Nitish Kumar *Editor*

Biotechnology for Biofuels: A Sustainable Green Energy Solution

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Preface

The depletion of petroleum-derived fuel and environmental concerns have prompted many millennials to consider biofuels as alternative fuel sources. But completely replacing petroleum-derived fuels with biofuels is currently impossible in terms of production capacity and engine compatibility. Nevertheless, the marginal replacement of diesel with biofuel could delay the depletion of petroleum resources and abate the radical climate change caused by automotive pollutants. Energy security and climate change are the two major driving forces for worldwide biofuel development and also have the potential to stimulate the agro-industry. The development of biofuels as alternative and renewable sources of energy has become critical in national efforts towards maximum self-reliance, the cornerstone of our energy security strategy. At the same time, the production of biofuels from various types of biomass such as plants, microbes, algae, and fungi is now an ecologically viable and sustainable option. This book describes the biotechnological advances in biofuel production from various sources while also providing essential information on the genetic improvement of biofuel sources at both the conventional and genomic level. These innovations and the corresponding methodologies are explained in detail.

Biotechnology for Biofuels: A Sustainable Green Energy Solution contains 11 chapters which covers the latest developments in the research on a promising biofuel crop Jatropha, discusses the application of nanotechnology and computational biology in biofuel production, addresses the role of microorganisms in biofuel production, catalytic approach for production of hydrocarbon-rich bio-oil from a red seaweed species, seaweed biomass and microbial lipids as a source of biofuel, and biomass of bamboo and sugarcane as a source of bioenergy.

Gaya, Bihar, India — Nitish Kumar

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Biofuels: Perspective for Sustainable Development and Climate Change Mitigation

1

Akshay Singhal and Prashant

Abstract

Soaring fuel prices and climate change are two major problems the world is facing now. There has long been argued that the commercial fuel stocks will only remain for some more time now. Since the human population depends vastly on the non-renewable sources of energy for much of its transport, creating alternatives to liquid fuels has become obligatory. Hence, there is global urgency to harness liquid fuels from non-fossil sources. This chapter argues that biofuels are a possible "greener" alternative to fossil fuels. They are globally sustainable and available. Because biomass is green and carbon neutral, it can lead to sustainable development and global environmental conservation. Apparent benefits of biofuels are indicated by the fact that a greater number of countries are willing to implement and plan increase share of biofuels in their energy requirements. In order to do so, substantial production increases are needed quickly to accommodate rising global demand. Technologies must be advanced to obtain biofuels from terrestrial as well as aquatic plants/algae. Policies both, nationally in respective countries as well as internationally, need to incorporate greater role of biofuel as a substitute to fossil-based fuels.

We bring in attention an approach to bring production and trade of biofuels under the carbon trade facility called Clean Development Mission. This will not only provide an added option to mitigate climate change but also better enhance global sustainability by encouraging biofuel production especially in developing countries.

Keywords

Biofuels · Energy crisis · Fossil fuels · Sustainability · Climate change

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1.1 Introduction

Energy plays a significant part in human lives. It is an indispensable driver for any country's socio-economic development. There are different ways to store, convert, and amplify the abundance of energy around us for our use. Scientists as well as policy makers have always been concerned with energy production. Energy sources can be divided into three components: fossil-centered non-renewable fuels, renewable, and nuclear based fuels. Fossil derived fuels are not replenishable and were formed in earlier geologic period millions of years ago. Some renewable sources of energy include: biofuels, hydroelectric, wind based, solar, geothermal, and marine sources.

The steady rise in prices of oil has obligated nations to explore these renewable solutions.

One such promising green energy source is biofuels. Researchers continue to work in the production of biofuels, as it is considered a potential replacement to non-renewable fuels (Weldemichael and Assefa 2016).

Biofuels' advantages over non-renewable fuels are (a) biofuels are quickly derived from the biomass, (b) they are renewable biodegradable in nature, (c) their carbon dioxide cycle combustion.

Biofuels mainly obtained from biomass are categorized as solid, liquid, and gaseous fuels. Depending on the type of biomass, biofuels are classified according to generations, viz. first, second, and third generation.

Biomass has been identified as the world's fourth largest available energy asset (Haykiri-Acma and Yaman 2010).

1.1.1 Fossil Fuel-Based Energy and Environment

Renewables in the form of coal, natural gas, and oil have been used to drive machinery and transport since the Industrial Revolution of the eighteenth century. Demand for fossil energy has increased considerably, and the world's energy need is likely to double by 2050 with projected population expansion and developing countries industrialization. Fossil fuels generate nearly 80% of the global energy supply. Oil accounts for approximately 40% of world's energy requirements and fuels 90% of the transportation sector. There are major problems with the persistent overuse of fossil fuels. The fossil fuel supply is limited and its deposits are located in a few parts of the world. It is increasingly difficult to obtain a secure supply of fossil fuel with growing global demand.

Moreover, fossil fuels are primarily made of hydrocarbons. Carbon dioxide, sulfur dioxide, and carbon monoxide are the primary by-products of combustion. Over the years, these gas emissions have negatively affected the atmosphere and spurred global change. Unpredictable weather, droughts, and wildfires are among the least damaging effects of global warming. Global carbon dioxide emissions must be mitigated by 50–85% by half of this century if rise in global temperature is to be controlled between 2 and 2.4 °C (Remme et al. 2011).

Through energy generation systems, fossil fuels are burnt which induces contamination in the environment. It also causes water and land pollution, but this concern is not as serious as air pollution. A pollutant is a material that is not a natural part of the atmosphere (generally a hazardous substance). If it occurs naturally, the concentration is unusually high. Carbon monoxide (CO), sulfur oxides (SOx), nitrogen oxides (NOx), and particulates (very small soot and dust particles) are the major air contaminants arising from fossil fuel emissions. In contrast, air pollution is caused by unburned hydrocarbons that either go into energy conversion systems before burning or evaporate into the atmosphere prior to combustion. Compounds of lead also caused air pollution for many years; however, the nearly total elimination of leaded gasoline has diminished this concern considerably. Other deleterious effects arise from the association of these key toxins with the environment. Acid rain and smog, the greenhouse effect, and high ozone levels in the atmosphere are several side effects (Radovic and Schobert 1997).

1.1.2 Energy Crisis

The energy crisis is a detailed and contentious issue. It is carrying on and becoming worse and worse. The explanation for this is that the complicated factors and mechanisms and attempts to solve it are not widely understood. There is a finite supply of natural resources. It may take thousands of years to replenish this even though they do occur naturally. Policymakers and individuals involved are seeking to promote the use of renewable energy sources and to reduce the reckless use of natural assets by increasing preservation.

There are many factors linked to the energy crisis. The fall in the available crude oil supply and the dramatic increase in demand for oil came mostly from China and India in the 2000s, when millions were pulled out of global poverty and were able to increase personal consumption. It acts as a cheap source of fuel for automobiles, electricity and a source of consumer goods once the crude oil is processed (Dudley 2011).

1.1.2.1 Causes of Energy Crisis

It might be easier to blame at one activity or company and accuse them for the whole energy crisis, but that would be a very simplistic and inaccurate view of the cause of this problem. Some of the causes are as follows:

Overconsumption: The energy crisis is the product of many different natural resource strains. Owing to overconsumption, which in effect can pollute oxygen in air and water, there is a pressure on fossil fuels oil, gas, and coal.

Overpopulation: The steady rise in the world's population and its demands for food and goods have a crippling effect on our energy supply.

Poor Infrastructure: Ailing power generation equipment infrastructure is also another reason for energy resource slump. Many companies that produce energy tend to use outdated technology that limits energy generation. Utilities are responsible for continuing to update the network and maintaining a high-performance level.

Unexplored Renewable Energy Options: The majority of countries where energy needs are fulfilled from non-renewable sources such as coal, renewable energy remain unused. Renewable sources of energy would reduce our reliance on fossil fuels and reduce greenhouse gas emissions as well.

Wastage of Energy: People do not understand the urgency of conserving energy in most parts of the globe. Small things like turning off lights when not in use, walking for short distances rather than driving, carpooling, using CFL instead of traditional bulbs, good insulation, etc. go a fair way in conserving energy.

Miscellaneous Factors: Many factors including civilian protests, increased taxes, political upsurge, extreme hot summers or chilling winters can lead to a sudden increase in energy demand and can limit supply. Moreover, a union strike in a company producing oil will certainly lead to a crisis of energy.

Sustainable development is a very broad term. It can mean anything from the use of renewable energy to the way cities should be spatially organized to better accommodate rapid urbanization. The most proper definition comes from the report of Brundtland Commission called the *Our Common Future:*

> Sustainable development is development that meets the needs of the present without compromising the ability of future generations to meet their own needs.

1.1.3 Overview of Biofuel Production

"Biofuels" or "Agrofuels" are fuels that are made from living plants. The theory is that because these fuels absorb as much CO when they grow, as they emit when they are burnt, they are basically "carbon neutral."

For several reasons, Biofuels have become increasingly popular as an alternative energy source, which include their ability to reduce greenhouse gas (GHG) emissions from transportation. The capability of biofuels to mitigate climate change depends on their GHG capacity compared to the liquid fossil fuels they come in place of (Khanna et al. 2011).

Compared to other technologies such as hydrogen, biofuels are a serious option to compete with oil in the transport system as biofuels techniques are now well evolved and accessible in several countries. Biodiesel as well as bioethanol can be blended with petroleum based products such as gasoline and diesel, which they replace and can be combusted in conventional combustion engines with mixtures comprising up to 10% of biofuels without engine alterations (Dufey 2006).

Biofuels can be used to serve various purposes such as transportation or heating requirements. Bioethanol is made of agricultural outputs, while biodiesel is produced from seeds of trees like jatropha. Biofuels are produced from biomass and are primarily usable for transportation. A wide variety of source can lead to their formation which includes forest resources, agricultural products as well as biodegradable wastes. Some of the products from which biofuels can be manufactured are shown in Fig. 1.1.

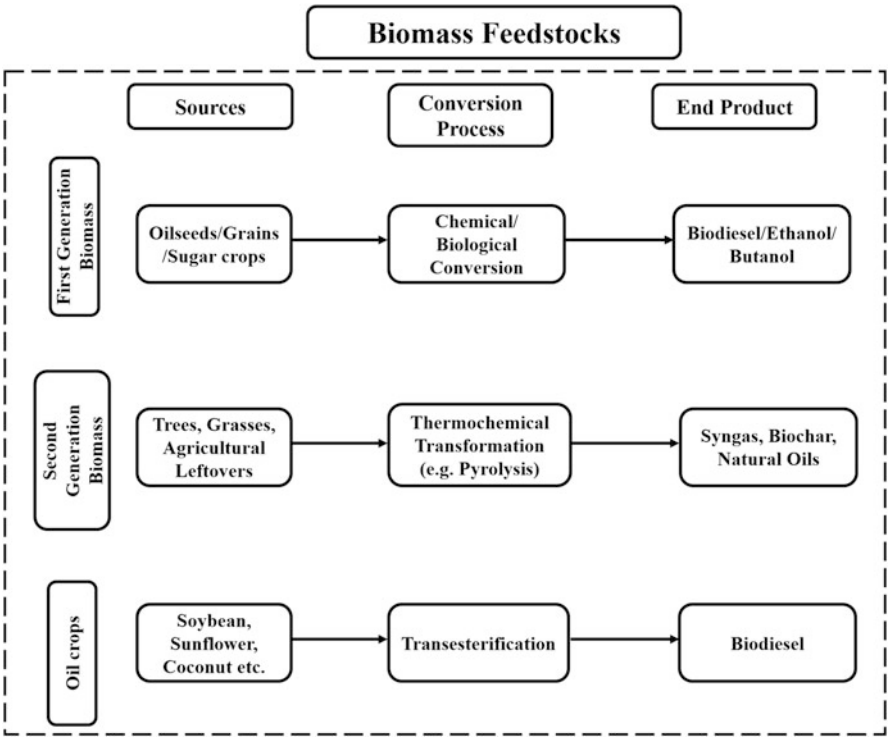

Fig. 1.1 General flowchart showing sources of biofuel, their conversion process and associated end product of the biofuel production process. Source: (Kour et al. 2019)

1.1.4 Biofuel from Terrestrial Plants

There are usually two subgroups of biofuels, bio-alcohol and biodiesel. Ethanol, which is a bio-alcohol, is made with help of bacteria and yeast by breaking down starch, whereas biodiesel is made from crops including soybean using their oil. Such vegetable oils are then converted to biodiesel upon treatment with alcohol. Generally, biofuel can be manufactured from any lignocellulosic plant. These plants could either be cereals or energy crops. Wastes generated from agriculture as well as municipal wastes can also be used.

1.1.4.1 Corn

Corn is the ruler of biofuels based on ethanol. In a similar way to beer making, corn rich in sugar is transformed into ethanol. Then the kernels are blended with yeast and hot water and harvested. Ethanol is made when the mixture is fermented by yeast. In existing car engines, this ethanol is then mixed with gasoline. Comparatively lesser carbon monoxide, NOx, and SOx is emitted by this mixture as compared to those on gasoline which also lessens smog in urban areas.

1.1.4.2 Sugarcane

Sugarcane is used to produce bioethanol. Many kinds of soil support growth of sugarcane. It requires high nitrogen and potassium fertilizers, albeit very little amount of phosphate. Availability of water is a usual limitation for sugarcane production. The weather is perfect for the processing of sugarcane in Brazil and other tropical regions.

With more than 514 million tons of sugarcane per year, Brazil is the world's leading producer of sugarcane and ethanol (FAO 2009). India, China, Thailand, and Pakistan are other major producing countries. Many countries (for example, Peru) have higher yields per hectare relative to Brazil. This is partly due to the widespread use of irrigation (Dufey 2006).

1.1.4.3 Rapeseed/Canola

For a long time, rapeseed oil is used to cook food and lamps. It is a key source of biodiesel. It is low in saturated fats and relatively high in oil content than most crops, making it the most efficient biodiesel feedstock on a world scale, making it a suitable crop for fuel production. This accounts for about 59% of the world's raw material for biodiesel (Pahl and McKibben 2008). In Europe, the average production of biodiesel by rapeseed is 1200 L per ton of rapeseed (FAO 2008).

1.1.4.4 Palm Oil

Palm oil is derived from the fruit of palm trees and is one of biodiesel fuels that is energy efficient. Biodiesel from palm oil is less polluting than petrol. In particular, it has helped develop Malaysia and Indonesia's economies. Palm oil accounts for approximately 10% of the rapidly expanding biodiesel supply, mainly from Indonesia and Malaysia (Pahl and McKibben 2008).

1.1.4.5 Jatropha

It is a toxic plant and a major player on the market for biofuels. The bushes increase rapidly, which does not need much water, and their seeds have an oil content of around 40%. India is presently the largest manufacturer of jatropha and this crop is the focus of its biodiesel industry. It has helped the nation to create economic benefits to rural farmers who can grow the crop on typically poor farmland. Jatropha plants can live on land destroyed by drought and pests for 50 years and still do well. The plant's seeds are crushed to release the biodiesel oil.

1.1.4.6 Soybeans

After rapeseed and palm oil, soya oil is the third most important biodiesel feedstock. In the USA, after rapeseed and palm oil, it contributes for 75–90% of biodiesel raw material. The output of soy biodiesel accounts for about 25% of global biodiesel production, and soy is the second largest biodiesel feedstock after rapeseed (Pahl and McKibben 2008).

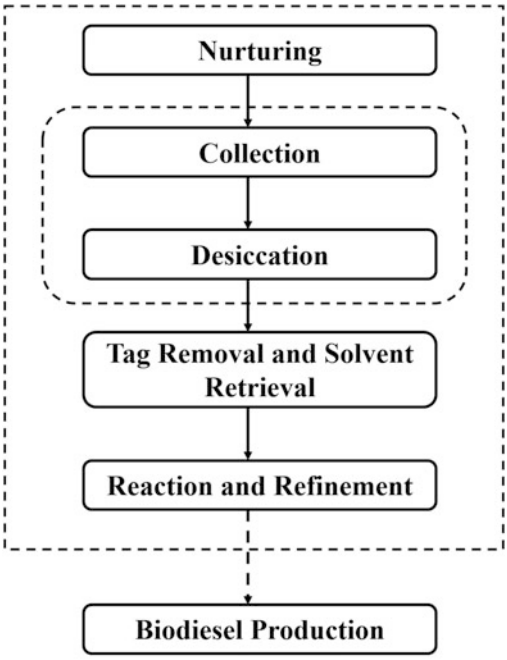

Fig. 1.2 General flow diagram depicting various pathways leading to production of Algae-Based Biofuels (ABB). The dotted arrow represent the pathway to end product, i.e. Biodiesel. Source: (O'Connell et al. 2013)

1.1.4.7 Eucalyptus

Firewood, charcoal, and hardwood pulp are the main uses of eucalyptus. Telephone poles, building, and mine prop and veneer can also use the timber. Many species produce honey and oils that can be used in the chemical industry. Lately eucalyptus has also been used in contemporary biofuel equipment, primarily for electricity, although the potential for second-generation ethanol is under review at present (van Bueren and Vincent 2003).

1.1.4.8 Poplar

Poplar (*Populus trichocarpa* or black cottonwood) has certain characteristics that make this species an ideal option for the production of renewable biomass energy, and also helps to reduce the need to use food crops as a raw material for the production of liquid fuel. Through a process that does not release additional carbon dioxide, energy can be extracted from them at cellulosic ethanol plants: cellulosic ethanol is a carbon neutral energy source.

About 273 L of fuel per ton of wood could be produced by hybrid poplar. It is possible to grow approximately 22 tons of poplar per hectare per year, containing 2730 L of ethanol.

1.1.5 Biofuel from Algae

There are many Algae-Based Biofuel (ABB) pathways (Fig. 1.2) with vastly differing possibilities and limitations, both land based and sea based. The former is more advanced than the latter. Some input sources such as combustion gas, salt water, and wastewater can be used in such ABBs. Climate conditions, such as annual solar irradiation and temperature, also affect ABB designs.

ABBs have significant implications for sustainability; some are special to algae. Fresh water use can be reduced in this and it is possible to use large amounts of land with low economic and ecological value. In sea-based systems, there are even more space opportunities and they are available for ABB development. The ability to capture GHGs and reduce their emissions is a key advantage of algae.

Studies on algae for oil manufacturing focuses primarily on microalgae—photosynthesizable species with a diameter of less than 0.4 mm, like diatoms and cyanobacteria—as compared to macroalgae, such as seaweed. Because of its less complex structure, rapid growth rate, and high oil content (for some species), microalgae is preferred for production of oil. Nonetheless, some work is being done on the use of seaweed for biofuels, possibly because of this resource's high availability.

At this time, ABB's main drawback is its lack of financial sustainability, as this sector is only in the nascent process and has a tough road ahead before it achieves market level. In addition, algae growing and processing systems require high (higher than agriculture) capital input. In the short and medium term, significant cost-cutting technologies are required and co-products of higher value need to be produced to achieve economic competitiveness and thus economic viability.

1.1.6 Biofuel from Wastes

Urban waste and certain biomass-rich waste products may be used as feedstock for the manufacturing of biofuels.

Municipal solid waste, food processing waste, and black liquor are three of the main categories.

Municipal solid waste–the component of municipal solid waste of biological origin (e.g., kitchen and garden waste, paper, cardboard) includes a very wide range of materials and total waste with significant opportunities to convert it into fuel through gasification or pyrolysis. There are wood by-products, such as construction/demolition wood (e.g., wood offcuts from building and wood recovered after demolition), manufacturing waste wood (e.g., crates and other objects from the packaging and pallet industry), and domestic waste wood (e.g., old furniture, fencing). Food processing waste—including waste from the dairy and sugar industries and from the production of wine and beer—can be converted through fermentation to ethanol. Waste cooking oils can be purified and used or converted to biodiesel as straight vegetable oil (SVO). In pyrolysis/thermochemical operations, lignocellulosic (woody) or mixed waste materials can be converted to bio-crude. It is

also possible to use thermochemical processes to manufacture bio-jet fuel, biodiesel, and bioethanol. The conversion process used depends on the quality of the usable nature and amount of waste and the end product. Smaller waste streams (e.g., orange rests from the production of orange juice) may also be of interest.

Green waste, such as forest residues or garden or park waste, can also be used to generate biofuel through various routes (e.g., biogas collected from biodegradable green waste and converted to syngas through gasification or hydrolysis for further processing into biofuels by catalytic processes).

Black-liquor—a by-product of the kraft process (which digests pulpwood into paper pulp by removing lignin, hemicelluloses, and other extractives from the wood to release cellulose fibers). Black liquor contains concentrated lignin and hemicellulose that can be gasified with very high conversion efficiency and syngas reduction potential that can be further processed for the production of bio-methanol or bio-methyl ether (BioDME). Pulp mills have used black liquor as an energy source since the 1930s. Most kraft pulp mills use recovery boilers to recover and burn much of the black liquor they produce, generating steam and recovering the cooking chemicals (e.g., sodium hydroxide and sodium sulfide used to separate lignin from the cellulose fibers needed for papermaking). This has aided paper mills reduce water pollution problems, reduce their use of chemicals through recycling and reuse, and become almost self-sufficient in energy by generating 66% of their own power needs on-site on average.

1.2 Biofuels and Sustainability

It is necessary to develop clean liquid transport fuels that can substitute finite fossil fuels to ensure future energy security. The manufacturing of biofuels, like all industrial processes, requires energy inputs and has an environmental impact. Biofuels of the first generation (bioethanol and biodiesel) also provide benefits in terms of reducing GHGs and replacing fossil fuel. Certain considerations need to be addressed when assessing the overall efficiency of biofuels, such as competition with food production and release of stored carbon and biodiversity effects as land is cleaned up for increasing energy crops.

The combined effects of climate change, persistent instability in fuel prices, the recent food crisis, and global economic deceleration have created a sense of urgency among policymakers, industry, and development practitioners in seeking viable and feasible solutions in the biofuels market (Amigun et al. 2010).

Biofuel land is used for energy supply. Biofuels compete with other criteria, such as food production, industrial energy, nature conservation, etc., as the bio-productive land area on our planet is small and diminishing. This not only results in higher prices for agricultural and forestry products, but also increases pressure on the environment (Stoeglehner and Narodoslawsky 2009).

1.2.1 Carbon Sequestration

Biofuels have the same definition as fossil fuels like coal. The energy of the Sun was collected in the form of naturally derived chemicals called hydrocarbons by biofuels and fossil fuels. The energy stored in fuels is the product of the photosynthesis capability of the plant—the production of sugar, starch, and other complex organic molecules using sunlight. Nevertheless, like fossil fuels, biofuels have the ability to be carbon neutral, which means that the loss of carbon dioxide to the environment caused by burning them is compensated by the absorption of carbon dioxide from biofuel plants as they grow (Plants that developed and photosynthesized millennia ago locked up the carbon in fossil fuels.). If a perfect balance existed between carbon dioxide absorption and emission, burning biofuels would not lead to an overall increase in carbon dioxide levels in the atmosphere, which is one of the main greenhouse gasses. Therefore, unlike fossil fuels, biofuels have the ability to help prevent global warming if they can substitute oil-based fuels such as gasoline and diesel fuel. They also have the added advantage of being fossil fuel replenishable.

There are many issues with the processing of biofuels that can significantly alter the carbon balance sheet.

Biofuel crops often need fertilizers and pesticides based on oil to begin with. Furthermore, the equipment used to grow, transport, and process the plant is frequently fueled by fossil fuel. There are several regions of ancient woodlands that are cut down to plant biofuel crops. This results in significant loss of global "carbon sequestration" in combating climate change. Biofuels, in short, are not some people's absolute panacea.

We did not look at the total costs and benefits of the entire manufacturing chain, from "farm to forecourt," which is sometimes referred to as life-cycle assessment and includes taking into account all aspects of the carbon budget from one end of the manufacturing process to the other. When this is done, the unrealistic arguments made by politicians and some activists about the benefits begin to look hopelessly optimistic. Eventually, the right type of biofuel crops grown in the right way and in the right place may be better for the environment in the longer term than burning fossil fuel. However, there are many issues that can be ignored, and people need to consider the manufacturing process's entire life cycle, including its effect on local populations and fauna.

1.2.2 Phytoremediation

The word phytoremediation is derived from the Greek prefix "phyto" meaning plant, and the Latin suffix "remedium" which means to clean or restore. The term usually refers to plant-based systems that use organic or genetically engineered plants to treat contaminated environments (Pandey et al. 2016). Large-scale energy crops can be grown in contaminated lands for remediation purposes as well as meeting biofuel requirements. Such crops can be used as alternatives to polluted land remediation. Miscanthus is one of the important phytoremediation crops. Miscanthus buffer strips

have been used to grow crops in order to improve water quality by extracting nitrate from fertilized agricultural fields in groundwater. The expected nitrate decrease was >60%, but would depend on the starting price of the nitrate and the vigor of the crop (Gopalakrishnan et al. 2012).

Jatropha curcas is another essential biofuel crop capable of soil phytoremediation. Remediating contaminated soil with non-consumable crops such as Jatropha curcas, due to lubricating oil from vehicles, offers an environmentally friendly and cheaper option for remediating polluted soil (Agamuthu et al. 2010).

1.2.3 Entrepreneurship Prospects in Biofuels

Since a few years, the rapid expansion of global biofuel markets has reflected a renewed interest in biofuels. Commonly cited reasons behind the current market growth of biofuels include: current high oil prices, opportunities to increase energy efficiency, and savings in currency through a reduced oil bill. But what is new about this renewed interest and what makes biofuels a serious option to partially replace oil as a transport fuel is its allegedly reduced emissions of greenhouse gases (GHG) (Dufey 2006).

With every passing day, the opportunities of entrepreneurship in biofuels are growing. Recently, several companies and government entities have invested considerable capital in commercially viable processing of algae. Often known as green crude oil, most companies use algae to create a range of hydrocarbon products using algae as a source of biofuel pumping water rich in borosilicate glass bioreactor tubes exposed to sunlight. Depending on the part of the cells used, hydrocarbons can be converted into various types of fuel. The lipid portion of algal biomass can be extracted and converted into biodiesel while the part of carbohydrate can be fermented into bioethanol. Early acting venture capitalists saw the advantages in a green and carbon neutral fuel that, unlike ethanol, did not compete with the global food industry. However, algae need no drinking water, they can be grown on brackish, sea, and even waste water. Other species are also growing rapidly: their short harvest period of 1–10 days allows for fast growing green oil. Maybe their biggest selling point is that they can produce 50 times more fuel per unit area than crops from biofuels. Also small-scale entrepreneurship in the production of biodiesel will popularize and encourage the development and use in diesel engines and stationary diesel sets of biodiesel derived from multi-feed stocks. It will therefore help to reduce the dependence of a nation on imported fossil diesel fuel. It immediately enhances the energy sector's sense of security.

1.2.4 Biofuels: Mitigation Towards Climate Change

Global warming is among the most vital problems currently facing mankind. Human beings are the primary witnesses of the changes our world is experiencing: from rising temperatures and sea levels or more greenhouse gas emissions to the resulting

gradual melting of the polar ice caps. In fueling the threat of climate change, fossil fuels have been a major culprit.

The main advantage of using biofuel as an alternative to fossil fuels is that greenhouse gas emissions are minimized by biofuels. Transforming biomass feedstocks into biofuels is an environmentally friendly process. This is how to use biofuels for transportation. By using bioethanol instead of gasoline, they help to reduce atmospheric CO_2 in three ways: (1) avoid gasoline-related emissions; (2) enable CO_2 content in fossil fuel to remain in storage; and (3) provide a route for CO_2 absorption through that new biomass of fuel. Thanks to their harmony with the natural carbon cycle, biofuels provide the transport sector with the most valuable option for removing greenhouse gases.

In addition to reducing GHG emissions, biofuels also have the potential to reduce emissions of significant toxic substances generally associated with conventional fuels. Engines running on biofuels or combining conventional fuels and biofuels tend to have lower particulate matter and CO emissions as well as lower sulfate emissions.

While bioethanol also shows a decrease in volatile organic compounds, ethanol and acetaldehyde emissions are higher. Biodiesel shows higher emissions of nitrogen oxide, while significant differences do not exist. Household air pollution is often reduced when crop-based biofuels replace other traditional forms of fuels widely used in the poorest countries, such as coal, fuelwood, and paraffin.

1.2.5 CDM and Biofuel

Under the auspices of the United Nations Framework Convention on Climate Change, the Kyoto Protocol (signed-1997, effective-2005) was negotiated as an international treaty to curb greenhouse gas emissions and set emission limits on developed counties. The goals were either to reduce their own GHG emissions or to use a versatile mechanism called the Clean Development Mission. This Clean Development Mission encourages developing countries to implement cleaner technology. As a result, the project owner may sell the resulting carbon credits to another country that can use such credits to reach their own GHG goal due to lower emissions of GHG. Not only does this process encourage the use of green technologies in developing countries and restrict climate change, but it also supports the idea of sustainable development (Kirkman et al. 2012).

It is curious that in today's age of energy security and climate change, both the Clean Development Mission and the development of biomass-densified liquid fuels are considered indispensable, but their combination is not very effective (Bakker 2006).

1.2.5.1 Criteria of Assessment

The requirements for determining if biofuel projects should be included in the context of the Clean Development Mission are taken from the standard project design document together with the approach taken by De Coninck & IEA Coal

Research. Clean Coal Centre (2005) examines the suitability of CDM clean coal technology.

Magnitude of Greenhouse Gas Reduction

A criterion against which to measure the GHG reduction must be calculated. Establishing a CDM project model has proved to be a complicated task in which many complexities play a role. It is necessary to determine the standard for reasonable costs with reasonable certainty. The issue of "leakage" must therefore be addressed: GHG reduction within the project boundaries should not result in an increase in GHG emissions outside the limits of the project.

Additionality

Additionality proof is the key element of the CDM/JI program. It must be explained clearly why registration of the project as a CDM or JI project is necessary to make the project feasible.

For example, it can be assumed that using CDM and JI will make the project financially viable for investors. Because of the essential nature of additionality for CDM and JI purposes and the scope for various interpretations at the same time, additionality and its meaning in particular remain sensitive issues for all stakeholders.

Monitoring of Emission Variables

In the years that the plan "generates" emission reductions, the variables that determine emissions (reduction) must be monitored correctly to ensure real climate gains.

1.2.5.2 Other Significant Contributions

Besides reducing GHGs, programs can offer other benefits: reducing (air) emissions, increasing energy supply protection by reducing dependence on fossil fuels, promoting jobs, and introducing new technologies. In short, projects involving renewable energy usually make a significant contribution to sustainable development.

1.2.5.3 Suitability of Biofuels Under CDM

Sutter (2003) measures the suitability of biofuels under CDM via certain requirements. They are:

Energy Security of Supply

Production and use of biofuels is widely recognized as a viable alternative to oil, primarily due to reduced dependence on imports from politically unstable countries. While the concept of energy security of supply should not be restricted solely to import dependency, in practice this is a popular theme.

Air Pollution

Depending on the type of fuel used, the impact of using biofuel on air pollution is pretty less. Biodiesel has little or no effect on PM and NOx emissions 14, but since the sulfur content is much lower than petrol, SO_2 emissions are declining, which is

important in the urban environment. Ethanol replacement with petrol leads to lower PM and slightly lower NOx levels, with little impact on SO_2. Biofuels substituted with diesel have the ability to decrease PM and NOx further. This co-benefit of using biofuels can become important due to the increasing pollution load in many Asian (mega) towns.

Jobs
Most research and recent CDM Project Development Reports point to a significant contribution in local employment to biomass and biofuel projects. It should be known that biofuel projects contribute to national and local jobs.

Natural Environment
The natural resource effect of the production of biofuel depends on the origin of the biomass. Palm oil wasted production in rainforest areas is indications of potential negative ecosystem impact. On the other hand, if biomass is derived from sustainably managed land or forest, the impact at habitats and water resources can be positive.

1.2.5.4 Barriers Related to Biofuel Inclusion in CDM

There is a succinct summary of many challenges that have been highlighted in ProBios (2006).

Regulatory Barriers
They apply to (local) environmental policy, waste use (for input energy), international biomass exchange, and biofuel conversion plant licenses.

Barriers in Innovation
Biomass processing technologies into biofuels are well known, but further developments and experience are required. End-use engine technology may be the limiting factor in the combination of lower percentages of biofuels. In order to allow higher blending shares, it is important to adjust to the current engine design.

Supplying Biomass
There are currently no conditions for biomass procurement to ensure continuous consideration of the biomass interest. It is necessary to identify supply routes from source to plant and end user. There is also rivalry between sources of biomass and uses other than biofuels, such as food production.

Potential for the Market
The high prices of biofuels are largely determined by the cost of feedstock and are therefore likely to remain relatively high compared to conventional fuels. Continued demand and supply of biofuels can also be difficult.

1.2.5.5 Biofuel Production Scenario in Developing Counties

The production of biofuels is highly promising where most people still live in rural areas and rely on agriculture. Developing biofuels would bring direct opportunities through local job development to developing countries—from increasing raw materials to manufacturing. Local production of biofuels in developing countries would also help to reduce dependence on high fossil fuel imports. Rapidly industrializing countries must be prepared to build specific regulatory regimes for their local equivalents, which can encourage environmental sustainability for these countries and prevent the reduction of unhealthy rainforests and similar high carbon environments.

In northern Zambia, oil is more than $2 a liter. This is because of high shipping oil prices on poor roads from the Indian Ocean to remote areas. Growing local plants can lower the price of diesel, supporting local people to develop small-scale alternatives and local development (Biofuels 2007). No state in modern history has reduced poverty, despite a massive increase in fuel consumption. Economic development includes the provision of transportation and electricity. The problem is that usually countries need to import oil for fuel, resulting in a negative trade balance that can lead to excessive debt, inflation, and exchange rate devaluation. For example, Brazil, one of today's largest ethanol-producing countries, has launched long-term projects to expand biofuel production as an alternative to oil. While the industry is dominated by large corporations in Brazil, the UN bioenergy report notes that the cooperatives of farmers often play a role and prove beneficial to small farmers.

Lately, instead of exporting mainly palm oil, Malaysia has encouraged national industries to grow the production of biodiesel for internal use. Nonetheless, palm oil prices have risen sharply, leaving Malaysian biorefineries without oil for domestic production because they are unable to afford the current price.

Liquid biofuels is the leading green alternative for the transport sector. They have achieved steady growth over the past 16 years. Biofuel production has increased tenfold since 2000–2017 from 16 billion liters to 143 billion liters.

In developing countries, the growth of global biodiesel production will be driven by policies in place in Argentina, Brazil, and Indonesia. It is expected to increase to 41.4 Bln L by 2025 from 31 billion liters in 2015. Advanced biofuels are not expected to take off during the process of prediction.

Markets in Brazil are expected to remain favorable for hydrous use of ethanol rather than petrol, and a persistent ethanol market, met primarily by domestic production, will therefore prevail over the outlook period. Indonesian biodiesel production will be primarily used to meet mandatory domestic demand.

India reportedly has six projects processing a cumulative of 650 million liters of biodiesel annually. The production capacity of existing plants ranges from 11 million liters to 280 million liters. Table 1.1 clearly highlights the rise in production as well as consumption of biofuels in India. India currently has six plants producing a total of 650 million liters of biodiesel per year. The production capacity of existing plants ranges from 11 million liters to 280 million liters. (India: Biofuels Annual 2019).

Table 1.1 India: biodiesel production from multiple feedstocks (Million Liters)

Calendar year	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
Beginning stocks	45	15	13	14	15	11	13	13	18	25
Production	100	111	126	132	138	152	158	170	185	190
Imports	0.0	0.0	0.0	0.3	1.7	0.8	2.7	7.1	25.2	11.5
Exports	0	0	0	3.9	41.5	33.1	41.7	7.6	23.1	19.7
Consumption	131	113	125	128	102	118	119	165	180	185
Ending stocks	15	13	14	15	11	13	13	18	25	22
Production capacity (Million Liters)										
Number of bio refineries	5	5	5	6	6	6	6	6	6	6
Nameplate capacity	450	450	460	465	480	500	550	600	650	660
Capacity use (%)	22.2	24.7	27.4	28.4	28.8	30.4	28.7	28.3	28.5	28.8
Feedstock use for fuel (1000 MT)										
Non-edible industrial	50	58	65	70	75	85	90	100	110	105
Used cooking oil	38	42	48	49	50	55	55	55	60	65
Animal fats & tallow's	6	6	7	7	6	5	6	6	8	10
			120	126	131	145	151	161	178	180
Market penetration (Million Liters)										
Biodiesel on road use	36	31	42	49	32	41	48	72	83	85
Diesel on road use	42,625	45,520	49,343	49,354	49,605	52,239	55,179	57,025	61,247	62,284
Blend rate	0.09	0.07	0.08	0.10	0.06	0.08	0.09	0.13	0.14	0.14
Diesel total use	71,041	75,866	82,238	82,256	82,674	87,064	91,965	95,041	10,2079	10,3807

In Brazil, the legislation states that anhydrous ethanol ranges from sugarcane to gasoline from 20 to 25%. Biodiesel mixing mandates are much lower (3%), but are expected to rise to 5% in 2013.

The biofuel draft strategy in South Africa is aimed at achieving an average market share of 4.5% (petrol and diesel) by 2013 for liquid road transport fuels. Pricing will be related to the BFP (common fuel price), which is a parity index for regional fuel product prices and is the essential element for regulating the fuel price. The biofuels industry will continue to enjoy a percentage reduction in the oil levy for all liquid biofuels that meet agreed criteria (Bekundaa et al. 2009).

Countries like Zambia and Mozambique are presently designing initiatives on biofuels and other countries in Africa are exploring biofuels strategies at a preliminary stage.

World production of biofuels rose by 3.5% in 2017, well below the 11.4% 10-year average, but the highest for 3 years. The largest increase (950 thousand tons of oil equivalent) was made by the USA. Global ethanol production grew by type of fuel at a similar rate of 3.3%, contributing more than 60% to total growth in biofuels. The production of biodiesel increased by 4%, mainly driven by growth in Argentina, Brazil, and Spain (BP 2018).

The production of liquid biofuels in developing countries remains very small, especially in Africa, where the sector is still in its early stages (Maltsoglou et al. 2013). Table 1.2 explains the yearly increase in production of biofuels in countries belonging to different parts of the world with the USA leading the way.

1.3 Biofuel Policies and Provisions in Indian

The following points are taken from the 2018 National Biofuels Policy, where they were discussed briefly. India's emphasis is on development goals that include shared national development vision, infrastructure upgrading and building capability, growth in the economy, prosperity, and social well-being. Energy security is seen as a crucial milestone in improving living standards. By the end of 2030, the nation aims to produce electricity by using a more eco-friendly fuel whose non-fossil based share is above 40%.

Government has prepared a road map to reduce import dependence in the oil and gas sector by implementing a five-pronged strategy that includes increasing domestic production, adoption of biofuels and renewables, energy efficiency requirements, enhancement of the refinery cycle, and market substitution. This provides a strategic role for biofuels in the Indian Energy Basket. Through coordinated programs such as the Ethanol Blended Petrol Program, the National Biodiesel Project, and the Biodiesel Blending Program, the government has made several efforts over the past decade to promote biofuels in the country. Drawing from previous experiences and overall supply status, the government has revised these structures by taking steps on rates, incentives, opening up alternative routes for ethanol production, supplying bulk or wholesale biodiesel to customers, concentrating on R&D, etc. These developments had a positive impact on the biofuels policy of the country.

Table 1.2 Yearly increase in production of biofuels in different parts of the world

Thousand tonnes of equivalent	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	**2010**	Change 2010 over 2009	2010 Share of total
US	2991	3288	3987	5226	6357	7478	9746	13,456	19,096	21,670	**25,351**	17.0%	42.8%
Canada	105	111	113	113	113	133	160	461	536	721	**996**	38.1%	1.7%
Total North America	3096	3399	4100	5339	6470	7612	9906	13,922	19,637	22,399	**26,355**	17.7%	44.5%
Argentina	4	9	9	9	9	9	29	228	632	1054	**1687**	60.0%	2.8%
Brazil	5212	5600	6149	7068	7135	7835	8729	11,323	14,132	13,932	**15,573**	11.5%	26.3%
Colombia	–	–	–	–	–	14	131	141	239	326	**351**	7.8%	0.6%
Jamaica	–	–	54	74	56	62	147	138	182	196	**196**	–	0.3%
Other S. & Cent. America	31	30	69	78	93	171	369	472	741	457	**457**	–	0.8%
Austria	18	18	22	26	48	70	105	220	263	354	**383**	8.3%	0.6%
Belgium	–	–	–	–	–	1	21	140	278	473	**454**	−4.0%	0.8%
France	315	315	337	368	385	439	798	1121	2012	2312	**2312**	–	3.9%
Germany	215	298	473	688	909	1788	2561	3181	2727	2728	**2930**	704%	4.9%
Italy	70	123	180	232	272	340	482	443	617	758	**670**	−11.5%	1.1%
Netherlands	–	–	–	–	6	3	22	80	77	241	**283**	17.6%	0.5%
Poland	–	–	–	27	23	84	158	116	279	393	**338**	−14.0%	0.6%
Portugal	–	–	–	–	–	1	79	153	136	202	**275**	36.3%	0.5%
Spain	70	70	134	184	221	288	248	320	356	958	**1179**	23.1%	2.0%
Sweden	–	14	31	32	43	48	54	99	118	173	**212**	22.8%	0.4%
United Kingdom	–	–	3	9	9	39	166	136	196	180	**180**	–	0.3%
Other Europe & Eurasia	57	113	126	138	166	301	406	536	1031	1825	**2135**	17.0%	3.6%

Total Europe & Eurasia	744	951	1305	1704	2081	3401	5103	6546	8091	10,597	**11,354**	7.1%	19.2%
Total Middle East	–	–	–	–	–	–	–	–	–	–	**–**	–	–
Total Africa	6	6	6	6	6	6	6	6	10	14	**14**	–	*
Australia	–	–	–	–	4	20	54	70	110	174	**246**	41.8%	0.4%
China	–	4	146	396	492	622	858	1076	1323	1399	**1399**	–	2.4%
India	82	85	91	94	99	114	134	92	148	82	**151**	84.5%	0.3%
Malaysia	–	–	–	–	–	–	48	110	197	250	**97**	−61.2%	0.2%
South Korea	–	–	1	2	4	9	39	74	140	217	**287**	31.9%	0.5%
Thailand	–	–	–	–	3	52	80	138	495	618	**647**	4.6%	1.1%
Other Asia Pacific	–	–	–	–	–	18	109	176	215	353	**448**	26.7%	0.8%
Total Asia Pacific	82	89	238	491	603	833	1323	1736	2628	3094	**3275**	5.9%	5.5%
Total world	**9176**	**10,084**	**11,930**	**14,767**	**16,452**	**19,944**	**25,743**	**34,512**	**46,294**	**52,098**	**59,261**	**13.8%**	**100.0%**
Of which: OECD	3841	4350	5406	7045	8549	11,013	15,054	20,494	27,728	32,569	**37,130**	14.0%	62.7%
Non-OECD	5336	5734	6523	7723	7903	8930	10,688	14,018	18,566	19,528	**22,131**	13.3%	37.3%
European Union	744	951	1305	1704	2073	3378	5052	6469	7944	9970	**10,447**	4.8%	17.6%
Former Soviet Union	–	–	–	–	11	22	28	49	129	645	**913**	41.5%	1.5%

The column in bold represents country wise production for the latest year (according to available data) while the row in bold represents overall global production of biofuel per year

(Source: BP statistical review of world energy 2018) Source: Includes data from F.O. Licht; US Energy Information Administration

Note: Consumption of fuel ethanol and biodiesel is included in oil consumption. * less than 0.06%

Biofuels in India is of strategic significance as it strengthens ongoing government policies like Make in India and Swachh Bharat Abhiyan and provides a great incentive to comply with the ambitious plans of multiplying farmers' incomes, growing imports, generating employment, creating wealth.

Overall, biofuels have come to the fore in the last decade and it is imperative to keep pace with developments in the biofuels sector. This strategy aims to put a renewed focus on international perspectives and the national scenario, mainly through the use of indigenous feedstocks for biofuel production. The strategy also concentrates on developing biofuel transformation innovations of the next generation based on new feedstocks and encourages the development of domestic feedstocks using the country's biodiversity. Vision, goals, strategy, and approach to the development of biofuels in India are established through technological structure, economic, organizational interventions, and mechanisms that enable them.

1.3.1 Vision and Goals

The program seeks to improve the use of biofuels in the energy and transportation sectors of the country over the next decade. The policy aims to use, develop, and promote domestic feedstock and its use in the production of biofuels, thus gradually replacing fossil fuels and contributing to national energy stability, mitigating climate change, and creating sustainable new job opportunities. Around the same period, the program will also promote the use of innovative technologies to produce biofuels. The purpose of the policy is to require biofuels to be on the market, thus increasing the percentage of blending. The percentage of gasoline ethanol mixture is currently around 2.0% and the amount of diesel biodiesel mixture is less than 0.1%. By 2030, a target of 20% ethanol in petrol and 5% biodiesel in diesel is proposed.

This purpose must be accomplished by:

(a) Strengthening the continued availability of ethanol/biodiesel by rising domestic production.
(b) Establishment of biorefineries of second generation (2 G).
(c) Creation of new biofuel feedstock.
(d) Introduction of new biofuel conversion technology.
(e) Develop the correct biofuel ecosystem and incorporate it with the main fuels.

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Nanoparticles for Sustainable Bioenergy and Biofuel Production

2

Muhammed Aasim, Egemen Foto, and Muhammad Sameeullah

Abstract

Nanotechnology is offering new technological improvements by using nanomaterials in various fields of science over the past three decades because of their distinctive properties in contrast to their bulk form. This technology can provide faster and more reliable methods to optimize energy resources from biological sources. Today, nanotechnology based products are found in daily life in a wide range of areas ranging from industrial measurement and detection devices, treatment systems, and wrinkle-resistant clothing to consumer-friendly products. Researches on nanotechnology are continuing to provide continuous improvement in life conditions by providing innovations in the fields of transportation, energy, agriculture, medicine, computer, and electronics. On the other hand, biofuel based on biological agents like algae, plants, etc. is another field which is gaining popularity and these biofuels are in use commercially. Recent advances in the biotechnology and nanotechnology open new window for researchers to enhance biofuel production. This study highlights the recent advances, contribution, and innovations in the field of nanotechnology to the development of biofuels.

Keywords

Biofuel · Biotechnology · Nanotechnology · Sustainability

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2.1 Introduction

Energy is the major factor controlling the socio-economic growth and to elevate sustainable human living standards (Walker et al. 2016; Arshad and Ahmed 2015). The global consumption of energy has increased substantially majorly dependent on fossil fuels to meet the demand (Pfenninger and Keirstead 2015). An estimation of around 80.3% fuel consumption has been covered by traditional fossil fuels (Escobar et al. 2009) resulting in serious resource depletion. Although, fossil fuels are cheap but causing the release of toxic greenhouse gases which are seriously detrimental for the environment (Chavez-Baeza and Sheinbaum-Pardo 2014; Friedlingstein et al. 2014). The whole world is now susceptible to global warming due to enormous CO_2 release from fossil fuels. This mighty challenge enforced the global communities to think about alternative renewable, eco-friendly, clean, and cost-effective energy resources like bioenergy. Biofuel offers reduced environmental pollution, increased socio-economic benefits (van Eijck et al. 2014; Creutzig et al. 2015), and controlling the depletion of fuel reservoirs (Smith 2013).

The "bio" in biofuel reflects biological feedstock processed for generating fuel, known as biofuel (Arshad et al. 2017) which offers the chance of reduced emission commitments under the Kyoto Protocol (de Alegría et al. 2016) especially for developed countries which are prone to more climatic changes in recent years. The biofuels are mainly grouped as primary biofuels comprised of plant/crop residue and animal wastes (Enagi et al. 2018) or secondary biofuels comprised of biomass and microorganisms (Sekoai et al. 2019). First generation biofuels are based on fermentation of starch from different edible crops (Sirajunnisa and Surendhiran 2016). The main issues associated with first generation biofuel are high cost of feedstocks, replacement of food crops with bioenergy crops, food scarcity and increase in food price (Hong et al. 2014; Sirajunnisa and Surendhiran 2016), agricultural related problems like land erosion, water contamination, and ecotoxicity (Singh et al. 2011a; John et al. 2011).

Second generation biofuels are comprised of biomass residues of different crops/plants (Leong et al. 2018) and can be further sub-grouped into three generations based on the biomass source (Fig. 2.1). For last two decades, cellulosic or lignocellulosic biomass (Sirajunnisa and Surendhiran 2016) has been used and considered as cheap, renewable resource, eco-friendly, and without posing any food security threat (Hong et al. 2014). However, high hydrolysis cost and low yield affect the production efficiency of second generation biofuels (Fu et al. 2010; John et al. 2011). The drawbacks of first and second generation biofuels like food security threat, increased agricultural inputs, and social challenges (Sirajunnisa and Surendhiran 2016; Ahmed and Sarkar 2018) forced the researchers to evolve new cost-effective and eco-friendly technology from new feedstock for biofuel production. Therefore, third generation biofuel (Fig. 2.1) is based on microalgae (Alaswad et al. 2015; Leong et al. 2018) due to high level of lipids and carbohydrates (Sirajunnisa and Surendhiran 2016). Third generation biofuels are preferred due to cultivation of microalgae under variable conditions (Khetkorn et al. 2017), no threat to food

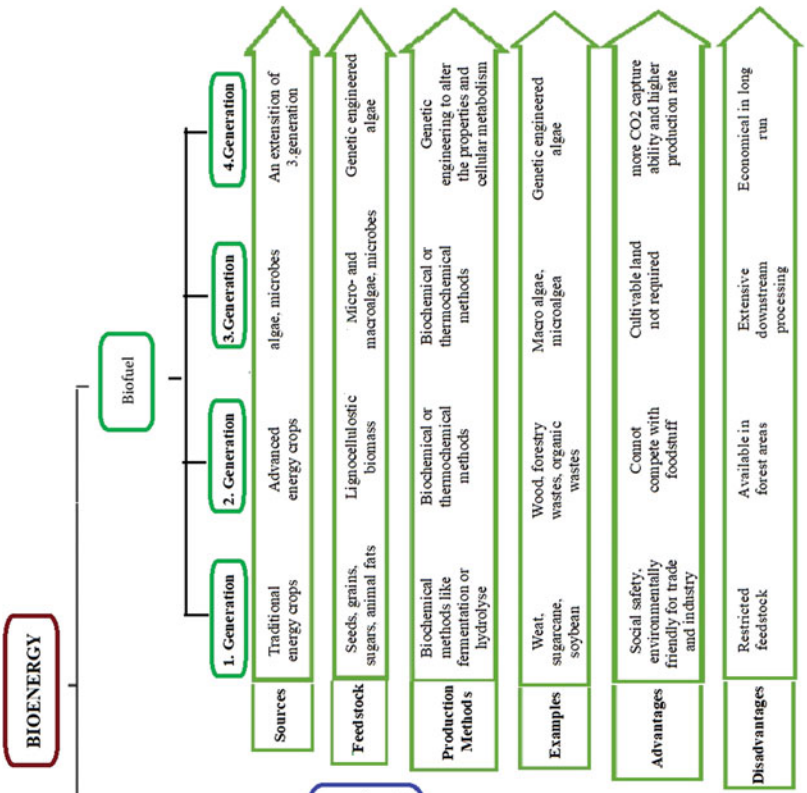

Fig. 2.1 An overview of biomass and biofuel based bioenergy

security (Ahmad et al. 2011), agricultural land, or water (Li et al. 2011), and generating variable biofuels and by-products (John et al. 2011).

Energy demand and its availability is one of the major issues of almost every country today (Waqas et al. 2018). These countries are continuously facing energy deficit due to limited resources, technology, and various political or economical reasons. Therefore, sustainable biofuel production become the main priority of such countries (Oh et al. 2018; Shields-Menard et al. 2018) to overcome the issues like energy crisis, increased energy prices, and environmental issues (Saravanan et al. 2018). Recent advances in the field of biotechnology enable scientists/researchers to develop alternative or new modern techniques (Nizami and Rehan 2018) to produce biofuels for sustainable energy production system. Sustainable biofuel energy system offers to tackle various environmental issues along with renewable energy production (Rai and Da Silva 2017). However, sustainable biofuel production is dependent on number of factors enlisted as biomass/feedstock pretreatment, process parameters and optimization, reactor designs, product quality and yields, capital costs, public acceptance, and market availability for various biofuels (Nizami and Rehan 2018).

The biofuels like biogas, biodiesel, and biohydrogen are synthesized through different processes using cheap and variable feedstock and considered as eco-friendly (Santoro et al. 2017). It is estimated that 90% of biofuel comprises mainly biogas, biodiesel, and bioethanol (Demirbas and demirbas 2011). Biodiesel is an important eco-friendly biofuel generated mainly from non-edible oils feedstock (Kirubakaran and Selvan 2017). Biodiesel technology is one of the most growing technology with growth rate of 7.3% per annum with industry worth of 54.8 billion USD by 2025 (Sekoai et al. 2019). Biogas is widely accepted sustainable biofuel synthesized through anaerobic digestion by microorganisms (Bundhoo and Mohee 2016) and comprises of CH_4, CO_2 and H_2S (Sekoai et al. 2019). In recent years, biogas production has substantially increased in developed countries while Germany is the leader in biogas market in Europe (Hijazi et al. 2016). Biohydrogen is one of the most prominent future biofuels due to high energy content, carbon-sequestration abilities, utilizing diverse feedstocks, bacteria, ambient temperature and pressure (Das et al. 2008). However, several factors like pH, temperature, substrate concentration, and hydraulic retention time control the efficiency of the whole technology but ultimate the result is the highly efficient biohydrogen (Sekoai et al. 2017) with other by-products like ethanol, butanol, and propanol (Sekoai et al. 2018). Bioethanol is another potential candidate for alternative fuel and renewable energy (Cesaro and Belgiorno, 2015) with 3–7% annual growth rate and estimated production was recorded 100 billion with expectation of two-fold during next decade (Aditiya et al. 2016). Bioethanol synthesized from second generation is not developed yet and has enormous potential from lignocellulosic feedstocks (Gaurav et al. 2017) due to no socio-economic issues and availability of feedstock (Sekoai et al. 2019). It is estimated that 200 billion tons/year plant biomass is produced which contains 90% lignocellulosic wastes and this abundant and cheap biomass can be utilized for generating renewable energy in future (Sekoai et al. 2019).

The feedstock used for generating biofuel determines the economics of biofuel industry (Elbehri et al. 2013). Comparison of biofuel prices with petroleum products varies with feedstock and region/country (Wesseler and Drabik 2016). Currently, the price of biofuel products is relatively higher than fossil fuels (Arshad et al. 2017). Feedstock is highly significant for commercial biofuel production and its availability is dependent on climate, soil, geographical locations, soil conditions, agricultural practices (Arshad et al. 2017), yield (Tabatabaei et al. 2015), and oil contents (Basumatary 2015).

Oil crops are considered as ideal feedstocks for biofuel production. Bart et al. (2010) reported more than 350 oil crops as potential feedstocks to generate biodiesel only feedstock accounts 75% of total biodiesel process cost (Atabani et al. 2012). In general, biodiesel feedstock comprises (i) edible (Westbrook et al. 2011) or (ii) non-edible oils (Gui et al. (2008), (iii) waste or recycled oils (Satyanarayana and Muraleedharan 2011), and (iv) animal and poultry fats (Mutreja et al. (2011). Microalgae are third generation feedstocks used for biodiesel using different strains (Popovich et al. (2012). Anaerobic digestion of organic matter by microbes generates biogas and it can be collected and utilized commercially. Biogas plants produce CH_4 gas along with CO_2 from plant biomass of different sources ranging from organic household waste, industrial waste, or bioenergy plants (Divya et al. 2015; Mao et al. 2015) containing large amount of carbohydrates, fats, and proteins (De Francisci et al. 2014). Apart from that, feedstocks containing sugars, starch, and lignocellulosic biomass are utilized for commercial bioethanol production through fermentation (Arshad et al. 2017).

Although, biofuel industry has achieved remarkable scientific breakthroughs and advancement in technology, the potential of second generation biofuels is still not triggered properly to compete with traditional fossil fuels (Cheng and Timilsina 2011; Sekoai et al. 2019). Expensive pretreatment technology (Zheng et al. 2014) and low yield (Balan 2014) due to compounds generated during fermentation (Ghimire et al. 2015) are the main hinders. Certain issues are almost associated with all types of biofuels and it reflects the optimization of new technologies to increase the biofuel production efficiency.

Recent advances in the field of nanotechnology/nanomaterials offer the researchers to incorporate this technology for process-efficient and cost-effective biofuel industry (Sekhon 2014) which implicates the use of green and catalytic chemistry along with engineering (Ramsurn and Gupta 2013). Nanotechnology offers the solution for many issues associated with biofuels production and enhancing efficiency due to structure, size and reactivity (Ghimire et al. 2015), high crystallinity intensity, catalytic activities, stability, and elevated adsorption capacity (Haun et al. 2010) which make them novel.

Application of nanotechnology in biofuel technologies include anaerobic digestion, gasification, hydrogenation pyrolysis, and transesterification for the production of biogas, fatty esters and renewable hydrocarbons, etc. One of the major area of application of nanotechnology/nanomaterials is the use of functional catalysts (Trindade 2011) due to its profound characteristics like adsorption capacity, catalytic

activity, durability, high degree of crystallinity, high surface areas, efficient storage, high recovery, reusability, and recycling potential (García-Martínez 2010).

Nanomaterials used in biofuel energy system include metal oxide nanocatalysts (Liu et al. 2007, 2008; Verziu et al. 2008; Gardy et al. 2017), mesoporous nanocatalysts (Yahya et al. 2016), and carbon-based nanocatalysts (Dehkhoda et al. 2010; Stellwagen et al. 2013; Mahto et al. 2016; Guan et al. 2017) for biodiesel production from wide range of feedstocks. Enzyme (biocatalysts) immobilization is another promising area in the biofuel industry using nano-encapsulation during lipase-catalyzed biodiesel and cellulosic ethanol production processes and for algal fuel production (Zhang et al. 2013). Nanoparticles (NPs) as fuel additives is another area where NPs like alumina and carbon nanotubes are successfully employed to enhance fuel blends performance (Trindade 2011) and combustion characteristics of biodiesel-operated engines (Basha and Anand 2011) with relatively less harmful emissions.

The objective of the chapter is to summarize the pivotal roles of NPs for biodiesel production from microalgal and bioenergy crops.

2.2 Nanoparticles and Lignocellulosic Feedstock

High demand of fuel for future, limited, and declining resources of fossil fuel opens a new era of renewable energy source of biofuels (Pandey et al. 2012; Srivastava and Jaiswal 2016). In recent years, the cost-effective biofuels production by using available resources like lignocellulosic biomass (Srivastava et al. 2015a, b) obtained from agricultural based industries (Sahaym and Norton 2008) is the target of researchers. The limitations associated with current biofuel production include high cellulase enzyme cost, which accounts 40% of total biofuel cost (Bhalla et al. 2013; Srivastava et al. 2015c), productivity, activity, and stability of cellulase enzyme at contrasting pH levels and components of medium (Yeoman et al. 2010). Application of NPs offer to elevate the productivity, hydrolysis, and stability of cellulase enzyme along with different biofuels production (Srivastava et al. 2015c). Application of NPs for biofuel production can be classified into six different groups like (i) cellulase production, (ii) cellulase thermal stability, pretreatment of biomass, (iv) waste management, (v) sugar and (vi) biohydrogen production (Srivastava et al. 2017).

Pretreatment of cellulosic and hemicellulosic fractions is the first step for cellulose conversion along with low cost biofuel production (Alvira et al. 2010). Application of NPs during pretreatment significantly affects the conversion rate and sugar contents. Wei et al. (2015) reported enhanced sugar contents when used Fe_3O_4 NPs. Similar type of results was achieved by Yang et al. (2015) when used Fe_3O_4-$RGOSO_3H$ NPs. After pretreatment, cellulases and hemicellulases enzymes are applied for releasing fermentable sugars (Bhalla et al. 2013; Rawat et al. 2014) for the production of cellulose by releasing cellulase. Different types of NPs have been used for enhancing cellulase production and to maintain its stability. Ansari and Husain (2012) used iron oxide (Fe_3O_4) NPs and achieved more cellulase production,

thermostability, and hydrolysis efficiency. Similar results were also attained by Verma et al. (2013a) when used zinc oxide NPs. Dutta et al. (2014) applied hydroxyapatite and reported enhanced cellulose production and stability with half-life at 80 °C. Srivastava et al. (2014a, b) checked the efficacy of nickel cobaltite ($NiCo_2O_4$) NPs and found 40% more cellulases production with elevated thermal stability (7 h; 80 °C). Application of two different iron based NPs by Srivastava et al. (2015c) revealed the 35% (Fe_3O_4 NPs) and 40% (Fe_3O_4/alginate) more cellulose production.

Efficient cellulase is highly significant for enzymatic hydrolysis and relatively slow process occurs at 45–50 °C with low sugar yield, incomplete hydrolysis, and microbial contamination (Wang et al. 2010). It is possible to increase thermal stability of cellulase by applying NPs (Jordan et al. 2011; Singh et al. 2016). Dutta et al. (2014) obtained reduced sugars at 80 °C by using calcium hydroxyapatite NPs. Srivastava et al. (2015a) used two different NPs and recorded relatively higher hydrolysis efficiency at 70 °C and 80 °C when used Fe_3O_4/alginate NPs and hydroxyapatite NPs, respectively.

Biohydrogen production is relatively complex process and can be facilitated by applying NPs (Chandrasekhar et al. 2015) but depending on various other factors like substrates, inorganic nutrients and operational condition, etc. (Wang and Wan 2009). Similarly, NPs can be useful for microorganisms under anaerobic conditions due to relatively easy electron transfer to acceptors (Beckers et al. 2013) or improved bioprocess kinetics (Xu et al. 2012). Incorporation of gold (Au) NPs significantly increased the biohydrogen fermentation and 36.3% more hydrogen yield compared to control group (Zhang and Shen 2007; Zhao et al. 2013). Han et al. (2011) reported the enhanced biohydrogen productivity by using hematite NPs which was reported to be due to immobilization of bacterial cells. Lower et al. (2001) reported 2–5 fold more production by using goethite (α-FeOOH).

The demand for lignocellulosic biomass degradation, sugar productvity, thermal stability and hydrolysis performance of cellulase for generating a cost effective technology is increasing substantially. However, this technology is still at initial stage and desires more precise work for sustainable biofuel production with the aid of nanomaterials. Nanomaterials are playing a significant role in biofuel production by altering the production process based on various physical characteristics (type/size/shape), structural morphology, and cost of NPs. However, there is need to focus on synthesis of NPs, compatibility rate, and interpreting the mechanism at the molecular level for the synergy between protein and nanomaterials.

2.3 Nanoparticles and Microalgal Biorefinery

Availability of promising feedstock is extremely important for biofuel production and characteristics like fast growth rates and high lipid contents turn microalgae into a leading feedstock for future energy (Sharma et al. 2011), also known as microalgal biorefineries (Lee et al. 2015). Microalgae are used for generating bioethanol due to possessing high carbohydrates and fermentable sugars (Nguyen and Van Hanh

2012), biodiesel and biohydrogen due to having high lipid contents (Ghirardi et al. 2000; Metzger and Largeau 2005). Apart from that, other products like nutrients, pharmaceuticals, and bioplastics can be achieved due to lipids, proteins and nucleic acids, polysaccharides, and pigments they contain (Moody et al. 2014; Kim et al. 2016). The ability of microalgae to be grown under variable conditions like saline or contaminated environments, fresh water without any extra demand of nitrogen makes them a significant candidate for future bioenergy production (Christenson and Sims 2011; Lam and Lee 2012; Rashid et al. 2014). The microalgal biorefinery can be splitted into four subsequential downstream processes comprised of (i) cultivation, (ii) harvesting, (iii) lipid extraction, and (iv) conversion (Seo et al. 2017). All these processes are subjected to different technological limitations and such type of limitations or problems can be minimized or over turned by applying NP engineering (Lee et al. 2015; Wang et al. 2015a) for improving mass production of biomass, lipid extraction output, biodiesel production, and cost-effectiveness (Seo et al. 2017).

2.3.1 Nanoparticle-Aided Cultivation

Microalgal cultivation accounts for 40% of total cost (Kim et al. 2013) and maintaining industrial scale lipid production (Pattarkine and Pattarkine 2012) and contamination of oleaginous microalgae when cultivated outdoor (Cho et al. 2013) are the major limitations. NPs can be used to boost photosynthetic cell growth and/or to induce intracellular accumulation of lipid without killing cell under stressed conditions (Lee et al. 2015). Indirect application of NPs includes the placement of localized surface plasmon resonances (LSPR) to outer side of closed photobioreactors for the adsorption and scattering of light at specific wavelengths (Pattarkine and Pattarkine 2012). Some studies revealed the enhanced light uptake by microalgae with the application of AgNPs (Torkamani et al. 2010), AgNPs, and AuNPs either single or in combination around photobioreactors (Eroglu et al. 2013). Direct use of NPs is the incorporation of NPs into culture medium for enhancing microalgal cultivation. The examples are synthetic nanoscale zero-valent iron (nZVI) (Kadar et al. 2012), iron NPs (Zhang et al. 2013), $MgSO_4$ NPs (Sarma et al. 2014), silica NPs (San et al. 2014), and TiO_2 (Kang et al. 2014). Although, NPs have been used successfully for microalgal cultivation, care must be taken prior to use as NPs may be toxic and lead to reduced microalgal growth.

2.3.2 Nanoparticle-Aided Harvesting

Microalgae harvesting is considered to be the major bottleneck of microalgae biorefinery governed by various biological and physicochemical characteristics like small size, high cell's dispersity, and concentration of culture at low upper end (Pienkos and Darzins 2009; Wang et al. 2015b). NPs-aided microalgae harvesting helps to enhance harvesting efficiency based on microalgal concentration,

energy consumption, toxicity, and cost-effectiveness (Lee et al. 2015). The different types of NPs used for enhancing harvesting efficiency are (i) functionalized magnetic NPs, (ii) aminoclay NPs, (iii) and multifunctional NPs for integrated use (Seo et al. 2017).

Magnetic NPs are generally preferable for microalgae harvesting due to characteristics like fast and high harvesting efficiency, automatable, scalable processing, and low contamination (Borlido et al. 2013). Some of the employed NPs are Fe_3O, Fe_3O_4–PEI (Polyethylenimine) (Hu et al. 2014), and diallyldimethylammonium chloride (PDDA)-coated Fe_3O_4 (Lim et al. 2012; Toh et al. 2012). Other magnetic NPs like cationic functionalized polyethylenimine (PEI) coated magnetic NPs (Prochazkova et al. 2013; Hu et al. 2014; Ge et al. 2015) or cationic polyacrylamide (CPAM)-modified Fe_3O_4 (Wang et al. (2014a), 3-aminopropyl triethoxysilane (APTES)—functionalized BaFe12O19 (Seo et al. (2014); PVP/Fe_3O_4 (Seo et al. 2015), chitosan/Fe_3O_4 (Lee et al. 2013a; Toh et al. 2014a) are highly recommended due to high harvesting efficiency.

Organophyllosilicates (Amine-group-rich) constituted aminofunctionalized phyllosilicate sheets and metal cations are intensively used in microalgae harvesting and known as aminoclays (Farooq et al. 2013a; Lee et al. 2014a). The most common aminoclays are Mg-aminoclay (Farooq et al. 2013a; Lee et al. 2014a), Fe-aminoclay (Farooq et al. 2013a), Al-aminoclay, Ca-aminoclay, (Lee et al. 2014a), humic acid/Mg-aminoclay (Lee et al. 2014a) or Mg-aminoclay-coated nZVI NPs (Lee et al. 2014b). Multifunctional NPs offer integrated use of microalgae harvesting and post harvesting stages like disruption of cells, extraction of lipids, and conversion of oils (Seo et al. 2017). The examples are aminoclay-conjugated TiO_2 composites (Lee et al. 2014c), PVP/Fe_3O_4 composites (Seo et al. 2015), and triazabicyclodecene (TBD)-functionalized Fe_3O_4@silica core-shell NPs (TBD-Fe_3O_4@Silica NPs) (Chiang et al. 2015). In recent years, efforts for recycling of these NPs for effective use and cost-effectiveness using different variables like pH (Seo et al. 2017) have been done. Recent development in the field of microalgae based harvesting using variable NPs and types of microalgae is presented in Table 2.1. Besides that, Table 2.1 also presents the direct use of photobioreactors and effects of some micronutrients on microalgae aided harvesting for different microalgae types and NPs.

2.3.3 Nanoparticle-Aided Lipid Extraction

Microalgae contain rigid cell walls which make it difficult to extract lipid and it requires energy-intensive or highly toxic organic solvents for pretreatment process (Lee et al. 2015; Kim et al. 2016). Traditional methodologies employed for lipid extraction from microalgae include solvent extraction or mechanical techniques/approaches (Kumar et al. 2015) with certain limitations like process cost, energy consumption, efficiency, quality, and stability of extracted lipid (Seo et al. 2017). Application of NPs although enhanced the lipid extraction efficiency but still need more extensive research work.

Table 2.1 Examples of direct or indirect uses of some NPs in microalgal harvesting, cultivation, and lipid extraction

Usage area	Type of NPs	Variety of Microalgae	Effect of NPs	Reference
Harvesting	Fe_3O_4	*Scenedesmus* sp.	Harvesting efficiency (%) was achieved by Fe_3O_4@SiO–NH_2 (>82%) followed by Fe_3O_4@PEI > Fe_3O_4@CTAB > Fe_3O_4 (I) > Fe_3O_4 (II)	Abo Markeb et al. (2019)
	Fe_3O_4	*Scenedesmus ovalternus, Chlorella vulgaris*	Perfectly suitable for microalgae harvesting for separation as magnetic forces	Fraga-García et al. (2018)
	Fe_3O_4 and $Y_3Fe_5O_{12}$	*Chlorella vulgaris*	Resulting in more than 90% harvesting efficiency by Fe_3O_4 and $Y_3Fe_5O_{12}$ (10 and 2.5 g/L, respectively) as flocculants	Zhu et al. (2019)
	Fe_3O_4 and Fe_3O_4@PAMAM	*Chlorella sp.*	This novel magnetic flocculants had cycling stability potentials to harvest of oleaginous microalgae efficiently	Wang et al. (2016)
	CaO	*Chlorella vulgaris*	When adding 60 mg/L NP, harvesting was increased 85%	Ma et al. (2016)
	Fe_3O_4 (Bare)	*Chlorella ellipsoidea*	Efficiency of harvesting was increased 85–99% when loading concentration of NPs was 300 mg/L	Xu et al. (2011)
	Fe_3O_4@PADAM	*Nannochloropsis sp.*	0.42 mg NP increased efficacy of harvesting 95%	Toh et al. (2014b)
	Fe_3O_4@CPAM	*Botryococcus braunii*	Harvesting was increased 90% by 120 mg/L NP	Wang et al. (2014b)
		Chlorella pyrenoidosa	High recovery, efficiency more than 93% at a dosage of 1500 mg/L and cells were separated ≥90% at a feed rate of 100 ml/min	Guo et al. (2018)
	Aminoclay-wrapped nZVI	*Chlorella sp.*	When adding 100 mg/L NP, harvesting was doubled	Lee et al. (2014d)
In directly used for photobioreactor	Ag	*Chlamydomonas reinhardtii, Cyanothece 51142*	Cell growth was increased. (>30%)	Torkamani et al. (2010)
	Ag and Au nanorod	*Chlorella vulgaris*	Both chlorophyll and carotenoid pigments accumulation were significantly increased by NPs	Eroglu et al. (2013)

Micronutrient supplement	nZVI	*Pavlova lutheri, Isochrysis galbana, Tetraselmis suecica*	Even though lipid contents of *P. lutheri* and *T. suecica* were increased, cell growth was normal	Kadar et al. (2012)
	$MgSO_4$	*Chlorella vulgaris*	Oil yield was increased	Sarma et al. (2014)
	Silica	*Chlorella vulgaris*	Cell growth was increased	San et al. (2014)
Inducer of lipid accumulation	TiO_2	*Chlorella vulgaris UTEX 265*	Efficacy of cells to produce lipid was increased. (≥15%)	Kang et al. (2014)

PAMAM Polyamidoamine, *PADAM* Polydiallyldimethylammonium chloride, *CPAM* cationic polyacrylamide, *nZVI* Nanoscale zero-valent iron

The NPs used for lipid extraction are hard, dielectric, magnetic, spinose, and enzymatic in nature. In order to enhance lipid extraction using NPs, aminoclays based NPs and specially engineered NPs such as Ca-APTES clay, Al-APTES clay, Mg-APTES clay, and Mg-N3 clay were practiced as flocculants for microalgae harvesting. Application of these flocculants exhibited decisive impacts on lipid yield, fatty acid methyl ester (FAME) contents and yield (Lee et al. 2013b). Lee et al. (2013c) presented the use of Fenton-like reaction of Cu-APTES, Fe-APTES clay, and Mn-APTES clay for lipid extraction. Whereas, aminoclays aided TiO_2 composites were used for photocatalytic reaction (Lee et al. 2014c).

2.3.4 Nanocatalysts for Greener Biodiesel

FAME (first generation biodiesel) is a sub-standard product owing to high unsaturated O_2 contents facing certain issues like cold flow property, stability storage, and engine compatibility (Park et al. 2015; Seo et al. 2017). Therefore, efforts had been made to increase biodiesel upgrades by adding different catalysts like sulfided Ni-Mo-, Co-Mo-, and Ni-W (Kumar et al. 2010; Peng et al. 2012a) or noble metal catalysts like Pt or Pd (Immer et al. 2010). The recent designed NPs based catalysts enable the researchers to convert lipids (microalgal oil) to biodiesel more efficiently. The examples are Ni-supported zeolites (Peng et al. (2012a), ZrO_2-promoted Ni catalysts (Peng et al. 2012b), AP-Ni-MSN (Aminopropyl-functionalized Ni-MSN) catalysts (Kandel et al. 2013), and Fe-MSN (Kandel et al. 2014). Application of NPs in microalgal biorefinery enables to boost harvesting performance, lipid yield, conversion and selectivity for green diesel.

Recent developments in the field of nanotechnology helped to engineer new functionalizing materials like surfactant-functionalized NPs or enzyme-functionalized NPs to boost lipid extraction by disrupting the rigid cell walls of microalgae. The surfactants are antimicrobial and biotoxic (Coward et al. 2014; Mohareb et al. 2015) in nature and show superior biocidal activity with relatively less environmental hazards and less skin irritation (Mohareb et al. 2015). The best example of surfactants NPs is the cetyltrimethylammonium bromide (CTAB) aided foam floatation which significantly boosted the lipid extraction efficiency (Coward et al. 2014) by causing cell deterioration (Huang and Kim 2013). Enzyme-functionalized NPs are used for pretreatment process and enable to boost lipid extraction efficiency but need specific requirements of pH, temperature, and incubation time (Cho et al. 2013). The examples are lysozyme or cellulose (Taher et al. 2014), lipase immobilized on alkyl-grafted $Fe_3O_4@SiO_2$ NPs (Tran et al. 2013).

2.4 Nanocatalysts for Biofuel Production

Biofuels are still expensive when compared with fossil fuels due to the high process cost (Azcan and Yilmaz 2013; Helwani et al. 2013) but still attractive for researchers due to their eco-friendliness. For cost reduction, catalysts are highly significant

during biofuel production process. Two types of generally used catalysts are (i) homogeneous or (ii) heterogeneous catalysts owing to certain advantages and disadvantages (Zuliani et al. 2018).

Homogeneous catalysts are employed for esterification and transesterification but with certain issues like neutralization of wastewater, recycling problems related with catalyst, and expensive equipment (Lam and Lee 2012; Chiang et al. 2015). Therefore, heterogeneous nanocatalysts are gaining attention for biodiesel and high density eco-fuels production owing to recyclable and cost effectiveness (Carrero et al. 2011; Lam and Lee 2012). Nanomaterials based nanocatalysts carry characteristics of both homogeneous (high activity) and heterogeneous (easy recovery) catalysts. These nanocatalysts are recoverable and recyclable in nature (Ma and Hanna 1999) and their catalytic properties (activity/selectivity) can be controlled by altering their physical characteristics like shape or size (Somorjai and Materer 1994).

The catalytic properties are also dependent on certain properties like acid-base, metal type or contents and porosity of the nanocatalysts (Zuliani et al. 2018). Metal oxides like CaO or MgO (Almerindo et al. 2011; Jeon et al. 2013; Bankovic-Ilic et al. 2017), hydrotalcites (Dias et al. 2012; Wang et al. 2012), zeolites (Costa et al. 2012; Narkhede and Patel 2013), zirconia (Zhang et al. 2014), and sulfated oxides (Vieira et al. 2013) are inorganic nanocatalysts, already documented for biodiesel synthesis. Recent advances on enzyme biocatalysts resulted in moderate reaction conditions, avoidance of saponification and smooth product purification (Zhao et al. 2015). These nanocatalysts can be classified as base (alkali), acid, and bi-functional nanocatalysts.

Alkali or base nanocatalysts (solid in nature) exhibit Bronsted basic and Lewis basic activity centers, having ability to accept proton or supply electrons to reactants. Base nanocatalysts are usually used for accelerating the reaction under moderate reaction conditions but need pure oil (Zuliani et al. 2018). The most used base nanocatalyst is calcium oxide (CaO) due to its elevated basicity and catalyst lifetime, low cost, and moderate reaction conditions (Ono 2003). In order to increase CaO activity, different methodologies were applied like doping with lithium (Kumar and Ali 2010; Kaur and Ali 2011), potassium fluoride (Wen et al. 2010; Hu et al. 2011; Kaur and Ali 2014), and zinc (Kumar and Ali 2013) or magnetic functionalization of CaO with strontium oxide to make CaO@($Sr_2Fe_2O_5$-Fe_2O_3) catalyst (Zhang et al. 2016). Other base nanocatalysts include hydrotalcite Mg/Al (Deng et al. (2011), zeolite (Xie et al. 2015), or magnetic nanocatalyst like Na_2O-SiO_2/Fe_3O_4 (Guo et al. 2012).

Acid nanocatalysts usually possess less activity but show relatively greater tolerance to polar impurities like water and FFAs, due to its hydrophobic surface. Acid nanocatalysts are used for catalyzing the alcoholysis of low-graded feedstock with more time needed. They have the ability to catalyze simultaneous esterification and transesterification for biodiesel production (Canakci and Van Gerpen 2001). Some of the examples of acid nanocatalysts include HUSY zeolite acid (Costa et al. 2012) and zirconia (Dehghani and Haghighi 2017), which are functionalized magnetic particles. Other examples of magnetic acid catalyst include sulfamic acid and sulfonic acid (Wang et al. 2015a).

Both base and acid catalysts are well known for their reactions like base catalysts accelerate the alcoholysis reaction, while acid catalysts show tolerance toward the purity (FFA content) of the feedstocks. Therefore, bi-functional nanocatalysts which are comprised of both acid and basic sites are used for one-step reaction for biodiesel production from low-grade oils with simultaneous esterification and transesterification (Kitakawa et al. 2013). Some of the examples of bi-functional nanocatalysts are Quintinte-3T (Kondamudi et al. 2011), Mo-Mn/γ-Al_2O_3-15 (75), and TPA/Nb_2O_5 (Srilatha et al. 2012). Use of these bi-functional nanocatalysts resulted in transesterification and esterification promoter (Kondamudi et al. 2011), efficient catalyst for waste cooking oil (Srilatha et al. 2012; Farooq et al. 2013b), and methanol for biodiesel production. It is concluded that nanocatalysts have great potential for high density biofuel production from different biomass.

2.5 Nanoparticles Aided Enzymes Immobilization

Commercial large scale biofuel production is dependent on enzymes but with some limitations like enzyme inactivation by solvents, high enzyme costs, and barriers to scale up (Watanabe et al. 2000; Shim et al. 2002). Immobilization of enzymes is considered as potential way to reduce biofuel system cost; easily separation and reuse ability and stability at extreme conditions (Ansari and Husain 2012; Hwang and Gu 2013; Verma et al. 2013b) make them suitable with improved product quality (Puri et al. 2013). These nanomaterials are considered as an alternative of conventional material for enzymes immobilization owing to higher enzyme loading, high biocatalytic potential, larger surface area (Gupta et al. 2011; Hwang and Gu 2013; Verma et al. 2013b), and enzyme counts bounded to NPs (Verma et al. 2016).

Magnetic/non-magnetic nanomaterials, pristine/functionalized nanomaterials, powder/suspension nanomaterials, and membrane nanomaterials have been employed for enzyme immobilization (Verma et al. 2013b). Nanomaterials like nanofibers are easy to handle with flexibility in reactor designing (Nair et al. 2007; Sakai et al. 2008) due to durability and easy separable properties. Other advantage of using nanofiber is the recovery of non-magnetic nanomaterials and controlling the dispersion (Kim et al. 2008) and reduced diffusional path (Jia et al. 2002). Huang et al. (2008) developed an enzyme immobilized fiber bioreactor which yielded continuous and steady hydrolysis. Nanocomposites are hybrid nanomaterials made by coating with inorganic or organic layer like silica, silane, or oleic acid, etc. on nanocores and these nanocomposites offer expedite grafting to variable functional groups for ideal immobilization of enzymes (Sen et al. 2010; Tran et al. 2012; Macario et al. 2013).

Immobilization of enzymes like cellulase and lipase on magnetic cellulose, silica, TiO_2, gold, and polymeric nanomaterials have been studied extensively (Cho et al. 2012; Huang et al. 2011; Pavlidis et al. 2012a, b; Verma et al. 2013b). Lipase enzymes were incorporated with covalent bonding (Xie and Ma 2010) or hydrogen bonding (Yu et al. 2005). Some examples of covalent immobilization of lipases include Fe_3O_4 NPs (Wang and Wan 2009), amino-functionalized magnetic NPs (Xie

and Ma 2009, 2010). Whereas, affinity method was adopted for immobilization of cellulases to gold-doped silica NPs (Cho et al. 2012), silica NPs (Chang et al. 2011), or silicon oxide NPs (Singh et al. 2011b). Immobilization of enzymes on NPs exhibits several benefits like elevated enzyme loading, multiple recyclings and avoiding enzymes denaturation compared to immobilization on larger materials, or un-immobilized enzymes (Verma et al. 2016).

2.6 Nanoparticles as Fuel Additives

Biodiesel is perceived as a renewable and eco-friendly fuel (Meng et al. 2013) due to certain advantages like low carbon monoxide, unburned hydrocarbons and smoke in comparison to petro-diesel. However, several factors regulate the engine performance like injection timing and pressure, engine speed and load, compression ratio or fuel blends, etc. (Rajesh et al. 2018) while using biodiesel (Ramadhas et al. 2004; Rajesh et al. 2018). Apart from that, characteristics like high viscosity and density along with low volatility of biodiesel generates various problems. Molecular size affects the combustion quality, whereas higher biodiesel viscosity may lead to inadequate automization performance and blockage of fuel entrances (Ramadhas et al. 2004). Such type of problems can be overcome by using fuel additives in order to enhance clean combustion with reduced exhaust emission and high engine performance.

Natural substances which are readily dissolvable in any kind of fuel (Rajesh et al. 2018) are known as fuel additives. These fuel additives affect physicochemical properties and combustion characteristics (Özgür et al. 2015; Imdadul et al. 2015) when added at relatively very low ppm to few thousand ppm. Most of these additives are antioxidants, biocides, corrosion inhibitors, and stabilizers. Some of the common fuel additives used in biofuel are metal based additives like Ba (barium), Ce (cerium), Cu (copper), Fe (iron), Ca (calcium), Mn (manganese) and Pt (platinum), or combinations of different metals like Ce-Fe or Pt-Ce. Oxygenated fuel additives like alcohols, ethers, or esters are used for increasing combustion quality and octane rating. Other additives include ignition promoter additives (alkyl nitrates), lubricant additives (unsaturated fat methyl esters), or antioxidant additives (butylated hydroxyanisole and pyrogallol propyl gallate). Although, these additives are very effective but researchers are always looking for new materials to increase the biofuel efficiency.

Recent advancement in nanotechnology enables the researchers to use metal based NPs as fuel additive commonly known as fuel borne catalysts (FBC) (Rashedul et al. 2014; Imdadul et al. 2015). CuO, $CuCl_2$, $CoCl_2$, $FeCl_3$, and $CuSO_4$ are commonly used FBC with biodiesel. These FBCs are used with biodiesel in order to improve thermo-physical properties (Dreizin 2000), brake power, SFC (Shahabuddin et al. 2012), and emissions performance (Kenneth et al. 2003; Farfaletti et al. 2005). Number of studies revealed the efficient use of nanomaterials as BFC and their impact on performance using different characteristics like heat release (Jones et al. 2011; Aalam et al. 2015a; Rao and Anand 2016), cylinder peak

pressure (Attia et al. 2014; Basha and Anand 2014; Aalam et al. 2015a), brake thermal efficiency (Selvaganapthy et al. 2013; Tewari et al. 2013; Banapurmath et al. 2014; Basha and Anand 2014), brake specific fuel consumption (Attia et al. 2014; Basha and Anand 2014; Rao and Anand 2016), and emissions like NOX (Tewari et al. 2013; Basha and Anand 2014; Ramarao et al. 2015), CO (Tewari et al. 2013; Banapurmath et al. 2014; Aalam et al. 2015a), HC (Tewari et al. 2013; Attia et al. 2014; Banapurmath et al. 2014; Basha and Anand, 2014; Ramarao et al. 2015), and smoke (Tewari et al. 2013; Basha and Anand 2014; Aalam et al. 2015a; Rao and Anand 2016). It is clearly evident that NPs as nano-additives are efficient tool for reducing the emission of pollutants and to enhance performance like the heat release rate and break thermal efficiency. This area needs more research work to find out the real potential of nanomaterials as biofuel additive. The possible impact of NPs as additives on the production of diesel and performance of biodiesel engines is summarized in Table 2.2.

2.7 Nanomaterials and Bioenergy Crops

Any plant based materials used for generating energy like liquid fuels, electricity, or heat are referred as bioenergy crops (Gao et al. 2006; Pandey et al. 2018). These bioenergy crops show wide range of adaptation to marginal soils with relatively low agricultural inputs with end result of huge biomass production (Linglan et al. 2008). However, the success of bioenergy crops is dependent on various factors like germination, faster growth and development, yield and tolerance to abiotic/biotic factors (Khodakovskaya et al. 2009; Pandey et al. 2018). Therefore, new technologies are always welcomed for enhancing yield of bioenergy crops. One of the major advancement in this area is the application of nanomaterials for enhancing total biomass of bioenergy crops by enhancing germination (Lin et al. 2009), or plant growth and development (Colvin 2003; Maynard et al. 2006; Ke and Qiao 2007; Lin et al. 2009; Sheykhbaglou et al. 2010; Khodakovskaya et al. 2011, 2012). The most commonly used NPs are carbon-based NPs like carbon nanotubes and carbon nanohorns and graphenes (Dugan et al. 1997; Basch et al. 2003; Colvin 2003; Maynard et al. 2006; Ke and Qiao 2007; Lin et al. 2009; Sheykhbaglou et al. 2010; Khodakovskaya et al. 2011, 2012; Kole et al. 2012). In some studies, detection and measurement of carbon nanotubes and carbon nanohorns were performed in different plant organs by microwave induced heating (MIH) technique (Maynard et al. 2006; Hyung et al. 2007). Recently, Pandey et al. (2018) reported the use of graphene and carbon nanotubes (CNTs) for enhancing biomass yield of different bioenergy crops named sorghum and switchgrass. Their results revealed the enhanced germination, shoot and root length, and seedlings biomass (only shoots) of both crops. Mixing of graphene in the soil enhanced the 28.11% shoot biomass of switchgrass. Whereas, CNTs enhanced the reproductive organs of both crops. Positive effects of nanomaterials on plant growth have been reported for other economic crops like barley, maize, and soybean (Colvin 2003). Application of NPs to bioenergy crops is considerably safe due to non-food crops (Pandey et al.

Table 2.2 Efficacies of some nanoparticles as additives on diesel and biodiesel engines (Modified from the reference Sezer 2019)

Type of fuel	Additives	Amount of additives in fuel	Size of NPs (nm)	Properties about the tested-engine		Effect of NPs (Variation degree %)						References
				Type	Volume (cc)	Cetane number	Inflammation temperature	Viscosity	Thermal value	Ignition temperature	Density	
Biodiesel	CeO_2	30 ppm/L	–	–	–	↑ 0.9	↓ 3.9	↓ 24.5	↑ 3.9	–	↓ 2	Rajalingam et al. (2016)
	KNT	100 ppm	15	a	661 cc	↑ 5.9	↑ 40	↑ 13.4	↓ 0.97	↑ 29.3	↑ 9.6	Thulasi et al. (2016)
	$FeCl_3$	5–50 µmol/L	10	a	661 cc	↑ 2.1–5.4	↓ 0–2.9	↓0.2–1.1	↑ 0.2–1	↓ 0–3.7	↓ 0.02–0.1	Yuvarajan and Ramanan (2016)
	n-Mn	4–16 µmol/L	–	a	395 cc	–	↓ 2.3–8	↓ 3.2–9.1	↑ 2.2–2.8	–	↓ 0.7–2.5	Celik (2016)
	KNT	100–300 ppm	18	b	661 cc	↑ 0–1.9	↑0.5–1.1	↑ 0.2–0.4	↑ 0.5–0.6	–	↓ 0.11–0.22	Balaji and Cheralathan (2015)
	CeO_2	25 ppm/L	–	c	1984 cc	↑ 24.4	↑ 229.3	↑ 4.4	↑ 21.3	↑ 172	↑ 0.4	Narasiman et al. (2015)
	n-G	25–50 ppm	–	b	661 cc	–	↓ 5.9–7	↑ 3.6	↓ 1.4–2.8	–	↑ 1.7–2.3	Bhagwat et al. (2015)
	Al_2O_3	30 ppm/L	51	a	661 cc	–	↓ 8.2	↑ 3.6	↓ 1.5	–	↑ 0.2	Arockiasamy and Anand (2015)
	CeO_2	30 ppm/L	–	d	1323 cc	–	↓ 10.6	↑ 4.8	↓ 2	–	↑ 0.3	Chaudhari et al. (2014)
	Ag-NPs	50 ppm n-	<150	b	661 cc	–	↓ 1.2	↑ 5.3	↓ 2.9	–	↑ 2.8	Banapurmath et al. (2014)
	Al-NPs	25–50 ppm	51	a	661 cc	↑ 1.9–3.8	↓ 1.2–3.5	↑ 1.1–1.9	↑ 0.9–1.7	–	↑ 0.1–0.2	Basha and Anand (2013)
	Fe_3O_4	%1	–	a	661 cc	↑ 8	↑ 2.1	↑ 3.7	↑ 2.8	–	↑ 5	Kannan et al. (2011)

(continued)

Table 2.2 (continued)

Type of fuel	Additives	Amount of additives in fuel	Size of NPs (nm)	Properties about the tested-engine		Effect of NPs (Variation degree %)						References
				Type	Volume (cc)	Cetane number	Inflammation temperature	Viscosity	Thermal value	Ignition temperature	Density	
	Al_2O_3	100–300 ppm	–	b	661 cc	↑ 0–1.9	↑ 0.5–1.1	↑ 0.2–0.4	↑ 0.5–0.6	–	↑ 0.1–0.2	Balaji and Cheralathan (2017)
Diesel	Al_2O_3	25–50 ppm	–	a	661 cc	–	↑ 4.1–8.3	↑ 1.5–2.7	↑ 0.3–0.6	↑ 3.8–7.7	↑ 0.2–0.3	Raj et al. (2016)
	Fe_3O_4	150–300 mg/L	70–120	b	661 cc	↑ 4.2–10.6	↓ 10.9–14.5	↑ 3.7–11.1	–	↓ 5–17	↑ 0.3–0.6	Mahendravarman et al. (2016)
	Al_2O_3	25–100 ppm	27–43	b	395 cc	↑ 0.9–1.5	↑ 10–15	↓ 0–2.8	–	–	↑ 0.08–0.12	Gumus et al. (2016)
	CuO	50 ppm	30–50			↑ 1.3	↑ 10	↓ 2.8	–	–	↑ 0.07	
	Al_2O_3	300 ppm	27–43	e	350 XL	–	↓ 5.7	↓ 2.8	↑ 0.04	–	↑ 0.1	Sungur et al. (2016)
	TiO_2	300 ppm	30–50			–	↓ 1.9	↓ 2.8	↑ 0.005	–	↑ 0.09	
	Al_2O_3	%0,01–0,1	500	f	–	↓ 6.5–13	↓ 1.2–16.9	↓ 32.5	–	–	↑ 0.09	Ooi et al. (2016)
	CeO_2	%0,01–0,1	200			↓8.7–10.8	↓ 18–22	↓ 0.6–33.1	–	–	↑ 0.01	
	CeO_2	50 cc/L	–	b	661 cc	–	↑ 14.8	–	↑ 0.36	↑ 16.1	↑ 0.2	Venkatesan and Kadiresh (2016)
	n-Al	25–75 ppm	<50	b	661 cc	–	↑ 3.6–12.7	–	–	–	↓4.7–12.5	Babu and Raja (2015)
	Al_2O_3	250–1000 ppm	40	b	661	–	↑ 1.9–15.4	–	↑ 0.09–0.6	↑ 4.8–22.6	↑ 0.2–1.1	Venkatesan and Kadiresh (2015)

	Fe_2O_3	25–50 ppm	16–27	b	553 cc	↑ 3–5.5	↑ 8.6–15.5	–	↑0.8–1.5	–	↑0.5–1	Aalam et al. (2015b)
	CeO_2	50 cc/L	–	b	661 cc	–	↑ 14.8	–	↑ 0.36	↑ 16.1	↑0.2	Venkatesan et al. (2014)

↑: Increased, ↓: Decreased, −: No definition, All of the type of engines were four stroke diesel except e and f. a: One cylindered and air-cooled engine, b: One cylindered and water-cooled engine, c: Three cylindered and air cooled engine, d: Two cylindered and water-cooled engine, e: Residential type hot water boiler, f: oven experiment set

2018). Besides CNPs, other NPs employed for plant growth and development of other plants were mostly on non-bioenergy plants. Therefore, more research is needed to check the efficacy of other NPs on bioenergy crops with aim to boost the biomass yield. Another important area regarding feedstocks (crops) used for generating biodiesel is the reaction conditions to generate more efficient production with the help of adding different NPs is summarized in Table 2.3.

2.8 Interaction of NPs with Biomass and Microorganism for Renewable Energy

Waste contaminations like municipal sludge are nutrient rich and considered as excellent biomass for generating bioenergy. Aerobic digestion (AD) process is used for disposing such type of biomass for generating renewable and cheap energy (He et al. 2016; Romero-Güiza et al. 2016; Bernat et al. 2017) along with environmental pollution (Faisal et al. 2019). Application of NPs significantly enhanced the organic matter-degrading bacterial activities which resulted in more degradation with high bioenergy production (Mao et al. 2015; Faisal et al. 2019). Besides that, NPs can be utilized for enhancing the efficiency of different types of biomass (algal) into bioenergy and other intermediates and by-products. It is also necessary to understand the characteristics of biomass, microorganisms, and their interaction with NPs. Biomass resources can be characterized on their source, biological, physical and chemical composition, particle size or pretreatments methods, etc. (Kumar et al. 2009). NPs can be used for detecting and separating the biological or chemical substances like metals, nutrients, algae, antibiotics, toxic chemicals, and microorganism present in the biomass (Faisal et al. 2019).

Conversion of biomass into bioenergy occurs as chemical, biological, or thermal process with the aid of NPs and factors like inorganic contaminants and size affects the conversion rate. On the other hand, most of the NPs exhibit antimicrobial activities and selection of proper NPs is important for efficient renewable energy production (Faisal et al. 2019). Algal biomass is considered as the potential biomass of future bioenergy, chemicals, and different economic extracts. Algae are the main component of aquatic bio-system and their growth is affected (Batley et al. 2012; Angel et al. 2013) by the presence of silver NPs (Ribeiro et al. 2014) where in some cases recorded above 5 g/L (Faisal et al. 2019). It is therefore highly recommended to investigate the risks related to NPs with target to their expected transformation, mobility, and interaction with other materials (Farré et al. 2011). Phytotoxicity or ecotoxicity is another area which must be targeted to check the efficacy of NPs.

Table 2.3 Reaction conditions of biodiesel production with various nanocatalysts and feedstocks

Feedstock	Nanocatalyst	Conditions of the reactions				Yield (%)	Reference
		Temperature (°C)	Time (min)	Methanol/ Oil ratio (%)	Catalyst loading/ concentration (wt)		
Sunflower oil	$MgO/MgAl_2O_4$	110	180	12	3	95.7	Vahid et al. (2018)
	$MgO\text{-}La_2O_3$	64.85	15	18:1	60	97.7	Feyzi et al. (2017)
	Sono-sulfated zirconia supported on MCM-41	60	30	9:1	5	96.9	Dehghani and Haghighi (2017)
	$MgAl_2O_4$	110	180	12	3	95	Rahmani Vahid and Haghighi (2017)
	Calcite/Au	65	360	0.3	9:1	97.58	Bet-Moushoul et al. (2016)
	$Ca/Fe_3O_4@SiO_2$	65	300	15:1	6	97	Feyzi and Norouzi (2016)
	$Cs/Al/Fe_3O_4$	58	120	14:1	4	94.8	Feyzi et al. (2013)
	MgO	170–270	40–120	1:4	300 mg	98	Verziu et al. (2008)
Soybean oil	$Ni_{0.5}Zn_{0.5}Fe_2O_4$ doped with Cu	180	60	1:20	%4	85	Dantas et al. (2017)
	Iron/cadmium and iron/tin oxide nanoparticles	200	60	10:3	1	84	Alves et al. (2014)
	MgO nanoparticles on TiO_2 support	225	60	1:18	0.1–7	95	Mguni et al. (2012)
	ZrO_2 loaded with $C_4H_4O_6HK$	60	120	16:1	6	98.03	Qiu et al. (2011)

(continued)

Table 2.3 (continued)

Feedstock	Nanocatalyst	Conditions of the reactions				Yield (%)	Reference
		Temperature (°C)	Time (min)	Methanol/ Oil ratio (%)	Catalyst loading/ concentration (wt)		
Waste cooking oil	Al_2O_3/Fe_3O_4	99.8	177	32.1	5	99.1	Bayat et al. (2018)
	$SO4^{2-}/ZrO_2$	148.5	93	12.7	2.9	93.5	Rahmani Vahid et al. (2018)
	ZnO	60	15	6:1	1.5	96	Varghese et al. (2017)
	Sulfamic and sulfonic acid-functionalized silica-coated crystalline Fe/Fe_3O_4	70	240	–	20 mg	95	Wang et al. (2015b)
Jatropha oil	CaO	60	133.1	5.15:1	0.02:1	98.54	Reddy et al. (2016)
	Lithium-impregnated calcium oxide (Li-Cao)	65	120	12:1	5	99	Kaur and Ali (2011)
	Hydrotalcite particles with Mg/Al	44.85	90	0.4:1 (v/v)	1 (anhydrous methanol = 40 mL, sulfuric acid = 4 mL)	95.2	Deng et al. (2011)
Rapeseed oil	$Na_2Si_2O_5$	65	120	30:1	0.4	97.8	Ghaffari and Behzad (2018)
	$K_2O/\gamma\text{-}Al_2O_3$	70	180	12:1	3	94	Heyou and Yanping (2009)
Canola oil	$ZnO/BiFeO_3$	65	360	15:1	4	95.43	Salimi and Hosseini (2019)
	KOH/calcium aluminate	65	240	12	4	91	Nayebzadeh et al. (2017)

Palm oil	KF/γ-Al_2O_3/honeycomb ceramic (HC) monolithic catalyst	140	33	18:1	6.96–24.126	96	Gao et al. (2015)
	TiO_2-ZnO	60	300	6:1	200 mg	92,2	Madhuvilakku and Piraman (2013)
Waste cottonseed oil	TiO_2/SiO_2	65	240	30:1	5	98	Kaur et al. (2018)
Bombax ceiba oil	CaO	65	70.52	30.37:1	1.5	96.2	Hebbar et al. (2018)
Castor oil	Ni doped ZnO	55	60	1.8	11	95.2	Baskar et al. (2018)
Rice bran oil	CaO	65	120	30:1	0.4	93.5	Mazaheri et al. (2018)
Tricaprylin	Carbon nanohorn dispersed with $Ca_2Fe_2O_5$	180	60	3 g methanol	0.12 g catalyst	100	Sano et al. (2017)
Microalgae oil	CaO	70	216	10:1	1.7	86.41	Pandit and Fulekar (2017)
Chinese tallow seed oil	KF/CaO	65	180	12:1	3	96.8	Wen et al. (2010)

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Bio-Hydrogen: Technology Developments in Microbial Fuel Cells and Their Future Prospects

3

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Abstract

The energy is the part of the human evolution; the innovation in the transportation and industrial evolution happened in this century made mankind to depend on fossil fuels invariably. The depletion of fossil fuel resources and global carbon footprint accumulation are worrying the global countries for the future environmental safety. The clear policies were amended to come out of releasing the global carbon footprint by many countries; even developing countries are making it compulsory for controlling or reducing greenhouse gases releasing in to environment. In this context hydrogen fuel is getting promising significance since it has high energy content per unit mass, and up on combustion it will not release any carbon footprint and considered to be complete green energy. Though there are many chemical and physicochemical methods available for the production of H_2, biological H_2 production will be superior since this method do not use harsh chemical process and do not need extreme conditions for the production. Hence, many research studies are put forward for the production of biological hydrogen production. In this book chapter we will have comprehensive discussion on these technologies developed for the hydrogen production till date. This chapter also included the next generation technologies which are in acceleration in engineering the strains for the enhancing the productivity and various other parameters like utilization of waste biomass and waste industrial affluent etc. This chapter also included with the list of aspects to be looked for the future development of H_2 as the next generation fuel energy.

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Biohydrogen · Microbial fuel cell · *E. coli* · Waste biomass and metabolic engineering

3.1 Introduction

Global demand for energy sources, depletion of the fossil fuel resources and critical worry on the greenhouse gas release pusing scientific community looking for the alternative sources of green energy which can check the environmental issues (Sudheer et al. 2010; Sudheer Pamidimarri and Reddy 2014). The global decay of the earth's environmental health and direct accumulation of the carbon footprints relesed by usage of fossil fuels; non-carbon energy source is said to be the way-out for the global crises of energy and to avoid the production of greenhouse gases (Hansel and Lindblad 1998). The non-carbon green fuels available in the present technology are hydraulic, wind, solar energies, and hydrogen fuel. Among these hydrogen fuel can readily answer the global environmental issues and have possibility to compensate the global energy demands (Dunn 2002).

Hydrogen (H_2) produced from biological sources is considered as the cleanest energy. Biological hydrogen is generated from the biological source by the process where green energy is generated by environmentally friendly way and was credited with zero emissions of pollutants. Globally at present H_2 is the most promising source in the succession of fuel evolution. Hydrogen fuel is encouraged throughout the globe because of several technical, socio-economic, and environmental benefits (Das and Veziro□lu 2001). H_2 gas is considered to be safer compared to the natural gas and better than domestic natural gas and is now universally accepted as environmentally safe. Moreover, hydrogen fuel could be generated from renewable source which can defy the greenhouse effect (Kumar and Kumar 2017). Presently, H_2 is produced from various sources like natural gas, heavy oils, naptha, coal, and electrolysis which in turn contribute to greenhouse emissions. Microbial cell factories, unlike the chemical or electrochemical counterparts, generate no effluents and are environmentally safe. Biological production of hydrogen catalyzed by microorganisms in an aqueous environment at the ambient temperature and atmospheric pressure is a complete green process (Lynd et al. 2009; Chaubey et al. 2013).

Globally, the major share of energy utilization is for the transportation and it occupies the share of 65%. Petroleum based fuels are the sole source of transportation fuel presently used, which is causing the local and comprehensive climate change and air congestion in the urbanized areas (Kumar and Kumar 2017). This is causing the alarming disturbance in the air quality and making the metro cities unsuitable for the living. If the same continue further, the future position of the urban areas in prospective of living standards will be deteriorated and countries need to spend the major section of economy for the health care. Hence, replacing the traditional transportation fuel (petroleum and coal based) with hydrogen fueled transportation system will improve the situation and can make the metro and urban

cities more human friendly (Das and Veziro?lu 2001; Maeda et al. 2012; Kumar and Kumar 2017).

In the present era of biotechnology, the concept of microbial fuel cell is rising since the biomass requirement of the microbial cells is more flexible and the productivity is reached near to the theoretical values. This whole cell based catalysis for the production of fuel energy is supposed to be the most efficient system which can answer the present energy crisis. Hydrogen production from the microbial fuel cell is said to be a good concept of green fuel since the hydrogen fuel combustion results in no greenhouse gases. Moreover, the energy content per mass of the hydrogen energy is 142 MJ kg^{-1} which is better than biofuels like bio-ethanol and biodiesel (Maeda et al. 2012). This book chapter presents the microbial hydrogen fuel cells, their significance and production mechanism, will discuss further about the different microbial sources of hydrogen production, the biomass requirement, and prospective utilization of lignocellulosic biomass or other waste biomass. A separate section is dedicated for the biotechnological approaches for the improvement of hydrogen production in *E. coli*. The concluding part will include the future prospective of the microbial fuel cells and possible strategies for enhancing the hydrogen production and aspects of hydrogen economy for the implementation.

3.2 Hydrogen Production Sources

Currently, hydrogen production is by three major processes; these include electrochemical, thermochemical, and biological process. Superiority of these methods is always under debate since each method is having its own credits and demerits (Stojić et al. 2003; Turner 2004). Biological or microbial based hydrogen fuel production is encouraged globally for their independence of non-renewable substrates. In this section brief account of each method and their merits and demerits will be discussed and detailed discussion is made on microbial based hydrogen fuel production.

3.2.1 Electrochemical Process

Electrochemical process is the first process to be designed for the production of hydrogen from the source of water via electrolysis. It is the simple splitting of the water in to corresponding components by using the electrical energy (Stojić et al. 2003). There are majorly two types of the process involved in the electrolysis; these are by alkaline electrolyzer and the polymer electrolyte membrane (PEM) electrolyzer (Marcelo and Dell'Era 2008). The efficiencies of these processes are about 56–73%. Though, the H_2 considered to be green energy source, however, the greenness of the process is mainly depending on the source of electricity utilized in the process. Hence the debate of the greenness of the process is still continuing. Utilizing solar energy for conducting the electrolysis is considered to be the best way for making whole process environmentally green. Considering the renewable source of electricity (via solar or wind power) the process can be most permissive in the

view of carbon footprint. However, the investment is needed for shifting towards hydrogen renewable energies. In economic stand point for the production, cost per unit is very high and is not a method of choice for the commercial production. Moreover, the investment needed for this is very high and this will be added to the production cost.

3.2.2 Thermochemical Process

Unlike the electrochemical process thermochemical process is more suitable for the bulk production and will have possibility of scale-up to the commercial level due to its higher productivity and efficiency (Ohta 1979; Freni et al. 2000; Funk 2001). There are various thermochemical methodologies used to produce H_2. These include thermal dissociation, thermal pretreatment (pyrolysis and gasification), and reforming. Among these three processes, only the thermal dissociation method uses direct splitting of water into corresponding elements and produces H_2 as same as in case of electrochemical process (Utgikar and Thiesen 2006). Later two methods use either hydrocarbons or organic biomass as starting material for the production of H_2 (Haryanto et al. 2005; Navarro et al. 2007). Thermal pretreatment method uses carbonaceous matter, and is first converted to smaller constituents which can be used for the production of H_2 in the second phase. Pyrolysis is the popular method for converting the rice husk or similar biomass into hydrogen. Gasification is similar to reforming, where it uses steam or oxygen for the conversion of carbonaceous material or biomass into gaseous product (Vasudeva et al. 1996; Marquevich et al. 2000; Demirbas 2004; Czernik et al. 2007). However, these methods are under debate since all these discussed methods rely on energy input which may not be from the source of green process. Hence, there are many efforts were made to integrate renewable energy like solar energy for the production of heat energy which can be used in the process (Fujishima et al. 2000). Moreover, the process reforming and pyrolysis process use the hydrocarbons as raw material whose sources are non-renewable; hence, long-term production technologies using renewable biomass must be developed for the sustainable production of H_2.

3.2.3 Biological Process

Biological production of H_2 is said to be the most prominent process since the technology involves complete green production and moreover the flexibility of starting material could be diverse based on the microbial source utilized for fermentation. The hydrogen producing microbes can be divided into two groups: photosynthetic and non-photosynthetic or fermentative hydrogen producers (Das and Veziro□lu 2001). Both processes use renewable raw material for the biomass generation and hydrogen production. Superiority of any method is not relevant since both photosynthetic and fermentative process have own advantages and

demerits. Hence the following section describes in details regarding biological H_2 production.

3.3 Microbial Hydrogen Fuel Cells

In contrast with electrochemical or thermochemical hydrogen production microbial fuel cells for the H_2 production is always given superiority because they are based on completely green process. Moreover, the process could be conducted in ambient condition without use of extreme temperatures and pressures. As mentioned earlier, among the photosynthetic and fermentative methods, much of the research is focused on the fermentative method because of the advantages like (1) this method does not depend on the presence of light for the H_2 production, (2) its higher production rates, and (3) a variety of carbon energy sources like organic matter, low-cost carbohydrates, cellulosic, lignocellulosic, cellobiose, and other waste biomass could be used as carbon source to grow the microbial cell mass for the production of H_2. In this section we will discuss both photosynthetic and fermentative methods of H_2 production.

3.3.1 Photosynthetic H_2 Production

Photosynthetic H_2 production is carried out by various bacterial, algal, and cyanobacterial species. These microbes use diverse pathways and various machinery for the generation of cellular energy and H_2 production, respectively. These photosynthetic H_2 producing bacteria can be grouped majorly into two groups based on oxygen generation. Majority of algal and cyanobacterial species use photosystems for harvesting the energy, and electrons are donated by photolysis of water, importantly the O_2 accept the electrons finally and these are called oxygenic photosynthetic H_2 producers (Barbosa et al. 2001; Kovács et al. 2006). The other group depends on various organic acids for the electron donors and use nitrogenases for the production of H_2 as a by-product during nitrogen fixation. In this section the mechanisms, advantages, technical limitations, and future prospective will be discussed in detail.

3.3.1.1 Oxygenic Photosynthetic H_2 Production

Photosynthesis is the basic functional aspect of plants, algae, and cyanobacteria. In the process of oxygenic photosynthesis H_2O is oxidized, generate O_2 and the electrons will be used by photosystems for the reduction of NADP. The protons released during photolysis combined with the electrons passed to membrane, upon electron transport by reducing NADPH or ferredoxin will be used for the production of H_2 by hydrogenases in many cyanobacteria and algae (Miyake et al. 1999) [Fig. 3.1]. In general, the photosynthetic system needs four electrons for a pair of electrons sequester from H_2O and reduce NADP or to generate couple of H_2 molecules. The major advantage of this process is, it utilizes the light energy for

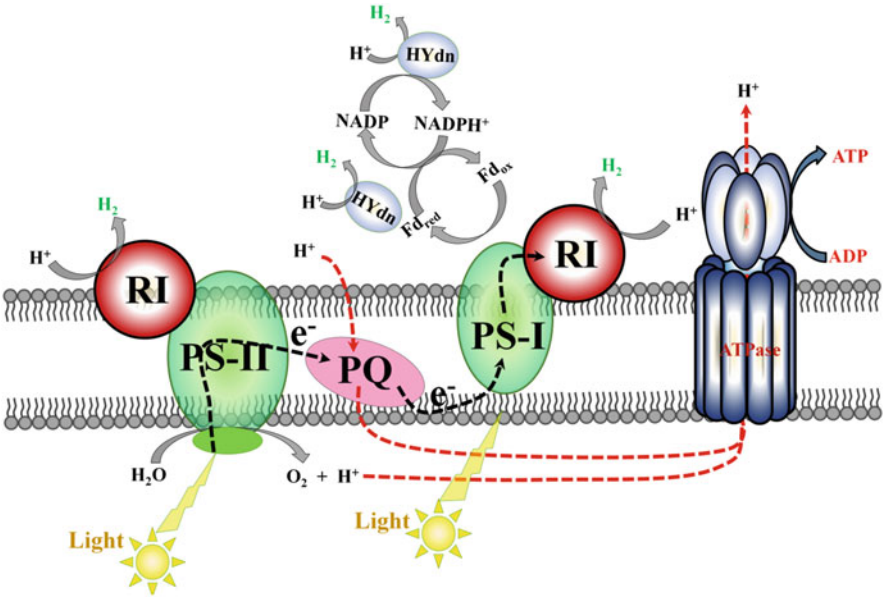

Fig. 3.1 Photosynthetic oxygenic H_2 production by microalgae and cyanobacteria. *RI* reactive intermediate, *PS-I* Photosystem 1, *PSII* Photosystem II, *PQ* Plastoquinone, *Hydn* Hydrogenase

the splitting of H_2O to O_2 and H_2 (Dutta et al. 2005; Lee et al. 2010a). This oxygen generating H_2 production system is the only green energy produced from the renewable light energy without emission of CO_2 and also it has a great importance of fixing the CO_2 and also generates the algal biomass which could be used for many biotechnological and fermentative applications (Miyake et al. 1999; Dutta et al. 2005). Although oxygenic photosynthetic H_2 production looks very promising, the major challenge in commercial implementation is especially in the context of engineering limitations for designing a suitable bioreactor for scale up to the level of industrial production. Since, the system needs the illumination of light, engineering a closed system with translucent glass reactor for the bulk production is necessary. Hence, there should be an innovative reactor model need to be designed for the bulk production and scale-up.

3.3.1.2 Non-oxygenic Photosynthetic H_2 Production

Though, the oxygenic photosynthetic hydrogen production system is under major discussion; a separate group of bacterial species called non-oxygenic photosynthetic H_2 producers comes under the group photosynthetic purple non-sulfur bacteria are also important group worth discussing in this section. The genera *Rhodobacter*, *Rhodopseudomonas*, and *Rhodospirillum* are the major representatives of photosynthetic purple bacteria that generate H_2 without generating O_2 (Lee et al. 2010a). These are the alternative photosynthetic H_2 producers in place of oxygenic photosynthetic H_2 producers. These utilize light as the energy source and organic acids

most commonly carboxylic acids as electron donors. Since H_2O does not act as the electron donor, hence no oxygen is released. The major benefit of this system is, in case of oxygenic photosynthetic H_2 production, the sensitivity of hydrogenases towards the presence of O_2 in high concentration will inhibit or lower in several folds the production efficiency. These non-oxygenic H_2 producers do not generate O_2 since this system uses nitrogenase in place of hydrogenases to generate H_2 (Masepohl et al. 2002). This system can effectively bypass the issue of hydrogenase sensitivity to the O_2 and can integrate with dark fermentation using organic acid containing effluents. This integrated system will be very valuable in harvesting energy from light; in addition it will help in effluent treatment and producing valuable green energy. The stoichiometry of moles of H_2 released during the fixation of mole of N_2 differs vastly. It ranges from 1 mol of H_2 produced while fixing 1 mol of N_2 by common Mo-containing nitrogenase to 9 mol of H_2will be produced while fixing a mole of N_2 by highly oxygen sensitive Fe-containing nitrogenase. Despite the unfavorable hydrogen production by nitrogen fixation, which may not be economically valuable; however, acceptable amount of H_2 production is possible if an efficient reactor system is developed based on the utilization of waste organic effluent. This could harvest natural light can bring an economically feasible system for H_2 production while treating effluent (Harwood 2008).

3.3.2 Hydrogen Producing Machinery (Hydrogenases/ Nitrogenases) in Photosynthetic hydrogen Production

The most common hydrogenases are Fe-Fe hydrogenases prominently present in most of the bacteria and eukaryote and followed by Ni-Fe hydrogenases present generally in *Achaea* and some species of bacteria. Among these Fe-Fe found to be more sensitive to oxygen compared to Ni-Fe hydrogenases. Fe-Fe hydrogenases are highly sensitive to oxygen and undergo denaturation even under trace concentrations of O_2 in the cell. Ni-Fe hydrogenases found to be more stable in the presence of O_2; in few cases up to minutes of exposure these remain stable and active (Stripp et al. 2009). Hence, Ni-Fe hydrogenase containing microbial source, in this case H_2 production in micro-oxygenic conditions is more preferable than Fe-Fe hydrogenases. Moreover, unlike Fe-Fe hydrogenases, Ni-Fe hydrogenases upon long time exposure to O_2 will get inactivate reversible rather than irreversible manner, hence, H_2 production can be revived by removal of O_2.However, the Fe-Fe hydrogenases have advantage of high rate of H_2 production compared to the Ni-Fe hydrogenases (Ghirardi et al. 2007). In case of scale-up production in industrial scale, the hydrogenases with O_2 stability will have better advantage, Ni-Fe hydrogenases are more preferred. These hydrogenases are taken as subject of studies in the aspects of molecular improvement and could be selected for the future protein engineering studies. The most promising virtue of enhancing the productivity is heterologous expression of more oxygen tolerant hydrogenases in efficient microbial system for the H_2 production. Introducing gene cluster of tolerant hydrogenase gene cluster into target organism can be beneficial system for enhancing H_2 production.

However, expression of active hydrogenases is very difficult since the maturation of the hydrogenase apparatus to involve multiple steps to produce active protein. Hence along with hydrogenase gene cluster, the maturation proteins also need to express in the heterologous system. Few studies reported in this regard (Maeda et al. 2008; Vardar-Schara et al. 2008); however, the successful bench scale studies need to be scale up to the industrial level for the real economic success. The reactor engineering is the major part of research to be concentrated for making these lab scale studies to get commercial success.

In evolution, purple bacteria generally produce H_2*via* nitrogen fixation; hence, the hydrogenases are replaced with nitrogenases and H_2 produced as by-product during nitrogen fixation. Nitrogenases catalyze high energy implicated, electron intensive N_2–fixation and there is no oxygen involvement in this process. Like in case of hydrogenases, nitrogenases are also oxygen sensitive and need to be protected from oxygen for their normal functions. Majorly two types of nitrogenases understood and they are Mo-containing nitrogenases and Fe-containing nitrogenases. In virtue of productivity Fe-containing nitrogenases produce high stoichiometric (9 mol) H_2production of per 1 mol of N_2 fixation. In this regard, Mo-containing nitrogen fixation found to be more energy intensive (use 16ATP) for the production of 1 mol of H_2 (Harwood 2008). Unlike in case of photo-chemical H_2 production, where the electron donor is by photolysis of water; purple bacteria needs organic acid for the electron to be provided to the microorganisms. Hence, the economic feasibility is under debate unless the carbon source is derived from the waste biomass or from organic effluent. So, key challenge here is to integrate the waste biomass and/or effluent carbon source with light harvesting bioreactor for efficient and economically viable hydrogen production by purple bacteria.

3.4 Fermentative Hydrogen Production

H_2 production via fermentation which does not need any light energy, more specifically it is also called as dark fermentation. The hydrogen is produced in the dark fermentation by taking H_2 as electron sink and is possible via anaerobic fermentation. These microbes are divided into two major groups; (1) Obligate anaerobe H_2 producers and (2) facultative anaerobe H_2 producers. The obligate anaerobes are strict anaerobes that will harvest the electron from pyruvate oxidation, then use these electrons for the oxidation of ferredoxin (Fd), further these electrons travel to the hydrogenases where H_2 will be produced. The best examples of this category are *Clostridium, Ethanoligenens*, *and Desulfovibrio*. The second group is facultative anaerobes which produce H_2 *via* formate oxidation. In this process formate is electron donor and produces hydrogen through formate hydrogen lyase. The major group of microbes fall under this system are *Enterobacter, Citrobacter, Klebsiella, Escherichia coli*, and *Bacillus* species (Brosseau and Zajic 1982; Kapdan and Kargi 2006). The dark fermentation takes up a pair of electrons and the ultimate sink of the electron is not always H_2. Only a part of electrons will be parted to produce H_2. In many cases only 17% of electrons are ended up in producing H_2 and other will be

accepted by other organic side products. The best example is, up on glucose fermentation by *E. coli* only the theoretical yields of H_2 are 2 mol per 1 mol of glucose and many other organic products act as electron sinks and will be accumulated in the culture medium. Ethanol and lactic acid are popular among those. To push maximum metabolic flux towards the H_2 production, many researchers made efforts in metabolic engineering and successfully made recombinant *E. coli* strain to make the H_2 production near to theoretical yields. Moreover, many organisms have hydrogenases which also conduct reversible reaction which utilize H_2 for the electron generation and utilize the protons for the reduction of co-factors (Hallenbeck 2012). Hence, the gene product needs to be removed in the cell via gene knockout for stabilizing the produced H_2. There are prominent studies conducted in this aspect and will be discussed in the preceding section in detail.

3.4.1 H_2 Production by Microbes and Productivity

Hydrogen energy by dark fermentation was studied from past couple of decades. However, the research was more confined to the laboratory. There are very limited studies promoted up to pilot scale level. Though the technologies demonstrated in the laboratory, the major success in scale-up will depend on the efficient bioreactor engineering. Many times though successful hydrogen is generated through the fermentation, instability to maintain the produced hydrogen is also a major issue since the microbial hydrogenases are equipped with reversible reaction to take up the H_2 back and release protons for reducing the co-factor. In nature dark fermentation occurs in a larger quantity utilizing the organic matter releasing H_2 in the environment by various processes. This process is called anaerobic digestion (Antonopoulou et al. 2008; Ren et al. 2011). During this process hydrogen is produced as a by-product; however, the produced product will be immediately utilized by other microbes producing methane and CO_2 as an end product. In this process many microbial communities are involved, namely hydrolyzers, acetogens, facultative anaerobic H_2 producers combined with methanogens and Archaea bacterial communities (Tapia-Venegas et al. 2015). Though the synthetic anaerobic digestion systems are reported for H_2 production by many researchers, these processes will be discussed in the later part of this section.

Pure cultures are always advantageous for study and implication in any microbial based fermentation system because of their consistent results, and easy for the storage and reproduction of the process. Pure cultures are significant in the aspect of metabolic control, easy for the establishing optimized conditions, also suitable for the molecular manipulations for enhancing the H_2 production by diversion of metabolic flux towards H_2 production either by addition of heterologous genes or knockout of the unwanted genes in the genome. In a dark fermentation process by a pure culture, the possible complete oxidation of glucose can result up to 12 molecules of hydrogen. However, this is true when only complete energy is released as H_2 gas. In dark fermentation the H_2 production in any microorganism is only a by-product during production of fermentation products like ethanol, acetate, formate, or butanol,

etc. In this dark fermentation the maximum yields of H_2 production can reach to 4 mole of H_2 from any hexose sugar. Moreover, sugar as a carbon source will be utilized for the biomass generation. Hence, even if theoretical stoichiometry is followed, still the H_2 productivity using glucose will not be economically feasible compared to other commercial system through which H_2 is generated presently. There should be a cheap and/or waste biomass should be implied to make the technology economic then it can compete with present technologies (Kim et al. 2006a; Ghimire et al. 2015) (Table 3.1).

3.4.2 Metabolic Pathway of H_2 Production in Microbial Cell

The simple hexose sugar glucose is a basic sugar used as carbon source by microbes. The microbes follow majorly two routes for the production of H_2. As mentioned earlier, H_2 is the by-product of dark fermentation and the final fermentation product is organic acids like acetic acid, butyric acid, lactic acid or alcohol like ethanol or butanol [Fig. 3.2]. In majority of microbes the glucose degradation leads to the pyruvate production via basic pathway of glycolysis. It results in the production of cellular energy, i.e., ATP and reduction of NAD to form NADH. This pyruvate now either converted in to acetyl-CoA and CO_2 or acetyl-CoA and formate. In the first case, the reduce ferredoxin molecule will be oxidized to produce H_2 by pyruvate ferredoxin oxidoreductase (PFOR). In the later situation, the formate was converted to H_2 and CO_2 by formate hydrogen lyase (FHL) system and whole pathway is called pyruvate formate lyase (PFL) pathway (Cai et al. 2011; Hallenbeck et al. 2012). The most popular organisms follow these pathways are *Clostridium* sp., being an obligate anaerobe follow the former one and *E. coli* as a facultative anaerobe will follow the later pathway produce H_2 from formate using FHL system. The productivity of these two pathways differs significantly. The production of H_2 by facultative anaerobes using FHL system depends on formate dependent [Fe-Fe] hydrogenases in most cases will not use NADH produced during glycolysis; hence, various products (ethanol or lactate) will be formed upon oxidizing the NAD. Hence, the final product of this pathway is only 2 moles of H_2 for 1 mole of glucose utilized. Unlike FHL system which follows PFL pathway, in case of PFOR pathway hydrogen production results by oxidation of reduced ferredoxin (Fd_{red}) with the help of ferredoxin dependent [FeFe] hydrogenase. Moreover, two more H_2 can also be generated by oxidation of NAPH with the help of NADH dependent [Fe-Fe] hydrogenase or NADH-Fd_{red} dependent [Fe-Fe] hydrogenase. Hence, here the productivity can be 2–4 mole of hydrogen from 1 mol of glucose. This shows the potentiality of the POFL pathway in efficient production of hydrogen (Tapia-Venegas et al. 2015).

Table 3.1 Yields of H_2 production using dark fermentation process with different carbon sources and different type of cultures (adopted from Łukajtis et al. 2018)

Substrate	Microorganism	Temp (°C), pH, hr. (h)	Hydrogen productivity	Hydrogen yields	Reference
Glucose (1%)	*E. cloacae*	36 °C, 6.0, 3.3 h	447 cm^3 $H_2/(dm^3.h)$	2.2 mole H_2/mole glucose	Kumar and Das (2000)
Glucose 7 g/dm^3	Mixed culture	36 °C, 5.5, 6 h	–	2.1 mole H_2/mole glucose	Kotsopoulos et al. (2006)
Glucose 4.85 g COD/dm^3	Mixed culture	70 °C, 7.2, 26.7 h	11.15 mM H_2/d	2.46 mole H_2/mole glucose	Kotsopoulos et al. (2006)
Glucose 10 g/dm^3	*Clostridiaceae* and *flexibacteraceae*	35 °C, 5.5, 3.3 h	640 cm^3 $H_2/dm^3.h$	4 mole H_2/mole glucose	Oh et al. (2004)
Glucose 10 g/dm^3	Mixed culture from compost	60 °C, 5.5	147 cm^3 $H_2/(dm^3.h)$	2.1 mole H_2/mole glucose	Morimoto et al. (2004)
Glucose 20 g/COD/ dm^3	*Clostridia sp*	32 °C, 6,6 h	7.42 mM H_2/ (gVSSh)	1.42 mole H_2/mole glucose	Lin and Chang (2004)
Lactose 29 $mmol/dm^3$	*C. termolacticum*	58 °C, 7, 35.7 h	2.58 mM H_2/ $(dm^3.h)$	1.5 mole H_2/mole hexose	Collet et al. (2004)
D-xylose 10 g/dm^3	*E. cloacae IIT-BT 08*	58 °C,7, 35.7 h	348 cm^3 $H_2/(dm^3.h)$	0.95 mole H_2/mole xylose	Kumar and Das (2000)
L-Arabinose 10 g/dm^3	*E. cloacae IIT-BT 08*	36 °C, 6, 37 h	360 $cm^3H_2(dm^3.h)$	1.5 mole H_2/mole arabinose	Kumar and Das (2000)
Sucrose 1 $g/COD/dm^3$	Mixed culture	26 °C, 6, 1 h	–	1.8 mole H_2/mole sucrose	Logan et al. (2002)
Sucrose 10 g/dm^3	*E. cloacae IIT-BT 08*	36 °C, 6	660 cm^3 $H_2/(dm^2.h)$	6 mole H_2/mole sucrose	Kumar and Das (2000))
Sucrose 20 g/dm^3	Mixed culture	35 °C, 6.7, 1 h	1.32 dm^3 $H_2/(dm^3.$ h)	–	Chang et al. (2002)
Sucrose 25 g/dm^3	Mixed culture	35 °C,5.5	1504 cm^3 H_2/h	2 mole H_2/mole sucrose	Mu et al. (2007)

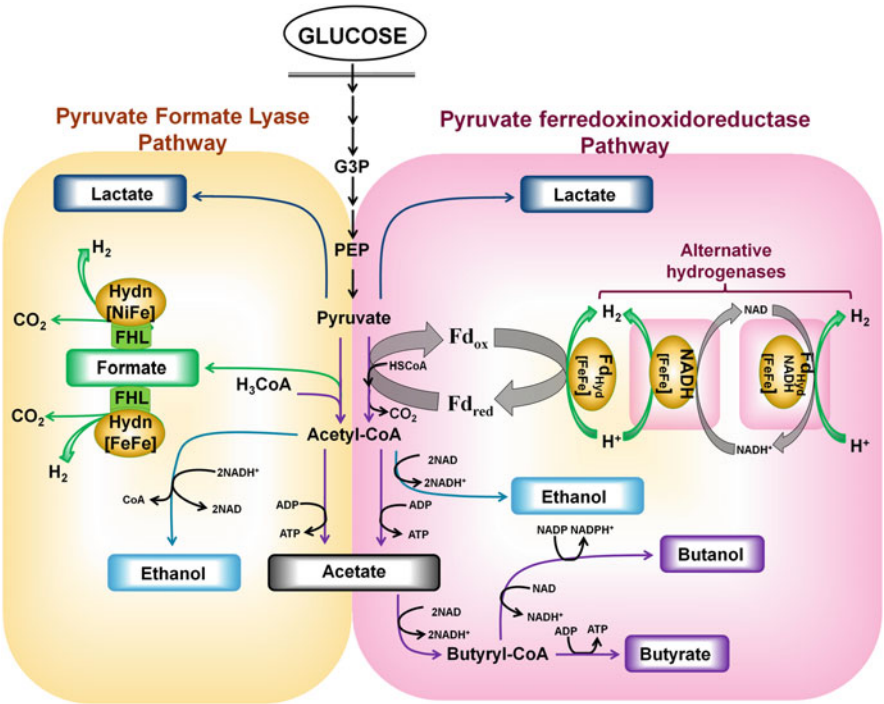

Fig. 3.2 Various pathways used by different microbial species for the production of hydrogen from basic hexose sugar (Glucose)

3.4.3 Dark Fermentation: An Economic Prospective of H_2 Production

Using simple sugars like glucose, sucrose, and lactose are generally studied in the lab scale for understanding the efficiency and stoichiometry of H_2 production. The majority reports demonstrated in the lab scale utilized these model sugars. These sugars are readily acceptable for many microbes and their utilization in metabolic pathway is well known, and manipulating the condition for the better productivity is convenient. However, in economic point of view, the production cost of H_2 using these sugars as carbon source cannot compete the present commercial H_2 production cost. Hence, the technology needs to be developed to replace these costly model sugars with low-cost renewable carbon sources. Utilization of lignocellulosic biomass, crude glycerol generated during biodiesel production, industrial waste water containing different organic acids which can directly enter into the metabolic pathway for the production of H_2, waste biomass having high content of biodegradable sugars looks very promising and many researchers conducted valuable studies utilizing these low cost or waste carbon source for the production of H_2. Dark fermentation found to be very promising concept of H_2 production by utilizing the

waste biomass for the H_2 production from industrial waste biomass [Table 3.2] (Łukajtis et al. 2018; Toledo-Alarcón et al. 2018).

Theoretically, dark fermentation is capable of producing the biological hydrogen from any waste biomass. In the literature, the reports are seen where the hydrogen was derived via biological means by dark fermentation utilizing renewable waste carbon source derived from agriculture, food industry, dairy whey, distillery industry, breviary, pulp and paper industry. Those waste biomasses are rich in starch, cellulose, and lignocelluloses which can be utilized as carbon source in anaerobic fermentation or by dark fermentation via mixed culture anaerobic digestion. The theoretical yield of the H_2 production in dark fermentation depends on the ultimate electron acceptor during the anaerobic fermentation. As shown in the Eqs. (3.1) and (3.2) theoretical yields of the H_2 production depends on the type of fermentation carried by the microorganism producing H_2.

$$C_6H_{12}O_6 + 6H_2O \rightarrow 2CO_2 + 2CH_3COOH + 4H_2 \tag{3.1}$$

$$C_6H_{12}O_6 + 6H_2O \rightarrow 2CO_2 + CH_3CH_2CH_2COOH + 2H_2 \tag{3.2}$$

Though the theoretical yields are either 4 or 2 moles of H_2 from the 1 mol of glucose, the final fermentation yields is always lower than the theoretical yields since the accumulation of different organic acids accumulated as electron acceptors. Moreover, the carbon sources are also used to build up the microbial cell biomass generation. With respective to the results obtained during the fermentation the experimental yields of the H_2 in anaerobic fermentation vary from 1 to 1.5 mole. In economic prospective the conversion of 60 to 80% biomass energy to H_2 said to be a cost-effective process. Possible use of organic acids accumulated during the fermentation for other process could decrease the cost of the production. A number of factors influence in the yields of the H_2 production and in this section few of the important factors were discussed (Levin et al. 2006; Hawkes et al. 2007).

3.4.3.1 Substrates for the Dark Fermentation

The carbohydrates are the major source for the microbes to use for their metabolism and produce H_2 in dark fermentation. Simple monosaccharides such as glucose, xylose, ribose, and disaccharides such as sucrose and lactose are the sugars readily utilized by most of the microbes and produce H_2. In the reports shows that the highest yield of 6 mole H_2 was obtained by utilizing mole of sucrose [83], in case of lactose up to 3 moles/mole of lactose. However, simple carbohydrates are not suitable carbon source in economic point of view because of their cost. Hence, use of these simple sugars makes unprofitable in industrial scale. Continuous and profitable production of the H_2 needs the use of renewable and non-edible sugars. Lignocelluloses or starch polymer derived from the various agriculture and food waste are the good source of alternative to simple sugars and also they act as renewable carbon source for the industry [Table 3.3] (Logan et al. 2002; Hawkes et al. 2007). The major hindrance in utilizing lignocelluloses is, in many instances these carbon sources are not suitable to use directly for dark fermentation due to their

Table 3.2 Hydrogen production achieved from wastewater by different industries (Adopted from Tapia-Venegas et al. 2015)

Substrate ($g_{COD}\ L^{-1}$)	Microbial species	Fermentation Temp (°C), pH, hrs condition	H_2 Production	Reference
Glycerol crude (1)	Activated sludge	40 °C; 6.5	4.90[a]	Mangayil et al. (2012)
Glycerol crude (5)	*Thermotoga neapolitana*	75 °C; 6.8	12.20[a]	Ngo et al. (2011)
Vinasse (0.25)	Hydrogen producers from a packed-bed reactor	25 °C; 5.5	24.97	Fernandes et al. (2010)
Domestic sewage	Hydrogen producers from a packed-bed reactor	25 °C; 5.5	6.01	Fernandes et al. (2010)
Glycerol crude (0.25)	Hydrogen producers from a packed-bed reactor	25 °C; 5.5	6.03	Fernandes et al. (2010)
Brewery (6.05)	Anaerobic sludge	35.9 °C; 5.95	6.12[b]	Shi et al. (2010)
Coffee drink (20)	Anaerobic digest sludge	35 °C; 5.5; 6 h	6.72[c]	Jung et al. (2010)
Cheese whey (40)	Anaerobic digest sludge	55 °C; 5.5; 3.5 h	22.00	Azbar et al. (2009)
Probiotic (9.48)	Mixed anaerobic consortia	37 °C; 5.5; 2 h	9.37[c]	Sivaramakrishna et al. (2009)
Condensed molasses (50)	Co-culture: C *sporosphaeroides*-C *pasteurianum*	35 °C; 7 2 h	9.27	Hsiao et al. (2009)
POME (100)	*C. bytyricum*	37 °C; 5.5 8 h	1.31[b]	Chong et al. (2009)
Distillery effluent (100)	Co-culture: C. *freundii*-E *aerogenes*-R. *palustris*	28–44 °C; 5–7 2 h	14.37[c]	Vatsala et al. (2008)
Cattle (2.4)	Sewage sludge	45 °C; 5.5; 2 h	13.05[b]	Tang et al. (2008)
POME (70–90)	Thermophilic microflora	60 °C; 5.5; 4d	11.66[c]	O-Thong et al. (2007)
Chemical and domestic sewage (2.75)	Anaerobic mixed microflora	29 °C; 6 h	1.25	Venkata Mohan et al. (2007b)
Dairy waste (3.5 g_{COD} $L^{-1}\ h^{-1}$)	Anaerobic mixed microflora	28 °C; 6; 24 h	0.46	Venkata Mohan et al. (2007a)
Citric acid (19.2)	Facultative anaerobic enrichment cultures	35–38 °C; 7; 12 h	4.37[c]	Yang et al. (2006)

Cheese whey (46.5)	*C. saccharoperbutylacetonicum*	30 °C; 6 h	7.03[d]	Ferchichi et al. (2005)
Confectionery (0.6) processing	Soil	23 °C; 6.1; 2 h	6.96[b]	Van Ginkel et al. (2005)
Apple processing (9)	Soil	23 °C; 6.1;	4.09[b]	Van Ginkel et al. (2005)
Potato processing (21)	Soil	23 °C; 6.1;	5.73[b]	Van Ginkel et al. (2005)
Rice whey (34)	Mixed bacteria flora	55 °C; 5.5; 2 h	11.14[c]	Yu et al. (2002)

[a]224 $g_{COD}\ mol^{-1}{}_{glycerol}$
[b]Considering a relation: V/mol = 24.44 L mol^{-1}, 25 °C and 1 atm
[c]192.06 $g_{COD}\ mol^{-1}{}_{hexose}$
[d]Considering a relation: 1.122 $g_{COD}\ g^{-1}{}_{Lactose}$

Table 3.3 Hydrogen production by dark fermentation with different renewable waste carbon resources (Adopted from Łukajtis et al. 2018)

Substrate	Organism	Liquid organic product	Temp (°C), pH condition	Hydrogen productivity	Hydrogen yield	Reference
Kitchen waste: 66% food waste, 27% vegetable waste, 0.96% tea waste, 1.09% egg shells, 1.36% packing materials, 3.61% ash	Mixed cultures	Butyric acid, acetic acid, propionic acid	pH = 5.5	N.D	72 cm^3 H_2/g VS	Jayalakshmi et al. (2009)
Organic municipal solid waste 110 g TVS/dm^3	Mixed culture	Butyric acid	50 °C, 5.5	5.7 dm^3 H_2/dm^3/d	N.D	Zahedi et al. (2013)
Organic municipal waste mixed with poultry slaughterhouse waste 70.86 g/dm^3	Mesophilic anaerobic sludge	Acetic acid	34 °C, 6.0	N.D	71.3 cm^3 H_2/g VS	Gómez et al. (2006)
Kitchen garbage	Anaerobic digester sludge	Butyric acid, acetic acid, ethanol	55 °C, 5.0	1.7 dm^3/H_2/dm^3/d	66 cm^3 H_2/g VS	Chu et al. (2012)
Synthetic food waste (Rice, vegetable, meat 30 g COD/dm^3	Anaerobic sludge from UASB treating cassava wastewater	Butyric acid, acetic acid, ethanol	37 °C, 6.0	0.9 dm^3 H_2/dm^3/d	55 cm^3 H_2/GVS	Nathao et al. (2013)
Potato steam peel 10 g glucose/dm^3	Mixed culture	Acetic acid, lactic acid	75 °C, 6.9	12.5 mM H_2/dm^3h	3.8 mole H_2/mole	Mars et al. (2010)
Kitchen waste from several cafeterias 50 g COD/dm^3	Anaerobic sludge from treatment plant	Butyric acid, lactic acid, acetic acid	55 °C, 5.5	79 mM H_2/l medium/d	N.D	Mohd Yasin et al. (2011)
Food waste: Pasta, bread, fruit, vegetable, fish, and meat	Mixed culture from aerobic sludge	Acetic acid, butyric acid	36 °C	N.D	70.34 cm^3/gVS	Alibardi and Cossu (2015)
Stimulated food waste fish 5% meat 10%; bread 10%; onion 5%; carrot 5%; cabbage 10%; potato 15%	Mixed culture from digested sludge	Acetic acid, butyric acid	34 °C, 5.5	0.23 N dm^3 H_2/kgVS	20.5 dm^3 H_2/kg VS	Redondas et al. (2012)

Mixed food waste from residential home	Anaerobic sludge from treatment plant	Butyric acid, lactic acid, acetic acid	50 °C, 7.5	54.2 cm^3 H_2/h	57 cm^3 H_2/gVS	Pan et al. (2008)
Raw cassava starch	Facultative anaerobic bacteria	Butyric acid, acetic acid, ethanol	35 °C	N.D	1.44 moles H_2/mole glucose	Wang et al. (2017)

polymer nature and slow microbial degradation process. The yields are also very slow which adds the operational cost for the production of H_2 (Hallenbeck et al. 2012). The researchers come with the idea of pretreatment using chemically or biological means. In case of biological pretreatment, the biomass is subjected to pretreatment with many fungal species that will release the simple sugars like xylose and ribose which could be easily integrated in to metabolism by microbial species for the H_2 production. The use of other waste carbon sources like organic waste derivatives, cheese whey, milk waste, crude glycerol obtained as by-product during biodiesel production could be best alternatives for the direct use in dark fermentation for the H_2 production. The glycerol after a simple purification by neutralizing with the mild acid followed by heating and filtration will result in the purified form which is devoid of contaminants derived during biodiesel preparation (Sudheer et al. 2018). In this study authors successfully demonstrated the utilization of crude glycerol generated during synthesis of biodiesel from *Jatropha* seed oil. This work proving the potential of utilization of waste crude glycerol as biomass for the many fermentation process and could be also implemented for the dark fermentation to generate H_2 gas (Hawkes et al. 2007; Ren et al. 2011).

Organic waste generated from domestic kitchen, food industry, bravery industry, and restaurants is also rich in carbon source in the form of simple sugars, cellulose, hemicelluloses, proteins, and lipid (Jayalakshmi et al. 2009). This waste biomass not only fulfills the carbon source but also some part as nitrogen supplement. These organic wastes are also very much suitable for the microbial fermentation. Moreover, the dark fermentation utilization of mixed culture fermentation results in the green manure rich in the form of simple nutrients. By using these waste biomasses to produce the H_2 gas will have two-way advantages. One is, released to environment these will be taken up by methanogens and result in release of methane which in turn increase the carbon footprint. Utilizing it for the H_2 production will result in green fuel (H_2) with nil carbon footprint upon combustion (Guo et al. 2008). Many researchers also consider the municipal waste also organic waste, since it is rich in carbohydrates, disaccharides, proteins, and peptides. In addition, sewage sludge is of rich in the microbial community and no need to add externally any microbial inoculum. However, the sludge should be pretreated to remove the hydrogen utilizers like methano-bacteria. Various methods are suggested to remove these methano-bacteria. The simple methods are treating the sludge by microwave or ultrasound, acid or alkaline treatment. Guo et al. have studied in details and found that sludge treated by microwave and ultrasound treatment provided highest yields of H_2 production (15 cm^3 H_2/g COD) (Valdez-Vazquez et al. 2005; Karlsson et al. 2008).

3.4.3.2 Microbial Type and Source

As introduced about the microbial types for the H_2 production in the earlier section, in this section the details of the microbial system for H_2 synthesis will be discussed in detail. The hydrogen gas production is purely of anaerobic fermentation and the cultures to be used should perform the anaerobic fermentation. This can be done by both obligate (strictly sensitive to oxygen) and facultative (grow in both in presence

and absence of oxygen) anaerobic bacteria. Dark fermentation can be carried in either pure cultures or mixed cultures. Both systems have their superiorities and disadvantages [Table 3.1]. The pure cultures fermentation is a single bacterial strain that will involve in the fermentation utilizing a metabolizable carbon sugars. The best example of bacterial genus is *Clostridium* sp. *Clostridium* sp. is an obligatory anaerobic bacterium that utilizes many simple sugars and produces H_2 via dark fermentation. The major characteristic feature of this species is, it performs the fermentation in variable carbon sources and also it has the ability to survive in difficult conditions such as high temperatures, pH, and presence of toxic substances. The major disadvantage with this species is; it produce the H_2 during the log phase and once reach to stationary phase the metabolic flux will be shifted towards accumulation of organic compounds. Depending on the substrate used for the fermentation, *Clostridium* produces H_2 along with accumulation of organic acids like acetic acid and butyric acid. Though wide variety of species like Methylotrophs, enteric bacteria like *E. coli*, *Enterobacter*, *Citrobacter*, *Alcaligenes*, *Bacillus* are capable of performing the dark fermentation as a pure culture; mixed culture fermentation has its superiority in H_2 production from a complex organic or carbon source derived from waste biomass (Kapdan and Kargi 2006; Hallenbeck et al. 2012; Łukajtis et al. 2018).

The mixed consortia under a strict controlled condition can perform dark fermentation on complex organic carbon source and produce H_2. These enriched consortia perform the dark fermentation utilizing broad spectrum of carbon source like industrial waste, animal waste manure, agricultural waste, sewage sludge, compost, and domestic kitchen waste. Upon the dark fermentation via mixed consortia will generate acetic acid, formic acid, butyric acid, and CO_2 along with H_2. The mixed culture fermentation has the advantage of utilizing the waste biomass like cellulose and lignocellulosic biomass directly without the pretreatment since metabolic cooperation one species with other will help in utilization of complex carbon sources. Hence, the mixed consortia based dark fermentation is the best way of utilization of waste biomass for the production of biohydrogen (Miyake et al. 1999; Logan et al. 2002; Ren et al. 2011; Łukajtis et al. 2018).

The other group of bacteria, i.e., the facultative anaerobes utilize oxygen for the generation of ATP and switch to anaerobic conditions in the absence of oxygen. The best example of hydrogen producing facultative anaerobes is Enterobacteriaceae group. The major system of hydrogen production in this group is via formate hydrogen lyase (FHL) system; where the hydrogen and CO_2 are released by utilizing formic acid as the substrate. The base pathway of formate generation studied via glucose metabolism; where maximum theoretical hydrogen yields are 2 moles of H_2 per mole of glucose. The final electron acceptor in the metabolism is most of the times organic acids or ethanol. Hence, at the end of the fermentation these organic acids are generated as end products along with hydrogen. To enhance the productivity and diverting the metabolic flux towards useful organic acids many researchers utilized molecular approaches, and details of this genetically modified strains for enhancing the hydrogen are described in the coming section (section details).

3.4.3.3 Fermentation Conditions Which Influence H_2 Production

Dark fermentation utilizing the mixed culture or pure cultures, the comprehensive reactions flow involved in the microbes for the production of hydrogen are thermodynamically favorable; however, they are controlled via biological regulators by various mechanisms in microbial cells and need to have favorable conditions to attain maximum productivity. The optimal growth and production conditions should be maintained to get maximum productivity during fermentation process. Three major factors which influence the fermentation conditions are (a) temperature, (b) pH, and (c) gas partial pressures. In this section we will give details of these conditions and how they influence the end productivity of H_2 in the fermentation.

Temperature

The crucial factor in any fermentation system is the temperature in which the fermentation system is operating. The productivity affected to the level of 100% or up to nil if favorable temperatures are not provided. There are no generalized temperatures defined for the H_2 production. It ranges from ambient (20 °C) to as high as 80 °C. The optimum temperatures depend on the type of organism and/or crucial bacterial species whose hydrogenase system responsible for the H_2production in context of mixed fermentation. Basically, bacterial species fall under three temperature groups and reports show that in each group of bacteria, ability of H_2 production is reported. The suitable growth conditions like low temperature (5–20 °C) in case of psychrophiles, ambient temperatures to moderately high temperature (25–45 °C) for mesophiles, and high temperatures (65–80 °C) for thermophiles (Levin et al. 2004).

Selection of optimum temperatures for biohydrogen production depends on species in the culture or mixed culture used for the fermentation. And also, the production of H_2 varies with the substrate used as carbon source. In many cases the cell growth and H_2 production temperatures differ since the optimum growth of the cell need not be the favorable temperature for the hydrogenase enzyme which produces H_2. Hence, crucial optimizations are very much necessary for the cell mass generation and H_2 production. Pakarinen et al. (Levin et al. 2004) found that 70 °C is the optimum temperatures for the maximum productivity of H_2 production; however, the cell mass generation is at the highest temperature of 50 °C. The multiple studies confirm that, thermophilic conditions are favorable for the substrates need to undergo hydrolysis during fermentation, and ambient conditions are sufficient for the simple sugars. This is because the high temperatures favor the hydrolyzing enzymes responsible for hydrolysis of complex substrates. One more reason for the enhanced productivity of H_2 in high temperatures is because of low solubility of gases at low temperatures; hence the growth inhibition of microbes will be minimum in low dissolved aqueous medium (Wong et al. 2014). Though in context of H_2 productivity, the high temperatures are favorable; however, in context of energy investment the profitability of process will be low (Azbar et al. 2009).

pH

In any fermentation system pH plays a significant role in cell growth and productivity, since all the metabolic processes are based on the enzyme activity of particular reaction at specific pH. The majority of the enzymes have a specific pH range; when the productivity of a target depends on multiple metabolic reactions, an optimum temperature needs to be studied to get a maximum productivity. The same concept is applicable to produce H_2. Moreover, pH affects the growth of microbes whether it is pure culture or mixed cultures. In mixed culture fermentation, lower pH value favors for the production of H_2 and limits the methanogens to utilize the produced H_2. However, maintaining at specific pH during fermentation is very important. The production of hydrogen is accompanied by the accumulation of organic acids (acetic, lactic, butyric, and propionic) which will lower the pH of the medium makes it unfavorable for hydrogenase complex to produce H_2 gas. Hence, the pH lower than 5 is not advised for the H_2 production (Bowles and Ellefson 1985). It is also noted that both initial pH and the operational pH are important; in case of batch fermentation, initial pH at neutral is favorable. In case of continuous mode, maintaining the nutral pH will favor the maximum productivity (Wang and Wan 2009; Jung et al. 2011). The initial and optimal operational pH to be maintained vary with the kind of microbial strains selected for the fermentation or source of microbial consortia (in case of mixed culture), kind of substrate selected, mode of fermentation (batch/continues) system will determine the pH to be applied for the best productivity.

In general the pH range for the H_2 production is reported to be in the range between 5.0 and 7.0 corresponding to the growth of the bacterial growth (Li and Fang 2007). The optimum pH differs with the substrate used for the fermentation; the neutral pH is suitable for the livestock waste, pH 6.5–7.0 is favorable for the crop/agriculture waste, pH 5–6 is good for the food waste (Liu and Shen 2004; Li and Fang 2007: Guo et al. 2010). However, some studies reported that 7–8 pH conditions also favorable for some mixed bacterial cultures, e.g. the studies of Liu and Shen explained that, the mixed culture fermentation of corn starch substrate gave best hydrogen production at pH 7 and 8 and the production was 103 and 120 mL H_2/g substrate, respectively.

Partial Pressure of H_2

The partial pressure of hydrogen (PPH) in the reactor is very crucial factor that affect the productivity. The hydrogen produced in the microbes is the result of the ferredoxin reduction up on oxidation enzyme hydrogenase. The hydrogenase also participates in reversible reaction up on higher availability of hydrogen gas, hence at high partial pressure of H_2 in the reactor the production rate will reduce and metabolic flux will move towards other products such as organic acids, ethanol, and butanol (Abo-Hashesh and Hallenbeck 2012; Hallenbeck 2012; Ghimire et al. 2015). There are two ways to deal with high PPH in the reactor and make system continue with high productivity. One is reducing the partial pressures of hydrogen produced in the reactor by sparging with inert gas most frequently nitrogen or removing of gas released in the system by application of vacuum. The earlier method

where reducing the PPH by sparging is effective, the results vary with the type of gas applied for sparging. Kim et al. (2006b) applied CO_2 as sparging gas and observed a better productivity compared with the nitrogen sparging. The yields were up to1.68 moles H_2/mole of $hexose_{consumed}$ compared to nitrogen sparging which yielded 0.95 moles H_2/mole of $hexose_{consumed}$. However, the major disadvantage of sparging system is the product will be diluted with the sparging gas and hydrogen separation will become tedious, time consuming, and require cost input. This all make the sparging system non-economic system which make final cost not competitive in commercial prospective. The alternative method as discussed is the removal of the generated gas in the reactor by applying vacuum. Theoretically this looks more beneficial than sparging; however, very limited studies were made in this aspect (Lee et al. 2012).

An alternative to above two methods is proposed by Teplyakov et al. (2002) and Nielsen et al. (2001) using activated selective membrane to hydrogen. The reactor equipped with the membrane system will remove the hydrogen which in turn will reduce the PPH. However, the membranes are effected with biofilms formed by microbes will have to be replaced often. Though many techniques are evolved to reduce the PPH, still much of the research is needed for handling high PPH in the reactor for the better productivity with inexpensive method which is economically competitive.

3.5 Engineered Bacterial System for Improving Hydrogen Productivity

In advance in the molecular biology, availability of genome sequencing system and evolution of various techniques for genome facilitated various researchers to engineer the available microbial sources rather than isolate new microbes with better productivity. The first choice of any researcher for microbial engineering is *E. coli* since much of the molecular information is explored and many tools were developed for the genome manipulation. Moreover, metabolic pathways were well characterized and information is available for easy manipulation for metabolic engineering. The majority of the work in strain engineering for understanding the microbial hydrogen production and/or improving the hydrogen productivity is made in *E. coli*. In this section much of the discussion will be made with the view of *E. coli*.

E. coli is a facultative anaerobe belongs to Enterobacteriaceae family have the intrinsic ability to produce hydrogen. The hydrogen producing apparatus of *E. coli* includes FHL (Formate Hydrogen Lyase) system. FHL system consists of hydrogenase 3 (*hyc*ABCDEFGHI) (Bagramyan and Trchounian 2003) and formate dehydrogenase-H (*fdh*F) (Axley et al. 1990). HycA protein acts as repressor of the FHL system. The FhlA will up regulate the FHL system and in turn will help in accumulation of H_2. However, *E. coli* consume hydrogen produced by the FHL system by hydrogenase 1 (*hya*ABCDEF) and 2 (*hyb*OABCDEFG). The efficient production of hydrogen by *E. coli* is controlled by the availability of formate to FHL

system. There are two formate dehydrogenases, such as, formate dehydrogenase-N and formate dehydrogenase-O and formate transporter (*Foc*A and *Foc*B) (Rossmann et al. 1991; Suppmann and Sawers 1994; Andrews et al. 1997). Moreover, the cells having sufficient amount of formate can divert metabolic flux to produce and enhance the H_2 productivity.The majority of the strain engineering aspects were designed based on the deletion of hydrogen utilizing genes, over expression of FhlA to upregulate the FHL system in turn to enhance the hydrogen production, and making formate available to the FHL system for increasing the productivity. In addition, the hydrogenases which utilize the produced hydrogen via the FHL system need to delete to avoid the reutilization of produced H_2.

3.5.1 Metabolic Engineering of *E. coli* for Better Productivity

Theoretically, the productivity of hydrogen is formed from basic energy molecules such as 2 mole of glucose and 1 mole of formate. Reaching to the theoretical yields in the system is practically not possible, since the microbial cell utilizes much of the carbon source for the growth and cell biomass generation. Hence, many studies are made in the view of hydrogen production always towards getting near to theoretical yields. Maeda et al. (2007a, b, 2008, 2012, 2018) contributed major input on the metabolic engineering of *E. coli* for the hydrogen production. Their studies first time reported to reach the theoretical values when formate was used as the substrate for the hydrogen production. In this study, Maeda et al. (2008, 2018) explained to the theoretical values (Maeda et al. 2012) of over-expressed *fhlA* and deleted the HycA repressor for enriching the FHL complex cell. The hydrogen uptake activity was eliminated by gene deletion of larger subunits (*hyaB* and *hybC*) of hydrogenase 1and 2, respectively. In addition the metabolic flux from formate to H_2 production was enhanced by deleting *fdoG* gene; this will inactivate the FDH which is responsible to convert formate into CO_2 without H_2 production (Maeda et al. 2008; Maeda et al. 2018).

Glucose is the being the starting carbon moiety and less expensive than formate, many researchers taken interest on metabolic engineering of *E. coli* utilizing glucose as the substrate to produce H_2. The basic principle most of the strategies were designed to increase the metabolic flux towards enhancing the formate availability to FHL system for the hydrogen production. As mentioned earlier, the base strain selected always with inactive hydrogenase 1 and 2 to eliminate reutilization of H_2 produced by FHL and FHL repressor (*hycA*) (*E. coli - hyaB*$^{-}$*, hybC*$^{-}$ *and hycA*$^{-}$). These mutations also showed that there is enhancement in H_2 production using glucose as the substrate (Penfold et al. 2003; Yoshida et al. 2006; Maeda et al. 2007; Turcot et al. 2008; Fan et al. 2009; Kim et al. 2009; Mathews et al. 2010). In addition, the H_2 production was further improved by over expressing FhlA with N-terminal truncation (Self et al. 2001; Turcot et al. 2008).

In *E. coli,* the glucose metabolism leads the formation of the phosphoenolpyruvate and pyruvate. The pyruvate is converted in to formate; subsequently, formate is transformed in to succinate and lactate as by-products. Hence, it is necessary to

divert the metabolic flux towards formate and eliminate the succinate and lactate accumulation during fermentation for enhancing H_2 production. The studies based on these were made by various researchers and enabled the recombinant *E. coli* to produce H_2 from glucose (Yoshida et al. 2006; Maeda et al. 2007; Manish et al. 2007; Fan et al. 2009; Kim et al. 2009). These studies targeted genes deletion of *ppc* encodes phosphoenolpyruvate, frd ABCD fumarate reductase, idhA lactate dehydrogenase (Maeda et al. 2007). Table 3.4 comprehended the information of various studied done in *E. coli* and productivity achieved by various engineered *E. coli* strains. In addition to these, expressing Fnr a global DNA-binding transcriptional global regulator also found to enhance the H_2 productivity (Fan et al. 2009). Comprehending all, the best H_2 productivity was obtained with the *E. coli* holding knockout of seven genes (hyaB, hybC, hycA, fdoG, ldhA, frdC, and aceE) five gene inactivation by (hyaAB, hybABC, hycA, ldhA, and frdBC/hycA, hya, hyb, ldhA, and frdAB) (Kim et al. 2009; Mathews et al. 2010) and three gene inactivation (hya, hyb, and ldhA) (Turcot et al. 2008).

As discussed in earlier section about the application of various carbon substrates like crude glycerol or lignocellulosic biomass, application of these components as carbon source will be economically beneficial. In this regard, glycerol fermentation was initially ruled out since the glycerol fermentation do not favor H_2 production. However, these studies made by Dharmadi et al. (2006) and Gonzalez et al. (2008) showed that at alkaline pH was favored the hydrogen production in the presence of potassium and phosphate. Despite the theoretical understanding of anaerobic fermentation by utilizing glycerol have the benefit of extra $NADPH^+$ generation; however, there are many genes whose expression will be shutdown which are based on glucose metabolism. Despite of handful studies on glycerol fermentation by *E. coli* are available; the information existing for hydrogen production is far from the understanding when compared to glucose and other monosaccharaides. This is because of contradictory studies by various researchers and also experimental yields are considerably limited.

Metabolic engineering is a good way to make *E. coli* to produce good amount of H_2 from glycerol. A powerful approach was made by Tran et al. (2014, 2015). In this study the knockout mutant of *E. coli* with seven genes which are mostly participating in enhancing the formate accumulation and blocking the metabolic flux in synthesis of by-products like methylglyoxal. The selected genes deleted are fumarate reductase (encoded by *frdC*), lactate dehydrogenase (*ldhA*), formate dehydrogenase (*fdnG*), phosphoenolpyruvate (*ppc*), nitrate reductase (*narG*), methylglyoxal synthase (*mgsA*), and the regulator of the transcriptional regulator FhlA (*hycA*). The resulted strain is able to produce the hydrogen near to the theoretical value (1 mole of H_2 for 1 mole of glycerol). Instead of targeted gene deletions, Tran et al. applied random mutagenesis for looking genes responsible for hydrogen production in glycerol fermentation (Tran et al. 2014, 2015). In this study four genes were identified which involved in hydrogen production. The individual mutant of the following four genes, namely *aroM, gatZ, ycgR,* and *yfgI* enhanced the hydrogen production up to 1.6fold. Moreover, the mutants not only enhanced the hydrogen production but also increased the growth rate of the mutant strains compared to wild type under glycerol fermentation in anaerobic conditions. In addition to adoptive

Table 3.4 Comparison of In-vivo Hydrogen production by engineered *E. coli* (Reproduced from Maeda et al. 2012)

Substrate	System	H_2 production rate (reported units)	H_2 production rate (converted units)
Protein engineering			
Formate	Protein engineering of HycE (truncation) of *E. coli*	9 μM H_2 (mg protein)$^{-1}$ h^{-1}	9 moles H_2 (mg protein)$^{-1}$ h^{-1}
Formate	Protein engineering of FhlA of E. coli	7 μM H_2 (mg protein)$^{-1}$ h^{-1}	7 moles H_2 (mg protein)$^{-1}$ h^{-1}
Metabolic engineering through modifying multiple native genes in E. coli			
Formate	Inactivation of HycA and overexpression of FhlA	23.6 g H_2 l^{-1} h^{-1}	254 μM H_2 (mg protein)$^{-1}$ h^{-1}
Formate	Inactivation of HyaB, HybC, HycA, FdoG and overexpression of FhlA	113 μM H_2 (mg protein)$^{-1}$ h^{-1}	113 μmol H_2 (mg protein)$^{-1}$ h^{-1}
Cheese whey	Inactivation of HycA and LacI	5.88 ml H_2 OD)$^{-1}$ h^{-1}	11 μM H_2 (mg protein)$^{-1}$ h^{-1}
Glucose	Inactivation of HycA, LdhA, FrdBC and overexpression of FhlA	13 mM (g DCW)$^{-1}$ l^{-1} h^{-1}	26 μM H_2 (mg protein)$^{-1}$ h^{-1}
Glucose	Inactivation of HyaB, HybC, HycA, FdoG, FrdC, LdhA, and AcoE	32 μM H_2 (mg protein)$^{-1}$ h^{-1}	32 μM H_2 (mg protein)$^{-1}$ h^{-1}
Glucose	Inactivation of Hyd1, hyd2, ldhA and overexpression of truncated FhlA	5.3 mM H_2 i^{-1} h^{-1}	24 μM H_2 (mg protein)$^{-1}$ h^{-1}
Glucose	Inactivation of HycA, HyaAB, HybBC, LdhA, and FrdAB	31.3 mM H_2 (gDCW)$^{-1}$ h^{-1}	63 μMl H_2 (mg protein)$^{-1}$ h^{-1}
Glucose + formate	Production of Hyd 1	3 ml H_2 100 ml^{-1}	0.8 μM H_2 (mg protein)$^{-1}$ h^{-1}
Glucose	Inactivation of HyaAB, HybABC, HycA, LdhA, and FrdBC	1.0 mM H_2(g DCW)$^{-1}$ h^{-1}	1.5 μM H_2 (mg protein)$^{-1}$ h^{-1}
Adaptive evaluation			
Glycerol	Chemical mutagenesis and adaptive evaluation	22 μM H_2 (mg protein)$^{-1}$	4 μM H_2 (mg protein)$^{-1}$ h^{-1}
Heterologous gene expression			
Glucose	Production of (Fe) hydrogenase from *E. cloacae*	0.96 mM h^{-1}	14.5 μM H_2 (mg protein)$^{-1}$ h^{-1}
Glucose	Production of HoxEFUYH hydrogenase from *Synechocystis sp.* PCC 6803	22 ± 3 μM H_2 (mg protein)$^{-1}$	4 μM H_2 (mg protein)$^{-1}$ h^{-1}
Glucose	Production of HoxEFUYH hydrogenase and the maturation proteins HypABCDEF and Hox W from Synechocystis sp. PCC 6803	8.4 μM H_2l^{-1}	0.004 μM H_2 (mg protein)$^{-1}$ h^{-1}
Glucose	Production of HydFEGA	420.3 μM H_2 min^{-1} l^{-1}	0.12 μM H_2 (mg protein)$^{-1}$ h^{-1}
Glucose	Production of HydFEGA and inactivation of lacR	1257.5 nM H_2 min^{-1} l^{-1}	0.34 μM H_2 (mg protein)$^{-1}$ h^{-1}
Glucose	Inactivation of lacR, production of hydFEGA hydrogenase from	9.6 mM H_2 (gDCW)$^{-1}$ h^{-1}	10 μM H_2 (mg protein)$^{-1}$ h^{-1}

(continued)

Table 3.4 (continued)

Substrate	System	H_2 production rate (reported units)	H_2 production rate (converted units)
	C. acetobutylicum, CpFdx ferredoxin form *C. pasteurianum* and YdbK		
Glucose	Production of HupSL hydrogenase from *Rhodobacter sphaeroides*	19.68 μl H_2 (ml culture)$^{-1}$ h^{-1}	1.1 μM H_2 (mg protein)$^{-1}$ h^{-1}
Starch	Inactivation of lacR, production of HydFEGA hydrogenase from *C. acetobutylicum*, CpFdx ferredoxin from C. Pasteurianum and YdbK pyruvate-flavodoxin oxidoreductase from *E. coli* and amyE from *B. subtilis*	30 μM H_2 culture^{-1}	0.65 μM H_2 (mg protein)$^{-1}$ h^{-1}
Sucrose	Inactivation of HycA and TatC and expression of the genes encoding ScrKYABR invertase from Bacillus subtilisG	1.38 ml H_2 (mg DCW)$^{-1}$ h^{-1}	3.9 μM H_2 (mg protein)$^{-1}$ h^{-1}
Single gene knockout or expression			
Formate	Inactivation of HycA	NA	100 μM H_2 (mg protein)$^{-1}$ h^{-1}
Formate	Production of FhlA	7 μM H_2 (mg protein)$^{-1}$ h^{-1}	7 μM H_2 (mg protein)$^{-1}$ h^{-1}
	Inactivation of HycA		
Glucose	Inactivation of FocA	14.9 μM H_2 (mg protein)$^{-1}$ h^{-1}	1.8 μM H_2 (mg protein)$^{-1}$ h^{-1}
Glucose	Inactivation of HybC	12.1 μM H_2 (mg protein)$^{-1}$ h^{-1}	1.4 μM H_2 (mg protein)$^{-1}$ h^{-1}

mutagenesis, Hu and Wood isolated a mutant strain named as HW2 which is holding the ability to produce 20 times more productivity and fivefold higher cell growth than original strain BW25113 Δ*frdC* (Hu and Wood 2010). Further transcriptome analysis of this strain showed that the isolated mutant defective in fructose-1,6-bisphosphatase (encoded by *fbp*), formate transportation (*focA*), and tagatose-1,6-bisphosphate aldolase (*gatYZ*). These studies gave a better picture on glycerol metabolism in hydrogen production; however, more comprehensive data is needed to link all these studies for elaborated understanding glycerol metabolism and hydrogen production for better productivity with less energy investment which can lead to a technology which can be as competitive as commercial production presently followed (Akhtar and Jones 2008a).

In addition, with the strategies based on the deletion of targeted genes, adoptive mutagenesis and random mutagenesis; few studies are also made for enhancing the hydrogen production by heterologous expression of various clusters of genes. Among these studies, expression of hydrogenases genes isolated from various strains in *E. coli* is important and results in enhanced H_2 production. In this regard the expression of hydrogenases derived from the microbial species like *Enterobacter cloacae* (Mishra et al. 2004; Chittibabu et al. 2006), *Ethanoligenens harbinense*

(Zhao et al. 2010), *Rhodobacter sphaeroides* (Lee et al. 2010b), *Clostridium acetobutylicum*, and *C. pasteurianum* (Akhtar and Jones 2008b, 2009) were heterologous expressed in *E. coli* BL21 with no ability to produce hydrogen. The heterologous expression of hydrogenases resulted in H_2 production by BL21 strain. Along with hydrogenases heterologous expression, few researchers also tried co-expression of other genes involved in the transportation of substrates and substrate utilizing enzymes which will divert in the core cellular metabolism tried for enhancing the hydrogen production. Few among these a significant study is expression of *scrB* (encode β-D-fructofuranosidefructohydrolase catalyzes the hydrolysis of sucrose 6-phosphate to β-D-fructose and α-D-glucose 6-phosphate) and *scrR* (encodes the negative repressor of the *scr* regulon) which enhanced the hydrogen productivity up to twofold from sucrose (Penfold et al. 2003).

3.6 Future Prospects

Hydrogen being the only green fuel which does not release any carbon footprint up on combustion is the next generation fuel for the future environmental outlook. To make this fuel as alternative fuel for the transportation and other industrial applications, the production cost must come down as competitive as commercial available hydrocarbon based fuels. The key points to be looked in the aspects of biohydrogen production is (1) innovative methodologies to be developed to utilize waste biomass and industrial waste water effluents, (2) isolating and developing efficient strains which could be used for hydrogen production utilizing more diverse carbon substrates, (3) engineering the microbial system for enhancing the productivity, resistance to growth retarding fermentative by-products, increasing growth rate, imparting ability to utilizing complex substrates, and accumulating useful by-products, and (4) innovative reactor designs. Looking at these aspects future research goals need be put forward to generate a sustainable biological hydrogen producing system prospective to forecast energy needs and for environmental safety.

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Recent Advances in Genetic Improvement of *Jatropha curcas:* A Potent Biodiesel Plant

4

Nitish Kumar and Swati Kamari

Abstract

Jatropha curcas is an ideal plant species for biodiesel production. It grows on the waste land and in adverse climatic conditions. The lack of hybrids and high yielding genotypes for yield and oil content is the main problem in large-scale cultivation of *J. curcas.* Therefore, genetic diversity assessment is pre-requisite for the development of superior variety through breeding program. Limited efforts have been carried out for the genetic improvement with both conventional breeding and biotechnology approaches. In vitro mass propagation is commonly used to multiply uniform plants of elite germplasm. Further regeneration or direct organogenesis from in vitro explant is pre-requisite for the development of transgenic plants. This chapter is the compilation of information on genetic diversity assessment, conventional breeding, and biotechnological approaches for its genetic improvement.

Keyword

Genetic diversity · Hybridization · *Jatropha curcas* · Micropropagation · Molecular marker · Transgenic

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4.1 Introduction

Jatropha curcas belongs to family Euphorbiaceae and has a great potential as a biodiesel crop. *J. curcas* has been observed to be the furthermost appropriate plant species for biodiesel production as it has ability to grow on waste land and adverse climatic conditions. The main constraint in cultivation of *J. curcas* is the low productivity, lack of knowledge about genetic diversity, suitable genotypes, and very narrow genetic base. Information about genetic diversity in the species is a pre-requisite for any breeding work. Due to the existence of natural hybridization among species, the genetic structure and taxonomical information of the *J. curcas* is not completely elucidated (Airy Shaw 1972). Sujatha et al. (2008) reported that the accessible genotypes lack evidence on the genetic base. Therefore, characterization and evaluation of genetic variability becomes necessary for the development of hybrid variety through conventional breeding. The available genotypes or germplasm with existence variability for various marketable characters is accessible, inadequate development has been carried out in generating newer varieties/cultivars which are resistant or tolerant to different abiotic and biotic pressures through conventional breeding. Hence, application of biotechnological techniques is also being employed for genetic enhancement of *J. curcas*.

This book chapter reviews conventional as well as biotechnological techniques such as tissue culture and genetic transformation employed for genetic improvement of *J. curcas*.

4.2 Taxonomy and Use

Approximately 170 species are known of *Jatropha* genus. Among them, 12 species, i.e., *Jatropha curcas, Jatropha integerrima, Jatropha glandulifera, Jatropha heynei, Jatropha gossypifolia, Jatropha multifida, Jatropha podagrica, Jatropha maheshwarii, Jatropha hastate Jatropha villosa, Jatropha nava, Jatropha tanjorensis,* are recorded in India and found growing on marginal and degraded lands making it appropriate to exploit degraded lands without challenging traditional crops for land. Among them, *J. curcas* is an ideal species for future production of biodiesel. *J. curcas* is a large shrub or small tree, which can attain height of 2–3 m, but under ideal conditions it can reach a height of 8–10 m. *J. curcas* is a diploid species having chromosome number 22b ($2n = 22$) and it is deciduous in nature and sheds leaves in hot climate. Approximately 35–40% oil is present in seed which attracted the attention of world as substitute biodiesel (Mandpe et al. 2005). Duke and Wain (1981) reported that, apart from biofuel, *J. curcas* has also medicinal value (Duke and Wain 1981). Seed cake which is produced after extracting oil is rich in nitrogen (6%), potassium (0.94%), phosphorus (2.75%), carbohydrates (17%), and proteins (19%).

4.2.1 Floral Biology

Inflorescences in *J. curcas* take place at the end of branches. The pattern of flowers in inflorescence is of racemose type in a biparous/dichasial pattern means flowers born at the end of main axis and at the same time it produces two sideways newer flowers. The sideways and following flowers grow in the same manner. It is well reported that J. *curcas* is a monoecious in nature, i.e., the female and male flowers are separate but are developed in the same inflorescence (Singh et al. 2010). In general, the inflorescence develops a main flower which is bounded by a bunch of male (Singh et al. 2010). However, some time, male flower was originated in place of female flower. The average time duration of full opening of the flower from initiation of floral bud is 1 to 1½ month. Before 10–15 days of flower opening, male and female flower can be differentiated from each other (Singh et al. 2010).

4.3 Genetic Diversity Analysis

The level of genetic differentiation and genetic variability in *J. curcas* germplasms deserves distinctive consideration due to its introduction history as an exotic species in several nations. In that condition, germplasm populations may cause in a multifarious genetic history, with various possible genetic blockages (Kjær and Siegismund 1996; Lengkeek et al. 2005). Sun et al. (2008) reported that narrow genetic diversity was observed in Chinese landraces of Jatropha, and only partial genetic diversity was found in Indian germplasm of Jatropha (Ranade et al. 2008; Basha and Sujatha 2007). Tatikonda et al. (2009) analyzed AFLP based genetic diversity of 48 landraces of Jatropha and observed 68% polymorphism. Ganesh Ram et al. (2008) studied genetic diversity of 12 Jatropha species based on RAPD markers and reported 80.2% polymorphism. Basha and Sujatha (2007) studied 42 germplasm of *J. curcas* and observed 42% and 35.5% polymorphism based of RAPD and ISSR markers, respectively, which shows various levels of genetic polymorphism/diversity in the Indian germplasm of *J. curcas*. Reddy et al. (2007) analyzed genetic diversity of 23 germplasm of *J. curcas* using RAPD and AFLP molecular marker and observed polymorphism percentage of 14–16% and 8–10% by RAPD and AFLP, respectively, which is narrow as compared to other previous studies. Low genetic diversity in African and Indian germplasm and high genetic diversity was observed in Guatemalan germplasm based on analysis of 225 germplasm (Montes et al. 2014). Mastan et al. (2012) studied elite accession of *J. curcas* and observed 56%, 57%, and 36% of polymorphism by RAPD, AFPL, and SSR molecular markers, respectively. Rafii et al. (2012) evaluated 48 germplasm of *J. curcas* and found 63% polymorphism percentage with RAPD molecular markers. 93% polymorphism percentage was observed in 20 germplasm of J. curcas using RAPD molecular marker (Kumar et al. 2013). Kaul et al. (2014) reported 59% and 60% polymorphism percentage using ISSR and RAPD, respectively, after evaluation of 29 germplasm of *J. curcas.* Murty et al. (2013) evaluated 19 germplasm using ISSR, RAPD, and DAMD (Direct amplification of minisatellite DNA marker) and

observed 96%, 91%, and 90% polymorphism percentage using RAPD, DAMD, and ISSR molecular markers, respectively. Osorio et al. (2014) found high genetic diversity (81%) within region, whereas narrow genetic diversity across genomic region (18%).

Mavuso et al. (2015) evaluated genetic diversity of 78 different accessions of *J. curcas* cultivated in Taiwan suing ISSR marker and observed 31.23% of the variability among populations and 68.77% within Jatropha populations which reflect low variation in Jatropha accessions in Taiwan. Vásquez-Mayorga et al. (2017) assessed the genetic diversity of 50 *J. curcas germplasm* from the Costa Rican using nrDNA-ITS, EST-SSR, G-SSR markers.

4.4 Conventional Breeding Strategies

The true breeding strategies of any crop plant depend mainly on the availability of genetic diversity of desired trait. Traits such as seed oil content, seed yield, early flowering, toxicity of seed, ratio of male and female flower, number of branching, uniform maturation of seed, and adaptation to abiotic and biotic stresses are considered applicable for development of hybrid verities (Abdelgadir et al. 2009). Seed yield could be enhanced by increasing female and male flower ratio and oil content could be increased by altering gene expression level of triacylglycerol and fatty acid synthesis. *J. curcas* is cross pollinated plant and exploitation of genetic diversity could be done by mass selection, recurrent selection, and inter specific hybridization.

4.4.1 Mass Selection and Recurrent Selection

The high-quality plants are selected based on morphological output and bulk seed is propagated to generate the next generation crop plant for genetic improvement. For improvement of desired trait, there is need a positive regression of offspring parent which mainly depends on environmental factors of parental population. Montes et al. (2014) studied levels of diversity by using 225 landraces of Latin America, Africa, and Asia and confirmed that low genetic diversity in African and Indian landraces and high genetic diversity was observed in Guatemalan and Latin American landraces. Recurrent selection is beneficial to overcome the shortages of mass selection in *J. curcas*. Development of hybrid verities mainly depends on identification of superior inbred line from population and further subjected to recurrent selection. This method is useful in incorporation of desired gene within population by maintaining variability. After getting the desired seed yield data, oil quality and content, resistance to insect pest and disease, the high performing genotypes is released as new varieties of by accepting the standard procedure (Punia 2007).

4.4.2 Inter-Specific Hybridization

In *J. curcas,* interspecific hybridization has enormous possibility for enhancing the agronomic traits and genomic attributes. The breeding program in *J. curcas* is mainly based on seed yield and oil content per unit area which depends on large number of female flower per bunch of inflorescence and number of capsule per plant. Based on phylogenetic analysis and importance of interspecific hybridization in Jatropha, Dehgan (1984) confirmed the cross ability barriers and phenological traits revealed that F1 hybrid except *J. multifida*× *J. curcas* were more vigorous than the parent. The species that might be crossed individually with *J. curcas* as female parent include *J. cinerea, J. capensis, J. macrorhiza, J. cordata, J. cathartica, J. podagrica, and J. multifida* (Sujatha 2006). Artificial hybrids developed between *J. curcas* and different Jatropha species except *J. podagrica* (Basha and Sujatha 2009). Parthiban et al. (2009) carried out crosses between *J. curcas* with other species. Cross between *J. integerrima and J. curcas* was fruitful as it developed hybrids with more number of seed set and other hybrids unsuccessful to harvest seeds due to presence of cross ability hurdles.

4.4.3 Breeding Between Toxic and Nontoxic Jatropha

Due to the toxic nature of *J. curcas* seed, consumption of seeds may cause several signs, counting diarrhea and vomiting (Abduaguye et al. 1986; Becker and Makkar 1998; Chimbari and Shiff 2008). The chemical nature of toxic compound which is present in *J. curcas* is phorbol esters that are present in high concentration in the seed of *J. curcas* (Makkar et al. 1997; Adolf et al. 1984; Rakshit et al. 2008). Phorbol esters are chemical which is well known reason to cause various diseases including and tumor promotion and inflammation (Haas et al. 2002; Goel et al. 2007). Therefore, breeding between toxic and nontoxic *J. curcas* may give remarkable prospects to reduce the concentration of phorbol esters. It is well reported that Mexican varieties of *J. curcas* contain very low amount of phorbol esters (Basha et al. 2009; Makkar et al. 1997; Martinez-Herrera et al. 2006; Makkar et al. 1998, 2008). The occurrence of *J. curcas* plant with low amount of phorbol esters is very interesting breeding material because it could make hybrid with low phorbol esters in *J. curcas*. Development of F1 hybrids between toxic and nontoxic *J. curcas*, and further backcrossing could be used to spot the genetic mechanism of toxicity. By localizing the locus accountable for (non) toxicity in the corresponding parent by the use of with molecular markers, it will be probable to use the markers for future breeding.

4.5 Biotechnological Approaches

The traditional methods of crop improvement seem to be the only time effective, alternate method, wherein biotechnological approaches such as tissue culture and genetic modification will be the utmost important in achieving the constraints of traditional methods.

4.5.1 Micropropagation

Development of efficient and effective micropropagation protocol is essential due to inconsistent and low yield and lacking of superior clones in *J. curcas*. Several studies were carried out on development of axillary bud proliferation and multiplication of shoot tips from different totipotent explants (Sujatha and Mukta 1996; Sardana et al. 1998; Lin et al. 2002; Rajore et al. 2002; Sujatha et al. 2005; Rajore and Batra 2005; Sharma et al. 2006; Qin et al. 2006; Datta et al. 2007; Kalimuthu et al. 2007; Shrivastava and Banerjee 2008; Thepsamran et al. 2008; Singh 2009). Sujatha and Mukta (1996) achieved shoot multiplication on MS medium containing combination of benzyl aminopurine (BAP) and indole-3-butyric acid (IBA). Kalimuthu et al. (2007) confirmed that combination of kinetin, BAP, and indole-3-acetic acid (IAA) is highly effective in multiplication of shoot from nodal segment and shoot tips. Datta et al. (2007) obtained best shoot proliferation on Murashige and Skoog's (MS) basal medium containing BAP (22.2 mM) and adenine sulfate (55.6 mM). This combination produced 6.2 shoots/nodal explant. Several studies also report that BAP is more effective in comparison to other cytokinins in proliferation and multiplication of shoot from nodal segments and shoot tip (Sujatha et al. 2005; Kalimuthu et al. 2007; Datta et al. 2007).

4.5.2 Regeneration

Several studies on regeneration was carried out and reported that thidiazuron (TDZ) was more effective as compared to other cytokinins in regeneration or direct organogenesis from leaf explants (Kumar 2009; Singh 2009; Kumar et al. 2010a, b, c, 2011a, b; Kumar and Reddy 2010, 2012; Singh et al. 2010; Sharma et al. 2011; Gopale et al. 2013; Zhang et al. 2013; Aishwariya et al. 2015; Liu et al. 2015, 2016). Several reports are also available on shoot regeneration through somatic embryogenesis in *J. curcas* (Sujatha and Mukta 1996; Sardana et al. 2000; Lu et al. 2003; Wei et al. 2004; Sujatha et al. 2005; Rajore and Batra 2007; Jha et al. 2007). All the above available protocols were based on callus mediated regeneration. Several reports have been reported on direct regeneration or organogenesis, i.e., without intervening callus using various explants (Deore and Johnson 2008; Kumar 2009; Dubey et al. 2010; Kumar and Reddy 2010, 2012; Kumar et al. 2010a, b, c, 2011a, b; Singh et al. 2010; Khemkladngoen et al. 2011; Sharma et al. 2011). The

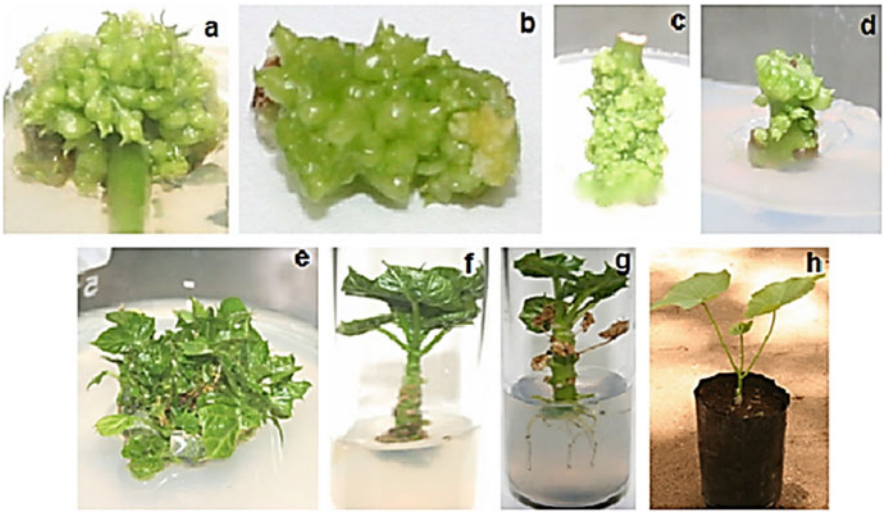

Fig. 4.1 Direct shoot bud induction from petiole explants of non-toxic *J. curcas*. Direct shoot bud induction from (**a**) in vitro petiole in horizontal position, (**b**) in vivo petiole in horizontal position, (**c**) in vitro petiole in vertical position, and (**d**) in vivo petiole in vertical position on MS medium with 2.27 M TDZ after 6 weeks. (**e**) Shoot proliferation on MS medium with 10 M Kinetin +4.5 M BAP + 5.4 M NAA after 4 weeks. (**f**) Elongation of shoot on MS medium with 2.25 M BAP and 8.5 M IAA after 6 weeks. (**g**) Development of roots on half-strength of MS medium with 15 M IBA + 11.4 M IAA + 5.5 M NAA + 0.25 mg/L activated charcoal after 4 weeks. (**h**) Regenerated plant in polybag. Source: Kumar et al. (2010a): Licence No. 4743471497135

efficiency of regeneration enhanced with increasing dose of TDZ (Deore and Johnson 2008; Kumar 2009; Kumar et al. 2010a, b, c, 2011a, b; Kumar and Reddy 2010, 2012; Sharma et al. 2011). It is also reported that in vitro explants showed more response as compared to in vivo explants (Kumar and Reddy 2010, 2012; Kumar et al. 2010a, b, c, 2011a, b; Sharma et al. 2011). The regeneration efficiency was observed in cotyledonary explants as compared to other explants (Sujatha and Mukta 1996; Kumar 2009). Effect of heavy metals such as copper and nickel on regeneration was studied (Sarkar et al. 2010; Khurana-Kaul et al. 2010). Sarkar et al. (2010) reported that the percentage of regeneration efficiency was decreased with addition of nickel to regeneration medium. However, Khurana-Kaul et al. (2010) found remarkable increase in regeneration efficiency with addition of copper sulfate to regeneration medium. Gopale et al. (2013) studied the importance of TDZ and reported that TDZ in MS medium showed more response as compared to BAP. Approx. 55% regeneration efficiency was observed on 2.27 μM TDZ. Liu et al. (2016) reported that at 0.3 mg/l of TDZ concentration 63% regeneration efficiency was observed. A representation of regeneration from petiole explant is represented in Fig. 4.1.

4.5.3 Somatic Embryogenesis

Somatic embryogenesis technique has an abundant prospective for true to type multiplication of plants. An efficient regeneration method through somatic embryogenesis was reported by Jha et al. (2007) and observed 58.5 somatic embryos per callus. It was reported that the size of embryo is crucial stapes for development of callus Varshney and Johnson 2010). Cai et al. (2011) reported an effective method of somatic embryogenesis in three different accession of Indonesia, China, and India. Devi et al. (2012) obtained somatic embryo from cotyledon explants and embryo axis on MS medium supplemented with picloram (1 mg/l).

4.5.4 Genetic Transformation

Genetic transformation technique appears to be the alternative approach and time effective in genetic improvement of crop plant. Several studies were carried out for establishment of *Agrobacterium*-mediated genetic transformation methods in *J. curcas* (Li et al. 2006, 2008; He et al. 2009; Hui Zhu et al. 2010; Kumar et al. 2010b; Mazumdar et al. 2010; Pan et al. 2010; Zong et al. 2010; Kajikawa et al. 2012; Novatiano et al. 2017). Li et al. (2008) studied *A. tumefaciens*-mediated transformation in callus using the strain LBA4404 and observed 55% explants produced phosphinothricin resistant calli on MS medium supplemented with BAP (1.5 mg/l), IBA (0.05 mg/l), phosphinothricin (1 mg/l), and cefotaxime (500 mg/l) after 4 weeks of transformation and about 13% transgenic plants were produced from transformed callus. He et al. (2009) studied various parameters affecting efficiency of transformation such as types of explant, co-culture time on co-cultivation medium, *A. tumefaciens* density, and concentration of acetosyringone and reported about 67% transformation efficiency. Kumar et al. (2010b) reported 28% transformation efficiency using the *Agrobacterium* strain LBA 4404 from salt resistance. Mazumdar et al. (2010) reported that juvenile explants are more responsive as compared to mature explants to *A. tumefaciens* -mediated transformation. Pan et al. (2010) reported 30.8% transformation efficiency using cotyledon explants. Zong et al. (2010) developed transgenic *J. curcas* plant having more number of lateral branching using lateral shoot inducing factor (LIF). Recently observed 23% of transformation efficiency when *Agrobacterium* and leaf explants was treated with vacuum filter paper. Another method of genetic transformation is microprojectile bombardment/biolistic method/particle gun method has been used in development of various transgenic plant in various laboratories. Transgenic *J. curcas* plant development using microprojectile bombardment is well reported (Purkayastha et al. 2010; Joshi et al. 2011). Kajikawa et al. (2012) developed a different system of selection of transformants using herbicide bispyribac sodium salt. Novatiano et al. (2017) transformed polyhydroxyalkanoate gene using Agrobacterium for production of bioplastic.

4.6 Conclusions and Prospects

J. curcas is known a very promising alternative option for manufacturing of biodiesel from waste land due to its adaptability and extensive distribution under various climatic conditions. Though, there are various hurdles in the marketable exploitation of *J. curcas* as biofuel plant due paucities of better-quality varieties for oil and seed yield and oil yield. Apart from institutional, socioeconomic, and agronomic constraints, strategic programs for crop improvement programs are missing worldwide. Therefore, genetic improvement of *J. curcas* can be carried out through evaluation of genetic diversity, conventional breeding, and biotechnological interventions.

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Catalytic Approach for Production of Hydrocarbon Rich Bio-Oil from a Red Seaweed Species

5

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Abstract

Macro algae represent a diverse group of multicellular marine organisms capable of performing the photosynthetic process and classified into three main categories due to the presence of specific photosynthetic pigment into their body: (1) Phaeophyceae (2) Rhodophyceae, and (3) Chlorophyceae, which in general term known as brown, red, and green seaweeds, respectively. There are more than 1000 species belonging to these groups of plants, having uses in food, pharma, textile, agriculture, and microbiology based industries, as they are the main sources of the key products. Due to different growth rate, hybrid nature of the products, higher contents of other cellular components, and poor quality of the obtained products, etc., only few species had occupied the industrial applications among which *Gracilaria, Eucheuma, Sargassum, Ulva, Laminaria* species are the key players. Nature has offered unique features to each species with respect to their possible application in the selective domain. The absence of lignin, higher rates of growth, no use of lands for cultivation as well as their higher CO_2 mitigation capabilities, seaweed biomass can find application in the energy sector as suitable energy resources. As compared to micro algal biomass, which has considered as alternative energy source of the third-generation biofuels macroalgal biomass has not been explored that much.

In this work, a red macroalgal biomass *Champia indica* was used for study towards its bioenergy prospective in terms of pyrolysis. Based on proximate,

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ultimate, TGA and DTG analyses; the biomass was pyrolysed to yield bio-oil, biochar and biogas. The bio-oil was further upgraded using ZSM-5 catalyst to give oil having calorific value of 34.6 MJ/Kg under optimum conditions.

Keywords

Seaweed · *Champia indica* · Biomass · Pyrolysis · Bio-oil · Thermochemical conversion · Catalytic upgradation · Biochar

5.1 Introduction

Increasing price of energy services, elevation in pollution level leading to the climate change, along with security concerned, have forced to revise the policy to meet the energy demand of the world (Chia et al. 2018). In present scenario, transport and energy sectors are mainly depending on the fossil fuel and cannot assessed as viable (Popp et al. 2014). The exploration for the environment-friendly and sustainable hydrocarbons resources is the prerequisite of the present day. Basic research towards the liquid transportation fuel synthesis from biomass conversion were adopted during the first US energy crisis which arises due to the consequence of the Yom Kippur, Iranian revolution, concerns around the security of imported petroleum, along with the contribution of carbon dioxide (CO_2) emissions (Blanch 2012). India is emerging as important primary energy consumers due to large population growth. The energy competency of India largely depends upon the import of coal (198.63 MT) and oil (202.85 MT) and reliance over oil imports, expected to be more than 90% by 2040 according to International Energy Agency (IEA 2017). The increasing energy import with rising populations, makes the development of bioenergy infrastructure and balance between energy as well as food security; as the Nation's key priority. The Indian government has set target to decline their fossil fuel dependency by increasing renewable energy capacity to 175 GW by 2022 ("Coal here to stay despite India's ambitious goals for renewable energy," 2019). Despite significant investments from national and international schemes, the present renewable energy standing is 37 GW ("Coal here to stay despite India's ambitious goals for renewable energy," 2019). These statistics highlight the need to diversify the country's energy portfolio to unconventional 2nd generation resources. As per announcement made on the eve of World Biofuel Day, it was proposed that biofuels would benefit the income of the rural sectors along with the energy security, creation of rural job as well as clean environment. The Indian government targets to improve towards a trillion biofuel budget, by investing ten thousand crores via state-run oil marketing companies, for setting up of various bio refineries for second generation biofuels. The statement comes in the milieu of cabinet approval of the policy for national biofuel to help India's efforts to shrink carbon emissions as well as dependency over energy imports.

5.1.1 Various Generation of Biofuel

It has been projected that biofuel based bio refineries can partially diminish those issues towards creation of more sustainable along with secure economies. Beside that it furthermore extended towards better opportunity of other raw materials to yield ethanol from various carbohydrate rich resources, e.g. sugar beet, corn, sugarcane, damaged grains sweet sorghum, cassava, etc., (Ho et al. 2014). Among various generations of the biofuel, consisting of edible feedstock based first-generation biofuels, waste lignocellulosic biomass and dedicated lignocellulosic feedstock based second generation fuel, and micro, and macroalgae based third generation, the latter one occupied important position in the biofuel research area (Chia et al. 2018). Although usages of algae to make fuels were discussed long back, an intensive work began with an oil crunch in the 1970s, enormous research plans in the USA and Japan were focused towards the growth of macroalgal energy production (Oswald and Golueke 1960). These marine plants have higher biomass vintages without demanding any arable land, having the cultivation potential in offshore (Roesijadi et al. 2008). The above features, in company with large-scale cultivation and processing methods can make the third-generation feedstock superior to that of previous generations (Daroch et al. 2013).

However, these biomasses may not be sufficient to sustain the ethanol demand; moreover, diverting food material for fuel may initiate Food vs Fuel debate (Popp et al. 2014). Thus, the inclusion of the 3rd generation biofuel materials is mandatory. Seaweeds in this regard have gained considerable attention at global scale as an alternative resource for energy production (Ghadiryanfar et al. 2016). It is mainly due to their prominent characteristics such as high carbohydrate content, no lignin, as well as high CO_2 fixation ability (Fei 2004).

5.1.2 Thermal Conversion of Biomass with Reference to Bioenergy Prospective

5.1.2.1 Pyrolysis

Pyrolysis techniques are the central chemical reaction method, defined as the chemical changes, which occurs when heat is applied to a sample of analysis in absentia of oxygen. In this thermochemical process biocrude, tars, charcoals, and gases are formed during the conversion (Bridgwater 2003; Czernik and Bridgwater 2004). The quantity and quality of the products vary dependent on the reaction constraints, e.g. nature of the catalyst, reactor types, pyrolysis temperature, heating rate as well as source (Vasudev et al. 2019). Catalytic pyrolysis abstains considerable courtesy for the deoxygenation of bio-oil to from various valuable components, e.g. aromatic compounds, to fulfill the petroleum industry demand. Protonation of hydrocarbons results for synthesis of aromatic compounds due to acidic sites of catalyst; through a series of reactions, e.g. cyclization, oligomerization as well as hydrogen transfer reactions (Lu et al. 2010; Norouzi et al. 2017). The formation of anhydro sugar from cellulose occurs due to acid catalyzed dehydration over the

acidic site of zeolites (Norouzi et al. 2017; Rachel-Tang et al. 2017; Rahman et al. 2018). The formation of C_2–C_6 olefins occurs from anhydro sugars, through various steps involving oligomerization, decarboxylation and decarboxylation, which further forms the aromatic compounds. Beside the cellulose, hemicellulose a non-crystalline key component of the biomass forms furanic compounds, e.g. low molecular weights compounds over the pores of zeolite.

Further decarboxylation and oligomerizing acid catalyst catalyzed reactions over the zeolite, sulfated zirconia, heteropoly acids lead to the formation of furfural from xylose, the latter one is a key product form hemicellulose. Formation of furfural is a key step towards preparation of various hydrocarbons, e.g. methyl furan, furan, furfuryl alcohol, furfural, 4-methyl furfural, furan-2-methanol, and 5-hydroxymethyl furfural (Rachel-Tang et al. 2017; Talmadge et al. 2014; Yang et al. 2014).

In general, all types of biomasses can be used as resource for bio-oil production through pyrolysis. However, quality of bio-oil is highly dependent upon proximate composition of respective biomass. Thermal gravimetric analysis (TGA) and pyrolysis–GC–MS analysis are the fast method to evaluate rate of degradation, kinetic analysis, and product analysis, respectively, during pyrolysis of the biomass (Huber et al. 2006). In addition, the pyrolytic parameters or conditions vary with the type of raw biomass employed. The gaseous components can be applied towards C-1 chemistry as per existing technology.

5.1.3 Hydrothermal Liquefaction (HTL)

Beside the above catalytic and non-catalytic pyrolysis, transformation of feedstock, i.e. biomass, another technique, e.g. hydrothermal liquefaction or upgradation is attaining the key role for the valorization of the bio-oil (Zhang et al. 2019). This upgradation process is a promising liquefaction process since it is applicable to various types of biomass feedstock. In this technique, the drying of feedstock is not essential as water used as one of the reactants is mainly suitable for wet biomass (Biller et al. 2012; Garcia Alba et al. 2011). It involves the reaction of biomass in water at high pressure and temperature in the presence or absence of the catalyst. The final products are biocrude, aqueous, gaseous fraction, and unconverted contents. This thermochemical technique for reforming of the biomass has energetic compensations. The use of high pressure, direct liquefaction process forms liquid oils having higher caloric values, along with a broad range of chemicals. The advantage of liquefaction is that the bio-oil produced is immiscible with water, has lower oxygen and therefore possesses higher energy value (Goudriaan et al. 2000). The bio-oil preparation from pyrolysis of biomass forms a mixture of C_5–C_{10} compounds including some oxygenated organic components.

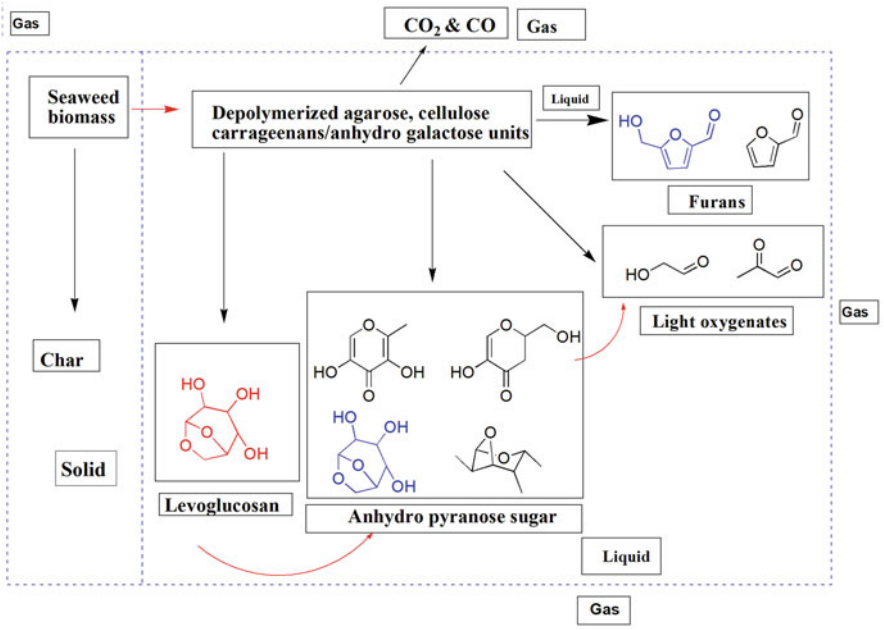
5.1.4 Macroalgal Biomass and Their Uses

Macro algae too are exceptionally diverse in their form, function, composition and offers distinctive prospect for their potential application in the field of agriculture, food, biofuel, cosmetics, pharmaceutical, and nutraceuticals domain (Kim et al. 2017; Rebours et al. 2014). Phycocolloids from seaweeds hold the second highest market share. These are macro algae belonging to the different groups based on their major photo pigments, e.g. red, green, and brown algae, and among the known species of seaweeds only a few (viz. species of *Undaria*, *Saccharina*, *Kappaphycus*, *Porphyra*, and *Gracilaria*) are cultivated for fulfilling the 97% demand of world's cultivated seaweed production (Kim et al. 2017). Among red seaweeds, only three seaweeds species, namely *Kappaphycus alvarezii*, *Gracilaria dura*, and *Gracilaria edulis* had reported to be promising sources of industrially important products such as κ-Carrageenan, Agar-Agar as well as agarose, respectively (Khambhaty et al. 2012; Marinho-Soriano 2001; Murano et al. 1990; Rao 1974). More than 60% biomass, after utilizing 30% of the total biomass of these cultivated macroalgal species, thrown as wasted after extraction of phycocolloids. This offers an opportunity to generate valuable higher energy compounds from these wastes (Ferrera-Lorenzo et al. 2014; Li et al. 2012b). This, therefore, opens up high possibility of developing these resources as suitable for bio-oil production either from the waste generated after phycocolloid production or raw biomass. Further, there are many more seaweed species, which need to be explored for their potentials to generate high value products. For instance, among red seaweeds, more than hundreds known species, only few macroalgal species are reported by the researcher for their valuable sources for production of carrageenan, agar, agarose as well as bioethanol (Eswaran et al. 2005; Khambhaty et al. 2012). These species possess the uniformity in the carbohydrate constituents and their cultivation technologies has been patented and commercialized by the Indian Researchers (Eswaran et al. 2005; Khambhaty et al. 2012). However due to difference in sugar profiling, different amount of methyl and pyruvate groups among other red seaweeds species, e.g. species of *Sarconema, Gracilaria and Champia*, etc., have put obstacles in their commercialization for biofuels/biochemical production (Kumar et al. 2012; Oza et al. 2011).

5.1.5 Bio-oil from Macroalgal Biomass Via Pyrolysis and HTL

There are a total of ~525-million-dollar commercial market for red seaweeds derived agar and carrageenan out of ~720-million-dollar total seaweed products (Rhein-Knudsen et al. 2015). Beside the other uses, seaweeds based pyrolysis and HTL, research is in initial stage, which can contribute more than existing market value for other known red seaweed species (Scheme 5.1). These seaweeds resources may fulfill the demand for biofuel preparation.

Among most seaweed producing nations, Ireland has agreed that for their nation, the biofuel production target for 2013 was set at 6% by volume, as compared to target for year 2010 for which it was 4% (Murphy et al. 2013). The production of



Scheme 5.1 Representation of the pyrolysis reaction of seaweed polysaccharides

bioethanol was the main candidate for preparation of biofuel. The production of bio-oil, biochar from the marine seaweed biomass via pyrolysis process in the last few years at International level is forecasted based on the report of different researchers, to point out the future prospective of this process. The HHV values of the various fractions, e.g. bio-oil, biochar, biogas along with algal biomass obtained via pyrolysis are significant as well as similar to the other known biomasses. Among the different known macroalgal biomasses, the reports containing the bio-oil preparation through pyrolysis and HTL by various researchers are *Cladophora socialis* (Ly et al. 2015), *Ulva prolifera* (Liu et al. 2013) along with species of *Enteromorpha* (Li et al. 2013; Zhao et al. 2013; Zhou et al. 2010; Li et al. 2012a, b), *Laminaria* (Bae et al. 2011), and *Fucus* (Ross et al. 2008). Classification of macroalgal species, viz. species of *Laminaria*, *Fucus*, and *Chorda* using a Van Krevelen diagram, proximate, ultimate, inorganic content, and calorific value analysis, were suggested as promising candidate for bio-oil potential (Ross et al. 2008).

Song et al. (2014) and Hua and Li (2016) had reported uses of green alga *Enteromorpha prolifera* by pyrolysis towards production of the bio-oil (Hua and Li 2016; Song et al. 2014). The optimum temperature for pyrolysis was found to be 300 °C and 80 min, for the green alga (Hua and Li 2016). The acid catalyzed, pyrolysis of the above species resulted to give 28% bio-oil having 29.5 MJ/Kg of high heating value, as reported by Yang et al. (2014). More than ~70% content of ketones, alkenes, and 5-methyl furfural as major components (Yang et al. 2014).

Francavilla et al. (2015) applied the cascade approach for the isolation of bio-oil through pyrolysis techniques after removal of phycobiliproteins from *Gracilaria gracilis*, a red seaweed species. They performed the fast pyrolysis of the residue after extraction of proteins at different temperature range between 400 and 600 °C for the production of the bio-oil and biochar. They observed a maximum yield of ~34.5% of organic bio-oil having a significant amount of nitrogen at the optimum pyrolysis temperature of 500 °C. They suggested that denitrogenation would improve the calorific value of the obtained bio-oil, to apply as a fuel (Francavilla et al. 2015).

Bae et al. (2011) reported the ~47% weight basis bio-oil yield, having major C_1–C_4 hydrocarbons at 500 °C from pyrolysis of *Undaria pinnatifida, Laminaria*, *and Porphyra tenera*, seaweeds species. They claimed that clear variation of main compounds present in bio-oils varies with the type of macroalgal biomass and was significantly different from those of land biomass, particularly with reference to nitrogen-containing complexes. They suggested that the acidic pretreatment would be more promising due to reduction of the ash content in the biomass (Bae et al. 2011).

Norouzi et al. (2017) had worked on the catalytic upgradation of the red seaweed species *Gracilaria gracilis* and they found that bio-oil derived from pyrolysis was more than 70% yield with respect to dry mass of the seaweed (Norouzi et al. 2017).

Ceylan et al. (2014) had explained the pyrolysis mechanism of a red macroalgal *Polysiphonia elongata* biomass based on the thermal behavior and kinetics parameter.

They observed that the main decomposition happened during the 225–485 °C, owing to the release of 78–82%, of the total volatile content, and was affected by the heating rate (Ceylan et al. 2014). Choi et al. (2014) studied the effect of the pretreatment, over the bio-oil production via pyrolysis, from *Saccharina japonica* as biomass. They reported that acidic treatment after removal of valuable components of the macroalgal species reduced the inorganic elements without affecting the properties of the pyrolysis oil as compared to control (Choi et al. 2014). Ferrera-Lorenzo et al. (2014) compared the conventional and microwave-based pyrolysis methodology for the bio-oil production from an industry. The significant variations in the presence of constituents were reported, based on the nature of pyrolysis carried out. In the microwave-based pyrolysis, low molecular weight compounds, aromatic and pyridine were the key components, whereas in conventional pyrolysis alkane, pyrrole, and phenolic derivative were the main products. The gaseous product was also varied during the type of the pyrolysis. Conventional pyrolysis contains higher content of methane and carbon dioxides as compared to microwave assisted pyrolysis which yielded higher syngas contents and lower amount of methane and carbon dioxides (Ferrera-Lorenzo et al. 2014).

Bio-oil yields vary in between 35 and 65% based on weight basis, depending on the macroalgal biomass and pyrolysis parameters. The bio-oil components of seaweed are greatly diverse as compared to terrestrial biomass based bio-oil, since they have the nitrogenous compounds derived from protein component during pyrolysis. In addition to nitrogenous compounds, other important components of bio-oil of seaweed are carboxylic acids, ketones, aldehydes hydrocarbon, alcohols along with

phenolic ones. To increase the energy value of the bio-oil, catalytic deoxygenation and or hydrogenation is key point for value addition of bio-oil as applicable bioresource. The same problem is also applied to macroalgal biomass like land plant biomass. For the same several researchers had tried to go for catalyzed pyrolysis of the seaweed feedstock. The bio-oil having a 7.1 MJ/Kg heating value mainly composed of methyl ester of long-chain fatty acid (C_{13}–C_{18}), benzene carboxylic acid along with diethyl phthalate, reported to form via microwave pyrolysis from green macroalgal biomass of *Ulva prolifera* (Zhuang et al. 2012). The production of bio-oil from the macroalgal biomass through catalytic and non-catalytic pyrolysis, is a one-step and cost-effective process. The various components obtained from the above pyrolysis process may further upgraded into valuable C_5–C_{10} organic compounds based on deoxygenation, hydrogenation process based on the need and possible process development (Zhuang et al. 2012).

The HTL based conversion of seaweed biomass for obtaining higher caloric value compounds was reported by various researchers around the world. The HTL of a green seaweed *Enteromorpha prolifera* in a batch reactor yielded about ~31.0 weight percentage of bio-oil, containing a diversity of fatty acid (C_3–C_{22}) esters, although the elemental analysis indicated that bio-oil have higher amount of oxygen (Zhou et al. 2010). Brown algal biomass of *Sargassum patens* was converted through HTL, to form bio-oil having HHV of 27.1 MJ Kg^{-1} (Li et al. 2012a). The key ingredients of the bio-oil were mainly phenol, lipid, esters, ethers, aromatic components, and water. The leftover biochar was found to be rich in higher ash and oxygen content.

Laminaria saccharina, a brown macroalgal biomass, using fast hydrothermal liquefaction techniques based production of bio-oil was reported by Anastasakis and Ross (2011), Anastasakis and Ross (2011). They claimed that a yield of ~20% with respect to biomass, bio-oil having higher heating values of 36.5 MJ kg^{-1}, similar to crude HHV, via HTL process. Another researcher group at Norway had also studied the effect of fast hydrothermal liquefaction on the same macroalgal biomass and they found that the bio-oil is mainly composed of dodecyl acrylate, phenol, 2,2-methylenebis(6-1,1-dimethyl)-4-methyl), and polyamide (Bach et al. 2016).

Zhuang et al. (2012) have studied the use of microwaves towards direct liquefaction. They claimed that by using ethylene glycol as solvent and sulfuric acid as a catalyst, yielded up to ~93% of bio-oil via the response surface methodology, from a green algal biomass of *Ulva prolifera*. The product obtained via the microwave pyrolysis were mainly phthalic acid esters, alkenes, and fatty acid methyl esters having a long-chain from C_{16} to C_{20} (Zhuang et al. 2012).

Among red seaweeds species seaweeds belonging to *Gracilaria* species, e. g. *Gracilaria gracilis* is studied by several researchers, while no such report is there of other *Gracilaria* species, e.g. *Gracilaria corticata, Gracilaria acerosa*, *Gracilaria dura*, *Gracilaria pudomensis* which are abundant species belong to this group. The above reports propose that macroalgal biomass can have screened and utilized as biomass feedstock, for production of transportation fuel and chemicals.

5.1.6 Macroalgal Biochar Potential

For the production of biochar, mainly agricultural waste resources, e.g. rice straw; woods, fruit peels, and forestry waste, are used (De Bhowmick et al. 2018). The macroalgal biomasses of *Undaria pinnatifida*; *Saccharina japonica*, *Sargassum fusiforme*; *Cladophora* sp. *Chaetomorpha* sp., etc., had extensively claimed to be potential biochar resources (Bird et al. 2011; Michalak et al. 2019; Zhou et al. 2018). All over the world, various researchers reported valuable work in the field of biochar derived carbon materials, and still various resources can be explored to fulfill the demand of the supply. Seaweed biochar has lower carbon content, cation exchange capacity as well as surface area, in comparison to lignocellulosic biomass derived biochar (Bird et al. 2011). Beside the above, they have higher pH along with inorganic nutrients elements, e.g. Ca, K, Mg, etc., suggesting their use in agriculture as soil additive (Jung et al. 2016). Its other applications include its use as an adsorbent for the removal of organic or inorganic pollutants, making them suitable for wastewater treatment (Michalak et al. 2019).

The uses of highly methylated agarose components, which are supposed to possess low melting point and gelling point, may be better seaweed biomass towards proposed bio oil production through pyrolysis. The irregular methylation pattern in the basic galactan skeleton of most of the red seaweeds makes them not to be useful for production of industrially applicable phycocolloids, i.e. production of agar, carrageenan, agarose, etc. Most of the biomass reported for bio-oil are land based, few from seaweeds and as per best of our knowledge no such report is there from red seaweeds *Champia indica* of Indian waters is an abundant biomass containing mainly the λ-carrageenan as the main constituent (Kumar et al. 2011). These seaweeds resources may fulfill the demand for biofuel preparation.

5.2 Experimental

5.2.1 Materials and Method

Champia indica was collected from Okha, Gujarat (India), washed with water followed by drying in oven at 70 °C for 24 h. The dried sample was powdered and sieved to size of 90–150 μm, further samples are stored in airtight zip bag for proximate, ultimate, TGA and FTIR analysis. Proximate analyses consisting of volatile matter, moisture, ash content, and fixed carbon (in weight %) were performed before the TGA analysis. TGA was carried out in Perkin Elmer made TGA-4000 model. Sample (3.0 mg) was loaded in platinum crucible, before the thermal analysis was performed. High-purity nitrogen gas at flow rate of 60 mL min^{-1} was used as carrier gas. Duplicate sets of non-isothermal experiments were performed with heating rates of 20 °C min^{-1}, in the temperatures range of 30–900 °C. During heating, the sample weight and furnace temperature were recorded. Based on the TGA result the pyrolysis was performed to get bio-oil, with and without catalyst discussed later on. The powdered biomass was mixed

with KBr in 4 mg to 600 mg ratio, and spectrum was recorded on a Perkin Elmer Spectrum GX (FT-IR System, USA) with a scan rate of 4 cm^{-1} in the range of 4000–400 cm^{-1}. Neat FTIR was recorded for the obtained bio-oil to characterize the various components present in the sample. Both the FTIR were normalized for analysis purpose. CHNS analysis of biomass, crude, and upgraded oil was recorded on Thermo Scientific FLASH 2000 CHNS/O Analyzer, using argon as carrier gas. The oxygen content was calculated based on difference method. The higher calorific values (HHV in MJ Kg^{-1}) on ash free basis were calculated from the elemental analysis using the following equation (Demirbaş 1997):

$$\mathrm{HHV}\left(\mathrm{MJ\,Kg^{-1}}\right) = 0.01 \times (33 \times \mathrm{C\%} - 142.3 \times \mathrm{H\%} - 15.4 \times \mathrm{O\%} - 14.5 \times \mathrm{N\%})$$

GC–MS to characterize the various organic components was done on Perkin Elmer GC-MS Clarus equipped with an Elite-5 column (30 m × 0.32 mm ID and 0.25 mm film thickness) and an electron ionization detector. The source temperature was set at 300 °C and carrier gas was helium of 99.999 purity. Three microliter injections at inlet temperature of 300 °C of upgraded bio-oil dissolved in methanol were made with an initial hold time of 4 min. A program with 5 °C/min oven ramp rate from 30 °C to 150 °C, hold time 4 min, 10 °C/min oven ramp rate from 150 °C to 280 °C, and final hold time of 5 min was selected. The ion fragmentation pattern was used to identify compounds based on the peaks pattern using NIST library.

5.2.2 Pyrolysis of *Champia indica* Biomass

A laboratory scale externally heated fixed bed pyrolysis batch reactor was used for production of bio-oil from *Champia indica* biomass. The effective length and diameter of the stainless steel made reactor are 45 cm and 16 cm, respectively. The reactor with biomass (100 gm) was heated electrically up to 450 °C with PID controlled electric heater. A nitrogen hole was used in the pyrolysis chamber to provide uniform heating across the cross-section of the reactor chamber and to create inert environment in the pyrolysis chamber. After the completion of the pyrolysis reaction, the collected liquid mixture was mixed with chloroform and filtered by suction filtration to remove the solid suspended matter. The collected liquid was separated into chloroform soluble and insoluble parts. The insoluble part was extracted with chloroform three times and all were mixed to get chloroform soluble bio-oil components. The chloroform soluble part was then recovered by evaporating the solvent and the remaining product after solvent evaporation was denoted as bio-oil. The mass balance of the various components, i.e. bio-oil, biochar, and biogas were done according to following equations. No attempt was made to get mass balance of chloroform insoluble components assuming that they were mostly water-soluble components.

$$\text{Biooil (wt\%)} = \frac{W_{\text{Biooil}}}{W_{\text{Biomass}}} \times 100$$

$$\text{Biochar (wt\%)} = \frac{W_{\text{Biochar}}}{W_{\text{Biomass}}} \times 100$$

$$\text{Gas (wt\%)} = 100 - \text{wt\% of (Biooil + Biochar)}$$

where W_{Biooil} is the mass of the bio-oil; W_{Biomass} is the mass of algal biomass utilized for pyrolysis; W_{Biochar} is the mass of solid residue after remaining after pyrolysis including the ash and removing the catalyst component. The yield of the gaseous product was obtained by the difference method.

5.2.3 Preparation of the Catalyst

The ZSM-5 zeolite ($SiO_2/Al_2O_3 = 55$) used in this work was purchased from Sigma Aldrich. It was calcined under air atmosphere at 500 °C overnight in furnace, exchanged with 1.0 M solution of NH_4NO_3, via the ion-exchange process, by stirring for 2 h at 80 °C. The exchanged catalyst was filtered, washed with water, dried at 120 °C for 10 h, and finally calcined at 500 °C overnight. The prepared catalyst was stored in the vacuum desiccator for further use.

5.2.4 Catalytic Upgradation of Bio-oil

In different sets of experiment, 10 ml of bio-oil was mixed with 30 ml of toluene, 10 ml of water, and 20–80 mg of the catalyst in an autoclave. To provide in-situ hydrogen demand and simultaneously removal of the nitrogen, 5 mL of formic acid was added to the mixture. After the reaction time (30–120 min) and reaction temperature (120–200 °C), the reactor was transferred to a cold-water bath to quench the reaction. The mixture was removed from the autoclave, and catalyst was separated from organic and aqueous phases via centrifugation at 600 rpm for 15 min. After separating the oil phase from water and evaporating the toluene, CHNS analysis of the upgraded oil was carried out to measure amount of nitrogen removed as well as to determine HHV of the upgraded bio-oil.

5.3 Results and Discussion

5.3.1 Proximate and Ultimate Analysis of Biomass

Proximate, ultimate analysis *Champia indica* along with some other red macroalgal biomass reported by other researchers are given in the Table 5.1. The volatile content of *Champia indica* was higher as compared to *Kappaphycus alvarezii* (Das et al. 2017) and *Polysiphonia elongata* (Ceylan et al. 2014). The ash content was higher

Table 5.1 Proximate, ultimate analysis and higher heating values of *Champia indica*, other red macroalgal biomass and biochar

	Proximate analysis (%)				Ultimate analysis (%)						
Macroalgal species	Moisture	Volatile matters	Ash	Fixed carbon	C	H	N	S	O[a]	HHV (MJ/Kg)[b]	References
Gracilaria skottsbergii	10.10	–	40.20	–	23.7	3.9	1.9	–	30.30	13.8	Francavilla et al. (2015)
Gracilaria gracilis	9.13	–	19.98	–	31.5	5.1	4.1	1.61	37.68	14.5	Norouzi et al. (2017)
Kappaphycus alvarezii	14.98	52.29	17.46	14.97	31.3	5.7	0.6	4.41	57.97	13.7	Das et al. (2017)
Agar waste	7.92	–	7.7	–	44.0	6.0	5.2	1.02	36.13	18.1	Ferrera-Lorenzo et al. (2014)
Polysiphonia elongata	11.55	48.20	27.50	12.80	35.8	5.9	6.9	–	51.40	15.6	Ceylan et al. (2014)
Champia indica	5	53.8	30.3	10.9	40.2	6.8	3.2	2.1	45.2	15.6	Present study
Biochar	–	–	–	–	60.6	1.5	1.4	0	36.5	16.3	Present study

[a]By difference

[b]Calculated according to the equation-HHV (MJ Kg^{-1}) = $0.01 \times (33 \times C\ \% - 142.3 \times H\ \% - 15.4 \times O\ \% - 14.5 \times N\%)$

than other red macroalgal biomasses, which may be due to sulfated nature of the carrageenan as the key polysaccharides of *Champia indica*. The value was much lower as compared to *Polysiphonia elongata* (Ceylan et al. 2014). The ash content was also lower as compared to brown macroalgal biomass, e.g. 36.82% ash was reported for *Sargassum* sp. (De Bhowmick et al. 2018). The low ash content is vital as due to high ash content results in clump formation in pyrolysis procedures. It also yields ineffective rate of heat transfer, suggesting the pyrolysis process of the above biomass can be performed through batch reaction mode.

5.3.2 Yield of Bio-oil, Biochar, Gas, Upgraded Oil and Their HHV

The bio-oil, biochar, and gas contents of the pyrolysis process were found to be 28, 40, and 32% with respect to biomass, respectively. The yield of upgraded bio-oil using catalyst was found to 80%, with respect to bio-oil suggesting the overall yield 22.4% of biomass. to According to the results, *Champia indica* biomass is supposed to be higher carbon and oxygen containing biomass having 6.8, 2.1, and 4.2 weight percentage of hydrogen, sulfur, and nitrogen, respectively. The bio-oil obtained from pyrolysis was rich in carbon content from 40.2% as compared to the biomass having 64.3% of carbon, along with removal of sulfur, and decrease in nitrogen content. The final residue in the form of biochar was ash rich component having HHV of 16.3 MJ/Kg, suggesting that it can be a good source of carbon rich micronutrient for soil application (Bird et al. 2011).

The upgradation via use of ZSM-5 as catalyst also further increases the content of carbon and hydrogen, through decrease of nitrogen and oxygen content. The trend in the upgraded bio-oil was increased with time, catalyst amount, and reaction temperature (Hosseinpour et al. 2017). The optimization of parameters results in the upgraded bio-oil having higher calorific values in between 27.1 and 34.6 MJ/Kg, suggesting that catalysis process is promising for getting better fuel in terms of calorific values. The best higher heating value of the upgraded oil was obtained for 50 mg of catalyst, 150 °C reaction, and 90 min reaction time (Table 5.2). During the process, the HHVs were increased to 34.6 MJ/Kg in the upgraded bio-oil as compared to 15.5 and 25.7 MJ/Kg for biomass and bio-oil, respectively. This suggest that the use of ZSM-5 according to given condition improves HHV of the pyrolysis based obtained bio-oil from red algal biomass.

5.3.3 Thermogravimetric Analysis

Fig. 5.1 shows the TGA (a) and DTG (b) result of at the heating rate of 20 °C per min, under nitrogen as carrier gas of *Champia indica* biomass. The residual weight at final temperature of 900 °C was ~23%, suggesting the biochar contains inorganic compounds, mainly due to sulfated carrageenan components. The TGA and DTG observation was found to be in good agreement with other reported macroalgal biomass.

Table 5.2 Reaction condition for upgradation of bio-oil using ZSM-5 catalyst and their higher heating values

S. No.	Reaction condition[a]			Yield (%) (w.r.t to bio-oil)	Ultimate analysis					HHV (MJ/Kg)[c]
	Catalyst amount (mg)	Temperature (°C)	Time (min)		C	H	N	S	O[b]	
Bio-oil	–	–	–	–	64.3	6.3	2.3	0.0	27.1	25.7
1	20	120	30	60	65.0	7.0	2.1	0	25.9	27.1
2	30	150	60	65	65.9	6.8	1.9	0	25.4	27.3
3	40	150	90	70	66.5	7.3	1.3	0	24.9	28.3
4	**50**	**150**	**90**	**80**	**76.2**	**8.3**	**0.1**	**0**	**15.4**	**34.6**
5	50	160	120	70	73.0	8.1	0.1	0	18.8	32.7
6	30	180	90	80	67.2	7.5	0.1	0.5	24.7	29.0
7	80	200	60	70	70.0	8.0	0.1	0	21.9	31.1

[a]Bio-oil (10 ml) + toluene (30 ml) + Water (10 ml) + Formic acid (5 ml)

[b]By difference

[c]Calculated according to the equation- $\text{HHV (MJ Kg}^{-1}) = 0.01 \times (33 \times \text{C \%} - 142.3 \times \text{H \%} - 15.4 \times \text{O \%} - 14.5 \times \text{N\%})$

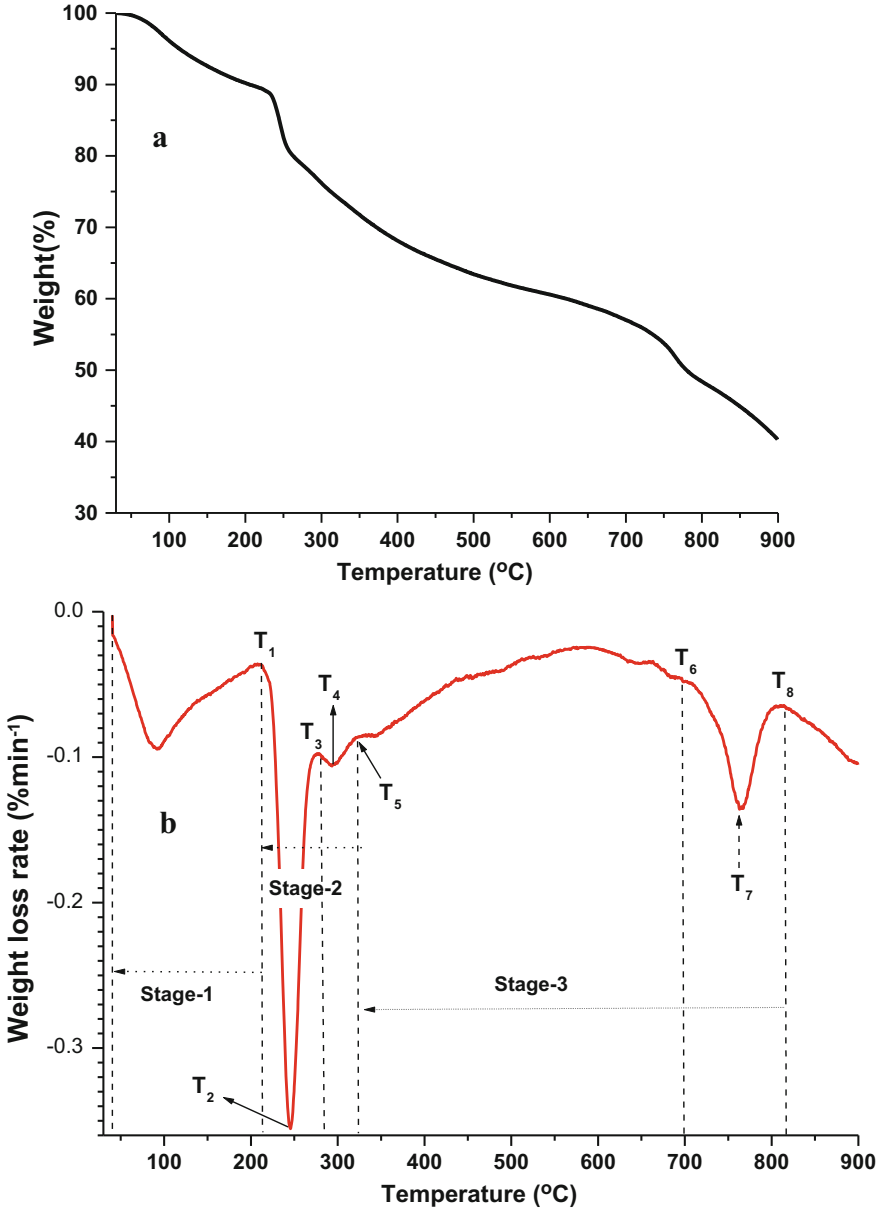


Fig. 5.1 (**a**) TGA and (**b**) DTG of *Champia indica* biomass during heating @ 20 °C/min

The DTG suggests that the pyrolysis of the biomass has been occurred through three stages (Fig. 5.1b). In the first stage, the degradation continues until 222 °C (T_1) from room temperature. This loss is mainly due to moisture and other low boiling temperature components (El-Sayed and Mostafa 2014). It accounts for a total of 10.4

weight percentage w.r.t to biomass (i.e., 100%). The average rate of reaction during this temperature was 1.23 weight percentage per minute. The second stage of degradation consists of two different zone of pyrolysis-based degradation suggesting that carrageenan and cellulosic components degrade at different pyrolysis temperatures. Since both are the carbon, hydrogen, oxygen rich polysaccharides, the degradation occurs within nearby temperature. The sharp mass loss was occurred at 246 °C (T_2) and 292 °C (T_4) for these two zones. The last stage of degradation continues until 900 °C, with another sharp mass loss at 763 °C (T_7), suggesting that secondary cracking may had occurred at high temperature (Ross et al. 2008). The metal oxides of the seaweed biomass formed during the conversion process play an important role during this stage of pyrolysis.

It was observed that above 700 °C, another weight loss was happened owing to additional devolatilization of the formed biochar. The above weight loss may be happened due to breaking of bonds of C–C and C–H, of the biochar. The decomposition features of *Champia indica* biomass pyrolysis are shown in Fig. 5.1(b) and were concluded that the main pyrolysis process occurs in between 210 and 275 °C for high heating rate, resulting in the formation of volatile matter as well as biochar. The higher heating rate of 20 °C min^{-1} boosts the heat transfer between sample and surroundings (Kim et al. 2012). The heating rate also affects the T_{max} of the biomass pyrolysis suggesting that with high heating rates, carbohydrate as well as protein components, starts decomposing simultaneously.

It is proposed that *Champia indica* can be a sensible applicant for biofuel production via pyrolysis. Carrageenan (alpha, lambda, iota, and pyruvated ones), cellulose, and proteins are the key constituents of red macroalgal biomasses with small amount of lignin. Due to multicomponent mixture, the pyrolytic process appears complex since it involves many reactions. The degradation of polysaccharides, e.g. carrageenan's, hemicellulose, and cellulose, occurs first at lower temperatures mainly for dehydration, followed by higher temperature decomposition. Since hydrogen bond has greater impact on degradation, cellulose and carrageenan present in the *Champia indica* degrade at a reasonably higher temperature as compared to hemicelluloses.

The constituents of macro algal biomass play important roles using thermochemical production of biofuels and biochemical. Although the macroalgae does not have so much lignocellulosic structures as compared to terrestrial plants, they have protein, lipids, carbohydrate containing alkali earth metals, and other inorganic ions; the trend of pyrolysis of macroalgal and terrestrial biomass are similar. The presence of higher ash contents has been reported to show the promotion of secondary cracking pyrolytic reactions in gaseous phase, which results in the lowering of the peak decomposition temperatures and yield of volatile matters.

5.3.4 FTIR Analysis

5.3.4.1 FTIR of *Champia indica* Biomass

The FTIR spectra of the *Champia indica* are shown in Fig. 5.2(a). The stretching vibrations in between 3600 and 3200 cm^{-1} were assigned to hydroxyl (–OH) group of alcohols and phenols, as well as secondary amines (-NH) groups of proteins (Bird et al. 2011). The stretching vibrations of CH, between 3000 and 2800 cm^{-1} (Gomez-Serrano et al. 1996), and CH deformation vibrations between 1490 and 1340 cm^{-1}, were assigned to the alkanes groups. Stretching vibration of carbonyl group (=CO) at 1642 cm^{-1} pointed to the presence of aldehyde and acid in the biomass, in lower amount (Jena and Das 2011). Additionally, the absorptions between 1300 and 950 cm^{-1} were attributed stretching of CO group present in the various alcohols and phenols. Other noticeable peaks among 900–800 cm^{-1} were assigned to carrageenan components of the biomass (Kumar et al. 2011).

5.3.4.2 FTIR Analysis of Biochar

The FTIR spectra of biochar are given in Fig. 5.2(b), and assigned peaks were divided into different ranges of wavenumber which include the C–H stretching, C=C stretching, C–H bending, and C=O bending. It was found that some of the peaks (i.e., O–H stretching and C=O bending) that originally present in the biomass were no longer observed from the spectra of biochar. The broad peak of biochar detected at 3451 cm^{-1} attributes to the O–H stretching that indicates the presence of chemical compounds with hydroxyl functional groups such as phenolic or aliphatic alcohol and carboxylic acids (Michalak et al. 2019). Absence of hydroxyl group in the FTIR spectrum of biochar confirms that –OH containing components were escaped in the form of volatile matter (CH_3COOH, CH_3OH etc.) through the disintegration process of hemicellulose, and via cracking of R–OH units during the pyrolysis. At 2800–3000 cm^{-1}, the peak presence in this range was due to chemical compounds having C–H stretching functional group. Presence of sharp peaks of C–H stretching was detected at 2925 and 2852 cm^{-1}, representing the asymmetric and symmetric C–H stretching, of an alkane compound in the biomass. These peaks mainly derived from the C–H stretching in $–OCH_3$, $–CH_3$, and $–CH_2–$ groups. However, the peak intensities of these functional groups were lower, signifying that the demethylation as well as transformation of $–OCH_3$ into carbon-containing species by the dissociation of the R–O–R bond during the pyrolysis. The peak at 1730 cm^{-1} directs the occurrence of compounds having the >C=O stretching, e.g. aldehydes, esters, ketones, and carboxylic groups (Jena and Das 2011). The absence of C=O stretching along with low intensity peak of C=O bending in the spectrum may be observed via the elimination of oxygenated compounds. The pyrolysis, through the decomposition of cellulosic, carrageenan components as volatile matter, resulted in the residual carbon as the biochar having lower oxygen content. This was concurred with the low oxygen content detected from the biochar (Table 5.1) although higher metal oxides are possible due to higher ash content of the biomass. The band in at 1642 cm^{-1} was assigned to compounds with C=C stretching, e.g. alkene or aromatic compounds (Jena and Das 2011). The

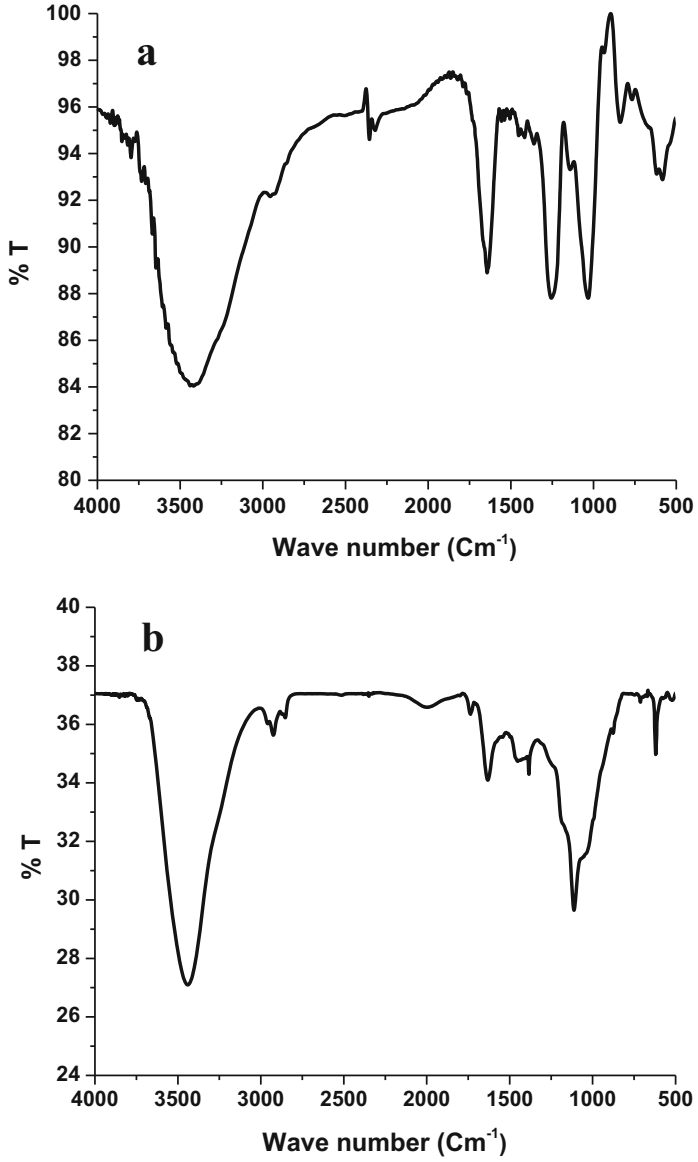


Fig. 5.2 FTIR spectra of (**a**) *Champia indica* biomass and (**b**) Biochar

spectra of biochar have better peak intensity specifying that the biochar has significant amount of alkene/aromatic compounds In conclusion the FTIR spectra of biochar showed that the oxygenated compounds were reduced after the pyrolysis. The resulted biochar would contain polycyclic structure components having higher aromaticity and low oxygen content.

5.3.4.3 FTIR of Bio-oil and Upgraded Bio-oil

From the FTIR spectra of bio-oil and catalytically upgraded bio-oil (Fig. 5.3), it was confirmed that the functional group transformation occurred during pyrolysis and upgradation process. The C–H vibration at 1350–1490 and 3000–2800 cm^{-1} (Gomez-Serrano et al. 1996) present in all the samples implying a high C and H content, which was also confirmed by the CHNS elemental analysis. The peaks relevant to be of acids, methyl, and alkyl groups as well as alkanes and were detected in the GC–MS analysis. The carboxylic fatty acids, in particular, were detected in the both the bio-oil and upgraded bio-oil samples from FTIR spectra, arising from the C=O bond stretching at 1700–1730 cm^{-1} and 1500–2000 cm^{-1} (Jena and Das 2011). Similarly, the carrageenan components at 1199–1400 cm^{-1} from the C–OH, C–C–H, and O=C–H bonds are non-existent after pyrolysis and upgradation process confirms that these bonds are thermally broken. The presence of nitrogen in the bio-oil after pyrolysis is visible as signal between 1600 and 1680 cm^{-1} and 1525–1575 cm^{-1} was seen which might be due to the N–H vibration, confirming the key metamorphosis between the fossil fuel and bio-oil obtained via pyrolysis. The amides, amines, and nitrogen-containing heterocycles that were present in the

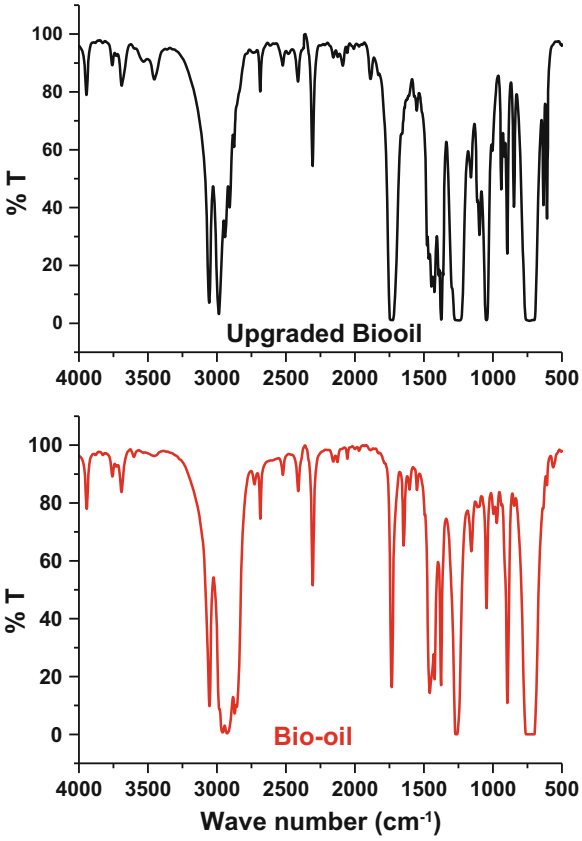


Fig. 5.3 FTIR spectra of bio-oil and upgraded bio-oil

bio-oil have decreased significantly in the upgraded bio-oil. A curious verdict obtained from the spectra displays the appearance of aromatic components in the 700–900 cm^{-1} range (Meesuk et al. 2012; Hu et al. 2018), which are mainly present in the fossil crude. Branched alcohol and phenol species were detected in the 1000–1260 cm^{-1} and 1210–1320 cm^{-1} shifts (Meesuk et al. 2012). The absence of moisture was confirmed by a lack of response in the 3000 and 3500 cm^{-1} range.

5.3.5 GC–MS Analysis of the Upgraded Bio-oil

The chemical composition of the upgraded bio-oil was determined using GC–MS analysis. Table 5.3 illustrates the major peaks identified using NIST library for various types of the components present in the upgraded oil, using mass fragmentation of the selected peak. The main intention was to identify the components present in the sample; therefore, Table 5.3 contains the assigned class of compounds at the given RT based on NIST data. Since only volatile compound in the samples was passed through the GC column and identified. According to the results given by GC–MS, the main volatile chemicals in the upgraded bio-oil were alkanes, furan, esters, alkenone, C-6 sugars and their derivative in various proportions.

5.4 Conclusions

The thermal degradation analysis based on TGA data of *Champia indica* biomass is supposed to occur through multiple step complex reactions involving many reactions. The degradation of the constituents components, e.g. carrageenan's, hemicellulose, and cellulose, occurs first at lower temperatures mainly for dehydration, followed by higher temperature decomposition. Bio-oil content, biochar content were 28 and 40% with respect to biomass, respectively. The biochar has higher ash content, suggesting it as mineral rich soil conditioner suitable for organic farming. The pyrolysis yielded bio-oil having HHV value of 25.7 MJ/Kg and further upgradation of the bio-oil using ZSM-5 zeolite as catalyst yielded hydrocarbon rich bio-oil. The upgraded bio-oil was found to have HHV of 34.6 MJ/Kg. The marine biomass of Champia *indica* biomass can be a promising feedstock for pyrolysis to meet the demand for alternative biomass resources.

Table 5.3 Identified products of upgraded bio-oil using ZSM-5 by GC–MS

S. No.	RT (min)	Compound
1	5.15	2,5-dimethylfuran
2	5.79	1-hydroxy-2-butanone
3	7.19	Butanedial
4	8.14	Furfural
5	9.06	Ethyl furan
6	9.48	2-furanmethanol
7	9.94	1-(acetyloxy)-2-propanone
8	11.00	1,3-dihydroxy-2-propanone
9	11.20	5-(hydroxymethyl)dihydro-2(3H)furanone
10	11.67	1-(2-furanyl)-ethanone
11	12.36	5-methyl-2(5H)-furanone
12	14.49	2H-pyran-2,6-3(H)-dione
13	14.56	5-methylfurfural
14	15.41	5-acetyldihydro-2(3H)-furanone
15	16.43	2,3-dimethyl-2-cyclopentenone
16	17.05	2-methylphenol
17	17.95	4-methylphenol
18	18.60	2,5-dimethyl-4-hydroxy-3(2H)-furanone
19	19.07	Galactopyranose
20	21.66	2,3-dihydrobenzaldehyde
21	22.41	1,4:3,6-dianhydro-D-glucopyranose
22	22.83	2-methoxy-4-methylphenol
23	26.27	4-methyl-1,2-benzenediol
24	27.07	3-methoxy-5-methylphenol
25	29.48	4-hydroxy-3-methoxybenzaldehyde
26	30.97	2-methoxy-4-propenylphenol
27	33.12	D-galactofuranose
28	34.77	3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone
29	36.27	2-methyl-2-cyclopentenone
30	36.55	1-(2-furanyl)-2-hydroxyethanone
31	36.75	3-ethyl-2-hydroxy-2-cyclopentenone
32	37.85	4-hydroxy-3,5-dimethoxybenzaldehyde
33	39.10	1-(4-hydroxy-3,5-dimethoxyphenyl)ethanone
34	39.61	4-hydroxy-2-methoxycinnamaldehyde
35	40.94	3,5-dihydroxy-6-methyl-2,3-dihydro-4Hpyran-4-one
36	42.32	4-biphenyl ethyl ketone
37	43.59	4-allyl-2,6-dimethoxyphenol
38	45.31	3,5-dimethoxy-4-hydroxycinnamaldehyde

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Conflict of Interest The authors declare there is no any conflict of interest.

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Seaweed Biomass and Microbial Lipids as a Source of Biofuel

6

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Abstract

The present chapter aims to highlight the promising alternative sources of bioenergy production. In this chapter, we discussed how seaweed biomass could be utilized for the derivatization of the biofuels. Seaweeds structure composed mainly of carbohydrates, which constitute some complex polysaccharides. Since they have a small amount of lignin content, it does not require complicated preprocessing like other generation biofuels that consume energy as well as time. There are needs to adopt some cost-effective technologies for efficient biomass conversion of available biomass into fermentable sugars. The chapter also focuses on the uses of conventional ethanolic microbes and oleaginous microbes. Some of the oleaginous yeasts were found to be producing a high amount of lipids that can be converted into biodiesel and are regarded as single-cell oil factories. However, the efficiency of production can be increased with metabolic engineering. Modification in the metabolic pathways and strain improvement can increase the bioenergy production. A new tool CRISPR-Cas9 in genome engineering has been discussed in brief that has significant effects on increasing the production of biofuel.

Keywords

Bioenergy · Biofuel · Biomass · Fermentation · Saccharification and seaweeds

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6.1 Introduction

Today the global population is heading towards the new era of development. For any developmental activities, energy is prime and the utmost demand. Each technology can be hiked with the support of an adequate amount of energy. Besides the development, the overgrowing population also demands a higher production of energy. However, global proliferation resulted in different environmental concerns like the increased emission of greenhouse gases (GHGs), *viz.* carbon dioxide, methane, nitrous oxide, etc.

Thus, in the research sector among the various challenges, one primary concern is energy security and sustainability. Apart from this, it also urges the researchers to find alternative and potential sources with sustainable and greener technologies for energy production. Fossil fuel or conventional fuel is a prime cause of environmental pollution. Hence to combat this issue, non-conventional fuel can be adopted. The non-conventional biofuel can be called green power and considered to be one of the best alternatives. It is considered to be the sustainable source of energy which is derived from the biomass. It can be processed and converted into transportation fuel. Biofuel can be used in a different form that is solid, liquid, or in the gaseous state. There are several advantages of biofuel over conventional fuel. They are greener in combustion and showed less greenhouse gas emissions. They are categorized in different generations based on their source and processing. Among all the generations, third-generation biofuels were given more emphasis, and this remained the focus of the present chapter.

Seaweeds are considered to be the untapped resource that comes in this generation. They are present in the intertidal and subtidal zones of the seacoast. These are regarded as the marine sea plants having high photosynthetic activity and growth rates as compared to terrestrial plants. They have a unique biochemical composition, which includes different types of polysaccharides. Due to their distinct composition, they have a wide range of the application and have high economic importance. Thus, it is considered to be the best candidate in this class. Globally the total coastline is of approx. 3,56,000 km (Central Intelligence Agency 2016) and global production of seaweeds is about 30 million tonnes (Ferdouse et al. 2018). India has a coastline of about 7500 km (Reddy et al. 2006) and enriched with many diverse species of seaweeds. Thus it provides a vast market of seaweeds availability. Seaweed biomasses are less explored with no competition for land and food. Unlike other generation biomass, they do not require complex pretreatments. These several advantages tend the researchers towards the more exploration of them. Apart from this, microorganism also plays an essential role in energy development. Certain microorganisms use biomass as their feeding material and synthesize fuel derivatives and other important industrial products of high economic values. From this characteristic, they can be considered as the powerhouse or the fuel generating factories. Seaweeds were composed of some complex polysaccharides. Hence low-cost pretreatment methods must be adopted for the conversion of the seaweed biomass into fermentable hydrolysate (Sudhakar et al. 2018; Behera et al. 2015). There is much biotechnological advancement performed in this area, like metabolic

engineering. These modifications are done by strain improvement, modifications in the enzymatic pathways, which improve their efficiency and increase production (Kavscek et al. 2015). Amidst all advancements, CRISPR (clustered regularly interspaced short palindromic repeats) is a breakthrough in the field of biotechnology. It is the novel technology and can serve as a boon in the derivation of the energy. Certain genomic modifications were done in the microorganisms like yeast and bacteria (Shapiro et al. 2018). With the help of this technology, one can speed up the process of modification as it tends to develop a tolerance for the various inhibitors produced during the complex pretreatment as well as specific genes responsible for the fuel and the pathway is upregulated (Dai and Nielsen 2015).

6.2 Biomass: Inexpensive and Non-exhausting Source of the Millennium

Globally, biomass comes under one of the largest available energy sources. It can be naturally available, economical source, and can be employed at any instance. Moreover, it also had an advantage that it can be converted into liquid transportation biofuel. Presently biomass available in India is around 500 million metric tonnes, and energy generation is probably around 18,000 megawatt (MW) (Gaurav et al. 2017). Typically, biomass is terrestrially generated from the agricultural, forest, and industrial residues. In the aquatic biomass, the prime source of biomass availability is in the form of marine organisms that includes seaweeds. The advantage of the aquatic biomass is that it can be produced in lesser time, and there is no competition for the land and the freshwater. Thus, seaweeds can be considered as ideal biomass for clean fuel development as it is less explored and can be recycled in a short interval. Biomass undergoing the non-programmed decomposition might release toxic pollutants into the environment. These can be minimized by developing strategies for efficient management of the biomass decomposition. Hence, various pollutants like GHGs can be reduced (Lee and Lavoie 2013; Behera et al. 2015). In doing so, present on-going research focuses on the development of the new energy sources with efficient utilization and decomposition of the biomass for the development of the green fuel.

6.3 Biofuel: Need as an Alternative to the Conventional Fuel

The world is moving day by day towards the increased hunger for development and growth. It has heightened the demand for energy; this urges the exploration of the new energy sources and technologies for sustainable consumption. Thus, to accomplish this, biofuel attracts prime attention to the development of bioenergy.

Biofuel is the fuel or energy derived from biomass through biological carbon fixation, which can be easily accessible from nature. Biofuel has several advantages over other conventional biofuels that are enumerated further. It comes with the objective of the cleanest combustion that is with less emission of CO_2 and other GHGs. They can be utilized in different forms that are solid, liquid, and gas. For

running our economy, liquid transportation fuel is required, which becomes one of the essential aspects of fuel properties. As mentioned previously, biofuel can be utilized in liquid form. In the context of liquid transportation fuels, the significant bioethanol producers in the world are the USA (1.7 Mt) and Brazil (1.5 Mt). A similar trend can be observed in the case of biodiesel in which both of these countries are the leading producers (Proskurina 2018). The biofuel can be categorized into different types, solid fuels include wood chips, charcoal, firewood, etc. In liquid bioethanol, biodiesel, biobutanol can be utilized and can serve as a liquid transportation fuel. In gaseous form, it exists like methane, biohydrogen. Secondly, by reuse, they can be classified into renewable, i.e., biofuels and nonrenewable like fossil fuels (Bhatt et al. 2018). Further, by generation, they can be classified into four generations and had been discussed in Sect. 6.4.

6.4 Generation of the Biofuels

The biofuels are categorized into four different classes based on the source from which these are generated and the conversion technologies. These generations are first-, second-, third-, and fourth-generation biofuels (Table 6.1).

6.4.1 First-Generation Biofuels

The primary feedstock of this generation is food crops. The energy is derived from the crops, which are mostly used as food. These crops are abundant in the sugar, starch, and some oleaginous crops also used for the production of the oil (Naik et al. 2010). The food crops which are used in the high proportionate are the soybean,

Table 6.1 Generations of biofuels and their sources

Generation of biofuels	Sources	Biofuel produced	References
First-generation of biofuels	Cereal and sugar crops like maize, barley, corn, sugarcane, sugar beet	Bioethanol and biobutanol	Rodionova et al. (2017)
	Oil crops like soybeans, rapeseeds, sunflower, palm coconut. Used vegetable oils	Biodiesel	Rulli et al. (2016)
Second-generation of biofuels	Oily crops like jatropha, Miscanthus, waste vegetable oils, lignocellulosic biomass straw, wood	Bioethanol and biodiesel	Antizar-Ladislao and Turrion-Gomez (2008)
Third-generation of biofuels	Microbes, microalgae, and seaweeds	Bioethanol and biodiesel	Behera et al. (2015)
Fourth-generation of biofuels	Genetically modified microalgae	Bioethanol and biodiesel	Abdullah et al. (2019)

sugarcane, and maize. The yield of this generation is higher, but there are many shortcomings related to it. Some of the significant drawbacks are like there is a competition between land and food along with freshwater and the use of pesticides. This generation has some advantages too as it uses direct and straightforward technologies like fermentation, transesterification, and anaerobic digestion (Sudhakar et al. 2018).

6.4.2 Second-Generation Biofuels

In this generation, energy is derived from the non-food feedstocks that are mostly agricultural and industrial wastes like lignocellulosic biomass that mostly comprises of the cellulose. The advantage of this generation biofuel is that it does not compete for the food as these feedstocks are not used as a food supplement. Thus, it adds the advantage to these types of fuels. However, there is a particular limitation in them like it requires complex pretreatment and processing before conversion into the fuel. The technologies required for preprocessing like thermochemical conversion and enzymatic treatments increase the costs, and hence the industrial scale-up becomes an issue (Dutta et al. 2014; Saladini et al. 2016).

6.4.3 Third-Generation Biofuels

In this generation of biofuels, low-cost feedstocks were included, which were mostly derived from the wastewater and low input fields like marine resources. The prime feedstocks are the microalgae and macroalgae. These do not require complex pretreatments as in the second-generation (2G) fuels as well as no use of freshwater (Khatri et al. 2019). Simple conversion technologies are required for fuel production as well as the lowest GHGs emissions (Dutta et al. 2014; Saladini et al. 2016).

6.4.4 Fourth-Generation Biofuels

In the fourth-generation biofuels basically organisms related to the high photosynthetic activity are utilized for the production of biofuels. Among the various organism, algae are the groups which have high photosynthetic activity. These marine microalgae are present in abundance in the marine environments like fresh, salty, or brackish seawater. In this generation, metabolically and genetically engineered microorganism was used for fuel production. Thus, this generation of biofuels can also be regarded as advanced biofuels. From this generation, biofuels can be derived in the form of gaseous biofuels, bioethanol, biodiesel, and biobutanol. Various strategies are involved in the enhanced production of the fourth-generation biofuels. Some of them are improving photosynthetic efficiency, light penetration, and photoinhibition reduction. Generally, microalgae were genetically modified with advanced biotechnological tools like Zinc finger nuclease (ZFNs), clustered

regularly interspaced palindromic sequences CRISPR/Cas9, and transcription activator-like effector nucleases (TALEN). These are customized endonucleases specially designed for the targeted genome editing. These modifications lead to the increment of the triacylglycerides (TAGs) production. Some of the species which are modified with these advanced engineering techniques are *Chlamydomonas reinhardtii, Cyanobacterium*, *Synechococcus elongates, Nannochloropsis oceanica,* etc. (Dutta et al. 2014). There are several advantages in the fourth-generation biofuel production like carbon sequestration, assimilation and wastewater treatment by bioremediation. Among various advantages, there are many limitations related to this generation of biofuels. Some of the limitations are lower production of the biomass, higher cost of the harvesting and production of the toxins from algal blooms in open pond culture. The release of the genetically modified organism in an open environment can lead to the emergence of the risk of health and environmental concerns (Abdullah et al. 2019).

6.5 Seaweeds as a Potential Source of Biofuel

Due to the alarming rate of globalization, industrialization, and the overgrowing population, the demand for energy increased. Subsequently, with this, the amount of GHGs emissions also got raised. It forces us to take initiatives towards the approach for the development of the sustainable source for the development of biofuels. Bioenergy is the most promising source of power, which is much greener than conventional fuels and comes with the motive of cleaner combustion (Marquez et al. 2015). Since biofuel has been developed from many sources as generated in the form of first-generation (1G) and 2G biofuels but each one of them has several disadvantages. The biggest problem includes competition between land, food, and freshwater. Secondly, the use of pesticides and complex pretreatment before fuel development also reduces efficiency and production. Since these feedstocks are preferred due to higher biomass generation but these possess significant disadvantages. Considering their limitation, seaweeds were identified as the ideal feedstock in the biofuel production. As it does not compete for the land and food with no requirement of the freshwater source and also available at a cheap cost (Jambo et al. 2016).

Seaweeds or marine macroalgae are the photosynthetic sea plants that do not have true leaves, root, and stem but have structures that resemble it. They are very diverse and are considered to be the prime producers in the sea (Sudhakar et al. 2018). As compared to the terrestrial biomass, they have high growth rates and high biomass yields. Seaweeds are considered to be one of the sustainable sources for biofuel. Therefore, seaweed cultivation should be given more emphasis as it can be quickly grown in saline water with no requirement of land and additional nutrients. Apart from the fuel seaweeds are utilized in many other industrial commodities (Tiwari and Troy 2015).

Seaweeds are very diversely present in nature so based on pigmentation seaweeds are classified into the three main groups, i.e., red, brown, and green seaweeds (Rioux and Turgeon 2015; Dawes 2016).

Green algae or the Chlorophyceae contain chlorophyll a and b in a similar ratio as in the higher plants and also the biomass chemical composition in a similar aspect. There are about 4500 species of green algae (1500 species in seawater). Some species reported for biofuel production are *Ulva* sp., *Chaetomorpha linum* sp., etc. (Bikker et al. 2016; Yahmed et al. 2016).

Red algae or the Rhodophyceae contains the chlorophyll and phycobilin proteins that are phycoerythrin and phycocyanin. Further, it is classified into two subclasses that are Florideophycidae and Bangiophycidae. There are about 4000–6000 species of red algae. Some species reported for the fuel production are *Gracilaria* sp., *Kappaphycus* sp., and *Gelidium* sp. (Kim et al. 2015; Hessami et al. 2018; Sukwong et al. 2018).

Brown algae or the Phaeophyceae contains the chlorophyll a and c, β-carotene, and the xanthophylls. There are around 1500–2000 species present (Jung et al. 2013). Some of the potential fuel-producing species as *Sargassum* sp., *Laminaria* sp., and *Saccharina* sp. (Pablo et al. 2019; Hou et al. 2015).

6.6 Proximate Composition of the Seaweeds

Seaweeds or marine macroalgae are well known for the reservoirs of the minerals, vitamins, and carbohydrates. They have high nutritional value so that they can be used as food supplements. These can be used for various purposes like in the fertilizers, pharmaceutical applications, production of biofuels, and industrially important products and chemicals. Macroalgae are composed of the water, polysaccharides, proteins, minerals, lipids, vitamins, dietary fibers, and some secondary metabolites. Generally, seaweeds have approximately 80–90% of water content, 5–15% of moisture content, 10–50% ash content, 35–60% of carbohydrates, 5–35% of proteins, and 1–10% of lipids (Jung et al. 2013).

6.6.1 Major Constituents (Proteins and Minerals)

The nutrients consist mainly of protein and minerals. The amount of proteins in seaweeds varies with different species. The crude protein was found to be different in the different varieties. It was seen that the lowest protein content was found in the brown species, moderate in the green seaweeds, but an exception was seen in the *Ulva* sp. and highest in the red seaweeds. In *Porphyra* sp., the protein content is seen as the highest (MacArtain et al. 2007). The protein content also varies in the seaweeds according to the seasons. Seasonal variations also contribute to the changes in the protein content.

Seaweeds are considered as one of the most abundant sources of mineral content. They contain significant amount of minerals that they absorb from the marine environment. These nutrients composed of essential minerals are important for human consumption. They also play a role in some vital reactions and act as a cofactor. These minerals are categorized into two groups, i.e., essential elements

(Na, K, Ca, and Mg) and trace elements (Fe, Zn, Mn, and Cu). The ratio of Na and K in seaweed varies from species to species. Environmental conditions have a direct effect on the health of the seaweeds because, in the case of the water toxicity, some heavy metals like arsenic, chromium, lead, etc. were also detected in some seaweed like *Ulva* sp. (Kamala-Kannan et al. 2008).

6.6.2 Minor Constituents (Lipids and Vitamins)

In minor constituents (lipids and vitamins), seaweeds show richness in the vitamin contents while the lipids account for a very low percentage in them. Many of the seaweed species were found to be rich in vitamin A, B_2, B_{12}, E, C, ascorbic acid, B_2. (Mabeau and Fleurence 1993; Chapman 2012). It was seen that the percentage of vitamin C was found to be highest in some green (*Enteromorpha* sp. and *Ulva fasciata*) and red seaweeds (*Eucheuma* sp.) and a significant amount of the vitamin A in *K. alvarezii*, *Palmaria palmata,* and *Porphyra tenera* (Peng et al. 2015). A higher percentage of vitamin B_1, B_2, and E were found in some red and brown seaweed. It was seen that some of the *Gracilaria* sp. and *K. alvarezii* showed a high amount of the β-carotene. It was seen that the amount of these vitamins varies in different seaweeds under different seasonal conditions (Misurcova et al. 2011).

Further, in minor constituents lipid contents are essential. Seaweeds are a good source of many essential fatty acids. They are present approximately 5–6% of dry weight (Kendel et al. 2015). In marine macroalgae, the lipid content is less, but their composition is profoundly affected by different environmental conditions like climate and geographical conditions. Seaweed lipids mostly comprised of the C-14 to C-22 chain of saturated and unsaturated fatty acids. Total lipids comprised of the nonpolar and polar lipids. Nonpolar lipids consist of monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). Seaweeds can be considered as an excellent health supplement because of the high content of the polyunsaturated fatty acids (PUFAs). These types of fatty acids are very beneficial for human consumption as it has many health benefits. In red and brown seaweeds, PUFAs were found to be higher, but in the case of green seaweed, *Ulva* sp. and some species of the *Caulerpa* show a higher percentage of this (Abomohra et al. 2018; Santos et al. 2019). Among red seaweeds, *Porphyra* and *Palmaria* show the higher content of ω-3 fatty acid (Koutsaviti et al. 2018).

Polar lipids constitute glycolipids, neutral, and phospholipids. These lipids present dominantly in seaweeds than the neutral lipids but these can be seen higher in the case of red and brown seaweeds.

6.6.3 Secondary Metabolites

In seaweeds apart from these major and minor constituents, one of the important constituents is the secondary metabolites. These are the biologically active compounds that play a specific function. Some of the essential secondary

metabolites present in the seaweeds are terpenes (monoterpene, sesquiterpenes) and these were found in all three types of seaweeds, for example, in green *Caulerpa* species and brown *S. Pallidum*. Phlorotannins are mostly found in brown algae predominantly in the *Sargassum* sp. The next important secondary metabolite in seaweeds is sterols; in marine algae, the commonly found sterols are of carbon skeleton number C26, C27, C28, C29. In red algae commonly found sterols are C27 and C28 and in brown C29 (Peng et al. 2015).

6.6.4 Carbohydrate Composition in Seaweeds

Carbohydrate is the main constituent of any organism and used as an energy source to carry out different life processes for them. In seaweeds carbohydrates are the central part of its constituents and present about 30–60% of the dried biomass. Based on their functions seaweed carbohydrates can be categorized into two types and these are structural and storage carbohydrates.

6.6.4.1 Storage Carbohydrates

Storage carbohydrates are the photosynthetic products and utilized as an energy reserve and also for osmoregulation. Some of the examples of storage carbohydrates are mannitol, sucrose, starch, etc. Floridean starch (α-1,4 glycosidic link glucose homopolymer), starch, laminarin are present in significant amount in the red, green, and brown seaweeds, respectively.

6.6.4.2 Structural Carbohydrates

Structural carbohydrates comprise of the cell wall polysaccharides. Their primary function is to maintain structural functions. Their amount can vary following physical or environmental conditions. These structural polysaccharides provide structural integrity to the seaweeds structure. The cell wall polysaccharides can be composed of several different units of sugars like glucose, galactose, xylose, and sulfated glycans. Some of the examples of such types of polysaccharides are the ulvan, agar, carrageenan, alginate, etc. Other than the basic functions, these polysaccharides can be used for various industrial applications.

Green Seaweeds

In green seaweeds, the structural carbohydrates mainly comprised of the cellulose, hemicellulose, and the ulvan. Ulvan is the sulfated water-soluble polysaccharides that mainly composed of the units of the D-glucuronic acid, L-rhamnose, D-xylose, and D-glucose. This type of polysaccharides is majorly found in the green seaweeds mostly in the *Ulva* sp. (Ito and Hori 1989). Water-insoluble polysaccharides are the cellulose and hemicellulose or the polymer branch of the (β1-4) D-glucose subunits. The sulphated polysaccharides found are the xyloarabinogalactans, glucuronoxylorhamnans, and glucuronoxylorhamnogalactans in the species of *Ulva, Monostroma, Caulerpa,* and *Codium* (Stiger-Pouvreau et al. 2016; Jung et al. 2013; Pereira 2011).

Red Seaweeds

Rhodophyta or the red algae mostly constitute the sulfated galactans. They contain the linear chains of the galactopyranose residues (Ito and Hori 1989). The major type of polysaccharides found is the agar and ~~the~~ carrageenan. They have various applications in the food industry, pharmaceutical, and biology. Agar is commonly called as the agar–agar, which is a hydrocolloid and is divided in the agarose and agaropectin. Agarose is the 1,4 linked α 3,6-anhydo-L-galactose and 1,3 linked β-D-galactose, i.e., Agarobiose. Agar is mostly found in the *Gracilaria* sp. viz. *Gelidium amansii, Gracilaria dura*, and *Gelidium cartilagineum* (Jung et al. 2013; Pereira 2011).

Carrageenan is made of D-galactose and 3,6 anhydrogalactose. It has three forms that are ι (iota), κ (kappa), and λ (lambda). These all differ by the position of the sulfate groups in them. These have various applications in the food, pharmaceutical, and also used for biofuel production. The most prominent species for the carrageenan extraction are the *Kappaphycus alvarezii*, *Eucheuma* sp., and *Chondrus crispus*.

Brown Seaweeds

Phaeophyta or brown seaweeds contain structural carbohydrates like laminarin, alginic acid, fucoidan, and mannitol. In the majority of members the cell wall constitutes the alginate or the alginic acid. Alginate is the polymer of the D-mannuronic acid and L-guluronic acid linked by the β1-4 linkage. Predominantly found in the species like *Sargassum* sp. and *Laminaria digitata*. Fucoidans are the sulfated fucans linked by the 1, 2-linked L-fucose-4-sulfate. Other sugars also include xylose, galactose, and uronic acids in *Fucus vesiculosus* and *Turbinaria* sp. (Jung et al. 2013; Pereira 2011).

Laminarin is a water-soluble polysaccharide and this is mostly found in the brown seaweeds. It is the linear chain of β-1.3-glycosidic bonds. This polysaccharide is found in the species like *Laminaria digitata, Saccharina latissima*, and some sp. of the *Sargassum*.

Mannitol is the sugar alcohol that is mostly found in the brown seaweeds. It is the six-carbon sugar that has various biological functions as it acts as the osmoregulators, osmoprotectant, and reactive oxygen species scavengers. It is commonly found in the species of the *Sargassum*, *Laminaria,* and *Saccharina*.

6.7 Availability of the Seaweed Biomass for the Biofuel Production

Two-thirds of the world's surface is covered with water and the total coastline around the globe is about 3,56,000 km. India has a vast coastline of about 7500 km. It ensures that there can be chances of the sufficient availability of the biomass. In 2010 the total production of seaweeds was approximately 15.8 million tonnes. But now seaweed consumption is drastically increased in the last few decades. It is possibly due to the exploration of the economic importance of the

seaweeds that led to massive consumption. Annually 21 million tonnes of seaweeds were utilized, and aquaculture obtains the majority of it. Top seaweed producing countries are China, Indonesia, Philippines, South Korea, North Korea, Japan, and Malaysia (Ferdouse et al. 2018). These countries primary focus is on the production of industrially important seaweeds (White and Wilson 2016; Ghadiryanfar et al. 2016; Sudhakar et al. 2018). Recently it was reported that the total production of seaweeds had been increased to 30.4 million tonnes. Approximately 1.1 million tonnes of the seaweeds are harvested from the wild habitat. Dominant harvested species are *Gracilaria* sp., Kelp*, Laminaria digitata,* and *Saccharina japonica* (Ferdouse et al. 2018). In the case of the farmed seaweed, around 29 million tonnes of seaweeds are farmed in 50 different countries. Dominant seaweeds cultivated are *Kappaphycus*, *Gracilaria* sp., *Nori, Porphyra* sp. *Undaria pinnatifida*, and *Sargassum* sp. In these countries, seaweed farming and harvesting are done for the food as well as for industrial purposes. India is a tropical country which is located in the southern region of Asia. It is surrounded by sea from three sides and thus has a vast coastline. There are several patches of rocky sea beds and have tidal and intertidal zones. India is enriched with the 271 genera and 1153 species of seaweeds. These are abundantly found in the regions of the Tamil Nadu and Andaman and Nicobar Islands also their presence can be seen in some regions of Gujarat, Mumbai, Goa, and Orissa. In India, the scenario is entirely different from other countries in the case of the utilization of seaweeds. This is because here the seaweeds are not consumed as food but may be utilized for agar production and other applications. Hence here the major cultivation focuses on the production of the *Gracilaria* sp. and *Kappaphycus.* Farming of these seaweeds is done in some parts of Gujarat, Kerala, and Tamil Nadu (Gaurav et al. 2017; Dhargalkar and Pereira 2005).

In India, the net harvest of the seaweeds is around 6.7–6.8 tonnes of the wet seaweed. Majorly harvested species are *Gracilaria* sp., *Sargassum* sp., *Turbinaria conoides*, *Kappaphycus alvarezii*, *Ulva* sp., and *Enteromorpha compressa* (Reddy et al. 2006). Since there is a diverse resource available, but yet much seaweeds are to be explored for fuel production.

6.8 Treatments to the Seaweed Biomass

Before the process of fuel conversion, seaweed biomass undergoes the process of the pretreatments for efficient saccharification. As compared to first- and second-generation seaweed biomass is easier for the pretreatment because this has comparatively low lignin content. The seaweed cell wall is made up of the complex polysaccharide; thus, it has to be broken down into the simpler compounds for effective fermentation. Different types of pretreatments for the seaweed biomass are physical, chemical, and enzymatic or combinations of all (Montingelli et al. 2015; Kadam et al. 2015; Marquez et al. 2015).

6.8.1 Physical Pretreatment

In the physical treatment from biomass, all the debris and mud were washed out. Usually, seaweed contains 80–90% of the water. Then it is dried at 60 °C, or sun drying can also be done until the constant weight is acquired. Then it is subjected to various physical treatments like milling, pyrolysis, sonication, and microwave-assisted methods. For the milling, the biomass is milled for about >0.5 mm size. In pyrolysis, thermal decomposition is done in the oxygen-free environment at high temperature (approx. 400–600 °C) to the solid, liquid, and gaseous phase. In the sonication, the cell wall is disrupted to release out the cellular constituents. In microwave-assisted extraction, the biomass is heated to 90 °C to 110 °C for about 10 to 40 min in case of the *Gracilaria* sp. (Yun et al. 2016; Cao et al. 2019).

6.8.2 Chemical Pretreatment

In the chemical pretreatment, the biomass is subjected to the solvents (acidic/alkali treatments, hot water treatments). Hot water treatment is applied for the agar and carrageenan extraction. The biomass is heated over 85 °C. For the agar, extraction dried and milled samples are (3 gm) soaked into the 150 ml distilled water and then autoclaved for the three h at 121 °C (*Geledium* sp.). After that, the crude sample is filtered with the cheesecloth. In the case of the carrageenan, it is subjected to a higher temperature, and then it is precipitated by potassium chloride or ethanol (Yun et al. 2016).

In the case of acid pretreatment, the weak acid hydrolysis is done by sulfuric acid or hydrochloric acid. It is used in different concentrations and time, and then it is autoclaved at 121 °C to 150 °C for about 90 min (Ge et al. 2011; Sudhakar et al. 2016). These methods are used in different seaweeds like *Codium fragile, Ulva sp., Caulerpa sp., Gracilaria sp., Geledium sp., Porphyra sp., Laminaria sp., Undaria pinnatifida,* etc. (Hong et al. 2014).

In the case of strong hydrolysis, 72% concentrated H_2SO_4 is used, and biomass is hydrolyzed for 30 min. Biomass is treated with $Ca(OH)_2$ or NaOH for alkaline hydrolysis and subjected for about 3–4 h then neutralized with HCl (Ge et al. 2011).

6.8.3 Enzymatic Treatment

Enzymatic treatment is comparatively more efficient than the chemical pretreatment, but it depends on the type of complexity of the cell wall. Seaweeds are made of complex polysaccharides like cellulose, alginate, ulvan, agarose, carrageenan, and fucoidan. Thus, different enzymes or combinations of different enzymes are used. It is less toxic to the environment; this adds the advantage over the chemical pretreatment. A mixture of enzymes is used to break the complex structure of the seaweed's polysaccharides into the simpler compounds or the simple sugars. The microorganisms can further utilize those for the derivation of the fuel. Another

advantage of this is that a higher proportion of the conversion that is up to 80% can be achieved with the help of the enzymes. For the enzymatic hydrolysis pH is being adjusted to 5.5 by 0.05 M citrate buffer. Various enzymes like Viscozyme L, Novoprime 959, Novoprime 969, or AMG 300 L (Kim et al. 2011) and cellulases (derived from the *Trichoderma reesei*) are being used for hydrolysis of seaweed biomass. These enzymes act like endo and exo-glucanases and ß-glucosidase (Jambo et al. 2016). There are several reports in which seaweed hydrolysis was done with the enzymatic method. Some of the examples for the enzymatic pretreatments are described further. For example, in the Acetone, Butanol and Ethanol (ABE) fermentation of the green seaweed *Enteromorpha intestinalis* microbial strain used was *Clostridium acetobutylicum*. The hydrolysate was prepared by the enzymatic saccharification using the celluclast 1.5 L and viscozyme L (Nguyen et al. 2019).

In red seaweeds like *Gelidium* and *Gracilaria* hydrolysis of the agarose or the agar is done by various marine bacterial enzymes. Agarases enzymes that are derived from certain marine bacteria like *Pseudoalteromonas atlantica* and *Alterococcus agarolyticus* are used for the hydrolysis of the agarose. Agarase is of two types that are α-agarase and β-agarase that differ in their cleavage pattern (Yun et al. 2015; Kim et al. 2013). Carrageenan degraded by the enzyme called carrageenases mostly derived from the marine bacteria, mostly gram-negative strains like *Pseudoalteromonas carrageenovora* and *Alteromonas fortis*. Based on the cleavage pattern, these are of three types, namely κ-carrageenases, ι-carrageenases, λ-carrageenases (Chauhan and Saxena 2016). In a report, it was seen that a red seaweed *Gracilaria verrucosa* was treated with the recombinant agarase. It was found that when enzymatic hydrolysis was done, it increased the amount of reducing sugar significantly (Kim et al. 2018). In the case of brown seaweed *Laminaria digitata,* two different enzymes celluclast and alginate lyase are used (Manns et al. 2014; Hou et al. 2015). In the *Laminaria japonica*, brown seaweed is hydrolyzed by using cellulase enzyme (Celluclast 1.5 L) and alginate lyase also used which produced the alginate oligosaccharides (Li et al. 2019). A brown algae *Cystoseira trinodis* was hydrolyzed by the crude fucoidanase produced by the marine algicolous fungus *Dendryphiella arenaria.* This report suggested that this fucoidanase resulted in the higher yield of the reducing sugar which can be subsequently utilized for bioethanol production (Hifney et al. 2018).

6.9 Technologies for the Conversion of Seaweed Biomass Into Biofuel

Before the fuel conversion technologies are being applied, seaweed biomass needs to be pretreated by removing dirt other impurities. Subsequently, it is hydrolyzed into simple fermentable sugars for efficient fermentation. Seaweed biomass is less complicated as compared to the terrestrial biomass. Hence, low input pretreatments methods are required for the conversion. The biomass is mainly comprised of the carbohydrate that is in the form of complex polysaccharides. Since they contain a very low amount of lipids, so direct oil conversion is less feasible. Hence, these

complex polysaccharides need to be hydrolyzed to simple sugars or monosaccharides. These monosaccharides are readily fermentable or taken up by various microorganisms for the objective of fuel production. The different technologies for the conversion of the biomass into biofuel are fermentation, anaerobic digestion, thermal liquefaction, and transesterification.

6.9.1 Fermentation

Simple sugars derived by the process of hydrolysis or saccharification can be directly utilized for the production of bioethanol. These fermentable sugars like glucose, galactose, mannose, etc. can be straight away utilized by the microbes to carry out their metabolic activities. With the production of bioethanol, some by-products are formed like CO_2 and H_2O. Some organisms that are employed for the production of bioethanol are yeast, fungi, and bacteria. Commonly for the bioethanol production yeasts are used. *Saccharomyces cerevisiae* is a commonly used strain for the production of bioethanol. Other than *S. cerevisiae,* some other strains are also capable of fuel production. The final product depends on the type of strain and the metabolic pathway that it follows. In ABE fermentation microbial strain used in *Clostridium*, which ferments the biomass and produces the mixture of acetone, butanol and ethanol. In another example, it was seen that fermentation of the *Ulva lactuca* was done with the *Clostridium acetobutylicum* and *Clostridium beijerinckii.* The biomass is prior subjected to the pretreatments and then to the commercial cellulase for the hydrolysis. This hydrolysate was further used as the carbon substrate for fermentation (Van der Wal et al. 2013). Some more examples of the ABE fermentation can be seen in brown seaweeds by the *Clostridium beijerinckii* in the *Laminaria digitata* (Hou et al. 2017). Another case study described the microbial fermentation of the brown algae. As the previous studies showed that the brown algae composed of some complex polysaccharides like laminarin, alginate, and mannitol. Thus, these types of polysaccharides may not be feasible to be used by all types of yeast. Hence some of the yeasts were screened which utilizes these types of complex polysaccharides. Some of the yeast strains screened for brown seaweed species like *Laminaria japonica* are *Pichia stipites, Saccharomyces cerevisiae, Kluyveromyces marxianus, Debaryomyces occidentalis, Brettanomyces bruxellensis, Pachysolen tannophilus, Schizosaccharomyces pombe,* and *Kloeckeraspora osmophila.* They utilized the seaweed biomass for ethanol production, but product formation depends on the type of component utilized in the biomass by each yeast (Lee and Lee 2012). In the case of mixed sugars microbes undergo different pathways depending on the sugar utilized. Like in the case of the conversion of the glucose and the galactose by *S. cerevisiae* different pathways were undertaken that is in case of the glucose conversion Embden–Meyerhof pathway and galactose conversion by Leloir pathway is used (Jambo et al. 2016). In red seaweeds, many species were reported for biofuel production. For example, some of the reports suggested that *Gracilaria sp.* was considered to be one of the potential

feedstocks for biofuel production (Wu et al. 2014). Another example of the red seaweed for bioethanol production is *Gelidium amansii* (Kim et al. 2015).

Recently two different approaches were used for the scale-up of the end products. This approach comprised of separated hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). In the SHF the hydrolysis and fermentation are done in the two stages. First, the biomass is hydrolyzed with the help of enzyme and the converted monomers were utilized for the fermentation. While in the case of the SSF simultaneously, the biomass is saccharified and converted monomers were utilized for the fermentation. In this, the yeast and the enzyme were added together and fermentation is done in the single step. In many seaweeds, this approach is used; for example, the bioethanol production from *Gelidium amansii* (Kim et al. 2015). Another example is of the green seaweed which undergoes the ABE fermentation. In this study the green macroalgae *Enteromorpha intestinalis* undergoes the ABE fermentation with the yeast *Clostridium acetobutylicum* (Nguyen et al. 2019).

6.9.2 Anaerobic Digestion

Anaerobic digestion is the process in which methane gas is produced by the methanogens by using simple sugar compounds. In this process, organic matter is decomposed by the microbial decomposition process in the oxygen-deficient environment (Alaswad et al. 2015). Major products formed by this process are methane and carbon dioxide with the effluent rich in a nutrient that can be further utilized as the fertilizer. Like the above-described methods in this method also the hydrolysis is done primarily, then it is subjected to the anaerobic digestion. The methanogens or the acidophiles use the biomass carbohydrates, protein, and other biochemical components. These monomers in the acetogenesis phase are converted into volatile fatty acids (VFAs) then into acetic acid, carbon dioxide, and hydrogen gas. Then some microbes convert into methane and CO_2 (Vasco-Correa et al. 2018). This method is cheap and easy to maintain as compared to other methods. Some of the examples are being discussed which showed that seaweeds biomass is used for the production of the methane by anaerobic digestion. In a report comparison was done between the two macroalgal species that are *Laminaria saccharina* a brown seaweed and *Palmaria palmata* which is a red seaweed in terms of methane production. In this study, it was seen that *P. palmata* was found to be better than *L. saccharina* due to its composition of the cations. *P. palmata* has a lower amount of the cations as compared to the *L. saccharina.* This means that the amount of sodium and potassium is higher in it which acts as an inhibitory factor in the anaerobic digestion (Jard et al. 2012). Another study suggested that brown seaweed *Laminaria hyperborean* was used for the production of the methane for about 8.4 ml mmol acetate^{-1} by some archaeal methanogens. Some of the different types of inoculum were prepared from different sources of animal and human wastes like sheep rumen, feces, and human sewage. Different combinations were tried for hydrolysis, and finally, the combination was made from all types of the wastes (Sutherland and Varela 2014). Another

example of biomethane production was seen from the five different types of the Irish seaweeds *Laminaria digitata, Saccharina latissima, Saccorhiza polyschides, Fucus serratus, and Ulva sp*. The inoculum was prepared from the bovine slurry. Out of this, *S. latissima* and *S. polyschides* showed a maximum amount of biomethane production followed by the *L. digitata* and *Ulva sp*. (Vanegas and Bartlett 2013a). In one of the study anaerobic digestion of the *Laminaria digitata* was done and the effect of the temperature was studied. In that, it infers that temperature plays an important role in the viability of the product (Vanegas and Bartlett 2013b). Some more algal species have experimented for the anaerobic digestion like *Saccharina japonica* (McKennedy and Sherlock 2015), *Sargassum muticum* (Milledge and Harvey 2016), *Ascophyllum nodosum* (Tabassum et al. 2016), and *Saccorhiza polyschides* (Tabassum et al. 2018).

6.9.3 Hydrothermal Liquefaction

Hydrothermal liquefaction (HTL) is the process in which the bio-oil from produced from the biomass with the value-added products in the form of the gaseous and solid form. In the solid form, the biochar can be derived, which can be used as fertilizer. In this process, the biomass is subjected to the reactor a high pressure or temperature with or without the present of the catalyst. It can serve as one of the technologies that convert algal biomass in the form of bio-oil. This process is best for the biomass that contains a large amount of water which was subjected to supercritical water gasification (SCWG) (Schumacher et al. 2011; Biller and Ross 2012). In this reaction, heating water under pressure and temperature change its dielectric constant, and density changes its solvent, and reactant properties and biomass decompose to new products (Anastasakis and Ross 2011). Some of the species reported for biofuel production by HTL are *Fucus serratus, Laminaria digitata, Alaria esculenta, Bifurcaria bifurcata* (Schumacher et al. 2011), *Enteromorpha prolifera, L. saccharina*, *A. esculenta,* and *F. vesiculosus* (Barreiro et al. 2015).

6.9.4 Transesterification

In this process the lipids are converted to the esters under the catalyst as the alcohol and then the biodiesel is derived. The lipid is extracted from biomass by chloroform and methanol extraction. Further it is subjected to the esterification reaction in the presence of the catalyst that is the alcohol. It converts lipids into the esters, and hence the biomass is converted and derived into biodiesel. This process is used on many types of seaweed for the production of biodiesel. Many such examples are described briefly. *Stoechospermum marginatum* is a brown seaweed from which directly bio-oil is derived from this process (Venkatesan et al. 2017). Another example was seen of the brown seaweed *Padina tetrastromatica* from which the lipids are extracted and further subjected to the transesterification and bio-oil is obtained (Ashokkumar et al. 2017).

6.10 Microbes as Single-Cell Oil Factory

Microbes have different industrial potential that dates back to the history. These are used for the production of different chemicals, antibiotics, enzymes as well as fuels. Various microbes are reported for the production of biofuels like bioethanol, biogas, and biobutanol. Apart from these various microbes are also used for the production of biodiesel. These types of microbes accumulate high lipids, and further, these lipids can be utilized for the production of biodiesel. Some fungi, yeast, and microbes were explored that accumulate more than 60% of the lipids as their biochemical composition. Some oleaginous microbes like *Arthrobactor, Rhodococcus opacus,* and *Acinetobacter calcoaceticus* can accumulate more than 80% of the oil content as their dry cell biomass (Dong et al. 2016). Among microbes, yeasts are the most promising agents that accumulate oil in the form of triacylglycerols (TAGs). These yeasts are often referred as the oleaginous yeasts.

6.10.1 Use of Oleaginous Yeast for Lipid Production

Microbes are used in fuel production for a very long time, but some of them can also be regarded as single-cell oil factories, particularly oleaginous yeast. Some of them can accumulate around 20% lipids as their dry cell weight. However, some strains are also reported to accumulate lipids more than 70% of their dry cell weight. The lipid accumulation depends on the strain type and the types of carbon sources on which they depend. Some strains *Rhodotorula, Cryptococcus, Yarrowia, Trichosporon, Lipomyces, Candida,* etc. are reported as single-cell oil-producing species for the production of the biodiesel (Ageitos et al. 2011). Many of these yeast species are reported for the production of the biodiesel utilizing the seaweeds as their carbon substrates (Table 6.2). *Cryptococcus curvatus* is reported as one of the most promising strains for the production of biodiesel from the brown seaweeds recently. These oleaginous yeasts utilize the hydrolyzed seaweed carbohydrates and accumulate oil in their cells. Some of the case studies have been described further. *Laminaria japonica* a brown seaweed is used as the carbon substrate by the oleaginous yeast for the production of biodiesel. *Cryptococcus curvatus* utilized the mannitol and alginate as the carbon substrate and produced more than 48% of the lipid content (Xu et al. 2014). In another study, the volatile fatty acids of the brown seaweed

Table 6.2 Some oleaginous yeast strains used for the production of biofuels utilizing seaweed biomass as a substrate

S.No.	Oleaginous yeast strain	Seaweeds substrate	References
1.	*Cryptococcus curvatus*	*Laminaria japonica*	Xu et al. (2014)
2.	*Rhodosporidium toruloides*	*Laminaria residues*	Zhang et al. (2016)
3.	*Rhodotorula glutinis*	*Laminaria residues*	Zhang et al. (2016)
4.	*Yarrowia lipolytica*	*Laminaria japonica*	Li et al. (2019)
5.	*Metschnikowia pulcherrima*	*Saccharina latissima*	Abeln et al. (2019)

Laminaria japonica are converted by *Cryptococcus curvatus* into microbial lipids that are around more than 61% (Xu et al. 2015). There was another report in which different oleaginous yeasts are screened using the substrate of the *Laminaria* residues. Among them, two strains *Rhodosporidium toruloides* and *Rhodotorula glutinis* are seen for high lipid accumulation (Zhang et al. 2016). One of the studies demonstrated that the simultaneous production of the alginate and biodiesel could be done. Kim et al. (2019) stated that in *Laminaria japonica* alginate is produced along with the biodiesel production using mannitol as the carbon substrate by the *Cryptococcus*. In *Laminaria sp*. production of biofuel has also been tried by other yeasts like *Yarrowia lipolytica* for the production of biodiesel (Li et al. 2019).

6.10.2 Method for the Conversion of Lipids Into Biofuels

Before the conversion of the yeast biomass into the biofuel, it needs to undergo various pretreatment measures for efficient lipid extraction. Several cellular barriers affect the lipid extraction procedure. Thus, it has to be ensured that appropriate cell disruption measures are to be taken for the extraction of lipids. Some of the pretreatments are discussed further, but these can vary on the type of the species and their biochemical composition.

6.10.2.1 Solvent Extraction

For lipid extraction by the solvent method, it must be ensured that the solvent chosen must be efficient that it extracts out the solute. This method depends on the partition coefficient of solvent. Commonly for the lipid extraction Bligh–Dyer method used in which chloroform–methanol is used for the extraction of lipids (Xu et al. 2014). However, sometimes it is less efficient and can be toxic; therefore, some other solvents are used which are likely to be less toxic, for example, hexane. The efficiency of the solvent extraction method also depends on the type of the lipid present, like for polar and nonpolar different solvents must be chosen.

Methods to Increase the Lipid Extraction

Various treatments are given for efficient lipid extraction that can be physical, chemical, and enzymatic. In the physical treatment, various methods are applied like bead beating, ultrasonication, high-pressure homogenization, pulse electric field, microwaves, osmotic shock, and subcritical water hydrolysis. In the enzymatic hydrolysis, the enzymes are used for the lipid extraction, and it is more efficient the conventional physical treatments. Enzymes like β-glycosidases, cellulases, β-glucanases, etc. are given. Chemical pretreatment comprises the acidic and alkaline pretreatments. In this dilute, mild acid or alkali treatments are given to the oleaginous yeast biomass, and then the lipids are being extracted. It is found to be more efficient than primary physical pretreatments.

6.10.2.2 Conversion of Lipids Into the Biodiesel

After various pretreatments are given to the yeast biomass for the efficient lipid extraction, further it is converted to the methyl esters. This process is called transesterification reaction. The lipids are converted into the methyl esters, in the presence of the catalyst that is the alcohol. Apart from the diesel, some other compounds are also derived like glycerol and other volatile fatty acids. There are various methods described for the transesterification reaction in the oleaginous yeasts like *Yarrowia lipolytica* (Louhasakul et al. 2018). In situ transesterification is applied to one of the developed methods. This method has an advantage over the other conventional transesterification methods because the conversion of biomass is done in a single step. In other methods, firstly, the biomass is converted into the lipids, then the lipids are transesterified. However, in this method simultaneously, the biomass conversion and transesterification are done. For biomass preparation treatment of the N-Lauroyl, sarcosine is given, which is an anionic detergent and helps in the disruption of the biomass of high-water content. This method is highly effective and lowers the cost and energy (Yellapu et al. 2017). There is one report in which *Rhodosporidium toruloides* lipid extraction was done for the biodiesel production. In this study, the oil from the biomass is extracted, and further, it is transesterified in the presence of lipase as the catalyst (Saran et al. 2017).

6.11 Role of Biotechnological Advancements in the Betterment of the Biofuel Technologies

Energy is an essential aspect of any development; hence, many attempts are made a concern to these sectors. It includes strategies to increase the production of fuel. Measures like identification of sustainable and cost-effective feedstocks, effective biomass conversion, and utilization and strain improvement come with the goal of efficient biofuel production.

For this, biotechnology provides better opportunities in the enhancement of biofuel production. Strain improvement can be made with the help of biotechnological tools. Metabolic engineering combined with genetic engineering of the microbial strain can be an effective option. This can be achieved by boosting up of the metabolic precursors or inserting superior strain's metabolic pathway in microbes (Kavscek et al. 2015). Biochemical pathways are modified or can be deleted which upregulates some enzymes or proteins leading to enhanced fuel production (Liu et al. 2018). Metabolic engineering increases the efficiency of different carbon substrate uptake and tolerance. Metabolically engineered microorganisms exhibit more product yield and diverse formation with simplified downstream processing (Liao et al. 2016). The diverse low-cost substrate can be utilized with the help of engineered microbes. Some of the metabolically engineered microbes are discussed further.

In the case of metabolically engineered bacteria recombinant *Zymomonas mobilis* TMY-HFPX is the metabolically engineered microbe with increased efficiency. Though this strain has effective glucose utilization, it follows the Entner–Doudoroff (ED) pathway which is more efficient than the Embden–Meyerhof–Parnas (EMP)

pathway which is used by *S. cerevisiae*. But *Z. mobilis* has inability of utilizing the pentose sugar thus this recombinant strain constructed such a way in which utilize xylose sugar and convert to the biofuel. This recombinant strain also show high ethanol yield, which is 90% of the theoretical conversion yield (solution of 295 g/L of glucose has 136 g/L of ethanol) (Majidian et al. 2018). Another case studied was observed in which *Pichia pastoris* was engineered for the production of the biofuels that is isobutanol and isobutyl acetate which is its ester. In this study, the endogenous biosynthetic pathway of the amino acid was exploited. In this biosynthetic pathway, an amino acid intermediate that is 2-ketoisovalerate was channeled to the 2-keto acid degradation. It resulted in isobutanol production.

By the overexpression of the endogenous l-valine biosynthetic pathway genes that strain was able to utilize glucose and can directly convert it into isobutanol that is around 0.89 g/L. Further, it was improved by the addition of the episomal-plasmid based expression system. Broad substrate range enzyme was also introduced, which is alcohol O-acyltransferase to generate isobutyl acetate ester (Siripong et al. 2018). Similarly, other recombinant bacterial strains like *Escherichia coli* KO11, *Bacillus subtilis* BS35 show high ethanol concentration. In the case of the yeast, several attempts were made in *Saccharomyces cerevisiae* for the generation of the recombinant strains with more catalytic efficiency and tolerance. In some of the examples, it was seen that lipid production was enhanced in some of the microbes. In a report it was seen that *Yarrowia lipolytica* was engineered to increase the production of the lipids (Niehus et al. 2018). In this study, Po1d strain was engineered for the xylose utilization in this XDH (xylitol dehydrogenase), and XR (xylose reductase) overexpressed. This strain is also able to produce a higher amount of lipids from the C5 sugars. Along with these modifications it was further engineered for the conversion of xylose to the acetyl-CoA expressing heterologous genes by alternative pathways (Niehus et al. 2018). Other yeast includes *Pichia stipites* and *Spathaspora passalidarum* metabolically engineered, which are xylose-fermenting yeast. Hosts should be engineered in such a way that fatty acid precursors like acetyl-CoA, malonyl-CoA, fatty acyl-CoA can be enhanced. Some microbes were also reported for tolerance development during the production of the biofuel. It was found necessary because during the fermentation, several toxic compounds were produced, which reduces the fermentation efficiency. Some of the modification includes the engineering of the gene associated with chaperones, transcriptional factors, membrane-modifying enzymes, and transport pumps. In some of the strains, such modifications can be seen like *Clostridium acetobutylicum, Escherichia coli,* and *Zymomonas mobilis* (Chubukov et al. 2016).

6.12 CRISPR Genome Editing Tools in the Improvement of the Yeast for Biofuel Production

In synthetic biology, the most recent tool used is the CRISPR/Cas9 system that is clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein Cas9 (CAS9). It is based on the defense mechanism used by the prokaryotes. In bacteria, they have a unique type of ancient defense mechanism of

adaptive immunity. This defense system results because of bacteriophage interference and other genetic elements under different environmental conditions. In order to combat these foreign attacks, one of the defense mechanisms followed by the bacteria is the CRISPR-Cas system. This mechanism is especially the RNA guided defense mechanism (Doudna and Charpentier 2014). There are two types of classes of the CRISPR-Cas systems that are class1 and class2 CRISPR-Cas systems. These distinctive CRISPR-Cas systems are based on the organization of the effector module. In class 1, CRISPR-Cas systems multi-protein effector complexes were utilized, and in class 2 CRISPR-Cas single-protein effectors were utilized. Different effector protein families are associated with these CRISPR-Cas systems. Based on this, further the class 1 CRISPR-Cas systems are divided into three types and twelve subtypes and class 2 CRISPR-Cas systems are divided into three types and nine subtypes. Class 1 CRISPR-Cas systems are present for about 90% in all types of prokaryotes, while class 2 CRISPR-Cas systems for about 10% (Makarova et al. 2017). In class I CRISPR-Cas systems type I, III, and IV CRISPR-Cas systems are involved. Class1 systems encode DNA helicase Cas3 and repeats are palindromic. Protospacer adjacent motif (PAM), located either 5′ or 3′ of the (proto) spacer, is required for both adaptation and interference. Type III and type IV systems often lack adaptation module genes and CRISPR arrays in their respective loci. The class 2 Cas system encodes the effector proteins, adaptation module protein, and accessory proteins. Type II and V include tracrRNA (trans-activating CRISPR RNA), partially complementary to the repeats and involved in CRISPR (cr) RNA processing and interference. Type VI consists only of an effector protein and a CRISPR array (Van Houte et al. 2016).

The primary mechanism of the type II system, which is the most extensively used CRISPR system. It utilizes the RNA-mediated endonuclease, which is the Cas9 protein. It makes the double-stranded break. Hence, two active regions were recognized that is the HNH domain and RuvC domain. For targeting the eukaryotic genome, the nucleus localization sequence (NLS) fused with the bacterial originated Cas9 protein from *Streptococcus pyogenes*. Apart from this most crucial component of this system is the single guide RNA (sgRNA) which directs the Cas9 protein to the targeted sites. The sgRNA consists of the crRNA that is the CRISPR targeting RNA and tracrRNA called trans-activating crRNA. The sgRNA targets the sequence binds to the PAM sequence (protospacer adjacent motif) 5′NGG3′ that distinguish between bacterial self DNA from non-self DNA. This complex is recognized by the Cas9, and double-stranded breaks were created. For the repairing of the DNA nonhomologous end-joining repair mechanism was followed (Cai et al. 2019). The benefit of this technology is that it can be applied for a single gene or multiple gene editing (Liu et al. 2019).

There are several reports in which the CRISPR/Cas9 system is used in yeast metabolic engineering, and hence there was a rise in the efficiency that can be seen (Stovicek et al. 2015; Shapiro et al. 2018). Further, some of the case studies have been described in which the stable gene modification was observed in many yeasts. The first attempt has been made in the *Rhodosporidium toruloides* in which multiple gene disruption was done. In this yeast two genes were deleted. *URA3* encodes for

the orotidine 5′-phosphate decarboxylase that enables for the conversion of the 5-fluoroorotic acid (5-FOA) 5-fluorouracil which is a toxic compound for the yeast. Another gene *CAR2* gene encodes for the phytoene synthase or lycopene cyclase protein responsible for the carotenoid biosynthesis. In this approach, multiple sgRNA are placed in a single array with the single guide RNA separated by the sequence of the tRNA. Further successful edition of both genes was seen in the single transformation. Hence this approach can be further used in the editing of multigene pathways for the production of the fuel and other products (Otoupal et al. 2019). There is another report in the *Saccharomyces cerevisiae* for the multiplex genome engineering. In that they reported first for the ALD4 gene with the CRISPR/Cas9 combinational engineering for the improved production of the ethanol. In this study, three genes were taken, i.e., the alcohol dehydrogenase (ADH) 2 gene, the glycerol-3-phosphate dehydrogenase (GPD) 1 gene, and the aldehyde dehydrogenase (ALD) 4 gene.

In the metabolic activity of the *S. cerevisiae*, pyruvate is decarboxylated into the acetaldehyde and alcohol dehydrogenase (ADH) reduces the acetaldehyde into the ethanol. Apart from this there is also the formation of the by-products like glycerol and acetate. These by-products act as the inhibitors in the ethanol formation. Many intermediate forming genes like the glycerol-3-phosphate dehydrogenase gene (GPD), the aldehyde dehydrogenase gene (ALD) are responsible for this. Hence these are needed to be disrupted for the high ethanol production. Glycerol is the glycolytic intermediate of the dihydroxyacetone phosphate (DHAP) catalyzed by enzymes that is NAD^+-dependent glycerol-3-phosphate dehydrogenase (GPD). It limits the ethanol formation by utilizing the carbon substrate; hence, it is needed to be eliminated. Interconversion of ethanol and acetaldehyde is catalyzed by the alcohol dehydrogenases (ADHs), this gene is also responsible for the oxidation of the ethanol to the acetaldehyde and aldehyde dehydrogenases (ALDH) are responsible for the oxidation of acetaldehyde into the acetic acid. This gene disruption increased the ethanol production efficiency that is 1.41 fold higher than the wild strains (Liu et al. 2019).

According to Zhu et al. (2019) modification was done by using CRISPR/Cas9 approach in *Candida glycerinogenes.* It is an industrial yeast and considered to be an ideal feedstock for the production of bioethanol. This strain has high resistance to the temperature and has high toxic tolerance, especially from the acetic acid and the furfural. However, the wild strain is incapable of utilizing of xylose. Hence the CRISPR/Cas9 system was developed for modifying this strain by the insertion of the xylB gene. This gene was knocked in this yeast system, which encodes the NAD^+-dependent xylose dehydrogenase, which led to the production of the xylonic acid along with the ethanol.

Another case study can be observed in which the endogenous CRISPR/Cas9 system was used for multiplex genome editing in the *Clostridium tyrobutyricum.* This strain was engineered for the high level of butanol production. In this study, butyrate production depended on the *cat1* gene, but disruption of this gene can lead to high butanol production because the introduction of the *adhE1* or *adhE2* can directly convert butyryl-CoA into the butanol. Thus, by using this approach, hyperbutanol

production was achieved. That is, it enables this mutant strain to produce around 26.2 g/l of the butanol (Zhang et al. 2018). There are some more examples of the yeasts like *Yarrowia lipolytica*, *Pichia pastoris, Scheffersomyces stipites,* etc. in which this CRISPR/Cas9 was used for genome modification in terms of high biofuel production.

6.13 Conclusion Remarks and Future Prospects

From this chapter, it can be seen that seaweeds can be considered as one of the low-cost substrate and most promising feedstock for the production of biofuel. It can be considered as an alternative to conventional fossil fuels and can help in lowering the direct dependence on it. It is more feasible in terms of pretreatment and processing as compared to the first- and second-generation of biofuels. Competition for land, food, and water are major drawback of first and second generation of biofuels. Fuel derived from seaweed biomass comes with the aim of cleaner combustion with less GHGs emissions. India is a tropical country with a vast coastline. So there is a need to develop more awareness for the seaweed cultivation, as it has the advantage of the high production in less period. Thus, the cultivation of seaweeds can provide a livelihood to people as well as efficient feedstock for industrial purposes. It shows that in the future, seaweeds production can be enhanced; hence, there is much potential in the seaweed market globally. Many countries have already accepted the mass production, and still, the government of developing countries needs to implement the advancement in the cultivation of seaweeds. Since seaweeds have the unique biomass composition therefore it can be utilized for various industrial purposes. The prime component is the carbohydrates in the form of polysaccharides. These can be broken down into simple sugars with the help of the combination of some chemicals and enzymes. These simple compounds in the form of sugars can be easily further utilized by microbes for the fermentation. Seaweeds also provide an opportunity for the setup of biorefinery as many important industrial chemicals, and other natural products can be derived from it. To run our economy, liquid transportation fuels are required; hence, several attempts were made to produce liquid fuel like bioethanol, biodiesel, and biobutanol from the seaweeds. Also, other types of biofuels like methane and biogas were produced using seaweeds as the substrate. Seaweeds are very diverse and less explored, so there is a need to look more insight into this area. Despite such attempts still, it requires more efficient technology to increase the feasibility in terms of cost and production.

Another phase shows the importance of microbes in fuel production. Some of the microbes are of great economic importance and can be utilized for the production of biofuels. Recently many attempts are made on them for the utilization of seaweeds biomass for fuel production. Recently microbes were made more efficient in terms of more product concentration, toxic tolerance, and more substrate utilization. In microbes, particularly the oleaginous microbes and yeasts are of much attention. These types of microbes utilize the biomass and accumulate the microbial lipids. These microbes are also referred as single-cell oil-producing species (SCO), which

accumulates lipids as their prime biomass constituents. In future microbial lipids, derived fuel will get more acceptances for a potential replacement of petroleum-based fuels. After the observation of much potentials to make them more economically viable several biotechnological approaches were made to improve their efficiency. Tools like genetic and metabolic engineering and synthetic biology, like the use of CRISPR/Cas9, made it possible to modify these microbes for the development of desired products at less cost and time. The metabolic pathway can be inserted or deleted accordingly; even multigenes can be modified or inserted in one step. In the future, such tools will be implemented for more stable production and high output. However, there are some challenges associated with it that there is a need for the development of the more efficient methods for lipid extraction. With this low cost downstream processing technologies has to be developed. It will impact the mass production and scale-up of it for the commercialization of the microbial fuels. Thus, there is a need to generate more awareness towards these alternatives for sustainable energy production.

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Microbial Biofuels: An Economic and Eco-Friendly Approach

7

Azmi Khan, Pratika Singh, and Amrita Srivastava

Abstract

Biofuels in recent years have turned out as an environment friendly and cost-effective approach to sustain the rising demand of energy for the growing population. Development of efficient methods for biofuel production using plants and microbes has gained considerable attention. Thus, a different generation of biofuels, i.e. first generation, second generation, third generation, fourth generation, and currently next generation of biofuels has evolved. Each generation overcame the limitations of the earlier generation and differs basically in the substrate being used for the production. For efficient biofuel production researchers and companies have evolved various methods and compositions and acquired respective patents. Also, machineries involved in biofuel production have evolved over time at the laboratory as well as the industrial level. Different countries have formulated various policies and laws to encourage the use of these renewable sources of fuels to overcome the problem of pollution. This chapter encompasses all these aspects related to biofuels with special emphasis on biofuel production utilizing microbes.

Keywords

Biofuels · Microorganisms · Biofuel patents · Generations · Policies

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7.1 Introduction

Rising demand for energy is one of the leading problems globally. The present resources are rapidly depleting and may soon be vanished. With respect to the current scenario, the utilization of renewable forms of energy is worth consideration. Fossil fuels have been enormously exploited and are extremely limited now. Inadequate distribution of resources such as petroleum poses economic as well as social crisis worldwide. Additionally, overexploitation of these resources would lead to an energy crisis in the coming generations. Moreover, excessive utilization of traditional fuels also brings about enhancement in the level of greenhouse gases (Singh et al. 2010). Therefore, alternative and economic energy sources that can be renewed and ensure lesser emission of harmful gases need to be searched. Consideration of biofuels based on cellular conversion of biomass into fuels may lead to formulation of sustainable forms of energy. Wastes derived from plant sources, mainly from agriculture and forestry sectors, and different industries contribute to biomass from which gaseous, liquid, and solid fuels can be derived and these are referred to as biofuels (Dufey 2006). The interest in producing various economically feasible, eco-friendly biofuels of microbial origin has risen in the recent past owing to variety of metabolic products that can be derived from different groups of microbes and used for biofuel production. Since the emission of greenhouse gases from fossil fuels used for electricity generation is 25% (IPCC 2014), there is an urgent need to look for eco-friendly, economically feasible, and natural renewable energy sources such as biogas, diesel, and alcohols with potential to replace the conventional fuels as a part of sustainable development. Diverse microbial groups such as microalgae, fungi, yeast, etc. serve as potential candidates for catalysis of biomass into biofuels (Xiong et al. 2008). However, due to the lack of extensive information on genetic regulation in their biochemical processes, biofuel generation through microbes is limited. Microbial biotechnology strategies are recently being explored for utilizing microbes to produce various versions of biofuels. Bacteria can easily convert sugars into ethanol or plant-derived lignocelluloses are readily employed by cellulolytic microbes such as *Clostridium thermocellum*. Algal research is currently being diverted for the extraction of biofuels. Microalga *Botryococcus braunii* is known for its biofuel convertible high hydrocarbon content of 40% (Mirza et al. 2008). Reports by WEO suggest that the USA, Brazil, and Europe will be the major biofuel producers in the future. Also, developing countries like India, Colombia, and China will pay a significant contribution to the total world production (Spiess 2011).

This chapter covers different aspects of microbial biofuels as a strategy to combat the energy crisis. The description includes production pathways involved and generations of biofuels followed by industrial trends in the recent scenario.

7.2 Bioalcohol as Biofuel

Alcohols including butanol, propanol, and ethanol are generated biologically via fermentation of plant-derived sugar components and are made mostly from sugar and starch rich crops. The production of bio alcohols is done using two methods: direct fermentation and indirect fermentation. In direct fermentation, starting plant material is identified first followed by isolation and development of associated fungi and designing suitable methods for the proficient derivation of sugar monomers from plant material. Genetically modified bacteria or yeast can further be engaged for conversion of sugars too. Indirect fermentation employs pyrolysis after which acetogenic bacteria are used to convert the produced gas to alcohol (Klasson et al. 1992; Elshahed 2010).

Ethanol production has been investigated in several organisms, i.e. *Zymomonas mobilis*, *Corynebacterium glutamicum, Pichia stipitis, Clostridium thermocellum, Clostridium phytofermentans,* and *Escherichia coli* (Gruszecki et al. 2005).

Indirect fermentation is based on the pyrolysis of plant substance for the production of Syngas. Latter mainly comprises of CO, CO_2, and hydrogen. Acetogenic bacteria further biotransform syngas to ethanol (Elshahed 2010; Leadbetter et al. 1999). Plant materials or even wastes generated from other sources which can be pyrolyzed prove useful in this approach (Gulati et al. 1996). Slower growth of involved microbes and lesser yield are among the key technical difficulties of this approach (Tanner 2008).

7.3 Biodiesel as Biofuel

Biodiesel has come up as a suitable replacement for diesel. These are non-petro-hydrocarbon produced with the help of microorganisms. Apart from microbes, lipids of plant origin, fats of animal origin, and even pre-utilized cooking oils can be used for producing biodiesel through the esterification of triglycerides with alcohol (Fukuda et al. 2001). The oil of microbes has the potential to be employed as a raw biomaterial for biological diesel production during transesterification (Meng et al. 2009). It consists of alkyl ester (methyl, ethyl as well as propyl group) of long chain fatty acid and can be used as pure biodiesel or in combination with petrodiesel. Different blends that are commonly used are: B100, B5, and B2, respectively, with 20%, 5%, and 2% of biodiesel mixed with rest amount of petrodiesel. Transesterification is the key process involved in biofuel production which is carried out either by catalytic or non-catalytic conversion of oils and fats to oxygen containing molecules. In the catalytic transesterification process, a catalyst triggers reaction between triglyceride (fat/oil) and an alcohol that produces esters and glycerol. Non-catalytic methods include supercritical method and alcohol and BIOX co-solvent process. Ethyl and methyl esters of certain fatty acids are the chief constituents of biodiesel (Behera and Varma 2018). Triglyceride oil can measure more than 80% of the algae dry biomass (Spolaore et al. 2006). Biodiesel derived from algal source is better in quality and is more sustainable than that

obtained from crops (Charles et al. 2007). Advantages of algae-based biodiesel production include rapid biomass production and better growth density thereby increasing the concentration of biodiesel (Elshahed 2010). Second, since they are photo-autotrophic in nature, they are non-competitive in nature. They also produce certain economically important compounds (Pittman et al. 2011).

7.4 Biohydrogen as Biofuels

Hydrogen gas is the cleanest biofuel. It does not emit carbon dioxide and produces good amount of energy on combustion making it an appealing alternative. Its easy conversion to electricity via fuel cell is an additional advantage. Fermentation and photosynthesis by several microbes are some of the biological processes that release hydrogen as a by-product (Elshahed 2010). It has highest calorific value among the known fuels and can be produced by numerous means (Levin et al. 2004). Photosynthetic microbes have the capability to carry out photolysis of water that leads to generation of hydrogen by the enzymatic activity of hydrogenase (Elshahed 2010). The process raises an issue to uncouple oxygen sensitive hydrogenase enzyme for commercial hydrogen production. This can be achieved by two steps: initially, the microbes can be placed under oxygenic environment to carry out the process of photosynthesis and then transferred to anoxygenic condition for biofuel (hydrogen) production. Second approach involves the use of nitrogenase enzyme fixation of N_2 gas by anoxygenic photoheterotrophic microbes like *Rhodopseudomonas palustris* (Rey et al. 2007). Hydrogen is produced as an essential by-product. Use of anaerobic fermenting bacteria *E. coli, Enterobacter aerogenes,* and *Clostridium butyricum* forms the third approach.

7.5 Biofuel Production Pathway Design

Biofuels are being produced nowadays by adopting different production pathway designs using various microorganisms including bacteria, algae due to the presence of metabolic pathways such as citric acid cycle, glycolysis, valine biosynthesis pathway and their ability to metabolize different substrate.

7.5.1 Biofuel Production Using Different Microorganisms

7.5.1.1 Biofuel Production Using Bacteria

Clostridium

In the biofuel production industry *Clostridium sp.* has been mostly used. In direct pathway such as in *Clostridium* that follows a biphasic fermentation of carbohydrate, butanol and ethanol are produced at solventogenic phase exploiting Embden–Meyerhof pathway by the cells growing earlier in acidogenic phase (Jang et al.

2012). *Clostridium ljungdahlii* an anaerobic bacterium that ferments sugars also produces ethanol in an anaerobic environment by using NAD^+-dependent acetaldehyde and ethanol dehydrogenases (Kopke et al. 2010). The amount of ethanol procured from *C. ljungdahlii* is enhanced by culturing it using two separate continuous bioreactors each for cell growth and ethanol production (Klasson et al. 1992). Cellobiose is a recommended substrate and *C. ljungdahlii* converts syngas (specifically CO) produced in the process to ethanol. Use of cellobiose gives a higher ratio of ethanol to acetate. Other than that, reducing agents like methyl and benzyl viologen enhance alcohol yield in *C. acetobutylicum* fermentation due to alteration in electron flow induced by such agents (Rao and Mutharasan 1987). Such alteration directs carbon flow from acid to alcohol leading to the formation of NADH from free hydrogen further increasing alcohol production.

In many cases, a native metabolic pathway has been genetically engineered from one species into another for efficient production as in the case of *Clostridium* and *E. coli*. Synthetic butanol pathway combining enzymes like NADH-dependent *trans*-enoyl-CoA reductase from different species altering the normal *Clostridium* butanol pathway has been engineered into *E. coli* that acts as a host leading to increase in n-butanol titer up to 8–12-fold (Bond-Watts et al. 2011; Shen et al. 2011). Apart from direct fermentation, generation of biofuels using microbes also follows indirect fermentation pathways whereby already processed substrates like plant materials are further exposed to microbes. For example, the production of methane, ethanol, and hydrogen is carried out by conversion of synthesis gas by utilizing the co-culture of *Rhodospirillum rubrum* and methanogens or by using *Clostridium ljungdahlii* alone (Klasson et al. 1992). For this synthesis gas or syngas isproduced majorly from carbonaceous feedstock like coal and from sources such as natural gas and biomass (Klasson et al. 1992; Speight 2019). Production of syngas requires gasification, i.e. partial oxidation of these sources. Following reaction occurs in the process:

$$[2CH] + O_2 \rightarrow 2CO + H_2$$

Escherichia coli

Escherichia coli has largely been employed for the production of biofuels including bioethanol and biodiesel. Since *E. coli* is a well-known model organism whose genetic regulation is well studied and subjected to modification, it has been genetically modified very well for the purpose of optimum and efficient biofuel production. Fatty acid methyl esters are produced from genetically engineered *E. coli* by expressing the enzyme fatty acid methyltransferase and the production was enhanced by deleting global methionine regulator *metJ* or by overexpression of methionine adenosyltransferase.

In yet another approach gene for NADH oxidizing system from *Zymomonas mobilis* has been inserted into *E. coli* resulting in efficient production of bioethanol from sources such as hemicellulose (Ingram et al. 1987).

Pseudomonas

Certain bacteria such as *Pseudomonas* isolated from microbial fuel cells are capable of converting their metabolic energy into electricity (Rabaey et al. 2005). These bacteria utilize redox mediators either produced by themselves or by other bacteria in their vicinity. These mediators shuttle electrons from bacteria and electron acceptors (anode), thus generating a significant power output.

Zymomonas Mobilis

Classically, the natural ethanologen *Z. mobilis* are known to utilize Entner–Doudoroff pathway that needs a lesser amount of ATP for ethanol production as compared to other pathways (Yang et al. 2016). Hexose utilizing *Z. mobilis* has been genetically engineered to produce ethanol from lignocellulose hydrolysate comprising of hexose–pentose mixture like glucose and xylose (Clarke et al. 2017). The culture condition with respect to pH and temperature for the recombinant *Z. mobilis* 8b has been optimized and thus a final enhancement in ethanol production was achieved.

Bacillus

Different *Bacillus Sp.* strains have been utilized for the purpose of biofuel production. Two strains, namely *B. thuringiensis* and *B. subtilis* were identified to produce 2450 and 2300 ml/L of biohydrogen and 1.55 and 1.03 g/L of ethanol from sugarcane molasses (Gabra et al. 2019).

For the purpose of biofuel production *B. subtilis* are being engineered. A global transcriptional repressor codY present in *B. subtilis* is known to control two monocistronic transcription unit, i.e. ilvD and ybgE and another operon comprised of seven genes, viz. LeuABCD involved in branched chain amino acid (BCAA) biosynthesis (Shivers and Soneshein 2004). To increase amino acid nitrogen flux codY was deleted in *B. subtilis* and together with some more modifications such as overexpressing LeuDH, a key deaminase enzyme, product formation by conversion of large polypeptides and proteins into advanced biofuels was enhanced (Choi et al. 2014).

7.5.1.2 Biofuel Production Using Algae

Since algae possess rich oil and fatty acid content in the form of triacylglycerol it is a major target of commercial biofuel production (Breuer et al. 2013). For biofuel production from microalga liquefaction, pyrolysis and gasification steps are required that separate hydrocarbons in liquid form (Fig. 7.1).

Direct liquefaction has been carried out in *Dunaliella tertiolecta* (Minowa et al. 1995). For separation of different products algal materials are autoclaved with nitrogen sparging at high temperature and then cooled. The gas obtained is initially separated and the remaining material is further extracted using dichloromethane to separate oil.

A cost-effective approach for this purpose is hydrothermal liquefaction that requires less energy since algae are rich in water content (Barreiro et al. 2013). In this process, microalgae are grown at large or small scale in industries or

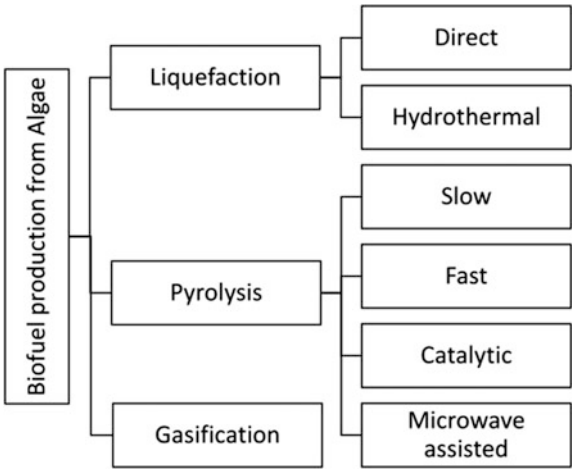

Fig. 7.1 Different approaches for the biofuel production from algae

laboratories. These microalgal cultures are harvested followed by separation of protein and lipids after its concentration and finally subjected to a thermochemical conversion generating crude biofuel (Alba et al. 2012). Generally, temperature ranging from 250 to 375 °C is employed. Also, a pressure ranging from 10 to 20 MPa is provided. In another system, an algal biorefinery has been developed where two different algal feedstock, one rich in lipid and another in algaenan, led to the production of biodiesel and hydrocarbon rich biofuel, respectively (Alba et al. 2012). Products from these two sources can further be mixed together generating a third type of energy fuel.

Four types of pyrolysis, i.e. thermal decomposition methods have been developed to generate bio-oils and fuels from microalgae making use of a variety of catalysts and temperature range, namely slow, fast, catalytic, and microwave assisted pyrolysis (Fermoso et al. 2017). The slow type of pyrolysis method employs a temperature increase of 10–100 °C/min up to 500–700 °C in a fixed bed bioreactor giving a maximum bio-oil yield of 31% when no catalyst is used (Pan et al. 2010; Grierson et al. 2011). In contrast, fast pyrolysis makes use of fixed, fluidized, or spouted bed reactor with a temperature increase of 10–200 °C per second up to 1000°C for a short time of 0.5–10 s yielding fatty acid rich bio-oil (Chen et al. 2015; Harman-ware et al. 2013).

Alga such as *Laminaria digitata* and *Fucus serratus* rich in C and H subjected to pyrolysis, in a continuous fluidized bed reactor with silica bed at 500 °C yields higher biochar than terrestrial biomass such as grape seed with low heating value (Yanik et al. 2013). Using a catalyst like zeolites, e.g. ZSM-5 or HZSM and sodium carbonate during pyrolysis at a temperature ranging from 400 to 650 °C results in a bio-oil yield of 19–45 wt % (Babich et al. 2011). For the microwave assisted pyrolysis a microwave absorber such as metal oxides, silica, or lignite char, etc. is utilized leading to the production of fast and enhanced amount up to 59 wt% of bio-oil (Xie et al. 2015).

In yet another step, i.e. gasification, heating of algal biomass is carried out in the absence of oxygen or air generating syngases. Syngases as described earlier is then utilized and converted to methanol or other fuel product. During gasification process firstly the algal biomass releases oxygenated vapors, water, and carbon dioxide at a lower temperature (Baker and Mudge 1984). After increasing temperature up to 850–1000 °C it further generates carbon monoxide, aromatics, phenols, and tars.

Marine eustigmatophyte *Nannochloropsis sp.* have been grown under artificial light in a 20-L alveolar panel with nitrogen deficient condition leading to increased lipid synthesis in turn increasing algal oil production (Rodolfi et al. 2009). For efficient growth and further oil procurement, a two-step cultivation process has been suggested. For this, in first step algae is cultured in a nutrient rich medium termed nutrient sufficient phase for proper growth and in second step nitrogen is made deficient called nitrogen deprived phase to increase lipid generation.

A special helical tubular photobioreactor called BIOCOIL™ is nowadays in use for semi-continuous algal culture of species including *Chlorella sorokiniana* enhancing lipid and fatty acid methyl esters (FAME) which are important components of biofuel and biodiesel production (Borowitzka 1999; Concas et al. 2016).

7.5.1.3 Biofuel Production Using Cyanobacteria

In another process for biodiesel production using microalgae or cyanobacteria, organisms are grown either with light in open or closed ponds or without light with different carbon sources for efficient generation of biomass. Harvesting or dewatering is carried out and further the biomass is concentrated. Finally, lipids, carbohydrates, and proteins are extracted by implying suitable conversion techniques depending upon which end products are to be procured. For example, lipids and carbohydrate conversion leads to the generation of biodiesel and gasoline (Blinová et al. 2015).

Synechococcus

In cyanobacteria metabolic engineering approaches have been utilized for optimum biofuel production. In one such approach a freshwater cyanobacterium *Synechococcus elongatus* PCC 7942 was used as host and a CoA dependent pathway responsible for the production of 1-butanol in *Clostridium* was inserted (Lan and Liao 2011). This pathway was however first modified before transfer. Classically, two molecules of acetyl-CoA condense in the presence of a thiolase enzyme giving acetoacetyl-CoA which further by another enzyme 3-hydroxybutyryl-CoA dehydrogenase (hbd) get reduced to 3-(OH) butyryl-CoA. By the action of 3-hydroxybutyryl-CoA dehydratase (crt), 3-(OH) butyryl-CoA gets converted into crotonoyl-CoA. A complex of butyryl-CoA dehydrogenase (bcd) and electron transferring protein A & B (EtfAB) hydrogenation of crotonyl-CoA occurs generating butyryl-CoA. In the next step, butyraldehyde is produced by the action of a bifunctional aldehyde alcohol dehydrogenase (AdhE2) and is further reduced to n-butanol in the presence of AdhE2 (Jang et al. 2012; Swidah et al. 2018: Fig 7.2a). The modified pathway involved five genes of which three, namely crt, adhE2, and

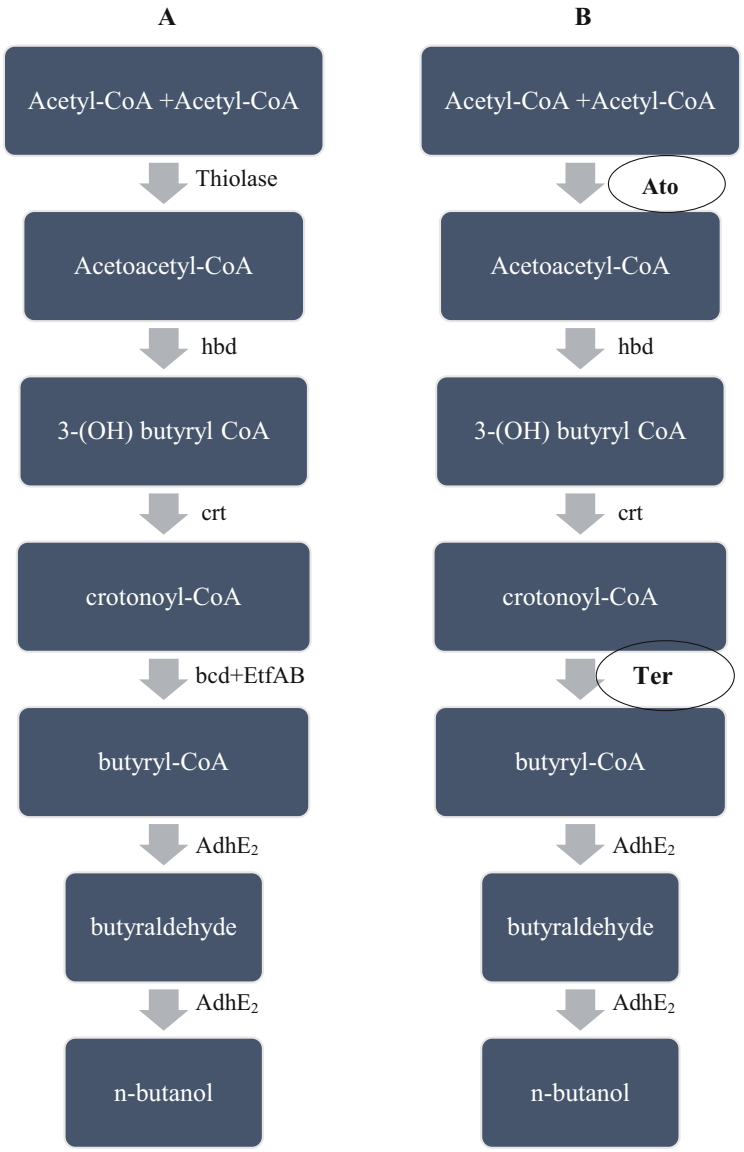

Fig. 7.2 Outline of coA dependent pathway in (**a**) *Clostridium* (**b**) recombinant *Synechococcus elongatus* PCC 7942 (Genes from sources other than *Clostridium* marked in bold)

hbd were taken from *Clostridium* itself while two genes were derived from different sources, namely *Treponema denticola* (trans-enoyl-CoA reductase) and *E. coli* (atoB replacing acetoacetyl-CoA thiolase of *Clostridium*: Fig. 7.2b). This resulted in

1-butanol production from the engineered autotrophic cyanobacterium (Lan and Liao 2011).

Synechocystis

In another cyanobacterium *Synechocystis sp.* PCC6803 genetic modification was carried out by introducing a pyruvate decarboxylase from *Z. mobilis* (Gao et al. 2012). Also, an alcohol dehydrogenase of the cyanobacterium was induced to overexpress together with interruption of its poly-β-hydroxybutyrate pathway causing a proficient production of ethanol (212 mg/L/day).

Arthrospira Platensis

Direct ethanol production by bioconversion using *Arthrospira platensis,* a free floating filamentous cyanobacteria results in high titer and yield by using $CaCl_2$ and lysozyme (Aikawa et al. 2018). This cyanobacteria uses as a feedstock in fermented using recombinant strain of yeast *Saccharomyces cerevisiae* BY4741 AASS/GASS that express α-amylase and glucoamylase gene (Inokuma et al. 2014).

7.5.1.4 Biofuel Production Using Fungi

Saccharomyces

Like *E. coli,* baker's yeast *Saccharomyces cerevisiae* has also been genetically engineered for optimum biofuel production. Already existing metabolic pathways have also been modified for enhancing biofuel production. Isobutanol in yeast is synthesized as a by-product of Ehrlich pathway (Hazelwood et al. 2008). By the action of Ilv2, 3, and 5 enzymes pyruvate gets converted to 2-ketoisovalerate (KLV). KLV further via Ehrlich pathway gets metabolized by the action of two enzymes, i.e. Aro10 and Adh2 to isobutanol (Brat et al. 2012). However, the yield is very less in conventional pathway. Thus, the gene responsible for encoding enzymes of valine biosynthesis *ILV2, ILV3, ILV5,* and *BAT2* has been overexpressed leading to increased isobutanol production (Wess et al. 2019). Also, re-localization and overexpression of Aro10, Adh2, and other mitochondrial enzymes have been carried out for the same (Brat et al. 2012).

Conversion of biochemical energy into bioelectrical energy has been earlier carried out using *S. cerevisiae* with the aid of biofuel cells (Halme and Zhang 1995). Fermentation was carried out in bioreactors using glucose as the source for carbon which acts as a substrate and leads to the generation of about 120 $\mu W/cm^3$ of power output.

Chrysoporthe Cubensis

Enzymes from fungal source such as cellulase are a prime target nowadays since such enzymes are involved in the conversion of cellulosic substrate like lignocellulose into sugars that can be further utilized for biofuel production. A plant *Chrysoporthe cubensis* has been reported to be an efficient cellulase and xylanase producer that saccharifies sugarcane bagasse releasing up to 320.8 mg/g of sugar monomers, i.e. glucose and xylulose (Falkoski et al. 2013).

Cellulase hydrolyses β-1,4-D glucan bond of cellulose, hemicellulose, lichenia, and cereal β-D-glucans to release glucose, cellobiose, and cello-oligosaccharide. Although fungi have better substrate utilization than bacteria still a majority of them lack all cellulose system components for efficient hydrolysis. Cellulase includes exoglucanases (EG; EC 3.2.1.4), cellobiohydrolases (CBH; EC 3.2.1.91), and β-glucosidases (BGC; EC 3.2.1.21) as reported by Rawat et al. (2014).

Fungal strains carry cellulose production via submerged fermentation (SmF). Scientists these days are looking for fungi found in harsh environmental conditions as their enzymes will possess high specific activity and better half-life. The cellulose system includes endoglucanase, beta glucosidase, and cellobiohydrolase.

Another enzyme endoglucanase cleaves reducing and non-reducing end of cellulose and cellobiohydrolase and leads to the release of cellooligosaccharides and cellobiose. These undergo synergic action that causes hydrolysis of cellulosic biomass and thus release sugars. The fermentation process causes the production of biohydrogen and bioethanol.

Trichoderma

In another approach hydrolysis of the biomass of *Sesbania bispinosa,* a plant of Fabaceae family by using cellulase derived from filamentous fungi *Trichoderma longibrachiatum* immobilized on magnetic nanoparticle increased the yield of bioethanol (Baskar et al. 2016).

Trichoderma reesei are also a prime producer of cellulase enzyme which are useful in biofuel production as discussed earlier.

Mortierella sp

Some other filamentous fungi such as *Mortierella vinacea, M. isabellina,* and *Aspergillus terreus* are being explored as a biodiesel producer from lignocellulosic biomass as it can even grow and tolerate acidic dilute H_2SO_4 hydrolysate of wheat straw and produce lipid (Zheng et al. 2012).

Apart from traditional microbes involved in biofuel production like the yeast *Saccharomyces cerevisiae* (Haghighi Mood et al. 2013), several molds have also been marked as ethanol producing microbes. Examples include *Mucorales, Mucor indicus, Mucor hiemalis, Mucor circinelloides, Rhizopus oryzae* and are able to ferment more variety of sugars than *S. cerevisiae* (Satari et al. 2015, 2016).

7.6 Apparatus Developed for Biofuel Production

For efficient biofuel production from different sources various bioreactors and fermenters are being developed nowadays.

7.6.1 Continuous Stirred Tank Reactor

Such tank-based reactors generally ferment syngas to produce biofuels like ethanol. In the continuous stirred tank reactor (CSTR) the coefficient of volumetric mass is optimized to achieve better production efficiency by increasing the amount of syngas and improving area for gas–liquid mixing (Munasinghe and Khanal 2010). A diffuser regularly introduces syngases into liquid and agitation is carried out using a stirrer that breaks larger bubbles into smaller making it easily available for microbes that ferment it.

Apart from such single stage CSTR, a two-step CSTR has been developed recently (Richter et al. 2013). This type of CSTR consists of a stage one CSTR with a working capacity of 1 L subjected to an agitation speed of 200 rotations per minute. This stage one CSTR is combined with another bubble column of 4 L working capacity. The bubble column is provided with a foam control system that upon detection of high foam level injects an antifoam solution. Other than that, few pumps are installed like a gas recycle pump, cell recycle pump, peristaltic pump, etc. together with microbubble spargers. In such type of 2-stage CSTR syngases have been fermented in the presence of *C. ljungdahlii* resulting in the generation of up to 0.37 gL^{-1} h^{-1}, with an added advantage of the recovery of carbon and hydrogen.

7.6.2 Biocoil™

BIOCOIL™ is a helical tubular photobioreactor that provides large surface area assisting optimum incidence of light energy/unit volume of culture in turn improving the efficiency of biofuel production of the feedstock (Watanabe et al. 1995). It is a patented bioreactor of Biotechna Grasser A.P. Ltd, London, UK (European patent No. EPO239272). Initially, the cyanobacterium *Spirulina sp.* have been tested for this purpose but nowadays these are also being utilized for biofuel production from different feedstocks including microalgae (Concas et al. 2016). In a BIOCOIL™ reactor, microalgae are cultured in a suitable nutrient rich broth and a continuous supply of CO_2 is maintained via flue gas bubbles using airlifts installed in the reactor (Concas et al. 2010).

7.6.3 Rotating Drum Bioreactor

A rotating drum bioreactor is made of perlite or diatomaceous earth (DE) coated rotating drum operating under vacuum (Ali et al. 2018). Such type of reactor model has been developed for solid-state fermentation, i.e. fermentation involving microbes growing on solid substrates in the absence of free liquid (Cannel and Moo-young 1980; Wang et al. 2010). Sweet sorghum stalk has been used as feedstock and the yeast *S. cerevisiae* were used for its fermentation in a cylindrical bioreactor having straight baffles at head, middle, and end of the drum. Yeast are grown in the substrate bed area which contains the substrate. A gas headspace is also

present and entire drum is rotated at regular intervals. Such bioreactor systems have led to the production of bioethanol by anaerobic fermentation.

7.6.4 Bubble Column Bioreactor

Bubble column bioreactor is a non-mechanical vertical type of photobioreactor. This type of bioreactor is made up of vertical and cylinder-shaped tubes with an inlet at the bottom for gases that upon entering form bubbles. Formation of bubbles inside the culture causes agitation of culture and transparent property of tubes allows entry of light in the system (Płaczek et al. 2017; Mohan et al. 2019).

7.6.5 Membrane Bioreactors

To overcome the limitations of toxic compound formation that come across during the conventional process of biofuel production bioreactors such as membrane bioreactor (MBR) are developed. In such system bioreactors consist of membrane units of different pore size that separates different components generated during biofuel production (Dubey et al. 2013). Various advancements have been carried out in developing such MBR units one of which is an anaerobic membrane bioreactor or AnMBR. These MBRs are operated in the absence of oxygen leading to complete separation of solid from liquid (Liao et al. 2006).

Dereli et al. (2012) have evaluated a pilot-scale AnMBR system having a reactor fitted with a recirculation tank and a membrane tank. In such system a top-entry mixer with a mechanical seal with oil lubrication was used. A continuous-fed batch of 5–15 min feed/hour at 37 °C was operated. This pilot-scale AnMBR has been proven well efficient for the treatment of ethanol thin stillage with respect to total dissolved solids and chemical oxygen demand.

Membrane bioreactors of aerobic type are however different from those of anaerobic type where aerobic MBR is operated under pressure rather than vacuum and has a liquid velocity greater than AnMBR resulting in lower membrane flux (Liao et al. 2006).

7.6.6 Soxhlet Extractors

Developed by Franz von Soxhlet (1879) a Soxhlet extractor is generally used for biodiesel production. It basically consists of a boiler/reflux prelocator for solvent circulation. A siphon mechanism working on atmospheric pressure evacuates solids from a thimble made of filter paper on a regular interval until desired product is obtained.

Lipids extracted using these extractors whereby a siphon evacuates a full Soxhlet chamber and after a full cycle of vapor formation followed by refining and final evaporation under rotation products are separated (Dong et al. 2016;

Graham et al. 2018). Using microalgae *Chlorella protothecoides* biodiesel and lipids are produced with Soxhlet extractor (Xiong et al. 2008). In the extractor n-hexane as a Soxhlet extraction solvent is used for repeated washing of the feedstock and then subjected to rotary evaporation yielding lipid up to 57.8% of cell dry weight.

7.7 Patents on Biofuels

Numerous methods and systems are being developed from more than a decade for efficient production of biofuels by different inventors of which many have been patented. Some of the patents are addressed in Table 7.1.

7.7.1 Biofuel Cells

A type of fuel cell invented by Dimitre Karamanev in 2011 is "biofuel cell" whereby ferric ion (Fe^{3+}) is regenerated by cathodic reduction from ferrous iron (Fe^{2+}) mediated by the action of chemolithotrophs such as *Acidithiobacillus ferrooxidans* and electricity generation takes place. This system is made of a cathode compartment containing ferric ion, anode compartment with hydrogen containing fuel, bioreactor containing microorganism of choice, pumps for pumping CO_2 and O_2 into bioreactor and ferric ion solution into cathode. The net reaction occurring in the cell is

$$4\,Fe^{2+} + 4H^{+} + O_2 \rightarrow 4\,Fe^{3+} + 2H_2O$$

Where the oxidation of ferrous ion is 5 lakh times faster than the classical reaction rate (Lacey and Lawson 1970). This patent for biofuel cell is owned by University of Western Ontario, Canada.

7.7.2 Methods for Biofuel Production

A method for the production of biofuel from organic sources is patented recently. In this patented method organic matters containing at least 60% carbon content are first treated with aqueous solvent in the presence of one or more catalyst and applying a temperature ranging from 250 °C to 400 °C in the presence of 100–300 bar pressure. The additional catalyst can preferably be an acid, transition-metal, or solid catalyst.

Such a method based on depressurization and cooling of the organic matter yields biofuel such as oils with gross calorific value of more than 35 MJ/kg and more than 8% wt db. hydrogen. Thus the method developed by Maschmeyer and Humphreys (2011) is useful in producing biofuel such as liquid hydrocarbon using organic materials which can be any lignocellulosic or carbonaceous material.

Table 7.1 List of patents associated with biofuel production

S. No	Patent name	Assignee and Country	Patent no.	Summary of patent	References
1	Biofuel cell	University of Western Ontario, Canada	CA2530914C	Microbial regeneration of ferric ions by chemolithotrophic microbes	Karamanev (2011)
2	Methods for biofuel production	Licella PTY LTD., Australia	WO2011123897A1	Depressurization and cooling of organic matter	Maschmeyer and Humphreys (2011)
3	Methods and systems for biofuel production	Sapphire Energy Inc, USA	US20100297749A1	Integrated biorefineries making use of non-useful resource	Aravanis et al. (2009)
4	Biofuel composition	Recycle LLC, Germany	DE112013000510T5	Production of methyl ether using plant oils	Panteleev et al. (2012)
5	Methods and compositions for producing chemical products from *C. phytofermentans*	QTEROS LLC, USA	US20110183382A1	Production of chemical compounds including biofuels by mutagenized *Clostridium sp.*	Schmalisch et al. (2010)
6	Biofuel Processing System	Texas A&M University System, USA	US20080280338A1	Biomass conversion system generating alcohol and methane	Hall et al. (2008)
7	Method and apparatus for producing synthesis gas from biomass	China	CN101918305A	Integrated pulp and biofuel production	Kukkonen et al. (2013)
8	Production of ethanol from cellulose using a thermophilic mixed culture	BIOLOGICAL ENERGY Corp A CORP OF DE, USA	US4094742A	Mixed bacterial culture producing ethanol	Bellamy (1977)
9	Hybrid process for the production of biofuel	Shaw Intellectual Property Holdings Inc, USA	US20100021980A1	Production of methane-based biofuel from biomass	McDonald et al. (2009)
10	Processes using antibiotic alternatives in bioethanol production	BUCK MAN LABORATORIES INTERNATIONAL, INC, USA	WO2011116042A2	Fermentation producing ethanol in the presence of nonoxidizing biocide	Wiatr et al. (2010)

(continued)

Table 7.1 (continued)

S. No	Patent name	Assignee and Country	Patent no.	Summary of patent	References
11	Process for producing a pretreated feedstock	Iogen Energy Corp, USA	US20080045762A1	Process for pre-treatment of feedstocks for biofuel production	Foody and Anand (2004)
12	Process for the biological production of n-butanol with high yield	Metabolic Explorer, Australia	AU2007316189B2	Process for the biological production of n-butanol with high yield	Soucaille et al. (2006)

7.7.3 Method and System for Biofuel Production

Patented by Aravanis and colleagues there is another method patented for biofuel production. This method proposes a system for biofuel production from non-useful sources such as lands unsuitable for farming. Termed as integrated biorefinery (IBR) this invention comprises of open pond production unit for cultivating photosynthetic organisms, processing unit for oil extraction from these non-vascular organisms, a refining unit that does cracking, transesterification and isomerization of the oil if required, a waste processing unit, and a conduit. The organism used can be an alga growing under CO_2 supply via flue gas.

7.7.4 Biofuel Composition

A different composition for the production of methyl ethers that can be utilized as biodiesel has been introduced. Invented by Panteleev et al. (2012) this method suggests using a mixture of traditionally used rapeseed methyl ether with glycerides of unsaturated fatty acids or ethers (Fatty acid methyl ether or FAME) for efficient biofuel production.

Ether containing biofuel can be produced by this mixture by first subjecting it to acid catalyzed etherification followed by neutralization of obtained solution and final recovery. However, this method is not very cost effective and might need additives such as cetane improvers.

7.7.5 Methods and Composition for Producing Chemical Products from *C. phytofermentans*

Schmalisch et al. (2010) have developed a composition using which a recombinant strain of *Clostridium* produces products in two stages. The first stage end products in this method are aspartic acid, malate, 1,4-diacid such as malic acid, glycerol, terpenes, etc. while in second stage the end products are ethanol, n-butanol, hydrogen, and other biofuels. Microorganism modified for this purpose is *Clostridium sp.* QD that upon mutagenic modification contains one or more heterologous or exogenous polynucleotides. These modifications are introduced in such a way that expression of certain enzymes needed for biomass hydrolysis is overexpressed leading to efficient production of both stage one and two products including biofuels.

Aerobic and anaerobic cycling converts the cellulose or lignocellulosic biomass that is utilized without any pre-treatment. However, in another instance a high/low pH using certain acid or alkali like caustic lime or soda may be used. A saccharification and fermentation process is also included via treatment of biomass by the microbes together with enzyme/s that breaks and detoxifies the lignocelluloses. This bioconversion process can also be carried out by a separate hydrolysis and fermentation process whereby in the first step enzymes such as xylanase,

hemicellulose, or glucanase break the lignocellulose material inside a bioreactor and the microorganism only ferments the released sugars.

A consolidated bioprocess varies from the separate hydrolysis and fermentation process in that the enzymes are produced by the microbes itself. Hence, in consolidated bioprocess conversion is aided by both exogenously supplied and microbial enzymes as well.

7.7.6 Biofuel Processing System

A system has been developed for the production of hydrocarbons from biomass and its further conversion to liquified fuels. Invented by Hall et al. (2008) it includes a biomass conversion system, a reactor that performs pyrolysis or gasification and a synthetic fuel creation system. Also, this system follows the Fischer–Tropsch process generating syngas in the initial stage from easily digestible biomass portion. Heat generated is utilized for biomass conversion.

7.7.7 Method and Apparatus for Producing Synthesis Gas from Biomass

This patent introduces an integrated apparatus for pulp and biofuel production. Waste from Kraft pulp mill, e.g. forest waste, bark, black liquor, etc. is utilized. Biomethanol is procured from purified black liquor by processes such as distillation. Biohydrogen is also purified in a stepwise manner that includes washing followed by scrubbing, stripping, liquid–liquid separation, and chromatographic approaches.

7.7.8 Production of Ethanol from Cellulose Using Thermophilic Mixed Culture

In this patented process of ethanol production developed by Bellamy (1977) fermentation was carried out using two bacterial strains of which one is gram negative *Sporocytophaga* and another is gram positive *Bacillus.* These thermophilic bacterial strains are subjected to mixed culture that generates a suspension when introduced to cellulose for treatment at a pH ranging from 7.2 to 7.8.

In the process a vacuum pressure ranging between 100 and 400 mmHg is applied at a temperature starting from 55 °C up to 65 °C. These all parameters are applied in the submerged condition in a closed fermentor and under continuous feeding mode.

7.7.9 Hybrid Process for the Production of Biofuel

For increasing feedstock efficiency and flexibility a hybrid process has been developed by McDonald et al. (2009). For the production of biofuel, a mash of biomass

with water is hydrolyzed by shockwaves greater than speed of sound exposing biomass component for fermentation by microorganism. The product obtained through this process is further distilled to produce ethanol, CO_2, and stillage. Stillage coming from the fermentation is further digested in a reactor vessel in the presence of α-amylase generating biogas under anaerobic condition.

Biomass utilized in such a hybrid system is primarily starch-based like corn, sugar based such as sugar beets or cellulose-based biomass.

7.7.10 Processes Using Antibiotic Alternatives in Bioethanol Production

This method developed by Wiatr et al. (2010) makes use of nonoxidizing biocide, i.e. dihalonitrilopropionamide. These stabilized oxidizers include a variety of chemicals such as stabilized hypochlorous acid, stabilized chlorine dioxide, slow releasing chlorine trione, etc. In this method a fermentable mash is produced from starch containing feedstocks and fermented in a closed vessel by yeast. The application of nonoxidizing biocide is done to control bacterial growth in such system during fermentation. Apart from ethanol solid contents are also produced that is further dried to obtain distiller dried grain product.

7.7.11 Process for Producing a Pretreated Feedstock

The method of pre-treatment of feedstock such as grasses, straw, etc. has been invented by Foody and Anand (2004) for the purpose of biofuel production. Feedstock is wetted in aqueous stream and then pressed to remove a portion of water and soluble substances. The pressed feedstock with 35% dry solid is passed through a nip point in a one roll press producing slurried feedstock containing 8–20% of dry solid. This slurried feedstock is further subjected to dilute acid pre-treatment inside a reactor at a pH ranging between 0.8 and 2 for 0.1–30 min. Further this pretreated feedstock is hydrolyzed using enzymes such as cellulase producing glucose. This glucose produced using the explained method of feedstock preparation can be used for efficient ethanol production.

7.7.12 Process for the Biological Production of n-Butanol with High Yield

This invention developed by Soucaille et al. (2006) makes use of recombinant bacteria modified in such a way that it lacks the conventional butyrate kinase activity. Fermentation of glucose, xylose, arabinose (5-Carbon), etc. and saccharides is carried out in fermenters using these modified bacteria preferably belonging to *Clostridia* class such as *C. acetobutylicum*. n-butanol is recovered and isolated through stripping and distillation.

Modification in *C. acetobutylicum* corresponds to the deletion of *buk* gene that is done by another patented process by Soucaille et al. (2006) in which an erythromycin resistance gene is also inserted together with the target deletion. Different pathways, namely butyrate pathway, lactate pathway, acetone pathway, and acetate pathways are removed decreasing hydrogen flux and hydrogenase enzyme expression is attenuated redirecting the reducing power towards n-butanol production increasing its yield.

Other than the above-mentioned patents there are about dozens of patents related to the enhancement of biofuel production. Considerable work is in progress in this regard and the coming decade hold promise for the generation of several new techniques and products.

7.8 Evolving Generations of Biofuels: A Close Look

Approaches have always been made to evolve the traditional process of fuel production from different sources. Such has been also the case of biofuel production which evolved time to time leading to the evolution of different generations of biofuels from first to fourth (Fig. 7.3). This evolution of biofuels mainly focuses and differs based upon the type of feedstocks used.

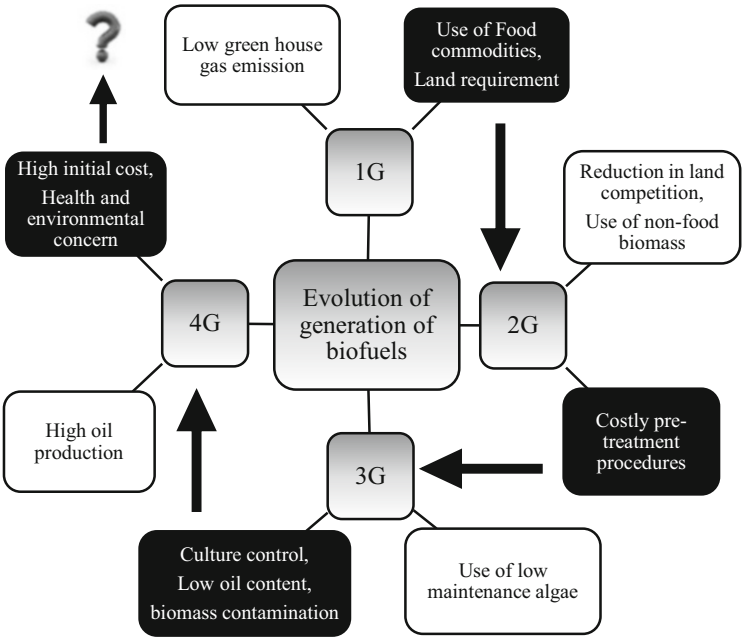

Fig. 7.3 Schematic representation of the generation of biofuels (White: Pros Black: cons)

7.8.1 Biofuels of First Generation

Production of biofuels of the first-generation (1G) majorly makes use of grains, sugar cane, corn plants, oil seeds, etc. that are rich in sucrose, starch, and lignocellulose (Malça and Freire 2006; Balat et al. 2008). Comparison of gasoline production from conventional crude oil and tar sand oils with sugarcane and corn-based bioethanol has been carried out by keeping into consideration the use of land, water, and the concept of ecological footprint (Chavez-Rodriguez and Nebra 2010). Lowest amount of greenhouse gas emission was from bioethanol from sugar cane and then from those produced from corn proving them more environment friendly than those from conventional means.

Non-flowering sugarcane NA56 has been extensively studied for bioethanol production by the action of two strains of *Saccharomyces cerevisiae* (Rolz and León 2011). Observations were made at particular intervals using cane bagasse corresponding to different growth stages and a feedstock of 307 days after plantation was recorded for maximal ethanol yielding among all other stages. The tuber crop sugar beet is also specially bred for the production of first-generation bioethanol accounting for a total of 30% yield (Haankuku et al. 2015).

For the production of first-generation bioethanol enzymes such as α and β amylase, glucoamylase, pullulanases, lytic polysaccharide monooxygenase, phytases, etc. are extensively utilized (Bertrand et al. 2016). These enzymes are responsible for starch hydrolysis in a stepwise manner. On a large scale 1G bioethanol and biobutanol production biorefineries are utilized whereby sugarcane juices and molasses are used (Dias et al. 2011). These juices are firstly utilized for ethanol production at different plant from where a clarified solution comes into fermentation unit of butanol plant. Butanol is then produced by pathways earlier explained using *Clostridium* in seed fermenters.

In an investigative analysis by Ajanovic and Haas (2010) it was concluded that only Brazil is capable of producing commercial bioethanol using sugarcane because of limiting policy conditions and shortage of lands in another EU countries. Even after evolution of different biofuel generations up to 2016, 1G bioethanol is still a major contributor to worldwide ethanol production (Bertrand et al. 2016). However, due to the involvement of food commodities this generation of biofuel has been labeled unsustainable (Mohr and Raman 2013).

7.8.2 Biofuels of Second Generation

These biofuels are generally produced using lignocellulosic feedstocks and non-food biomass such as bagasse of sugarcane, straw, municipal wastes, etc. (Sims et al. 2010). Biochemical and thermochemical pathways using enzymes, microorganisms, and pyrolysis lead to the production of second generation of ethanol and synthetic diesel.

From lignocellulose sources biofuels are produced either by biochemical or thermochemical pathways (Sims et al. 2010). In biochemical pathway utilizing

microbes, biofuel such as ethanol is produced via fermentation while in thermochemical pathway synthetic diesel or ethanol is produced via gasification or pyrolysis. Such biomass to liquid conversion follows the route of Fischer–Tropsch in which higher hydrocarbon compounds are produced in sequential reaction from carbon monoxide and hydrogen of syngas produced from biomasses (Snehesh et al. 2017).

Apart from lignocellulose materials, microalgal systems are exploited and considered under second generation (Schenk et al. 2008). Production of biofuels, such as bioethanol, biodiesel, and syngases, by utilizing such system reduces land competition overcoming the challenges of 1G biofuels (Sims et al. 2010). *Chlamydomonas reinhardtii* in an aerobic–anaerobic cycle produces biohydrogen by utilizing sunlight for the generation of hydrogen from water (Melis et al. 2000). In this process initially water breaks into $2H^+$ and 1/2 O_2 and further in a sequential reaction mediated by hydrogenase enzyme H_2 is produced.

Production of 2G biofuels also has certain limitations such as requirement of certain costly pre-treatment of potential substrates, e.g. alkaline pre-treatment of kapok fiber for efficient bioethanol production (Tye et al. 2012). In this method Kapok fiber is pretreated in a stationary digester made of stainless steel. A treatment of 60 min each with a solid-to-liquid ratio of 12:1 is carried out at each step. Firstly, water treatment is given at a temperature of 150 °C followed by sulfuric acid treatment at 120 °C and then final treatment is given with alkaline sodium hydroxide at 120 °C. At the end of all the treatments this treated kapok fiber is neutralized by washing it with tap water and then dried prior to further use. Another limitation of 2G biofuels includes the total production cost that fluctuates depending upon time and type of feedstock thus limiting its competitive advantage over traditional oils.

7.8.3 Third-Generation Biofuels

Search for cost-effective approaches for biofuel production led to the evolution of third-generation biofuels. This generation solely depends on utilization of oleaginous microbes that encompass microalgae, bacteria, fungi, and yeast for producing biodiesel, bioethanol, syngas, biobutanol, methane, etc. (Leong et al. 2018). High biomass, shorter doubling time, and high oil content made microalgae efficient target for biofuel production (Chisti 2007). However, the fatty acid content varies depending upon the species and their growth phase (Pratoomyot et al. 2005). Thus, there is crucial need of recognizing and selecting algal species that possess substantial oil content and that can be grown suitably under industrially feasible conditions.

Culture of microalga in closed system is costly since it requires artificial light indoors, thus, an open culture system utilizing sunlight provides better approach (Borowitzka 1999). However, this approach also has limitations of inability to control culture environment. Also, some algal species such as *Chlorella* require specialized raceway system minimizing culture overgrowth. Such open culture

system, however, presents with low oil content and chances of biomass contamination (Dutta et al. 2014).

7.8.4 Fourth-Generation Biofuels

Similar to third generation of biofuels the subsequent evolution of fourth generation of biofuel makes use of algae but with genetic modifications that serve to curb the limitations of third-generation biofuel production. This approach makes use of expressed sequence databases and genome sequences either nuclear or from chloroplast and mitochondria (Radakovits et al. 2010). More than 30 species including *Chlorella vulgaris, Chlamydomonas reinhardtii, ulva lactuca, Cuphea sp.*, etc. have been genetically modified up till now modifying their metabolic pathways such as those responsible for lipid biosynthesis (Huang et al. 1996; Chow and Tung 1999; Chen et al. 2001; Eichler-Stahlberg et al. 2009).

Such modifications lead to high yield of biofuels since it enhances CO_2 capture ability, lipid content as well as algal biomass making it more feasible even though the initial cost is high (Singh and Gu 2010). Together with algae other microorganisms like cyanobacteria are being modified and designed for efficient development of solar biofuels and electrobiofuels (Aro 2015). Current research is aimed at developing more feasible approaches. However, disposal and use of such genetically modified organisms and their effect on health and environment upon accidental exposure is a huge concern (Abdullah et al. 2019).

7.9 Biofuel Production in India: Sustainability and Cost

The Indian economy has been growing decently since its independence and the growth rate has increased at the rate of seven per cent since 2000 (EIA 2013). To keep pace with this economic growth energy demand is equally thriving, thus biofuels are considered to be excellent alternative options. Global oil supplies are uncertain and its increased demand led India to look for an alternative that can be produced locally. Moreover, apart from energy requirements, the purpose also includes environment sustainability and agricultural and food security (Lapola et al. 2009). To harness the biofuel sector in India, the government has introduced several programs like ethanol blended petrol program and biodiesel blending program in the year 2003 under the National Biofuel Mission (NBM) (Fig. 7.4). The purpose of these programs is to blend ethanol or biofuels with petrol commercially. Ministry of New and Renewable Energy (MNRE) in December 2009 proposed blending 20% biofuels with high speed diesel and petrol. This will increase the requirement of bioethanol which can be fixed by cultivating more sugarcane than the present sugarcane productivity. However, this will create burden on land, resources, and water demand. Thus, the Indian Ministry of Petroleum and Natural Gas (MoPNG) is able to achieve 5% ethanol blending in petrol across nine states and

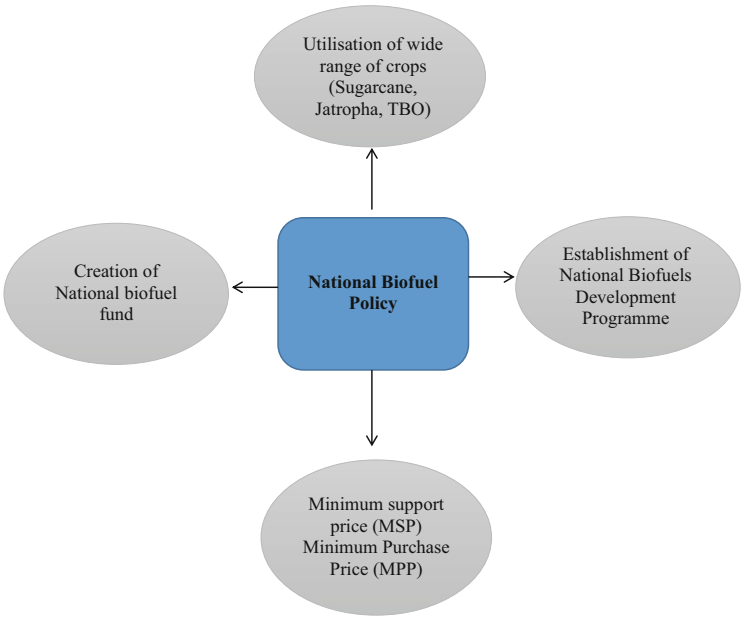

Fig. 7.4 Important aspects of National Biofuel Policy

five union territories. This was later extended to cover 20 states and 8 union territories in 2016.

The key features of the National Biofuel Policy in India:

- To ensure the minimum requirement of biofuels in the country. The target includes 20% blending in 2019.
- To motivate growing non-edible oilseeds crop plantation on degraded land and discouraging plantation on agricultural land.
- Announcement of Minimum Support Price (MSP) for farmers producing biodiesel oilseeds so that optimum price can be given to them. In addition to this, Minimum Purchase Price (MPP) based on the actual cost of production and import price of bioethanol by oil making companies is encouraged.
- Financial supports like grants, subsidies for new and second-generation feedstocks, latest technologies, etc. Biodiesel and bioethanol production is exempted from all sorts of central taxes except for concessional excise of 16% on bioethanol.
- Moreover, in order to attract Foreign Direct Investment (FDI) 100% foreign equity is allowed on biodiesel technologies and projects when biofuels are used domestically.
- The status of state independence has allowed them to set a target and enjoy their own biofuel policy. For example, Chhattisgarh biodiesel development authority and Tamil Nadu provide *Jatropha* seedlings at subsidized rate enabling its

smooth cultivation in wasteland. Similarly, the Government of Odisha with the help of Odisha Renewable Energy Development Agency (ORCDA) and Odisha Forest Development Corporation (OFDC) provides subsidies and acts as bridge between biofuel program and other organizations.

The economic feasibility of biofuel production and its associated technologies depends primarily on feedstock, capital investments, etc. where feedstock cost occupies approximately 40–80% of the total production of biofuels (Carriquiry et al. 2011). India's bioethanol and biodiesel program depend on molasses and *Jatropha* cultivation, respectively. India stands as the second largest producer of sugarcane and a large producer of ethanol prepared from sugarcane molasses around the globe. The production increased by six times from 1950 to 2011, i.e. the yield increased from 33.4% to 70% t/ha. Although sugarcane production is prevalent in almost all states more than 75% of sugarcane cultivation is concentrated in Uttar Pradesh (120.55 Mt), Maharashtra (81.9 Mt), Karnataka (39.66 Mt), and Tamil Nadu (34.25 Mt). It is estimated that 85–100 kg of sugar and 35–45 kg of molasses can be obtained from 1 ton of sugarcane (Bhattacharya 2010). Maximum ethanol yield is concentrated in Uttar Pradesh (0.96 BL), Maharashtra (0.65 BL), Karnataka (0.31 BL), and Tamil Nadu (0.27 BL).

Similarly, biodiesel production depends on using non-edible oils extracted from *Jatropha*, *Pongamia,* and other tree-borne oil seeds grown in unproductive land. However, Indian government does not interfere with the growth of these oil seeds and agricultural crops.

7.10 Industrial Trends: Current Status

Ongoing researches have presented a plethora of opportunities for the production of biofuels using resources that are cost effective and environment friendly. However, the feasibility and commercial exploitation of such resources need to be assessed on the industrial level.

Biofuel production has increased only by 4% up to 2017 including sugar and starch-based ethanol, hydrogenated vegetable oil, etc. (2019 report of International Energy Agency). This 4% increase numerically accounts for about 83 million tonnes of oil equivalents (Mtoe). However, a growth of about 2.5% per year is being speculated and a target has been set to 284 Mtoe. Bioethanol industries are vastly located at Brazil that uses sugarcane molasses and starch (IEA 2004; Antoni et al. 2007). These industries generally initiate ethanol production using *S. cerevisiae* that upon batch fermentation also leads to the generation of products such as methanol.

Biofuel production from lignocellulosic biomass is commercially limited due to a lack of enzymes that might efficiently convert the cellulosic components into its reducing sugars (Taha et al. 2016). Nowadays, biomasses are being subjected to specialized biorefineries for integrated production of food, chemicals as well as biofuels at industrial scale (Kamm and Kamm 2007). Food crops such as *Cassava*

are being used for bioethanol and other biofuels production at the industrial level (reviewed by Zhang et al. 2016).

Recent trends in the production of biofuels at industrial level encompasses development of certain policies in different countries including those of European Union, the USA, etc. For example, Brazil encourages its citizens to use hydrous bioethanol in place of gasoline while in the USA additional incentives are provided for using cellulosic bioethanol (Kojima et al. 2007). According to the Energy Policy Act, 2005 the US Congress has also extended biodiesel fuel excise tax credit through 2008.

Following the footsteps of developed countries, some underdeveloped and developing countries such as sub-Saharan African countries are also taking into account the use of l and industrial advances are being approached in countries including Ghana, Burkina Faso, Mali, etc. (Sekoai and Yoro 2016). South Africa has implemented a 5-year pilot phase plan and mandates use of bioethanol and biodiesel together with conventional fuels (Banks and Schäffler 2006). On the national level, for biofuel production with low carbon emission, India joined United States Partnership to Advance Clean Energy and majorly uses broken rice, pearl millet, and sorghum at ethanol distilleries of Haryana (Packiam et al. 2018). These distilleries also make use of *S. cerevisiae* that produces bioethanol by saccharification or fermentation of the feedstocks (Wu et al. 2006).

7.11 Conclusion

Production of fuels from renewable sources leading to the generation of biofuels is a major area of research nowadays to curb energy crisis as well as pollution worldwide. Biofuels generated from different plant sources and with the aid of microorganisms have been categorized as bioalcohols, biodiesels, biohydrogens, etc. For more than decades reactors, fermenters, and other apparatuses are being developed. A large number of patents are also being filed and issued from different countries including the USA, Canada, Australia, etc. for efficient biofuel production. Also, depending upon the substrate being utilized for production, biofuels have evolved from time to time leading to evolution of different generations from first to fourth up till now. Each generation has been an advancement over the previous one and overcomes the limitations of preceding generation. Currently, industries and companies are being set up and policies are being developed in different countries for feasible, sustainable as well as environment friendly production of biofuels.

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8 Biofuels: Sources, Modern Technology Developments and Views on Bioenergy Management

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Abstract

Increasing energy demands and the rising global carbon footprint are forcing mankind to look for alternative green fuels. Fuels derived from biological sources are considered to be green fuels since they do not release toxic pollutants upon combustion. The global accumulation of the carbon footprint and accelerated demands on energy are pushing us to look for alternative green fuels based on renewable resources. Hence, identification of potential sources of green fuels produced by biological means and utilization of these resources for commercialization provide the context of the priorities for future energy needs. The two major concepts considered for next-generation green fuels are (i) fuels that do not increase the carbon footprint (e.g. hydrogen fuel) and (ii) utilization of photosynthetic processes to fix CO_2 and produce biofuels. Keeping these two priorities in mind, this chapter provides a detailed discussion of various biofuels available for mankind, which can replace traditional hydrocarbon-based fossil fuels. These biofuels could help in reducing the global carbon footprint. The chapter gives information about the various biological sources for production of biodiesel and microbial sources for production of liquid fuels. This chapter also discusses the concept of microbial fuel cells, the importance of biohydrogen, aspects of molecular engineering of organisms to enhance productivity, fabrication of microbial systems for production of biofuels and the prospects for biofuel production by utilizing modern biotechnology tools.

Keywords

Biofuels · Microbial fermentation · Biodiesel · Biohydrogen and microbial fuel cells

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8.1 Introduction

Greenhouse gas (GHG) accumulation, increasing demands for energy resources and depletion of petroleum resources are making mankind look for alternatives to hydrocarbon-based fossil fuels. The future global economy will be energy based, and each nation is in the process of looking at different sources of renewable energy such as solar energy, wind energy and biofuels. Though solar energy and wind energy are clean and renewable resources, there are technical issues that need to be resolved before we can completely depend on these sources. Moreover, solar and wind sources can fulfill only part of our requirements; hence, contributions from other alternative renewable sources are much needed to cope with increasing demands. Use of fuels from various sources of biological origin can effectively resolve issues such as depletion of hydrocarbon-based fossil fuel resources, increasing energy demands and the growing carbon footprint worldwide, which are leading to global environmental turbulence. Biofuels generated from various biological means are attracting attention from many countries because of their environmental benefits and their renewable nature. Moreover, for sustainable growth of this bioenergy sector, these sources must not compete with food crops, as such competition would have an adverse impact on food security. The biofuels presently available commercially are derived from vegetable oil, which is, upon transesterification, converted to biodiesel and can replace conventional petrodiesel. On-road tests using biodiesel have shown reduced carbon emissions in comparison with petrodiesel. Despite this success, there are two major issues with these renewable biodiesel sources. One is that they compete with food crops for land agriculture, and the other is the cost of their production. To reach the goal of large-scale biofuel production without causing problems for food security, the best way to attain energy security is by utilizing non-edible vegetable oil for biodiesel production. In this context, biodiesel derived from *Jatropha* seed oil has the potential to overcome these issues because this crop does not compete for land that is utilized for agriculture of food crops, as *Jatropha* grows on marginal land and in the semi-arid tropics (Sudheer et al. 2012). Moreover, biodiesel generated from *Jatropha* seed oil has been found to be high quality with very low carbon emissions (Ghosh et al. 2007).

Microbial fuel sources have been found to have the potential to meet growing demands for energy. Microbial-based biofuels have the advantage of overriding the concept of food security, since they do not compete with food crop land for their cultivation. Moreover, many microbial species use agricultural waste such as lignocellulose biomass for their cell biomass generation and produce biofuels via dark fermentation (Tuck et al. 2012). In the past couple of decades, application of microorganisms for production of various types of biofuels utilizing waste biomass has been steeply increasing (Liao et al. 2016). These microbial systems are highly diverse, can utilize diverse substrates for their metabolism and can generate cell biomass and useful products that can be directly utilized for energy or can be converted into fuel. Selection of the microbe type, selection of a suitable substrate (preferably inexpensive or waste biomass) and the strategy used for energy production play important roles in creation of next-generation green fuels.

This chapter gives a comprehensive outlook on various types of biofuels from different biological sources, the different processes by which biofuels are generated and the technological advancements that have been made in the bioenergy field. It also discusses next-generation biofuels produced by genetically engineered organisms and their future prospects, the predicted problems that need to be resolved for each biofuel type and the synergic efforts that need to be made to meet future demands for energy with consideration of environmental issues.

8.2 Plant-Based Biofuels

Plant-based biofuels were in use long before machines and the automobile industry evolved. With the identification of hydrocarbon-based fossil fuels, the emergence of the coal-based energy economy and the availability of these fossil fuels at minimal cost, mankind moved towards dependence on fossil fuel–based energy. However, increasing energy demands, depletion of fossil fuel resources, the rising price of crude oils and our increasing carbon footprint leading to global warming, are now making us to look for green energy resources (Reddy and Sudheer 2010; Mastan et al. 2012; Rahman 2012). There are two ways of utilizing plant-based raw materials for production of biofuels: (i) microbial fermentation and (ii) utilization of vegetable oils for generation of biodiesel via transesterification. Indirect utilization of plant carbohydrate–based raw materials for production of biofuels by microbial fermentation is discussed later sections in this chapter. The next subsection discusses preparation of vegetable oil–based biodiesel, its advantages, its future prospects and issues that need to be resolved.

8.2.1 Biodiesel

Traditionally, different types of plant-derived biomass were utilized to provide energy for domestic use. However, in the modern era, the application of energy in various fields was expanded, and in many instances, the fuel source needed to be of high calorific value to reach the required temperatures. Hence, most traditional biofuels were replaced with hydrocarbon-based fuels. To fulfill the modern fuel sector's energy needs without causing environmental issues, we need to innovate traditional processes to make usage of fuel environmentally friendly, and devise new strategies to produce fuels from renewable biomass that can be substituted for hydrocarbon-based fuels. Biodiesel is one renewable fuel source whose potential has been experimentally demonstrated for replacement of petrodiesel without the need for any engine modifications (Ghosh et al. 2007; Reddy and Sudheer 2010; Sudheer et al. 2010). Biodiesel can be generated from any vegetable oil via a procedure called transesterification.

The biodiesel consists of mono-alkyl esters of fatty acids obtained after transesterification of vegetable oil. The transesterification reaction includes alcohol as a co-substrate in the presence of a suitable catalyst, forming fatty acid methyl

esters (FMEs), commonly called biodiesel. Biodiesel can be used in a blended form with conventional diesel; alternatively, a refined quality of biodiesel (e.g. biodiesel derived from *Jatropha*) is suitable for direct use in commercial diesel-run engines without any modification (Ghosh et al. 2007; Sudheer et al. 2010; Rahman 2012). Transesterification is also commonly known as alcoholysis, where alcohol is used as one of the substrates to obtain methyl or ethyl esters. Of these, methyl esters are more popular since use of methanol will reduce the final cost of the product. In this reaction, triglycerides participate in the esterification reaction with the alcohol substrate in the presence of a suitable catalyst to form fatty acid alkyl esters (biodiesel) and glycerol as a by-product (Fig. 8.1). After the transesterification reaction, this mixture of products is resolved and separated by downstream processing. With regard to the catalyst, in most commercial cases, sodium hydroxide is used as the alkali catalyst. The issue with this process is that the alkali water released after downstream processing needs to be neutralized or treated. An alternative is conducting the reaction under acidic conditions in the presence of sulfuric acid, sulfonic acid or hydrochloric acid. Acid catalysis is preferred when the triglyceride content of the vegetable oil includes high levels of free fatty acids. However, the problem with acid catalyst is that it corrodes the reactor. There are also non-conventional heterogeneous catalysts—such as amorphous zirconia, titanium and potassium zirconia—that can catalyze the transesterification reaction. These catalysts have an additive advantage in downstream processing, as they can be separated from the reaction mixture easily. This reduces the cost of biodiesel production (Fukuda et al. 2001; Demirbas 2005; Rahman 2012).

Both alkaline and acid catalysis have their own disadvantages in downstream processing. However, these are the successful methods for biodiesel production on a commercial scale. Researchers have conducted enzymatic transesterification reactions to produce biodiesel. The enzymatic catalysis method is a realistic alternative where the conversion rate is reported to be up to 92% with use of soybean oil. An excess of alcohol and smaller amounts of lipids in the reaction reduce the enzyme activity, and alcohol also has a deteriorating effect on the enzyme. Glycerol, as a major by-product, also blocks the free or immobilized enzyme (Noureddini et al. 2005; Modi et al. 2007). However, with the present costs of enzyme production and technology implementation, enzyme-based transesterification is not economical and cannot compete with acid/alkaline catalysis. All of these factors need to be addressed for successful implementation of enzymatic transesterification at the industrial level. Technology that bypasses these issues will become a potential system for production

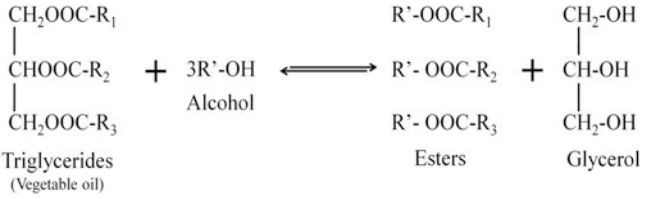

Fig. 8.1 Chemical process for transesterification of vegetable oils

of biodiesel. This process will be greener and also will cut down the final cost of the diesel that is synthesized. However, a lot of effort needs to be put into innovating/integrating lateral technology by utilizing modern protein engineering and nanotechnology to make potential enzyme-based catalytic molecules for transesterification.

8.2.1.1 *Jatropha* Biodiesel

Biodiesel can be produced from any vegetable oil, but the unique physicochemical characteristics of biodiesel derived from *Jatropha curcas* seed oil have been found to be very impressive both for direct use and for blending. *Jatropha* seed oil comprises about 72% unsaturated fatty acids, the major proportion of which is oleic acid, followed by linoleic acid. Hence, the viscosity of *Jatropha* oil is naturally low in comparison with that of other oils such as soybean oil, cottonseed oil and sunflower oil (Table 8.1). Hence, after transesterification, the resulting biodiesel is reportedly able to be used directly without the need for any engine modifications (Ghosh et al. 2007). As shown in Table 8.2, the physicochemical properties of *Jatropha* methyl ester (JME)—the so-called *Jatropha* biodiesel—are very comparable to those of traditional petrodiesel (Table 8.1). This feature is helpful for a direct shift from use of petrodiesel to use of JME. In addition, its high flashpoint (160–170 °C) and cetane number make JME more efficient and more environmentally friendly than conventional petrodiesel. Moreover, the fact that *Jatropha* oil is nonedible (because it contains toxic ingredients such as curcin and phorbol esters) makes the "food versus fuel" dilemma irrelevant. Besides, *J. curcas* is naturally drought resistant and can grow on marginal land and wasteland. Along with these features, its inherent advantages such as its high oil content, low-cost agriculture and natural resistance to pests and diseases make JME the best candidate for promotion as commercial biodiesel production.

Table 8.1 Fatty acid composition of various vegetable oils used for biodiesel synthesis (Sudheer et al. 2012)

Fatty acid	*Jatropha* oil	Soybean oil[a]	Cottonseed oil[a]	Palm oil[a]	Sunflower oil
Capric (%)	0.1		–	–	–
Myristic (%)	0.1	0.1	0.7	1	0.2
Palmitic (%)	15.1	10.2	20.1	42.8	4.8
Palmitoleic (%)	0.9	0.1	–	–	0.8
Stearic (%)	7.1	3.7	2.6	4.5	5.7
Oleic (%)	44.7	22.8	19.2	40.5	20.6
Linoleic (%)	31.4	53.7	55.2	10.1	66.2
Linolenic (%)	0.2	8.6	0.6	0.2	0.8
Arachidonic (%)	0.2	0.3	–	–	0.4
Behenic (%)	0.2	0.1	–	–	–
Lauric (%)	–	0.1	0.1	0.1	0.5

[a]Because of rounding, the percentages listed do not add up to 100

Table 8.2 Physicochemical properties of *Jatropha* methyl ester (Sarin et al. 2007; Sudheer et al. 2012)

Property	Value
Flashpoint (°C)	163
Viscosity at 40 °C (cSt)	4.4
Sulfated ash (% mass)	0.002
Sulfur (% mass)	0.004
Cloud point (°C)	4
Copper corrosion rating	1
Cetane number	57.1
Water and sediment (vol%)	0.05
Conradson carbon residue 100% (% mass)	<0.01
Neutralization value (mg, KOH/g)	0.48
Free glycerin (% mass)	0.01
Total glycerin (% mass)	0.02
Phosphorus (% mass)	<0.001
Distillation temperature (°C)	295
Oxidation stability (h)	3.23

The ability of *J. curcas* to grow on wasteland bypasses the issue of food security, since its cultivation does not compete with food crops for agricultural land. This makes JME a promising alternative to petrodiesel. Moreover, the by-products obtained during JME synthesis—such as crude glycerol, seed cake and empty seed capsule shells—can be used as raw materials for different applications, which can also help to reduce the cost of JME production. Apart from these advantages, JME (which comes under the EN 14214 norms) will simplify operational norms and also its production can be decentralized on a small scale, according to seed production centers, which will also cut down transportation costs. Unlike petrodiesel, JME does not come under the explosive and petroleum act (Govt. of India); hence, it is possible to decentralize its production with low capacity of up to two tons per day. This will aid economic empowerment of local populations and also encourage independent economic growth in rural areas. The operational conditions required for the production of JME are ambient and make it easy to establish small-scale production. Many studies are currently in progress to make the whole system environmentally friendly with zero-waste management. Test vehicle runs conducted by the well known automobile manufacturer Daimler Chrysler, using two Mercedes Benz C220 vehicles without engine modifications, showed that in all conditions, JME was very comparable to, and competitive with, petrodiesel in terms of mileage. Moreover, no negative remarks were reported during the test runs. Emission tests showed dramatic reductions in carbon and particulate emissions, which were found to be 96% and 80% lower, respectively, than those seen with petrodiesel. Commercial success of JME will be possible if the production cost of JME comes down to the same cost as petrodiesel, and this could be possible if each by-product obtained during JME synthesis is converted into some type of valuable product and wasteland is utilized for *Jatropha* agriculture for seed production. Though JME is the best option for renewable energy, the refined agricultural technology and

protocols required for mass propagation of explants for agriculture have not yet been commercialized. Moreover, lack of extensive field research studies and non-availability of defined and characterized germplasm for agriculture are limiting the progress of replacement of petrodiesel with JME. Hence, more efforts need to made by the scientific community, public and private funding needs to be provided for development and improvement of agricultural protocols for application of waste-land agriculture.

8.2.2 Algal Biodiesel

The alternative to vegetable oils for biodiesel production is algae-derived energy-rich oils—in most instances, derived from microalgae. Microalgae are single-celled organisms, including a diverse group that has the ability to accumulate energy-rich oils in the cells. In the modern era of energy management systems, algal oil is being looked at as a good prospect because of the flexible growth conditions tolerated by algae, their efficiency in sequestering CO_2 by photosynthetic fixation and their accumulation of oil, which could be used for diesel production. Hence, many researchers are attracted to microalgal cultivation for oil extraction (Falkowski et al. 1998). Microalgae have the ability to accumulate large amounts of lipids—from 20% to 80% of total dry mass, which is several times higher than the amounts accumulated by higher plants (Schenk et al. 2008; Amaro et al. 2011). The major advantages of microalgae are that they need only very limited land space for their cultivation and the land need not be cultivable/fertile. Though microalgae are aquatic species, less water is needed for their cultivation than required for irrigation of land plants. Microalgae need only fresh water or marine water (in the case of marine algae) with additive nutrient salts; most often, those salts are inexpensive. Hence, many researchers and industries are looking at opportunities for application of algal oils for biodiesel production. Many factors influence the productivity of biodiesel from microalgae. A few are discussed briefly in the following subsections.

8.2.2.1 Algal Species and Types

The choice of species for production of algal biomass for lipid extraction is very critical. Diverse species of microalgae accumulate lipids at rates of up to 70 to 80% of total dry mass; in some instances, accumulation is up to 90% of total dry mass has even been reported. However, many other factors need to be considered before production is scaled up. Table 8.3 lists various freshwater and marine algal species and their capacity for lipid accumulation. Next to the lipid percentage accumulation, the next most significant factor to be considered is the growth rate. If the growth rate is slower, even though the final lipid accumulation is high, the maintenance of the culture system will add to the cost of the final product. Hence, species selection needs to be more innovative, and a species with moderate lipid accumulation and a fast growth rate should be preferred than a species with high lipid accumulation but a slow growth rate. However, the algal species selected for cultivation should thrive in broad growth conditions. This will allow flexibility in reactor design and it will allow

Table 8.3 Percentages of lipid accumulated by various species of microalgae grown in fresh and marine water (Amaro et al. 2011)

Water	Microalga	Lipid content (%, w/wDW)
Fresh water	*Botryococcus* sp.	25.0–75.0
	Chaetoceros muelleri	33.6
	Chaetoceros calcitrans	14.6–16.4/39.8
	Chlorella emersonii	25.0–63.0
	Chlorella protothecoides	14.6–57.8
	Chlorella sorokiniana	19.0–22.0
	Chlorella vulgaris	5.0–58.0
	Chlorella sp.	10.0–48.0
	Chlorella pyrenoidosa	2
	Chlorella sp.	18.0–57.0
	Chlorococcum sp.	19.3
	Ellipsoidion sp.	27.4
	Haematococcus pluvialis	25
	Scenedesmus obliquus	11.0–55.0
	Scenedesmus quadricauda	1.9–18.4
	Scenedesmus sp.	19.6–21.1
Marine water	*Dunaliella salina*	6.0–25.0
	Dunaliella primolecta	23.1
	Dunaliella tertiolecta	16.7–71.0
	Dunaliella sp.	17.5–67.0
	Isochrysis galbana	7.0–40.0
	Isochrysis sp.	7.1–33
	Nannochloris sp.	20.0–56.0
	Nannochloropsis oculata	22.7–29.7
	Nannochloropsis sp.	12.0–53.0
	Neochloris oleoabundans	29.0–65.0
	Pavlova salina	30.9
	Pavlova lutheri	35.5
	Phaeodactylum tricornutum	18.0–57.0
	Spirulina platensis	4.0–16.6

ambient conditions for growth, thereby reducing the cost input for cultivation and will reduce the final product cost (Schenk et al. 2008; Amaro et al. 2011).

Popular microalgal species for biodiesel cultivation are *Chlorella*, *Dunaliella*, *Nannochloris*, *Nitzschia*, *Nannochloropsis*, *Neochloris*, *Isochrysis*, *Phaeodactylum* and *Porphyridium* spp. These species are known for their fast growth rates and moderate oil content (25 to 50% of total dry mass). Among these, marine species of *Chlorella* are the best choice for commercial cultivation since they have a high specific growth rate and use of salt water avoids contamination by other microbes. Use of marine water for cultivation will help conserve fresh water, which can be utilized for food agriculture. Not only the oil content of the species play a significant

part but also the type of lipid accumulation is important, since the quality of biodiesel depends on the types of fatty acids accumulated in the cells (Chen et al. 2018). For example, *Botryococcus braunii* is currently under the study for the potential quality of its oil, with a high content of long-chain fatty acid (oleic acid), which can result in a good quality of biodiesel. However, the growth rate and biomass productivity of this species are very low. Hence, multiple criteria such as the growth rate, biomass accumulation in terms of the unit volume, resistance to environmental turbulence, nutrient requirements and rate of uptake, light requirements, ability for CO_2 sequestration, nitrogen and phosphate requirements, biomass-harvesting requirements etc. need to be considered in strain selection.

8.2.2.2 Cultivation of Microalgae for Biodiesel Production

Microalgal species for cultivation will be selected mostly on the basis of the proposed geographical area for cultivation. These microalgae will be screened initially for the suitability of the environmental conditions, their energy-harvesting ability and carbon source provision in the selected area. Growth optimization is greatly needed for biomass generation; most often, microalgae grow via autotrophic nutrition by harvesting light and fixing CO_2. However, some species shift their nutrition from photoautotrophic to heterotrophic or mixotrophic nutrition on the basis of the pH and other culture conditions. The best example of this is spirulina, which shifts its growth from photoautotrophic to heterotrophic or mixotrophic nutrition on the basis of pH variations and light availability (Mata et al. 2010). Likewise, several different types of microalgae utilize external carbon sources in dark conditions via heterotrophic cultivation (Xu et al. 2006). In a study using heterotrophic fermentation in dark conditions, it was shown that *Chlorella protothecoides* was able to accumulate oil at a rate of 55% of total dry mass. The biodiesel produced from the extracted oil had a high heating value of 41 MJ kg^{-1}, with a density of 0.864 Kg L^{-1}, and was considered good-quality biodiesel. Another significant feature of this work was that instead of glucose, the researchers used maize seed powder hydrolysate as a carbon source, making the fermentation very economical.

8.2.2.3 Cultivation Methods

The best choice of cultivation method has been a matter of debate for microalgal cultivation and still has not been established. The choice of reactor type, such as open pond cultivation or a closed photobioreactor, is often debated; both types have their pros and cons to be considered for implementation on a large scale. Open pond cultivation is relatively good in terms of the required investment for building and operation, but contamination and maintenance of the growth conditions (especially temperature control) pose significant challenges. Hence, in the case of open pond conditions, it is important to select a suitable strain that can sustain its growth despite temperature fluctuations. However, the major issues associated with open pond cultivation are contamination by other microbes in the culture and the large amount of land space required for commercial production (Costa et al. 2019).

In contrast to open pond cultivation, a closed photobioreactor needs only limited space. This type of reactor also provides ease of harvesting by various methodologies (Scott et al. 2010). There are many types of reactor designed for culturing microalgae; popular ones are tubular reactors, flat-plate reactors, annular reactors and vertical reactors. These reactors are aerated by mechanical stirring, bubbling and airlifting (El-Shishtawy et al. 1997).

Light is another crucial factor that regulates growth and biomass production. At present, the maximum efficiency of light harvesting by microalgae by any means (natural/artificial light) is up to 12% and the maximum efficiency of open pond systems is up to 7%. Thus, researchers are looking for innovative ways to cultivate microalgae and further increase their efficiency to attain good cell biomass and oil accumulation. Small-scale laboratory experimentation utilizing fluorescent lamps for illumination has shown improvements in cell biomass and oil content, and hopping that data can be reproduced in the scale-up. The reactors also need to be designed to capture natural light as efficiently as a laboratory system. To achieve this, researchers have designed various types of reactors, the most popular are tubular reactors, plate reactors, bubble column reactors, annular reactors and plate airlift reactors. All of these reactors are made with transparent materials to allow natural light to get inside, where the microalgae are circulated linearly and circularly.

8.2.2.4 Aeration Systems

Microalgae cultures need to be aerated continuously not only for CO_2 capture for photosynthesis but also because aeration via different methods mixes the cultures and avoids clumping and settling in the reactors, thereby increasing the biomass and accumulation of oil in the cells. Studies have been conducted in which researchers used CO_2-rich exhaust from industry for aeration purposes and found that the biomass and lipid accumulation increased in the cultures. This is a potential method for CO_2 sequestration and will reduce the carbon footprint. The best results were obtained with *Nannochloropsis oculata*, *Scenedesmus obliquus* and *Chlorella kessleri* strains. In particular, the later two species showed high potential for CO_2 fixation (Fujishima et al. 2000; Chiu et al. 2008). However, an open reactor system is not suitable for this type of CO_2 capture.

8.2.2.5 Harvesting Technologies

The major cost involved in production of microalgal fuel is associated with harvesting of the grown cultures from the medium. The harvesting process is very tedious, and no single method is readily suitable for industrial application. Moreover, the methods currently used are energy intensive and time consuming. Many different types of cell-harvesting systems have been designed; the most popular among them are centrifugation, membrane-based filtration, flocculation, electrolysis and magnetophoretic separation (Christenson and Sims 2011; Farooq et al. 2013). Flocculation is the most popular method and used for the harvesting of microalgae cultures in scale-up systems. This method has three advantages: (i) it is easily applicable to most species, (ii) it is less costly and (iii) it is environmentally friendly. Recently, with the advent of nanotechnology, magnetic nanoparticle–based

flocculation technology has been increasing in popularity for its efficiency in harvesting and the fact that it does not depend on use of traditional chemical flocculants (aluminum sulfate and cationic polymers). When chemical flocculants are used, they are retained as contaminants in the harvested biomass and necessitate treatment to rid the biomass of the flocculants prior to oil extraction. Magnetic nanoparticle–based flocculation also adds the advantage of reusability; hence, it is suitable for scale-up and industrial applications (Kim et al. 2015; Seo et al. 2016).

In addition to cell harvesting, further processing is required for disruption and oil extraction. In the case of microalgae, various types of disruption methods have been used. These are mostly chemical methods; however, a few methods are based on combinations of physical and chemical disruption. In any case, each of these methods has its own advantages and setbacks for application on an industrial scale. Extensive studies by Ji-Yeon Park and colleagues (Seo et al. 2016) have resulted in novel Fe_3O_4 nanoparticles decorated with cationic surfactants, representing the best innovation in this context. These novel engineered nanoparticles assist not only in cell harvesting but also in cell disruption, since they are coated with detergent. This method has been reported to be the best one, and laboratory-scale experiments have demonstrated high-quality oil extraction and suitability for biodiesel preparation.

From an industrial perspective, the success of microalgae-based biodiesel systems is very encouraging; however, the relevant methodologies are still at the developmental stage. Hence, both industry and government should put more funding into this sector to develop innovative methodologies that can be applicable on an industrial scale. The production costs of microalgae-based biodiesel can be divided into two aspects: one is enhancement of the productivity of the biomass and the second is the harvesting technologies and downstream processing used to produce biodiesel. Still, there is a long way to go to attain a potential microalgal system that is suitable for mass cultivation and industrial production of microalgal biodiesel at a level sufficient to meet demands and replace conventional petrodiesel.

8.3 Microbial Fuels

Unlike plants and microalgae, microbes grow under heterotrophic nutrition by utilizing various organic and inorganic substrates, and accumulate diverse types of products, many of which could be used for fuel and energy (Fig. 8.2). There are many metabolic pathways by which microorganisms produce different types of biofuels, and these are listed in Table 8.4. These products either can be used directly as fuel or can be blended with traditional fuels. Their diversity of metabolism, flexibility in coping with different growth conditions, and adaptability in utilizing diverse substrates for metabolic assimilation make microorganisms a good choice for future energy needs. In the present era, utilization of waste biomass for production of microbial fuel is a good concept, since this approach will reduce not only the production cost of the fuel but also direct and indirect carbon emissions into the environment. The best example is lignocellulosic biomass (most of which is

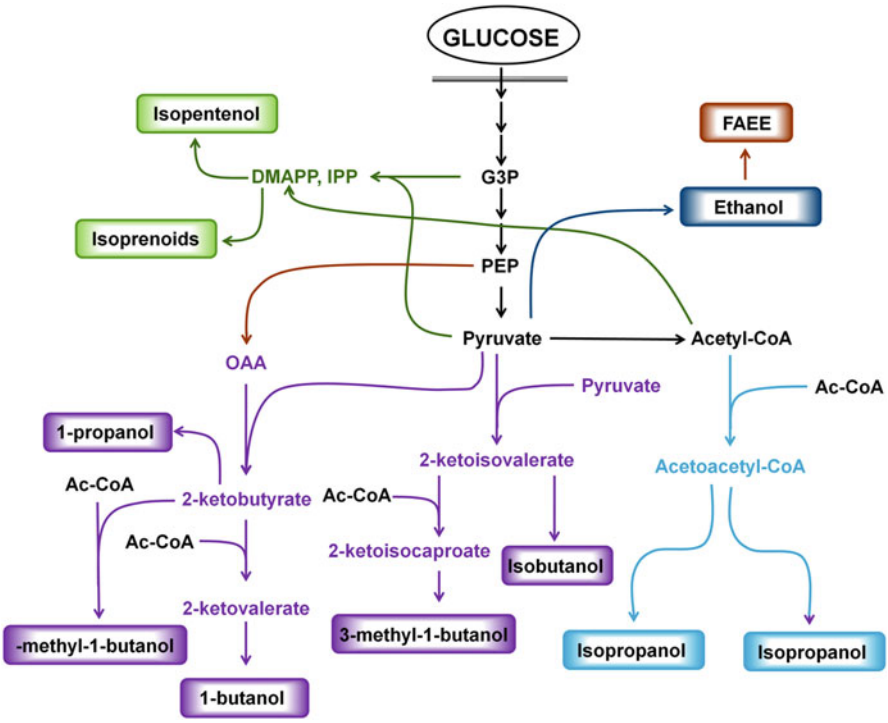

Fig. 8.2 Overview of biofuel production by microbial fermentation. *Ac-CoA* acetyl-coenzyme A, *DMAPP* dimethylallyl pyrophosphate, *G3P* glyceraldehyde 3-phosphate, *IPP* isoprenyl pyrophosphate, *PEP* phosphoenolpyruvate

Table 8.4 Various types of biofuels produced by microbial fermentation (Kumar and Kumar 2017)

Microorganism	Biofuel	Biofuel yield (mg L^{-1})
Clostridium acetobutylicum	Butanol	3000
Clostridium thermocellum	Isobutanol	5400
Escherichia coli	Butanol	30,000
Escherichia coli	Ethanol	25,000
Saccharomyces cerevisiae	Fatty acids	380
Saccharomyces cerevisiae	Isoprenoid	40,000
Pseudomonas putida	Butanol	50
Cryptococcus vishniaccii	Lipids	7800
Zymomonas mobilis	2,3-Butanediol	10,000
Zymomonas mobilis	Ethanol	–
Caldicellulosiruptor bescii	Ethanol	700
Trichoderma reesei	Ethanol	10,000
Yarrowia lipolytica	Fatty acids	55,000
Synechococcus sp.	Limonene	40
Synechococcus elongates	1,3-Propanediol	280

agricultural waste), which is considered to be waste biomass. If it is not utilized, it is used by soil microbial florae to generate organic acids that are released into the soil. These organic acids are then taken up by methanogens to generate methane, which is a GHG several times more potent than CO and CO_2 (Yvon-Durocher et al. 2014). Hence, use of this lignocellulosic biomass for microbial fermentation and biofuel production will have dual advantages: (i) it will produce green fuel via renewable technology and (ii) it will avoid collateral emission of GHGs either by microbial recycling or by the incineration practices usually followed by farmers.

Utilization of lignocellulosic biomass in fuel production involves multiple steps, which include primary degradation of the polymer and complex sugar components into simple soluble sugars. This can be achieved by physical, chemical or biological pretreatments. However, innovative biological pretreatments—which include direct utilization of microbial species to degrade the biomass, simple enzymatic hydrolysis (using enzymes extracted from microbes) and microbial consortium–based bioprocessing—release simple sugars that can be utilized for biofuel production. These simple sugars can be used for microbial fermentation for accumulation of biofuels. One recently introduced technology utilizing complex organic waste for biofuel production—more specifically, methane production in a single step via utilization of a mixed microbial consortium—is becoming popular. In this technology, complex organic waste is degraded by multiple microbial species working in metabolic cooperation in multiple steps. In this process, eubacteria participate in degradation of complex organic matter and accumulate different organic acids, and archaebacteria, like methanogens, generate methane. The methane can be used directly as fuel gas or converted into other biofuels (such as methanol) by biological means utilizing methylotrophic bacteria (Liao et al. 2016).

8.3.1 Alcohols for Bioenergy

Alcohols are liquid biofuels, which are energy-dense fuels accumulated by microbes during fermentation. Various different types of microorganisms perform anaerobic fermentation and accumulate various types of alcohols. Among these, ethanol is the most ancient and most explored one. C3 and C4 alcohols can be produced by many bacterial species, most commonly by *Clostridium* spp., and are used as biofuels or auxiliary blending fuel with traditional petrofuels. Ethanol is also produced by aerobic fermentation via a pyruvate decarboxylation process. The microbe best known for ethanol production by carbohydrate fermentation is yeast (*Saccharomyces cerevisiae*), which belongs to the eukaryotes. *Zymomonas mobilis* is a natural ethanologenic bacterium that accumulates ethanol via aerobic respiration. The major advantages of *Z. mobilis* are, comparatively more resistant to ethanol accumulation and has a higher rate of specific ethanol production. However, yeast is more robust, tolerant of acidic conditions and easy to use in industrial applications (Kremer et al. 2015). The keto acid pathway is a key metabolic pathway in accumulation of various types of alcohols. In various microbial systems, a longer-chain keto acid is decarboxylated to form long-chain alcohols. Branched-chain alcohols are also

formed from the keto acid pathway by decarboxylation of 2-keto acids. Various species of *Clostridium* can produce different types of C3 and C4 alcohols. Among these, butanol (which has high energy density of 29 MJ/L) is considered to be the best alternative to traditional petroleum fuels. Butanol is used as a transportation fuel upon blending with gasoline to a certain percentage. Experts and researchers have said that with minor engine modifications, butanol could replace petrodiesel. However, the cost of production, the choice of raw material (the carbon source) for microbial fermentation and the yield are major issues to be resolved (Lee et al. 2008). *Clostridium* can produce both butanol and isopropanol, and experimentation data from laboratory-scale studies have shown that *Clostridium acetobutylicum* (1.8 g/L) can achieve near-theoretical yields in which 1 mole of glucose results in nearly 1 mole of butanol production (Chen and Hiu 1986). However, there are three major issues associated with use of *Clostridium*: (i) it is a strict anaerobe, (ii) the specific growth rate of *Clostridium* is very slow and (iii) the fermentation conditions need to be controlled significantly for accumulation of alcohols in the medium.

Apart from the core sugar metabolic pathway via pyruvate intermediate, some microorganisms utilize an amino acid biosynthetic pathway for production of higher alcohols. In this pathway, during biosynthesis of amino acids, several keto acids are generated and by decarboxylation of these keto acids, higher alcohols are accumulated during fermentation. *S. cerevisiae* generates these alcohols via the Ehrlich pathway. In this pathway, the keto acids are converted to their corresponding aldehydes by decarboxylation and further to alcohols via reduction (Hazelwood et al. 2008), and the organisms are able to produce alcohols such as 3-methyl-1-butanol from 2-keto-4-methylpentanoate, 2-phenylethanol from phenylpyruvate, 1-butanol from 2-ketovalerate etc.

Branched-chain and aromatic alcohols are also good alternatives to traditional gasoline and diesel; these complex alcohols could also be used as jet fuel. These types of alcohols can be generated by re-routing the isoprenoid biosynthetic pathway. In this pathway, isoprenoids are formed by addition of five-carbon isoprenyl pyrophosphate (IPP) and the isomer dimethylallyl pyrophosphate (DMAPP); the products that are formed are converted to their corresponding pyrophosphates (geranyl pyrophosphate (GPP, C10), farnesyl pyrophosphate (FPP, C15) or geranylgeranyl pyrophosphate (GGPP, C20)). From these, branched-chain and cyclic alkenes are generated. Upon oxidation, these alkenes can be converted into alcohols and could be used as fuel alternatives.

8.3.2 Biohydrogen Production

Biological hydrogen production can be achieved by two diverse microbial systems. One uses photosynthesis and the other involves fermentative hydrogen production. The former is achieved by photosynthetic microalgae, cyanobacteria and a few bacterial species. In this process, the organisms use the photosynthetic system to capture light energy and this harvested energy is used for photolysis of water to generate H_2. The best part of this is that at the end, the harvested electrons are

accepted by O_2, and this process is called oxygenic photolysis for H_2 production (Barbosa et al. 2001; Kovács et al. 2006). There is another type of H_2 production by photosynthesis, called non-oxygenic photosynthetic hydrogen production. This type of H_2 generation is executed by purple non-sulfur bacteria, and the best examples of this group are *Rhodobacter*, *Rhodopseudomonas* and *Rhodospirillum*. In these bacteria, as in another groups, H_2 is generated via photolysis of water; however, the end electron accepter is an organic acid instead of O_2. Hence, this system is known as non-oxygenic photosynthetic H_2 production. Though production of H_2 by the photosynthetic method looks very promising, the major issue is that it needs a supplementary light source. Much research is still needed to produce an innovative reactor design for utilization of sunlight for H_2 production. The key factor that could fulfill the promise of an efficient and economical method of H_2 production is utilization of waste biomass and integration of it into an efficient photoharvesting reactor system.

Utilization of waste biomass for generation of clean energy is very useful and will provide clean energy at a competitive cost. This type of H_2 production without the need for light is called dark fermentation. In this system, bacterial cells utilize soluble sugars for fermentation and, with the help of hydrogenases or nitrogenases, H_2 is released. The theoretical yield of H_2 production is 2–4 moles for 1 mole of glucose consumption. However, the yield depends on the end electron acceptor during the fermentation. Achievement of theoretical values is very difficult; moreover, much of the carbon source is utilized for cell mass generation. Hence, the system would be commercially viable if we could utilize waste biomass for bacterial growth and H_2 production. Use of lignocellulosic biomass–derived soluble sugars, as in pentose sugar fermentation, will be very profitable for H_2 production; this has potential prospects for industrial-scale production. Many bacterial species such as *Escherichia coli*, *Enterobacter*, *Citrobacter*, *Alcaligenes* and *Bacillus* are capable of fermenting simple sugars for production of H_2, but their efficiency has never reached minimum theoretical values. However, application of sugars extracted from waste biomass or waste organic acids from feedstock will have good applications in the context of cost reduction for H_2 production (Hallenbeck et al. 2012; Łukajtis et al. 2018).

8.3.3 Microbial Fuel Cells

In the recent past, there has been much discussion about generating electricity by using microbial living cells to harvest electrons released during their metabolism. This concept attracted significant interest when researchers showed that organic waste and industrial effluent are very suitable for electron generation and could be harvested to generate electricity. Also, with minor modification, the same strategy could be implemented to generate hydrogen gas. In both cases, the energy generated is completely green, with zero carbon being emitted into the environment. From a biological perspective, both bioelectricity and biohydrogen production involve similar principles under which the selected energy is generated. The following

subsections briefly discuss this concept of microbial fuel cells (MFCs) for bioelectricity and biohydrogen production.

8.3.3.1 Microbial Fuel Cells for Bioelectricity Production

MFCs are devices that oxidize organic and inorganic matter to produce electricity. In this process, electrons harvested upon oxidation of organic/inorganic matter are transferred to the anode and flow through the cathode to produce electricity. This setup contains an anaerobic anode chamber in which carbohydrate substrates or other organic/inorganic substances are used, which are suitable for oxidation by suitable bacteria or a mixture of bacteria, and generate bioelectricity. The most prominent bacterial species studied for production of bioelectricity are *E. coli* and *Saccharomyces* (Logan and Regan 2006). However, these bacteria have a non-conductive lipid and peptidoglycan layer, which hinders direct transfer of electrons to the anode. Hence, special mediators are needed to pass electrons to the anode, and a few studies have focused on access of electrons to the anode (Chaturvedi and Verma 2016). Some bacteria such as *Geobacter sulfurreducens* and *Shewanella oneidensis* harbor special machinery that helps in the transfer of electrons from the cells to a conductive anode surface through the outer cellular membrane. These transferred electrons could be utilized for electricity generation. This system was explored by Kracke et al. (2015) to make MFCs and was used not only to generate bioelectricity but also to produce biohydrogen. However, use of MFCs for bioelectricity production is still at the developmental stage; their low-energy output is not sufficient for current real-world needs. The theoretical yield of an MFC is only up to 1.2 V. The infrastructure and reactors needed for commercialization have not yet been developed, and it will take more time to make MFCs that can generate sufficient energy for real-world applications.

8.3.3.2 Microbial Fuel Cells for Biohydrogen Production

Use of MFCs for biohydrogen production is made possible by a bioelectrochemical system. This system works with the bacterial species and is based on anaerobic respiration in contact with the anode, where oxidation of acetate occurs, and a reduction process occurs at the cathode to produce H_2 gas by accepting electrons donated by the oxidation process. An ion-specific membrane is necessary to separate the cationic and anionic electrodes for creation of an electron gradient. When oxygen is removed from the cathode, hydrogen is released by the protons accepting the electrons, and this hydrogen release can be achieved either by provision of a small amount of electricity or by an electrogenesis process. This can be attained by oxidation of an organic substrate by bacteria, as described earlier. Unlike electrolysis of water, bacterial electrolysis of an organic substrate is an exothermic reaction and, upon oxidation of the organic acids or another organic substrate, will provide energy to the bacteria; hence, this process could be used for both electricity production and H_2 production with a slight reactor modification. Application of electricity for hydrogen is needed in this process because in natural conditions of organic acid oxidation, the electrical potential generated by the bacteria is -0.3 V, and this is not sufficient for hydrogen generation by protons and electrons. Hence, with addition of

an extra 0.25 V, the productivity of this process is 2.9 moles of hydrogen from 1 mole of acetate, which is a good yield in comparison with any other process by which H_2 is generated by biological means. The net gain of energy, in terms of energy investment, is nearly equal to 0.5 mol of hydrogen. This shows that with this process, the net gain of energy is almost 5.8 times greater than the overall energy input. Hence, this method looks very promising as an alternative future energy source. However, previous investigations have been successful only at the laboratory scale, and much more research is needed to scale up to the pilot scale and further to the industrial scale (Logan and Regan 2006).

Biological electricity production and H_2 production via MFCs looks very promising at the laboratory scale; the real challenge is making the system successful in a commercial-level scale-up. The second most important factor is the carbon source used for cell mass generation and as an organic substrate to generate electrons. A good way of utilizing waste biomass (such as lignocellulosic biomass or crude glycerol) as a carbon source for bacterial culture growth will reduce the cost of the final product. Industry-generated organic waste that can be utilized for substrate oxidation and electron generation will make the system very economical, and its utilization for this purpose will have beneficial impacts on environmental protection and water recycling. The real challenges for implementation of this process in industrial production are the cost input for the infrastructural setup and creation of a standard and successful reactor design system, which is still not available. Hence, further research input is vital for reactor engineering and study of suitable bacterial species for application in commercial production.

8.4 Recombinant Microbes for Biofuel Production: Prospective Future Energy Resources

Many microbial strains have been isolated and characterized for application in biofuel production. Each type of microbe has been characterized in terms of its inherent aptitude for producing certain types of biofuels. However, there are a few issues that need to be addressed for better productivity and strain viability. In this regard, many researchers are trying to exploit modern recombinant technology for strain improvement by genetic engineering and metabolic flux re-circuiting for higher product formation. In the past couple of decades, many strains have been engineered to enhance productivity. In addition, many researchers have even tried to produce biofuels in non-native hosts by introducing metabolic pathways via heterologous expression of a group of genes. To enhance productivity or introduce a pathway for biofuel synthesis in non-native hosts, molecular information and information on the proteins and/or accessory proteins involved in regulation of product synthesis are essential. In this area of research, two diversified versions have been developed: (i) genetic engineering of native hosts for strain improvement for better productivity and/or for broadening physiological conditions for better performance in a reactor and (ii) engineering of a non-native microbe for producing target biofuels by introducing pathways or diverting metabolic pathways via heterologous

expression of a group of genes and/or knockout of a few native genes for metabolic flux re-circuiting.

8.4.1 Engineering of Native Microbial Systems

The major difficulties involved in genetic engineering of native strains are the limited availability of molecular tools for DNA manipulation and the limited availability of information on the complex physiology of the strains. Hence, only a few studies on genetic engineering of a native host have been reported. The most studied microbe is yeast, which is known for production of ethanol. *S. cerevisiae* is an ancient microbial strain, which has been explored for alcohol production over several millennia. Apart from producing ethanol for the purpose of biofuel, it is well known for producing many other compounds such as low molecular weight flavored biochemicals. In the present context, ethanol production by *S. cerevisiae* has been well characterized, and the major limitation for enhancing its productivity is ethanol toxicity to the host cells (Stephanopoulos 2007). Hence, many studies were aimed to understand ethanol toxicity and concluded that ethanol tolerance in yeast is not governed by a single gene or a couple of genes; instead, it is a complex phenotype controlled by multiple genes (Stephanopoulos 2007). Mutational screening studies have identified multiple genes that control ethanol tolerance at 6% ethanol. The major breakthrough in enhancing glucose/ethanol tolerance is an approach named global transcription machinery engineering. In this work, mutagenesis of gene codes for Spt15p (a transcription factor) and further selection of dominant mutants showed enhancements in both tolerance and ethanol productivity (Alper et al. 2006). Further, the same research group, led by Stephanopoulos, showed that with up-regulation of potassium and proton pumps, *S. cerevisiae* showed good tolerance to ethanol accumulation in the medium; moreover, the strain was able to utilize xylose for ethanol fermentation, hence broadening the application of this strain for generation of biofuels (ethanol) from lignocellulose-derived pentose sugars (Lam et al. 2014).

Z. mobilis is another prominent bacterium that produces ethanol by sucrose, glucose and fructose fermentation. *Z. mobilis* is known for its superiority to yeast, and its characteristic features are (i) its high ethanol tolerance, (ii) the fact that it can accumulate ethanol in aerobic conditions, (iii) its dynamic rate of sugar metabolism and (iv) its high specific growth rate in comparison with yeast. Despite these superiorities, the native strain cannot utilize C5 carbon for ethanol production (Yang et al. 2016); hence, it cannot utilize the major soluble sugars extracted from lignocellulosic hydrolysates. Multiple researchers have put effort into strain improvement for utilization of pentose sugars (either mixed sugar or sole pentose sugar) for production of ethanol. The most significant finding of these studies was adoption of a strain for pentose sugar fermentation. Through engineering of the *xylA*/*B* operon, the *tal* and *tkt* genes from *E. coli* were transformed into *Z. mobilis*, conferring the ability to utilize xylose for ethanol production (Zhang et al. 1995). By exploring various methods of mutagenesis and metabolic engineering of

Z. mobilis, researchers successfully improved the strain to utilize glucose, xylose and arabinose derived from lignocellulosic biomass hydrolysate after pretreatment (Zhang et al. 1995; Yanase et al. 2012; Zhang et al. 2013).

Apart from yeast and *Z. mobilis*, many other microbes have been explored for production of biofuels. The majority of these efforts have been focused on generating native strains that can utilize lignocellulose-derived sugars to produce biofuels or vice versa. The best example is *Caldicellulosiruptor bescii*, a thermophilic bacterium that can utilize cellulose as a carbon source. This bacterium cannot natively generate ethanol; however, through diversion of its metabolism from lactate production via deletion of lactate dehydrogenase and heterologous expression of acetaldehyde-alcohol dehydrogenase from *Clostridium thermocellum*, it is able to produce ethanol at a rate of up to 700 mg L^{-1} (Chung et al. 2014). As discussed above, the other approach used to convert ethanol-producing strains to utilize lignocellulose was achieved through heterologous expression of cellulolytic enzymes (Tsai et al. 2009; Yamada et al. 2010).

8.4.2 Engineering of Non-native Strains for Biofuel Production

The choice of making non-native host like *E. coli* to produce biofuels is since the host system has robust tools for genetic engineering. Moreover, the genetic information regarding its physiology and metabolism has been well studied, and this will provide an extra benefit in fabrication of a system to achieve the desired product. The best example of this aspect is production of higher-chain alcohols by *E. coli*. As described earlier in this chapter, higher-chain alcohols are always in demand since they are compatible and can replace gasoline and petrodiesel. Traditional production of these higher-chain alcohols is possible by utilization of *Clostridium*. *E. coli* was engineered to produce higher-chain alcohol, although it does not naturally have the genes required for production of higher-chain alcohols. To make this possible, genes for the Ehrlich degradation pathway were introduced into *E. coli*. Introduction of alcohol dehydrogenase (2-keto acid decarboxylase (KDC) and alcohol dehydrogenase (ADH)) into *E. coli* made it possible to produce six higher-chain alcohols (2-methyl-1-butanol, 1-butanol, isobutanol, 1-propanol, 2-phenylethanol and 3-methyl-1-butanol). This strategy was extended to produce these alcohols in *S. cerevisiae* (Atsumi et al. 2008).

Non-native host systems are engineered not only for production of biofuels but also for production of fatty acid ethyl esters (FAEEs) by provision of vegetable oils in the medium. This is achieved by exploration of a whole-cell biocatalysis system, where the whole cell acts as a catalyst for performing a multiple-step catalytic process utilizing the substrate provided in the medium (Sudheer et al. 2017, 2018). This type of process was initially developed in *E. coli* by introduction of the ethanol-producing pathway from *Z. mobilis* (*pdc*, *adhB*), along with acyltransferase from *Acinetobacter baylyi*. Upon providing oleic acid, this engineered strain produced FAEEs from the oleic acid by utilizing ethanol produced from glucose by recombinant *E. coli*.

In recent years, many groups have put forward their efforts to make engineered *E. coli* capable of producing next-generation biofuels that could readily replace hydrocarbon-based fossil fuels. In one of these studies, Choi and Lee (2013) were able to make recombinant *E. coli* produce various types of short-chain alkanes. The β-oxidation pathway of the host was blocked by deletion of *fad*E, avoiding degradation of fatty acyl-coenzyme A (acyl-CoA). Further, to enhance fatty acid biosynthesis, the genes responsible for unsaturated fatty acid biosynthesis were down-regulated by deletion of the *fadR* gene. With introduction of acyl-CoA reductase and fatty aldehyde decarboxylase from *C. acetobutylicum* and *Arabidopsis thaliana*, respectively, the resulting engineered strain was able to ferment glucose as the substrate to accumulate short-chain alkanes consisting of nonane, dodecane, tridecane, 2-methyl-dodecane and tetradecane, together with small amounts of other hydrocarbons.

Genetic engineering is being explored not only to produce liquid biofuels but also for hydrogen production and bioelectricity production. In the case of MFCs, electroactive biofilm–producing bacteria can participate in direct transfer of electrons to the anode surface upon oxidation of organic biomass. Hence, researchers have examined possibilities to make genetically modified microbes generate the required biofilm-producing ability (Angelaalincy et al. 2018). Genetic engineering of bacteria with the ability to transfer electrons by a direct transfer method will be advantageous for reducing the cost of the bioreactor for MFCs. These engineered systems not only will benefit the production of electricity but also will have applications in bioelectrocatalytic H_2 production.

Only a few examples of genetic engineering for biofuel production have been discussed in this section, and this discussion is only the tip of the iceberg; the applications of this potential technology is exponential in the area of biofuels. These genetic engineering strategies will have the potential to enhance productivity several-fold. However, these technologies need to be further evolved to make them an efficient replacement for hydrocarbon-based fuels to meet future transportation fuel needs and the needs of industrial applications.

8.5 Concluding Remarks

Use of current petroleum-based energy resources and other fossil fuels is causing global disruption because they emit greenhouse gases. Moreover, they are non-renewable and limited energy resources, and the current acceleration in the use of these fuel resources is arousing fears of exhaustion of all such reserves in the near future. Hence, there is a great need for alternative renewable energy resources that do not add carbon footprint, in order to safeguard future generations and help preserve climatic stability. Though many research studies on biofuels have been performed and considerable knowledge on development of biosystems for energy generation has been accumulated, the magnitude of development is still lacking due to limited national policies and research funding. Many countries have introduced special government policies for research funding allocation in this area; however,

industrial investment in research and development of biofuels is still limited. Moreover, more innovative strategies need to be developed for utilization of waste biomass (lignocellulosic biomass) and industrial effluent (organic effluent for bioelectricity and bioelectrolytic hydrogen production), and scale-up studies are still needed to make further progress. More knowledge input from interdisciplinary areas, such as knowledge on aspects of reactor engineering, is needed for establishment of a successful system. This will aid the development of multiple types of biofuels, which could be made available for diverse needs and provide supplementary energy for our future needs without compromising environmental safety.

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Integrating Omics and Microbial Biotechnology for the Production of Biofuel

9

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Abstract

Renewable energy sources are being found around the world which replaces the increasing demand and using up of fossil fuels. Many microalgae species generate necessary and sufficient quantities of polysaccharides, hydrocarbons, and other useful products. However, in comparison to non-renewable production from fossil fuels, the manufacturing of large-scale algal products is not a simple process. It has been seen that microalgae is naturally to be more effective in producing compounds that can replace fossil fuels. However, to make the process economically feasible, it requires optimization of the strains through genetic engineering and systems biology tools. The strain improvement can also be done with the help of metabolic engineering which is part of microbial biotechnology, which may enhance the productivity of the microorganism. Recently bioinformatics and systems biology tools explored the algal genome sequencing which can also help us to deeply understand the metabolic system of the algae to produce the renewable compounds and to optimize biofuel production. The present review article focused on major computational tools and approaches developed can encourage us to identify target genes, pathways, and reactions of particular interest to biofuel production in algae. Since the use of these tools and methods in algal biofuel studies has not been completely adopted, the aim of this review is to discuss how to utilize the system biology approach and metabolic engineering for future implementation in algal research in the production of algal biofuel.

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Fossil fuels · Microalgae · Renewable energy · Bioinformatics · Biofuel

9.1 Introduction

9.1.1 Biofuels Overview

Biofuels consist of liquid or gaseous fuels which can be produced from biomass high in sugar (such as sugarcane, sugar beet, sweet sorghum), starch (such as corn and cassava), or oils (such as soybeans, rapeseed, coconut, sunflowers, and palms). Ethanol and biodiesel are the two most frequently used biofuels. Generally, biofuels are used as a transport fuel. In the world the production of biofuels has been growing over the last decade from 16 billion liters in 2000 to around 110 billion liters in 2013. By the end of 2011, worldwide demand for biofuels was low as compared to conventional transport fuels (e.g. gasoline, petrol, and diesel) (International Energy Agency 2011). On the other hand, nowadays ethanol-based fuel gels have also been used for cooking purpose. Improvement in the algal fuel and bio-product technology to the economic level is attainable by overcoming the related challenges and constraints in microalgae production (Khan et al. 2018). Governments and industry worldwide also play an essential part in the further growth of microalgae projects by enhancing the bio-economy to generate green jobs, energy security as well as clean the environment (Chia et al. 2018).

9.1.2 Classification of Biofuel Sources

The classification of biofuels is based on the natural product available in nature, natural by-products, and synthetic products (Fig. 9.1). Natural biofuels are usually derived from organic sources which include mainly vegetable, animal waste, and landfill gas. Primary biofuels represent usually natural by-products to come from plants/trees like woods mainly used for cooking, brick kiln, and heating purpose or in electricity production.

The secondary biofuels like bioethanol and biodiesel are produced by the processing of biomass and are based on the microorganisms. These types of biofuels are also used in transport sectors (Nigam and Singh 2011).

As the new technologies come in advances the secondary type of fuels is further subclassified into their generations which is based on the production, quality, and uses. Such type of biofuels is subclassified as: (a) first-generation biofuels, (b) second-generation biofuels, and (c) third-generation biofuels (Dragone et al. 2010).

The first-generation carbon neutral biofuel was not considered suitable for economically, environmentally, and politically concern because large amount of biofuel production requires more agriculture land at the cost of space for humans and animal

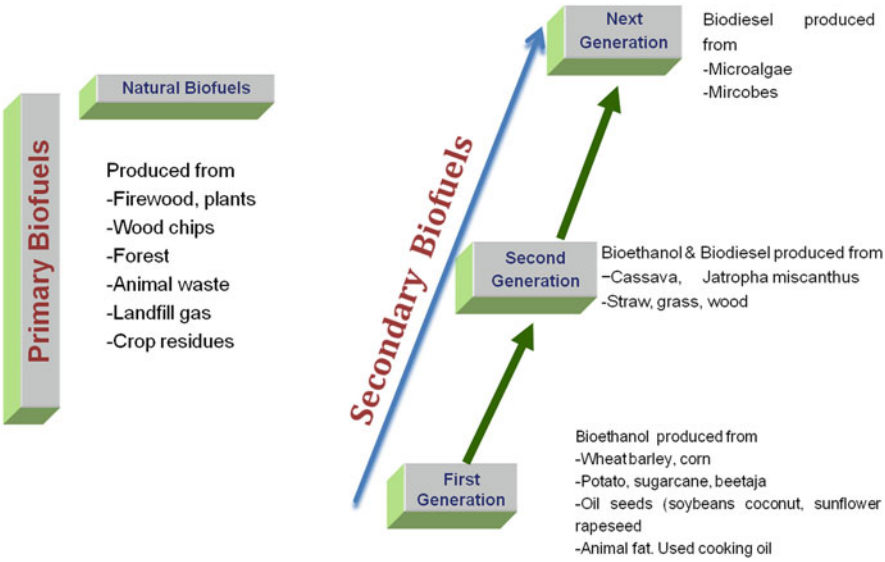

Fig. 9.1 Classification of biofuels and its production sources (biomasses) (adapted from Dragone et al. 2010; Alam et al. 2012)

feedstock. The increased pressure on agriculture land for food production leads to severe food shortages, especially in developing countries of Africa, Asia, and South America where over 800 million individuals suffered from hunger and malnutrition owing to serious food shortages. With the growing world's population, the demand for food is increasing while the agricultural land is decreasing (Schenk et al. 2008). Increasing usage of first-generation biofuels can raise the food price beyond the reach of the underprivileged. Therefore, the production of the first-generation biofuel has been stopped and researchers focused on second-generation biofuels.

The primary goal here is to produce biofuels using lignocellulosic biomass, the woody part of plants which do not affect the production of food and feedstock for human as well as animals (Dragone et al. 2010). The second-generation biofuel major sources are predominantly based on agricultural wastes (e.g., trimmed branches, wood chips, leaves, straws, etc.), wastes after forest harvesting, wood processing residues (e.g., sawdust), and non-edible components of corn, sugarcane, beet, etc. However, converting the woody biomass into fermentable sugars requires sophisticated and expensive technologies for the pretreatment with special enzymes making second-generation biofuels economically not profitable for commercial production (Dragone et al. 2010; Brennan and Owende 2010).

Hence, the research focuses on the production of third-generation biofuels. Microalgae are the primary component of the third-generation biofuel. The production of third-generation biofuel is presently regarded as an alternative renewable energy resource, which overcomes the disadvantages of biofuels of the first and second generation (Nigam and Singh 2011; Dragone et al. 2010; Chisti 2007; Li

Table 9.1 Classification of biofuel sources by different characteristics (source from UBET, FAO Classification Biofuel, FAO 2004)

		Biomass from woods	Biomass from Herbs	Biomass from fruits and seeds	Others (including mixtures)
Energy by cultivation	Direct	WOODFUELS	AGROFUELS		ORGANIC WASTES • Animal by-products • Horticultural by-products • Landscape management by-products
		• Energy forest trees • Energy plantation trees	• Energy grass • Energy whole energy grain • Cereal crops		
By-Products∗		• Thinning by-products • Logging by-products	Crop production by-products:		
			• Straw stones, shells, husks		
	Indirect	• Wood processing • Industry by-products • Black liquor	• Fiber crop • Processing by-products	• Food processing • Industry by-products	• Biosludge • Slaughterhouse by-products
End use materials	Recovered	• Used wood	• Used fiber products	• Used products of fruits and seeds	MUNICIPAL BY-PRODUCTS • Kitchen waste • Sewage sludge

The ∗word "by-products" refers to the solid, liquid, and gaseous residual wastes obtained from the operations of biomass treatment by using different procedures including physical, biochemical, thermal, and others

et al. 2008). On the other hand, Food and Agricultural Organization (FAO) also classified the biofuels which is shown in detail in Table 9.1.

9.1.3 Microalgae Diversity and its Biotechnological Potential

Microalgae are simple photosynthetic prokaryote or eukaryote and one of the most diverse of all organisms. All aquatic ecosystems including oceans, lakes, rivers, snow, glaciers, also at rocks, and other difficult surfaces inhabit microalgae (Reijnders et al. 2014).

There are considerable variations observed in physiology and metabolism between distinct phyla of microalgae (Merchant et al. 2007; Dorrell and Smith 2011). Because of genetic diversity, it provides an excellent opportunity to identify new biotechnological pathways by genomes analysis of the diverse species. Therefore, microalgae have an essential role in the biosynthesis of a range of industrially usable products like hydrocarbons and polysaccharides (Borowitzka 2013; Scott et al. 2010).

Moreover, due to fast growth rates, microalgae are suitable microorganism for large-scale fermentation, and also useful for the sustainable process development

(Wijffels et al. 2010). Algae are naturally the possible source of triacylglycerides (TAG) which are the precursor molecule for biodiesel (Merchant et al. 2012) and give higher potential yields greater than competing agricultural processes (Mata et al. 2010). Evaluations of advanced technologies establish that microalgae are a commercially viable source for biofuel production (Passell et al. 2013; Jorquera et al. 2010; Bodjui et al. 2019).

Metabolic algae network reconstructions can provide information on a genetic modification that can be utilized to improve the microalgae strains for increasing the production of metabolic products. These metabolic products are extremely useful as biofuel components.

Many varieties of computational tools have been developed to identify bioengineering strategies based on the genetic and thermodynamic property which can aid in biofuel production of the improved algal strain. Although a significant number of algal genomes were completely sequenced, however, a couple of metabolic network models have been recreated for these species which restrict the algal bioengineering progress (Koskimaki et al. 2013).

A metabolic network is a system of an organism that transforms carbon and energy sources into energy, biomass, and byproducts. Such type of chemical transformation involves the electron acceptors and donors mechanisms. Metabolic system engineering can give rise to higher yields of or useful by-products. In this way, a mechanistic understanding of metabolism is critical for various disciplines including biofuels research (Zhang and Hua 2015).

The metabolic network modeling based on constraint programming is a popular powerful technique of metabolic assessment (O'Brien et al. 2015). In this strategy, all annotated metabolic genes in an organism are initially combined with enzymes and responses to this reaction acquire the gene–protein–reactions (GPRs) product. The GPRs are utilized to recreate a genome-scale metabolic network model (GSMNM), which is then used to enumerate the flux distribution across the entire network in any specified condition for the organism (Yilmaz and Albertha 2017).

The usefulness of metabolic network models spans across several types of applications. These models assist to contextualize high-throughput observational data, for instance, integrating gene expression data with metabolic pathways under different growth conditions (Usaite et al. 2006). Metabolic models can also reveal targets for metabolic engineering approaches, which can promote enhanced production of target metabolites (Zelle et al. 2008) or increase the respiration rates preferably (Izallalen et al. 2008). With the accessibility of large and diversified sets of biological information, metabolic network models can further provide a structure to omics data and validate downstream hypotheses to be formulated and tested. In addition, cross-species metabolic comparisons represent another convenient means for such reconstructions that identify differentially activated metabolic pathways and other comparative analyses can be accomplished (Oberhardt et al. 2009).

Herein we discuss the metabolic network reconstruction models and main computational tools that contain the potential to contribute to the improvement of algal strains for biofuel production. In addition, a number of valuable tools are described

which come up with successful strategies for algal biofuels improvement accompanied by major embellishment potential.

9.2 Metabolic Network Model Reconstruction

The metabolic network reconstruction based on genomic and large-scale experimental data can help to understand and predict the process of metabolism and pathways. A considerable number of computational applications and biological databases have been developed to exclusively facilitate in the metabolic network reconstruction. Additionally, novel analytical applications and approaches are being developed along with the extension of relevant databases and resources. Some of the available databases and tools for algal metabolic network reconstruction are given in Table 9.2 (Oberhardt et al. 2009).

The metabolic network reconstruction requires valuable information on gene–protein–reaction combination to reconstruct evidence-based, species-specific networks. Protein database resources and tools help to link information between genes, enzymes, EC numbers, substrates, proteins, and pathways. These include BRENDA (Schomburg et al. 2013), ExPASy (Artimo et al. 2012), and UniProt (Universal Protein Resource) (Consortium 2011). A number of publicly available databases of the metabolic pathway exist which can help to design maps of metabolic networks and explore more about metabolic pathways. For example, BioCyc, MetaCyc (Caspi et al. 2014), KEGG (Kyoto Encyclopedia of Genes and Genomes) (Kanehisa et al. 2012), Reactome (Crof et al. 2011), and BiGG (Schellenberger et al. 2010); COBRA, common tools for metabolic reconstruction (Becker et al. 2007;

Table 9.2 Web sources for metabolic network reconstruction databases and tools (Koussa et al. 2014)

Database and tools	Link
Functional annotation tool for algal	http://pathways.mcdb.ucla.edu/algal/index.html
BiGG	http://bigg.ucsd.edu/
BioCyc	http://biocyc.org/
BioMart	http://www.biomart.org/index.html
BRENDA	http://www.brenda-enzymes.info/
COBRA	http://opencobra.sourceforge.net/openCOBRA/
ExPASy	http://www.expasy.org/
KBASE	http://kbase.us
KEGG	http://www.genome.jp/kegg/
Model SEED	http://www.theseed.org/wiki/Main_Page
MetaCyc	http://metacyc.org/
Pathway Tools	http://pathwaytools.org/
Reactome	http://www.reactome.org/PathwayBrowser/
UniProt	http://www.uniprot.org

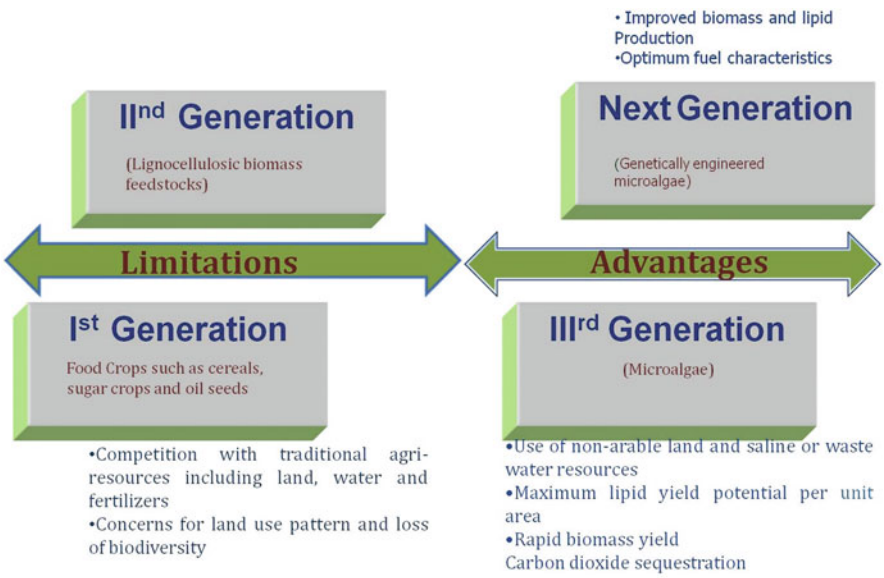

Fig. 9.2 The advantages and limitations of different biofuel feedstock derived from renewable resources (Misra et al. 2013)

Schellenberger et al. 2011; Torleifsson and Tiele 2011), Model SEED (Devoid et al. 2013), and Pathway Tools (Karp et al. 2009).

As microalgal biofuels give significant promise to contribute to the growing worldwide demand for alternative sources of renewable energy; however, the lipid manufacturing capabilities of microalgae need to be considerably improved in order to make algal-based fuels competitive with petroleum. Recent advancement in algal genomics, coupled with other "omic" methods, has accelerated the potentiality in the identification of genes and metabolic pathways to detect the potential targets for the biofuel production and with the advent of genetic engineering, the genetically modified microalgal strains with less content of lipid molecules were developed. In this context, many current investigations have conclusively recommended the oleaginous fast-growing microalgae retaining several potential benefits over land plants. Growing microalgae can be the best alternative source as raw materials for third- and next-generation biofuels production (Chisti 2007; Lam and Lee 2012; Mata et al. 2010). The advantages and limitations of different biofuel feedstock obtained from renewable resources are given in Fig. 9.2.

Although as compared with other eukaryotes and higher plants, the understanding of the biosynthetic pathways of lipids in microalgae remains still incomplete (Hu et al. 2008). Access to various microalgal genome sequences currently brings resources of possibilities for application of "omics" approaches to find an answer to algal lipid metabolism and recognize potential gene targets for the growth of potentially engineered strains with optimized lipid content (Fig. 9.3) (Misra et al.

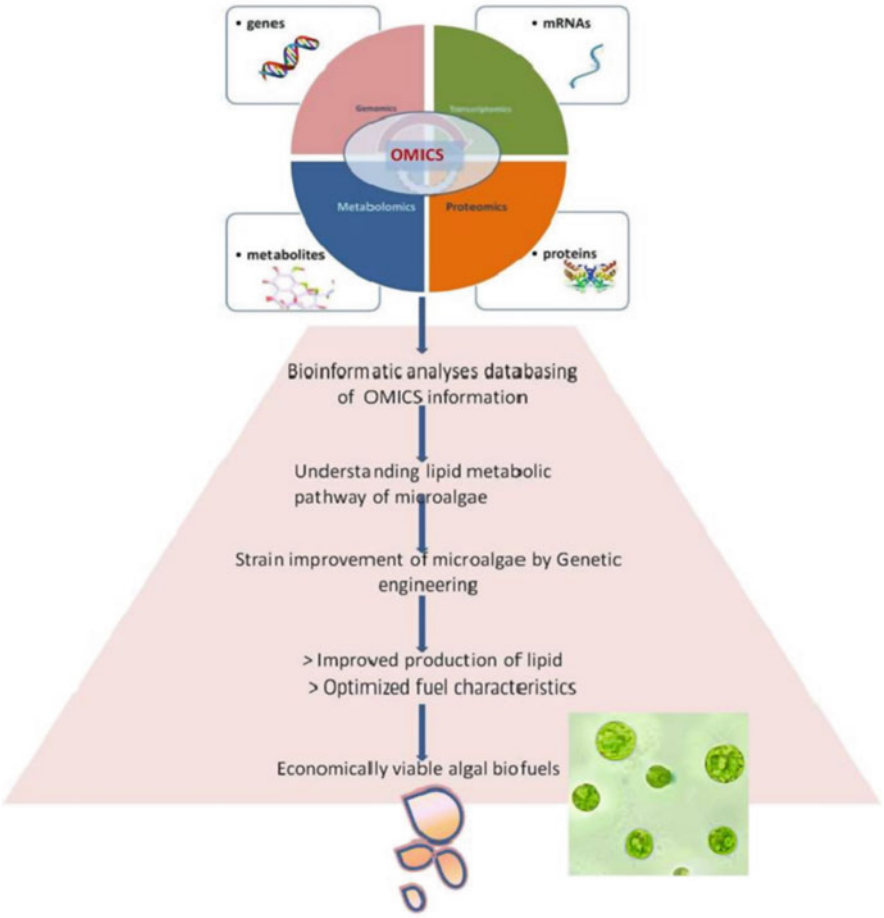

Fig. 9.3 An integrated approach for the development of biofuel production from microalgae (Misra et al. 2013)

2013; Beer et al. 2009; Georgianna and Mayfield 2012; Mukhopadhyay et al. 2008; Rodriguez-Moya and Gonzalez 2010; Yu et al. 2011).

A detailed synopsis about the recent developments of genomics study in microalgae is provided, followed by a number of available bioinformatics resources to explore the metabolic pathways of lipids in microalgae. There has been some latest research reported the extensive use of various "omic" techniques for determining and characterizing of putative genes responsible for microalgal biofuel production is also examined.

9.3 Microalgal Genomic Sequences

Due to the development in sequencing technology the next generation sequencing (NGS) technology revolutionized the genomic research. With the help of NGS entire genome of the living being can be sequenced within a single day (Grossman 2005; Radakovits et al. 2010; Tirichine and Bowler 2011). To date, the whole genome sequences of several microalgae have been generated. These include the *Cyanidioschyzon merolae* 10D (Matsuzaki et al. 2004), *Phaeodactylum tricornutum* CCP1055/1 (Bowler et al. 2008), *Thalassiosira pseudonana* CCMP1335 (Armbrust et al. 2004), *Guillardia theta* CCMP2712 (Curtis et al. 2012), *Chlamydomonas reinhardtii* CC-503 (Merchant et al. 2007), *Ostreococcus tauri* OTH95 (Derelle et al. 2006), *Ostreococcus lucimarinus* CCE9901 (Palenik et al. 2007), two strains of *Micromonas pusilla*, RCC299 and CCMP1545 (Worden et al. 2009), *Bathycoccus prasinos* RCC1105 (Moreau et al. 2012), *Volvox carteri* UTEX2908 (Prochnik et al. 2010), *Chlorella vulgaris* NC64A (Blanc et al. 2010), *Coccomyxa subellipsoidea* C-169 (Blanc et al. 2012), *Ectocarpus siliculosus* EC32 (Cock et al. 2010), *Aureococcus anophagefferens* CCMP1984 (Gobler et al. 2011), *Nannochloropsis gaditana* (Radakovits et al. 2012), and *Bigelowiella natans* CCMP2755 (Curtis et al. 2012), *Ostreococcus sp*RCC809 (Robbens et al. 2007; Lanier et al. 2008; Misumi et al. 2008), *Dunaliella salina* CCAP19/18 (Hong et al. 2017), *Galdieria sulphuraria* (Barbier et al. 2005; Jain et al. 2014), *Chondrus crispus* (Jonas Collén et al. 2013), *Fragilariopsis cylindrus* CCMP1102 (Mock et al. 2017), *Pseudo-nitzschia multiseries* CLN-47 (Cao et al. 2016), *Emiliania huxleyi* CCMP1516 (Radakovits et al. 2010; Tirichine and Bowler 2011). Several organelle (mitochondria or/and plastid) genomes in microalgae have also been sequenced, including those for *D. salina* CCAP19/18 (Smith et al. 2010), *Botryococcus braunii* (Weiss et al. 2010, 2011), *Nephroselmis olivacea* (Turmel et al. 1999), *Chaetosphaeridium globosum* (Turmel et al. 2002), *Mesostigma viride* (Lemieux et al. 2000), *Cyanophora paradoxa* (Stirewalt et al. 1995), *Cyanidium caldarium* (Glockner et al. 2000), *Gracilaria tenuistipitata* (Hagopian et al. 2004), *Porphyra purpurea* (Reith and Munholland 1995), and *Odontella sinensis* (Kowallik et al. 1995).

9.4 Application of "Omics" in Biofuel Production

The application of bioinformatics includes sequence analysis, homology modeling, structural analysis, protein function analysis, evolutionary analysis, metabolic networking and can also be applied for microalgae. Computational approaches like maximum likelihood, Bayesian, and maximum parsimony are used to find phylogenetic relationship. This step provides help in deciphering any possible link between other groups of the organism which it belongs and can also help to identify its function as well. Some software that are often used in the field of algae bioinformatics are BLAST, FASTA, EMBOSS, Clustalw, and RasMol.

9.4.1 Others Commonly Used Tools and Software Used in Algae Bioinformatics

9.4.1.1 Software for Gene Prediction

AUGUSTUS is the best example of algae gene prediction and functional annotation tool. AUGUSTUS is the most accurate and precise program for the species for which it is trained. It is the most precise *ab initio* program. For example, AUGUSTUS was the most precisely gene finder program among the ab initio programs and tested in ENCODE genome annotation assessment project (EGASP) on the human ENCODE regions (Mario et al. 2006). It includes a training program to evaluate the parameters by known genes training set and in order to find the values of the meta parameters, like splice window sizes, that increase the levels of prediction accuracy. AUGUSTUS is useful to correctly interpret the large-scale experimental data of alternative splicing and alternative transcripts obtained from *Chlamydomonas reinhardtii* and *Chlorella* NC64A (Lopez et al. 2011).

9.4.1.2 Sequence Alignment Software

SAM—Sequence Alignment and Modeling system (SAM), A set of various utilities software tools for biological sequence analysis.

SeaView—A graphical multiple sequence alignment editor.

ShadyBox—The first GUI based multiple sequence alignment drawing program for Major Unix platforms.

JAligner—A Java implementation of biological sequence alignment algorithms.

9.4.1.3 Comparative Genomics Software

VISTA—VISTA is a collection of programs, databases, and servers for extensive comparative analysis of genomic sequences.

9.4.1.4 Phylogenetics and Evolution Software

PhyloDraw—A drawing tool for creating phylogenetic trees.

PHYLIP—A free set of programs for inferring phylogenies.

phyloXML—phyloXML is an XML language for the analysis, exchange, and storage of phylogenetic trees (or networks) and associated data (for, e.g., *Chlorophyta* and streptophytic algae).

9.4.1.5 Algae Bioinformatics Resources

There are a few important databases available for researchers working on algae bioinformatics. Some of the following are given:

PLMItRNA

PLMItrna database consisting of mitochondrial transfer RNA molecules and genes of Viridiplantae (green plants) has been extended to include algae. Currently, the database includes 609 genes and 34 transfer RNA entries related to Viridiplantae (27 Embryophyta and 10 Chlorophyta) and photosynthetic algae (one Cryptophyta, four Rhodophyta, and two Stramenopiles) (Guglielmo Rainaldi et al. 2003).

AlgaeBASE

AlgaeBase (https://www.algaebase.org) is an algae database which contains information related to different species of algae found in freshwater, terrestrial, or marine.

UBC databases

Information from the UBC databases are used by researchers around the world to study DNA, species variation, plant chemistry, bioinformatics, and other related fields. The algae collection *herbarium of* University of British Columbia (UBC) is a complete algal database which provides information about all diverse specimens of algal collection. Other open software links are also available which provide information related to algal informatics.

9.4.1.6 Programming Aspects of Algae Bioinformatics

In algae bioinformatics, bioinformatician require programming languages to process biological data, for example, Perl, R, XML, Java, etc.

BioPerl

Programming languages PERL is a scripting, powerful, and dynamic language most suitable for bioinformatician to process the biological task easily. Perl supports better programming styles and enables fast data collection and analysis to answer queries like the number of genes in a certain chromosome. The BioPerl project contains many modules for processing biological data that is helpful for resolving the biological data query. It was one of the first repositories of biological modules which made it more usable. It is a useful tool in genomics, bioinformatics, and life science research.

BioJava

BioJava is freely available for the public that offers a Java-based tool to process biological information. It provides statistical and analytical procedures. It is a compiler for common file formats and enables the manipulation of sequences and 3D structures. The objective of the BioJava project is to help rapid growth and applications development for bioinformatics. It can be widely exercised in algae bioinformatics.

BioXML

BioXML is an easily and automatically parseable way to present data on the web. This is a resource to gather XML documentation and document type definition (DTDs) for biology in one location. It is a useful platform for database interchange.

Bioconductor

Bioconductor is a freely available open source software which provides graphical and statistical methods for the genome analysis. Bioconductor package used the R language of programming. It facilitates the metadata analysis of genome.

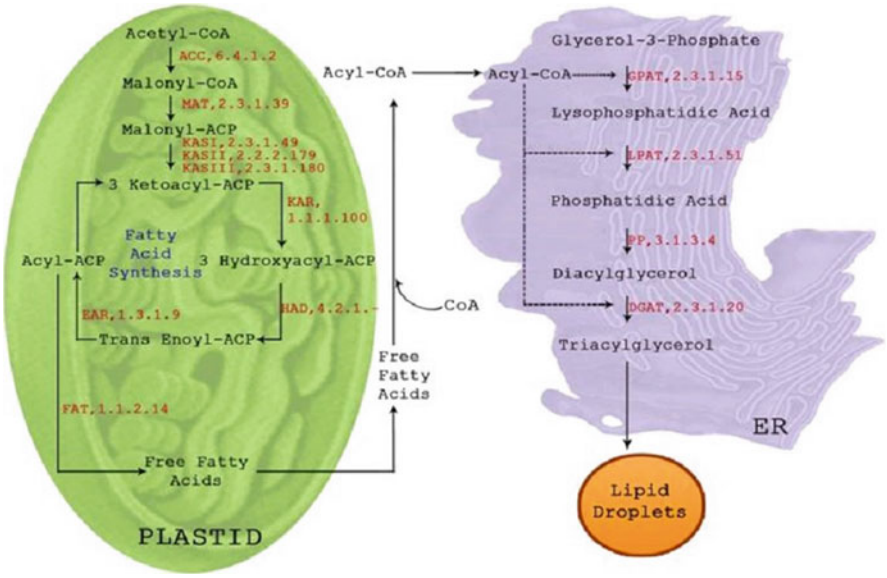

Fig. 9.4 An overview of triacylglycerol (TAG) biosynthetic pathway in microalgae (adapted from Misra et al. 2013)

9.4.2 Advantage of "OMics" in Metabolic Engineering

Omics is an emerging area of science which can be used to explore the metabolic pathway of the algae and related genes. For example, triacylglycerol (TAG) synthesis is a complex and multistep pathway in which different cellular compartments are involved. The synthesis begins with acetyl-CoA, a primary precursor molecule of the pathway. The pathway continues through the biosynthesis of fatty acid which leads to the formation of complex lipid molecules. At the end of the pathway, modification of saturated fatty acid processed into the TAG bodies. The summary of the biosynthesis pathway of TAG in microalgae is shown in Fig. 9.4.

The use of high performance approaches for gene expression analysis and availability of whole genome sequences in public domain has facilitated the characterization of genes and enzymes responsible to regulate the metabolic pathways of microalgae. However, the molecular process responsible for lipid accumulation in microalgae does not have sufficient understanding as compared to higher plants (Hu et al. 2008). To create engineered microorganisms with the required fuel-grade characteristics, the most possible targets for metabolic pathway reconstruction are the genes and enzymes.

Recently, several studies reported the successful application of "omics" approach into the identification of the expressed genes and different enzymes which support the metabolic pathways in different organisms which may also be involved in algal lipid accumulation (Guarnieri et al. 2011; Lei et al. 2012; Liu and Benning

2012; Misra et al. 2012; Nguyen et al. 2011; Radakovits et al. 2012; Riekhof et al. 2005; Rismani-Yazdi et al. 2011; Smith et al. 2012; Valenzuela et al. 2012). It was reported that the comparative genome-wide analyses of algal species showed the variations in their fatty acid composition. These observations were exploited by using phylogenomics approach to determine the variation in gene contents and differential subcellular localization of TAG synthesis between species of higher plant such as *Arabidopsis* and algal species, thereby resolving the several important questions related to algal evolution (Misra et al. 2012, 2013; Sato and Moriyama 2007).

Detailed knowledge of the algae metabolic process and their accurate role in biofuel precursor manufacturing will be helpful. Most of the omics related observations can assist to identify the potential gene target for the improvement of microalgae lipid production. In silico research helped in the prediction of candidate genes to determine the composition of fatty ester in microalgae (Chi et al. 2008; Hashimoto et al. 2008).

9.4.3 Role of Omics Approach: Integration of Synthetic Biology with Omics Approach to Generate Biofuel

The synthetic biological strategies give the following benefits to the bioenergy industry, as compared to the traditional bioproduction approaches:

- Improving the process of production to improve production quantity, quality, and concentration;
- Enables the production of new, less toxic, more accessible, easier to manufacture and superior quality biofuels;
- Providing time and money savings by decreasing the costs of feedstock, improving the optimization, and streamlining the manufacturing process facilitate the use of renewable and more accessible feedstock sources;
- Reduce carbon footprint through the use of natural or waste feedstocks and environmentally friendly procedures.

Therefore, the integrating omics approach to synthetic biology provides certain following solutions for producing biofuel:

- Fragments of the Gene: High quality gene blocks, which can be used separately or as part of a complete gene assembly and pathway.
- Synthesis of High-Throughput Gene: GenBuilder™ high performance assembly technology and NGS multiplex sequencing QC synthesized custom orders of any size of the gene.
- Combinatorial Assembly Library: A powerful source for new protein discovery and the development of novel microbial strains and metabolic pathways.
- CRISPR/Cas9 Genome Editing: The CRISPR (clustered regularly interspaced short palindromic repeat)/Cas9 (CRISPR-associated nuclease 9) is an emerging novel technology for targeted genomic engineering. It is a very powerful technique in genome editing and useful in the development of novel microbial strains.

9.5 Conclusion

This review summarizes the potential usefulness of emerging omics approach which could provide a better understanding of genome structure and lipid metabolism of different microbial species. Extensive knowledge of metabolic pathways of lipid accumulation in combination with genetic engineering strategies improves the production of biofuels in microalgae.

Several recent studies reported the use of omics-based approaches to discover the basic cellular and genetic mechanism involved in the precursors of biofuel synthesis from diverse species of algae. Despite these attempts, there are still several difficulties to be accepted as challenges to create cost-effective petroleum competitive fuels derived from algal products.

In general, there are insufficient quantities of biofuel relevant genomic data for different types of oleaginous microalgae. Therefore, we have to access more algal genomes that facilitate identifying the novel genes and enzymes involved in metabolic pathways which are suitable for the optimal production of biofuels.

With the advancement of genome sequence and omics techniques, additional bioinformatics resources including databases for the organization, visualizing and logical interpretation of big data sets are required.

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An Overview on Biomass of Bamboo as a Source of Bioenergy

10

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Abstract

Biomass and derived biofuels are the main sustainable and renewable sources of energy. Traditionally it is used as energy required source in developing countries from ancient period for their domestic needs. Biomass is easily available across the world and a cheaper source of energy, as well as combustion of biomass produces less quantity of greenhouse gases. This chapter documents different aspects of biomass, lignocellulosic conversion methods of bamboo biomass to fuel, namely different thermochemical routes (combustion, gasification, pyrolysis, and liquefaction) and biochemical route. Bamboo is a faster growing plant, which could be one of the useful sources of energy. The considerable downside of bamboo cultivation is vegetative propagation and major land requirement are some challenges to be resolved and further research is needed to fulfill the need of our increasing demand for energy.

Keywords

Bamboo · Biomass · Renewable energy · Bio-fuel · Thermochemical conversion · Biochemical conversion

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10.1 Introduction

Renewable energy is the platform to provide cleaner and sustainable energy for producing biofuels and bioenergy in order to avoid carbon footprint, non-renewable energy sources, and other environmental issues due to fossil fuel. Consequently, biomass represents an appropriate energy source to generate energy in a controlled manner. Biomass energy can be explained as the energy obtained from organic matter based mass of the living organisms of plant origin, terrestrial or marine/cultivated by humans, animals and microorganisms. In short biomass indicates living matter owing to an organic matrix. The main key features of biomass (cheap, renewable, abundant, biocompatible, and biodegradable) increase its versatility to produce energy and fuel. Biomass fulfills the major requirement of renewable and sustainable source of energy in the global energy cycle. Therefore, biomass has gained considerable focus as a renewable energy resource for bioenergy and biofuel to address various energy and environmental issues caused by the use of fossil fuels (Hameed et al. 2019). To reduce the dependency on fossil fuel as well as CO_2, biomass fuel is greatly favored (Sims 2003; Villeneuve et al. 2012). Biomass is raised as a fourth largest energy source after coal, oil, and natural gas and the most significant renewable energy alternative today which can be preferred to generate different forms of energy. On combining with other renewable energy sources biomass contributes all the energy services essential to current society and when compared to other renewable sources, biomass resources are general and extensive across the world (Ladanai and Vinterbäck 2009). Some records showed that 10% of the overall energy production was contributed by biomass and waste. The main application of biofuel is to replace fossil fuel and overall reduction of release of carbon dioxide and ultimately help to undertake global warming (Omer 2017).

Modern statistics specify that biomass and waste contributed to approximately 10% of the entire primary energy fabrication (IEA 2018). The bio-energy or biomass energy possess sources including crops, landfill gases, organic components of industrial wastes, different kinds of residues, wood (presently the leading biomass resource), and even algae, among other potential sources (Azevedo et al. 2019). Currently, biomass is contributing overall around 14% of the total world's energy supply, subsequently to coal, oil, and natural gas (Asif and Muneer 2007). Many types of vegetation (agriculture/forest) such as switchgrass, willow, poplar, straw, corn stover, and wood wastes are the form of biomass feedstock which provides bioenergy production. Lainez et al. (2018) emphasized numerous potential biomass sources such as wastes, residues, and by-products from crop and animal production systems, industrial and municipal solid wastes from human activities as well as other non-conventional and promising sources of biomass coming from the cultivation of algae and microorganisms. However, biomass is most importantly composed of C, H, and O which are oxygenated hydrocarbon. When biomass undergoes geological and biological process coal is obtained as the main and end product.

Decomposition of the organic matter leads to the production of biogas as matter converted in primary gases like methane (CH_4), carbon dioxide (CO_2), and nitrogen

(N_2). Organic matter includes anaerobic digesters, wastewater treatment plants emit biogas from these decomposing waste.

Each ecosystem is considered in requisites of areas, signify net carbon production per year and standing biomass carbon that enclosed in biomass on the earth plane and does not embrace the carbon storage in biomass underground. Biomass by green plants including land and aquatic-based vegetation is produced through photosynthesis by converting sunlight to plant material where solar energy unit react with moisture and CO_2 to form carbohydrate and oxygen. Due to universal availability of the biomass makes it strategic resource which can be used in shortage of traditional energy resource. The energy obtained from biomass is complex and required agricultural production based on principles of global sustainability which directly means that usually food (primary products) and residues turn out to products with biological potential directly related to cosmetic, pharmaceutical industries as well in food. The resources of bio-energy are exclusively useful for the provision of rural power supplies, wind machine and solar driers, which can be constructed using local resources.

Bamboo is a potential feedstock to approach future energy production. India ranked second in the production of bamboo after China in the world. In India bamboo covers approximately 136 species and 23 genera exclusively distributed in 12.8% of total forest area which is approximately 9.57 million hectare (Dwivedi et al. 2019; Yeasmin et al. 2015). Arundinaria, Bambusa, Chimonobambusa, Dendrocalamus, Dinochola, Gigantochloa are the chief genera of Indian bamboo (FSI 2017). Thick walled bamboo of *Bambusa* genus widely distributed in India includes 37 species (Nath et al. 2018) and exclusively grown for home gardens in clumping manner. India covers more than 50% of bamboo species distributed in the Northeast area especially in Assam, Manipur, Arunachal Pradesh, Nagaland, Meghalaya, Mizoram, Tripura, Sikkim, and West Bengal. Other bamboo bearing places in India are Madhya Pradesh, Chhattisgarh, Western Ghats, etc. (FSI 2017). Total rainfall and interference of human being are two chief causes of distribution of bamboo in particular region. Bamboo plays important role in day-to-day life of human being, it is poor man's timber (because used in construction purposes such as doors, windows, floor, and pillars) and other utilizations in medicinal, charcoal formation (by mature culms), in making of artistic musical instrument like flute, pickle formation, and it is also considered as green gold. In Southeast Asia knife made by bamboo used to cut the placenta of newborn (Yeasmin et al. 2015). Tropical woody bamboo contain superior genomic DNA content as compared to woody bamboo calculated by flow cytometric study (Yeasmin et al. 2015).

10.2 Chemical Characteristics of Biomass

The residual biomasses are mainly composed of polysaccharide or lignin. Polysaccharides with high molecular weight are composed of cellulose and hemicellulose with lignin considered as main constituents of woody biomass. According to De Jong (2015) biomass is composed of cellulose, hemicellulose, lignin, and

Table 10.1 Biomass composition and chemical properties

Biomass component	Bermuda Grass (Herbaceous) [% mass]	Poplar (Woody) [% mass]	Pine (Woody) [% mass]	Refused fuel (Waste) [% mass]	Carbon content [% mass]	HHV [MJ/kg] [% mass]
Cellulose	32	41	40	65	40–44	17
Hemicellulose	40	33	25	25	40–44	17
Lignin	4	26	35	3	63	25
Protein	12	2	1	4	53	24
Ash	5	1	1	17	0	0

HHV High heat value
Source: Global Climate & Energy Project (2002)

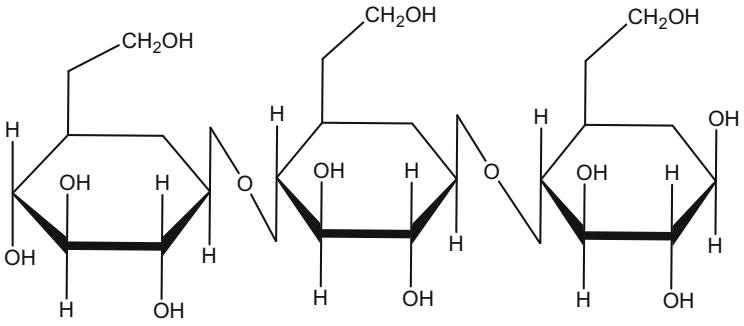


Fig. 10.1 Structure of cellulose

major components (i.e., bio-organic polymers, inorganic species, and extractives). Table 10.1 includes the composition and chemical properties of biomass.

10.2.1 Cellulose

Cellulose is the most versatile organic compound found on the earth. It is fibrous and provides strength to the woody biomass and forms a major structural component of cell wall of higher plants, so that it can be harvested from plant sources. Cellulose is said to be the main part of lignocellulose which is made up of a linear chain of D-glucose linked by β (1-4)-glycosidic bonds to each other.

Cellulose is a polydisperse linear polysaccharide consisting of many glucose monosaccharides having formula $(C_6H_{10}O_5)_n$ with up to 10,000 monomer units and it contains 1,4-β-glycosidic linked D-glucose units. Figure 10.1 shows a monomer unit of cellulose. The formation of the cellulose is as a result of natural polymerization of glucose molecule in large number. It contains components of cotton (95%), flax (80%), jute (60–70%), and wood (40–50%) (Rudnik 2013). It can be purchased from market in a variety of shape, size, and crystallinity. Cellulose can be procured from many agricultural by-products such as sugarcane, sorghum

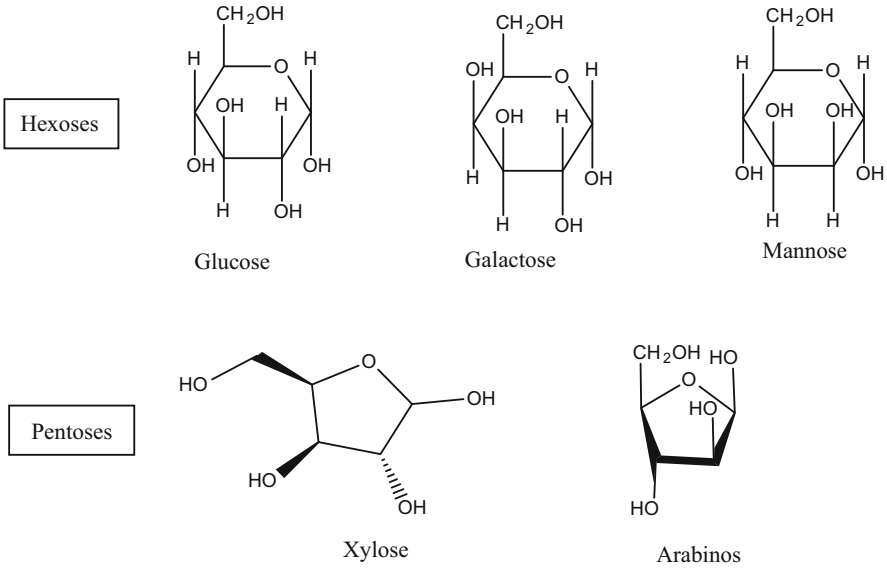
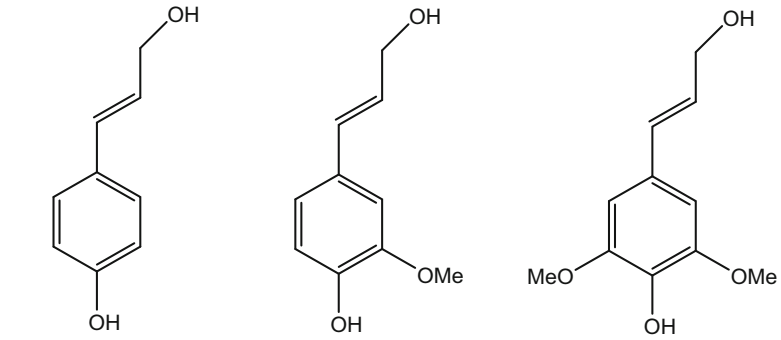


Fig. 10.2 Structure of constituents of hemicellulose

bagasse, corn stalks, and straws of rye, wheat, oats, and rice. Human beings are unable to digest cellulose because the enzyme required to digest cellulose lacks in them. Cellulose breaks generally at temperature 240–360 °C, some parts are generally converted to tars and chars which solidified further whereas some converted to volatile.

10.2.2 Hemicellulose

Hemicellulose is considered as the second most abundant biopolymer in the plant kingdom after cellulose (Ren and Sun 2010). Hemicellulose comprises 20–30% of lignocellulose biomass and they contain similar monomeric units like cellulose but the difference is that hemicelluloses are branched polymer of pentose (xylose and arabinose) and hexose (glucose, galactose, mannose, and rhamnose) sugar, whereas cellulose is a linear polymer. The structures of pentose and hexose sugars are mentioned in Fig. 10.2. They can be classified as xylanes, glucuronoxylan, arabinoxylan, mannan, and glucomannan. They are found in hardwood, softwood including grass, herbs, cereals, and grains. It is made up of different sugar units situated at different proportions of substituent, such as wheat straw carries different branches such as arabinose, xylose, and uronic acid. Hemicelluloses in form of polysaccharides in plant tissue can be extracted by aqueous alkali and water (Glasser et al. 2000). The glycosidic linkages in hemi-cellulose at position 2, 3, 4, and 6, the reason of disorderly arranged amorphous polymer, which makes it more soluble in



Coumaryl alcohol Coniferyl alcohol Sinapyl alcohol

Fig. 10.3 Structure of common monolignols

water. Hemicellulose decomposes at 200–260 °C as a result percentage of volatile components is more as compared to tars and chars.

10.2.3 Lignin

Lignin is a complex organic amorphous polymer having high molecular weight with interconnected dendritic structure surrounds cellulose and hemi-cellulose, which is very stable and difficult to separate. In the formation of lignin *p*-hydroxy-cinnamyl alcohols get dehydrogenated into monolignols, for example, *p*-coumaryl, coniferyl, and sinapyl alcohols (Bonechi et al. 2017). Lignin provides strength and rigidity to plant and generally present in between cells and cell wall. The structures of some common monolignols are mentioned in Fig. 10.3.

Lignins are amorphous in nature and form highly branched three dimension chain. The breakdown of the lignin takes place at the temperature range of 280–500 °C into phenols and gives a maximum fraction of char.

10.2.4 Biomass Categorization

Biomass can be roughly categorized into two main categories: aquatic and terrestrial biomass.

10.2.4.1 Aquatic Biomass

This category comprises of a diverse group of aquatic plants, microalgae, and macroalgae such as seaweed most of them are primitive and provided value-added chemicals as well as produce fuels because they possess advanced photosynthetic efficiency, higher biomass production, and faster growth when compared to ligno-cellulosic biomass types (De Wild 2015). The value chain of aquatic biomass has been discussed in the flow chart of Fig. 10.4.

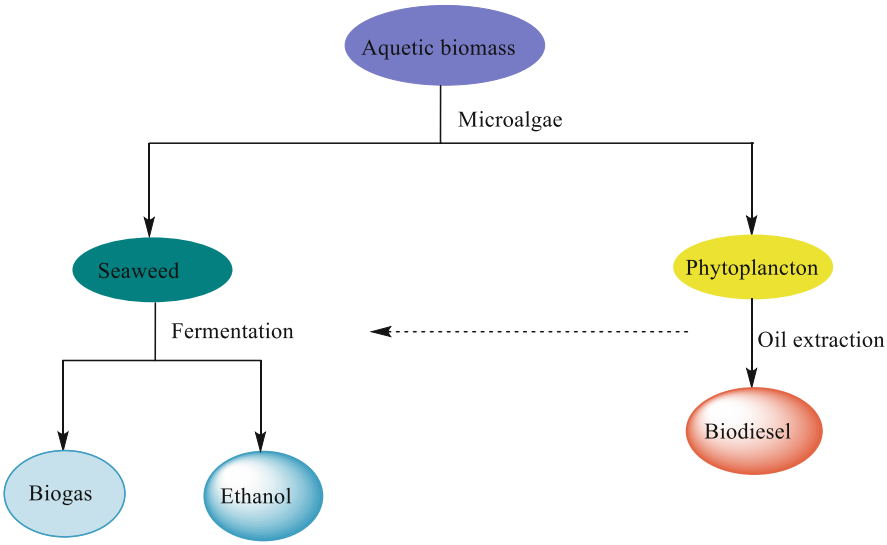


Fig. 10.4 Value chain of aquatic biomass (EIBI Aquatic biomass)

Aquatic biomass is wet in nature, upon harvesting and hydrothermal liquefaction can change fractions to feedstock from lipids to valuable bio-crude (Biller 2018). Duckweed and water hyacinth biomass are classified as aquatic plants not as algae because they block sunlight to the underwater. Algae from marine origin contain a large amount of ash content in the form of salt and other mineral matter as high as 60 wt% on a dry basis. Ecologically, marine plants can be categorized into two main categories: phytoplankton and benthos. Phytoplanktons are mostly microscopic and unicellular such as diatoms, dinoflagellates, coccolithophorids, and certain blue-green algae, floating on the upper layer on water body. The diatoms are rich in carbohydrate content which can be used as a good source of fuel.

10.2.4.2 Terrestrial Biomass

Terrestrial biomass is used for energy production, this biomass includes lignocellulose residue, organic residue, oil crops, grassy starch crops, and sugar crops. The terrestrial biomass can be achieved by many sources some of them are energy crops, municipal solid waste, agriculture biomass, and forestry biomass. Terrestrial biomass generally consists of cellulose, hemicellulose, and lignin, while algae consist of varying amounts of protein, starch like carbohydrates and lipids. Figure 10.5 includes different feedstocks for biomass power production.

Agriculture crops are termed as agriculture residues which can be classified as primary and secondary residues. Residue which is obtained in the field at the time of yield is said to be primary or field based residue, whereas assembled residues during processing are termed as secondary or processing based residue (Kumar et al. 2015). Table 10.2 describes the differences between terrestrial and aquatic biomass.

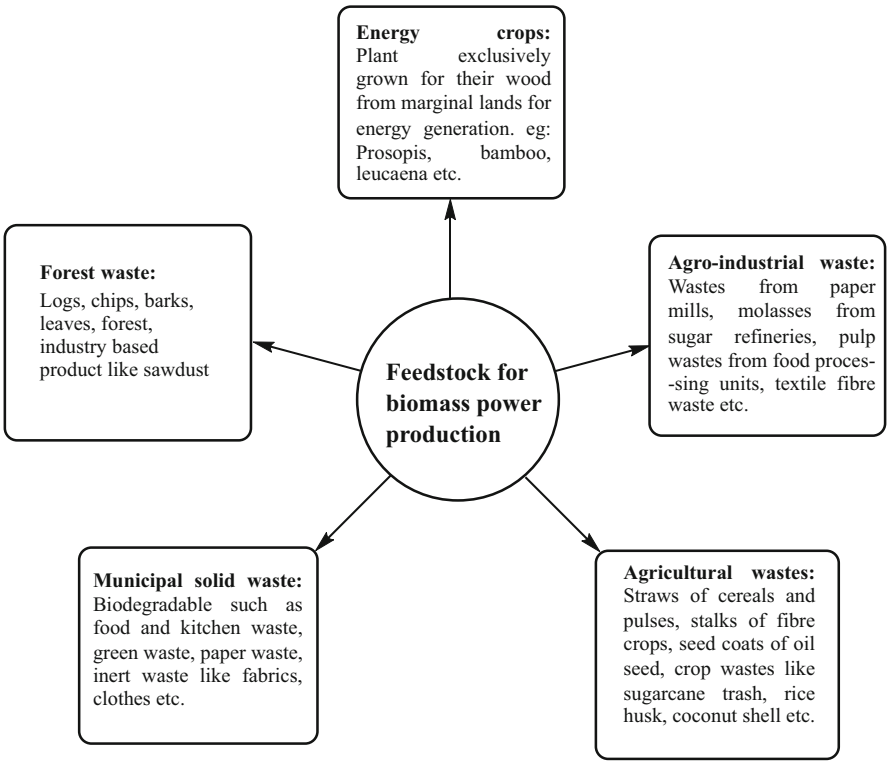


Fig. 10.5 Classification of available biomass resources in India (Kumar et al. 2015)

Table 10.2 Differences between terrestrial and aquatic biomass (Aresta and Dibenedetto 2010)

Terrestrial biomass	Aquatic biomass
• Light efficiency 1.5–2.2% • Requires land and water for growing (environmental and economic costs) • Biomass is generally rich in lignocellulosic components • Open area more than greenhouse cultivation • Cereals and seed plants are mostly used • Soil additives may be required • Productivity depends on soil quality (for a given plant)	• Light efficiency 6-8% (or higher when irradiated bioreactors are used) • May not require land for cultivation (coastal ponds, offshore basins) can be grown in process and municipal waters • Low lignocelluloses content. Lipid/protein/ polysaccharide content can be adjusted • Easy to grow in bioreactors (light-temperature adjustment); decoupling from climatic conditions

10.2.5 Biofuel

Bioethanol plays a major role in the production of biofuel and it can be achieved by various feedstocks such as fermented sugars of carbohydrate, starch containing plants like sugarcane, rice, potato, wheat, barley, sweet sorghum, corn, vegetable

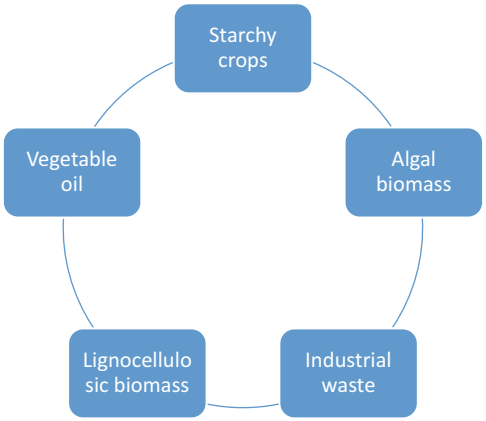


Fig. 10.6 Categorization of bioethanol feedstock

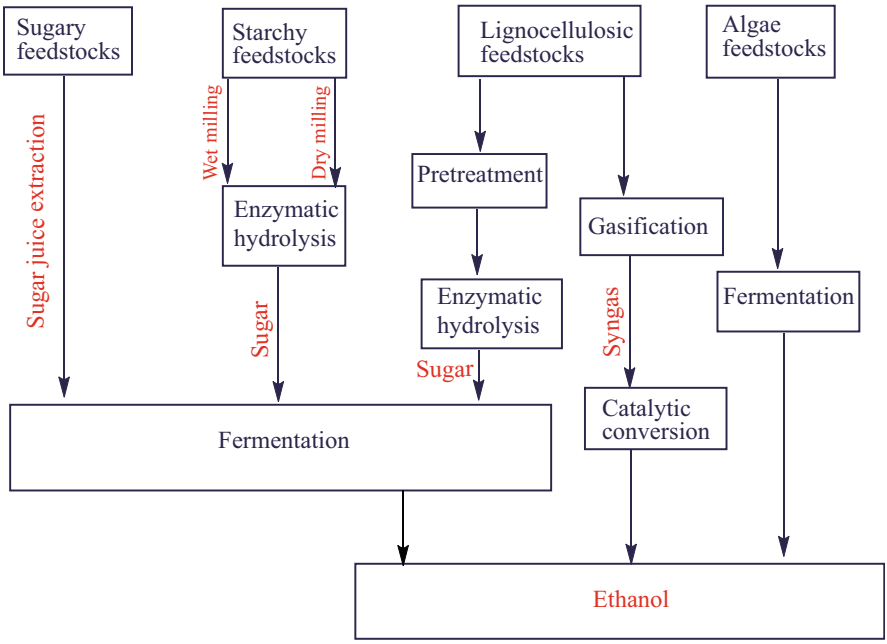


Fig. 10.7 Route of bioethanol production (Halder et al. 2019)

oil such as mustard oil, canola oil, olive oil, carbohydrate-rich strains of algal biomass and lignocellulosic biomass such as woody and herbaceous biomass including forest residues (Azad et al. 2015, 2016). Bioethanol producing feedstocks mentioned in Figs. 10.6 and 10.7 describe about the possible route for the production of bioethanol. Dave et al. (2019) reported that algal-derived bio-ethanol having high heat of vaporization and octane rating, which makes it eco-friendly and sustainable energy resource.

Numerous biomasses used as energy sources such as maize ethanol and soybean biodiesel were developed in the USA as energy feedstock in the past decades (Sims et al. 2006).

Bamboo is one of the promising renewable biomass feedstock for the production of bioethanol from bamboo biomass. Dried biomass of bamboo contains some components like holocellulose (70.0%), lignin (28.1%), protein (2.4%), lipid (2.6%), and ash (1.4%). According to Sun et al. (2011) hydrolysis of holocellulose is the key step to produce ethanol from lignocellulosic biomass. Holocellulose is the polysaccharide fraction of cellulose and hemicellulose obtained after removing lignin and extractives. The percentage of holocellulose does not decrease with the age of bamboo biomass. Ethanol can be produced by bamboo biomass followed by hydrolysis of concentrated sulfuric acid and oligosaccharide, separation of acid and sugars, exclusion of color compounds leads fermentation of ethanol. The saccharification by concentrated sulfuric acid conducted using acid to substrate ratio 1.4 at 80 °C resulting the efficacy of sugar recovery reached approximately 82%. Hence the fermentation yield obtained up to 92% and ethanol productivity achieved 8.2 g/l/h (Sun et al. 2011).

10.2.5.1 Bio-Oil

Biomass is a major feedstock to produce bio-oil which is eventually an alternative source of energy. The conversion of biomass to bio-oil can be obtained by two methods: hydrothermal liquefaction and flash pyrolysis. Bio-oils are generally brown liquids with characteristic odor owing to distinctive combination of many organic compounds and water. It can be extensively obtained as raw bio-oil from fast pyrolysis of biomass feedstock. It is acidic (pH = 2–4) and corrosive in nature and shows instability towards temperature as well as chemicals. Bio-oil can be achieved by petroleum waste, animal manure, and oil crops (sunflower, olive, palm, coconut, and groundnut), cereals, starch and sugar crops (potato, sugar beet), and cellulose crops, these are some common multipurpose energy crops used to generate various kinds of energy product. Bio-oil contains lots of properties which make it more compatible over heavy petroleum oil such as high viscosity, ash content, oxygen content, water content, etc. The main challenge to convert bio-oil from biomass causes relatively high cost with poor quality and yield makes it less preferable and to improve production there is still research on. Bio-oil can be used as a renewable source of energy in many fields such as fuel oil in turbines, engines, furnaces.

10.2.5.2 Biogas

Biogas can be produced by the decomposition of organic matter such as biodegradable waste, landfill sites, municipal waste, manure, and sludge waste. Generally methane (gives clear flame on heating) and carbon dioxide create a major ratio of biogas and usually used for domestic uses such as cooking and heating. The biogas is composed of different gases such as methane (50–70%), carbon dioxide (25–50%), nitrogen (10%), and other gases (H_2, O_2, H_2S) in trace amount (Renato et al. 2013).

Fig. 10.8 Bunched bamboo tree

Table 10.3 Bamboo biomass stock in some Asian countries (million tonnes) (Lobovikov et al. 2007)

Country	1990	2000	2005
China	643	811	907
India	239	243	252
Pakistan	0.059	0.091	0.130
Republic of Korea	0.406	0.309	0.310

10.2.6 Bamboo Biomass

For future energy perspective bamboo is also a promising biomass. Herein, we are discussing overview on bamboo biomass:

Bamboo extensively grows in India and China due to great ecological balance, growth pace, and its versatility. Bamboo belongs to the family of grass, has the fastest growing strategy than any other heartwood tree and it produces new shoots after each harvest. Worldwide bamboo forest distribution is almost 22 × 106 ha, among them 13.96 × 106 ha is distributed in India and predictable annual production rate in the country is around 13.5 × 109 tons (FSI 2011). In India the whole growing stock of bamboo is around 180 × 106 tons, including bamboo grown in forest and non-forest areas. An image of bunch of bamboo is shown in Fig. 10.8.

According to Yen (2016) bamboo is a superior carbon storage species which develop shoot to mature culms within 5 years. Bamboo comprises of strong, light, and flexible woody stem that has many applications including in construction, textiles, paper making, bamboo mats, furniture, and some species are used as a source of food as well. Table 10.3 shows the biomass stock in some Asian countries and Fig. 10.9 represents the pie chart of contribution of bamboo resources by continents, whereas Fig. 10.10 represents countries with the largest distribution of

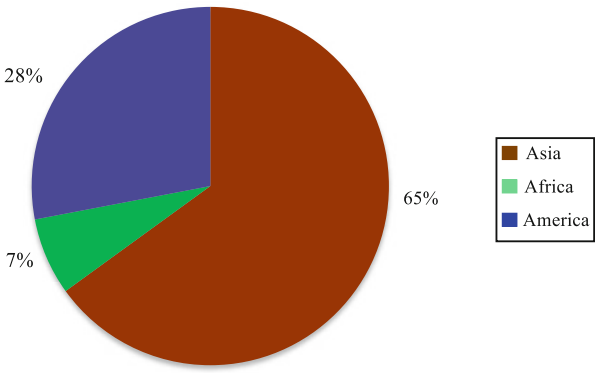


Fig. 10.9 Contribution of world bamboo resources by continents (Lobovikov et al. 2007)

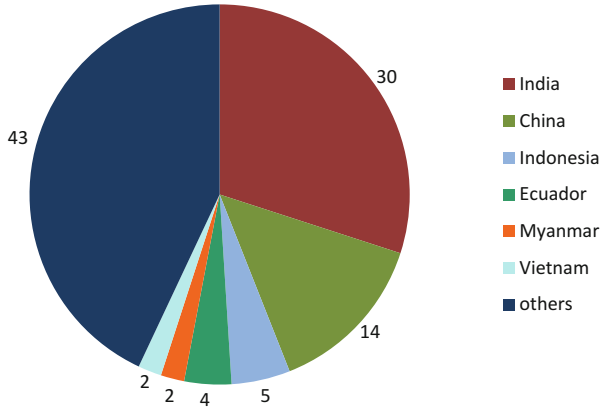


Fig. 10.10 Countries with largest bamboo resources (Lobovikov et al. 2007)

bamboo. Charcoal obtained from bamboo is used for domestic purposes as well as fuel for gasifiers. Northeastern states (Assam, Manipur, Mizoram and other) in India, having power plants for electricity production, which are exclusively based on bamboo.

10.2.7 Value of Fuels and Bamboo Lignocellulose as Raw Material

Lignocellulosic biomass is a natural and renewable feedstock of energy and is abundantly available as raw material on the earth surface. This comprises of cellulose, lignin, and hemicelluloses (already discussed in the previous section).

Lignocellulose includes biomass obtained from plants which is versatile biopolymer on the earth. Lignocellulosic biomasses are rich in carbohydrates and available with low and stable price. In addition, they are mainly waste materials and non-competitive with food chain. According to Johansson et al. (1993) lignocellulosic biomass is predicted to provide approximately 38% of the world's direct fuel and 17% of the world's electricity by 2050. Lignocellulose is composed of cellulose

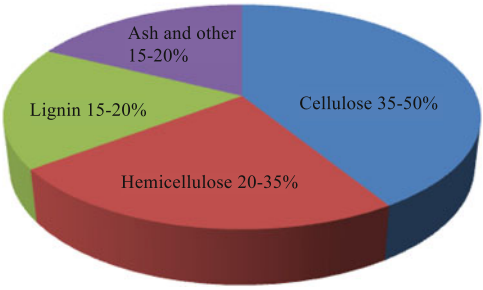


Fig. 10.11 Composition of the lignocellulose

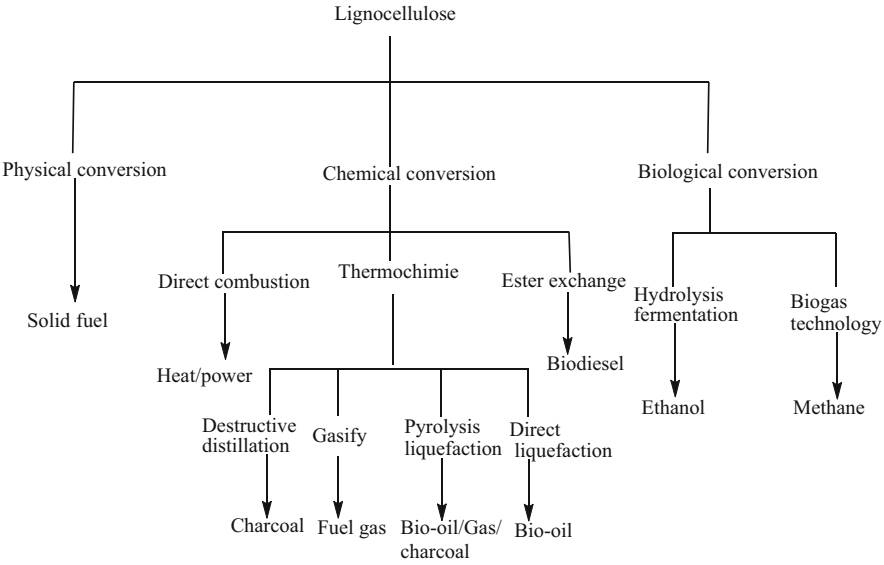


Fig. 10.12 Different forms of energy obtained through different pretreatment methods (Chen et al. 2017)

(35–50%), hemicellulose (20–35%), lignin (15–20%), ash, and some other compounds (15–20%) (mentioned in Fig. 10.11), among them lignin is recalcitrant component and the second most abundant organic compound in nature (Feng and Lin 2017). This substituent has been already explained in Sect. 10.2. In the bamboo biomass silica and potassium are major ash forming minerals. A flow chart given in Fig. 10.12 shows different forms of energy obtained through lignocelluloses via different pretreatment methods.

10.2.8 Fuel Analysis of Bamboo

Bamboo has fascinated a lot of researcher's consciousness between various non-wood lignocellulosic bioresources. In the series of advanced utilization of

Table 10.4 Fuel analysis of *Bambusa vulgaris, Bambusa vittata, and Bambusa heterostachya*

Fuel property	*Bambusa*	*Bambusa vittata*	*Bambusa heterostachya*
Moisture content (%)	12.39	16.02	9.84
Ash content (%)	2.96	2.45	1.20
Calorific value (%)	17.29	17. 24	17.84

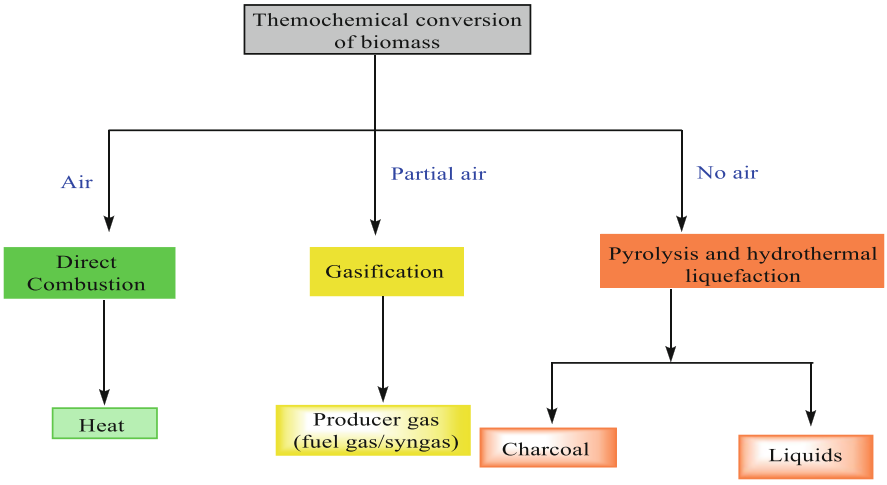
Source: Sarfo (2008)

bamboo the effectivity of pretreatment was affected by bamboo's properties such as pulp and paper, wood plastic composites, and chemical component (cellulose, hemicellulose, and lignin) of biomass and also the chosen pretreatment method. Bamboo has enormously lot of fuel characteristics such as low alkali index as well as ash content. As compared to other plants bamboo has low moisture content, i.e. 8–23% (Scurlock et al. 2000) as well as it contains high heat value (HHV) than other agricultural crops. Table 10.4 shows fuel properties of various bamboo species *Bambusa vulgaris, Bambusa vittata, Bambusa heterostachya*.

10.2.9 General Conversion Methods of Bamboo Biomass to Fuel

10.2.9.1 Thermochemical Routes for Biomass Conversion to Fuels

Thermochemical process is a general biomass conversion route at high heating rate. This conversion route includes direct combustion of biomass into heat, liquid fuel, and other forms of fuel generator to provide heat for thermal and electricity production. The thermochemical route is classified into three main categories: combustion, gasification, and pyrolysis and liquefaction (mentioned in the flow chart depicted below).



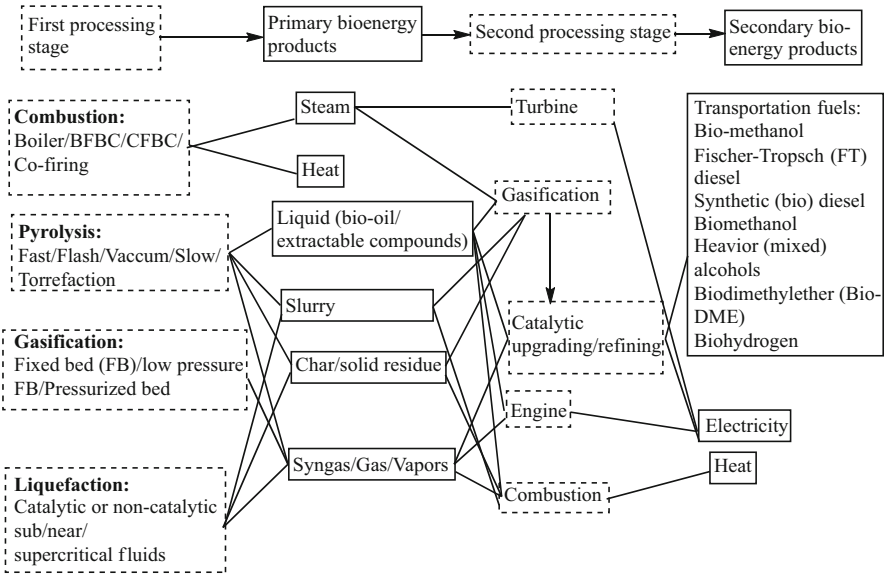


Fig. 10.13 An overview of thermochemical processes for the conversion of woody biomass into bioenergy products (Gorgens et al. 2014)

A pictorial representation of thermochemical processes for the conversion of woody biomass into bioenergy products is presented in Fig. 10.13. Thermochemical conversion involves some extent of heat in conversion process as well as method varies with degree and method of burning. The liberated heat is used in many forms including in industries, steam turbines and to generate electricity also. The thermochemical conversion is classified into combustion, pyrolysis, gasification, and liquefaction.

According to a study woody and dry biomass are converted to bioenergy by thermochemical path. There are numerous methods used to convert energy from bamboo biomass. Bamboo belongs to this category so for the bamboo biomass we will focus mainly on combustion, pyrolysis, and gasification.

Combustion

Combustion refers to direct heating and this is the most common method used in conversion process. This method exhibits three main fundamental feedstock, such as fuel, air (as oxidant of the feedstock) and the application which required for a particular temperature from a heat source (Gorgens et al. 2014). The combustion process takes place in the presence of excess of oxygen to burn the biomass which can be varied with different temperature and oxygen circumstances. Usually combustion process operates at very high temperature range about 800–1000 °C, which is sufficient to burn any biomass but if the biomass having more moisture content

more than 50%, biological conversion process is preferable. Dry bamboo biomass can be used as a source of energy like in firewood, cooking, and it is preferred by the people in rural area especially where domestic gas and electricity is not available and industrially it has many applications. The reaction of biomass combustion can be summarized in the equation below:

$$CH_yO_x + \left(1 + \frac{y-2x}{4}\right)(O_2 + 3,76\,N_2) \longrightarrow CO_2 + \frac{y}{2}H_2O + 3,76\left(1 + \frac{y-2x}{4}\right)N_2 + \text{Heat}$$

Overall reaction of biomass combustion (**Source** : **Kerlero de Rosbo and De Bussy 2012**)

The main components of combustion are H_2O and CO_2, the combustion process of bamboo biomass fall out into an assembly called boiler, where heat transfer through steam gas to other gases as well as to fluid, are discussed in Fig. 10.14. As a result the gases are exhausted and fluid (water/air) is further used in turbines for the generation of electricity. The boiler section uses medium to high pressure >20 bar for generation of large scale steam (Kerlero de Rosbo and De Bussy 2012).

Pyrolysis

Pyrolysis is the most efficient and environmentally benign process which converts biomass to energy sources. It can be defined as a moderate temperature (600–750 K) organic substances breaking down under thermochemical condition where oxygen is limited or absent. Pyrolysis is a phenomenon which converts biomass into solid (charcoal), liquid (bio-oil), and gas fuels. Pyrolysis is the precursor of gasification and combustion of solid fuels. Figure 10.15 represents the pyrolysis pathway of biomass.

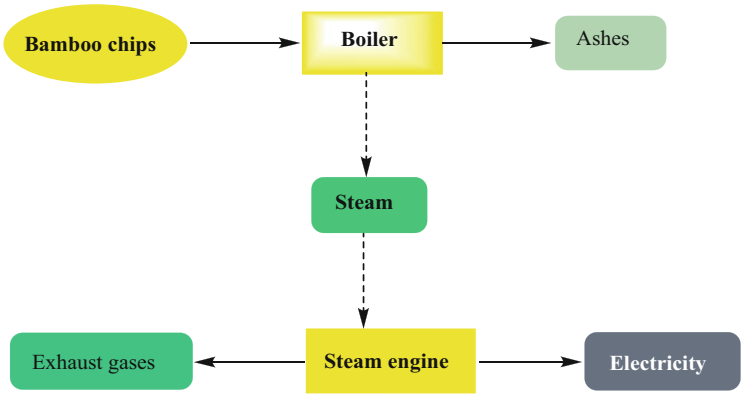


Fig. 10.14 Combustion process from bamboo biomass (Kerlero de Rosbo and De Bussy 2012)

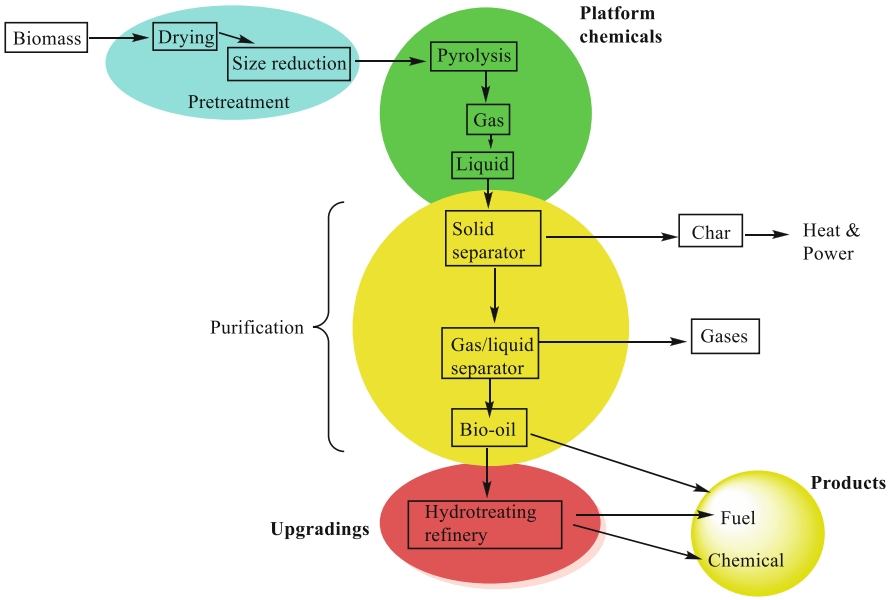
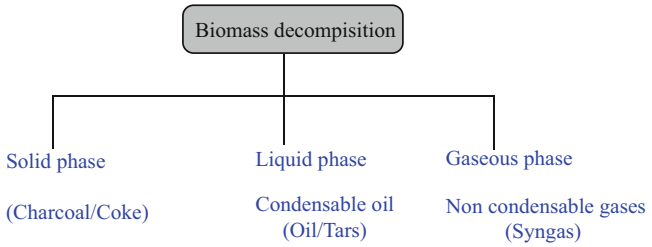


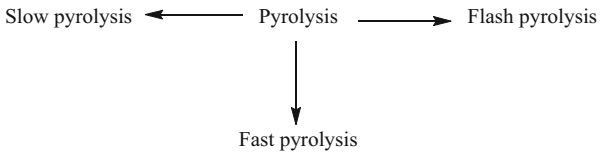
Fig. 10.15 Pyrolysis pathway of biomass (Alonso et al. 2010)



It is extensively used to convert high value added products such as syngas, liquid oil, and solid char from biomass. The bio-oil contains acid, ketone, aldehyde, sugar and esters produced by pyrolysis, can be obtained at optimum temperature of 800 K.

Classification of Pyrolysis

Several types of pyrolysis have been developed with time such as vacuum pyrolysis, pressurized pyrolysis, fast pyrolysis, flash pyrolysis, torrefaction pyrolysis, and slow pyrolysis (Gorgens et al. 2014). But it can roughly classify on the basis of maneuver state and the classification is depicted as:



- Slow pyrolysis: It takes several times to burn the organic material in the absence of oxygen and the favored route for the production of biochar and bio-oil. The operating temperature of slow pyrolysis is approximately between 550–950 K. In this process organic matter partially evaporates and charcoal remains as the main product (80%). Carbonization is another term usually used for slow pyrolysis.
- Fast pyrolysis: It is an anaerobic thermochemical technique at an elevated temperature of about 577–977 °C and gives liquid bio-fuels as major product and biochar with biogases are remain as minor products.
- Flash pyrolysis: It is a process which provides biomass crude oil equivalent to petroleum through high yield efficiency. The flash pyrolysis operates at an elevated temperature of about 777–1027 °C and gives unwanted pyrolytic water as the final product (Panwar et al. 2012).

There are lots of factors which affect the thermal conversion and product formation such as nature and intrinsic composition of substrate, pyrolysis pressure, temperature, residence time, and heating rate. Thermal destructive distillation of the biomass occurs at 500 °C and at limited oxygen condition. According to Kerlero and Bussy the following equation represents conversion in solid, liquid, and gaseous products through pyrolysis of biomass (MM: Mineral material).

$$(\mathrm{C, H, O, N, MM, H_2O})_{\mathrm{Biomass}} \rightarrow (\mathrm{C, MM})_{\mathrm{Solid}} + (\mathrm{CO, H_2, CH_4, CO_2, N_2})_{\mathrm{Gas}} + (\mathrm{C_n, H_m\ H_2O})_{\mathrm{oils/tars}}$$

Dehydration of bamboo chips leads to dry bamboo biomass which on medium heating gives fuel gas or syngas from thermal decomposition as shown in Fig. 10.16. Thermal degradation process degrades hydrocarbon, liquid oils, and saturated as well as unsaturated hydrocarbons like methane and other gases with calorific value 4–7 MJ/m^3. Syngas contains different gases such as carbon monoxide, carbon dioxide, methane, nitrogen, and hydrogen which can be used as fuel for power production by using boiler and gas engine. The pyrolyzed oils are purified by "biorefinery" to produce biofuels.

Gasification

The gasification takes place in the presence of air/oxygen to produce heat either through exothermic reaction or endothermic reaction. Gasification of biomass conversion is one of the older techniques, employed from the past few decades and famous in European countries where they used in the blast furnace. This process converts biomass to combustible gases with the same amount of energy. Gasification is fractional combustion of biomass in order to generate gas and char in the initial

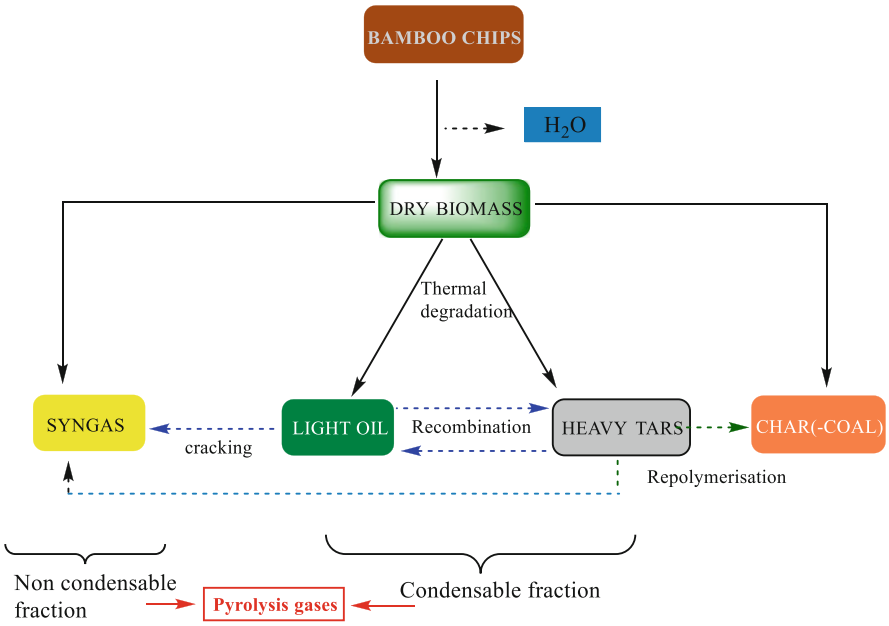


Fig. 10.16 Pyrolysis reactions and products (Kerlero de Rosbo and De Bussy 2012)

phase, consequently reduction resulting primarily in gases like CO_2 and H_2O through the charcoal into CO and H_2 with methane and further hydrocarbon. The gasification process is completed at a very high temperature of 1000 K. Gasification produces syngas or producer gas (CO_2, CH_4, and N_2) by partial combustion of biomass (Huber et al. 2006). The obtained syngas is further improved by Fischer–Tropsch (FT) synthesis to liquid fuels like gasoline and diesel, this synthesis is already used in South Africa in bulk level (Dry 2002). The gasification method is particularly appropriate for lignocellulosic feedstocks. Figure 10.17 shows the distinctive method of biomass gasification. The gasification of bamboo is discussed in Fig. 10.18.

An additional advantage of gasification is that 15% of the biomass would also be available as a by-product in the form of high grade charcoal of bamboo. Apart from the solid and liquid conversion of bamboo biomass there is an alternative route from lignocellulose biomass to gaseous fuel. The resulted fuel can be used in engines, gas turbines which ultimately lead to the production of electricity. There are two broad categories of conversion method of gaseous fuel thermochemical and microbial conversion. Thermochemical conversion consumes more energy which is a faster conversion method at high temperature which converts organic fraction biomass to gas fuel whereas microbial conversion is an anaerobic conversion which consumes very less energy and requires mild temperature to execute.

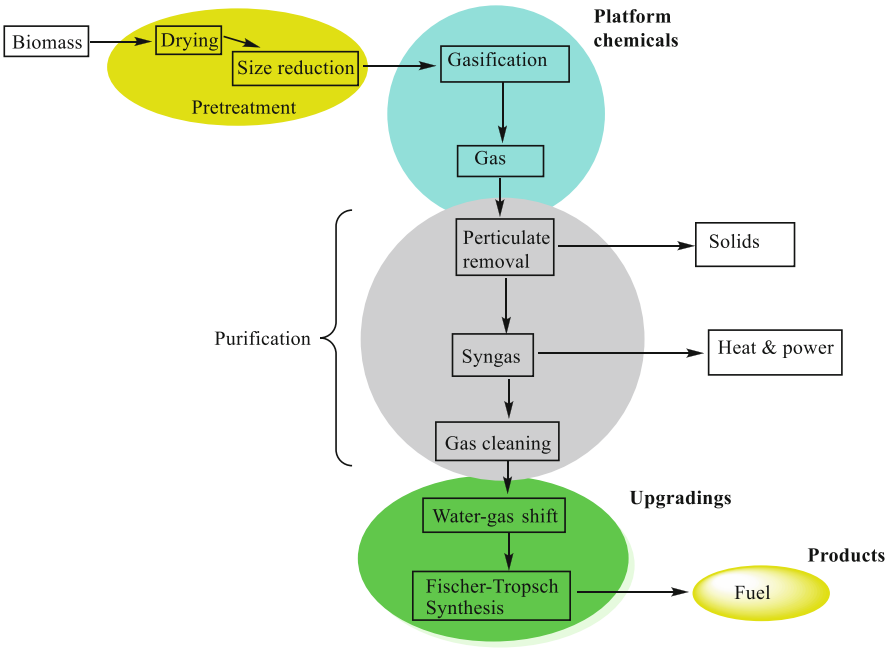


Fig. 10.17 Schematic methodology of biomass gasification (Alonso et al. 2010)

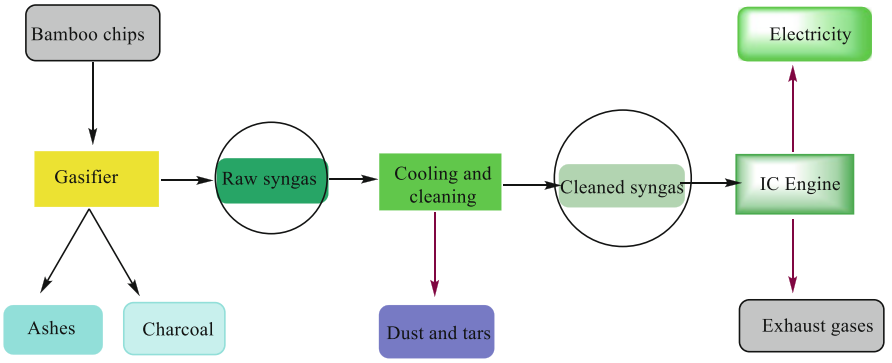


Fig. 10.18 General process of gasification (Kerlero de Rosbo and De Bussy 2012)

Liquefaction

Liquefaction is the process extensively used for the production of bio-oil and can be achieved directly or indirectly. Direct liquefaction gives oil and liquid tars by rapid pyrolysis and hydrothermal liquefaction, whereas indirect liquefaction gives liquid and gaseous products by adding catalyst followed by consecutive production of intermediates. The biomass decomposition followed by catalysis produces macro and micromolecules, these micromolecules again polymerize together into bio-oil.

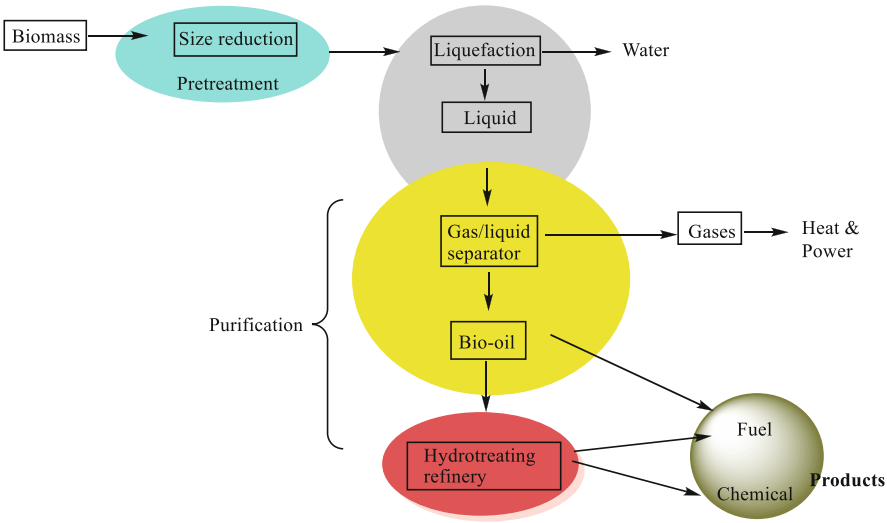


Fig. 10.19 Schematic representation of biomass liquefaction (Alonso et al. 2010)

The carbonate salts of alkali metals such as sodium and potassium carbonate are used as a catalyst in mixture of aqueous biomass for liquefaction at temperature (500–700 K) and high pressure 20 atm (Alonso et al. 2010). One of the major drawbacks of liquefaction is that the liquid product holds less oxygen, hence less suitable for further use. Figure 10.19 explains the pictorial representation of the liquefaction of biomass.

10.3 Biochemical Routes for Biomass Conversion to Fuels

Biochemical conversion of biomass includes conversion of biomass to reduced sugar by enzymatic hydrolysis followed by microbial conversion to fuel products (Truong and Anh Le 2014). Anaerobic and fermentation are two most common conversion processes following by biochemical routes. When degradation of biomass takes place under anaerobic condition or in the absence of oxygen by microorganism, which produces biogases such as methane and CO_2 are fall into anaerobic conversion, whereas decomposition of biomass proceeds via yeast and bacteria to produce ethanol are known as fermentation process as discussed in flowchart (Fig. 10.20).

10.4 Conclusion

At present most of the existing source of energy are non-renewable, such as petrol, diesel, natural gas, crude oil, etc., which ultimately causes environmental pollution and have limited source. So it is a need of hour to develop and discover new

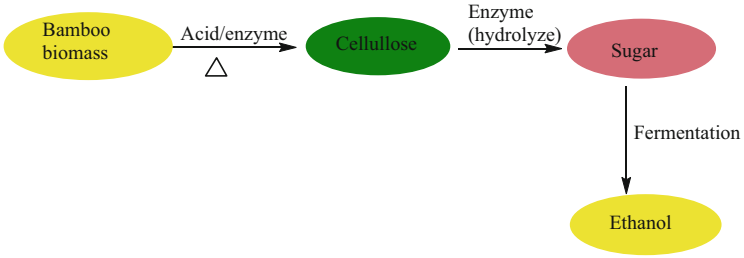


Fig. 10.20 Flowchart of producing ethanol from bamboo biomass

renewable sources, which can fulfill the increasing demand of civilization. In this connection, biomass is a significant renewable source of energy, which exclusively produced fuels and chemicals. Currently these biofuels are used as significant traditional source of energy in domestic area especially in developing countries. Biofuels such as lignocellulosic biomass and algae are preferred as second and third generation biofuels and employed for the production of fuels and chemicals too. Bioethanol is the most important class of liquid fuel, which is non-petroleum, based sustainable and renewable fuel having less energy particles than petrol with high octane number. Consequently, the presence of oxygen in bioethanol resulted in clean combustion (Krylova et al. 2008), its beauty of the biofuel. The biofuels from lignocellulosic feedstocks can be achieved by two methods: In the first method whole biomass is converted into upgradeable gaseous or liquid fuel followed by the thermochemical route. The production of synthetic gas is typically carried out by the gasification process, while pyrolysis and liquefaction conversion method are totally used to produce bio-oils. The other biochemical route successively converts biomass to biofuel especially to bioethanol.

Bamboo biomass as bioenergy crop for the purpose of bioenergy production is a completely new approach. One of the main purposes is that being a renewable energy source it can replace fossil fuel to produce biofuels like solid, liquid, and gas. To convert bamboo biomass to biofuel various methods such as thermochemical (combustion, pyrolysis, and gasification) and biochemical have been used. With some drawbacks in the production of bamboo biomass such as land and water consumption, it has major advantages including domestic and industrial uses, electricity, and biofuel production. In the production of bamboo, it requires vegetative propagation which makes it an expensive object. However the uses of bamboo biomass as energy crop project is in initial stage, the largest production of bamboo in some countries, like India and China requires some attention and research to use bamboo biomass as an energy source. India owes second position in bamboo production, and looking towards future propensity in various fields such as agriculture, forest as well as in industries it can be develop as an alternative field crop resource.

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Conflict of Interest The authors declare no conflict of interest.

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Advances and Challenges in Sugarcane Biofuel Development

11

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Abstract

Biofuel produced from the plant biomass shows greater alternative source of renewable energy and better than the fossil fuels in reducing the greenhouse gas emission from the burning of fossil fuels. Sugarcane is one of the best candidates for biofuel production which has been used successfully to produce bioethanol extensively in Brazil and also in other countries worldwide. Sugarcane is a perennial monocot with C4 photosynthesis, having a fast growth rate without any serious maintenance and can be harvested four to five times by multiplying using the ratoons. Sugarcane is one of the primary crops as a source for both food and bioenergy, with Brazil, India, and China contributing more than 60% of the world's total production. The diminishing resources of fossil fuel coupled with augmented research interest for an environmentally sustainable and renewable source of energy in the form of sugarcane. Industrial levels of biofuel production have been achieved in Brazil and the USA, however more concerted efforts needs

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to be directed towards deployment of second-generation biofuel production by utilizing lignocellulosic biomass.

Keywords

Sugarcane · Biofuel · Lignocellulosic biomass

11.1 Introduction

Exploration of alternative energy sources has shown renewed special research focus that could curtail or replace the usage of fossil fuels (Waclawovsky et al. 2010). The ever increasing energy demands can be supplanted by plant feedstocks, which are excellent sources of renewable energy resources. The availability of renewable bioenergy resources can go a long way in securing the energy needs of a country in sustainable economic manner, thereby diverting the precious resources from oil-based import economy towards a self-sustainable one. The encouraging trends in adoption of bioenergy-based utilization would go a long way in mitigating the adverse effects of greenhouse gases (GHGs). Besides, it offers socio-economic and environmental benefits. Several agricultural crops and plants have been explored and identified for biofuel purpose, like sugarcane (*Saccharum* spp.), maize (*Zea mays*), soybean (*Glycine max*), willow (*Salix* sps.), switch grass (*Panicum virgatum*), rapeseed (*Brassica napus*), wheat (*T. aestivum*), sugar beet (*Beta vulgaris*), palm oil (*Attalea maripa*), manioc (*Manihot esculenta*), miscanthus (*Miscanthus* sps.), potato (*Solanum tuberosum*), sweet potato (*Ipomoea batatas*), and barley (*Hordeum vulgare*); sorghum (*Sorghum bicolor*), cassava (*Manihot esculenta*), and hemp (*Cannabis sativa*) (Cho 2011; Davis et al. 2013; Balat 2010; Leite and Leal 2007; Solomon and Bailis 2014).

Sugarcane is one of the most energy efficient crops being grown in more than 100 countries (Fig. 11.1) having very wide adaptability range supported by its C4 photosynthetic system resulting into large biomass production per unit area having the desirable traits of high yield along with low input requirements and better processing capabilities (Verheye 2010) as well as reducing greenhouse gases (Matsuoka et al. 2009). Worldwide, it is grown on an area of 25.9 million ha, and its total production is ~1.84 billion tons with a fresh cane yield of 70.9 tons ha^{-1} (FAOSTAT 2019) (Fig. 11.2) The largest acreage of sugarcane lies in Brazil contributing 41% of world production (758 Mt), followed by India (306 Mt—16%) and China (104 Mt—5.6%) (FAOSTAT 2019) (Fig. 11.3). Sugarcane was originally domesticated around 8000 BC in Papua New Guinea. Commercial sugarcane is the cross of *Saccharum officinarum* with wild *Saccharum* spp., i.e., *S. spontaneum*, *S. robustum*, *S. barberi*, *S. sinense*, and *S. edule* (Talukdar et al. 2017; Allen et al. 1997; Jeswiet 1929). Commercial sugarcane suffers from high level of pollen sterility, and propagation through vegetative cuttings is the method of choice (Allsopp et al. 2000). Disaccharide sugar is the main product of sugarcane. Juice extracted by crushing of the canes is clarified at high temperature in the

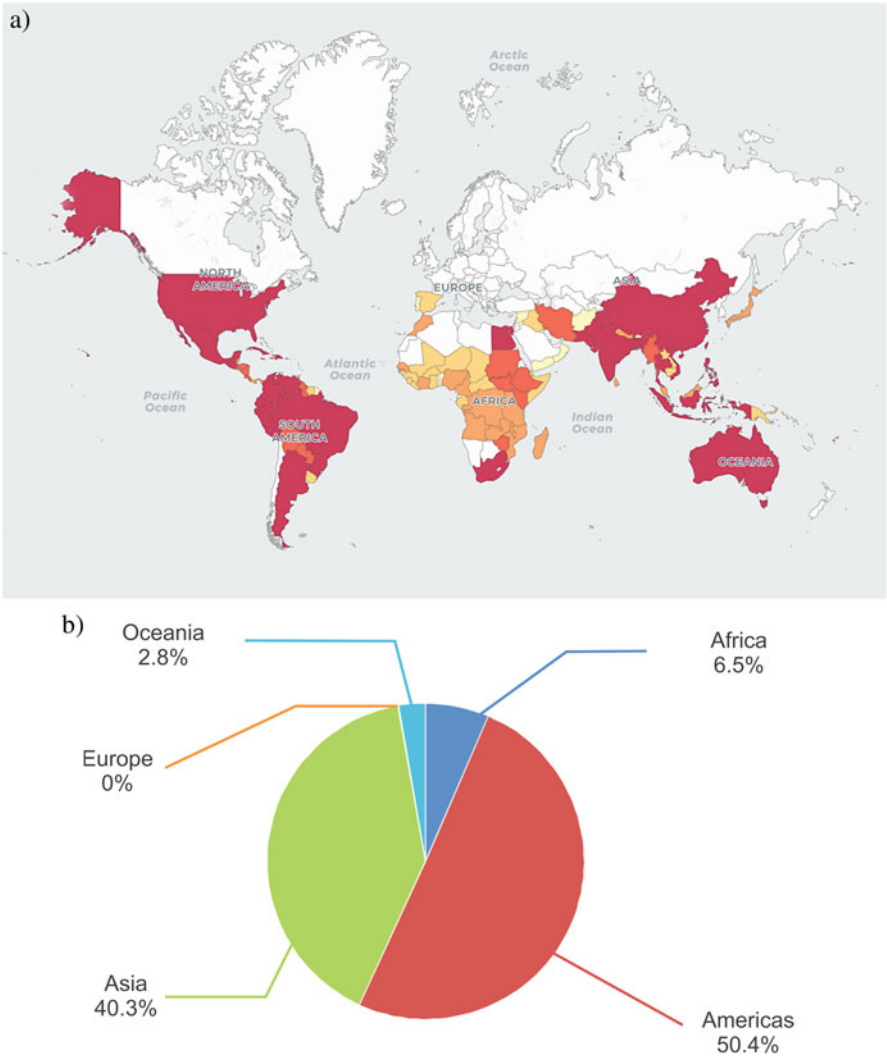

Fig. 11.1 (**a**) Major sugarcane-producing countries. (**b**) Global distribution of sugarcane

presence of lime, which forms complexes with phosphorus in juices, precipitating with impurities supported by flocculants (Mackintosh 2000).

Bioethanol obtained through sucrose fermentation of sugarcane (*S. officinarum*) often referred as "noble cane" is referred to as "first-generation" bioethanol production. Production of biofuel through fermentation of the lignocellulosic plant cell wall biomass of sugarcane is referred to as "second-generation" bioethanol production. Third- and fourth-generation bioethanol are derived from algal sources and

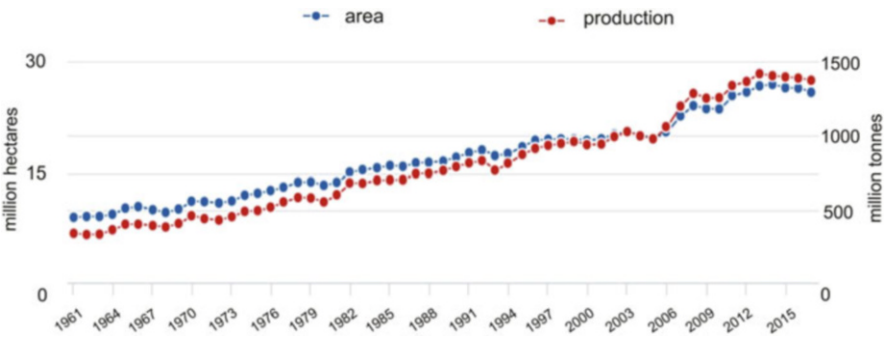

Fig. 11.2 Sugarcane area and production around the world (1961–2017) (FAOSTAT 2019)

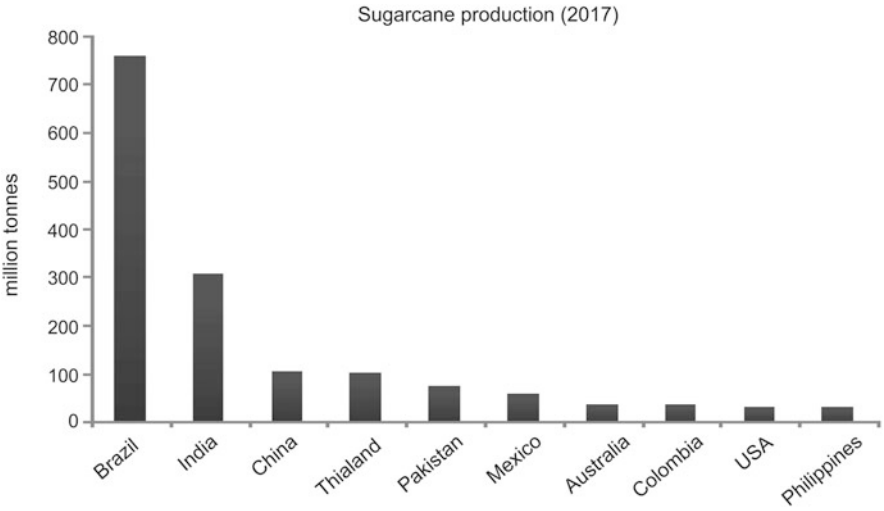

Fig. 11.3 Leading sugarcane-producing countries (FAOSTAT 2019)

genetically modified microalgae, respectively (Buckeridge et al. 2010; Carvalho et al. 2013).

11.2 First-Generation Bioethanol Production

The first-generation bioethanol is sourced from easily extractable sugar or starch sources. Here, sugarcane offers an obvious advantage with ~20% juice content with production levels of 8000 L/ha which is twice that of maize, thereby requires half the land requirement (Lima and Natalense 2010). Sugarcane undergoes chopping and shredding in traditional mills to extract the broth. First-generation bioethanol produced from the sugarcane by fermentation of sugar obtained from its juice and left-

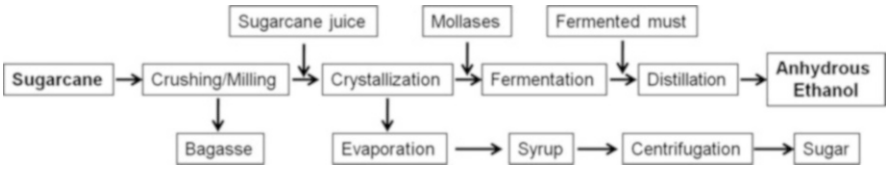

Fig. 11.4 Block flow diagram of a sugarcane-based first-generation bioethanol production

over plant material after extracting the juice (bagasse) is burned to produce steam for electricity generation, to produce fertilizers, or to produce heat in the sugar mills (Pandey et al. 2000) (Fig. 11.4). The impurities and contaminants laden extracted broth are removed as bagasse with aid of filters (physical treatment), and clear broth undergoes chemical treatment wherein soluble impurities are coagulated using CaO and phosphoric acid with pH7.0, followed by decantation and concentration to 20–22° brix in evaporators for better fermentation (Santos et al. 2012). Sulfitation is an additional step in bioethanol production to purge the color from the formed sludge. Under anaerobic condition, the most crucial step of bioethanol production is accomplished by yeast (*Saccharomyces cerevisiae*) which metabolizes sugars to bioethanol. Fermentation process at commercial scales involves: (1) Simple Batch: Yeast is added to the fermenter, with the yeast fermentation process lasting till the presence of nutrients. The process is slow and needs to be cleaned and reloaded with each batch. Supplements and inoculums are incorporated at the start of the reaction, with constant agitation that supports the growth and fermentation process. To moderate pH, chemicals and antibiotics are added to the medium (Maxon and Johnson 1953; Zhang 2009). Often fermenters are operated in series at commercial level to sustain the high demand of bioethanols (Gomez-Pastor et al. 2011). The status of the growth of yeast is regularly monitored. (2) Fed Batch: The fermentation involves the addition of supporting nutrients to the fermenter with the products remaining till the end of reaction. The fed-batch system offers an advantage over the batch process: higher productivity level of ethanol along with lower content of residual sugars, thereby self-inhibition by the presence of substrates and products is minimized. The process requires less fermentation period, reduced toxicity levels to the growing yeast cells, and prevalence of optimum growth conditions (Stanbury et al. 2003). Higher inoculum load is inversely correlated to reduced yeast cell viability (Laluce et al. 2009). (3) Multistage Continuous Process: These fermenter systems are designed to operate continuously and are fed by sugarcane juice to maintain continuous flow towards the distillation units. Often four or more reactors are operated in series. The major advantage this system offers is very high levels of ethanol production coupled with lower operational running costs (Deindoerfer and Humphrey 1959). The drawbacks include higher chances of contamination, therefore requires large amounts of sulfuric acids and antibiotics (Domingues et al. 2000).

11.3 Second-Generation Bioethanol Production

The second-generation biofuels involve the use of lignocellulosic materials. Lignocellulose comprises of cellulose (homo-polymer of glucose units), hemicelluloses (hetero-polymers of D-mannose, D-glucose, D-xylose, L-arabinose, D-galactose, mannuronic acid, and glucuronic acid units), and lignin (phenylpropane units). These three components are responsible for the rigidity of plant cell (Brodeur et al. 2011; Hendriks and Zeeman 2009; Ogeda and Petri 2010; Sarkar et al. 2012). The idea of employing sugarcane straw from crop residues while not competing with food production is building up the buzz. Bioethanol yield through this method can be augmented as much as 100% with a yield of ~300 L of bioethanol from one ton of bagasse. After harvest, the sugarcane straw (comprising 40% cellulose, 30% hemicelluloses, and 25% lignin) is shredded and processed by hydrolysis. The plant cell wall is degraded into monosaccharides to be used as a feeder for fermentation process (Piacente et al. 2015). The hydrolysis of cellulose is catalyzed by cellulase enzymes to produce mono- and disaccharides followed by fermentation to bioethanols. Since the process is slow, a pretreatment is often undertaken (Fig. 11.5).

Pretreatment helps disrupt the cellulose structure, breaking down hemicelluloses and modification/removal of lignin (Mosier et al. 2005). The methods include physical, chemical, and biological pretreatments (Alvira et al. 2010). Physical processes include steam explosion, mechanical reduction in size, and hot water application, often added in combination with catalysts to improve efficiency (Agbor et al. 2011). Physicochemical methods include CO_2/SO_2-steam explosion, acid-steam explosion, and ammonia fiber explosion (Agbor et al. 2011). Chemical pretreatments involve the use of dilute acids like H_2SO_4 and HCl; dilute alkalis like

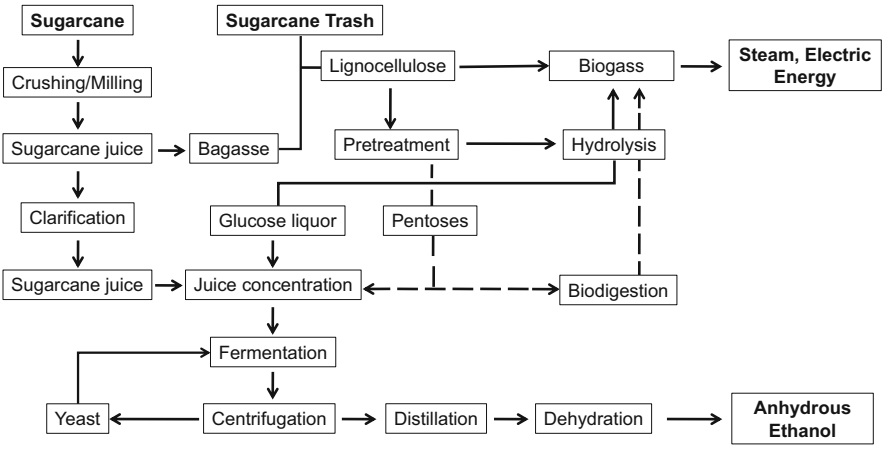

Fig. 11.5 Block flow diagram of a sugarcane-based second-generation bioethanol production (Adapted from Dias et al. 2013)

NaOH, ammonia; oxidizing agents like hydrogen peroxide and peroxyacetic acid; organic acids like formic acid and acetates; and inorganic salts like $FeCl_3$ and $CaCl_2$ (Ngyen et al. 2010; Brandt et al. 2013; Zhang et al. 2012, 2013). To improve reaction efficiency one or more methods are used in combinations.

11.4 Yeasts in Bioethanol Fermentation

Saccharomyces cerevisiae, the most commonly employed ethanol producing yeast, offers distinct advantages in terms of owing to its high ethanol production from hexoses, low cost and easy availability, high tolerance to ethanol, and other inhibitory compounds and ability of fermenting wide range of sugars. Studies conducted for ethanol production by *S. cerevisiae* from different substrates at varying treatment and optimization conditions are compiled in Table 11.1.

The commonly used *Saccharomyces cerevisiae* yeast in industrial fermentation processes lack the ability to metabolize pentoses such as xylose and arabinose. These pentoses are present in large quantity in hemicelluloses, which forms a major component of plant biomass (De Souza et al. 2013, 2015). Bio-prospecting for new strains of pentose-fermenting microbes has gained prominence as a source for the development of recombinant yeast strains with improved fermentation abilities (Zhang and Geng 2012; Harner et al. 2015). Some of the new yeast species identified for fermentation of pentose from diverse sources are listed in Table 11.2. The whole genome sequencing of these newly identified strains of pentose metabolizing strains will divulge the new genes of biotechnological importance for the development of recombinant strains of *S. cerevisiae.*

11.5 Biotechnological Approaches

The second-generation biofuels are produced from the lignocellulosic material of the plants. In the current scenario, uses of sugarcane bagasse for second-generation biofuels emerged with great potential. The bottleneck in uses of lignocellulosic material is the production cost, preventing this technology from the commercialization on a large scale (Halling and Simms-Borre 2008) due to use of expensive microbial enzymes for pretreatment of the bagasse fibers to remove the recalcitrant components (Yuan et al. 2008). For accelerating higher biofuel production from sugarcane, requires a strategic shift to incorporate both first- and second-generation biofuels production. This strategic shift can be achieved by the implications of biotechnological practices such as improving the sugarcane yield, increasing the sugar content, developing the faster-growing cultivars, modified bagasse lignocellulosic fiber quality which requires less or cheap pretreatments and faster biodegradable property of these fibers (Hoang et al. 2015). Transgenic approaches to engineer any organism have the unique ability that it can be applied independent of closeness or relativeness of the source of the genes, i.e., a gene from any organism can be transferred to any organism from other kingdoms.

Table 11.1 Bioethanol production by *Saccharomyces cerevisiae* from different feedstock at varying pretreatment and optimization conditions (Mohd Azhar et al. 2017; Tesfaw and Assefa 2014)

S. cerevisiae strain	Feedstock	Pretreatment	Enzymatic hydrolysis	Ethanol produced (g/l)	References
MTCC 173	Sorghum stover	NaOH	Cellulase	68.0	Sathesh-Prabu and Murugesan (2011)
MTCC 174	Rice husk	NaOH	Crude unprocessed enzyme	14	Singh et al. (2014)
RL-11	Spent coffee grounds	H_2SO_4	Cellulase	11.7	Mussatto et al. (2012)
ATCC 26602	Wheat straw	H_2O_2	Cellulase	10	Karagoz and Ozkan (2014)
L2524a	Empty palm fruit bunch fibers	NaOH	Cellulase	64.2	Park et al. (2013)
KL17	Galactose and glucose	–	–	96.9	Kim et al. (2014)
Y5	Corn stover	Steam explosion	Cellulase and glucosidase	50	Tian et al. (2013)
ATCC 6508	Sweet potato chips		α-Amylase and glucoamylase	104.3	Shen et al. (2012)
DQ1	Corn stover	H_2SO_4	Cellulase	48	Chu et al. (2012)
CHY1011	Cassava starch	–	α-Amylase and glucoamylase	89.1	Choi et al. (2010b)
TISTR 5596	Sugarcane leaves	H_2SO_4 or $Ca(OH)_2$	Cellulase	4.71	Jutakanoke et al. (2012)
Y5	Corn stover	Steam explosion	Cellulase	40	Li et al. (2011)
TISTR 5596	Starch cassava pulp	–	α-Amylase and glucoamylase	9.9	Akaracharanya et al. (2011)
CHFY0321	Cassava starch	–	α-Amylase and glucoamylase	89.8	Choi et al. (2010a)
DQ1	Corn stover	Steam explosion	Cellulase	55	Bi et al. (2011)
Var. ellipsoideus	Corn meal	–	Heat stable α-amylase and glucoamylase	79.6	Nikolić et al. (2010)
ZU-10	Corn stover	H_2SO_4	Cellulase	41.2	Zhao and Xia (2010)

Table 11.2 Novel yeast species isolated and identified for xylose and arabinose fermentation

Yeast species	Isolation	Pentose substrate	References
Scheffersomycesshehatae and *S. stipitis*	Gut of Guatemalan passalid beetles	Xylose, arabinose	Kurtzman et al. (2011)
Meyerozyma guilliermondii	Termites (*Nasutitermes* sp.) in the Amazonian habitat	Xylose	Matos et al. (2014)
Scheffersomyces shehatae	Natural habitats in Brazilian forest	Xylose	Martiniano et al. (2013)
Sugiyamaella xylanicola, Scheffersomyces queiroziae, and *Scheffersomyces stipitis*	Rotting wood of Atlantic rainforest	Xylose	Morais et al. (2013)
Spathaspora brasiliensis, Spathaspora roraimanensis, Spathaspora suhii, Spathaspora xylofermentans	Rotting wood of the Brazil forest ecosystem	Xylose	Prompt (2012)
Spathaspora passalidarum, Scheffersomyces stipitis	Rotting wood samples of the Amazonian forest ecosystem	Xylose	Cadete et al. (2009)
Scheffersomyces insectosa, Scheffersomyces lignosus	Baotianman Nature Reserve, China	Xylose	Ren et al. (2014)
Zygoascushellenicus, *Candida blankii, Candida saraburiensis*	Agricultural residues	Xylose	Nitiyon et al. (2011)
Spathaspora passalidarum and *Candida jeffriesii*	Gut of passalid beetles in the USA	Xylose	Nguyen et al. (2006)
Candida tropicalis, *Candida parapsilosis*, *Candida mengyuniae*, *Sporopachydermia lactativora*, *Trichosporon asahii*	Rectum of Murrah buffalo and Swamp buffalo in Thailand	Xylose	Lorliam et al. (2013)

Among the several monocots which are being used for biofuel, sugarcane was extensively studied through the genetic transformations to improve its potential (Hoang et al. 2015 and the references therein). The genetic modification of sugarcane plants which have a desired ratio of cellulose to noncellulose content; transgenically expressing some of the cellulolytic or hemicellulolytic enzymes prior to which are being used for pretreatment before its conversion to ethanol; improving the pest and disease resistance by expressing disease resistant genes; improving the abiotic stress tolerance; or improving the agronomic performance by incorporating some of the regulatory genes enhancing the growth parameters (Khan et al. 2019; Hoang et al. 2015; Sticklen 2006; Yuan et al. 2008; Matsuoka et al. 2009; Arruda 2012). In line with changing the carbohydrate composition, changing the cell wall carbohydrate would facilitate in achieving the easier processing of the biomass in the form of the end products for biofuel generation (Harris and DeBolt 2010).

11.5.1 Biomass Improvement

Increasing biomass yield of sugarcane would also enhance the quantities of ethanol produced from the same area of cane cultivation. It was showed that the *ScGAI* gene regulates the growth and development of the sugarcane culm by modulating the ethylene signaling pathway (Garcia Tavares et al. 2018). They showed that silencing the *ScGAI* gene increases the internode length, bigger height, and increased carbon allocation to the stem (Garcia Tavares et al. 2018). For second-generation biofuel the sugarcane bagasse fibers composed of lignocellulosic materials are being used. The lignocellulosic biomass yield is about 22.9 tons dry weight per hectare per year and thus the total available estimated dry weight of sugarcane lignocellulosic material worldwide is approximately 600 million tons (Van der Weijde et al. 2013) and combined bioethanol yield of 9950 L per hectare can be achieved (Khan et al. 2019 and the references therein). Hence, increasing the biomass potential is another promising strategy for producing higher amounts of biofuels from sugarcane.

11.5.2 Abiotic and Biotic Stress Tolerance

Drought is one of the most devastating abiotic stresses causing severe damage to crop productivity. Similar to several other crops, scarcity of water can negatively affect the growth of the sugarcane and could result in decrease of the biomass yield by 50% (Inman-Bamber 2004). Many sugar molecules in plants serve as an osmolyte to increase the solute concentration intracellular and thus promoting the efficient water uptake during the mild drought stress. Trehalose is one of the good examples which functions as an osmolyte and has been reported to protect the cellular structure from dehydration induced damages (de Jesus Pereira et al. 2003). Developing genetically modified sugarcane which expresses the genes of trehalose biosynthetic pathway showed better growth, improved drought tolerance, and produced higher sugar content than the WT plants (Zhang et al. 2006). Similarly, overexpression of a drought responsive transcription factor cloned from *Arabidopsis At*DREB2A CA in sugarcane upregulates the expression of stress responsive genes, maintains better relative water content and photosynthetic efficiency, and performs better vegetative sprouting (Reis et al. 2014). Moreover, transgenic sugarcane overexpressing another transcription factor BcZAT12 cloned from *Brassica carinata* enhanced both salinity and drought stress tolerance (Saravanan et al. 2018). To improve the salinity stress tolerance in the sugarcane, transgenic sugarcane overexpressing *Arabidopsis* vacuolar pyrophosphatase (*AVP1*) or Δ1-pyrroline-5-carboxylate synthetase (*P5CS*) gene has been developed which showed the improved endurance against the salinity stress (Kumar et al. 2014; Guerzoni et al. 2014).

On the other side, genetically engineered sugarcane to mitigate the diseases caused by the biotic factors or fighting against the pests were also developed and tested. Transgenic sugarcane resistant to the yellow leaf virus has been developed very early as in 1997 (Khan et al. 2019; Arencibia et al. 1997, 1998, 1999). Glufosinate resistant sugarcane was developed by expressing the phosphinothricin

acetyltransferase (*bar*) gene and by spraying the glufosinate, the weeds are selectively killed without having negative effect on the transgenic sugarcane (Manickavasagam et al. 2004). To fight against several pest and insects, Monsanto has already developed the transgenic sugarcane using the Bt technology and it is being used commercially (Maldonado et al. 2010).

As above discussed, approaches are useful for the improvement of the yield potential for both first- and second-generation biofuel from the sugarcane, in the following sections we would emphasize the specific genetic engineering approaches used for either first- or second-generation biofuels.

11.5.3 Increasing Cellulose Content

Obviously, it is clear that modifying the cell wall composition of the sugarcane by increasing the cellulose and hemicellulose content will increase the fermentable sugars produced from the same amount of the materials. Transgenic sugarcane plants expressing the cellulose synthase gene *CsCesA* from a marine invertebrate *Ciona savignyi* increased the cellulose synthase activity and also the cellulose content in the transgenic plants (Ndimande 2014). Additionally, the hemicellulosic glucose content and the uronic acid content of the transgenic sugarcane have also been increased with the decline of lignin content (Ndimande 2014).

11.5.4 Enhanced Sucrose Accumulation

Sugar is the first product of photosynthesis which is further modified in different structural, nutritional, protective, or storage metabolites in the plants. Enhancing the sugar synthesis either by increasing the photosynthesis efficiency or by manipulating the sugar synthesis or sugar degradation pathway has not been successful so far. Because an increase in any of these components sends feedback signals to the photosynthesis and thus the photosynthesis is inhibited. To overcome the feedback inhibition of sugar synthesis, the pathway has been modified, where the natural sugar product of photosynthesis is modified in a different form of sugar. The modified form does not send any feedback signal and is relatively more stable. These modified sugars were designed in such a way that it can be used for food as well as for the biofuel sector. Isomaltulose (IM) is a stable sugar which shows slower digestion property than the sucrose and non-hygroscopic (Khan et al. 2019; Lina et al. 2002). Expression of bacterial sucrose isomerase (*SI*) in vacuole of sugarcane accumulated the IM in the vacuole without affecting the cellular sucrose concentration and thus doubled the total sugar concentration of the sugarcane juice (Wu and Birch 2007). Interestingly, the transgenic lines also showed increased photosynthesis, sucrose transport, and increased sink strength (Wu and Birch 2007). Targeted expression of the *Saccharomyces cerevisiae* invertase gene (*SUC2*), which has been expressed in the apoplast of the sugarcane callus/liquid culture cells, showed the rapid conversion of sucrose to hexose and increased hexose concentration in the medium (Ma et al.

2000). Alternatively, several other strategies like improving the photosynthetic capacity by expressing cyanobacterial genes, metabolic engineering for modifying the photorespiratory pathways, Calvin–Benson cycle, or modifying the sugar forms in the sink tissue will increase the photosynthetic efficiency of the sugarcane and would also result in higher sugar yield (Lin et al. 2014; Shih et al. 2016).

Second-generation biofuel generation was adopted to avoid the competition between the crops for feeding the growing population or for the fuel. The second-generation biofuel is being produced from the lignocellulosic biomass of several grasses with a higher growth rate and rich potential of yield and can be grown in the marginalized lands. Traditionally, the lignocellulosic fiber of the sugarcane bagasse obtained after extracting the juice is being used in the fertilizer industries or in sugar mills for producing heat, steam, and electricity (Pandey et al. 2000). Including the sugarcane lignocellulosic materials along with the sugar for bioethanol production would make the breakthrough by enhancing the total yield of bioethanol of 9950 L per hectare (Hoang et al. 2015; Somerville et al. 2010). Producing ethanol from the bagasse lignocellulosic material is not as convenient and cost-effective as from the sugar derived from the sugarcane. Enzymatic degradation of lignocellulosic biomass to fermentable sugar requires several enzymes in huge quantities. For example, 15–25 kg cellulase is required for the processing of a ton of biomass (Carroll and Somerville 2009; Fan and Yuan 2010). These degrading enzymes are derived from microbial sources and thus the requirement of these huge quantities of enzymes making the whole process expensive. The presence of recalcitrant material in the cell wall arises additional bottleneck preventing the enzymatic access to the cellulose or hemicellulose for their degradation. A new approach adopted to tackle these issues was to express these enzymes required for pretreatment of the lignocellulosic materials stably in the leaf of the sugarcane or metabolic engineering of the cell wall content to reduce the recalcitrant material. Transgenic sugarcane lines with reduced lignin content, higher cellulose to noncellulose ratio, and expressing the lignocellulosic processing enzymes inplanta has been successfully reported (Khan et al. 2019 and the references therein).

11.5.5 Modifying the Cell Wall Content of the Sugarcane

Removal of recalcitrant compounds in the bagasse lignocellulosic fibers is required before they can be used for bioethanol production. Sugarcane bagasse constitutes of cellulose, hemicellulose, and lignin at the ratio of 50, 25, and 25% of dry weight, respectively (Khan et al. 2019; Hoang et al. 2015; Loureiro et al. 2011; Mutwil et al. 2008; Pauly et al. 2013). Lignin of the cell wall is one of the large barriers which prevent the access of the cellulase to the cell wall. The biosynthetic pathway of the lignin is complex which involves 10 enzymes (Whetten and Sederoff 1995), and monolignol, the starting material for the lignin biosynthesis pathway whose biosynthesis in plants is linked with 28 unigenes (Bottcher et al. 2013). A wise strategy can be applied to suppress these genes or a candidate gene regulating these pathways to reduce the lignin content in the sugarcane bagasse. It is important to be noted that the

lignocellulosic fibers serve as the skeleton of the sugarcane (Khan et al. 2019) and precaution must be taken that the modification of the lignin content should not affect the plant growth and development. Some examples of modifying the lignin biosynthesis pathway for the purpose to reduce recalcitrance of lignocellulosic fibers come from the studies where enzyme like caffeic acid O-methyltransferase (COMT) expression of the lignin biosynthesis and cinnamyl alcohol dehydrogenase (CAD) enzyme expression of the monolignol biosynthesis were suppressed (Jung et al. 2012; Sticklen, 2006). In these studies, it was found that the growth and development of the plants were not affected in the controlled growth conditions, while the reduction of the lignin content resulted in a significant increase in the fermentable sugar content without any pretreatment (Khan et al. 2019; Jung et al. 2012; Sticklen 2006). Field trial study of these COMT-suppressed transgenic lines in the USA revealed that the lignin content of the transgenic was reduced by 12% as compared to the WT plants and reduction of lignin content has reduced the hydrolysis time by one-third and enzyme consumption decreased by 3- to 4-fold (Khan et al. 2019; Jung et al. 2012). Using the similar strategy to engineer another biofuel grass, switchgrass has shown better efficiency of cellulase treatment and increased production of glucose and bioethanol (Fu et al. 2011; Saathoff et al. 2011).

Alternative to reducing the lignin content of the cell wall, approach where changing the composition of the lignin polymer composition can also be employed. It has been reported that the lignin in angiosperm is composed of guaiacyl, syringyl, and p-hydroxyphenyl units derived from the monolignols (Vanholme et al. 2010), where syringyl units are better-degrading type than that of recalcitrant guaiacyl-rich lignin (Papes et al. 2015). Changing the syringyl and guaiacyl levels by manipulating the gene expression has a minor effect on the plant development (Vanholme et al. 2010) and the genetically modified sugarcane having altered cell wall lignin composition can be easily processed, adding advantage in terms of cost-effectiveness of the second-generation ethanol production (Maldonado et al. 2010).

11.5.6 *In-Planta* Processing

The idea of expressing cellulolytic and hemicellulolytic enzymes in sugarcane using genetic engineering is to degrade or digest the cell wall cellulose and hemicellulose within the sugarcane plants after harvesting, so that the highly cost consuming pretreatment process can be mitigated. Maize *PepC* promoter-controlled expression of the cellulolytic fungal cellobiohydrolase I (CBH I), CBH II, and bacterial endoglucanase (EG) shows stable expression in different cellular compartment of the leaf in transgenic sugarcane (Harrison et al. 2011). It was shown that the accumulation of exo- or endoglucanase in the transgenic plants had no any negative impact on the growth of the transgenic sugarcane plants (Harrison et al. 2011). But this strategy also comes with the challenges and the detailed knowledge to overcome these challenges are still limited. To achieve the full purpose of this strategy, extensive knowledge of several inducible promoters are required, so that these enzymes are expressed only after harvesting of the biomass. Use of constitutive

promoter was limited due to occurrences of transgene silencing in the sugarcane caused by its complex genome structure (Harrison et al. 2011). Expression of these enzymes at the early developmental or growth stages could also be devastating and may negatively impact the growth and development of the transgenic sugarcane plants (Dale 2007; Harris and DeBolt 2010; Maldonado et al. 2010).

11.6 Genetic Engineering of Sugarcane for Biodiesel

The lipid in plants is stored in the form of triacylglycerols (TAGs) which have the relatively higher energy content than that of the carbohydrates (Durrett et al. 2008). The TAGs are converted to biodiesel by modifying the acyl chains of TAGs to fatty acid methyl esters (Ohlrogge and Chapman 2011). Oil-seed crops tend to have relatively higher content of the TAGs but the use of oil seeds or fruits for the biodiesel product negatively impacts the food produced from those crops and thus focus has been diverted towards use of the vegetative biomass of the crops without affecting the food productivity (Chapman et al. 2013). Being a C4 grass, sugarcane has efficient photosynthetic capability and extensive production of the vegetative biomass drew attention of the scientific communities to explore the possibility of biodiesel production from the sugarcane. Genetic engineering approaches are focused to upregulate the lipid biosynthesis pathway in the sugarcane by rerouting the carbon flux (Vanhercke et al. 2014; Zale et al. 2016). TAGs accumulation up to 19% dry weight of the total biomass production in the tobacco has been achieved by expressing three genes, namely WRINKLED1, DGAT, and Oleosins (Vanhercke et al. 2014; Zale et al. 2016). Similar strategy was adopted in sugarcane which resulted in accumulation of 5% TAGs and 10% total fatty acids (Huang et al. 2015; Zale et al. 2016). As most of the biomass in sugarcane is contributed by the stem, the metabolic engineering using the stem-specific promoters could have large impact on the TAGs production in sugarcane (Khan et al. 2019 and the references therein). It will be an additional breakthrough in the biofuel industry if the metabolic engineering for TAGs synthesis in sugarcane would be successful which has a great potential for biodiesel production due to its huge biomass production rate.

Disadvantages of Sugarcane-Based Biofuel Production The main drawback that questions the sustainability of sugarcane-based biofuel production is the competition between the land usage for food production and biofuel production. The possibility of horizontal land expansion is not possible. This would lead to deforestation and loss of soil diversity. The forest is a great carbon sink, so loss of forest would lead to global warming. Sugarcane also requires substantial inputs of fertilizers and water that lead to eutrophication. The use of pesticides and machine leads to soil pollution and erosion. The other disadvantages are the GHG emissions from agricultural inputs and farming operations. Therefore, the alternatives to sugarcane-based biofuel which would be more sustainable like third and fourth generation biofuel should be discussed.

Alternatives to Sugarcane-Based Biofuel: 3rd- and 4th- Generation Recently, the idea of algal biomass-based biofuels also called third-generation biofuel is getting more acceptances. The algae have higher energy conversion efficiency and surface area-to-volume ratio as compared to sugarcane. Hence the amount of lipid is more in the algae, and biofuels from algae usually relies on the lipid content of the microorganisms, for example, Chlorella has high lipid content (around 60 to 70%; Liang et al. 2009) and high productivity (7.4 g/L/d for *Chlorella protothecoides*; Chen et al. 2011). However there are geographical and technical challenges associated with algal biomass production. First, algae production requires a large amount of water with specific nutrient and temperature condition. Second, the harvesting of algae, removal of water from them, and lipid extraction need technical skills. The idea of using 3rd generation biofuels is setback by the cold countries and countries lacking enough fresh water. At present, extensive research to improve both the metabolic production and separation of fuels from non-fuels is underway.

To meet up such challenges and in order to develop biofuel that can be used universally, the use of nonarable lands and solar energy towards the sustainable development of biofuels is proposed. Such biofuels are also called fourth-generation biofuels and can effectively reduce greenhouse gas emissions and mitigate climate change. They include photobiological solar fuels and electrofuels. It is also based on redesigning the genome of algae and cyanobacteria in such a way that their energy conversion efficiency increases (also called photon-to-fuel conversion efficiency (PFCE)) (Berla et al. 2013; Hays and Ducat 2015; Scaife et al. 2015). Photosynthetic microorganism can be used as biocatalyst for the production of hydrogen by photosynthetic water splitting (water oxidation). This can become a large contributor to fuel production on a global scale, both by artificial photosynthesis (Inganäs and Sundström 2016) and by direct solar biofuel production technologies. However, the production of photobiological fuel and electrofuel requires synthetic biology approach which is still in its beginning stage and requires a lot of optimization.

11.7 Conclusions

Sugarcane is characterized by narrow genetic base with a complex genome and low levels of fertility. To realize the full potential of sugarcane as a bioenergy crop, more efforts need to be directed towards improvements in biomass addition coupled with sucrose accumulation, imparting tolerance to biotic and abiotic stresses. The emerging biotechnological tools of genetic transformation primarily through *Agrobacterium*-mediated genetic transformation are likely to emerge as major force to supplement the classical breeding approaches towards sugarcane crop improvement which is hampered by laborious and long development period. With the availability of whole genome sequence information of sugarcane coupled with ever evolving bioinformatics tools, the enigmatic goal of achieving the plant type with most desirable traits will be within reach. Recent technique of genome editing

and successes in the other crops offers new scope and dimension to sugarcane crop improvement.

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