bubbler, agitation rate, type of surfactants and also on temperature. Static mixers installation could reduce the bubble size which increases the surface area and leads to increase the contact area in between liquid and gas. Whereas fine spargers produce low liquid flow rate, and big bubble formation leads to low or poor mass transfer. Eriksen et al. (1998) explained a setting up of dual orifice with membrane-perforated sparger in a closed reactor, which resulted in efficient air and $CO_2$ supply separately and attained proper mixing. They achieved fivefolds supply of $CO_2$ to liquid phase as compared to conventional spargers. In the total cost of the reactor, usually the highest percentage is occupied by energy consumed and the mixing mechanism. In bubble column reactors, the sparger working efficiently mixing and formed small size bubbles thereby efficient $CO_2$ mass transfer takes place.

The specialty of this type of reactor with no moving parts is that it provides elevated diffusion area volume ratio, excellent mass and heat transmittance, excellent release of $O_2$, and also small capital cost. This was scaled up by perforated plates that are set inside the reactor, resulting in break-up of coalesced particles and are redistributed. An airlift reactor is also one type of bubble column reactors; it has internal draft tube which enhances the mixing and mass transfer coefficient. In Fig. 4.5, the left side designated three zones: air-riser up, bubble down-comer, and disentanglement zones in air lift reactor.

Air bubbles are sprinkled by sparger through the bottom of the reactor, maybe inside or outside of the draft tube. The air bubbles go up (rise) and lead to fluid flow vertically, due to the opposite action against this, and the fluid flow will be downwards causing down flow. Upon the continuous supply of air bubbles, there is an effective movement that leads to improved mixing. Further, this movement facilitates in uniform distribution in one direction with same velocity, thereby no or less coalescence, and no fusion of bubbles ensuing greater k$L_a$ (the volumetric mass transfer coefficient) than bubble-column reactors. The above positive parameters result in greater mass yields in airlift reactors than other stirred reactors. Several researchers are examined on improved mixing and mass transfer in flat-panel reactors (Wondraczek et al. 2013; Hoekman et al. 2012; Posten and Schaub 2009; Tredici and Materassi 1992).

#### 4.6.2.3 Water Consumption

Water utilization is an important parameter as it is precious along with reactor options. Algae are able to grow in various water sources, i.e., fresh drinking water, brackish water (saline), and also in waste water (Wogan et al. 2008) effluents. Based upon the type of water (pure or saline), strains of microalgae are classically divided into two categories: freshwater algae and salt water algae. The overall productivity

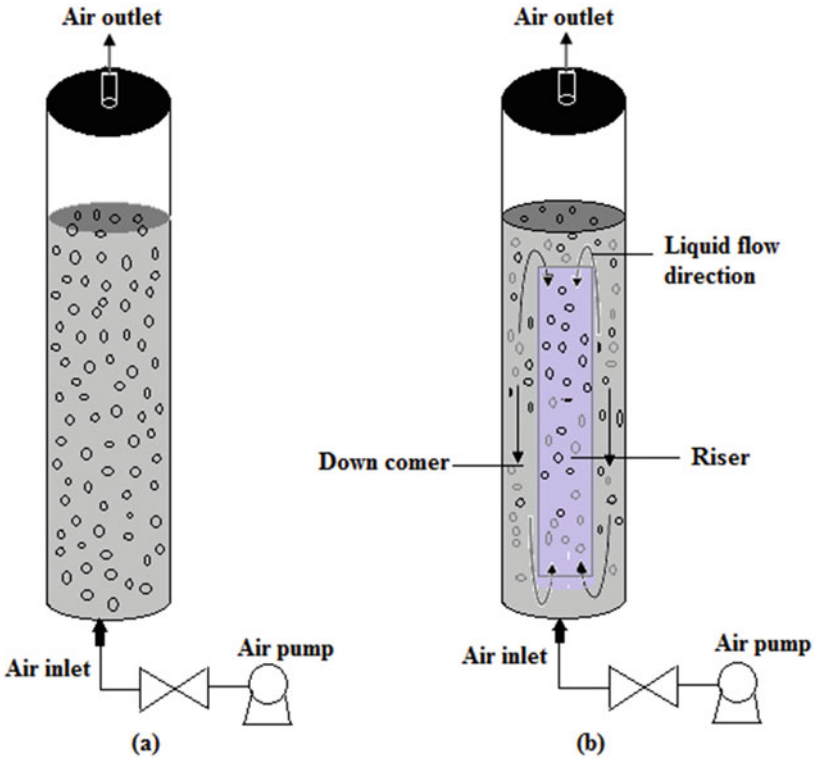


**Fig. 4.5** Description of the bubble column PBR (**a**) and airlift PBR (**b**)

and individual content (lipids, carbohydrates, etc.) productivity in each strain are influenced by the level of salinity. Abu-Rezq et al. (1999) reported on the saline conditions of high-quality marine algae for the production, i.e., *Nannochloropsis* salinity range was 20–40, *Tetraselmis* 20–35, and *Isochrysis* 25–35 (Rao et al. 2007).

Waste water having rich nutrients (nitrogen and phosphorus) with high concentrations may be treated further when used as medium for algae cultivation. In open ponds, due to evaporation of water, high concentration of nutrients will be formed; high concentrations of these nutrients causing for growing of unwanted algae and may be undergone eutrification. If it occurs in the salt water ponds, where the salinity of the pond reaches the extreme and above-tolerance levels. In PBRs, by minimizing the evaporation/loss of water and minimizing these extreme concentrations of nutrients in waste water, the unwanted algae growth can be avoided, and these nutrients can be utilize efficiently for the particular strain of algae growth (Aslan and Kapdan 2006). One of the huge costs-saving approaches in algae cultivation is usage of waste water as medium. Chinnasamy et al. (2010) used corporate industry waste water as medium for the cultivation of algae in vertical tank reactors, polybags, and also raceway ponds for mass cultivation of algal consortium. They

attained huge proteins and lesser carbohydrates in algal consortium. Yang (2011) reported the same as above results that the overall biomass productivity in poybags is more than the vertical tank reactor and raceway. Attractively, with diverse positioning, the polybag reactor can attain the highest yields in commercial systems (large scale) and diminish running costs.

#### 4.6.2.4 $CO_2$ Consumption

Carbon is essentially required for photosynthesis, i.e., organic and inorganic carbon ($CO_2$), among them inorganic carbon is the best in commercial aspect. For photosynthesis of algae, sufficient $CO_2$ is essential along with light, nutrients, water, and high partial pressures ($pCO_2$). Several studies revealed that excess partial pressure of $CO_2$ is detrimental for microalgae. Lee and Tay (1991) reported that exposure of $CO_2$ with high partial pressures on *Chlorella pyrenoidosa* leads to declining the rate of growth. They suggested that the supply of $CO_2$ is through a gas permeable membrane to provide controlled release for the entire culture and prevent high partial pressures of $CO_2$. Even though the laboratories routinely aerate 5–15% of $CO_2$, the available concentration from 1 to 5% is often enough for maximum growth (Suh and Lee 2003). Flue gas is the best for carbon source as it reduces the greenhouse gas emissions and also low cost. Thermal power stations are good sources for exhaust gas which exhibit up to 13% of carbon dioxide, and it is of lower cost compared to pure $CO_2$ supply. In the photobioreactor, infrared analyzer is used to measure the $CO_2$ concentration in the gas phase through which the flow of flue gas is regulated. Schenk et al. (2008) reported that $pCO_2$ is 0.15 kPa is the minimum requirement to avoid the limitation for $CO_2$ kinetics, and the required $CO_2$/g dry biomass is 1.7–1.8 g (Chisti 2007). Doucha et al. (2005) explained that even flue gas contains CO and NOx along with $CO_2$; however they may not interfere in the photosynthetic efficiencies even with low mass transfer of $CO_2$, and interestingly the productivity was similar to pure $CO_2$ supply. For further decreasing the cost of $CO_2$ in biofuel production by microalgae, Carvalho et al. (2006) suggested an option that is the use of hollow fiber membranes for interrupted supply of $CO_2$ and enhanced mass transfer.

#### 4.6.2.5 $O_2$ Removal

In algal photosynthesis, oxygen is delivered as byproduct, the maximum allowable is as 10 g $O_2$ $m^3$ $min^{-1}$ in a typical tubular reactor. Likewise, in a photobioreactor, the dissolved oxygen when excess than the minimum allowable levels leads to photo-oxidative damage in the presence of intensive irradiation ultimately causing to the drastically reduced productivity. In general, $O_2$ concentration should be 400% of air saturation. In case of open ponds, oxygen is evolved from the medium easily, but in photobioreactors, the oxygen pressure is built inside, so periodically oxygen should be pumped out of the reactor. In tubular reactors, it is very difficult to do it, which

limits the scale-up process. Molina et al. (2011) designed the airlift zone by algae culture frequently returned in the tubular reactor where oxygen was stripped by air. In the airlift zone, gas–liquid separator prevents recirculation air bubbles to horizontal loop. The fluid travel time through the extent of the degasser should be the same as oxygen bubbling rising time.

#### 4.6.2.6 Nutrient Supply

Nitrogen and phosphorus are the main nourishments to manage the growth rate and yield of lipid in algae. The essential other nutrients required for microalgae are carbon, oxygen, hydrogen, sulfur, chlorine, sodium, calcium, potassium, and magnesium. The micronutrients are iron, silicon, cobalt, nickel, copper, manganese, molybdenum, boron, vanadium, and selenium. In N-8 medium (common medium) containing deficient quantities of Mg, S, Fe and N which results huge cell mass. Even in separate supplementation of these elements not showed better yields, but the four elements reasonable boosting reported enhanced performance. Chisti (2007) explained the need for the excess nutrients like phosphorus which reacts with metal ions. Applying stress condition by providing limited nutrients can enhance the lipid percentages with biomass. But the stress application may limit the rate of growth and decrease the overall lipid production. Rodolfi et al. (2009) illustrated three types of nutrient supply: (1) nutrient-sufficient, (2) nutrient-limited, and (3) nutrient-deficient. They applied the above three condition on few strains, among them *Nannochloropsis* showed exceptional results, lipid production was enhanced, when applied deficient scenario (N-deficient environment) after grown formerly with nutrient sufficient medium. Xinxin et al. (2019) studied the consequences of N-limitation on fatty acids and lipid quantity on *P. tricornutum*, *Isochrysis galbana*, *Rhodomonas baltica*, and *N. oceanica*, and higher yields are achieved. Various media are tabulated and their compositions with quantities are discussed in detailed in the cultivation section.

#### 4.6.2.7 Temperature

Temperature is one of the critical parameters which play a vital role for large-scale culturing of microalgae. Because the daily variations may be seen upon the presence of illumination and at dark cycles, which leads to the reduction in biomass production and algal lipid capacity. Microalgae showed decline in cell volume with an enhanced temperature (upon extra illumination), and the maximum growth occurred at the temperature range was noted as 20–30 °C. Venkata Subhash et al. (2014) revealed that several algae species tolerate the temperatures up till 15 °C lesser than their optimistic point, with lower growing rate. But the temperature is fewer degrees

more than the tolerable point causing to death of microalgae as it is an environmental parameter (Huang et al. 2010).

Zhu et al. (2013) critically explained that temperature choice may vary from species to species. For example, *Scenedesmus* and *Chlorella* were adapted in the temperature range of 5–35 °C; the best range of temperature is 25–30 °C. Kurpan et al. (2015) experimented on *Isochrysis galbana*, *N. oceanica*, and *P. tricornutum* for biodiesel and PUFAs. *Isochrysis galbana* and *Phaecodactylum tricornutum* yielded high TAG at 20 °C and 30 °C, respectively, and *Nannochloropsis oceanic* yield was noted as very less. They stated that, if an ideal temperature was not maintained during the course of cultivation, the biochemical pathways are changed, which leads to improper build-up of lipids. Singh and Singh (2015) reported an optimum temperature of 20–30 °C for the algae, i.e. *Nannochloropsis*, *Chlorella*, *Scenedesmus*, *Botryococcus*, *Spirogyra*, *Neochloris*, *Chlamydomonas*, *Haematococcus*, and *Ulva* species. The studies in the literature report that the optimum growth temperature depends on algal species and geographical origin, where an optimum growth can be attained (Goldman and Carpenter 1974). When the tolerable temperatures exceeds, the response of microalgae against temperature variation could affect (1) nutritional necessities, (2) nature of metabolism and rate, and (3) cell composition (Richmond 1999). Torzillo et al. (1991b) examined the temperature consequences on *S. platensis* at outdoor environments at different temperatures, i.e., 25 and 35 °C (from May to September). They reported that there is 14% increase in biomass productivity at 35 °C, (1) when the temperature decreases in the period and the average biomass productivity is decreased, and (2) during night, there is a loss of biomass significantly at 25 °C (Torzillo et al. 1991a).

According to geographical regions, microalgae cultivation in outdoors (open ponds and race ponds) may be exposed to extreme temperatures. Even though microalgae competent grows at various temperatures, favorable growth is restricted with a slight range of temperature in particular to each strain. Abu-Rezq et al. (1999) reported the best range of temperatures for *Isochrysis*, *Nannochloropsis*, and *Tetraselmis* were 24–26 °C, 19–21 °C, and 19–21 °C, respectively. Daily and seasonal temperature fluctuations also obstruct the algae growth. In the absence of controlling units in PBRs can result high temperatures, then evaporate cooling method is frequently adapted to reduce that much magnitude from required temperatures. Further at low temperatures especially at night times owing respiration could loss the biomass. So, maintenance of temperature is required for attaining optimum growth in large scale PBRs and in open ponds.

#### 4.6.2.8 pH

pH is an important parameter that influences the microalgae metabolism in lipid production. It influences the fluid chemistry like accessibility of nourishments,

organic acids and $CO_2$. Further in marine environment react with water forms ($H_2CO_3$) carbonic acid, readily dissociate into bicarbonate ($HCO_3^-$) and again detached into carbonate ion ($CO_3^-$) and protons ($H^+$) leads to decrease pH ultimately damage the algae growth (Bautista-Chamizo et al. 2018). Bicarbonate ($HCO_3^-$) is utilized by microalgae through active transport leads to catalytic conversion of cation exchange and formed as $CO_2$ and $OH^-$ (Seyed et al. 2018). Bicarbonate ($HCO_3^-$) is utilized by microalgae through active transport, catalytic conversion of cation exchange forms of $CO_2$ and $OH^-$ (Seyed et al. 2018). Each and every algal strain has a lesser pH array, so commercial pH regulators must be preferred in reactors to attain the maximum growth. Xinxin et al. (2019) studied the consequences of N-limitation on profile of fatty acids and lipid quantity on *P. tricornutum*, *Isochrysis galbana*, *Rhodomonas baltica*, and *N. oceanica* and maintained the optimal pH as 8.5. Sharma et al. (2018) studied on *Lyngbya confervoides*, *Chroococcus turgidus*, *Nostoc commune*, *Chlorella* sp., *Skeletonema costatum*, and *Chaetoceros calcitrans* and reported that the suitable pH is the neutral pH. They also stated that the best pH for *N. oculata* and *Chlorella sorokiniana* was noted as 8.5 and 8, respectively. For lipid production by *Scenedesmus obliquus,* it is noted as neutral pH (Breuer et al. (2013), for *chlorella sorokiniana* optimum pH was 6 (Qiu et al. 2017). Liao et al. (2018) stated that lower pH leads to decreased growth rate, and this effect is reversible and at high intensity. Ramanna et al. (2017) explained about pH as it is specific to strain, set the pH prior to illumination flux to keep away from photo-inhibition which causes irreversible damage to essential proteins.

### *4.6.3 Other Considerations*

When designing a photobioreactor configuration, it is essential to judge the whole process of the production (Table 4.10). Our aim is the highly dense biomass production, in the biomass lipid content and oil downstream processing with good quality. The downstream processing and reactor design integration are very important that impact on the product excellence and cost. Chisti (2008) recommended that the genetic engineering may improve microalgae biomass yield and lipid quantity in cellular level and may have the supreme enhancement in the economics of biofuels. Genetic engineering could improve fuel production in different ways, i.e., upgrading in photosynthesis, enhanced biomass yield, augmented lipid quantity, and better temperature tolerance of algae. In addition, genetic engineering could increase tolerable levels of algal cells to light saturation, photo-inhibition, and photo-oxidation. Geography is important for area selection, assessing the viability of biofuel production, as few regions of the globe are best than other places. In USA, southwest locations are good; Texas is well suitable for large-scale production.

**Table 4.10** Various reactor configurations for microalgae with biomass productivity

| Reactor type | Microalgae species | $CO_2$ feed gas (%) | [a]Specific growth rate (/h) or [b]Biomass productivity ($g/m^3/h$) | References |
|---|---|---|---|---|
| Open pond reactors | *N. saline* | 5 | 1.25[b] | Matsumoto et al. (1995) |
| | *Chlorella* sp. | 6.8 | – | Doucha et al. (2005) |
| | *Nannochloropsis saline* | 15 | 4.1[b] | Doucha et al. (2005) |
| Batch reactors | *Chlamydomonas reinhardtii* | 30[b] | 0.06 ± 0.01[a] | Yang and Gao (2003) |
| | *Spirulina platensis* | 0.03 | 0.0082 ± 0.002[a] | Kumar et al. (2009) |
| | *Chlorella pyrenoidosa* | 100[b] | 0.09 ± 0.09[a] | Yang and Gao (2003) |
| | *Scenedesmus obliquus* | 60[b] | 0.06 ± 0.04[a] | Yang and Gao (2003) |
| | *Chlorogloeopsis* sp. | 5 | 0.0007 ± 0.0060[a] | Kodama et al. (1993) |
| Bioreactors | *Porphyridium* sp. | 2–3 | – | Nakamura (2004) |
| | *Euglena gracilis* | 11 | 4.8[b] | Chae et al. (2006) |
| | *Chlorella vulgaris* | 1 | – | Fan et al. (2007) |
| Membrane reactors | *Nannochloropsis* sp. | 1 | 4.2–5.8[b] | Carvalho and Malcata (2001) |
| | *Spirulina platensis* | 2–15 | 3–17.8[b] | Kumar et al. (2009) |
| | *Chlorella vulgaris* | 1 | 4[b] | Ferreira et al. (1998) |
| | *Chlorella vulgaris* | 1 | – | Fan et al. (2007) |
| | *Chlorella vulgaris* | 0.045 | – | Fan et al. (2008) |

[a]Specific growth rate is cell density improvement upon the time (hour)
[b]Biomass productivity is biomass weight in reactor per hour. If the growth rate or biomass productivity is increased the lipid content also will be increased. That is the reason researchers are measuring the growth rate from the reactor by taking sampling

## 4.7 Conclusions

This chapter discusses the various essential elements required for the cultivation of the microalgae. The basics of the microalgae classification, components present, and cultivation requirements have been explained. A review on the components (from carbohydrates to bioactive compounds) along with the compositions, cultivation, media, and various media used for the cultivation of microalgae with metabolic

pathways are consolidated in the tables and figures. In this chapter, around 55 media compositions are tabulated and their specialties and applications for the microalgae as feedstock are explained.

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# Chapter 5
# Microalgae as an Efficient Feedstock Biomass for Biofuel Production


**Kishore Kumar Kadimpati, Sujatha Sanneboina, Narasimha Golla, Sridevi Ayla, Ramesh Kumpati, and Wojciech Skarka**

**Abstract** Solar, biomass, and wind are the main renewable energy resources to fulfill the need of modern society. Biofuels include bio-diesel, bio-methane, bio-ethanol, bio-methanol, bio-ethers, and bio-hydrogen. The nonfood feedstocks such as agricultural wastes, municipal wastes, microalgae, and other microbial sources are most suitable to produce biofuels. Microalgae cultivation for biofuel production can utilize the wastewater as substrate, reduces the greenhouse effect (sequestration of $CO_2$), and also releases $O_2$. By utilizing this technology, one can produce bio-ethanol, bio-methanol, biodiesel, and bio-hydrogen along with oxygen release. Microalgae contemplated as substrates for the generation of bio-diesel together with other sources of biomass, such as lignin-cellulose materials, organic wastes that are characterized by high yielding potential, are not utilized as a source of human food. Various steps involved in the bioprocessing of the valuable products and downstream processing techniques along with their merits and demerits have been revealed in this chapter.

**Keywords** Microalgae · Lipid productivity · Photobioreactors · Circular economy

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## 5.1 Introduction

The world population was 7.7 billion in 2019, according to the United Nations Population statistics (United Nations 2019), and it may reach 8.5 and 9.7 billion by 2030 and 2050, respectively. With the population increment, the fossil fuel consumption will also increase, and the fossil fuels are exhausting worldwide. The present fossil fuel reserves are soon becoming exhausted and not sufficient for the increasing demand. Air pollution, greenhouse effect, global warming, and exaggerated fuel price have led to the emergent search for renewable energy. There is an emergent need for the investigation of an alternative to fossil fuels to fulfill the societal need of energy and fuels. Solar, biomass, and wind are the main renewable energy resources in this regard. Among them, biomass is the best option as feedstock for sustainable production of biofuels. Various biofuels include bio-diesel, bio-methane, bio-ethanol, bio-methanol, bio-ethers, and bio-hydrogen. Additionally, first-generation fuels are obtained from plant products such as soybean, wheat, maize, and sweet sorghum and animal and human food. Further, they have negative impact on food and water industry since they are part of animal and human feed. In this point of view, the present chapter suggests the use of nonedible parts of plants like Jatropha, grass, silver grass, etc. as second-generation feedstock for the production of biofuels. This plant cultivation occupies an excessive land area, huge water consumption for their growth, and harmful pesticides usage.

The nonfood feedstocks such as agricultural wastes, municipal wastes, microalgae, and other microbial sources are most suitable to produce biofuels. Among them, microalgae as third-generation biofuel has much more attention due to involving waste water as nutrients and carbon dioxide sequestration capacity. Several countries have been mobilizing their scientists and rising research grants for biofuel research and development of Biofuel projects. In this context, several biofuel funded projects have been implemented by the USA, Australia, and the European Union. The USA funded four projects in various areas on biofuels, i.e., Florida (2013), Massachusetts (2011), Mexico (2009), and Arizona (2008). Whereas, in the European Union, four research project funds were released; among them, three commenced between 2011 and 2016 and the fourth one was implemented in 2012–2017 (European Biofuels Technology Platform 2016). The world leaders of biofuel production and consumption are the USA, Brazil, Sweden, France, and Germany. Biofuel production from microalgae not only produces the fuels but reduces the greenhouse effect, utilizes the waste water as nutrients, and further produces oxygen. This technology can produce bio-ethanol, bio-methanol, biodiesel and bio-hydrogen along with oxygen. Microalgae are promising biomass/material for the production of all the above-mentioned biofuels efficiently, cost-effective, and with higher yields. Microalgae can be grown in waste water and ocean water without negative impact on fresh water assets (Yang et al. 2011); algae fuels are environmental friendly even if spilled into the environment as they are biodegradable (Energy-Arizona 2007). The cultivation of microalgae, nutritional requirements, and usage of photobioreactors are discussed in the previous chapter. In this chapter,

the pathways of biofuels from the lipid productivity, biomass productivity, production of lipids, harvesting, extraction, and purification process have been explained with the focus of transforming to fuels. Several works are also exemplified for the explanation of the several parameters from the literature.

## 5.2 Biofuels from Microalgae Biomass

Microalgae can produce the precursors for biofuels generation, i.e., biodiesel, hydrogen, ethanol, and methane which are rapidly biodegradable and perform more efficiently than the fossil fuels. Microalgae have the perspective to generate elective beginning for energy as biofuels. Microalgae biochemical constituents and metabolism influence the production of biofuels, i.e., *Porphyridium cruentum* has dry weight of 40–57% w/w of carbohydrates, *Schizochytrium* species have 50–77% w/w of lipids, and *Spirulina maxima* has 60–71% w/w of proteins. Several species of green algae, i.e., *Botryococcus braunii* and *Chlorella protothecoides*, contain high amounts of terpenoid hydrocarbons and glyceryl lipid, etc. which can be converted into major crude oil. Several algae act as a potential source for the triterpenic hydrocarbons, isobutanol, isobutyraldehyde, and bioethanol production (Fig. 5.1). Hydrocarbons from microalgae can be transformed into gasoline, kerosene, and diesel. Biogas is chiefly made of $CH_3$ (65–75%) and $CO_2$ (25–35%). Practically, anaerobic digestion progresses by (1) the formation of monosaccharides through hydrolysis by hydrolytic bacteria, (2) through fermentation organic acids are formed from monosaccharides, (3) by the action of acetogenic bacteria acetate are formed, and (4) methanogenic bacteria will acts on acetate for the formation of methane and carbon dioxide.

Table 5.1 summarizes the various types of microalgae and their uses for the biofuels. The usual lipids are composed of glycerol molecule along with three fatty acids bounded; some of the fatty acids are linked at the third position by phosphate and carbohydrates called as phospholipids and glycolipids, respectively. These fatty acids contain a long unbranched carbon chain and are classified as saturated and monounsaturated or polyunsaturated fatty acids. Rangarao and Ravishankar (2007) extracted hydrocarbons from outside the cells of *Botryococcus braunii* and achieved excellent oil yield. Through biomass gasification in the presence of air, oxygen bio-syngas is produced. In the absence of oxygen directly produce hydrogen by various microalgae and is discussed in the section of H2 production. Few workers, i.e., Markou et al. (2012) and Chen et al. (2013), reported that 50% or above of starch is yielded from microalgae. Song et al. (2013) evaluated the ten microalgal strains for biodiesel production which is tabulated in Table 5.2.

Kurpan et al. (2015) examined the influence of light intensities and temperature on *Isochrysis galbana*, *Nannochloropsis oceanica*, and *Phaeodactylum tricornutum* for cell growth, specific growth rate, oil content, and oil productivity for biodiesel production. The illumination intensities fixed at 50, 300 and 600 μmol photons $m^{-2}$ $s^{-1}$ for cultivation, Nannochloropsis oceanica showed less productivity than other

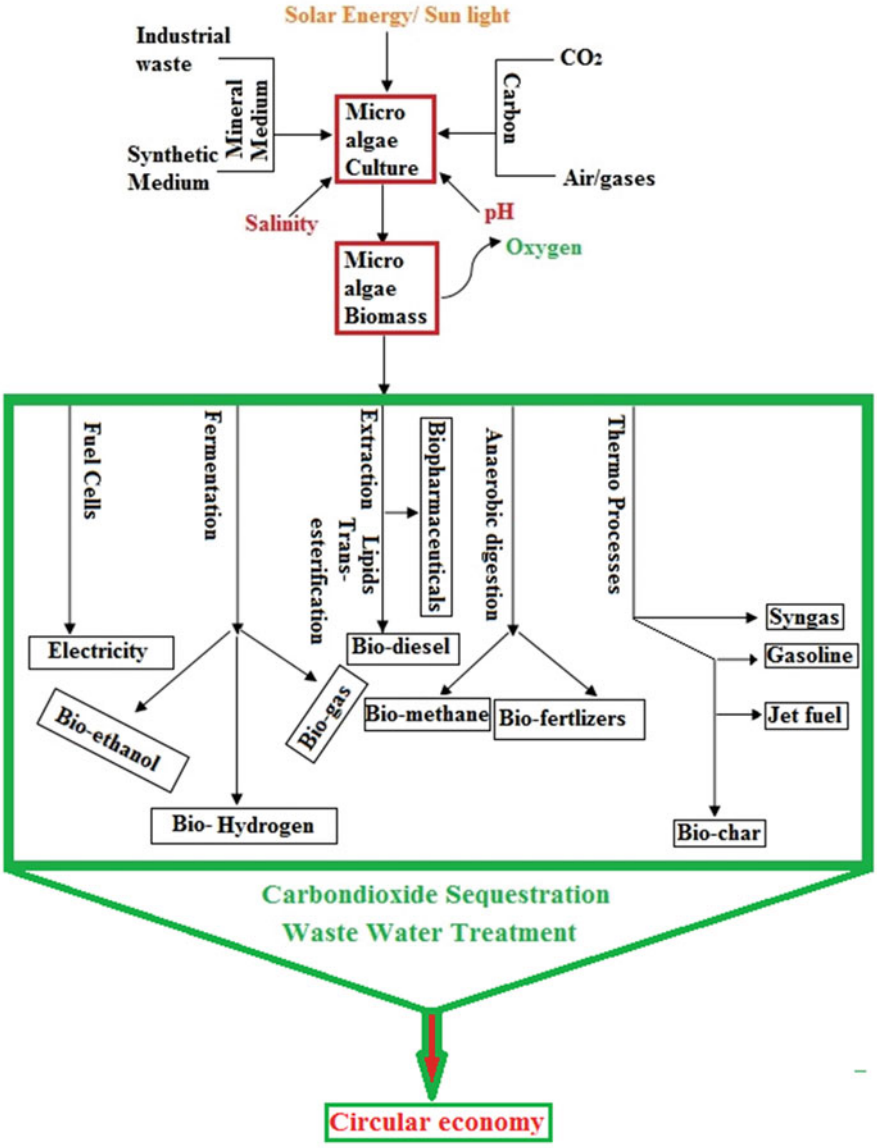


**Fig. 5.1** Flow diagram of the processes in the production of biofuels from microalgae

two microalgae. All are cultivated photoautotrophically at 20 and 30 °C in f/2 medium with 50 μmol photons $m^{-2}$ $s^{-1}$. The specific growth rate was noted as 0.84 ± 0.038, 0.63 ± 0.009, and 0.87 ± 0.034 for *Isochrysis galbana*, *Nannochloropsis oceanica*, and *Phaeodactylum tricornutum*, respectively. Triacylglycerol (TAG) productivity (mg $L^{-1}$ $d^{-1}$) was noted highest at intermediate

**Table 5.1** Various microalgae as feedstock for biofuel manufacture like bio-ethanol, syngas, methane, and bio-oil

| Type of the product | Name of the microalgae | References |
|---|---|---|
| Syngas | *Chlorella vulgaris* | Onwudili et al. (2013) |
| | *Nannochloropsis* sp. | Khoo et al. (2013) |
| | *Nannochloropsis oculata* | Duman et al. (2014) |
| | *Nannochloropsis gaditana* | Sanchez-Silva et al. (2013) |
| | *Spirulina platensis* | Stucki et al. (2009) |
| | *Saccharina latissima* | Onwudili et al. (2013) |
| | *Tetraselmis* sp. | Alghurabie et al. (2013) |
| Gas/oil/char | *Emiliania huxleyi* | Wu et al. (1999) |
| | *Chlorella* sp. | Babich et al. (2011) |
| | *Chlorella vulgaris* | Wang et al. (2015c) |
| | *D. tertiolecta* | Grierson et al. (2009) |
| | *Nannochloropsis* sp. | Pan et al. (2010) |
| | *Synechococcus* | Grierson et al. (2009) |
| | *Tetraselmis chuii* | Grierson et al. (2011) |
| | *Chlorella protothecoides* | Rizzo et al. (2013) |
| | *Microcystis aeruginosa* | Miao et al. (2004) |
| Methane | *Chlorella vulgaris* | Buxy et al. (2013) |
| | *Spirulina* sp. | Zamalloa et al. (2012) |
| | *Scenedesmus obliquus* | Zamalloa et al. (2012) |
| | *Arthrospira maxima* | Inglesby and Fisher (2012) |
| | *Euglena gracilis* | Nguyen et al. (2015) |
| Bio-ethanol | *Chlorella vulgaris* | Hirano et al. (1997) |
| | *Chlamydomonas reinhardtii* | Choi et al. (2010) |
| | *Chlorococcum* sp. | Harun et al. (2010) |
| Bio-oil | *Chlorella* sp. | Barreiro et al. (2013) |
| | *Chlorella vulgaris* | Biller et al. (2012) |
| | *Chlorogloeopsis fritschii* | Biller et al. (2012) |
| | *Nannochloropsis* sp. | Barreiro et al. (2013) |
| | *Nannochloropsis oculata* | Biller et al. (2012) |
| | *Nannochloropsis gaditana* | Biller and Ross (2011) |
| | *Spirulina platensis* | Biller et al. (2012) |
| | *Tetraselmis* sp. | Eboibi et al. (2014) |
| | *Bacillariophyta* sp. | Huang et al. (2016) |
| | *Cyanobacteria* sp. | Huang et al. (2016) |
| | *Desmodesmus* sp. | Alba et al. (2012) |
| | *Scenedesmus dimorphus* | Biller et al. (2012) |
| | *Porphyridium cruentum* | Biller and Ross (2011) |
| | *Phaeodactylum tricornutum* | Barreiro et al. (2013) |

stationary growth phase in *Isochrysis galbana* (14.27 ± 7.17) and late phases in *Nannochloropsis oceanica* (9.79 ± 0.74) and *Phaeodactylum tricornutum* (17.25 ± 2.14). Among the light intensities, 300 μmol photons $m^{-2}$ $s^{-1}$ is the best for the TAG productivity.

**Table 5.2** Lipid productivity and specific growth rate of 10 selected microalgae (Song et al. 2013)

| Algae species | Culture medium | Specific growth rate, $k$ ($d^{-1}$) | Biomass concentration, DM (mg $L^{-1}$) | Lipid productivity (mg $L^{-1}d^{-1}$) |
|---|---|---|---|---|
| *Kirchneriella lunaris* | BG-11 | 0.21 | 293.79 | 8.82 |
| *Selenastrum capricornutum* | BG-11 | 0.20 | 97.90 | 6.94 |
| *Staursatrum* sp. | BG-11 | 0.10 | 78.38 | 0.84 |
| *Chlorella vulgaris* | BG-11 | 0.17 | 225.00 | 7.96 |
| *Scenedesmus obliqnus* | BG-11 | 0.16 | 206.40 | 6.57 |
| *Batrachospermum sirodotia* | BG-11 | 0.13 | 49.23 | 1.18 |
| *Lyngbya kuetzingii* | BG-11 | 0.26 | 234.98 | 7.75 |
| *Isochrysis sphacrica* | F/2 | 0.14 | 255.56 | 8.69 |
| *Navicula* sp. | D1 | 0.19 | 71.33 | 3.67 |
| *Phaeodactylum tricornutum* | F/2 | 0.17 | 256.18 | 26.75 |

Rodolfi et al. (2009) examined 30 microalgae strains from fresh water and marine habitat and tested for biomass productivity and lipid productivity using various types of nutrient media (Table 5.3). They also tried with nutrient starvation technique, explained about the two-stage cultivation of microalgae in which primarily sufficient nutrition was available for some time, and later nitrogen/phosphorus starvation or stress was induced for enhanced yield of lipids. Here, some of the microalgae along with habitat, lipid production, and nutrient media are summarized in Tables 5.4 and 5.5. Lipid contents present in the microalgae cultivated are presented in Table 5.6 along with the habitats. The other fatty acid contents, i.e., eicosapentaenoic and decosahexaenoic, are particular attention on production by microalgae as it is an alternative technology. A researcher from the University of Almeria (Spain) has fabricated and improved an outdoor tubular photobioreactor for the production of 96% eicosapentaenoic (EPA) from *Phaeodactylum tricornutum*.

Yan and Schenk (2011) summarized (Table 5.7) few microalgae along with their production capacities of eicosapentaenoic and decosahexaenoic acids. The production of lipid from microalgae research has been carried out for decades under the nitrogen-deficit medium. Those results concluded that nitrogen-deficient medium at stationary phase of the growth significantly enhanced the lipid yield. A number of microalgae alter their carbon storage model which mainly supports the production of neutral lipids in the form of TAG (Rodolfi et al. 2009).

Jia et al. (2015) achieved 44.2% of glycerolipids from 8.3% when provided N-deficient medium was used for the *N. oceanica* cultivation and correlated with

**Table 5.3** Lipid productivity of various microalgae species

| Name of the microalgae | Lipid productivity (mg/dL) | References |
|---|---|---|
| *Isochrysis* sp. | 21.1 | Benemann et al. (1980) |
| *Chaetoceros calcitrans* | 17.6 | Rodolfi et al. (2009) |
| *Chaetoceros muelleri* | 21.8 | Rodolfi et al. (2009) |
| *Chlorella sorokiniana* | 44.7 | Rodolfi et al. (2009) |
| *Chlorella vulgaris* | 36.9 | Rodolfi et al. (2009) |
| *Chlorococcum* sp. | 53.7 | Rodolfi et al. (2009) |
| *Ellipsoidion* sp. | 47.3 | Rodolfi et al. (2009) |
| *Nannochloris* sp. | 76.5 | Yamaberi et al. (1998) |
| *Nannochloropsis* sp. | 61.0 | Rodolfi et al. (2009) |
| *Neochloris oleoabundans* | 125 | Rodolfi et al. (2009) |
| *Pavlova lutheri* | 50.2 | Rodolfi et al. (2009) |
| *Pavlova salina* | 49.4 | Rodolfi et al. (2009) |
| *Phaeodactylum tricornutum* | 44.8 | Rodolfi et al. (2009) |
| *Porphyridium cruentum* | 34.8 | Rodolfi et al. (2009) |
| *Tetraselmis* sp. | 22.7 | Huerlimann et al. (2010) |
| *Chlorella vulgaris* | 14.7 | Li et al. (2008) |
| *Chlorella emersonii* | 50.0 | Illman et al. (2000) |
| *Chlorella protothecoides* | 1214 | Xiong et al. (2008) |
| *Chlorella vulgaris* | 35 | Liang et al. (2009) |
| *Chlorella protothecoides* | 2400 | Chen and Walker (2011) |
| *Chlorella protothecoides* | 11,800 | Wang et al. (2014) |
| *Chlorella vulgaris* | 11.2–40 | Mata et al. (2010) |
| *Chlorella sorokiniana* | 44.7 | Mata et al. (2010) |
| *Chlorella vulgaris* | 52–56 | Wang et al. (2014) |
| *Chlorella sorokiniana* | 29–56 | Wang et al. (2014) |

glycerolipids containing membrane. They were noted EPA at N-repletion as 3.6 μmol $g^{-1}$ DW and the same condition when N-depletion as 21 μmol $g^{-1}$ DW in TAG content. TAG accumulation and profile changes in fatty acids on N-deficient medium were noted in green and red algae by several workers (Yang et al. 2013; Sharma et al. 2012; Valenzuela et al. 2012). Several researchers studied and compared the N-deficient and N-rich environments in one of the cultivation methods (Chen et al. 2015a, b; Griffiths et al. 2012). Some investigations are based only on the lipid content in batch cultures with various temperatures and nitrogen concentrations in the medium (Converti et al. 2009; Fakhry and El Maghraby 2015). Some of the cultivation conditions along with the lipid content (cell dry weight %) on various microalgae are shown in Table 5.8.

**Table 5.4** Microalgae used for the production of lipid with nutrient medium and habitat

| Name of microalgae | Habitat | Nutrient | Total lipid (%) of biomass | References |
|---|---|---|---|---|
| *Spirulina* sp. | Fresh water | Zarrouk's medium | 20 | Anitha and Narayanan (2012) |
| *Chlorella* sp. | Fresh water | BBM medium | 26 | Anitha and Narayanan (2012) |
| *Chlorella pyrenoidosa* | Fresh water | Bold's basal | 29.68 | Shekh et al. (2013) |
| *Phaeodactylum tricornutum* | Fresh water | F/2 medium | 61.43 | Song et al. (2013) |
| *Ettlia* sp. (YC001) | Fresh water | BG11 Agar medium | 42 | Yoo et al. (2013) |
| *Aurantiochytrium* sp. (KRS101) | Marine water | Defined medium | 38.1 | Ryu et al. (2013) |
| *Chlorella protothecoides* | Terrestrial | Basal medium | 27 | Wen et al. (2013) |
| *Endogenous chlorella* sp. | Fresh water | Brewery wastewater | 23 | Farooq et al. (2013) |
| *Chlorella vulgaris* (UTEX-265) | Fresh water | TAP medium | 42 | Farooq et al. (2013) |
| *Ettlia texensis* | Fresh water | BBM | 35 | Isleten-Hosoglu et al. (2013) |
| *Synechococcus* sp. (PCC7942) | Marine water | BG-11 liquid | 29 | Silva et al. (2014) |
| *Chlorella* sp. (KMN1) | Marine water | BBM | 27.11 | Tale et al. (2014) |
| *Chlorella* sp. (KMN2) | Marine water | BBM | 31.52 | Tale et al. (2014) |
| *Chlorella* sp. (KMN3) | Marine water | BBM | 20.27 | Tale et al. (2014) |
| *Scenedesmus* sp. (KMN4) | Marine water | BBM | 28.63 | Tale et al. (2014) |
| *Monoraphidium* sp. (KMN5) | Marine water | BBM | 34.93 | Tale et al. (2014) |
| *Chlorococcum* sp. ( IMMTCC-1) | Fresh water | BBM | 8.7 | Nayak et al. (2011) |
| *Chlorella* sp. (IM MTCC-2) | Fresh water | BBM | 22.7 | Nayak et al. (2011) |
| *Scenedesmus* sp. (I MMTCC-3) | Fresh water | BBM | 11.04 | Nayak et al. (2011) |
| *Scenedesmus* sp. (I MMTCC-7) | Fresh water | BBM | 14.6 | Nayak et al. (2011) |
| *Chlorella* sp. (IM MTCC-8) | Fresh water | BBM | 6.1 | Nayak et al. (2011) |
| *Chlorella* sp. (IM MTCC-9) | Fresh water | BBM | 4.9 | Nayak et al. (2011) |

(continued)

**Table 5.4** (continued)

| Name of microalgae | Habitat | Nutrient | Total lipid (%) of biomass | References |
|---|---|---|---|---|
| *Micractinium* sp. (ME05) | Fresh water | BG-11 medium | 10.7 | Onay et al. (2014) |
| *Scenedesmus* sp. (ME02) | Fresh water | BG-11 medium | 12.3 | Onay et al. (2014) |
| *T. variabilis* | Brackish water | BG110 | 12.1 | Bruno et al. (2012) |
| *I. galbana* | Marine water | F/2 medium | 21 | Kurpan et al. (2015) |
| *P. tricornutum* | Marine water | F/2 medium | 28 | Kurpan et al. (2015) |
| *Botryococcus braunii* | Fresh water | Secondary domestic wastewater | 36.14 | Sydney et al. (2011) |
| *B. braunii* (AP103) | Fresh water | Modified Chu's 13 | 19 | Ashokkumar and Rengasamy (2012) |
| *Isochrysis galbana* | Marine water | Enriched artificial sea water f/2 | 16.47 | Picardo et al. (2013) |
| *Ettlia* sp. | Fresh water | Sugar factory wastewater | 42 | Moon et al. (2013) |
| *P. tricornutum* | Marine water | F/2 medium | 28 | Kurpan et al. (2015) |
| *Chlorella vulgaris* | Fresh water | Modified basal | 27 | Mitra et al. (2012) |
| *Dunaliella* | Marine water | ESAW | 17.1 | Richardson et al. (2010) |
| *Phaeodactylum* | Marine water | Ukeles | 6.1 | Richardson et al. (2010) |
| *S. obliquus* (YSL02) | Fresh water | Bold basal | 29 | Abou-Shanab et al. (2011) |
| *Chla. pitschmannii* (YSL03) | Fresh water | Bold basal | 51 | Abou-Shanab et al. (2011) |

## 5.3 Harvesting

In open ponds and reactors, microalgae are generally cultured in liquid medium, due to the micron size of the algae; the intended selection of separation of algae in suspension form and the separation process of microalgae is called as harvesting. Several processes are typically practiced for the separation of microalgae, i.e., centrifugation, flocculation, coagulation, floatation, sedimentation, filtration, microscreening (Brennan and Owende 2010), electroflotation, electrophoresis, and ultrasound (Heasman et al. 2000). Among them flocculation, centrifugation, and screening are the most used ones because they are energy efficient and inexpensive; electroflotation, electrophoresis, and ultrasound are less likely used in rare conditions. Harvesting is a tough task, and it occupies 20–30% of the whole cost of the

**Table 5.5** Microalgae used for the production of lipid with nutrient medium and habitat

| Name of microalgae | Habitat | Nutrient | Total lipid (%) of biomass | References |
|---|---|---|---|---|
| *Chlorella vulgaris* | Fresh water | BBM | 27 | Richardson et al. (2010) |
| *Aphanothece* | Fresh water | BGN | 8 | Richardson et al. (2010) |
| *Phormidium, Scenedesmus* | Fresh water | BBM | 14.1 | Richardson et al. (2010) |
| *Neochloris oleoabundans* | Fresh water | Bristol | 56 | Gouveia and Oliveira (2009) |
| *Chlorella* sp. | Marine water | Walne's | 26 | Hsieh and Wu (2009) |
| *Chlorella vulgaris* | Fresh water | Basal | 38 | Cheng et al. (2009) |
| *B. braunii* | Fresh water | Modified Chu | 25.7 | Yoo et al. (2010) |
| *C. vulgaris* | Fresh water | BG11 | 11.9 | Yoo et al. (2010) |
| *Scenedesmus* sp. | Fresh water | BG11 | 11.9 | Yoo et al. (2010) |
| *Chlorella vulgaris* | Fresh water | N-deficient medium | 40 | Gouveia and Oliveira (2009) |
| *Scenedesmus obliquus* | Fresh water | N-deficient medium | 35 | Gouveia and Oliveira (2009) |
| *Neochloris oleoabundans* | Fresh water | N-deficient medium | 35 | Gouveia and Oliveira (2009) |
| *Spirulina maxima* | Fresh water | N-deficient medium | 9 | Gouveia and Oliveira (2009) |
| *N. oculata* (NCTU-3) | Marine water | Modified f/2 | 29.7 | Chiu et al. (2009) |
| *N. oleoabundans* | Fresh water | Bristol | 16.5 | Da Silva et al. (2009) |
| *S. obliquus* | Fresh water | Bristol | 12.5 | Da Silva et al. (2009) |
| *Botryococcus braunii* | Fresh water | Modified chu13 | 0.005 | Chiu et al. (2009) |
| *Chlorella vulgaris* | Fresh water | BG 11 | 0.020 | Chiu et al. (2009) |
| *Scenedesmus* sp. | Fresh water | 50% BG 11 | 31–33 | Xin et al. (2010) |
| *Botryococcus braunii* | Fresh water | BG 11 | 13.5 | Chinnasamy et al. (2010) |
| *Chlorella saccharophila* | Fresh water | BG 11 | 18.10 | Chinnasamy et al. (2010) |
| *Dunaliella tertiolecta* | Marine water | Modified BG 11 | 15.20 | Chinnasamy et al. (2010) |

(continued)

**Table 5.5** (continued)

| Name of microalgae | Habitat | Nutrient | Total lipid (%) of biomass | References |
|---|---|---|---|---|
| *Pleurochrysis carterae* | Marine water | Modified BG 11 | 12 | Chinnasamy et al. (2010) |
| Consortium | Fresh water | BG 11 | 12.20 | Chinnasamy et al. (2010) |
| *Chlorella vulgaris* | Fresh water | Artificial wastewater | 42 | Feng et al. (2011b) |
| *C. vulgaris* (YSL04) | Fresh water | Bold basal | 26 | Abou-Shanab et al. (2011) |
| *S. obliquus* (YSL05) | Fresh water | Bold basal | 28 | Abou-Shanab et al. (2011) |
| *Chla. Mexicana* (YSL07) | Fresh water | Bold basal | 29 | Abou-Shanab et al. (2011) |
| *C. vulgaris* (2714) | Fresh water | Modified medium | 40 | Heredia et al. (2011) |
| *Chlorella vulgaris* | Fresh water | N11 medium | 55 | Mallick et al. (2012) |
| *Chlorella vulgaris* | Fresh water | Kessler and Czygan | 30 | Abomohra et al. (2013) |
| *Scenedesmus obliquus* | Fresh water | Kessler and Czygan | 60 | Abomohra et al. (2013) |
| *B. braunii* | Fresh water | BG11 medium | 40 | Abomohra et al. (2013) |
| *Chlorella vulgaris* | Fresh water | Thin stillage (TS) | 43 | Mitra et al. (2012) |
| *Chlorella vulgaris* | Fresh water | Soy whey (SW) | 11 | Mitra et al. (2012) |
| *I. galbana* | Marine water | F/2 medium | 21 | Kurpan et al. (2015) |

biofuel production on commercial scale (Singh et al. 2013). So in the selection of the process, one should consider the factors such as cell size, density, yield of the biomass, energy efficiency, cost, and output. After separation, the microalgae undergoes several processes to extract the contents inside the algae cell. The selected process should have minimal negative impact on the further extraction process. The process necessitates bulk sedimentation and thickening or separation of biomass into slurry.

### *5.3.1 Sedimentation*

Sedimentation required the settling of microalgae to the bottom of the fluid mainly due to gravity and to attain the complete separation of biomass along with

**Table 5.6** Lipid content of microalgae cultivated in fresh water and marine waters

| Name of the microalgae | Habitat | Lipid content by weight (%) | References |
|---|---|---|---|
| *Anabaena cylindrica* | Fresh water | 4–7 | Demirbas and Fatih Demirbas (2011) |
| *Chlamydomonas reinhardtii* | Fresh/ marine | 6 | Sachdeva et al. (2016) |
| *Chlorella vulgaris* | Fresh water | 49–52 | Kumar et al. (2019) |
| *Dunaliella bioculata* | Marine | 8 | Sajjadi et al. (2018) |
| *Chlorella pyrenoidosa* | Fresh water | 38 | Wen et al. (2014) |
| *Nannochloropsis* sp. | Marine | 30 | Khatoon et al. (2014) |
| *Neochloris oleoabundans* | Fresh water | 35–54 | Demirbas and Fatih Demirbas (2011) |
| *Chlorella sorokiniana* | Fresh water | 22–24 | Sajjadi et al. (2018) |
| *Nannochloropsis granulata* | Marine | 28.5 | Chua and Schenk (2017) |
| *Porphyridium cruentum* | Fresh water | 9–14 | Demirbas and Fatih Demirbas (2011) |
| *Nannochloropsis oculata* | Marine | 45 | Chua and Schenk (2017) |
| *Scenedesmus dimorphus* | Fresh water | 10 | Bordoloi et al. (2016) |
| *Prymnesium parvum* | Marine | 22–38 | Demirbas and Fatih Demirbas (2011) |
| *Scenedesmus obliquus* | Fresh water | 30–50 | Sajjadi et al. (2018) |
| *Tetraselmis* sp. | Marine | 20–50 | Khatoon et al. (2014) |
| *Scenedesmus quadricauda* | Fresh water | 1.9 | Khatoon et al. (2014) |
| *Dunaliella salina* | Marine | 6–25 | Sajjadi et al. (2018) |

gravitational forces when additionally considered density difference is in between biomass and fluid. The fluid and biomass density difference can be maintained by the change in the pH of the suspension (Liu et al. 2013) and may be addition of alum/ iron oxide in the suspension (Wang et al. 2014a). Microalgae harvesting can be performed by utilizing sedimentation followed by flocculation. The rate of sedimentation of microalgae depends on the settling velocity of algae (Schenk et al. 2008) and cell size (aggregation of cells to bulky size).

**Table 5.7** Few microalgae used for the production of fatty acids, EPA and DHA

| Name of the microalgae | Eicosapentaenoic acid (% of total fatty acids) | Decosahexaenoic acid (% of total fatty acids) | References |
|---|---|---|---|
| *Isochrysis galbana* | 0.9 | NA | Yan and Schenk (2011) |
| *Nannochloropsis* sp. | 30.1 | NA | Yan and Schenk (2011) |
| *Chaetoceros calcitrans* | 34 | NA | Yan and Schenk (2011) |
| *Tetraselmis suecica* | 6.2 | NA | Yan and Schenk (2011) |
| *Chaetoceros muelleri* | 12.8 | 0.8 | Yan and Schenk (2011) |
| *Pavlova salina* | 19.1 | 1.5 | Yan and Schenk (2011) |
| *Skeletonema costatum* | 40.7 | 2.3 | Yan and Schenk (2011) |
| *Chroomonas salina* | 12.9 | 7.1 | Yan and Schenk (2011) |
| *Chaetoceros constrictus* | 18.8 | 0.6 | Yan and Schenk (2011) |

### 5.3.2 Centrifugation

The density difference and particle size are two important factors to separate the two liquids or solid–liquid phases by centrifugal forces, whereas in sedimentation, the gravitational forces are mainly involved along with density difference in two phases. Algae more than 5 μm size have thick cell walls and also high density and could be separated easily by centrifugal forces. Molina et al. (2003) performed experimentation and developed a prototype pond system that carried 500–1000 g of microalgae and achieved 80–90% of microalgae separation in 2–5 min. Schenk et al. (2008) considered centrifugation as the secondary harvesting method because it was time consuming, and it may be preferred after filtration at 100–200 g/L. Extraordinary research was performed on centrifugation methods and their effectiveness, and reliability was documented in the literature (Huang et al. 2010; Heasman et al. 2000). Several researchers have suggested and judged centrifugation as a reliable process to recover microalgae without damage of their contents (Girma et al. 2003; Heasman et al. 2000; Sim et al. 1988) and can be used in commercial scale and also

**Table 5.8** Lipid content of microalgae in nitrogen starvation condition

| Name of the microalgae | Cultivation conditions | Lipid content by cell dry weight (%) | References |
|---|---|---|---|
| *Chlorella pyrenoidosa* | 120 rpm shaking incubator under continuous cool white light illumination | 58 | Ratnapuram et al. (2018) |
| *C. reinhardtii* CC1010 | Constant illumination (2000 lx) at a distance of 50 cm for alternate photoperiod | 61 | Karpagam et al. (2015) |
| *Chlorella regularis* | 14 h light and 10 h dark, temperature 25 ± 1 °C with 160 rpm agitation | 42.3 (excess phosphorus) | Fu et al. (2017) |
| *Chlorella sorokiniana* | Mixotrophic, cool white light intensity 100 μmol $m^{-2}$ $s^{-1}$, 25 °C, shaking at 150 rpm | 44 | Li et al. (2015a, b) |
| *Chlorella protothecoides* | 180 h in fed-batch culture at 30 °C | 39.2 (hyperosmotic stress) | Wang et al. (2017) |
| *Chlorella vulgaris* ESP-31 | Mixotrophic on modified Bristol's medium | 40–53 | Yeh and Chang (2012) |
| *Isochrysis zhangjiangensis* | Addition of nitrogen at 24 h interval (high lipid yields under nitrogen rich conditions) | 53 | Feng et al. (2011a, b) |
| *Nannochloropsis oceanica* DUT01 | 14 h light and 10 h dark. Light intensity 60 μmol $m^{-2}$ $s^{-1}$, f/2 medium containing 37.5 mg/L $NaNO_3$ combined with 1/5 fresh medium replacement | 64 | Wan et al. (2013) |
| *Nannochloropsis oculata* | Batch mode, sudden starvation of nitrogen on low initial biomass | 50 (43% TG) | van Vooren et al. (2012) |
| *Neochloris oleoabundans* | 25 °C, 300 rpm, exponentially fed-batch cultures 0.042 $h^{-1}$ growth rate | 53.8 | Morales-Sánchez et al. (2014) |
| *Pseudochlorococcum* sp. | Low light intensity of 20 μmol photons $m^{-2}$ $s^{-1}$, orthophosphate (Pi) used as AGPase inhibitor and 6-methoxy-2-benzoxazolinone (MBOA) used as α-amylase inhibitor | 52.1 (low light) | Li et al. (2011a, b) |
| *Scenedesmus obliquus* NIES 2280 | 6-day cultivation, maintained in the dark, stirred at 100 rpm and maintained at 24 ± 2 °C | 38–48 (sufficient phosphorus) | Shen et al. (2015) |

for the separation of secondary metabolites from microalgae. According to the centrifugal forces generated, centrifugation methods are classified as follows: (1) hydro-cyclone, (2) solid bowl decanter, (3) nozzle type, and (4) solid ejecting disk methods.

### 5.3.3 Flocculation

This operation collects the scattered particles to form mass units and settle down at the bottom. Microalgae cell contain negative charge on the surfaces and also contain various functional groups, i.e., carboxylic acids which are caused by dispersibility of the fluids (Hadjoudja et al. 2010). In principle, microalgae cell surface containing negative charge will be neutralized by chemical process and forms the agglomerates which leads to settle down. Several chemical and physical methods are used to form floccules; they are called as flocculating agents. Multivalent metal particles, i.e., alums like ferric sulfate, ferric chloride, aluminum sulfate, and aluminum chloride, are used as flocculating agents. Further organic flocculants, electrolytes, and synthetic polymers are also used in flocculation process (Zheng et al. 2012). But during further extraction process, these metal particles may interfere in the quality of the product (Sim et al. 1988). Therefore, natural flocculating agents (chitosan, cationic starch) are preferred to neutralize the negative charges on the surface of the algae cells. Several researchers described that metallic hydroxide precipitate was formed when pH of the growth medium was increased, which led to charge neutralization on cell surfaces, and the floccules were formed (Yahi et al. 1994; Vandamme et al. 2012; Wu et al. 2012) and influenced the hydrolysis of multivalent metal ions in the growth medium.

pH is a vital parameter which affects the physiochemical properties of algae cells especially ionization of functional groups. If the pH is decreased by the addition of acids, the carboxylate ions interacted with protons and neutralize the surfaces and agglomeration forms leads to settle down of the biomass. Liu et al. (2013) reported and explained how protons neutralized the outer surface negative charges of the algae cells. They successfully improved the harvesting the algae cells, i.e., *Chlorococcum nivale*, *Chlorococcum ellipsoideum*, and *Scenedesmus* sp. at pH 4. Instead of synthetic, inorganic flocculating agents, Divakaran and Pillai (2002) effectively utilized chitosan as bioflocculating agent for the harvesting of *Chlorella*, *Spirulina*, *Oscillatoria*, and *Synechocystis* sp. Simply they altered the pH of the medium in the harvesting process at pH 7 for fresh water algae and lower pH for marine algae. Heasman et al. (2000) also reported that chitosan was used as bioflocculant for harvesting *Thalassiosira pseudonana*, *Isochrysis* sp., and *Tetraselmis chui* with 40 mg $L^{-1}$, and for *Chaetoceros muelleri*, 150 mg $L^{-1}$ was utilized for complete flocculation.

### 5.3.4 Coagulation

In this process, the microalgae moving to positive charge due to negative charges present on microalgae by the electrolytic induction, thereby formation of aggregates (Vandamme et al. 2011). Coagulation process can be seen in three mechanisms: (1) electrolytic oxidation, (2) deterioration of suspension, and (3) accumulation of

destabilized phases. One can choose the one of the mechanisms or combined mechanisms for formation of flocs. Vandamme et al. (2011) reported that around 80–95% of algae is removed by using electrolytic flocculation. In this method, multivalent metal anodes generally iron or aluminum are utilized to produce ionic flocculants ($Al^{3+}$ and $Fe^{3+}$). Later, as in the flocculation, the agglomerate again forms flocs by neutralizing the surface charges of algae cells (Gao et al. 2010). The coagulation process entails the generation of agglomeration by debauchery of the reactive anode, weakening of colloidal suspensions, and assemblage of destabilized suspensions, resulting in the formation of algal flocs. The EC process is a more efficient chemical flocculation technique compared to conventional processes of direct interaction of the aluminum sulfate with algal suspensions (Aragón et al. 1992). The flocculated microalgae are separated from water, by sedimentation or flotation.

### *5.3.5 Floatation*

Gas or air is converted into bubbles, send them through microalgae suspension, these bubbles get attached to algae solid particle and bring them accumulated upwards to the surface (Uduman et al. 2010). Microalgae separation cost is reduced because during flotation little energy is consumed for bubble formation. Flotation is a smart and quicker technology (Singh et al. 2011) when compared to other unit operation especially for microalgae separation and centrifugation which require more energy compared to flotation. The reaction of air bubbles with microalgae depends on the type of air, size of air bubbles (<500 μm), biomass, and contact angle of liquid phase. Air of gas bubbles drifts up into smaller particles, and large contact angle is more suitable for air adherence to the biomass (Matis et al. 1993). Commonly used flotation is differentiated depending upon the bubbles formation processes such as (1) air dispersed flotation, (2) dissolved air flotation, and (3) electrolytic flotation. In flocculation, several chemicals and flocculating agents are used to separate microalgae biomass which requires more energy and is expensive, further biofuel quality decreased due to these exogenous matter. Especially flotation is as effective as flocculation in microalgae harvesting, efficiently with low or no surfactants (Coward et al. 2013; Wiley et al. 2009). Rubio et al. (2002) demonstrated that due to the availability of modern instrumentation with high technological throughput, the flotation is potential and effective. Several researchers implemented flotation process for microalgae harvesting from suspension (Christenson and Sims 2011; Wiley et al. 2011).

### 5.3.6 Filtration

This is a physical technique which contains a filter medium through which liquids and gases can pass and the solids are separated, and the filter medium can be prepared by diatomaceous earth/cellulose (Brennan and Owende 2010) can be used for filtration. This technique is mostly used in case of large size algae (>70 μm), and it is not appropriate for *Dunaliella*, *Scenedesmus*, *Chlorella*, etc. Various types of filtration units are available, i.e., micro-filtration, dead-end filtration, vacuum filtration, tangential flow filtration (TFF), pressure filtration, and ultrafiltration (Petruševski et al. 1995). According to the reports cited in the literature, tangential flow filtration (TFF) and pressure filtration consume less energy and is efficient (Danquah et al. 2009). The disadvantage of filtration process is that it requires regular change of membranes and filters which is not much viable economically (Mohn 1980). Ultrafiltration and membrane filtration are other options for filtration process, but pumping and replacement of membranes increase the cost (Pittman et al. 2011). Mohn (1980) tried to harvest *Coelastrum proboscideum* with vacuum filtration and reported it is as inappropriate. Some researchers tried with polymer membrane filtration, the performance depending upon the hydrodynamic conditions, properties, and concentration of microalgae.

### 5.3.7 Electrophoresis

By the influence of electric field, the negatively charged microalgae move to the other end of the tray, moving forward. In electrophoresis, the tray is filled with medium which can permit the electrical field and the buffer system is present. This method is the best method to achieve separation without addition of any chemical. So, this technique is environmentally compatible, safe, and energy and cost efficient (Mollah et al. 2004). Sandbank et al. (1974) have revealed that electroflotation used for diversified microalgae harvesting.

### 5.3.8 Ultrasonication

By employing energy at frequency of >20 kHz, vibrations are produced through the medium to disturb the microalgae samples. Ninety percent efficiency can be achieved by using this method, and it is the best method (Bosma et al. 2003) with the flow rate range of 4–6 L/day. Li et al. (2011a, b) reported that ultrasound waves are used for the elimination of toxic algae, namely *Microcystis aeruginosa*, by coagulation. Various harvesting techniques along with energy usage and possible yields are shown in Table 5.9.

**Table 5.9** Various harvesting techniques along with energy usage and possible yields

| Harvesting technique | Energy usage (kW h $m^{-3}$) | Highest yield (% solids) | References |
|---|---|---|---|
| Centrifugation | 8.00 | 22.0 | Girma et al. (2003) |
| Gravity sedimentation | 0.1 | 1.5 | Shelef et al. (1984) |
| Filtration (natural) | 0.4 | 6.0 | Semerjian and Ayoub (2003) |
| Filtration (pressurized) | 0.88 | 27.0 | Semerjian and Ayoub (2003) |
| Tangential flow filtration | 2.06 | 8.9 | Danquah et al. (2009) |
| Vacuum filtration | 5.9 | 18 | Girma et al. (2003) |
| Polymer flocculation | 14.81 | 15.0 | Danquah et al. (2009) |
| Electroflotation | 5.0 | 5.0 | Azarian et al. (2007) |

## 5.4 Lipid Extraction

Lipids occupy more percentage of quantity in microalgae, and many researchers throughout the world are working on the enhancement of the lipid yield. Lipid extraction and separation are important processes for biodiesel production and contains 2–60% of lipids per total microalgae dry weight. Lipid synthesis by microalgae in photosynthesis process is oxygen dependent, alignment of 16 carbons to 22 carbon chain length (Hu et al. 2008). These carbon chain and oxygen alignment depend on various factors such as (1) light intensity, (2) $CO_2$ concentration, (3) nutrient concentration, (4) salinity, and (5) temperature which influence the quantity and quality of lipid accumulation in microalgae cells. Triglycerides (nonpolar lipids) are stored in microalgae and phospholipids, and glycolipids (polar lipids) are also stored inside the cells for involving in the formation of cellular and chloroplast membranes (Guckert and Cooksey 1990). Depending on the microalgae species, the oil content varies, and few are tabulated in Table 5.10.

So, nonpolar fraction of triglycerides is the most required lipids for the production of biodiesel. Lipid oils extracted from microalgae contain triglycerides and fatty acids and are used for esterification to alcohol esters. These alcohol esters blend with diesel upto 30% without any alteration in engine performance (Rand et al. 1983). Several techniques are available for the extraction of lipid from microalgae, majorly dividing into three classes, namely mechanical methods, chemical and solvent systems, and lipid extraction by enzymatic process.

**Table 5.10** Oil content in the selected algae species (modified from Chisti 2007)

| Name of the algae | Oil content (%dw) | Habitat | References |
|---|---|---|---|
| *Ankistrodesmus* TR-87 | 28–40 | | Chisti (2007) |
| *Botryococcus braunii* | 29–75 | Fresh water | Chisti (2007) |
| *Chlorella* sp. | 29 | Fresh water | Chisti (2007) |
| *Chlorella protothecoides* | 15–55 | Fresh water | Chisti (2007) |
| *Cyclotella* DI-35 | 42 | Fresh water | Chisti (2007) |
| *Dunaliella tertiolecta* | 36–42 | Marine | Chisti (2007) |
| *Nannochloris* | 31 | Marine | Chisti (2007) |
| *Nannochloropsis* sp. | 46 | Marine | Chisti (2007) |
| *Phaeodactylum tricornutum* | 31 | Marine | Chisti (2007) |
| *Scenedesmus* TR-84 | 45 | Fresh water | Chisti (2007) |
| *Porphyra red alga* | 33 | Marine | Chisti (2007) |
| *Tetraselmis suecica* | 15–32 | Marine | Chisti (2007) |
| *Diatoms Nualgi* | 21–31 | Marine | Chisti (2007) |
| *Neochloris oleoabundans* | 35–54 | Marine | Chisti (2007) |
| *Schizochytrium* | 50–77 | Marine | Chisti (2007) |

### *5.4.1 Lipid Extraction by a Mechanical Process*

Cell wall and cell structure are tough barriers which resist to access the biomolecules present inside the microalgae to solvents, in which the biomolecules cell wall disruption process is essential prior to extract them from the cell. The usual methods of cell disruption are (a) pressing with expeller, (b) bead beating, (c) homogenizers, (d) lyophilization with grinding, (e) grinding when frozen in liquid nitrogen, (f) ultrasonication, and (g) freezing and thawing simultaneously.

(a) Pressing with expeller

The screw-type pressing machine is used. Dry or wet biomass is fed on one side. High pressure is applied by screwing on the plate. After pressing the oil, other waste biomass exits through the other side. This is an efficient mechanical crushing process, and it is the oldest method. One disadvantage was observed that uncontrolled application of high pressure may lead to decrease in the quality and quantity of the lipid.

(b) Bead beating

The algae cells are broken by the vibration or agitation of the container with beads, in which high-speed impact action mechanism takes place in the chamber in that 80–85% of the volume is occupied by glass beads, leads impacted on algal cells and producing heat (Ranjith et al. 2015). Sometimes this heat may peak to 90 °C which may damage the quality of lipids. To avoid the generation of heat, the instruments are covered with cooling jackets. Lee et al. (2012) also explained that the bead size used in the container also influences the damage of oil; they recommend the bead size as 0.5 mm.

(c) Homogenizers

In this process, the principle involved is high pressure pump (550 atm) is integrated with an adjustable valve along with restricted orifice, and the cells are forcefully pressurized through the entry which leads to the disruption of cells. By the high pressure shear forces are generate causing completely disrupt the cells. This mechanical process is familiar in large-scale production in aquaculture.

(d) Lyophilization with grinding

Lyophilization is a drying process of the algae biomass in which the algal slurry to be dehydrated is frozen and ice crystals sublime by warming without thawing. This sublimes directly converts into vapor at partial pressure of water below 4.6 mm of Hg where the water triple point. It contains three phases: solidification of material by freezing, sublimation drying up to below 20% w/w (preliminary drying), and desorption of moisture to final value below 1% w/w. In the second stage, the vapor is produced from the interface (outer surface of cake) and passed through the channels from the ice crystals which are formed on freezing, at this escalates the drying process. In the presence of liquid nitrogen, this process is very quick, but small ice crystals are formed. Ultimately here most water from wet biomass to ice and convert all solute molecules into solids.

(e) Ultrasonication

Ultrasonication is a physical technique to disrupt the cells in which the ultrasound waves at 20–100 MHz stretch and compress the molecular spaces of the medium and generates cavitation effect on algae cells. It can be managed continuously without causing shear stress and generation of temperature with high efficiency. Zhang et al. (2016) used ultrasonication for the production of bio-diesel by using *Trichosporon oleaginosus*. Gerde et al. (2012) explained that increasing the sonication waves increases automatically the intracellular component extraction, but lipid oxidation occurs, thereby poor quality of lipid are generated. Natarajan et al. (2014) confirmed that ultrasonication is a good process for the rigid-walled cell microalgae like *Chlorella* sp.

(f) Microwave technique

Microwave technique is also preferred because it is rapid, offers heating, and requires low amount of solvents and shorter heating time (Dai et al. 2014). Microwave energy increases the lipid yields and requires low time for extraction due to rapid energy transfer and also reduces thermal gradients. Guldhe et al. (2014) compared these two methods on *Scenedesmus* sp. and concluded that microwave technique shows higher lipid yields.

(g) Freezing and thawing

In this freeze/thawing technique, first the filtrate is frozen in one part of distilled water and thawed with acetone (nine parts) to achieve a 90% (v/v) concentration. During freezing, ice crystals are formed that disrupt the cell membranes and are effectively extracted. This method is preferably useful in relatively high rigid cell walls for more extraction examples are *Cyanobacterium, Synechococcus spp*.

### 5.4.2 Lipid Extraction by Chemicals and Solvents

In the extraction process, chemicals and solvents are common to extract the contents from inside of the cell to outside. They are (a) hexane solvent, (b) two solvent systems, (c) soxhlet, (d) supercritical fluid (methanol or $CO_2$), (e) subcritical water, (f) accelerated solvent, (g) milking, (h) enzymatic, and (i) transesterification extraction methods.

Lipid extraction can be performed by using the solvents, i.e., methanol, ethanol, chloroform, and hexane. The usual method practiced industrially for extraction is two-phase solvent system and chloroform phase is fractionated the lipid (Liu et al. 2016). However, all the solvents are not safe, for example, chloroform and hexane are toxic, and unwanted components are solubilized which affects the product quality and may adversely affect the environment (Jeevan et al. 2017). The solvent system chloroform/methanol (2:1) was used for the lipid elicitation as per Folch method (Folch et al. 1957). The sample was extracted with the solvent system, and the dissolved organic phase was dried with a stream of nitrogen, and then the residue was reconstituted in isopropanol/chloroform before analysis. Bligh and Dyer method (Bligh and Dyer 1959) is a common method utilized for lipid extraction with two-phase solvent system, and lipid is fractionated by chloroform solvent. The ideal solvent must consider the requirements, i.e., highly specific to lipid, nontoxic, enough volatility, low energy intake for distillation, higher lipid yield, and no interference with other nonlipid components. Novel environmentally friendly solvents are introduced for lipid extraction from microalgae, i.e., acid combination with solvents, supercritical fluid, nanoparticles, biological enzymes, and ionic liquids. Table 5.11 lists various solvents utilized for the extraction of lipid from microalgae as feedstocks.

#### 5.4.2.1 Acid-Mediated Solvent System

Acid-mediated solvents are used with heat for cell disruption by catalyzing the hydrolysis at elevated temperatures (Lee et al. 2014). Park et al. (2014) reported that lipid extraction from *Chlorella vulgaris* at temperature of 120 °C for 1 h with 1% $H_2SO_4$ and achieved the enhanced yield. Lee et al. (2014) also found that the increased concentration of $HNO_3$ leads to declined yield when extraction is performed on *Nannochloropsis salina*. The utilization of acids with appropriate concentration should be investigated for lipid extraction from microalgae.

#### 5.4.2.2 Supercritical $CO_2$ Fluid Technology

Supercritical $CO_2$ fluid technology (SCF) is an eco-friendly technology with higher selectivity, lower toxicity, and shorter extraction time compared with organic solvents. In this technology, SC-$CO_2$ is utilized with other co-solvents which is in

**Table 5.11** Solvent systems used in lipid extraction from microalgae along with conditions

| Name of microalgae | Solvent system | Lipid yield (%) | Extraction conditions | References |
|---|---|---|---|---|
| *Acutodesmus obliquus* | $C_2H_5OH$-$C_6H_{14}$ (1:2) | 92% (Soxhlet) 59% (ultrasonic) | 60 °C, at 12 h (Soxhlet) & 2 h (ultrasonic) | Escorsim et al. (2018) |
| *Heterochlorella luteoviridis* | $C_2H_5OH$ | 83% | 75 mL/100 mL ethanol solution, electric field (90 V) | Jaeschke et al. (2016) |
| *Botryococcus braunii* | $CHCl_3$-$CH_3OH$ (1:3) | 98.9 wt% | 5 h (Butt tube systems) | Hidalgo et al. (2016) |
| *Nannochloropsis gaditana* | $C_6H_{14}$ | 69.1 wt% | Homogenization at 1700 bar and temperature (20–22 °C) | Jiménez Callejón et al. (2014) |
| *Chlorella pyrenoidosa* | $CHCl_3$-$CH_3OH$ (2:1) | 19.74% | Stirred 700 rpm at 20 °C | Dóca et al. (2011) |
| *Chlorella vulgaris* | $CH_3OH$-$CHCl_3$-$H_2O$ (25:12.5:5) | 52.5% | Sonicated for 40 min | Araujo et al. (2013) |
| *Chlorella vulgaris* | $CH_3OH$ | 20.7% | Sohxlet at 373 K for 96 h | Yang et al. (2015) |
| *Chlorella* sp. | $CH_3OH$-$CH_3COO$ $CH_2$ $CH_3$ (2:1) | 18.1% | 60 °C, 2 h | Wu et al. (2017) |
| *Scenedesmus* sp. | $C_6H_{14}$ | 16.3 ± 0.2 wt % | 235 °C, 31 bar | Shin et al. (2014) |
| *Schizochytrium* sp. | $CO_2$ expanded $C_2H_5OH$ | 87 wt% | Pressure 6.9 MPa, 313 K, ethanol flowrate 1.0 ml/min, $CO_2$ flowrate 6.0 ml/min | Wang et al. (2015a, b, c) |
| *Botryococcus braunii* | $CO_2$ expanded $C_2H_5OH$ | 24 wt% | 35 °C and 7.2 MPa | Paudel et al. (2015) |
| *Chlorella* sp. | $K_2HPO_4$+ $C_2H_5OH$ | 69 ± 2% | Three phase partitioning 2 h | Li et al. (2015a, b) |

gaseous state at ambient pressure, and high temperatures lead to easy separation and recovery. Further, no need to follow up the usage of solvents and recycle $CO_2$, avoiding the greenhouse effect (Mouahid et al. 2013). SCF extraction efficiency depends on the intrinsic factors, i.e., pressure, temperature, and extrinsic factors, i.e., sample characteristics and interaction between SC-$CO_2$ and targeted compounds (Sharif et al. 2014). This technology simple, scale-up also easy and limited movable parts, hence gained attention for lipid extraction from microalgae, but it showed higher cost of equipment (Reverchon and Marco 2006). Chatterjee and Bhattacharjee (2014) used SCF technology for the separation of antioxidant fraction

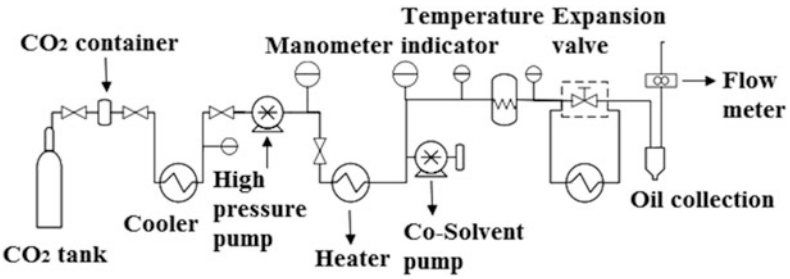


**Fig. 5.2** Schematic diagram of the SCF technology for lipid extraction from microalgae. (Adapted from Solana et al. 2014)

from algae, *Phormidium valderianum*, and optimized the experimental parameters and concluded that SCF is much suitable for neutral lipid extraction but not able to solubilize the phospholipids.

Solana et al. (2014) used SCF technology for lipid extraction from microalgae and compared Nannochloropsis salina, Chlorella protothecoides, and Scenedesmus obliquus, when increased the pressure at constant temperature and achieved higher yields. Taher et al. (2014) also revealed similar results in lipid extraction and concluded that lower temperatures and higher pressures yielded higher yields of lipids from microalgae. In case of low polarity, the addition of polar solvents as polarity modifiers, i.e., ethanol, attains enhanced lipid yields. Zinnai et al. (2016) extracted huge quantity of LC-PUFAs by SCF (Sc-$CO_2$ oil extraction) technology from *Schizochytrium* sp. The schematic diagram in Fig. 5.2 explains the SCF technology.

#### 5.4.2.3 Ionic Liquids

These contain organic cation and inorganic polyatomic anion, generally they are a solution of nonaqueous salt. Ionic liquids are noninflammable, do not have detectable vapor pressure, eco-friendly in nature, liquids in the temperature range of (0–140 °C), and can be used as green solvents (Swapnil 2012). Regardless of their properties, few works are reported on the utilization of ionic liquids for extraction of lipids from microalgae. Salvo et al. (2011) reported on lipid extraction process using 1-butyl-3-methylimidazolium as ionic liquid, in which the cell walls are lysed and then two immiscible layers are produced, i.e., hydrophobic and hydrophilic in upper and lower phases, respectively. Due to density difference in two phases, the upper hydrophobic layer is easily wiped out and fractionated.

### 5.4.3 Enzyme-Assisted Extraction

Few microalgae have rigid cell wall contains cellulose, protein combination and unable to receive the solvent inside to extract the cellular components present inside the cells. For this to extract the valuable components from the microalgae cells other than mechanical and chemical methods needed to achieve the efficient extraction. A novel technique is the enzyme-assisted extraction which enzymatically lyses the cell walls and enhances the lipid extraction from the microalgae. This technique is specific, mild, environmentally friendly, and higher moisture-containing feedstock (microalgae) but is time consuming. *Nannochloropsis* sp., marine algae cell wall containing cutan related compounds and the extraction is not suitable by physical and chemical methods. *Nannochloropsis* sp. can accumulate omega-3-fatty acids (PUFs), astaxanthin, carotenoids, and zeaxanthin which can be separated by using enzyme-assisted extraction. In the same way, *Neochloris oleoabundans* cell wall disruption occurred when using cellulase enzyme along with papain at high pressure homogenization process; they achieved 95.41% cell rupturing and 92.60% of lipid recovery (Wang et al. 2015a, b, c).

### 5.4.4 Surfactant-Assisted Extraction

This extraction process is nontoxic and biodegradable and has much attention toward the lipid extraction without equipment. Microalgae membranes possess negative charges, surfactants easily interact and cell disruption taking place at hydrophobic domain (Nasirpour et al. 2014). Few microalgae have more interaction with detergent others are not, surfactant like trilaminate *Chlorella emersonii* has more interaction than *Chlorella vulgaris* (Corre et al. 1996). *Botryococcus braunii* contains algaenan which hydrolyzed by trilaminar structure, altered the composition of cell wall (Simpson et al. 2003). Huang and Kim (2013) also cell disrupted with the cetyltrimethylammonium bromide (CTAB) and achieved higher percentage of lipid recovery.

### 5.4.5 Osmotic Pressure

Osmotic pressure can alter the exterior and interior integrity of the microalgae cells, very fast, and effective when compared to other extraction technology. This is in principle two types (1) Hyperosmotic and (2) Hypo osmotic. Hyper osmotic entail the salt in higher concentration at the outside of the cells, whereas hypo osmotic condition the salt concentration is very low. In both cases the cellular contents flow integrity of fluids are happens, i.e. in hyper osmotic the cell wall damage occurs there by the contents are came out from the cell and second one accumulation of fluids

from outside leads to high pressure inside the cell and busted the cell walls there by the contents are came out from the cell. Hence, it is very fast and also it is an eco-friendly and cheap method compared with other technologies. Most of the researchers preferred the hypo-osmotic principle in lipid extraction process from microalgae (Yoo et al. 2012). Fresh water microalgae, i.e., *Botryococcus* sp., *Scenedesmus* sp., and *Chlorella vulgaris*, were examined by researcher for effectiveness of lipid elicitation by osmotic pressure techniques (Ranjith et al. 2015).

## 5.5 Transesterification

Lipid extraction can be done from microalgae in either wet or dry condition, and dry biomass can give higher yields, but for the drying process, high energy is required which may be expensive. Due to this, most of the extraction processes are preferred in wet condition, but moisture content may not allow the organic solvents to interact with the cellular contents. Balasubramanian et al. (2013) worked on both dry and wet biomass to extract the lipids from microalgae and concluded that drying method affects total fatty acid content and has no positive impact on the yield of the lipids. Another alternative technology eliminates the utilization of various solvents in the extraction process and also requires less energy in situ transesterification process directly from biomass. The biomasses is either wet or dry in the transesterification process, and alcohol added acts as transesterification reactant and extraction solvent (Zhu et al. 2017). The co-solvents like chloroform may significantly enhance the yield by the formation of a homogenous system between alcohol and microalgae oil. Transesterification process directly synthesizes biodiesel without the loss of lipids along with other by-products, i.e., ethyl formate, glycerol carbonate, ethyl levulinate, and diethyl ether (Sivaramakrishnan and Incharoensakdi 2018). Various solvents and co-solvents are used in the direct biodiesel production (transesterification) along with conventional techniques, and their conditions are consolidated in Table 5.12.

Microalgae as feedstock for biofuels, i.e., *Chlorella* sp., *Nannochloropsis* sp., and *Spirulina* sp., are noted for the direct biodiesel production. Lemões et al. (2016) discovered the higher yields of methyl and ethyl esters than the conventional transesterification process. Ghosh et al. (2017) explained the combination of acid catalyst in transesterification, and extraction yielded more biodiesel in a two-step process on *Chlorella* sp. MJ11/11. Chen et al. (2015a, b) performed sequential transesterification process from biomass and confirmed the requirement of higher catalyst for direct biodiesel production. In single-step transesterification process, the biodiesel yield depends on various parameters, i.e., catalyst application, microwave and ultrasonic techniques, and lastly utilization of supercritical alcohols. Acid or base catalysts are conventionally used in biodiesel production, and base catalyst is noted for higher yields (Sivaramakrishnan and Incharoensakdi 2018) than acid catalyst due to faster reaction rate, but it is suitable for lower fatty acid content lipids (Lee and Saka 2010).

**Table 5.12** Biodiesel production from microalgae using conventional methods

| Name of the microalgae | Transesterification conditions | Biodiesel yield (%) | References |
|---|---|---|---|
| *Chaetoceros gracilis* (wet) | 100 mg biomass, 2 mL methanol, add chloroform to form single-phase solution, 1.8% sulfuric acid catalyst, 80 °C for 20 min | 84 (FAME) | Wahlen et al. (2011) |
| *Chlorella* sp. | Ultrasonic power 137 W, reaction time 100 min, molar ratios of methanol to oil of 83 and chloroform to oil of 30, 0.08 Mol sulfuric acid concentration | 81.2 | Karimi (2017) |
| *Chlorella pyrenoidosa* (wet) | 1 g biomass, 4 mL chloroform, 4 mL methanol, 0.2 mL sulfuric acid catalyst, microwave assisted for 30 min | 10.51 | Cheng et al. (2013) |
| *Nannochloropsis gaditana* | 4 kg biomass in 16 L of water, 36.4 L hexane, 36.4 L $C_3H_5OH$ 18.2 L 98% sulfuric acid catalyst, 2 h, vacuum distillation | 85.5 ± 2.6 (FAME) | Torres et al. (2017) |
| *Nannochloropsis gaditana* | 0.75 g biomass, 4.06 M sulfuric acid catalyst with 6.67 mL (ethyl acetate)/g dried algae, heated at 113.6 °C for 2 h | 97.8 wt% (FAEE) | Park et al. (2017) |
| *Nannochloropsis oceanica* (wet) | 0.2 g biomass, 1 mL methanol, 2 mL chloroform, 0.4 mL sulfuric acid catalyst, 95 °C for 120 min | 91 (FAME) | Im et al. (2014) |
| *Nannochloropsis* sp. (wet) | Mixture of biomass (20% water), methanol, and sodium hydroxide catalyst, microwave assisted at 50 °C for 10 min | 75 | Chee Loong and Idris (2017) |
| *Nannochloropsis* sp. | 1:400 M ratio of lipid to methanol 1:1 vol $CH_3OH$ and n-hexane, heated for 4 h maintained 60 °C | 90.9 | Dianursanti Religia and Wijanarko (2015) |
| *Nannochloropsis gaditana* | 5 g biomass, 1.98 mL of 1, 2-dichloroethane/ g biomass, 4.69 mL ethanol, heated at 185.08 °C for 3 h | 92 (FAEE) | Kim et al. (2017) |
| *Nannochloropsis gaditana* | 0.15 g dry biomass saturated to 80 wt% moisture, 1.5 mL methanol, 0.1 mL chloroform, 0.3 mL hydrochloric acid catalyst, heated at 95 °C for 2 h | 90 | Kim et al. (2015) |

The utilization of heterogeneous catalyst permits easy recovery as alcohols, and it is a promising technique for biodiesel commercialization from microalgae. Calcium oxide (CaO) recycles shell wastes of egg, mollusk, oyster, crabs, and chicken bones gaining a great deal as a basic catalyst can use for biodiesel production and increases the commercial value of sea food wastes (Kings et al. 2017; Mazaheri et al. 2018). Usage of lipase enzyme is a new trend in enhanced yield of biodiesel production from microalgae. Guldhe et al. (2016) selected lipase-producing fungi *Aspergillus niger* immobilized in polymers and used for lipid transesterification from microalgae *Scenedesmus obliquus* and achieved 90.82% yield. Various types of heterogeneous catalysts, their conditions, and yields are summarized in Table 5.13. Further, the

**Table 5.13** Biodiesel synthesis from microalgae using heterogeneous catalysts

| Name of the microalgae | Biodiesel yield (%) | Transesterification conditions | References |
|---|---|---|---|
| *Acutodesmus obliquus* | 86.41 | 1 g biomass, biomass to methanol (w/vol) ratio 1:12, 1.7% (w/w) calcium oxide catalyst from waste egg shell mechanically stirred at 140.6 rpm for 3.6 h, 75 °C | Pandit and Fulekar (2017) |
| *Botryococcus* sp. | 88 | 0.1 g biomass, 0.5 mL of *Candida antarctica* lipase B (Novozyme CAL-B) immobilized on Celite with dimethyl carbonate ultrasonicated at 40 °C for 6 h | Sivaramakrishnan and Incharoensakdi (2017) |
| *Chlorella* sp. | 47 FAME | 0.3 g biomass, 12 mL $g^{-1}$ methanol to biomass ratio, LiOH pumice catalyst (20 wt% at 12 mL $g^{-1}$) mechanical stirring at 500 rpm for 3 h, 80 °C | de Luna et al. (2017) |
| *Chlorella pyrenoidosa* | 84.6 | 1 g biomass, 4 mL methanol, 4 mL chloroform, 5 wt% sulfonated graphene oxide, microwave irradiated at 90 °C for 40 min. | Cheng et al. (2017) |
| *Nannochloropsis gaditana* | 99.5 FAME | 3 g biomass, 13.8 $cm^3$, 0.32 ratio of catalyst to oil mass, lipase catalyst from *Candida antarctica* with 21.3 $cm^3$ t-butanol stirred and incubated at 40 °C for 56 h | Navarro López et al. (2016) |
| *Nannochloropsis* sp. | 28 | 1 g biomass, 45 mL solvent (methanol/methylene dichloride = 3:1), 10% mg-Zr solid catalyst heated at 65 °C for 4 h | Li et al. (2011a, b) |
| *Nannochloropsis* sp. (dry) | 37.1 (MW) 20.9 (Soni) | 1 g biomass, methanol to chloroform (1:2 v/v), 0.3 g strontium oxide catalyst, microwave (MW) and sonication (Soni) assistance at 60 °C for 5 min. | Koberg et al. (2011) |
| *Scenedesmus obliquus* | 98.28 | 0.1 g biomass, 1 ml hexane, 20:1 methanol to oil molar ratio, 15% chromium-aluminum catalyst mechanically stirred (200 rpm) at 80 °C for 4 h | Guldhe et al. (2017) |

contemporary usage of ultrasonic microwave assisted with catalyst during transesterification enhances the efficiency of biodiesel yield. Teo and Idris (2014) succeeded higher yields with microwave-assisted transesterification of *Nannochloropsis* sp. and showed good cetane number, lubricating properties. Martínez et al. (2017) also reported that *Spirulina* sp. showed higher yields of biodiesel through the ultrasonic-assisted transesterification. Hence, heterogeneous catalyst transesterification is a good option as it avoids the catalyst recovery and can reduce the biodiesel production cost (Cheng et al. 2017).

### 5.5.1 Supercritical Conditions

Supercritical conditions also applied in transesterification process without catalyst to avoid the pollutants at elevated temperatures outside the critical temperature of alcohol to occur homogenous reaction (Jazzar et al. 2015a). Methanol cracks the walls of microalgae cells and permits the solvent dispersion easily into lipids when at supercritical conditions (Mohamadzadeh et al. 2017). Ethanol also replaces methanol in microalgae transesterification process; the same yields were noted in both alkyl esters and fatty acids (Lemões et al. 2016). Several works were reported about the supercritical ethanol, methanol, and hexane for biodiesel production from microalgae which are presented in Table 5.14. Instead of methanol, several long chain alcohols, i.e., isopropanol and butanol were introduced due to toxicity. These alcohols get best cold flow characteristics, cetane number, and oxidation stability of biodiesel. Reddy et al. (2014) stated that microwave-assisted supercritical esterification of *Nannochloropsis* (CCMP1776) with methanol as solvent showed best cetane number and oxidation stability. Zhou et al. (2017) reported that supercritical $CO_2$ recovered high value contents from the filtrates after transesterification of *Chlorella* sp. and *Chrysophyta* sp. Similarly Jazzar et al. (2015b) concluded on single-step isolation by supercritical conditions with methanol on biodiesel production from *Nannochloris* sp. and *Chlorella* sp. using transesterification.

**Table. 5.14** Biodiesel production by microalgae after supercritical transesterification

| Name of the microalgae | Biodiesel yield (%) | Transesterification conditions | References |
|---|---|---|---|
| *Nannochloropsis gaditana* | 45.8 (FAME) | 255–265 °C, 50 min, supercritical methanol to algae ratio (10:1) | Jazzar et al. (2015a) |
| *Nannochloropsis gaditana* (CCMP–1775) | 59.28 | Methanol to wet biomass (vol/wt) ratio 6:1, temperature 225 °C, and reaction time of 90 min | Sitthithanaboon et al. (2015) |
| *Nannochloropsis oculata* | 67 (FAEE) | 265 °C, 20 min, ethanol to algae ratio (9:1) at supercritical conditions | Reddy et al. (2014) |
| *Nannochloropsis salina* | 65 (FAEE) | 280 °C, 25 min, ethanol to algae ratio (9:1) at supercritical conditions | Patil et al. (2013) |
| *Nannochloropsis* sp. | 21.79 wt% | 265 °C, 50 min, methanol to algae ratio (10:1) at supercritical conditions | Jazzar et al. (2015b) |
| *Nannochloropsis* sp. | 62 | 50 °C, 200 bar, and 24 h reaction in supercritical $CO_2$ (SC–$CO_2$) medium | Taher et al. (2015) |
| *Nannochloropsis* (CCMP1776) | 85.75 | 1200 psi, methanol to biomass (12:1), 30 min | Patil et al. (2012) |
| *Chlorella* sp. | 45.62 wt% | 265 °C, 50 min, methanol to algae ratio (10:1) at supercritical conditions | Jazzar et al. (2015b) |
| *Chlorella protothecoides* | 90.8 | 320 °C, 152 bar, 31 min, methanol to oil ratio (19:1) | Nan et al. (2015) |
| *C. vulgaris* CCAP | 7.06 ± 1.03 | Hexane/biomass ratio (6:1) | Abedini Najafabadi et al. (2015) |

Xu et al. (2006) suggested that biodiesel must technically resemblance petroleum-based diesel in properties without pollution including viscosity, density, flash point, heating value, solidifying point, and cold filter plugging. Microalgae biodiesel complies all the parameters in the range recognized by the American Society for Testing and Materials (ASTM 2012) and the International Biodiesel Standard for Vehicles (EN14214) (Antolin et al. 2002). The future of algal biofuels depends on the organization of cost-effective technologies for commercialization. By 2020 UAE proposed that 10% of its vehicle transport run with biodiesel. The USA also proposed that by 2022 its road transport will occupy 20% of biodiesel-based vehicles, and it is providing Renewable Transport Fuel (RTFC) Certificate to fulfill the requirement (Wise and Cole 2015). Renewable energy is predictable to dominate by the year 2070 (Adeniyi et al. 2018). As biofuel production is expecting the circular economy by utilizing the waste water resource as cultivation medium and $CO_2$ as carbon source, many thermal plants release $CO_2$. For microalgae growth, nitrogen- and phosphorus-loaded wastewater is suitable, and it is anticipated that approximately 16.67 times more carbon than in gas carbon as $CO_2$ (Bilanovic et al. 2016). Mehrabadi et al. (2016) suggested that a method to reduce microalgae biodiesel production costs is the utilization of biomass from wastewater treatment in high-rate algae ponds. Several companies such as Sapphire Energy, Heliae, and Solazyme shifted from the commercialization of microalgae biofuels to other fields (food, nutrition, water treatment) to stay afloat (Su et al. 2017). This suggests that microalgae cultivation solely for biodiesel production is impossible.

At present, biodiesel production is economically feasible because in several countries, the government policies sustain the subsidies, tax credits, and import tariffs. Further reduction in equipment taxes and surcharges facilitates more viable, but as per the experiences from other companies along with biofuels some other products also to be maintain for the smooth running of the firm. Further in agriculture-based countries, preliminary training, learning sessions, and awareness programs about microalgae cultivation have to be planned for formers and young entrepreneurs. All these measures along with beneficial renewable energy policies, if implemented, may then be reaching the targets of the all countries to industrialize and compete with fossil petrol products.

Several countries made their policies feasible to renewable energy production and implement their prestigious projects for biofuel production using microalgae as feedstock. Brazil recently introduced tax exemptionsand loans for biodiesel production using microalgae as feedstock in non-arable lands (dos Santos Alves et al. 2017). The USA introduced Energy Independence and Security Act (EISA 2017), which offers loans and grants for the commercialization of microalgae-based biodiesel production. Several research grants (2015) were released from EU, implementing a BIOFAT project worth €10 million in Italy and Portugal. Lee (2011) predicted that algal biofuel could supply 7.1% of the developed world fuel demand by 2040 and 0.5% in developing countries with strong government support. Kovacevic and Wesseler (2010) estimated that algae biofuel could cost 51.60 euros/GJ by 2020 if technological advancements remained linear and crude oil remained at $100/barrel.

## 5.6 Conclusions

Various bioreactors and their designs were explained along with key factors affecting the biomass productivity. Several photobioreactors, their performance and various configurations were tabulated as well. Biofuel production along with the processes depicted in the figure and several techniques useful for the production from the nutrients to extraction process are clearly explained. For a clear explanation, several research works were exemplified according to the parameters studied. A wide discussion on the various harvesting methods along with their applications, advantages, and disadvantages were explained. Further research is required on several challenges in optimization of wastewater utilization such as cultivation medium including wastewater composition, toxic contamination, presence of solids particles, and effective light transmission. Additionally the research has to be about the new technologies that could reduce the cost and process. As discussed in the above sections, competitiveness could be more enhanced by the optimal maximum of the extraction and purification of all high-value contents through tumble biorefinery. Various works are in progress through the genetic transformation of microalgae to enhance the biomass productivity, product accumulation. Biodiesel from microalgae also provides a more sustainable and environmentally friendly alternative to fossil fuel.

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# Chapter 6
# Microalgae Potential Feedstock for the Production of Biohydrogen and Bioactive Compounds


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**Abstract** Urbanization and industrialization increase the energy demand, and fossil fuels are depleting drastically due to energy consumption by industrial and domestic purposes. There is a need to increase the production of energy by sustainable renewable sources. Marine microalgae are potential sources of biofuels and feedstock for the production of other bioactive compounds. Microalgae can be easily cultured in photobioreactors for the production of several types of biofuels. This chapter describes about the production of biohydrogen through photolysis followed by dark fermentation. Several types of photobioreactors used in the production of biohydrogen, suitability of microalgae as feedstock, and other microorganism used in the dark fermentation are discussed. The end product of biohydrogen after combustion is only water vapor; hence, there is no air pollution. Because of this nature of hydrogen gas, much attention has been paid by several researchers for the production of biohydrogen. Several bioactive compounds produced by microalgae, possibility of scale up, and industrialization have been revealed. Various parameters involved in the process, bioreactor types, and their design has to be discussed. Further the estimated cost of the pilot project for various biofuel products and the

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impact of circular economy has to be discussed with scientific attention at global scale.

**Keywords** Microalgae · Biohydrogen · Dark fermentation · Bioactive compounds

## Notations

| | |
|---|---|
| AA | Arachidonic acid |
| Ac | Acetic acid |
| ATP | Adenosine triphosphate |
| DHA | Docosahexaenoic acid |
| EPA | Eicosapentaenoic acid |
| GLA | γ-Linolenic acid |
| HMF | 5-Hydroxymethylfurfural |
| LED | Light-emitting diode |
| PBR | Photobioreactor |
| PUF | Polyunsaturated fatty acid |
| TS | Total solids |
| VS | Volatile solids |

## 6.1 Introduction

The thermal conversion of coal to energy leads to air pollution, greenhouse effect, global warming, climate change, and exaggerated fuel/energy price which have led to the emergent search for renewable energy. There is an emergent need for the investigation of energy source alternative to fossil fuels to fulfill the societal needs of energy. The end product of biohydrogen after combustion is only water vapor; hence, there is no air pollution (Batista et al. 2014). Because of this nature, much attention has been paid by several researchers for the production of biohydrogen. Several methods have been reported for hydrogen production, i.e., (1) natural gas modification, (2) coal/biomass gasification, (3) photoelectrolysis, (4) water electrolysis, (5) photobiological and decomposition at high temperatures, and (6) dark (bacterial) fermentation. Among these methods, natural gas with thermo-chemical process offered 48% of universal hydrogen needed. Das et al. (2014) reported that the biohydrogen conversion from oil, coal gasification, and water electrolysis are occupied as 30, 18 and 3.9% of total demand. Water electrolysis is the best technique with high purity; this process is highly tough and not cost-effective. Biohydrogen production is environmental friendly, requires low energy, and can be done in ambient conditions (Das and Veziroğlu 2001). Researchers are continuously developing the processes in photo-biological production of hydrogen; their substantial advances (Torzillo et al. 2015; Márquez-Reyes et al. 2015) and dark fermentation are gaining much attention by researchers (Roy et al. 2014; Batista et al. 2014;

Ortigueira et al. 2015a). Hence, this chapter describes about the biohydrogen production using microalgae in biophotolysis and dark fermentation.

## 6.2 Hydrogen Production

Biohydrogen production is classified into three divisions: (1) water photolysis, (2) photo-fermentation, and (3) dark-fermentation process. In fermentation process, biological transformation of organic compounds to hydrogen occurred under the illumination (photosynthetic) and independent (acidogenic) metabolic pathways (Liu et al. 2009a; Lo et al. 2010). Further, these organic substances can be utilized from renewable sources (organic waste), and waste reduction is also possible, which fulfills the circular economy principles.

### *6.2.1 Photofermentation*

Photosynthetic bacteria can produce hydrogen using organic acids, i.e., acetic acid and butyric acid as substrates, they have high theoretical yield and low by-product release ($CO_2$). The photosynthetic bacteria also generate $H_2$ from nonvolatile acids, i.e., malate, succinate, and lactate, and also from glucose. Therefore, photosynthetic bacteria also utilize the metabolic products of dark fermentation for the production of hydrogen (Liu et al. 2010; Wang et al. 2010).

### *6.2.2 Dark Fermentation*

Anaerobic bacteria such as *Clostridium*, *Bacillus*, and *Enterobacteria* are execute the dark fermentation using glucose, starch, molasses and sucrose as carbon source and emits the hydrogen along with other by-products i.e. butyrate, saccinate, malate, acetate, ethanol and $CO_2$. The major soluble metabolites, i.e., acetate and butyrate are almost favorable metabolic pathways for $H_2$ release (Fang et al. 2005). In the dark fermentation, the theoretical yield of hydrogen is 4 mol and 2 mol $H_2$/mol of glucose as substrate when the end products are acetic acid and butyrate (Logan 2004), respectively. Nguyen et al. (2010) reported on *Thermotoga neapolitana*, a hyperthermophile, giving higher yield as 1.8–3.2 mol $H_2$/mol glucose.

### 6.2.3 *Hybrid System Using Photosynthetic and Dark Fermentative Bacteria*

From dark fermentation organic wastes are used for $H_2$ generation, this process is cheap, eco-friendly and generates economy from wastes. The by-products of the dark fermentation are acetic acid and butyric acids and are further employed by photosynthetic bacteria for $H_2$ production under illumination (Shi and Yu 2006). Hence, the utilization of photosynthetic bacteria and anaerobic fermentative bacteria in one system is considered as efficient hybrid system for energy conversion from waste waters containing organic compounds (Tao et al. 2007).

$$2CH_3COOH + 2H_2O_{3.0} \overset{\text{Light Energy}}{\rightarrow} 4H_2 + 2CO_2 \tag{6.1}$$

The theoretical maximum yield of dark fermentation is 4 mol $H_2$/mol glucose and 2 mol HAc/mol glucose (acetic acid). Photosynthetic bacteria could utilize organic acids from dark fermentation as carbon source. As per Eq. (6.1), the theoretical maximum yield is 4 mol $H_2$/mol HAc. Hence, a combination of dark and photo fermentation is able to maximize hypothetical hydrogen yield as 12 mol $H_2$/mol glucose and 6 mol of $H_2O$ (Eq. 6.2).

$$C_6H_{12}O_6 + 6H_2O_{3.0} \overset{\text{Light Energy}}{\rightarrow} 12H_2 + 6CO_2 \tag{6.2}$$

## 6.3 The Key Enzymes Associated with Hydrogen Production by Photosynthetic Bacteria

In biohydrogen production, the key enzymes nitrogenase and hydrogenase are utilized by photosynthetic bacteria. Gloe et al. (1975) explained that the nitrogenase concerns in the absence of nitrogen and oxygen and presence of ATP to allow the $H_2$ evolution. In the lack of $N_2$, ammonia is not produced (Eq. 6.3) but $H_2$ evolution continues, in the presence of $N_2$, down trend of nitrogenase activity leads to $H_2$ evolution is suppression (Eq. 6.4).

$$2H^+ + 2e^- + 4ATP \rightarrow H_2 + 4ADP + 4Pi \tag{6.3}$$

$$N_2 + 8e^- + 8H^+ + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP \tag{6.4}$$

The nitrogenase preliminary inhibitors are ammonium and molecular oxygen, but the oxygen irreversibly inhibits nitrogenase enzyme. Practically, ammonium is utilized as nitrogen source which is essential for the growth of photosynthetic bacteria, but when it is too higher, the nitrogenase activity is hastily inhibited

**Table 6.1** The variation in $H_2$ yield obtained from integrated dark photofermentation process

| Carbon sources | Dark fermentation | Photofermentation | $H_2$ yield (mol $H_2$/mol C) | References |
|---|---|---|---|---|
| Glucose | *Enterobacter cloacae* DM11 | *Rhodobacter sphaeroides* O.U.001 | 6.31–6.75 | Nath et al. (2008) |
| Sucrose | *Clostridium butyricum* CGS5 | *Rhodopseudomonas palustris* WP3–5 | 5.45 | Lo et al. (2010) |
| Sucrose | *Clostridium pasteurianum* | *Rhodopseudomonas palustris* WP3–5 | 7.1 | Chen et al. (2008c) |
| Sucrose | *Microflora* | *Rhodobacter sphaeroides* SH2C | 6.63 | Tao et al. (2007) |
| Sucrose | *Microflora* | *Rhodobacter sphaeroides* ZX-5 | 6.26 | Zong et al. (2009) |
| Cellulose | *Cellulomonas* sp. | *Rhodobacter capsulatus* | 6.2 | Nandi and Sengupta (1998) |
| Cassava starch | *Microflora* | *Rhodopseudomonas palustris* | 2.92 | Su et al. (2009) |
| Glucose | *Rhodopseudomonas palustris* P4 | *Rhodopseudomonas palustris* P4 | 4.8–5.6 | Oh et al. (2004) |
| Molasses | *Caldicellulosiruptor saccharolyticus* | *Rhodobacter capsulatus hup_* (YO3) | 6.85 | Özgür et al. (2010) |
| Glucose | *Ethanoligenens harbinense* B49 | *Rhodopseudomonas faecalis* RLD-53 | 6.32 | Liu et al. (2009b) |
| Glucose | *Escherichia coli* HD701 | *Rhodobacter sphaeroides* OU001 | 2.4 | Redwood and Macaskie (2006) |
| Glucose | *Lactobacillus delbrueckii* NBRC13953 | *Rhodobacter sphaeroides* RV | 7.1 | Asada et al. (2006) |

owing to product inhibition (Chen et al. 2008c) effect. Hence, one can consider that the inhibition of nitrogenase is reversible when ammonia is removed. Table 6.1 shows the variation in $H_2$ yield obtained from integrated dark-photo fermentation process.

Akkerman et al. (2002) explained that light is substantially needed for the synthesis of ATP, and the wavelengths of light required are 522 and 860 nm. Hence, in the design of photobioreactor for $H_2$ production, consider fixing appropriate light source with the required wavelengths. The benefits of photosynthetic bacteria for photo-hydrogen production are:

(a) We can achieve greater theoretical yield of $H_2$.
(b) In the photosynthesis, $O_2$ is not evolved, in that way the difficulty of $O_2$ inhibition can be reduced.
(c) Able to utilize a wide range of light of wavelength between 400 and 900 nm (Chen et al. 2006).

(d) Able to utilize the end products of dark fermentation and other renewable sources, i.e., organic waste water as carbon source (Liu et al. 2009a) to attain greater $H_2$ yields.

## 6.4 Medium Constituents and Cultivation Environments for Photosynthetic Bacteria

Carbon: Carbon is a typical substrate essential for cellular growth and biohydrogen release (Fang et al. 2005). The dark fermentation soluble metabolites are generally utilized as carbon source, i.e., acetate, propionate, butyrate, and lactate (Wang et al. 2010). The photofermentation capacity is assessed by (1) the rate of hydrogen production and (2) the hydrogen yield. Rate of hydrogen production reveals the kinetics of hydrogen production, and hydrogen yield represents the ratio of real hydrogen production to the utmost theoretical hydrogen production ensuing from the entire conversion of the carbon to hydrogen and carbon dioxide (Eqs. 6.5–6.8).

$$C_3H_6O_3 + 3H_2O \rightarrow 6H_2 + 3CO_2 \tag{6.5}$$

$$C_4H_6O_5 + 3H_2O \rightarrow 6H_2 + 4CO_2 \tag{6.6}$$

$$C_4H_8O_2 + 6H_2O \rightarrow 10H_2 + 4CO_2 \tag{6.7}$$

$$C_2H_4O_2 + 2H_2O \rightarrow 4H_2 + 2CO_2 \tag{6.8}$$

Oh et al. (2004) reported that hydrogen was yielded in the range of 60–70% when acetate was provided as carbon source to *Rhodopseudomonas palustris* P4. Tsygankov et al. (1994) used succinate as carbon source on an isolate Rhodobacter sphaeroides RV was yielded $H_2$ 75% and lactate used on Rhodobacter capsulatus B10 attained $H_2$ production rate as 120 mL $L^{-1}$ $h^{-1}$. Some of the carbon sources and their theoretical yields via photo fermentation are summarized in Table 6.2.

Nitrogen: Nitrogen is an important nutrient essential for cell growth and is usually required in limited quantities. Ammonia is a rich source of nitrogen, and if it is present in excess amount in medium, it inhibits the $H_2$ production. So, the optimized concentration of nitrogen is preferable as nitrogen source for the fruitful operation of photofermentor especially in the integrated process of $H_2$ production (Lo et al. 2008). Shi and Yu (2005a) utilized glutamate as the nitrogen source for the enhanced production of $H_2$; however, it is costly than ammonia. Instead of glutamate, lower concentration of $NH_4$ is the best as non-inhibitory range preferable for practical applications.

**Table 6.2** Various carbon sources used for $H_2$ generation and their yields

| Name of the photosynthetic bacteria | Carbon source | $H_2$ yield (%) | References |
|---|---|---|---|
| *Rhodopseudomonas palustris* WP 3–5 | Organic acids | 2.2–49.6 | Lee et al. (2002) |
| *Rhodopseudomonas sphaeroides* O.U.001 | Malate | 22.0 | Ginkel et al. (2001) |
| *Rhodopseudomonas palustris* DSM 131 | Benzoate | 62 | Fißler et al. (1995) |
| *Rhodobacter sphaeroides* RV | Succinate | 75.0 | Tsygankov et al. (1994) |
| *Rhodobium marinum* A-501 | Glucose | 7.2 | Ike et al. (1999) |
| *Rhodopseudomonas palustris* P4 | Acetate | 60–70 | Oh et al. (2004) |
| *Rhodopseudomonas palustris* | Acetate | 14.8 | Barbosa et al. (2001) |
| *Rhodopseudomonas faecalis* | Acetate | 64.8 | Liu et al. (2009a) |
| *Rhodopseudomonas capsulatus* B100 | Acetate | 53.0 | Ooshima et al. (1998) |
| *Rhodobacter capsulatus* | Acetate (butyrate) | 62.5 | Fang et al. (2005) |
| *Rhodopseudomonas sphaeroides* RV | Lactate | 26.1 | Kondo et al. (2002) |
| *Rhodobacter sphaeroides* RV | Lactate | 20–73 | El-Shishtawy et al. (1997) |
| *Rubrivivax gelatinosus* L31 | Lactate | 50.5 | Li and Fang (2008) |
| *Rhodobacter capsulatus* JP91 | Lactate | 52.7 | He et al. (2006) |
| *Rhodopseudomonas palustris* WP 3–5 | Acetate and butyrate | 53.8 | Chen et al. (2008a) |
| *Rhodopseudomonas capsulate* | Acetate, propionate, butyrate | 32.6 | Shi and Yu (2005b) |
| *Rhodopseudomonas capsulate* | Acetate, propionate, butyrate | 45.0 | Shi and Yu (2006) |

## 6.5 Various Parameters Influencing the Biohydrogen Production

**pH of the Culture Medium**: The culture medium pH establishing the active sites as ionic form and activity of enzymes, so usually affects the characteristics of biochemical reactions. In $H_2$ production nitrogenase and hydrogenase plays vital role at the optimum pH 7.1–7.3 and 6.5–7.5 (Koku et al. 2002) respectively.

**Temperature**: The temperature in photo-fermentation usually used in the range of 30–40 °C, *Rhodopseudomonas palustris* CQK 01 is noted as 27.5–32.5 °C (Wang et al. 2010), *Rhodobacter capsulatus* JP91 and IR3 the optimum temperature was noted as 30 °C (He et al. 2006). If the temperatures are alter slightly hydrogen production and substrate conversion efficiency declined.

**Light Intensity**: Light is an important parameter that releases electrons from the reducing power and ATP synthesis. Sufficient availability of ATP plays a vital role

in $H_2$ production and required light energy principally at the wavelengths of 522, 805 and 850 nm (Chen et al. 2006). The most favorable illumination strength for the biohydrogen rate of production and yield was 10,000 lux (Argun and Kargi 2010).

**Cofactors**: Iron and molybdenum are important cofactors if supplied in appropriate quantities to the medium in photo-hydrogen production. These microelements are essential for the synthesis of nitrogenase in appropriate quantities (Koku et al. 2002). Lee and Yu (2005) reported that the increase in iron concentration in the medium improved the efficiency of the production of the photo-hydrogen, whereas with molybdenum, the efficiency of the production was decreased. However, iron supplementation and small quantities of molybdenum are favorable for photo-hydrogen production.

## 6.6 Photobioreactor Design for Hydrogen Production

In the design of photo-bioreactor, three main parameters need to be considered: (1) appropriate use of light intensity and wavelength, (2) light conversion, and (3) maintenance of cell number in prolonged tenures. For light source, halogen and tungsten filaments are used in conventional reactors, but decrease in light intensity exponentially due to increased cell number and metabolic products. Ultimately light energy conversion efficiency has been decreased in conventional photo-reactors (Nakada et al. 1995). So, improving light conversion efficiency and maintaining cell concentration in continuous photo-bioreactor are a big challenge. To overcome these limitations and problems, the researchers are designed innovative photobioreactors for hydrogen production. Several photo-reactors with lots of modifications have been designed for efficient light energy conversion and maintenance of the cell culture in the reactor. They are: (1) solar energy excited optical fiber photobioreactors, (2) photobioreactors with immobilized cells, (3) the plate-type photobioreactors, and (4) the LED photobioreactors.

### *6.6.1 Solar Energy Excited Optical Fiber Photobioreactors*

The reactor container is illuminated with the sunlight transmitted through optical fiber. Chen et al. (2008b) proposed a photo-bioreactor, the sunlight was transmitted via side-light optical fibers, which were straightly immersed into the culture with higher light intensity and uniformed distribution. The Fresnel lenses capture the sunlight, the lens rotates toward the position of the sun, and transmits through the side-light optical fibers to gain the maximum light intensity inside the reactor. Further, internal illumination also fitted to maintain uniform distribution of light and intensity and also avoids the shielding effect of the cell density. With these

fitments with a bioreactor, they achieved higher $H_2$ production (22.7 mL $L^{-1}$ $h^{-1}$) and yield (62.3%) using *Rhodopseudomonas palustris*.

### 6.6.2 Photobioreactors with Immobilized Cells

In view of solving the problems and making innovative ideas in the design of reactors, workers also reported the interest to use the porous material as carrier of cells in the fermentation broth that enhances the cell growth and photo-$H_2$ production (Eroğlu et al. 2008). These carriers may show more surface active sites for biofilm formation which may lead to higher cell concentration. Further, these carriers also act as buffer system to protect from extreme conditions, i.e., high pH shock, $NH_3$-N concentration, organic acids may also be useful for the better operational stability of photo-bioreactor. *Rhodopseudomonas palustris* cells were immobilized with natural polymers, i.e., agar, alginate, agarose, k-carrageenan (Fißler et al. 1995). Another report noted that *Rhodobacter sphaeroides* were entrapped in a two-layer gel for cell growth and light penetration (Nakada et al. 1996). Porous glass beads were used to enhance the surface to volume ratio for cell attachment (Tsygankov et al. 1994), and achieved higher growth rate, and $H_2$ yield was 1.3 mL/h/mL porous glass and 75%, respectively. Tian et al. (2009) tried with polyvinyl alcohol-boric acid gel for the entrapment of *Rhodopseudomonas palustris* CQK 01 and achieved higher growth rate as 80.6 mL/g cell dry weight/h. Xie et al. (2010) revealed that co-culturing of entrapped *Rhodopseudomonas faecalis* RLD-53 with *Ethanoligenens harbinense* B49 achieves higher yields as 3.10 mol $H_2$/mol glucose. However, light penetration is hindered due to the high dense cell growth in carriers, leading to low light supply. This can be eliminated by the proper mixing of the culture medium to avoid the light blocking effects because of utilizing immobilized carriers in photo-bioreactors.

### 6.6.3 The Plate-Type Photobioreactors

These plate-type photobioreactors have superior features, i.e., small light path, great surface area illumination, low cost, and easy to clean. These plate-type photobioreactors show limitations by showing hydrodynamic stress due to difficulties to control the temperature and biofilm formation on bioreactor surface. Kondo et al. (2006) fabricated user-enabled grid, medium layer, and membrane to weaken a strong incident light and uniform diffusion in the photobioreactor to achieve the enhanced performance of $H_2$ production.

### 6.6.4 The LED Photobioreactors

Instead of halogen/tungsten lamps, LED light shows that narrow spectral band width and specific wavelengths are more advantageous for photosynthetic absorption of light. Using LED lights is a good strategy, as small as small and can fit easily in any photobioreactor for enhanced light energy conversion. Chen et al. (2006) explained that for photosynthetic bacteria, the major absorption bands are located at the wavelength range of 522–860 nm, and predominantly the wavelength range 800–900 nm is a requisite, which is necessary for ATP-consuming photo-hydrogen production. Further, LED lights have other benefits such as low energy consumption, long life, low heat generation, and cheap. Tian et al. (2009) reported that LED is used as light source for $H_2$ production by using *Rhodopseudomonas palustris* CQK 01, to attain the greatest production rate, yield, and light conversion as 3.816 mmol/$m^2$/h, 0.75 mol $H_2$/mol glucose, and 3.8%, respectively. The process conditions in the production of bio-$H_2$ with microalgae as feedstock via photolysis are summarized in Table 6.3.

## 6.7 Biomass Pretreatments Influence the $H_2$ Production

Before the extraction of cellular contents from the cells, the biomass undergoes disruption process; through the pretreatment, it is easy to disrupt the cells. In biodiesel production, several techniques were used for the disruption of cells to integrate the lipids to outside environment including chemical methods. Here, in the production of bio-$H_2$ release, the cellular biomolecules hydrolyze the complex sugar molecules of homo- and heteropolysaccharides, which is an important process. In this process, the dehydration of sugars and the development of inhibitors like furfural and HMF (5-hydroxymethylfurfural) should be avoided. Generally, acid pretreatments are practiced in the process; this may release the inhibitors and reduce the $H_2$ production efficiency. Yun et al. (2013) conducted pretreatment experiments on *Chlorella vulgaris* biomass and explained the relation between the concentration of dilute acids and HMF formation. They observed that the concentration of HCl increased from 0.1 to 3% which leads to enhanced HMF concentration from 0.23 to 4.3 g/L. They also optimized the conditions, i.e., 1.6% HCl for 35 min thereby increased the $H_2$ production to 36.5 mL/gTS.

Several physical methods are also employed in the cell disruption process, i.e., sonication, microwave irradiation, autoclaving, lyophilization, and ultrasonication. Jeon et al. (2013) reported that the sonication of biomass *Scenedesmus obliquus* for 15 min enhanced 12% sugar solubilization than non-sonicated biomass. Xia et al. (2013b) explained the importance of the temperature effect on the addition of acid hydrolysis, and they found that on acid hydrolysis at 140 °C the reduced sugar recovery was (0.29 g/gTS) higher than the achieved (>0.05 g/gTS) at 100 °C. Nayak et al. (2014) explained about the *Anabaena* sp. biomass pretreatment by amylase

**Table 6.3** $H_2$ production from microalgae feedstock via photolysis

| Environmental conditions | Microalgae and strain | $H_2$ yield | References |
|---|---|---|---|
| Flat glass PBR, sulfur supplement (25 μM) in TAP-S medium, 300 μE/$m^2$/s, argon sparged | *Chlamydomonas reinhardtii* cc-124 | 5.94 μmol mg/chl/h | Kosourov et al. (2002) |
| Flat plate PBR, argon sparged, TAP-S medium, 120 μE/$m^2$/s | *Chlamydomonas reinhardtii* Immobilized CC-1036 pf18 mt+ | 6.4 μmol/mg/chl/h | Laurinavichene et al. (2006) |
| Flat plate PBR, 70 μmol/$m^2$/s in TAP-S medium | *Chlamydomonas reinhardtii* strain L159I-N230Y | 5.77 mL/L/h | Torzillo et al. (2009) |
| Flat panel PBR, medium: BG 11, argon/$N_2$ (20/80) and 100% argon sparged, 44 μmol/$m^2$/s | *Nostoc* PCC 7120 Δ hup W | 0.71 mmol/mg/chl/h | Nyberg et al. (2015) |
| Indoor helical tubular PBR, Allen and Arnon medium, 12 h/12 h light and dark cycles, air +2% $CO_2$, 332 μE/$m^2$/s | *Anabaena variabilis* PK84 | 19.2 mL/h/PhBR | Borodin et al. (2000) |
| Indoor tubular PBR, argon sparged, medium: BG 11, 456 μE/$m^2$/s | *Anabaena* PCC 7120 | 1.4 mL/h/PhBR | Lindblad et al. (2002) |
| Indoor tubular PBR, argon sparged, medium: BG 11, 456 μE/$m^2$/s | *Anabaena* AMC 414 | 13.8 mL/h/PhBR | Lindblad et al. (2002) |
| BG 11 with optimized nutrients, $N_2$ atmosphere, dark condition | *Synechocystis* sp. PCC 6803 | 0.81 μmol/mg/chl/h | Burrows et al. (2008) |
| Glass vial, 750 mM β-mercaptoethanol, BG11-S, argon sparged, 24 h dark | *Synechocystis* sp. PCC 6803 | 14.32 μmol/mg/chl/min | Baebprasert et al. (2010) |
| Glass reactors, medium with benzoate (600 mg/L), argon sparged, 4000 lx | *Lyngbya* sp. | 17.05 μmol/gchl 1/h | Shi and Yu (2016) |
| Erlenmeyer flask, 30 μmol/$m^2$/s for 18 h, BG 11medium (nitrogen-deprived) | *Aphanothece halophytica* | 13.8 μmol/mg/chl/h | Taikhao et al. (2013) |
| Glass bottles, Tris–HCl buffer medium, argon sparged, 24 h dark | *Gloeocapsa alpicola* | 25 μL/h/mg dw | Troshina et al. (2002) |
| Outdoor tubular PBR, Allen and Arnon medium, air +2% $CO_2$, sunlight | *Anabaena variabilis* PK84 | 45.8 mL/h/PhBR | Tsygankov et al. (2002) |

along with thermophilic fermentation produced 1600 mL/L hydrogen. The hydrogen (1600 mL/L) achieved was 0.3-, 2.6-, and 8.0-folds higher than that obtained from other pretreatments, i.e., dilute acid, high temperature (autoclave), and sonication, respectively. Several pretreatments with process conditions along with $H_2$ yields are shown in Table 6.4.

**Table 6.4** Pretreatments of microalgae biomass in $H_2$ production process

| Process conditions | Microalgae | Pretreatment | $H_2$ yield | References |
|---|---|---|---|---|
| 35 °C, heat-treated activated sludge, batch process | *Nannochloropsis oceanica* | 1% $H_2SO_4$, 140 °C microwave for 15 min | 39 mL/gVS | Xia et al. (2013b) |
| 35 °C, heat-treated activated sludge, batch process | *Arthrospira platensis* | 1% $H_2SO_4$, 140 °C microwave for 15 min, glucoamylase degradation | 96.6 mL/gTS | Cheng et al. (2012) |
| 35 °C, heat-treated activated sludge, batch process | *C. vulgaris* | Hydrolytic extracellular enzyme solution | 43.1 mL $H_2$/gTS | Yun et al. (2014) |
| 35 °C, heat-treated activated sludge, batch process | *C. vulgaris* | Cellulase, pectinase, and hemicelluloses degradation | 135 mL/gVS | Wieczorek et al. (2014) |
| 35 °C, heat-treated activated sludge, batch process | *C. vulgaris* | 1.6% HCl, 35 min | 36.5 mL/gTS A | Yun et al. (2013) |
| 35 °C, heat-treated activated sludge, batch process | *Cyanobacterial* blooms | pH 13 (6 mol/L NaOH) for ½ h | 94 mL/gVS B | Cai et al. (2015) |
| 35 °C, heat-treated activated sludge, batch process | *Scenedesmus obliquus* | Ultrasonication for 15 min at 45 °C | 56 mL/g biomass | Jeon et al. (2013) |
| Batch, 37 °C, *Clostridium butyricum* | *Scenedesmus obliquus* | Dried (80 °C, 16 h) and autoclaved (121 °C, 15 min) | 90.3 mL/gTS | Batista et al. (2014) |
| Batch, 58 °C, *C. butyricum* DSM 10702 | *Scenedesmus obliquus* | Dried at 70 °C, ground (<0.5 mm) | 116.3 mL/gVS | Ortigueira et al. (2015a) |
| Batch, 30 °C, *Enterobacter aerogenes* | *Scenedesmus obliquus* | Dried and autoclaved (121 °C, 15 min) | 56.8 mL/gVS | Batista et al. (2015) |
| 35 °C, heat-treated activated sludge, batch process | *Cyanobacterial* blooms | 170 °C for 20 min | 113 mL/gVS | Cai et al. (2015) |
| Semicontinuous, 35 °C, Heat-treated anaerobic sludge | *Chlorella pyrenoidosa* | 1% $H_2SO_4$, 135 °C for15 min | 56.1 mL/gVS | Xia et al. (2014a) |
| Batch, 35 °C, *C. butyricum* CGS5 | *C. vulgaris* ESP6 | 1.5% HCl, 121 °C, 20 min | 81 mL/gTS | Liu et al. (2012) |
| 35 °C, heat-treated activated sludge, batch process | *C. vulgaris* | 0.79% HCl, ultrasonication (49,600 J/gTS) | 41.2 mL/gTS | Yun et al. (2013) |

## 6.8 Other Environmental Factor Influence on $H_2$ Production

### 6.8.1 Effect of Thermophilic Conditions

In thermophilic conditions, the $H_2$ yield ranges from 56 to 135 mL/gVS, and when compared with mesophilic conditions, the yield was noted in the range of 36.5–113 mL/gVS. Jeon et al. (2013) evaluated the temperature conditions with the same biomass and pretreatment methods and reported that the $H_2$ yield was higher in thermophilic than the mesophilic conditions. Liu et al. (2013) used mixotrophic *C. vulgaris* for $H_2$ production on dark fermentation effluent and exhibited the same carbohydrate concentration and $H_2$ yields than autotrophic microalgae.

### 6.8.2 Effect of Batch, Sequencing Batch, and Semicontinuous Reactions

The dark fermentation (DF) batch system completes by hours to days. Ortigueira et al. (2015a) evaluated the $H_2$ production in dark fermentation using *Clostridium butyricum* with *S. obliquus* as feedstock and found that complete sugar exhaustion occurred after 24 h, and maximum $H_2$ production was noted at 48 h. Further, *Spirogyra* biomass pretreated with dilute acids and at higher temperatures hydrolyzed in a batch and SBR (sequencing batch reactor) yielded similar $H_2$ production (Ortigueira et al. 2015b), semicontinuous process showed as potential irrespective of operation mode.

### 6.8.3 Presence of Methanogenic Microorganisms

In dark fermentation, methanogenic bacteria are present as an inoculum, due to this methane also produced frequently. In case of untreated biomass at wet condition after inefficient inoculum treatment, methane was produced rather than $H_2$ production (Kumar et al. 2016). Several researchers have reported that methane production from residuals of dark fermentation is noted in the range of 109–394 mL $CH_4$/gVS (Yang et al. 2011; Cheng et al. 2014; Xia et al. 2013a). Frigon et al. (2013) reported on methane production using *Scenedesmus*, which yielded 410 mL $CH_4$/gVS, whereas in case of mixed microalgae in secondary effluents, methane production reached 390 mL $CH_4$/gVS (Cea-Barcia et al. 2015). By photofermentation (Xia et al. 2013a; Cheng et al. 2012; Kim et al. 2006) and by anaerobic digestion (Cheng et al. 2014; Xia et al. 2013a; Yang et al. 2011), several gaseous biofuels can be produced,

and a combination of these above two techniques enhanced $H_2$ yield as 337 mL $H_2$/g DW using *Arthrospira platensis* biomass.

## 6.9 Bioactive Compounds

### *6.9.1 Introduction*

Manufacturing nutritious food from algae is an interesting alternative technology and is essential. As we discussed in previous chapters about the components present in the microalgae, it has been suggests that there is a need to develop and synchronize the technology for the commercial production of nutraceuticals from microalgae which is already existed. Several researchers also discussed about the essentiality, development of technology, and the production of high-value products from microalgae (Spolaore et al. 2006; Enzing et al. 2014; Leu and Boussiba 2014). The important bioactive compounds along with other fractions from the cultivation of microalgae are illustrated in Fig. 6.1.

Microalgae-based biofuel and biohydrogen production units are facing several difficulties as they are incapable to get superior revenue due to declining biomass production, increased production, and operational costs. Richardson et al. (2014) assessed the financial and economic viability of biofuel firms, and they suggested that co-production of high value-added products with biofuel production will be the

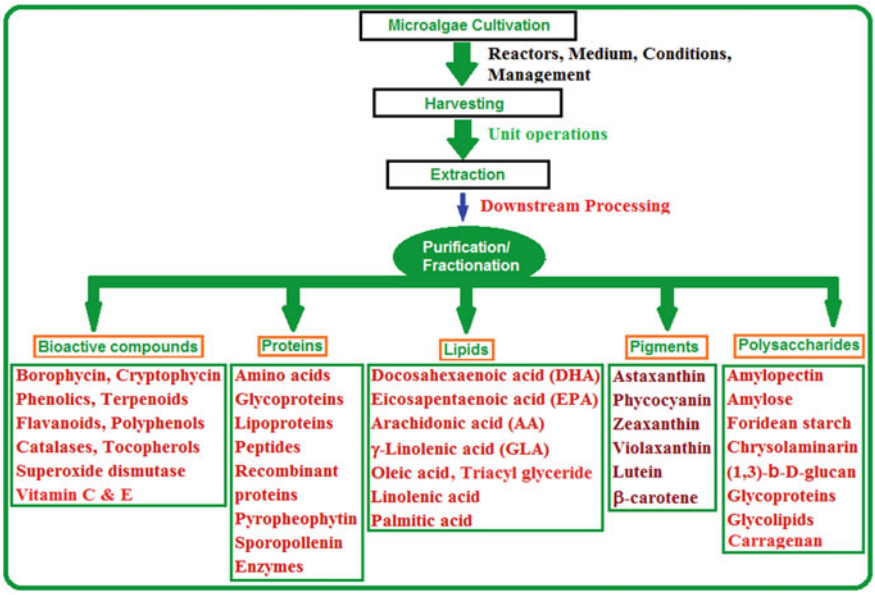


**Fig. 6.1** Flowchart of the bioactive compounds from microalgae

best for industries; they can grow and run with good potential of financial and economic feasibility. Microalgae secondary metabolites are produced upon the exposure of environmental stress conditions as they do not have specific function in the cell, but they can exhibit special characteristics. Astaxanthin, canthaxanthin, beta-carotene, lutein, zeaxanthin, lycopene, etc. are produced from microalgae and are extensively used in cosmetics, foods, and as supplements. By 2021, the carotenoid market is predicted to enhance from $1.24 billion USD to above $1.53 billion USD (Markets and Markets 2017).

### *6.9.2 Various Bioactive Compounds*

Various bioactive compounds in purified form have been reported as a good resource from microalgae, i.e., sulfated polysaccharides (de Jesus Raposo et al. 2013), omega-3 fatty acids (Haimeur et al. 2012), carotenoids (astaxanthin, fucoxanthin, carotene) (Gammone et al. 2015; Peng et al. 2011), marennine (Pouvreau et al. 2008), and polyphenols (Goiris et al. 2012). Among them few metabolites have been verified as exhibited biological activities like anticancer, potent antioxidant, antiviral, and anti-inflammatory activities (Lauritano et al. 2016; Xia et al. 2014b; Lee et al. 2006). Therefore, microalgae and its derived products have immense potential in human diet as supplements for the treatment, management, and prevention of abnormal physiological as natural source alternative to synthetically derived dietary supplements (Beetul et al. 2016). Biotin, vitamin $B_2$ (riboflavin), vitamin $B_3$ (nicotinic acid), vitamin $B_5$ (pantothenate), and vitamin $B_9$ (folic acid) (Manoj et al. 2018). Almost all vitamins are useful in the energy generation, as co-enzymes, and in the prevention of liver disease, pellagra, and ulcerative colitis. Antioxidants such as catalases, polyphenols, superoxide dismutase, and tocopherols are also very rich in microalgae and are also used as antioxidant supplements (Table 6.5).

Microalgae is a good resource for vitamins, i.e., vitamin A (retinol), vitamin $B_1$ (thiamine), vitamin $B_6$ (pyridoxine), vitamin $B_{12}$ (cobalamin), vitamin C (ascorbic acid), vitamin E. Several polyunsaturated fatty acids (PUFA), i.e., docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), arachidonic acid (AA), γ-linolenic acid (GLA), etc., are produced by microalgae which are essential fatty acids used for nutritional supplements, infant formulas, and in aquaculture. Various kinds of carotenoids or pigments are synthesized by microalgae, i.e., β-carotene, astaxanthin, lutein, zeaxanthin, canthaxanthin, chlorophyll, and phycocyanin. β-Carotene used in vitamin A deficiency, astaxanthin used in the treatment of Alzheimer's disease, and lutein and zeaxanthin both are utilized in the management of eye diseases.

In several species of microalgae, whole biomass containing huge protein content, health benefits along with good nutritional values is considered as "super foods" in the market (Pulz et al. 2013). *Arthrospira* formerly noted as *Spirulina* is being marketed in developing countries as well as developed countries as it contains γ-linolenic acid, phycocyanin, and rich and quality proteins. Further, several researchers revealed that it exhibits antioxidant, anti-inflammatory, anticancer, and

**Table 6.5** Bioactive compounds like pigments and antioxidants from microalgae

| Name of the algae | Bioactive compound | References |
|---|---|---|
| *Dunaliella salina* | β-Carotene | Fujitani et al. (2001) |
| *Haematococcus pluvialis* | Astaxanthin | Ciccone et al. (2013) |
| *Chlorella zofingiensis* | Astaxanthin | Ciccone et al. (2013) |
| *Chlorella zofingiensis* | Astaxanthin | Markou and Nerantzis (2013) |
| *Scenedesmus* sp. | Lutein | Sánchez et al. (2008) |
| *Muriellopsis* sp. | Lutein | Del Campo et al. (2001) |
| *Chlorella sorokiniana* | Lutein | Cordero et al. (2011) |
| *Nostoc linckia* | Borophycin, cryptophycin | Singh and Dhar (2011) |
| *Nostoc spongiaeforme* | Borophycin, cryptophycin | Singh and Dhar (2011) |
| *Spirulina platensis* | Phenolics, terpenoids, alkaloids, phycobilins | Singh and Dhar (2011) |
| *Spirulina maxima* | Phenolics, flavonoids | AbdEl-Baky et al. (2009) |
| *Nostoc muscorum* | Phenolics, terpenoids, alkaloids, phycobilins | Mostafa (2012) |
| *Isochrysis* sp. | Phenolics | Goiris et al. (2012) |
| *Chlorella vulgaris* | Phenolics | Cha et al. (2011) |
| *Nannnochloropsis* | Phenolics | El-Baky et al. (2009) |
| *Arthrospira platensis* | Phycocyanin | Romay et al. (2003) |
| *Chlorella pyrenoidosa* | Phycocyanin | Romay et al. (2003) |
| *Chlorella stigmatophora* | Phycocyanin | Guzman et al. (2003) |
| *Spirogyra* sp. | Phycocyanin, polysaccharides, phenolic acid, tocopherols | Amaro et al. (2013) |
| *Spirulina fusiformis* | Diacylglycerols, astaxanthin, lutein, β-carotene, zeaxanthin | Singh and Dhar (2011) |
| *Haematococcus pluvialis* | Lutein, β-carotene, zeaxanthin | Markou and Nerantzis (2013) |
| *Chlorella* sp. | Carotenoids, sterols, PUFs | Ibáñez and Cifuentes (2013) |
| *Chlorella ellipsoidea* | Zeaxanthin, violaxanthin | Amaro et al. (2013) |

antiviral activities (Abu Zaid et al. 2015; Tang and Suter 2011; Hernández-Corona et al. 2002). In case of green algae, *Chlorella* extract contains vitamins, amino acids, minerals, glycoproteins, minerals, β-glucans, and nucleic acids and is considered as "growth factor" (Liu and Hu 2013). This *Chlorella* extract also confirmed the

activities of antitumor, antioxidant, antibacterial, and also declining cholesterol range in the body (Reyna-Martinez et al. 2018; Medina-Jaritz et al. 2013; Ryu et al. 2014).

### 6.9.3 Peptides and Polyunsaturated Fatty Acids

Long-chain polyunsaturated fatty acids (PUFs) are required for normal physiology for humans, and lack of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the diet leads to damage in cardiovascular health, poor fetal development during pregnancy, enhanced inflammatory processes, and Alzheimer's disease. Fish oil is a rich source for the PUFs, alternatively microalgae are rich sources for the production of PUFs, i.e., EPA and DHA.

Few microalgae that are able to produce EPA include *P. tricornutum*, *Nitzschia* sp., *Monodus subterraneus*, and *Nannochloropsis* sp. Some of the microalgae reported are rich sources for DHA, i.e., *Isochrysis galbana*, *Thraustochytrium* sp., *Schizochytrium* sp., and *Aurantiochytrium* sp. The protein content in microalgae is rich and good quality with essential amino acids which in mammals cannot be synthesize. Further, some of the various sources of microalgae for the production of PUFs are summarized in Table 6.6.

### 6.9.4 Anti-inflammatory Agents from Microalgae

Various microalgae produced secondary metabolites as they can exhibit antioxidant role, and they are also potential anti-inflammatory agents. Various microalgae genera have also been reported about their chemical and anti-inflammatory compound release such are rhodophyta, chlorophyta, etc. Guzman et al. (2003) already noted that anti-inflammatory response in a rat paw edema test by two species, i.e., *Phaeodactylum tricornutum* and *Chlorella stigmatophora*. Sulfated polysaccharides were extracted from the *Porphyridium* sp., which exhibited anti-inflammatory activity comprising 10 sugars along with inorganic sulfate and glycoproteins. Matsui et al. (2003) explained that the anti-inflammatory activity of *Phaeodactylum tricornutum* and *Chlorella stigmatophora* extracts showed 25–30% activity when compared with the standard drug indomethacin. Few bioactive compounds with the microalgae sources are given in Table 6.7.

Fan and Chapkin (1998) also revealed the two species *Spirulina* and *A. flos-aquae* containing two important dietary factors in considerable amounts as ω-3-α and ω-6-γ linolenic acids which are involved in the inhibition of arachidonate compounds and prostaglandins. Few researchers worked on the *Chlamydomonas debaryana* biomass, extracted the oxylipin and lyophilized, and used for the anti-inflammatory activity on rat models (Lavy et al. 2003; Lee et al. 2013; Ávila-Román et al. 2016). Further, *Dunaliella bardawil* extract was used in acetic acid-induced rat models on

**Table 6.6** Bioactive compounds like peptides, polyunsaturated fatty acids, etc. from microalgae

| Name of the algae | Bioactive compound | References |
|---|---|---|
| *Dunaliella salina* | β-Carotene, linolenic and palmitic acid | Palavra et al. (2011) |
| *Botryococcus braunii* | Linear alkadienes ($C_{25}$, $C_{27}$, $C_{29}$, $C_{31}$, etc.) | Palavra et al. (2011) |
| *Porphyridium* sp. | Sulfated polysaccharides | Tannin-Spitz et al. (2005) |
| *Phaecodactylum tricornutum* | Sulfated polysaccharides | Guzman et al. (2003) |
| *Chlorella stigmatophora* | Water soluble extracts | Guzman et al. (2003) |
| *Phaecodactylum tricornutum* | Water soluble extracts | Guzman et al. (2003) |
| *Graesiella* sp. | Water soluble extracts | Trabelsi et al. (2016) |
| *Porphyridium purpureum* | Arachidonic acid | Su et al. (2016) |
| *Porphyridium cruentum* | Arachidonic acid | Giménez et al. (1998) |
| *Parietochloris incise* | Arachidonic acid | Solovchenko et al. (2008) |
| *Nannnochloropsis* sp. | Eicosapentaenoic acid (EPA) | Sukenik (1999) |
| *Phaecodactylum tricornutum* | Eicosapentaenoic acid (EPA) | Molina Grima et al. (1999) |
| *Chlorella minutissima* | Eicosapentaenoic acid (EPA) | Singh and Dhar (2011) |
| *Poryphyridium cruentum* | Eicosapentaenoic acid (EPA) | Asgharpour et al. (2015) |
| *Crypthecodinium cohnii* | Docosahexaenoic acid (DHA) | Horrocks and Yeo (1999) |
| *Schizochytrium* sp. | Docosahexaenoic acid (DHA) | Horrocks and Yeo (1999) |
| *Ulkenia* sp. | Docosahexaenoic acid (DHA) | Horrocks and Yeo (1999) |
| *Spirulina platensis* | Oleic acid, Linolenic acid, | Plaza et al. (2009) |
| *Chlorella pyrenoidosa* | Peptides | Wang and Zhang (2013) |
| *Nannnochloropsis oculata* | Peptides | Samarakoon et al. (2013b) |
| *Arthrospira maxima* | Peptides | Samarakoon et al. (2013a) |
| *Tetraselmis suecica* | Peptides | Samarakoon et al. (2013a) |
| *Botryococcus braunii* | Peptides | Samarakoon et al. (2013a) |
| *Porosira glacialis* | DHA, EPA | Artamonova et al. (2017) |
| *Attheya longicornis* | DHA, EPA | Artamonova et al. (2017) |

bowel inflammation, and it was found that it has anti-inflammatory activity (Lavy et al. 2003; Lee et al. 2013). Actual chemical compounds are unknown in the above extracts responsible for the anti-inflammatory activity. However, carotenoids, PUAs, PUFs, lipid-soluble phytochemicals, etc. reported in the literature have showed anti-inflammatory activity (Kaulmann and Bohn 2014).

**Table 6.7** Bioactive compounds from microalgae responsible for anti-inflammatory activity

| Microalgae | Bioactive compounds | References |
|---|---|---|
| *Porosira glacialis* | Docosahexaenoic, eicosapentaenoic acids | Ingebrigtsen et al. (2016) |
| *Attheya longicornis* | Docosahexaenoic, eicosapentaenoic acids | Ingebrigtsen et al. (2016) |
| *Cylindrotheca closterium* | Glycol- and phospholipid-rich fraction | Lauritano et al. (2016) |
| *Odontella mobiliensis* | Polyunsaturated aldehydes (PUAs) | Lauritano et al. (2016) |
| *Pseudo-nitzschia pseudodelicatissima* | Polyunsaturated aldehydes (PUAs) | Lauritano et al. (2016) |
| *Chlorella ovalis* | Polyunsaturated aldehydes (PUAs) | Samarakoon et al. (2013b) |
| *Nannochloropsis oculata* | Sterol-rich fraction of extract | Samarakoon et al. (2013b) |
| *Amphidinium carterae* | Polyunsaturated aldehydes (PUAs) | Samarakoon et al. (2013a) |
| *Tetraselmis suecica* | Polyunsaturated aldehydes (PUAs) | Jo et al. (2010) |
| *Chlamydomonas debaryana* | Oxylipin-containing extract | Ávila-Román et al. (2016) |
| *Dunaliella bardawil* | Oxylipin-containing extract | Lavy et al. (2003) |
| *Haematococcus pluvialis* | Astaxanthin | Ma and Chen (2001) |
| *Pavlova lutheri* | Mixture of MGDGs and DGDGs | Robertson et al. (2015) |
| *Isochrysis galbana* | Mixture of MGDGs and DGDGs | de los Reyes et al. (2016) |
| *Chlorella marina* | Lycopene | Renju et al. (2013) |
| *Dunaliella tertiolecta* | Phytosterols | Caroprese et al. (2012) |
| *Scenedesmus* sp | Xanthophyll family of carotenoids | Qin et al. (2008) |
| *Haematococcus lacustris* | 3,3′-Dihydroxy-β,β′-carotene-4,4′-dione) | Del Campo et al. (2004) |
| *Porphyridium* sp. | Sulfated polysaccharides | Ginzberg et al. (2000) |
| *Gyrodinium impudicum* KG03 | p-KG03, sulfated exopolysaccharide | Yim et al. (2005) |
| *Chlorella stigmatophora* | Hydrosoluble components, sterols | Guzman et al. (2003) |
| *Phaeodactylum tricornutum* | Hydrosoluble components, sterols | Guzman et al. (2001) |

### *6.9.5 Antibacterials*

Several bioactive compounds are present in the microalgae, and they exhibit antimicrobial activity. Several microorganism are showing drug resistance toward the antibiotics, and few are multidrug resistant which make the challenge to health professionals. Microalgae could fulfill this challenge and exhibits killing of microorganisms with minimal quantity of extract. Many studies reported that the secondary metabolites of microalgae could produce antibacterial and antifungal activities. Polyunsaturated fatty acids (PUFs) are produced from several microalgal strains, and *Dunaliella primolecta* and *Chlorococcum strain* have also produced PUFs which

exhibit antibacterial activity against methicillin-resistant *Staphylococcus aureus*. The expected mechanism for antibacterial activity is that bioactive fatty acids may inhibit the fatty acid synthesis in bacteria and bacterial membrane, leading to leak out the cellular contents. Najdenski et al. (2013) extracted exopolysaccharides and phycobiliproteins from red microalgae *Rhodella reticulata* and *Porphyridium aerugineum*, respectively, and tested for antibacterial activity against Gram-positive bacteria, *Streptococcus pyogenes* and *Streptococcus aureus* (Table 6.8).

### *6.9.6 Antiviral and Anticancer Activities*

Microalgae have more attention and interest toward the antiviral and anticancer activities. Most of the antiviral activities found in microalgae are due to the presence of sulfated polysaccharides and other bioactive compounds, and it is easy to extract the contents from the cells. Further, virions are worst pathogens causing several human diseases, and mortality is also high worldwide due to elderly and most of the immunosuppressive patients. Microalgae have significant value in the production of cytotoxic bioactive compounds and exhibit anticancer activity. Carotenoids and pigments already reported evidence on the antioxidant and anticancer activities. In this concern, astaxanthin, polyketides, and polyunsaturated aldehydes have much attention in the anticancer activity. A few bioactive compounds are listed along with the microalgae source in Table 6.9.

## 6.10 Microalgae Preservation

Preservation of algae species for the longer time is needed without contamination with bacteria, fungi, and virus. Algae suspension was centrifuged, and the paste was preserved by several methods: (a) preservation by lower temperature, (b) preservation by spray drying, (c) preservation by freeze drying, and (d) microencapsulation of algae.

### *6.10.1 Preservation by Lower Temperature*

Microalgae can preserve for longer periods, centrifuged microalgae suspension and the pastes are kept at low temperature +4 °C. The cooled microalgae *Skeletonema costatum* and *Chaetoceros calcitrans* pastes were preserved for longer periods successfully.

**Table 6.8** Bioactive compounds from microalgae with antibacterial and antifungal activities

| Name of the microalgae | Bioactive compound | Target microorganism | References |
|---|---|---|---|
| *Fischerella ambigua* PTCC1635 | Parsiguine | *Staphylococcus epidermidis* | Ghasemi et al. (2004) |
| *Nodularia harveyana, Nostoc insulare* | Norharmane (9H-pyrido(3,4-b) indole), 4,40-dihydroxybipheny | *Escherichia coli* ATCC8739, *Pseudomonas aeruginosa* ATCC9027 | Volk and Furkert (2006) |
| *Cyanobacterium* sp., *Cyanobium* sp., *Gloeotrichia* sp., *Phormidium autumnale* | Aeruginosin, cyanopeptolin, microcystin, cylindrospermopsin | *B. cereus, S. typhimurium, E. coli, P. aeruginosa, S. pasteuri, M. luteus* | Silva-Stenico et al. (2013) |
| *Skeletonema costatum* | Cylindrospermopsin | *Listeria monocytogenes* | Terekhova et al. (2009) |
| *Chlorella vulgaris, Dunaliella salina* | Algal extracts, phenolic compounds | *E.coli*, *Klebsiell*a sp., *Bacillus* sp., *Pseudomonas* | Syed et al. (2015) |
| *C. ellipsoidea, C. pyrenoidosa C. protothecoides* | Saturated and unsaturated Fatty acids | *Propionibacterium acnes* | Sibi (2015) |
| *Chlorococcum* HS-101 | Alpha-linolenic, polyunsaturated fatty acid | *B. subtilis, B. cereus, S. aureus, Enterobacteria* | Ohta et al. (1995) |
| *Chlorococcum humicola* | Pigments (carotenoid, chlorophyll) | *B. subtilis, S. aureus, E. coli* | Bhagavathy et al. (2011) |
| *Dunaliella salina* | β-Ionone neophytadiene | *P. aeruginosa* | Mendiola et al. (2008) |
| *Tetraselmis suecica* | Fatty acid | *Proteus* sp., *S. pyogenes* | Bai and Krishnakumar 2013) |
| *Porphyridium aerugineum* | Phycobiliprotein | *S. aureus, Salmonella typhi* | Najdenski et al. (2013) |
| *Chaetoceros muelleri* | Unsaturated fatty acid | *B. subtilis, S. aureus, E. coli* | Mendiola et al. (2007) |
| *Haslea karadagensis* | Marennine-like pigment | *Polaribacter irgensii, Pseudoalteromonas elyakowii* | Gastineau et al. (2012) |
| *Haslea ostrearia* | Marennine | *P. irgensii, P. elyakowii, V. coralliilyticus, V. tubiashii* | Gastineau et al. (2014a, b); Falaise et al. (2016) |
| *Amphidinium* sp. | Karatungiols | *Aspergillus niger* | Washida et al. (2006) |
| *Lyngbya majuscula* | Lyngbyabellin A, B | *Candida albicans* | Milligan et al. (2000) |
| *Pithophora oedogonium* | Ethanol extract | *Penicillium viridicatum* 1101 | Danyal et al. (2013) |
| *Gloeocapsa* sp. | Exopolysaccharides | *Candida albicans* | Najdenski et al. (2013) |

(continued)

**Table 6.8** (continued)

| Name of the microalgae | Bioactive compound | Target microorganism | References |
|---|---|---|---|
| *Haematococcus pluvialis* | Butanoic acid, methyl lactate | *Candida albicans* | Santoyo et al. (2009) |
| *Fischerella ambigua* PTCC1635 | Parsiguine (cyclic polymer) | *Candida krusei* ATCC 44507 | Ghasemi et al. (2004) |
| *Nostoc insulare* | Norharmane | *Candida albicans* | Volk and Furkert (2006) |
| *Nostoc* sp., *Anabaena* sp. | Hassallidin, scytophycin | *C. albicans, A. flavus* | Shishido et al. (2015) |
| *Nannochloris* sp. | Aponin | *Ptychodiscus brevis* | Halvorson et al. (1984) |

**Table 6.9** Bioactive compounds from microalgae with antiviral and anticancer activities

| Microalgae | Activity identified | Bioactive compounds | References |
|---|---|---|---|
| *Navicula directa* | Antiviral | Sulfated polysaccharide | Lee et al. (2006) |
| *Phaeodactylum tricornutum,* | Antiarrythmic | Adenosine | Prestegard et al. (2014) |
| *Haslea ostrearia* | Antibacterial, antiviral | Marennine | Gastineau et al. (2014a, b) |
| *P. tricornutum* | Anticancer | Ester nonyl 8-acetoxy-6-methyloctanoate (NAMO) | Samarakoon et al. (2014) |
| *Skeletonema marinoi* | Anticancer | Decadienal, octadienal, and heptadienal (PUAs) | Sansone et al. (2014) |
| *Nannochloropsis granulata* | Anti-inflammatory | Digalactosyldiacylglycerols and monogalactosyl | Blunt et al. (2015) |
| *Chlorella ovalis* | Anti-inflammatory | Digalactosyldiacylglycerols and monogalactosyl | Samarakoon et al. (2013a) |
| *Amphidinium carterae* | Anti-inflammatory | Digalactosyldiacylglycerols and monogalactosyl | Samarakoon et al. (2013a) |
| *Azadinium spinosum* | Allelopathic | Polyketides (PKSs) | Meyer et al. (2015) |
| *Alexandrium ostenfeldii* | Antipredator, allelopathic, anticancer | Polyketides (PKSs) | Meyer et al. (2015) |
| *Skeletonema marinoi* | Antiproliferative | Polyunsaturated aldehydes (PUA) | Lauritano et al. (2016) |
| *Coolia monotis* | Allelopathic, anticancer | Polyketides (PKSs) | Meyer et al. (2015) |
| *Prorocentrum lima* | Allelopathic and anticancer | Polyketides (PKSs) | Meyer et al. (2015) |

### *6.10.2 Preservation by Spray Drying*

This is a common method for the preservation of microalgae in aquaculture. Boeing (1997) reported on *Schizochytrium*, *Tetraselmis suecica*, and *Chaetoceros* without

significant loss of growth rate. In recent days, the spray drying is practiced for the preservation of microalgae *Spirulina* and *Haematococcus pluvialis*.

### 6.10.3 Preservation by Freeze Drying

Freeze drying is an efficient technique for long shelf life of algae preservation without loss of shape, size, and biochemical composition. Albentosa et al. (1997) performed the comparative study on *Phaeodactylum tricornutum*, *Isochrysis galbana,* and *Tetraselmis suecica* as seed culture of different ways of preservations. The above microalgae observed as the fresh, concentrated, freeze dried, and frozen can utilize as seed culture. In the case of *Thalassiosira pseudonana* concentrates, it was possible to extend the shelf life from a few days to more than 1 year, which makes it possible to utilize excess and off-season algal production.

### 6.10.4 Microencapsulation of Algae

Another way to preserve the microalgae for conservation is microencapsulation. It is the best technique that utilize microcapsules as enclosures without loss of the nutrients in the inside fluid medium. Joo et al. (2001) reported that four species, i.e., *Chlorella minutissima*, *Dunaliella bardawil*, *Pavlova lutheri*, and *Haematococcus pluvialis,* were immobilized in Ca-alginate capsules.

## 6.11 Economic Concerns to Circular Economy

Microalgae are potential sources for biofuels as feedstock and also for various types of pigments, biochemicals, etc. as renewable and can be cultivated throughout the geographical regions of the world. The productivity of various valuable products depend on the cultivation conditions and downstream processing. The waste water containing organic, domestic waste is considered as medium for the microalgae cultivation and produced several biofuels. This technology rises the circular economy as waste water acts as medium. For microalgae cultivation, non-agricultural land can be utilized; power plant gaseous effluents $CO_2$ and sun light can be utilized as a source of carbon energy source respectively for the production of biofuels and valuable products. Through this at the same time reduce the greenhouse effect, effective utilization of land and waste water treatment could rise into valuable products and met the circular economy principles. A commercial plant was established in Sarnia, Ontario, with this technology as the project named "Solutions4$CO_2$" with the aim of capturing $CO_2$ and waste water as nutrients in

the area of 50,000 $ft^2$. They are producing omega-3 fatty acids for biopharmaceuticals and biofuel production (advancedbiofuelsusa 2020).

According to various researchers, the economic viability of the use of microalgae as renewable source of biofuels and eco-friendly feedstock depend on upgrading the technology for enhanced production with low cost price of biodiesel (Wijffels and Barbosa 2010; Bleakley and Hayes 2017). Norsker et al. (2011) explained the economic analysis on the usage of microalgae for the production, mainly concentrated on the type of bioreactor to achieve enhanced biomass productivity, lipid productivity, and biofuel. In Europe, the weather condition favors the use of tubular photobioreactor which is the most economic as the sum of capital investment and operational costs around a unit is noted as 4.15 €/kg (dw) of algal biomass. Further, another important parameter is downstream processing which is occupies the maximum cost for purification. Conventional solvent are toxic and maximum cost occupying in the total cost of the product, hence revise and think about the use of green solvents with eco-friendly nature with high lipid quality with limited steps in the process. The recyclable green solvents can be used in single step, low energy required, time saving and low or no by-products. In the same way, transesterification process can directly extract the lipids in single or two steps which actually reduce the total cost in DSP. This economic viability mainly depend on the cost of microalgae biomass production, downstream processing. Microalgae biomass productivity, extraction, and conversion of biochemicals, fuels, and intermediates under optimized conditions will be economically feasible. In this background, production of bio-oil, biofuels, bio-chemicals from microalgae is a promising economic viability and rose the circular economy.

### *6.11.1 Future Prospective in Microalgal Research for Biofuels*

The biorefinery idea and implementation in downstream processing may increase the sustainable biofuel production, biochemicals, and biomaterials, decreasing the production cost, ultimately leading to improved economic viability. Various works are in progress in genetic transformation of microalgae area to enhance the biomass productivity and product accumulation in the microalgal cells. The most attractive solution is to use genetically engineered algae with high biomass productivity and production accumulation. The first work reported was on red marine seaweed, *Porphyra yezoensis* (Cheney et al. 2001). Recent efforts also reflect the genetically improved microalgae especially fresh water algae, *Chlamydomonas reinhardtii* (Lauersen et al. 2016; Morales-Sanchez et al. 2017). The French Institute for

Research and Automatic Control (Inria), led by Dr. O. Bernard (The Biocore Team), has been developed evolution engineering for enhanced productivity.

Several countries have made their policies feasible to renewable energy production and implemented their prestigious projects for biofuels production using microalgae as feedstock. Brazil has recently introduced tax exemptions and loans for biodiesel production using microalgae as feedstock in non-arable lands (dos Santos et al. 2017). The USA has introduced Energy Independence and Security Act (EISA 2007) which offers loans and grants for commercialization of microalgae-based biodiesel production. Several research grants (2015) were released from EU for implementing a BIOFAT project worth €10 million in Italy and Portugal. Lee (2011) predicted that algal biofuel could supply 7.1% of developed world fuel demand by 2040 and 0.5% in developing countries with strong government support. Kovacevic and Wesseler (2010) estimated that algae biofuel could cost €51.60/GJ by 2020 if technological advancements remained linear and crude oil remained at $100/barrel. Bio-$H_2$ manufacturing plants essentially needed technological breakthroughs, realization, rational investment cost (machinery and infrastructure), and operational and management cost (Riis et al. 2006).

### *6.11.2 Future Prospects on Bioactive Compounds*

All the above are commercially value-added products, used in medical and nutritional purposes. The main obstacles in the separation of valuable products are energy requirements for the processes, downstream processing, and investment costs. These are causing for the high cost for the production, further the conventional downstream processing technology targets only one type of component and damage the others. For example, several techniques used for the extraction of lipids cause denaturation of proteins present in the microalgae cells (Marieke et al. 2013). There is an emergent requirement to fulfill this gap; research is needed to separate all the valuable products at once (mild extraction), and at later stages, it is required to invent the processes which are suitable for segregation and recovery of all valuable products without damage of other components. This may be reduce the cost of the product, decrease the damage of the other components and also available the products containing good quality and sufficient quantities in the market. Still there is a growing interest in addressing the biological and economic viability challenges connected to the large-scale cultivation and biorefinery process to make sure the sustainable production of bioactive compounds with health benefits.

## 6.12 Conclusion

Several methods available for the preservation of microalgae strains have been explained. Production of lipid, conversion technology of various factors affecting the lipid production, and biomass productivity are clearly explained. Production of biofuels like bio-ethanol, syngas, and bio-oil using microalgae as feedstock has been tabulated well. The basic concepts of the various products along with hydrogen production are depicted. Various conditions, medium and special photobioreactors used for the $H_2$ production are explained well. Overall, the use of microalgae as biofuels feedstock is technically sound and economically feasible. Here prospects and challenges are also discussed well for the better biofuel production using microalgae as feedstock. The basic concepts in biohydrogen production and bioactive compounds reported in the literature are tabulated with source microalgae and are explained well. Economical concerns with circular economy and feasibility of commercial production for replacing the fossil fuels are expected to be studied hopefully in the coming decade.

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# Chapter 7
# Algal Biofuels: An Economic and Effective Alternative of Fossil Fuels


**Nisha Bhardwaj, Komal Agrawal, and Pradeep Verma**

**Abstract** Microalgae, cyanobacteria are vital organisms for sustainable production of various value-added products, e.g. food, chemicals and biofuels are also well known to meet out high energy requirements. These organisms can be a sustainable tool for reducing our current dependency on fossil fuels with growing world populations and environmental concerns. In recent times, the huge exploitation of algae as third-generation feedstocks for producing biofuels, e.g. biodiesel, biohydrogen, bioethanol and bioethanol, are underway. The biofuels have similar combustion properties, the energy content that is present in the fossil fuels furthers their transportation, and the storage is well suited with the existing infrastructure. The metabolic and genetic engineering of algal cultures can be manipulated for the advancement in the development of promising strains to produce alternative biofuels. This chapter includes the detailed account of various aspects of biofuel production using valuable algal feedstock, such as open and closed cultivation, stock availability, intercellular components (carbohydrates and lipids, etc.), challenges and future prospective.

**Keywords** Algae · Cultivation · Feedstock · Value-added products · Biofuel

## 7.1 Introduction

The depletion of fossil fuels with the rise in global energy demand (70%, whereas only 30% electricity) has led to countless initiations towards finding an efficient alternative for fuel used in manufacturing, transportation, and domestic applications. The use of fossil fuels, oil extraction and natural gas has been considered the society's primary resource of energy which eventually leads to global warming

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(Change 2014). Hence, the bio-based methods can serve as an effective alternative that can convert biomass into economic and sustainable value-added by-products. Biofuels are the fuels obtained from biological sources, e.g. plants, microorganisms and animals (Chowdhury and Loganathan 2019). The first-generation biofuels are obtained from edible agricultural crops and the second-generation biofuels from non-edible feedstock. As the feedstocks are non-sustainable and less economically feasible, the third-generation biofuels obtained from algal biomass gained more importance as it is more viable and sustainable as compared to other sources which have limited economic requirements (Chowdhury and Loganathan 2019). In contrast to land plants, algae are fast-growing microorganisms (100 times faster reproduction capability) that accumulate lipids inside the cells of some algal strains which are used for bioconversion into biodiesel (Mutanda et al. 2011; Pittman et al. 2011). Algal biofuel does not affect the food safety concern, and there is no competition for agricultural land area as it does not require fertile land; further, it can be simultaneously processed with the treatment of wastewater as it also contains good amount of important nutrients, which eventually make the process sustainable (Chisti 2007; Mata et al. 2010). Apart from these advantages, after the extraction of lipids, the residues from algal biomass can be utilized to produce biohydrogen, bioethanol and biomethane (Harun et al. 2010). The genetic manipulation is quite easier with the microbial resources to produce highly competent strains (Khan and Fu 2020). Among various studied microorganisms, algal resources are more commonly used for biodiesel and bioethanol production, as they can easily grow in open ponds, simple Continuous Stirred Tank Reactor (CSTR) system or large photobioreactors (Hulst 2013). Various end-products such as feedstocks, pigments, enzymes and pharmaceuticals are produced during biofuel generation process (Mata et al. 2010). As a result, biorefinery process carries double advantages in waste management and generating useful end-products. Therefore, a detailed study in the field of biofuel production with all the recent technologies will be useful for the researchers focusing on the biorefinery aspects. It includes various aspects of biofuel production using algal feedstocks, an open and closed cultivation system, intercellular components (carbohydrates and lipids, etc.), challenges and future prospective (Fig. 7.1).

## 7.2 Sources of Algal Biomass

Algae are aquatic photosynthetic organisms that emit oxygen. The structure of algae cannot be easily defined as it does not fit under a single monophyletic group although it is simple structure with no roots, leaves or stem, unlike land plants. They occupy specific habitat as they are a group of universal but individual species. They are found in different forms, e.g. attached with plant substrates, some in motile forms like animals, in suspended water, loosely grown on trees, animals, and soil and also in symbiotic association with another organism such as corals and lichens (Kumar et al. 2019). Algae consist of 40% lipids which is rapidly converted to biofuel, thus it

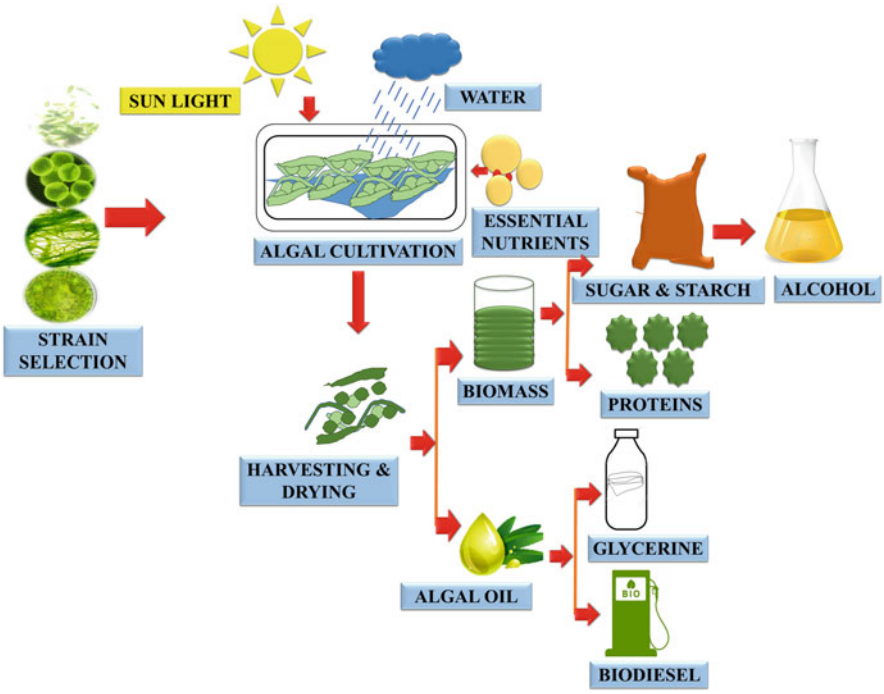


**Fig. 7.1** Various aspects of algal biofuel production process

leads to the economic and environmentally friendly process and also algae cultivation is easy, hence making the process globally attractive (John et al. 2011). The classification of biomass obtained from algae can be done as sugar, triglycerides, and proteins that can be used for the production of value-added products. Therefore, biorefinery can be used as an efficient replacement of petroleum refinery (Kumar et al. 2019).

## 7.3 Micro- and Macroalgae

Microalgae consisting of diatoms, golden and blue or green algae and macroalgae involve green, brown and red seaweeds, bacteria and yeast. All these organisms have specific characteristics to remove greenhouse gas and adsorb $CO_2$. The biofuel production using microalgae includes various steps such as the selection of species-specific microalgae cultivation, pre-treatment, harvesting including acid transesterification, anaerobic digestion and separation of products (Assacute et al. 2018). Macroalgae are also called as seaweeds, commonly available in the intertidal region and subtidal region of the ocean. Similar to microalgae, macroalgae are also selected, cultivated and harvested, followed by the extraction of biodiesel, enzyme

**Table 7.1** Algal culture currently used for biofuel production

| Algal culture | Products | References |
|---|---|---|
| *Chlorella vulgaris* | Biofuel and by-products | Cheung et al. (2020) |
| Natural algae from Taihu lake | Bioethanol | Zhou et al. (2020) |
| *Desmodesmus* sp. ASK01<br>*Chlorella* sp. ASK14<br>*Scenedesmus* sp. ASK16<br>*Scenedesmus* sp. ASK22<br>*Chlorella* sp. ASK25<br>*Chlorella* sp. ASK27 | Biofuel production, feedstock and dairy effluent treatment | Pandey et al. (2019) |
| *Chlorella vulgaris* YH703 | Biofuel | Yun et al. (2019) |
| *Nannochloropsis oculata*<br>*Euglena gracilis*<br>*C. protothecoides*<br>*C. sorokiniana* | Two-stage biofuel production | Nagappan et al. (2019) |
| *Chlorella vulgaris* | Microalgal cultivation and wastewater polishing | Chong et al. (2019), Zhu et al. (2019) |
| *Chlorella sorokiniana* | Enhanced production of algal biomass and microbial fuel cell power generation | Das et al. (2019) |

production, fermentation of alcohol and anaerobic digestion. The complexity of macroalgae is more as compared to microalgae (Assacute et al. 2018) (Table 7.1).

## *7.3.1 Microalgae*

### 7.3.1.1 Chlorella

It is one of the well-known green microalgae containing high protein content and can be utilized for the consumption by humans and when grown under stress conditions can accumulate lipids in the large amount (Guccione et al. 2014). The cost of cultivation of *Chlorella* is more, due to its open pond growing condition under photoautotrophic situations (Ramaraj et al. 2016). In 2000, the major autotrophic production of algae was initiated, the system used for the process was in glass tubing (500 km) in Klotz, Germany, and approximately 100 tonnes of *Chlorella* was produced annually (Pulz and Gross 2004). *Chlorella* species comprises high starch

content which can be exploited for bioethanol production in the presence of 50% sulphur (Brányiková et al. 2011). *C. vulgaris* and *C. pyrenoidosa* are two main species which are used commercially (Brányiková et al. 2011). In addition to high starch content, C*hlorella* biomass is also rich in minerals, carbohydrates and proteins, which can be further utilized for the other value-added products' production after the extraction of biofuel (Brennan and Owende 2010).

#### 7.3.1.2 *Botryococcus braunii*

It is a pear-shaped, bloom-forming green microalga that grows in the form of cluster and can be utilized for the biodiesel production, e.g. *B. braunii* which secretes lipid in the extracellular medium. Blooming is responsible for high quantity and quality of biodiesel production (Lassing et al. 2008). The strain selection depends on the production of lipids in high amounts; however, its growth is very slow when not grown under optimized conditions, in contrast to microalgae which have cytoplasmic lipids (Hirose et al. 2013).

#### 7.3.1.3 *Pleurochrysis carterae*

It is a unicellular microalga with an unusual capability of calcification which occurs at subcellular level for the production of calcified scales. *P. carterae* can be utilized commercially for the production of biodiesel because it is a fast-growing microorganism with less contamination risk along with high content of lipid (Rahbari 2009).

#### 7.3.1.4 *Dunaliella salina*

It is a biflagellate green microalga belonging to *Dunalliellacea* family. *D. salina* is mostly seen in marine waters (high salt concentrations). It is also considered as a food source due to high carotenoid and its antioxidant activity. Due to fatty acid methylation, e.g. palmitic and linolenic acids, it can also be used as biodiesel production (Oren 2005).

### *7.3.2 Macroalgae*

#### 7.3.2.1 *Gracilaria chilensis*

It is a red macroalga that produces a higher amount of biomass in comparison to other macroalgae (Wi et al. 2009). Because of high polysaccharides content, *G. chilensis* can be efficiently used for bioethanol production using hydrolysis as well as other value-added by-products production after the extraction of biomass.

#### 7.3.2.2 *Sargassum angustifolium*

*Sargassum angustifolium* is a brown alga found in Persian Gulf and is utilized for the biodiesel production. After biofuel extraction, it is mostly utilized for sodium alginate production, and the obtained biomass is further used for the production of bioethanol using the fermentation process. The biomass after acid pre-treatment can also be utilized as an alternative of yeast during ethanol fermentation (Ardalan et al. 2018). The pre-treatment process completely damages the intricate structure of macroalgal biomass and evolves nitrogen gas which can be utilized for fermentation that will reduce the nutrients' cost (Yazdani et al. 2015).

#### 7.3.2.3 Sea Lettuce: *Ulva lactuca*

It is a green macroalga and renewable gas fuel which is used for bioethanol production and commonly refers to green tides because of high secretion of nitrogen (eutrophication) or as algal blooms because of high lipid content (Allen et al. 2013). Generally, sea lettuce constitutes very less cellulose that is utilized for biomethane production using anaerobic digestion (Vergara-Fernández et al. 2008). It is mostly grown in the shallow basins, whose topographs protect the washout of algae, and it also keeps nitrogen and urea pollutant from starting the algal growth.

## 7.4 Nutritional Requirements for the Algal Biomass Production

Algal biomass comprises various metabolites (primary and secondary) such as carbohydrates, proteins, lipids and pigments, which either belong to cell structural components or involved in several metabolic processes (Markou and Monlau 2019). In their composition, mostly carbon is present along with various other elements, such as nitrogen, phosphorus, sulphur and potassium, in varying concentrations. Lipids, proteins and carbohydrates are the three crucial metabolites of the algal biomass with a total biomass content exceeding 80%, which greatly varies between different species (Tibbetts et al. 2015). *Chlorella* and *Scenedesmus* are the species that have high protein content ($\gg$35%), and *Porphyridium* has high carbohydrate content (>40%). Lipids and carbohydrates are the carbon-rich compounds, whereas proteins are nitrogen-rich compounds (approximately 16% nitrogen content). Similarly, other metabolites such as enzymes and pigments contain other nutrients, e.g. iron, zinc and cobalt (Markou and Monlau 2019). Hence, for the proper growth of the algal biomass, all the nutrients are required in specific concentrations as it is directly proportional to the growth rate. Generally, algae have flexibility regarding their nutritional requirements as it can grow within suboptimal conditions of one or more elements (Beuckels et al. 2015; Thrane et al. 2017).

## 7.5 Energy Requirements for Life Cycle of Algal Biofuels

Tremendous researches have been performed for algal biofuel production, and still, experiments are in process for the further development of the process towards scale-up and commercialization (Markou and Monlau 2019). Although, it may negatively affect the growth due to the relation between cell growth and particular nutrients' intracellular content as they are directly proportional to each other. Therefore, 'subsistence quota' which is an intracellular nutrient content threshold is introduced where algae are repressed and ceased for further growth (Droop 1968). To get the maximum possible lipid or carbohydrate accumulations, the nutrient starvation can be optimized up to the optimum concentrations (Markou and Monlau 2019).

### *7.5.1 Carbon*

In the algal biomass, the most abundant component is carbon which accounts for approximately 45–55% of the total biomass and also increased up to 65% under appropriate environmental conditions. Naturally, microalgae are phototrophic organisms which use light energy for producing organic molecules by fixing inorganic carbon ($CO_2$). They are mostly marine microbes, where the carbon (inorganic) is available in the solubilized form (Markou and Monlau 2019).

The cultivation of microalgae depends mainly on atmospheric carbon dioxide, and this source is prone to carbon limitations as the rate of transfer of carbon dioxide to media is low as compared to the uptake by microalgae. Therefore, it is necessary to supply $CO_2$ to improve the productivity of the microalgal culture to fulfil the cell's carbon requirements (De Godos et al. 2014). $CO_2$ can be either found from the pure gas which is commercially available or created from various flue gases (Van Den Hende et al. 2012) after the removal of potentially harmful components, e.g. volatile heavy metals (Huang et al. 2016). Some algal species can also utilize various organic carbon, e.g. glucose and acetate, in the presence or absence of light (mixotrophic/heterotrophic mode) (Morales-Sánchez et al. 2015). The most commonly used organic carbon molecules are monosaccharide, e.g. glucose, volatile fatty acids, e.g. acetic acid, urea and glycerol (Perez-Garcia et al. 2011). Mixotrophic mode for algal growth plays a very important role in wastewater treatment by utilizing the organic compounds and reducing the organic load. For growing mixotrophically, microalgae need wastewater organic compounds in a fermentable form.

### *7.5.2 Nitrogen*

The second most abundant element is nitrogen for the microalgal biomass. Nitrogen is the main component of nucleic acids, amino acids, proteins and other secondary

metabolites which comprise around 5–10% of algal biomass. When compared to land plants, microalgae consist of comparatively more protein content (30–60%); hence, nitrogen requirement is also high, which can be utilized in both inorganic and organic forms, e.g. the two important forms are $H^3/NH^{4+}$ (ammoniacal nitrogen) and $NO^{3-}$ (nitrate-nitrogen). Certain species of cyanobacteria are able to fix atmospheric nitrogen (Peccia et al. 2013).

Various harmful effects are caused by ammoniacal nitrogen (in toxic form), i.e. free ammonia because it freely penetrates into the cells and causes serious damages to the photosynthetic machinery or to the various metabolic processes (Markou et al. 2016). Because of being a gaseous molecule, usually, free ammonia inclines to slip out from the cultivation medium, which results in the loss of essential nutrients. To overcome this problem, it is necessary that the cultures in which ammoniacal nitrogen is added should be pH controlled or gradual addition of the nutrient must be done in fed-batch cultivation (Ji et al. 2015; Markou 2015).

### *7.5.3 Phosphorus*

It is also required for the microalgal growth and is present in various important organic molecules, e.g. RNA and DNA, ATP, and approximately 0.5–1% maximum up to 3% is present in the microalgal biomass (Richmond 2004). The cells utilize these in orthophosphate form, and it can also be as organic bounded phosphorus after the mineralization of phosphatase (Dyhrman and Ruttenberg 2006). It is often preventive in algal cultivation systems which are grown on wastewater. Phosphorus may form complexes in the presence of cationic metals and humic substances, and get precipitated in the presence of alkaline pH medium which eventually leads to the low phosphorus bioavailability (Cembella et al. 1982; Li and Brett 2013). The accumulation of a considerably higher concentration of intracellular polyphosphate granules by microalgae for their metabolic processes may be utilized by them when the surrounding phosphorus becomes depleted, and the process is called as luxury uptake (Powell et al. 2009; Shively 1988).

### *7.5.4 Other Nutrients*

Other additional components are also required for proper growth of microalgae, e.g. potassium, magnesium, sulphur, chlorine, iron, calcium, manganese, cobalt, copper, boron and zinc. These nutrients are required in many processes, e.g. oxygen metabolism, ATP reactions for carbon fixation, nitrogen assimilation, electron transfer and synthesis of chlorophyll, DNA and RNA (Markou and Monlau 2019).

## 7.6 Algal Cultivation Strategies

Various methods have been used for several years for the innovations in algal biomass production. These various cultivation strategies have been developed to increase the productivity of algal biomass, and some of these cultivation strategies are discussed below.

### *7.6.1 Open Pond Photobioreactor*

Considering the commercialization of algal biofuel with minimum energy input and economic approach, phototrophic cultivation method seems to be the most favoured process due to abundantly available sunlight at no cost. Along with this, phototrophic alga is capable to capture flue gas $CO_2$ and can effectively act as a superior carbon bowl which offers an advantage to the cultivation process. A perfect cultivation system must have the following characteristics: (1) efficient lighting area, (2) optimum liquid–gas transfer, (3) easy to operate, (4) low-level contamination maintenance, (5) economic, and (6) smallest land requirement (Xu et al. 2009). Open pond and closed-photobioreactor cultivation method has limitations when used in those countries where appropriate sunlight concentration is not available throughout the year (Lam and Lee 2012).

### *7.6.2 Raceway Pond System*

It can be considered as the most effective method for the cultivation of a huge amount of algal biomass, mainly because of economic and easy operation process. This pond system (Fig. 7.2) is generally formed of a closed-loop, oval-shaped recirculation channel, in which circulatory mixing is done by paddle wheels in order to prevent the algal biomass from sedimentation. The $CO_2$ source is sprayed at the raceway pond bottom. There are some reports stating that the artificial light is incorporated in the raceway pond system, although it is practically not possible and also affects the economic efficiency of the process (Singh et al. 2011). This pond (depth 0.2–0.5 m) is made up of concrete earth covered with plastic bags (white), considering algae to take satisfactory sunlight exposure (Brennan and Owende 2010; Chisti 2007).

However, the raceway ponds have various advantages, e.g. low energy requirement and cost of process however, there are also some limitations such as more water loss because of evaporation and more chances of microbial contamination because of continuous exposure (Schenk et al. 2008). Therefore, the consistent cleaning and maintenance of the raceway pond are required to maintain the optimum conditions for proper algal growth.

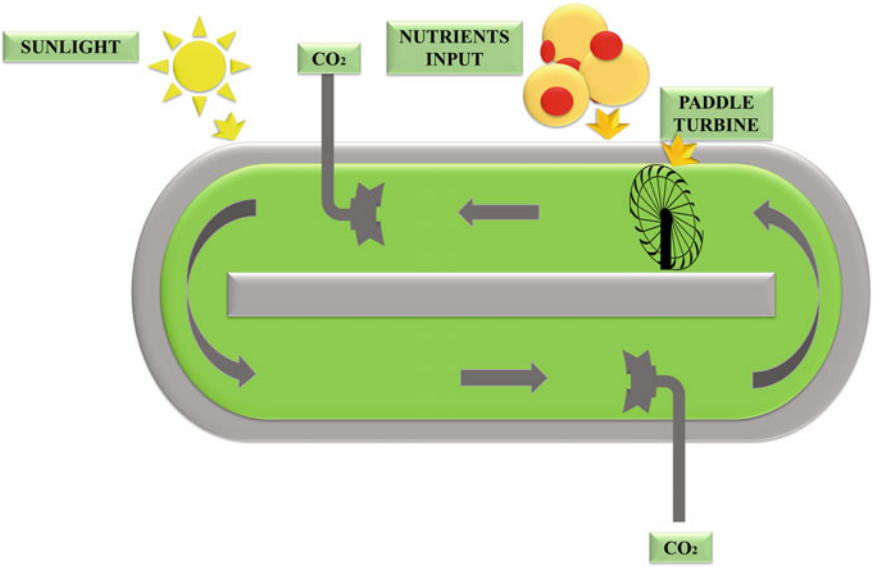


**Fig. 7.2** Raceway pond system for algae

Considering the proper working of raceway pond system, the production of maximum algal biomass and large lipid content are not only the points to be considered, whereas other factors such as high growth rate, easy cultivation and increased survival capacity in extreme environments are also need to be considered. The best example is *Chlorella*, which can efficiently grow in a nutrient-rich medium. Similarly, *Spirulina* and *D. salina* can grow effectively at high bicarbonate/pH concentrations and highly salinity medium, respectively (Borowitzka 1999; Brennan and Owende 2010).

### *7.6.3 Closed-Photobioreactor*

Later, considering the restrictions of the raceway pond system, closed-photobioreactors (Fig. 7.3) were designed to confirm the efficient optimal conditions for maximum biomass yield. The cultivation parameters in a closed-photobioreactor system are controlled strictly, and chances for contamination are lowered in the cultivation system, which helps in the cultivation of single strain of algae for long duration, and water resources can be reused multiple time (Brennan and Owende 2010; Chisti 2007). The flexibility of the cultivation system is more than the raceway pond because it can be used for the optimization of biological and physiological characteristics favouring the proper growth of algal strains (Mata et al. 2010). This process has gained interest among the researchers because optimization of physical

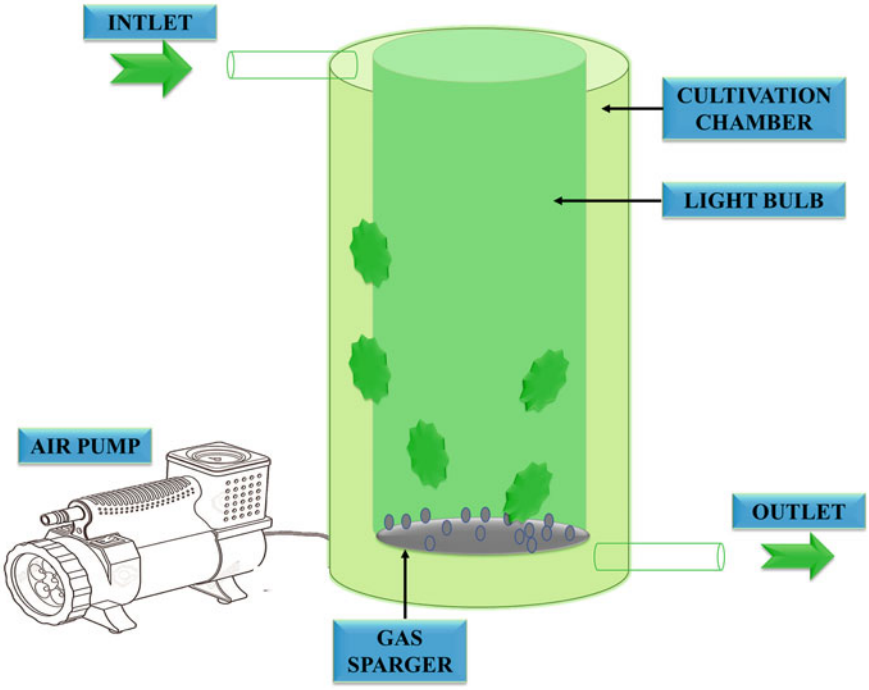


**Fig. 7.3** Closed-tank photobioreactor

parameters such as $CO_2$ concentration, cultivation pH, intensity of mixing, temperature, and level of nutrient can be carried out easily in closed-photobioreactor system (Brennan and Owende 2010). Various closed-photobioreactors have been reported such as air-lift tubular, flat plate and column. Apart from having several advantages the air-lift tubular photobioreactor also have some limitations such as use of huge electricity volume to control heavy-duty pumps for the adequate mixing and optimal gas–liquid transfer rate, which may lead to a negative energy balance in the algal biofuel production, when precautions have not been taken for the reduction of energy input. However, the energy input has not included the energy used for other processes such as artificial lights at night-time, algal biomass harvesting and drying, water treatment, extraction of lipid, and conversion to biodiesel (Stephenson et al. 2010; Razon and Tan 2011). If these factors have also been included, the overall energy balance for algae cultivation will become even more negative. An air-lift tubular photobioreactor is costlier as compared to the column type and flat-plate which make them more reasonable for commercialization.

### 7.6.4 *Hybrid Cultivation System*

The hybrid cultivation system is a more advanced form of the bioreactor for the cultivation of algal biomass which was designed after reviewing the challenges of the open ponds and closed photobioreactors (Huntley and Redalje 2007). Brennan and Owen (Brennan and Owende 2010) reported that the hybrid cultivation system mainly work in the process where hybrid two-stage cultivation was required.

The first stage in which the closed-photobioreactor cultivation conditions are controllable to attain large biomass volume in the nutrient-sufficient cultivation medium, and in the second stage, open ponds which allows environmental stress such as nutrients lacking environments is applied on the cultivated algae to increase the lipid production. However, the hybrid cultivation system strongly affects the financial feasibility of the process towards the commercialization of algal biomass to biofuels (Kunjapur and Eldridge 2010). This kind of combined cultivation system generally involves higher processing costs as compared to any single bioreactor.

## 7.7 Harvesting and Drying of Algal Biomass

Harvesting of algal biomass is defined as separating the algal culture from water for producing biofuels. This process contains two different steps, first harvesting in bulk for the separation of microalgae from the suspension of bulk using gravity sedimentation, flocculation, and flotation; and second, thickening for the concentration of microalgae slurry via centrifugation and filtration (Brennan and Owende 2010; Chen et al. 2011). The harvesting process is challenging because of cell small size, i.e. generally 1–20 μm and water suspension (Lam and Lee 2012; Suali and Sarbatly 2012). Although the algae are grown in closed-photobioreactor, the mass ratio of algal biomass and water is very low (Chen et al. 2011) which usually lies between the range of 0.00035 and 0.027 assumed to produce around 0.05 and 3.8 gm/L per day of biomass during cultivation for 7 days. During the scaleup of a closed photobioreactor, approximately 73 tonnes of water needs to be processed while harvesting 1 tonne algal biomass (Lam et al. 2019).

As the water requirement is quite extensive, it is necessary to search an effective harvesting process for the commercialization for the production of algal biofuel. Various researches have been reported for the current harvesting techniques and algal biomass drying which consume a significant energy input for producing algal biofuel (Sander and Murthy 2010). The study evaluated two types of thickening methods for algal slurry without previous bulk harvesting: filter press and centrifugation which resulted in 88.6% and 92.7%, respectively, for the total energy input of the process. Hence, it can be considered as only centrifugation or filtration for algal biomass harvesting is still difficult for the commercial application, due to high energy utilization and high processing price. Whereas, bulk harvesting processes,

e.g. flocculation, suggest another approach for algal biomass harvesting with low energy input and economic viability.

## 7.8 Biofuel Conversion

The biomass feedstock conversion into biofuels and additional value-added by-products is a challenging and costly process. Before the conversion into biofuel of other by-products, harvested and dried algal biomass is broken into different components such as lipid, protein, carbohydrate and mass residues using fractionation or extraction methods. Additional processing methods are required to further change the components into various liquid, solid and gaseous biofuels and other products.

Algal biomass can be utilized for producing various fuels like syngas, hydrogen, ethanol, methane, diesel, butanol, jet fuel, acetone and charcoal by using various chemical conversion methods such as fermentation, photobiological, gasification, anaerobic digestion, liquefaction, pyrolysis and transesterification (Azizi et al. 2018; Gavrilescu and Chisti 2005). Hydrothermal liquefaction (HTL) of microalgae biomass was found to be an efficient conversion technology, where wet biomass is treated under high pressure and temperature and the water present in the wet biomass has been considered as the reaction mixture (Han et al. 2019). Dilute acid pre-treatment was stated as one of the effective process for the active utilization of algal biomass (Dong et al. 2016).

## 7.9 Improvement of Algal Biofuels Using Biotechnological Strategies

In algal and cyanobacterial species, the cell size, composition of lipid and starch content varies; hence, it is assumed that not all the species can be used for biofuel production. Among them, some are suitable for the production of biofuel because they can naturally synthesize and also accumulate an adequate amount of starch and lipids, which can further be enhanced by genetic manipulations (Urtubia et al. 2016). Various algal and some *Chlorella* species, such as *Synechocystis* sp. (PCC 6803) and *Phaeodactylum tricornutum*, have been studied extensively for biotechnological engineering to improve their biofuel producing potentials (Xue et al. 2017; Yang et al. 2019). They have high potential for the biotechnological applications due to simple genomes, efficient for easy transformation, appropriate strains diversity and availability of complete genome sequences (Majidian et al. 2018). These properties can help in the biosynthesis of starch, increasing the ability of carbon capture, biohydrogen production, lipids enhancement and accumulation (Yunus and Jones 2018).

Genetic engineering process involves overexpression of those enzymes which have their involvement in fatty acid and lipid biosynthesis (Takemura et al. 2019;

Tan and Lee 2017; Yunus and Jones 2018), assembly and biosynthesis of TAG (Fukuda et al. 2018; Xin et al. 2017), targeting the blocking competitive pathways and biosynthesis of lipid/starch catabolism (Kao and Ng 2017; Shin et al. 2019). Several reports are also available regarding the gene-targeted transcription factors which are involved in the regulation of lipid biosynthetic pathways (Ajjawi et al. 2017) or enhancement of reducing agent NADPH availability to improve the content of fatty acid in *P. tricornutum* (Xue et al. 2017).

Overexpressing the key genes responsible for carbon fixation pathways in microalgae for the enhancement of the ability of carbon capture and accumulation of lipid is an efficient strategy to capture $CO_2$ excess for enhanced lipid accumulation (Huang et al. 2017; Oh et al. 2018). Carbonic anhydrase that exists in various algal strains is a well-known enzyme for $CO_2$ and bicarbonate interconversion catalysation. The reports on genetic engineering of carbonic anhydrase have been proven as an active participant in $CO_2$-sequestration. In *Nannochloropsis oceanica*, the carbonic anhydrase is considered as an important constituent of the carbon concentrating mechanism (Gee and Niyogi 2017; Tan et al. 2018) (Table 7.2).

## 7.10 Economic Aspects of Algal Biofuels

The techno-economic analysis is a primary assessment tool that is used for the estimation of the process cost and also for the determination of the economic viability of the algal biofuels (Hall 1986). This analysis assimilates the engineering process and thermodynamic modelling, processing cost, analysis of sensitivity and risk calculation (Bowyer et al. 2018; Langholtz et al. 2016). The price of biofuel ranges from $0.44/L to $8.76/L (Benemann et al. 1987; Richardson et al. 2012) which is assessed from the available literature, independently generated laboratory-scale experiments, growth rate-related assumptions, lipids yield, nutritional and energy necessities (Chia et al. 2018). The estimated cost of equipment and the economic process were done by Aspen Process Economic Analyzer (PEA) software (Rahimi and Shafiei 2019). The economic viability of microalgae suggested that the present cost per litre of algal biofuel is comparatively higher than conventional fossil fuel. Therefore, further studies can be performed to minimize the cost in the near future (Chowdhury et al. 2019).

## 7.11 Challenges and Future Perspective

Algal culture biomass for the biofuel production with marine resource is quite challenging due to its complexity and harvesting problems. The process also involves various pre-treatment processes, fermentation from microorganisms, and risk of contamination; hence, reduction in the biofuel cost is one of the main challenges (Balan 2014). If the cost-related problems are solved, biofuels can be

**Table 7.2** Different genomic approaches for the improvement of the production of biocomponents from algal cultures

| Algal species | Observations | References |
|---|---|---|
| *Chlorella* sp. | • Gene downregulations under UVR stress condition in various metabolic pathways for energy conservation, carbon resource reallocation and oxidative damage countering<br>• Whole genome transcriptome analysis of Antarctic *Chlorella* sp. (growth temperature: 4 and 33 °C) | Poong et al. (2018a, b) |
| *Chlamydomonas* sp. | Gene involved in polyunsaturated fatty acids (PUFA) encoding in the ICE-L transcriptome, synthesizes enzymes, cell membrane transport and molecular chaperon proteins | Liu et al. (2016) |
| *Chlorella vulgaris* | Fatty acid and TAG biosynthetic machinery upregulation was obtained under oil-accumulating conditions | Guarnieri et al. (2011) |
| *Chlamydomonas acidophila* | • Cadmium exposure enhances expression of transposon in a green alga<br>• The induction of genes for oil biosynthesis is done under heavy metal stress | Puente-Sánchez et al. (2018) |
| *Chlamydomonas reinhardtii* | The third fatty acid of TAG is originated from phosphatidylethanolamine or diacylglyceryl-*O*-4′-(*N*,*N*,*N*,-trimethyl)-homoserine betaine lipid species, and the candidate genes were provided by the comparative transcriptomic analysis which is also included in DAG acyltransferase and DGTT1phospholipase A2 homologue | Légeret et al. (2016) |
| *Dunaliella acidophila* | High constitutive gene expression methods involved in oxidative stress and response reactive oxygen species | Puente-Sánchez et al. (2016) |
| *Dunaliella parva* | During carbohydrate metabolism, glycolysis and the TCA cycle could be affected by nitrogen limitation and later hinder energy production | Shang et al. (2017) |
| *Dunaliella tertiolecta* | The de novo assembly integration suggested the long alterations in the expression patterns (13 861 protein-coding transcripts) with the growth phenotypes | Shin et al. (2015) |
| *Aurantiochytrium* sp. | The glucose utilization rate was accelerated by gibberellin and also involves in the fatty acid metabolites synthesis | Yu et al. (2016) |
| *Nannochloropsis oculata* | Organic carbon and nitrogen obtained from the pigments and protein breakdown were mainly channelled into fatty acid synthesis. | Tran et al. (2016) |
| *Tetraselmis* sp. | Organelle-specific responses varies with the temperature variations | Shin et al. (2016) |
| *Nannochloropsis oceanica* | N-condition TAG synthesis involves in<br>• Upregulation of putative diacylglycerol acyltransferase (*DGAT*) genes (seven)<br>• Downregulation of other *DGAT* genes (six)<br>• Rise in the Kennedy pathway genes | Li et al. (2014) |
| *Phaeodactylum tricornutum* | Betaine lipids are reported as the main source for the development of triglyceride, sedoheptulose accretion during *Phaeodactylum tricornutum* nitrogen starvation | Popko et al. (2016) |

represented as "future fuel" for the upcoming years for transportation purposes. The advancements in the modern era with new abilities, companies, and job prospects are likely to appear in the future of biorefineries that require further inventions in the above-discussed points (de Jong and Jungmeier 2015).

## 7.12 Conclusion

Fossil fuel resource combustion is the major cause of global warming as it releases high atmospheric carbon dioxide. In contrast to this, the utilization of organic waste resources as nutrients can be helpful in the development of a sustainable environment via biofuel production. Therefore, biofuel from marine sources serves several advantages of consuming maximum carbon dioxide, providing high energy production, using cost-effective fuel sources. The algal biomass is found to be an efficient alternative of fossil fuel for the development of sustainable environmental as the process is economic and also produces various value-added potential by-products. Further, biotechnology-based methods offer an effective path for enhanced $CO_2$ capture and higher biofuel production. Therefore, it will be beneficial to use advance gene manipulation techniques, e.g. using synthetic biology and artificial intelligence for further enhancement of the production of algal biofuels on industrial-scale.

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# Chapter 8
# Nanocatalysts to Improve the Production of Microbial Fuel Applications


**Siva Sankar Sana, Punita Kumari, Zeba Usmani, Minaxi Sharma, Surya Sudheer, D. Dinesh Kumar, Zhijun Zhang, and Huizhen Li**

**Abstract** Fossil fuel which is the current source for energy supply is responsible for environmental pollution. Recently, renewable source comes as a substitute for this because of the depletion of fossil fuel resources and thus its inability to meet the future energy demand. Renewable energy now used is the wind, solar, biomasses, hydrogen and geothermal source. Among them, biomass is more ideal because of its efficiency in converting to liquid biofuel directly. Main conversion routes of biomass to biofuels include pyrolysis, gasification, liquefaction, hydrolysis, anaerobic transesterification and digestion. In all the methods, development of high-quality product with the optimized process is needed, which requires the use of modern science like nanotechnology. Over the last few decades, uses of active nanocatalyst for biofuel productions have been rapidly increasing. Nanocatalyst with size less than 5 nm shows a good catalytic property.

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Additionally, porous catalysts developed with controlled size can be attributed to its good property like higher crystallinity, surface areas and shape-selective characters. Recent research mainly focuses on developing effective nanocatalyst for improved conversions, for getting milder operation condition, and mostly to reduce the biofuel production costs. This chapter gives an overview of different biofuel producing methods, advancements of use of nanocatalyst for biofuel production and its merits and drawbacks in various fields.

**Keywords** Biofuels · Classification · Production · Nanocatalyst · Applications

## 8.1 Introduction

Nowadays, among all different technologies, nanotechnology research has arisen as an advanced technology that deals with biology, physics, medicine, chemistry, engineering, agriculture, material science and military. The word 'nano' is derived from the Greek word 'Nanos' meaning dwarf, which implies the thing is one billionth in size (Sajid et al. 2015). Nanometre-sized materials or nanostructures are at the forefront of gifted material research, and its applications are gradually expanding into all sort of day-to-day life.

Nanoparticles show new characteristics relied on particular attributes including shape, size and distribution. In the past, an increase in the production of nanometre-sized particles with controlled morphology and efficient features was observed, which makes it an extensive research area. Nanometre-sized particle consists of atomic as well as molecular assembly of nanometre range dimension with different shape, size and enhanced surface characteristics which can be engineered meticulously (Roy et al. 2013). Production of nanomaterials, particularly nanometre-sized metal particles using nanotechnology, attains global interest because of their potential use in the biomedical and physiochemical field because of the large surface to value ratio, and their surface areas are hundred times more than their weights (Tiwari et al. 2011). The unique features modify not only physical properties but also chemical properties which includes, like mechanical property, satirical and biological properties, electrical and thermal conductivity, catalytic activity, melting point and optical absorption in comparison to bulk-sized material of same chemical constituents (Vijayaraghavan and Ashokkumar 2017).

Because of their enhanced Rayleigh scattering, surface-enhanced Raman scatterings, surface plasmon resonances in metal nanometre-sized particles, quantum size effect of semiconductors and superparamagnetism in magnetic material, nanometre-sized particles possess several advantages when compared to bulk scale materials. Therefore, nanometre-sized particles assumed to be the backbone of next-generation electronics, optoelectronics and different bio-chemical and chemical sensor (Narayanan and Sakthivel 2010).

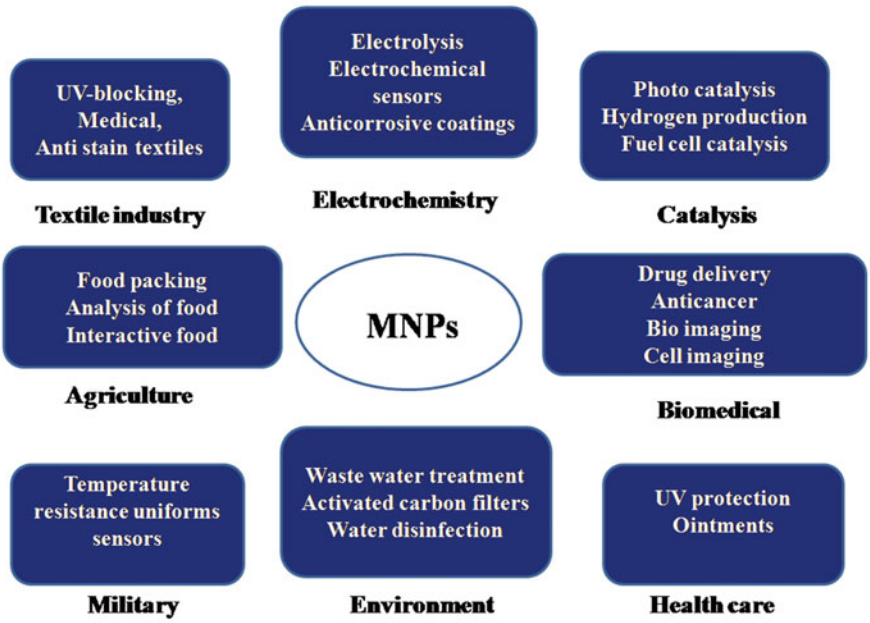


**Fig. 8.1** Application of MNPs in different fields

Because of its smaller dimension and exceptional properties of the surface, nanometre-sized particles have displayed good potential for miscellaneous application. Nanomaterials are of small size having one dimension of 100 nm at least. They have been used in biomedical applications, textile (Gyeongwon et al. 2017), agriculture (Chen 2018), military (Kharat et al. 2006), health care (Muhammad et al. 2018) and also fuel cell applications (Omer et al. 2011) (Fig. 8.1).

## 8.2 Nanomaterial Classification

1. Zero dimensional (0D): All the three dimensions of materials are in nanoscale, e.g. nanoparticles. Here, all the electrons are fully confined, whereas all the electrons are fully delocalized in 3D nanomaterials.
2. One dimensional (1D): Two dimensions of materials are at the nanoscale, e.g. nanowires and nanotubes.
3. Two dimensional (2D): At least one dimension of materials is at the nanoscale, e.g. thin films. In the case of 1D and 2D nanomaterials, electron confinement and delocalization coexist.
4. Three dimensional (3D): All three dimensions of the materials are not at the nanoscale. Figure 8.2 shows the examples for different classes of nanomaterials.

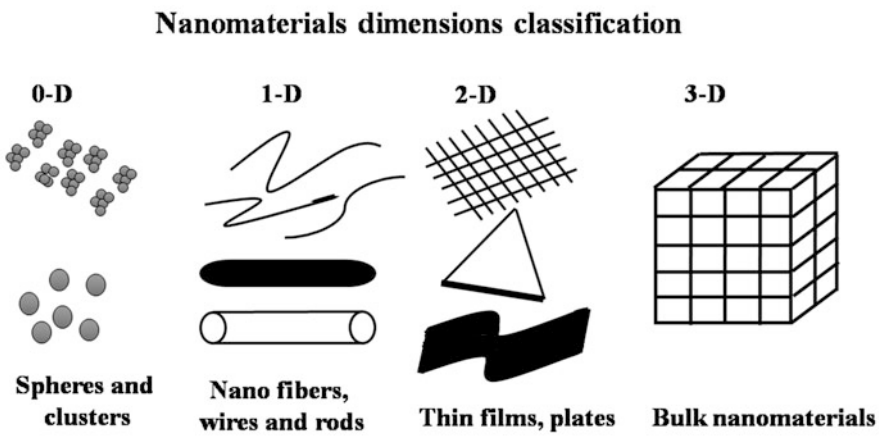


**Fig. 8.2** Classification of nanomaterials. (a) 0D: nanoparticles or nanoclusters, (b) and (c) 1D: nanowires, nanorods and nanotubes, (c) 2D nanostructure: thin-film/nano coatings, (d) 3D bulk materials

## 8.3 Nanoparticle Synthesis Techniques

The two suggested approaches for the synthesis of nanoparticles are bottom-up and top-down. Nanomaterial or framework is produced in the bottom-up strategy by gathering atom by atom or molecule by molecule (Samberg et al. 2010). The primary aspect of this method is that nanomaterials create no structural abnormalities and homogeneous chemical composition. By physical, mechanical or electrical techniques, the bulk material is decreased in size in the top-down approach. There are some constraints in the size and structures of the acquired nanomaterial in these strategies. Surface abnormalities have a crucial effect on the conduct of physical characteristics and surface chemistry. Several biological, chemical and physical methods were developed for the synthesis of nanomaterials depending upon the sort and nature of the material to be prepared and response circumstances.

In the bottom-up approach, biological and chemical methods are used for the production of nanometre-sized particles. The chemical approach of nanometre-sized particle fabrication contains photochemical reduction, chemical reduction and electrochemistry technique. In the 'top-down' approach, bulk materials are broken down to smaller particles by physical and chemical methods. In this approach, there are certain limitations in the structure and size of the nanomaterials obtained. In general, 'top-down' strategy is the lithographic method for the production of desired pattern in micro- and nanometre size. In comparison to 'bottom-up pathway', 'top-down' strategies offer high reliability and also control the shape and size of the particle, but they involve high expenditure on tools and less access to fabrication facility (Prasad

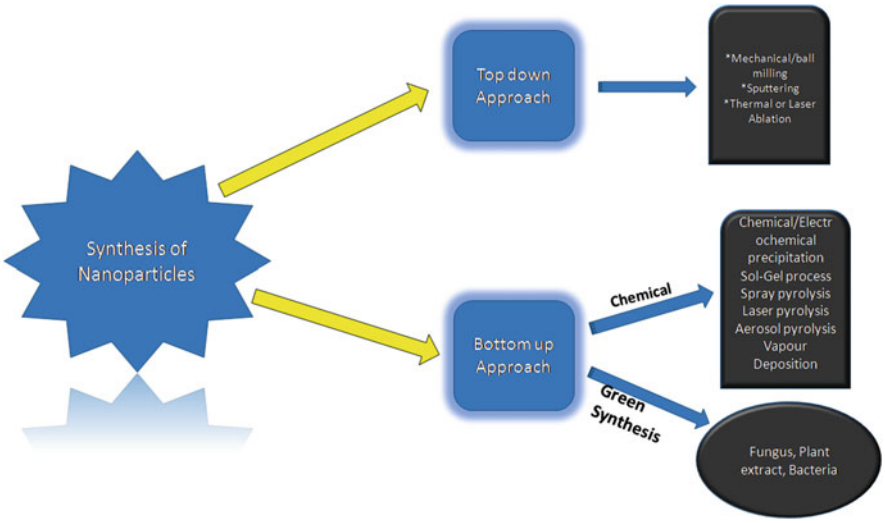


**Fig. 8.3** Top-down and bottom-up approach of metal nanoparticles synthesis

et al. 2019). The physical method includes evaporation-condensation, sputtering deposition, electron beam deposition, laser pyrolysis, combustion flame, electron beam lithography, anodization, ion and plasma etching, melt mixing, flame spray pyrolysis, grinding, high energy ball milling and laser ablation (Prasad et al. 2019).

Inorganic nanosized particles are commonly prepared by using phenomena such as nucleation and crystal growth. 'Bottom-up' approaches are of low-cost, readily accessible and productive but lack precise control in particle morphology, dispersity and size. The chemical method includes sol-gel, chemical reduction, spinning, microemulsion or colloidal, polyol, hydrothermal, electron beam deposition, combustion flames and plasma-enhanced chemical vapour deposition (Prasad et al. 2019) (Fig. 8.3).

### *8.3.1 Classification of Biofuels*

According to the commercialization of biofuel technology, biofuel can be classified into two groups: conventional and advanced biofuel. The commercialization degrees are as follows: commercial > early commercial > demonstrations > researches. The conventional biofuel included sugar- and starch-based bioethanols, transesterification-based biodiesels and anaerobic digestions of biomethane. Vegetable oils are at early commercial stages for the synthesis of advanced biofuels, while cellulosic bioethanols, biomass-to-liquid bioethanols, microalgae biodiesels and biohydrogen are still at the research stage. We note that the advance biodiesel is not mostly available presently, but it can be fully commercialized in future.

Some researcher classified biofuel into two groups: first-generation and second-generation biofuel. The first generation of biofuel includes bioethanols, biodiesels and biogases. The liquid biofuel mainly includes saturated/unsaturated edible plant oil from oilseed of corn, sunflowers and soybeans. Oils from fruit of coconut, olives and palms are also taken. The second-generation biofuel includes lipid-based biofuel, which is formed from waste vegetable oil, animal fat, insect and oleaginous microorganism. The first-generation biofuel is commercialized currently, where the second-generation biofuel is underdevelopment. The first-generation biofuel and second-generation biofuel are thus similar to conventional biofuel and advance biofuel, respectively. The second-generation biofuels include biofuel of bioethanols, biodiesels, dimethyl-ethers, biosyngas FT biodiesel, biodiesels, etc. These biofuels are mainly from nonfood feedstock like wood grass, cereal straws, forest residue, dedicated energy crop and municipal solid waste (Saini et al. 2015).

The third-generation biofuel includes biofuel of biomethane, biodiesels, bioethanols, biobutanol, vegetable oil gasoline, jet fuel, and aviation fuels that are obtained from cultivated aquatic feedstock like algae or cyanobacteria. The secondary biofuel has three generations of biofuel (first-, second- and third-generation biofuel). The drawback of the first- and second-generation biofuels like disturbing food chains, using higher amounts of water, deforestation and incrementing greenhouse gas emission leads the researcher to find a different solution. That is how the third-generation biofuel become popular.

## *8.3.2 Another Classification of Biofuel*

### 8.3.2.1 Solid Biofuel

(a) **Solid Biomasses**
Solid biomasses refer to raw solid biomass feedstocks that are for biofuel productions, like agricultural residue, forest residue, crop and solid waste. Raw solid biomass feedstocks are also biofuels. Solid biomasses include oil crop, lignocellulosic biomasses, solid waste, etc.; all these materials can convert to biofuel and can also be used as biofuel directly.

(b) **Biochar**
In comparison to the fossil fuel, the raw feedstock, for example wood, has high combustion emission and low energy content. Also, the fire temperatures from wood combustions would be very less (e.g., below 850 °C), making it harder for melting metal. To overcome these drawbacks, biochar are used. Biochars are also termed as charcoals. It is produced by heating biomasses feedstocks at 400 °C in vacuum. It is also produced from torrefaction, gasification, hydrothermal carbonization, etc.

#### 8.3.2.2 Liquid Biofuels

Liquid biofuels are more in use because they are easier for storage, they are high ranged in combusting, they are not explosive, they had higher energy to mass ratio, they are stable for longer storages, easily transportable and cost-effectiveness. Liquid biofuel includes bioethanol, biomethanol, biopropanols, biodiesels, jet fuel, etc. Among them, the important ones are as follows:

(a) **Bioethanol**

Bioethanol is also termed as ethylic alcohol or ethanol. It has the formula $CH_3CH_2OH$, which is the same organic compound that is used in an alcoholic beverage. Microbial fermentations of different feedstocks like sugarcanes, corns, grains, agricultural wastes, and forestry wastes form sugary or starchy materials. The bioethanols can be used as gasoline substitutes in engine directly. Ethanol has a low LHV (21.1 MJ/L) than gasoline (30–33 MJ/L). Thus, more ethanols are needed for obtaining the output. However, higher octane numbers of ethanol allow high engine compression ratios used, which lead to improve thermal efficiencies and increase power, thereby decreasing the differences in fuel consumptions.

(b) **Biodiesel**

Bioethanol is substituted for gasoline where biodiesels are substituted for diesel. Biodiesel is usually a colourless liquid mixture of longer-chain fatty acid methyl ester derived from vegetable oils, animal fats, algal lipid or waste greases through transesterification in the presence of alcohols and alkaline catalysis. Biodiesels encompass alkyl fatty acid ester of short-chain alcohol (methanol or ethanol), and its chemical structures are a chain of 12–22 carbon atoms and 0–2 double bonds. The chemical structures of biodiesels are varying from that of diesel. Biodiesel contains carbons, hydrogens and oxygen. The advantage of biodiesel includes its high flash points which make it safe for storages, uses, transportations and high percentage numbers which mean that it has good ignition qualities. The disadvantage of biodiesels includes its low calorific values which mean more biodiesels will be taken for supplying same outputs, high pour points which mean it tends to get up in cold weathers and high viscosities which have a negative effect on fuel spray atomizations (Guo et al. 2015).

#### 8.3.2.3 Gas Biofuels

Advantages of gaseous biofuel are (a) it is more reactive, (b) requires lower oxidant, (c) requires simple reactor, (d) is easy to control, (e) yields only lower amount of waste, etc., and thus gaseous biofuels are more applied. Some important gaseous biofuels are biogas, biohydrogens, biosyngases, etc. (Guo et al. 2015).

(a) **Biogases**

Biogas also termed as biomethane is produced from anaerobic digestions or biological fermentation of organic substances, for example, liquid manures and

other digestible biomasses feedstock. It is observed that natural gases are made of methane (95%) and ethane (5%) where biogases usually contain methane (45–65%) and carbon dioxides (30–40%). The high content of carbon dioxides (30–40%) contributes to much low energy of biogas in comparison to the natural gases. Still, biogases have some advantage than natural gases: (a) the raw feedstocks can be renewed, it is economical, it does not add any GHG to atmosphere, and it helps in alleviating the waste management problem. Consequently, biogas is used as substitutes for natural gases in the motor vehicles, cooking, house heaters and electricity generators.

(b) **Biohydrogen**

Hydrogen is a colourless or odourless gas and is a simple combustible gas in nature. Biohydrogen can be widely explained as hydrogen from a renewable source. The sources are energy crop, crop residue, livestock residue, agricultural residue, forest residue, algae biomass, municipal waste, industrial waste, waste oils, wastewaters, etc. There are two methods for the biohydrogen production: (i) thermochemical conversions (ii) biological conversions. Hydrogen produced from thermochemical conversions is termed as biohydrogen due to original raw feedstock presence as biomasses.

(c) **Biosyngas**

Different from biogases and biohydrogen, syngas is produced from biomass gasification. Biosyngas is a mixture of $H_2$, CO, $N_2$, $CO_2$ and hydrocarbon. Some moisture, $H_2S$, $NH_3$ and tar may exist. Based on gasification agents used, biomass gasification can be air gasification, e.g. oxygen, steam and $CO_2$ gasification. Since the air is readily available, and it is a simple method, air gasification is more used among all. Since biogas contains nitrogen, it can dilute the syngas. For improving the qualities of biosyngases, oxygen, steam, carbon dioxide, or supercritical water can be used, replacing air oxygens, and steam is commonly used. Oxygen can reduce the effects of $N_2$, thus increasing the volume fraction of different gases. However, it is costly and tar in them can be higher (Tabatabaei and Mollahosseini 2015; Du et al. 2016).

## 8.4 Biofuel Production Methods

### *8.4.1 Gasification*

Gasification is considered to be economical at capacity beyond 5 kW (Kirubakaran et al. 2009). Thus, there is continuous interests in producing energy from biomasses through gasification. Gasification is partial temperature oxidation, which leads to a higher proportion of gas product with little amount of chars, ashes and condense compound. The gas produced can be standardized in its qualities and is more easy to use compared to the primary biomasses. In better words, the gases can be used in power gas engine and gas turbine. Gasification adds value to the lower- or negative-value resource by transmission to fuel. Thus, gasification is an effective way to

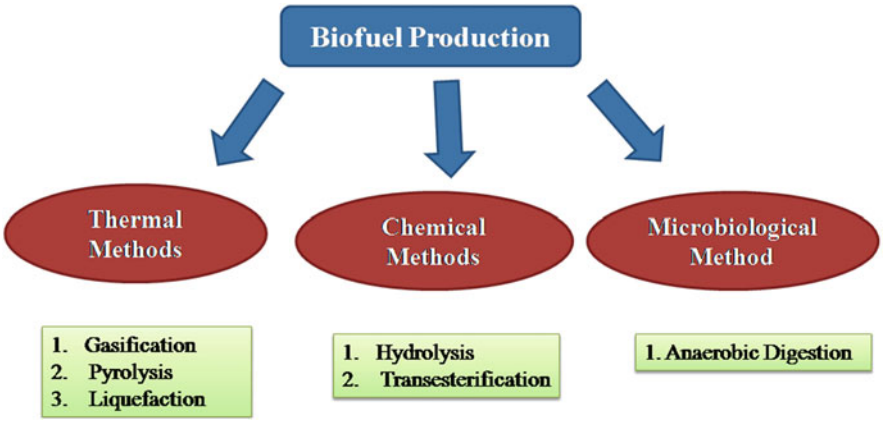


**Fig. 8.4** Different generation methods of biofuels

convert energy in biomass, and it is an alternative to reuse waste materials. It may contain impurities like tar, $NH_3$, HCl, $H_2S$ and $SO_2$, which are formed during gasification processes and generally lead to the problem in the downstream application (Chiang et al. 2013) (Fig. 8.4).

In terms of impurity, tar causes a lot of problems. This contaminant has to be removed by other methods like physical filtrations, wet scrubbings or catalytic hot gas cleanings. Physical filtration is the simplest methods but suffers from some demerits like blocking pore of filters and incapabilities for separating gaseous impurity. The gasification process consists of the following stage: drying stage, where moisture in biomasses is decreased by heating at 100–200 °C.

### *8.4.2 Pyrolysis*

In pyrolysis, biomasses are heated up and decomposed in the absence of oxygen into biofuels, charcoals and another chemicals, and this happens at slow heating rates. Different findings confirm that hydrothermal pyrolysis of manures with biodiesels crude glycerol or free fatty acid (FFA) could lead to a higher yield of bio-oil at high temperatures. Reaction kinetics of biomass pyrolysis is hard to interpret because biomass contains biomass, which is hemicellulose, celluloses and lignins. Decompositions of these three components occur at different temperatures and rates. This conventional slow pyrolysis is applied for production of charcoal. However, fast pyrolysis at higher temperature with short residence time is preferred (Demirbas 2007a, b). In fast pyrolysis, the major producers are gases which are removed continuously. Tars are generally produced at lower temperature while gases are released at higher temperature. However, solids, liquid aerosols, condensed melts, vapours, gases and aromatic chars make fast pyrolysis a complicated method.

Microwave pyrolysis is one alternative for this and is a thermochemical technology where biomass is heated by microwaves irradiation. Microwave-assisted pyrolysis is mostly used for wood processing (Miura et al. 2004), corn stovers, rice straws, coffee hulls, microalgae, pine sawdust and wheat straws. It is a fast and uniform heating method.

### *8.4.3 Liquefaction*

Liquefaction is a thermochemical process, where organic compounds present in specific feedstocks is converted to liquid product. However, in hydrothermal liquefaction, water acts as reactants and catalysts, and this makes a meaningful difference from pyrolysis, and thus biomass can be directly changed without taking energies for drying steps, as is the case of pyrolysis (Toor et al. 2011). It can be noted that hydrothermal liquefaction HTL cannot complete with pyrolysis with yield. Still, it has different fundamental privilege like relative stable oil products and aqueous environments, which do not need energies in drying of biomass.

### *8.4.4 Enzymatic Hydrolysis*

In this process, the glycosidic bond between sugar unit is cleaved for forming sugar like glucose and less hydrolysed oligomer. The hydrolysing of celluloses is mostly used because plant biomass is made of celluloses, and glucose produced is an important intermediate (Huang and Fu 2013). Hydrolysis needs pre-treatment step for expediting the breakdown of polymer for different sugars useful for fermentations. By that method, hemicelluloses can be reduced. Enzymatic hydrolysis is useful to get yield, but it is costly, and its reusability is not practical. Hydrolysis is usually performed by cellulases, β-glucosidase, xylanases, etc. This enzyme acts on pre-treated biomasses at the moderate condition and results in the release of the more significant amount of sugar. Acid-based hydrolysing can be used under higher temperature and pressure conditions when dilute acid is used at low temperatures and pressures when hydrolysis is done using concentrate acid. Cellulose hydrolysis however has some disadvantages because of concentrated acid causing toxicities, and thus dilutions can efficiently hydrolyse hemicellulose, and it is found that the alkali pre-treatments eliminate the requirements for size reductions and remove lignin effectively.

### 8.4.5 *Transesterification*

Plant oil contains FFAs, sterols phospholipid, water, odorant and other impurities, and thus, the oils cannot be used as fuel (Murugesan et al. 2009). Overcoming these light chemical modifications is termed as transesterifications, pyrolysis and emulsifications (Murugesan et al. 2009). Transesterifications help in the production of biodiesels from vegetables and animal fat. Transesterification is the replacement of alcohols from an ester by a different processes similar to hydrolysis; alcohols are used instead of water (Meher et al. 2006). For achieving an effective transesterification reaction, the catalyst selection is vital. Due to their simple use and need of lesser time for lipid conversions, the homogeneous catalysts are advantageous and used more in biodiesel industry. However, it needs highly pure feedstocks and complicates downstream processing. Thus, catalysts like solid acids, alkaline catalyst, enzyme, supercritical catalyst system and ionic liquid catalyst got more attention (Borges and Dıaz 2012).

Enzyme like lipase is non-toxic and biodegradable. Alkaline-catalysed transesterifications are done at a temperature below the boiling points of methanols, that is, 65 °C under atmospheric pressures. High temperatures and pressers can help in attaining reactions faster but is expensive. The disadvantages of the reactions are energy intensives, difficulties of glycerol recoveries, difficulties of removals of acid or alkali catalysts from products, and so on (Fig. 8.5).

### 8.4.6 *Anaerobic Digestion*

Anaerobic digestion (AD) is to treat different industry and domestic organic wastes and biogas productions as energy carriers (Madsen et al. 2011). AD represents a system having active microbial communities capable of efficiently processing organic waste under specific condition. These microbes are sensitive in processing

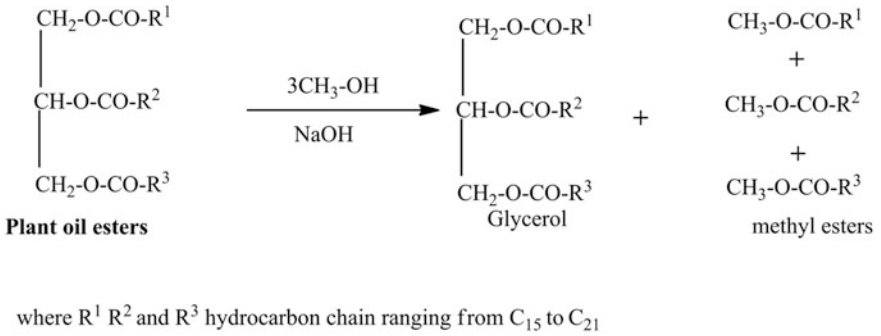


**Fig. 8.5** Vegetable oil transesterification

conditions, and thus, biogas productions by anaerobic digestion processes will be controlled by proper management. Temperatures, organic loading rates, retention times, etc. are factors influencing anaerobic digestions (Lin et al. 2014).

## 8.5 Nanocatalysts in Biofuel Production

One major challenge of today is nanosciences in developing sustained and renewable energy production scheme. In the first decades of the century, the emerged field in catalyst sciences, named nanocatalysis, seems to attract a wide range of attentions. Generally, high surface to volume ratio of nanoparticle in comparison to bulk material makes them a good catalyst. They have attractive special chemical and physical characteristic features due to the size and high density of active catalytic sites (Gokdai et al. 2010; Liu et al. 2012; Glaser 2012; Budarin et al. 2013). Metal oxide nanostructure catalysts could be appropriately classified as follows:

(a) Alkali earth metal oxides (e.g. MgO, CaO)
(b) Transition metal oxides (e.g. ZnO, NiO)
(c) Mixed metal oxides (e.g. $TiO_2$–ZnO)
(d) Supported metal oxides (e.g. KF/$Al_2O_3$, KF/CaO, CaO/$Fe_3O_4$, Cs–Ca/$SiO_2$–$TiO_2$, KF/CaO–MgO, Li/CaO)

They are generally classified into heterogeneous group catalysts and homogeneous catalysts. Biofuel can be grouped to bioethanols and biodiesels. Bioethanols are common biofuels, accounting for 90% or more of total usages. Using starch and sugar biomasses as feedstocks, it can be made depending on enzyme conversions of starchy biomass into sugar and fermentation of six-carbon sugar with final distillations of ethanol to fuel grades. While using lignocellulosic material as feedstocks, bioethanol production includes biomass pre-treatments for releasing celluloses and hemicelluloses, hydrolysis for releasing fermentable five- and six-carbon sugar, sugar fermentation, separations of solid residue and non-hydrolysed celluloses and distillations to fuel grades. In the current years, heterogeneous catalysis for biofuel making is vastly studied. Different metal oxides have been analysed for transesterification processes of oil and have come up as potential heterogeneous catalyst; these include transition metal oxides, alkali earth metal oxides supporting metal oxides and mixed metal oxides. Metal oxide constitutes positive metal ion (cation), which acts as Lewis acids, and negative oxygen ion which acts as Bronsted bases. Nanostructure uses have improved catalytic performances of ZnO (Reddy Yadav et al. 2018) (Fig. 8.6).

Nanocatalyst plays a vital role in increasing product qualities and achieving optimal operating condition. Nanocatalysts with higher specific surface areas and higher catalytic activities solve the common problem of heterogeneous catalyst like mass transferring resistances, time consumptions, fast deactivations and inefficiencies. In this respect, attempts for developing a new type of nanocatalyst are increased. The roles of nanoparticle in the catalytic process are important in social,

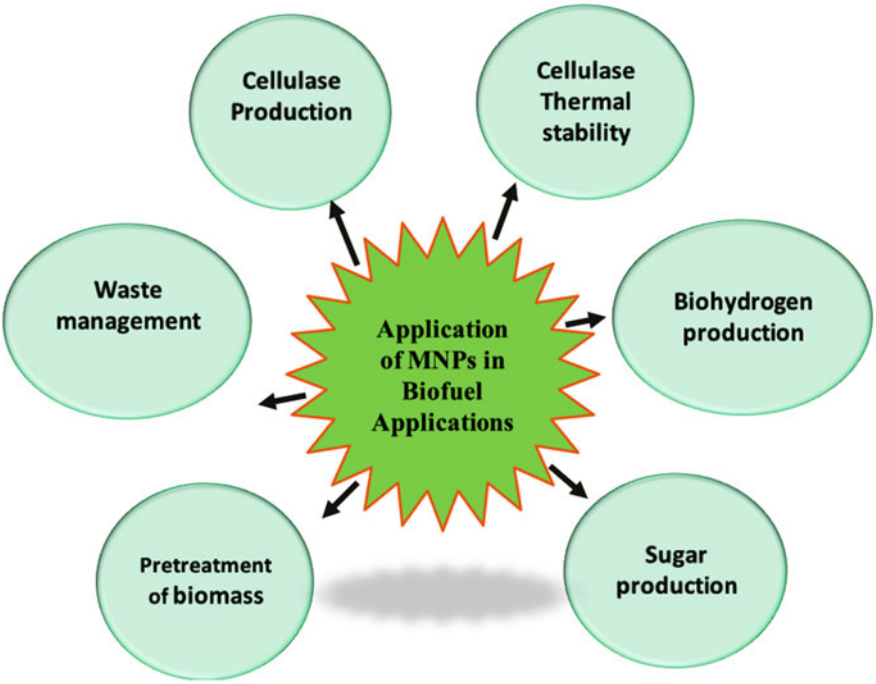


**Fig. 8.6** Applications of nanocatalysts in biofuel production

technological, environmental and scientific points of view due to its characteristics like selectivity, activity disabilities and recover abilities. The vital characteristics that nanocatalyst satisfies relates to 100% selectivity, higher activities, lower energy consumptions and longer lifetimes. The expected advantage of nanocatalysis in the chemical industry are improved economy, energy efficiency, reduced global warming, optimum feedstock utilizations, safe catalyst and reagent and minimal chemical wastes. The activated surface increases when catalyst sizes are lowered. So, with smaller particles, the covers to volume ratios and reaction efficiencies are high, and catalyst amount decreases. Another property is spatial organizations of the active site in catalyst. An important catalyst selectivity property is the geometrical and electronic structure, which plays essential roles in enzyme uses. This makes heterogeneous catalyst quite energy saving in nature. Because of the catalytic property of the nanoparticles, which relates to higher activities, surface reactivity, larger pore sizes and larger surface areas in comparison to macroscopic catalysts, they are a good candidate as the catalyst for the production of environment-friendly fuel like biodiesels.

## 8.6 Nanoparticles in Biomass Pre-treatment

Nanomaterials can be used to enhance biofuel production, for instance by using nanoparticles to enhance biofuels like biohydrogen or bioethanol (Srivastava et al. 2014). Different methods include the use of nanomaterials as one of the key approaches to increasing the production of biofuel. The benefit of employing nanomaterials will add importance to the method of producing biofuel to make it much more viable by decreasing expenses and having favourable impacts on the environment. Approximately all biofuel production techniques face certain constraints that impede their method of marketing. The primary difficulties in the production of biofuels are the development of such biofuels that are incredibly efficient in terms of cost-effectiveness and higher energy performance. The use of metal nanoparticle plays a crucial role in proving this method economically viable in this respect.

## 8.7 Use of Nanoparticles in the Production and Stability of Cellulase

The use of nanomaterials has now appeared as the latest region for the generation of bioenergies to improve enzyme stability (Jordan et al. 2011; Srivastava et al. 2014). Lately, only few prospective and successful studies in this region have been revealed. Enhanced production of cellulase in the presence of hydroxyapatite nanoparticles using bacterial strains was noted in the research by DD. The extremely thermostable enzyme used for this study maintained its half-life at 80 °C. The existence of calcium hydroxyapatite nanoparticles indicates enhanced thermostability of ~35% for purified cellulose and xylanase enzymes extracted from bacteria.

Srivastava et al. (2014) found an increase in cellulase production, its thermal stabilities and productivities of sugar in the existence of nanocomposite $Fe_3O_4$ or alginates. These authors used thermotolerant fungal species to report increased sugar productivities. They used *Aspergillus fumigatus* AA001 in the presence of $Fe_3O_4$ or alginate nanocomposites for olid-state fermentation. In addition, the development of cellulase enzymes along with their thermal stability has also enhanced relative to control in the presence of intact $Fe_3O_4$ nanoparticles. Cellulase production improved by 35–40% in the presence of bare $Fe_3O_4$ nanoparticles and $Fe_3O_4$ or alginate nanocomposite, respectively. Furthermore, $Fe_3O_4$ or alginate cellulase allowed to treat with nanocomposite demonstrates its thermal stability at 70 °C for 8 h while maintaining 56% of relative activity. These findings indicate that nanoparticles can play a vital part in changing the whole process of bioconversion.

Enhanced production of cellulase along with thermal stability was also reported through solid-state fermentation of thermotolerant *Aspergillus fumigatus* (Class: Eurotiomycetes) NS in the presence of nickel cobaltite ($NiCo_2O_4$) nanoparticles (Srivastava et al. 2014). Cellulase production is improved by 40%, while enzymatic

activity is increased by 19.8%, 49% and 53%, respectively, in xylanase, endoglucanases and β-glucosidase. In addition, crude cellulases show thermal stabilities for 7 h, 80 °C with $NiCo_2O_4$ nanoparticle, when controls (cellulose non-treated) show stability at the same temperature for up to 4 h.

## 8.8 Nanocatalyst for Biomass Gasification

In biomass gasification, prevention of tar and char formations is crucial. Tar is a complex mixture of a hydrocarbon having an aromatic compound of the singular ring to five rings with oxygen having hydrocarbon and complex polycyclic aromatic hydrocarbon. The boiling temperatures of tars are higher, and it is condensed at a temperature below 350 °C which created the main problem like corrosions or failures of engine and blockage of pipe and filter. Tar can be poisonous for catalyst (Chan and Tanksale 2014). The effects of catalyst on gasification product are significant. Catalyst not only decreases tar contents but also improves the qualities of gas products and conversion efficiency. The presence of catalysts decreases char yields during the final steps of gasification processes while it increases char formations during evolution stages (Li et al. 2009). Potassium, sodium and calcium have found to be an efficient catalyst for char gasification in steam. Other metallic species can be useful for biomass conversions; some elements present in waste biomasses can prevent char gasification by poisoning the catalyst. Catalyst tar cleaning is attractive due to its property that no energy is needed (Table 8.1).

## 8.9 Conclusion

This chapter clearly states that nanocatalyst has been found as the best agent for the production of petroleum or diesel biofuel, and it provides an environmental-friendly method in biofuel productions, which conducts high catalytic conversions and selectivity, economical method, mild operating condition and long-time persisting catalytic activity, etc. are the observed advantages. It is found that nanocatalyst leads to increased performance rather than a commercial catalyst. This study discusses the importance of biofuels, the challenges in the areas of biofuel productions, efficient use of nanocatalysts for biofuel applications and its merits and limitations in application fields. However, there are still certain resistance and difficulty exciting in different application fields of nanocatalysts like catalyst reusability which insist more investigation and researches in future.

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**Table 8.1** Recent development of nanocatalysts for biofuel applications

| S. No. | Nanocatalyst | Size (nm) | Source | Fuel | References |
|---|---|---|---|---|---|
| 1 | $Fe_3O_4$@$SiO_2$ | 20 | *Aspergillus niger* | Biofuel | Baskar et al. (2019) |
| 2 | Ru/$SiO_2$-$ZrO_2$ | 4.1 | Lignin oil | Phenolic compounds | Riyang et al. (2020) |
| 3 | Cu-$TiO_2$ | 80–150 | Palm oil | Biodiesel | Arghyadeep and Siddhartha (2020) |
| 4 | CaO@MgO | 20–50 | Waste edible oils | Biodiesel | Rauf et al. (2020) |
| 5 | Fe-W@$ZrO_2$ | 25.5 | Waste cooking oil | Biodiesel | Fatah et al. (2015) |
| 6 | $TiO_2$-$C_4H_5KO_6$ | 26–179 | Linseed oil | Biodiesel | Indu et al. (2018) |
| 7 | $Fe_2O_3$ | 200 nm | Microalgae | Biodiesel | Srijoni et al. (2019) |
| 8 | γ-$Fe_2O_3$ | 75.89 | Algae oil | Biodiesel | Fatih et al. (2019) |
| 9 | $K^+$/MgO | | Sunflower oil | Biodiesel | German et al. (2019) |
| 10 | Co-ZnO | 27.8 | Non-edible oil | Biodiesel | Manash et al. (2019) |
| 11 | $TiO_2$-ZnO | ~12 | *Ulva lactuca* | Biodiesel | Sivaprakash et al. (2019) |
| 12 | ZnO | 50–70 | Algal oil | Biodiesel | Kalavathy and Baskar (2019) |
| 13 | CaO | | Cooking oil | Biodiesel | Majid et al. (2019) |
| 14 | Zn-CaO | 14.3–65.6 | *Calophyllum inophyllum* oil | Biodiesel | Naveenkumar and Baskar (2019) |
| 15 | KOH/ $Ca_{12}Al_{14}O_{33}$-C | 15.1 | Canola oil | Biodiesel | Hamed et al. (2019) |
| 16 | CaO/MgO | 63.3 | Waste cooking oil | Biodiesel | Abdelrahman et al. (2019) |
| 17 | $K_2CO_3$/γ-$Al_2O_3$ | | Sunflower oil | Biodiesel | Sadykov et al. (2019) |
| 18 | Al-MCM-41 | | Sunflower oil | Biodiesel | Neda et al. (2019) |
| 19 | $CaTiO_3$ | 37.19 | Scum oil | Biodiesel | Yatish et al. (2018) |
| 20 | CaO | 32 | *Butea monosperma* oil | Biodiesel | Yatish et al. (2018) |
| 21 | $CeO_2$/$SiO_2$ | 15 | Waste engine oil | Liquid fuel | Navid et al. (2017) |
| 22 | MgO/$MgAl_2O_4$ | 13.8–25 | Sunflower oil | Biodiesel | Hamed et al. (2019) |
| 23 | $Ni_{0.5}Zn_{0.5}Fe_2O_4$ | 40–110 | Fatty acids | Biodiesel | Dantas et al. (2019) |
| 24 | MgO/$MgAl_2O_4$ | | Sunflower oil | Biodiesel | Behgam and Mohammad (2017) |
| 25 | Ag-ZnO | 20–30 | Terminalia bellirica oil | Biodiesel | Reddy Yadav et al. (2018) |

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# Chapter 9
# Microbial System: An Emerging Application in the Bioenergy Production


**Veer Singh, Nidhi Singh, Nazish Tabassum, and Vishal Mishra**

**Abstract** Environmentally safe and cost-effective renewable fuels are attained from various types of biological sources such as agricultural waste, microbial biomass, and plant residues. Nowadays, bioethanol and biogas are the main biological fuels and play an important role in the energy sector. These fuels can be considered as alternative energy sources of fossil fuels in the future. Biofuels can be classified into various classes such as first generation, second generation, third generation, and fourth generation on the basis of their production sources. They are generally produced from several biological raw materials through aerobic and anaerobic digestion of biomass. The fermentation process requires a suitable microbial system and raw materials (algal biomass, agricultural waste, and plant residues). Microorganisms such as algae, bacteria, and fungi can utilize organic materials and also convert biomass into several bioenergy products. Nowadays, the effective and suitable microbial system can be obtained by genetic modification and metabolic pathway modification methods. Additionally, suitable microbial systems can be obtained from environmental sources such as soil and water by using metagenomic techniques. The recent research in the production of bioethanol, biogas, biomethanol, biodiesel, and biohydrogen has been discussed in this chapter.

**Keywords** Microbial system · Bioenergy · Generation of biofuels · Metagenomics

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## 9.1 Introduction

Fossil fuels are the major energy source worldwide and play an important role in the energy sector. These fuels produce several types of harmful gases during combustion of the fuels. These gases cause environmental pollution and harmful effects on human health. The sources of the fossil fuels are also very limited. These sources will deplete after certain period of time. Hence, it is very needful to develop an alternative option of fossil fuels which can fulfil our energy needs (Singh et al. 2020). The biofuels (biogas, biodiesel, and biohydrogen) are the cost-effective and eco-friendly fuels derived from the various types of biomass. Hence, biofuels are the best option which can replace the need of the fossil fuels in the coming future. Biofuels are classified into different categories (based on the utilization sources for the production of the biofuel) such as first generation, second generation, third generation, and fourth generation. First- and second-generation biofuels are derived from the lignocellulosic materials of the biomass (Oh et al. 2018). These biofuels are derived from various types of biomass such as plant residues and agricultural wastes (Demirbas 2011). The third-generation biofuels are produced from the algal or microalgae biomass and are considered as good alternative option of energy worldwide in the future. The fourth-generation biofuels are based on the third-generation biofuel sources (Peng et al. 2016).

Genetically modified microorganisms are the main sources of the fourth-generation biofuels (Peng et al. 2013). Modified genome of the biofuel producing organism responsible for enhanced production of the biofuels and beneficial for energy sector (Wang et al. 2010). These biofuels can help reduce of toxic level of gases in the environment as well as minimize the health problem in the living organisms (Ramasamy et al. 2015). Microalgal biomass can accumulate up to 100 times more oils compare than other terrestrials crops such as soybean (Yun et al. 2014). Hence, biomass derived from microalgae is more appropriate than biomass derived from other biofuel-producing crops. The growth of microalgae highly depends on the presence of water, sunlight, carbon dioxide, and nutrient composition of the growth medium (Kim et al. 2012).

Biogas is the second most valuable biofuel categories which plays an important role in the bioenergy production sector (Kim et al. 2017). Biogas can be obtained by digestion of organic materials using suitable microbial species (Knuckey et al. 2006). The main components of biogas are methane and carbon dioxide. Some other gases like hydrogen sulfide and nitrogen gas are also present in biogas (Laamanen et al. 2016). The typical quantities of biogas components are 45–70% methane, 30–40% carbon dioxide, and 1–15% nitrogen and other gases. The composition of these gases in the biogas is highly dependent on the raw materials used for the production of biogas (Park et al. 2019; Peng et al. 2013). Burning of the methane and hydrogen with oxygen is reported to release sufficient amount of energy which can be used for cooking or other purposes (Peng et al. 2015; Ra et al. 2015).

Biofuel is considered as renewable source of energy because it is derived from the biological materials present in nature. These biological materials are found in the liquid or solid form which can be converted to utilizable biofuels through various biomass conversion process (Cheng et al. 2015). There are several disadvantages in the biofuels production such as low efficiency, and raw materials of some biofuels are based on the food materials such as corn, rice, sugar beets, and oilseed (Demirbas 2008).

Nowadays, bioethanol is the main additive used in the petroleum fuels. In future, researchers are working to increase the amount of bioethanol in petroleum (Adeniyi et al. 2018). Addition of bioethanol in the petroleum fuels can decrease the amount of carbon monoxide and hydrocarbon during the burning process in diesel-powered vehicles. Biofuel can be considered as an alternative option of energy producers which can replace the demand of petroleum products in the coming future. Biofuel is in the form of alcohol including ethanol, methanol, propanol, and butanol. Fossil fuel increased $CO_2$ accumulation due to its burning in the atmosphere. Lignocellulosic feedstock produces bioethanol based on enzymatic hydrolysis. Lignocellulose contained 30% hemicellulose, 44% cellulose, and 20% lignin. Bioethanol reduces the negative environmental impacts produced by fossil fuel usage, and it is produced by the fermentation of raw materials that contain fermentable sugars. Biomass-derived simple sugar such as glucose as well as sucrose is considered as the major raw material for the production of biofuel in many countries. The conversion of plant biomass into disaccharides and finally into biofuel is a complex process (Sebastian et al. 2013; Arroussi et al. 2015).

Biomass can be converted to biofuel sources by suitable microorganisms. The suitable microorganisms can be isolated or modified by genetic engineering techniques and can optimize the biochemical pathways for effective degradation of raw materials (Park et al. 2011; Lee et al. 2013). The initial concentration of sugar in the microbial growth medium affects the growth of microorganism as well as the production of ethanol. The immobilization is the method by which microbial cells are immobilized on the surface of the solid substrate. This process also affects the production of bioethanol (Jin et al. 2012). Genetic modification is more advantageous for biofuel production. Different methods such as random chemical mutagenesis, genome shuffling, and ultraviolet exposure have been used to obtain microbial mutants. The suitable microbial strain is selected by using different selection methods and can be applied for the production of biofuel (Blatti et al. 2012). Other characteristics like species adaptation, cell protection, and tolerance capacity need to be optimized during the biofuel production because high temperature and high concentration of the ethanol are main stressors in the bioreactor. Hence, the presence of ethanol and variable temperature ranges affect the production of biofuels (Abreu-Cavalheiro and Monteiro 2013).

In the environment, 99% of microorganisms, as yet, are uncultured. Metagenomics is the technique by which DNA samples are collected and analyzed from environmental sources followed by complete genome cloning of the whole microbial community. The constructed metagenomic libraries are used for further analysis and application in various fields (Asada et al. 2012). Isolation and

characterization of microbial communities directly from natural sources are performed to understand the human-related disease including skin, mouth, and gut sample and plant soil–microbes interaction by taking soil samples (Attwood et al. 2019). Handelman and collaborators describe the importance of soil microbes from uncultured microorganism mining and their novel chemical compounds. Metagenomics is a culture-dependent as well as culture-independent technique based on sequencing or expression as well as data analysis available on the online database. DNAs are isolated from various environmental sources such as soil and water followed by DNA sequencing to identify the unknown microbial species present in the samples. Metagenomic profiling/identification of populations using phylogenetic markers (such as 16S RNA for bacteria and archaea) is mainly based on the PCR-based approach. Metagenomics approaches are the analysis of microbe with the application of industrial enzymes in biofuel production such as microbial glycoside hydrolase from target screening (Alves et al. 2018).

## 9.2 Classification of Biofuels

Biofuels are classified based on the source of production into primary and secondary. The primary category of biofuels is derived from natural bioresources like plants, animal wastes, and lignocellulosic materials, whereas the sources of secondary biofuel are directly from bacteria, microalgae, and fungi. The secondary biofuel is divided into four generations. First-generation biofuels are produced from starch-rich biomaterials which include sugarcane waste, wheat or wheat straw, oats, potato, sweet potato, corn, and animal fats.

### *9.2.1 First-Generation Biofuels*

The first-generation biofuel is also called as a conventional biofuel. Biodiesel, ethanol, and biogas are the three major types of first-generation biofuels and are used at the commercial level. These biofuels play an important role in the bioenergy sector, and the technology used for the production of these biofuels is well stabilized. This generation of biofuels is derived from the transesterification of vegetable oils and fatty acids. Biodiesel is used as an energy source through minor modifications in the diesel engine. Biogas and biomethane are the gaseous form of fuel and are very crucial for the bioenergy sector. Due to high demands and limited production of edible oils and food materials worldwide, it is very difficult to use edible oil or food materials for the production of biofuels (Alptekin et al. 2014). Some crops have excellent potential for the production of biofuels. One of the best advantages of these crops is that it is cultivated in unreproductive fields. Jatropha is a very well-known oilseed crop cultivated worldwide for the production of biodiesel. It can be cultivated on the unreproductive lands and requires less additional nutrients as compared to

other crops. Jatropha considers a major source of biofuels as well as some other value-added products (Naik et al. 2010).

### 9.2.2 Second-Generation Biofuels

Second-generation biofuels are derived from non-food biomaterials. These biofuels derived from a different type of biomass such as lignocellulosic biomass, agriculture products, and food crop waste material (Naik et al. 2010). Nowadays, these fuels are not cost-effective due to various techniques used for the production of these biofuels. Plant biomass is composed of mostly plant cell wall, which is composed up to 75% of polysaccharides (Eisentraut 2010), and these polysaccharides are good sources of valuable sugar that have good potential for biofuel production. Second-generation biofuel is derived through advanced process and utilization of lignocellulosic biomaterials for biofuel production.

### 9.2.3 Third-Generation Biofuels

Third-generation biofuels are generally derived from algal biomass. They are an excellent source of vitamins, proteins, and carbohydrates as well as supply 60% of oxygen to all living beings by algae in many ecosystems. Algae produce crude oil which can be easily converted into diesel and gasoline. Some algal species may be categorized into microalgae and macroalgae. The third-generation biofuels have many advancements in terms of efficiency as compared to first- and second-generation biofuels. They obtain low-cost materials and based on completely renewable energy sources. Therefore, these third-generation biofuels are cost-effective. At the same time, these energy sources are renewable and do not contribute to any unwanted or harmful effects in our climate (Alam et al. 2015).

### 9.2.4 Fourth-Generation Biofuels

It is the latest biofuel technology containing two parts of the new generation of biofuels. One part is based on the engineered biofuels producing crops that act as carbon-capturing machines. The second part is based on the "solar to fuel" concept. The fourth-generation biofuel is advantageous because it has a high production yield and requires non-arable land. These biofuels also include electro fuel (Abdullah et al. 2019). Solar to fuel method based on the microorganisms (cyanobacteria) and presence of the sun as well as $CO_2$. In this method, cyanobacteria are placed in the flat panel, and these panels are filled with the water. The panels are set up facing the

sun, the same as solar panels. The microorganisms (cyanobacterial) take solar energy from the sun and capture $CO_2$ from the environment. This is a more cost-effective technology as compared to third-generation biofuels (Abdullah et al. 2019).

## 9.3 Sources of Biofuel Production

Biofuels are basically produce from the dead biomass of marine or terrestrial algae (macroalgae and microalgae), plant biomass, agricultural waste, and waste derived from various industries like sugar industry. Different sources of biofuels are given below.

### *9.3.1 Agricultural Waste*

Farmers cultivate various types of crops like wheat and rice in their field every year, and large quantity of wastes are generated in the form of dry biomass. These dry biomass (crop residue) remains left in the field which enhances the fertility of the soils. In India, large number of farmers burn the crop residue in the field, which decreases the fertility of soil and causes air pollution during burning of crop residues (waste) (Kakucs and Kun-Szabo 2009; Brosowski et al. 2016; Papilo et al. 2017). The crop biomass or agricultural wastes have been utilized in the production of biofuels. These biofuels are synthesized through the degradation of agricultural waste by microbial catabolic reaction (Knapek et al. 2015; Baum et al. 2013). Other sources such as milk whey sugar (left during cheese production) and organic compounds generated during livestock operation provide the suitable option for biofuel production. These operations minimize the cost of the fuel production as well as are eco-friendly for the environment (Zhai et al. 2015). Plant biomass is also utilized for the production biofuels as well as in the generation of heat during combustion. These biomasses minimize the need of the fossil fuel and natural gases (Gan and Smith 2012).

### *9.3.2 Microalgae Biomass*

Microalgae are the single-cell algae present in nature, and these are good source for various types of biofuel production. Microalgae can be grown in fresh water as well as marine water. They intake large amount of carbon dioxide from atmosphere and maintain the level of the carbon dioxide in the atmosphere. Hence, microalgae play an important role in global warming (Biffinger and Ringeisen 2008). These microalgae can be grown very fast (doubling time 6–25 h) and require less nutrients. Microalgae are rich in energy sources because they contain 40–50% oils in their dry

weight of biomass. *Botryococcus* spp. can accumulate up to 50% of lipid content of its dry weight of biomass (Kojima and Zhang 1999; Parker et al. 2008). Various types of algal species are present in the nature which provide a large number of options to the researchers for characterization and identification of algal species and their genetic information (Mandotra et al. 2016; Ho et al. 2013). The microalgae also have the potential for the treatment of municipal waste. The nitrate and phosphate contents present in the waste can be removed before discharge as effluent. The process of cultivation of microalgae in wastewater has been found suitable for bioremediation along with biofuel production. This combination of bioremediation-cum-biofuel production is more advantageous and cost-effective (Douskova et al. 2009; Hannon et al. 2010).

Improved technology of biofuel production plays an important role in the biofuel industry. The effective protocol is used for the cultivation of microalgae such as maintaining the photoperiod as well as balanced nutrient in the growth medium. Design of the photobioreactor by expert engineers also minimizes the power consumption and the cost of biofuel production (Borowitzka 2013). Cultivation of microalgae in the open system is responsible for the decrease in the cost of biofuel production, but this creates the risk of contamination. Closed system provides a control growth of microalgae and is responsible for low risk of contamination. Hence, recently developed technologies are very important for the cultivation of microalgae, cell harvesting, and extraction of oils from the microalgal biomass (Chisti 2008; Rosenberg et al. 2008; Lehr and Posten 2009; Beer et al. 2009).

## 9.4 Approaches for Microbial Strain Improvement

Nowadays, the research has been focused on the economic successes of technology as well as selection and utilization of effective and cheap technologies (Elshahed 2010). Lignocellulosic materials from various agricultural crops such as maize are considered as potential feedstocks for biofuel production. Some challenges are created like selection of suitable microorganisms for pretreatment process and efficiency of biofuel production. Microorganisms have different biomass degradation capability due to the expression of biomass degradation enzymes (extracellular) by microorganisms. Ideal organisms have been used for the conversion of biomass such as complex sugar converted into simple sugar molecules. Various growth factors like temperature, pH, and presence of the nutrient also affect the biomass conversion efficiency of microorganisms. Some inhibitors are produced during biomass treatment process, and these inhibitors create toxic effect which affects the biomass conversion process (Wu et al. 2014). Therefore, selection of suitable microorganisms is very important for biofuel production (Mukhopadhyay 2015). *E. coli* has been used as model organism in the current research to improve the efficiency of bioconversion of biomass. Various metabolic engineering techniques

have been applied to enhance the production of biofuels. Metabolic pathway modification and regulation can help in the adaptation process of microorganism against physical and biochemical factors (Huffer et al. 2012; Sarchami and Rehmann 2014). Most of the biofuel-producing enzymes have low efficiency of hydrolysis of waste which is responsible for the less production of biofuels. Protein engineering techniques can be used to solve these issues. Protein engineering approaches are based on the post-translation modification in the protein molecules, and these modified proteins can be used in the biofuel production industry (Wen et al. 2009; Xue et al. 2017).

## 9.5 Metagenomic Approach for the Isolation and Characterization of Microorganisms

Metagenomic is a culture-independent as well as culture-dependent process for the identification of novel biofuel enzymes from unculturable microorganism. The novel biofuel enzymes are isolated from metagenome of soil microorganisms. In the metagenomics methods, it is very important to select appropriate environment site for the collection of suitable microorganisms which involve in the biofuel industry (Sotoft et al. 2010). The isolation and characterization of microorganisms from environmental sample generally involves isolation and characterization of microorganism (Fig. 9.1). Molecular biology and computation biology play active role in the isolation and characterization of microorganisms from various environmental

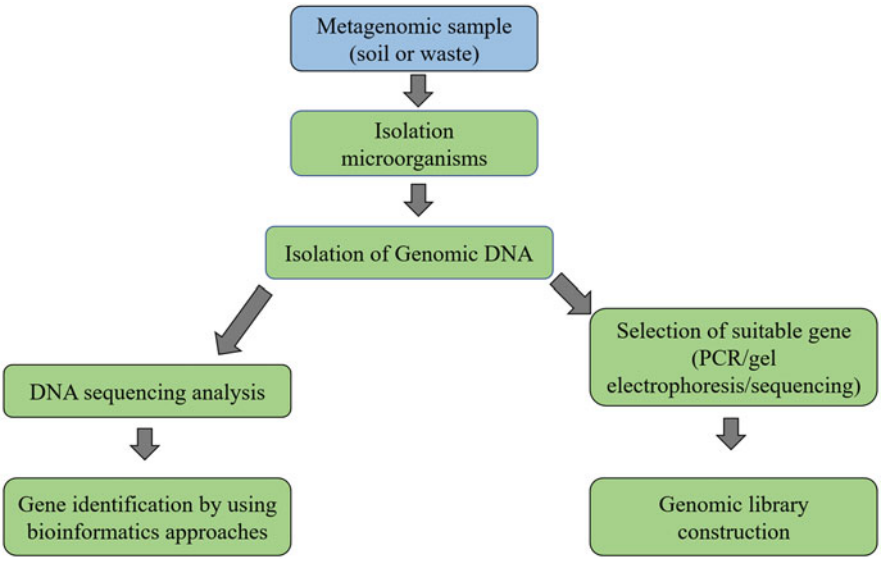


**Fig. 9.1** Isolation and identification of novel microorganism from environmental samples

samples. Genetic engineering methods are used for the construction of genomic library of targeted genes responsible for the production of biofuel enzymes. The targeted gene is constructed in the suitable host by using transformation method (Masran et al. 2016). Vectors are also constructed for protein expression. Hence, suitable metagenomic library is constructed for further application. From this library, gene encoding novel enzyme is isolated (Schmieder and Edwards 2011). The isolation and identification of novel biofuel enzymes involve a series of metagenomic approaches.

### *9.5.1 Sample Collection and Isolation of Genomic DNAs*

Suitable environmental site selection for the metagenomic sample collection play an important role in the isolation of novel enzymes. Sample processing is also crucial and first step of metagenomic analysis. DNA extracted from the environmental sample which is representative of cell and a large amount of nucleic acid obtained for library preparation and sequencing (Schmieder and Edwards 2011).

### *9.5.2 Host Selection and Vector Construction*

Suitable host microorganism is selected as host for the construction of metagenomic library. The desired DNA fragments are inserted into the vector DNA, and these desired DNAs are transferred into the host cell with the help of vector for maintaining a genomic library of metagenomic samples (Schmieder and Edwards 2011).

### *9.5.3 Metagenomic Library Screening*

Metagenomic can be based on sequence-driven and function-driven analysis of uncultured microbial community. They have been used to identify novel biocatalysts from the metagenomic samples. Microbial communities are a collection of archaea, bacteria, viruses, and fungi (Sebastian et al. 2013). Various screening methods have been used for the metagenomic library screening such as function-based screening (Sebastian et al. 2013), sequence-based screening (Xing et al. 2012), and SIGEX screening (Yun and Ryu 2005).

### 9.5.4 DNA Sequencing for Metagenomic Sample

The region of interest or the subset of the genome of microbial DNAs has been determined by using various sequencing methods. In general, if the size of the gene is rather large, then high-throughput sequencing is required. In this situation, Illumina Hiseq sequencing will be better for sequencing DNA. Whereas if the throughput required is lower and/or the read length plays an important role, then the Illumina Miseq or even the PacBio may be the most appropriate (Chen 2014).

Next-generation sequencing is based on the large amount of DNA. Next-generation metagenomic sequencing has been used for comprehensive study of genes present in environmental sample. Metagenomic sequencing potentially viable for the assessment of various microorganisms and sufficient in different niche allows effective screening of the library. The metagenomic library used for the identification of novel biocatalyst has potential application in the biofuel industry (Wang et al. 2019). Various sequencing methods have been used to study genetic makeup of metagenomic DNAs. Oxford nanopore sequencing technologies were designed by Oxford Nanopore Technologies (Eisenstein 2012). MinION sequencing has emerging application in metagenomic taxonomic identification by accessing the low-complexity metagenomic community and can produce high-quality reads to introduce 99% exact taxonomic composition (Brown et al. 2017). Second-generation sequencing is Illumina and Ion Torrent sequencing technologies that produce many short reads (150–400 bp) (Wang et al. 2019). 454 Life Sciences produced the first successful second-generation sequencer, i.e., 454 DNA sequences. About one million sequences with reading length 400–500 bp are produced through a sequencing-by-synthesis approach (Agah et al. 2004). Other important sequencing methods such as Illumina genome analyzers, ion torrent, third-generation sequencing, single-molecule real-time (SMRT) sequencing, and applied biosystem also have important application in the identification of genomic DNAs (Chen et al. 2014; Stadermann et al. 2015).

## 9.6 Possibilities of Bioenergy Production

### 9.6.1 Electricity Generation

Organic compound is converted into electric power generation and has been reported in various literature. The electrical power generation mainly depends on the microbial fuel cell (MFC) and design of the various reactor. Electric power production by MFC basically depends on the hydrolysis and oxidation process of organic materials. The organic waste materials are obtained from various sources such as plant biomasses, rice straw, wheat straw, and other agricultural wastes. The electricity is generated under the fermentation process in the absence of the oxygen. The physical and biochemical parameters also affect the growth of microorganism as well as

electricity generation. The design of the electrodes also affects the electrical power generation by MFC (Karluvalı et al. 2015). MFC devices for the bioremediation-cum-electrical power generation is more advantageous compared to other MFC. The food- and agriculture-based effluent is more suitable for the bioremediation-cum-bioenergy production. Fuel cells utilize the waste component for their growth as well as energy production. This process is based on low cost and eco-friendly nature (Mekawy et al. 2015; El-Chakhtoura et al. 2014).

### *9.6.2 Biogas Generation*

Biogases are generally produced in the absence of oxygen. The organic materials decompose in the oxygen-deficient environment and produce a mixture of biogases. Methane is the main component of the biogases which are produced during decomposition of organic biomass by methanogenic bacterial species (Srivastava et al. 2019). The biogas can be used in the synthesis of heat, generation of electricity, and in the vehicles as fuels. Various substrates can be used in the production of biogases. The selection of the suitable substrate also depends on the types of bioreactor used in the biogas production process. Efficiency of biogas production also depends on the profile of microbial community structure used for pretreatment process and various growth parameters like availability of nutrient as well as temperature and pH (Schnurer 2016).

Agriculture wastes like rice straw and wheat straw are cost-effective and present abundantly. These wastes also have good potential for biogas production with some issues like complex structure creates difficulty in the degradation of cellulosic structure. Only limited number of microbial species are present in the nature which have potential application in degradation of cellulosic biomass. Microbial community is isolated from wastewater treatment plant. These microbial species have potential application in the digestion of waste along with biogas production. Some microbial species such as *Clostridium cellulolyticum* have potential application in the degradation of cellulosic biomass (Sun et al. 2016).

### *9.6.3 Bioethanol Generation*

Microalgae have been widely used for the production of biofuel due high lipid content and fast growth rate compared to macroalgae. Cultivation, harvesting, and oil extraction technology are involved in the production of biofuels from microalgae (Wen et al. 2016). Microalgae have high photosynthetic efficiency and low carbon dioxide emission into the environment, which help in minimizing global warming. Microalgae perform high growth rate and can accumulate up to 60–70% lipid content in their biomass. The lipid content varies species to species and it also affects by several environmental factors like temerature, pressure, humidity and

nutrition behaviour. They can survive in the adverse environment which is suitable to grow and minimize the cost of cultivation. Biodiesel, bio-oil, methane, biohydrogen, biomethanol, and bioethanol are the best examples of biofuels obtained from microalgae (Milano et al. 2016).

Biomasses of the microalgae and macroalgae are the good source of green energy generation. They can be utilized in the production of huge quantity of biodiesel, bioethanol, and biohydrogen. Other biological materials such as rice straw, palm oils, sugarcane crops, and wheat grain straw have been used for the production of biofuels. But microalgae are more advantageous due to short growth period and can grow in non-arable and sterile land (Tan et al. 2015).

## 9.7 Conclusion

Bioenergy is derived from natural renewable sources and considered as green and clean fuels. Biofuels exist in both liquid (bioethanol, biodiesel, biomethanol, and bio-oil) and gaseous (biogas and biohydrogen) states. The limited sources of the fossil fuels are available on the Earth and will be utilized completely in the future. Therefore, it is extremely important to find out cheap and effective source of energy in future. The biofuel can be considered the alternative option of the fossil fuels and can fulfil all the energy needs of world. Biogas is the second-ranked energy sources generated from the algal biomass as well as agricultural wastes. It can be used in cooking (alternative of LPG) and in the generation of heat in various industries. Microalgae can accumulate large amount of lipids in their cell mass (up to 70% of dry biomass) and have potential application in the biofuel production. Biofuels are also produced from other biological materials such as agricultural waste and domestic waste by using fermentation process. The various microbial species involve in the degradation of complex biomass to simple substance and finally produce biofuels. Bio-electricity is also generated during the degradation or utilization of raw materials. The production of bioelectricity highly depends on the size of the MFC as well as the composition of growth medium. It is a very advantageous process used for wastewater treatment-cum-bioenergy production. The application of genetic engineering and metabolic engineering is beneficial for the improvement in the microbial community for the production of biofuels. The metagenomic is the culture-dependent as well as culture-independent technology used for the identification of novel microbial community which have potential application in the biofuel production. The authors concluded that bioethanol, biomethanol, biogas, and bio-oils produced from sustainable resources like microalgal biomass and various types of organic wastes (agricultural and plant residues) are very advantageous to the environment, farmers, and industries.

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# Chapter 10
# An Introduction of Metagenomics and Its Application in Microbial Fuel Production


**Nidhi Singh, Veer Singh, Divya Mishra, and Mohan Prasad Singh**

**Abstract** Petrolium fuels are considered as non-renewable fuels. These fuels are also responsible for increased $CO_2$ level in the environment due to the burning of fossil fuels. Biofuels have been considered as an alternative option that can replace the need for fossil fuels and minimize environmental pollutions. Biofuels can be produced using biomass derived from microalgae or macroalgae, bacterial biomass, and lignocellulosic biomass (plant and agricultural wastes). The use of lignocellulosic materials and evading the use of food materials for biofuel production are a viable strategy. Lignocellulosic materials are the most abundant resource on the Earth and easily available worldwide. These lignocellulosic materials can convert into simple sugars, and these sugars further are used for ethanol production. The cellulolytic enzymes can be digested using cellulosic materials into biofuels and some other value-added products. Various highly-effective techniques and pathways have been evolved, but the use of the enzymes for degradation of biological wastes has been isolated only from limited culturable microorganisms. Most of biomass-degrading microorganisms are not suitable for biofuel production at an industrial level while conventional techniques for identifying and cloning their individual enzymes are inefficient. The metagenomics methods are genomic analysis techniques of isolation of microorganisms from various environmental sources and discover novel more effective microbial enzymes for biomass degradation. This chapter focuses on the process of biofuel production, metagenomic tools for the identification of novel enzyme and metagenomic applications for biofuel production.

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**Keywords** Biofuel · Biomass · Metagenomics · Microbial enzymes · Renewable energy

## 10.1 Introduction

Fossil fuels such as coal, petroleum ions, and liquid petroleum gases (LPG) are available in nature in a limited amount because these sources of energy are nonrenewable. These fuels have towering costs and cause several types of environmental pollutions such as air, soil, and water pollution. When fossil fuels burn, they produce energy and various types of gases including carbon dioxide, sulfur dioxide, and other various types of gases. Carbon dioxide is the major gas that plays important role in the greenhouse effect and also responsible for maintaining Earth's temperature. An increase in the level of this gas causes an rising in the temperature of the Earth which affects the distribution of living organisms. Air pollution decreases the quality of air and affect to the ozone layer. The ozone layer protects us from ultraviolet radiation. UV radiation causes various types of health effects in humans and other living organisms. Water pollution by these fuel waste materials is responsible for the addition of many types of toxic substances in the water sources which affect the distribution and life of living aquatic organisms. These wastes generated from these fuels also cause loss of fertility in the soil. The availability of fossil fuel is limited, and after a certain time, these sources may be exhausted, then biofuel may fulfill the requirements of energy sources. Therefore, biofuel production is very needful for the future as an alternative energy source (Palaniappan 2017).

Biofuel is a source of energy produced directly or indirectly from biomass of plants, algae or animal residue. Feedstock materials such as leaves, grasses and corn stalks are cellulosic materials used for the production of ethanol. All biofuels are considered as renewal and basic energy sources. Biofuels are basically type of chemical energy which derived from photosynthetic conversion of solar energy and used for various purpose. Biofuel is considered a better source of energy in the future compared to fossil fuels because sources of fossil fuels are limited and maybe exhausted after a certain time. Therefore, biofuel is an alternative source of renewable energy while fossil fuels are a carbon-based energy source. Biofuel depends on two or more microbial groups that live symbiotically which transform cellulosic into sugars by the fermentation process of sugar into ethanol followed by sugar into ethanol by the fermentation process (Kang and Lee 2015). Microbes also produce methane and hydrogen as a source of bioenergy.

Biofuels are also known as renewable fuels. They are produced from biowaste or dead biomass. They are found in the gas or liquid form derived from the solid biological waste. There are various issues with biofuel production such as low production efficiency and raw materials of some biofuels are based on the food materials. Biofuel produced in the form of ethanol from corn, rice, sugar beets, and biodiesel from oilseed are also used in the food industries. The crops are high-quality agricultural plant (Demirbas 2008). Nowadays, bioethanol is used in the petroleum

as an additive. Biomethanol can be obtained from biomass using biosynthetic natural gas. Biodiesel is produced from transesterification of oils or fats. In vehicles, it can be used as fuel. It is used to reduce carbon monoxide and hydrocarbon by using as a diesel additive from diesel-powered vehicles. Biofuel can be considered as an alternative option of energy which can replace the demand of petroleum products in the coming future. Biofuel in the form of alcohol includes ethanol, methanol, propanol, and butanol. Fossil fuel increased $CO_2$ accumulation due to its burning in the atmosphere. Biofuel protects the environment. Lignocellulosic feedstock produces bioethanol based on enzymatic hydrolysis. Lignocellulose contained 30% hemicellulose, 44% cellulose, and 20% lignin. Bioethanol reduces negative environmental impacts produced by fossil fuel usage bioethanol produced from the fermentation of raw materials that contain fermentable sugars. Biomass-derived simple sugars such as glucose as well as sucrose are considered as major raw materials for the production of biofuel in many countries. The conversion of plant biomass into disaccharides and finally into biofuel is a complex process (Sebastian et al. 2013).

In some environment, 99% of microorganisms, as yet, are uncultured on Earth. Metagenomic analysis recovered a direct collection of DNA from environmental samples followed by complete genome cloning of the whole microbial community. The constructed metagenomic library is used for function and sequence. Isolation and characterization of metagnomic microbial communities directly from natural sources to understand the human-related diseases (Attwood et al. 2019). Handelsman and collaborators describe the metagenome which is the importance of soil microbes from uncultured microorganism mining of novel chemical compounds which comprise microbial diversity is more than 90%. Metagenome analysis is culture-dependent as well as culture-independent methods based on sequencing or expression as well as data analysis available on the online database. DNA isolation from environmental sources is followed by DNA sequencing of genetic materials to identify the unknown sample. Metagenomic profiling/identification of populations using phylogenetic markers (such as 16S RNA for bacteria and archaea) usually relies on a PCR-based approach. Metagenomics approaches are the analysis of microbe with the application of industrial enzymes in biofuel production such as microbial glycoside hydrolase from target screening (Alves et al. 2018).

## 10.2 Classification of Biofuel

Biofuel is classified on the basis of raw materials used for production and its production efficiency. Biofuel is mostly divided into two classes on the basis of source of production, primary and secondary. The primary category of biofuels is derived from natural biofuel sources such as plants, animal wastes, and other lignocellulosic materials. The second type of biofuel directly comes from microbial sources such as bacteria, microalgae, and fungi. The second type of biofuel is divided into four generations (the fourth generation is basically correlated with the

third generation). First-generation of biofuels are produced from starch-rich biomaterials such as sugarcane waste, wheat or wheat straw, oats, potato, sweet potato, corn, and animal fats. Various types of plant residues and grasses have important role in the biofuel industries. The aglae and microorganism are also good sources of biofuel production. Researchers are working in various areas of bioenergy production and trying to improve the production of bioenergy through various modifications at the genetic level in the algae and bacterial strain. This field of biofuel production is considered as a fourth-generation biofuel. The yield of biofuel production generally depends on the microorganisms used for the biofuel production process and optimum condition for the growth of selected microorganisms or algae. The components of media also affect the growth of microorganisms as well as the production of biofuel. Genetic engineering techniques are promising methods for modification at the genetic level in the biofuel-producing species. These techniques are responsible for enhancing the efficiency of biofuel production (Dragone et al. 2010; Abdelaziz et al. 2013). Various types of biofuel have been shown in Fig. 10.1a, b.

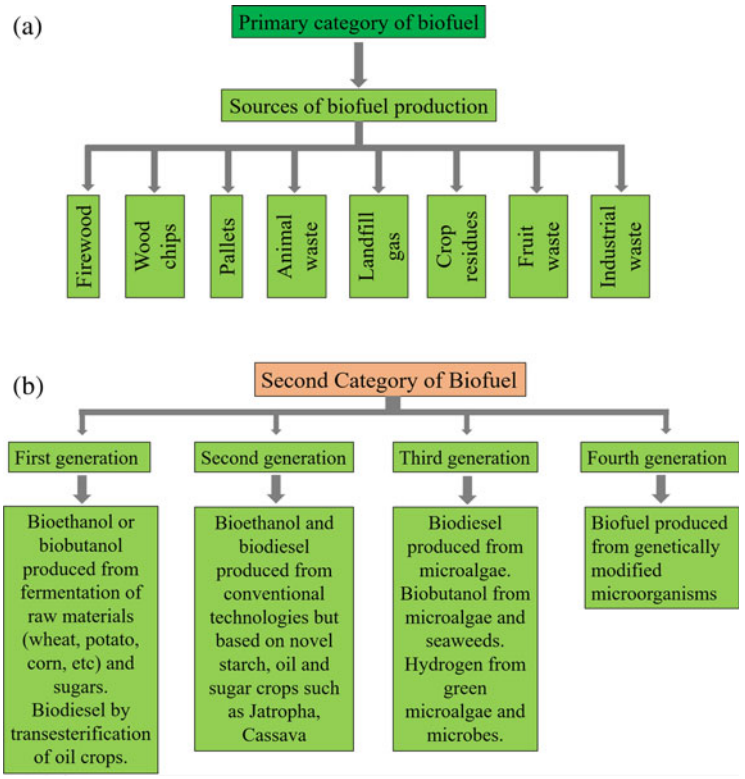


**Fig. 10.1** (**a**) The primary category of biofuel and its sources of production. (**b**) The second category of biofuel, generations of biofuel, and sources of biofuel production

### 10.2.1 First-Generation Biofuels

This category of biofuel also known as a conventional biofuel is resultant of food crops such as corn starch, sugarcane, and soyabean oil; the resultant derived from food crop is changed into biofuel by using a various processes such as transesterification and fermentation. The three major types of biofuels such as biodiesel, ethanol, and biogas are produced under first generation and used at the commercial level. These biofuels have important role in the bioenergy sector and the technology used for the production of these biofuels is well stabilized. Biodiesel is the alternative option of petroleum-derived diesel in the future. Nowadays, biodiesel is also used in the energy sector. It is derived from the transesterification of vegetable oils and fatty acids. The biodiesel is used as an energy source through minor modification in the diesel engine. Thereby, it can be considered as better option of energy in future.

Bioethanol is the alternative option of gasoline in the coming future. It is produced from natural cost-effective sources through fermentation process. It is also used as raw sources for the production gasoline. Biogas and biomethane are gaseous forms of fuel and are very important for the bioenergy sector. Biogas is generally used with a combination of gasoline and used as energy sources. Anaerobic digestion of feedstock is also one of the major pathway for the production of biogas.

Nowadays, biogas, bioethanol, and biodiesel are produced from raw materials that already used in food purposes. It is very difficult to use edible oil/vegetable oils or food materials for the production of biofuels due to the increasing demands of edible oils and food materials worldwide and limited production of these edible products (oils and food materials) (Alptekin et al. 2014). There are some crops used for the production of biofuels. These crops have excellent potential for the production of biofuels. One of the best advantages of these crops is that these crops can cultivate on unreproductive fields. These oilseed crops have muptiple applications such as biomass derived from these crops can be used in the production of various types of value-added products. The whole crops biorefinery has been shown in Fig. 10.2. The uses of industrial oilseed crop Jatropha have been discussed in Fig. 10.2. Jatropha is a very well-known oilseed crop cultivated worldwide for the production of biodiesel. It can cultivate on the unreproductive lands, and it requires less additional nutrients compared to other crops. Jatropha is considered as a major source of biofuels as well as some other value-added products (Naik et al. 2010).

### 10.2.2 Second-Generation Biofuels

Second-generation biofuels are derived from non-food biomaterials. These biofuels are derived from a different type of biomass such as lignocellulosic biomass, agriculture products, and waste food crop material (Naik et al. 2010). Nowadays,

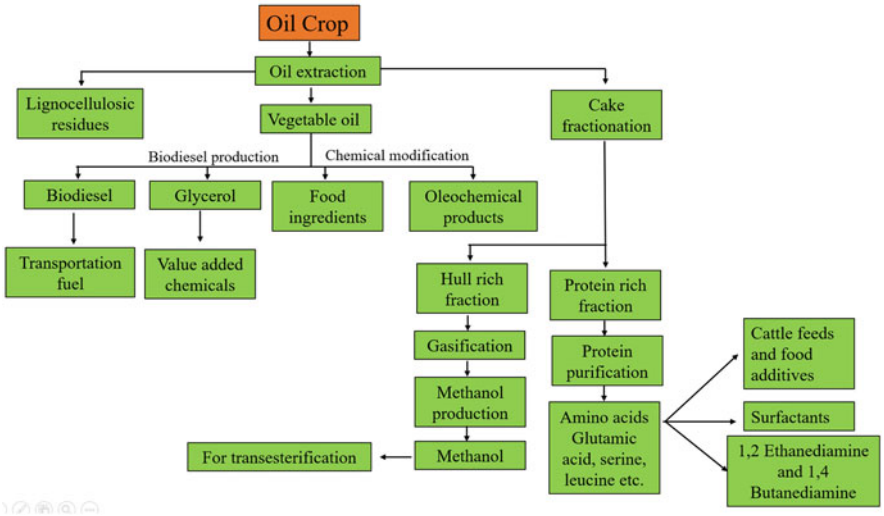


**Fig. 10.2** Whole crop biorefinery

these fuels are not cost-effective due to various techniques used for the production of these biofuels. Plants are the major and most abundant sources in the Earth. These plant sources are used as raw materials for biofuel production and considered as basic and well-known most utilizable biological raw materials for the second-generation biofuel production. Plant biomass is composed of mostly plant cell wall, which is composed up to 75% of polysaccharides (Pauly and Keegstra 2008). These polysaccharides are sources of valuable sugar that have good potential for biofuel production. One of the most known examples of these polysaccharides is present in the stem of wheat. The contents of sugar/polysaccharides are much higher in the stem than the contents of sugar present in the wheat grains. Other agriculture wastes such as paddy straw also have great potential for biofuel production. Biofuel production from agriculture waste can satisfy the demand for liquid fuel. There is a great interest in the field of bioenergy sector to the generation of biofuels and for increasing the production of biofuel using biomass crops as feedstock (Alptekin et al. 2014).

Lignocellulosic materials are the major source of biofuels. Various methods such as hydrolysis as well as fermentation (bioethanol production) and gasification (bio-diesel production) are used for biofuel production. Forestry crops like poplar, some grasses, and residue from the wood industries or forestry or agriculture are considered as potential raw materials for biofuel production at industrial level. The second-generation biofuel is derived through advanced process and utilization of lignocellulosic biomaterials for biofuel production. However, this process of biofuel production is under development. Bioethanol is the best alternative option of gasoline, and it knows as flexi-fuel vehicles due to the full substitute for gasoline. Sugar extracts from lignocellulosic feedstock materials and these simple sugars are

converted into ethanol. Produced ethanol (biodiesel) is the full substitute for diesel. Syngas is produced from the gasification of lignocellulosic materials, and this syngas converts into liquid hydrocarbons. These liquid hydrocarbons produced from syngas are mostly diesel and some other fuels. Biologically derived synthetic gas also knowns as Bio-SNG is also produce from lignocellulosic materials which is used in the gasoline vehicles with slight adaptations. Methane is considered biogas and plays an important role in the bioenergy field. Methane also produces from the lignocellulosic materials. Syngas is produced from lignocellulosic materials by gasification and finally syngas transformed into biogas. Bio-DME (dimethyl ether) is the fuel that can be used in diesel vehicles with slight adaptations. Bio-DME is also produced from the lignocellulosic materials using the gasification process (Balat 2006).

### *10.2.3 Third-Generation Biofuels*

The third-generation biofuels are generally derived from algal biomass. Algae are found anywhere on the plant as well as the aquatic system. They are an excellent source of vitamins, proteins, and carbohydrates as well as 60% of oxygen supply to all living beings in many ecosystems. Algae produce crude oil which can be easily converted into diesel and gasoline. Some algal species may be categorized into microalgae and macroalgae. Microalgae produce lipids by the carbon metabolic pathway, and these lipids convert into biofuels using various technologies. Algae grow in a suitable environment and extract lipid/oils from them. The extract algal oils convert into the various biofuels using a similar first-generation and second-generation process. These oils can also be refined into other fuels that can fulfill the requirements of petroleum-based fuels. Figure 10.3 shows the general steps of the generation of third-generation biofuels from algae (Alam et al. 2015).

The third-generation biofuels have many advancement in terms of efficiency compared to the first- and second-generation biofuels. They obtain low-cost materials based on completely renewable energy sources. Therefore, these third-generation biofuels are cost-effective. Algae and microalgae are more advantageous because they can grow in that area which unsuitable for the first- and second-generation biofuel crops. Algae can tolerate environmental stress and can easily grow in the salted lakes or seawater. These algae can also grow in sewage water. One of the challenges is finding an energy source that can satisfy our energy needs. At the same time, these energy sources are renewable and do not contribute to any unwanted or harmful changes in our climate (Alam et al. 2015).

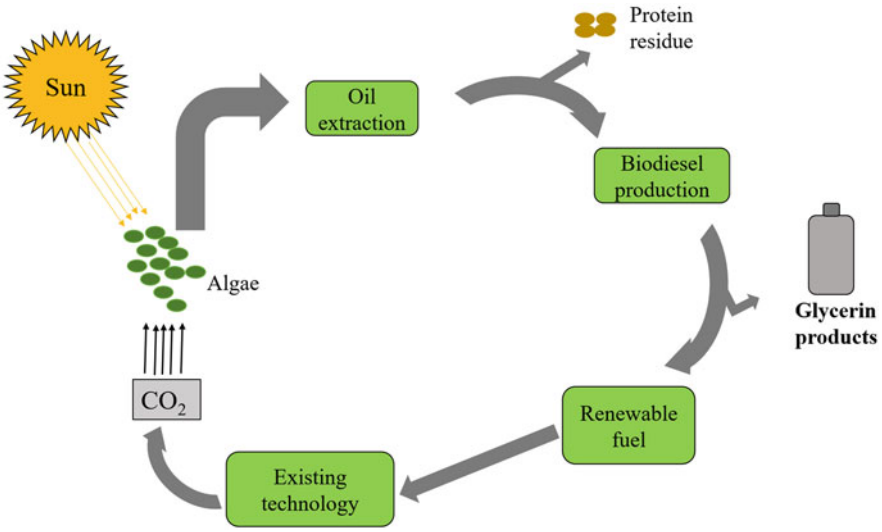


**Fig. 10.3** Third-generation biofuel production

### *10.2.4 Fourth-Generation Biofuel: The Latest Biofuel Technology*

There are two major parts of the new generation of biofuels. One part is based on the engineered biofuels producing crops that act as carbon-capturing machines. The second part of the fourth-generation biofuels is based on the "solar to fuel" concept. The fourth-generation biofuel is advantageous because it has a high production yield and requirement of non-arable land. These biofuels also include electro fuel (Abdulla et al. 2019).

Genetically modified microorganisms/crops produce a large amount of biomass compared to unmodified crops. These genetically modified crops also have high lipid content in their biomass, and this enhanced the lipid content used for biofuel production. The raw materials derived from these crops are converted into fuels using the first- and second-generation techniques. The overall process of engineered crops based on the fourth generation of biofuel production is summarized in Fig. 10.4 (Abdulla et al. 2019).

Solar to fuel method based on the microorganisms (cyanobacteria) and presence of the sun as well as $CO_2$. In this method, cyanobacteria are placed in the flat panel, and these panels are filled with water. The panels are set up facing the sun, the same as solar panels. The microorganisms (cyanobacterial) take solar energy from the sun and capture $CO_2$ from the environment. Cyanobacteria synthesized carbon source which are used as raw materials for biofuel production. Cyanobacteria can produce 15,000 gallons of diesel per acre annually. This is four times greater than the

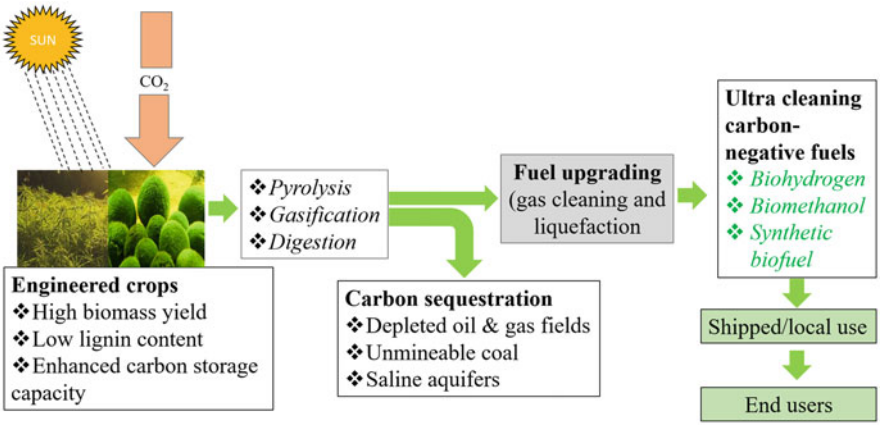


**Fig. 10.4** Fourth-generation biofuel sources and production

biodiesel produced from algae. Therefore, this advanced process is more cost-effective compared to the third-generation biofuels (Abdulla et al. 2019).

## 10.3 Production of Biofuel

Plant, algae, bacterial biomass, and other waste materials such as agricultural wastes are used as raw materials for the production of biofuels. Bioethanol, biomethanol, and biohydrogen are produced from a variety of algal, fungal, and bacterial biomass. These biofuel products are partially mixed with fossil fuel (4–5%) such as petrol and diesel. In the next few years, biofuel may be considered as major energy product and independently used in the energy sector. Hence, biofuel can be considered as the best alternative of petroleum by-products (Ong and Bhatia 2010; Chanakya et al. 2013; Swain 2014). Pacific biodiesel is the first biodiesel plant in the USA, and this plant mainly focuses on biodiesel production from cooking oils. Biodiesel production was boosted after 2001 due to the price hike of petroleum oils. Biodiesel has many advantages like emission of less toxic waste, inexpensive and independent from the fossil fuel (Chanakya et al. 2013; Swain 2014). Various types of techniques are used for the conversion of biomass and the production of utilizable biofuels. Biomass conversion processes are shown in Fig. 10.5.

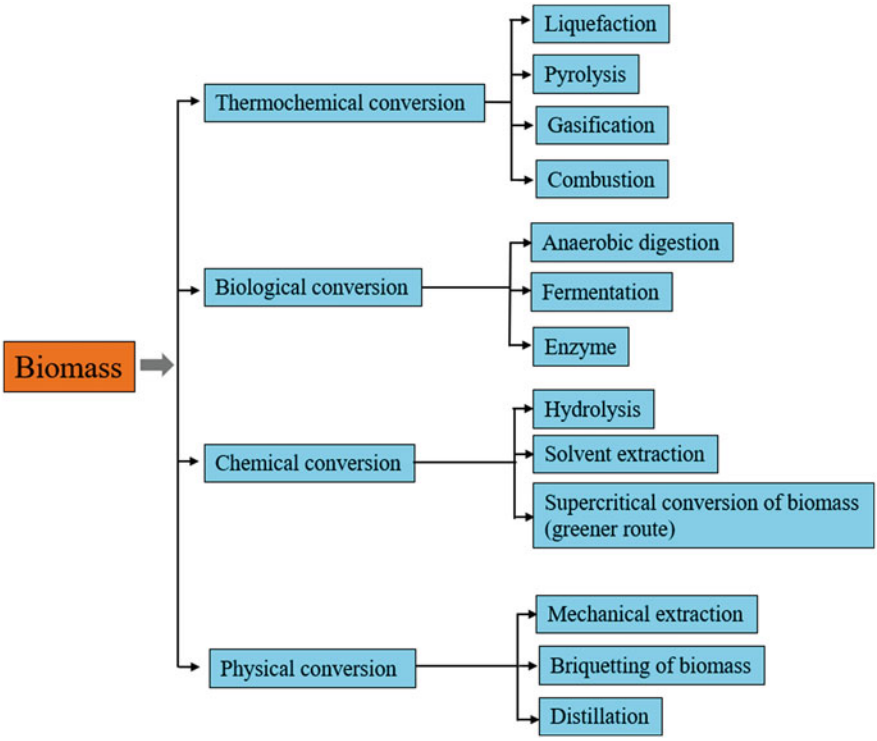


**Fig. 10.5** Biomass conversion process

### *10.3.1 Biofuel Production Process for the First-Generation Biofuels*

Biofuel production methods depend on the source of biomass used for the production, reactor types, reactor volume, and reactor parameters such as temperature, pH, and mixing. A variety of biological materials and production methods have been used for the production of biofuel. A few examples have been given below. Hydrotreatment is a technique in which vegetable oil and other lipid materials are used for biodiesel production purposes. It is a complex reaction process in which various types of reactions are involved (Kamm and Kamm 2004).

#### 10.3.1.1 Transesterification

Fatty acid methyl esters (FAME) are generally derived from vegetable oils. These FAME are known as biodiesel, and it is very advantageous due to the environmental-friendly nature of biofuel. Biodiesel is an alternative option of petroleum-based diesel and is derived from renewable biologically based raw materials such as

vegetable oils and animal fats. The vegetable oils and fats react and convert into a mixture of methyl esters with the help of homogenous and heterogeneous catalysts. These methyl esters are considered as biofuels such as biodiesel and glycerol. Glycerol has various applications and considered a high-value co-product (Kulkarni et al. 2006; Meher et al. 2006).

#### 10.3.1.2 Homogeneous Catalysis

Transesterification is the reversible process and the reactions start with the mixing of reactant and catalyst (Kulkarni et al. 2006).

#### 10.3.1.3 Heterogeneous Catalysis

The main application of these catalysts are conversion of free fatty acid to methyl ester through transesterification reaction when the contents of free fatty acid are very high. The conversion of fatty acids to methyl ester decreases due to the saponification reaction. Solid acid catalyst generally used for the conversion of high contents is free fatty acid. Solid acid catalysts are responsible to catalyze the transesterification of free fatty acid present in the vegetable oils (Kulkarni et al. 2006).

#### 10.3.1.4 Ethanol Conversion Process

Carbohydrate raw materials are considered as major sources of bioethanol production through fermentation of sugars present in the biomass. The biomass is classified into three major classes.

(a) Sugar crops: These crops are rich in sugar materials and widely used for the production of ethanol. These crops are beetroots, sugar cane, wheat, various types of fruits, and palm juice.
(b) Starch containing crops: These crops are rich in starch which is used as a raw material for the production of ethanol. These crops are wheat grain, barley grain, rice grain, sweet sorghum, corn, etc. The roots of various plants such as potato and cassava are also used for start production. The starch materials are considered a good source for bioethanol production.
(c) Cellulosic biomass: These materials are derived from forest wood and wood waste, agriculture wastes, and plant leaves. These materials are easily available on Earth. Therefore, cellulosic materials are cost-effective raw materials for bioethanol production.

Ethanol derived from various types of grains such as corn, barley, and wheat is known as grain ethanol. Ethanol derived from the lignocellulosic materials such as paddy straw, wheat straw, and grasses are known as biomass ethanol. Both the types of ethanol are produced through biochemical pathways (Shelley 2006).

#### 10.3.1.5 Fermentation Process

This process is explained as a metabolic process in which organic materials are converted into desirable products by an enzymatic reaction. These enzymes used in the fermentation process are produced from several microorganisms. There are two basic types of fermentation process generally used for the production of biofuels: (a) aerobic and (b) anaerobic depending on the presence and requirement of oxygen for the fermentation reaction. Many microorganisms in nature are capable to produce ethanol from sugar and starch raw materials. The microbial species are classified into three categories which apply in ethanol production: (a) yeast (*Saccharomyces* species), bacteria (*Zymomonas* species), and mold (mycelium). These microorganisms are very selective for the fermentation process, and some microorganisms are specific to hexoses or pentoses or mixture of both. Many researchers are working to develop a microbial strain that can produce ethanol using any carbohydrates (Lee et al. 2007).

#### 10.3.1.6 Anaerobic Digestion of Biomass

In this process, solid waste is digested in the absence of oxygen. This process is very similar to the biogas production process. Anaerobic microorganisms are able to breakdown the organic materials into a mixture of methane or carbon dioxide. This reaction occurs in the absence of oxygen. Nowadays, the cost of energy is increasing with respect to time and needs to search for alternative sources of energy. The biogas is a fuel that has high heating value and considered a low production cost. Hence, this biofuel can be considered as an alternative option of fossil fuel. During the biogas production process, a large amount of wastes are generated. These waste materials can be used as biofertilizer for organic cultivation (Lee et al. 2007).

### *10.3.2 Biomass Conversion Process for Second-, Third-, and Fourth-Generation Biofuels*

Two major processes are accessible for the production of biofuels: one process is based on the thermochemical processing and the second depends on the biochemical processing of biomass. The thermal degradation of biomass into various useful products. The obtained products and their types also depend on the biomass involved in the conversion process, the presence of oxygen (various concentrations), and the duration of the thermal treatment process. The thermal process is more advantageous than the biochemical process due to the easy conversion of biomass into valuable products. This review is mainly focused on the conversion of lignocellulosic waste into various biofuel products.

#### 10.3.2.1 Physical Conversion

Basic vegetable oils and fatty acids are obtained from the oil crop seeds through mechanical force. This mechanical force can be applied by various instruments, and the well-known one is a screw (expeller). The oil is partially extracted using this mechanical technique and remaining oil in the cake like materials can be extracted using a solvent extraction process.

Distillation is an important process for extracting essentials oil and separation of volatile materials present in the extraction. In this process, raw biofuels are separated on the basis of their boiling points. The extracts are heated at several temperatures, and the vapors are collected. The collected vapors are allowed to condense into liquids. Therefore, various valuable products and impurities can be separated on the basis of temperature (Stevens and Verhe 2004).

#### 10.3.2.2 Thermo-Chemical Degradation of Biomass

Biomass can be transformed into biofuels by basically two methods. These methods are thermo-chemical or biochemical methods. The thermo-chemical method of biomass conversion includes various processes such as pyrolysis, combustion, gasification, and liquefaction. When the biological material is heated in the absence of oxygen, it produces syngas that consists of hydrogen and carbon monoxide. It can be directly used as fuel or converted into different types of liquid and gas which are used as energy sources. Thermal conversion is a very simple method used for the production of biofuels (Lee et al. 2007).

***Direct combustion***: Direct combustion is the type of chemical reaction between fuels and air. This is basically known as burning. Carbon dioxide and water are produced during the combustion process.

***Gasification***: It is the well-known method generally used for the conversion of biomass into biofuel. Gasification methods contain two types of biomass conversion process. These methods are catalytic and non-catalytic methods. The non-catalytic method occurs at high temperature, and the catalytic method occurs at low temperatures.

***Liquefaction***: Liquefaction is the process of biomass conversion that occurs in the presence of alkaline solution, butanol, propanol, or direct liquefaction. The products of liquefaction are water-insoluble oils. These products require solvents and reducing gases such as carbon monoxide or hydron or catalysts for the complete conversion of biomass into biofuels (Rowlands et al. 2008).

***Pyrolysis***: It is the thermal degradation of biomass in the absence of oxygen. The major products of pyrolysis are charcoal, bio-oil, and gases (fuel gases) (Demirbas 2004). Pyrolysis can be divided into conventional pyrolysis, fast pyrolysis, and flash pyrolysis.

Conventional pyrolysis method is based on the slow heating rate, and the time of heating varies between 45 and550 s. The conventional pyrolysis process completes

in three steps. The first step occurs at low temperature and is also known as pre-pyrolysis, i.e., the breakage of week bonding and elimination of water molecules. The second step is also considered as the main step which is conducted at a high heating rate and leads to produce various products. The char decomposition occurs in the third step. This step occurs at slower rate compared to other steps and produces carbon-rich products in solid form (Demirbas 2004).

Fast pyrolysis is performed between the temperature range of 850–1250 K and a fast heating rate compared to the conventional pyrolysis method. The biomass decomposes into gaseous and liquid products. The major products of fast pyrolysis are 60–75% bio-oils, 15–25% solid chars, and 10–20% non-condensed gases (Shafizadeh 1982).

Flash pyrolysis is different from the conventional pyrolysis and slightly different from the fast pyrolysis. It occurs at high temperatures, a fast heating rate, and a short period of time. Conversion of biomass into bio-oils is the main process of flash pyrolysis. These bio-oils can be converted into bioslurry with the addition of char. Bioslurry can be easily moved into syngas by the gasification method. Syngas is a well-known product used as an energy source (Demirbas 2004; Mohan et al. 2006).

#### 10.3.2.3 Chemical Conversion

Chemical Hydrolysis

Chemical hydrolysis is an important method used for the conversion of biomass into usable production. Various conditions like surface to volume ratio, the temperature of the solution, time of hydrolysis reaction, and concentration of acid in the reaction mixture play an important role in the hydrolysis process of biomass. The smaller particles are considered as better for biofuel production due to large surface area. The large surface area of biomass particles is responsible for the more reaction sites at the time of the conversion process. The concentration of the solid particle in the liquid medium affects biofuel production efficiency. The high liquid–solid ratio is responsible for the faster reaction of biofuel production (Jensen et al. 2008).

Solvent Extraction Method

Solvent extraction is the technique different from the other unit operations. Extraction refers to a process by which the desired product is obtained from the raw materials. The raw substrate materials allowing to mix into the suitable solvent and then recovering the desired product from the solvent mixture. Both extraction and separation are important for the solvent extraction methods during the conversion of biomass and extraction of desired products from it (Dewarte et al. 2007).

Supercritical Water Conversion (SWC) of Biomass

The supercritical fluids are neither liquid nor gas at the supercritical condition. A supercritical fluid is a method of biomass conversion and alternative option to acid hydrolysis, enzymatic hydrolysis of complex sugar to simple sugar. Supercritical water can easily and rapidly convert cellulose into simple sugar and biomass into mixtures of lipids, ethanol, methanol, and organic acids. In supercritical and near about critical point, the water molecules are broken down into hydrogen ions and hydroxyl ions, and these ions are dissolved in the biomass. The dissolved water ions can easily break the biomass into their components. The cellulose and hemicellulose convert into simple sugars such as glucose and oligosaccharides (Sasaki et al. 1998). Supercritical water conversion and its stream utilization are shown in Fig. 10.6.

### *10.3.3 Algae Biodiesel*

Algae are the photosynthetic organisms that fix atmospheric $CO_2$ in the form of biomass (Shapouri et al. 1995). Algal biomass is considered as raw material for the biofuel production process. Microalgae is the best alternative option for the production of diverse types of biofuel such as biohydrogen, bioethanol, and biodiesel due to high yield, fast growth rate, high lipid and sugar contents, and easy to grow (cultivate on both arable and non-arable land) (Pradhan et al. 2009). However, biodiesel production from microalgae is much expensive due to the tremendous requirement of energy source and maintenance of the growth condition for the cultivation of algal species. Algal biofuel production is a very complex process. A variety of mechanisms are involved in the production process (Fig. 10.7). Many researchers are working for the reduction of production cost and enhancement of the production of biofuels (Soon 2000; Koutinas et al. 2007).

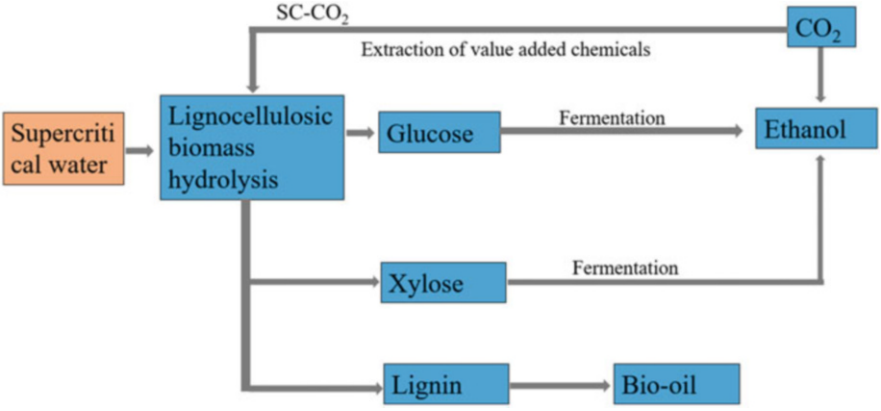


**Fig. 10.6** Supercritical water conversion of biomass

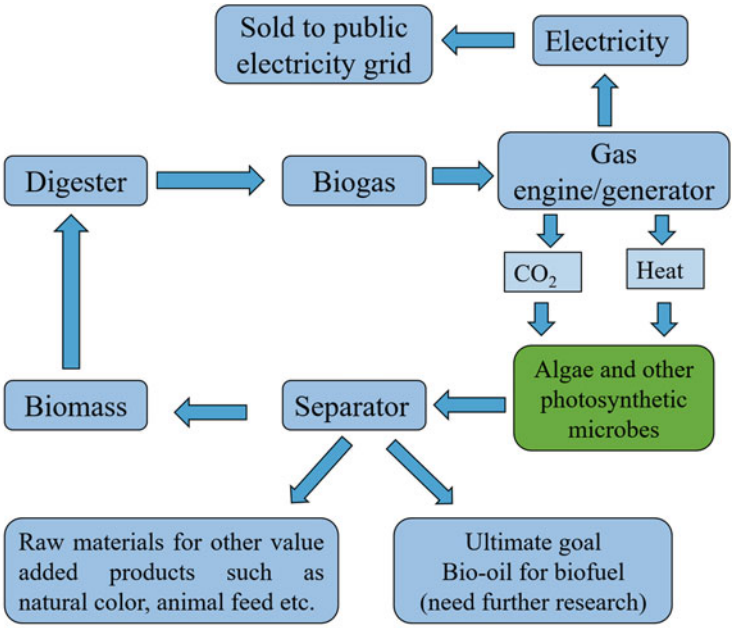


**Fig. 10.7** The schematic diagram represents the processing of algae for the production of biofuel

### *10.3.4 Biohydrogen and Biogas*

Biohydrogen can be the best option for energy sources in the future. It is an eco-friendly and a cost-effective biofuel. Biohydrogen has many advantages compared to other fuels. It has high efficiency due to the high octane number. Biohydrogen is generated during the photolytic reaction in the photosynthetic microorganisms such as microalgae and plants. It is also generated by the dark fermentation process in an anaerobic microorganism. Various types of biohydrogen production processes have been discussed in this chapter (Kaparaju et al. 2009).

Biogas is formed in the absence of oxygen when organic materials are degraded. Methanotrophic bacteria break down carbon organic material (Kaparaju et al. 2009).

## 10.4 Metagenomic Methods for the Identification and Characterization of Gene Encoding Novel Enzymes for Biofuel Production

Metagenomic is a culture-independent process, and it discovers novel enzymes from unculturable microorganisms. The novel enzymes are isolated from metagenome, and pretreatment of environmental samples isolated the DNA from environmental

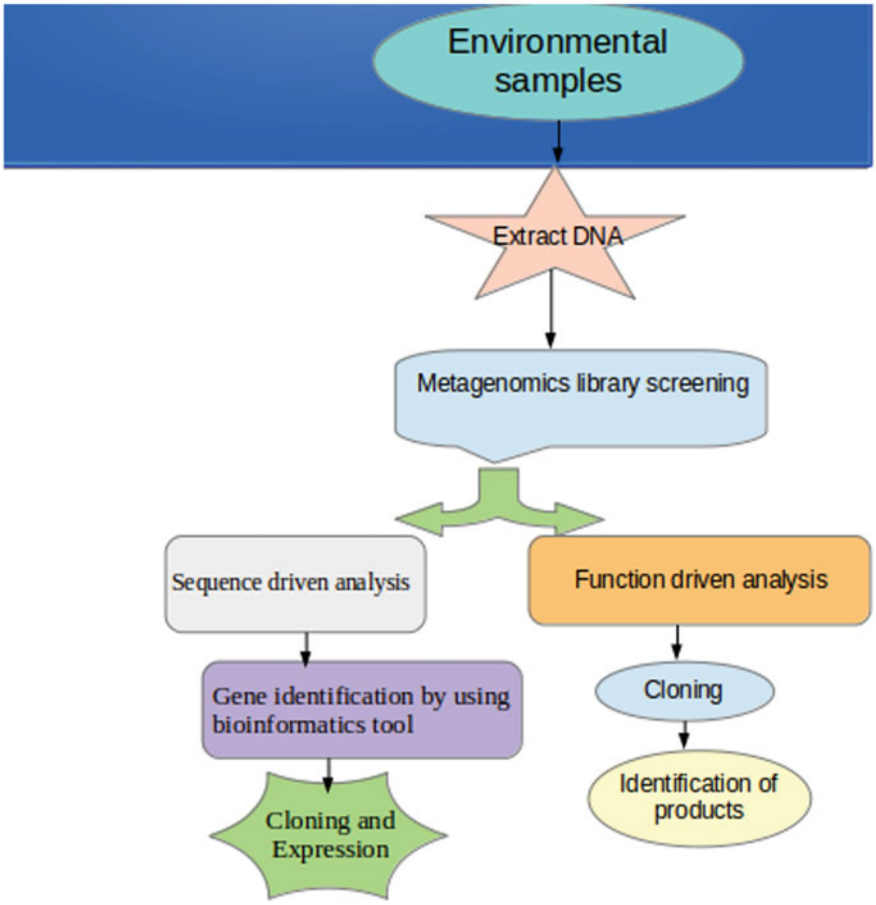


**Fig. 10.8** Identification of novel biocatalyst from environmental samples

samples (Fig. 10.8). Vector and host are selected for gene transformation and cloning. Vectors are also constructed for protein expression. Hence, suitable metagenomic library is constructed for further use. From this library, gene encoding novel enzyme is isolated (Schmieder and Edwards 2011) (Fig. 10.9).

### *10.4.1 Sample Processing and Isolation of DNAs*

In the metagenomic process, sample processing is the most crucial and first-step process, and DNA extracted from the environmental sample which is representative of cell and a large amount of nucleic acid is obtained for library preparation and sequencing (Schmieder and Edwards 2011).

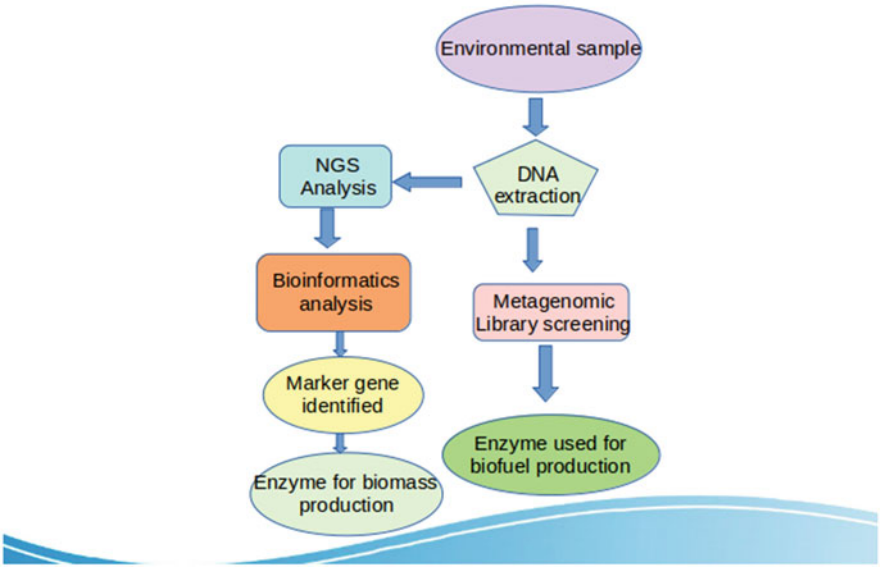


**Fig. 10.9** Identification of gene encoding novel enzyme in metagenomic pipeline

### *10.4.2 Selection of Host, Organisms, and Vectors Construction*

Suitable microorganisms select for the metagenomic library. The DNA fragments inserted into the vector DNA and these constructed vector transfer into the host cell for maintaining a genomic library of metagenomic samples (Schmieder and Edwards 2011).

### *10.4.3 Metagenomic Library Screening*

Metagenomic can be based on sequence driven and function-driven analysis of uncultured microbial community. They have been used to identify biocatalysts from the metagenome. Microbial communities are a collection of archaea, bacteria, viruses, and fungi (Sebastian et al. 2013).

#### 10.4.3.1 Function-Based Screening

Function-driven analysis is the identification of gene function which expresses the desired function and identifies the phylogenetic analysis of cloned DNA. It is the identification of metagenomic in medicine, agriculture, or industry by using useful

active protein. In the metagenomic process, microbes produce antibiotics, enzymes, and novel bioactive compounds. Metagenomic applications include the discovery of antibiotics and industrial enzyme, personalized medicine, and bioremediation (Sebastian et al. 2013).

#### 10.4.3.2 Sequence-Based Screening

Random sequencing of the clone can use a complete clone sequence that contains a phylogenetic marker which indicates the taxonomic classification that is the probable origin of DNA (Xing et al. 2012).

#### 10.4.3.3 SIGEX Screening

Substrate-induced gene expression (SIGEX) screening substrate supplied exogenously is used to increase the expression of the enzyme. The substrate of the enzyme increases the efficient expression of the enzyme. This type of screening reduces the unexpressed target gene of library members. From induced gene (Yun and Ryu 2005).

### *10.4.4 DNA Sequencing for Metagenomic Sample*

A target of such an experiment could be a chromosome, a set of genes or a region of interest in a particular gene. The size of the region of interest or subset of the genome will determine the technology and approach to be used. In general, if the subset of the genome is rather large and high throughput sequencing is required, we recommend the use of the Illumina Hiseq. Whereas if the throughput required is lower and/or the read length plays an important role, then the *Illumina Miseq* or even the *PacBio* may be most appropriate (Chen 2014).

Next-generation sequencing generated a large amount of data than Sanger sequencing. The next-generation metagenomic sequencing has been used for comprehensive sampling of the gene present in environmental samples. Metagenomic sequencing potentially viable for assessment of various microorganisms and sufficient in different niche, allow effective screening of the library. The metagenomic library used for the identification of novel biocatalyst and high-throughput sequencing method is useful due to higher genetic diversity as well as higher gene library capacity. The next-generation platforms and their applications have been given in Table 10.1 (Wang et al. 2019).

**Table 10.1** Next-generation sequencing platform

| Company | Platform | Library | Sequencing principle | Nucleotide modification | Reference |
|---|---|---|---|---|---|
| Roche | Roche 454 FLX Titanium | emPCR | Pyrosequencing | None (which is added as thiol derivative dATP) | Chen et al. (2014) |
| Illumina | Illumina HiSeq1000 | Bridge-PCR | Sequencing by synthesis | End-blocked fluorescent | Chen et al. (2014) |
| Ion Torrent | Ion PGM | emPCR | Semiconductor-based sequencing | None | Chen et al. (2014) |
| Applied Biosystem | SOLiD5500 | emPCR | Sequencing by ligation | 2-base encoded fluorescent oligonucleotide | Wang et al. (2019) |

#### 10.4.4.1 Second-Generation Sequencing

Second-generation sequencing have Illumina and Ion Torrent sequencing technologies that produce many short reads (150–400 bp) (Wang et al. 2019).

#### 10.4.4.2 454 Sequencing Platform

454 Life Sciences produced the first successful second-generation sequence, i.e.,454 DNA sequences. It is based on the investigation of the release of pyrophosphate during the addition of nucleotide (Agah et al. 2004). About one million sequences with reading length (400–500 bp) are produced through a sequencing-by-synthesis approach. It uses the emulsion PCR (emPCR) technique to remove the complementary strand. This system is used for the identification of genes that identify species through metagenomics studies (Wang et al. 2019).

#### 10.4.4.3 Illumina Genome Analyzers

This type of next-generation sequencing uses the sequence by synthesis method. Short DNA fragment is bound to micro-well and to form a cluster by amplification method and each nucleotide is the fluorescent label is washed across flow cell and incorporated reversible to the DNA sequence. Incorporated nucleotide sequence generates fluorescence signal detected by high-sensitivity camera of A, C, G, and T during each cycle. Illumina produces short read lengths of up to 300 bp (Chen et al. 2014).

#### 10.4.4.4 Ion Torrent

In this next-generation sequencing technology, beads are attached to DNA fragment, and in micro-wells, single beads are placed. In the wells, each one of four nucleotides flows and the complementary strand gets incorporated and voltage change can be measured by release an $H^+$ ion. Ion torrent technology produces 400 bp reads length (Chen et al. 2014).

#### 10.4.4.5 Applied Biosystem

SOLiD (Sequencing by Oligonucleotide Ligation and Detection) is developed by Life Technologies Applied Biosystems in 2007. This type of next-generation sequencing generates billions of nucleotides reads at on time. The new amplified DNA fragment is generated by using emPCR with this sequencing platform, and both the ends of the fragment are ligated with adaptors. For metagenomic analysis of bacterial consortium, SOLiD 3 Plus has been used (Wang et al. 2019).

#### 10.4.4.6 Third-Generation Sequencing

Second-generation sequencing has suffered from lower number of short sequences ranging from 35 to 400 bp. Third-generation sequencing has been used to overcome the disadvantage of second-generation sequencing. It includes PacBio and Oxford Nanopore Technologies that produce longer reads (6–20 kb). Three commercially available third-generation DNA sequencing technologies are Pacific Biosciences (PacBio) Single-Molecule Real-Time (SMRT) sequencing, the Illumina Tru-seq Synthetic Long-Read technology, and the Oxford Nanopore Technologies sequencing platform (Agah et al. 2004).

#### 10.4.4.7 Single-Molecule Real-Time (SMRT) Sequencing

SMRT sequencer can read large number of nucleotide basepairs up to 64.5 kb as well as an optimum reading length of 10–15 kb. Metagenomic methods with SMRT sequencing was used to study the digestion of complex carbohydrate such as lignocellulose. *Actinobacteria* as well as *Proteobacteria* possessed capability of selective degradation for lignocellulose materials. Currently, the PacBio SMRT sequencers were applicable for the determination of components of coral-associated microbial diversity by using 16S rRNA-based sequencing (Stadermann et al. 2015).

#### 10.4.4.8 Oxford Nanopore Sequencing Technologies

Firstl, nanopore DNA sequencer was designed by Oxford Nanopore Technologies (Eisenstein 2012). MinION is based on effective, real-time, long-read sequencer designed for an easy library preparation. GridION generated 100 GB of data per run as well as applicable for real-time process. MinION sequencing has application in metagenomic taxonomic identification by accessing the low-complexity metagenomic community. MinION can produce high-quality reads to introduce 99% exact taxonomic composition (Brown et al. 2017).

### *10.4.5 Bioinformatics Tools Applicable in the Analysis of Metagenomic Sequencing Data*

Next-generation sequencing method generated a huge amount of data which are short read lengths that require the design of bioinformatics tools for the development of metagenomic technologies. Data analysis is followed by assembling of DNA, prediction of genes, and annotation of genes (Fig. 10.10). DNA assembly is a

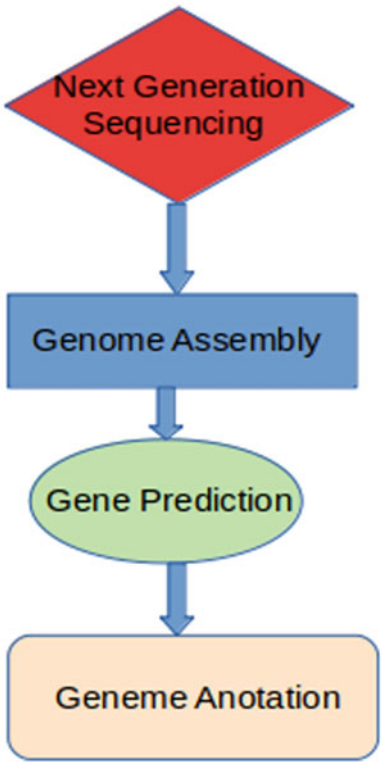


**Fig. 10.10** Metagenomic bioinformatics pipeline

**Table 10.2** Metagenomic tools for sample analysis

| Analysis method | Software | Website |
|---|---|---|
| Metagenomic assembly | MetaQUAST<br>Velvet<br>Megahit<br>MetAMOS | http://bioinf.spbau.ru/metaquast<br>**http://www.ebi.ac.uk/~zerbino/velvet**<br>https://github.com/voutcn/megahit<br>https://github.com/treangen/MetAMOS |
| Metagenome gene prediction | MetaGeneAnnotator<br>Glimmer-MG<br>FragGeneScan<br>GeneMarks | http://metagene.nig.ac.jp/<br>http://www.cbcb.umd.edu/software/glimmermg/<br>https://omics.informatics.indiana.edu/FragGeneScan/<br>http://exon.gatech.edu/GeneMark/genemarks.cgi |
| Protein domain database | InterProScan | https://www.ebi.ac.uk/interpro/ |
| Pathway database | KEGG<br>WikiPathways<br>MetaCyc | https://www.genome.jp/kegg/<br>https://www.wikipathways.org/index.php/WikiPathways<br>https://metacyc.org/ |
| Data sharing and online portals | MG-RAST<br>EBI Metagenomics<br>IMG/M<br>EDGE | https://www.mg-rast.org/<br>https://www.ebi.ac.uk/metagenomics/<br>https://img.jgi.doe.gov/<br>https://bioedge.lanl.gov/ |

process in which contigs as well as scaffolds were developed from reads. In metagenomics data analysis de novo assembler is used (Wang et al. 2019).

Gene prediction process is used for assembled data based on similarity search or to locate the gene elements in genomic content. Gene annotation can be used for the identification of protein function and their metabolic pathways (Table 10.2). Metagenomic data analysis new tools are designed such as MetaVelvet or Meta-IDBA for assembler and MG-RAST or CAMERA are used as annotation tools. Phylogenetic marker gene is used for taxonomic identification and composition of the microbial community (Knief 2014).

## 10.5 Application of Metagenomics for Biofuel Production

Metagenomic is the genetic analysis of the microbial community. Metagenomic is an uncultured analysis of the metagenome. The metagenomic application includes the discovery of antibiotics and industrial enzyme, personalized medicine, and bioremediation. Many microbes made new medicine by degrading waste products or made new eatable food. Organic substances produce biofuel products that contain bioethanol, biodiesel, biobutanol, and biogas. The enzyme plays an important role in industrial biofuel production. Metagenomics cleaning up environmental contamination such as the waste from waste treatment and gasoline leaks on lands or oil spills in the oceans and toxic chemicals (Sebastian et al. 2013).

### *10.5.1 Application of Metagenomic Enzymes for Biofuel*

Biofuel products including bioethanol, biodiesel, biobutanol, as well as biogas produced from organic substances such as sugars, oil crops, starch, agriculture wastes, animal wastes, and lignocellulosic biomass. Enzymes are identified by metagenomic studies for the production of biofuel from various methods such as next-generation sequencing strategies. Different novel enzymes are identified for biomass degradation such as amylolytic enzymes, β-glucosidases, endoglucanases, ligases, as well as xylanases. The biodiesel production enzymes are lipolytic enzyme. These enzymes are highly effective, active, stable, as well as more specific for substrate at various temperature, pH as well as ionic strength. Lignocellulosic enzyme for biofuel production: plant biomass is efficiently utilized for biofuel production by the lignocellulosic enzyme. The overall enzymatic digestion mechanisms are shown in Fig. 10.11 (Xing et al. 2012).

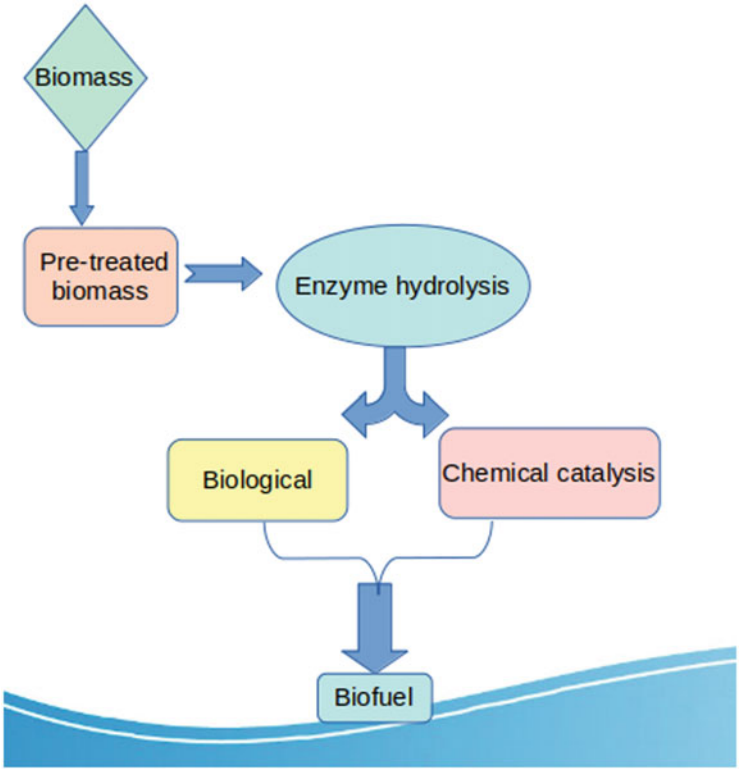


**Fig. 10.11** Mechanism of biofuel production

## 10.6 Conclusion

The survey of alternative resources of energy is most important due to the shortage of energy sources and the depletion of fossil fuels. Therefore, biofuel is looked at as the best option which can replace the need for fossil fuels in the future. Hence, these fuels are knowns as renewable as well as eco-friendly fuels. These fuels derived from microbial, algal, and agricultural lignocellulosic waste by enzymatic digestion. Microbial enzymes have important application in the degradation of biomass and production of biofuels as well as few other value-added products. However, the availability of industrial enzymes is very limited, and few enzymes have very low enzymatic activities. Therefore, characterization of novel and more effective enzymes from natural sources is very needful. These novel enzymes can easily digest biomass and become more beneficial to biofuels production. The metagenomics approach provides a novel idea to identify and characterize more effective microbial enzymes from the natural sources. DNA sequencing as well as gene screening methods also plays important role in the characterization of enzymes from metagenomic sources which have higher enzymatic activities and have potential application in the biofuel industries. In summary, the metagenomic approach is playing a promising role in isolation, identification, and selection of suitable microbial species from diverse natural environments. The novel enzymes isolated from selected microbial species have potential applications in the degradation of lignocellulosic biomass and the production of biofuel.

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