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

Manish Srivastava
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Bioenergy Research: Basic and Advanced Concepts

Springer

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

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Foreword

Green energy production via sustainable route is the most appropriate way to reduce harmfully impact of fossil fuel-based pollutant. Additionally, it will also minimize the risk of fossil fuel limitations. To apply green fuels for day-to-day life commercially, there are number of issues which need to be fixed permanently for long-term viability of these fuels on commercial scale. Though green energy production area is not new, still several roll backs are stick which hinders their large-scale commercial production. In green fuel category, numbers of bioenergy options are available, and vast research has been performed; there is urgent need to focus on the up-to-date research outputs in order to evaluate whether the work is on the front of "Lab to Land" or still we are under bottles, which need to be addressed immediately for sustainable bioenergy life adaptation.

Publication of the book entitled *Bioenergy Research: Basic and Advanced Concepts* is one of the important effort by editors of the book in the series of improving bioenergy production technologies.

I am glad to write this message and congratulate editors of the book for their restless efforts as this book proves boon for the people engage in the area. The book holds 10 striking detailed chapters which showed their depth discussion toward the recent developments in the area of bioenergy research. Apart from present development review, the book also presents sustainable solution to overcome the existing roll back in the proposed area. The book covers recent insight in the research of various existing potential bioenergy options from their basic to future prospects only in terms of improving this option at commercial scale. The book will be definitely an asset for the people involved in academic, research, and industries.

I appreciate the efforts of *Dr. Manish Srivastava*, *Dr. Neha Srivastava*, and *Dr. Rajeev Singh* for bringing out the book entitled *Bioenergy Research: Basic and Advanced Concepts*.

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Contents

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Chapter 1
Downstream Processing of Biofuels

Arpit Shrivastava, Abhishek Dutt Tripathi, Aparna Agarwal, and Veena Paul

Abstract The rapid reduction in the availability of petroleum and the impact these related fuels have on the atmosphere have contributed to a growing use of biofuels. Biorefineries promise to transform biomass accurately into biofuels and bioproducts in the expedition for sustainable utilization of energy which is renewable. Separation and purification stage are also termed as downstream processing which is indeed quite critical for manufacturing high-quality biofuel. Separation and purification methods can also be categorized as equilibrium, affinity-dependent, membrane, solid-liquid, and reaction-driven separation processes depending on the complexity of their operation. This chapter discusses various ancient tactics, recent advances while linking and conflicting the diverse methods of downstream processing of first, second, and third generation of biofuels.

Keywords Biofuels · Downstream processing · In-stream recovery · Hydrodynamic fluid techniques · Harvesting methods · Cell disruption techniques

1.1 Introduction

1.1.1 Biofuels and Their Importance

As stated by the International Energy Agency (OECD 2011), overall global demand for electricity has increased by more than 78% over the past three eras. The consumption of fossil fuels is causing significant environmental issues globally, and there has been considerable focus on eliminating alternative green sources for their use. In general, owing to the global warming effect triggered by greater than

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Table 1.1 Physical and chemical properties of biofuels (Michael et al. 2011)

Fuel	Gasoline	Ethanol	Butanol	Diesel	Biodiesel
Energy density [MJ/kg]	45.0–47.0	28.0–30.0	36	45	40.0
Mileage [%]	100	61–66	83–91	100	90–100
Air-fuel ratio	14.6	9.0	11.2	15.0	13.8
Research octane number (RON)	91–95	129	96	–	–
Motor octane number (MON)	81–89	102	78	–	–
Cetane number (CN)	–	–	–	50–60	45–70
Vapor pressure [hPa]	35–90 (at 20 °C)	58 (at 20 °C)	6.7 (at 20 °C)	–	–
Flashpoint [°C]	< -20	12	35–37	55–60	100–190
Enthalpy of vaporization [MJ/kg]	0.36	0.92	0.43	–	–
Kinematic viscosity [mm^2/s]	0.4–0.8 (at 20 °C)	1.5 (at 20 °C)	3.6 (at 20 °C)	1.2–3.5 (at 40 °C)	2–9.5 (at 40 °C)

before use of fossil fuels in combination with restricted fossil fuel supplies and inconsistent prices caused by unpredictable political uncertainty, significant attention has recently been paid to alternative renewable energy sources (Sarkar and Shimizu 2015). As substitutes for transportation fuels derived from petroleum, alternative options are attracting growing worldwide prominence to help address oil costs, climate efficiency, and global warming issues related to fossil fuels. Often the word biofuel is used to describe any fluid fuel derived from plant resource that could be used as a replacement for fuel derived from petroleum. Biofuels may include pretty common fuels, such as ethanol from sugarcane or soya oil which is diesel-like, to less acquainted fuels some of them are dimethyl ether (DME) or Fischer-Tropsch liquids (FTL) produced from biomass of lignocellulosic origin (Callegari et al. 2020; Faiz et al. 1996). Biomass provides a variety of biofuels, such as butanol, ethanol, biodiesel, methanol, renewable diesel, methane, and syngas (Bharathiraja et al. 2017; Oluyede and Phillips 2007). FAO defines biofuels as "Fuel [s] produced directly or indirectly from biomass" while *Biomass*: "Biologically derived material except material found in geological formations and are transformed to fossil (FAO 2004)." Various physical and chemical properties of biofuels in comparison to traditional fuels are shown in Table 1.1.

1.1.2 History of Biofuels

Biofuels use is new innovation, fueling by plant oils or ethanol was common after the combustion engine was invented. Oil lamps comprising of vegetable and animal oil have been in use since evolution started (Michael et al. 2011). By this time the first US patent proposed for alcohol as a lamp fuel was given to S. Casey in 1834

(Kovarik 1998). About 1850 million of distilleries yield approximately 24 million liters (90 million gallons) of "Camphene" (a combination of turpentine and alcohol fragranced by camphor oil) each year (Kovarik 1998). Also, in automotive industry, biofuels have been used since its early days. Even with biofuels was performed the development of the leading combustion engine, the "Otto cycle" interestingly, the initial support for Otto hailed from Eugen Langen, who operated a sugar refining business with bonds to Europe's alcohol markets (Kovarik 1998). In addition, Henry Ford's first automotive concept, the "Quadricycle," could be powered with ethanol as fuel in the 1880s, and his "Model T," the "Tin Lizzie," the most successful car built between 1908 and 1927, was initially intended to run on pure ethanol (Michael et al. 2011). In reality, Dr. Rudolf Diesel established the diesel engine to function on a variety of fuels like water-suspended coal dust, heavy mineral oil, and vegetable oil. The first tests on Dr. Diesel's engine were disastrous failures. However, his engine was functioning on 100 percent peanut oil while he exhibited this engine at the 1900 World Exhibition in Paris. Dr. Diesel was vocal in 1911 he stated "The diesel engine can be fed with vegetable oils, which will contribute significantly to the growth of the countries that use it." In 1912, Diesel told that, "Today, the use of vegetable oils for motor fuels appears somewhat unrelated." Though such oils will become as vital as petroleum and the current coal tar products in the course of time. No doubt this assertion has come to stay. Since Dr. Diesel died prematurely in 1913, his engine has been updated to run on the infesting petroleum energy we currently known as "diesel" (Agarwal 2007). His theories on agriculture and his innovation nevertheless formed the basis for a community powered by safe, sustainable, locally produced coal. Countries all over the place now use this form of fuel again thanks to its renewable energy and lack of emissions.

1.1.3 Different Generations of Biofuels

First-century claimed biofuels were produced by corn starch and sugarcane. This also grounds the problem of the so-called "food and energy challenges" as the production scale grows (Misra 2014). Leguminous plant as a bioenergy belonging to P. J. H. Hurter and Mabb. family (widely source Leguminosae) such as *Vachellia nilotica* (L.) well-known by the taxonomic synonym *Acacia nilotica* (Lam.) Wild.) (babool), *Dalbergia sissoo* Roxb., *Peltophorum pterocarpum* (DC.) K. Heyne (yellow flame (Jacq.) R. Br. ex G. Don (locust bean), *Delonixregia* (Boj. ex Hook.) Raf., etc. can be castoff as a basis of carbohydrate. Hulls of all these seed-containing plants can be used as a carbohydrate base, and could also be used as a substratum in the fermentation practice. These plants' pods release large amounts of reduced sugars after enzymatic treatment. Nilotica (synonym *A. nilotica*) pods when treated with 4% amylase reducing sugar content was heightened whereas *P. pterocarpum* at 4% amylase enzyme displays the lowermost yield of reducing sugar (Gulalkayi et al. 2012), Indian rosewood tree (*Perkia biglobosa*) (Rasool and Hemalatha 2016).

Additionally, the manufacturing process of second-generation biofuels from lignocellulosic biomass is currently under consideration. On the other hand, it involves energy-intensive degradation of biomass comprising lignocellulosic by pretreatment (Kumar and Sharma 2017). Biofuels of the second generation may be described as biochemical or thermochemical systems used to turn the biomass into a liquid. Second-generation ethanol or butanol can entirely be processed by means of biochemical technology (Devi et al. 2019). Second-generation thermochemical renewable energy sources may remain extra prevalent to readers, but there are multiple fuels now generated extensively from fossil fuels utilizing production processes who are in some contexts extremely similar to what is used for bioenergy production. These fuels comprise fluids from Fischer-Tropsch (FTL), methanol, and dimethyl ether (DME). Worldwide, several projects are underway to commercialize biofuels of the second generation. And in case of biochemical fuels, there is a need for breakthroughs in microorganism science and engineering designed to handle different feedstocks, accompanied by large-scale demonstrations to prove economic viability. It may take some 10–20 years before commercial production starts radically. And in the true meaning of thermochemical fuels, various equipment components required for biofuel processing are now commercially targeted at fossil fuel conversion applications, and the method is very oblivious to the particular feedstock input, needing fewer creation and demonstration efforts. The commercial production of thermochemical biofuels can commence in 5–10 years.

While some consideration has been given to the third generation of renewable energy of photosynthetic organisms such as cyanobacteria and algae, the rate of cell transformation is very small and thus the metabolite productivity is relatively lower (Sheehan 2009). Microalgae are monocellular or basic multicellular organisms and can be prokaryotic or eukaryotic by nature. Microalgae by nature poses the ability to prosper in fresh or salt waters. Due to the standard cellular structure of the microalgae they can skillfully convert organisms to our solar power. Planet microalgae is believed to be among the earliest living life forms on earth. There is a huge microalgae variant, and some 300,000 microalgae species. Among these different ranges of microalgae, almost species ensure about 80% oil content. Microalgae have the potential to be used as biodiesel harvest.

Even though the transport sector pays much concern to biofuels, the use of biofuels for cooking is a feasible use of broad global significance, especially in developing countries' rural areas. For all cases, the combustion of cooking biofuels will create emissions of contaminants that are lower (or much lower) than the emissions from solid fuel cooking. Around 3 billion people in developed countries cook with solid fuels and experience major damage to their health from the resulting indoor air pollution. Biofuels may thus conceivably be influential in illuminating the health of billions of individuals. It is worth remarking that the scale of biofuel production required to meet the cooking energy needs is much lesser than that essential to meet the transport fuel requirements (Chen et al. 2015).

1.1.4 Biofuel Development Across the Globe

Internationally, the execution of a biofuels markets soon flickered a food contrasted with fuel argument among critics. Whereas the supporters see biofuels as part of a potential renewable and clean oil, some protested that fuel would not come from edible plants. However, the demand for biofuels remained steady. The world market for ethanol and biodiesel production and wholesale prices was sized at over USD 136 billion in 2016. The demand is projected to rise to nearly US $154 billion by 2024. India has a rising ethanol market which is further mature than the biodiesel industry. Ethanol Blended Petrol (EBP) program was launched in India in 2003, which approved 5% blending of ethanol with gasoline. In India biodiesel production is focused primarily on non-edible sources, since there are less amounts of edible sources. Such origins are not edible: jatropha, mahua, karanja, neem, etc. Global energy demand is projected to increase by 28% by 2040 compared to 2015, according to projections. Fig. 1.1 shows the current global scenario of biofuel production.

To encounter the ever-rising plea for electricity, the rising environmental need is to draw on cleaner, reliable, sustainable energy sources. Renewable energy is projected to double between 2015 and 2030, making it the fastest-growing energy source in the world.

Demand for biodiesel will increase more rapidly, mainly in the Asia/Pacific, Central and South America and the Africa/Mideast region (Gashaw and Lakachew 2014). The growing demand for biodiesel in developing countries will in many cases reflect setting consumption mandates designed to reduce necessity on imported petroleum products and offer supplementary support for the local production of

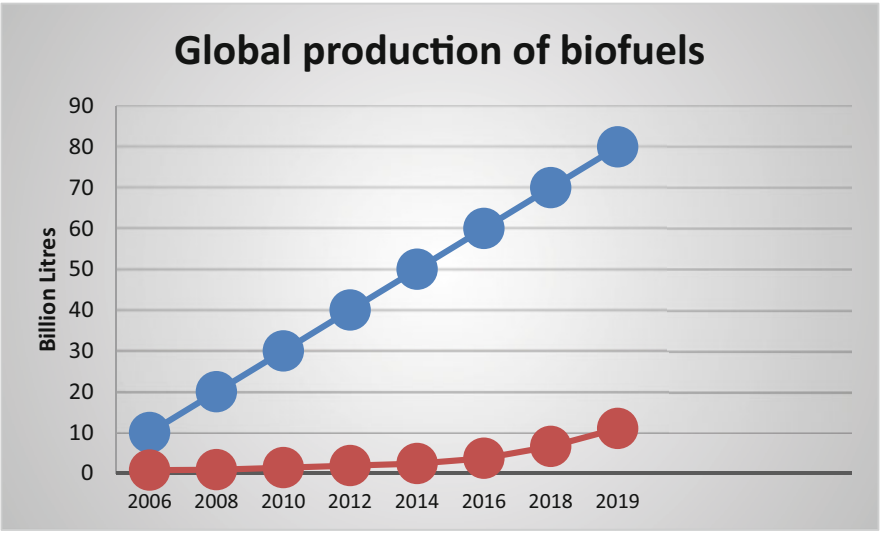

Fig. 1.1 Global production of biodiesel and ethanol

biodiesel and biodiesel crops. In terms of volume, Western Europe will continue to boost demand for biodiesel, backed by efforts by EU member states to meet the EU's target of 10% renewable transport fuel by 2020. Nonetheless, the rate of growth in biodiesel demand in the following year, the EU will slow down because EU diesel fuel standards limit the amount of biodiesel that can be blended without the need for a new fuel mark. North American demand for biodiesel will decline from an especially strong base year through 2018, but will endure to raise later (Sarkar and Shimizu 2015; Rezania et al. 2019).

1.1.5 Specifications for Biofuels

Biofuels must meet clear physical and chemical specifications for use in present ignition engines and for the use of current distribution networks. Next, specifying accumulation is of particular prominence (Muller and Young 2013; Dahman et al. 2019). The transport biofuels ought to be fluid at ground temperature and atmospheric pressure for gasoline, biodiesel, and sustainable diesel. Gaseous biofuels, such as hydrogen and methane (biogas), will require modern system technology and upgraded engines. Biofuels must moreover have the same features that petrochemical fuels would have (Muller and Young 2013).

1.2 Production of Bioethanol

Colorless liquid ethanol (ethyl alcohol [CH3CH2OH]) that has a molecular weight of 46.7 g/mol is soluble in water, acetone, ammonia, and other organic solvents (Baeyens et al. 2015). Ethanol can be produced by fermenting sugar, also known as bioethanol, both chemically from petrochemical sources through ethylene hydration and naturally through plant biomass. Bioethanol's most important benefits are that biomass can be reprocessed, and can possibly provide long-term viable fuel supply (Amelio et al. 2016; Ibrahim 2013). Bioethanol currently denotes one of the most prevalent petroleum-based fuel alternatives. Oil is well-thought-out more ecologically pleasant than petroleum-based fuel and can save the planet from pollution (Ibrahim 2013). The processing cycle of bioethanol depends on the feedstock; it depends on technologies ranging from the minor conversion of sugar by fermentation to the multi-stage transformation of lignocellulosic biomass into ethanol. Every industrial fermentation can be divided into three main phases, i.e., upstream followed by fermentation and downstream processing which is shown in Fig. 1.2.

Fig. 1.2 Schematic diagram of fermentation process

1.2.1 Downstream Processing of Biofuels

The isolation of ethanol from a mixture of water and ethanol is problematic as of the presence of an azeotrope in the mixture. The two old-style methods of separation of high-purity ethanol are: extractive distillation and azeotropic distillation; other three developing methods are: distillation through salt, distillation of pressure swing, and lastly pervaporation. In the distillation stage, ethanol will be isolated and clears the head of the column from the other constituents as an azeotropic blend. This is an extremely energy-intensive processing phase and involves using internal heat smartly. The ethanol-water mixture is subsequently dehydrated to 99.5 wt. % which is one of the main aims by pressure swing adsorption (PSA). The residue collected at the bottom of the distillation column is considered a stillage. This is a mixture of soil, lignin, and other organic materials which are not used during the SSF cycle. This stillage then needs to undergo a division of solid-liquids, and then it is separated into solids that are insoluble in nature and a portion of the liquid. After a drying stage at the cogeneration plant, the solids, which mainly contain lignin, are used for process steam and power generation (Lassmann et al. 2014). The key downside is the amount of tremendous energy required for providing heat to vaporize liquid and condense the vapor back into liquid next to the condenser (Plessas et al. 2007).

Currently, integrated fermentation/separation coupling systems had already gained extensive attention; it has been shown to be used adequately to recover bioethanol from the feed stream as it is formed and to restrict end-product inhibition to boost overall performance levels. Various separation methods have been developed in this context to be integrated with the fermentation process such as pervaporation, adsorption, gas stripping, vacuum fermentation, and solvent extraction.

1.2.1.1 Pervaporation

Among the numerous membrane approaches, the most effective separation technology commonly used to isolate azeotropic mixtures, mixtures of organic into organic, solutions, and recover dissolved organics from aqueous solutions is pervaporation (Zentou et al. 2019). It is quick, extremely selective and low toxicity to fermenting microorganisms in contrast to conventional techniques employed (Wei et al. 2014). Other membranes made of cellulose acetate and polydimethylphenyleneoxide had selective water permeation, whereas the prospect of removing alcohol from aqueous solution by PV using silicone membranes was demonstrated by Kimura and Nomura

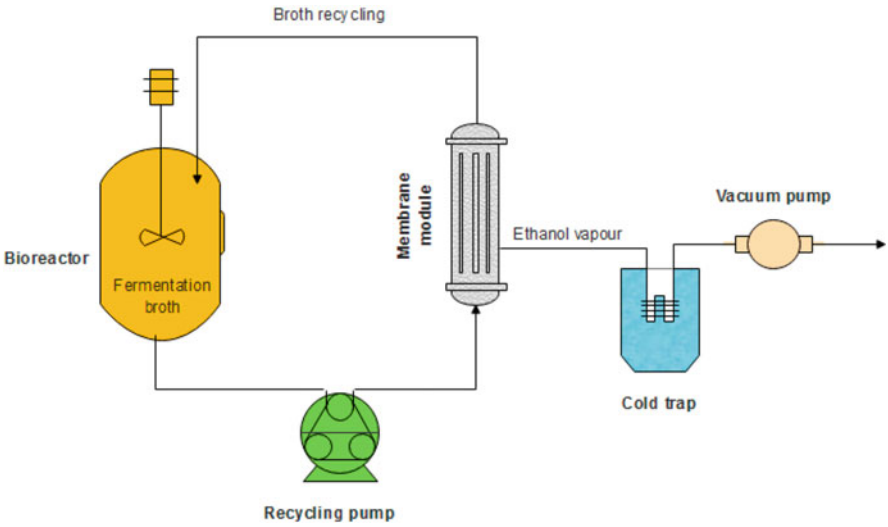

Fig. 1.3 Diagram for fermentation process coupled with pervaporation separation unit

(1982). Pervaporation is a liquid mixture membrane separation process in which the initial solution comes into contact with the inner surface of a membrane cell and permeates the outer surface in the form of low partial pressure vapors. Therefore, pervaporation is sometimes referred to as an extractive distillation process whereby the membrane has the role of part third. However, to separate liquid mixtures in the pervaporation phase process is not based on the vapor and liquid system equilibrium as in the distillation process, but variations in the solubility coefficient and mixture components diffusivity matter the most. Here, the equilibrium of the vapor-liquid system directly influences the driving force of the cycle and, ultimately, the characteristics of the separation (Belyaev et al. 2003). Mostly, the pervaporation system includes a feed tank, heater, feed pump, vacuum pump, a membrane module, and cold trap condenser, as schematized in Fig. 1.3.

In the case of alcohol elimination from the atmosphere, the use of a hydrophobic alcohol-selective membrane would result in a permeate enriched with alcohol. In addition, water and fermentation broths have been studied extensively again for regeneration of ethanol and butanol by pervaporation (Vane 2005). Summary of some applied pervaporation techniques and its specifications is shown in Table 1.2.

1.2.1.2 Gas Stripping

Fermentation broth is made alcohol-free by moving the alcohol into a gas stream which is theoretically pleasing due to its virtual easiness, the choice of using the carbon dioxide discharged during the gas stripping process is less energy-intensive and is extensively used, but has very poor solvent selectivity. Butanol removal takes

Table 1.2 A summary of some applied pervaporation techniques and its specifications

Membrane materials	Feedstocks	Operating conditions	Separation factor	Total flux	Reference
IPN membranes	Ethanol-water mixture	30 °C mm hg, 30 wt. %, NA	2.5	0.1 to 1.0 tor	Lee and Kim (1988).
Polyetherimide	Acetic acid and ethanol along with ethyl acetate and water mixtures	Temperature was varied and 2.67 × 10–3 bar pressure was fixed at permeate side	7–248	2.67 × 10–3 bar (i.e., 2.0 torr)	Park (2004)
PDMS	Banana waste	Temperature was 25 °C, at 19.5 mm hg pressure, 3.8 wt. %, 19.8 L/h was the feed rate	10.36	0.01	Bello et al. (2014)

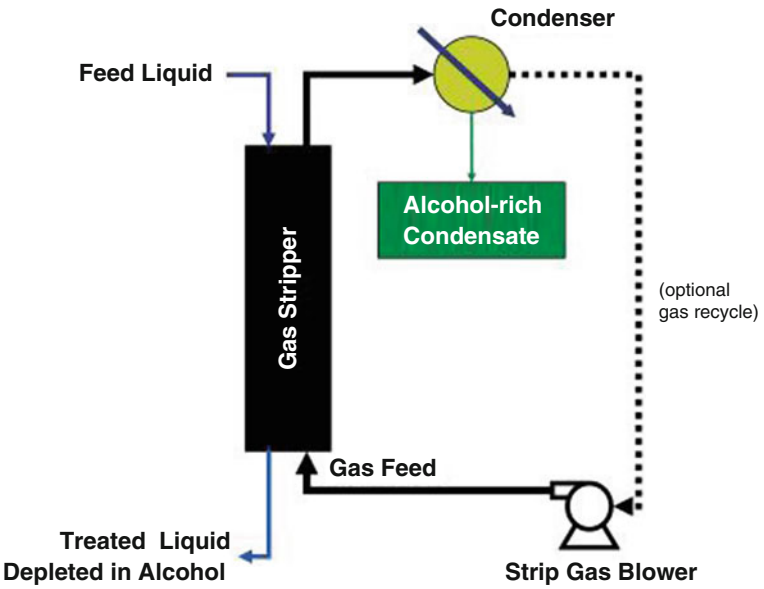

Fig. 1.4 Schematic model of a gas-stripping cycle using condensation to extract alcohol

place with other solvents and huge amounts of water, it is therefore important that the liquid stream is condensed either by distillation or LLE before separating and purifying individual solvents which should be purified by distillation. A typical gas stripping system is shown in Fig. 1.4. The energy requirement for gas removal decreases with increased concentration of solvents and vacuum application increases butanol selectivity. The working standard is generally that of a gas-liquid counter-current contactor. In the gas phase, at equilibrium, the ratio of alcohol to water is determined by a gas-liquid partitioning activity comparable to the distillation

process. The alcohol versus inert gas ratio is a good stripping temperature feature (alcohol or water) as a volatile compound has partial pressure in the gaseous phase (Vane 2008).

1.2.1.3 Distillation

The elementary distillation component is a central module for the distillation of composite separation of many components. The vapor increasing due to the boiling of the fluid in the still is essentially richer in more volatile component than the residual liquid in this phase (Kraemer et al. 2011). The vapor composition that leaves the liquid phase is thermodynamically balanced with the liquid phase (Nakao et al. 1987). Frequently, it is used in the processing of bioethanol production at the biorefinery. This supplements noteworthy energy charge to the production cycle. The estimation showed that ethanol distillation consumes energy that is equal to half or more of production energy in the entire process, and the heat recovery system comprises diverse heat exchangers. The easiest concept involves only one heat exchanger that further heats the wash up to the boiling point, such that a splitting spine can segregate the methanol and water. However, current distillation concentrates ethanol with salt, which is also known as "hydrous" or "hydrated" ethanol, up to its azeotropic level (about 95% ethanol). Continuous azeotropic ethanol dehydration produces "anhydrous" alcohol (99.6% ethanol). Furthermore, the distillation yields a lasting flow identified as vinasse that can be assessed as an additional product (Bateni et al. 2017). Since distillation is among the most energy-consuming stages in ethanol production, the studies have proposed numerous energy-saving techniques, such as heat-integrated, membrane-based, feed-splitting, and ohmic-assisted distillation methods, to overcome this concern. Some recent distillation techniques are discussed in Table 1.3 (Gavahian et al. 2019).

Heat-Integrated Distillation

Haselden (1958) first implemented the HIDiC method for gas separation processes. The main emphasis of heat-integrated distillation is to maximize the use of energy that was originally applied to the distillation device. The configuration of the system used to achieve improved distillation that saves energy varies conferring to approach. This technique has been reported to be able to reduce the energy consumption of biofuel production by up to 40%. Recently, i-HIDiC has been shown to be energy-efficient than the general HIDiC which comprises individually the reboiler and condenser as well as the internal heat integration arrangement. However, when the feed rate has increased beyond the fixed amount, the ideal HIDiC is not economical. But in such a case, the HIDiC configuration is ideally further used to accomplish a suitable heat balance, which is to say, to operate the column with no reboiler and a condenser, the supply combination must be preheated before being introduced into the optimized heat-integrated distillation column. This preheating

Table 1.3 A description of the energy-saving approaches recently suggested to bioethanol distillation

Technique	Key findings	Reference
Heat-integrated distillation	Distillation is one of the most energy-intensive stages in the process of ethanol, the studies have suggested various energy-saving techniques, including heat-integrated, membrane-based. Energy requirements were identical for operations with four separation processes and three distillation columns (between 7.7 and 11.7 MJ fuel/kg)—acetone, butanol, and ethanol (ABE). The most economical process was the double-effect system (DED) with four columns (0.12–0.16 $/kg-ABE)	Ponce et al. (2015), (Diaz and Tost 2018)
Membrane-based technologies	Fermentative biofuel (bioethanol) production in a multi-staged membrane integrated bioreactor system commencing sugarcane juice (SCJ) via *Saccharomyces cerevisiae* (NCIM 3205). The retained fructose is also converted to ethanol at 18.0 g L^{-1}, with an output of about 72%. Hybrid system for the development and purification of bioethanol was developed and operated under various operating conditions. Diluted ethanol (3 wt. %) Was produced by fermentation in the first stage and concentrated by poly(dimethylsiloxane) membrane to 80 wt. % ethanol–water mixture	Pal et al. (2018), Song et al. (2017), Nigiz and Hilmioglu (2016)
Feed-splitting ohmic-assisted distillation	Reduction of 27.5% of the energy demand heterogeneous azeotropic distillation of ethanol–water process Two solutions of second-generation bioethanol, with ethanol concentration 90 and 50 wt. %. The results were compared with pure ethanol after diverse separation practices (distillation and flash, correspondingly) 99.9 vol %	Tavan and Shahhosseini (2016), Ramis et al. (2017)

configuration for feed can also be applied to the HIDiC, if necessary. If the I rectifying column hot overhead vapor outlet is reused as a possible hot use for preheating feed, the distillation system is called the I amplified (int-i-HIDiC).

Membrane-Based Downstream Separation

Membranes, like membrane reactor, membrane distillation unit, membrane contactors, membrane adsorption, and membrane crystallizer units, are used as

process components in chemical production processes. The application of pressure, chemical, or thermal membrane systems such as microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), pervaporation (PV), and membrane distillation (MD) is used in biorefining processes. The final phase of downstream purification and bioethanol processing using microporous membrane distillation can be implemented with ease (Pal et al. 2018). Utilizing microporous hydrophobic membranes including polyvinylidene fluoride (PVDF), polytetrafluoroethylene (PTFE), and polypropylene, membrane distillation for the final step of downstream purification and bioethanol recovery can be successfully applied (Kumar et al. 2019; Lipnizki 2010).

Ohmic-Assisted Hydrodistillation

The term ohmic-assisted hydrodistillation (OAHD) relates to a section system for ohmic heaters and condensers. While the latter uses energy volumetrically (based on Joule's law), this collects and settles the heating section vapor produced. The key areas of the ohmic heater section are the source of energy, the electrodes, and a non-electroconductive chamber (flask) (Gavahian et al. 2019) This basic design can be combined with many other segments such as safety systems, variable transformers for controlling input power, frequency and variable transformers, data acquisition systems, thermocouples, voltmeter, and other sensors (Aditiya et al. 2016).

1.2.1.4 Diffusion Distillation

Initially, diffusion method was introduced by Fullarton and Schlünder (1986) who suggested diffusion separation technique through internal gas voids and then gets condensed. The combination is vaporized before the boiling point, and mixture's stability is affected by diffusivity and also of inert gas. Numerous predistillation combinations of alcohol and water, viz. binary isopropanol-water and isopropanol-methanol-water were tested in their study, and the experiments were performed with variance of condensation, evaporation temperatures, inert gasses, and annular widths of the wetted-wall column (Aditiya et al. 2016).

1.2.1.5 Salting out Method

Miscibility of water and ethanol is due to their current intermolecular forces, the sturdiest of them is the hydrogen bonding. For instance, an electrolyte (in the above case K_2CO_3) is introduced to water, the electrolyte solubility makes water inaccessible to bond with ethanol to hydrogen. The ethanol solubility reduces due to the nonexistence of hydrogen bonding interfaces with water. As a side benefit, organic dye that was previously yellow due to the acidic solution is mixed with ethanol into the organic process and changes color (Shakhashiri 1985).

1.2.1.6 Adsorption

There is a solid adsorbent material in adsorption which will adsorb the alcohols in the fermenter. The extractant is usually enclosed in a packed-column contactor that assists as both adsorber and desorber. On the other hand, the solid extractant is not stimulated through the system causing a cyclic loading and unloading process of the adsorbent. This solid will show a greater sorption selectivity for alcohol than water and high distribution coefficients for sorption equilibrium resulting in a high separation factor distillation by adsorption method. To collect the excess water content, use the variance in molecular sizes of the ethanol and water combination. In this type of distillation, molecular sieves separate ethanol from the combination according to the size of the sieve openings. The 4 Å diameter ethanol molecules are sequestered from the water molecules through sieve having 3 Å in diameter, meanwhile water molecules are characteristically of 2.5 Å in diameter and size (Kumar et al. 2010). Dual beds of molecular sieve in majority are mandatory in typical adsorption distillation process. The water vapor molecules fill the empty space of the molecular sieve and are adsorbed in the column where ethanol water vapor is fed and injected into the first bed. Once the mixture vapor stream starts to flow, water molecules are absorbed uninterruptedly until the bed can absorb the complete mass of water molecules, sorting out the dehydrated and anhydrous feed. If the bed is filled with particles of water, the additional bed substituting the hydrated bed will be switched using the robotics system or control valve to help. The bed is reformative with certain different absorption cycles, and it can be reused. Zeolite is one of the instances of absorbent material that makes the reusable property. Aditiya et al. (2016) reported that activated carbon (252 mg per g) showed maximum adsorption while bone charcoal (206 mg per g) and silicate (97 mg per g) exhibited lowermost adsorption. Though, silicalite presents the assistance of complete desorption at low energy consumption (1948 kcal/kg) (Qureshi et al. 2005). Certain bio-adsorbents (based on lignocelluloses and starch) were produced to extract water from the alcohol and water vapor mixture (Chang et al. 2006). These adsorbents are less able to differentiate, but can be regenerated at lower temperatures.

1.2.1.7 Extraction Liquid-Liquid

An extractant should have the highest possible selectivity for alcohol in relation to water, resulting in a greater factor of separation and a more concentrated product stream. Also important is the equilibrium distribution coefficient, since it defines the quantity of extractant needed to remove the desired alcohol. A typical diagram of this type of extraction is demonstrated in Fig. 1.5.

Ideally, there should be no solubility between the extractant and the soil, but some soil-soluble extractant is likely to be lost because the extractant has a high alcohol affinity which should be examined carefully as it can cause problems for the bioreactor, the products, and even the wastewater treatment plant. It should be easily

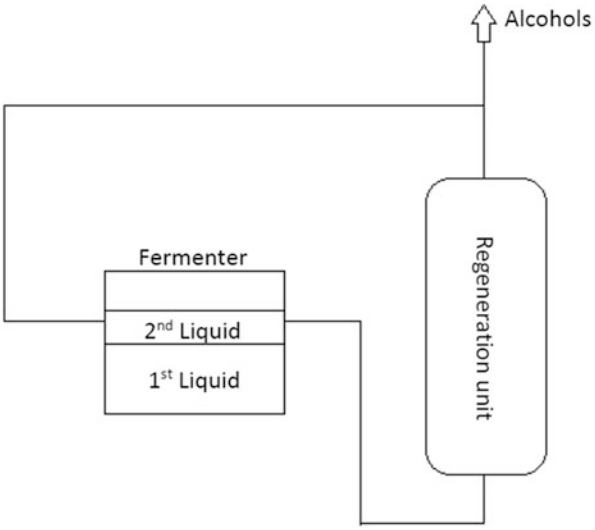

Fig. 1.5 Schematic diagram of an extraction liquid-liquid process with solvent regeneration

Table 1.4 Energy requirement of butanol recovery systems (N/A: not available data) (Felgueira et al. 2015)

Recovery system	MJ/kg	MJ/kg	MJ/kg
L-L extraction	8.8	7.7	N/A
Pervaporation	13.7	N/A	9
Gas stripping	21.7	31	21
Adsorption	8.1	1.3	33
Steam stripping + distillation	24.1	N/A	N/A

separated from the aqueous cycle, typically by gravity settlement or continuous centrifugation. Energy requirement of different systems for recovery of biofuels is discussed in Table 1.4.

1.2.2 In Situ/In-Stream Recovery Techniques

This method involves extracting discriminatory reaction products while fermenting. There are diverse methods for the creation of an integrated recovery method for products. This system comprises of a fermentation unit, coupled with a separation unit for the product shown in Fig. 1.6 (Woodley et al. 2008). Although the solutes that are inhibitory in nature are extracted straight into the solvent process in this system, certain authors also suggest that in situ substance removal also known as extractive fermentation may not be suitable for large-scale production (Kraemer et al. 2011).

- Poor mass alteration (relatively slow than butanol production) through solvent phase
- Emulsion formation done with agitation

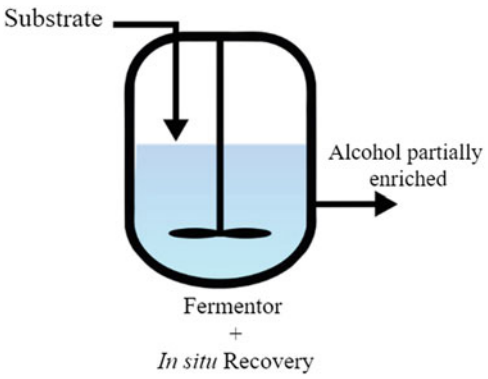

Fig. 1.6 Fermentation in situ recovery process

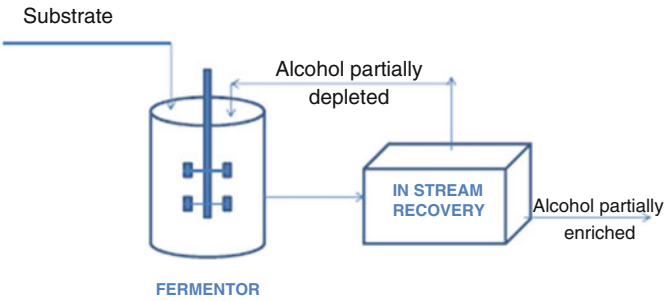

Fig. 1.7 Fermentation *in-stream* recovery

- Cell inhibition by solvent (interface toxicity) and the loss of interface cells
- Physical defending by depiction of cells to the interface: real coefficients of distribution in fermenter lesser than in non-cell experiments
- Precipitates transmitted water into the solvent stage
- Problematic control over the whole process

1.2.2.1 In-Stream Recovery

Fermentation and primary separation are accompanied simultaneously but in two distinct vessels. This means that the fermentation broth is uninterruptedly pumped through another column containing the selective phase or material which allows a fraction of the product to be separated, the alcohol-depleted stream is reverted back to the bioreactor. A typical diagram is shown in Fig. 1.7.

The pros and cons of both modules are definitely among adsorption, liquid-liquid extraction, stripping and more, depending on the type of separation unit used. The integrated product recovery techniques of the aqueous broth alcohols may be based

on the difference between the physical or chemical properties of the various alcohols and water, or their interaction with an auxiliary agent or material.

Enrichment of the end product varies depending on the selection criteria of the ISPR (in situ process removal) methodology but methods with lower separation factors may still attain a similar or higher concentration of the product in the concentration when operated at higher residual product concentrations. Consequently, the concentration of the concentrates is closely related to the concentration of the product in the fermentation broth.

It can be beneficial in different ways, such as enriching the end product concentration contributing to lower downstream prices, enhancing productivity by removing the inhibition stock, decreasing stream flows as it hits higher product concentrations and thereby raising product yield, decreasing side reactions by removing the product.

1.2.2.2 Vacuum Fermentation

Cysewski and Wilke (1977) first introduced this technique to reduce the inhibitory effect of ethanol throughout the fermentation progression. They demonstrated that rapid and whole fermentation was possible with condensed sugar media by holding the bioreactor below inert atmosphere, throughout the vacuum fermentation route, bioethanol is continually extracted through fermentation broth by applying vacuum pressure beneath the fermenter to enable ethanol to fade at fermentation temperature and consequently to be condensed utilizing condensation cooling or chilling water. Ethanol concentration can be regulated at low levels during the vacuum fermentation phase, reducing or decreasing the inhibitory effect of ethanol on the breakdown of the leaves and the fermentation method. A few reviews of recent researches are discussed in Table 1.5.

1.2.3 Comparison of Various Biofuels Recovery Techniques on the Basis of Economics

Reliable downstream processing of broth for fermentation biofuels is usually a trade-off between rate of recovery, cost, and durability of the device. In relation to overhead, an appropriate strategy for the recovery of biofuels should be economically viable and implemented into the industrial scale (Fig. 1.8). Vane (2008) have carried out a detailed economic study of various energy-consuming ethanol recovery techniques. Operating costs during ethanol processing depend on the decisions made about the cost of feedstock, the cost of enzymes, and the kind of pretreatment to be used. Therefore, merely performing an analysis in terms of energy consumption is not enough. Hence, more factors such as infrastructure and operating expenses, for efficient downstream processing, must also be included.

Table 1.5 Review of biofuel removal by vacuum fermentation (*C* concentration of substrate, *P* pressure in vacuum, *T* temperature)

Parameters	Aim of the study	Findings	References
Glucose $C = 60$ g/L $P = 711–737$ mHg $T = 35$ °C	Synchronized acetone butanol and ethanol fermentation employing *Clostridium beijerinckii* 8052 and vacuum extraction of in situ butanol	In the recovered stream, the concentration of ABE was higher than in the fermentation broth (from 15.7 to 33 g/L). The inclusion of the vacuum with the bioreactor led to an increase in a 100 percent increase in acetone, butanol, and ethanol productivity	Mariano et al. (2012)
Agricultural wastes $C = 600$ g/L $P = 175$ M bar $T = 60–80$ °C	Alcoholic fermentations were performed, on substrates apple, kiwifruit, and peaches wastes; and corn threshing residue (CTR). *Saccharomyces bayanus* was chosen as starter yeast	Greatest production of ethanol was achieved with CTR (10.22% (v/v) and apple (8.71% (v/v)) among fruits. Distillations to harness warm water from a cogeneration plant is checked at low temperatures and under vacuum	Cutzu and Bardi (2017)
Rice bran $C = 2.5–7.5$ g/L P=N.A $T = 30 \pm 2$ °C	The research proposed further use of rice bran as energy source with the ability to affect the production of bioethanol with *Saccharomyces cerevisiae* (1–5 g/L) and rice bran (2.5–7.5 g/L) at various concentrations	The average yield/s was (0.577 g and 0.375 g, correspondingly for rice bran) at the point of highest rice bran concentration and least cell concentration was detected for time 12 h and 72 h.	Moreira et al. (2019)

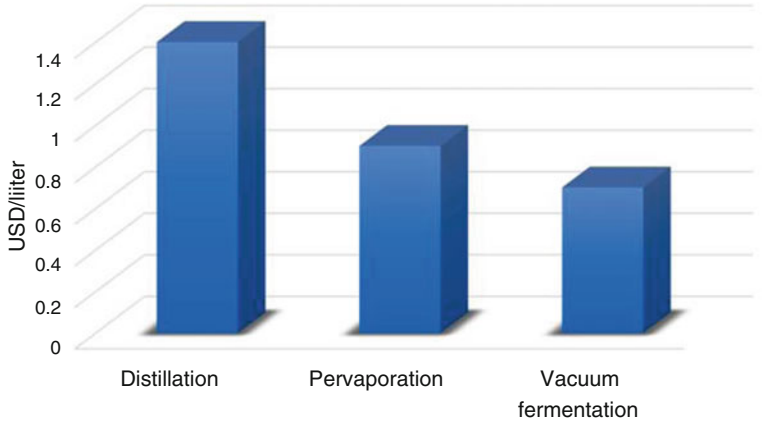

Fig. 1.8 Assessment of ethanol assembly outlays using various recovery techniques (Zentou et al. 2019)

1.2.4 Downstream Processing of Third Generation of Biofuels

Microalgae have gained substantial attention recently as an enticing source for the industrial development of unconventional biofuels, predominantly biodiesel and aeronautics energies. Moreover, to its capability for advanced biofuels and bioproducts as an alternative biomass, the gathering of diluted crops of algae from large crop volumes desirable for production of biofuels and bioproducts is a considerable barrier to the algal biofuel's economic feasibility (Kim et al. 2013). Although sedimentation along with centrifugation are now surmounted to capacities that will require straight application to the production of algal biofuel, their economics for biofuel production are not optimistic. Despite the numerous advantages associated with biofuel production using microalgae, the commercial feasibility of the microalgae-based biofuels industry is nearly equivalent with that of either the petroleum industry or the bioethanol industry. One of the key motives for the great algal biofuels production expense is the absence of an extremely inexpensive method which incorporates the several phases involved with harvesting, extracting, and transforming biomass to biodiesel. As per Kim et al. (2013), for most downstream production, a secondary or thickening phase is necessary to increase the concentration of solids. For this secondary concentration stage, the final solid content required varies and 50% solids is the range mostly for downstream practices that will be able to withstand substantial moisture range.

1.3 Harvesting Method

1.3.1 Settling/Sedimentation/Gravity Sedimentation

Among all the methods to extract algal cultures, easiest way is naturally settling of cells due to gravitational force. It has been applied to various algal strains so far and is generally applicable in high bacterial load wastewater treatment which includes nutrient rates to support clumping and settling. The density of cell and the radius of algal cell influence the system flow rate and its usefulness, in turn improved by lamella separators and sedimentation tanks (Chen et al. 2011). Settling is also done for the introduction of flocculant/coagulants. While this is indeed the slowest possible segregation alternative, it is still the one with the lowest energy demand.

1.3.2 Centrifugation

Most microalgae can be retrieved by centrifugal force from dilute suspension. Centrifugation offers both enhanced harvesting performance and improved

microalgal biomass concentration within a short period of time. It is also recommended for expensive products, such as food or aquaculture applications, to recover superior class of algae with least or no contamination of the initial product with chemical and bacterial substances. Centrifugation for main and secondary dewatering is commonly believed to be viable only for high value submissions (Grima et al. 2003). Centrifuges are usually set for optimizing capture efficacy. Cost-efficient microalgal harvesting, consequently, the full acquisition output cannot match (Barros et al. 2015).

1.3.3 Filtration

To maintain the algae and let the intermediate to flow over the screen devoid of unnecessary clogging, a faster screens or filters with large gage pores which is also cost-efficient can be used. Examples of macrofiltration technology are die-cast filters and filter presses. This harvesting method applies to a small number of large sized or filamentous algae, properties that do not cause them to form impermeable cakes, flow through the pores or clog the screens. Screens having huge pores accurately capture the biomass offered in significant proportions.

1.3.4 Sedimentation

The initial step of removing the algae from the water is sedimentation. The algae are allowed settling along with densify until tension is over. Other methods, however, probably must also be required to achieve full segregation. Gravity sedimentation is a process separating a suspension of the feed into a concentrated slurry and a transparent liquid. Harvesting at natural gravity by sedimentation can be done through lamella separators and sedimentation tanks. Gravity sedimentation relies on the capacity of the suspended matter to settle down, which is determined by the density (Stokes radius) of the algae and the sedimentation velocity. This approach is ideally well-matched for wastewater treatment and applies to algae with a 70 mm diameter, such as Spirulina. Lamella separators, due to the orientation of the plates, can provide an improved settling area compared to traditional thickeners. The microalgal suspension is continuously pumped while discontinuously extracting the slurry.

Separating microalgae from sedimentation tanks is a costly operation. Nevertheless, the reliability is poor without flocculant addition (Milledge and Heaven 2013).

1.3.5 Membrane Separation

To a vacuum flask funnel is connected in the laboratory. The contents on the funnel are poured out onto the filter and allowed to dry some on the pipe as the vacuum is pulled on. This method could be targeted for collecting low-density microalgae but is generally performed on a minor scale. Yet there is membrane fouling that is the biggest downside. There are three reforms: (1) reverse-flow vacuum, (2) direct vacuum above the filter along with stirring blade, and (3) belt compression.

1.3.6 Flocculation

It is a process in which some chemical substance is added to a water alga mix which results in aggregation of clumping of algae together with colloid formation. Chemical flocculants contain chloride in the alum along with ferric. Chitosan is an organic flocculant but it costs quite high. In autoflocculation, injection of CO_2 into system causes algae to flocculate alone. Flocculation is sometimes used in conjunction with a compressor filter, as mentioned in the last paragraph, that can be caused in various ways. First, where a charged particle attracts a particle charged in the opposite direction forming a layer. Second, connecting the surface of two different particles forming a connection between them. Major factors such as cell concentration, surface properties, flocculant concentration, pH, ionic strength, and microalgal growth step play chief roles in the flocculation cycle. Coagulants distort the loads and physical characteristics of the suspended cells, so that agglomeration is not tolerated. Flocculants focus on promoting the production of larger, destabilized masses from algal biomass. Chemical flocculation, autoflocculation, electroflocculation, and bioflocculation are all instances of practices which rely on cumulative and rapid flocculation. All these flocculation processes, on a large scale, requires vast space and are costly due to the expenses of coagulants, flocculants, and operators (Bosma et al. 2003) and various chemical modifications (e.g., autoflocculation pH adjustments).

1.3.6.1 Chemical Flocculation

Chemical flocculation adds to the culture medium flocculating along with coagulating agents to accelerate cell aggregation. This method acts as a pretreatment with microalgae in conjunction with other methods, for instance, dissolved flotation of air (DAF). While this enhances pace of cells collection, to achieve this rate there is the additional dosage of chemicals complication at the desired concentration. They are typically used to neutralize particle charges within the solution, and flocculants are the chemicals utilized in collection of the particles. Such chemical coagulants and flocculants exacerbate the overall cycle by introducing extra costs, often

incorporating metals or other additives to be released into the resultant biomass, and complicating the downstream recycling of the materials into main and co-products. Adverse impacts were offset in many ways, together with the used flocculants (e.g., polyacrylamide and starch) that are biodegradable in nature and electroflocculation in which flocculant is not added directly.

1.3.6.2 Auto and Bioflocculation

While diverse phenomena occur, it is normal to denote auto and bioflocculation principally as the same. Autoflocculation (flocculation alone by rise in pH) is an appealing alternative as it is cost–efficient, lower energy requirements, low or no toxic to microalgae, and no need for flocculants. This process can occur naturally with limited CO_2 supply in microalgae cultures that are wide-open in sunlight during warm and sunshiny day. Microalgae eliminate CO_2 absorbed by photosynthesis in the culture medium and thus raise its pH content (Barros et al. 2015). Auto and bio-flocking are both methods which use regular measures hence forcing the cells to aggregate in floccs. In auto-flocculation, environmental factors such as salinity, temperature, or pH are engineered to affect the surface charges of the cell and enable the cells to come into close association so that they can stay in small groups at last.

1.3.6.3 Inorganic Flocculants and Coagulants

Typically, iron or aluminum-based inorganic flocculants and coagulants are used to counter the surface load. This approach includes a large response of the inorganic flocculant that supplements the sludge, adding responses and handling (to extract the chemicals) to the OpEx (Moheimani et al. 2015). In addition, the process is pH-sensitive and typically works greatest at higher pH but varies with strain and crop condition. Not all strains of algae react equally to a specific chemical, therefore alteration will be needed to suit the organism for harvestment (Chen et al. 2011). Chemical flocculants may result in posing a problem with the downstream processing of biomass for feed, stock feed for anaerobic digestors, and remaining ions can pose a problem with the use of digestates for land use for soil modification (Casey et al. 2011).

1.3.6.4 Organic Flocculants and Coagulants

It is also possible to use organic flocculants and coagulants, bridging polymers having high molecular weight (e.g., starch and chitosan), which respond to large aggregates with cells in the crop and aid in speeding flocculation process (Edzwald 1993). Generally, it is claimed that biomass is not contaminated by these biodegradable polymers I comparison to coagulants inorganic in nature, and that cationic polymers are preferable over neutral along with anionic polymers. Cationic

polyelectrolytes (e.g., Dow C-31) initiated flocculation of algal cells while nonionic as well as anionic polymers demonstrated ineffectiveness.

Flocculation is often achieved by smearing upright ultrasound waves and by flocculating the electrocoagulation. While the use of hanging ultrasonic waves works well in the laboratory, its large-scale handling is very difficult, and the high amount of metal ions in the end result is a disadvantage of flocculating electrocoagulation.

1.3.6.5 Electroflocculation/Electro-Coagulation/Electrolytic Aggregation

The need for an electrical field to change the surface characteristics of the algal cells and to induce flocculation carries the ability for better harvesting because, in principle, the process does not entail the addition of chemicals and could effectively be performed as a continuous process. The obstacles to the general applicability of these techniques are the development of electrodes that do not connect metals to the device and the cost of the requisite electricity.

Electrophoresis is one of the harvesting processes in which no chemical substance is added for segregation of substances. Charged algae in the solution escape due to electric field. Hydrogen, generated water electrolysis, sticks to and takes to the surface of the microalgae flocks. Compatibility with environmental, flexibility, security fussiness, energy efficacy, and cost-effectiveness are a few profits of this method. The major drawback of this approach is the fouling of cathodes and devices that are impaired by high temperatures due to higher amount of power required (Pragya et al. 2013).

1.3.7 Flotation

It is a process in which algal particles and air froths are exposed to one another and the buoyancy of the air froth brings to the surface and it can be the algae. Flotation methods can catch particles below 550 μm, these methods are made especially suitable for single-cell microalgae. There are a few alternative approaches used for harvesting such as flotation: dissolved air flotation (DAF), electrolytic flotation, dispersed flotation, and flotation of ozone.

1.3.7.1 Dissolved Air Flotation (DAF)

DAF is based on the introduction of coagulants or flocculants, but the harvest is enhanced by the use of fine bubbles from the bottom of the DAF device to collect small aggregates and to carry them to the tank surface. The froth containing the cells is skimmed off at the tank surface and deposited in a far more concentrated solution (100-fold rise in solids) (Milledge and Heaven 2013). This supersaturates the culture

of the suspension and causes bubbles to be nucleated as the pressure decreases which raise the particles to the surface. The pressure applied produces bubbles which range from 10 to 100 mm. Dispersed air flotation requires a higher pressure drop for generating bubbles, and generates bubbles by continuously passing air through a porous material. While less energy is consumed it is more costly (Moheimani et al. 2015).

1.3.7.2 Froth Floatation

It is a separation and harvest technique that involves segregation of algal cultures from water. This is a method use since many years in the coal and mine cleaning technology. It is based on materials differing in density. Air bubbles are usually inserted within the device. Often a supplementary organic chemistry or change in pH boosts the separation. It is achieved in a long column containing the solution for feed which is ventilated below. A durable column of foam is produced from a side arm close to the top of this column (Levin et al. 1962). It is an advanced technique which can be too costly for commercial usage at this stage. The probability also exists of merging with froth flotation and flocculation. If alum is added to algae as a flocculant, for example, air is simmered over to isolate the flocculant by mass. Also, it can unite with a compressor filter.

1.3.7.3 Dispersed Flotation

700–1500 μm bubbles formation takes place in dispersed flotation by mechanical high-speed agitator coupled with air injection arrangement. Cationic N-cetyl-N-N-trimetyl ammonium bromide (CTAB) and some other surfactants were used to eliminate *Scenedesmus quadricauda* whereas non-ionic X-100 and anionic nature SDS did not function (Chen et al. 2011).

Continuous-flow centrifuge is among the mostly utilized techniques. It is productive and collection of algae along with some other particles is the main motive of this technique. However, it is used more frequently for the creation of algae-based value-added products and usually not applied for generating fuel.

Besides to improve the shelf-life, moisture must be removed from the algae along with separation techniques. Algae are extracted through a sequence of routes from water including the separation phase.

1.3.7.4 Ozone Flotation

In one of the variants of the flotation, ozone bubbles are introduced into "disseminated flotation of ozone," which were found to be effective in the *Scenedesmus obliquus* harvest (Cheng et al. 2011). With this culture there was no conceivable distinction along with traditional air flotation. Separation in ozone air-flotation was

due to the algal biomolecules release (proteins and polysaccharides) produced by ozone that assisted flocculation (Cheng et al. 2011). Proteins generated due to ozonation from the cells probably allowed for froth formation and cell segregation.

1.3.7.5 Electrolytic Flotation

Water hydrolysis powered by electric fields generates H_2 bubble formation that attach microalgae with each other and bring them to the upper surface to speed up harvest process (Mollah et al. 2004). The process of electrolytic flotation was conducted simultaneously in batch mode, and continuous mode higher power correlated positively with improved separation of algal cells in eutrophic lake water was reported (Alfafara et al. 2002). The benefit of this technique is that no additional chemicals are required to induce separation.

1.3.7.6 Foam Flotation

It includes the mechanism of extracting the active surface chemicals from the water and by drawing water from solid and liquid mixtures. Foam generation is by addition of a surface-active chemical to the solid-liquid mixture and air bubbles injection for creating a steady foam. Depending on the algae to be collected, bubbles may be of various sizes, usually between 10 and 3000 mm in diameter.

1.3.8 Magnetic Separation

Magnetic algae separation was suggested in the past and is currently under investigation. The principle consists of passing algal cells that are magnetized in nature (or aggregates magnetic in nature) through a magnet and extracting them from culture medium directly.

The magnetite and aluminum sulfate were used in the initial magnetic separation methods applied to the culture that is attached with the algal cells and then subsequently an electromagnet aids in extraction. In neutral and slightly acidic pH waters they were more active (removal from 79 to 94%) but less effective with higher pH water (55 to 64%) (Bitton et al. 1975). This method has the pH sensitivity concern, along with the reliance on magnetic materials and flocculants added.

1.3.9 Ultrasonic Separation

Ultrasonic waves have been used to induce algae agglomeration and to improve algal differentiation in various formats. The mechanism of separation is based on a

smooth, acoustically induced aggregation coupled with the increasing sedimentation (Bosma et al. 2003). The idea is to use ultrasonic energy to start concentrating the algae at a specific position or node to promote fast recovery. Ultrasonic separation can be implemented continuously, cells remain viable (experiencing low amount of stress), is free from fouling, has limited active parts that breakdown during the operation, and space requirement is relatively low. However, this method was considered less economical than centrifugation for commercial harvest of algal cells mostly due to the cooling system necessity and concentration factor which is relatively low paralleled to centrifugation and micro-filtering. It could be useful in a system comprising of a metabolite secretion from alga, and extracting living cells for reprocessing in the development of additional substance while collecting the metabolite from the media would be advantageous.

1.4 Cell Disruption Techniques

Usually, the composition of microalgal cell walls varies with different organisms except for *Euglena* and *Dunaliella*, because they do not have a cell wall. Cell walls consist of glycoproteins, cellulose, fat, xylan, uronic acid mannose, alganic fibers, and minerals, for example, calcium or silicate. Application of microwave techniques, sonic waves, pounding by bead, high temperature application by autoclave, size reduction, shock with the help of osmosis, homogenization, freezing, dehydration, and 10% (weight per volume) sodium chloride addition are numerous methods of cell disruption (Amaro et al. 2011). Simple mechanical grinding is easy and quick but the rise in temperature due to grinding will reduce certain compounds. Grinding can also be achieved with liquid nitrogen, making the process comparatively simpler. Breaking the cells by application of direct physical force is one of the mechanical methods and benefits include that these methods can be applied comprehensively irrespective of species to a biomass. Additionally, chances of the target products being damaged or degenerated during cell disruption are very low. Harrison (1991) offered various cell disruption options, such as high-pressure homogenization, pounding through bead, and grinding through mortar and pestle that belong to mechanical class, but there are limited significant methods.

1.4.1 Bead Beating

It is better known as beadmill or ballmill, is among the simplest techniques of disruption of cell that split cells by filling target cells and beads made of quartz or any other type of metal in a closed vessel and then application of shaking force. Disturbance is caused by bead's contact or frictional force which results in grinding. Extracting DNA from biological samples is one of the most general applications of bead mill (Robe et al. 2003). Bead beating could indeed destabilize a cell very

quickly, also without any preparation application on in-situ humid biomass is possible. For instance, grinding or simple pressing with algal paste or a diluted algal suspension with water content higher than 60% cannot be used proficiently. In contrast to other processes ultrasonic can be applied to wet biomass more effectively. The process of cell destruction is accompanied by extraction of the lipids. Most extensively used solvents include chloroform along with methanol. Other extraction solvents include acetone, ethanol, acetone, and iso-propyl alcohol showing various choices of efficiencies in extraction of lipid.

1.4.2 High-Pressure Homogenization

Charles Stacy French invented high-pressure homogenization. In this process of cell disruption, the feed is passed through a very tiny orifice under high pressure which results in hydraulic force creation and results in cell disruption. HPH was widely used in extraction and sterilization of the inner constituents of micro-organisms. Numerous benefits include formation of little heat, risk of thermal degradation is also lower, lower reactor cooling rate, dead volume is almost nil, and simple scale up.

1.5 Extraction of Lipid

It is a critical step to the use of algae to produce fuels. It is probably an affordable choice too. For release of the preferred products the algae cells must be subjected to cell disruption. Methods include: 1) mechanical methods such as bead beating, 2) electric field application, 3) application of sonic waves, 4) shock through osmosis, and 5) expelling through manual press. Biological and chemical methods include: 1) solvent extraction (single solvent, co-solvent, and direct reaction by trans-esterification), 2) supercritical fluids, and 3) enzymatic extraction.

1.5.1 Single Solvent Extraction

Among the commonest extraction techniques is the single extraction of solvents. A lipid-like solvent, for instance, hexane, petroleum ether, or other solvent (which is merely a light solvent and petroleum based in nature) is used. This method is commercial. Extraction proceeds at high temperatures and with high pressure. The benefits include an increased rate of mass transmission and solvent availability, and a decreased immiscible solvent dielectric constant. A co-solvent method is a little different to use. A solvent is chosen on two parameters. Option should include: 1) a co-solvent polar in nature that disturbs the algal membrane cells, and 2) a co-solvent with lesser polarity to better suit the extracted lipids polarity (alkanes fits in

criterion). Co-solvent extraction has numerous examples; in 1959, Bligh and Dyer developed one process. The solvents are chloroform and alcohol, and mostly chloroform stage achieved better lipids dissolution. The interfaces consist of water and methanol>methanol and chloroform>lipid and chloroform. Co-solvents of other combinations includes: (1) hexane/and sopropanol, (2) dimethyl sulfoxide (DMSO) and petroleum ether, and (3) hexane and ethanol.

1.5.2 Supercritical Extraction

It is equivalent to a solvent extraction technique. The biggest distinction includes that the solvent is sustained until predefined temperature or pressure specifications are achieved in respect solvent properties and enable extraction of the constituents. This is often applied on minor scale, and might not be of industry interest. Supercritical fluid extraction facilitates extraction of one solvent from another using a supercritical fluid, for example, carbon dioxide. The separable part is the extractant, on the other end is matrix. Diagram of supercritical fluid extraction is shown in Fig. 1.9. Preferably strong matrices are used but liquid matrices are also employed. When heated above its critical temperature, the fluid reaches a critical point and is compressed beyond its critical pressure limit. These fluids have the benefits that include viscosities which resemble gas, densities which are liquid alike, along with intermediate diffusivity. Supercritical CO_2, as it is non-flammable, harmless, inexpensive, and easy to isolate, is widely used for lipid extraction.

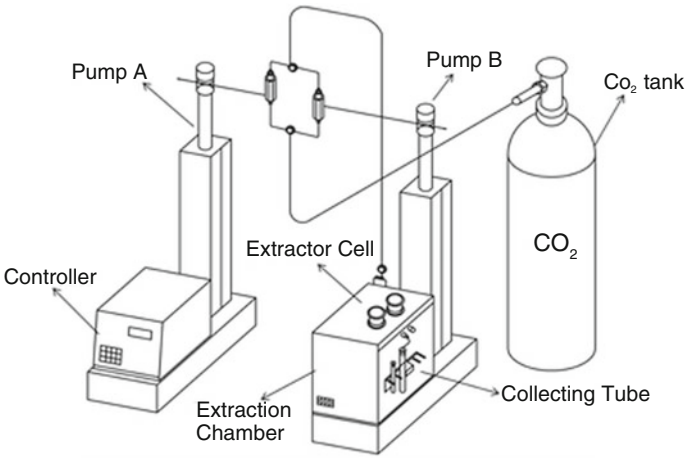

Fig. 1.9 Supercritical fluid extraction apparatus

1.5.3 Enzymatic Extraction

Is also related to extraction with solvent, with the exception of that an enzyme is utilized instead of a solvent to isolate the materials; the trans-esterification reaction is also aided for turning lipids into fatty ester methyl esters (FAMEs) via alcohol in presence of catalyst. The benefits of using this approach are the fast extraction of medium-chain triglycerides that are volatile in nature, and the absence of antioxidants to preserve lipids.

1.5.4 Extraction Through Ultrasound

Ultrasound is generated by mechanical waves. Ultrasound and sound are differentiated according to wave frequency. Ultrasound frequencies are beyond human levels of hearing (20 kHz-10 MHz), while sound waves are lower than the frequencies that human can hear (16 Hz to 20 kHz). Ultrasound is of two types: high intensity and low intensity. Higher intensity and lower frequency ultrasound can modify the properties of food, while high-frequency, low-intensity ultrasound is used for nondestructive research, mostly for quality evaluation.

1.5.5 Microwave-Assisted Extraction

Through microwave heating, the sample being considered is heated evenly with a non-contact source of heat. Like in conventional heating, during microwave heating the heat reaches the inside of the sample. Two facets of considerable importance are transfer of heat and transfer of mass traditional and microwave heating.

1.5.6 Ionic Liquids for Extraction

It contains cation- and anion-forming salts. They can be in a fluid stage, till 140 °C. It is well known for its excellent electrochemical and thermal stability. They are highly conductive and have a vast array of miscibilities. The algal cell wall of cellulose can be dissolved by ionic liquids.

1.6 Hydrodynamic Fluidic Devices

At the Palo Alto Research Center, a hydrodynamic, fluid segregation method was developed; fluidic shear was the main basis as the culture stream flows through a curved form in the fluidic unit shown in Fig. 1.10. This device is a curved channel over which the culture is strapped, dragging from the channel walls exercises forces separating particles from the suspended solution such that separated condensed and diluted streams come out toward the end of the separator. The machine can operate uninterruptedly, does not require any filters, is normally wide enough to prevent fouling and does not need extra resources for parting. They also showed an assistance from the usage of flocculants with tiny algae though this process. Due to the vast scale of the fluid channels, a system like this would also have little or no fouling.

1.7 Direct Biofuel Production from Algae

Other fuels can be obtained directly from algae aside from separating the lipids to make diesel fuel. Those involve alcohols including butanol, ethanol, and hydrogen along with methane. Alcohols may be derived through algae using heterotrophic (carbon nutrients obtained from bio-materials), through fermenting starch into alcohols, like ethanol along with butanol. *Chlorella vulgaris* and *Chlamydomonas perigramulata* are marine algae which are used for this. Procedures involve the accumulation of starch through photosynthesis, subsequent anaerobic fermentation for the processing of alcohol under dark conditions, and alcohol extracted straight from algal culture media. Photo fermentation and dark fermentation directly from the algae also produce hydrogen. Methane can be generated through anaerobic algal conversion. It may be united with other procedures (e.g., use the residue after extracting lipids). Encounters include high biomass content of protein, which in turn lead to inhibition of NH3 and could be resolved by co-digestion by high carbon co-substrate.

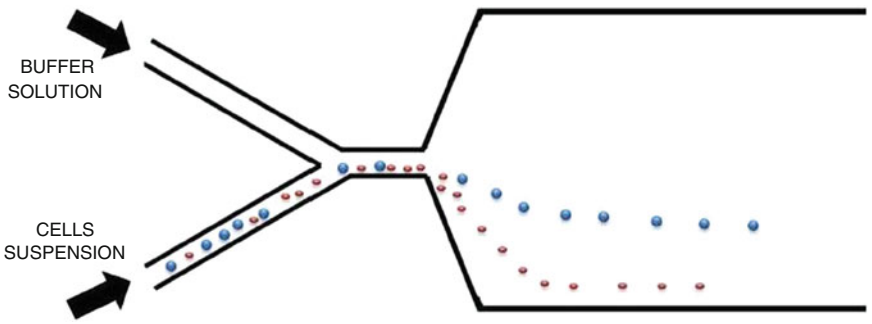

Fig. 1.10 Operation principle for the hydrodynamic separation (Torino et al. 2017)

1.8 Conclusion

Downstream processing of biofuels is a crucial step when producing biofuels. Purification in the industry is achieved primarily through distillation. Owing to many benefits some of which are ethanol and butanol's great separation capabilities, and ease of use, distillation is quiet an efficient and favorable separation practice in industries related to bioethanol production. But, other alternative methods, such as adsorption, pervaporation, gas stripping, and vacuum fermentation, are developed for biofuel recovery that are more efficient in energy and also economical. The harvesting of dilute algae crops from large crop volumes required to generate biofuels and bioproducts is a major barrier to the commercial feasibility of algae biofuels. Microalgal harvesting methods are very diverse, and the eventual use of the collected biomass determines the choice of a particular system.

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Chapter 2
Application of Microorganisms for Biofuel Production

Nidhi Jaiswal, Aparna Agarwal, and Abhishek Dutt Tripathi

Abstract Increase in global energy demand, rise in crude oil prices, depletion of resources and environmental challenges have resulted in the need of biofuels which are a renewable, sustainable, efficient, cost-effective and eco-friendly source of energy with the potential to replace conventional petroleum-based fuels. Biofuel production using lignocellulosic biomass has now been promoted and the process is made cost-effective by the use of microorganisms which are currently being researched for important biofuel production like biodiesel, bioethanol, biogas, etc. from various substrates. The isolated strains of different microorganisms have been studied as well as genetically modified species have been developed, to improve their metabolic capabilities to utilize the organic feedstock in order to produce biofuel as its final products. The present chapter outlines the role of microorganisms in the conversion of biomass into biofuels, their potential to replace conventional fuels, along with different approaches to be applied to improve biofuel production.

Keywords Biofuels · Biomass · Microorganism · Fermentation · Biodiesel · Biobutanol

2.1 Introduction

The increasing demand for natural resources such as crude oil has led to increase in industrialization and globalization, which increases global warming. Non-renewable natural resources like coal, crude oil and natural gas account for 80% of the total energy consumed all over the world, out of which nearly 58% is being used up in transport sector. The non-renewable source of fuels is getting exhausted and caused greenhouse emissions which have led to adverse effects in the environment such as

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diminishing of glaciers, global warming, climate change, rising sea level and decline of biodiversity.

This has led to the development of renewable fuels: biofuels which are sustainable, effective and are economical natural energy source with lesser carbon dioxide emissions. They are one of the most important sustainable fuels which limit gas emissions, improve air quality and also provide a regular income to the farmers.

2.2 Biofuels: Definition, Classification and Characterization

Biofuels are predominantly produced through modern technologies from biomass (biological raw material) and convert them to solid, liquid or gaseous fuels. Biomass is the fourth largest renewable energy resource in the world which is natural and inexpensive, and has been used to produce biofuels. The EIA (U.S. Energy Information Administration) explains the word biomass is primarily used for the production of liquid and gas biofuel which are particularly used as transportation fuels. Biofuels are considered as a source of renewable energy as these biofuels are produced from biomass which can be regenerated at a faster rate.

Microbial biotechnology approach and microorganisms are being used to produce different types of biofuels such as biohydrogen, bioethanol, biogas, biodiesel, etc. from different types of raw material such as lignocellulosic biomass, carbohydrates, oil crops, vegetables, etc.

2.2.1 Characteristics of Biofuels

1. Source of renewable energy
2. Cost-effective
3. Eco-friendly and as an alternative to petroleum fuels and non-renewable energy resources
4. Considered as carbon neutral
5. Sustainable
6. Non-toxic

2.2.1.1 Classification of Biofuels According to Generations

First-generation biofuels are produced from crops, sugars, waste oils or fats employing traditional technology. These are converted into biodiesel (made from vegetable oil after pressing), bioethanol (produced from grains with high sugar) using trans-esterification or fermentation by yeast. The most commonly used first-generation biofuels are biodiesel, bioalcohols (bioethanol, biobutanol), biogas, biochar, and syngas.

Second-generation biofuels are produced from lignocellulosic matter and waste material of crops, residue obtained from agricultural produce particularly wheat straws and corn husks. Other waste biomass includes non-edible part of agriculture residues and by-products of plants. These can be produced by feedstock, can also be grown on arable land (which is not a general practice) or using agricultural residues. The conversion technology is also biochemical or thermochemical method of converting the biomass into biofuel. Other feedstock sources such as forestry residue, seed crops, waste oils and fats, solid waste etc. can also be used. Common examples are biodiesel, biohydrogen and biomethanol.

Third-generation biofuels are algal biofuels produced from algae by extraction of oil. Sources can be marine reserves, seaweeds, cyanobacteria and microorganisms. Its production cost is low and biofuel is high-yielding as compared to conventional first-generation biofuels.

Fourth-generation biofuels are biofuels that do not require destruction of biomass for conversion to biofuel. These types of biofuels are electrofuels and photobiological solar fuels produced by using cheap, inexhaustible resources such as solar energy (Aro 2016; Chiaramonti 2011; Mbaneme-Smith and Chinn 2015) (Tables 2.1 and 2.2).

2.3 Technology for Production of Biofuels

The extraction of biofuel from marine/agricultural biomass can be done initial drying step. Biofuel production from wet biomass, i.e. agricultural biomass is done by hydrothermal treatments, hydrolysis by application on enzymes, and microbial fermentation to produce different end products such as bioethanol/biohydrogen/biobutanol. The extraction method from dry biomass, i.e. marine biomass or seaweed, involves different steps such as direct combustion, pyrolysis, gasification and finally trans-esterification to biodiesel.

2.3.1 Pretreatment

The pretreatment is the foremost essential step in biofuel production. Pretreatment is done by physical, physicochemical, chemical or biological process. The physical pretreatment involves milling; chemical pretreatment involves acid or alkali treatment, hot-water treatment, steam treatment, microwave and solvent extraction, and biological pretreatment involves enzymatic and microbial treatment. Since the breakdown of lignocellulosic material is not easy, pretreatment is an essential step to separate cellulose, lignin and hemicelluloses.

Table 2.1 Conversion technologies for producing biofuels (Babu et al. 2013)

Biofuels	Biofuel source	Conversion technology	Products
First generation	Fats and oils	Trans-esterification Hydrogenation	Biodiesel FAME
	Starches and sugars (wheat, barley, corn, potato, sugarcane, sugar beet)	Conventional alcohol fermentation	Ethanol Butanol
Second generation	Lignocellulosic biomass (cellulose, hemicellulose, lignin) (from agricultural and forest residues and municipal waste)	Biochemical/physical conversion: – Enzymatic hydrolysis and fermentation – Anaerobic digestion – Novel approaches	Bioethanol Biobutanol DME Advanced biodiesel Value-added products Methane
		Thermochemical conversion: – Pyrolysis – Gasification – Liquefaction	Syngas Bio-oil Solar fuels Value-added products
Third generation	Macroalgae, microalgae, microbes	Biochemical conversion: – Photobiological hydrogen production – Fermentation – Anaerobic digestion	Bioethanol Biohydrogen Biogas
		Chemical conversion**:** – Trans-esterification	Biodiesel
		Thermochemical conversion: – Combustion – Liquefaction – Gasification – Pyrolysis	Electricity, biogas Syngas

2.3.2 Enzyme Conversion Technology

Cellulose degrading enzymes, i.e. cellulase are involved in the breakdown of cellulose to monosaccharide units, i.e. glucose. The first step is the degradation of the cellulose by breaking of glycosidic bonds by the action of the enzyme endoglucanase (1,4-ß-D-glucan-4-glucano-hydrolases; EC no. 3.2.1.74). The enzymes act on cellulose in a random manner. In the second step exoglucanase enzyme (EC 3.2.1.91 and EC 3.2.1.74) acts on both the ends of the cellulose (i.e. reducing end having free anomeric hydroxyl group as well as non-reducing

Table 2.2 Properties of different types of biofuels (Prakash et al. 2016)

Fuel	Gasoline	Biobutanol	Bioethanol	Biodiesel	Biomethanol
Energy density (MJ/I)	32–35	29.2	29.2	–	16
Air fuel ratio	14.6	11.1	11.2	–	6.4
Specific energy (MJ/kg air)	2.9	3.6	–	–	3.1
Mileage (%)	100	61–66	83–91	–	–
Research octane number (RON)	91–99	96	96	–	106
Motor octane number (MON)	81–89	78	78	–	92
Vapour pressure (20 °C:hPa)	35–90	58	6.7	–	–
Enthalpy of vaporization (MJ/kg)	0.36	0.43	0.43	–	1.2
Cetane number	Below 15	12	8	40–55	3
Density (g/mL)		0.81	0.79	0.86	0.80
Auto-ignition temperature (°C)	257.2	385	434	200–220	470
Lower heating value (MJ/kg)	–	35.1	26.8	42.5	19.9
Boiling point (°C)	30–215	117	78.4	300–350	64.5
Flashpoint (°C)	−43	35	12.8	135–150	–
Kinematic viscosity (at 20 °C: mm^2/s	0.4–0.8	1.5	3.6	–	–
Saturation temperature (°C)	–	20	20	–	20
Saturation pressure (kPa)	–	0.6	5.93	–	11.83

end) and degrade the cellulose resulting in the formation of two products which is cellobiose and glucose. The last step in the enzymatic hydrolysis is complete with the conversion of cellodextrins and cellobiose by the action of enzyme ß-glucosidase (BGL) (EC 3.2.1.21) and formation of glucose as the end product (Table 2.1) (Gaurav et al. 2017; Chiaramonti 2011; Blanch 2012; Ephraim et al. 2020; Srivastava et al. 2020a, b).

2.4 Microbial Production of Biodiesel

Biodiesel is the first-generation liquid biofuel, used alone or blended with diesel up to 5% (v/v). Production of biodiesel is done through the chemical processes involving trans-esterification of triacylglycerols (from vegetable oil or animal fat) with alcohols (methanol or ethanol) and leading to the formation of monoalkyl esters of long chain of fatty acids, namely fatty acid methyl esters (FAMEs) and fatty acid ethyl esters (FAEEs). Thus, two steps are involved for biodiesel generation, i.e. extracting oil or lipid and trans-esterification of extracted lipid in the presence of alcohols. The latter step may require the presence of catalysts.

The oil for biodiesel production can be extracted from various feedstocks which are widely available. The first-generation biodiesel is obtained from various edible seed oils, like rapeseed, soybean, palm and sunflower which are used commonly; however, peanut, linseed, safflower, etc. and animal fats can also be used for extraction. Because of the increasing cost, non-edible vegetable oils and other economically important plants with the advantage of rapid growth and high seed productivity are used such as tung, cotton, castor oil, jojoba and jatropha. This is the second-generation biofuel produced from herbs, woody plants, waste cooking oils, etc.

The production cost of biodiesel is high because of raw materials that are used for its production. Apart from this, the catalyst needed during the trans-esterification reaction also contributes to its increased cost. In order to achieve high efficiency and lower down the cost, homogeneous catalysts, for example, sodium hydroxide, sulphuric acid, etc. are commonly used for commercial production of biodiesel but their recovery and reuse is very difficult and costly too. This can be overcome by the use of heterogeneous catalyst, but with a disadvantage of difficulty in preparation, and unstable activity (Atabani et al. 2012).

2.4.1 Microbial Production of Biodiesel

Currently, the use of microorganisms (e.g. microalgae, bacteria, fungi and yeast) is considered a promising alternative for the biodiesel production because they are able to accumulate huge amounts of lipids with increased yield and they also use a smaller proportion of arable land. Among various microorganisms' storing oils, only those microorganisms are available for biodiesel production which are oleaginous, i.e. those that can accumulate lipids named single cell oil (SCO) to up to 20% or above of their total cellular dry weight. Thus, the oils extracted from fast stable growing microbes are then trans-esterified through simple-chain alcohols, and producing a value quality biodiesel. Various factors determine the yield (oil content) and composition including types of organism, selection of substrate, culture condition (such as nitrogen, pH, agitation rate, temperature and length of incubation, etc.). Also, the amount of lipid accumulation in microorganism is regulated by their genetic composition, as the accumulation of lipid can differ extensively among species and individual strains (Akinsemolu 2018; Huang et al. 2010; Pandey et al. 2019).

2.4.1.1 Microalgae

Microalgae, prokaryotic or eukaryotic photosynthesizing microorganisms, characterized by rapid growth, are potentially the most favourable crude material to supply a high percentage of lipids. Microalgae could be grown in photobioreactors and can be harvested within days of cultivation, thereby leading to the efficient production of

biodiesel as compared to other having traditional crops. They are considered as the third generation of biofuels and best alternative, overcoming the flaws of first and second generations. Carbon dioxide can be converted into organic compounds more efficiently by microalgae (while using light energy) with higher photosynthetic efficiency and higher production of biomass. Microalgae can grow extremely rapidly with high oil yields, and can also double the biomass within 24 h, thus becoming a promising source of biodiesel production. Since algae use huge amounts of carbon dioxide and also biodiesel contains less sulphur when obtained from algae, it reduces the threat of global warming by reduction of emissions of CO_2 hydrocarbons and SO_2. Microalgae also produce an abundance of proteins and carbohydrates that can result in the formation of valuable co-products like methanol fuel production (Spolaore et al. 2006).

Microalgae have a unicellular or simple multicellular structure, namely blue-green (cyanobacteria), green, red, brown algae and diatoms. Prokaryotic microalgae, i.e. blue-green algae are commonly used for biodiesel production. Although there are a number of species of microalgae that can accumulate lipid, this must be taken into consideration that only few species are utilized for biodiesel production. There is variation in the lipid content in algal cells from 20% to 60% of dry weight biomass, and can reach approximately 80% in some genera, i.e. *Botryococcus, Neochloris oleabundans, Nannochloropsis* and *Schizochytrium*. Microalgae oils are an abundant source of unsaturated fatty acids, i.e. linolenic acids, linoleic, oleic; palmitoleic acid, essential amino acids, like valine, leucine, isoleucine, etc. Various species of microalgae such as *C. protothecoides*, *B. braunii* and *C. chlorella* may produce more lipids depending on the culture media used, mineral salts (nitrogen, phosphorus, iron, etc.) and change in temperature (Xu et al. 2006; Wang et al. 2008).

Several steps are required for biodiesel production. Firstly, biomass production is done through the growth of algal cells and then cells are isolated to the culturing medium. Isolation is followed by extracting lipids from algal biomass and then fatty acids are trans-esterified followed by fractioning on chromatographic columns. Figure 2.1 presents the stages of biodiesel production from microalgae (Chisti 2007; Frac et al. 2010; Anwar et al. 2019).

2.4.1.2 Production of Biomass from Microalgae

Biomass production from algae is influenced by various factors like light (sunlight would be preferred to reduce the production cost), carbon dioxide, water, mineral salts (nitrogen, phosphorus, iron, etc.) and temperature (within range of 20–30 °C). Algal biomass has around 50% of carbon in its dry matter as it utilizes atmospheric carbon dioxide necessary for algal growth.

Various methods can be used for growing algae on large scale such as open ponds and photobioreactors. The former utilizes sunlight as the immediate source of solar energy and are cheaper but the yield is lower as compared to photobioreactors. Photobioreactors are built of translucent materials and have higher harvesting efficiency as it permits growing of exactly those microalgal species that are required in order to obtain a greater amount of oil compared to algae culturing in ponds.

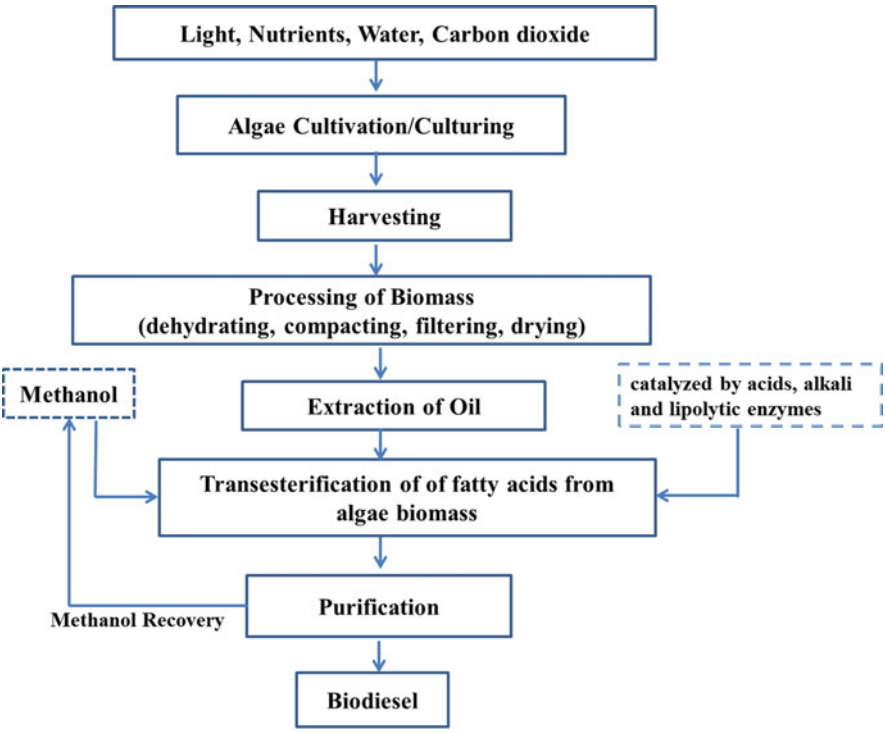

Fig. 2.1 Stages of biodiesel production from microalgal biomass (adapted from Roy and Das 2015; Rodionova et al. 2017)

Various types of photobioreactors are vertical column photobioreactors, cylindrical photobioreactors and flat or panel-type photobioreactors. Artificial lighting is usually used when bulk production algal biomass is done. Photobioreactors also have an advantage of a better control on various parameters such as temperature, pH, CO_2 concentration, etc. but they are costlier, therefore, a combination of open pond and photobioreactors can be profitable.

Various harvesting methods are used in order to separate the algal biomass from culture suspension which in turn depends on the nature of the microalgae, its shear sensitivity, culture medium and cost involved. Various separation methods can be applied such as centrifugation, flotation, filtration, etc. with the addition of chemical agents to algal biomass to induce algal flocculation for harvesting. Macro-filtration (for larger microalgae), micro-filtration and ultra-filtration are some of the filtration techniques which are widely used.

Biodiesel production requires the culture of algae which in turn depends on various factors like the content of free fatty acids and triglycerides, growth rate of algal biomass, availability of nutrients, resistance to changes in environmental conditions (light, temperature), ease of isolation and processing of biomass. Therefore, selecting the most appropriate procedure of algal production according to

specific species is an important consideration so that reduction in production cost can be achieved (Gouveia and Oliveira 2009; Lourinho and Brito 2015; Mata et al. 2010).

2.4.1.3 Trans-Esterification

After extraction, the oil which is composed of triglycerides when combined with methanol is the trans-esterification reaction, forming FAMEs (biodiesel) and glycerol (waste product). Trans-esterification is catalysed by acids, alkali and lipolytic enzymes. Alkaline catalysis is commonly used as it is faster than the acid-catalysed reaction. Also, because of the high cost of lipolytic enzymes, they are not used commonly.

As subjection of triacylglycerol (1 mol) with methyl alcohol (3 mol) results in the formation of 3 mol of methyl esters of fatty acids and 1 mol of glycerol, therefore, the surplus amount of methanol is applied so as to carry the reaction in the direction of methyl esters, towards biodiesel. After termination of trans-esterification process, distillation of excess methanol and its returning back into the process takes place. The most appropriate temperature is 60–70 °C; however, higher temperatures can also be applied with higher pressures, but it can increase the cost.

During the reaction of methanolysis, the oil used should be completely dehydrated and lacking free fatty acids as it leads to the soap formation that affects or reduces the activity of the catalyst and also cause hindrance in the isolation of the FAME and glycerol. Also, the contact between the alcohol and triacylglycerol can be improved by vigorous stirring of the reaction system because of poor solubility of methanol in oil (Meher et al. 2006; Fukuda et al. 2001; Li et al. 2008).

2.4.2 Bacteria

Bacteria are also preferred as an important source for biodiesel production as they are able to grow rapidly with easy culture method, unlike microalgae which require larger spaces to culture with prolonged fermentation period. The limitation being lower lipid accumulation with an average content of about 20–40% as compared to microalgae because most of the bacteria are not oil producers.

However, some bacterial strains can be used to produce lipids to about 60–70% of their cellular dry weight, in order to obtain the esters that can constitute biodiesel. Some species of *Actinomycetes*, as well as other bacterial genera of *Acinetobacter*, *Mycobacterium* and *Streptomyces* can be used as these bacteria are capable of producing triacylglycerol intracellularly to great scales from simple sources of carbon under growth-restricted conditions. To overcome the major problems of biodiesel production including geographical and seasonal restrictions of producing plant oil, and production cost, an extensive research is now been carried out to engineer *Escherichia coli* to produce FAEEs for biodiesel production. Microdiesel is thus considered as an important future fuel totally created by bacteria *E. coli*.

Furthermore, interest has grown following molecular engineering in order to modify well-investigated microbes (i.e. *Escherichia coli* and *Saccharomyces cerevisiae*) into bioenergy cell plants. This could be achieved by initiating an ester synthesizing route by directly esterifying the bioethanol with acyl-moieties of coenzyme A with the potential to result in the immediate product of fatty acid ethyl esters (FAEEs).

2.4.3 Yeast and Fungi

Yeasts and fungi are also the suitable oil-producing microorganisms that accumulate lipids rich in polyunsaturated fatty acids (oleic acids, linoleic acids) together with frequently found other fatty acids (palmitic or palmitoleic acids). Some major oleaginous yeast like *Rhodosporidium*, *Rhodotorula, Cryptococcus*, *Candida*, *Lipomyces*, etc. are utilized for biodiesel production as they accumulate intracellular lipids and has many advantages over other renewable sources like high productivity (up to 65% of cellular dry weight) and less affected by seasonal variations for growth.

Similarly, some species of fungi are also capable of producing a great amount of lipids (approximately 70%) that includes *Humicolalanuginosa* and *Mucorcircinelloides* species. Various procedures are followed to form fungal esters and resulted in the formation of FAME that can be utilized as biodiesel. Thus, yeasts and fungi seem to be the potential alternative non-conventional source of energy (Nigam and Singh 2011; Dahman et al. 2019; Meng et al. 2009; Khan et al. 2020).

2.5 Bioethanol

Bioethanol, an important source of fuel, is produced using plant material or food crops containing large amounts of starch and sugars, such as corn, potato, sugarcane, and cassava. Ethanol is dominating the biofuel industry with an advantage of higher octane number and similar energy content and so serves as the most promising alternatives as a transportation fuel. It is sometimes combined with gasoline for fuel production or replaces gasoline to reduce carbon dioxide emission. It causes reduced emissions of toxic gases like sulphur oxide, carbon monoxide, nitric oxide, etc. due to low-temperature combustion as oxygen is present in its molecular form. Therefore, blending can significantly reduce greenhouse gas emissions.

2.5.1 Substrates for Bioethanol Production

There are several feedstocks which are utilized for bioethanol production, such as common crops like sugarcane, sugar beet, sorghum, etc.; and starchy feedstocks like

maize, wheat, etc. The major limitations of using feedstock are its cost, cost of enzymes and cost of ethanol recovery which ultimately results in higher production cost. So, attention is now given on the use of non-feedstock, agricultural wastes or lignocellulosic feedstocks, like stalks, sawdust, wood and bagasse, which can be used as a substrate for production of bioethanol by using advanced technology, in order to produce the fuel with least carbon emission and to eliminate food security issues (Ingale et al. 2014).

Lignocellulose is basically the part of the plant that remains undigested and it is a mixture of lignin, hemicellulose and cellulose that can be hydrolysed into sugar and fermented to ethanol directly. It contains hexose (glucose, galactose) and pentose sugars (xylose, arabinose). Sugarcane bagasse (the pulpy fibrous residue which is left after the sugarcane is crushed to extract the juice) is a by-product of sugar industry which is available in an abundant amount and rich in cellulose that can be hydrolysed for bioethanol production. Lignocellulose is the most abundant renewable resource and alternative for bioethanol production. As it covers the wastes that can be collected from agriculture, forest and industries, the land use for bioethanol production would be less. The use of lignocellulose has many advantages as it is less expensive and can be produced with lower input of fertilizers, pesticides and energy. On one hand, the production cost of bioethanol is affected by the purchase price of feedstock with lignocellulose being less expensive as compared with prices of corn, sugarcane, etc. On the other hand, the conversion cost of lignocellulosic biomass using current technology is high which in turn can be overcome by incorporation of improved and advanced technology (Jiang et al. 2017; Lamsal et al. 2015; Ramachandra and Hebbale 2020; Alfonsín et al. 2019).

2.5.2 Stages of Bioethanol Production

Production of bioethanol from lignocellulosic biomass is done in majorly three important steps—the first step includes the pretreatment of substrates while the next step is the saccharification process, i.e. converting polysaccharides present in biomass into fermentable sugars. Fermentation is the final step wherein conversion of released sugars into alcohol takes place by microorganisms in absence of oxygen. The bioethanol produced can then be finally purified by distillation (Figs. 2.2 and 2.3).

Pretreatment is the primary and important step in bioethanol production from lignocellulosic biomass. This process eases separation of complex carbohydrate molecules like cellulose, hemicellulose and lignin into their constituent simple sugars, thus, preparing the biomass for the hydrolysis. Therefore, pretreatment is necessary to make them available easily to the enzymes required for saccharification. The structure of cellulose (6-carbon sugar) and hemicellulose (5-carbon sugar) is complex and after depolymerization, it requires efficient microorganisms for bioconversion of fermentable sugars into bioethanol.

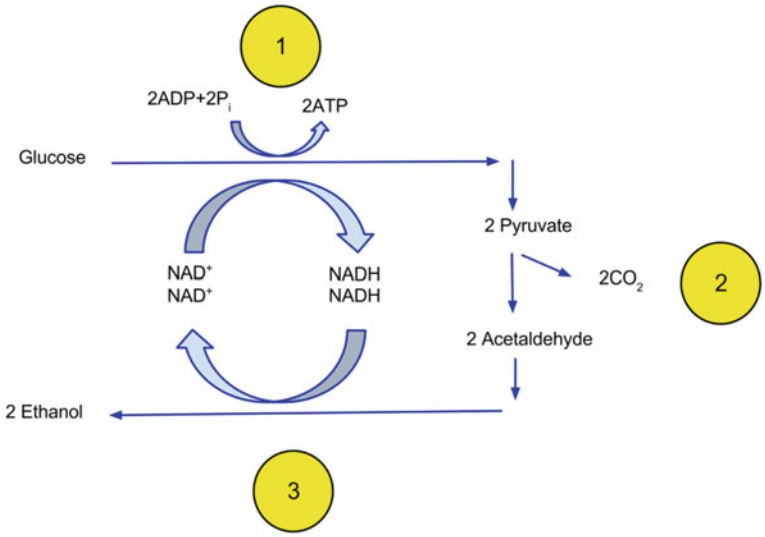

Fig. 2.2 Ethanol fermentation (Adapted from Open source)

Pretreatment of plant biomass can be achieved through various methods such as physical or physical-chemical method like steam explosion, ammonia fibre explosion, CO_2 explosion; chemical method, like ozonolysis, acid hydrolysis, alkali hydrolysis, organo-solvent; and biological method, like biomass treatment by fungus. Pretreatment process leads to reduction of crystallinity in cellulose, breakup of lignin, removal of hemicellulose, etc. depending on the pretreatment method used.

The most frequent method used is steam explosion as inclusion of sulphuric acid, sulphur dioxide or carbon dioxide in steam explosion can result in hemicellulose elimination completely and effectiveness of enzymatic hydrolysis can also be improved by preventing the production of inhibitory compounds. Hydrolysis rate can be increased by CO_2 explosion because of carbonic acid formation and degradation of lignin and hemicellulose can be achieved through ozonolysis. The acid hydrolysis is first diluted and then can be used for pretreatment process as concentrated acid is corrosive and damaging in nature and requires special reactors which are resistant to corrosion. Alkaline hydrolysis is also one of the methods which results in saponification of intermolecular ester bonds cross-linked and increase in the porosity of lignocellulosic biomass. Biological pretreatment processes carried out by various microorganisms such as fungi have many advantages as they require less energy, mild environmental conditions and produce various enzymes which attack cellulose, hemicellulose and lignin for their degradation; however, the hydrolysis rate is very low (Sun and Cheng 2002; Luque et al. 2016; Luque and Clark 2010; Rastogi and Shrivastava 2018; Lin and Tanaka 2006; Devarapalli and Atiyeh 2015; Peralta-Yahya and Keasling 2010; Balan 2014).

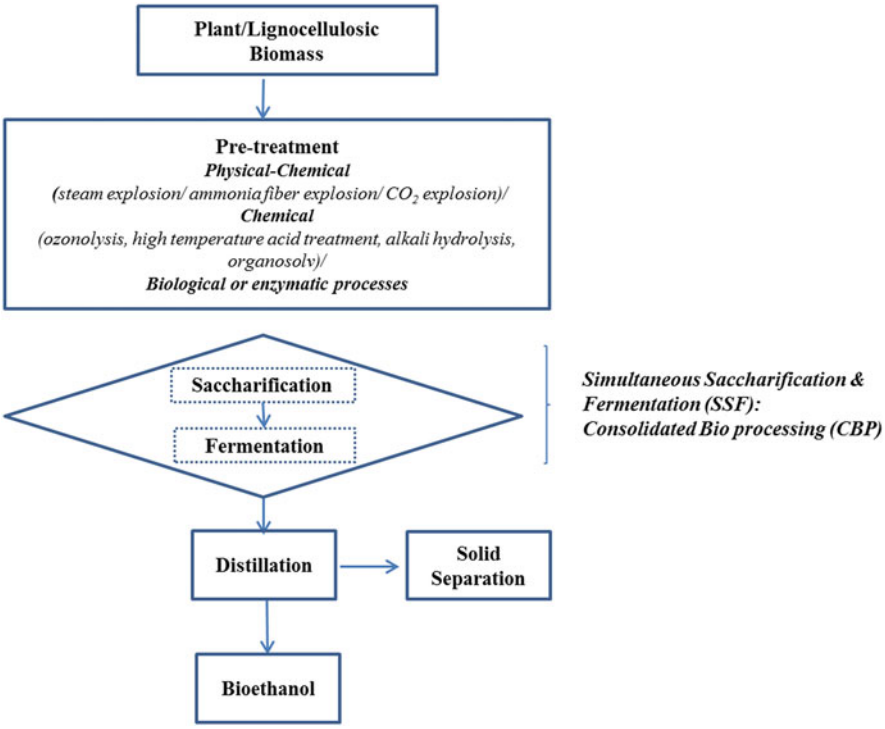

Fig. 2.3 Production of bioethanol from lignocellulosic biomass (adapted from Lin and Tanaka 2006; Balat et al. 2008)

2.5.3 Microbiological Production of Bioethanol

Enzymatic hydrolysis is a preferred procedure where cellulose is hydrolysed into fermentable sugars and it can be catalysed by cellulase enzymes. Production of cellulase enzymes can be done by bacteria (*Clostridium, Bacillus, Thermomonospora, Bacteriodes, Streptomyces*, etc.) and fungi (*Sclerotium rolfsii, Phanerochaete chrysosporium* and species of *Trichoderma, Aspergillus, Penicillium*). Several aspects like porosity, cellulose fibre crystallinity, lignin (which is not fermentable) and hemicellulose content, etc. affect the hydrolysis of cellulose. Thus, hydrolysis can be improved by reducing the cellulose crystallinity and removing lignin and hemicellulose with an increase in porosity during the pretreatment process.

The application of agricultural wastes or residues and bagasse (a by-product of sugar industry) to produce bioethanol has been shown in recent studies. The major drawback was the cost of cellulase enzymes used in the saccharification process, and ultimately leads to increased production cost. To achieve high product yield and reducing production cost, a lot of research has been conducted where enzyme

cocktails are used for better saccharification and engineered microorganisms are used so as to improve the yield. Various organisms (bacteria, yeast, mould) have been investigated for ethanol production, i.e. *Zymomonasmobilis; Corynebacterium glutamicum; Pichia stipites; Clostridium thermocellum; Clostridium phytofermentans; Saccharomyces cerevisiae* and *Escherichia coli*. Bioethanol, mainly produced by *Saccharomyces cerevisiae*, was thought to have the capacity to be used as an alternative fuel because it limits the emission of toxic gases. One of the disadvantages is its inability to use pentose sugars, resulting in less productivity. However, *Escherichia coli* and *Clostridia* are the alternatives for ethanol production as these organisms can use pentose as well as hexose sugars. *Clostridium* has the capability to produce 1-butanol during the butyrate and butanol-acetone anaerobic route that proceed at the same time of ethanol production.

Algae, which are regarded as a promising substrate for production of biodiesel, can also be used for bioethanol production as it contains sufficient sugar and protein components. The conversion of biomass into ethanol requires a group of microorganisms that produce the enzyme cellulase to hydrolyse the lignocellulosic biomass. In an effort to produce large amounts of energy, enzyme-producing microorganisms are immobilized on a stable surface for saccharification and fermentation of sugar to convert into ethanol with high levels of efficiency and in a cost-effective manner. In the case of brown algae, the absence of lignin results in comparatively easy saccharification which is a great advantage. However, the presence of alginate is one of the limitations that can be controlled by the utilization of alginate by using the metabolically engineered microorganisms (Dave et al. 2019).

Hydrolysis of lignocellulosic biomass can be achieved by hydrolytic enzymes so that microbes can utilize them for ethanol or butanol production, but their high cost causes hindrance in mass production of biofuels. However, some species of microorganisms namely *Neurospora, Monilia, Paecilomyces, Fusarium,* etc. have been reported recently for their capability of direct fermentation of cellulose into ethanol by the process of simultaneous saccharification and fermentation (SSF). Thus, an alternative approach could be the consolidated bioprocessing (CBP) as it offers cellulase enzyme production and cellulose saccharification along with fermentation by microbes in one sole step thereby eliminating enzymes production and purification steps.

Consolidated bioprocessing can be achieved by adopting two different strategies. One strategy is to use genetic techniques to increase the biofuel yield by cloning the cellulase coding sequences into those microorganisms that are incapable of utilizing cellulose directly. Microorganisms, thus, are genetically engineered to breakdown cellulose by creating new cellulase production systems, resulting in improved production and activity of enzymes and reduction in the cost. Another strategy is native cellulolytic strategy in which microorganisms are used for the efficient hydrolysis of cellulose, but with a limitation of low biofuel productivity. The process of fermentation is advanced and improved by the engagement of several mesophilic and thermophilic microorganisms, but compared to former ones, thermophilic microorganism present greater possibility in consolidated bioprocessing by achieving direct production of biofuel from lignocellulosic biomass with numerous

advantages which include increased hydrolysis rate, wider carbon sources, lesser microbial contamination, decrease in cooling costs and facilitating downstream product recovery. They can also be used for metabolic engineering as they provide many thermostable genes. Thermophile such as *Clostridium thermocellum* is an anaerobic CBP microorganism which is capable of degrading and fermenting crystalline cellulose to ethanol. One such anaerobic thermophile is *Thermoanaerobacterium saccharolyticum* that can form various fermentation products like ethanol, acetate, butyrate, carbon dioxide, hydrogen, etc. Development of new technologies has improved the bioethanol production and a concerted effort is required to further improve the developed technology and overcome the challenges which include maintenance of a stable performance by genetically engineered microorganisms. Development of new productive technologies is also required so as to achieve improvement in processing and utilization of lignocellulosic biomass (Sheoran at al. 1998; Soccol et al. 2019).

2.6 Microbiological Production of Hydrogen

Hydrogen is a colourless, odourless and most abundant gas present in the atmosphere. Hydrogen gas is considered as an ideal energy carrier. As a fuel it is one of the most promising fuels. It has high energy yield (120–142.9 MJ/kg) than other hydrocarbon fuels. Upon combustion there are no greenhouse gas emissions as water vapour is the combustion product. Its advantages are use of organic waste, less energy input, sustainability, reduced global carbon dioxide emissions, eco-friendly and also the efficiency as fuel in vehicles is 35–50% which is higher than the traditional fuels (Rojo 2008).

Biological hydrogen process is a process technology which involves the production of hydrogen using microorganisms. The process is more efficient as the reaction occurs at atmospheric temperature and pressure and consumes less energy.

2.6.1 Substrate Involved in Fermentation

Substrate particularly starch and/or cellulose-rich or carbohydrate-rich are required for biohydrogen production. Industrial effluents and biomass of dairy, bakery can be used as raw material.

Other substrates include agriculture products, waste biomass and lignocellulosic material.

2.6.2 Microorganisms Involved in Biohydrogen Production

Different microorganisms which are involved in the biohydrogen production are cyanobacteria, anaerobic bacteria and fermentative bacteria.

Anaerobic bacteria such as *Clostridia* sp. perform dark fermentation and produce H_2. Cyanobacteria are photosynthetic bacteria having basic nutritional requirements. They grow in presence of air, water, salts and light as energy source and carry out photoconversion of water to hydrogen. Fermentative bacteria are *E. coli*, *Citrobacter*, and *Enterobacter* which convert the substrate to hydrogen.

Different methods and pathways are available to produce hydrogen by the application of microbes (biologically); photofermentation, dark fermentation, biophotolysis, and microbial electrolysis cell. Dark fermentation is the fermentation of organic compounds of the biomass with the help of micro-organisms anaerobically. Biophotolysis is the application of phototrophic bacteria such as cyanobacteria and green algae that converts H_2O to H_2 and O_2 in the presence of sunlight. Photofermentation is by photosynthetic bacteria in the presence of light source convert H_2O to H_2 and O_2 (Fig. 2.4, Table 2.3).

$$\text{Biomass} + O_2 \rightarrow CO + H_2 + CO_2 + \text{energy}$$

2.6.3 Pretreatments for the Feedstock

Different pretreatment methods have been used for the feedstock before they can be used for biohydrogen production as these are not easily biodegradable. These

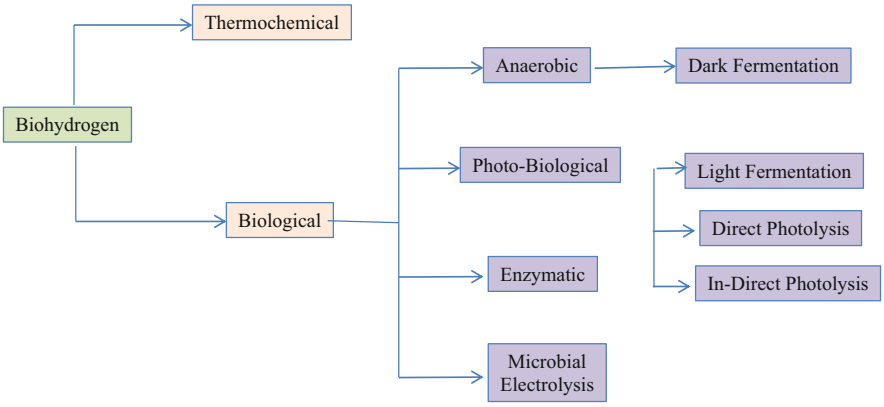

Fig. 2.4 Different modes of hydrogen production (Veeravalli et al. 2019; Srivastava et al. 2020a, 2020b)

Table 2.3 Different biohydrogen processes with their advantages and disadvantages (Saratale et al. 2019)

Process	Microorganisms	Advantages	Disadvantages
Biophotolysis	Blue green algae and Cynaobacteria	• Cheap source • Uses solar energy	• Oxygen inhibits the hydrogen production
Photo fermentation	Purple non-Sulphur photosynthetic bacteria	• Substrate is completely utilized • Waste effluents can be used	• Low volumetric rates of production
Dark fermentation	Strict anaerobes and facultative anaerobes	• High volumetric rates of production • Different substrates can be used	• Low yield of the product
Microbial electrolysis	*Geobacter*, *Pseudomonas* and *Shewanella*	• Complete substrate utilization	• Extra voltage needed • Expensive

methods will ensure the availability of the substrates and further increase the hydrogen production and yield.

Various methods which can be employed are physical, physicochemical, chemical and biological processes. Common physical methods which are used are wet and dry milling, however the processing cost is very high.

Chemical pretreatments include acid, alkali and organosolv solvents. Acid treatment is most commonly researched method and improves the accessibility of enzymes for fermentation. Alkali treatment is done to remove lignin from the lignocellulosic biomass. The main disadvantage of this method is of high processing cost.

Steam explosion and carbon dioxide explosion is one of the most widely researched physicochemical methods. It involves quick depressurization of pressure steam which leads to breakdown of lignocellulosic material to cellulose, hemicellulose and lignin.

Certain unusual chemicals such as N-methylmorpholine-N-oxide and cholinium taurate have also been used for pretreatment purposes (Veeravalli et al. 2019; Srivastava et al. 2020a, b).

2.6.4 Dark Fermentation

Hydrogen is generated from electrons through fermentation via 2 stages (1) oxidation of substrate to pyruvate and (2) pyruvate is converted to volatile fatty acids (VFAs) and alcohols.

Organic mass after pretreatment contains glucose which yields two molecules of pyruvate. This pyruvate moves into the acidogenic pathway that coupled with hydrogen production. Other pathways which generate hydrogen are acetic acid

pathway, butyric acid pathway, ethanol pathway, and acetone pathway; among all these, acetone and acetic acid pathways generate high amounts of biohydrogen.

Clostridium sp. is the genera which are important in dark fermentation which converts carbohydrate of the organic waste into hydrogen. *Clostridia* are obligate anaerobic and spore-forming organisms. The important genus of Clostridia, which are involved, are *Clostridium buytricum*, *Clostridium thermolacticum, Clostridium pasteurianum, Clostridium paraputrificum M-21,* and *Clostridium bifermentants*. Hydrogen gas is produced by the *Clostridia* sp. during the log phase of growth.

Another important genus responsible for hydrogen production is Enterobactericeae family. It metabolizes glucose by acid fermentation pathway and 2,3-butanediol fermentation pathways. In both the pathways, carbon dioxide and hydrogen are produced along with the other end products as ethanol and 2,3-butanediol.

Thermophilic organism such as *Caldicellulosiruptor saccharolyticus* produces high concentrations of hydrogen nearly about 83–100%.

Hydrogen gas is a common product in anaerobic bacterial fermentations and is an interesting by-product in future large-scale industrial fermentation (Antoni et al. 2007; Show et al. 2011; Srivastava et al. 2019; Shao et al. 2020).

2.6.5 Photofermentation

Photofermentaion is decomposition of organic compounds to hydrogen by the application of photosynthetic microorganism in the presence of sunlight. Photosynthetic bacteria include *Chlorobi*, *Proteobacteria, Chloroflexi* and *Heliobacteria*.

Most researches focus on hydrogen produced by purple non-sulphur bacteria because of high hydrogen production rate, ability of the purple non-sulphur bacteria to convert volatile fatty acids to CO_2 and H_2 and use different substrates such as glucose, sucrose and succinate.

The most extensively used purple non-sulphur bacteria in photo-fermentative hydrogen production are *Rhodobacter sphaeroides* O.U001, *Rhodobacter capsulatus*, *R. sphaeroides*-RV, *Rhodobacter sulfidophilus*, *Rhodopseudomonas palustris* and *Rhodospirillum rubrum.*

Two enzymes which are important in the hydrogen production are hydrogenase and nitrogenase. Nitrogenase enzyme is important in hydrogen production under anoxygenic conditions. It produces hydrogen gas from acetic acid by photofermentation (Fig. 2.5) (Argun and Kargi 2011) (Kapdan and Kargi 2006).

$$(CH_2O)_2 \rightarrow \underset{\uparrow \text{ATP}}{\text{Ferridoxin}} \rightarrow \underset{\uparrow \text{ATP}}{\text{Nitrogenase}} \rightarrow H_2$$

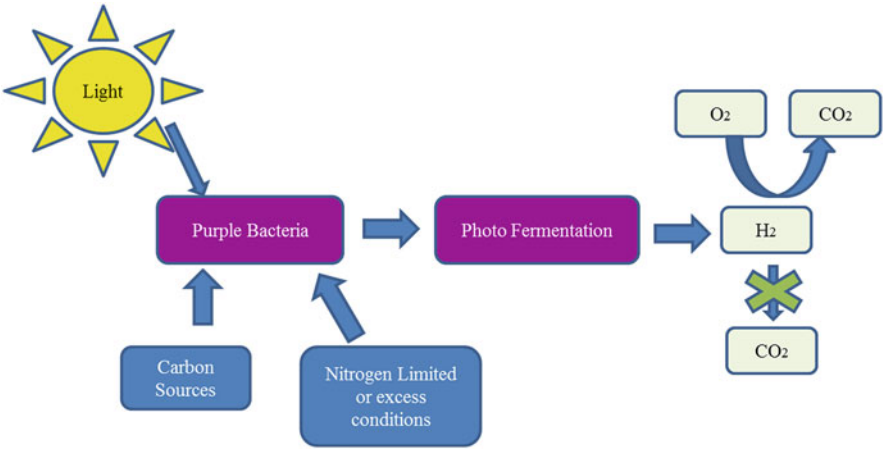

Fig. 2.5 Photofermentation (Kapdan and Kargi 2006)

2.6.6 Biophotolysis of Water Using Algae and Cyanobacteria

It uses the same principle of photosynthesis of plants. Photoautotrophic microorganisms such as cyanobacteria and algae are capable of generating hydrogen during photolysis process. It utilizes energy source as sunlight and a carbon source as carbon dioxide for the photolysis process. Biophotolysis is of two types: direct and indirect biophotolysis, depending on flow of electrons to hydrogen-producing enzymes.

Microalgae, blue green algae, cyanobacteria and seaweeds are capable of producing biohydrogen at certain conditions because they have hydrogenase enzyme.

Cyanobacteria is nitrogen-fixing bacteria, perform photosynthesis same as in plants. They occur in marine environment and soil ecosystems. Cyanobacteria performs autotrophic growth through photosynthesis. It is capable of biophotolysis, converting water to hydrogen and oxygen utilizing sunlight as a light source. Blue green algae metabolizes hydrogen by the action of two enzymes hydrogenase and nitrogenase enzymes. Nitrogenase enzymes catalyse the hydrogen production and reduction of hydrogen to ammonia. Hydrogenase finally produces hydrogen, however hydrogenase decreases hydrogen yield also by taking it up.

2.6.6.1 Direct Biophotolysis

Direct photolysis is an important and attractive process for biohydrogen production as energy utilized is solar energy which metabolizes the substrate to hydrogen and water. In direct biophotolysis, light is absorbed by PS-II to generate electrons, protons and oxygen molecules. The electrons generated in photosystem-II are transported to electron transport chain using solar energy to photosystem-I to

produce hydrogen. The flow of electrons is from photosystem-II to photosystem-I to ferridoxin and then to hydrogenase enzyme to produce biohydrogen. Hydrogenase enzyme produces hydrogen and oxygen as end products from substrate, oxygen produced in turn inhibits the hydrogenase activity which ultimately decreases the hydrogen production.

$$2H_2O \xrightarrow{\text{solar energy}} 2H_2 + O_2$$

Examples of cyanobacteria which have been able to produce biohydrogen are *Anabaena* sp., such as *Anabaena cylindrical* and *Anabaena variabilis.*

Chlorella vulgaris YSL01 and YSL16 strains of green algae can also produce hydrogen using oxygen and carbon dioxide as a source of carbon.

The main limitation of this method is the sensitivity of hydrogenase enzyme to oxygen which is produced during photosynthesis resulting in low biohydrogen yield. Other limitation is low light conversion efficiencies make it inefficient for large-scale bioreactors.

2.6.6.2 Indirect Biophotolysis

Indirect biophotolysis is in two steps: First, carbohydrates are produced through photosynthetic system and secondly, converted to hydrogen production under anaerobic conditions. The two different types of indirect biophotolysis are viz., temporal separation and spatial separation. This classification is dependent on separation of photosynthesis and fermentation processes. In temporal separation microorganisms producing hydrogen perform photosynthesis and accumulate carbohydrates under light radiation and then placed in dark and anaerobic condition for fermentation of endogeneous carbohydrate to release hydrogen. By this process, oxygen generated in presence of light does not inhibit hydrogenase enzyme during the next phase, i.e. in dark fermentation (Das 2001; Das and Veziroğlu 2001; Liu et al. 2008; Rout 2020).

Green algae, cyanobacteria, *Synechocystis, Synechococcus* sp. and *Gloebacter* sp. can perform indirect biophotolysis.

Heterocyst-forming cyanobacteria are able to form biohydrogen through spatial separation in indirect biophotolysis. These heterocysts are nitrogen-fixing cyanobacteria which are capable of providing an anaerobic condition for hydrogen production such as protecting the nitrogenase enzyme from oxygen inhibition (Kotay and Das 2008; Sindhu et al. 2019; Ding et al. 2016).

2.6.7 Hybrid System Using Photosynthetic and Fermentative Bacteria:

Hybrid systems comprise of two systems as dark fermentation with photo-fermentation process which involves both non-photosynthetic and photosynthetic bacteria. Dark fermentation produces hydrogen at high rates with simple reactor design along with the by-products as volatile fatty acids. These volatile fatty acids become substrate for the second stage in hybrid system as photofermentation which convert them to hydrogen. This will ultimately increase the hydrogen concentration and overall yield.

The combination of the two systems enables to have high hydrogen production yield and also decreases the amount of the light energy required for photosynthetic bacteria (Lam et al. 2019).

2.6.8 Microbial Electrolysis Cell

Microbial fuel cells convert organic substrate to electricity to exoelectrogens. Exoelctrogens are microorganism which convert organic substrate to electrons not hydrogen. Hydrogen can be produced by modifying microbial fuel cells to microbial electrolysis cells by adding small voltage to the fuel cell produced by bacteria and creating anaerobic conditions. Microbes will decompose organic substrate in the anode chamber and this anode act as an electron acceptor. There are two stages: first is dark fermentation in which microbes produces hydrogen and second is electrohydrogenesis.

Microbial electrolysis cell uses a wide range of organics as substrates along with microbes such as *Geobacter*, *Pseudomonas* and *Shewanella* spp. Microbial electrolysis cells generally give high hydrogen rate in terms of electron recovery (Singh and Das 2019).

Biohydrogen produced by different methods have advantages over thermochemical methods as they can be produced at less atmospheric temperatures and less energy-intensive, environment friendly but also utilized renewable energy resources (Das and Veziroğlu 2001; Merlin Christy et al. 2014; Rahman et al. 2015; Bardhan et al. 2019; Lazaro and Hallenbeck 2019; Usman et al. 2019).

2.6.9 Biohydrogen Production from Algae

Algae are third-generation renewable feedstock for the production of hydrogen. They are beneficial over other feedstock with the fact that it requires no land to be used and also they have high photosynthetic efficiencies. Algae are of two types: microalgae and macroalgae. Macroalgae are seaweeds mainly observed in marine

environment and are of three types: green, red and brown depending on the pigment composition. Microalgae are simple aquatic single-celled or multi-celled photosynthetic organisms and can be cultivated in fresh, sea and waste water. Microalgae is beneficial as high photosynthetic efficiencies will lead to the formation of organic compounds and this will further convert to hydrogen giving high production efficiencies (Saratale et al. 2019; Show et al. 2019).

2.7 Microbial Production of Biogas/Biomethane

Natural gas is a fossil energy source which consists of hydrocarbons mainly methane CH_4, it contains hydrocarbon liquids and non-hydrocarbon gases such as CO_2 and water vapour formed beneath the earth surface by buried plants and animal. Natural gas is extensively used as fuel in various operations like heating, generating electricity and industrial manufacturing of chemicals.

Biogas is a renewable energy source like solar or wind energy and used as an important substitute to natural gas. Biogas is produced biologically by anaerobic decomposition of organic wastes. It is an environment friendly fuel. Raw biogas consists of 60–65% methane, 30–35% carbon dioxide, small percentages of water vapour, hydrogen and hydrogen sulphide (Table 2.4). Further purification of raw gas leads to removal of H_2, H_2S and CO_2 to make it more pure and upgraded to be used as biomethane in the pipeline.

2.7.1 Feedstock for Biogas Production

All types of biomass containing proteins, fat and carbohydrates can be used as biomass for biogas production. Organic waste, agricultural waste, municipal biowaste, energy crops and industrial waste are common feedstocks used.

Anaerobic microorganisms during anaerobic decomposition degrade various organic compounds present in biomass such as carbohydrate, protein and lipids. Methane content in the biogas is dependent on the carbon content of the biomass. High lipid content is not preferable for biogas production. Micro- and macronutrients such as phosphorus, potassium, magnesium, and sulphur are important during

Table 2.4 Composition of biogas (Panwar et al. 2014)

S. no.	Component	Percentage (%)
1.	Methane	50–65
2.	Carbon dioxide	35–50
3.	Nitrogen	0–5
4.	Hydrogen	0–1
5.	Hydrogen sulphide	0–3
6.	Carbon monoxide	0–1

the growth of anaerobic microorganisms during methane production. Apart from micro- and macronutrients, trace elements such as iron, cobalt and nickel are also vital for cell growth. Nickle is very important as is required as cell component cofactor F_{430} which is involved in methane production. Iron is required as constituent of electron carriers. Zinc is another important trace element which is required for the synthesis of several enzymes. For optimal growth of methanogenic bacteria, the cells require cobalt to build up the Co-containing corrinoid factor III.

2.7.2 Biological and Chemical Process

Anaerobic decomposition is a complex biochemical process consisting of consecutive and interactive reaction carried out by anaerobic symbiotic microorganisms. It is the decomposition of biomass with the help of anaerobic microorganism. It is controlled by various factors such as relative humidity, temperature, pH, oxygen carbon dioxide concentration and substrates. Biogas is colourless, odourless gas and burns same as liquefied petroleum gas to give same blue flame. Bacteria and methanogenic Archaea are involved in the anaerobic decomposition of the organic matter of the feedstock for biogas production. The four key biochemical stages of anaerobic decomposition are hydroloysis, acidogenesis, acetogenesis and methanogenesis (Figs. 2.6, 2.7, and 2.8).

2.7.3 Hydrolysis

The organic polymers present in the feedstock proteins, lipids carbohydrates are hydrolysed to its monomer units and smaller units by the action of the various hydrolytic microorganisms such as *Clostridia, Micrococci, Bacteroides, Butyrivibrio, Fusobacterium* and *Selenomonas* sp. These microorganisms secret

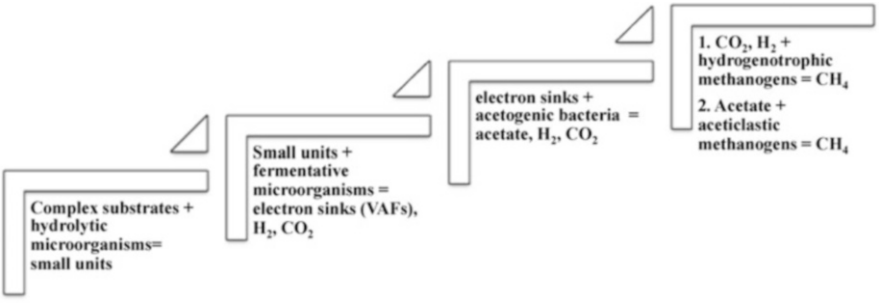

Fig. 2.6 An overview of microbial processes involved in anaerobic digestion (Adapted from Caruso et al. 2019)

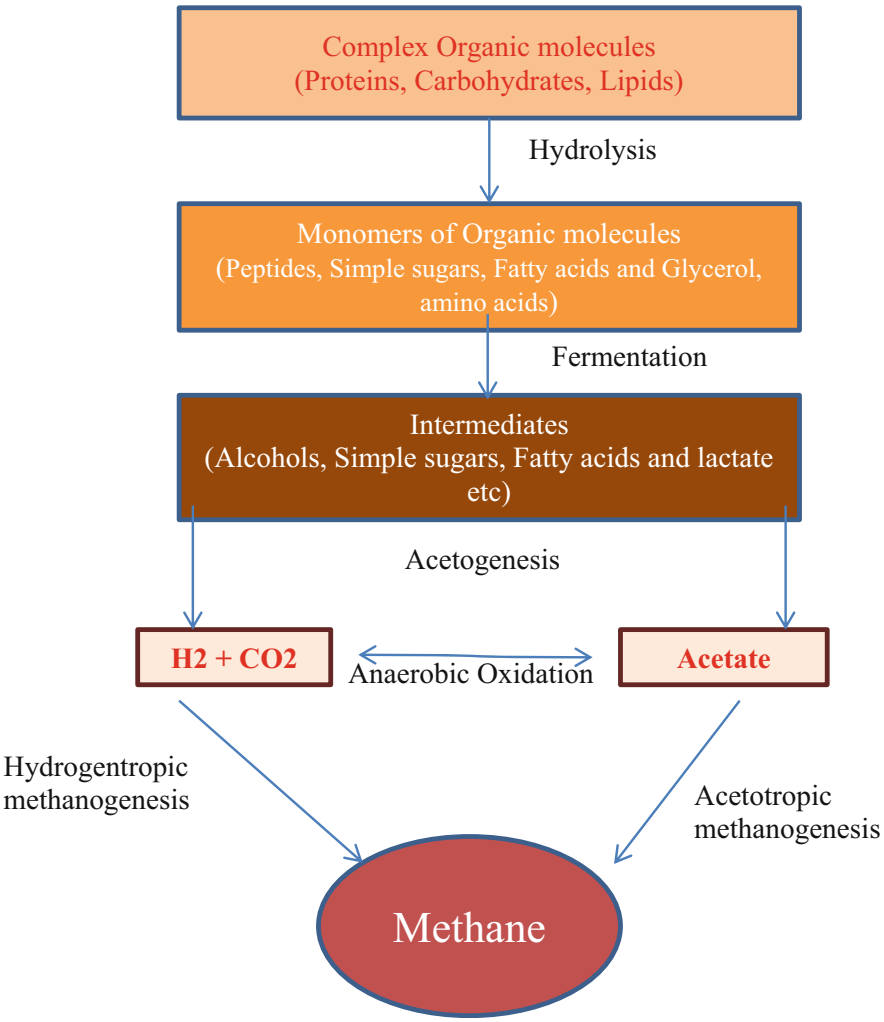

Fig. 2.7 Biogas decomposition from organic matter (Caruso et al. 2019; Panwar et al. 2014)

extracellular enzymes which hydrolyse the polymers. This step occurs in two phases firstly the bacteria colonise on the surface of the soil and these anaerobic microorganisms release extracellular enzymes and produce monomers, the second phase is these organisms utilize monomers as substrates.

The anaerobic decomposition of the lignocellulosic matter is a limiting factor. Lignocelllulosic matter has to be pretreated with acid or alkali or any other cost-effective method.

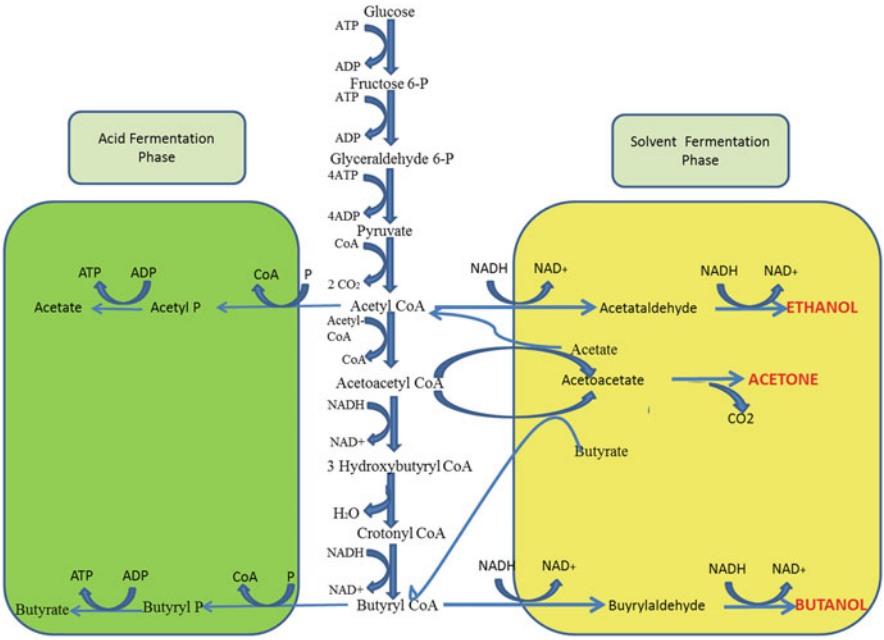

Fig. 2.8 ABE fermentation pathway by *Clostridia* (Adapted from Open Source Ecology)

2.7.4 Acidogenesis

Among all the biochemical reactions in the biogas production, acidogenesis usually is the fastest reaction. Acidogenic microorganisms which are involved are *Streptococcus*, *Lactobacillus, Bacillus, Escherichia coli* and *Salmonella* which decompose fatty acids, sugars and amino acids. The fast reaction rate is due to the high growth rate of the acidogenic bacteria and another important characteristic is that they can tolerate low pH up to 5. End products of acidogenesis are alcohols, carbon dioxide, hydrogen, ammonia, and organic acid such as propionic acid, butyric and acetic acid. Organic acid produced from sugars leads to decrease in pH which is favourable for the growth of acidogenic and acetogenic bacteria. Amino acids also serve as energy source for the growth of the facultative fermentative bacteria. Concentrations of butyric and acetic acid are important in the acidogenesis stage as they are the precursors of the methane formation.

2.7.5 Acetogenesis

Acetogenic bacteria are strict anaerobes with an optimum pH of 6. These are slow-growing bacteria and sensitive to changes in organic load. Acetogenesis is referred as degradation of lactate, ethanol, propionate, butyrate and higher volatile acids by obligate hydrogen-producing acetogenic bacteria as these cannot be hydrolysed by methanogens. Acetogenic bacteria are generally *Syntrophomonas wolfeii* and *Syntrophobacter wolinii.* This step is very important with respect to the production of biogas. Acetogens make syntrophic association with the methanogens as they require partial hydrogen pressure for their degradation.

2.7.6 Methanogenesis

In methanogenesis, the methane is produced by two different types of methanogens, both of which produce methane from different substrates. One group is acetoclastic methanogens which convert acetic acid to methane. Other groups are hydrogen utilizing methanogens which convert hydrogen and carbon dioxide to methane. Methanogenic organisms belong to Archaea. The hydrogenotropic methanogenesis is the most common metabolic pathway. The hydrogen utliizing methanogens *such as Methannospirillum hungatei, Methanoculles receptaculi* are fast growing than the acetoclastic methanogens, e.g. *Methanosarcina thermophile* that is the reason hydrogen utilizing methanogens are more common (Merlin Christy et al. 2014; Guo et al. 2015; Bhatia et al. 2017; Srivastava 2020).

The key features of the biogas produced by anaerobic digestion are it does not deplete fossil fuels, does not contribute to greenhouse emissions, and uses low cost digesters.

Biogas is composed mainly of methane, carbon dioxide along with small quantities of hydrogen sulphide, carbon monoxide and water vapour. Removal of water vapour and hydrogen sulphide is important before its use. Biogas finds applications in heating, fuel cell, dual engines and power systems (Weiland 2010; Klocke et al. 2008; Caruso et al. 2019; Tsavkelova and Netrusov 2012).

2.8 Microbial Production of Butanol

Biomass-derived alcohols such as biobutanol are next most sustainable alternative to internal combustion engines as it has similar energy density of 30 MJ/L and same octane number (97–103) as gasoline (energy density of 33 MJ/L, octane number as 90–105). Butanol has four carbon alcohol with the molecular formula $C_4H_{10}O$. Biobutanol has been considered as high heating value fuel because of long chain. Butanol has following benefits over other fuels; energy density is

relatively high among other gasoline alternatives, lower Reid vapour pressure (RVP is used to measure volatility of gasoline and other petroleum products) leading to decrease volatility and emissions, increased energy security to be able to produce from a large variety of feedstock, fewer emissions (Al Makishah 2017).

It is less corrosive than bioethanol, gets easily mixed with gasoline, less evaporative than ethanol and also generate less emissions. It is less hygroscopic and less hazardous. Among the different isomers n-butanol is a fermentative product (Jiang et al. 2019; Tigunova et al. 2020).

2.8.1 Feedstock for Biobutanol Production

Biobutanol is produced from substrate; sugar, starch or lignocellulosic biomass by the process of microbial fermentation. Commercially butanol is produced from different feedstock such as starch/sugar-based crops, corn starch, beet molasses and cassava.

2.8.2 Microorganisms Involved in Butanol Production

Microalgae is another important feedstock used for biobutanol production. Microalgae usage has advantages over other feedstock as high efficiency and simple photosynthetic nutritional requirements. Microalgae with high concentrations of carbohydrates are required for butanol production as glucose is the primary substrate for its production. The production of the butanol is by two-stage cultivation of microalgae; first stage will be increasing biomass concentration by providing high amounts of nutrients and second stage involves limiting the nutrients and increasing the carbohydrates which in turn leads to production of butanol. Limiting nutrient is generally sulphur which helps to accumulate carbohydrate. Other factors which effect the butanol production from algae are light, osmotic stress and availability of carbon dioxide (Gottumukkala et al. 2019).

The different strains used in this fermentation are *Neochloris aquatic* CL-M1, *C. acetobutylicum* ATCC 824, 55025, etc. *C. acetobutylicum* is hyper-butanol producer and able to produce 19.1 g/L butanol utilizing glucose as substrate. *C. beijerinckii* BA101 is another strain of Clostridium which is hyper-butanol producer producing 19 g/L butanol in synthetic media. Clostridia utilize various sugars such as glucose, sucrose, xylose, etc. for production of butanol.

2.8.3 Production Process

Butanol is produced through a unique fermentation process called as acetone-butanol-ethanol (ABE) fermentation. ABE fermentation pathway is the typical feature of the genus *Clostridium* and certain strains involved in butanol production by this pathway are *Clostridium acetobutylicum* (Weizmann's organism), *Clostridium saccharoperbutylacetonicum* and *Clostridium beijerinckii. Clostridium* sp. is a rod-shaped organism with anaerobic growth capable of forming heat-resistant endospores and having G+ type of bacterial cell wall. During fermentation three major products are formed viz., acetone, butanol and ethanol are produced in 3:6:1 ratio (Green 2011; Pugazhendhi et al. 2019).

2.8.4 Pretreatment Process

Pretreatment is an important step before the fermentation. Pretreatment converts cellulose to glucose and various other sugars which is ultimately utilized during the fermentation and govern the fermentation efficiency.

First-generation feedstock generally employs simple pretreatment of biomass as this feedstock has high concentration of sugar and also this is easily accessible. Acid treatment, heat sterilization and deproteinization can be used to treat first-generation feedstock.

Second-generation feedstock is more complex as compared to others as they have high amount of lignin, cellulose and hemicellulose which requires comprehensive pretreatment so as to release the sugars for the fermentation process.

Third-generation biomass is less complex and is a good substrate for biobutanol production.

2.8.5 Physical Treatment

Physical methods employed for pretreatment are milling such as wet milling and dry milling, microwave, pyrolysis, etc. The main objective of physical method is to reduce the particle size of the feedstock which ultimately leads to increase in the surface area. Increase in surface area further increases the efficiency of the process; however, the disadvantage of the physical methods is that it is a high energy consuming process.

2.8.6 Physicochemical Method

Different physicochemical pretreatment methods are steam explosion, liquid hot water, ammonia fibre explosion, and carbon dioxide explosion.

Steam explosion is one of the most sustainable methods among all physicochemical methods as drawback with other methods is that it leads to lignin solubilization. Steam explosion method involves two steps: auto-hydrolysis which allows the formation of acetic acid at high temperatures and de-pressurization will break the bonds present in the complex structure. Major drawback of this method is that due to incomplete hydrolysis of lignin carbohydrate complex it leads to the formation of inhibitory compounds.

2.8.7 Chemical Method

Chemical pretreatment methods have been useful lignocellulosic biomass. The various methods are acid pretreatment, alkali pretreatment, and organosolv pretreatment. Acid pretreatment method employs the use of dilute acid. This method is most preferred for non-cellulosic feedstock with no lignin content. Alkali pretreatment can be used for biomass with high lignin content, however it requires longer time for release of sugars. Organosolv process involves the use of organic solvent which aids in hydrolysis of lignin (Kushwaha et al. 2019).

2.8.8 Production Process

Acetone-butanol-ethanol (ABE) fermentation process takes place in two phases: acidogenesis and solventogenesis (Fig. 2.7).

In the first phase of acidogenesis, *Clostridia* which is a solvent-producing heterofermentative ananerobic organism exponentially grows and produces different end products such as butyrate, acetate, carbon dioxide and hydrogen with minute quantities of acetoin and lactate. Pyruvate is produced from the sugar which acts as the carbon source via glycolytic pathway and converted to acetyl Co-A and carbon dioxide using pyruvate:ferredoxin oxidoreductase enzyme. Two molecules of acetyl Co-A will combine together to form butyl Co-A with the use of different enzymes thiolase, 3-hydroxybutyryl-CoA dehydrogenase, crotonase, and butyryl-CoA dehydrogenase.

Acetyl Co-A and butyl Co-A are important intermediate compounds in the formation of acid and solvents during the acidogenesis and solventogenesis. During glycolytic pathway, ATP and NADH are generated. ATP which is produced is essential for the growth and for recontinuation of the glycolytic pathway for the continuous cycle NADH is reoxidized to NAD+.

Next phase is the solventogenesis which starts with the completion of the log phase or the exponential phase of growth of microorganisms and also the acid-producing pathways (acidogenesis) get converted to solvent producing pathways (solventogenesis).

Important factors which effect the solventogenesis are pH drop, acid products, temperature, oxygen and nutrient limitation as this is the end of exponential phase. During the initiation of solventogenesis, acetyl Co-A and butyryl Co-A are converted to ethanol and butanol by respective dehydrogenases (Amiri and Karimi 2019).

The main limitation of *Clostridia* sp. during the ABE fermentation and butanol production is that the growth is repressed by increase in the concentration of butanol produced during the fermentation.

The second phase of fermentation process is limited by a number of factors such as inhibition of substrate, toxicity of butanol, slow growth of the microorganisms and all these factors leading to lower cell density in the medium.

There are certain challenges with respect to the butanol production by *Clostridia* sp. which needs to be worked upon which are low yield, cost of substrate, low productivity of butanol because of inhibition and energy-intensive process for recovery (Pratto et al. 2020; Cao et al. 2016; da Conceição Gomes et al. 2019; Bardhan et al. 2019; Tsai et al. 2020).

2.8.9 Applications

Biobutanol is used in the production of fuels for spark ignition engines (internal combustion engines) along with the gasoline as it is miscible with gasoline. It is generally used directly as fuel in automobile engines. It is a non-toxic, non-corrosive and biodegradable fuel which do not pose any environmental impact. The primary use of biobutanol as fuel is in the spark ignition engines (internal combustion engines) because of the fact that biobutaol has higher energy that bioethanol because of the higher number of carbon atoms in butanol which gives higher energy, high polarity and high combustion values. It is non-hygroscopic in nature which makes it as safe to store product. Apart from this it can also be used as industrial solvent and chemical feedstock. Other applications include pharmaceuticals, resins, herbicides, paints and coatings (Dharmaraja et al. 2020; Patakova et al. 2011; Sindhu et al. 2019; Tigunova et al. 2020; Verardi et al. 2020).

2.9 Syngas Fermentation

Syngas is mainly composed of carbon dioxide, hydrogen and carbon monoxide. It can be majorly produced by two ways which are Fischer-Tropsch (FT) synthesis using metal catalysts and microbiological fermentation. The feedstock which are preferred are lignocellulosic feedstock, agriculture residue and agriculture

Table 2.5 Composition of syngas (Dharmaraja et al. 2020)

S. no.	Gas	Percentage (%)
1.	Carbon monoxide	30–60%
2.	Hydrogen	25–30%
3.	Carbon dioxide	5–15%
4.	Methane	0–5%

by-products and also forest residues. Fischer-Tropsch (FT) process is the most common process for the synthesis of syngas to liquid fuels such as bioethanol. The process requires application various metal catalysts such as cobalt, nickel, etc. The main limitations of syngas fermentation by Fischer-Tropsch (FT) process are the application of expensive catalysts, sensitivity of the catalysts with the inert gases such as sulphur and high pressure.

Biological method of fermentation of syngas leads to production of alcohols and organic acids with the use of microorganisms. The main advantages of biological production of syngas over FT process are the low reaction temperature, less sensitivity to inert gases, high specificity in terms of microorganisms and no metal poisoning. Composition of syngas is shown in Table 2.5 (Wu and Tu 2016; Yasin et al. 2019).

2.9.1 Microorganisms Involved

Soil and intestinal tract of animals contain the acetogenic bacteria, for example, *Clostridium ljungdahlii* and *Clostridium carboxidivorans* which have been isolated and used for biological production of biofuels such as syngas. These organisms are chemolithotrophic (chemolithotrophic means that the energy is obtained from the oxidation of inorganic compounds) and utilize one carbon compounds such as carbon monoxide and produce methanol and butanol. Other acetogens are *Clostridium ljungdahlii, Clostridium autoethanogenum, Eubacterium limosum, Clostridium carboxidivorans P7, Peptostreptococcus productus* and *Butyribacterium methylotrophicum* (Anggraini et al. 2018).

2.9.2 Fermentation

Syngas fermentation leads to the production of ethanol and butanol. Acetyl Co-A enzyme is one of the key enzymes in the biological processes. Acetyl Co-A is an intermediate metabolite which synthesizes complex compounds and yields acids and alcohols. Acid which is produced supplies energy for the synthesis of cell mass. The ability of acetogenic bacteria to convert acids to alcohols is the fundamental rule for biofuel production.

The biological pathway involved in the syngas fermentation is Woode Ljungdahl pathway, which is also termed as acetyl-CoA pathway. The Woode Ljungdahl pathway controls the reaction of carbon monoxide and hydrogen to produce two products as acetate and acetyl-CoA. Woode Ljungdahl pathway has two branches which are methyl branch and carbonyl branch and forms intermediate acetyl CoA depending upon the type of substrate.

In methyl branch, the formate dehydrogenase enzyme reduces carbon monoxide to formate. Formate reacts with tetrahydrofolate using a formyl-THF synthetase enzyme with an consumption of ATP, producing formyl-tetrahydrofolate. Methyl-tetrahydrofolate is reduced to formyl-tetrahydrofolate by the application of different tetrahydrofolate-dependent enzymes such as methenyl tetrahydrofolate cyclohydrolase, methylene-tetrahydrofolate dehydrogenase, and methylene-tetrahydrofolate reductase. Then, the methyl group is transferred to a corrinoid iron-sulphur protein. Lastly, the enzyme complex acetyl CoA synthatase catalyses the formation of acetyl CoA.

Acetic acid formation:

$$4\ CO + 2\ H_2O \overset{\text{yields}}{\rightarrow} CH_3COOH + 2\ CO_2$$

$$2\ CO_2 + 4\ H_2 \overset{\text{yields}}{\rightarrow} CH_3COOH + 2\ H_2O$$

Ethanol formation:

$$6CO + 3H_2O \rightarrow C_2H_5OH + 4CO_2$$

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

In the carbonyl branch of the Woode Ljungdahl pathway, carbon dioxide dehydrogenase enzyme reduces carbon dioxide to carboxyl group. Carboxyl group reacts with the methyl group to produce acetyl-CoA, which undergo further series of reactions to produce biofuels. In the fermentation phase, alcohol dehydrogenase enzyme catalyses the reaction of acetaldehyde to alcohol which is ethanol. In the complete process, first is the non-growth phase where acetyl-CoA converts to ethanol, otherwise the acetyl-CoA converts to acetate (Bengelsdorf et al. 2013; Munasinghe and Khanal 2010; Caruso et al. 2019).

Syngas fermentation is one of the promising biofuel technologies because of several advantages such as no pretreatment is required for biomass substrates, can tolerate high amounts of sulphur compounds, high reaction specificity, cost-effective process and CO/H_2 ratio is flexible. However, there are some limitations also such as low volumetric productivity and sensitivity to organisms.

2.9.3 Application

The key benefits of syngas are high reaction specificity, fermentation process can occur at low temperature and pressure also can tolerate compounds with high sulphur content. However, the limitations are that the reaction gets inhibited due to growth of microorganisms and has low volume productivity. Syngas can be used to produce different kind of products such as fertilizers, solvents and biofuel.

For example,

1. Electricity generation
2. Nitrogen in pressure cylinders and fertilizers
3. Ammonia for production of polyurethran and nylon
4. Methanol for production of pharmaceuticals, paints, resins, etc.
5. Diesel production

2.10 Conclusion

Biofuels have turned out to be an attractive replacement of fossil fuels as they are renewable, sustainable, environment friendly, cost-effective/ economic and contributes less greenhouse emissions. Microbial biofuel production will have a great role in the coming years as it will increase fuel yield along with reduction in the nature conservation. Microorganisms convert feedstock and into different types of biofuels. Production cost and chemical transformation is a costly process. Biotechnological approach in the application of microbes for biofuel production is important as it will decrease the processing cost, increase the feasibility of the process, strengthening the global economy as well as make it more viable in terms of the commercial process.

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Chapter 3
Influence of Significant Parameters on Cellulase Production by Solid-State Fermentation

M. Subhosh Chandra, P. Suresh Yadav, Pallaval Veera Bramhachari, and Narasimha Golla

Abstract Cellulases become an area of unique attention in bioremediation methods owing to their capability to breakdown cellulose. Development of cost-effective, high titer of attractive enzymes by fungi is a challenge. The overproduction of dynamic enzymes which cut various β-1,4-glycosidic bonds still wreck a challenge and is the key blockage for the cellulosic biomass transformation. Microbes are an eye-catching topic for production of cellulases because of their enormous prospective for production of cellulase, enzyme intricacy, and severe habitation variability. Microbial cellulolytic enzymes are ideal because of their immense advantages in number of industries. In fact, trend for cellulolytic enzymes is undeniably expanding for their use in bioremediation, pharmaceuticals, pulp and paper, waste management, food processing, and so on. Future research is ensuing into enhanced scientific information in addition to the achievement of summit of the rising demands of cellulase and associated enzymes for production of eco-friendly textiles, detergents, bio-pulping, and bio-alcohols. Furthermore, it is opening novel paths for exploitation of a variety of agricultural residues and pollutants as a basis of renewable energy in lieu of throwing away to cause environmental degradation. In years to come, newest knowledge of outstanding cellulolytic enzymes and acceptance of various biotechnological approaches will undoubtedly bring immense vision in the field of green chemistry. Hence, the present book chapter focused on fungal cellulases in bioremediation and factors affecting cellulases production by solid-state fermentation (SSF).

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Keywords Cellulolytic enzymes · Lignocelluloses · Process parameters · Solid-state fermentation

3.1 Introduction

The rising of energy requirement and ecological evils reasoned by utilization of nonrenewable fossil fuels, it has become a burning need to set up substitute energy sources. Bioethanol generation by cellulosic substrates is anticipated as an option for sustainable reserve for renewable fuel, which can alleviate the pressure of energy calamity, and also assist to decrease greenhouse gas discharge (Pervez et al. 2014). Lignocellulosic residues contain cellulose; hemicellulose and lignin bound each other in a composite structure. Attributable to complex structure of the cellulose is in fact challenging to microbial and enzymatic activity. Cellulose is a most copious, renewable biopolymer, and is a cheap energy source (Zhang et al. 2009). However, utilization of this natural biopolymer for production of useful materials through saccharification process primarily relies on the action of cellulolytic enzymes and also the cost of cellulase enzyme. The high cost of cellulases is the major hurdle for profit-orientation of biomass, biorefineries (Zhang et al. 2006; Zhu et al. 2009). Moreover, production of bioenergy and its products from cheap renewable lignocellulosic biomass would bring gain to local financial system, environment, and public energy security (Zhang 2008). The booming strategy is to enhance the cellulolytic enzymes production by using hyper cellulase-producing strains or locally existing cheap raw material and optimizing culture conditions. Nevertheless, the factors affecting lignocellulose-degrading enzyme knowledge are indispensable for understanding the optimal conditions that take place in unique circumstances. This chapter primarily emphasizes on factors affecting cellulolytic enzymes production in SSF from fungal sources.

3.2 Cellulose

Cellulose is a widespread polymer of plant cell walls that was first documented by "Anselm Payen" in 1838. It happens in nearly pure form in cotton fiber and in mixture by other substances, including lignin and hemicelluloses, in forest biomass, plant leaves, and stalks. It was accepted that cellulose is a polymer that consists of repeating units of glucose, a simple sugar. They detached from connected plant substances that take place in mixture with cellulose by dissolving them in concentrated sodium hydroxide. They referred undissolved remains as an α-cellulose. The soluble materials (referred as β-cellulose and γ-cellulose) were afterward shown not to be celluloses, but rather, comparatively simple sugars and other carbohydrates. The α-cellulose of Cross and Bevan is what is frequently meant when the name "cellulose" is applied at present.

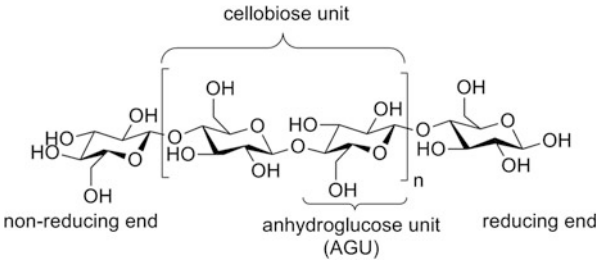

Fig. 3.1 Chemistry of cellulose chain

Cellulose is the most abundant renewable carbon source and a potentially vital source for production of industrial products (Muthuvelayudham and Viruthagiri 2006, 2007). Enzymatic hydrolysis is an economic process in the conversion of cellulose to simply sugars (Kotchoni et al. 2003). Native cellulose is an unbranched homo-polysaccharide consisting of D-glucose residues linked by β-1,4-glucosidic bonds to form a linear polymer chain (Fig. 3.1). The smallest repetitive unit in cellulose is cellobiose, which contains two glucose units. Cellulose is regarded as an expensive reserve hugely due to it decomposed into soluble cellobiose and glucose when β-bonds are breakdown (Dorland Newman 2003). This method is known as "cellulose hydrolysis." Pure cellulose is commercially available in several forms, which include cotton, filter paper, avicel, etc., and these forms are normally used as substrates to assess the efficiency of whole cellulase systems. However, their physical heterogeneity complicates enzyme studies.

3.3 Cellulases

Cellulases have a broad variety of applications in bioenergy in specific biofuel. Cellulase enzyme consists of three main constituents, viz., endoglucanase, exoglucanase, and β-glucosidase efficiently alter lignocellulosic substrates to fermentable sugar. The production of cellulase is mainly by two approaches, i.e., submerged fermentation (SmF) and solid-state fermentation (SSF). However, SmF is expensive and less profitable for biofuels production. In addition, microbial cellulase production endures from different obstacles. Since the low cost, production of cellulase using SSF by fungi is most advantageous. Cellulose is mainly present in lignocellulosic substrates and cellulases (endoglucanase and exoglucanase) can hydrolyze it to cellobiose, which can be changed to glucose by β-glucosidase (Fig. 3.2). The major parameters that influence the cellulase production in SSF is as follows:

Cellulases are differentiated by an array of enzyme components whose correct number differs from organism to organism. For instance, all three classes of enzymes were identified and occur in multiple and isozymic forms, though the number of isozymic forms produced by various species or even strains of same species can vary (De Vries and De Visser 2001). Three exoglucanases (Exo I, Exo II, Exo III) have

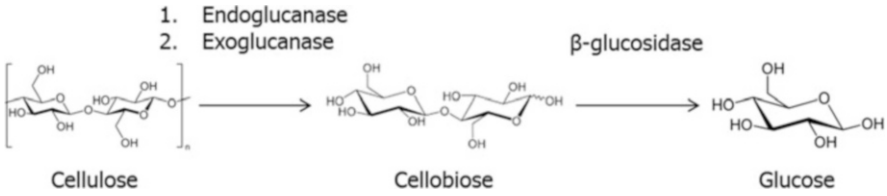

Fig. 3.2 Mechanism of cellulose degradation by cellulase

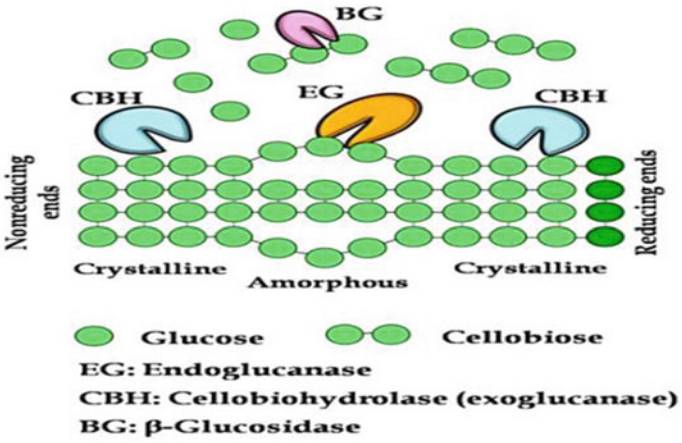

Fig. 3.3 Efficiency in cooperation of members of cellulase enzyme system

been purified from *Aspergillus nidulans* (Bagga et al. 1990). Two cellobiohydrolases have been identified in *Aspergillus ficuum* (Hayashida and Mok 1988) and *Aspergillus terreus* (Ivanova et al. 1983). Similarly, two immunologically dissimilar cellobiohydrolases (CBH I and CBH II) were identified in *Trichoderma* spp. specifically *reesei* (Kubicek and Pentilla 1998). The organization of native cellulose and its hydrolysis by different endoglucanases and cellobiohydrolases is demonstrated schematically in Fig. 3.3. These enzymes operate in a mutualistic approach. The endoglucanase breaking linear cellulose polymers, generating reducing and non-reducing ends that, in turn, can be breakdown by exoglucanase. Exoglucanases, in turn, work to eliminate cellulose and open more internal sites for endoglucanase binding.

The role of these two enzymes, particularly CBH, is restrained from cellobiose. The breaking of cellobiose to glucose using β-glucosidase very much decreases this inhibition and permits continuous cellulase activity. This standard synergism among exoglucanase and endoglucanase was extended to cover a variety of cellulolytic fungi (Wood et al. 1995; Wood and McCrae 1996). Still, synergistic interactions (exo-exo) among isozymic forms of exoglucanase happened in solubilizing crystalline cellulose (Henrissat et al. 1985).

3.4 Composition of Lignocelluloses

Lignocellulosic substrates are a major sustainable reserve, consisting of about 50% of plant matter generated by photosynthesis and most copious renewable organic matter which is a renewable resource (Gavrilescu 2004; Gavrilescu and Nicu 2004). It comprises of three kinds of components, cellulose, hemicellulose, and lignin that are strongly connected and chemically bound by non-covalent and covalent cross-linkages. The main part is cellulose, followed by hemicellulose and lignin (Fig. 3.4). The composition and percentages of these compounds differ among plants and the chemical composition of some of lignocellulosic materials is presented in Table 3.1 (McKendry 2002; John et al. 2006; Prasad et al. 2007; Carmen 2009).

3.5 Influence of Important Parameters on Production of Cellulase

3.5.1 Lignocellulosic Substrates

The unique characteristic of lignocellulosic substrate used is the significant thing that affects SSF and its assortment relies on various factors typically allied with the cost and accessibility of solid substrate and as a result engages the screening of numerous agricultural wastes. Cellulolytic enzymes production is affected by different parameters like pH, temperature, nature of substrates incubation time, etc. (Srilakshmi and Narasimha 2020). The solid biomass not only provides nutrients to the organism but also acts as a harbor to microbial cells (Vastrad and Neelagund 2011). The particle size and moisture content are the most vital, among numerous factors, which are key

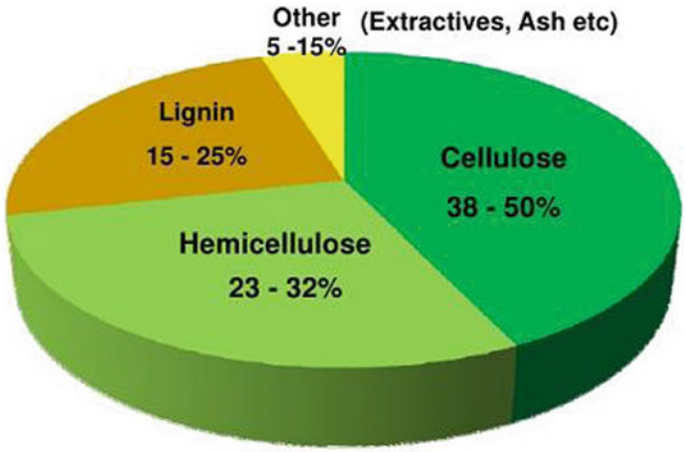

Fig. 3.4 Composition of various components of lignocellulosic feedstock

Table 3.1 Cellulose, hemicellulose, and lignin content in lignocellulosic materials

Lignocellulosic materials	(% of total dry weight)		
	Cellulose	Hemicellulose	Lignin
Hardwood steams	40–50	24–40	18–25
Softwood steams	45–50	25–35	25–35
Wheat straw	33–40	20–25	15–20
Grasses	25–40	35–50	10–30
Corn corbs	45	35	15
Nut shells	25–30	25–30	30–40
Paper	85–99	0	15
Switch grass	30–50	10–40	5–20
Sorted refuses	60	20	20
Leaves	15–20	80–85	0
Waste paper from chemical pulps	60–70	10–20	5–10
Cotton seed hair	80–95	5–20	0
Primary waste water solids	8–15	NA	NA
Solid cattle manure	1.6–4.7	1.4–3.3	2.7–5.7
Sugar cane bagasse	25–45	28–32	15–25
Rice straw	29.2–34.7	23–25.9	17–19
Corn Stover	35.1–39.5	20.7–24.6	11.0–19.1
Bamboo	49–50	18–20	23

NA not available

for the growth of microbes and their activity in a specific substrate (Aydınoglu and Sargin 2013; Harmanpreet et al. 2016).

A good number of agricultural residues are studied for cellulase production including wheat bran (Chandra and Reddy 2013), rice husk (Suresh et al. 2016), groundnut shells (Sridevi et al. 2008), fodder (Shruthi et al. 2019), cellulose bagasse (Long et al. 2012), egg shell waste (Verma et al. 2012), water hyacinth (Zhao et al. 2011), wheat bran (Maurya et al. 2012), sugarcane bagasse (Singhania et al. 2006), natural and pretreated lignocelluloses (Sridevi et al. 2008), domestic wastewater sludge (Alam et al. 2008), oil palm of empty fruit bunches (Alam et al. 2009), soya hull (Brijwani and Vadlani 2011; Herculano et al. 2011), saw dust (Narasimha et al. 2006; Guruchandran and Sasikumar 2010), rice straw (Hideno et al. 2011; Liang et al. 2012; Rahnama et al. 2016), potato peel (Santos et al. 2012), cassava baggase (Singhania et al. 2006), ground nut shell waste (Vyas et al. 2005), and pea seed husk (Srilakshmi et al. 2017). Various lignocellulosic substrates used for cellulolytic enzymes synthesis by fungi in SSF are presented in Table 3.2.

A number of solid substrates were employed for cellulolytic enzymes production in SSF. Banana waste, rice straw, wheat straw, and corn stalks were utilized as lignocellulosic substrates for cellulolytic enzymes production by *A. niger, F. oxysporum, F. avenaceum, C. acremonium,* and *A. flavus* NRRL 5521 under SSF (Azzaz et al. 2012; Azzaz and Azzaz 2013). In a study, Abdullah et al. (2016) standardized the cellulase production by municipal solid waste as a solid substrate

Table 3.2 Lignocellulosic substrates used for cellulolytic enzymes production by fungi in SSF

S. No.	Fungi	Lignocellulosic substrates	References
1	*Aspergillus unguis*	Groundnut fodder	Shruthi et al. (2019)
2	*Aspergillus protuberus*	Rice husk	Suresh et al. (2016)
3	*Aspergillus niger*	Wheat bran Saw dust	Chandra et al. (2007, 2010), Chandra and Reddy (2013), Narasimha et al. (2006)
4	*Microporus* sp. KA038	Green tea waste	Nguyen et al. (2019)
5	*Penicillium* sp.	Corn cob	Francis et al. (2018)
6	*Perpurieocillium hiacinum*	Pea seed husk	Srilakshmi et al. (2017)
7	*Trichoderma reesei*	Rice bran, rice husk, and rice straw	Nazanin et al. (2019)
8	*Trichoderma longibrachiatum*	Wheat bran	Hind et al. (2017)
9	*Trichoderma reesei*	Municipal solid waste	Abdullah et al. (2016)
10	*Aspergillus terreus* RS2	Rice straw	Shaymaa and Amira (2020)
11	*Aspergillus niger* ATCC 16888	Copra waste	Chysirichote (2018)
12	*Trichoderma harzianum*	Corn cob	Sonika et al. (2015)
13	*Aspergillus nidulans* AJSU04	Coir pith	Anuradha et al. (2014)
14	*Trichoderma viride* GSG12	Rice bran	Nadagouda et al. (2016)
15	*Penicillium funiculosum*	Pretreated sugar-cane bagasse	Maeda et al. (2011)
16	*Rhizopus oryzae*	Palm kernal cake	Othman et al. (2013)

with *T. reesei* and *A. niger*, compared and found that *T. reesei* showed the maximum production of cellulase. Azzaz and Azzaz (2013) demonstrated the use of banana waste, rice straw, wheat straw, and corn stalks as lignocellulosic substrates for production of cellulase by *A. niger, F. oxysporum, F. avenaceum, C. acremonium,* and *A. flavus* NRRL 5521 under SSF. Badhan et al. (2007) reported the production of maximum cellulase activity was noticed with rice straw by *Myceliophthora* sp. IMI 387099. Nadagouda et al. (2016) utilized the rice bran and optimized fermentation parameters for the production of cellulolytic enzyme by *T. viride* GSG12. Similarly, Damisa et al. (2011) employed CMC as a solid substrate for production of cellulase enzyme by *A. niger* isolated from various sources and found that *A. niger* isolated from rice-growing field showed high efficiency. Similarly, higher cellulase production was noticed in the mixture of wheat bran and rice bran (Praveen Kumar Reddy

et al. 2015). Castor bean was employed as solid support for cellulase production with *A. japonicus* URM5620 by SSF and optimized the various physical parameters using a full factorial design (2^4) (Herculano et al. 2011).

Solid substrates like castor husk, sugarcane bagasse, sesamum husk, groundnut fodder, rice husk, tea residue, and sawdust were screened for cellulolytic enzymes production by *A. unguis* in SSF. Notably, the highest production of enzymes differed from substrate to substrate, though based on the next excellent substrate and local accessibility of groundnut fodder-assisted highest enzyme activity contrasted with other substrates (Shruthi et al. 2019). In a related report, Chandra et al. (2007) found that synthesis of cellulases by *A. niger* on substrates, i.e., groundnut fodder, wheat bran, rice bran, and sawdust in SSF was compared. Among the tested substrates, wheat bran served as an excellent solid substrate for maximum production of cellulase. Agricultural residues including carrot, onion, potato, and sugar beet peels were used for synthesis of cellulolytic enzymes (Mushimiyimana and Tallapragada 2015). The agricultural residues produced cellulase which was attained on sixth day on potato peel and seventh day on carrot, onion, and sugar beet peels. The higher cellulase enzyme was noticed with sugar beet in all parameters. Cellulase was found to be produced from *S. fungicidicus* RPBS-A4 by rice bran (5–6%), as a top substrate among tested (Akurathi and Thoti 2018). Interestingly, pineapple and orange peels were used as substrates for cellulolytic enzymes production. Maximum enzymes were obtained on fifth day after *A. niger* was cultivated in the medium with pineapple peel, orange peel, and carboxymethyl cellulose (CMC) whereas it was attained on third day for pineapple peel and fifth day (Amaeze et al. 2015). Wastes like waste paper, cotton ginning, wheat bran, sugarcane bagasse, and cellulose use SSF for production of cellulase (Komal and Anjali 2015). Substrates including rice bran, corn bran, sugarcane bagasse, rice straw, and saw dust were chosen for investigating the best solid support for higher cellulase production. Among the substrates, corn bran showed high cellulase activity (107.7 U/gds) in unoptimized condition; therefore, corn bran was chosen as substrate for the statistical standardization of various experiments for enhancing cellulase activity (Faisal and Benjamin 2016).

3.5.2 Carbon Source

Carbon sources play a significant task in all chemical reactions of the cell and cellulolytic enzymes production by the microbes. Cellulase-producing organisms are grown on medium consisting of diverse carbon sources. Sethi and Gupta (2014) screened various carbon sources namely fructose, glucose, sucrose, lactose, and maltose at 1.0% concentration to enhance the cellulase production by four various fungal strains, i.e., *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., and *Microsporium* sp. They observed that *A. niger* in presence of glucose, fructose, and maltose brought about the highest cellulase production compared with other carbon sources. In another study, the effect of various carbon compounds viz., maltose, corn cob,

wheat bran, sucrose, and filter paper was studied for the production of exo, endo-1,4-β-D-glucanases by eight different species of *Trichoderma* under SSF and observed that corn cob was the most efficient carbon source for cellulolytic enzymes production followed by wheat bran, filter paper, sucrose, and maltose. Similarly, Nathan et al. (2014) isolated a total of 12 fungal strains from mangrove plant debris and soil sample and exploited a variety of carbon sources (dextrose, sucrose, xylose, and CMC) for better production of cellulase enzyme and observed maximum cellulase activity by glucose. Srilakshmi et al. (2017) studied the influence of eight arrays of carbon sources including xylose, fructose, glucose, maltose, galactose, lactose, and cellulose at 3% on the cellulase production by *P. lilacinum* (NCBI accession number: KT387301) incubated for 7 days on rotatory shaker. Out of the eight carbon sources used, 3% xylose was proved to be the top carbon source for higher FPase (1.15 IU/ml) and CMCase (2.09 IU/mL) production. Maltose and cellulose were noticed as poor carbon sources for FPase and CMCase production.

Studies have reported lactose is an extremely used soluble carbon source and a good inducer for cellulase production by *T. reesei* (Amore et al. 2013). The highest enhancement of endoglucanase (17.21 U/gds) and exoglucanase (1.99 U/gds) titers was observed by using lactose. The most enzyme activity was recorded on manitol and sugar beet peel as substrates (2.32 U/ml) noticed by Mushimiyimana and Tallapragada (2015). Wheat bran yielded higher cellulase enzyme activity reported by Jain and Jain (2016). The medium was added with banana agro-waste as the carbon source produced high levels of cellulase observed by Shah et al. (2015). Maltose acts as the best carbon source (1%), studied by Akurathi and Thoti (2018).

3.5.3 Nitrogen Source

Generally, nitrogen requirement of microbes is met by the substrate itself in a number of cases, while supplementation of extra nitrogen compounds in form of organic or inorganic is frequently requisite. The result of nitrogen source on the production of cellulolytic enzymes is changeable; rely on fungi and compound assessed (Kachlishvili et al. 2006). Additionally, enzyme production was influenced considerably in various concentrations of nitrogen sources (Panagiotou et al. 2003). Therefore, optimization of nitrogen source for better synthesis of cellulolytic enzymes in SSF is required.

Nathan et al. (2014) studied the different organic and inorganic nitrogen sources like peptone, beef extract, sodium nitrate, and ammonium nitrate to enhance the cellulase activity by *T. viride* VKF3 under SmF, and peptone was observed to be greatest nitrogen source for exoglucanase higher activity on seventh day of incubation and down tendency was recorded on more incubation. In another study, Sethi and Gupta (2014) optimized various nitrogen sources (ammonium sulfate, urea, yeast extract, and peptone) for better production of cellulase from four diverse fungal strains, i.e., *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., and *Microsporium* sp. Among the studied four varieties of nitrogen sources ammonium sulfate served as

the best nitrogen source for *A. niger*. Effect of various nitrogen sources including ammonium sulfate, potassium nitrate, ammonium nitrate, yeast extract, peptone, malt extract, tryptone, and beef extract at 0.2% level on production of exo, endo-1,4-β-D-glucanases production by *P. lilacinum* was studied and found that ammonium sulfate had the highest impact on FPase (1.11 IU/ml) and CMCase (1.95 IU/mL) production (Srilakshmi et al. 2017). However, different researchers have recorded organic nitrogen sources effect in the maximum production of cellulases compared to inorganic (Jeya et al. 2010; Deswal et al. 2011). Supplementation of peptone was observed to increase growth and cellulase production (Chandra et al. 2009).

Malik et al. (2010) examined the inorganic nitrogen sources like Ammonium sulfate supported maximum production of Fpase (0.926 U/ml/min), CMCse (1.68 U/ml/min). Similarly, 0.3% Ammonium sulfate with 20% Apple waste enhanced the production of cellulase activity of 2.28 IU/ml/min by *A. fumigatus* JCF (Elsa et al. 2015). It was noticed that better cellulase activity can be attained by ammonium sulfate as the nitrogen source (Sethi and Gupta 2014). Almost the same results were observed by Bhattacharya et al. (2014) when compared to organic nitrogen sources and the rest of inorganic nitrogen sources NH_4SO_4 acted as the good nitrogen source for production of cellulolytic enzyme during SSF. Various inorganic nitrogen sources viz., ammonium phosphate, ammonium phosphate dibasic, ammonium nitrate, ammonium oxalate, ammonium sulfate, and urea were used as nitrogen sources to examine their result on the production of enzymes by *A. oryzae* FK-923. In this study, they concluded that urea and ammonium nitrate were more appropriate for cellulase enzyme synthesis (Hassan et al. 2016). Nonetheless, potassium nitrate and sugar beet peel also acted as the best nitrogen sources for higher production of cellulase (Mushimiyimana and Tallapragada 2015). Peptone yielded higher cellulase enzyme activity (Jain and Jain 2016). According to Akurathi and Thoti (2018), yeast extract was noticed to be the better nitrogen source for maximum cellulase production.

3.5.4 pH

Among physical parameters, the pH of growth medium plays a central role by enhancing phenetic differences in microorganisms and enzyme production. The pH alter noticed in the growth of microorganisms also influences product constancy in the medium. The optimum pH differs by various microbes and enzymes. Better focus is given to standardizing the primary pH of moist solid medium (Fadel et al. 2013).

Sethi and Gupta (2014) optimized the initial pH by operation factorial-design technique for four different fungal strains, i.e., *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., and *Microsporium* sp. to enhance cellulase enzyme production. They observed that maximum enzyme activity (0.95 U/mL) for *P. chrysogenum* at pH 5.0 and followed by *A. niger* (0.85 U/ml). In a similar manner, Hassan et al.

(2016) standardized initial pH for production of four different hydrolytic enzymes by *A. oryzae* FK-923 and observed the initial pH series from 4.5 to 5.5 supported the highest degree of enzymes production, i.e., 116.8, 129.2, 148.8, and 686.2 U/g for FPase, CMCase, β-glucosidase, and xylanase, respectively. In another study, Pandey et al. (2015) standardized initial pH for production of exo, endo-1,4-β-D-glucanases by eight different *Trichoderma* sp. and found that better pH for enzyme production was noticed ranging from 4 to 6. Nathan et al. (2014) observed that at neutral pH CMCase showed higher yield on third to fifth day followed by a quick decrease in enzyme production and FPase exhibited the highest yield at neutral pH on the fifth day of incubation under SmF for *T. viride* VKF3 strain. Similarly, maximum secretion of exo and endo-1,4-β-D-glucanases of 1.22 IU/mL and 1.82 IU/mL was observed at pH 5.5, respectively, by *P. lilacinum.* In this cellulase activity enhanced with raise in pH up to 5.5 and later declined at higher pH (Srilakshmi et al. 2017). Optimum pH for cellulase production by *A. terreus* and *T. reesei* was reported at pH 5.5 (Shahriarinour et al. 2011).

Optimum pH is 6.5 (1.68 U/ml) for maximum production of cellulase (Mushimiyimana and Tallapragada 2015). Highest enzyme production such as endoglucanase (15.93 IU/gds), cellobiohydrolase (3.59 IU/gds), and β-glucosidase (41.59 IU/gds) was recorded at initial medium pH 5.0 on third day (Jain and Jain 2016). Higher enzyme production was noticed at pH 6.0 (Shah et al. 2015). Notably, solid-state processing augmented the production of enzyme at pH 9.0 (Akurathi and Thoti 2018). pH 5.0 was found to be the best pH for the production of cellulolytic enzyme (Amaeze et al. 2015). The optimal pH for higher production of cellulase is pH 5 (Faisal and Benjamin 2016). Ire et al. (2018) studied that the most favorable pH value for production of cellulase using *Penicillium* sp. was pH 5 with utmost cellulase activity of 37.32 IU/mL.

3.5.5 Temperature

Temperature, like any other physical parameters, plays an important role in solid-state fermentation system. A number of researchers have studied various temperatures for maximum cellulolytic enzymes production both in the flask and in fermenter levels by *Aspergillus* sp. and *Trichoderma* sp. telling that the best temperature for cellulase production also relies on strain distinction of organism (Yoon et al. 2014). Incubation at elevated temperature influences fungal growth which effects enzyme production. As the enzyme is a prime metabolite produced in the exponential growth phase, incubation at higher temperature could lead to meager growth and therefore decrease in enzyme activity. However, the best possible temperature for cellulase synthesis usually drops between 25 and 30 °C. It is notable that SSF performed at higher temperature shows an unfavorable result on cellulase production, since enzymes produced could be denatured. This recommends that an attractive temperature should be a compromise among optimal temperature for cellulolytic enzymes synthesis and fungal development too (Yoon et al. 2014).

Hassan et al. (2016) optimized the incubation temperature for production of different hydrolytic enzymes, i.e., FPase, CMCase, β-glucosidase, and xylanase by *A. oryzae* FK-923 and observed that 30 °C is the optimum temperature for maximum secretion of enzymes, i.e., 96.4, 98.2, 118.2, and 625.6 U/g, respectively. Sethi and Gupta (2014) optimized the incubation temperature for four different fungal strains, i.e., *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., and *Microsporium* sp. to enhance cellulase enzyme production and found maximum secretion of cellulase enzyme by *A. niger* at 40 °C. Strikingly, Nathan et al. (2014) found CMCase activity was more at 25 °C on the ninth day while FPase had peak activity at 55 °C on the fifth day by *T. viride* VKF3 under SmF conditions. In another study, Srilakshmi et al. (2017) observed that maximum FPase (1.14 IU/ml) and CMCase (2.03 IU/ml) activities were found during the initial incubation temperature of 30 °C. Liang et al. (2012) observed that 32 °C is most fitting for production of cellulase on rice grass by *Aspergillus* sp. in SSF. Saini et al. (2017) observed that the higher production of enzymes was attained at 30 °C, resulting in 11.71 U/gds CMCase and 1.31 U/gds FPase activities by *T. reesei* grown on Parthenium biomass. Pandey et al. (2015) optimized the incubation temperature for production of exo, endo-1,4-β-D-glucanases by eight different *Trichoderma* sp. and found that the better temperature for enzyme production was noticed between 30 and 40 °C.

Zhao et al. (2011) noticed that 28 °C was the optimal temperature for production of cellulase under SSF by *T. reesei* SEMCC-3217 with water hyacinth as a solid substrate. An augment or decline in temperature from optimum resulted in the considerable decrease in cellulolytic enzymes production by fungi. Poor growth of the *T. reesei* was observed at elevated temperature which showed a negative effect on growth and can be interrelated with reduced enzyme secretion (Sethi and Gupta 2014); 25–35 °C is, in general, the suitable range of temperature in SSF (Mrudula and Murugammal 2011) and high temperature is anticipated to denature enzymes because of heating effects (Yoon et al. 2014). Mushimiyimana and Tallapragada (2015) explored optimal incubation temperature is 40 °C for higher cellulase production. The standardization of incubation temperature for production of enzyme in SSF explained that enzyme production was enhanced from 20 °C to 30 °C during third day of incubation. The production of endoglucanase (20.53 IU/gds), cellobiohydrolase (4.75 IU/gds), and β-glucosidase (56.98 IU/gds) was recorded by Jain and Jain (2016). The maximum enzyme production was noticed at a temperature of 28 °C (Shah et al. 2015). SSF improved the production of enzyme yield as studied by various authors at different temperatures viz. 40 °C (Akurathi and Thoti 2018), at 30 °C (Amaeze et al. 2015) and at 40 °C (Faisal and Benjamin 2016). The optimal temperature for cellulase production by *Penicillium* sp. with corncob is at 30 °C with higher cellulase activity of 37.32 IU/ml reported by Ire et al. (2018). Faisal and Benjamin (2016) observed that the highest cellulase production was at 40 °C. Incubation temperature of 45 °C was the optimal for maximum production of cellulase (Budihal and Agsar 2015).

3.5.6 Moisture Content

Moisture content plays an imperative function in the SSF system. The initial moisture level influences solid substrate affecting aeration, nutrients solubility, heat and gas transfers, and substrate swelling by microorganisms (Ellaiah et al. 2004). The moisture content demands in SSF vary based on enzyme produced, substrate, and microbe, particle size of substrate as well as the pattern of particles (Kalogeris et al. 2003). The decrease in enzyme activities at higher initial moisture level might be a steric impediment in inter-particular spaces and impair oxygen transport (Sandhya and Lonsane 1994).

The elevated moisture level improved fungal growth and cellulolytic enzymes production when agro-wastes were carbon sources during SSF (Kalogeris et al. 2003; Panagiotou et al. 2003). Lesser moisture content leads to less growth, a lower level of substrate enlargement, and higher surface area, while higher moisture content decline porosity, which would affect lesser oxygen transfer, heat dissolution, and improved development of aerial mycelium respectively. Moisture level in solid substrate influences aeration and nutrients solubility which is essential to sustain organism's growth and metabolites (Ahmed 2008).

Hassan et al. (2016) reported that hydrolytic enzyme production was improved with rising the moisture level in sugarcane bagasse pith by *A. oryzae* FK-923 and higher production of enzymes were recorded when moisture level was 80% and enzyme production with 96.4, 98.2, 118.2, and 625.6 U/g for FPase, CMCase, β-glucosidase, and xylanase, respectively. Hassan et al. (2016) found 70% moisture for cellulase production on rice grass with *Aspergillus* sp. SEMCC-3248 in SSF. Zhao et al. (2011) evidenced 75% moisture was used for the synthesis of cellulase in SSF by *T. reesei* SEMCC-3217. Kim et al. (2014) observed that 40–50% moisture was best for production of cellulases by *Penicillium* sp. in SSF of oil palm empty fruit bunch. But, more water in the medium could create clumping of medium, hinder aeration, and growth of hyphae, which could also effect in decline production of an enzyme (Gao et al. 2008).

The best moisture level for higher cellulase production with *T. reesei* mutant was observed to be 55–70%, but *T. reesei* Rut C30 was evidenced to produce cellulase maximum at 79% of moisture content (Das et al. 2008). Enhancement of initial moisture level from 60 to 80% in actual fact increased the enzyme activity and higher enzyme activity was attained with 80% moisture level (Gao et al. 2008). When moisture level is lower than the demand level, the solubility of nutrients is narrow and it hampers the efficient nutrients take-up with fungi (Kumar et al. 2011). On contrary, when the moisture level is very high, particles of substrate were bound with a thick layer of water. As a result, particles are likely to attach collectively wherein this confines air diffusion among particles and surrounding (Deswal et al. 2011). Additionally, the risk of contamination is larger if higher moisture level is used in SSF as situation supports the growth of hostile microbes. The sum of moisture required is straightly connected to the structure of lignocellulosic substrate. The porosity and specific surface area of solid particles administer the effectiveness of air

diffusion and water-holding ability of substrate (Chen 2013). Jain and Jain (2016) studied that the highest production of endoglucanase (20.05 IU/gds), cellobiohydrolase (4.65 IU/gds), and β-glucosidase (52.45 IU/gds) on the substrate to moisture ratio was 1:3 at 30 °C temperature and pH 5. Initial moisture level 60% and 65% were optimal for maximum production of cellulase observed (Faisal and Benjamin 2016; Budihal and Agsar 2015).

3.6 Cellulase in Biomass Hydrolysis and Biofuel Production

The drastic increase in word population along with increases ultimatum of energy, exhaustion of fossil fuel, and increased greenhouse effect from traditional fuel, there is an urgent necessitate to build up or look for inexpensive, renewable, and sustainable sources of energy (Ahmed et al. 2017). Hence, cellulase plays a vital role in biofuel production and reduces energy crisis and environmental contamination (Horn et al. 2012; Sharada et al. 2014). Though the conversion of lignocellulosic substrates into sugars use multiple enzymes for full hydrolysis, of which cost is high, making biorefining approaches reasonably unfeasible. Therefore, the search of potent enzymes like cellulase with novel properties showed high thermostability, acidophililicity and high solvent tolerant could assist to conquer cost impediments. Cellulases advantage in biomass saccharification and biofuel production is presently the focus of various experiments supported by various organizations across the globe (Budihal et al. 2016; Srivastava et al. 2015).

3.7 Future Perspectives and Conclusions

The increasing demand of energy and natural products combines with an increase in the demand of industrial enzymes such as cellulases being important enzymes in the conversion of biomass and biofuel production. The main obstacle in the production of biofuel and other products from lignocellulosic substrates is the lack of efficient economically feasible cellulase. Cellulase finds potential applications in various biotechnological industries. In the recent past, enzymatic hydrolysis and enzyme-based methods are ideal than chemical methods due to environmental friendly, high yield, low cost, easy, and safety. Hyperproduction of microbial enzymes with high specific activity can be achieved by manipulating their genes via genetic engineering. Most of the enzymes of microbial origin are still unknown and there are numerous openings for finding potential applications in a broad range of industries, particularly in the bioenergy process. In fact, SSF in light with environmental trouble because of filling up of lignocellulosic biomass has to be exploited with an economic and industrial approach.

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Chapter 4
Influence of Xenobiotics on Fungal Ligninolytic Enzymes

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Abstract White rot fungi (WRF) (belonging to the Basidiomycota family) are considered as the most efficient microorganisms to degrade lignin polymer through secretion of lignin-modifying enzymes such as oxidases (laccase) and peroxidases (lignin peroxidase and manganese peroxidase). Non-specific nature of these LMEs has a wide range of industrial and environmental applications including biodegradation and bioremediation of xenobiotics. Environmental pollution was generally caused by the extensive use of xenobiotics in the ecosystem. Massive studies on bioremediation of pollutants by bacteria and actinomycetes are highly noticed. It was recognized that very fewer research reports have existed on the influence of xenobiotics on the growth of highly environmentally adapted fungi as well as white rot fungi (WRF). Hence, the present book chapter mainly reveals the effect of xenobiotics on growth and secretion or production of LMEs by WRF and their participation in the bioremediation of xenobiotics. This chapter initially revealed the chemical nature of xenobiotics and their toxicity impact on WRF biomass. Furthermore the effect of pesticides such as malathion, lindane, and diuron on white rot fungal (*Pleurotus ostreatus, Phanerochaete chrysosporium, Ganoderma lucidum*) growth as well as secretion of ligninolytic enzymes and minimization of xenobiotics including PAHs and dyes by the WRF was clearly explained. This chapter provides information about how to reduce the harmful impact of xenobiotics in the environment by using LMEs and improve the applications of enzymatic technology.

Keywords Xenobiotics · White rot fungi · Ligninolytic enzymes · PAHs · Dyes

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Abbreviations

CPF	Chlorpyrifos
CYP	Cytochrome P450
HCH	Hexachloro cyclohexane
LAC	Laccase
LE	Ligninolytic enzymes
LiP	Lignin peroxidase
LMEs	Lignin-modifying enzymes
MnP	Manganese peroxidase.
MSM	Mineral salts medium
PAHs	Polyaromatic hydrocarbons
PCP	Pentachlorophenol
SmF	Submerged fermentation
SSF	Solid-state fermentation
TCP	2, 4,6 trichlorophenol
TNT	Tri-nitrotoluene
WRF	White rot fungi

4.1 Introduction

The white rot fungi (WRF) have the most prolific wood biodegraders that existed in nature, which possess the remarkable capacity to convert lignin polymer to carbon dioxide (CO_2) effectively (Hatakka 2001; Abdel-Hamid et al. 2013). They facilitate degradation of lignin through secretion of LMEs such as oxidases (e.g., Laccase (LAC)) (Ozer et al. 2019), and peroxidases (manganese peroxidase (MnP) (Carmona-Ribeiro et al. 2015; Agrawal et al. 2018a), lignin peroxidase (LiP)) (Pollegioni et al. 2015; Castro et al. 2016) and make the cell wall polysaccharides accessible to other organisms for utilization and play an essential role in carbon recycling on the earth (Chowdhary et al. 2019; Kumar and Chandra 2020; Kumar and Verma 2020; Gunjal et al. 2020). Recent reports on LEs producing WRF are listed in Table 4.1.

The entry of different kinds of pesticides into the environment occurs due to anthropological and industrial activities. A big ecological issue is an environmental contamination caused by xenobiotics, especially, insecticides and their bioremediation products which can create elaborate ecological disturbances (Guliy et al. 2003). A wide variety of xenobiotics (polycyclic aromatic hydrocarbons (PAHs), organochlorines (OC), organophosphates (OP), explosives, and dyes) have been exposed to non-target organisms. But broad range usage and accumulation of these xenobiotics in the ecosystem may cause toxic effects to the growth of several ecologically constructive and non-target microbes in the environment including WRF (Chishti et al. 2013).

Table 4.1 Secretion of ligninolytic enzymes by WRF

S. No.	Name of the white-rot fungi	Produced LMEs	References
1.	*P. Chrysosporium* *Lasiodiplodia theobromae*	LiP LAC	Cao et al. (2020)
2.	*Trametes trogii* 4_6	LAC MnP LiP	Kostadinova et al. (2018)
3.	*Stereum ostrea*	MnP LiP LAC	Usha et al. (2014)
4.	*Agaricus blazei*	LAC	Valle et al. (2015)
5.	*T. Trogii*	LiP MnP LAC	Krumova et al. (2018)

The xenobiotics effects on WRF, particularly biomass and secretion of LMEs, are least understood. However, by the treatment of xenobiotics such as PAHs, chlorophenols like pentachlorophenol (PCP) and synthetic dyes with WRF (*Anthracophyllum discolor*) secrete these LEs extracellularly and mainly MnP was identified under the treatment of these xenobiotics (Tortella et al. 2008; Elgueta and Diez 2010; Rubilar et al. 2011; Acevedo et al. 2011; Elgueta et al. 2012). According to the studies of Coelho-Moreira et al. (2013) at the end of 10-day cultivation, herbicide concentrations (Diuron) up to level 80 μmol/L (18.6 μg/mL) influence the growth of *Phanerochaete chrysosporium* when evaluated with the fungal growth obtained without herbicide treatments. An increased concentration of diuron at 100 μmol/L (23.2 μg/mL) *P. chrysosporium* shows highly reduced biomass productions (Coelho-Moreira et al. 2013). The pesticide diuron enhanced only LiP the activity of *P. chrysosporium* whereas MnP production was lowered in the same culture under the influence of diuron (Coelho-Moreira et al. 2013). Similarly, Coelho-Moreira et al. (2013) observed that with/without diuron treatments, LAC was not identified under any conditions at any point in experimental studies. Exposure of different types of xenobiotics—PAHs, polychlorinated phenols (DCP, TCP, PCP), chlorinated guaiacol, pesticides, chlorinated biphenyls; stable polymers,2,4-dichloroaniline, dioxins, nitrates, and dyes to certain WRF had an influence on the secretion of LMEs (Tortella et al. 2008; Torres et al. 2016; Kaur et al. 2016; Lazim and Hadibarata 2016; Shanthi Kumari et al. 2019; Peter et al. 2019). A variety of environmental pollutants (PAHs, pesticides, dyes, PCP) can be efficiently oxidized/degraded in vitro by both WRF and its LMEs. Several recent reports have well documented on the bioremediation of pollutants by the ligninolytic enzymatic aspects as well as the involvement of WRF (Rabinovich et al. 2004; Tortella et al. 2005; Anastasi et al. 2013; Ghosal et al. 2016; Tripathi and Dixit 2016). The unspecific existence of WRF enzymes has been identified as key factors in their capability to oxidize certain aromatic intricate polymers with lignin polymer like chemical structures (Tišma et al. 2010; Mendonça Maciel et al. 2010).

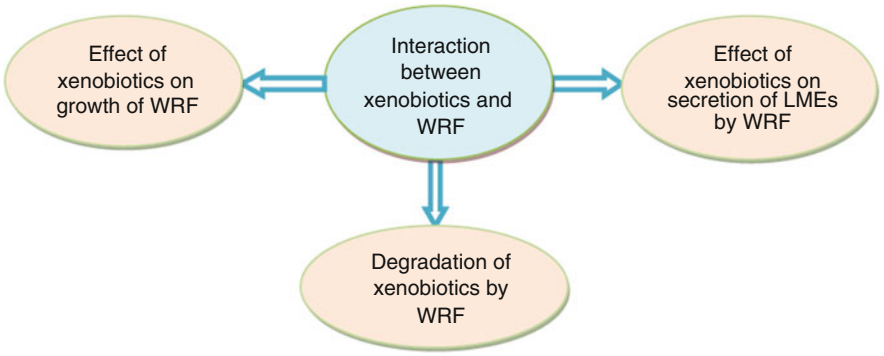


Fig. 4.1 An overview of the interaction between xenobiotics and white rot fungi

The white rot fungi (WRF) produces the laccases, and extracellular peroxidases have been recognized as key enzymes taking part in the oxidation of toxic aromatic polymers (Mester and Tien 2000; Zahmatkesh et al. 2010; Mendonça Maciel et al. 2010). Some of the organic compounds have been degraded by the participation of cytochrome P450 (CYP) enzymes in WRF (Ning and Wang 2012; Kelly and Kelly 2013; Zhang et al. 2015; Zahmatkesh et al. 2016). The catalytic property CYPs such as oxidation/hydroxylation/epoxidation reactions which are directly involved in the bioremediation of pollutants and these CYPs are recognized as hemo-membrane-bound proteins (Neve and Ingelman-Sundberg 2008; Aranda 2016).

However, the interaction between xenobiotics and the WRF on the secretion of LMEs has not been evaluated and information is virtually lacking on the impact of pollutants on growth and production of LE by the WRF. Hence the present book chapter mainly focused on the effect of xenobiotics/pesticides on the growth (biomass) and secretions of LMEs by WRF and also explained the intern reaction of WRF concerned in the bioremediation of xenobiotics. Interaction of these xenobiotics to WRF is represented in Fig. 4.1.

4.2 Effect of Contaminants (Xenobiotics) on the Biomass of WRF

WRF can exhibit positive (enhanced growth) or negative (inhibition of growth) effect on growth against different concentrations of xenobiotics.

4.2.1 *Effect of Insecticide: Malathion*

Malathion is an organophosphorus insecticide that is widely used in agriculture, forestry system, and housing landscaping. Malathion is extensively used in pest control programs such as to control sucking insects and chewing insects in various field crops and also broadly used as a substitute for DTT compound to control insects of household, parasites of animals, mosquitoes, flies, and head body lice (Chambers 1992; Barlas 1996). Singh et al. (2012) studied the toxicity effect of malathion on microorganisms. The chemical structure of malathion is given in Fig. 4.2.

According to the studies of Ganash et al. (2016), organophosphorus insecticide—malathion at concentrations within a range of 25–100 µg/ml caused inhibition to the growth of *P. ostreatus*. And the percentages of growth inhibition exerted by malathion at concentrations of 25, 50, and 100 µg/ml on *Pleurotus ostreatus* were 5, 36, and 65%, respectively.

4.2.2 *Effect of Organophosphorus Insecticides (Diazinon, Profenofos, and Malathion)*

The O-(4-bromo-2-chlorophenyl) O-ethyl S-propyl phosphorothioate is commonly called as profenofos (PFF) is an OP pesticide group of substance and it is broadly used as a non-systemic acaricide and foliar insecticide (pesticide). Profenofos was still successfully used to control different types of pests including mites, sucking insects, and chewing insects on a variety of agricultural crop-fields (Reddy and Rao 2008). Extensive usage of this toxic PFF has created significant implications for the ecosystem (Hina et al. 2015). Diazinon [*O*, *O*-diethyl *O*-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothioate] is also broadly used as a pesticide. According to the study by Tomlin (2006), diazinon is an inhibitor of cholinesterase action and also acts as a non-systemic acaricide. Pesticide nature of this diazinon inhibits the respiratory and stomach actions in insects. Hence it is widely used for the control of a broad range of insects in the agroforestry system. Similarly, Tu (1970) reported the impact of diazinon on fungi. The chemical structure of diazinon and profenofos is shown in Fig. 4.3.

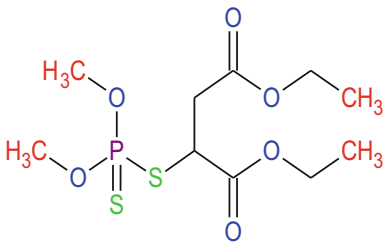
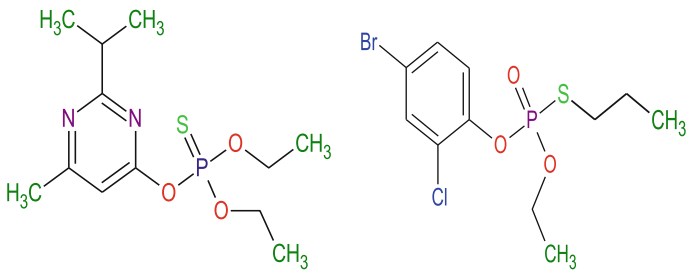


Fig. 4.2 Malathion chemical structure



a) b)

Fig. 4.3 Chemical structure of (**a**) diazinon and (**b**) profenofos

According to the studies of El-Ghany and Masmal (2016) on the impact of assessment of insecticides (diazinon, profenofos, and malathion), the growth of fungal cultures, *T. harzianum,* and *M. anisopliae* lowered significantly with higher concentrations of organophosphorus pesticides (diazinon, profenofos, and malathion). Of the four fungal cultures (*Fusarium oxysporum*, *Curvularia lunata*, *T. Harzianum,* and *M. anisopliae*) tested in this study, *Fusarium oysporum* exhibited the highest tolerance to insecticides at higher concentrations as evident from better growth yields (El-Ghany and Masmal 2016).

4.2.3 Effect of Hexachlorocyclohexanes (HCH)

The mixture of chemical isomers of 60 to 70% (α-hexachlorocyclohexane), 5–12% (β-hexachlorocyclohexane), 10–12% γ-hexachlorocyclohexane, and 6–10% δ-hexachlorocyclohexane are commonly known as hexachlorocyclohexanes (HCH). From this γ-HCH, generally called as lindane, has pesticide activity and it has been extensively utilized along with other isomers of commercial formulations (Breivik et al. 1999; Li 1999). Hexachlorocyclohexane (HCH) isomers have been identified as toxicants, noted as environmental persistence, and also exhibited potential carcinogenic effects. In certain soils, evaluated levels of these contaminants were identified (Willett et al. 1998; Macrae et al. 1984). γ-HCH structure is presented in Fig. 4.4.

Recently, Abbas and Yadegar (2015) reported inhibition of spore germination and biomass of *T. harzianum* at higher concentrations of pesticides. Independent of the fungal species, the delta-HCH (hexachlorocyclohexane) isomer had the greatest adverse effect on the mycelial development, followed by miner effects by γ-HCH, ß–HCH, and α-HCH isomers, respectively (Quintero et al. 2008). The highest inhibition of mycelial biomass was observed in the dose–response effect at 5 and 10 $mg \cdot L^{-1}$ of the respective delta-HCH isomers. A concentration of 10 $mg \cdot L^{-1}$ delta-HCH mycelia biomass was entirely reduced in *Stereum hirsutum* and inhibited more than 80% of mycelial growth in *Lentinus tigrinus*, *Phlebia radiata*, *Polyporus*

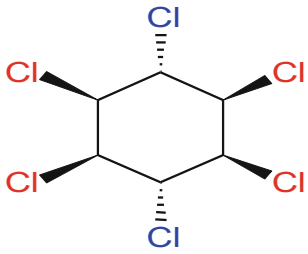


Fig. 4.4 γ-HCH Hexachlorocyclohexane (lindane)

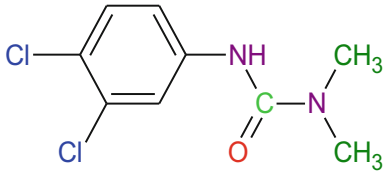


Fig. 4.5 Diuron chemical structure

ciliatus, and *Phanerochaete sordid* were also observed at this concentration. In the same level concentration, γ-HCH reduced 60% of the growth of *P. sordida*, *P. radiata,* and decreased 40% growth of mycelia *Bjerkandera adusta* and *P. ciliatus.* Among these fungal species, *P. chrysosporium* and *B. adusta* were the most tolerant of HCH isomers (Quintero et al. 2008). Incubation of the WRF basidiomycete—*P. chrysosporium* inoculated into sterile-soil mixed with lindane (γ-HCH) at 0.8 μg/g^{-1} yielded biomass of 110 mg (Mougin et al. 1997).

4.2.4 *Influence of Lindane*

Growth of fungal cultures of *P. sordid* and *C. bulleri* in the liquid medium was not influenced by the treatment of 8 ppm concentration-level lindane (Singh and Kuhad 2000).

4.2.5 *Effect of Diuron*

Phenyl urea herbicide diuron is used extensively in a variety of field crops, especially the sugarcane cultivation area. Diuron compound mode of action is the inhibition of photosynthesis by blocking of photosystem II electron transportation mechanism in photosynthetic organisms. Under natural conditions diuron can be degraded abiotically via photodegradation and hydrolysis reactions, in the ecosystem, these reactions take place at very low levels (Giacomazzi and Cochet 2004). In Fig. 4.5, diuron chemical structure is given.

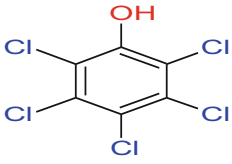
Addition of salt enhanced toxicity of diuron to the growth of a jelly fungus *Dacryopinox elegans*, whereas glycerol suppressed toxicity of diuron toward the growth of the same culture (Arakaki et al. 2013). In another experimental study concentrations of diuron below 19 μg/ml in the medium did not affect the biomass production of *P. chrysosporium* (Coelho-Moreira et al. 2013). A higher concentration of diuron beyond 19 μg/ml resulted in inhibition of the biomass of the same fungal culture (Coelho-Moreira et al. 2013).

4.2.6 Effect of Chlorophenols

A chlorophenol is a phenolic organochloride (OC) consisting of one or more chlorine atoms that are covalently bonded to the compound. In an environment generally, Chlorophenols with five types have been identified (mono- to pentachlorophenol). Chlorophenols are produced by electrophilic halogenation of phenol with chlorine. Pentachlorophenol (PCP) is an organochlorine compound and used as a pesticide and a disinfectant. Pentachlorophenol chemical structure is given in Fig. 4.6.

Tortella et al. (2008) isolated 11 fungal cultures from different locations of forests in Chile and tested the growth in the presence of a variety of chlorophenols including pentachlorophenol with measurement of the diameter of the colony of the culture on solid medium. Tortella et al. (2008) reported that the growth of two fungal cultures (*Galerina patagonica* and *Inonotus* sp.) in the presence of DCP (2,4-dichlorophenol) at 25 mg^{-1} was comparable to the growth of the same cultures in the absence of DCP indicting 100% tolerance. Fungal cultures, *Lenzites betulina*, *Phanerochaete chrysosporium, S. hirsutum,* and *T. versicolor* exhibited moderate tolerance with about 50% inhibition in growth at 25 mg L^{-1} of DCP. In contrast tolerance of the same cultures was further reduced with an increase in DCP concentration to 50 mg/L^{-1}. Other chlorophenols such as 2,4,6-trichlorophenol (TCP) and pentachlorophenol (PCP) were severely toxic to the fungal cultures with detection of growth in respect of only one or two cultures—*Lenzites betulina* and *Stereum* sp. Growth of these cultures in the presence of DCP, TCP, and PCP even at 50 mg L^{-1} was improved after adaptation to DCP at 25 mg L^{-1} for 25 days (Tortella et al. 2008).

Fig. 4.6 Pentachlorophenol



4.2.7 Effect of Diuron and Bentazon

Coelho et al. (2010) reported that herbicides bentazon and diuron were added into the medium after 3 days of growth of the WRF—*Ganoderma lucidum* on glucose basal medium; further incubation of this culture for 7 more days after the incorporation of herbicides resulted in inhibition of growth of the *Ganoderma lucidum*. The extent of suppression of cultural growth of *Ganoderma lucidum* was dependent on the dose of herbicides employed and at the highest concentration of 80 μM of diuron, about 50% growth was observed in comparison to that in control. Growth of the fungus *Ganoderma lucidum* was slightly inhibited at the lower level concentrations (5 mM bentazon and 30 mM diuron) of the herbicides treatment, while at the increased concentrations (80 mM diuron and 20 mM bentazon), the growth was severely reduced so that less than 60% of the mycelial growth was obtained after 10 days of the incubation period (Coelho et al. 2010).

4.2.8 Effect of Fungicides (Thiram, Zineb, or PCP) and Heavy Metals

Barajas-Aceves et al. (2002) reported that inhibition of growth in terms of ergosterol content within a range of 30–70% occurred upon exposure of *Coriolopsis gallica* 8260 to fungicides (Thiram, Zineb, or PCP) heavy metals and during 7 days of the incubation period (Barajas-Aceves et al. 2002).

4.2.9 Effect of Polyaromatic Hydrocarbons (PAH)

PAHs are a kind of pollutants that are frequently discharged into the ecosystem as a result of incomplete incineration of organic materials such as oil, gasoline, coal, municipal and industrial wastes (Juhasz and Naidu 2000; Ijoma and Tekere 2017; Kadri et al. 2017). Structurally PAHs consist of three/additional benzene fused rings, arranged in the angular, cluster, or linear arrangements (Di Toro et al. 2000). Continuous exposure to PAHs causes acute toxic effects in humans such as nausea, vomiting, and eye irritation. Higher-level concentrations of PAHs exhibit carcinogenic, mutagenic, and teratogenic, genotoxic effects. PAHs can also cause suppress immune reactions, skin inflammation, during the pregnancy affect the embryonic developments, liver and kidney damage (Rostami and Juhasz 2011; Rengarajan et al. 2015). The chemical structures of phenanthrene and pyrene are given in Fig. 4.7.

The growth of the WRF—*Pleurotus ostreatus* increased with higher concentration of polyaromatic hydrocarbons (PAH) in culture broth (Torres et al. 2016). There was a rise in biomass yield from 1.253 g at 26 μg/ml concentration of PAH to 1.67 g at 80 μg/ml concentration of PAH upon the growth of *P. ostreatus* for 15 days

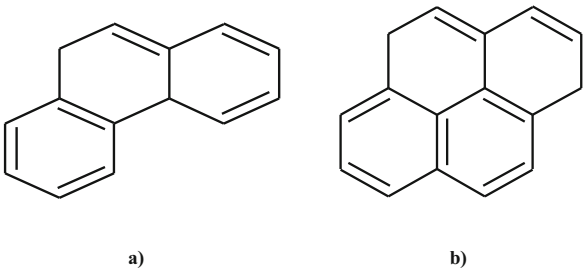


Fig. 4.7 Chemical structures of (**a**) phenanthrene and (**b**) pyrene

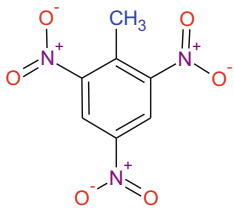


Fig. 4.8 2,4,6-trinitrotoluene

(Torres et al. 2016). In contrast, mycelial biomass of *Ganoderma lucidum* decreased with an increase in the concentration level of PAH in medium (Ting et al. 2011). When PAH concentration was increased from 2 μg/ml to 100 μg/ml in the fungal culture broth, the biomass of *G. lucidum* decreased from 0.18 to 0.10 g after 10 days (Ting et al. 2011). Growth of *Polyporus* sp. on pyrene in mineral salts medium (MSM) was influenced by the treatment of surfactants, Tween 80 (Lazim and Hadibarata 2016). Tween 80 was relatively more effective in enhancing the growth of *Polyporus* sp. on pyrene. The enhancement in the growth of *Polyporus* sp. on pyrene by Tween 80 was attributed to the higher solubility of pyrene in the presence of surfactant and the increasing availability of pyrene to the culture.

4.2.10 Influence of 2,4,6-Trinitrotoluene (TNT)

Nitro-aromatic explosive 2,4,6-tri-nitrotoluene (TNT) and its metabolites cause contamination of water and soil and also which can create major ecological problems in the worldwide. TNT exhibits mutagenic and toxic effect on microorganisms, plants, animals, and humans (Lewis et al. 2004; Claus 2014). The 2,4,6-trinitrotoluene chemical structure is shown in Fig. 4.8.

Nitro-aromatic explosive—2,4,6 trinitrotoluene at lower concentrations up to 6 μg/ml (0.03 mM) was innocuous to *Cerena unicolor*, but trinitrotoluene at a higher concentration of 60 μg/ml (0.3 mM) was toxic to the same culture as reflected by 75% inhibition of growth at the end of second-day incubation (Kachlishvili et al. 2016). *C. unicolor* recovered from the toxicity of trinitrotoluene at a higher concentration by 6 days as reflected by biomass. Effect of xenobiotics (at concentration level) on the growth of WRF is given in Table 4.2.

Table 4.2 Influence of xenobiotics (at concentration level) on the growth of WRF

S. No.	Xenobiotic	White rot fungi	Growth response Positive/ Negative	References
1.	Malathion	*Pleurotus ostreatus*	– (100 ppm)	Ganash et al. (2016)
2.	Diuron	*Phanerochaete chrysosporium*	+ (19 ppm) – (beyond 19 ppm)	Coelho-Moreira et al. (2013)
3.	PAHs	*Pleurotus ostreatus*	+ (26 ppm–80 ppm)	Torres et al. (2016)
4.	PAHs	*Ganoderma lucidum*	+ (2 ppm) – (100 ppm)	Ting et al. (2011)
5.	2,4,6-trinitrotoluene	*Cerena unicolor*	+ (6 ppm) – (60 ppm)	Kachlishvili et al. (2016)

Notes: Positive: Enhanced or increasing the growth of WRF, Negative: Inhibit the growth of WRF

4.3 Effect of Xenobiotics on the Secretion of LMEs by WRF

4.3.1 Effect of Malathion

Some xenobiotics enhance the secretion of LMEs in WRF. Ligninolytic enzymes – LiP, manganese peroxidase, and LAC by *P. ostreatus* were stimulated upon growth for 10 days culture medium amended with lower concentrations of Malathion at 25 and 50 μg/ml (Ganash et al. 2016). At 25 μg/ml of the concentration of Malathion, the productivity of lignin peroxidase, MnP, and LAC by *P. ostreatus* at the end of 10-day incubation was 0.51, 0.53, and 4.30 U/ml, respectively, and was increased than that of the respective enzyme secreted by the same culture of control (absence of Malathion). The productivity of LMEs by the same culture at a higher concentration (100 μg/ml) of Malathion was inhibited in comparison to control (Ganash et al. 2016).

4.3.2 Effect of Lindane

Ganoderma lucidum GL-2 strain is grown on the rice-bran substrate at 30 °C and pH 5.6 treated with 4 ppm lindane in the liquid medium, as well as in SSF, induced production of LMEs (Kaur et al. 2016). In SmF, 100.13 U/ml of LAC, 50.96 U/ml of MnP, and 17.43 U/ml of LiP enzymes were noticed whereas the SSF system gave yields of 156.82 U/g of laccase, 80.11 U/g of MnP, and 18.61 U/g of LiP enzyme activities (Kaur et al. 2016). Further rise of lindane to 40 ppm concentration in both SmF and SSF led to the suppression of the secretion of LMEs by *G. lucidum.* However, there was an increase in the secretion of extracellular protein content by *G. lucidum* at higher (40 ppm) concentration of lindane (Kaur et al. 2016).

4.3.3 Effect of Isoproturon

Growth of *P. chrysosporium* on the straw of wheat treated with isoproturon at70 μg/g in SSF resulted in secretion of LMEs of 0.306 U/g (MnP) and 0.20 U/g (LiP), respectively, at a peak time interval (Castillo et al. 2001).

4.3.4 Effect of Herbicides Diuron and Bentazon

Herbicides bentazon and diuron were strong inducers of LAC for *Ganoderma lucidum* (Coelho et al. 2010). The production of LAC activity in culture filtrates of 7 days incubation of *Ganoderma lucidum* grown by the treatment of herbicides bentazon and diuron at 20 and 80 μg/ml, respectively, was 170 and 207 Ug^{-1} (Unit/dry biomass) as against 20 Ug^{-1} of laccase production by the same culture on herbicide-free medium. The influence of diuron and bentazon on the secretion of another ligninolytic enzyme MnP by *Ganoderma lucidum* was less pronounced in absolute terms. The MnP activity was enhanced by the treatment of diuron at 80 μg/ml from 0.7 Ug^{-1} (control) to 8.6 Ug^{-1}. Coelho et al. (2010) have also observed that the activity of LiP in the supernatant of *G. lucidum* grown on medium with/without herbicides was not detected. The electrophoresis analysis of extracellular enzymes in culture filtrate of *G. lucidum* in the presence/absence of herbicides indicated that laccase isoform 2 was induced while laccase isoform 1 was suppressed.

4.3.5 Effect of Diuron

Supplementation of diuron (7 μg/ml) increased the lignin peroxidase activities from 47 U/L (at day 7) to 88 U/L at the tenth-day interval. The maximal manganese peroxidase activity was noticed in *P. chrysosporium* treated with diuron (7 μg/ml), at 5 days of incubation; in the absence and presence of diuron, MnP activities were 20.0 U/L and 22.4 U/L. It was also noticed that MnP activities in the culture filtrates in the absence/presence of diuron after 10 days of incubation were 15.4 and 29.4 U/L, respectively (Coelho-Moreira et al. 2013). Laccase was not at all detected in the culture filtrate of *P. chrysosporium* grown in the presence of diuron (Coelho-Moreira et al. 2013).

4.3.6 Effect of Chlorpyrifos

Chlorpyrifos [O,O-diethylO-(3,5,6-trichloro-2-pyridyl)phosphorothioate)] is considered as one of the OP pesticides which is widely used for the control of a variety

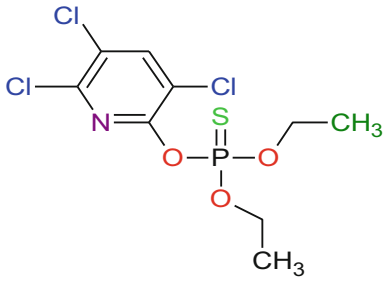


Fig. 4.9 Chemical structure of chlorpyrifos

of insect in an agroforestry system (Solomon et al. 2014). Extensive use of this pesticide can create ecological disturbances and also causes a toxic effect on the variety of living organisms in the environment (Giesy et al. 2014; Fu et al. 2015). The structure of CPF is given in Fig. 4.9.

According to the study of de Sousa Fragoeiro (2005), the test isolates exhibited a capacity for production of ligninolytic activity in the presence of pesticide mixture at 30 ppm level. In this study, *T. versicolor* produced very high activity of laccase with 680 U/ml in soil extract medium in the presence of pesticide mixture at 30 ppm level. Suppression of growth of *P. chrysosporium* at higher concentration of chlorpyrifos at 30 ppm in mineral salt, the medium was recognized by Rajakumar and Umamaheswari (2014). Wali et al. (2020) reported that compared to the control samples, the presence of chlorpyrifos at 100 mg/L concentrations suppressed production of laccase enzyme in *Pleurotus sajorcaju* at any point in time intervals.

Shanthi Kumari et al. (2019) studied the influence of chlorpyrifos (at single concentration 20 ppm level) on the secretion of three LE (LAC, MnP, LiP) in white rot fungus *Stereum ostrea* under submerged fermentation and stationary conditions. Enhanced secretion of LE was noticed in 20 ppm concentration CPF-amended culture medium rather than control (without CPF) cultures of *Stereum ostrea* under SMF. Maximum activities of LAC (214.362 U/ml), MnP (82.74 U/ml), and LiP (8.05 U/ml) were, respectively, noticed on CPF-amended medium against the same condition of without CPF culture medium. Maximum LAC (138.064 U/ml), MnP (51.84 U/ml), and LiP (6.44 U/ml) were recorded on the tenth day of incubation. Reduced secretions of LMEs were observed in CPF-amended culture medium rather than control samples under stationary conditions. Enhanced growth of *Stereum ostrea* was also noticed in CPF-amended culture medium under the same SmF conditions. In Fig. 4.10, Shanthi Kumari et al. (2019) presented the growth of *Stereum ostrea* in the presence/absence of CPF under SmF and stationary conditions on the tenth day of incubation. Additionally in the same Fig. 4.10 authors identified laccase as an enzyme in liquid culture medium treated with CPF.

Severe toxicity of chlorpyrifos at the highest concentration (40 ppm) toward secretion of intracellular proteins including ligninolytic enzymes by *S. ostrea* and at the same the concentration of CPF inhibited the growth of the fungal culture (Shanthi Kumari 2014).

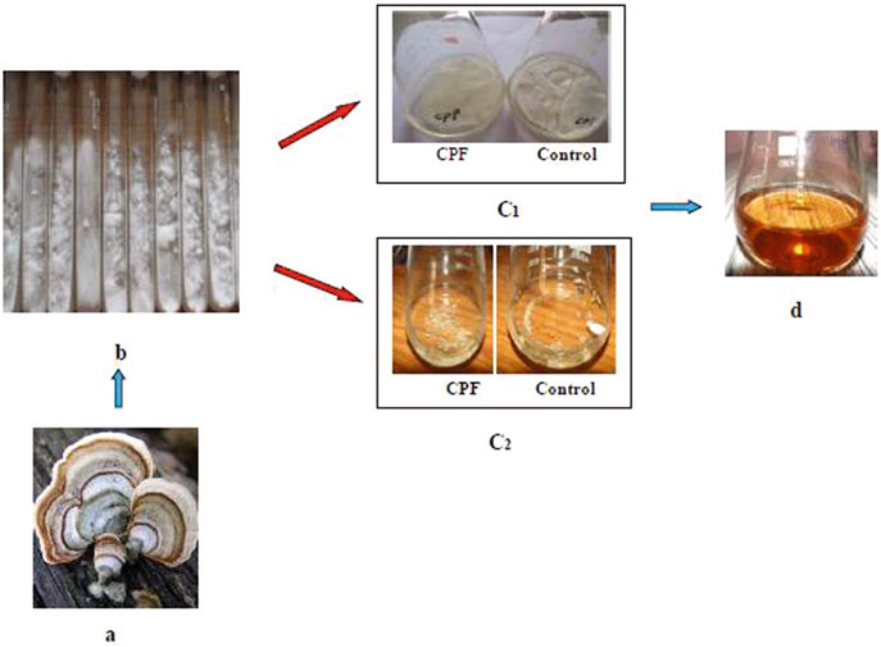


Fig. 4.10 (**a**) White rot fungi, (**b**) Growth of 6 days old culture of *Stereum ostrea.*(**c1**) Growth of *Stereum ostrea* in the presence and absence of CPF under stationary. (**c2**) Growth of *Stereum ostrea* on the tenth day of incubation in the presence and absence of CPF under shaking conditions. (**d**) Identification of LAC enzyme from the culture filtrate of CPF-amended medium (color change of the medium from colorless to dark red color indicates the presence of LAC enzyme)

4.3.7 Effect of 2,4,6-Trinitrotoluene (TNT)

The LE of WRF was dependent on medium composition (Kachlishvili et al. 2016). They have tested five WRF cultures, from that *Cerena unicolor* 300 produced the highest yields upon growth either on glycerol medium or mandarin peel treated with 2,4,6-trinitrotoluene (TNT) at 0.2 mM. Yields of LMEs such as laccase and MnP on mandarin peel + TNT were relatively higher than on glycerol + TNT. But, inhibition of MnP production by *Cerena unicolor* 300 occurred within 5 days after exposure to the highest concentration (0.4 mM) of TNT. This culture made recovery not only from the toxicity of TNT at the increased concentration after 6 days of incubation but also turned around in MnP production over control (Kachlishvili et al. 2016).

4.3.8 *Effect of Fluorene*

Ligninolytic enzymes laccase and MnP occurred to the extent of 138 and 44 U/L in the culture broth of *Polyporus* sp. have grown on fluorene in the presence of surfactant-Tween 80, respectively (Lazim and Hadibarata 2016).

4.3.9 *Effect of Dyes*

Textile industry effluents—a dynamic combination of chemicals, among which colorants (Dyes) are of special concern—impose major ecological challenges. Moreover, various dyes have shown to be carcinogenic and mutagenic effects (Weisburger 2002). Azo dyes polluted with wastewater have shown to be either toxic or maybe biologically transformed into hazardous or carcinogenic compounds (Ventura-Camargo and Marin-Morales 2013). In Fig. 4.11, chemical structure of colarene red azo dye is given.

LAC has been identified as the major LE with least and negligible detection of MnP when all three species (*Pleurotus ostreatus, Pleurotus sapidus*, and *Pleurotus florida*) of *Pleurotus* were grown on dyes (coralline navy blue, coralene red azo dye, and coralene azo dye) at different concentrations within a range of 20–200 g/ml (Kunjadia et al. 2016). In this study, the highest LAC activity was produced on the eighth day of culture in the medium spiked with 20 g/ml concentration of dye. *Trametes versicolor* secreted laccase, lignin peroxidase, and MnP to the extent of 16, 2, and 6.5 U/g at the end of the 30-day incubation period when grown on banana peel amended with basic red 46 dye in SSF (Zuleta-Correa et al. 2016). Textile industry's effluents-real dye was decolorized *Leptosphaerulina* sp. under conditions of glucose/nitrogen supplementations. These decolorizations were associated with significant productions of LAC (650 U/L) and MnP (100 U/L) (Placido et al. 2016). The influence of xenobiotic on the secretion of LE by WRF is given in Table 4.3.

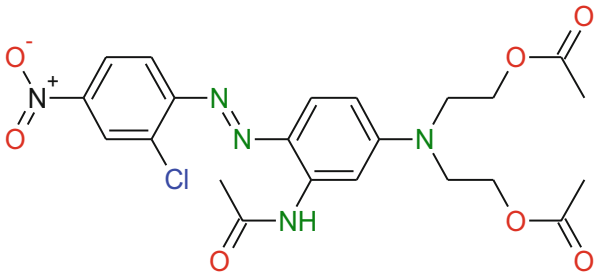


Fig. 4.11 Coralene red azo dye

Table 4.3 Effect of xenobiotics on LMEs by WRF

S. No.	Xenobiotics	White rot fungi	Influence of xenobiotic on ligninolytic enzymes positive/negative effect		References
1.	Malathion	*P. ostreatus*	LAC	+ (25 ppm–50 ppm) – (100 ppm)	Ganash et al. (2016)
			MnP	+ (25 ppm–50 ppm) – 100 ppm	
			LiP	+ (25 ppm–50 ppm) – (100 ppm)	
2.	Dyes	*Leptosphaerulina* sp.	LAC	+	Placido et al. (2016)
		Trametes versicolor	LAC	+	Zuleta-Correa et al. (2016)
			MNP	+	
			LiP	+	
		Pleurotus sp.	LAC	+	Kunjadia et al. (2016)
			MnP	–	
3.	Diuron	*P. Chrysosporium*	LiP	+	Coelho-Moreira et al. (2013)
			LAC	–	
4.	Trinitrotoluene (TNT)	*Cerena unicolor*	LAC	+	Kachlishvili et al. (2016)
			MnP	–	
5.	Bentazon and diuron	*Ganoderma lucidum*	LAC	+ (20 ppm Bentazon and 80 ppm Diuron)	Coelho et al. (2010)
			MnP	+ (20 ppm Bentazon and 80 ppm Diuron)	
			LiP	–	
6.	Fluorene	*Polyporus* sp.	LAC	+ (10 ppm)	Lazim and Hadibarata (2016)
			MnP	+ (10 ppm)	
7.	Lindane	*Ganoderma lucidum* GL-2	LAC	+ (4 ppm) – (40 ppm	Kaur et al. (2016)
			MnP	+ (4 ppm) – (40 ppm	
			LiP	+ (4 ppm) – (40 ppm	
8.	Chlorpyrifos	*Stereum ostrea*	LAC	+ (20 ppm)	Shanthi Kumari et al. (2019)
			MnP	+ (20 ppm)	
			LiP	+ (20 ppm)	
9.	Dichlorophenoxyacetic acid (2,4-D)	*Lentinus crinitus (L.)*	LAC	+	Serbent et al. (2020)

Note: Positive respond indicates enhanced secretion of lignin-modifying enzymes under the influence of xenobiotics. Negative respond indicates inhibition on the secretion of lignin-modifying enzymes under the influence of xenobiotics

4.4 Biodegradation of Pollutants by WRF

The WRF *Ganoderma lucidum* was more effective in removing herbicide bentazon than herbicide diuron as evident from leftover residues in the medium (Coelho et al. 2010). *G. lucidum* was more effective in degrading lindane in liquid fermentation than in SSF (Kaur et al. 2016). In liquid medium, about 80% of initially added lindane (4 ppm) was degraded at the end of 28-day incubation as against 38% degradation in SSF. Inoculation of pesticide-fortified soils with the WRF *T. versicolor* and *P. chrysosporium* increased bioremediation of insecticides (simazine, trifluralin, and dieldrin) in soil microcosms (Fragoeiro and Magan 2008). At 14 days incubation period in straw cultures of *P. chrysosporium* was able to bioremediate 91% of the herbicide-isoproturon (Castillo et al. 2001). *C. versicolor*, *H. fasciculare,* and *S. hirsutum* caused the highest remediation of terbuthylazine, atrazine, and diuron (86%) in liquid culture, but poorly degraded metalaxyl to less than 44% (Bending et al. 2002). They also demonstrated the deterioration of pollutants when WRF grew on-farm "biobed" organic matrix and showed some differences to that of submerged cultures. After 42 days, in biobed matrix *H. fasciculare* and *C. versicolor* were able to bioremediate about a third of the poorly available compound chlorpyrifos, among the tested WRF, *S. hirsutum*, shown to be the most effective oxidizer of the pesticides. PCP biodegradation in contaminated field soils (100 to 2137 mg kg^{-1} PCP) by *T. versicolor* (3 to 175 g kg^{-1} inoculum) was evaluated by Ford et al. (2007). Furthermore, Schmidt et al. (2005) observed a clear link with the amount of *T. Versicolor* fungal inoculums used and colonization of fungal cultures in soil bioaugmented for bioremediation.

Soil contaminated with PCP biodegradation by immobilized WRF *Anthracophyllum discolor* and *P. chrysosporium* on wheat straw was studied by Rubilar et al. (2011). In the presence of a contaminant, high-level MnP activities and fungal biomass was recognized in their studies. Moreover, 75% of pollutant degradation was consequently identified in immobilized fungal cultures on wheat grains in the soil samples (Rubilar et al. 2011). Efficient bioremediation of atrazine by immobilized *A. discolor* on formulating pelletized support was evaluated by Elgueta et al. (2016). The list of recent studies on the bioremediation of xenobiotics by white rot fungi is given in Table 4.4.

4.4.1 LE Involved in Bioremediation of Xenobiotic Compounds

WRF is the most efficient organisms to secrete LMEs (LAC, MnP, LiP) for the mineralization of lignin polymer. Oxidative and non-specific nature of these enzymes shows the potential application in the bioremediation of environmentally toxic pollutants. Among these ligninolytic enzymes laccase enzyme plays a major role in the oxidation of various xenobiotic compounds and also there are several

Table 4.4 List of bioremediation of xenobiotics by white-rot fungal cultures (WRF)

S. No.	White rot fungi	Xenobiotics	References
1.	*Ganoderma lucidum GL-2*	Lindane	Kaur et al. (2016)
2.	*Phlebia brevispora* and *P. lindtneri*	Lindane	Xiao and Kondo (2020a)
3.	*P. ostreatus*	Aldrin	Setyo et al. (2017)
4.	*Trametes versicolor*	Carbofuran	Ruiz-Hidalgo et al. (2014)
5.	*Anthracophyllum discolor*	Atrazine	Elgueta et al. (2016)
6.	*Coriolopsis* sp.	Dye	Cheng et al. (2016)
7.	*Phlebia acanthocystis*	PCP	Xiao and Kondo (2020b)
8.	*G. lucidum*	Pyrene and phenanthrene (PAHs)	Agrawal et al. (2018b)
9.	*Phlebia brevispora*	PAHs	Harry-asobara and Kamei (2019)
10.	*Pleurotus* spp.	Azo dye	Kunjadia et al. (2016)

reports that have been reported in the degradation of pollutants by laccase enzymes. Soil and water contaminating with a variety of pollutants such as OP pesticides, and azo dyes, aromatic compounds (phenylenediamine derivatives, benzenethiols, phenols, aminophenols (anilines), and trichlorophenols), and PAHs can be efficiently oxidized by a broad substrate range of laccases (Xu 1996; Amitai et al. 1998; Kues 2015; Sharma et al. 2018). Kadri et al. (2017) reported that both lignin and non-lignin type compounds as well as PAHs were completely mineralized by the involvement of LiPs. Pozdnyakova (2012) demonstrated that PAHs such as phenanthrene, anthracene fluoranthene, pyrene as well as a variety of derivatives of these PAHs can be effectively oxidized by the secretion of MnP by WRF *A. discolor.* An MnP from the WRF, *Trametes* sp. displayed a strong capability of mineralizing PAHs as well as azo and indigo dyes (Zhang et al. 2016). Some recent reports of WRF-secreted LMEs involved in the remediation of a variety of pollutant are presented in Table 4.5.

Koroleva et al. (2015) described the molecular level degradation of herbicide atrazine by laccase-HBT redox-mediated system.

4.5 Conclusions

Lignocellulosic material is the only natural resource on the earth which has a broad prospective as biofuel material. Excess of lignocellulosic material discharged from agro-waste industries causes environmental pollution. The major toxicant in lignocellulosic materials is lignin. The complex structure of this lignin is not easily bioremediation by microbial communities. But WRF and their highly effective ligninolytic enzymatic system have efficiently degraded/detoxified/oxidized the complex lignin in lignocellulosic material. Due to this reason, WRF and their LMEs are highly used as clean technological agents as well as act as an initiator

Table 4.5 Bioremediation of pollutants by the involvement of LMEs

S. No.	Source of enzyme	The enzymes involved in remediation	Selected pollutant	References
1	*Ganoderma lucidum*	MnP	Endocrine-disrupting nonylphenol and triclosan	Bilal et al. (2017)
2	*Pleurotus ostreatus D1 Agaricus bisporus F-8*	Versatile peroxidase (VP) LAC	PAHs	Pozdnyakova et al. (2018)
3.	*P. Chrysosporium*	LiP	PAHs	Pozdnyakova (2012)
4	*Irpex lacteus*	LAC	Dyes (azo, indigo dyes)	Qin et al. (2014)
5	*Trametes* spp.	LAC	Dyes and PAHs	Zhang et al. (2016)
6.	*Coriolopsis gallica*	LAC	Halogenated pesticides	Torres-Duarte et al. (2009)
7.	*Trametes versicolor*	LAC	PAHs	Bautista et al. (2015)

for the bioconversion of lignocellulosic materials into various helpful bioenergy products (e.g., bioethanol, lactic acid, microbial polysaccharides). The non-specific nature of these ligninolytic enzymes not only degrades lignin but also oxidizes the compounds of the lignin type, such as phenolic compounds, PAHs, and pesticides. Industrial and environmental applications of LMEs and WRF have been extensively reported in recent reviews. But it was also identified that very few recent research reports are available on how to interact with this WRF with xenobiotic compounds. Hence, the current chapter analyzed the effect of various pollutants on the growth and production of ligninolytic enzymes (LE) by WRF and also analyzed the intern action of WRF in the degradation of xenobiotics**.** All the above studies concluded that different kinds of xenobiotic compounds influence the growth and secretion of LE by WRF depending upon the concentrations of residues. From the above references, it was noticed that at a lower concentration of xenobiotics treatment shows enhanced both biomass and secretion of LMEs in WRF. It was also identified that at higher concentrations of xenobiotics treatments inhibit the growth as well as the production of LMEs in WRF. WRF has massive applications in the bioremediation of a variety of environmental pollutants. Non-specific and oxidative property of LMEs in WRF is the major key factor involved in the bioremediation of xenobiotics. This analysis supports the importance of WRF and its LMEs in the area of biotechnology and environmental applications.

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Chapter 5
Challenges in Bioethanol Production: Effect of Inhibitory Compounds

Faiza Kausar, Muhammad Irfan, Hafiz Abdullah Shakir, Muhammad Khan, Shaukat Ali, and Marcelo Franco

Abstract In today's world, the need for sustainable fuel production is increased. For the production of cost-effective fuels for many purposes, the concern has shifted toward the use of biomass, including plants. The use of biomass for bioethanol production has proved beneficial in terms of cost, of production, but the main challenges encountered are of the production of inhibitors. Production of bioethanol from biomass involves first, second, and third generations of feedstock. The pretreatment of second-generation biomass, i.e., lignocelluloses, results in the formation of inhibitory byproducts. The inhibitors include furans, weak acids, and phenolic compounds. These inhibitors result in the increase of cost for the whole processing. This review is focused on process, the compounds that have inhibitory role and are extracted from biomass rich in lignocelluloses in the duration of pretreatment, their mechanism of action, and how to minimize their effects on fermentation process.

Keywords Pretreatment · Lignocelluloses · Bioethanol · Biomass · Inhibitors · HMF · Furfural · Phenolics

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5.1 Introduction

Biofuels like bioethanol have been seen to gain the peak of attention as they may be acting as the substitute to fuels that are based on petroleum, and may protect the reserves of oil and also may reduce the greenhouse gases and atmospheric carbon dioxide (Parawira and Tekere 2011; Sarawan et al. 2019). To some extent, it is used for the replacement of a mixture of ethanol and gasoline, E85 (85% ethanol and 15% gasoline) and E15 (15% ethanol and 85% gasoline). This fuel is a liquid based in nature which can possibly be produced from many kinds of biomass and conversion techniques (Vohra et al. 2014). The focus of research has been changed to non-food biomass (Deshavath et al. 2017). In case of second generation of bioethanol production, the biomass type is lignocellulose, and it is rich in cellulose which is same as sugar and starch as it is also a glucose polymer (Tran et al. 2019). Also, lignocellulose consists of lignin and hemicellulose, lignin is not cellulosic in nature so is not fermentable (Nguyen et al. 2018).

For making the cellulose available from lignocelluloses and increasing the rate of fermentation, pretreatment is performed. But this pretreatment is associated with the production of derived microbial inhibitors (Hou et al. 2019).

A large number of biomass feedstock overlapping the three generations, i.e., first, second, and third, had been utilized for production of biofuel. The feedstocks involved in the first generation are the ones that are rich in sucrose, e.g., sugar beet, sweet sorghum, sugarcane, and fruits, and also involve the feedstocks rich in starch, e.g., wheat, corn, potato, rice, barley, cassava, and sweet potato. The next generation of biofuel is related to substances rich in lignocelluloses, e.g., straw, grasses, and wood. The last generation of biofuel comes from biomass of algae, i.e., macroalgae and microalgae (Azhar et al. 2017; Soccol et al. 2019). The most efficient method is the second-generation bioethanol production. The 2G feedstocks are chemically composed of carbohydrates and lignin, e.g., minerals, ash, salts, pectin, cellulose, and hemicelluloses (Ravindran and Jaiswal 2016). Polysaccharides are present in lignocelluloses-rich materials, which are seen to be un-accessible for further processings like bioconversion. For this problem, pretreatment has become an important step in making the cellulose accessible to enzymes (Hou et al. 2019; Kumar et al. 2019; Watanabe et al. 2019).

Lignocelluloses have many polymers in it, and the main polymers included are lignin, cellulose, and hemicellulose (Alonso et al. 2012); they also contain some other molecules in small amounts, e.g., minerals, acetyl groups, phenolic compounds, and some others are present in trace amounts (Agarwal et al. 2017). The percentages of these molecules differ based on their origin. However, the generalized percentages of the components of lignocellulosic biomass are summarized in Fig. 5.1 (Madadi et al. 2017a).

Cellulose and hemicellulose (carbohydrates) together make almost 70% of LB. Their high percentage in LB is the main benefit of utilizing these in bioethanol production (Cheng et al. 2008). Only these carbohydrate components are the basic ones for the bioethanol (and other biochemical) production after fermentation.

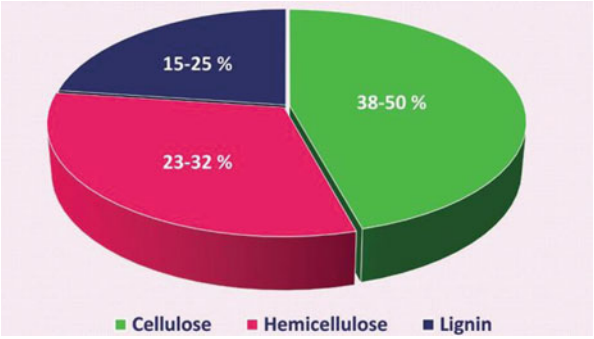


Fig. 5.1 Percentages of different polymers in LB (Ingle et al. 2019)

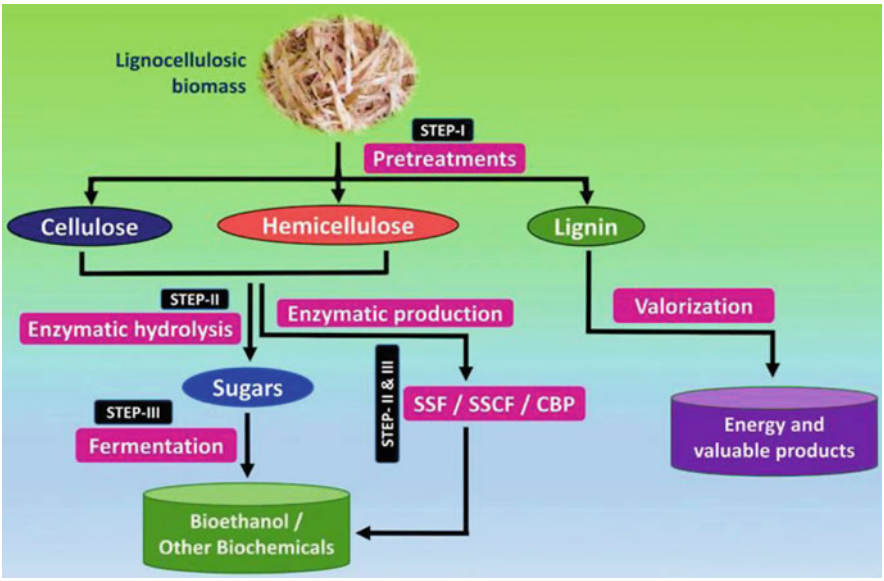


Fig. 5.2 A schematic representation of bioethanol production from LB (Ingle et al. 2019)

Production of bioethanol from LB is carried out by the following three steps:

1. Pretreatment
2. Hydrolysis
3. Fermentation

However, in some cases, steps (2) and (3) can be combined to carry out SSF (simultaneous saccharification) or SSCF (simultaneous saccharification and co-fermentation). Figure 5.2 is representing the steps involved in bioethanol production from LB. The non-carbohydrate components (lignin) go through the valorization step to make other value-added products.

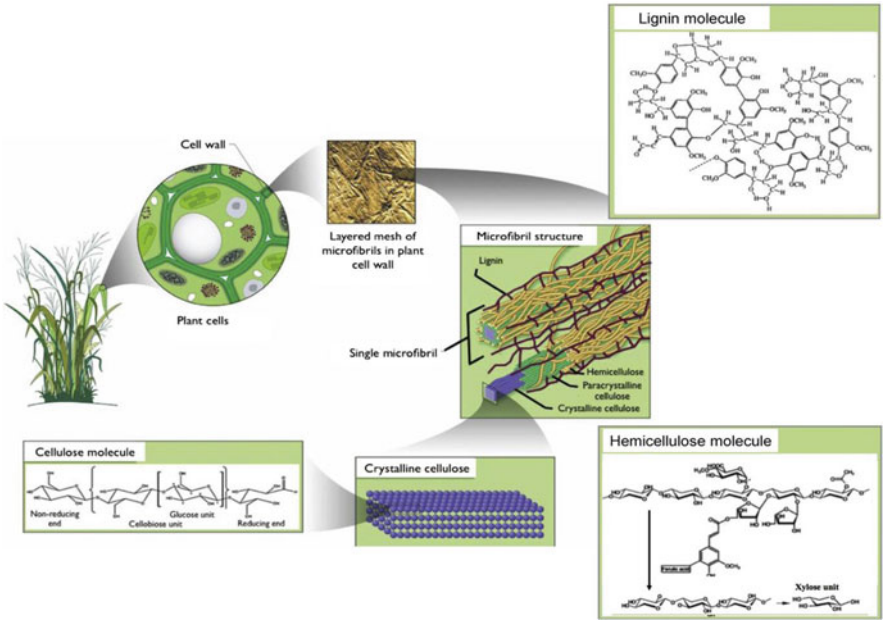


Fig. 5.3 Structure of lignocellulose (Seidl and Goulart 2016)

The reason why pretreatment should be carried out lies in the structure and composition of LB. Cell walls of plants are resistant to degradation by micro-organisms because of the strength and robustness provided by the defensive structure of lignocellulosic biomass (Fig. 5.3). More strength and toughness is provided by the bonding (cross-linked) between hemicellulose and cellulose with lignin through the strong ether and ester linkages (Alonso et al. 2012). For conversion of such complicated structures to simple ones, the chemistry of its inner side must be understood completely, so that the conversions to bioethanol and other products can be carried out in a convenient way (Chandel et al. 2015). The most crucial step in bioethanol production is pretreatment as it converts the un-accessible products to accessible products (i.e., simpler sugars) by presenting them to the enzymes (cellulases) (Mosier et al. 2005). The cost estimated for pretreatment is almost 40% of the total process cost (Sindhu et al. 2016).

For carrying out pretreatment in some ideal ways, the following criteria should be met:

The pretreatment method should

1. Boost the simpler sugars formation from lignocellulosic biomass or may produce them by hydrolysis
2. There should be no loss or degeneration of those sugars
3. Process used for pretreatment should be effective cost-wise
4. Byproducts must not be formed (Chandel and Da Silva 2013; Kumar and Sharma

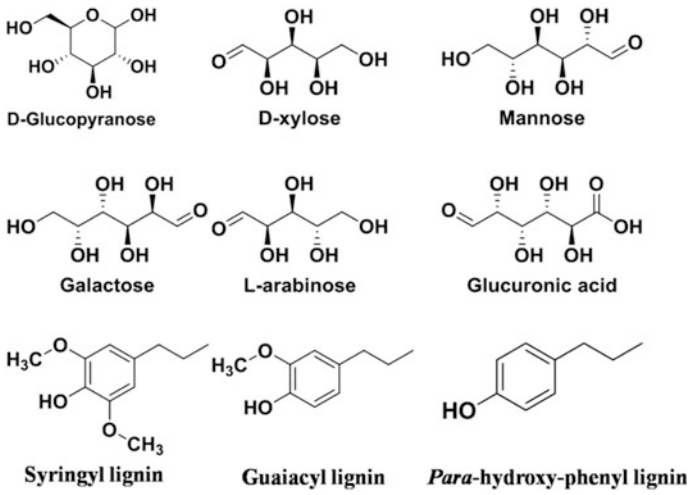


Fig. 5.4 Structural units of cellulose, hemicellulose, and lignin (Chen et al. 2017)

2017; Kumar et al. 2009)

The process of pretreatment affects the structure and chemical makeup of LC biomass by making changes to its macrostructure and microstructure. Also, it makes changes to LC structure by making it more representable to microbes (An et al. 2015). During this change in structure process, cellulose surrounded by lignin and hemicellulose, these two polymers are broken down, the structure of cellulose is altered to make cellulose available to enzymes. Similarly, lignin and hemicellulose are removed and degraded respectively. This process makes interaction of enzyme substrate better which improves the hydrolysis of sugar and makes the process efficient (Chandra et al. 2015; Michalska et al. 2012; Palmqvist and Hahn-Hägerdal 2000). The structures of lignin cellulose and hemicellulose are complex as shown in Fig. 5.4.

There are two basic strategies to make the lignocelluloses susceptible to be converted into bioethanol, i.e., pretreatment and hydrolysis.

1. Pretreatment

- Physical pretreatment
- Chemical pretreatment
- Physico-chemical pretreatment
- Combined pretreatment
- Pretreatment by biological ways (Kumari and Singh 2018; Madadi et al. 2017b)

2. Hydrolysis

In this stage, the biomass that had undergone pretreatment is converted into glucose which is fermentable to be used for bioethanol synthesis (Machineni 2019).

5.1.1 Pretreatment Explained

The different methods of pretreatment (Fig. 5.5) are explained in detail as below:

5.1.1.1 Mechanical Pretreatment

Mechanical pretreatment of biomass is the milling and grinding, for reduction of the sizes of particles. The high cost of the process as compared to the worth of final product is the challenge accompanied by this process. The final results of the very process are reflected by the temperature range, pressure, time, and feedstock. Mechanical pretreatment is not enough alone, it must be used along with the chemical methods for improving the yields of fermentable carbohydrates (Tu and Hallett 2019).

Pretreatment of lignocellulosic biomass with mechanical methods is crucial for the improvement of their receptiveness to enzymes, placement of particles, and their affectivity for further conversion by biological means. This mode of pretreatment is also helpful in improving the flow characteristics, increase in the pore sizes, making the surface are suitable enough to be acted upon, and increasing the bulk density (Barakat et al. 2014).

The reduced sizes of particles make the surface area larger and the crystalline property of cellulosic materials is reduced which is also helpful. Once after harvesting biomass, preconditioning proves helpful in making the lignocellulosic biomasses come in the raw form of the size 10–50 mm. Further conversion by chipping makes their sizes lower to 10–30 mm, at the end by milling and grinding the sizes can be more lower as 0.2–2 mm (Agbor et al. 2011; Hsu 1996).

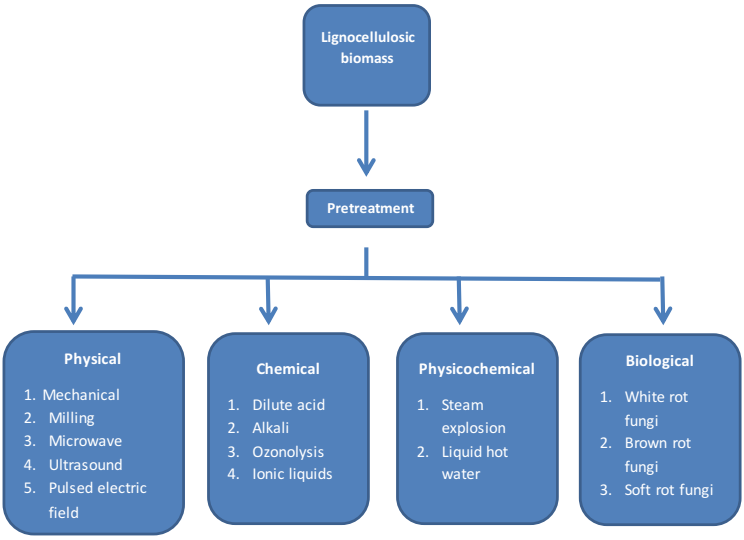


Fig. 5.5 Different strategies for pretreatment (Arora et al. 2020)

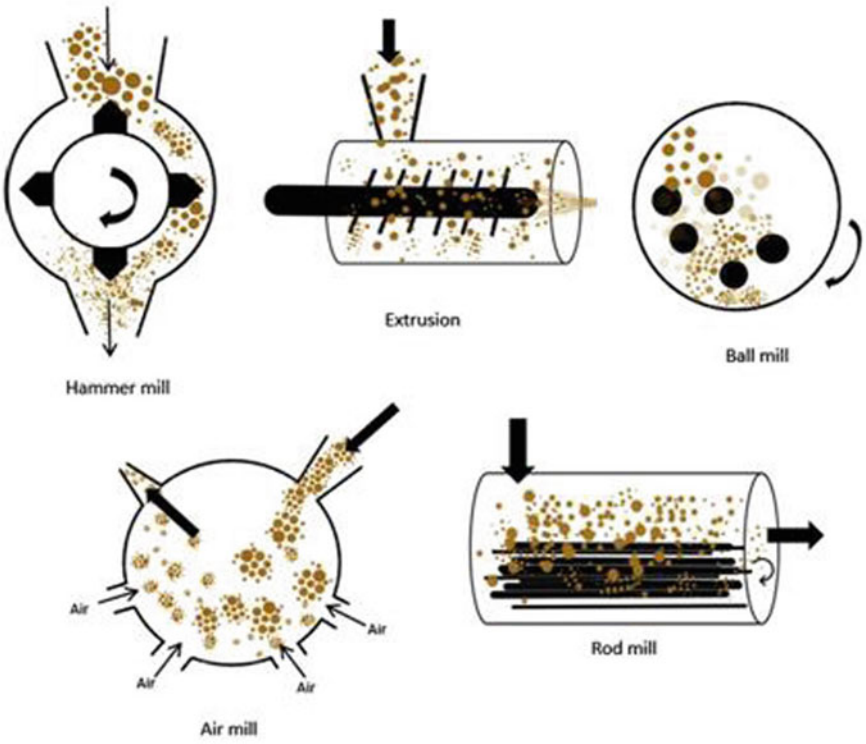


Fig 5.6 Physical methods of pretreatment (Jędrzejczyk et al. 2019)

The purpose of using mechanical pretreatment finds its success in converting the larger sizes to extremely fine ones and also reduction in the crystalline property of cellulose. Milling methods that reused include: pin mill, vibratory mills, knife mills, extruders, and hammer mills (Cheng and Timilsina 2011; Sun and Cheng 2002).

Among all the mills mentioned above, hammer mills are the ones that have easier mode of operation, have ability to make a greater range of particle sizes, and also are cost-effective (Dey et al. 2013). The modes of action by which hammer mills reduce the sizes of particles are impact and shear. The limitation is that we cannot process the larger stalk or straws by these mills. For processing such materials they are first processed by other mills like knife mills to make their sizes compatible with other mills so that they may be processed without any difficulty as that of choking (Bitra et al. 2009). Figure 5.6 (Jędrzejczyk et al. 2019) explains the process of milling.

5.1.1.2 Chemical Pretreatment Methods

- *Dilute acid pretreatment*: By using this method of pretreatment we are actually hydrolyzing the hemicelluloses and it also plays role in making the celluloses susceptible to degradation by enzymes easily. H_2SO_4 (strong acids) are used in dilutions for lignocelluloses. The samples are kept soaked in the acid solution with the rise of temperature up to 160 °C for 10 min. When pretreatment is done with such strong dilute acids then the reduction in hemicelluloses (xylosyl and galactosyl) groups can be seen but the contents of lignin will still be higher (Zhou et al. 2014). In one of the studies, the pretreatment strategy was applied to shells of *Jatropha curcas*, where, 70% of cellulose was transformed enzymatically by using this approach (Martín et al. 2015).
- *Alkaline hot water pretreatment*: The comparison of pretreatment strategies shows that the method of alkaline pretreatment is most advantageous as it gives lower inhibitor generation and low cost for the reactor (Zhuang et al. 2016). This approach of liquid hot water pretreatment utilizes high pressure while biomass is kept in auto-ionized water. As a result of auto-ionization and applied pressure, H_3O+ ions are generated that result in the hydrolytic disruption of hemicelluloses, celluloses, and some of the lignin. In one of the studies by Xiao et al. (2014), pretreatment of bamboo with LHW at 200 °C, he received a 3.8-fold increase in glucose content through hydrolysis y enzymatic methods. But when the temperature was raised to a high level, a drastic decline in the pH of water was observed and more of the carbohydrates were degraded (Timung et al. 2015). Moreover, deacetylation of the biomass is introduced by alkali, which eliminates the suppression of acetyl groups on enzymatic hydrolysis (Yang et al. 2019).
- *Mild alkaline methods*: As only cellulose and hemicellulose can be digested for ethanol production so we have to eliminate lignin from the biomass. For this purpose, the best method is the use of mild alkalis. This method produces the least inhibitors and lesser hemicellulose solubilization; also temperature range will be reduced for processing.

  NaOH and KOH are the best alkalis for this pretreatment but they are costly which is a serious concern. Some other alkaline solutions for this method are NH_3 and $Ca(OH)_2$ that can be used in AFEX, ARP (ammonia recycled percolation), and alkaline pretreatment (Yang and Wyman 2008).
- *Organosolv pretreatment*: This method involves the addition of a mixture of organic liquid and water and adding it to lignocellulosic biomass. Usually, organic liquids are used with water in 1:1, the common organic liquids include: C_2H_5OH (ethanol), CH_3OH (methanol), and C_3H_6O (acetone) (Blanch et al. 2011). The mixture of LG and organic compound is heated so that it may dissolute lignin and help in giving refined cellulose. Besides the above-mentioned organic compounds, formic acid and acetic acid can be used as mixtures in 30% volume by volume, respectively, along with water (Sindhu et al. 2012). In this method, the resultant dissolved products are lignin and hemicellulose, but cellulose remains in solid form. This yields three classes: residual cellulose, liquid hemicellulose, and solid lignin (Pan et al. 2006).

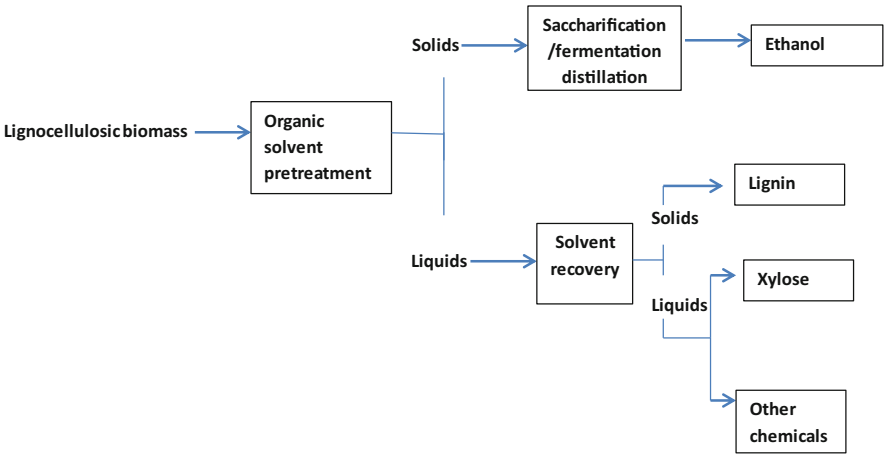


Fig. 5.7 Flow chart of Organosolv pretreatment (Bhutto et al. 2017)

The process is shown in Fig. 5.7 (Zhang et al. 2016). Mineral acid is used as a catalyst sometime to either reduce the operating temperature or enhance the delignification process. Catalytic OS process can be carried out at 100–150 °C to break the hemicellulose lignin bonds. Catalyst increases the rate of delignification, and solubilization of the hemicelluloses fraction and higher yield of xylose are obtained (Hu et al. 2008). Organic acids such as formic acid, oxalic, acetylsalicylic, and salicylic acid also can be used as catalysts (Bitra et al. 2009).

Organosolv pretreatment is the same as pulping by this very method, but the later one requires a higher degree of lignin removal (Zhao et al. 2009b). For saving the inhibition of the microbes that are necessary for the hydrolysis and fermentation by the solvents, these solvents need to be removed. If we see the aspect of low-cost expenses and recovery, then we can use low boiling point alcohol. But for considering the easy recovery, we have to use high pressure which proves to be costly. For minimizing the explosion and fire hazards by the use of violent organic solvents one has to use very strict measures for avoiding such mishaps.

Zhang et al. (2016) have suggested that for commercial purposes the pretreatment conditions should be up to the mark so that the whole process may be cost-effective. For the reduction of water and solvent in the system, LSR (liquid to solid ratio) is the best criterion; by its help one can minimize the solvent and water content in the system, as a result cost is reduced effectively.

Organosolv pretreatment has many beneficial effects; as in producing the proportions of lignin, cellulose, and hemicellulose so scientists consider it as the most effective and developing pretreatment technique. Also, the benefits of easier recovery and reusability make it an effective method. Lesser inhibitory compounds, i.e., HMF and furfural are produced because the conditions are milder (pressure and temperature) (Kim and Pan 2010).

- *SO_2-catalyzed steam explosion*: In this pretreatment method, SO_2-catalyzed steam is used. SO_2 is a gas which gives H_2SO_3 upon dissolving in water. This

method of SO_2–catalyzed acid hydrolysis is more favorable as it gives the least inhibitors as compared to other methods and also gives a more easily digestible substrate. As compared to dilute acid (H_2SO_4)-catalyzed pretreatment this method is effective at lower temperatures (Dechman and Foody 2020).

- *Oxidative delignification*: This method of pretreatment is the common one. The reagents that are used include oxygen, ozone, hydrogen peroxide, and chlorine. This process might be accompanied by the production of free radicals from reagents, which prove helpful in evacuating lignin from LG biomass. When LG biomasses are treated with oxidative methods, the reduction in lignin contents is significant and loss of cellulose is at the least. Pretreatment with ozone results in the significant yields of hydrolysis as compared to the hydrolysis from the untreated biomass.

  Removal of lignin is carried out at suitable conditions that results in almost no inhibitor formation (Sun and Cheng 2002). All the lignin can be removed from LG biomass by this very method leaving behind the hemicellulose and cellulose which can be easily fermented to ethanol (Qi et al. 2009). This process of pretreatment is somehow costly so, can be used in assistance with some other methods, to eliminate lignin from LG biomass (Qi et al. 2009).
- *Ionic liquid pretreatment*: Ionic liquids are such salts that have two fractions: negative ions that are inorganic and positive ions that are organic. These salts have the ability that their characteristics can be changed accordingly through adjustment of the positive and negative ions (Zavrel et al. 2009). The mode of action of strong ionic liquids is such that they dissolve LG biomasses thus make a uniform dissolvent (Zavrel et al. 2009). Li et al. (2010) studied the impact of several ionic liquids on the hydrolysis of corn cob. These studies showed that those salts that have chloride and phosphate in them result in higher percentages of reducing carbohydrates. The reason why this happens lies in the fact that the ionic liquid dissolution makes the surface area of the larger LGs smaller which leads to the conversion of larger sugars to smaller ones. Enzymes cannot survive in ionic liquids any longer due to their pH sensitivity, so hydrolysis steps cannot be performed in such liquids.

The uniform solutions of lignocellulosic biomasses are made by using the anti-solvents of ionic liquids so that reformed lignocellulosic biomass is formed. These modified solutions of LC biomasses can be easily attacked by enzymes and also they have lower proportions of crystalline structures as compared to untreated biomasses (Zhao et al. 2009a). The cellulose becomes free from the LC biomass so, it can be easily and efficiently processed by the enzyme (cellulase). The LC materials swell by the treatment of ionic liquids which may aid in the increase in the rate of their hydrolysis in the next stage, but the non-treated biomass have lower rates of hydrolysis (Cao et al. 2014b). Due to the structure of the lignin present in LC biomass, the solubility of these LCs needs severe conditions to dissolve them in ionic liquids. Also, ionic liquids are costly, so there is a need of research to make the solubility better and the process cost-effective (Sun et al. 2016).

5.1.1.3 Physico-Chemical Pretreatment

- *AFEX pretreatment*: AFEX is a kind of pretreatment method in which volatile NH_4 is used for treating cellulose by the expansion of ammonium fibers. This method was developed by Bruce Dale to minimize the challenges to processing of lignocelluloses and increase the rate of destruction of biomass into simpler sugars that can be easily fermented. In contrast to other pretreatment methods that are aqueous in nature, this one is dry in nature process, and results in no significant rise of biomass composition, and no washing is needed leading to no significant waste and expenditure (Chundawat et al. 2020).
  This technique of pretreatment has been applied to different biomasses including: perennial grass, switchgrass, corn stover, bagasse, wheat straw, and alfalfa. Alizadeh et al. (2005) performed switchgrass pretreatment by AFEX using the best possible conditions of pretreatment. This study showed that the pretreatment with AFEX results in ethanol yield which is much higher than that obtained without the treatment with AFEX. Uppugundla et al. (2014) have also reported similar results by this technique. The limitation of this pretreatment strategy is that it cannot be effective with the LC biomasses having very high lignin contents, e.g., nutshells and wood (Kumar et al. 2009).
- *Hydrothermal pretreatment*: When one moves toward the use of thermal properties of LC materials, the stabilities of each component should be kept in mind as there is a wide range of thermal stabilities for each biomass. Hemicellulose is easily decomposed as compared to lignin and cellulose. So it is removed from the process earlier to the disruption of cellulose through hydrothermal techniques. This pretreatment strategy is beneficial as it makes the enzymatic digestibility of LC biomasses better. When the temperature of hydrothermal pretreatment is higher than 240 °C, then cellulose is adversely disrupted, so the range of temperature of this process should be in the limit of 160–240 °C (Cao et al. 2014a; Sun et al. 2014b). Xiao et al. (2014) in their study observed the effects of different temperature ranges (i.e., 140–200 °C) on the composition changes of bamboo chemical nature; they also examined the hydrolysis characteristics at the varied conditions. By introducing the extreme temperature ranges, small amounts of lignin and celluloses were denatured and most of hemicelluloses were eliminated from the process. Pretreatment at 200 °C resulted in about 76% conversion into glucose but only 16% conversion was seen in the non-treated biomass (Xiao et al. 2014). The liquids remaining after hydrothermal pretreatment can be further treated to convert them to some other chemicals. When beech wood was treated by this pretreatment strategy its enzymatic digestibility increased from 7% by weight to 70% by weight, also its surface area and pore size was increased (Nitsos et al. 2013). This pretreatment strategy is inviting due to the conveniences including: low cost, lesser inhibitors production, no need of catalysts, and the most important one is that fresh or wet LC biomasses can be used.

  As hydrothermal pretreatment does not needcatalysts usually, but such methods are also developed that make use of catalysts for efficient elimination

of lignin or hemicelluloses and also these catalysts help in optimizing the simple carbohydrates from the complex ones (celluloses and lignin) (Sun et al. 2014b). If diluted alkali is used as a catalyst in this mode of pretreatment, then it is observed that in sugarcane bagasse the contents of lignin were reduced, and the yield of glucose plus the hydrolysis rates were increased (Miura et al. 2012). Sulfuric acid was also used as a catalyst for hydrothermal pretreatment in LG biomass to make their enzymatic hydrolysis easy (Lu et al. 2009).

- *Steam explosion*: For the pretreatment of lignocellulosic biomass, researchers commonly use the strategy of steam explosion. This pretreatment is operated by exposing the chipped LC material to higher pressure with steam at elevated heat (for multiple seconds to minute), after that pressure is dropped to 760 torr. Due to the sudden decrease of high pressure, most of the LC materials are converted into fibers. Also, lignin and hemicellulose get disrupted and can be eliminated from the mixture (Pan et al. 2005). This process has many benefits as environmentally friendly, energetically efficient, and lesser hazardous chemicals production (Alvira et al. 2010).

  Steam explosion is applied to LC materials without adding any other chemicals. When wheat straw was treated with this method, at 170–220 °C, the results were observed to check the hydrolysis behaviors (Horn et al. 2011). The observation was that when the temperature was kept at 210 °C then the hydrolysis products obtained were at the maximum. Similarly, glucose yields were also produced at the same percentages when the conditions were harsh, but the inhibitors of aromatic nature were produced which affected the fermentation process. So the removal of inhibitor is necessary. Simple wash with water is also effective for steam pretreated LC biomasses having inhibitors in the solution. As many inhibitors are produced in this pretreatment process for the next stages when the conditions of the method are severe, so some impregnating reagents are used for improved separation of hemicelluloses from the mixture at milder conditions. Those reagents may include sulfuric acid and sulfur dioxide. Boussaid et al. (2000) studied the changes in the chemical composition and the behavior of enzymatic hydrolysis when sulfur dioxide was impregnated on LC material, under different conditions. With severe conditions of steam explosion method, the digestibility of material to enzymes was enhanced but the negative side was that severe conditions resulted in lesser production of simple sugar of hemicelluloses. However, the mild conditions for steam explosion pretreatment enhanced both glucose levels and enzymatic digestibility for the later stages (Boussaid et al. 2000). In a study by Martín et al. (2002), the fermentation abilities and effects of enzymes on hydrolysis were compared at 25 °C temperature for a time duration of 10 min, with sulfuric acid and sulfur dioxide as impregnated agents in sugarcane bagasse LC material. With sulfuric acid as, the yield of glucose was higher but that of total sugars was lower, on the other hand, with sulfur dioxide the yields of xylose and total sugar were higher. Sulfuric acid had played its role in the inhibition of inhibitors, enzymatic hydrolysis catalysis, and polysaccharides hydrolysis (De Bari et al. 2007; Varga et al. 2004).

5.1.1.4 Biological Pretreatment

Biological pretreatment makes use of white, soft, and brown rot fungi for degrading the hemicelluloses and lignin of lignocellulosic biomasses (Saritha and Arora 2012; Sindhu et al. 2016). Soft rot fungi and white fungi target the lignin and cellulose while brown rots target only cellulose (Cheng and Timilsina 2011). Basically, these fungi use their enzymes (lignases), and break lignins with these enzymes. The process parameters of the method besides composition of biomass and nature of biomass including moisture content, duration of incubation, rate of aeration, pH, temperature and the most important, type of microbeare effective in pretreatment (Sindhu et al. 2016). Sindhu et al. (2016) have explained the review of features of biological pretreatment like enzymes involved and parameters and also the future prospects. The duration of incubation for delignification is longer in the biological pretreatment method, for this purpose greater space is needed which is a limitation for use in industries. By using a suitable microbial selection, this factor can be minimized (Sindhu et al. 2016). The second limitation of this strategy is that the rate of hydrolysis is slower in comparison to other techniques (Saritha and Arora 2012). When the sugar concentrations will be lower, then apparently the yield of ethanol will also be lower. The inhibitors are also formed that need to be removed or detoxified (Arora et al. 2016). Shirkavand et al. (2016) have suggested to make an effective and efficient pretreatment strategy, the idea was to make a combined pretreatment process. Combined pretreatment methods use the combinations of many pretreatment methods which maximize the use of LC material at their best (Sun et al. 2016).

5.1.1.5 Combined Pretreatments

Use of oxidative delignification, alkaline method, and biological pretreatment results in the removal of lignin, while alkaline, acidic hydrothermal, and steam explosion methods result in removing hemicelluloses from LC materials. The surface area accessible to enzymes is increased by ammonia fiber explosion method and steam explosion. The combination of these pretreatment strategies can be effective in improving hemicellulose and lignin recovery and digestibility of LC materials. So multiple combinations of pretreatment methods, e.g., ionic liquid method with supercritical carbon dioxide method, mild acid with the biological method (Ma et al. 2010), biological combined with mild chemical or physical methods, alkali combined with hydrothermal (Yu et al. 2009), alkaline peroxide with hydrothermal or steam explosion method (Chen et al. 2008; Cuevas et al. 2014), alkali pretreatment with dilute acid pretreatment (Lee et al. 2015), have been made to pretreat multiple LC biomasses.

Cuevas et al. (2014) investigated the effects of alkaline peroxide pretreatment combined with hydrothermal pretreatment method on prunings of almond trees; the main things were changes in components and enzymatic digestibility. Sixty percent

lignin was eliminated by alkaline peroxide method, also the digestibility by enzymes was also made better by this method as compared to the hydrothermal process used alone. By using combined pretreatments the cost of the process is increased but it helps in making the hydrolysis better. A better pretreatment method is the one that is cost-effective and at the same time have the ability to make LC digestibility and their usability better. Hemicelluloses are linked with lignin in LC biomasses and they need to be removed in the initial step of combined pretreatment. The efficient methods of removing hemicelluloses from the LC biomass are steam explosion, hydrothermal, and alkaline pretreatment methods, removal of hemicelluloses must be the first step. On the other hand, biological, oxidative, alkaline, and Organosolv are better choices for removing lignin from the biomass. Oxidative and Organosolv methods are costly than the alkaline method. Also, oxidative delignification helped by alkaline methods is the best choice for making chemical pulp with lower lignin percentage. Lignin is advantageously removed from LC material by biological pretreatment but the efficiency of removal is lower so its use may be limited. Till now the most effective and efficient lignin removal strategy is alkaline pretreatment. Keeping in view this factor, hydrothermal, steam explosion, and dilute acid methods combined with the alkaline method become the most advantageous ways to pretreat LC biomasses. Lee et al. (2015) studied the role of alkaline (NaOH) and dilute sulfuric acid methods combined to pretreat corn stover for improving its digestibility by enzymes. Report showed that dilute sulfuric acid hydrolyzed xylan to 74.6–77.3%, and sodium hydroxide in the second step removed lignin up to 89.4%. This combined pretreatment increased the enzymatic digestibility of corn stover. Enzymatic hydrolysis 97.9% and 75.9% glucose and xylose were obtained, respectively. Sun et al. (2014b) used combination of NaOH and hydrothermal pretreatment to pretreat fiber from *Eucalyptus urophylla* and also examined digestibility by enzymes. All the hemicellulose was converted to liquid in 30 min during the initial step of pretreatment (temperature was higher than 180°). But lignin was not converted at such high temperature and alkaline conditions. In this experiment, enzymatic hydrolysis of *Eucalyptus urophylla* was increased because almost 50–60% lignin was dissolved into alkaline solution. Taking the energy consumption and recovery of hemicelluloses and lignin into consideration, an optimum 66.3% of cellulose was converted into glucose in the final enzymatic hydrolysis process. Sun et al. (2014a) also investigated the feasibility of steam explosion combined with alkali pretreatment. The material pretreated by this method had been seen to give a lower percentage of xylan, i.e., 8.32–20.85, but the concentration of lignin was reduced by the steam explosion method. In the alkaline pretreatment method, high pressure resulted in the removal of lignin. Steam explosion when used alone results in enzymatic hydrolysis by 7.9–33.1%; on the other hand, when alkaline pretreatment was combined with steam explosion method, it resulted in 45.7–63.9%. When such a combined pretreatment method is used it results in hydrolysate that can further be dehydrated to prepare furfural, this is due to the high content of hemicellulose in the hydrolysate. Solutions of alkalis, having higher ratios of lignin can be burned or concentrated for the provision of energy and

Table 5.1 The comparison of different pretreatment methods for lignocelluloses (Chen et al. 2017)

Pretreatment methods	–	Advantages	Disadvantages
Physical pretreatment	Mechanical splintered Microwave High-temperature pyrolysis	Reduction of particulate size and cellulosic crystalline structure Easily operated, energetically productive, lesser time Faster cellulose decomposition	Hemicellulose and lignin not be removed, need high energy Costly Less production, more energy needed
Chemical pretreatment	Organosolv pretreatment Alkaline pretreatment Oxidation pretreatment Dilute acid Ionic liquid pretreatment	Cellulose, hemicellulose, and lignin in pure form 25 °C temperature (room), destruction of lignin Eco-friendly, effective lignin removal Faster process Eco-friendly, high range of temperature	Costly, affects environment and fermentation Degradation of sugar is low Costly Inhibitors formed, temperature and pressure are higher Costly
Physicochemical pretreatment	Electrical catalysis Steam explosion AFEX method CO_2 explosion	No inhibitory compounds, cost-wise suitable, surface area increased, lignin removal, easy cleaning Hemicellulose dissolved, lignin changed, costly Area of surface increases, no inhibitors production	Lower efficiency, Lignin and hemicellulose not affected, pressure high Pressure and temperature are high More costly, no efficiency for raw forms, higher lignin
Biological pretreatment	–	Cellulose and lignin are degraded, high energy demand	Hydrolysis proceed at lower rate

recovery of chemicals. On the other hand, lignin can also be separated and used as high-value feedstock to overcome the cost of pretreatment (Cherubini 2010).

By taking into consideration this aspect, combined pretreatment makes digestibility of LC biomass better, but also helps in recovering hemicellulose and lignin for the production of high-value products.

Table 5.1 is the summary of various pretreatment strategies with their pros and cons (Chen et al. 2017).

5.2 Effect on Lignocellulosic Structures

In the physical as well as chemical lignocellulosic cell wall structures, the reagents are applied for the treatment along with enzymes. This results in the different forms of products as well yield differently (Kim et al. 2015; Zhang et al. 2016; Zheng et al. 2014). There are a lot of pretreatments that work differently, i.e., to dissolve

cellulose polymer, also the pretreatment methods separate the lignin and hemicelluloses in the cell wall. Many of the bonds breakdown by the hydrolysate components application and also the carbohydrates become chemically modified, the components used for the pretreatment include various methods such as the ionic compounds, milling, and Organosolv (George et al. 2015; Han et al. 2015; Narron et al. 2016).

In high severe environments, the applied pretreatment features show great commercial level advantages. It is also depicted that along with applications also keep an eye on the disadvantages (George et al. 2015; Han et al. 2015; Narron et al. 2016). Hereby understood that this pretreatment effectively eliminates the lignin components from the cell wall. Chemicals such as alkali and other acids are used for the pretreatment methods for the modification of cell wall (components, i.e., degradation of pentoses along with the breakdown of several chemical components in the cell wall) (Ji et al. 2016; Lima et al. 2014). These modifications lead to the wide applications in industrial as well in commercial companies, i.e., fermentation processes and in many enzymatic hydrolysis that yield efficiently and greatly promotes product yield (Rasmussen et al. 2014).

All the pretreatment methods are accompanied by the production of certain inhibitors formation from the above-mentioned polymers (lignin, cellulose, and hemicellulose). The inhibitors that are produced as a result of pretreatment include: HMF, furfurals, week acids, and phenolic compounds (Jacobsen and Wyman 2000). These microbial inhibitors are the main challenge to bioethanol production.

Some of the pretreatment strategies, their modes of actions on lignocellulosic biomass and presence or absence of inhibitor formation are summarized in Table 5.2 (Abraham et al. 2020; Harmsen et al. 2010; Ravindran and Jaiswal 2016).

5.3 Hydroxymethyl Furfural (HMF)

Common pretreatment techniques (e.g., steam and acid) seem to produce microbial inhibitors, these inhibitors may include 5HMF (5-hydroxymethylfurfural) and furfural, released into the hydrolysate and acting in the reduction of bioethanol yield, through the fermenting yeast inhibition (Crigler et al. 2020).

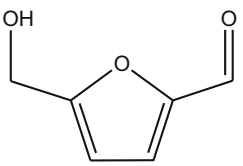
HMF and furfural are released by the loss of water molecules from sugar either in high temperature or acidic environments; HMF comes from hexose sugars and furfural comes from pentose sugar. It is not understood that these inhibitors inhibit the cells, but the enzymes responsible for fermentation and glycolysis are known to be inhibited, with the induction of reactive oxygen, and a decrease in the population of reduced redox cofactors (Crigler et al. 2020). HMF tends to negatively affect the metabolism of yeast and prolong the process of ethanol production (by fermentation) (Sukwong et al. 2019). Chemical structure of HMF is depicted in Fig. 5.8 (Menegazzo et al. 2018).

Production of HMF is easy by losing three molecules of water from hexose material, in a reaction catalyzed by acid. But HMF synthesis is not that simple, but complicated as it involves many other reactions.

Table 5.2 Pretreatment strategies and inhibitors formation (Abraham et al. 2020; Harmsen et al. 2010; Ravindran and Jaiswal 2016)

Pretreatment	Mode of action	Inhibitors formation
Mechanical method	• Cutback in the sizes of particles linked with surface area increase	+
Dilute acid pretreatment	• Increase in the pore sizes/volumes of plant cell walls • Significant redistribution and disruption of lignin • Nearly complete removal of hemicelluloses	_
Alkaline hot water	• Expansion in size of pores in plant material • Removing hemicelluloses and depolymerization of lignin • Preserving most of the cellulose	_
Alkali	• Increase in the central surface area due to blistering of cellulose • Eradication of lignin • Substitution of uronic acid on hemicelluloses and acetyl groups removal	++
SO2-catalyzed steam explosion	• Partial lignin transformation and lignocellulose removal • Pore size and volume expansion • Particle size reduction linked with increase in surface area	+
Organosolv	• Increment of approachable surface area and pore volume • Significant removal of hemicelluloses and lignin	++
AFEX	• Ammonolysis of lignin carbohydrate solubilization formation of nanoporous, and ester linkages formation relocation of cell wall, interconnected networks	++

+ = positive characteristic: low fermentation inhibitors; − = negative characteristic: high amount of fermentation inhibitors.



HMF

Fig. 5.8 Structure of HMF (Menegazzo et al. 2018)

Theoretically, hexoses can be transformed into HMF by a reaction involving three steps as illustrated in Fig. 5.9:

Step 1: hydrolysis of any polymer which is glucose-based (which may be starch or cellulose) into glucose (Bronsted acid catalyzes this reaction).
Step 2: conversion of glucose to fructose (Lewis acid catalyzes this reaction).
Step 3: loss of water molecules by fructose and conversion to HMF carried out by Bronsted acid (Menegazzo et al. 2018).

The mechanism of the procedure is depicted by in Fig. 5.9 (Ranoux et al. 2013).

Studies have shown that furfural along with acetate and phenolic compounds is the major inhibitory compound, observed in the pretreatment of corn stover hydrolysate for *Z. mobilis* in dilute acid pretreatment. And the inhibitory activity is related to the hydrophobic nature of these inhibitors (Yang et al. 2018).

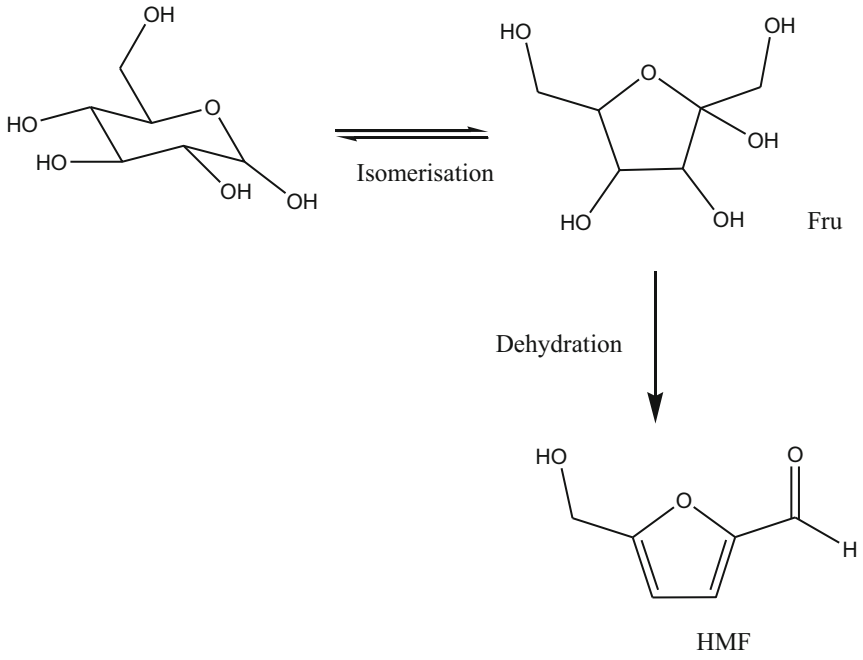


Fig. 5.9 Mechanism of acid-catalyzed dehydration of glucose to form 3-deoxyglucosone and hydroxymethyl furfural (Ranoux et al. 2013)

These inhibitory compounds result in the damage of microbes by the reduction of their biological and enzymatic actions, protein inhibition, RNA inhibition, and DNA breakdown (Zha et al. 2014). Synthesis of these inhibitory compounds and their resulting toxic effects shows a negative impact on the hydrolysis rate by enzymes, this is negative inhibition of bioethanol fermentation (Malav et al. 2017).

Hydroxymethyl furfural is not much inhibitory to microbes as compared to furfural, but it has the ability to prolong the lag phase and inhibit cell growth. It stays for a longer time than furfural because it is converted at a faster rate, i.e., four times that of hydroxymethyl furfural, resulting in the prolonged microbial action (Liu and Blaschek 2010).

5.4 Furfural

The formation of furfural occurs from pentose sugars, e.g., xylose. There is an array of catalysts responsible for xylose dehydration into furfural. An important factor for enhancement of selectivity to furfural is Bronsted acid. Lewis acids are also seen to have a positive effect in this process as it speeds up the conversion of xylose into xylulose, which can then be further dehydrated into furfural at a faster rate

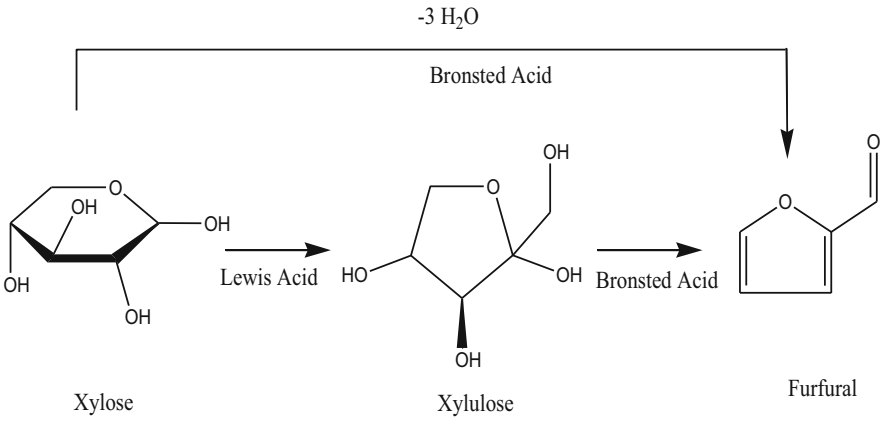


Fig. 5.10 Mechanism of furfural formation by xylose dehydration (Li et al. 2016)

(Steinbach et al. 2017). The mechanism of furfural formation is shown in Fig. 5.10 (Li et al. 2016).

Furfural is toxic and can act along with weak acids to inhibit metabolism, cell growth, and ethanologenic bacteria's ability for carrying out fermentation (Modig et al. 2002). Also, the weak acid (acetic acid) can synergistically work with furfural for inhibitory action (Wang et al. 2020). Furfural and acetic acid contrived the activity of cellulase, when their concentrations were 4 g/L and 13 g/L, respectively (Kim et al. 2011). Inhibition by furfural compounds result in the delay of lag phase and therefore will contrive the complete process of fermentation. Furfurals do not significantly inhibit the yield of ethanol in *Zymomonas mobilis* and *S. cerevisiae* (Behera et al. 2014). By increasing the size of *S. cerevisiae* inoculum, the inhibitory role of furfural on fermentation can be minimized (Taherzadeh et al. 1999). But when multiple inhibitors are together, they can adversely affect the fermentation process by killing the microbes by affecting their growth. In case of *Scheffersomyces stiptitis*, furfural at 0.5 g/L did not affect cell growth but at 2 g/L it was dangerous for the growth of cell (Roberto et al. 1991). Similar results on ethanol production and yield were observed when hydrolysate of wheat straw was fermented by *S. stiptitis*. Furfural at 0.25 g/L did not have any impact on the production of ethanol and growth of cells, but at a high concentration of 1.5 g/L constrained the yield of ethanol and production by 90.4 and 85.11% respectively (Nigam 2001). Synergy was observed among furfural, acetic acid, and derivatives of lignin that decreased production and yield as compared to the combined inhibition of single compound (Nigam 2001).

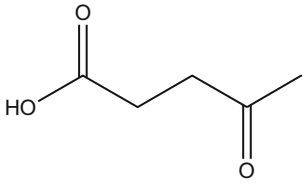
Other inhibitory effects of furfural include crippling effects on mitochondrial membranes and vacuole, actin, and chromatin and bring about the accretion of reactive oxygen in *S. cerevisiae* (which is mostly used as fermentation organism) (Yee et al. 2018). The integrity of cell membranes in permeabilized cells is affected by furfural presence (Da Silva et al. 2017).

Furfural inhibits yeast but the mode of inhibition is not exactly understood. However, it constrains the main enzymes of glycolysis, e.g., phosphofructokinase, hexokinase, and triosephosphate dehydrogenase (Malav et al. 2017; Weil et al. 2002). Concluding the effects of furfurals on cells, it has been observed that furfurals avert the energy levels of the cells by the reduction of inner cell's ATP and NADPH levels through inhibition of enzymes, by consuming the cofactors, damage of genetic materials, membrane damage, and few proteins (Almeida et al. 2007). However, cells have the ability to bear the low levels of furfurals and also can convert them into furoic acids and furfuryl alcohols which are the less toxic forms of furfurals. Furoic acid (is the oxidized form of furfural) is produced by the usage of ALDH (Wikandari et al. 2019), while furfuryl alcohol (is the reduced form of furfural) is produced from furfural by using ADH (Horváth et al. 2001).

5.5 Weak Acids

During the rehydration of HMF water is incorporated into the ring of furan at position C2-C3 during the existence of acid acting to speed up the reaction as a result LA (livulinic acid) and FA (formic acid) are formed (Kang et al. 2018; Mosier et al. 2005). There is a limiting condition when there is a high amount of weak acid it depletes bioethanol production from biomass hydrolysate; on the other hand, a low amount of acid will have a positive impact. In the medium, if the concentration of formic acid and acetic acid is low then it would increase the amount of ethanol and vice versa (Fu et al. 2014; Pérez et al. 2002). The structure of Livulinic acid is shown in Fig. 5.11 (Peng et al. 2010).

When acetate is produced then from inside of the cell it cannot cross back the cell membrane through simple diffusion, therefore, aggregates inside the cell which results in enhanced turgor pressure and oxidative stress. Other deleterious effect is that pHi is reduced and inhibits the activities of normal metabolism (Palma et al. 2018). When weak acids enter into the cell, they follow dissociation and result in the lowering of pHi (intracellular pH). After an increase in pHi, the cell tends to bring the normal pHi back by exporting the positive hydrogen ions from the cells, at the cost of ATP. This process results in the reduction of cell growth rate (Cola et al. 2020). The acidity by weak acids is because of acidification of cytosol, through the



Livulinic Acid

Fig. 5.11 Structure of Livulinic acid (Peng et al. 2010)

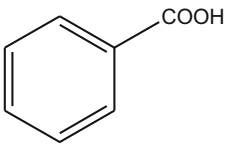
accumulation of anions thus lipid-soluble acids move across the membrane and dissociate in the cytoplasm resulting in the pH lowering. This acidity causes suppression of phosphogluconate pathway, enhanced turgor pressure, aggregation of proteins, oxidative stress, lipid peroxidation, and plasm and vacuolar membrane disruption which affects the trafficking across membranes and leads to the ultimate cessation of cell (Brandt et al. 2019).

During the fermentation process, the lag phase is short, i.e., almost 4 h, which shows the adaptation of yeast cells to the spiked broth which allows them for normal development for the fermentation process. The inhibitory compounds are more actively present in the lag phase for obtaining greater contact with yeast cells. Moreover, it is concluded that the small amount of inhibitory acids is somehow beneficial for ethanol production (fermentation itself), as it will be playing the role of catalyst for the provision of energy needed for the ATP production. On the other hand, higher concentrations of acids will cause a decrease in pH, therefore affecting the fermentation and leading to cell lysis (De Klerk et al. 2018).

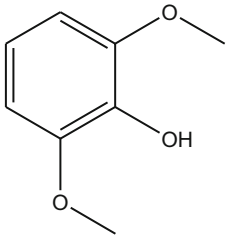
5.6 Phenolic Compounds

When side chains of phenylpropanes are cleaved oxidatively it leads to phenolic acids, e.g., vanillic acids, 4-hydroxyphenolic, and syringic acids, structure of syringic acid and benzoic acid are shown in Fig. 5.12 (Liu et al. 2020). These compounds have been found to come from lignin or the hydrolytic reactions of esterified phenols, this fact is supported by the presence of syringyl, guaiacyl, and 4-hydroxyphenyl (Jönsson and Martín 2016).

In the pretreatment of eucalyptus green liquor for ethanol production, the inhibitory compounds produced were syringic acid, syringaldehyde, vanillin, and acetosyringone. These inhibitors were observed to affect the metabolic pathways



Benzoic Acid



Syringic Acid

Fig. 5.12 Phenolic acids (Liu et al. 2020)

of *S. cerevisiae* PE-2. As in the case of acid inhibition, these phenolics also are not much harmless in lower concentrations, but when their concentration rise, inhibitors need to be removed (Lyra Colombi et al. 2018). Toxicity due to vanillin is the leading cause of cost reduction of bioethanol production (Ito et al. 2020). Moreover, phenolics mostly do not decrease the yield of ethanol, but can reduce the rate at which ethanol is produced. When they affect the activity and integrity of cell membranes then they can also reduce the yields and growth rates. The aspect of microorganism's resistance to such inhibitors is dependent on the compositions of: specific fatty acids, membrane composition, headgroups of phospholipids, and content of proteins (Heipieper et al. 1994; Wikandari et al. 2019).

Other effects of phenolic inhibitors include their negative effects on cellulases. The observation was made on the basis of the presence and absence of vanillin in the cellulolytic solution. With vanillin, the cellulose was converted by a value of 26% but in absence of vanillin it was 53% (Qin et al. 2016). Other phenolic compounds like ferulic acid and p-coumaric acid also showed reduction of glucose production from glucose by 16% and 30% respectively. Also, those phenolic compounds that had been taken from the pretreated biomass had their effects on the functioning of enzymes. Adsorption of cellulases to hydroxyl groups and derivatives of lignin had their contribution to inhibitory effects (Kim 2018).

As the phenolic compounds have lower molecular weight so they can easily enter the cell membranes of microbes by disturbing their internal structures, also they are much stronger inhibitors as compared to other inhibitory compounds, e.g., weak acids, furans, other byproducts (Jönsson and Martín 2016; Klinke et al. 2004). Ezeji et al. (2007) indicated that p-coumaric acid and ferulic acids are the most harmful of all the phenolic inhibitors, when checked with *Clostridium beijerinckii* (BA101) strain, the growth of this strain of bacteria was reduced by 74% in the presence of 1 g/L of these two inhibitory compounds. In some other research, it was found out that these two inhibitors reduced the growth of saccharomyces cerevisiae by 80% (Adeboye et al. 2015). The fluidity of the membranes of cells can also be affected by these phenolic inhibitors by decreasing the levels of potassium. Phenolic inhibitors also affect the stability of the cell membranes that may result in DNA breakage that will affect RNA and hence protein production, accumulation of carbohydrates, and disturbance in growth of cells (Kim 2018).

There is not much information regarding the nature of inhibitory mechanisms by phenolic compounds. It can be suggested that a multiple site inhibition mechanism may be there, in which many aromatic molecules would be joined together and cooperate with the enzyme to bring about not only inhibition but also inactivation. Hemicellulasses and cellulases both are equally influenced by phenols derived from lignin (dos Santos et al. 2019).

Table 5.3 dos Santos et al. (2019) represented the summary of inhibition mechanisms of furfural, weak acids, and phenolic compounds.

Table 5.3 Inhibitory effects of inhibitors in the bioethanol production process

Inhibitors	Mechanism of inhibition	References
Furfural	• Pyruvate dehydrogenase, alcohol dehydrogenase, aldehyde dehydrogenase • Integrity of cell membrane negatively influenced by the age of culture and presence of furfural • Alteration in flux of cell energy to repairing of damages and reduced levels of cell's internal ATP and NADPH	Da Silva et al. (2017), Wang et al. (2018)
Weak acids	• Causes drop of pH irreversibly and stopping the cell process eventually cell death occurs • Disturbs the function of cell membrane	Ndukwe et al. (2020), Oshoma et al. (2015), van der Pol et al. (2014)
Phenolic compounds	• Prolongs lag phase, diminishes ethanol production, xylitol, lactic acid, and H2 fermentation • Damages cell membrane and cell communication is negatively affected • Kills microbes responsible for fermentation	Favaro et al. (2019), Fletcher et al. (2019), Ladeira-Ázar et al. (2019), Sivagurunathan et al. (2017), Wang et al. (2018)

5.7 How to Minimize Inhibitory Compound Formation

For the minimization of the toxic effects of inhibitors from lignocellulosic biomasses, different methods like physical, biological, and chemical detoxification are in use. But the use of any detoxification method may be responsible for the increase of the cost of production of the entire process and the time involved too shall increase this way.

5.7.1 Removal of Inhibitory Compounds

To resolve the inhibition issue most used method is conditioning or detoxification in which inhibitors are removed in hydrolysate and solid fractions (Jönsson et al. 2013). Many conditioning methods have come out in this era, which consist of treatment with chemical additives (Alriksson et al. 2011), addition of sulfite, activated carbon treatment, liquid-liquid extraction, and lignin-blocking agents (Eriksson et al. 2002). In chemical additives method, the main purpose is to aggregate, precipitate, or absorb the unwanted compounds from hydrolysates. By this method, inhibitors get maintained at low effective concentrations by which inhibitory effect on enzymes and microbes get minimal (Ciesielski et al. 2014). However, the effectiveness of this method depends on many factors, e.g., dosage of additive, concentrations of inhibitors, properties of feedstock, and conditions before treatment. Bovine serum albumin when used as a lignin-blocking additive was highly efficient in decreasing

unproductive adsorption of enzymes to other molecules hence improve the enzymatic hydrolysis of pretreated hardwood. When pre-culture with bovine serum albumin was done at 50 mg/g solids prior to enzyme digestion resulted in giving 90% conversion yield (Kim et al. 2015). Only 30% yield was obtained in control. The lignin effect was studied more and it revealed, as enzyme activity decreased and lignin to exposed enzyme ratio was increased, a very noticeable enzyme inhibition was seen. It was mainly due to the nonproductive binding of enzyme to lignin (Ko et al. 2015). In the release of strong lignin and lignin-derived compounds the major contributor is severity factor of pretreatment, which could be more severe to enzyme activities. For example, 1% (w/v) lignin-free cellulose conversion in presence of 0.5% isolated lignin at 8 mg enzyme protein/g glucan resulted in giving 58% product but when the isolated lignin from higher severity factors of log R0 = 11.39–12.51 was added, the cellulose conversion to glucose was fallen by 51%. Activated carbon is also very useful in binding and sequestering of many furan derivatives, acetic acid, and phenolics in the slurry. Particularly, activated carbon can remove all phenolics efficiently. Recent work concluded that most of the phenolics were reduced from 132 AU to 8 AU after treatment with activated carbon (Kim et al. 2016). It is in the chemical property of carbon that it can absorb soluble hexose and pentose that results in loss of fermentable sugars (Kim et al. 2013). Aghazade et al. demonstrated in their another attempt that liquid-liquid extraction LLE was able to extract 90% acetic acid, using ethyl acetate solvent that gave 11% higher ethanol yield. This method is not favorable for industries as it requires additional solvent supplements and extraction processes, it provides a new scalable technique and protocol to alleviate inhibitory compounds in pretreated lignocellulosic masses. The main challenge with implementing a detoxification approach is that these protocols require an additional independent step that may rise the concern of capital evaluation. Cellulose ethanol production is currently available around $2.5/gallon according to recent techno-economic analysis.

Ethanol production properties are acid pretreatment, simulation with different agricultural feedstocks, detoxification with activated carbon, enzyme hydrolysis fermentation with pichia stipites and *S. cerevisiae* and distillation (Duque et al. 2015).

5.7.2 Biological Detoxification

For the implementation of a detoxification process, which is an environment friendly way, we can use harsh chemicals and expensive processing materials that will help in avoiding energy-intensive processing conditions. During this process, lignocellulosic-derived inhibitors could be alleviated or eliminated before enzymatic hydrolysis and fermentation by microorganism pretreatment (Cannella et al. 2014; Cao et al. 2015). Prior to enzyme digestion and microbial fermentation, several microorganisms, such as *Coniochaeta ligniaria*, *Paecilpmyces variotii*, *Urebacillus thermosphaericus*, and genetically modified *S. cerevisiae* were suggested and

evaluated the alleviation of the inhibitors. *C. ligniaria* NRRL30616 was an ideal candidate identified by (Nichols 2005). It is considered ideal because it had increased tolerance to inhibitory compounds and could metabolize these inhibitors (mainly furans and acetate) as a carbon source and energy. *C. ligniaria* can be used to reduce the inhibitors that are formed during diluted acid, pretreatment of different biomass, such as switchgrass, reed canary grass, alfalfa stem, corn stover, and rice hull, resulting in confirmation, ethanol productions with a short lag phase (Nichols et al. 2010). To improve the ethanol production by a recombinant bacterium, *Escherichia coli* FBR5E, we can use the C5 sugars such as pentose and arabinose in the biologically detoxified hydrolysates with *C. ligniaria*. This strain of *E. coli* can ferment both C5 and C6 sugars, but we cannot use it in the presence of the pretreated hydrolysates due to its sensitivity to inhibitory compounds (furfural, HMF, and acetic acid). The FBR5 strain of *E. coli* could consume both C5 and C6 sugars, when the diluted acid pretreated and detoxified corn stover hydrolysates were used as a substrate for microbial fermentation, but could not in the non-biologically detoxified hydrolysates (Nichols et al. 2008). Detoxification in the liquid hot water pretreated corn stover hydrolysate is another example that showed the best cellulose conversion to glucose by the combination of biological detoxification followed by maleic acid or activated charcoal plus enzyme treatment (Kim et al. 2016).

5.8 Drawbacks of Biological Method

It is time-consuming and more time is consumed in microbial growth. And this growth as a result can affect sugars. Currently, 1070 oxidoreductase, 926 dehydrogenases, 227 decarboxylases, and 23 genes related to oxidative stress are found in the first genome of C. ligniaria. These achievements can prove very beneficial in the coming genetic and metabolic engineering (Duque et al. 2015).

5.8.1 Adaptation of Microbes

Many of the inhibitory components like that of hydrolysate samples interact with the microbes so that arises the evolution of that treated microbe. In the fermentation process whenever a treated microbe with an inhibitory component is applied, it modifies microbe that becomes highly endurable to the organic molecules like aldehydes, benzene components, etc.; these organic components had a great impact on yield (Almario et al. 2013). The genetically modified strains of S. cervisiae effectivily utilized the pretreated bagasse hydrolysates and gave better yield. The high yield and productivity was due to the availability of sugars from biomass. Due to this modified strain used in the fermentation makes the process fast but the final product is quite similar to that strain which is not modified (Martín et al. 2007). This revolutionized that the most evolved modified microorganism increase the

productivity and other studies also depict that modified and non-modified microbes are not compatible (Liu et al. 2005). Great yield productivity is depicted by the screening of selected inhibitory *S. cerevisiae* strain that is tolerant.

More recent attempts, in addition depict that the microbial fermentation test using bagasse hydrolysate results in the ethanol production rate almost 7.7 times higher in comparison to the control experiment, i.e., test (benchmark *S. cerevisiae* strain) (Favaro et al. 2013).

5.8.2 Genetic Engineering

In the process of fermentation many DNA recombinant strains are prepared by metabolic engineering techniques to overwhelm inhibitory problems. The genetically/metabolically engineered strain *S. cerevisiae* had improved the yield of ethanol while using along with the furfural hydrolysate inhibitory component during the fermentation (Hasunuma et al. 2014).

In view of this study, it is identified that there are some genes that are involved in the pentose phosphate pathway that modifies the strain. High overwhelming ability against inhibition effect to sugarcane bagasse hydrolysates has been reported in most recent studies on the fungus strain as well as bacterial *Escherichia coli* (Wang et al. 2013).

In recent studies, it is inferred that the chemically as well physically modified microbe strain has great enhancing qualities like the cell efficient growth and also the ethanol, i.e., synthetic pigments as well as production. These modified microbes strains have appropriate enhancing features (Kim 2018).

5.8.3 Some Other General Strategies

The ability of *S. cerevisiae* to endure the inhibitors for a short period of time occurs at the cost of prolonged lag phase and reduction of bioethanol yield (Fosso-Kankeu et al. 2015).

Strains of *S. cerevisiae* collected from grape marc from a winery showed them to be highly resistant to furans and aliphatic acids. Also, 90 strains of *S. cerevisiae* species were seen to have an estimate of resilience to formic acid, acetic acid, furfural, vanillin, and HMF (Hawkins and Doran-Peterson 2011). By using such strains for fermentation, the inhibitor formation can be minimized (Wimalasena et al. 2014).

For minimizing the effect of furfural it can be converted to furfuryl alcohol or 2-furoic acid, with anaerobic conditions, this conversion will be beneficial as it will save any change in the final concentration of ethanol (Da Silva et al. 2017).

In one of the studies of minimizing inhibitors (furfural and acetic acid), which greatly inhibit ethanol production, it was observed that the addition of biochar to the

Table 5.4 Strategies applied for detoxification and removal of fermentation inhibitors (Kim 2018)

Process selected	Main effects	Considerations
Modification and choice of biomass	Feedstock screened or engineered so that it will produce lesser unwanted compounds	Suitable residues from agriculture, engineering and selection time needed
Detoxification by biological ways	Microorganisms are used	Sugars lost, time taking
Genetic engineering	GMOs needed for hydrolysates of LC biomass	GMOs needed
Detoxification	Chemical substitutes, e.g., polymers, BSA, alkali	Requirement of chemicals, involve some supplementary procedure
Microbial adaptation	Inhibitory environment may have evolution of adaptive microbes	May not be applicable to other materials (conditions of pretreatment)

broth can increase ethanol yield. The increase of ethanol production by biochar addition is only for fermentation by *Zymomonas mobilis* strain ZM4. This solution to inhibition of fermentation proves to be effective as it showed much higher increase in the biochar treated fermentation than in non-treated broth (Wang et al. 2020).

In another case, 78 terrestrial yeast species were compared with 166 marine yeast strains, to analyze their tolerance to inhibitors produced from lignocellulosic biomass (e.g., acetic acid, furfural, formic acid, salts, and vanillin). Terrestrial yeast species were having less tolerance to inhibitors, while marine strains were more tolerant to ethanol inhibition (Greetham et al. 2019).

The chemical methods employed for detoxification may involve the use of polymers, reducing agents, and alkali (Jönsson and Martín 2016). To remove 5-HMF and furfurals from the hydrolysis mixture of lignocelluloses, some other methods that are used include ion-exchange, over-liming, adsorption using active charcoal, and conversions by using enzymes (Zhang et al. 2010).

Table 5.4 is the summary of detoxification of the hydrolysis products by the use of different methods other than biological ones, for different LH (lignocellulosic hydrolysates). Each method is specific for a specific inhibitor from hydrolysate.

5.9 Conclusion

During bioethanol production from lignocellulosic feedstock, the main challenges encountered are of the formation of toxic inhibitory compounds. These inhibitory compounds result in the toxicity of the whole fermentation process and render the process somewhat un-economical. Production of the inhibitory compounds occurs due to the pretreatment process. These problems of toxicity can be solved by maintaining proper pretreatment conditions, i.e., controlled temperature, pressure, and appropriately adjusted pH. Moreover, general methods of detoxification like,

physical, chemical, and biological have proven to be effective. Advancement is the resistance of the micro-organisms to the inhibitory compounds that may be naturally occurring or engineered ones. Adopted technology should be economical and highly effective toward inhibitors.

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Chapter 6
Engineering of *Zymomonas mobilis* for Enhanced Biofuel Production

Muneeba Khalid, Nasheen Rubab, Wajiha Afzal, Muhammad Irfan, Misbah Ghazanfar, Hafiz Abdullah Shakir, Muhammad Khan, Shaukat Ali, and Marcelo Franco

Abstract *Zymomonas mobilis* strains are examined as the model organism in the industries because of having many potential advantages. Different research showed that different strains of *Z. mobilis* produced high amount of ethanol and the sugar because it can easily utilize xylose and arabinose in addition to glucose. We can improve the strains of *Z. mobilis* such as by adaptive laboratory evolution (ALE) by many methods. It is a very important method for the improvement of different attributes of common industrial strains. These strains have used as advanced model organism for genetic method and inverse metabolic engineering. The ED pathway of *Z. mobilis* gives an alternative way for the production of bio refineries and valuable byproducts like sorbitol, succinic acid, levan, and isobutanol. The metabolic engineering using *Z. mobilis* gives advanced biofuel production. The techniques adopted for strain improvement and future guidelines for this were discussed.

Keywords Engineering · *Zymomonas mobilis* · Biofuels · Fermentation

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6.1 Introduction

Fuel ethanol is recently produced from sugar-based feedstock, which is known as first generation (1G) biofuel. Due to expanding population, there is competition between food and first generation biofuel, so alternative sources are much needed for the production of biofuels. Basic consequences of 1G biofuel production on eatable things protection have continued throughout the centuries (de Andrade Ramos et al. 2016). Many of the lignocellulosic biomass, especially crops debris is not related to food and mostly accessible, and it shows an acceptable source material for producing second generation (2G) biofuel (Gupta and Verma 2015).

Microbial strains are the hallmarks of biofuel production. Some strains of *Saccharomyces cerevisiae* are very efficient in utilization of starch based sugars for the production of biofuel while cellulosic based sugars yield low productivity due to fermentation inhibitors (Andre et al. 2016; Gombert and van Maris 2015).

The xylulose converted into xylulose 5-phosphate by *Saccharomyces cerevisiae* an intermediary of pentose phosphate pathway that is able to form G3P for biofuel production by glycolysis mechanism. The *Saccharomyces cerevisiae* can be established by xylose mechanism by the uses of many genes that are encoding enzymes in microorganisms which use pentose, e.g. *Acetobacter aceti* (Seo et al. 2017), as well as overexpression of xylulokinase, to stabilize hexose and pentose sugar to produce bioethanol. The heterologous pathway constructed from various bio-parts could be engineered into *Z. mobilis* (Fig. 6.1) (Wang et al. 2018).

Zymomonas mobilis as a potential candidate for bioethanol has some benefits, like high intake of sugar, high yielding of bioethanol, low production of biomass, and unwanted addition of O_2 during the process of fermentation (Panesar et al. 2006). Extensive studies on *Zymomonas mobilis* for the past 30 years made a criticism as an

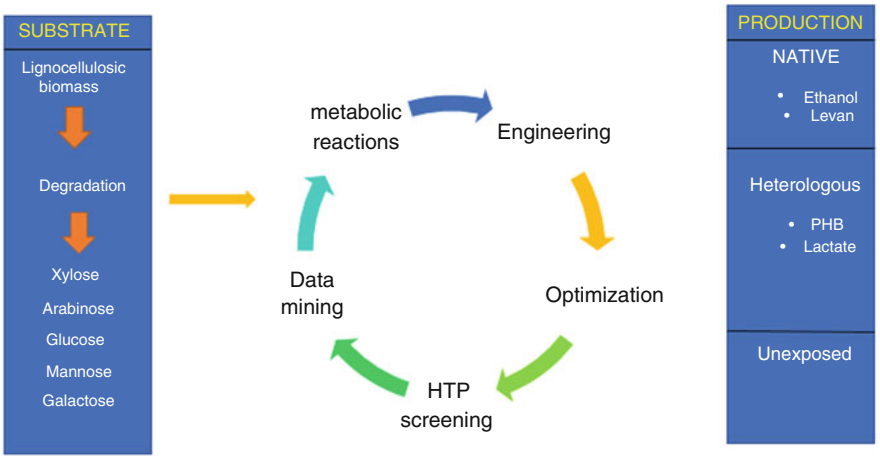

Fig. 6.1 *Z. mobilis* development as a cell factory for lignocellulosic biofuel (modified from Wang et al. 2018)

ethanolgenic industrial strain for biofuel production. While on the other side, advances in research technologies (that involves gene expression system, plasmid vector, gene mutation, gene function, gene knockout, transposon system) help in better genetic development of industrial biotechnology (Rogers et al. 2007).

Lignocellulosic ethanol production is restricted by low concentration of ethanol which results in high distillation costs and one of its required conditions for ensuring energy usage and economic balance of lignocellulosic ethanol is distillation (Koppram et al. 2014; Sun et al. 2020).

6.2 Attractive Physical Characteristics of *Zymomonas mobilis* for Biotechnology

Z. mobilis is a common microorganism that lives without oxygen. It is an ethanologenic bacterium that has gram-negative cell wall. It has many useful industrial features. For example, it is considered that *Zymomonas mobilis* is safe (GRAS), that represent high tolerance efficiency of bioethanol more than 6% (v/v), it can make bioethanol with variable range of pH (low pH, 3.5–7.5). Like an anaerobic bacteria, the *Zymomonas mobilis* does not need to regulate aeration when fermentation process occurs, so lessens the production value (He et al. 2014; Jackson et al. 2007; Yang et al. 2016a, b).

A natural ethanologen is the optional anaerobic bacterium *Zymomonas mobilis* and past studies have greatly defined its physiology in relation to commercial processes such as the manufacture of biofuel (Wang et al. 2018). Only small quantities of carbon substrates are integrated in *Z. mobilis* as biomass and this is an enticing biocatalyst in refining systems (Wang et al. 2018; Kalnenieks et al. 2008).

When compared the EMP glycolysis mechanism with other species like *Escherichia coli* and *Saccharomyces cerevisiae* just 1 mole of ATP resulted via glucose of every ED pathway. Previous studies showed that the ED metabolic pathway has lessen relation with heat and needs low enzymatic protein than the EMP pathway that needs to hold equal flux (Lee and DeVries 2013). An active ED pathway is combined with 2 alcohol dehydrogenases (Adh) and 1 pyruvate decarboxylase that make glycolysis mechanism for *Zymomonas mobilis*. It is noted that the pentose phosphate pathway (PPP), tricarboxylic acid (TCA) cycle, and EMP pathway are not completed in *Zymomonas mobilis*. Because in this microorganism most of the enzymes that are used in these pathways are not recognized (Table 6.1).

Z. mobilis breathing chain exhibits unique strength and cellular growth with a suggested physiology to regulate ratio of low $NADH/NAD^+$ for active cellular growth and glycolysis (Hayashi et al. 2015; Rutkis et al. 2014). In the presence of oxygen, *Zymomonas mobilis* used the O_2 like electron receptor that terminates the active respiratory chain which includes cytochrome b, NADH dehydrogenase type II (Ndh), terminal oxidase, and coenzyme Q10 which are electron carriers other small

Table 6.1 Comparison of physical characteristics of *Z. mobilis* to *E. coli* and *S. cerevisiae* (modified from Wang et al. 2018)

Categories	*Z. mobilis*	*E. coli*	*S. cerevisiae*
Taxonomy	Gram negative	Gram negative	Eukaryotic
Ethanol	5.67	0.60	0.67
Metabolic pathway	ED pathway	EMP pathway	EMP pathway
Respiratory chain	High oxygen	ATP accumulation	ATP accumulation
		Stops PFK	Stops PFK
Genome size	2.14 Mb	5.15 Mb	12.12 Mb
Growth condition	Anaerobic	Aerobic	Aerobic
Ethanol tolerance	16%	06%	15%

or unknown places (Sootsuwan et al. 2013). After comparison it is noted that *Zymomonas mobilis* consumes high oxygen and yielding low ATP (Agrawal et al. 2017; Zhang et al. 2019).

Interestingly, advance studies shown that *Zymomonas mobilis* produces bioethanol by using N_2, which lessens the production value of biofuel because of less value of N_2 gas like nitrogen source (Yang et al. 2016a, b). These all characteristics make *Zymomonas mobilis* as a best source of industrial microorganism.

6.3 Sequence Detection of Various Genes of *Zymomonas mobilis*

Besides the industrially enticing chemistry, *Z. mobilis* has never discussed its cellular structure and unlike other industrial microbes such as *E. coli, B. subtilis*, and *Saccharomyces cerevisiae*. The lack of *Z. mobilis* cell biology could be a bottleneck for fully exploiting its metabolic processes and considering that growth of cell and division are result of glycolysis, which also produces ethanol as a major end product. Therefore, better understanding is required to direct sound metabolic engineering for *Z. mobilis* in regulation of cell geometry focused on the biorefinery (Randich and Brun 2015; Brenac et al. 2019; Fuchino et al. 2020).

Gene deletion methods were developed for gene regulation, *Z. mobilis* metabolic engineering was also greatly improved, and various approaches have been used to inactivate particularly *Z. mobilis* genes, including injection mutation, plasmid-based suicide mutation construction, site-dependent FLP recombinase, and fusion-PCR-based construction techniques. Many genes including (pdc, ZMO1360), (adhB, ZMO1596), (cytC), etc., have been chosen as targets for improving some particular phenotype (Table 6.2). Advances in quality sequencing innovations and particularly next-generation sequencing (NGS) procedures give new chances to increase the potential of *Z. mobilis* strains.

Genome comparison using open reading frames showed that *Zymomonas mobilis* has a close resemblance to *Novosphingobium aromaticivorans* (Seo et al. 2005)

Table 6.2 Some genes knockout in Z. *mobilis* (modified from He et al. 2014)

Inactive genes	Methods	Description	References
Nha [AZMO0117]	Insertional mutant	When $C_2H_3NaO_2$ present growth of cell decrease	Yang et al., (2010)
Ndh [ZMO1113]	Insertional mutant	Growth of cell is high, in aerobic conditions yield of ethanol is high	Hayashi et al. (2011) and Kalnenieks et al. (2008)
Pdc [ZMO1360]	Homologous recombination	Succinate conc. Is high in the presence of glucose, low ethanol	Seo et al. (2009)
Gfo [ZMOo689]	Homologous recombination	In the presence of heat, osmotic and ethanol stress production of ethanol occur	Sootsuwan et al. (2013)
cytC	Insertional mutant	At high temperature growth is reduced	Charoensuk et al. (2011)
cytB [ZMO0957] cytB [ZMO1572]	Insertional mutant	When cultivated anaerobically it has low respiration capacity	Strazdina et al. (2012)

which is according to an earlier phylogenetic study on the sequencing of RS rRNA (Leksawasdi et al. 2001). With the exception of genome sequence of ATCC 31822 and ATCC 31823 bases have 26 and 30 connections, respectively. The strains were fully sequenced and the genome sequences of the first 9 phases were compared with the *Zymomonas mobilis* transmission (Yang et al. 2016a, b). The NRRL B-1960 may be a strain which was recently known having 2,045,798 bp of chromosome and two plasmids with 11 different genes (Chacon-Vargas et al. 2017).

Additionally, the entire chromosome sequence of the *Zymomonas mobilis* plasmid (CP023715) is the xylose-derivatives using its derivatives 2032 and 8b are identified and characterized recently. Chromosome sequencing of *Zymomonas mobilis* recognized 65 single nucleotide proteins and 2400 bp insertion concerning to the *Zymomonas mobilis* chromosomal sequence (AE008692.2). Four essential plasmids are also identified which vary in size from 32 to 39 kb (CP023716-9 for pZM32, pZM33, pZM36, and pZM39 respectively), also for total storage of 150 predict open reading frames (Zhang et al. 2015).

The genomic size of all strains varies from 2.01 to 2.22 Mb for two to eight parasites that is half the genomic size of *E. coli*. Additionally the central metabolism of *Zymomonas mobilis* looks simpler than *E. coli* that is originated from the essential genes and metabolism (Widiastuti et al. 2011). Genome sequencing technology provides opportunities for basic understanding and facilitates the event of growth complexes (He et al. 2009). Seo et al. (2005) reported the primary *Z. mobilis* sequence ZM4 that contains a 2,056,416 bp of round chromosome with five circular plasmids. Other important information about genome and translation, transcription found since 2005 and so on. The milestone about all these is given in Fig. 6.2.

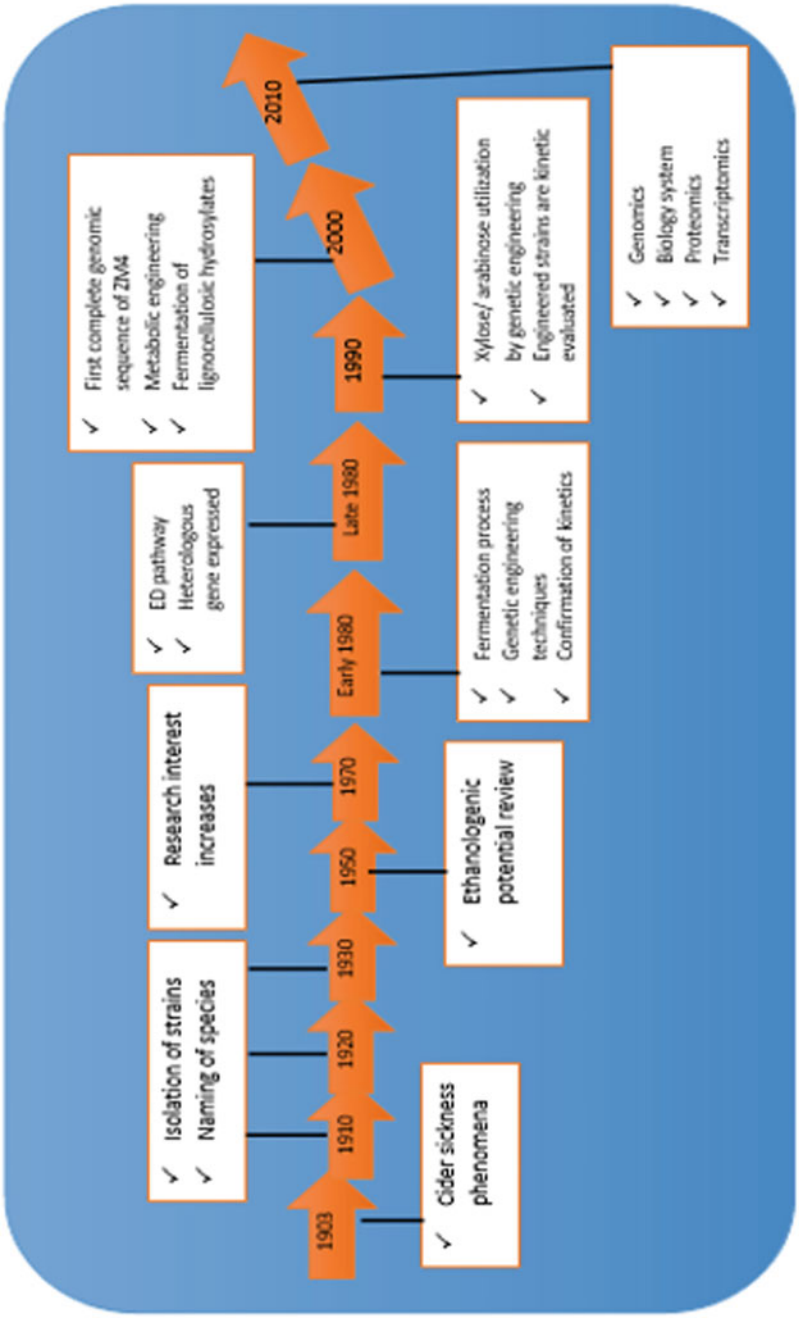

Fig. 6.2 Selected milestones in *Z. mobilis* (modified from He et al. 2014)

Complete genetic sequencing of other *Z. mobilis* strains has also been reported since 2005 (Desiniotis et al. 2012).

6.4 Improvement of Strain by Adaptable Laboratory Evolution (ALE)

Evolutionary laboratory development is a scientific approach to the analysis of evolutionary phenomena in a controlled laboratory setting is very crucial. The principles on which research in laboratory evolution are based date back to researchers such as Antonie van Leeuwenhoek, Louis Pasteur, Robert Koch, and most particularly Charles is a common approach in biomedical research to give knowledge into the basic mechanisms of molecular evolution and adaptive changes that occur in microbial communities under defined growing conditions throughout long-term selection.

Evolutionary engineering also known as compatible lab development and also called as whole-cell controlled development is a predominant method for betterment of industrial strains and evaluate these complicated tolerance phenotypes due to its clarity and efficiency. ALE is a very beneficial method for the improvement of different attributes of common industrial strains. Traditionally, strain improvement was achieved mainly through mutagenesis and selection that are still very beneficial in *Z. mobilis.* Adaptable Laboratory evolution has turned up just like a significance process for strain development in metabolic engineering and in escalation (Amarendran et al. 2016; Dragosits and Mattanovich 2013).

It has been used fruitfully in classic entities, for example, *Escherichia. Coli* (6364) *Saccharomyces cerevisiae* (6568). This strategy was also used by Agrawal et al. to select an extremely effective xylose-fermentation *Z. mobilis* A3 strain. Such two studies opposed the idea of using the ALE. Thus evolutionary laboratory development was already accomplished by William Dallinger about a 100 years ago (Bennett and Hughes 2009) and throughout the middle from the last century (Silver and Mateles 1969) there has been an increasing number of such experiments, especially over the last 25 years.

Through microbial ALE, a microbe is cultured for extended periods under clearly defined conditions, in the range of weeks to years, which enables the collection of improved genetic variations. Microbial cells provide important benefits for ALE studies: (a) the majority of microbial cells have basic nutrient requirements; (b) they can be easily grown in the laboratories. Dynamic laboratory evolution approach as an effective synthetic biology technique to improve some features of *Z. mobilis*, for example, inhibitors tolerance or surface usage in the future.

Further studies showed that many other modifications could also be used symbiotically for advancement of the strain. ALE approach was also selected for the betterment of *Z. mobilis* strain. For example, a mutation process introduced for

acetic acid-tolerance in *Z. mobilis* is a type of adaptable mutants that are used in bioethanol production (Agrawal et al. 2012).

Further, studies determined all the ALE processes could be used as a forceful transfiguration engineering approach in the improvement of some kind of characters of *Zymomonas mobilis*. ALE strategies have also been utilized recently for enhancement of the pressure by *Z. mobilis*. For instance, a developed dynamic mutation technique has been used to monitor acetic acid tolerance. Although various engineered *Z. mobilis* strains have also been previously developed by incorporating desired genes as discussed above, the conversion process of cellulosic organic matter into ethanol is also a major task in the production of ethanol.

While managed efficiently for even more than 25 years, several recent studies have participated in the emergence of transcript and cheap next-generation sequencing technologies which actually implemented this methodology to engineer pathogenic microorganisms for biological processes. Improvement and development of the nutritional and stress metabolism of related model organisms have been gained over the past two decades, although some other aspects, such as niche-specific variations from non-renewable cell factories, are not fully understood. The status and its future projections underline the significance and potential of adaptive laboratory production as a biotechnological strategy.

6.5 Escalation in the Surface Implementation Variety of *Zymomonas mobilis*

Many researches have been conducted on the production of bioethanol from starch and sugars by *Zymomonas mobilis*, but production of bioethanol from starch and sugars sources is threat for food supplies (He et al. 2013) and environmental degradation (Pimentel et al. 2005). Nowadays, lignocellulosic feedstocks have been proved as an alternate source of sugars for bioenergy production (Balat and Balat 2009). *Z. mobilis* has ability of fermentation of many sugars like pentose and hexose from lignocellulosic source hydrolysate into ethanol.

Research history of *Z. mobilis* is described in Fig. 6.3. A recombinant *Z. mobilis* CP4 (pZB5) strain was produced by introducing two operons. Almost 86% ethanol yield was obtained by fermentation of pentose sugar using *Z. mobilis* (Leksawasdi et al. 2001). Co-fermenting of 6C sugar glucose, xylose, and aldopentose sugar arabinose to ethanol resulted in 72.5% ethanol yield using *Z. mobilis* like co-culture ATCC 39676 (pZB4L) and ATCC 39676 (pZB206) (Picataggio et al. 1998).

Both xylose and xylose-fermenting strain had a tremendous effect on the arabinose exertion strain. A single *Z. mobilis* 206C (pZB301) in 1998 fermented mixture sugars to ethanol and offered 82–84% theoretical yield (Zhang et al. 1998). Nevertheless, by antibiotic-resistant plasmid, all types of recombinant strains were composed of extension of many antibiotics to control cohesion for the sake of increased

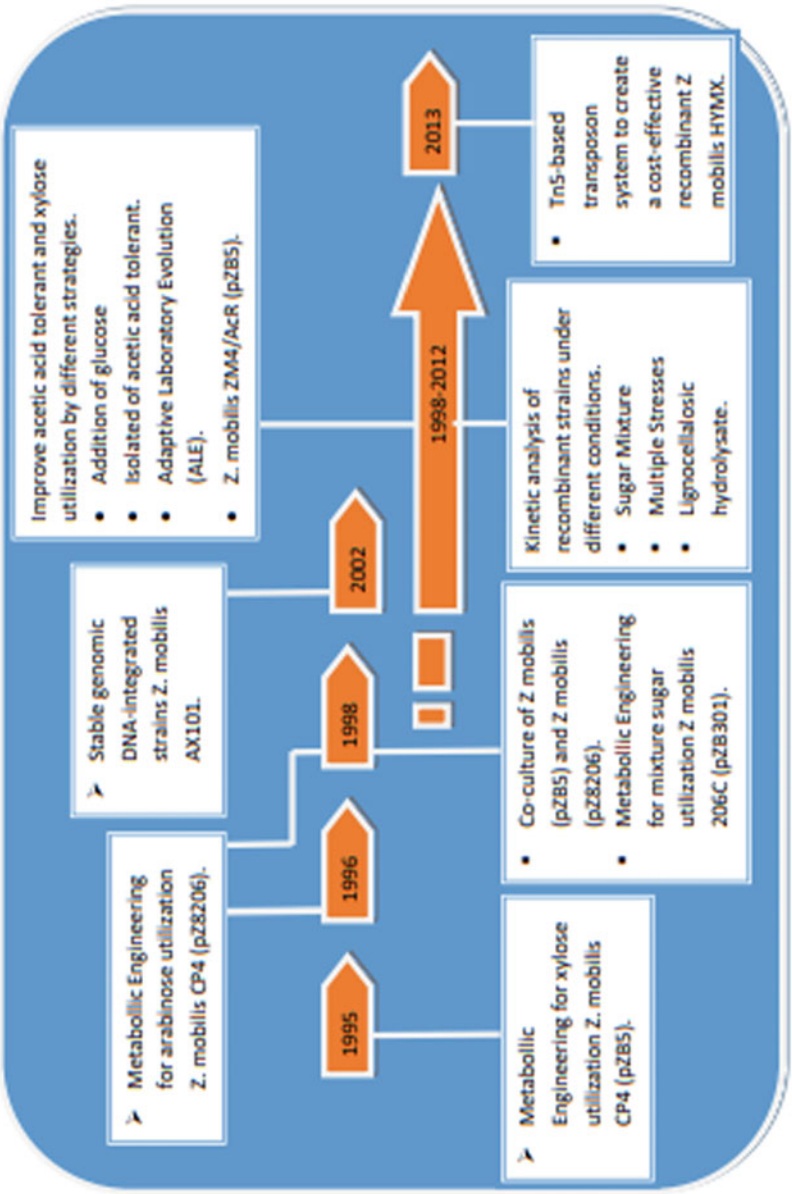

Fig. 6.3 Research history of *Z. mobilis* for the production of ethanol (modified from He et al. 2014)

and desirable fermentation products, and also for the improvement of genetic stability.

There are seven genes that are important for the pentose relevance and were incorporated into the genome of *Zymomonas* and another stable *Z. mobilis* AX101strain in 2002, which could ferment a hexose and mixture of pentose across preferred coherent (Picataggio et al. 1998). If a strain is competent for co-fermentation of all the three sugars, it means that all recombinant strains were sensitive to acetic acid pressure. Further studies regarding nuclear magnetic resonance (NMR) illustrated that acetic acid also can hinder the effectiveness of application of xylose in *Z. mobilis* ZM4(pZB5) (Rogers et al. 2007).

6.6 Modifying Laboratory Transformation of Ethanologenic *Zymomonas mobilis* Strain that Is Being Tolerant to Acetic Acid Inhibitors

During production of cellulosic ethanol, acetic acid from lignocellulosic hydrolysate is the predominant inhibitor to *Zymomonas mobilis*. *Z. mobilis* is sensitive to acetic acid inhibitors, this issue can be minimized by applying analytical engineering approaches due to inferior standard of their own molecular mechanical actions. Further studies suggested that adaptive laboratory evolution (ALE) approach was recycled for advancement of acetic acid-tolerant pressure. Later these three curved development resulted in four derived mutants; ZMA7-3, ZMF3, ZMF3-3.

These mutants show more improved capacity that was profitably achievement for the ALE method. And on the basis of cell growth, consumption of glucose and yield of ethanol, two devoting strains; ZMA7-2, ZMF3-3 were obtained that indicate greater resistance under 7 g/1 acetic acid. The finest strain is *Z. mobilis* ZMF3-3, that offered 94.84% yield of theoretical alcohol. Different approaches were applied for the improvement of acetic acid tolerance. Lawford et al. (1998) evolved a method for extension of more glucose in ethanoic acid that consists of media culture for the sake of advancement of fermentation performance of recombinant *Zymomonas* (Table 6.3).

Recombinant plasmid pZB5 also transformed into an ethanolic-acid-tolerant strain and a mutant (recombinant *Z. mobilis*) (pZB5) strain modified to acetate tolerance (Jeon et al. 2002). Overexposure of enzyme xylulokinase in a xylose-metabolizing recombinant strain was also established as a result of another recombinant strain (pZB5, pjX1) (Jeon et al. 2002). The adaptable laboratory evolution approach has been used for the improvement of ethanolic-acid ability (Wang et al. 2016). Productivity of xylose application (Agrawal and Chen 2011) in *Z. mobilis* is described above. The confined mutant CP4 (pZB5) M1-2 strain can metabolize (pentose sugar) xylose more immediately than glucose. Furthermore, sequenced data scanning showed mutations in both glucokinase (glk) and glucose facilitator (glf) genes.

Table 6.3 Different genes that improve the inhibitor tolerance in *Z. mobilis* (modified from Wang et al. 2018)

Genes	Function	Host	Resistance	References
Hfq (ZMO0347)	Global regulator	ZM4	Acetate, HMF	Yang et al. (2010)
nhAa (ZMO0119)	Sodium proton antiporter	ZM4	$C_2H_3NaO_2$	Liu et al. (2017)
ZMO1696 ZMO1885	NADH oxidase, alcohol dehydrogenase	ZM4	4-Hydroxyben zaldehyde	Yi et al. (2015)
irrE	Regulatory protein	*E. coli*	NaCl	Zhang and Lynd (2010)
ZMO1875	Unknown protein	ZM4	NaCl	Skerker et al. (2013)
himA (ZMO1122)	Aldo/keto reductase	ZM4	NaCl	Wang et al. (2016)
ZMO1771	Alcohol dehydrogenase	ZM4	Corn Stover hydrosylate	Wang et al. (2017)

Mohagheghi et al. (2004) established another constituent of ZM4 (pZB5) and named it *Z. mobilis* 8b and obtained 82–87% ethanol output. Previously, the distinctive fermentation of many recombinant strains had also been explored. Basically, the performance of fermentation of better recombinant strains from different sources are being used for the production of alcohol. *Z. mobilis* AX101, *S. cerevisiae* 424A(LNH-ST), and *E. coli* KO11 were analyzed for the first time with cellulosic material. Besides this the most important is *Z. mobilis* AX101 that showed the greatest rate of glucose consumption and lowest byproducts yield (Lau et al. 2010).

Such results also show that the metabolic pathways of *Z. mobilis* AX101 and *E. coli* KO11 are more efficient in ethanol fermentation from the comprehend pathway of yeast (Lau et al. 2010). Different kinds of raw lignocellulosic materials, e.g. sugarcane and its dry pulpy fibrous residues, oat hull, agro-industrial waste (Ruanglek et al. 2006), corn stover (Mohagheghi et al. 2004; Su et al. 2013), bamboo residues (He et al. 2013), and many other kind of hydrolysate have been produced by Arkenol Technology for further manufacturing of ethanol by *Z. mobilis.*

These studies also explained basis for alcohol production in future. Different types of engineered *Z. mobilis* strain have also been fortunately build up by establishing desirable genes as mentioned above, which covert cellulosic biomass into alcohol. Further studies established the method of ALE, which can also be used as a wonderful metabolic engineering appliance for metabolic engineering in *Z. mobilis.*

Moreover, two best strains that can also be used as innovative hosts for next metabolic engineering in upcoming bioreactors and cellulosic ethanol. Most eminently, two strains can also be used as inventive-resistant model organisms for the sake of genetic mechanism on the "omics"(such as study of genomics, proteomics, and metabolome) level that have been proven to be beneficial for providing some innovative information for inverse metabolic engineering (Agrawal and Chen 2011).

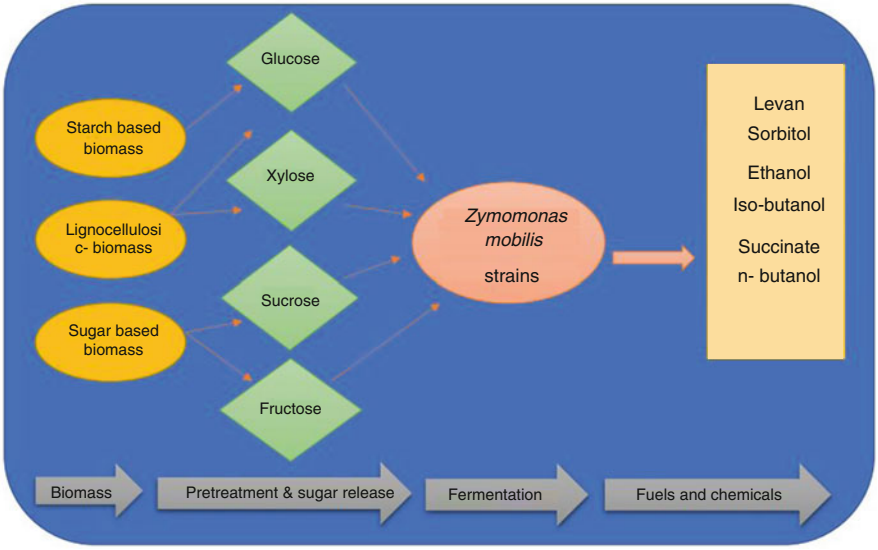

Fig. 6.4 General process of biofuel production (modified from Yang et al. 2016a, b)

Fuels and chemicals have been synthesized in the past from renewable resources but there were some issues regarding environmental pollution and limitations; therefore, another platform is needed for this microorganism and its metabolic engineering. For this purpose, employed strains should be optimized so that various available feedstocks like agricultural residues, industrial wastes, energy crops, sugar cane, forest residues, starch, etc., can be used successfully for industrial applications (Fig. 6.4).

Z. mobilis subspecies are considered as model organisms for industrial researches (Wang et al. 2018) due to their potential benefits, i.e. biomass production is low, they need no special treatment as oxygen is added in control during fermentation (Yang et al. 2016a, b), high ethanol yield, high sugar uptake, easily utilize xylose and arabinose in addition to glucose (Zhang et al. 1995), not get contaminated by bacteriophages, ethanologenic, osmotolerant, and genome is simple, small, and sequenced.

Using these strains and adopting recombinant techniques helped in strain improvement that will make great advancement in industrial biotechnology (Panesar et al. 2006). The following techniques were used for strain improvement; using plasmid as vector, transposons, expression vectors, transformation, knockout of genes, mutagenesis of transposon, adaptive laboratory evaluation, metabolic pathway engineering. The major fields of genomics and transcriptomic also helped in strain improvement.

6.7 Functional Genes in *Z. mobilis*

Although microbes encounter various stresses, for example, environmental factors, substrate chemical composition, production of toxic compounds, and other fermentation byproducts, *Z. mobilis* is very tolerant towards these stresses but pretreatment of biomass makes the process easier and thus high yield is obtained (Winkler and Kao 2014). BLAST is used to match the sequence of *Z. mobilis* to the other sequences that are given to the NCBI, as the result of the detection of genomic sequence the ring form structure appear in which different strains are used which are showed by the different colors (Fig. 6.5).

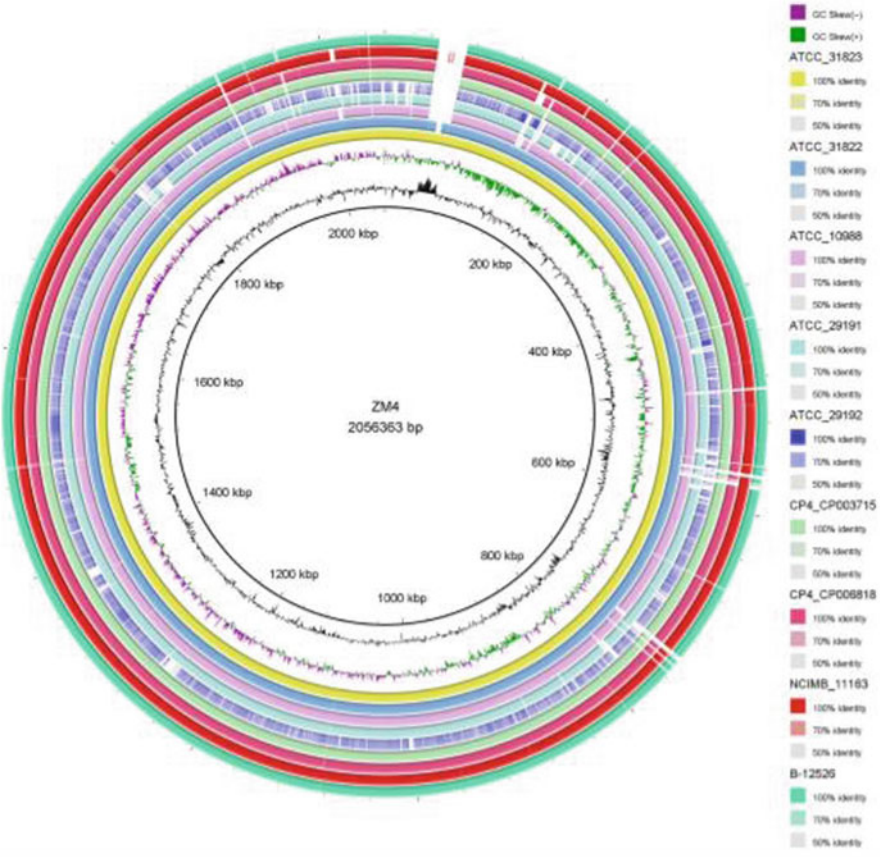

Fig. 6.5 Genomic analysis of *Zymomonas mobilis* taken from (Yang et al. 2016a, b)

6.7.1 *How Z.* mobilis *Is Unique*

- As *Z. mobilis* is a facultative anaerobe but has ED pathway that is characteristic of obligate aerobes.
- *Z. mobilis* has characteristic of energy uncoupled mechanism of growth for high ethanol production.
- *Z. mobilis* has advantage of essential genes as pdc and adh genes for ethanol production. The carbon diversion of this gene to other essential byproducts can be challenging (Yang et al. 2010a, b). Although *Z. mobilis* can be easily modified genetically. The genome editing tools as CRISPR-cas 9 can make *Z. mobilis* more effective and sophisticated.

6.7.2 *Pretreatment of Biomass*

Pretreatment is conducted to remove lignin and other plant cell wall residuals so that enzymatic activity can be done easily and plant biomass with rich cellulose and hemicellulose can be obtained (Harmsen et al. 2010). Pretreatment of grasses and hard woods gives potential sugar as byproducts such as xylose and arabinose so that enzymatic hydrolysis becomes easier. The main focus of pretreatment is to improve the consumption of lignocellulosic feedstocks and to get higher yield of ethanol.

A pretreatment approach AFEX is a physio-chemical process that involves the use of liquid ammonia and steam explosion (high temperature and pressure) (Bals et al. 2011). Another widely used pretreatment approach is DA pretreatment. It is beneficial as it is cost effective (Mathew et al. 2016). As compared to DA, AFEX requires higher energy requirement and use of liquid ammonia makes it costly approach but AFEX gives less inhibitor formation and more yield is obtained with less sugar consumption (Harmsen et al. 2010).

6.7.3 *Biomass Feedstocks*

The feedstock grain and corn give the starch based carbon sources. Besides lignocellulosic feedstocks other energy crops as sugar beet, sweet potato, sweet sorghum, and sugar cane can be used (Behera et al. 2012; He et al. 2013; Yang et al. 2013). A wide range of carbon sources can be used by *Z. mobilis* for ethanol production.

1. Algal biomass, e.g. biomass obtained from *Spirogyra hyalina*.
2. Agricultural wastes, e.g. sugar cane, molasses, waste paper sludge, and corn residues.
3. Industrial wastes, e.g. soyabean and meal.
4. Energy crops.
5. Energy plants, e.g. switch grass.

6.7.4 Strategies to Overcome Toxic Compounds

For greater ethanol production, strategies are employed by knowing the chemical composition of toxic compounds and their effects on the host microbes. The chemical components that behave as inhibitors in toxic compounds are acetate, phenolic aldehydes, and furfural compounds (Franden et al. 2009, 2013; Wang et al. 2014; Yi et al. 2015). In the pretreated AFEX of corn stover and switch grass, phenolic acids and amides are the inhibitors (Keating et al. 2014; Serate et al. 2015).

Methods adapted to lessen the toxicity of pretreated hydrolysates are

1. Deacetylation and disc refining.
2. Deacetylation and mechanical refining.

These methods overall increase the bioethanol production and reduce the toxicity of pretreated biomass and digestibility (Chen et al. 2016). When corn stover is fermented and DMR process is applied the ethanol titer obtained is 86 gL^{-1} (Chen et al. 2016). The second approach employed for high ethanol production is genetic approach. Both forward genetics and reverse genetics have been used. In forward genetics chemical mutagenesis and transposon mutagenesis are performed (Panesar et al. 2006).

6.7.5 Strain Evaluation and Fermentation Strategies

For the purpose of strain development high throughput strain evaluation is used. These include techniques such as Bioscreen C, Biolog's phenotype microarrays, and BioLector system. Biolog's phenotype microarray gives phenotype profiling of about 2000 *Z. mobilis* strains so that physiology of *Z. mobilis* can be studied easily (Bochner et al. 2010). Bioscreen C system gives the advantage of studying cellular growth of two 100 well plates at 0.4 ml scale (Table 6.4) (Franden et al. 2009; Yang et al. 2010b).

6.8 Fermentation Systems

These are used for strain evaluation and are advantageous at both mini and micro scales, e.g. Biolector Micro Bioreactor system. This system has various modules for monitoring various functions and to study the different fermentation parameters as dissolved oxygen and pH (Buchenauer et al. 2009; Funke et al. 2010; Blomberg 2011; Rohe et al. 2012). Fermentation strategies were applied depending on the product titer and yield. Batch, fed batch, and continuous cultures can be applied depending on the mode of application.

Table 6.4 Examples of different fermentation platforms, processing strategies, and cultivation techniques have been applied on *Z. mobilis* for ethanol, fructose, and levan production (modified from Yang et al. 2016a, b)

Product	Strain	Substrate	Initial carbon ($g\ l^{-1}$)	Fermentation strategy	Condition (pH, temperature, r. p.m.)	Time (h)	Titer ($g\ l^{-1}$)
Ethanol	ATCC 10988	Glucose	100	Batch	pH: 4.5, 37 °C	12	50.6
	MCC 2427	Sugarcane molasses	216	Batch	pH: 5.1, 3 °C	44	58.4
	10,225	Kitchen garbage	70	Batch	pH: 4.0, 30 °C	40	52
	NRRL-806	*Eucalyptus globulus*	79.5	Batch	pH: 5.5, 30 °C, 150 r.p.m.	27	37
	CP4	Sugarcane bagasse	80	Batch-SSF	pH: 5.0, 30 °C	36	60
	8b	Paper sludge		Batch-SSCF	pH: 5.8, 30 °C, 300 r.p.m.	120	46.3
	PTCC 1718	Carob pods	180	Batch-ASSF	pH: 5.3, 30 °C	40	1.8
	CP4	Glucose	295	Batch-VHG	pH: 6.0, 32 °C	60	78
	TMY-FHPX	Glucose	295				136
	TMY-FHPX	Glucose	295				145

Batch fermentation system is the simplest method as nutrients were supplied in the beginning and wastes were collected at the end. As it is the simplest method, has low capital investment and is a quick approach to study the early stages of developmental process (King and Hossain 1982; Lawford et al. 1988; Ishikawa et al. 1990; Szambelan et al. 2004; Patle and Lal 2007). But it has limitation because it has low productivity and low cell density. The toxic compounds accumulated in batch fermentation offer low cell growth (Arcuri 1982; Jain et al. 1985; Edye et al. 1989; Lawford et al. 1998; Bravo et al. 2000; Amutha and Gunasekaran 2001; Silbir et al. 2014).

Z. mobilis has four important genes ZM001139, ZM0141, ZM01792, and ZM 01140 that encode functional enzymes A1s, IIvD, and IIvC. These enzymes form 1-valine from pyruvate (He et al. 2012). A gene for the production of alcohol dehydrogenases is also present in *Z. mobilis* and is very important. These alcohol dehydrogenases are important for the production of 2, 3-butanediol production. Different studies revealed the important and functional role of these Adh enzymes in formation of different alcohols (Fig. 6.6).

In these aspects, fed batch and continuous fermentation systems are advantageous to give high ethanol productivity and growth. A continuous fermentation using flocculated *Z. mobilis* WR6 gives the ethanol titer of 47 gl^{-1} and volumetric productivity of ethanol is 80 gl^{-1} (Fein et al. 1983). Immobilized *Z. mobilis* gives

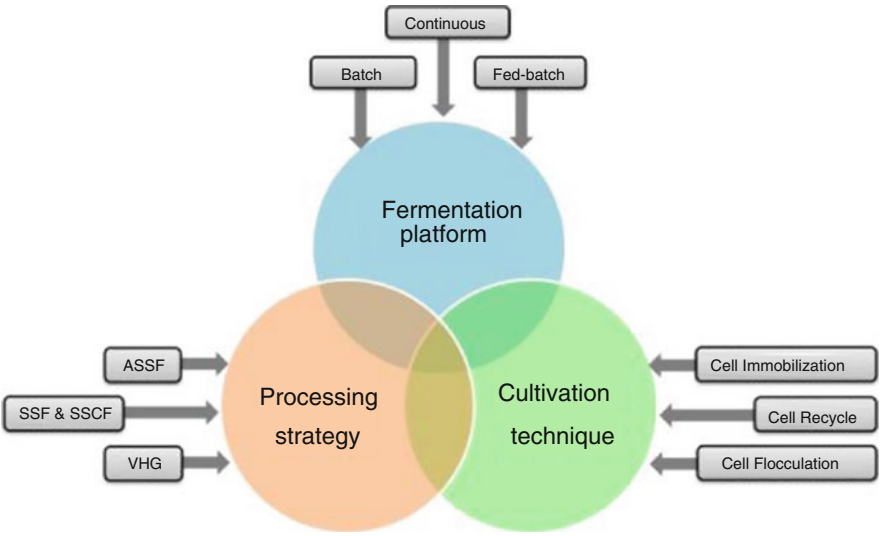

Fig. 6.6 Fermentation platform for *Zymomonas mobilis* taken from (Yang et al. 2016a, b)

more cell density and productivity as they grow in biofilms and increase the surface area for normal metabolism and reusability than free encapsulated *Z. mobilis* (Niu et al. 2013).

Economic bioethanol is produced using *Z. mobilis* in other industrial approaches described below:

1. Advanced solid state fermentation technology.
2. Solid submerged fermentation technology.
3. Co-fermentation technology (Lawford et al. 1997; Zhang et al. 2010; Das et al. 2013; Saharkhiz et al. 2013).

6.9 Biosynthesis Pathways

Metabolic pathways adopted for the synthesis of biofuels is given in (Fig. 6.7).

6.10 Valuable Byproducts of *Z. mobilis*

6.10.1 Isobutanol Production

Another liquid fuel other than bioethanol produced by genetically engineered strains is isobutanol. *Z. mobilis* was made genetically engineered by incorporating genes of alcohol dehydrogenase (adha A) and 2-ketoisovalerate decarboxylase (kiv d) which

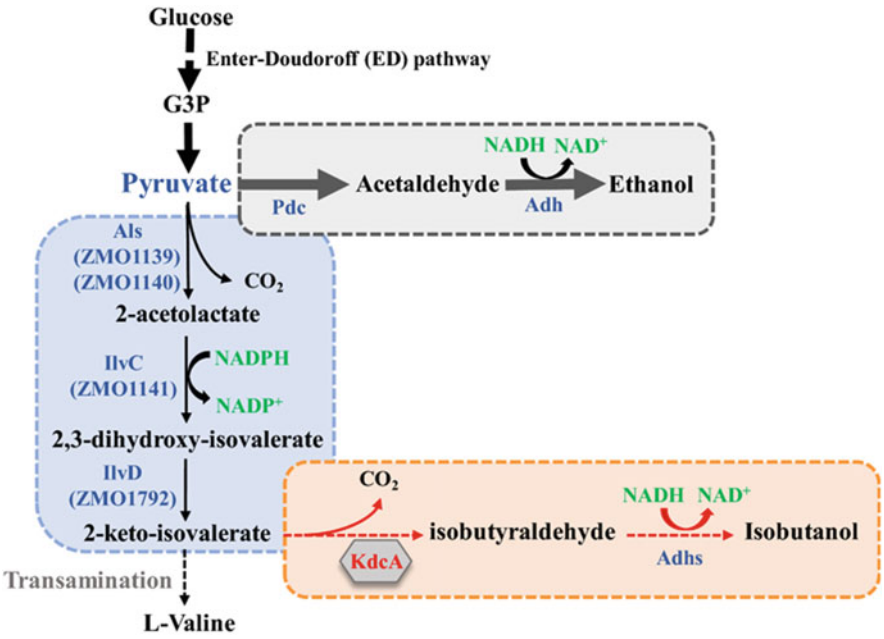

Fig. 6.7 Metabolic pathway for biofuel production (taken from He et al. 2012)

led the *Z. mobilis* to effective production of biofuel. An achievement of higher yield production is obtained by incorporation of key genes of desired pathways for desired production (Smith et al. 2010).

6.10.2 Levan Production

Levan is produced when *Z. mobilis* was cultured on sucrose medium (Bekers et al. 2001). Levan has antitumor activities. Different studies reveal that sucrose fermentation gives ethanol and levan. So a purpose to obtain bioethanol gives a useful bio-products using *Z. mobilis*. Sucrose is bio-converted into ethanol and levan yield of 0.22 gl^{-1} (Beker et al. 1990). Different studies state the fundamental role of Adh genes and ato AD and Pdc genes for the ethanol production using a metabolic pathway at genome scale.

6.10.3 Substrate Utilization Range

The substrates like sugarcane, corn, starch, glucose, and molasses have been used for the ethanol production *by Z. mobilis*. Lignocellulose feedstock's are considered

better alternatives of corn, sugarcane for ethanol production (Balat and Balat 2009; Rogers et al. 2007).

6.11 Strategies for Strain Improvement of *Z. mobilis*

Now there is demand of renewable resources, sustainable biofuels technologies, and lignocellulose substrates to obtain high yield of biofuel. The strategies adopted are:

6.11.1 Conventional Mutagenesis

Selective mutants of *Z. mobilis* were made using mutagenic agents like caffeine, EMS (ethyl methane sulfonate), UV lights, etc., for bioethanol industry. Mutants of *Z. mobilis* obtained were auxotrophic, osmotolerant, sucrose-hyper tolerant, fructose-negative, and antibiotic sensitive strains which showed desired applications in bioethanol industry (Wang et al. 2013).

6.11.2 Transposon Mutagenesis

This is successfully done by using broad host range plasmids like Tn951, Tn5, (Wang et al. 2013) and Tn1725 using *Z. mobilis*. Carey et al. (1983) stated that plasmid PGC91.14 successfully expressed in *Z. mobilis* at 30 °C. Transposon mutagenesis is an effective tool for the ethanol production. Using TN5 transposed *Z. mobilis* recombinant plasmid replicon fusions were also helpful (Zhang et al. 2013).

6.11.3 Adaptive Laboratory Evolution (ALE)

This is effective for strains optimization selection and adaption. Metabolic engineering is also helpful (Zheng et al. 2009). Selective mutants were made using adaptive mutation protocols for the bioethanol production. Agrawal et al. followed these protocols for the xylose-fermenting *Z. mobilis*. Substrate utilization and inhibitor tolerance are limitations and can be overcome by using mutants made from ALE procedures (Kerr et al. 2011).

6.11.4 Conjugation

Conjugation is another mechanism that gives higher yield using *Z. mobilis*.

6.11.5 Recombination

Research showed that five genes of cellulolytic enzymes from bacteria were successfully incorporated in the *Z. mobilis* that directly ferments the cellulosic feedstock's into ethanol. By introducing additional cellulose genes in the *Z. mobilis* to make it recombinant so that it can serve as potential CBP platform organism (Zhang et al. 2013).

6.11.6 Recombinant Strains of Z. mobilis

Recombinant strains were made by introducing genes of *E-coli* in *Z. mobilis* for better ethanol yield. Good yield of ethanol is obtained by introducing xylose using genes and metabolic pathway genes. Another recombinant strain of *Z. mobilis* was made for arabinose fermentation (Mohagheghi et al. 2004).

6.11.7 Co-Fermentation

Co-fermentation of all three sugars glucose, arabinose, and xylose to ethanol occur using *Z. mobilis strains* ATCC 39761 (PZB4L) and ATCC 39676 (PZb206). Co-fermentation is another approach for the effective bioethanol production (Wirawan et al. 2020).

6.11.8 Consolidated Bioprocessing Approach (CBP)

CBP was constructed by incorporation of five genes of *E. coli* for the arabinose metabolism. CBP approach is used for effective ethanol production. Its mechanism involves enzyme synthesis, substrate assimilation, and fermentation in single step using lignocellulolytic microbes. E1 and GH12, these are two cellulolytic enzyme genes that were successfully incorporated and expressed (Linger et al. 2010).

6.11.9 Gene Knockout

This genetic tool is used for gene deletion from the organism. Gene knockouts is done using insertion approach so that functional capacity of mutant gene is enhanced using suicidal plasmids, site directed FLP recombines, PCR based approaches, and transposons so that intentionally specific genes can be inactivated. Literature review gives some genes that were targeted for the desired phenotype, i.e. aldo-keto reductase, oxidoreductase, cytochrome related genes, hydroxylamine reductase, NaOH dehydrogenase, alcohol dehydrogenase, and pyruvate dehydrogenase (Zhang et al. 2013).

6.11.10 Genomics

This technique is a promising approach for strains improvement. *Z. mobilis* genome was sequenced in 2005. This strain has 2,056,416-bp chromosome that is circular and also contains extrachromosomal material called plasmids. In 2005 other strains of *Z. mobilis* complete genome sequence were also reported. Genomics approach is a great help in targeting a particular gene for a specific purpose (Widiastuti et al. 2011).

6.11.11 Transcriptomic

Transcriptomic study of *Z. mobilis* is a better way to understand gene function. Different techniques like DNA array or DNA sequencing approaches have been used in differential gene expression under different stress conditions. Transcriptomic profiling done in the past was of greater help in the gene regulation. Different studies were done using ZM4 growing in ethanol stress media, heat shocked media, high ethanol concentration, and rich and minimal media. Different mutants were made using genomic profiling like ACR that is sodium acetate tolerant mutant.

6.11.12 Using Shuttle Vectors

A gene important for the production of different alcohols especially isobutanol is cloned into shuttle vector PEZ15A sp. By incorporating tetracycline inducible promoter sequence and then introducing it in *Z. mobilis*, a recombined strain ZM4-Kit can be formed. This can be confirmed by gene Sanger Sequencing. Different experiments were performed to check the impact of kdcA gene expression due to pet promoter for isobutanol production and it was recorded that isobutanol

production was increased by increasing the tetracycline concentrations (Yang et al. 2019).

6.12 Heterologous Biofuel Production

A heterologous KdcA gene is integrated into the chromosome or can be integrated into plasmid of *Z. mobilis* for the isobutanol synthesis (Yang et al. 2019). Two recombinant strains ZMQ1 and ZMQ2 were made for stable isobutanol production by the introduction of kdcA gene either into the chromosome locus or into the native plasmid locus. This integration basically shifts the production of ethanol to the isobutanol. A1s gene reduces ethanol production and enhances the isobutanol production. Such plasmid were constructed that shifts the carbon flux for the ethanol production to isobutanol (Yang et al. 2016a, b). Different studies reveal the overexpression of kdcA gene and Als-ilvC-ilvD operon that shifts the ethanol production to the isobutanol biosynthesis. These studies give the strategy to use metabolic pathway of valine so that biofuels can be synthesized from pyruvate.

6.13 Conclusion

Z. mobilis is a natural anaerobic ethanologenic gram-negative bacterium with many desirable industrial characteristics. For example, *Z. mobilis* is generally considered safe (GRAS), shows high tolerance for ethanol up to 16% (v/v), and can produce ethanol at a wide pH range (3.5–7.5, especially low pH). Advances in gene sequencing technologies and especially next-generation sequencing (NGS) techniques provide new opportunities for gaining a basic understanding of *Z. mobilis* strains.

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Chapter 7
Sustainable Production of Hydrogen by Algae: Current Status and Future Perspectives

Rahul Kumar, Ragini Gothalwal, Swati Mohapatra, Pallav Kaushik Deshpande, and Ramchander Merugu

Abstract With energy costs reaching historical highs, biohydrogen as an alternate fuel is progressively attracting attention. Production of gas from algae is being thought of as an alternate to other forms of fuels. However, industrial production of biohydrogen remains not practically possible as a result of low biomass concentration and expensive downstreaming processes. Biohydrogen is a zero-emission fuel and is an alternate to conventional fossil fuels. Nowadays, photobioreactors and outdoor systems are being used for gas production from algae. Numerous factors affect the production of hydrogen. These factors need to be optimized for enhancing algal biohydrogen. Significance and challenges of algal hydrogen production are discussed in this communication.

Keywords Algae · Immobilization · Bioreactors · Microalgae · Macroalgae · Biomass · Biohydrogen · Sustainability

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7.1 Introduction

The world is facing energy crisis due to ever growing population. By the year 2025, the world's oil consumption is expected to rise by 60%. Hence, Mallick (2002) opined that there is a need of developing sustainable and cost-effective methods for energy needs. The depletion of fossil fuels and industrialization is the reason for this crisis (Medipally et al. 2015). Saifuddin and Parthasarthi (2016) felt that governments have now become proactive in addressing the need for new potential fuels for meeting these demands. Dragone et al. (2010) felt that renewable energy sources can be explored to overcome the energy crisis. Biohydrogen does not produce any emissions (Chang and Lin 2004). Algal biohydrogen can replace fossil fuels and needs to be optimized to enhance hydrogen production rate (Moreno-Garrido 2008; Dincer 2012). Prince and Kheshgi (2005) have highlighted the importance and efficiency of photobiological production of hydrogen. Complex polymers such as lignin, cellulose, and hemicelluloses are difficult to be degraded and increase the process costs (Azwar et al. 2014). Gaffron and Rubin (1942) discovered hydrogen production from the green alga *Scenedesmus* under anaerobic conditions. Algae are unicellular as well as multicellular autotrophic aquatic life forms. Cyanobacteria and green microalgae are the most accepted microorganisms for the production of biohydrogen (Kotay and Das 2007). Hydrogen can be considered as a future energy fuel and has the "highest energy content per unit weight" (Das and Veziroglu 2008). Rajkumar et al. (2014) studied the potential of algae for biofuel production. Biofuels of first and second generation have low production capacity which is a major limitation (Saqib et al. 2013). Redwood et al. (2008) have suggested using co-cultures for maximizing H_2 production. Behera et al. (2015) described the utility of algae as third-generation biofuel. Aresta et al. (2005) felt that the aquatic biomass represents an important strategy for large-scale industrial application without environmental concerns. Thermal processing of biomass for renewable fuel generation was studied by Bridgewater (2003). Algae play a major role in maintaining the water quality and in controlling microbial growth. In this chapter, status of algal biohydrogen production, its sustainability, potentials, and the challenges of algal biohydrogen production are reviewed.

7.2 Hydrogen Production by Algae

Algal production of hydrogen has generated significant interest since the mechanism of gas production from algae by sulfur deprivation was discovered (Ghirardi et al. 2009; Melis 2007). Barsanti et al. (2008) have survive that alga will survive over different ranges of pH, concentration, temperature, and different light intensities. Das et al. (2011) suggested that microalgae will have potential applications in

biofuel production, bioremediation, and greenhouse gas sequestration. Several useful bioproducts like lipids, proteins, vitamins, pigments, polysaccharides, and antioxidants are generated from algae (Brennan and Owende 2010). The influence of freshwater pretreatment on *S. muticum* as a feedstock was investigated by Milledge et al. (2018a). Yuan et al. (2018) extracted polysaccharides from the algae *Ulva prolifera* using microwave technology and studied the properties and activities of them. The commercial cultivation of microalgae for novel natural useful ingredients has increased over the years. The connection between gas production and electron transport pathway in sulfur-limited *Chlamydomonas reinhardtii* was studied by Antal et al. (2009). Macro and microalgae are used for bioenergy production (Carlsson et al. 2007) as they are extremely productive, simply harvestable, and economical compared to different other sources used for bioenergy production. Skjanes et al. (2013) analyzed the potential of microalgae in biohydrogen production and other high-value bioproducts. The optimum temperature is 20–24 °C, though this might also differ with the culture medium and the species cultured (Brown et al. 1989). Most typically cultured microalgae withstand up to 27 °C temperature, while the temperature less than 16 °C slows down algal growth. The temperatures above 35 °C are inhibitory for a wide variety of species. Algal species isolation is not easy due to the fact of the small cell dimension and the association with different epiphytic species. Bacteria can be removed from the algae by way of washing or plating with medium containing antibiotics. The various types of algal culture methods include indoor or outdoor, open, axenic, batch, continuous, and semicontinuous. Indoor cultures allow the control over operating conditions such as temperature, nutrient level, illumination, and contamination. The axenic cultures are free of any contamination and are sterilized. The limitations of the continuous system are that these systems are highly expensive as constant illumination is required.

Counting chambers are of two kinds, namely, Fuchs-Rosenthal and Burker, which are used for a variety of cell sizes and concentrations. For a unique algal species, dry weight per cell can also differ notably in accordance to the stress and tradition conditions. Microalgae can additionally be viewed as a rich source of ascorbic acid (Brown and Miller 1992). The dietary value of microalgae can differ significantly according to the culture conditions. Culture medium used significantly affects the metabolite production in various species of microalgae. Palanisamy et al. (1991) have studied the application of algal cultures in shrimp hatcheries. Walsh et al. (1987) have cultivated marine microalgae for producing bivalve seed. Principles for the cultivation of microalgae in photo-bioreactors were designed by Posten (2009). Challenges and opportunities in microalgal bioreactors were explained by Xu et al. (2009). The microorganisms in mixotrophic cultures grow quicker and may synthesize hydrogen through autophytic and heterotrophic pathways (Ceron Garcia et al. 2005). Dragone et al. (2010) have felt that mixotrophic conditions with inorganic carbon and organic carbon such as aldohexose, glycerol, and acetate can

enhance hydrogen production. Hydrogen production by photoautotrophic sulfur-deprived *Chlamydomonas reinhardtii* incubated under high light intensity was reported (Tolstygina et al. 2009). Enhanced photoproduction of hydrogen in *Chlamydomonas reinhardtii* D1 mutant was studied by Torzillo et al. (2009). The continuous production of hydrogen by salt addition in sulfur-limited cultures of *Chlamydomonas reinhardtii* was reported by Kim et al. (2010). Hydrogen photoproduction by sulfur-limited cultures of *Chlamydomonas reinhardtii* was also studied under completely different growth conditions (Kosourov et al. 2007). Kruse et al. (2005) reported enhanced hydrogen production in engineered green algal cells. Laurinavichene et al. (2004) studied hydrogen production by sulfur-limited *Chlamydomonas reinhardtii* at different light intensities. Mechanism and challenges involved in production of algal biofuels were reviewed by Singh et al. (2011). Under sulfur deprivation, *C. reinhardtii* cellular division and growth is inhibited, and RuBisCO enzyme breakdown takes place (Zhang et al. 2002a). The process of isolation of an ideal algal strain can be done by first isolating the algal species in a specific medium. Biomass-based feedstock was proposed by Wang and Yin for biohydrogen production. Michalak (2018) processed seaweeds for biofuel production. Gallagher et al. (2018) investigated the macroalgal species variation during the process of dewatering. Chemical composition changes of the algal biomass during various seasons in the algal species *Saccharina latissima* were studied by Sharma et al. (2018). Sandbakken et al. (2018) proposed the biomass of *Saccharina latissima* as carbon source for biofuel production and suggested that it can be preserved using acid. The algal species can be separated by means of plating, serial dilution, or micropipetitng. Once the specific algal species is identified, then it can be reconstructed toward producing hydrogen. Kumar et al. (2020a) have reviewed the processes involved in the generation of various kinds of algal biofuels and biorefinery model in detail. They suggested measures to be taken for making the process feasible and sustainable. Chen et al. (2020) reported a generation rate of 0.44 μmol H_2 h^{-1} in an engineered biological system for about a month. Zaidi et al. (2020) have optimized the use of nickel nanoparticles for enhancing biogas generation from green algae *Enteromorpha* by response surface methodology. The have reported that concentration of 1 mg/L Ni nanoparticles resulted in higher biogas yield. Xia et al. (2020) have suggested the use of co-fermentation of microalgae *Arthrospira platensis* with macroalgae *Laminaria digitata* for enhancing hydrogen production. Fakhimi et al. (2020) have reviewed the prospects of using different types of co-cultures of bacteria and algae for enhancing biohydrogen production. Kumar et al. (2020b) have reviewed the advantages and disadvantages of various molecular techniques for identifying microbes involved in hydrogen generation. Various approaches can be used such as genomics, transcriptomics, proteomics, metabolomics, etc. to see that a genetically engineered strain of the algae is developed for hydrogen production (Fig. 7.1).

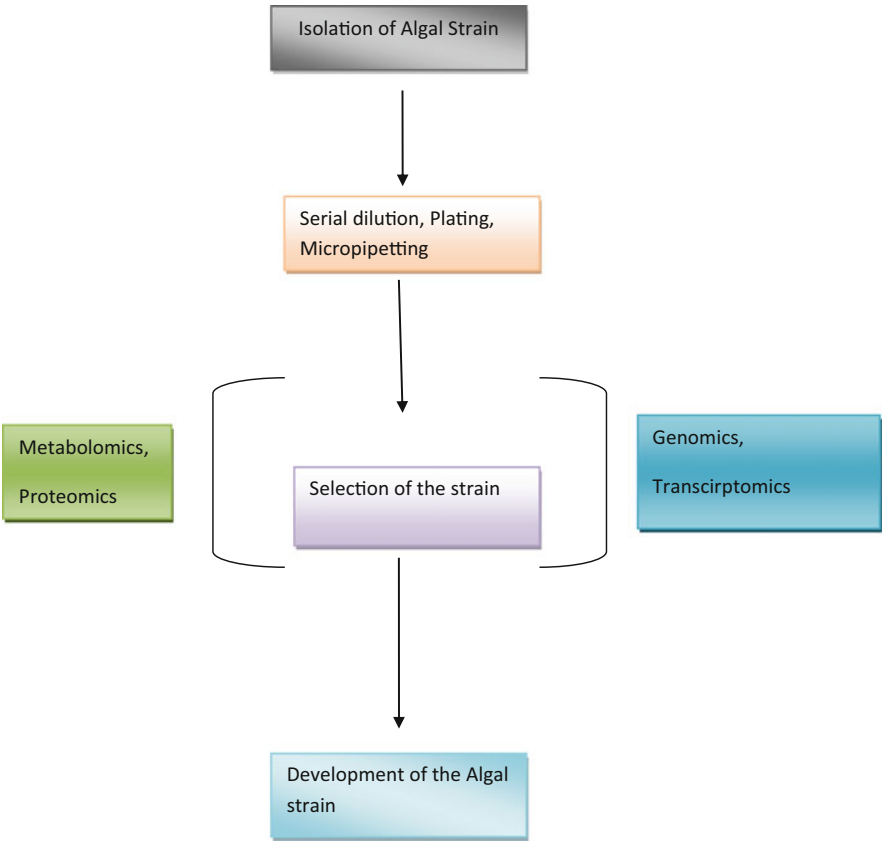

Fig. 7.1 Isolation and development of Algal strain for hydrogen production

7.3 Microalgae for Hydrogen Production

Lee (1997) have reported that some species of microalgae *Arthrospira*, *Chlorella*, *Isochrysis*, *Dunaliella*, *and* Chaetoceros can be used for biohydrogen production. High amounts of lipids in microalgae can be converted into biofuel (Avagyan 2008). Xiong et al. (2008) observed that microalgae such as *Chlorella* species can shift its nutrition mode. Williams and Laurens (2010) have reported that the major biomolecules in microalgae are carbohydrates, proteins, lipids, and nucleic acids. Microalgae have a rapid growth rate which makes them attractive renewable energy source for biohydrogen production (Tredici 2010). Algae use sunlight for growth without competing for agricultural resources (Pirt 1986). They have a short-generation time, and the algal oil can be converted into biofuel (Sudhakar Premalatha 2012). Searchinger et al. (2008) investigated US croplands for algae harvesting and drying for biofuel production. Microalgae are tiny factories which remove atmospheric carbon dioxide (CO_2) (Sumi 2009). Microalgae have benefits

like genetic diversity and possess numerous physiological and biochemical characteristics apart from generation of abundant carbohydrates and lipids. Still, several microalgae needs to be explored for cultivation, and there metabolism needs to be understood for any genetic modification to generate strains which are capable of higher hydrogen production (Wang et al. 2014). Borowitzka (1999) felt that industrial biomass production of microalgae through phototrophic approach is the most viable and economical, and many factors like light intensity, pH, temperature, salinity, vapor content, and nutrients have an effect on the microalgal cultivation. Ras et al. (2011) reported that *Scenedesmus*, *Spirulina*, and genus *Ulva* have the potential to produce biogas. Taihu blue alga was investigated for biogas production with corn straw as a carbon source (Zhong et al. 2012). Production of biogas from macroalgal waste streams for bioenergy generation was investigated by Tedesco and Stokes (2017) in Eire. Vergara-Fernandez et al. (2008) reported that algal species like *Macrocystis pyrifera* generated biogas in anaerobic reactor. Mussgnug et al. (2010) reported that the microalgal species *C. reinhardtii* and *Scenedesmus obliquus* can be used as substrates for fermentative biogas production. They additionally reported that heat, salt levels, and macromolecule content have an effect on the biogas yield. Pretreatment could enhance the biogas production (Kavitha et al. 2017). Bayro-Kaiser and Nelson (2016) mutagenized *C. reinhardtii* to come up with mutants that exhibited temperature-sensitive photoautotrophic growth. Eilenberg et al. (2016) generated the HydA enzyme and reported that the in vivo enzymatic activity of the Fd-HydA enzyme is more than that of the native HydA and shows higher gas tolerance. Under sulfur deprivation, hydrogen formation from algae was reported (Skjanes et al. 2013).

Batyrova and Hallenbeck (2017) reported about the genetically modified *Chlamydomonas reinhardtii* strain cy6Nac2.49, which was suitable for hydrogen generation. Krassen et al. (2009) studied the stepwise assembly of photosystem I and hydrogen evolution. Melis and associates discovered that sulfur limitation caused production of hydrogen in light by *Chlamydomonas reinhardtii* (Melis et al. 2000). Satoh et al. (2002) studied the regulation of energy balance in photosystems. Techno-economic analysis of microalgal biofuels was done by Stephens et al. (2010). The effect of pH and a methanogenic matter addition on hydrogen production was studied by Kumar et al. (2016). The promising way forward for microalgae as a renewable source of energy was reviewed by Khan et al. (2018). Melis et al. (2000) have reported that microalgae that produce polysaccharide will also produce biohydrogen along with methane in anaerobic conditions. A review of algal biohydrogen production was done by Rathore and Singh (2013). Genetic improvement of microalgae for biohydrogen production was investigated by Oncel et al. (2015a). Anaerobic membrane bioreactors for biohydrogen production were studied by Aslam et al. (2018). Show et al. (2012) reviewed the current status of biohydrogen production. Li et al. (2020) have studied microalgae for biofuel production. Shaikh Abdur et al. (2017) have reviewed the possibilities of growing microalgae in wastewater. Investigations on the cultural conditions required for growing *Chlorella vulgaris* were optimized by Daliry et al. (2017). Saba et al. (2017) have used bacteria and algae in microbial fuel cells for generation of

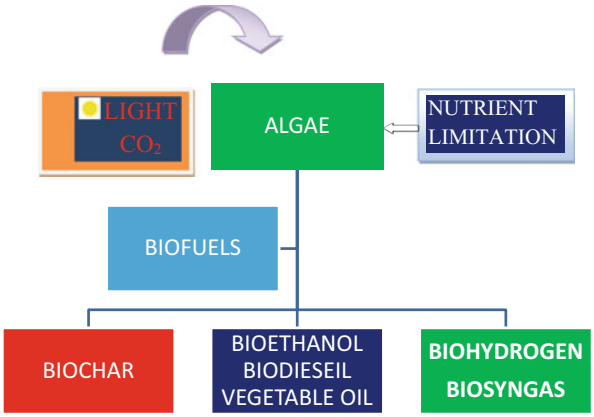

Fig. 7.2 Hydrogen production from algae

bioelectricity. Pradhan et al. (2017) and Raheem et al. (2018) have studied the use of microalgae for mitigation of carbon dioxide levels and biofuel production. Shuba and Kifle (2018) and Adeniyi et al. (2018) have opined that microalgal biofuel can be a potential alternative to the fossil fuels. The production of hydrogen from algae is shown in Fig. 7.2.

7.4 Macroalgae for Hydrogen Production

Macroalgae refers to a group of benthic marine algae and seaweeds. Macroalgae lack shoots, vascular tissues, flowers, and roots. With the exception of a few, macroalgae grow attached to hard surfaces; many species do not grow in mud due to lack of roots to cling to. Compared with high vascular plants, macroalgae have more complex ways of life and a wider range of reproductive methods. Most algae reproduction takes place by way of releasing sexual spores. Macroalgae has four distinct phyla members with a different history of evolution. Macroalgae absorbs, retains, and releases nutrients, thus contributing to the restoration of nutrients in the natural coral reefs. Many macroalgae play an important role in the formation of solid structures by the addition of calcium carbonate ($CaCO_3$). Crustose calcareous algae (CCA) similar to porolithon bind to the nearby surface and provide an erosion barrier. Direct calcareous algae such as *Halimeda*, *Dotea*, *Amphiroa*, and *Galaxa* are involved in filling the areas between corals. The white sand of the sea and sea lakes comprises most of the calcium carbonate sediments. In *Halimeda*, calcium is deposited as aragonite. Calcification can also be an adaptation to face up to wave shock and to provide mechanical support. Macroalgae have a vital place in reef degradation and mainly in ecological phase shifts. Dominance by macroalgae may make a contribution to reef degradation with the help of overgrowing corals, inhibiting coral recruitment, and contributing to coral diseases (Diaz-Pulido and McCook 2008). Macroalgae have three types of life cycles: (1) haplontic life cycle, (2) diplontic life

cycle, and (3) diplobiontic life cycle (Diaz-Pulido and McCook 2008). Marine macroalgae such as Rhodophyta, Ochrophyta, and Chlorophyta phyla are widely studied due to their applications (Barsanti et al. (2008)). Traditionally, they were cultivated from the sea directly. Lately, macroalgae cultivation methodologies have been improvised (Sahoo and Yarish 2005; Kim et al. 2017). Macroalgal species belonging to *Undaria*, *Sargassum*, *Saccharina* (*Laminaria*), *Kappaphycus alvarezii*, *Porphyra*, *Eucheuma denticulatum*, *Gracilaria*, *Gelidium*, *Saccharina*, and *Lessonia* have applications in food and medicine. Main genera were *Kappaphycus*, *Gracilaria*, *Eucheuma*, *Porphyra*, *Saccharina japonica*, and *Sargassum fusiforme*. The idea behind the use of macroalgae for bioenergy generation is that they are rich in polysaccharides which make them potential candidates for bioenergy generation (Kraan 2013). There process would become economically feasible only if it is coupled with production of valuable bioproducts (Balina et al. 2017). Brown algae contain alginates and fucoidans and laminarin, while red algae contain agar, carrageenans, xylans, and mannans. Xylans, sulfated galactans, and ulvanes are present in green algae. Hydrogen produced by macroalgae is attractive for renewable energy due to their rapid growth (Luning and Pang 2003). The most cultivated are *Undaria pinnatifida*, *Laminaria japonica*, *Gracilaria*, *Eucheuma*, *Porphyra* and *Kappaphycus*, *Enteromorpha*, *Monostroma*, *Laminaria japonica*, *Porphyra*, *Eucheum*, and *Enteromorpha*. Gendy and El-Temtamy (2013) suggested that microalgae-based energy fuels are eco-friendly and nontoxic. Kraan (2010) observed that some macroalgae gather a high quantity of carbohydrates for the assembly of biofuels. Park et al. (2011) have reported that *Gelidium amansii* can produce biohydrogen by anaerobic fermentation. Prospects of hydrogen production by algae were reviewed by Prince and Kheshgi (2005). Anabaena was pretreated with the enzyme to generate biohydrogen (Nayak et al. 2014). Sparging the cultures of *Mastigocladus laminosus* with gases was used for biohydrogen production (Miyamoto et al. 1979). Aerobic and anaerobic phases, light intensity, and mixing speed of *Chlamydomonas reinhardtii* were investigated by simulation of environmental conditions by Oncel et al. (2015b). Various algae used for hydrogen production are tabulated (Table 7.1).

7.5 Mechanism of Hydrogen Production by Algae

Hydrogen can be produced by the following methods: dissociation of water in the presence of sunlight into hydrogen and oxygen which is termed direct photolysis (Johnston et al. 2005).

$H_2O \rightarrow H_2 + ½O_2$.

Microalgae can carry out photosynthesis in the presence of light (Ghirardi et al. 2000). Indirect photolysis splits water molecules in sunlight forming oxygen

Table 7.1 Algae used for hydrogen production

Name of the algae	References
Mastigocladus laminosus	Miyamoto et al. (1979)
C. reinhardtii	Winkler et al. (2002)
Chlamydomonas reinhardtii	Melis et al. (2000)
Scenedesmus oblique	Winkler et al. (2002)
Platymonas subcordiformis	Guan et al. (2004)
Chlamydomonas reinhardtii	Fedorov et al. (2005a)
Chlamydomonas reinhardtii	Tsygankov et al. (2006a)
Chlamydomonas reinhardtii	Jo et al. (2006)
Anabaena variabilis	Liu et al. (2006)
Chlamydomonas reinhardtii	Laurinavichene et al. (2006)
Chlorella sorokiniana	Chader et al. (2009)
Chlorella sp.	
Chlamydomonas reinhardtii	Torzillo et al. (2009)
Chlorella salina	Chader et al. (2009)
Chlamydomonas moewusii	Greenwell (2010)
Chlamydomonas reinhardtii	Faraloni et al. (2011)
Chlorella sp.	He et al. (2012)
Chlorella pyrenoidosa	Voloshin et al. (2016)
Scenedesmus obliquus	He et al. (2012)
Nannochloropsis sp.	
Chlorella protothecoides	
Gelidium amansii	Park et al. (2011)
Laminaria japonica	Shi et al. (2011)
Stigeoclonium sp. AARL G030	Duangjan et al. (2017)
Actinastrum gracillimum AARL G033	
Dictyosphaerium cf. *ehrenbergianum* AARL G004	
Micractinium sp. AARL G009	
Chlorella sp. AARL G014	
Acutodesmus acuminatus AARL G092	
Coelastrum indicum AARL G043	
Coelastrum microporum AARL G007	
Desmodesmus armatus var. *bicaudatus* AARL G019	
Desmodesmus armatus AARL G083	
Desmodesmus communis AARL G072	
Desmodesmus denticulatus AARL G024	
Desmodesmus hystrix AARL G080	
Desmodesmus maximus AARL G026	
Desmodesmus opoliensis AARL G089	
Desmodesmus perforatus AARL G027	
Dimorphococcus lunatus AARL G048	
Monoraphidium cf. *obtusum* AARL G016	
Pectinodesmus pectinatus AARL G097	
Pediastrum boryanum AARL G062	

(continued)

Table 7.1 (continued)

Name of the algae	References
Pediastrum duplex var. *duplex* AARL G060	
Pediastrum tetras AARL G063	
Scenedesmus acunae AARL G087	
Scenedesmus obtusus AARL G020	
Selenastrum bibraianum AARL G052	
Verrucodesmus verrucosus AARL G079	
Ulothrix cf. *tenerrima* AARL G029	
Chlamydomonas sp. AARL G031	
Pandorina morum AARL G010	
Closterium ehrenbergii AARL G056	
Closterium moniliferum AARL G041	
Cosmarium lundellii AARL G053	
Euastrum denticulatum AARL G001	
Gonatozygon aculeatum AARL G047	
Staurastrum muticum AARL G116	
Staurastrum tetracerum AARL G011	
Staurodesmus cuspidatus AARL G059	

followed by carbon dioxide fixation and production of hydrogen gas by means of hydrogenase enzyme.

$12H_2O + 6CO_2 + \text{lightenergy} \rightarrow C_6H_{12}O_6 + 6O_2C_6H_{12}O_6 + 12H_2O + \text{lightenergy} \rightarrow 12H_2 + 6CO_2$.

Blue-green algae have significant advantages as hydrogen generation and oxygen evolution are separated (Das and Veziroglu 2008). In dark fermentation, hydrogen production takes place by fermentative microorganisms in a dark environment. Purple nonsulfur bacteria (PNS) produce hydrogen by photofermentation (Das and Veziroglu 2008; Merugu et al. 2010; Kadari et al. 2018).

$CH_3COOH + 2H_2O + \text{light} \rightarrow 4H_2 + 2CO_2$.

The reactions of hydrogen production from water are given below (Das and Veziroglu 2008). In indirect biophotolysis, the inactivation of [FeFe]-hydrogenases by oxygen is eliminated by separating oxygen and while hydrogen is evolved (Manish and Banerjee 2008). In the first stage, microalgae are allowed to fix carbon dioxide into carbohydrate, while in the second stage, called as anaerobic dark fermentation, carbohydrate is converted by nitrogenase enzyme to produce hydrogen (Pilon et al. 2011).

$6H_2O + 6CO2\ C_6H_{12}O_6 + 6O_2$.

$C_6H_{12}O_6 + 6H_2O\ 6CO_2 + 12H_2$.

Under sulfur deprivation and exposure to light, hydrogen production was observed for a period of several days (Fedorov et al. 2005b; Kim et al. 2010). Vignais (2008) has reported that hydrogen production in algae is catalyzed by oxygen-sensitive FeFe hydrogenases that are under the control of the gene HYDA

(Böck et al. 2006; Posewitz et al. 2004). Inactivation of algal Fe-Fe hydrogenase takes place when exposed to oxygen (Erbes et al. 1979; Stripp and Happe 2009), represents a major challenge, and needs to be overcome for efficient hydrogen production. Stripp and Happe (2009) reported that carbon monoxide leads to reversible inactivation of algal FeFe hydrogenase by binding to the H-cluster of 2Fe domain and may have a protective effect on the hydrogenase. The two hydrogenases involved in hydrogen production are coded by *HYDA1* and *HYDA2* genes (Forestier et al. 2003). With increasing light intensity, chlorophyll concentration increases resulting in more number of electrons being generated. These electrons combine with protons to form hydrogen (Rashid et al. 2013). One of the limitations of direct biophotolysis is the duration of hydrogen evolution which it is only up to few minutes. This is due to the simultaneous production of hydrogen and oxygen molecules (Melis 2007).

The role of periplasmic hydrogenases within the sulfate-reducing microorganism *Desulfovibrio vulgaris* was studied by Caffrey et al. (2007). Ghirardi et al. (2000) opined that microalgae would be a promising supply of biohydrogen. Dasgupta et al. (2010) have described the recent trends in hydrogen production by photobiological processes and photobioreactors. Hydrogenase was found to be inactivated in extracellular extracts of *Chlamydomonas reinhardtii* by oxygen (Erbes et al. 1979). continuous production of hydrogen by *Chlamydomonas reinhardtii* was observed by Fedorov et al. (2005a). Flynn et al. (2002) have generated oxygen-tolerant phenotypes by mutations in hydrogen-producing *Chlamydomonas reinhardtii*. The structure of NiFe and FeFe hydrogenases was described by Fontecilla-Camps et al. (2007). Under anaerobic conditions, two [Fe]-hydrogenases were expressed in *Chlamydomonas reinhardtii* by Forestier et al. (2003). [FeFe] hydrogenase evolution from a genomic perspective was reviewed by Meyer (2007). Hydrogenase (Cpl) from *Eubacterium pasteurianum* was crystallized by Peters et al. (1998). A completely unique FeS cluster in Fe-only hydrogenases was discovered by Nicolet et al. (2000). The events taking place during algal hydrogen production are shown in Fig. 7.3

7.6 Factors Affecting the Production of Hydrogen by Algae

Higher yields of hydrogen were observed with immobilized anaerobic microflora by Zhang et al. (2008). Hannon (2010) suggested that much more improvement is required for algal fuel technology for making the process viable. Duangjan et al. (2017) have compared the hydrogen generation capabilities of microalgae under auxotrophic and mixotrophic cultural conditions. Microalgae isolated from wastewater of fisheries under different light intensities and atmospheric gas conditions was investigated by Pholc han et al. (2017). Maswanna et al. (2020) have used green alga Tetraspora sp. CU2551 for successful hydrogen generation using algination immobilization by limiting sulfur. Genetically engineered microalgae were grown at a larger scale for assessing risks for the environment (Beacham et al. 2017). The

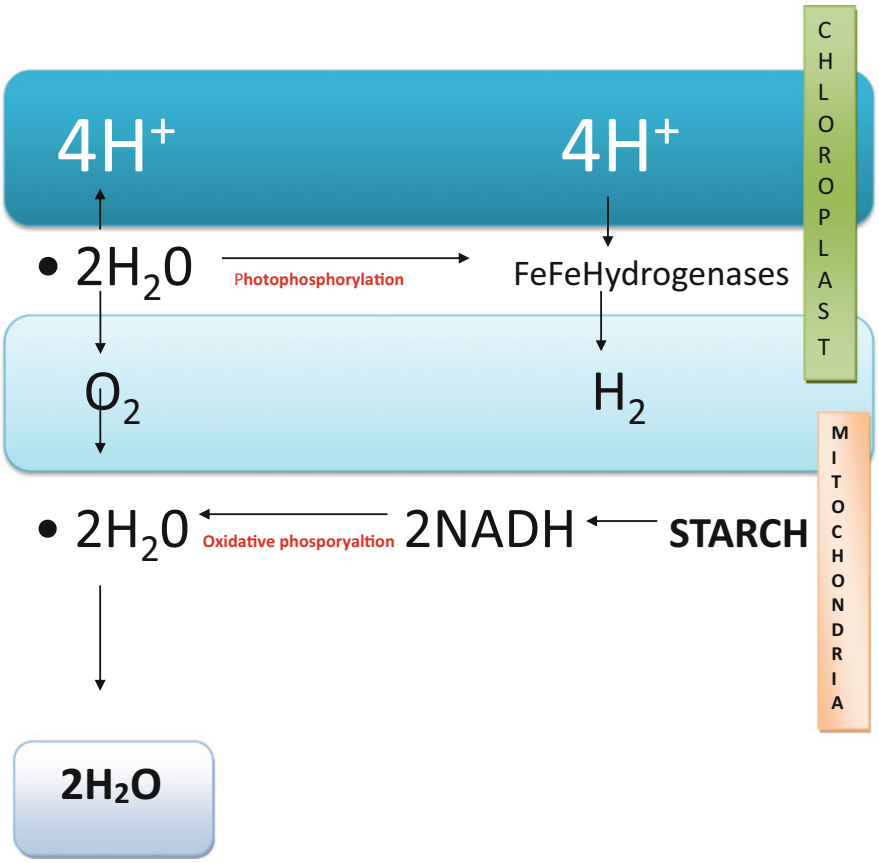

Fig. 7.3 Events during algal hydrogen production

factors affecting hydrogen production are given in Table 7.2. Different kinds of immobilization are shown in Table 7.3.

Milledge et al. (2018b) reported that phenolic compounds are responsible for inhibition of anaerobic digestion. Large energy inputs are required for disrupting algal biomass to be developed (Prajapati et al. 2016). The most important factor which influences the production process would be that of harvesting algal biomass (Pienkos and Darzins 2009). The use of chemical flocculants can make the harvesting process economical compared to process like centrifugation (Pienkos and Darzins 2009). Some bacteria associated with diatoms lead to their aggregation, e.g., *Thalassiosira weissflogii* (Gärdes et al. 2011). Bacterium HW001 isolated from Permian groundwater was found to aggregate *Nannochloropsis oceanica* IMET1 (Wang et al. 2014). Bioflocculants from the bacterial culture supernatants (Manheim and Nelson 2013; Ndikubwimana et al. 2016) were found to be useful in of algal cell aggregation. Many other filamentous fungi have also been reported to be efficient bioflocculants (Wrede et al. 2014). *Aspergillus fumigatus* was found to be a good

Table 7.2 Factors affecting the production of hydrogen

Name of the factor	References
pH	Goldman et al. (1982), Khanal et al. (2004), Khanal et al. (2004), Lee et al. (2007), Kosourov et al. (2007), Antal et al. (2003), Song et al. (2011), Lam and Lee (2012), Juneja et al. (2013), Banu et al. (2018a), Yang and Wang (2018), Kumar et al. (2016), Daliry et al. (2017), Kumar et al. (2019)
Nutrients	Lin and Lay (2004), Lo et al. (2008), Sharma and Arya (2017), Juneja et al. (2013), Devi and Mohan (2012), Kong et al. (2010), Nguyen et al. (2010), Bai et al. (2015a), Hernandez et al. (2009), Kumar et al. (2018), Onwudili et al. (2020), Zhu et al. (2013a)
Temperature	Schroda (2004), Salvucci and Crafts-Brandner (2004), Singh and Singh (2015), Ras et al. (2011), Bechet et al. (2017), Morgan-Sagastume and Noyola (2006)
Light intensity	Nishiyama et al. (2006), Krzemińska et al. (2014), Tsygankov et al. (2006b), Phlips and Mitsui (1983), Rashid et al. (2013), Phlips and Mitsui (1983), Uyar et al. (2007), Kim et al. (2006), Huesemann et al. (2013)
Pretreatment	Yin and Wang (2019), Yang and Wang (2018), Zhang et al. (2020), Muñoz-Páez et al. (2020), Nagarajan et al. (2020), Fonseca et al. (2020), Chang et al. (2020), Margareta et al. (2020), Banu et al. (2018b), Kumar et al. (2019), Radha and Murugesan (2017)
Substrate concentration	Kim et al. (2006), Wei et al. (2011), Chen et al. 2020, Show et al. (2012), Bala Amutha and Murugesan (2011), Antal et al. (2020), Fakhimi et al. (2020), Kannah et al. (2019)
Salt concentration	Orosa et al. (2001), Oren et al. (2008), Hadi et al. (2008)

fungal bioinocculant (Muradov et al.) Khan and Fu (2020) have suggested that algae are important for energy security and recommended biotechnological approaches for improved production of biofuel precursors such as fatty acids and engineering of hydrogenases for biofuel generation.

7.6.1 Nutrients

Nutrient limitation is commonly the limiting issue for growth as new adaptation ways are adapted by algae. Polyose as a carbon supply for gas production was investigated by Lo et al. (2008) and Lo et al. (2009). Under nutrient limitation, photosynthetic activity is diminished causing photodamage to PSII. Lin and Lay (2004) observed that iron plays a very important role in protein activity for hydrogen evolution. Sharma and Arya (2017) have suggested that nutrients play a significant role for the commercial production of microalgae biomass. Juneja et al. (2013) showed that the basic requirements of various microalgae are carbon dioxide, phosphorus, and carbon. Devi and Mohan (2012) discovered that the nutrients affect the buildup of carbohydrates and lipids in microalgae. Nutrient deprivation causes photoinhibition in algae. Kong et al. (2010) suggested cultivating *C. reinhardtii* in industrial and household wastewater. However, due to lower concentration of

Table 7.3 Immobilization methods for algal immobilization

Name of the organism	Matrix	References
Chlamydomonas reinhardtii	Calcium alginate beads	Hahn et al. (2007)
Chlamydomonas reinhardtii	Alginate films	Antal et al. (2016), Kosourov and Seibert (2009), Kosourov et al. (2012)
Nannochloropsis sp.	Alginate beads	Cheirsilp et al. (2017)
Phaeodactylum tricornutum	Alginate	Moreira et al. (2006)
Chlorella vulgaris	Sodium alginate and sodium carboxymethylcellulose	Rushan et al. (2019)
Chlorella vulgaris	Sodium alginate and gelatin	Rushan et al. (2020)
Chlamydomonas reinhardtii	TEMPO-oxidized cellulose nanofibrils (TEMPO CNF)	Jamsa et al. (2018)
Chlorella vulgaris UTEX 1803	Polyurethane	Gallegos-Suárez et al. (2016)
Chlorella vulgaris	Magnetic nanoparticles	Taghizadeh et al. (2020)
Scenedesmus obliquus	Calcium-alginate gel	Guoan et al. (1995)

nitrogen and phosphorus in wastewater, algal biomass production was less. Hence, concentrated wastewater was used which increased the biomass yield. Nguyen et al. (2010) studied starch accumulation in *C. reinhardtii* cells and found that it was utilized by a hyperthermophilic bacterium, *Thermotoga neapolitana*, as substrate for hydrogen evolution. *Ulva lactuca* is used for biogas production where the ratio of C/N is between 20 and 30. The carbon limitation can be overcome when heterotrophic bacteria are cultivated with algal species, e.g., *Chlorella* sp. (Bai et al. 2015b). *Bacillus pumilus* ES4 was reported to fix nitrogen resulting in enhancement of algal cultivation of *Chlorella vulgaris* (Hernandez et al. 2009). The requirement of phosphorus is essential for the growth of the algae. Algae take up inorganic phosphorus coming from organic phosphorus in which bacteria play a major role by the use of phosphatases (Zhu et al. 2013b). Li et al. (2020) reported hydrogen production using *Chlorella pyrenoidosa* strain IOAC707S under nitrogen limitation. When the cultures were nitrogen limited, photosystem II photochemical activity efficiency and oxygen production decreased. The transcriptome showed that under nitrogen limitation induction of hydrogenase enzyme took place and the metabolism shifted toward hydrogen evolution analysis. Onwudili et al. (2020) have conducted studies on three algae *Chlorella vulgaris*, *Spirulina platensis*, and *Saccharina latissima* for hydrogen generation. They were processed under supercritical water gasification in a batch reactor. The generation of hydrogen was found to be twice the amount of hydrogen produced in the presence of sodium hydroxide. Among the

Table 7.4 Hydrogen production under sulfur limitation

Name of the organism	References
Chlorella autotrophica	He et al. (2012)
Chlorella protothecoides	He et al. (2012), Pongpadung et al. (2015), Pongpadung et al. (2018)
Chlorella Salina	Chader et al. (2009)
Chlamydomonas reinhardtii	Melis et al. (2000), Zhang et al. (2002b), Laurinavichene et al. (2006), Fedorov et al. (2005b), Tsygankov et al. (2006c), Kosourov et al. (2007), Faraloni et al. (2011), Torzillo et al. (2009)
Chlorella sorokiniana	Chader et al. (2009)
Nannochloropsis	He et al. (2012)
Platymonas subcordiformis	Guan et al. (2004)
Tetraselmis striata	He et al. (2012)
Tetraspora	Maswanna et al. (2020)

three, *Saccharina* produced the more hydrogen gas. Secondly, the study pointed out that the nutrients of the "process waters" from *Saccharina* could be useful for cultivation of microalgae. Kumar et al. (2018) pretreated *Ulva reticulata* with the surfactant and disperser. Azman et al. (2016) investigated hydrogen production from deoiled rice bran feedstock. Bharathiraja et al. (2016) reviewed the feedstocks for biohydrogen and biogas production. Hydrogen production under sulfur deprivation is shown in Table 7.4.

7.6.2 pH, Temperature, and Pretreatment

All algae maintain a neutral intracellular pH, but some algae can survive at high or low pH. At lower pH, 50% of the ATP has been observed to be consumed. Most microalgal species grow well at a pH range between 6.0 and 8.76. *C. vulgaris* can survive a range of pH, while other algae are sensitive (Lam and Lee 2012). Daliry et al. (2017) reported maximum biomass production at pH 9–10 in *C. vulgaris*. Juneja et al. (2013) ascertained that increasing the hydrogen ion concentration can increase the salinity of the growth media and raise the destructive measures for algal cells. Khanal et al. (2004) studied the effect of pH on hydrogen production process and reported that any small change in pH can affect the production of acetate. In this process, Song et al. (2011) observed that initially pH decreases and after 24–72 h pH increases. They reported that microalgae grow at a pH range between 5.0 and 9.0. Increasing the culture pH can increase the rate of hydrogen production (Khanal et al. 2004). At lower pH, it is produced by hydrogenase. This occurs through a pathway

called pyruvate–ferredoxin–oxidoreductase pathway which suppresses Fe-hydrogenase activity (Gong et al. 2008; Kumar and Das 2000; Lee et al. 2002). Lee et al. (2007) explained that pH affects the activity of hydrogenase and nitrogenase. He observed that at a pH of 5.0, the enzyme gets inactivated. Kosourov et al. (2007) reported higher rates of hydrogen gas at pH 7.7 which decreased at pH 6.5 during cultivation of sulfur-deprived cultures of the green algae *Chlamydomonas reinhardtii*. Goldman et al. (1982) reported that marine algae requirements for pH are different from those of fresh algae and the levels of nitrate are major factors affecting pH. Antal et al. (2003) observed the optimal pH of 6.0–7.5 was required for hydrogen generation in *Gloeocapsa alpicola*. Biosurfactant and microwave were used for sludge treatment at basic pH by Banu et al. (2018b). Kumar et al. (2019) used microwave for pretreatment of biofuel production. Ionizing radiation under acidic conditions was used as a pretreatment method for enhancing biohydrogen (Yang and Wang 2018). Temperature is a key factor that is responsible for the growth of microalgae, as it influences rate of photosynthesis. Bechet et al. (2017) reported that optimum temperature enhances algal growth, but beyond this algal growth is retarded. Temperature causes a downturn in the action of ribulose-1,5-bisphosphate (Rubisco) affecting photosynthesis, and its activity increases with a rise in temperature up to an acceptable level and then reduces (Salvucci and Crafts-Brandner 2004). At lower temperatures, algal enzymatic reactions become slower causing oxidative stress. Algae may produce more of a given enzyme, or optimal enzymatic activity may be shifted toward lower temperature to compensate for slower enzymatic reactions (Morgan-Kiss et al. 2006). Singh and Singh (2015) observed that the favorable temperature range required for the growth of most algal species is 20–30 °C. The importance of temperature in growth and survival of algal systems was described by Ras et al. (2011). Banu et al. (2018a) have investigated the use of microwave for disintegration of waste. Ultrasonics and microwave were combinedly also used for biomass disintegration (Kavitha et al. 2018). Temperature range of 20–32 °C was found to be optimum for the growth of *Chlamydomonas reinhardtii* (Schroda 2004). Ibrahim et al. (2020) have conducted hydrothermal liquefaction (HTL) experiments on microalgae *Galdieria sulphuraria* in a membrane reactor using $Pd_{77}Ag_{23}$ hydrogen-selective membrane. This membrane was reported to recover hydrogen and aid in the conversion of biochar to fuels. Zhang et al. (2020) have studied the alkaline and thermal treatment of brown seaweed for production of pure biohydrogen with reduced carbon dioxide formation. They have observed that Ni/ZrO_2 catalyst improved the secondary hydrogen generation through steam methane reforming and water-gas shift reactions. Muñoz-Páez et al. (2020) have studied acid agave bagasse hydrolyzates as substrate for hydrogen production. The effect of increasing concentrations of acid hydrolyzates from Agave on hydrogen production and stability of granular biomass in an expanded granular sludge bed (EGSB) reactor and suspended biomass in an anaerobic sequencing batch reactor (AnSBR) fed with acid hydrolyzates were investigated. The hydrogen production from acid agave hydrolyzates was higher for EGSB reactor than for the AnSBR, but was less stable. Rebello et al. (2020) have performed life cycle analysis of various pretreatment strategies used in anaerobic digestion process for biofuel

production. They concluded that these fuels are better than petro-based fuels and have less environmental implications. Nagarajan et al. (2020) have reviewed the different kinds of pretreatment methods for algal hydrogen generation. They have suggested that carbohydrates present in algae are effective carbon source for dark fermentation. Further, they stated that mechanical methods for hydrogen generation have high extraction efficiency but are energy-intensive; while chemical methods are less energy intensive, they generate chemical compounds which inhibit fermentation (Nagarajan et al. 2020). Fonseca et al. (2020) optimized the pretreatment process for *Kappaphycus alvarezii* biomass which was used by the *Clostridium beijerinckii* Br21 to produce hydrogen and hence suggested that macroalgae biomass can be used as feedstock for hydrogen production. Chang et al. (2020) studied the effect of microwave power on hydrogen production using microalgae which was pyrolyzed. With the increase in microwave power, the yield increased twice. The potential of using macroalgae *Ulva* sp. was reported by Margareta et al. (2020). In that study, macroalgal biomass was used as feedstock for enhancing hydrogen production through dark fermentation. Biohydrogen generation rate of 812 mL/L/h was seen. The green macroalgal biomass *Ulva* sp. was subjected to mild acid-thermal combined pretreatment for the effective release of fermentable sugars for biohydrogen production. The impact of pH and BESA addition on gas production by mixed microalgae biomass was investigated by Kumar et al. (2016). Dadak et al. (2016) had done the eco-exergy analysis for gas production. Yin and Wang (2019) suggested pretreatment for bioenergy recovery from algae. They studied various pretreatment methods and concluded that all the pretreatment methods could enhance the hydrogen production, while the combined pretreatment showed significant enhancement. Hydrogen yield of 17.5 mL/g TS_{added} was seen in heat-base pretreatment and heat-acid pretreatment with the highest total energy conversion efficiency of 35.4% (Yin and Wang 2019). Kosourov et al. (2011) immobilized a tla1 mutant (CC-4169) in thin alginate films and cultured it under sulfate-limited and low-intensity light conditions. They found that hydrogen production rates were significantly reduced. Radha and Murugesan (2017) increased the biohydrogen production by using different pretreatment processes in the marine macroalgae *Padina tetrastromatica*.

7.6.3 Substrate and Salt Concentration

Kim et al. (2006) observed the consequences of substrate concentration on production of hydrogen. Although some algae have adapted to tolerate a very high salt concentration, for example, the halophilic *Dunaliella salina* (Oren et al. 2008), most algae thrive in either freshwater, brackish water, or marine environments. When salinities increase above optimum for growth, algae may suffer from hyperosmotic stress, leading to impaired electron transfer between antenna pigments, and in PSII and PSI reaction centers, again leading to photoinhibition and oxidative stress. Among several different adaptive responses to salinity, stress is the production of osmolytes or production of secondary carotenoids (Hadi et al. 2008). In few cases,

algae exposed to high salinities produce high amounts of carotenoids for protection (Orosa et al. 2001). Wei et al. (2011) used different monosaccharides for hydrogen generation. Glucose, fructose, galactose, and sucrose substrates at a concentration of 200 mg/L were used by Chen et al. (2020) in *Anabaena* sp. strain to investigate the biogas production. *Chlamydomonas reinhardtii* could produce hydrogen at a rate of 1.7 mol/mol with acetate as carbon source (Show et al. 2011). Bala Amutha and Murugesan (2011) have observed that *Chlorella vulgaris* MSU 01 strain isolated from a pond produced hydrogen. They optimized the media with different carbohydrates and amino acids. Feedstock of corn stalk was studied for growing algae and generation of hydrogen. Antal et al. (2020) have studied the role of hydrogen generation under sulfur limitation in the acclimation of *Chlamydomonas reinhardtii* CC-425 cells and compared it with hydEF-1 mutant lacking hydrogenase activity. They suggested that under sulfur deprivation, the active hydrogenase plays a key role in the algae acclimation to anaerobic phase by modulating intracellular redox and maintaining pH. Fakhimi et al. (2020) reported synergistic hydrogen production using co-cultures of bacteria and algae. They have observed an enhancement of about 60% hydrogen when photobiological and fermentative production was combined in *Chlamydomonas* and *Escherichia coli* co-cultures using glucose substrate. Kannah et al. (2019) pretreated rice straw and used modeling approaches for hydrogen production.

7.6.4 Light Intensity

When the light intensity is high, the overexcitation of the chemistry equipment takes place resulting in the formation of reactive chemical element species (ROS). High strength causes injury to PSII by inhibiting the synthesis of the D1 supermolecule of the PSII reaction center (Nishiyama et al. 2006). Krzemińska et al. (2014) investigated the influence of sunlight on the expansion rate and algal biomass formation. Modeling of the outdoor or indoor algal culture system light intensity plays a significant role (Huesemann et al. 2013) in optimizing hydrogen production. Alabi et al. (2009) found that at different light intensities, microalgae cannot grow to their full potential. Enhanced hydrogen production after 4 h light exposure in anaerobic condition was seen by Tsygankov et al. (2006c). The role of light in hydrogen production is still being debated (Uyar et al. 2007). Kim et al. (2006) found that hydrogen production depends on the rate of consumption of sulfate by algae. They observed that the consumption of sulfur was maximum at a light intensity of 200 mmol/m^2/s. The effects of light on microalgae biohydrogen needs to be explored at various stages of the process (Rashid et al. 2013). Phlips and Mitsui (1983) have studied the effects of environmental factors in *Oscillatoria* sp. strain Miami BG7 and reported that biohydrogen production is greatly influenced by light intensity. Nitrogen depletion was essential for the initiation of hydrogen production which was observed at the linear phase of growth. Phlips and Mitsui (1983) observed that the

rate of production was low at lower light intensities and highest temperature limit for hydrogen production was about 46 °C.

7.7 Bioreactors for Algal Hydrogen Production

Algal bioreactors are used in cultivating both micro- and macroalgae for biohydrogen production. Algae bioreactors are of two types, namely, open reactors and enclosed reactors. Enclosed reactors are called photobioreactors. The bioreactors are based on the photosynthetic reaction of algal cultures using carbon dioxide and sunlight energy. Tubular photobioreactors are fully closed and can be used for large scale (Molina et al. 2001). They are made of plastic or glass (Miron et al. 1999). In flat plate photobioreactors, compactness is the major advantage (Posten 2009). The U-turns use small amount of space, and the thickness of the wall is thin when compared to other kinds of bioreactors (Pulz and Scheibenbogen 1998). The reactor panels are illuminated mainly on one side by direct sunlight so that maximum absorption of the light energy takes place (Janssen et al. 2003). The airlift and bubble-column bioreactors are also used for cultivating algal cultures. They are used for biofuel production, wastewater treatment, and industry. They are economical and compact and can be easily handled (Miron et al. 2002). Anto et al. (2020) too have discussed different configurations of reactors which are used to decrease risk of contamination and methods of increasing biomass of the algae. They have highlighted the importance of temperature, size, innoculum nutrient concentration, light intensity, CO_2, and mixing for hydrogen generation.

Various types of bioreactors used for algal hydrogen production are shown in Table 7.5.

Hydrogen productions from selected species of chlorophyta under sulfur deprivation in bioreactors were investigated by Posten (2009). Many factors are necessary for designing bioreactors with optimum properties such as light intensity penetration, agitation, and gas exchange. Another limitation of bioreactors is that the severe inhibiting impacts of some materials like rubber and latex materials apart from metals could inhibit the growth of the organisms (Jin et al. 1996; Singh and Rai 1991; Williams and Robertson 1989). The major disadvantages are that the scaling

Table 7.5 Bioreactors used for algal hydrogen production

Type of bioreactor	Reference
Photobioreactors	Doenitz et al. (1988), Evens et al. (2000)
Continuous stirred tank reactor (CSTR)	Luo et al. (2011), Kosourov and Seibert (2009), Younesi et al. (2008), Ding et al. (2010)
Fixed-bed bioreactor	Fang and Liu (2002)
Membrane bioreactor	Ntaikou and Lyberatos (2010)
Multi-stage bioreactors	Kosourov et al. (2012)
Hybrid bioreactors	Show et al. (2011)

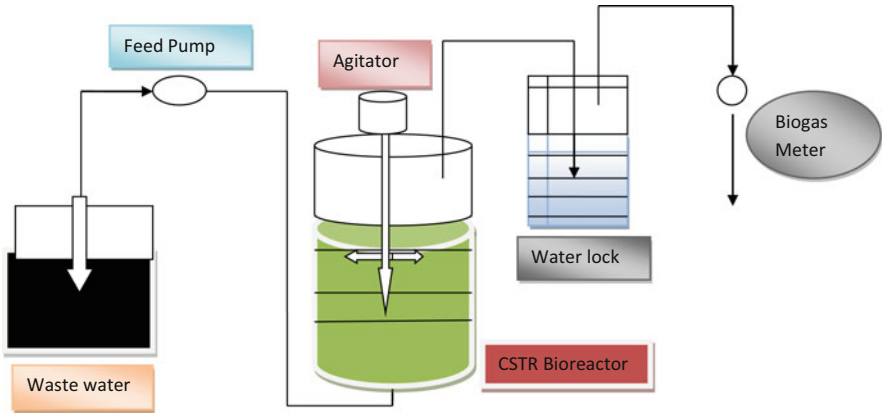

Fig. 7.4 A CSTR bioreactor for hydrogen generation

up of the process is very expensive (Sierra et al. 2008). Zijffers et al. (2008) developed Green Solar Collector (GSC) for microalgal production and were found to capture light efficiency by 57%. However, the main limitation of this is that the fabrication of these kinds of bioreactors is expensive. The optical fibers used in this bioreactor can be sterilized. Apart from this, they are also stable to agitation which is a major advantage. Constant productivity (Gordon 2002) and continuous illumination allow for scaling up of the process. Parmentier et al. (2020) studied a new electrocoagulation-flotation set-up with a tubular coaxial reactor using *Chlorella vulgaris*. They reported only lower energy consumption rates compared to other kinds of similar reactors. This kind of setup can be used for harvesting algae at larger scales. Figures 7.4, 7.5, and 7.6 show the construction of a continuous stirred tank bioreactor, membrane bioreactor, and fixed bed reactor used for hydrogen production.

7.8 Current Status of Algal Hydrogen Production.

The challenges and potential of biofuel generation from algae have been reviewed by Hannon (Hannon et al. 2010). The advantageous option of algal biohydrogen is that they are renewable and reduce greenhouse emissions. This is the most important aspect when it comes to biohydrogen produced by algae. Compared to petroleum based fuels algal based fuels generate lesser sulfur emissions. Algae use carbon dioxide and fix over four hundredth of the world's carbon (Falkowski et al. 1998). In this way, large amounts of carbon dioxide are being sequestered by the marine algae. Algae will double within 6 h, and biomasses can be easily generated due to the shorter generation period which is most advantageous when compared with bacterial biomass (Sheehan et al. 1998). Macroalgal hydrogen production is not economical with the prevailing technology unless it is coupled with bioremediation or

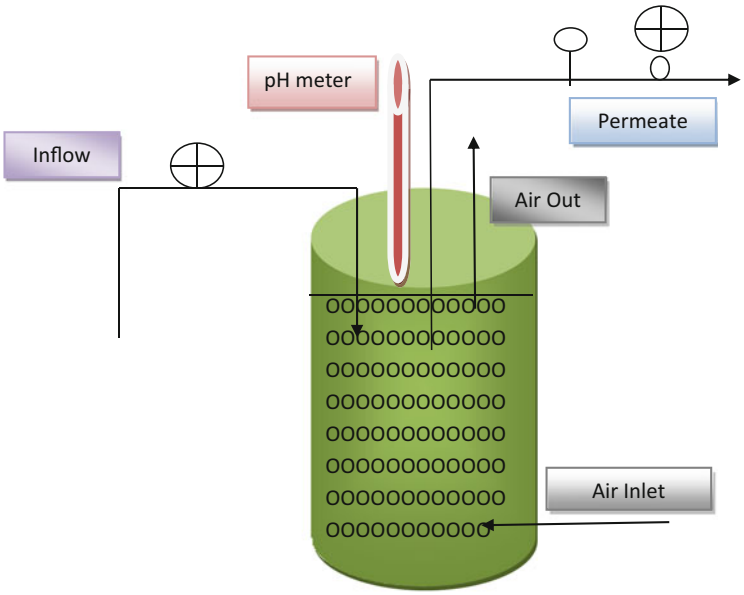

Fig. 7.5 Membrane photobioreactor (lab scale)

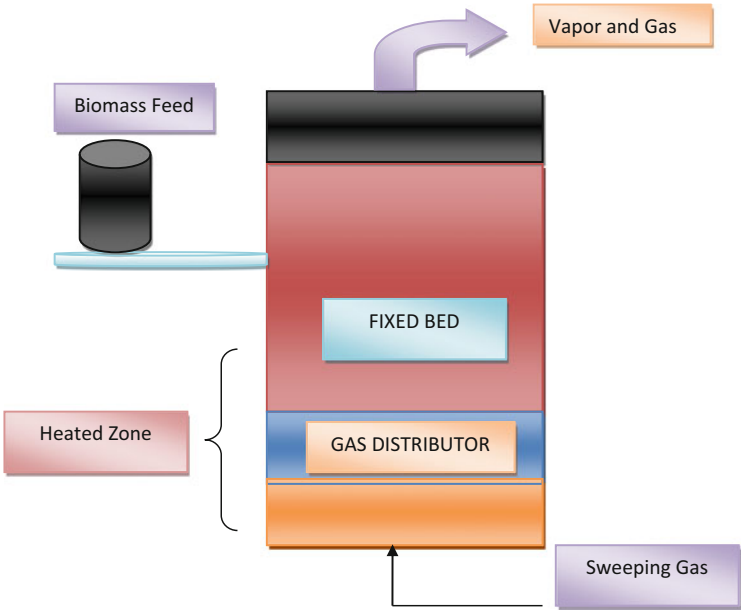

Fig. 7.6 Fixed bed reactor for hydrogen production

production of different useful byproducts (Savage 2011). Hence, the algal hydrogen production should always be coupled with the production of other useful bioproducts which the algae produce. These can minimize the expenses incurred during the scale up of the process of hydrogen production. The only way forward for enhancing algal biological hydrogen production is with advances in recombinant DNA technology and improvements in the design of the bioreactor (FAO 1997). Recombinant DNA methods should be effectively used for generation of transgenic algae which will be able to withstand abiotic and biotic stress. Designs in the bioreactor should be improvised such as using solar cells to entrap light efficiently and minimizing nutrient loss so as to generate higher rates of hydrogen. Techno-economic analysis of cultivating algae for generation of hydrogen is important as it gives an indication about the feasibility of the production process. Selections of efficient strains which are reengineered for hydrogen production are presently needed. Jmel et al. (2018) studied different pretreatment techniques on biomass of *Ulva lactuca*. Chiaramonti et al. (2017) studied the effect of pyrolysis on microalgae for biofuel generation. Liao et al. (2018) studied the various kinds of bioreactors which could be used for generation of bioenergy from microorganism. Banu et al. (2019) studied the effect of homogenization and sodium tripolyphophate on sea eelgrass for enhancing biofuel production. Ran et al. (2008) have observed an enhancement in the quantity of biohydrogen in *Platymonas subcordiformis* when carbonyl cyanide m-chlorophenylhydrazone (CCCP) was added. However, the study also opined that the process may not be sustainable as there would be an interruption of the proton gradient in thylakoid membranes. Pankratz et al. (2020) have evaluated the sustainability of microalgae production in cold climates in two kinds of cultivation systems, namely, open pond raceway (OPR) and photobioreactor (PBR) cultivation. They suggested that coupling cultivation with supercritical water gasification is advantageous when compared to the same over pyrolysis of diluent for reduced emission of greenhouse gases during hydrogen generation. Downregulation of the light-harvesting proteins will lead to more hydrogen production in the bioreactor (Oey et al. 2013). The second method of enhancing hydrogen production would be to lock the electron transport chain (Laurinavichene et al. 2004). Rezvani et al. (2020) have studied bioremediation potential of *Chlorella vulgaris*, *Ettlia* sp., and *Chlamydomonas reinhardtii* for nitrate removal along with hydrogen production. The observed removal rate of nitrate is 86 mg/L/d. Phosphorus removal was less efficient. Kolbe et al. (2020) suggested using algal generated hydrogen for running cars. The size, shape, and feasibility for decentralized hydrogen production were studied for overcoming the limitations being faced by hydrogen-powered cars in the market.

Bacteria and fungi associated with algae are tabulated in Table 7.6.

Asadi et al. (2017) have done investigations on the feasibility of biofuel generation, its properties, and life cycle and conducted techno-economic analysis for boosting the biological hydrogen production. The choice of the algal strains chosen, selection of the site, and cultural conditions have to be optimized. It is best to

Table 7.6 Bacteria and fungi associated with algae

Name of the algae	Microorganisms associated with algae	References
Chlorella sorokiniana IAM C-212	*Microbacterium trichotecenolyticum*	Watanabe et al. (2006)
Chlorella ellipsoidea	*Brevundimonas* sp.	Park et al. (2008)
Thalassiosira rotula	*Roseobacter* sp. and *Hyphomonas* sp.	Grossart and Simon (2007)
Chlorella vulgaris	*Bacillus pumilus*	Hernandez et al. (2009)
Scrippsiella trochoidea	*Marinobacter* sp. strain DG879	Amin et al. (2009)
Phaeodactylum tricornutum Utex 646	*Alphaproteobacteria* sp. strain 29	Bruckner et al. (2011)
Chlorella vulgaris	Tap water bacteria	Lakaniemi et al. (2012)
Dunaliella sp. SAG 19.3	*Alteromonas* sp. and *Muricauda* sp.	Le Chevanton et al. (2013)
Thraustochytrid sp.	*Aspergillus fumigatus*	Wrede et al. (2014)
Lobomonas rostrata	*Mesorhizobium loti*	Grant et al. (2014)
Chlorella vulgaris	*Rhizobium* sp.	Kim et al. (2014)
Chlorella vulgaris	*Flavobacterium* sp., *Rhizobium* sp., *Hyphomonas* sp.	Cho et al. (2015)
Botryococcus braunii	BOTRYCO-2	Tanabe et al. (2015)
Chroococcus sp.	*Aspergillus lentulus*FJ172995	Prajapati et al. (2016)
Chlorella vulgaris	*Pleurotus geesteranus*	Zhou et al. (2018)
Chlorella vulgaris	*Ganoderma lucidum*	Zhou et al. (2018)
Chlorella vulgaris	*Pleurotus ostreatus*	Zhou et al. (2018)
Scenedesmus obliquus	*Ganoderma lucidum*	Zhou et al. (2018)
Scenedesmus capricornutum	*Ganoderma lucidum*	Zhou et al. (2018)
Nannochloropsis oceanica	*Mortierella elongata*	Du et al. (2018)

cultivate the algae in their own habitats rather than growing them in vitro and introducing them to the field (Leite et al. 2013). Advantages are bioremediation along with hydrogen production, while the disadvantages are nitrogen limitation and pretreatment of sample (Mathews and Wang 2009). Radakovits et al. (2010) felt that several technical needs have to be addressed before going for large-scale production of these algae. According to Das and Veziroglu (2008), the efficiency of the process is about 10%. Water molecule may be split by biophotolysis (*Chlamydomonas reinhardtii*) generating hydrogen and oxygen from water. Marxen et al. (2005) and Grima et al. (1999) have clearly elucidated the major challenges involved in the production of hydrogen by algal systems which are as follows:

1. Minimizing contamination: Most of the algae live with different kinds of microorganism during their life span (Dittami et al. 2014). Hence, this relation is important for the growth of the algae. Beneficial association is essential, but

excessive contamination would lead to lower growth and lesser productivity of the algal systems (Hom-Diaz et al. 2015). During cultivation of algae, open ponds are more contaminated rather than closed systems (Carney and Lane 2014). Although the association may be beneficial in some cases where waste can be remediated (Cavaliere et al. 2017). Algae can be infected by bacteria, viruses, and fungi (Carney and Lane 2014). The most contaminating species which are encountered during the biohydrogen process are the bacteria belonging to these families, namely, *Proteobacteria*, *Alphaproteobacteria*, *Bacteroidetes*, *Betaproteobacteria*, and *Gammaproteobacteri*a (Carney et al. 2016; Sambles et al. 2017; Fulbright et al. 2018). Some bacteria belonging to the genus *Alteromonas*, *Vibrio*, *Flavobacterium*, *Saprospira*, *Pseudomonas*, *Cytophaga*, and *Pseudoalteromonas* are reported to cause rot symptoms in algae (Ashen and Goff 2000). Some bacteria are reported to be involved in the bleaching of the *Delisea pulchra* (Zozaya-Valdés et al. 2017). *Microbacterium* sp. LB1 was reported to cause *Choricistis minor* algal cell lysis (Ivanova et al. 2014). Fungal contamination was also observed which is generally lethal to algal growth (Hoffman et al. 2008). Contamination can be prevented by using physical filtration (Carney and Lane 2014), change in pH and temperatures (Ras et al. 2011), and using different kinds of chemicals (Lee et al. 2002).

2. Provision of carbon dioxide and light: The minimum requirements apart from the nutrients are the requirement of carbon dioxide and light which needs to be provided to the system. The light entrapment efficiency and levels of carbon dioxide in the cultivating vessel need to be increased so that proper growth and biomass generation take place. Algae present in the outermost layers in a bioreactor are more illuminated compared to algae growing in the inner most layer. Sometimes due to excess light the growth of the outermost layer of algae can be inhibited due to excessive illumination causing photoinhibition. Secondly, the effect of self-shading also reduces the light reaching the inner most layers of the reactor. Apart from this, sulfur deprivation to the cultivating cultures is the major factor which needs to be looked into as it induces the generation of hydrogen. Hence, illuminating the inner and outermost layer of algae at required intensities is a major limitation which needs to be controlled for scaling up and making the process more economically viable. Once the cells are grown, they should be shifted to a mode of diverting electrons for the generation of hydrogen. For this to be achieved, a gene encoding the PSI-hydrogenase proteins needs to be manipulated so that electrons are diverted for generation of hydrogen instead of carbon dioxide fixation to form carbohydrates.
3. Minimizing space requirements: The amount of land available is the major constraint for the establishment of a refinery for the production of bioenergy, and secondly, even if the land is available, it should be supported with good transportation infrastructure (Cai et al. 2011). The major issues which need to be addressed would be the location of the land, transportation services, topography of the land, the presence of water resources, availability of nutrient sources, and suitable climatic conditions (Cai et al. 2011). Pate et al. (2011) also concluded that among all other demands for generation of algal hydrogen, land requirements

were the easiest to manage. The most important factor which needs to be understood is the productivity factor of the feedstock. More productive algal strains would require less space, and this is one of the best ways of mitigating the space constraints (Pate et al. 2011).

4. Reducing capital and production costs: Neda Fakhimi et al. (2019) were able to enhance the hydrogen production by combining mixotrophic combination of algae with bacterial cultures. The amount of hydrogen production was found to increase by 60% when *Chlamydomonas reinhardtii* along with the bacterium *Escherichia coli* was used. Sewage water can be employed for hydrogen generation and simultaneous bioremediation which makes the process sustainable and economically viable. Tools such as computational fluid dynamics (CFD) may be used to design better bioreactors which can be used for efficient scale up of the process. Modularization is one of the approaches which is a better option for scaling up the process. In this method, small bioreactors are used. Intensity of the light, nutrient supply, and carbon dioxide can be effectively managed. If any problem arises in any of the module, it can be detached. The problem of contamination of the system can be handled. The capital costs can be reduced if the production of hydrogen is lined to sequestration of carbon dioxide. To establish a viable process, techno-economic analysis and life cycle analysis are presently needed.
5. Controlling cultivation conditions: This is one of the major challenges which need to be addressed for generating biohydrogen. During the process, evaporation of water is a major constraint which needs to be addressed. In open ponds, large amounts of water are evaporated, and salt levels increase (Yang and Wang 2018). Unless this is not addressed, the cultivating conditions cannot be controlled in a bioprocess (Harto et al. 2010). The management of water and salt is the essential areas which have to be addressed (Gerbens-Leenes et al. 2009). Darzins et al. (2010) suggested that coastal regions are suitable if freshwater is not available for salt water algal production (Darzins et al. 2010). Water requirements for cultivation of algae are comparatively higher when compared to that of other petroleum-based fuels (NRC 2011). Pate et al. (2011) opined that marine water, wastewater, and water from other industrial wastes should be used to make the cultivation process viable in place of freshwater.
6. Future Perspectives: The choice of pretreatment technology for biohydrogen production method depends on substrate composition. The development of different pretreatment technologies will result in enhancements of hydrogen production and by effective solubilization of the substrate. Lee (2016a) opined that biohydrogen is economically possible and may be commercialized with success. A study by Lee (2016b) reported that biohydrogen will replace fossil fuels with less economic burden. Ogden et al. (2004) suggested that the use of generated algal hydrogen in hydrogen electric vehicles will be a major interesting application of hydrogen production using biomass. Stanislaus et al. (2017) investigated hydrogen production from Ipomoea aquatica using digested sludge as inoculums and reported that the energy consumed was lesser than energy produced in the process, which shows a positive energy balance. Unless a process is less energy

intensive, it would not be feasible for it to be viable. The development of biohydrogen economy may be possible because it provides energy security and environmental safety. Moreover, hydrogen economy is the only way forward as this is a zero emission fuel and produces water and oxygen during the process. Hybrid or biorefinery concept will result in commercialization of biohydrogen production. Metabolic engineering will play an important role in biohydrogen production and can substantially increase the yield of biohydrogen. Methods for generating stable transformed algal lines (Coll 2006) have led to the possibility of metabolically engineering algae for production of biohydrogen apart from other useful byproducts during the process (Leon-Banares et al. 2004; Rosenberg et al. 2008). High light intensity can lead to photoinhibition apart from producing toxic photoproducts including peroxide and hydroxyl radicals (Taiz and Zeiger 2006; Long et al. 1994). One of the alternatives to overcome some of the challenges is the use of transgenic algae, but the safety aspects in outdoor reactors raise public and environmental concerns. The transgenic algae generally are designed in such a way that it withstands any abiotic stress of temperature, pH, etc. In this process, the algal strains are modified genetically with DNA from other organisms. This in turn leads to environmental and public health concerns. Hence, transgenic algae, to be used in enclosed photobioreactors, should be equipped with spill containment technology. Only when such safety measures are taken, accidental spills of the transgenic algal cultures will not take place. Hence, safety measures have to be considered for sustainable generation of algal hydrogen.

7.9 Conclusions

For potential and sustainable biohydrogen production, we have to look for potential algal species which can produce valuable bioproducts throughout hydrogen production under sulfur deprivation. The media requirements for cultivation of algal biomass for enhanced hydrogen production have to be optimized. The design of bioreactors for hydrogen production from algae should be made in such a way that all the process parameters are taken into account. The expression of hydrogenase genes under different cultural conditions in algal species needs to be studied. Lastly, a lot of research has to be focused on generation of algal mutants which are able to tolerate abiotic stress such as temperature, salt, and light intensity. Work also needs to be done for isolating algal strains which can grow with less amounts of water.

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Chapter 8
Bioprocess Parameters for Thermophilic and Mesophilic Biogas Production: Recent Trends and Challenges

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Abstract The latest advancements in technology have led to the progress in designing more efficient anaerobic digestion (AD) systems which have incorporated modifications such as feedstock pretreatment methods, bioprocess improvements, techno-economic gas upgrading, and superior digester designs among others. The different types of feedstocks being used, the mechanism of biogas production, the operation of a biogas plant, and the different types of digesters used for anaerobic digestion are explained. The various process parameters like pH, temperature, electrical conductivity, etc. are also discussed. Challenges in anaerobic digestion along with the advantages and disadvantages of biogas generation are deliberated. Further, the microbial population involved in various stages of process is presented. In this chapter, the existing state of biogas technology highlights the latest advancements in its applications as well as production.

Keywords Biogas · Anaerobic digestion · Feedstock · Mechanism · Process parameters

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8.1 Introduction

The continuing use of fossil fuels is responsible for many ecological concerns. This necessitated a shift to focus from fossil fuels to more sustainable biofuels. Fossil fuels are depleting and are responsible for environmental pollution. Moreover, about 88% of present-day energy requirements are being met by fossil fuels. As they are depleting, research efforts are initiated for alternative fuels which are sustainable and environment friendly (IEA 2015). Kothari et al. (2010) opined that biogas is best suited for tropical climates as an environmental friendly and can aid in sustainable development. It can be used for heart generation and electricity generation. It can provide source of fuel to the rural population where there is less access to electric power. It can be used as a substitute to firewood and charcoal. Anaerobic digestion is a procedure where many diverse microbes transform organic waste to biogas. These microorganisms can survive in anaerobic environments where the transformation of organic matter to biofuel will take place. Wetlands generally are the most commonly found areas where the presence of these microorganisms will be seen. Apart from these freshwater sediments, digestive tracts of animals also host such environments for the growth of the microorganism. This process is exploited for the production of biogas using similar conditions where the necessary conditions for the growth of anaerobic bacteria like pH, temperature, and anoxic conditions are maintained. A part from this process is also used for the preparation of biofertilizers from agriculture and domestic waste. Anaerobic digestion is used for waste treatment and biogas production (De Baere et al. 2010). It is a sequence of biological techniques that use a different kind of bacteria to break down organic matter into biogas, mainly methane and mixture of different gases (like carbon dioxide, hydrogen in anoxygenic conditions) (Antoni et al. 2007). A biogas unit consists of reception tank, digester, gas holder, and an overflow tank. The improvement of reactor for anaerobic digestion generation has undergone further advancements over the years. Ribas et al. (2009) reported 70% COD elimination along with 70% methane content in biogas with the aid of a mesophilic SBBR reactor while treating sugar cane-vinasse. Almeida et al. (2017) studied configuration of different types of reactors. He observed that the removal efficiency of COD increased by 97%. The effect of physicochemical parameters on the biogas plant efficiency was also reported (Chen et al. 2017; Hong and Haiyun 2010; Hussain et al. 2017; Liu et al. 2016). A thermophilic digester functions at temperatures more than 50 °C generating biogas. It has some benefits such as that it does not need agitation and is quicker in fermentation than a mesophilic digester. Vinasse produced at more than 70 °C can be used for this kind of biodigester. The main types of biogas production plants are fixed-dome plant and floating-drum plants. In fixed dome type, the digester is fixed with a gas holder. The costs incurred in operation are quite low, and the life span of these kinds of digesters is generally about 20 years.

In conventional systems the major limitations are high space requirement, low OLR/high HRT, low treatment efficiency, and biomass washout. Anaerobic digestion can be classified into two types, namely, wet digestion and dry digestion based

on solid content present. Later an even more effective technology came into being combining both the modes called co-digestion. Wet anaerobic digestion systems are used to treat sewage water and industrial effluents which contain low amounts of solids. In dry digestion, high solid content substrates (25–40%) are treated (Verma 2002). Heat and nutrient transfer is good in wet processes when compared to dry processes (Luning et al. 2003; Wellinger et al. 1993). In the process of dry digestion, municipal solid waste (MSW) and energy crop residue digestion are generally done. These systems could reach higher organic loading rate values resulting in smaller volumes of digestate and hence are more economical when compared to wet digestion processes. Co-digestion is the process of transformation of various feedstocks. In contrast to conventional methodology used for anaerobic digestion process, mixtures of substrates are used as feedstock. Of late, this procedure was adopted by many countries. Mathias (2014) proposed the use of four types of anaerobic digesters, namely, "continuously stirred tank reactors (CSTR); upflow anaerobic sludge blanket (UASB) reactors, upflow anaerobic filter (UAF) digesters, and baffled digesters." The digester to be used in the process is dependent on the major type of the substrate which would be treated in the process. Substrates with more amounts of total solids are treated in continuously stirred tank reactors (CSTRs). Other types of feedstocks especially dissolved organic solids are treated in upflow anaerobic sludge blanket (UASB) reactors, anaerobic filters, and fluidized bed reactors (Mathias 2014). The process takes place in a single step in which the substrates are digested till we reach a solid dry content between 8% and 15%. According to Langeveld et al. (2016), the major advantages of co-digestion when compared to other types of digestion strategies are enhanced biogas yields and lower emission of greenhouse gases, process stability, homogenization, high nutrient recycling, and continuous production of biogas in all season.

The feedstocks are treated at very high temperatures for hydrolysis of substrate to make it more homogeneous. Figure 8.1 shows the conversion of food waste to biogas and the intermediate steps involved in it. It also removes contaminants present in the feedstock and to produce a uniform biomass. The refined organic substances are treated at high temperatures to enhance biogas generation. This process also helps in the pasteurization of the waste. The process generally involving treatment at temperatures about 70 °C with hydraulic retention time (HRT) of 1 h is done to pasteurize the waste as required by national and international regulations. The slurry obtained after pasteurization is cooled. The temperature should be equal to that of the digester operating temperature. Using a heat recovery system, the excess heat is recovered. It will be then used to treat the unpasteurized organic waste. Pathogenic microorganisms are eliminated through the process of thermal treatment. Thermal treatment of high lignocellulosic contents will result in higher organic transformation efficiencies especially when the organic waste is heated up to 165–170 °C for half an hour. In anaerobic contact process, the limitations are high space requirement and not suitable for high organic rate loading. Moreover, no phase separation takes place, and the tank must be always closed to prevent foul smell. In case of fluidized bed reactor (FBR), difficulties in maintaining optimum mixing and difficult to start-up conditions are seen. It would also be difficult to scale up the

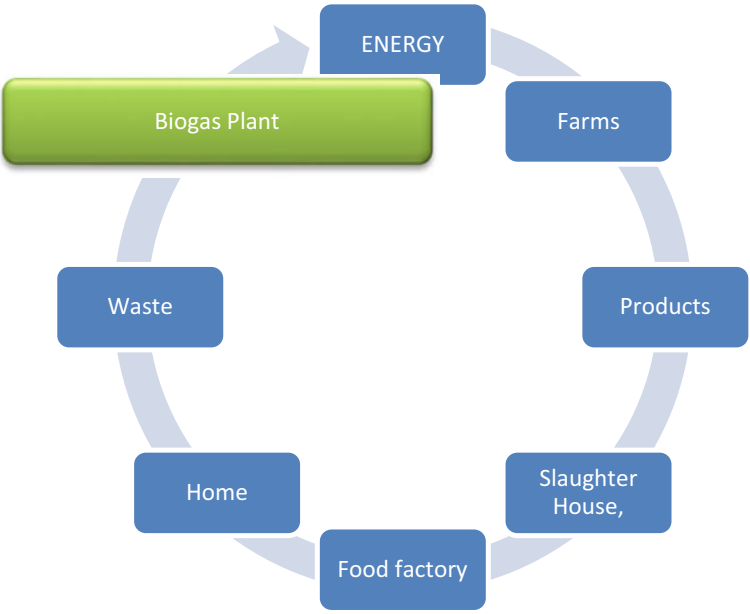

Fig. 8.1 Conversion of food waste to biogas

process to industrial scale. In case of upflow anaerobic sludge blanket (UASB), the disadvantages of operation are that performance is based on the granule formation which in turn depends on the type of wastewater being used and phase separation does not take place. The process of start-up will be delayed if suitable innoculum is not selected. In anaerobic baffled reactor (ABR), variable system hydraulics difficulties and biomass growth are seen. Expanded granular sludge blanket (EGSB) is energy intensive, has poor process stability, is the absence of phase separation, and is not appropriate for wastewaters containing more solid contents. In anaerobic filters, the major disadvantages are no phase separation takes place, problems with mixing, not appropriate for wastewaters with solid contents, and high energy requirement (Akunna 2018). Wet fermentation systems are those in which only 15–25% solids are present. System in which more than 30% high solids are present is called dry fermentation. The slurry is digested in wet fermentation. Many digesters comprise a single reactor vessel but can be divided into two stages with more than one reactor vessel. Hayes et al. (1979) observed that plug-drift digesters use slurries. Bruins (1984) has reported that at low concentration of total solids, problems with floating and settling layers are seen and suggested that this can be overcome by vertical mixing inside the pipe. During this process, the phenomenon of hydrolysis and that of methanogenesis is separated in the pipe. Hydrolysis occurs first followed by methanogenesis. In this type of system, the SRT is the same as that of HRT.

8.2 Thermophilic and Mesophilic Anaerobic Digestion

Thermophilic anaerobic digestion will take place at temperatures above 50 °C. The biggest advantage of thermophilic digestion is the decrease in retention time which could be as low as 10 days when compared to mesophilic reactors where the retention time is about 20 days. The advantages are that mixing energy requirements are less and overall heat loss per unit volume of material processed also is less apart from pathogen reduction. In the third process, hydrolysis stage is the rate-limiting step. This is overcome in the thermophilic digester which operates at high temperature range so that hydrolysis takes place efficiently. Thermophilic anaerobic digestion generated higher amounts of biogas production. The disadvantage is that there tends to be accumulation of volatile fatty acids which decrease the biogas yield. The thermophilic anaerobic digestion process is also instable. Other limitations are that the water quality gets worse, fluctuation in temperatures, and sensitivity to toxic heavy metals (Khemkhao et al. 2012). The process is energy intensive as more energy is required for raising the initial temperature. The anaerobic process which operates at mesophilic temperature range (35–38 degree centigrade) is called mesophilic digestion. This temperature range can produce class A biosolids. Thermophilic digesters need lesser time to process feedstocks but are difficult to operate and are expensive. Kushkevych et al. (2020) have investigated the diversity of various thermophiles which are occurring in mesophilic biogas plants located in Czech Republic. They found 19 thermophilic genera using 16S rRNA gene sequencing. Most of the thermophilic population was found in substrate containing primary sludge and biological sludge, and less were found in maize silage and liquid pig manure. Bolzonella et al. (2020) have treated agrowaste using a thermophilic post-hydrolysis process in a digester operated for 3 days to increase the production of biogas by 30%. Dai et al. (2020) have proposed a thermophilic mixed culture fermentation (TMCF) for enhancing the production of methane and hydrogen with a high substrate degradation rate and low gas solubility. Lei et al. (2020) have investigated thermophilic anaerobic digestion (TAD) of *Arundo donax*, an energy crop with high cold tolerance to understand the relation among microbial population and their functions during the process of fermentation. They have observed *Firmicutes* with three dominant genera of *Tepidiphilus*, *Sedimentibacter*, and *Gelria* during the thermophilic anaerobic digestion process apart from *Methanoculleus* and *Methanosarcina*. Wu et al. (2020) compared the process of anaerobic digestion of municipal sludge with high (10%) solid content under both mesophilic and thermophilic conditions. Thermophilic digestion was better than mesophilic anaerobic digestion for biogas production. Mesophilic anaerobic digestion showed more microbial diversity than thermophilic anaerobic digestion.

Ryue et al. (2020) reviewed the usual and promising methods for improving process stability in thermophilic anaerobic digestion. Zhang et al. (2020) used a mixing strategy for treating food waste and chicken manure under thermophilic conditions using a mesophilic innoculum. They observed that methane yield in the continuous stirred reactor was 71.3% more when compared to intermittent agitated

reactor. Hirota (2020) investigated production of methane in wet and semi-dry anaerobic digesters. Maximum levels of methane gas were seen for 30 days in both thermophilic conditions. In anaerobic digestion, new studies of using hyper-mesophilic temperatures were reported by Moestedt et al. (2014). The range of organic loading rate is 3–5 kg VS/m^3/d. Hyper-mesophilic temperatures between 40 and 44 °C have been explored for different kinds of substrates (Westerholm et al. 2015). van Lier et al. (1993) and Lindorfer et al. (2008) have earlier observed process instability when hyper-mesophilic conditions were used for anaerobic digestion process for mesophilic microorganism. However, Moestedt (2015) has reported higher biogas yield in digestion of food and slaughterhouse waste in Linköping biogas plant. Biogas produced during the process of anaerobic digestion are made up of material such as PVC-coated fiber fabric, etc.. Labtut et al. (2014) have done a comparative study between mesophilic and thermophilic processes and concluded that a mesophilic digester was stable regardless of the organic and influent composition, while thermophilic digester performed better at high organic loading rates. They have also observed that the stability of thermophilic digester was dependent on influent composition when compared to mesophilic digester. Performing anaerobic co-digestion of food waste with lignocellulosic wastes can overcome the limitations of their respective mono-digestions. Mahdy et al. (2020) evaluated the influence of hyper-thermophilic pre-hydrolysis stage on methane recovery using sewage sludge and microbial populations present in them. Bacteroidetes and Cloacimonetes populations were more, while there was reduction in the population of Firmicutes. Prem et al. (2020) studied the microbial community dynamics when proteinaceous wastes were treated in mesophilic and thermophilic batch reactors. They have observed that in mesophilic samples, acetoclastic methanogenesis took place where phenylacetate (PAA) levels favored the growth of *Psychrobacter* spp., while phenylpropionate (PPA) favored the growth of *Haloimpatiens* spp. Lopez et al. (2020) assessed the microbial quality of sewage sludge which was treated in three different plants: two anaerobic and one aerobic plant. Out of the three, one was anaerobic mesophilic, one was anaerobic thermophilic, and the last plant was aerobic thermophilic. They have observed that anaerobic thermophilic treatment could decrease the concentration of the *Enterococcus* sp., while aerobic thermophilic could decrease the concentrations of *E. coli*.

8.3 Mechanism of Biogas Production

The groups of microbes involved in anaerobic digestion are poorly understood. Angelidaki et al. (2011) have reported that the bacterial communities involved in anaerobic digestion can be divided into fermenting bacteria, anaerobic bacteria, and methanogens. The oxidizing microorganisms oxidize these reduced substances to hydrogen, formate, acetate, and carbon dioxide (Angelidaki et al. 2011). Propionate accumulation is seen in cases of process imbalance (Angelidaki et al. 2006). Wang et al. (2012) have reported that ratio of 1.25 between propionate and acetate may lead

to failure of biomethanation process. Clostridium and Megasphaera species have been reported to convert lactic acid to propionic acid (Prabhu et al. 2012; Tracy et al. 2012). Biogas has lower emission rates compared to that of any other fossil fuel, subsequently leading to less environmental pollution (Vijay et al. 2006). The need for international sustainable waste management has resulted in renewed research interest in agro-waste and biowaste-based biofuels (Weiland et al. 2009; Deublein and Steinhauser 2008). Boe et al. (2012) reported that the feedstock composition with excessive lipid or protein content shows high correlation with foam formation during anaerobic digestion. Other parameters, like temperature, digester design, and form of the mixing, are responsible for foam formation (Barber 2005). Foaming may cause blockage of mixing systems due to the presence of solids in the foam (Ganidi et al. 2009). Excess financial costs are incurred due to foaming (Barjenbruch et al. 2000). In anaerobic digestion method, four processes are involved (Bharathiraja et al. 2014), namely, hydrolysis, acidogenesis, acetogenesis, and methanogenesis. In the hydrolysis step, carbohydrates, proteins, and lipids are hydrolyzed to single chain monomers and dimers like sugars, amino acids, and fatty acids. In step 2 (acidogenesis), the monomers and dimers from hydrolysis are turned into propionic acid, butyric acids, and valeric acids. In the case of step 3 (acetogenesis), acetic acid, hydrogen, and carbon dioxide are formed. In the last stage (methanogenesis), acetate is converted into methane and CO_2; whole hydrogen is used up. Methanogenic microorganisms are sensitive to oxygen and are less versatile when it comes to substrate utilization. Methane is generated through acetoclastic methanogenesis using acetate. Hydrogen produced will be the remaining 1/3 of the total biogas produced. Belay et al. (1986) and Lovely and Klug (1983) have observed methane production from substrates such as formate methanol and methylamines. Wolfe (2011) reported that methanogens need a higher pH at later stages of the process compared to initial stages. Richards et al. (2016) reported that *Methanococcus maripaludis* has a doubling time of just 2 h. Research by De Vrieze et al. (2012) found that *Methanosarcina* spp. is a more robust methanogen when compared to other methanogenic populations which are involved in methanogenesis. They have reported that it is capable of variations in pH and also concentrations of acetate, ammonia, and sodium. Dhamodharan et al. (2015) and Li et al. (2015) have developed many kinetic models to describe the processes involved in anaerobic digestion.

Anaerobic digestion takes place in three stages, that is, hydrolysis, acidification, and methane formation. The acidogens produce hydrolytic enzymes and transform soluble organics to volatile fatty acids and alcohols. Breakdown of carbohydrates, proteins, and lipids into sugars, amino acids, and fatty acids takes place in hydrolysis. This is carried out by specific enzymes of hydrolytic bacteria. In the hydrolysis stage, these microorganisms were observed, namely, *Peptococcus*, *Ruminococcus*, *Eubacterium*, *Bacillus*, *Butyrivibrio*, *Proteus vulgaris*, *Micrococcus*, *Staphylococcus*, *Acetovibrio*, *Clostridium*, *Lactobacillus*, *Streptococcus*, etc. The monomers released during hydrolysis are converted by fermentative bacteria into carbon dioxide, pyruvate, hydrogen or formate, ammonia, volatile fatty acids, lactic acid, and alcohols. In acetogenesis, some compounds generated during acidogenesis are

oxidized to carbon dioxide, hydrogen, and acetic acid by metabolic action of acetogens. Volatile fatty acids and alcohols are then transformed by acetogenic bacteria into acetic acid, hydrogen, and carbon dioxide. During acidogenesis, *Desulfovibrio*, *Lactobacillus*, *Butyrivibrio*, *Bacillus*, *Desulfuromonas*, *Pelobacter*, *Sarcina*, *Staphylococcus*, *Selenomonas*, *Pseudomonas*, *Streptococcus*, *Clostridium*, *Eubacterium*, *Desulfobacter*, *Veillonella*, etc. are seen. In the stage of acetogenesis, *Syntrophomonas buswelii*, *Clostridium*, *Methanobacillus omelionskii*, *Syntrophomonas wolfei*, *Syntrophomonas wolinii*, etc. are involved. Methanogenesis leads to the formation of CH_4. Seventy percent of methane produced is from acetic acid by acetoclastic methanogenic bacteria. During methanogenesis, *Methanosarcina* and *Methanosaeta* were generally observed. Hydrogenophilic methanogens such as *Methanoplanus*, *Methanobacterium*, *Methanospirillium*, *Methanobrevibacter*, etc. are also seen (Wheatley 1991; Stronach et al. 1986). Methanogenic bacteria then use acetic acid or hydrogen and carbon dioxide to generate methane. Yang et al. (2004) have reported that the yield of biomethane is never greater than 60% of theoretical yield. The possible reason for this decrease is the presence of other compounds which do not undergo degradation and are resistant such as lignin, cellulose, or some complex proteins in the waste:

$$4CH_3COOH \rightarrow 4CH_4 + 4CO_2$$
$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$$
$$4CH_3OH \rightarrow 3CH_4 + CO2 + 2H_2O$$
$$CH_3OH + H_2 \rightarrow CH_4 + H_2O$$

8.4 Microorganisms in Anaerobic Digestion

Different groups of bacteria such as *Methanoculleus bourgensis*, *Peptoniphilus sp.*, *Ruminiclostridium cellulosi*, *Herbinix hemicellulosilytica*, *Clostridium bornimense*, and *Clostridium ultunense* participate in various anaerobic digestion stages (Mauset al. 2014, 2016; Hahnke et al. 2014; Koeck et al. 2015; Tomazetto et al. 2016; Manzoor et al. 2013; Sun and Schnürer 2016). *Methanoculleus* species are known to be one of the most biologically involved organisms in methanogenesis (Nettmann et al. 2010; Wirth et al. 2012; Maset al. 2014). *M. bourgensis* is an important microbial species in the process. Certain genes involved in methanogenesis and osmolytes production were found in the *M. bourgensis* MS2T, and much of the genetic information commonly seen in methanogenesis in biogas plants was found in its genome (Maus et al. 2016). Hahnke et al. (2015) used the Illumina MiSeq system to sequence the anaerobic *Porphyromonadaceae* bacterium, which was isolated from an anaerobic digestion plant. They suggested that the bacterium may play a role in both hydrolysis and acidogenesis stages, as its genome showed the presence of genes which can produce proteins capable of breakdown of complex carbohydrates and production of fatty acids (VFAs). Koeck et al. (2014) sequenced *Ruminiclostridium cellulosi* DG5, a thermophilic, anaerobic, and cellulolytic bacterium which was responsible for lignocellulose degradation. The

enzymes included mainly belong to hydrolase group that are most engaged in hydrolysis and regenerating glycosidic bonds. *Herbinix hemicellulosilytica* was isolated from a thermophilic biogas reaction and was capable of breaking down cellulose at higher temperatures (Koeck et al. 2015).

High-performance genomics and metagenomics sequences are used to investigate the bacteria present in the biogas generation. In order to improve the biogas digestive function, the presence of highly efficient microbial communities, hydrolyzing polymers varying from methane, is essential. Further understanding has limitations as a large part of biodiversity is unaffected (Tian et al. 2016). Thus, the identification and designation of microbial pathways of biogas production is an important function (Stark et al. 2014). NSG strategies and "omics" have significantly reduced costs and improved the reliability and consistency of the sequence data generated. These benefits make it possible for tens of amplicon samples immediately after hundreds of amplicon samples for a single operation without the need for the initiation and cultivation of individual microorganisms (Vanwonterghem et al. 2014; Delmont et al. 2012). Different metagenomics techniques, such as denaturing/Moche gradient gel electrophoresis (Connaughton et al. 2006; Liu et al. 2009a, b), terminal restriction fragment length polymorphism (T-RFLP) (Carballa et al. 2011; Ziganshin et al. 2013), sequence (Dong et al. 2015), fluorescence in situ hybridization (FISH) (Nettmann et al. 2010), and p4osequing (Li et al. 2013), were used for studying microbial populations in biogas digestion. These studies have been done on large microbial communities, lab small (Li et al. 2013), and small-scale reactors (Dong et al. 2015; Tian et al. 2016). Hassa et al. (2020) have analyzed the genome sequence of *Methanothermobacter wolfeii* SIV6 isolated from a thermophilic industrial-scale biogas fermenter and reported an operon encoding different subunits of the enzyme methyl-coenzyme M reductase which catalyzes the rate-limiting step during methanogenesis. The different kinds of microbes isolated from biogas treatment plants are tabulated in Table 8.1.

Table 8.1 Microorganisms isolated from biogas treatment plants

Name of the organism	Type of feedstock	References
Methanoculleus bourgensis	Sewage sludge	Maus et al. (2015)
Porphyromonadaceae	Maize silage; pig and cattle manure	Hahnke et al. (2015)
Clostridium bornimense M2/40	Maize silage and wheat straw	Hahnke et al. (2015)
Ruminiclostridium cellulosi DG5	Cellulolytic biogas plant	Koeck et al. (2014)
Peptoniphilus sp.	Maize silage	Tomazetto et al. (2014)
Clostridium Bornimense M2/40T	Maize silage and wheat straw	Tomazetto et al. (2016)
Clostridium ultunense	Acetate-oxidizing sludge	Manzoor et al. (2013)
Clostridium sp.	Slaughterhouse waste	Sun and Schnürer (2016)

8.5 Process Parameters Affecting Anaerobic Digestion

The anaerobic digestion operation depends on the temperature which is one of the primary factors which affects the production of biomethane. Other factors which are important in the process are pH, alkalinity, and toxicity. At the temperatures range of 35–37 °C Lettinga and Haandel (1993). Mesophilic organism's growth will take place. Anaerobic digestion occurs at three different kinds of temperature which are psychrophilic (10–20 °C) conditions, mesophilic (20–40 °C) conditions, and thermophilic (50–60 °C) conditions. Based on the growth rate of the bacteria at these temperatures, retention time of the process differs. Since the growth of bacteria is slower at lower temperatures, a longer retention time is required for psychrophilic anaerobic digestion when compared to mesophilic or thermophilic digestion. The local construction regulations of the place where the digester is being built has to be kept in mind. Different kinds of pretreatment methods are show in Table 8.2.

The following parameters are generally used for process design and operational control during anaerobic digestion.

1. Hydraulic Retention Time (HRT).
   HRT = Volume of Aeration Tank (V)/Influent flow rate (Q).
2. Organic Loading Rate (OLR).
   OLR = Q × So/V.
3. Solids Retention Time (SRT).
   Θc = VX / (Q-Qw) Xe + Qw Xw
4. Hydraulic loading rate (HLR).
   HLR = Q/A
5. Specific biogas yield.
   Ybiogas= Qbiogas / Q(So-Se)

Table 8.2 Anaerobic digestion pretreatment methods

Pretreatment	Feedstock	References
Physical	Straw	Motte et al. (2014)
	Fruit and vegetable waste three sonication times of 9, 18, and 27 min, operating at 20 kHz	Zeynali et al. (2017)
	Olive mill solid residue	Rincón et al. (2013)
Chemical	Cotton stalk residues	Zhang et al. (2018)
	Agriculture straw	Song et al. (2014)
	Sunflower oil cake	Monlau et al. (2013)
Biological	Food waste	Lim and Wang (2013)
	Chicken feathers	Patinvoh et al. (2016a)
	Paddy straw	Phutela and Sahini (2012)
	Organic waste	Wagner et al. (2013)
Thermal	Wheat straw	Rajput et al. (2018)
	Hay	Bauer et al. (2014)

6. Specific biogas production rate (BPR).
   BPR= Qbiogas/V
7. Treatment efficiency.
   % COD removal = So-Se/So X 100

Even the reactor volumes have to be larger for the psychrophilic digestion. If the pH values are between 6.5 and 7.5, the rate of production of biomethane will be less. Hence, hydrogen carbonate is added to the reactor to maintain optimum pH for higher methane generation. Numerous compounds such as volatile fatty acids, ammonia, sodium, calcium, heavy metals, sulfide, and xenobiotics have a detrimental effect on the production of the methane. Anaerobic digestion involves a diverse group of microbes such as methanogens which are sensitive to cultural conditions under which they grow. Hence, the cultural conditions have to be optimized to see that the maximum production of biogas takes place. Secondly, some organic as well as inorganic compounds present in the substrate can be toxic to the entire process of anaerobic digestion (Boe et al. 2012). The factors that affect biogas production are as follows:

(a) pH

pH plays a major role in anaerobic digestion. As the process is divided into different stages, pH at various stages has to be maintained differently so that the microbial growth at different stage is not inhibited. During the hydrolysis stage, the pH should be maintained between 5.0 and 6.0, while the pH required during the phase of acidogenesis stage is between 5.5 and 6.5. In the stage where the actual production of methane takes place which is called methanogenesis, the pH required is about 6.8–7.2. When the pH is not optimized as required, volatile fatty acids will be generated. The presence of these will inhibit the growth of the methanogenic microorganism. Changes in volatile fatty acid (VFA) levels are always measured as it is a good indication of the stability of the operation. The concentration of the volatile fatty acids (VFAs) will change based on process parameters like HRT, OR, or temperature.

(b) Temperature

A constant process temperature is essential for a successful anaerobic digestion process (Jain and Kalamdhad 2018). Increased temperature leads to increased metabolism and an increase in nutrient requirement. The various performance enhancers are explained by Carlsson et al. (2012). The different approaches being used are seeding, particle size reduction, ultrasonic pretreatment, addition of metals, thermal pretreatment, and alkali pretreatment. Chen et al. (2017) have proposed that temperature is a vital parameter that could influence the work of an anaerobic digester. Digester working in thermophilic condition is reported to have the fastest reaction rates compared to other operating conditions, thus leading to more generation of biogas (Mao et al. 2015). However, the disadvantage of operating in such high temperatures is that inhibition of the process may take place due to increase in production of

ammonia which is toxic to other groups of microorganisms (Weiland et al. 2009). Martinez-Sosa et al. (2011) and Smith et al. (2013) have also observed lower methane production under psychrophilic conditions. Fouling smell was also increased when the temperature of the digester was lowered (Gao et al. 2014). Microbial growth depends on the temperature being maintained at various stages of the process in the digester. Ennouri et al. (2016) treated urban and industrial sludge samples and found that treatment at temperature of about 120 °C leads to higher biogas formation. Bowen et al. (2014) reported those temperatures less than the optimal required led to lower substrate utilization which indirectly affects the digestion process. Kundu et al. (2014) confirmed that increase in process temperatures is associated with lower negative effects compared to lower temperatures. Similarly, Westerholm et al. (2017) have also reported that increased temperatures are beneficial for the bioprocess to take place while studying thermophilic-to-mesophilic temperature adaptation. During the process of scale up, it would be difficult to control the temperature at the required level as the ratio between surface area and volume of the digester will be decreased. Heat exchangers like cooling coil, cooling baffles, vessel wall, and external loop are generally used for controlling excess heat so as to control the temperature. Stanton number describes the ratio between "heat transfer capacity through coils and convection capacity in cooling water." This is very useful for designing a heat exchanger. The various devices which are used for temperature monitoring are bimetal thermometers, liquid thermometers, thermistors, crystal window tape, infrared detectors, etc. Clemens (2006) suggested that for maintaining temperature in biogas digesters, temperature control devices have to be used. Matsakas et al. (2020) evaluated a novel pretreatment method for enhancing methane production using hybrid system of organosolv-steam explosion fractionation. The approach was used for obtaining pretreated solid which is highly digestible from birch and spruce woodchips.

(c) Feedstock.

The non-lignocellulosic liquid feedstock which is generally used for anaerobic digestion process is palm oil mill effluent (Sri Rahayu et al. 2015). Guardia-Puebla et al. (2014) treated coffee wastewater and reported methane gas production of about 61%. They have also studied the influence of OLR and HRT in the treatment of coffee wet wastewater in a UASB reactor. Chicken feather was pretreated and was found to be effective as 75% of the feather was transformed into protein after 8 days (Patinvoh et al. 2016b). Janke et al. (2015) used vinasse as a feedstock, but lower yields of biogas were found. They suggested a reactor design with higher OLR and lower HRT. Pig and cattle manure were used as feedstock for the production of biogas (Matulaitis et al. 2015). The process showed that pig liquid manure gave more biogas yields compared to pig solid manure and cattle manure. The solid feedstock for anaerobic treatment includes food residues (Yong et al. 2015). Zhang et al. (2007) has suggested that lignocellulosic wastes are abundant renewable organic resources with 200 billion

tons production every year. Kang et al. (2014) opined that the abundant lignocellulosic wastes found in nature make them a good feedstock for biogas production and can add approximately 1500 MJ/year of energy. Although they are difficult to be digested (Himmel and Picataggio 2009). The lignocellulosic feedstock which was used for anaerobic digestion was silage maize (Mumme et al. 2011). Cadavid-Rodríguez and Bolaños-Valencia (2016) used grass silage for anaerobic digestion and found that maximum methane was seen when the total solids were at 4% composition. Liew et al. (2012) studied the use of wheat straw, corn stover, yard waste, and leaves for biomethane production through anaerobic digestion and found that corn stover was the best feedstock for generation of methane followed by wheat straw, leaves, and yard waste. Sugarcane bagasse was treated with alkali to remove lignin which improved the rate of lignin removal. The maximum methane yields were found to be about 221.8 mL/g-VS (Kumari and Das 2015). Battista et al. (2016) used the lignocellulosic materials in coffee wastes by pretreating them with sodium hydroxide and observed a higher biogas production with pretreated coffee waste. Forestry residues were also used as feedstock for biogas production by pretreatment (Teghammar et al. 2014). Oil palm fiber from a Colombian palm oil mill was studied for generation of biogas (Garcia-Nunez et al. 2016a). Different types of agricultural residues from maize, coffee, cotton, sugarcane, and bananas were found to be suitable as feedstock for biogas production in Kenya (Santa-Maria et al. 2013; Nzila et al. 2017). Co-digestion of food waste and straw at 35 °C was studied by Yong et al. (2015). Brown and Li (2013) and Xu and Li (2012) have reported that co-digestion of food waste and lignocellulosic wastes helps maintain a carbon/nitrogen ratio, reduction of the start-up time, and volatile fatty acid accumulation thereby improving the overall biomethane production. Lott et al. (2020) produced high purity methane by adding H_2 and CO_2 through the process known as ex situ biogas upgrading in which agro-municipal residues such as cow manure (CM) and the organic fraction of solid municipal waste (OFSMW) were used. Agata et al. have used mild thermal pretreatment of kitchen waste and concluded it was helpful in the solubilization of macromolecules and proposed it as a promising option for enhancing biogas production. Rasapoor et al. (2020) reviewed the challenges involved in improving biogas generation and suggested balancing the waste composition, optimizing nutrient, and using additives like biochar, carbon, and phenazine for direct interspecies electron transfer (DIET). Lim et al. (2020a) studied the influence of seed sludge on microbial diversity and performance of thermophilic digestion of food waste. Lim et al. (2020b) proposed the use of biochar for overcoming process instability during start-up of the anaerobic digestion process. They observed that biochar addition enhanced the methane production by 18%. When biochar was added, the growth of electroactive *Clostridia* and other electroactive bacteria was seen, while in its absence, biochar promoted the growth of *Clostridia* and syntrophic acetate oxidizing bacteria. The types of feedstocks are shown in Table 8.3.

Table 8.3 Types of feedstocks used for anaerobic digestion process

Name of the feedstock	Reference
Palm oil mill effluent	Langeveld et al. (2014), Sri Rahayu et al. (2015)
Slaughter waste	Patinvoh et al. (2016b)
Vinasse	Janke et al. (2015)
Potato effluent	Hung et al. (2006), Verheijen et al. (1996)
Coffee wastewater	Segura-Campos et al. (2014)
Pig slurry	Matulaitis et al. (2015)
Wheat straw	Liew et al. (2012)
Sugarcane bagasse	Kumari and Das (2015)
Coffee parchment	Battista et al. (2016), Syarief et al. (2012)
Oil palm fiber	Garcia-Nunez et al. (2016b)
Banana flower stalks	Santa-Maria et al. (2013), Nzila et al. (2015)
Grass silage	Cadavid-Rodríguez and Bolaños-Valencia (2016)
Corn Stover	Liew et al. (2012) and Li et al. (2011)
Coffee pulp	Battista et al. (2016) and Syarief et al. (2012)
Forestry residues	Hoyne and Thomas (2001)
Fruit bunches	Garcia-Nunez et al. (2016b), Zhang et al. (2012)
Banana leaves	Santa-Maria et al. (2013), Nzila et al. (2015)
Banana pseudostems	Santa-Maria et al. (2013), Nzila et al. (2015), Kalia et al. (2000)

(d) Nutrients and Electrical Conductivity

Weiland (2001) reported that the carbon, nitrogen, phosphorus, and potassium are essential for the process of anaerobic digestion as bacterial growth depends on the various nutrients supplied. These nutrients are required at different ratios 500/15/5/3 (C/N/P/S) for hydrolysis and acidogenesis and while 600/15/5/3(C/N/P/S) for methanogenesis. Minimal amounts are required for sulfur and phosphorous compared to other macronutrients. The limiting nutrient was found to be nitrogen, and the carbon/nitrogen (C/N) ratio of 20 to 30 is required (Deublein and Steinhauser 2008; Polprasert 2007). Apart from this, cobalt, nickel, iron, and zinc are required for stimulating methanogenesis. Keratin-rich wastes are produced worldwide by several industries. Angelidaki and Sanders (2004) have observed that if all the insoluble protein (keratin) is converted into soluble protein, the methane potential of keratin wastes is as high as 0.496 Nm3/kgVS. Wu et al. (2020) have investigated the effect of copper salts, cupric sulfate, and cupric glycinate on anaerobic digestion of swine manure. They observed that addition of these salts improved the production of methane by 28.78%. The presence of *Clostridia* and *Methanobacterium* were observed in higher amounts. Lackner et al. (2020) have studied the influence of sulfur addition on microbial community when cellulose was used as substrate in thermophilic digestion. Sulfate addition of 0.5 to 3 g/L caused a decrease in methane generation by 73–92%, while higher sulfate concentrations had no additional inhibitory effect. Upon addition of sulfate, dominance of Firmicutes and decreased concentrations of Bacteroidetes and Euryarchaeota were seen.

The levels of methanogens were reduced, while the levels of sulfate reducing bacteria increased. "Electrical conductivity (EC)" is an estimation of salt content which is measured by an electrical conductivity meter. EC can be used to know if there is an accumulation of any salt taking during anaerobic digestion process. It is important as there are many salts which when accumulated within the process may inhibit the process and thus may decrease the yield of the biogas. It is also used to measure the salts present during the loading of the solid or liquid waste so that its addition does not inhibit the process. To overcome this, generally dilution of the input wastewater is done to keep the value of the electrical conductivity at a minimum.

(e) Toxicity.

Compounds of sulfate and sulfur found in the reactor influence both acetogens and methanogens. This is due to the presence of sulfate-reducing bacteria (SRB) which can use various substrates for survival and are more versatile. Sulfate-reducing bacteria present in the wastewaters convert sulfates, sulfite, and thiosulfate into sulfide. The presence of sulfur compounds reduces the methane yield. At pH 8.0, sulfide remains in the solution, and below pH 8.0, hydrogen sulfide is seen. At pH of 7.0, about 80% of the sulfides is present as hydrogen sulfide. Inhibitory effect of sulfides occurs when the ratio between COD and sulfides is less than 7.7 (Speece 2008). Decreases up to 50% in biogas yield are seen when sulfide concentrations are between 50 and 250 mg/L. This toxicity can be overcome by (Pohland 1992) dilution of the influent, addition of iron salts for precipitating sulfide from solution, or biological sulfide oxidation. Ammonium at 100 mg/L was found to be toxic to the anaerobic digestion process. Salt accumulation can lead to cell death and depends on microbial acclimatization (Ollivier et al. 1994; Appels et al. 2008; Feijoo et al. 1995). Chromium, iron, cobalt, zinc, and nickel have also been reported to be toxic at relevant concentrations (Chen et al. 2008). Phenolic, chlorophenols, halogenated benzenes, and N-substituted aromatic compounds are inhibitory to microorganisms as it interacts with cell membrane (McDonnell 2007). The addition of excess chemical when operating the reactor leads to chemical foaming. The other type of foam is caused due to excess production of biomass in the reactor called biological foam which is usually brown in color. A baffle is used to prevent scum production on the medium where the biomass is generated. Scum is formed due to variation in temperature, mixing, light, and less than four percent of total solids present in the reactor. Both foam and scum formation damage the gas pipes and result in reduction of biogas yield. For regular monitoring of the anaerobic digestion process, fatty acids and total alkalinity are considered. Volatile fatty acids are produced which may cause a change in the pH of the reactor and hence lead to lesser biomass production and biogas. Acetic acid, propionic acid, butyric acid, etc. produced are generally utilized to produce methane. However, at the same time if there levels are high, they tend to cause a change in the pH which needs to be adjusted. This is generally done by

means of adding bicarbonate into the digester to keep the pH stable (Boe 2006; Lahav and Morgan 2004). Otherwise there would be a sudden pH drop in the digester. The ratio between fatty acids and total alkalinity is taken into consideration while adjusting the pH of the digester (Deublein and Steinhauser 2011). The ratio should be typically between 0.2 and 0.6, while high pH can also result from the production of ammonia which is mainly seen during the digestion of the protein waste. Methanogenic organisms present in the digester are sensitive to the levels of ammonia. Reducing the input of high protein wastes and addition of iron oxide and clay minerals are reported to reduce the levels of ammonia produced during the process of digestion (Clemens 2013). Sanchez et al. (1996) have reported that iron, nickel, cobalt, copper, and zinc can be responsible for inhibition and cause the failure of the digester. Heavy metals at higher concentration than 10−4 M are inhibitory in nature. This could be due to replacement of metal ions bound with enzymes as prosthetic groups with these ions, causing enzyme inactivation (Chen et al. 2017). The input waste should be properly segregated before the digester is loaded so that any industrial wastes containing metals as such will be separated. The level of EC should be 25–30 dS/m for better operation of the digester. Higher levels of electrical conductivity caused due to the presence of salts can be controlled by dilution with water. The presence of higher amounts of organic matter in the waste material being digested in the reactor can lead to acidification decreasing methane production. When the reactor is in the initial stages, organic loading rate should be increased till a range where efficient production of biogas takes place (Fig. 8.2).

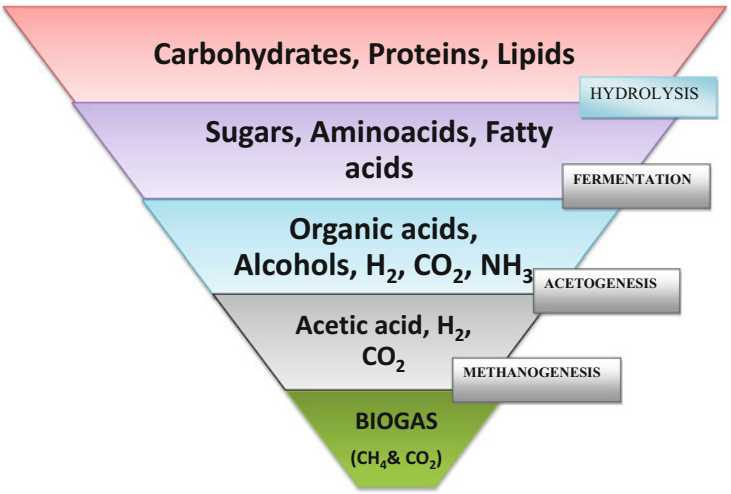

Fig. 8.2 Mechanism of biogas production

8.6 Reactor Design

Bouallagui et al. (2005) have explained different types of bioreactors which are commonly used in the industry: "batch, continuous one-stage system, or continuous two-stage/multi-stage systems." Some additional modifications are made to the existing models to design "anaerobic sequencing batch reactor (ASBR), upflow anaerobic sludge blanket (UASB) reactor, tubular reactor, plug-flow systems, and anaerobic filters." Khalid et al. (2011) have opined that among all the reactors. Batch reactors are quick, economical, and simple to operate. The digester tanks used are made of steel and concrete. Among the different types of construction materials being used, concrete constructions are more advantageous compared to others. Generally, the digester tanks are constructed with a lifetime of about 15–20 years. Hydrogen sulfide formation may lead to corrosion of the tank. The mixing system is important as it maintains a homogenous digestate during the process of anaerobic digestion. Longer stirring times are required during the initial phases of operation compared to the later phases of the operation. Ward et al. (2008) have suggested that the design of digester has to address three major issues for competent and economical formation of biogas. Firstly, it should have the capability to handle a high organic loading rate. Secondly, it needs to have a short hydraulic retention time, and lastly it should be able to produce higher volumes of good quality biogas. In this process, the highest methane production is seen in the beginning. Figure 8.3 shows the construction of a thermophilic anaerobic digester used for methane production. In the process of continuous digestion, these are fed continuously; after digestion, the digestate is discharged leading to a steady state for constant gas production rate. These types of systems are dependent on substrates which can be pumped into the system without any mechanical hindrance. If it is not possible, a semi-continuous process is experimented where the feedstock is fed at several times. "Continuous stirred-tank reactors (CSTR) (Fig. 8.4) and plug-flow reactors (PFR)" are the two most commonly used reactors, while others are less used. The plug flow reactors are generally used for dry digestion with the feedstock which contains a lot of solid mass (Patinvoh et al. 2016a). On the other hand, CSTRs are used only in systems where there is continuous supply of feedstock to the reactor such as in wet digestion systems. The decision to use either of the other mentioned systems depends upon the solid contents which are present in the feedstock. Mostly, CSTR design is used in single-stage systems favoring acidogens and methanogens. These are economical and easy to operate (Vandevivere et al. 2003). In the case of two-stage reactors, the process of acetogenesis is separate from that of acidification and takes places in two stages. The first phase favors growth of acidogens, and the pH is generally kept low and acidic. In the case of second phase, the pH is increased favoring the growth of methanogens (Ince 1998). Chaudhary (2008) has observed that the rate-limiting issue in the second stage is the growth rate of microorganisms. Hence, biomass retention times are longer in this phase (Verma 2002). Chaudhary (2008) have noticed that these kinds of systems are more stable compared to single-stage systems. Griffin (2012) has opined that better process control and optimization can

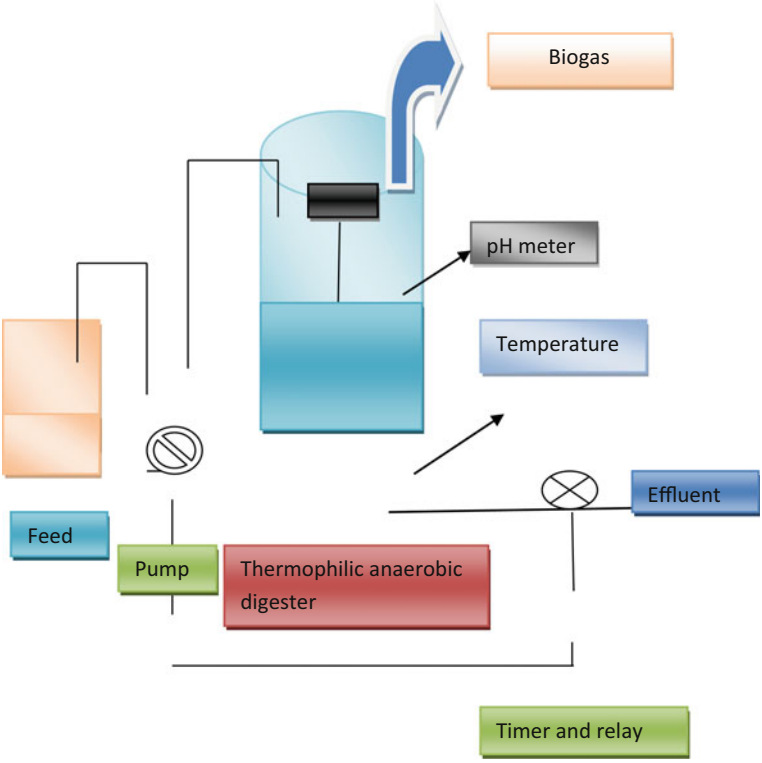

Fig. 8.3 Thermophilic anaerobic digester

take place in multistage reactors. Westerholm et al. (2020) have compared mesophilic and thermophilic industrial-scale plug-flow digesters. The high-solid treatment (HST) demonstrated showed good biogas yields from food waste. In thermophilic HSTs, the abundance of Clostridia group MBA03 while in mesophilic HST abundance of Cloacimonetes was seen. Figure 8.5 shows the construction of a floating drum digester.

8.7 Advantages and Disadvantages of Anaerobic Treatment

The advantages of the process (Gerbens and Zeeman 1999) include provision of energy source through methane recovery, consumption of lower amounts of energy, reduction of solids to be handled, sludge production, raw waste stabilization, less odor, retention of the fertilizer nutrients nitrogen (N), phosphate (P), and potassium (K). The volume of the reactor is generally small and can handle higher loading rates. This process requires less amounts of energy compared to the aerobic process of treatment of waste as biomass generation required is comparatively lower than

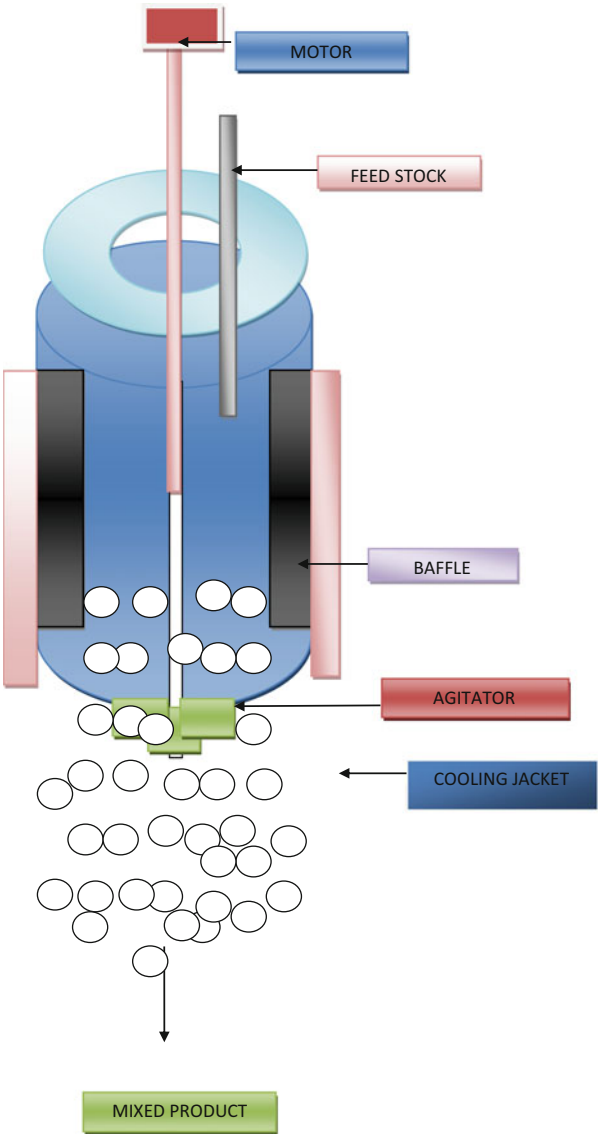

Fig. 8.4 Continuous stirred tank reactor (CSTR)

aerobic process. Since the biomass required is less, the nutrient concentration required is very less. After a shutdown period, when nutrients are added the plant operation starts quickly. The process generates slurry and a fibrous fertilizer. The process is versatile for treating different types of wastes and is eco-friendly. The process generates methane, which can be used as biofuel. Moreover, the process is not energy intensive. The major disadvantage of anaerobic treatment process is that it is not capable of removing inorganic pollutants which are present in the waste and

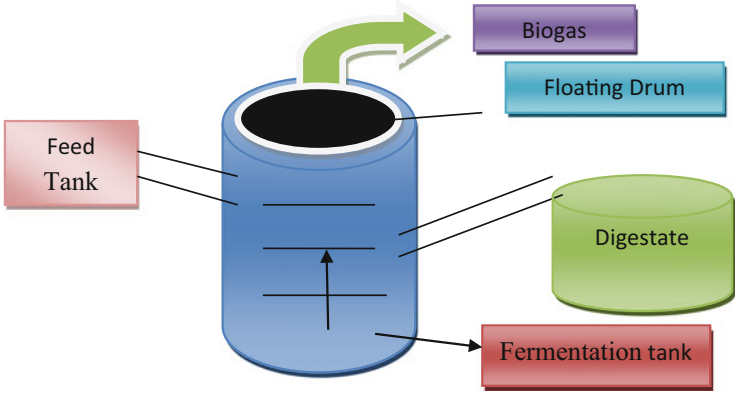

Fig. 8.5 Floating drum digester

any pathogenic organism present in the waste. Only when the reactor is run under thermophilic conditions where high temperatures are used, pathogenic bacteria will not survive; the effluent released may lead to zoonotic diseases if pathogenic organisms survive. Anaerobic processes cannot handle if excess amounts of industrial effluents containing waste are treated as they contain mostly heavy metals which may hinder the process of digestion. It is always better to see that the feedstock is homogenous and steady. The amount of investment for maintaining an anaerobic digestion plant is high. It is not efficient as that of gasification procedure which is used for conversion of carbon to biogas. The anaerobic treatment can be accompanied by odor due to the formation of sulfide. This is one of the most seen disadvantages which are commonly found during the process of anaerobic digestion due to which the area around the biogas plant gets exposed to this foul smell. Moreover, there cannot be any inhabitation because of the smell which emanates from these plants. One of the effective solutions to this problem is to employ a microaerophilic posttreatment step, to convert sulfide to elemental sulfur. This will reduce the odor emanating from the plant.

8.8 Challenges in Biogas Production

The challenges faced are based on the type of waste being treated in the anaerobic digestion. For example, for municipal solid waste, aerobic treatment is preferred compared to anaerobic treatment. This is because it has lower concentrations of biodegradable COD and an effluent which is of better quality as it may be released back into the atmosphere. In the case of industrial effluents which have more concentrations of biodegradable COD, the process will be less expensive. Although the presence of heavy metals should be less in these effluents. Biogas production is challenging considering that there are many factors which need to be optimized and

the complex interplay between different microorganisms which are present in different stages of anaerobic digestion. All of these factors affect the production of biogas and lead to its inhibition. The gas produced should be further analyzed and purified. The identification of waste composition, nutrient content of the feedstock, pH, temperature, and reactor design are some of the crucial factors which have to be optimized for enhancing the quality and quantity of biogas being produced (Rasapoor et al. 2020).

A major limitation of current computational enzyme design approaches is the lack of community-wide objective assessment. Recent studies focus on combining processing technologies such as multiple-stage or high-pressure technologies (EBTP-SABS 2016). To improve the AD efficiency, the influence of temperature, pH, C/N ratio, mixing ratios, additives, and other parameters on AD has been studied intensively (Abouelenien et al. 2014; Zhai et al. 2015; Dong et al. 2015). During the AD process, alkalinity is a better indicator of process performance. This can be managed by adjusting pH value; therefore, pH adjustment could provide a way to improve the self-buffering capacity of AD systems to meet the requirements of the microbial populations (Zhang et al. 2016). It affects the activities of the specific acidogenic microbial populations and methanogenic bacteria (Zhang et al. 2012) and consequently influences the process stability (Zhai et al. 2015). They also included different substrates and operational conditions (Jiang et al. 2013; Zhang et al. 2009). Mao et al. (2015) have studied the process performance of anaerobic co-digestion of swine manure and corn straw. Sustainability of the process is the major concern, and many factors have to be taken into consideration when a biogas plant is being established. Apart from those mentioned above, permission from Government agencies for establishing the biogas plant is required. Many factors such as social, environmental, and economic elements have to be considered for sustainability. This process involves a technology which is very simple. One of the major limitations would be to educate the rural population about the benefits of using biogas. Presently, the technology is not very much feasible to be adopted by the rural population as the production depends on number of factors. Many improvements should be made in the production process and the reactor design so that the process becomes feasible and can be adopted by several rural households. Biogas sector requires a long-term vision and good quality control systems, and training mechanisms are essential (Sovacool and Ramana 2015). Public private partnership should be encouraged so that this can facilitate the rural population for start-ups in this area. The development challenge is to seek grants and equity loans from government agencies to support biogas production in rural economy. There are a number of elements such as the migration of the rural population to the urban areas. The limitations of biogas sector include inadequate planning, lack of infrastructure, lack of skilled human resources, and high input costs.

The selection of the feedstock is important as some of the feedstocks will have an inhibitory effect on the process which is called substrate-induced inhibition. This is seen in the processes where the substrate or its byproducts formed after some stages of anaerobic digestion hinder the growth of the microorganism which is helpful for carrying out further stages of the digestion. Hence, the substrate should be properly

analyzed, and then optimal conditions for completion of the digestion should be investigated. Many researchers have reported such kinds of inhibitions due to substrate. The substrates which were generally found to hinder the process include pesticides, limonene, furans, metals, and antibiotics (Lallai et al. 2002; Wilkins et al. 2007; Alvarez et al. 2014; Yangin-Gomec and Ozturk 2013). Zabed et al. (2020) have reviewed the production of biogas from microalgae and opined that commercial production of microalgae-based biogas is still in its immature stage and a state-of-the-art technology for producing microalgal biogas is the need of the hour. Excess amounts of proteins and lipids can also cause substrate-induced inhibition. For example, excess amounts of proteins may generate ammonium and hydrogen sulfide which will inhibit microbial growth and change the pH. To overcome these kinds of obstacles, co-digestion is preferred and can lower the toxicity of the substrate or its metabolites. Protein at higher concentrations may result in the formation of ammonia which is toxic for microbial growth (Angelidaki and Ahring 1994). Sousa et al. reported that long chain fatty acids can inhibit the growth of methanogens. Lansing et al. (2008) reported that eutrophication of aquatic ecosystems inhibits the growth of plant and predators which are phototrophic in nature depend on the inorganic carbon levels depleted along with an increase in pH. Certain heavy metals such as nickel, zinc, copper, lead, chromium, cadmium, and mercury also have harmful effect on the environment (Demirel et al. 2013).

Mizuki et al. (1990) reported that limonene (65–88 g/L) can effectively inhibit the anaerobic digestion process. Furans such as hydroxymethylfurfural are produced during the dehydration of carbohydrates present in lignin (Barakat et al. 2012). They are inhibitory to microorganisms present within the digestion process. Monlau et al. (2013) and Barakat et al. (2012) reported that 5-HMF is more inhibitory than other furan compounds and the concentration of the compound should be above 6 g per liter. Pharmaceutical and industrial wastewater consists of antibiotics and pesticides which can be inhibitory to the process (Ji et al. 2013). A raise in the C/N ratio of the feedstock can minimize the production of ammonia by the metabolism of excess protein present in the feedstock (Zeshan et al. 2012). As the ecosystem involved is very complex, anaerobic treatment process needs to be explored further, and the process should be optimized. Only when this is achieved, the process of anaerobic digestion will become sustainable. As long as this is not realized, the process will continue to be a matter of research (Fagbohungbe et al. 2017).

8.9 Conclusions

The use of biogas health and sanitation benefits, ecological and societal benefits. Compressing and bottling biogas would be of really help in commercializing the biogas sector. The use of biogas has been on decline due to urban migration. Many changes are needed such as research and development for optimizing the process parameters and design of bioreactors which are efficient and economical. The present state of giving subsidies to fossil fuels by the Governments should stop so

that there is a shift toward investment and research in biogas sector. Biogas as such can have many applications apart from mitigation of greenhouse gas emissions which include different kinds of agricultural operations. If all the above can be done, the process would definitely become economical and create employment for rural population.

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Chapter 9
Microbial and Bioinformatics Approach in Biofuel Production

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Abstract Due to the increasing world population and ever developing technology, the need and demand for energy are increasing day by day. In parallel with this, the tendency to use renewable alternative energy sources instead of limited fossil fuel reserves is increasing worldwide. Lignocellulosic biomasses which are abundant in nature with renewable energy potential are preferred in biofuel production. These raw biomaterials are transformed into forms that can be used in biofuel production processes by various pretreatment techniques. The physical and chemical methods commonly used in the pretreatment of the substrate have some limitations. However, microbial methods for hydrolysis of biomass are quite remarkable. In this study, we focused on the pretreatment of biomass and microbial enzymes used in biofuel production process.

Furthermore, due to the increasing applications of molecular interaction simulations in this field, at a small scale, we demonstrate how the molecular docking technique is able to reveal the interactions between the xylanase enzyme (both wild type and mutant) isolated from *Thermotoga petrophila* RKU-1 and its substrate, xylobiose. In conclusion, molecular interaction simulations (molecular docking and molecular dynamics) contribute to the fields of bioengineering and genetics as powerful bioinformatics tools and offer a unique opportunity to study, at the atomic level, how enzyme-substrate affinity change as a result of induced mutations in the protein structure.

Keywords Biofuel · Biomass pretreatment · Microbial enzymes · Cocktail enzymes · Binding free energy · Molecular docking · Molecular dynamics

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9.1 Biofuels

Energy plays a central role in the improvement of technology and quality of life. In recent years, many countries have turned to alternative energy sources due to the limited reserves of fossil fuels and their negative effects such as increased greenhouse gases, global warming, unsustainability, and high prices. All these drawbacks of fossil fuels (coal, petroleum, and gas) have increased the attention for sustainable, renewable, economic, and alternative fuel sources like bioethanol, biodiesel, and biohydrogen that are being produced from low-cost biomass (Mood et al. 2013; Leong et al. 2018; Amoozegar et al. 2019).

According to Prasad and coworkers, population growth in the last 25 years has increased the total energy consumption by approximately 200% (Prasad et al. 2019). Factors such as the increase in global population, global warming, depletion of fossil fuels, and a rise in demand for energy have led to the search for cost-effective and renewable alternative energy sources (Das et al. 2015). Some advantages and disadvantages arising from the use of biofuels can be seen in Fig. 9.1 (Leong et al. 2018; Prasad et al. 2019; Zabed et al. 2019).

In addition, the need and demand for sustainable energy sources have increased due to industrialization, urbanization, globalization, and economic development (Bhatia et al. 2017; Leong et al. 2018). Therefore, immediate measures should be taken worldwide such as lowering greenhouse gas emissions, decreasing environmental problems, and increasing the quality of life (Hajjari et al. 2017; Majidian et al. 2018; Prasad et al. 2019).

Reduction in fossil resources, increasing environmental problems, and global warming are serious troubles that concern all communities. For this purpose, environmentally friendly, renewable alternative fuels and energy sources are being developed in order to avoid the threat of such problems (Kardooni et al. 2018). In this context, interest in the transformation of biomass into biofuels is rising (Pimentel et al. 2009).

The excessive use of fossil fuels causes a large-scale imbalance in the global energy need and leads to the search for alternative sources to meet the energy needed. Furthermore, as a result of the burning of fossil fuels, the CO_2 level rises in the atmosphere, which is one of the biggest factors of global warming. In order to deal with such negative situations, there is a need for clean energy infrastructures that are readily accessible, renewable, and greener for nature (Corral Bobadilla et al. 2017; Leong et al. 2018). Although fossil fuels are currently our main energy sources, biofuels with renewable energy sources have also gained more attention recently and replaced fossil fuels (Majidian et al. 2018). Since they are obtained from biological material, biofuels are preferred as suitable alternative energy sources that can be preferred against the disadvantages of fossil fuels (Bhatia et al. 2017).

Conversion of raw materials to biofuels by microorganisms has been a highly effective, cost-effective, and interesting method in recent years (Barnard et al. 2010). Using microorganisms in biofuel production stages is an important factor that reduces costs. In addition to the contribution of microorganisms to biofuel

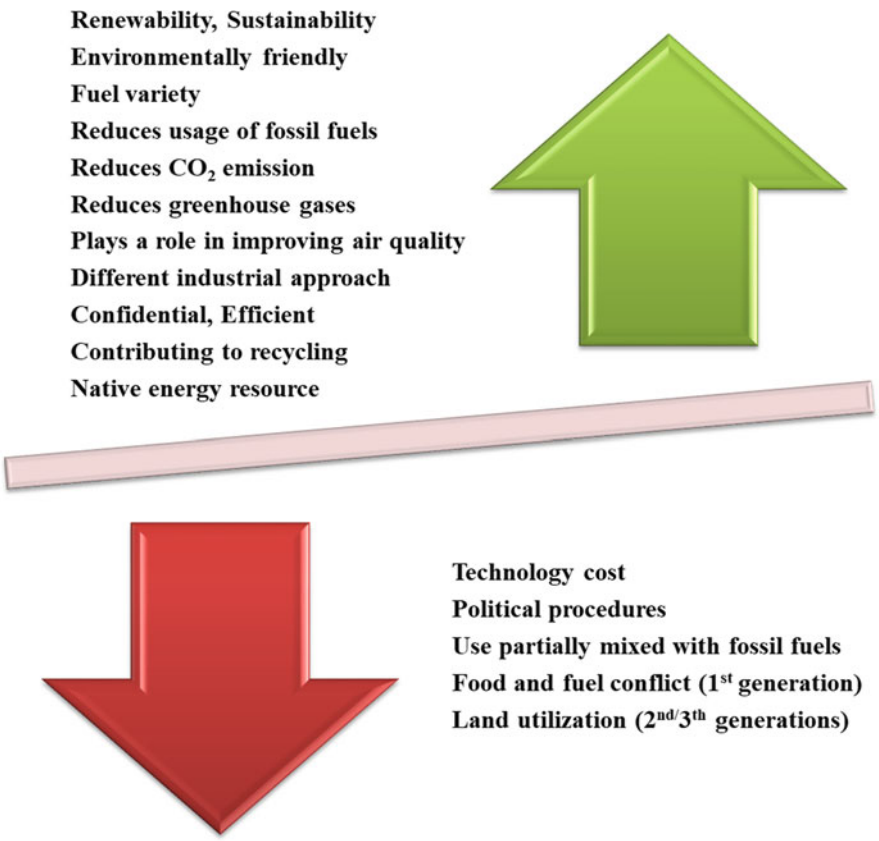

Fig. 9.1 Some advantages and disadvantages of using biofuels

production, some of them are used as raw materials in these processes (Amoozegar et al. 2019). Microorganisms that are resistant to harsh conditions such as high acidic/alkaline pH, low/high temperatures, and high salt concentrations that can be encountered in biofuel production stages can be used in biomass degradation and biofuel synthesis stages (Woolard and Irvine 1995).

In nature, there are abundant biological resources that can be converted into biofuels and can be an alternative to fossil fuels. Thanks to the use of biofuel types obtained with the use of these organic biomass resources, the rapidly depleting fossil fuel resources will be conserved, and environmental and air pollution will be significantly reduced (Das et al. 2015).

Biofuels produced worldwide are bioalcohols (bioethanol and biobutanol), biodiesel, and biogas. A wide variety of raw materials are used in biofuel production processes, and the fuels obtained are classified according to the type of raw materials. These raw materials include agricultural harvesting wastes, urban wastes,

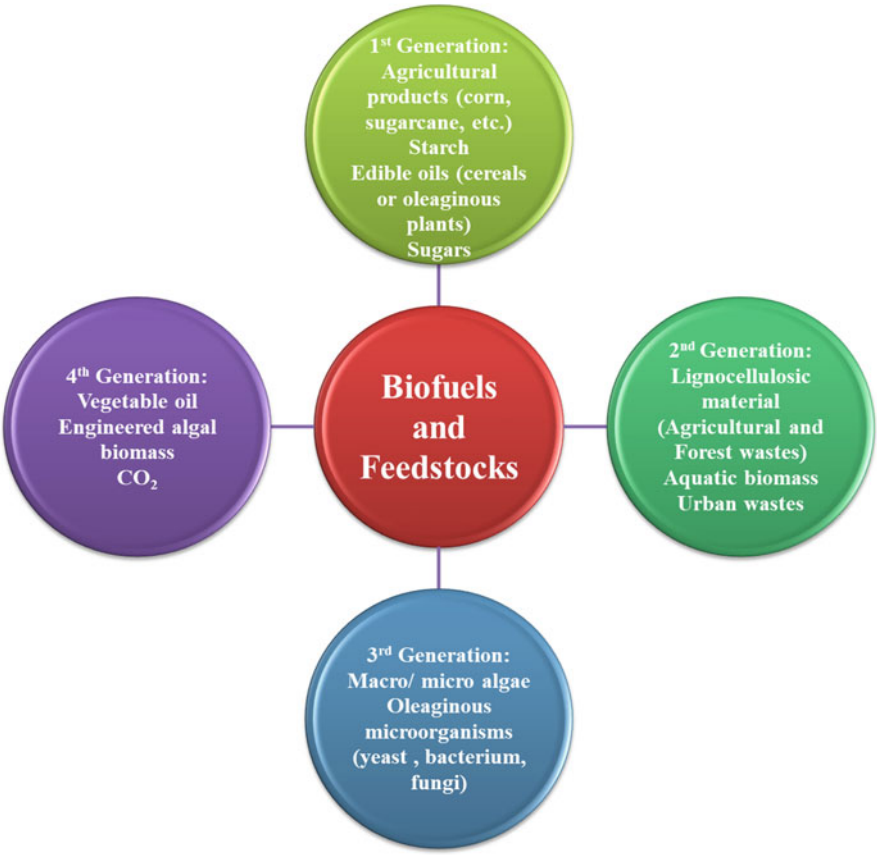

Fig. 9.2 Generations in biofuel production (Lü et al. 2011; Mizik 2020)

industrial wastes, edible/animal oils, and plants used for food (Palmer and Brigham 2016; Bhatia et al. 2017).

We can divide biofuels into four main generations based on the structure of the biomass used and the methods of conversion of this raw material to biofuel (Demirbas 2009) (Fig. 9.2). *First-generation biofuels* are obtained from fatty plants such as starch, sugar, sugar cane, corn, beets, sorghum, wheat, soybean, sunflower, palm, and coconut, which are food sources. *Second-generation biofuels* are produced from lignocellulosic materials that are not consumed as food (Amoozegar et al. 2019). Algal biomass is used as a raw material in *third-generation biofuel* production, whereas CO_2 and advanced technologies are used in *fourth-generation biofuel* production (Lü et al. 2011; Mizik 2020).

Lignocellulosic materials, predominantly preferred for biofuel production, are very productive raw materials, abundant in nature, and not harmful like fossil fuel sources (Prasad et al. 2019). Due to durable structural feature, they cannot be easily degraded by microorganisms. In order to release sugars which are present in the

structure of the lignocellulosic material, it is necessary to apply a series of pretreatment methods to the material in question. These processes are physical, chemical, physicochemical, and biological (Kumar and Sharma 2017).

Biofuels which are highly preferred compared to fossil fuels are obtained by using organic substrates (sugar, starch, agricultural and animal wastes, etc.) in microbial processes. Bioalcohol, biodiesel, and biogas production methods developed in this context are very low cost (Amoozegar et al. 2019).

Some studies have revealed that the United States, Brazil, and various European countries prefer plants consumed for food (sugarcane, corn, barley, and wheat) to produce first-generation fuels (Lopes et al. 2016; Bhatia et al. 2017). However, the use of preferred vegetable sources for nutrition in the production of first-generation biofuel has triggered some nutritional problems (Mizik 2020). For this reason, researchers have turned to second-generation biofuels produced with the use of the entire plant as a raw material. The use of this renewable lignocellulosic biomass makes the second-generation biofuels more advantageous. This is because, in smaller agricultural lands, more plant materials can be obtained by using less fertilizer and raw material can be obtained under more economical conditions (Demirbas 2009; Bhatia et al. 2017). Environmental problems, energy-related costs, and problems in food production and consumption are therefore reduced, thanks to the use of these waste materials (Wang et al. 2018; Amoozegar et al. 2019).

As outlined in a study published in 2017, in the coming years the rise in fuels used in transportation will increase the demand for biofuels by 55% compared to the amount in 2004. The main approach to produce low-cost biofuels is to obtain fuel by using lignocellulosic raw material, so that the costs stemming from substrate and biofuel production process can be minimized (Srivastava et al. 2017).

9.2 Pretreatment of Biomass

The main purpose of pretreatment applications is to separate the biomass, to weaken the bonds between cellulose fibrils by disrupting the crystalline structure of the cellulose, to modify the lignin component in the lignocellulosic structure, to increase enzyme accessibility to biomass by increasing the surface area of the lignocellulose structure, and also to release different sugar molecules and get more efficiency from lignocellulosic biomass (LCB) for bioethanol production (Mood et al. 2013; Singh and Satapathy 2018).

The principal gains of the pretreatment are as follows (Singh and Satapathy 2018):

1. It facilitates attacking by enzymes
2. It avoids the formation of inhibitory compounds
3. It provides hemicellulose and cellulose recovery
4. Reduction in size and the cost of materials for construction of fermentation reactors can be achieved
5. Pore size of the biomass can be increased.

Due to the different structural properties of the biomass used in biofuel production processes, various pretreatment methods have been developed. They are generally classified as physical, chemical, physicochemical, and biological (Alvira et al. 2010; Zabed et al. 2019).

9.2.1 Physical Methods

Physical preprocessing methods can be summarized as chipping, grinding, milling, sonication, microwave usage, and extrusion. The goal of these kinds of mechanical force pretreatment methods is to increase the surface area-to-volume ratio of the material and the degree of polymerization by reducing the particle size and crystallinity of the lignocellulosic feedstock. Also, the yield from biomass can be increased in this way (Alvira et al. 2010; Mood et al. 2013).

According to some studies, the particle sizes of the raw material used can be reduced to 10–30 mm by chipping and further to 0.2–2 mm by grinding or milling (Sun and Cheng 2002; Abdullah et al. 2020).

The microwave method is an alternative to the conventional heating technique and can help increase the effectiveness of enzymatic hydrolysis by disrupting the lignocellulosic structure (Mood et al. 2013), is a widely used biomass pretreatment method, requires little energy and short process period, and is advantageous in terms of ease of application (Moodley and Kana 2017). This method transforms most of the biomaterial that is applied to gaseous products consisting of H_2, CH_4, CO, and CO_2 (Huang et al. 2016a).

Ultrasonic waves in the sonication process help to increase the hydrolysis of enzymes by breaking down the hemicellulose and cellulose structures in the biomass (Gabhane et al. 2014).

In the extrusion method, which is a thermophysical pretreatment method, materials are exposed to processes such as mixing, heating, and shearing, so that physical and chemical changes occur while they are passing through the extruder (Alvira et al. 2010; Mood et al. 2013).

9.2.2 Chemical Methods

Chemical pretreatment techniques applied to biomass include acidic, alkaline, and ionic liquids and oxidizing agents (Abdullah et al. 2020). Thanks to these applications, the lignin and hemicellulose structures in the structure of the lignocellulosic material are resolved, thereby facilitating the access of the hydrolytic enzymes to cellulose used in the process (Rajak and Banerjee 2016; Bhatia et al. 2017).

Acid hydrolysis is a pretreatment method that provides high sugar yield from lignocellulosic material and is frequently used (ye Lee et al. 2013). In this method, it facilitates the access of the enzymes to the cellulosic unit by providing the

hemicellulosic structure to be disrupted and solubilizing the lignin (Hendriks and Zeeman 2009; Abdullah et al. 2020). Diluted and concentrated acids used in acidic pretreatment are as follows: hydrochloric acid, sulfuric acid, acetic acid, formic acid, phosphoric acid, nitric acid, oxalic acid, and maleic acid (Maurya et al. 2015; Bhatia et al. 2017). However, as mentioned in a research conducted in 2010, concentrated acids are less preferred in bioethanol production because they form inhibitory compounds. The industrially preferred diluted acid method can be applied at both high temperatures (180 °C) in lesser time intervals and lower temperatures (120 °C) in a longer time scale (Alvira et al. 2010; Bhatia et al. 2017).

The alkali pretreatment method applied using various alkalis, such as NaOH, KOH, $Ca(OH)_2$, NH_4OH, and Na_2CO_3, is very effective in the solubility of lignin compared to hemicellulose and cellulose (Singh and Satapathy 2018) because the solubility of hemicellulose and cellulose is weaker in this technique compared to other pretreatment methods (Carvalheiro et al. 2008). This method helps the access of the enzyme to the material as it removes acetyl and uronic acid groups from the structure of hemicellulose and cellulose (Mood et al. 2013).

Chemicals such as hydrogen peroxide (H_2O_2) and ozone (O_3) are used in pretreatment with oxidizing agents. Ozone gas is a strong water-soluble oxidant and contributes to facilitating the use of cellulose by disrupting the structure of hemicellulose and lignin (Balat 2011). Ozonolysis with ozone affects aromatic ring structures (Maurya et al. 2015; Bhatia et al. 2017).

However, a deficiency of this method is that it is not suitable for all raw materials containing lignocellulosic structure (Singh and Satapathy 2018). It has been used for wastes from various agricultural products, such as wheat and rye straw, and has a low yield (García-Cubero et al. 2009; Alvira et al. 2010).

9.2.3 Physiochemical Methods

Physiochemical pretreatment methods can be summarized as hot water, ammonia fiber/freeze explosion (AFEX), steam explosion, and CO_2 explosion, respectively (Bhatia et al. 2017). Biomass, which is subjected to other physical pretreatment methods, is separated into its components by the steam-explosion method. In this frequently used pretreatment method, the material is saturated using high pressure (0.7–8.0 MPa) in the reactor, causing the temperature to increase by 160–260 °C. When the pressure suddenly decreases, the fiber structure of the biomass is destroyed and the crystallinity of cellulose increases. Thus, hemicellulose and lignin are dissolved, and the structure of cellulose becomes more accessible (Bhatia et al. 2017; Abdullah et al. 2020). This pretreatment method is generally preferred in ethanol and biogas production (Singh and Satapathy 2018). Water is used in the liquid hot water method in place of the steam, which is similar to the steam explosion application and applied under pressure with high temperature (Mood et al. 2013). According to the AFEX method, the hot liquid ammonia at 90–100 °C for 30 min and the high pressure are applied on the LCB, and its structure is disrupted.

Therefore, with the crystallization of cellulose, the surface area where enzymes can act on biomass is expanded (Bhatia et al. 2017; Singh and Satapathy 2018). Another method, carbon dioxide (CO_2) explosion application, is based on the use of high pressure (1000–4000 psi) supercritical CO_2. CO_2 used in this method, which is similar to AFEX and steam explosion methods, hydrolyzes cellulose, hemicellulose structure with high pressure and provides the delignification by entering very small pores of lignocellulosic structure. Hence, the surface area of the substrate for enzymatic processes is increased (Alvira et al. 2010; Agbor et al. 2011).

9.2.4 Biological Methods

Biofuels obtained as a result of the biological transformation of lignocellulosic biomass with biological pretreatment have been highly demanded as a promising alternative to fossil fuels in recent years (Saha et al. 2016; da Silva and Ferraz 2017). Biological pretreatment is an environmentally friendly, a reliable, an inexpensive, and a green method used in the conversion of lignocellulosic raw material to biofuel since it does not require chemical treatment compared to other pretreatment methods (Singh et al. 2008a; Mood et al. 2013) and is carried out by means of microorganisms and enzyme systems. Therefore, biological pretreatment methods can be divided into two main groups as microbial pretreatment and enzymatic pretreatment. In addition, the preprocessing time depends on the structure and composition of the biomass used and the type of microorganism preferred. For example, the lignin removal of the lignocellulosic material takes a long time. Pretreatment methods using fungal organisms take longer than bacterial or enzymatic pretreatments (Zabed et al. 2019).

According to some researches, white-rot fungi (*Ceriporia lacerate*, *Cyathus stercoreus*, and *Pycnoporus cinnarbarinus* (Kumar et al. 2009), *Ceriporiopsis subvermispora*, *Phanerochaete chrysosporium*, and *Pleurotus ostreatus* (Shi et al. 2008)), brown rot fungi (*Coniophora puteana* (Zabed et al. 2019), *Serpula lacrymans* (Sánchez 2009), and *Gleophyllum trabeum* (Bhatia et al. 2017), and soft-rot fungi (*Paecilomyces* sp., *Daldinia concentrica*, and *Cadophora* spp.) (Bhatia et al. 2017) can easily break down lignin and hemicellulose structures by its high delignification performance (Sánchez 2009).

A number of researchers have stated that the use of white-rot fungi is more efficient in the biological pretreatment of lignocellulosic material than other processes (Balat 2011). White-rot fungi, involved in lignin degradation, synthesize lignin-degrading enzymes such as peroxidase and laccase in the biological pretreatment of lignocellulosic material (Kumar et al. 2009).

However, in delignification, fungi that are capable of degrading lignin are more preferred rather than bacteria (Rashid et al. 2017). Furthermore as reported in a research, some bacterial strains, such as *Bacillus* sp. AS3, *Bacillus circulans*, *Sphingomonas paucimobilis*, *Cellulomonas*, and *Zymomonas* sp., produce less ligninolytic enzymes than fungi (Bhatia et al. 2017). According to a study, among some aerobic bacteria that can degrade lignin are actinomycetes, γ-proteobacteria,

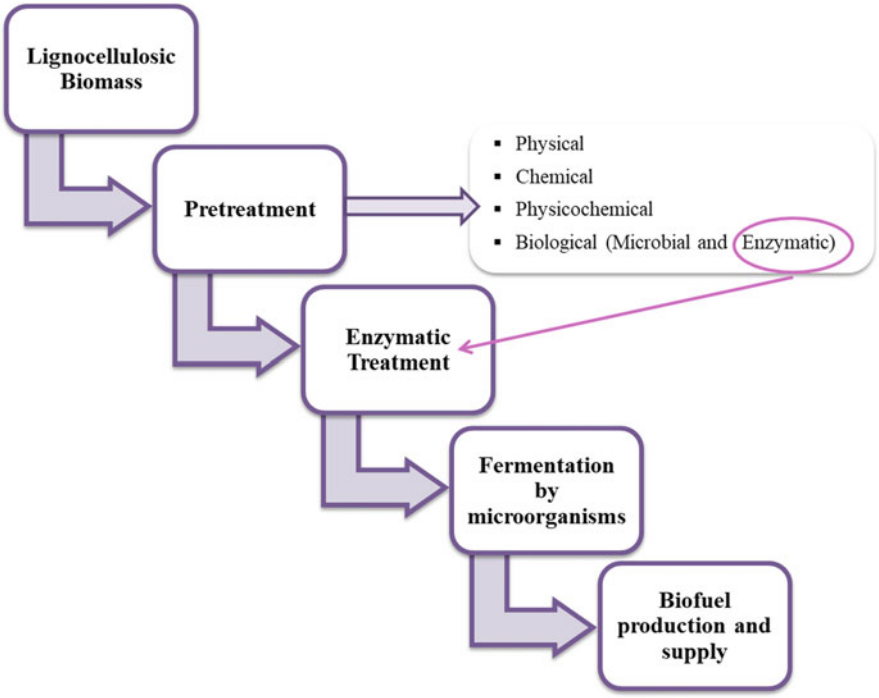

Fig. 9.3 The general process of biofuel production from lignocellulosic biomass

and α-proteobacteria (Bugg et al. 2011b). Besides these, bacteria such as *Sphingobium* sp., *Bacillus* sp., and *Rhodococcus jostii* also produce enzymes that can act in delignification (Xu et al. 2018; Zabed et al. 2019).

The presence of bacteria in biological pretreatment has some benefits to shorten the pretreatment time, such as having faster metabolic activity than fungi, multiple and rapid reproduction, easy genetic manipulation, and low cost (Carrillo-Reyes et al. 2016; Yan et al. 2017).

In the enzymatic pretreatment method, partially, fully purified, or raw hydrolytic and ligninolytic enzyme groups are used (Asgher et al. 2013). Both single enzymes and enzyme cocktails (see in Sect. 9.5) isolated from bacteria and fungi are preferred in these methods. It is advantageous to use enzyme mixtures in order to obtain more efficiency from the hydrolysis of various biopolymers in the structure of biomass to be used for biofuel production (Ehimen et al. 2013; Zabed et al. 2019).

Following the appropriate pretreatment method, the biomass is reduced to fermentable sugars by enzymatic hydrolysis and converted into biofuel by microorganisms (Kumar and Sharma 2017; Wang et al. 2018). Figure 9.3 shows the biofuel production process flow using lignocellulosic raw materials.

9.3 Lignocellulose

Lignocellulose is the most commonly found carbohydrate source in nature, and represents a renewable energy source (Yeoman et al. 2010; Abdel-Hamid et al. 2013). Lignocellulose is a very complex, hard, and persistent substrate. This carbohydrate source abound in plants (lignocellulosic biomass) which has a significant contribution to the reinforcement of the plant cell wall is composed of three main polymers: (Bugg et al. 2011a) cellulose (30–50%), hemicellulose (25–30%), and lignin (10–35%) (Fig. 9.4) (Bugg et al. 2011a; Achinas and Willem Euverink 2016).

Lignocellulosic biomass raw material has a significant potential for biofuel production, and it exists in different forms such as herbaceous and wood-like energy plants, forest wastes, and agricultural wastes (such as sugar cane bagasse, crop residue, rice straw, banana waste, rice husk) (Lin and Tanaka 2006; Sindhu et al. 2016).

Depending on their physical and chemical contents, wood-like biomasses contain more lignin than the agricultural biomass; this in turn makes them more resistant to microbial degradation (Prasad et al. 2019).

Hardwoods (angiosperms) are one of the wood-like biomasses that have higher density compared to the softwoods (gymnosperms). Due to the small variations in the cellulose, hemicellulose, and lignin compounds that they contain, hardwoods have higher xylan and lower mannan contents compared to softwoods (Álvarez et al. 2016).

A series of effective enzymatic processes are required in order to degrade the lignocellulosic biomass and use its end products in biofuel production. For this purpose, the components of the lignocellulosic material and the enzymes that take part in their breakdown should be determined first.

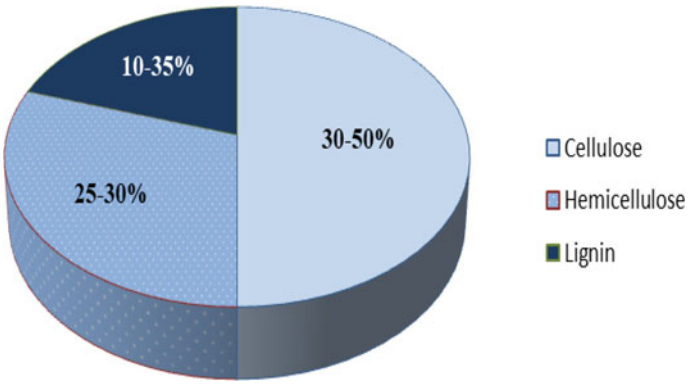

Fig. 9.4 Basic components of lignocellulosic structure

9.3.1 Cellulose and Cellulolytic Enzymes

Cellulose $(C_6H_{10}O_5)_n$ is a polymer that constitutes the 40% of the plant cell wall (Yeoman et al. 2010). It occurs by the way of binding the glucose units with β-1,4 glycosidic bonds, through hydrogen bonding and van der Waals interactions (Horn et al. 2012; Chatterjee et al. 2015).

While the glucose units are synthesized as a chain in nature, in the biosynthesis area they merge on their own and turn into elementary fibril units consisting of approximately 30 cellulose chains. When these are packaged in larger units, they form microfibrils that create cellulose fibrils (Lynd et al. 2002). Hydrogen bonds bind the chains together with in-chain and interchain bonds and provide a hard structure (Kannam et al. 2017).

Unlike the other polysaccharides, celluloses can be created in crystal forms. Crystal cellulose molecules in nature are in Iα and Iβ forms. Iα form is thermodynamically more stable and mostly found in terrestrial plants. Crystal form can change over time, and it can turn into amorphous forms (Horn et al. 2012; Sorieul et al. 2016).

Due to microfibril schemes, this crystal form creates a packing method which does not allow the small molecules such as enzyme or water to get in, and thus, it limits the activity of the enzymatic hydrolysis (Cosgrove 2005; Yeoman et al. 2010).

Cellulases, in the glycoside hydrolase family, are enzymes that can hydrolyze the crystal structure of cellulose into small oligosaccharides and then into glucose (Carrillo-Reyes et al. 2016) and have a broad substrate specificity (Yeoman et al. 2010). These enzymes are produced by microorganisms (bacteria, fungi, archaea), plants, and animals (except mammals) (Zhang and Zhang 2013). They catalyze the hydrolysis of the β-1,4 bonds in cellulose via two catalytic mechanisms: retaining and the inverting mechanisms (Davies and Henrissat 1995; Mosier et al. 1999; Sindhu et al. 2016). Moreover, they have a carbohydrate-binding module (CBM) which binds to the catalytic area with a flexible binder. This module takes part in binding the enzyme to the crystal cellulose and increasing the cellulase activity (Hervé et al. 2010; Reyes-Ortiz et al. 2013).

There are three main enzyme groups for effective hydrolysis of the cellulose (Elleuche et al. 2015; Carrillo-Reyes et al. 2016).

9.3.1.1 Endoglucanases (Endo-1,4-β-Glucanes or 1,4-β-D-Glucan-4-Glucanohydrolases, EC 3.2.1.4)

These types of enzymes randomly separate the β-1,4 glycosidic bonds in the amorphous regions of the cellulose, which causes a rapid decrease in the polymer length and thus the occurrence of oligosaccharides in different lengths. Moreover, they cause a gradual increase in the number of released reducing ends (Sun and Cheng 2002). Some microorganisms synthesizing endoglucanase enzymes are shown in Fig. 9.5 (Bauer et al. 1999; Li et al. 2003; Yang et al. 2010).

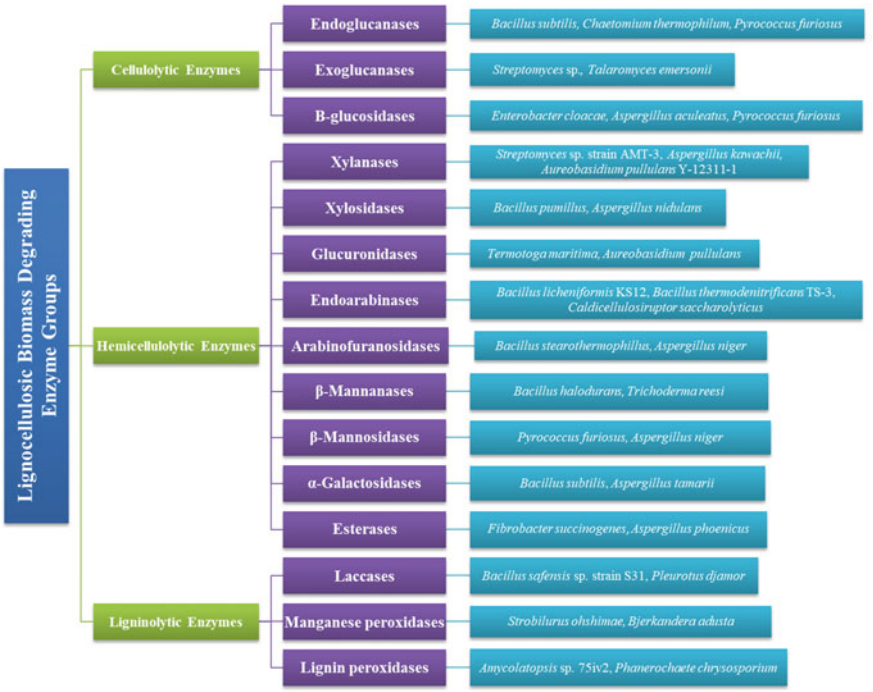

Fig. 9.5 Lignocellulosic biomass degrading enzyme group

9.3.1.2 Exoglucanases (Cellodextrinase or 1,4-β-D-Glucan Glucanohydrolases, EC 3.2.1.74) and Cellobiohydrolases (Exo-1,4-β-Glucanases or 1,4-β-D-Glucan Cellobiohydrolases) (EC 3.2.1.91)

Exoglucanases hydrolyze the cellulose chains (mostly cellobiose) from the reducing and non-reducing ends. As a result of this, the reducing ends are rapidly released, but no significant change occurs in the polymer length (Zhang et al. 2006). At the end of the hydrolysis, they produce glucose (glucanohydrolase) or cellobiose (cellobiohydrolase) as the final product (Carrillo-Reyes et al. 2016). Figure 9.5 includes some samples of exoglucanase producing microorganisms (Park et al. 2001; Tuohy et al. 2002).

9.3.1.3 β-Glucosidases (Cellobiases or β-D-Glucoside Glucohydrolase, EC 3.2.1.21)

β-glucosidases are enzymes that turn cellodextrin and cellobiose into glucose. Due to the reduced final product inhibitions of the endoglucanases and exoglucanases, the hydrolysis of the cellulose is generally increased by adding these cellobiases

(Lynd et al. 2002; Zhang and Zhang 2013). Some microorganisms producing β-Glucosidase can be seen in Fig. 9.5 (Yeoman et al. 2010; Sakamoto et al. 2012; López-Mondéjar et al. 2019).

Besides these three mentioned enzymes, there are also non-hydrolytic proteins (non-acting on ß-1,4 glycosidic linkages) called swollenin which contribute to the degradation of cellulose. These proteins contribute to the accessibility of the cellulases to cellulose chains, by loosening the cellulolytic fibril networks (Saloheimo et al. 2002; Binod et al. 2011; Sindhu et al. 2016).

Microorganisms have developed various adaptations for the complete hydrolysis of the cellulose. Cellulolytic filamentous fungi and actinomycetes are able to diffuse into cellulolytic substrates along with the fiber extensions, and the enzymes in these systems do not constitute great stable complexes with respect to molecular weight. Therefore, they are called non-complex systems (Lynd et al. 2002). Despite this, the anaerobic bacteria do not have the ability to effectively diffuse into the cellulosic material. For this reason, since the ATP is limited, other microorganisms have had to develop an alternative mechanism called cellulosome for synthesizing cellulase (Schwarz 2001; Doi and Kosugi 2004).

9.3.2 Complex Cellulose Systems (Cellulosome)

Cellulosome is a multienzymatic extracellular enzyme complex that exists in anaerobic bacteria and can degrade cellulose, hemicellulose, and pectin (Schwarz 2001; Duan et al. 2009; Carrillo-Reyes et al. 2016). Cellulosome is also described as the cellulose-binding factor which binds the anaerobic bacteria cells to cellulose particles and minimizes the diffusion loss of the hydrolytic products (Stern et al. 2015). Typical ruminal bacteria types such as *Ruminococcus*, *Butyrivibrio*, and *Clostridium* are examples that form cellulosomes (Schwarz 2001; Doi and Kosugi 2004).

The main component of the cellulosome complex is a catalytic or non-catalytic essential protein called scaffolding (Doi and Kosugi 2004; Brás et al. 2012). Catalytic scaffold contains the multiple dockerin protein which has degradative enzymes. Non-catalytic scaffold contains numerous copies of the cohesin modules and carbohydrate-binding modules (CBM). This CBM is responsible for connecting cellulase on the cellulosic substrate (Dassa et al. 2017; Haitjema et al. 2017).

The non-covalent interaction between cohesin-dockerin plays roles in arranging the assembly of cellulosomes and helping the scaffolding to bind the enzyme subunits into the complex (Bégum and Lemaire 1996; Mechaly et al. 2001; Prasad et al. 2019).

The primary examples of the enzymes that are included in the cellulosome complex are cellulases (endoglucanases, exoglucanases, cellobiohydrolases), xylanases, mannanases, and pectate lyases. The presence of these enzymes shows that the cellulosomes can break down all the cell wall compounds (Haitjema et al. 2017).

9.3.3 Hemicelluloses

Hemicelluloses are heterogeneous polymers which consist of the pentose (xylose and arabinose) and hexose sugars (mannose, glucose and galactose) and sugar acids that are present in the primary and secondary cell walls of the plants (Saha 2003; Scheller and Ulvskov 2010; Sorieul et al. 2016). They are energy rich structures due to the sugar mixtures in their composition. They are attached to lignin sheaths through covalent bonds and to the cellulose by hydrogen bonds (Bailey et al. 1992; Sethi and Scharf 2013; Amoozegar et al. 2019).

Hemicelluloses found in different plant species contain a wide variety of polysaccharides such as glucomannan, galactomannan, galactoglucomannan, glucuronoarabinoxylan, xyloglucan, mixed-linkage glucans, ß-D(1–4) bonds, arabinogalactan, and ß-(1–6) glycosidic bonds (Buchanan et al. 2015; Sorieul et al. 2016). Besides, softwood hemicelluloses contain mostly glucomannan, whereas the hardwood hemicelluloses contain rather more xylan (Saha 2003; Huang et al. 2016b).

For this reason, according to the complex structures of the hemicelluloses and the kind of pretreatment, quite a lot of hemicelluloytic enzymes are needed for the breakdown of the substrate (Yeoman et al. 2010; Álvarez et al. 2016). These hemicelluloytic enzymes contain endoxylanases (EC 3.2.1.8), xylosidases (EC 3.2.1.37), xyloglucanases (EC 3.2.1.151), endomannanases (3.2.1.78), mannosidases (EC 3.2.1.25), fucosidases (EC 3.2.1.63), arabinofuranosidases (EC 3.2.1.55), glucuronidases (EC 3.2.1.31), xylan esterases (EC 3.1.1.72), ferulic acid esterases (EC 3.1.1.73), and p-coumaric acid esterases (EC 3.1.1.B10) (Bosetto et al. 2016; Razeq et al. 2018; López-Mondéjar et al. 2019).

Therefore, in contrast to cellulases, the hemicellulases are a broader spectrum of enzymes.

9.3.3.1 Xylan and Xylolytic Enzymes

Xylan is the hemicellulose that forms the ~¼–⅓ of plant biomass and is the most abundant component in the cell walls of most plants (Prade 1996). Also, it is a kind of polysaccharide that consists of xylopyranosyl units bound with ß-1,4 glycosidic bonds (Polizeli et al. 2005).

In a general sense, a xylan polymer usually contains a linear backbone of the ß-1,4-D-xylopyranoside residues which can change places with acetyl, arabinofuranosyl, and 4-0-methyl glucuronyl groups (Yeoman et al. 2010; Amoozegar et al. 2019).

Most of the xylans contain variable groups in their main and side chains. This variability depends on the source of the plant material (Puls 1997; Wainø and Ingvorsen 2003).

The complete hydrolysis of xylan requires the synergistic action of various enzymes, in proportion to its complex structure (Yeoman et al. 2010).

Xylanases (EC 3.2.1.8)

Xylanases are the enzymes that catalyze the hydrolysis of xylan which is a heterogeneous polysaccharide (and the fullest extent of these enzymes is called the xylanolytic enzyme system) (Subramaniyan and Prema 2002).

As shown in Fig. 9.5, the xylanases can be synthesized by certain molds (Ito et al. 1992), bacteria (Nascimento et al. 2002), and yeasts (Mandal 2015).

Like celluloses, xylanases are also a part of the glycoside hydrolase (GH) family and are mostly derived from GH10 and GH11. However, there are also some xylanases included in other GH families (CAZy; http://www.cazy.org/).

These enzyme families are similar with respect to the way they depolymerize the xylan via Koshland type (two-phased catalysis: the products are separated with the retained stereochemistry of the anomeric configuration). Family 10 enzymes give products with less molecular weight (tetramer) than the family 11 enzymes (pentamer) (Christov et al. 2000; Decker et al. 2017).

Crystal structures of many GH10 and GH11 family members have been clarified. It has been stated that the xylanases of the GH family 10 consist of $(\alpha/\beta)_8$ tim-barrel fold and the xylanases of the GH family 11 generally consist of β-sheets (Manikandan et al. 2005; Yeoman et al. 2010).

Due to the complex nature of the xylans, their enzymatic hydrolysis is harder than that of other plant polysaccharides, so the enzymes need to work together. ß-glycosidases and endoxylanases mediate the depolymerization of the xylan backbone (Decker et al. 2017).

Moreover, according to the xylan type, debranching enzymes such as α-glucuronidase (EC 3.2.1.139), acetyl xylan esterase (EC 3.1.1.72), and ferulic and coumaric acid esterases (EC 3.1.1.73) also help moving away the side groups in xylan structure (Begemann et al. 2011; Amoozegar et al. 2019).

Xylosidases

ß-D-xylosidases are the enzymes that hydrolyze the xylooligomers in various lengths to the xylose (Sindhu et al. 2016; Decker et al. 2017), and they are in GH families of 3, 30, 39, 43, 51, 52, and 54 (Yeoman et al. 2010). As shown in Fig. 9.5, xylosidases are isolated from a series of certain fungi (especially the Aspergillus genus) (Martin Perez et al. 2017) and bacteria (Panbangred et al. 1984).

In a similar fashion to the ß-glucosidases that are in the cellulose systems, ß-D-xylosidases are crucial in moderating the final product inhibition of xylanases which is caused by xylobiose (Yeoman et al. 2010). As the concentrations of the final product (xylose) increases, the performance of the ß-xylosidases typically gets inhibited (as is the case with the ß-glucosidases). Zanoelo and co-workers have pointed out that the thermophilic ß-xylosidase, isolated from fungus (*Scytalidium thermophilum*), develops a tolerance to final product inhibition (Zanoelo et al. 2004).

It is obvious that this type of feature has great importance for the bio-catalyzers in the biofuel industry (Yeoman et al. 2010).

Glucuronidases

α-D-glucuronidases are enzymes that exist in family 67 and participate in the hydrolysis of the α-1,2 glycosidic bonds between 4-0-methyl-α-glucuronic acid and the terminal, non-reducing xylopyranosyl end unit of small oligosaccharides (Mierzwa et al. 2005; Decker et al. 2017).

α-1,2 bonds are the areas that slow down the hydrolysis in the enzymatic hydrolysis process of xylane. Xylan α-1,2 glucuronidases use paranitrophenyl α-D-glucuronide as the substrate, but this enzyme is specified for a glucuronamide with an α (1→2) link (Saraswat and Bisaria 1997).

As indicated in Fig. 9.5, this kind of enzymes can be obtained from various microorganism groups (Khandke et al. 1989; Suresh et al. 2003).

9.3.3.2 Endoarabinases and Arabinofuranosidases

Endoarabinases and arabinofuranosidases are the enzymes that play catalytic role in the degradation of the hemicellulosic biomass.

Endoarabinases in GH43 family function by making synergic effect with α-L-arabinofuranosidases in the hydrolysis of the arabinan which is between the hemicellulosic and pectic polysaccharides (Verhertbruggen et al. 2009; Prasad et al. 2019).

Contrary to α-L-arabinases, α-L-arabinofuranosidases are exolytic enzymes of the GH55 family (Wongratpanya et al. 2015) that hydrolyze α-1,2, α-1,3, and α-1,5 glycosidic bonds of arabinofuranosides in arabinan, as well as the α-1,2 and α-1,3 bonds in arabinoxylan and arabinogalactan. (Matsuo et al. 2000; Yeoman et al. 2010).

There are very few examples of characterized arabinases, which are obtained only from some bacteria (Hong et al. 2009; Seo et al. 2010), whereas the arabinofuranosidases are obtained from both bacteria and fungi (Kaji and Tagawa 1970; Gilead and Shoham 1995) (Fig. 9.5).

9.3.3.3 Esterases (EC 3.1)

In addition to pectin, plant cell wall polysaccharides that consist of hemicellulose compounds such as xylans, mannans, and glucomannans in particular are usually acetylated and sometimes feruloylated, with O-linked acetyl groups (Yeoman et al. 2010). In a study on the xylans in the hardwoods, it has been stated that the 60–70% of xylose residues have been esterified with acetic acid (Shao and Wiegel 1995).

For this reason, to obtain efficient results in the hydrolyzation of the xylans and to catalyze the hydrolysis of ester bonds, some enzymes from the carbohydrate esterase (CE) family are needed (acetyl esterase [EC 3.1.1.6], acetyl xylan esterase [EC 3.1.1.72], and ferulic acid esterase [EC 3.1.1.73]) (Linden et al. 1994; Karnaouri et al. 2019).

These enzymes enable the cleavage of the acetic acid and phenolic acid units in the xylan molecule. Moreover, the cleavage of acetyl, feruloyl, and p-coumaryl groups from xylan is an important step in the cleavage of lignin. At the same time, breaking down the ester bonds between hemicellulose and lignin contributes to the resolution of lignin (Subramaniyan and Prema 2000).

The enzyme examples in this group obtained from different microorganisms are included in Fig. 9.5 (Kuhad et al. 1997; Sunna and Antranikian 1997).

9.3.3.4 Mannan and Mannolytic Enzymes

Mannan is a compound that is present in bacteria, fungi, and yeasts (Singh et al. 2018). Besides being an essential part of hemicellulose, it is also a storage polysaccharide in the cell walls of the algal species and in the seeds of some plants (Kaihou et al. 1993; Ojima 2013; Decker et al. 2017). Mannan can be found in four different forms such as linear mannan, galactomannan, glucomannan, and galactoglucomannan (Moreira and Filho 2008).

While the mannan which is a linear and water-soluble polysaccharide consists of a linear ß(1→4) mannopyranose polymer, galactomannans consist of the galactose residues that are α-1,6 linked to the mannan backbone (Mestechkina et al. 2000; Chaubey and Kapoor 2001).

α-1,6 linked galactose side groups in the galactomannans prevent the pair-bond among the adjacent polymers, which in turn allows a more amorphous structure that holds the water and contributes to its water solubility (Van Zyl et al. 2010).

Essentially, glucomannan is a ß (1→4) linked polysaccharide that is present in the root of the konjac plant (*Amorphophallus konjac*) and consists of slightly breanched-chain D-glucosyl and D-mannosyl backbone (Katsuraya et al. 2003). Furthermore, it is known that the man:glc ratio in its structure is ∼1.6:1 (Cescutti et al. 2002).

Finally, the galactoglucomannans contain galactose residues that are α-1,6 linked on D-glycosyl and D-mannosyl residues with the ratio 3:1:1 mannose:glucose: galactose. Due to high polymerization, acetylated galactoglucomannans which constitute the most of hemicellulose in softwoods fulfill a structural function similar to the xylans in hardwoods (Willför et al. 2003).

Mannanases

Mannan-degrading enzymes take part in the glycosyl hydrolases synthesized by fungi and bacteria (Singh et al. 2018). Certain microorganisms producing mannanase are given in Fig. 9.5 (Kuhad et al. 1997; Vijayalaxmi et al. 2013).

The main enzymes that catalyze the hydrolyzation of linear mannans and glucomannans are as follows:

- β-Mannanase (EC 3.2.1.78)
- β-Mannosidases (EC 3.2.1.25)
- β-Glucosidases (EC 3.2.1.21) (Dhawan and Kaur 2007).

Endo-acting ß-mannanases are hydrolases that impact the inner glycosidic links of the mannan backbone chain by releasing the short ß-1,4 mannooligosaccharides (Shallom and Shoham 2003), and they are located in the GH families of 5, 26, and 113 (Yeoman et al. 2010).

On the other hand, exo-acting mannosidases are hydrolases that release the mannose away from oligosaccharides by attacking its terminal links on the non-reducing ends in addition to transforming mannobiose into mannose units that are included in the GH family 1, 2, and 5 (Moreira 2008; Van Zyl et al. 2010; Yeoman et al. 2010).

With the help of ß-mannases, ß-glucosidases release the 1,4 glucopyranose units at the non-reducing end of the oligomers that are formed as a result of the degradation of glucomannan and galactomannan, and they are in GH families of 1 and 3 (Mccleary and Matheson 1987; Bhatia et al. 2002; Cairns and Esen 2010).

Moreover, to remove the side groups from galactomannan, glucomannan, and galactoglucomannan, auxiliary enzymes are required, specifically α-galactosidase and acetyl mannan esterase (Tenkanen et al. 1995; Ganter et al. 2001; Moreira 2008).

9.3.4 Lignin and Ligninolytic Enzymes

Lignin, which is the most abundant source of raw material in nature after cellulose and consists of phenylpropanoid units linked through covalent bonds, is the main structural component of the plant cell wall (Li et al. 2009; Sriharti et al. 2017). In addition to having an important mechanical role for the plants, it also helps the plants to gain resistance against microbial attacks (Vance et al. 1980; Li et al. 2009).

The enzymes that participate in the degradation of lignin are laccases and peroxidases (lignin peroxidase and manganese peroxidase) (Kuhad et al. 1997; Plácido and Capareda 2015; Sindhu et al. 2016).

1. Laccases (EC 1.10.3.2) are extracellular glycoproteins that contain copper (Mayer and Staples 2002; Alcalde 2007). Due to their low substrate specificities, they enable the deterioration of phenolic structured compounds (Zouari-Mechichi et al. 2006). The potential of the laccases to degrade lignocelluloses depends on the phenolic compounds (e.g., 3-hydroxyanthranilic acid; 2,2′-azino-bis-(3 ethylbenzothiazoline-6-sulfonic acid; vanilin)) that function as redox mediators (Camarero et al. 2004). When the mediators are absent, the activity of the laccases is limited (Saloheimo et al. 2002; Fillat and Roncero 2009; Plácido and Capareda 2015). Laccase enzyme does not require additional compounds such as manganese or hydrogen peroxide for its activity; therefore, it is the most interesting among the environmental applications. Moreover, in hypersaline environments, they synchronously carry out actions such as degradation and color removal of lignin (Molitoris et al. 2000).

   Various microorganisms catalyze the enzymatic break down of lignin are shown in Fig. 9.5 (Althuri et al. 2017; Siroosi et al. 2018).

2. Lignin peroxidases (LiPs, EC 1.11.1.14) are monomeric heme-glycoproteins (Orth and Tien 1995) that use H_2O_2, and at first, they were obtained from a kind of fungus called *Phanerochete chrysosporium* (Venkatadri and Irvine 1993). It catalyzes the oxidation of nonphenolic aromatic compounds (Conesa et al. 2002; Sindhu et al. 2016). These enzymes are shown in Fig. 9.5 (Wong 2009; Brown et al. 2012).

3. Manganese peroxidases (MnP), (EC 1.11.1.13) are also extracellular glycoproteins like lignin peroxidases, which contain heme group and utilize H_2O_2 (Asgher et al. 2008; Sindhu et al. 2016). They exert effect on phenolic and nonphenolic compounds through lipid peroxidation reactions (Binod et al. 2011; Sindhu et al. 2016).

   The basic function of the MnP is oxidizing Mn^{2+} ions to Mn^{3+}, then later, Mn (III) complexes that occur as a result of the chelation process carried out by organic acids (such as malate or oxalate), oxidizes various substrates of the MnP (Hofrichter 2002). Some examples of microorganisms producing this enzyme can be seen in Fig. 9.5 (Homma et al. 2007; Romero et al. 2007). Both group of enzymes (laccases and peroxidases) turn phenolic compounds and aromatic amines into radicals (Higuchi 1989; Kersten et al. 1990).

However, in addition to the differences in their prosthetic groups, the laccases differ from the peroxidases also because they have lower oxidation potentials (Hayashi and Yamazaki 1979; Farhangrazi et al. 1994).

9.4 Other Enzymes

In addition to the basic microbial enzymes involved in the biofuel production process mentioned above, there are some enzymes that contribute to this process. Pectinases, LPMOs, amylases, pullulanases, and proteases described below are among such groups of enzymes (Fig. 9.6)**.**

9.4.1 Pectin and Pectinolytic Enzymes

Pectin has a linear backbone consisting of α-1,4 linked D-galacturonic acid residues which can be methylated and interchanged with L-rhamnose, arabinose, galactose, and xylose. Furthermore, apart from celluloses and hemicelluloses, pectin is another structural polysaccharide that ensures the integrity of the plant tissues that are on the plant cell wall matrix (Kashyap et al. 2001; Celestino et al. 2006).

Pectinolytic enzymes can be evaluated in two different groups: esterases (pectinesterases, EC 3.1.1.11) and depolymerases (hydrolases: endopolygalacturonase, EC 3.2.1.15; exopolygalacturonase, EC 3.2.1.67 and lyases: pectate-lyase, EC 4.2.2.2; pectin lyase, EC 4.2.2.10) (Soares et al. 2001; Celestino et al. 2006; Elleuche et al.

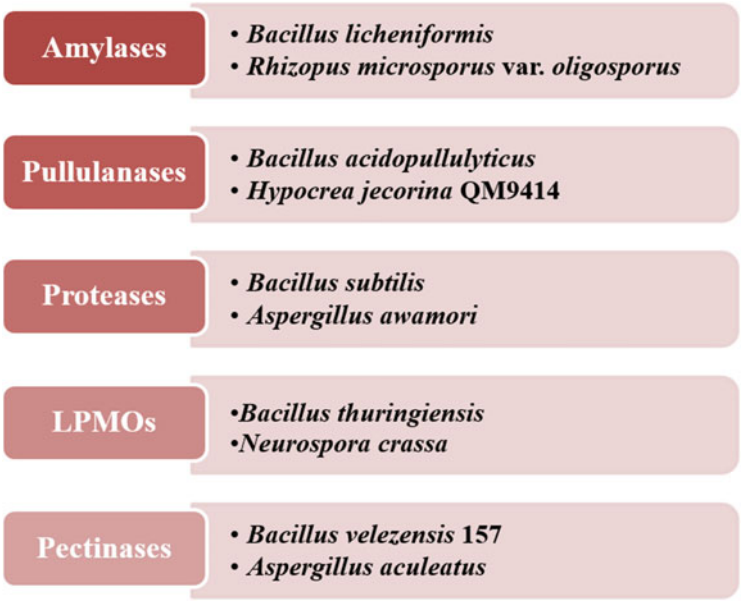

Fig. 9.6 Some potential auxiliary microbial enzymes that can be used in the biofuel production process

2015). Some microorganisms producing pectinase are included in Fig. 9.6 (Kim et al. 2014; Chen et al. 2018).

While pectin esterase detaches the methyl group from pectin, depolymerase enzymes cleave the α-1,4 links among the galacturonic acid residues in the main chain via a trans mechanism (lyases) or acid-base catalysis mechanism (hydrolases) (Mckie et al. 2001; Parisot et al. 2003; Celestino et al. 2006).

9.4.2 Starch and Amylolytic Enzymes

Starch is a heterogeneous polysaccharide that consists of an insoluble linear compound-amylose and a soluble branched polymer-amylopectin (Bertoldo and Antranikian 2002; Elleuche and Antranikian 2013).

At the same time, it is one of the most easily accessible and low-cost feedstock sources and a carbohydrate reserve in plants and can be used in biofuel production (Tanaka and Kondo 2015).

Amylose (15–25% of the polysaccharide) is a glucose polymer that consists of α-1,4 glycosidic links, whereas amylopectin (75–85% of the polysaccharide) consists of both α-1,4 and α-1,6 links (Elleuche and Antranikian 2013).

Since starch has a complex structure, it must be converted to smaller units (such as glucose or maltose) in order to be used as a carbon source in biofuel production

and other industrial areas. To provide the bioconversion of starch, a variety of enzyme combinations are required. These combinations consist of α-amylase, β-amylase, glucoamylase, isoamylase, and pullulanase (Haki and Rakshit 2003).

Some microorganisms that biosynthesize amylase (Somda et al. 2011; de Barros Ranke 2020) and pullulanase (Awg-Adeni et al. 2013; Orhan et al. 2014) are indicated in Fig. 9.6.

9.4.2.1 α-Amylases (EC 3.2.1.1)

α-amylases are extracellular enzymes also known as α-1,4-glucan-4-glucanohydrolase or glycogenase (Cherry et al. 2004). The members of α-amylases are in GH families of 13, 57, and 119 (Elleuche and Antranikian 2013). This endo-acting amylolytic enzyme produces some oligosaccharides such as maltose, maltotriose, and dextrins by randomly hydrolyzing the α-1,4 linkages of starch and similar long-chain carbohydrates (Sivaramakrishnan et al. 2006; Amoozegar et al. 2019).

Microbial α-amylases are the most preferred among the industrial α-amylases (De Souza 2010; Zhang et al. 2017).

9.4.2.2 β-Amylases (EC 3.2.1.2)

β-amylases are known as α-1,4-D-glucan maltohydrolase or saccharogen amylase. They are exo-acting enzymes that allow the constitution of β-maltose by hydrolyzing the second α-1,4 glycosidic linkage at the non-reducing end of the starch. All the known isozymes are in GH14 family (Vaidya et al. 2015; Saini et al. 2017) with the exception of ß-amylase [GH57] from *Pyrococcus furiosus* (Elleuche and Antranikian 2013).

9.4.2.3 Glucoamylases (EC 3.2.1.3)

Glucoamylases are also named γ-amylase, amyloglucosidase, and glucan 1,4-α-glucosidase. It enables the formation of the glucose units by hydrolyzing the α-1,4 glycosidic links at the non-reducing ends in the amylose and amylopectin. At the same time, they also hydrolyze α-1,6 glycosidic links, and they produce glucose as the final product (Xu et al. 2016; Saini et al. 2017). Seldomly, they hydrolyze α-1,3 glycosidic linkages (Kumar and Satyanarayana 2009).

These enzymes are categorized in GH15 family (Bourne and Henrissat 2001). Apart from the other amylase forms (α-amylase, β-amylase), glucoamylases are very active and stable in acidic environments (Saini et al. 2017).

9.4.2.4 Isoamylases (EC 3.2.1.68)

Isoamylases are also known as glycogen-6-glucanohydrolase. They hydrolyze especially the α-1,6 glycosidic linkages in the branched polysaccharides such as amylopectin, β-limit dextrin, or glycogen (Van der Maarel et al. 2002; Hii et al. 2012; Elleuche and Antranikian 2013).

However, they are unable to hydrolyze the pullulan or branched oligosaccharides that have α-1,6 linkages. For this reason, they exert little effect on α-limit dextrin (Bertoldo and Antranikian 2001).

9.4.2.5 α-Glucosidases (EC 3.2.1.20)

α-Glucosidases are also known as α-D-glucoside glucohydrolase. Usually, they take part in the last step of the degradation of starch (Lévêque et al. 2000).

In contrast to glucoamylases, they prefer disaccharides and oligosaccharides as the substrate, and they act on the α-1,4 glycosidic linkages in these structures (Bertoldo and Antranikian 2002). They belong to the GH families of 4, 13, 31, 63, 97, and 122 (Henrissat 1991).

With some exceptions, their optimum pHs are quite acidic and they are thermostable enzymes (Lévêque et al. 2000; Elleuche and Antranikian 2013).

9.4.2.6 Cyclodextrin Glycosyltransferases (EC 2.4.1.19)

They are also known as CGTases or α-1,4-D-glucan α-4-D-(α-1,4-D-glucano) transferase. They form non-reductive cyclodextrins (in α, ß, γ forms) via α-1,4 glycosidic linkages by degrading starch, amylose, or oligosaccharides (Gawande et al. 1999). Besides bacteria (e.g., *Anaerobranca gottschalkii*), they are also found in archaea (e.g., *Thermococcus* sp.) (Bertoldo and Antranikian 2002).

9.4.3 Pullulan and Pullulanolytic Enzymes

Pullulan is an exopolysaccharide synthesized by fungus *Aureobasidium pullulans* (Wu et al. 2010). It is a linear homopolymer consisting of maltotriose subunits linked with α-1,6 glycosidic linkages and isopanose/panose subunits linked with α-1,4 glycosidic linkages (Singh et al. 2008b). Moreover, it is renewable, biologically degradable, and nontoxic (Prajapati et al. 2013) and plays a significant role as a model substrate in the determination of the activities of the enzymes that hydrolyze the branched structures of starch. The α-1,6 glycosidic linkages in its structure are similar to those in the branch points of amylopectin.

Pullulanases are also known as α-dextrin 6-glucanohydrolase. They hydrolyze endo-acting branched structures that enable maltotriose formation by hydrolyzing the α-1,6-D-glycosidic linkages in the structure of pullulan, starch, ß-limit dextrin, amylopectin, and similar oligosaccharides (Asha et al. 2013).

While the thermophilic pullulanases are in GH 13 or GH 57 families, mesophilic pullulanases are usually in the GH 13 family (Nisha and Satyanarayana 2013).

They are separated into five different categories according to their substrate specificity and reaction final products.

9.4.3.1 Type I Pullulanase (Pullulan α-1,6-Glucanohydrolase, α-Dextrin-Endo-1,6-Glucanohydrolase, EC 3.2.1.41)

Type I pullulanases are the enzymes that form maltotriose and linear oligosaccharides by hydrolyzing the 1,6 glycosidic linkages in pullulan, amylopectin, glycogen, α-limit dextrin, and ß-limit dextrin (Kim et al. 1996; Nisha and Satyanarayana 2016).

9.4.3.2 Type II Pullulanase (Amylopullulanase, EC 3.2.1.1./41, α-Amylase-Pullulanase, EC 3.2.1.1)

Type II pullulanases are the enzymes that hydrolyze the α-1,4 and α-1,6 linkages that exist in the structure of starch and pullulan. It releases glucose units by hydrolyzing the α-1,6 glycosidic linkages in the structure of pullulan.

They can convert the polysaccharides to simple sugars in the absence of enzymes such as α-amylase or ß-amylase. Since they have both of these dual catalytic activities, they are also called amylopullulanase or α-amylase-pullulanase (Van der Maarel et al. 2002; Hii et al. 2012; Nisha and Satyanarayana 2016).

9.4.3.3 Pullulan Hydrolase Type I (Neopullulanase, Pullulan-4-D-Glucanohydrolase, EC 3.2.1.135)

Pullulan hydrolase type I enables the constitution of the panose (6–0-α-D-glucosylmaltose) units by hydrolyzing the α-1,4 glycosidic links in the structure of pullulan (Bertoldo and Antranikian 2001). They can hydrolyze the α-1,4 and α-1,6 glycosidic links of starch and similar polysaccharides with low efficiency (Hii et al. 2012).

9.4.3.4 Pullulan Type II Hydrolase (Isopullulanase, Pullulan-4-Glucanohydrolase, EC 3.2.1.57)

Pullulan type II hydrolase enables the constitution of isopanose (6-0-α-maltosylglucose) units by hydrolyzing the α-1,4 links in the structure of the pullulan (Niehaus et al. 2000).

Contrary to neopullulanases, this enzyme does not hydrolyze starch or dextran (Van der Maarel et al. 2002; Hii et al. 2012).

9.4.3.5 Pullulan Type III Hydrolase (EC 3.2.1._)

Pullulan type III hydrolases produce maltotriose, panose, maltose, and glucose units by hydrolyzing the α-1,4 and α-1,6 glycosidic linkages in the structure of pullulan (Li et al. 2015; Liu et al. 2016). They also convert starch, amylose, amylopectin, glycogen, and cyclodextrin to maltose and maltotriose (Van der Maarel et al. 2002; Hii et al. 2012).

While the type I and II pullulanase and type I pullulanan hydrolase enzymes are produced by bacteria and archaea, types II and III pullulan hydrolases are produced respectively by fungi and hyperthermophilic archaea (Nisha and Satyanarayana 2016).

9.4.4 Proteases (EC 3.4)

Proteases are another group of enzymes that are involved in the pretreatment of lignocellulosic biomass (Srivastava et al. 2020) and microalgae (Carrillo-Reyes et al. 2016). Proteases belonging to the glycoside hydrolase family are at the top of the global enzyme market (Rao et al. 1998; Haki and Rakshit 2003). They have a wide substrate specification (Mienda et al. 2014) and catalyze the cleavage of the peptide linkages in proteins (Theron and Divol 2014; Elleuche et al. 2015).

They are divided into two main groups regarding the location of the peptide linkage they affect: exopeptidases (aminopeptidases and carboxypeptidases) and endopeptidases (serine proteases, sistein/thiol proteases, aspartic/acid proteases, threonine proteases, and metalloproteases) (Rao et al. 1998; Pushpam et al. 2011; Gurumallesh et al. 2019). Certain protease synthesizers are included in Fig. 9.6 (Arifeen et al. 2009; Choi et al. 2014).

Proteases are typically used in the biofuel production to generate a nitrogen source for the fermentation of the yeast. Besides, they can contribute to the fermentation by separating starch-gluten complexes, increasing the accessibility of the amylases to the starch, and changing the chemistry of the grain (Alvarez et al. 2010; Bhari and Singh 2016).

9.4.5 *Non-hydrolytic Cellulose-Degrading Enzymes (LPMOs: Lytic Polysaccharide Monooxygenases)*

Lytic polysaccharide monooxygenases (LPMOs) are Cu-dependent redox enzymes that require oxygen (Mohanram et al. 2013; Laurent et al. 2019) and help to increase the degradation of some persistent polysaccharides such as crystalline cellulose (Quinlan et al. 2011) and chitin (Langston et al. 2011).

LPMOs are very important for the pretreatment of the biomass (Levasseur et al. 2013). LPMOs known to act on cellulose and chitin in the beginning have later been identified to react also with a series of polysaccharides such as starch (Leggio et al. 2015), xyloglucan, cellodextrins, and glucomannan (Borisova et al. 2015; Bennati-Granier et al. 2015; Johansen 2016).

The discovery of this enzyme group has been started in 2010 when Vaage-Kolstad et al. introduced the oxidative enzyme (CBP21) that is located in the backbone of chitin and can break glycosidic bonds (Vaaje-Kolstad et al. 2010). In the beginning, the LPMOs obtained only from the fungi were classified in the GH61 family. Later, when GH61 family was designated as oxidative enzymes, this enzyme group was reclassified and placed into the auxiliary activity families (AA) (Quinlan et al. 2011; Beeson et al. 2012; Hemsworth et al. 2015). LPMOs can be accessed via CAZy database [www.cazy.org] (Levasseur et al. 2013; Lombard et al. 2014).

Similar to glycosidic hydrolase enzyme producers, the LPMOs can be predominantly obtained from certain fungi (Vaaje-Kolstad et al. 2010; Hemsworth et al. 2015), bacteria (Forsberg et al. 2014; Zhang et al. 2015; Eijsink et al. 2019), and viruses (Chiu et al. 2015; Johansen 2016; Filiatrault-Chastel et al. 2019) (Fig. 9.6).

The strength of the LPMOs to increase the activity of the GHs has been one of the driving forces which made the investigations on LPMOs press forward. In the presence of an external e^- donor (oxygen), LPMOs (special oxidoreductases) oxidize the glycosidic bonds (Vaaje-Kolstad et al. 2010; Quinlan et al. 2011; Forsberg et al. 2014) and give way to the cleavage of the polysaccharide chain, which in turn significantly contributes to the accessibility of the hydrolytic enzymes (hydrolases) to the substrate (Vermaas et al. 2015; Laurent et al. 2019).

In the pretreatment with LPMO, compared to cellulases, it has been determined that the degradation of the highly resistant crystalline cellulose is completed faster and entirely (Eibinger et al. 2014).

It was observed that the yield from different celluloses (such as bacterial cellulose, microcrystalline cellulose, cellulose pretreated with phosphoric acid) increased 6–8 times in the combination of AA9 (AA: auxiliary activity) and CDH (cellobiose dehydrogenase) (Langston et al. 2011; Hemsworth et al. 2015). These tremendous activities of the LPMOs are significant development in biomass conversion. However, there are some points that need to be solved or considered in industrial applications:

- Because LPMOs have oxidative property, they are not compatible with various simultaneous saccharification and fermentation schemes that require reduction conditions (Olofsson et al. 2008; Rana et al. 2014).
- The oxidized sugar products that enzymes release are not effectively used by microorganisms; hence microbial growth can be inhibited (Kaur et al. 2006; Decker et al. 2017).
- The autocatalytic inactivation of LPMO: The effect of the substrate is very important in the stability of LMPOs. LPMOs are protected from auto-oxidative inactivation when attached to the substrate. LPMOs are inactivated when the amount of substrate is reduced (Courtade et al. 2018).
- The stability of the enzyme: Since the LPMOs are extracellular enzymes, they are usually stabilized by disulfide links. In the presence of Cu, they are thermostable above 60 °C (Hemsworth et al. 2013; Hemsworth et al. 2015).
- Hydrogen peroxide production of the oxidative enzymes may cause them to lose their activities (Valderrama et al. 2002).
- Due to the fact that most of the substrates are insoluble, some analytical problems arise (Forsberg et al. 2019).
- The kinetic data is scarce, and obtaining these data experimentally is hard (Forsberg et al. 2019).
- Adding LPMOs in commercial cellulolytic enzyme cocktails for the saccharification of pretreated plant residues economically contributes to the process cost (Teter 2012; Johansen 2016). However, phenolic compounds and high concentration of oxygen may cause the inactivation of the cellulase cocktails due to LPMO (Kim et al. 2011; Ximenes et al. 2011).

9.5 Enzymatic Cocktails

Although the search for producing alternative fuels out of lignocellulosic biomass has begun long ago, there are still many challenges in applying and maintaining the process commercially. Various enzymes are needed to transform lignocellulosic materials or microalgae into fully fermented sugars (Yeoman et al. 2010; Chandel et al. 2012; Zabed et al. 2019). However, a single microbial organism that can sufficiently produce all the required enzymes for producing the biomass conversion has not been defined yet (Adsul et al. 2020).

Industrial enzyme production (depending on the enzyme source, production approaches, country, etc.) is a costly process; furthermore completing the process in short time is important (Klein-Marcuschamer et al. 2012; Binod et al. 2019). In recent years, many researchers and enzyme-producing biotechnology companies (e.g., Dupont-Genencor, Dyadic and Novozyme) have focused on "enzymatic cocktails" in order to develop enzymatic hydrolysis processes (Agrawal et al. 2018; Sanhueza et al. 2018).

Preparation of an appropriate enzymatic preparate requires knowledge about particularly the biomass composition, followed by the type of the applied

pretreatment, choosing appropriate enzymes and strains, choosing non-catalytic proteins, the chemicals, etc. (Adsul et al. 2020). There is a wide variety of biomasses (sugar cane, corn cob, wheat straw, banane waste, microalgae, etc.), and each of the biomasses has different heterogeneity (depending on the compounds they contain and the amount of these compounds) (Kenney et al. 2013; Williams et al. 2016).

Moreover, depending on the type of the applied pretreatment (with alkali/acid/steam, etc.), the chemical composition and structure of the biomass vary (Li et al. 2014; Satlewal et al. 2018). Again, choosing the appropriate strain(s) is also important for the supply of the enzymes (appropriate for the chemical composition/structure of the biomass) which will function in the hydrolysis of the biomass.

Natural microbial strains can produce sufficient enzymes for their vital functions, but may not meet demands on industrial scales (Adsul et al. 2020). To reach high levels of expression (increasing the enzyme production efficiency), strain improved through genetic modifications (e.g., cloning and mutation) is required (Juturu and Wu 2014; Singh et al. 2017; Singhania et al. 2017).

LPMOs (see Sect. 9.4.5) have become the focus of interest in the degradation of the pretreated lignocellulosic biomass (Eibinger et al. 2014; Couturier et al. 2018).

It has been pointed out that the LPMO/AA9 helps the biomass to liquify rapidly and thus increases the accessibility of the other enzymes to the biomass. Furthermore, it has been stated that adding optimum AA9 to the cellulase preparate decreases the general protein/cellulase load by 5–6 times (Sun et al. 2015) and the hydrolysis of the biomass which holds more lignin in its structure after the pretreatment is conducted more effectively with the synergic effect of the cellulase preparate and AA9 (Hu et al. 2014).

It has been reported that some non-hydrolytic proteins affect the decomposition process of the cellulose, which is characterized by the dispersion of the microfibrils and the swelling of the macrofibrils. These effects are a decrease in the crystallinity of the cellulose, an increase both in the cellulose surface area and in the accessibility to cellulose (Arantes and Saddler 2010; Chen et al. 2010; Adsul et al. 2020). For this reason, these proteins function as the factors that increase the cellulase activity. The functions of these proteins (plant-like expansin, swollenin, and CBM (see Sect. 9.3.2)) can be explained with a couple of examples.

As demonstrated in the study of Kim et al., expansin (B5EXLX1), isolated from Bacillus subtilis and expressed heterologously in Escherichia coli, increased the synergistic effect by 240% and the overall enzymatic activity 5.7 times when used with cellulases (Kim et al. 2009).

Moreover, it has been stated that, when fungal (*Orpinomyces* sp. strain CIA) swollenin expressed in high amounts is included in a cellulolytic cocktail, depending on the pretreatment type, it provides up to 7% improvement in the hydrolysis of the corn stover (pretreated with liquid, alkali, acid and ionic, respectively) (Morrison et al. 2016). Besides these proteins, a small heat-shock protein (cbHsp 18) obtained from bacteria has also been found to contribute to the hydrolysis of bioenergy feedstock by increasing the thermal stability of the glycoside hydrolases (Su et al. 2012).

Finally, admixtures like e^- donors such as ascorbic acid which are necessary for the function of the AA9 and other chemicals such as bovine serum albumin (BSA), surface-active agent, lignosulfonate, etc. which are used for preventing the cellulases from bonding to lignin inefficiently can be added to the mixture (Wang et al. 2013; Adsul et al. 2020).

As mentioned, although the preparation of the enzyme cocktail is not very easy, it is a requirement for being able to efficiently and economically (less amount of enzyme, less time, high substrate loading) turn biomass into biofuels or value-added products.

Below, a number of examples of some microbial enzymes used in the production of various biofuels, their sources, and different substrates are demonstrated in Table 9.1.

9.6 The Role of Genetic Engineering and Bioinformatics in Biofuel Production

Due to the need for increased energy demand, sustainable economy, and clean environment, the interest in biofuel production has been increasing in recent years (Popp et al. 2014). Therefore, the production of chemicals and fuels from renewable biomass has accelerated significantly today. Since the lignocellulosic material is the most abundant plant-based raw material in nature, the mostly produced substance as biofuel is ethanol (Sun and Cheng 2002). Although not as much as ethanol, the interest in butanol has been increasing again because of its high air-to-fuel ratio and high energy density (Durre 2007).

One of the most obvious difficulties encountered in the conversion of lignocellulosic material to bioethanol is the high cost of enzymes used in the process (Sangkharak et al. 2011; Singh and Sharma 2012). In addition, cellulases, xylanases, and laccases from different microbial sources differ in their thermostability and catalytic efficiency. These differences also greatly affect the hydrolysis of the components (lignin, cellulose, and hemicellulose) present in the lignocellulosic material (Liu et al. 2012; Zhang and Zhang 2013; Santhi et al. 2014).

In order to overcome such difficulties in biofuel production and to develop enzymes that effectively break down the components of lignocellulosic material, molecular modeling, molecular docking, and molecular dynamics analyses have now been frequently used in combination with genetic and protein engineering.

Cho and coworkers investigated butanol selectivity in a variety of clostridial aldehyde/alcohol dehydrogenase (AAD) variants using random-mutagenesis approach (Cho et al. 2019). Researchers have used molecular dynamics and molecular docking methods to determine the extent to which mutation-induced changes in wild-type and mutant AAD variants affect the substrate (butanol) specificity. In molecular dynamics simulations, more water molecules were determined to be localized near the active regions of enzyme variants, and the molecular docking

Table 9.1 Some microbial enzymes used in the production of various biofuels

Enzyme(s)	Substrate(s)	Microorganism(s)	Biofuel(s)	Reference(s)
Laccase	Sugarcane bagasse	*Cyathus stercoreus*	Ethanol	Chandel et al. (2007)
Lipase	Microalgae	*Microbacterium* sp.	Biodiesel	Tripathi et al. (2014)
Cellulase	Newspaper	*Cytophaga hutchison*	Bioethanol	Byadgi and Kalburgi (2016)
Cellulolytic and xylanolytic enzymes	Saccharum biomasses	*Sporotrichum thermophile* BJAMDU	Bioethanol	Bala and Singh (2019)
Laccase	Saccharum biomasses	*Ganoderma lucidum* MDU-7	Bioethanol	Bala and Singh (2019)
Cellulase	*Equisetum arvense* (horse-tail) waste	*Streptomyces fulvissimus* CKS7	Bioethanol	Mihajlovski et al. (2020)
Cellulase	Sesame seeds residues	*Bacillus cereus*	Bioethanol	Abada et al. (2018)
Lipase	Algae oil	*Bacillus* sp.	Biodiesel	Sivaramakrishnan and Muthukumar (2012)
Cellulase and xylanase	Wheat straw	*Trichoderma reesei*	Bioethanol	Tabka et al. (2006)
Feruloyl esterase (FAE)	Wheat straw	*Aspergillus Niger*	Bioethanol	Tabka et al. (2006)
Laccase	Wheat straw	*Pycnoporus cinnabarinus*	Bioethanol	Tabka et al. (2006)
α-Amylase	Corn meal	*Bacillus licheniformis*	Bioethanol	Mojovic et al. (2006)
Glucoamylase	Corn meal	*Aspergillus Niger*	Bioethanol	Mojovic et al. (2006)
Amylase	Rice water waste	*Bacillus licheniformis*	Bioethanol	Chethana et al. (2011)
β-Glucosidase	Bagasse and rice straw	*Pseudomonas* sp. CL3	Biobutanol	Cheng et al. (2012)
Endo-glucanase	Bagasse and rice straw	*Clostridium* sp. TCW1	Biobutanol	Cheng et al. (2012)
Pectinase	Microalgal biomass	*Aspergillus aculeans*	Ethanol	de Farias Silva et al. (2018)
Laccase	Wheat straw slurry	*Pycnoporus cinnabarinus*	Bioethanol/ biogas	Moreno et al. (2013)

analysis performed with acetaldehyde and butyraldehyde showed that the volume of substrate binding regions was expanded. As a result, using the random-mutagenesis method, the researchers have increased the butanol production capacity (by increasing butanol selectivity) of the *Clostridium acetobutylicum* species and managed to significantly reduce the amount of ethanol produced by the same enzyme.

Nimbalkar and colleagues conducted a research to increase the butanol concentration produced by the enzyme butanol dehydrogenase (BDH) (Nimbalkar et al. 2018). For this purpose, they added trace elements such as nickel chloride and sodium selenite into the reaction mixture and investigated changes in the butanol production efficiency of BDH.

They also performed homology modeling and molecular docking analysis to investigate the correct placement and molecular interactions between trace elements, NADH (cofactor), and substrate (butyryl aldehyde) with the 3D structure of butanol dehydrogenase. Molecular docking analyses were instrumental in visualizing possible substrate-inhibitor interactions in the BDH enzyme.

In another study, a comparative sequence alignment, molecular modeling, and molecular docking study was conducted to understand in detail the enzyme-substrate interaction in cellulose hydrolysis and to find out the binding free energies of five different microbial cellulase enzymes (three bacteria and two fungi) with β-D glucose. Furthermore, using an in silico approach, the researchers designed and validated the deleterious mutations (E133A and H98A) in cellulase enzyme from *Dickeya dadantii* (Paul et al. 2020).

In the molecular docking analysis, it was found that *Streptomyces* sp. Endoglucanase-1 interacted with β-D glucose through Val114, Ala255, Val288, Val289, and Ser301 residues and displayed the most negative binding free energy (−5.61 kcal/mol).

Dodda and coworkers using homology modeling modeled the structure of cellobiohydrolases (CBHs) of *Aspergillus fumigatus* NITDGPKA3 to predict its catalytic activity, and they applied molecular docking and molecular dynamics simulation to reveal the structural and functional mechanism of the enzyme. As a result of molecular docking analysis with cellulose, they reported that Gln248, Pro287, Val236, Asn284, and Ala288 were the main residues in the hydrolysis of glucose (Dodda et al. 2016).

Wickramasinghe and coworkers, to hydrolyze xylan into xylooligosaccharides, genetically modified *Pichia stipitis* (a pentose sugar fermenting yeast species) via cloning and heterologous extracellular expression of EXN1 gene from *Trichoderma virens* species. For this purpose, the 3D structure of the recombinant protein encoded by the EXN1 gene was designed by homology modeling. Furthermore, molecular docking and molecular dynamics methods were also used to investigate EXN1-xylan interactions (Wickramasinghe et al. 2017).

Based on molecular docking results, it was concluded that the presence of glutamic and aspartic acid in the active site of the enzyme in question may mediate catalytic activity by retaining or inverting mechanisms, and the molecular dynamics simulations indicated a stable EXN1-xylan complex throughout a short 15-ns simulation time. Therefore, the researchers concluded that the enzyme-substrate association was stable, and the modeled EXN1 enzyme was realistic.

The cellulase enzyme breaks the β-1,4-glycosidic bonds and degrades the cellulose into glucose. Cellulases, which have the great potential, are among the third widely used enzymes in ethanol production, and they also play a vital role in degrading biomass. Selvam and coworkers performed docking analysis of

cellobiose, cellotetraose, cellotetriose, and laminaribiose to find substances that can be used as a substrate (carbon and nitrogen source) for cellulase (Selvam et al. 2017). To this aim, they first modeled the 3D structure of the cellulase of *Acinetobacter* sp. by homology modeling and confirmed the structural and atomic properties of the resulting enzyme using the Ramachandran plot. Cellotetraose showed the highest score (−7.8759 kJ/mol) in terms of Gibbs free energy (ΔG) in binding to cellulase among those potential substrates analyzed by molecular docking.

Replacing fossil fuels with renewable resources such as lignocellulosic biomass is an important alternative in obtaining biofuels and fighting climate change. For this purpose, Gomez and coworkers characterized an endo-β-1,4-xylanase Xyl2 of *Fusarium oxysporum* as a promising glycoside hydrolase enzyme for the industrial degradation of xylan (Gomez et al. 2016). Using molecular docking technique, to further understand Xyl2 substrate binding and catalytic mechanism, they performed docking of a β-1,4-xylopyranoside hexasaccharide (XYP6) with the Xyl2 structure. In the docking experiment performed (pH 7.5), Glu176 and Tyr72 residues of Xyl2 showed catalytically effective conformation, and the lowest energy pose of Xyl2-XYP6 complex was in agreement with the hydrolysis of the β-1,4-glycosidic bond.

Increasing the hydrolytic performance of hemicellulases is crucial to degrade lignocellulosic biomass in second-generation biorefinery. For this purpose, You and coworkers designed the variant XYL10C-ΔN by removing the N-terminal 66 amino acids from the construct in order to develop near-perfect xylanase candidates (You et al. 2018). They found that the enzymatic activity of the most efficient xylanase variant increased by 1.8-fold and its thermostability remained within the same level compared to the wild type. In their docking experiment to understand the structure-function relationship of the enzyme, Asn269, Tyr272, His302, and Arg336 residues were determined to have been evolutionarily conserved in relation to the substrate (long-chain xyloheptaose) and were indispensable in substrate-binding.

Thermophilic xylanases are more suitable due to their stability in industrial applications. In this context, Chauhan and coworkers subjected *Bacillus aestuarii* SC-2014 strain to EMS- and MNNG-induced mutagenesis to enhance the xylanase activity of the enzyme (Chauhan et al. 2020). They cloned, sequenced, and performed the molecular docking experiment of wild-type and mutant xylanase gene products to reveal differential atomic interactions of wild-type and mutant enzyme-binding pockets with their corresponding substrate. As a result, the H121D mutation (histidine → aspartic acid) made the binding pocket acidic and charged and subsequently enhanced the xylanase activity. Furthermore, the Y99K (tyrosine → lysine) and the H121D (histidine → aspartic acid) mutations were determined to enhance the thermostability of the mutant protein.

Xylan accounts for about 35% of the dry weight of the plant cell wall and is an important, abundant, and renewable bioresource. Thermophilic xylanases with high catalytic activity draw attention in the biofuel, food, and feed industries. In this context, Wang and coworkers produced the recombinant TlXyn11B protein by cloning the *Talaromyces leycettanus* JCM12802 GH11 xylanase gene (Tlxyn11B) in *Pichia pastoris* GS115 strain (Wang et al. 2017). They obtained high specific activity (8259 ± 32 U/mg with beechwood xylan as substrate) and excellent pH

stability (from 1.0 to 10.5) by creating a saturated mutation in amino acid residues S3 and D35 in the recombinant enzyme obtained. They also performed structural analysis of TlXyn11B and its mutants, S3F and D35I, by homology modeling and MD simulations. As a result, they determined that the overall structural rigidity (improved thermostability) was increased in S3F and D35I double mutants (S3F/D35I).

In conclusion, molecular docking analysis (including molecular dynamic simulations) and 3D enzyme/protein modeling tools offer unprecedented advantages that no pure experimental approach can provide in studies such as enzyme-substrate affinity or enzymatic yield increment through random/site-directed mutagenesis in biotechnology. In this context, the superiority of in silico design versus *experimental* approaches can be summarized as follows:

1. Possible noncovalent (non-bonded) interactions (hydrogen, electrostatic, hydrophobic, halogen and others) occur during enzymatic catalysis between the ligand and the receptor cannot be experimentally demonstrated at the atomic level.
2. Neither all enzymes are experimentally isolated nor their crystallographic structures are determined. In addition, it is almost impossible to produce an enzyme from the scratch which is functional in the cell. Therefore, the catalytic properties or affinity of each enzyme to its substrate may not be studied experimentally.
3. Due to the probabilistic nature of mutagenesis, a lot of experiments may be required to increase enzymatic yield through amino acid substitutions.

Therefore, when molecular docking, molecular dynamics, and 3D molecular modeling methods are used in conjunction with special visualization software, they essentially provide an invaluable atomistic perspective in protein-ligand interactions, and molecular dynamic simulations add the time scale into these interactions, providing us with valuable information about the stability of protein-ligand complexes.

However, as these three different approaches mentioned above have reached a very professional level today, and although the software produced or web servers designed for this purpose are relatively easy to use, computer-assisted protein-ligand modeling and the interpretation of the interactions between protein-ligand complexes still require a solid background.

Finally, with a small-scale molecular docking analysis that we performed for this review, we have demonstrated how useful molecular docking applications are in atomic-level demonstration of enzyme-substrate interactions in biofuel production. In these examples, the best poses from docking analyses of xylanase (10B from *Thermotoga petrophila* RKU-1, PDB ID: 3NJ3) enzyme and its substrate xylobiose are demonstrated (Santos et al. 2010). The examples clearly explain the interaction mode and binding energy of both wild-type and mutated xylanase enzyme with its corresponding substrate, xylobiose. 87.5% of the amino acids of xylanase in the substrate binding region consist of polar amino acids. Therefore, for trial purposes, all amino acids in this region have been replaced by hydrophobic alanine (except tryptophan) to show how amino acid substitution events in the binding site will affect the binding affinity of the ligand and to show how useful molecular docking analysis is in site-directed mutations.

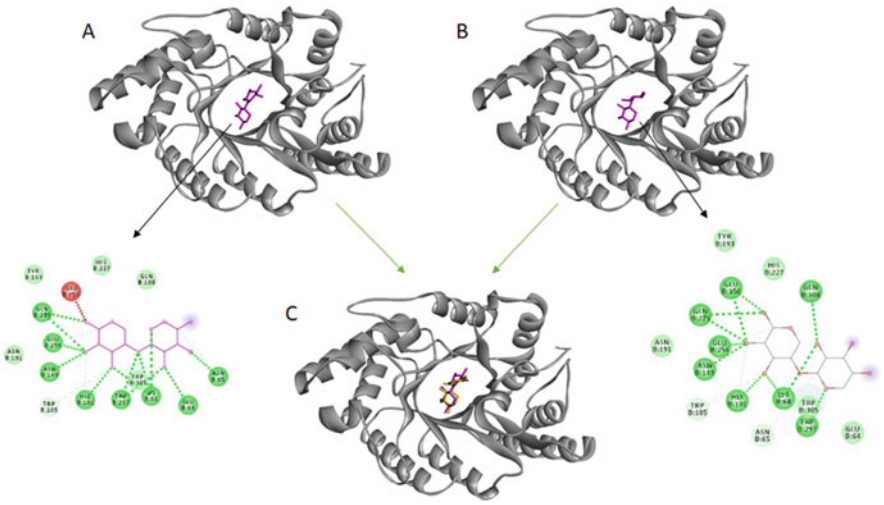

Fig. 9.7 Comparison of the redocked xylanase-xylobiose and control xylanase-xylobiose complexes. (**a**) Original crystallographic structure, (**b**) the top-ranked docking pose after redocking, (**c**) the superimposed poses of original crystallographic structure and the top-ranked docking pose resulted from redocking experiment. It should be noted that the ligand pose from redocking experiment (**b**) is nearly identical to the ligand pose in the control xylanase-xylobiose complex (**a**)

Considering Figs. 9.7 and 9.8, the binding conformation of the mutated xylanase protein and the xylobiose ligand appears to be significantly fluctuated relative to the control. In addition, when attention is paid to Table 9.2, the binding free energy of xylobiose in the mutant xylanase enzyme decreased significantly, and there were also significant differences in the amino acids it interacts with. Consequently, molecular docking analysis is a powerful tool in calculating the strength of receptor-ligand interactions and to show the extent to which the interactions of mutated proteins with their respective ligands have changed. Finally, the workflow to be followed in a classical molecular docking simulation is briefly summarized in Fig. 9.9.

9.7 Conclusion

As a result, the widespread use of biofuels as renewable energy sources instead of fossil fuels to protect the environment and natural resources and to meet the increasing energy need is promising. In particular, the use of various microbial enzymes in the hydrolysis of lignocellulosic raw materials obtained from renewable resources used in the production of second-generation biofuels attracts great industrial and biotechnological interest. In addition to all these, genetic engineering and bioinformatics offer unique advantages in increasing efficiency in biofuel production

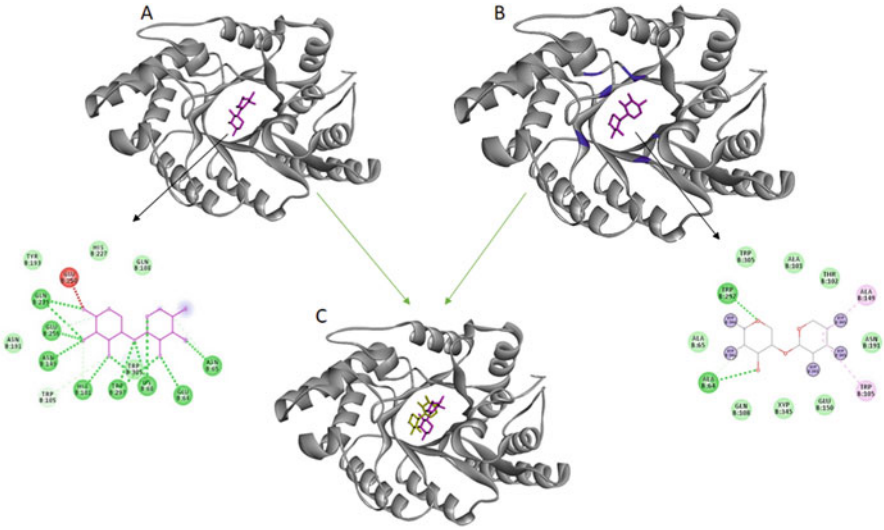

Fig. 9.8 Comparison of the control xylanase-xylobiose and mutant xylanase-xylobiose complexes. (**a**) Original crystallographic structure, (**b**) the top-ranked docking pose of xylobiose with mutant xylanase, (**c**) the superimposed poses of original crystallographic structure and the top-ranked docking pose resulted from mutant xylanase-xylobiose docking analysis. It should be noted that in the docking analysis performed with mutant xylanase (**b**), the ligand xylobiose shifts substantially (**c**) compared to the ligand pose in the control group (**a**). The mutated residues are marked in blue (**b**)

Table 9.2 RMSD, binding free energies, and ligand interactions obtained by docking analysis of native (wild-type) and mutant xylanase enzymes with the substrate xylobiose

	RMSD	Binding free energy (kcal/mol)	Ligand interactions
Xylanase-xylobiose (control)	–	−7.90[a]	Glu64, Asn65, Lys68, His101 Asn149, Gln225, Glu256, Trp297
Xylanase-xylobiose (redocking)	1.97 Å	−8.54	Asn65, Lys68, His101, Trp105, Gln108, Asn149, Glu150, Gln225, Glu256, Trp297
Xylanase-xylobiose (mutant)	7.02 Å	−5.95	Ala64, Trp105, Ala149, Trp297

[a]According to (Yang and Han 2018)
RMSD Root-mean-square-deviation

by paving the way for the development of enzymes that can catalyze more effectively and have high affinity to the substrate.

In this context, molecular docking and molecular dynamic simulations as bioinformatics tools have found a solid place in this field with an increasing prevalence in the last two decades. Two of the most important factors underlying this are that

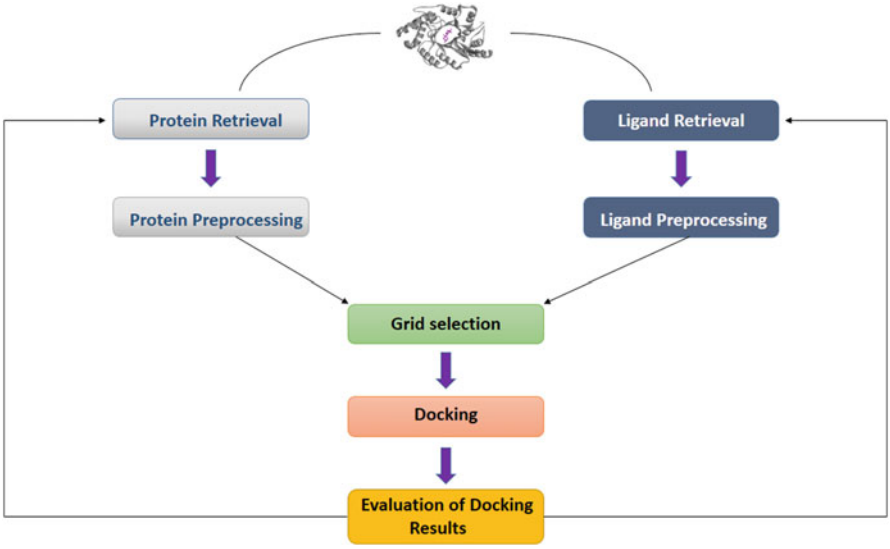

Fig. 9.9 The workflow of a classical molecular docking experiment

docking and dynamic simulations provide results that are highly consistent with experimental approaches and provide a unique visualization opportunity of enzyme-ligand interactions. From this point of view, it can be concluded that molecular interaction simulations that are architecturally developing more and more each day will always maintain their place as a complementary tool to experimental biology.

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Chapter 10
Substrate Characterization in the Anaerobic Digestion Process

Pietro Bartocci, Sara Massoli, Mauro Zampilli, Federia Liberti, Yan Yunjun, Qing Yang, Haiping Yang, Hewen Zhou, Eid Gul, Gianni Bidini, and Francesco Fantozzi

Abstract Anaerobic digestion, for the production of biogas and digestate, can be a powerful technology to obtain a gaseous fuel used for combined heat and power generation and for transportation (if upgraded to biomethane) and simultaneously obtain also a fertilizer. Anaerobic digestion is a biochemical process in which wet biomass is converted into gas by bacteria. To optimize the process at both research and industry level, it is necessary to characterize in detail the substrate before and during the process. Analytical methods applied to anaerobic digestion start from substrate characterization (which can be done through proximate and ultimate analysis and calorimetry, but also through the analysis of COD, alkalinity, pH, FFA, and other inhibitors); then also the microbial community has to be monitored during the process, and gas analysis has to be performed to determine the heating value and also the contaminants inside it (e.g., siloxanes). All these measurements have to be carefully standardized and are discussed in this chapter to provide information to researchers and operators in the field of anaerobic digestion.

Keywords Biogas · Anaerobic digestion · Methanation · Substrate · Analytical methods · Microbia

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10.1 Biogas Raw Materials Characteristics

Anaerobic digestion is the technology usually employed for the treatment of liquid animal effluents (e.g., pig, poultry, and cattle), compost, and sewage sludge obtained from wastewater treatment plants working in aerobic conditions. In the 1970's ecological and environmental movement, the need for a sustainable waste management is joined together with the development of renewable energy forms. In this framework, the use of anaerobic digestion became more and more diffuse for treating both municipal and industrial wastes. Thus, biogas plants have been constructed to stabilize the organic fraction of municipal solid waste (OFMSW) and semi-solid and solid wastes and have grown in numbers as an alternative to landfills or aerobic composting (Steffen et al. 1998). The advantage of the treatment is to produce two useful products which are: energy and a fertilizer.

Different raw materials are currently fed to anaerobic digestion plants to obtain biogas and digestate, with different composition and chemical and physical characteristics. Here we take into consideration pig slurry, cow manure, chicken manure, farmyard manure, agricultural residues, energy crops, and wastewaters.

Pig breeding farms with usually more than 1000 animals produce a liquid slurry with a total solid content between 2 and 10 wt%.

Cow slurry is usually gathered by a scraper system from feedlots. The addition of straw in the feedlots causes a slight increase in the total solid content (see Table 10.1).

Usually chickens are grown in large-scale breeding farms. The manure of chicken has high NH_4–N, and the total solid content is equal to 20 wt%. In chicken sheds, water-dissolved ammonia is excreted usually in crystal form. The high ammonia content can inhibit the anaerobic digestion process, causing also high NH_4 emissions in the feedlots during manure storage.

Table 10.1 Biogas substrate characteristics (Steffen et al. 1998)

Feedstock	TS (%)	VS (%)	C:N	Inhibiting substances
Pig slurry	3–81	70–80	3–10	Presence of antibiotics
Cow slurry	5–121	75–85	6–201	Presence of antibiotics
Chicken slurry	10–30	70–80	3–10	Presence of antibiotics
Whey	1–5	80–95	n.a.	
Ferment slops	1–5	80–95	4–10	
Leaves	80	90	30–80	Pesticides
Wood shavings	80	95	511	
Straw	70	90	90	
Wood wastes	60–70	99.6	723	
Garden waste	60–70	90	100–150	Pesticides
Grass	20–25	90	12–25	Pesticides
Grass silage	15–25	90	10–25	
Fruit wastes	15–20	75	35	Pesticides
Food remains	10	80	n.a.	Disinfectants

Conventional farmyard manure is produced in smaller farmhouses. In this context, straw is used for the animal beds, and the total solids of these residues range from 10 to 30 wt%. The farmyard manure digestion needs significantly greater retention times and can require to perform a pretreatment of the manure when it is inhomogeneous. Commonly, supplementary operative difficulties, like formation of scum layer, are observed. Other bedding tools, such as wood chips, are barely degradable anaerobically.

Garden wastes and agricultural harvest residues can also be utilized as feedstocks in farm-scale digesters, providing useful fertilizer in form of digestate. This kind of residues includes leaves, corn stover, clover harvest remainings, stems, spoiled vegetables or low-quality fruits, silo leachate, and straw.

Corn silage is the energy crop which is mostly used in anaerobic digestion (Nordberg and Edström 1997). Corn cultivation can be performed also in rotation with other crops such as triticale or oats. An advantage of silage is represented by the fact that it can be used and stored over long periods of time.

Wastewaters are produced in the food industries, where important amounts of agricultural raw materials are treated. Usually, agricultural industrial wastes and by-products contain proteins, sugars, and fats which have important yields of biogas. Effluents from the olive oil industry, the dairy industry, fruit juices industry, and alcohol distilleries can be also used as raw materials for biogas production. Anaerobic digestion can be performed on other industrial effluents, which are not coming from the agroindustry and also on municipal wastewaters. Table 10.1 shows the most important characteristics of anaerobic digestion substrates.

10.2 Biogas Raw Materials and Inoculum Characterization

10.2.1 Introduction

The different steps which have to be made to characterize the materials are shown in Fig. 10.1. The first one is the sampling of the material. The sample is required to have characteristics that are representative of the bulk materials from where it has been taken. After sampling, the materials should be properly stored in the lab and prepared for analysis (e.g., by drying it or milling it). Then classical analysis comprehends pH measurement, TS and VS measurements, chemical oxygen demand (COD) measurement, total nitrogen measurement, total organic carbon (TOC) measurement, trace elements, and S and P measurement, alkalinity. The most important measuring techniques for the above-cited parameters are reported in (Wellinger et al. 2013), while fundamental knowledge on food analysis is reported in (Nielsen 2017). As we know, food waste can be a significant raw material for anaerobic digestion (Bartocci et al. 2020; Liberti et al. 2018); so in this case also the food composition appears to be important. This is also in light of the fact that biogas yields can be predicted with modeling tools, such as those developed in (Hafner et al. 2018a) which use as inputs the substrate content of carbohydrates, lipids, and

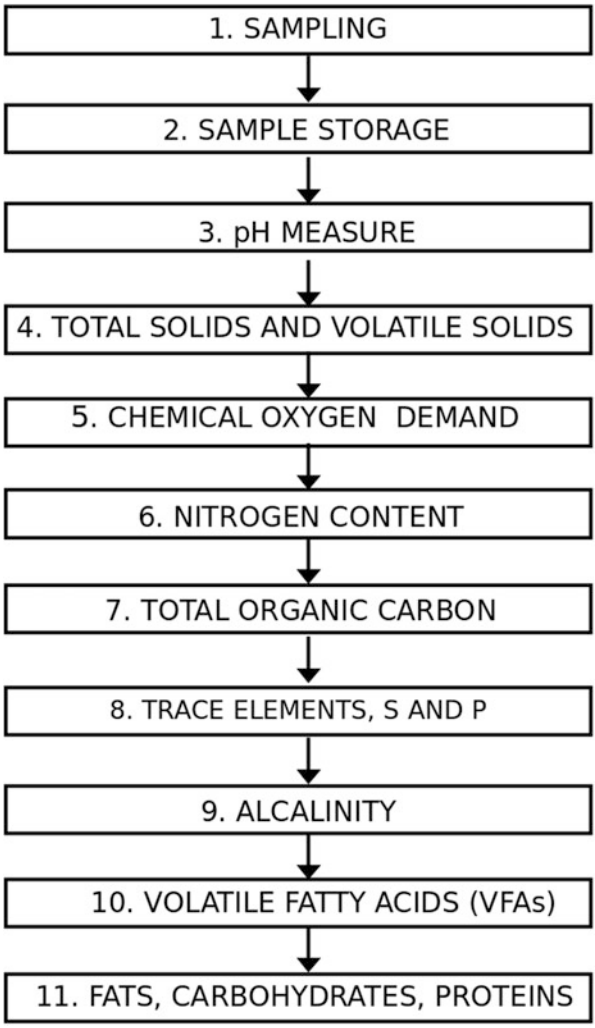

Fig. 10.1 Main analysis performed to characterize anaerobic digestion feedstock

proteins. As we see from (Koch et al. 2020a), methane yields of waste food can be predicted from the composition of the raw material, and the model can be calibrated with the results of BMP tests. According to Boyle (Boyle 1977), in fact the following yields can be obtained for the different food components:

- Cellulose and starch: 414 L_{CH4}/kg_{VS} (Angelidaki and Sanders 2004; Batstone et al. 2002).
- Sugars: 392 L_{CH4}/kg_{VS} (Moletta et al. 1986; Ntaikou et al. 2010).
- Proteins 534 L_{CH4}/kg_{VS} (based on the following empirical formula $C_4H_{6.1}O_{1.2}N$) (Batstone et al. 2002; Miron et al. 2000).

– Lipids: 1006 L_{CH4}/kg_{VS} (based on the following empirical formula: $C_{51}H_{98}O_6$ of tripalmitin) (Batstone et al. 2002; Kleerebezem and van Loosdrecht 2006).

Predictive biogas models have been developed also by (Raposo et al. 2020).

An interesting overview on feedstock analysis and biogas potential yield measurement is presented in (Weinrich et al. 2018).

10.2.2 Sampling

Sampling of biogas feedstock can be performed according to the standards VDI 4630 (2016) and ISO 5667-13 (2011) and the document developed by Petersen (2005). In order to obtain correct results, according to VDI 4630 (2016), the sampling procedure has to be based on the principles reported in Table 10.2.

Experimental errors must be avoided. They generally take place during the sample collection, the sample preparation and treatment, and the analysis. If the material is homogeneous, one sample is enough. If the material shows a phase separation, one sample from each phase should be taken. For the solid materials, the procedure shown in Table 10.3 can be performed.

Table 10.2 Principles behind a correct sampling procedure

Step number	Action
1	Identify the objective of the investigation
2	Identify the source of the material
3	Characterize the projected sample
4	Analyze the sampling parameters
5	Analyze the deviation of sample characteristics, due to the influence of the time of sampling and of the location
6	Consider the requirements for protection and security for the personal taking the sample

Table 10.3 Sampling procedure for solid sample

Step number	Action
1	Take a large sample from the bulk material
2	Spread it on a surface and then mix it
3	Implement a cross in the middle of the sample
4	Remove two opposite quarters
5	Spread the other two quarters
6	Mix again
7	Then divide the sample with a cross
8	Remove two quarters
9	Repeat again till the quantity which is required for the analysis is remained

For samples which are in liquid phase, these have to be mixed well before sampling. In the case in which the sample is taken using a valve, the material which exits first has to be excluded. If the sample is taken in pipes, a vertical pipe is preferred than a horizontal one, to avoid the deposits of sediments.

10.2.3 Sample Transport, Storage, and Preparation

The sample is then put in sealed vessels. It has to be preserved by cooling it to 4 °C during transport and storage, which is preferable to be quite short. If the storage time is forecasted to be long, it is preferable to store the sample at −20 °C and so freeze it to preserve its original characteristics. Physical impurities can be separated from the sample, previously taking note of their masses. For some kinds of analysis, preparation of the sample can be done through drying or milling. To improve the sample blending performance, water can be added.

10.2.4 pH Measurement

The pH defines the basicity or acidity of an aqueous solution. Usually, it is measured in liquid raw materials, using a standard potentiometric electrode (standards EN 12176 (1998) and APHA 4500-H+ B (National Environmental Methods Index, 4500-H+B n.d.)). For solid samples, before the measuring, they can be mixed with water. The typical pH values in anaerobic fermentation are generally slightly above neutral. During the process, the pH is maintained constant by the buffer capacity inside the reactor, which is due to mainly the presence of CO_2 (which is adsorbed in gaseous form into the liquid), ammonia (which is present in liquid phase), and water, that is also present inside the reactor. If the pH value is very high or too low (e.g., <pH 6.8, >pH 7.5), in this case the buffer capacity is probably not sufficient to control effectively the pH, so it can be needed a neutralization step before feeding the feedstock to the biogas plant. It is quite frequent to have a small acidification of the feedstock, for example; in that case, the pH value can be controlled with the addition of bases (e.g., $Ca(OH)_2$, Na_2CO_3, NaOH) in the reactor (Bischofsberger et al. 2005). The pH is measured initially on the substrate before using it in an anaerobic digestion test to know if it has to be corrected and if acidification is already ongoing. Then, pH has to be measured also during the anaerobic tests to see how the process is developing and if the buffer capacity of the substrate is properly working (see Fig. 10.2). Usually substrates like food waste can cause in general acidification of the mixture in the reactor; for this reason, also two-phase anaerobic digestion can be performed.

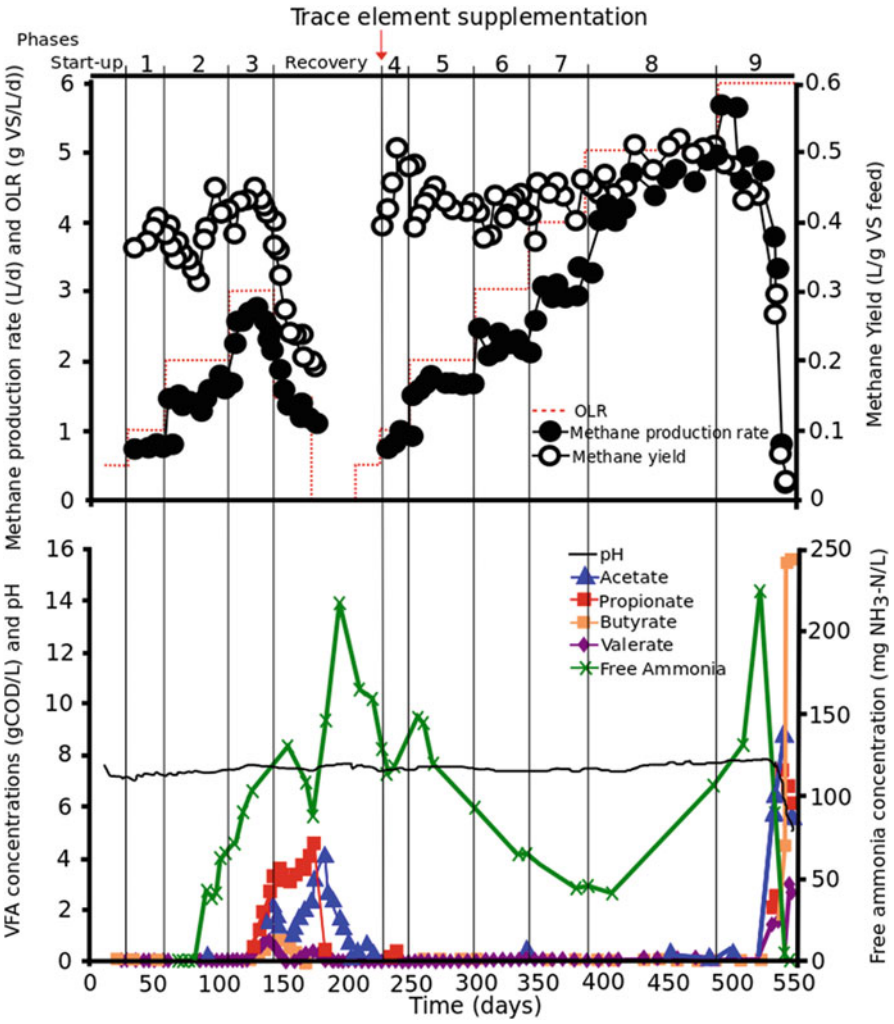

Fig. 10.2 pH trend in anaerobic digestion, together with other parameters (Jo et al. 2018)

10.2.5 Total Solids (TS) and Volatile Solids (VS)

TS or VS values are usually expressed in mass percentage and determined through proximate analysis. Total solid determination is performed by drying the sample until its weight reaches a constant value. The temperature is usually set to 103–105 °C (standards EN 12880 (2000) and APHA 2540 B (2540 SOLIDS 2017)). Through the drying process, the total solids are determined, but we do not know how much would be the low molecular weight organic volatiles (which are represented by low boiling substances, like volatile acids and alcohols) that are not

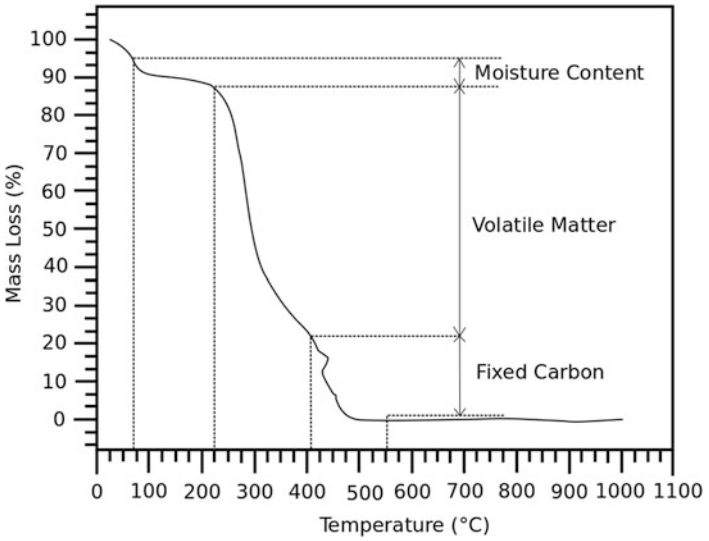

Fig. 10.3 TGA diagram which highlighted the main biomass components (El-Sayed and Mostafa 2014)

measured. They can represent an important fraction of wastewaters. The total solid content is used to understand if the feedstock contains sufficient moisture. If the moisture content (which is complementary to 100% to the solids content) is low, dilution has to be performed using the following:

- Fresh water
- Adding other more liquid feedstocks
- Recycling part of the digestate or its liquid fraction, once the solid–liquid separation has been performed

If the liquid content is too high, this will mean that too few nutrients are fed to the biogas reactor which will not work in an efficient way.

For calculating the amount of organic matter in the raw material, the volatile solids (expressed in %) or organic dry matter (expressed in g/l) should be determined. This determination is usually carried out using a thermogravimetric balance (see Fig. 10.3) (The related standards are EN 12879 (2000) and APHA 2540 E (2540 SOLIDS 2017)). Obviously, in this measurement are not counted the low molecular weight organic compounds which evaporate during drying.

10.2.6 Chemical Oxygen Demand (COD) and Biochemical Oxygen Demand (BOD)

Especially for wastewater treatment and anaerobic digestion processes, different types of chemical oxygen demand (COD) can be considered, based on its biodegradability, particulate, or soluble state. We distinguish so biodegradable and nonbiodegradable COD:

- The biodegradable COD can be readily biodegradable and non-readily biodegradable;
- The nonbiodegradable COD can be soluble nonbiodegradable or particulate nonbiodegradable.

The chemical oxygen demand indicates the organic matter content of a feedstock which can be chemically oxidized. The analysis can be performed through the titration of unreacted potassium dichromate (Standards DIN 38414 (DIN 38414-8 1985) and APHA 5220 B (5220 CHEMICAL OXYGEN DEMAND (COD) 2017) apply). Also spectrophotometry can be used or online UV-photocatalytic oxidation (Dan et al. 2005). The COD is a direct representation of what is the maximum energy contained in the chemical boundaries of the organic matter in the raw material. This is in fact the measurement of the presence of potential reacting substances which can undergo the anaerobic degradation process and produce biogas, at the net of the energy needed to sustain the microbia converting biomass. Some of the COD is not biodegradable, for example, the complex organic molecules. Raposo shows an improved process for determining the COD of substrates with high total solid content. Some reference data on different matrices are proposed in Table 10.4 (Raposo et al. 2008).

In reality, as above mentioned, COD is constituted by different fractions. As presented in (Myszograj et al. 2017), the fractions of COD are indicated in the following equation:

Table 10.4 COD content of different matrices (Kim et al. 2010)

Type of waste	Feedstock	Standard deviation	Digestate	Standard deviation
Sludge of municipal wastewater treatment plant	20,400	141	12,800	1992
Sludge of livestock wastewater treatment plant	25,200	6505	15,833	2650
Sludge of food wastewater treatment plant	26,300	1273	18,233	961
Organic rumen substance originated from a slaughter house	26,750	5869	17,000	2600

$$\text{CODTot} = \text{SS} + \text{SI} + \text{XS} + \text{XI} + \text{XH} + \text{XA} + \text{XP gO2/m3} \quad (10.1)$$

where:

Ss—represents the soluble and ready to be biodegraded substrates, gO2/m3
SI—inert organic soluble material, gO2/m3
XS—substrates in particulate form which are slowly biodegradable, gO2/m3
XI—organic material made by inert particulate, gO2/m3
XH—heterotrophic organisms, gO2/m3
XA—autotrophic nitrifying organisms, gO2/m3
XP—decay products, gO2/m3

If the biomass microbial fraction is not considered, the model can be simplified into the following form (Myszograj et al. 2017):

$$\text{CODtot} = \text{SS} + \text{SI} + \text{XS} + \text{XI gO2/m3} \quad (10.2)$$

Examples of COD fraction percentages contained in raw municipal sewages in Italy are $S_I = 6$; $S_S = 15$; $X_I = 8$; $X_S = 56$; $X_H = 15$; $X_S + X_H = 71$. COD fractions for sewage sludge available in other geographic areas are presented in (Myszograj et al. 2017; Ekama et al. 1986; Kappeler and Gujer 1992; Rieger et al. 2001; Pasztor et al. 2009; Xu and Hultman 1996; Melcer et al. 2003).

10.2.7 Concentration of Nitrogen

The nitrogen concentration of a raw material which is used for anaerobic digestion is usually determined through the total Kjeldahl nitrogen (TKN) analysis (ISO 5663 (1984); ISO 11261 (1995); APHA 4500–Norg B (National Environmental Methods Index, 4500-NorgB n.d.)). In this analysis, the sample is first mixed with sulfuric acid and a catalyst and then boiled to obtain ammonia. In the second step, the mixture containing the sample is added with a base to change the pH and cause the conversion of ammonium ions into ammonia (NH3). This is separated by distillation from the solution with basic pH, and it is brought a solution with acid pH to make it condense. Then the amount of ammonia can be determined using, for example, potentiometric acid titration or the photometric phenate method (ISO 5663 (1984) and APHA 4500-Norg B (National Environmental Methods Index, 4500-NorgB n.d.)).

The determination of ammonia nitrogen (i.e., NH_4–N) is similar to that already explained in the Kjeldahl method, but without considering the first boiling step which is necessary for organic nitrogen degradation (DIN 38406(E5) (DIN 38414-8 1985); APHA 4500-NH3 (National Environmental Methods Index, 4500-NorgB n.d.)).

The analysis of the TKN in the raw material is important, to understand if the content of nitrogen is sufficient for the development of anaerobic bacteria.

Usually during the anaerobic digestion process, about 60–80% of the TKN is transformed to ammonia. Too much ammonia production can exert also an inhibiting effect, so this also has to be avoided. This problem is more important when substrates rich in proteins are digested, such as wastes of slaughterhouses, rape seed cake, stillage, and residues obtained from the meat processing industry. The TKN essay does not measure obviously all the nitrogen contained in the feedstock because nitrates and nitrites are not detected.

10.2.8 Total Organic Carbon

In a sample, the total carbon is composed by the following:

- The total inorganic carbon (TIC) which is basically represented by the dissolved carbonate, carbon dioxide, and bicarbonate
- The total organic carbon (TOC) which represents the organic carbon, which can be both dissolved or present in form of particulate matter

Therefore, TOC can be either directly analyzed and measured or determined by subtraction of TIC from the total organic carbon.

The standard procedures to determine TOC content are reported in EN 1484 (1997) or APHA 5310 (5310 TOTAL ORGANIC CARBON (TOC) 2017). Sample preparation is very important for biogas feedstocks. We can have different cases:

- Solid feedstock can be usually dried
- Liquid samples, such as polluted watery samples (without particulate matter), can be inserted directly into a TOC analyzer
- In the case of liquid samples with particulates (which is the most frequent in case of anaerobic digestion), a solid–liquid separation step is necessary, then followed by a separate analysis of the two obtained phases.

The first step in the TOC analysis is the total inorganic carbon (TIC) removal. For this sake, a sample is acidified with CO2-free gas. The second step is oxidation of the total organic carbon. This can be done in two ways:

1. Combustion in case of solid sample
2. Using UV ray photo-oxidation or chemical oxidation in case of a liquid sample

Once the sample has been oxidized, the produced CO2 is measured using infrared sensors or thermal conductivity sensors.

The final information given by the TOC analysis is the total organic carbon; the researcher has to take into consideration that this total organic carbon can be composed also by non-digestible carbon, such as the one contained in lignin.

10.2.9 Trace Elements

The methods to be followed in the TE (trace elements) analysis are reported in the EN 13346 (2000) (sludges) and in the ISO 11885 (wastewater) (2007), as in APHA 3120 C (National Environmental Methods Index, 3120 B n.d.). Drying and milling of the biogas feedstocks is recommended before the analysis.

After drying, the sample is digested using boiling aqua regia (EN 13346 2000). Filtration is practiced on the sample after digestion. The determination of the trace elements can be done then by using different instruments:

- AAS (atomic absorption spectroscopy)
- ICP-OES (inductively coupled plasma—optical emission spectroscopy).
- ICP-MS (inductively coupled plasma—mass spectroscopy)

This analysis usually is used to determine the concentrations of many trace elements. The useful ones in the anaerobic digestion are represented by Co, Ni, Mn, Fe, Se, Zn, and Mo. These are used to build up enzymes, nucleic acids for the bacteria, and vitamins. Scarcity of useful trace metals can be encountered if single substrates are digested (e.g. corn silage), while the problem can be avoided, for example, with co-digestion.

On the other hand, toxic heavy metals can exert a toxic effect on bacterial growth, so those concentrations also have to be checked.

10.2.10 Sulfur

According to ISO 11885 (2007), the total sulfur content can be analyzed using ICPOES. The preparation process for the biogas feedstock sample is almost the same as for the trace element analysis. However, the sulfur wavelength is very low with respect to the trace metals which have been previously described. This can make detection not easy and reduce its accuracy. The effect of high sulfur concentration on the anaerobic digestion process is mainly negative and responsible for the formation of H2S, which can inhibit the microbial fauna but also damage the internal combustion engine used to covert biogas in electricity and heat. The damage caused by H2S can be also increased by the high presence of NH3 so the two gases have a synergistic negative effect (Chen et al. 2008).

10.2.11 Phosphorus

Phosphorus content can be measured according to the methods reported in the following norms: ISO 6878 (2004), APHA 4500-P (4500-P PHOSPHORUS 2017), and DIN 38414 (S12) (DIN 38414-8 1985). Methods can be colorimetric

or based on optical emission spectrometry (OES). In the first step, as previously seen, the samples of feedstock are dried and milled; then they are digested using a mixture of nitric acid and sulfuric acid. The aim is to solubilize all the existing phosphorous. Then using molybdate, which is an acid complexing agent, the blue complex antimony phosphorous molybdate is produced. This allows the colorimetric detection of phosphorous. To determine the total phosphorous, another approach is defined in ISO 11885 (2007) (wastewater) and EN 13346 (2000) (sludges). When the sample is digested, the phosphorous concentration can be analyzed by AAS, ICP-MS, or ICP-OES.

10.2.12 Alkalinity

Alkalinity is a typical measurement of water and wastewater quality. It is the capacity to neutralize acid components. It is defined as the sum of all the bases which can be titrated. Because the alkalinity of many feedstock is mainly influenced by the concentration of carbonate, bicarbonate, and hydroxide, it indirectly provides an information on the concentration of these compounds. The final measured value may include also the contribution from other compounds, such as borates, phosphates, silicates, and other bases, if contained. Properly operating anaerobic digesters typically have supernatant alkalinities ranging from 2000 to 4000 mg calcium carbonate (CaCO3)/L (Pohland and Bloodgood 1963). Alkalinity is measured mainly with the titration method, in which hydroxyl ions, which are present in the sample, due to the dissociation or hydrolysis of the solutes, react with acid compounds, which are added. The final alkalinity value depends also on the pH considered as an end-point (2320 Alkalinity n.d.). Alkalinity can be also determined with laboratory test kits, such as the ORION total alkalinity test kit (produced by Thermo Scientific).

10.2.13 Volatile Fatty Acids

The measurement of VFA is typically performed during the anaerobic digestion process to check how this is evolving. An example of online monitoring system for VFA is shown in Fig. 10.4.

For this purpose, different online titration processes can be employed, a comparison of which is proposed in (Holten Lützhøft et al. 2014).

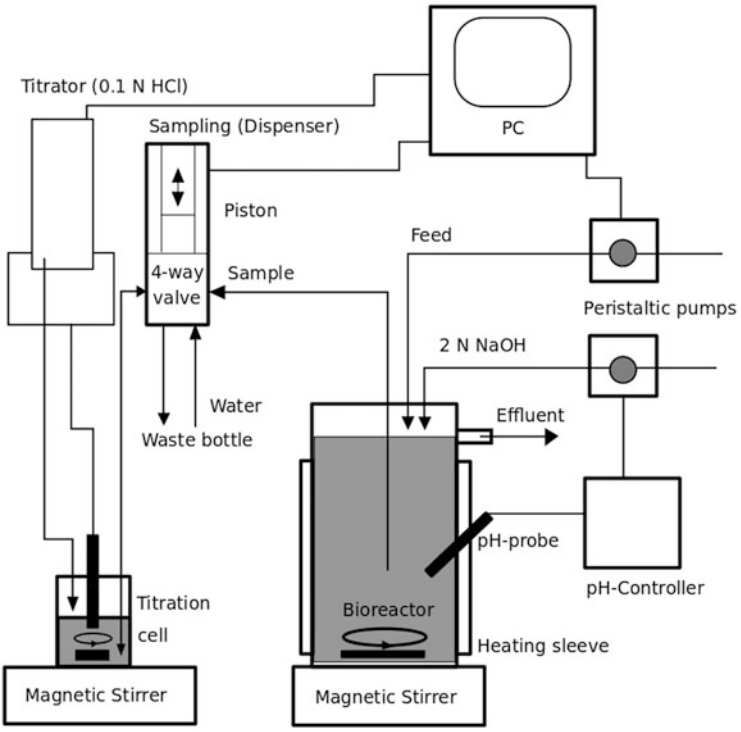

Fig. 10.4 Online monitoring of VFA (Feitkenhauer et al. 2002)

10.2.14 Carbohydrates

To understand the methane yield and model, the anaerobic digestion process, in case of food feedstock, to determine the concentration of carbohydrates, protein, and lipids can be beneficial. The total carbohydrate content has to be calculated by subtraction to 100% of the percentage of proteins, fats, moisture content, and ash (Nielsen 2017).

10.2.15 Fats

The determination of the total lipid content of a feedstock is determined by extraction with organic solvents. An optimal solvent to be used in fat extraction should have the characteristics shown in Table 10.5 (Nielsen 2017).

Among the most used solvents, we find ethyl ether and petroleum ether but also pentane and hexane.

Table 10.5 Solvent characteristics

Number	Feature
1	High affinity for lipids
2	Low affinity for proteins and carbohydrates
3	It has to evaporate readily and to leave no residue
4	Low boiling point is required
5	Nonflammable and nontoxic solvents are preferred
6	Efficient penetration of sample particles
7	Low cost solvents are preferred
8	Non hygroscopic materials are also preferred

Two possible extraction processes can be applied:

- Continuous solvent extraction method: Goldfish method
- Semi-continuous solvent extraction method: Soxhlet method

These are quite well-known processes which do not need to be discussed further.

10.2.16 Proteins

To measure proteins, different methods can be employed, among them:

- Nitrogen-based methods
- Infrared spectroscopy
- Colorimetric methods
- Methods using ultraviolet absorption
- The determination of nitrogen which is nonproteic

Among those, one of the most used is the Kjeldahl method.

10.2.17 Summary

For the biogas feedstock analysis, the available normative is of fundamental importance to standardize the process. Table 10.6 shows a brief overview of the most significant available standards and approaches.

Table 10.6 Overview of the most important methods for the characterization of feedstock to be converted in anaerobic digestion processes

Type of Analysis	Standard
Sample taking	VDI 4630
	ISO 5667-13
pH value	EN 12176
	APHA 450-H + B
TS/DM	EN 12880
	APHA 2540 B
VS/ODM	EN 12879
	APHA 2540 E
COD	DIN 38414 (S9)
	APHA 5220 B
TKN	ISO 5663
	ISO 11261
	APHA 4500-Norg B
NH4-N	Din 38406 (E5)
	APHA 4500-NH3 B
	APHA 4500-NH3 C
	APHA 4500-NH3 F
S	ISO 11885
P	EN 13346
	DIN 38414 (S12)
	ISO 6878
	APHA 4500-P B
	APHA 4500-P E
Trace element	ISO 11885
	EN 13346
	APHA 3120 C
BMP	EN11734
	DIN 38414 (S8)
	VDI 4630
TOC	EN 1484
	APHA 5310

10.3 BMP Tests

10.3.1 Introduction

Generally, the biomethane potential (BMP) test is usually used to measure the methane generation from different feedstock, both solid and liquid. A number of norms were developed to standardize the BMP tests, such as ASTM D 5210 (1992) (ASTM 2007), ASTM D 5511 (1994) (ASTM 2018), DIN 38414 TL8 (1985) (DIN 38414-8 1985), ISO 14853 (1998) (ISO 14853 2016), ISO 11734 (1995) (ISO 11734 1998), and ISO 15985 (2004) (ISO 15985 2014). In 2006, the Association of German Engineers issued the first description of the comprehensive methodological

guideline VDI 4630 (2016) titled "Fermentation of organic materials." The procedures and standardized practices dealing with BMP analysis are continuously improving; see (Weinrich et al. 2018; Holliger et al. 2016). For the BMP test protocols, detailed guidelines were published by many authors (Raposo et al. 2011); Cresson et al. 2015), and international tests among different laboratories have demonstrated that the results of the biomethanation potential measurements can vary considerably between different laboratories, which require highly standardized BMP test protocols and procedures. This means that each aspect of the BMP analysis has to be highly repeatable and standardized as follows:

– The identification, the characterization, and the preparation of the inoculum;
– Substrate pretreatment, substrate preparation, and its storage;
– Test parameter setup;
– Data statistical analysis and data reporting;
– Final result interpretation.

In order to authenticate BMP test outcomes, the following essentials must be satisfied:

- All experiments must be repeated at least three times.
- Together with the BMP of the substrate, also positive controls (performed using standardized materials, like microcrystalline cellulose and tributyrine) and blank assays must be assessed. The positive controls represent tests where the final results are standardized and known; while the blank tests are useful to assess the inoculum production and so to subtract this to the final methane yield obtained in the test, this makes the test result not dependent from the inoculum which has been used.
- The duration time of the BMP test is not predetermined before starting, but it is determined based on the daily methane production; when this parameter is lower than 1% of the total volume of methane, which has been accumulated during the whole period, the test is stopped (the parameter is also called BMP1%).
- The final BMP results consist of the value of methane production expressed in volume of dry methane produced, referred to as standard conditions (273.15 K and 101.33 kPa) per mass of volatile solids (VS) contained in the feedstock. The unit of measure is the following: $NLCH_4kgVS^{-1}$;
- The BMP positive control and the final results of the substrate analysis are then elaborated subtracting the methane yield obtained from the blank tests, to obtain the real production of the substrate and of the positive control assays. This has to be done also taking into consideration the standard deviation of the blank tests, which is expressed in the following equation:

$$\mathrm{BMP} = \mathrm{BMP}_{\mathrm{average}} \pm \sqrt[2]{(\mathrm{SD}_{\mathrm{blank}})^2 + (\mathrm{SD})^2}$$

where BMP is the biochemical methanation potential of the substrate, BMP average is the average of the results obtained from the three times repeated analysis, SDblank

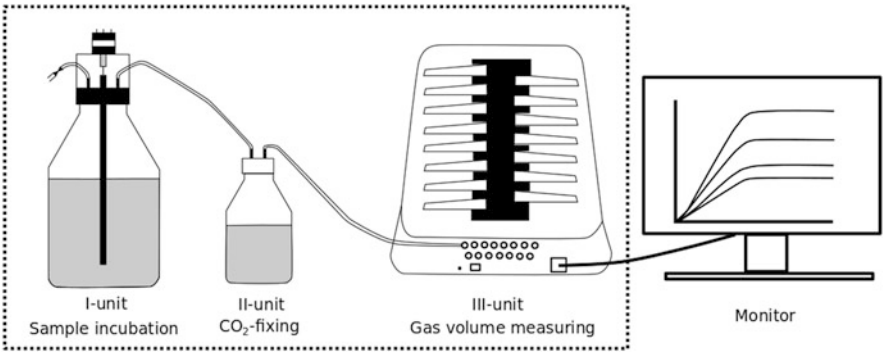

Fig. 10.5 AMPTS II® apparatus; (I) incubation unit; (II) CO2 capture unit; (III) Flow Cell System (Amodeo et al. 2020; AMPTS II n.d.; Liu et al. 2016)

is the standard deviation of the blank tests, and SD is the standard deviation of the tests performed on the substrate.

- If any one of the subsequent conditions is fulfilled, the test results must be rejected:
  - If the RSD is higher than 5% of the final value of the blank or of the positive control, even after eliminating one single outlier
  - If the RSD is higher than 5%, of the final value of the homogenous substrate, even after eliminating one single outlier
  - If the RSD is higher than 10%, of the final value of a heterogeneous substrate, even after eliminating one single outlier
  - If the BMP is higher than 85% and 100% of the value of the positive control and of the theoretical BMP value (e.g., for cellulose: <352 NLCH4 kgVS-1 and > 414 NLCH4 kgVS-1)

The Dixon's test will be used to reject a single outlier from triplicate measurements. If it is possible to have a substrate inhibition condition, then tests with different inoculum-substrate ratios (ISRs) have to be performed in parallel.

An example test apparatus for BMP tests is presented in Fig. 10.5 (Amodeo et al. 2020). This presents the AMPTS II system (AMPTS II n.d.) which is composed by a set of 15 bottles or reactors of the volume of 0.5 L each. Each reactor is connected to a CO2 trap which consists of a vessel which is filled with NaOH (estimated capture efficiency is about 98%). Once the carbon dioxide has been retained, the pure flow of methane is measured through a flow cell array, using the displacement of liquid and buoyancy phenomena.

10.3.2 Recommendations to Perform BMP and Validate Correctly the Results

This part presents the diverse items that intensely affect the results of BMP tests. To increase the possibility of finding authenticated and reproducible BMP test results, some recommendations are made, referring especially to the inoculum, the substrate, the test setup, data analysis, and reporting.

10.3.2.1 Inoculum

The inoculum must be obtained from an anaerobic digester, which is active at the time of sampling and is digesting complex organic substrates. Sampling of the inoculum should be performed at steady state.

Quality control of the inoculum is performed through its characterization, which is mainly based on the following:

- The analysis of pH
- The analysis of volatile fatty acids (VFA) concentration
- The analysis of ammonium concentration
- The analysis of alkalinity

Another way to check inoculum quality is that of analyzing the operational parameters of the digester, from which the inoculum itself is taken. Optimal values for operational parameters are as follows:

- pH: >7.0 and <8.5
- $NH4^+$: < 2.5 gN_NH4 L^{-1}
- VFA: < 1.0 gCH3COOH L^{-1}
- alkalinity: > 3 gCaCO3 L^{-1}

All the routines to analyze the inoculum activities are presented in the work of Angelidaki et al. (2009). The inoculum usually has the most important characteristic that of producing low quantities of methane, so to have a reduced content of organic load. The final aim of the inoculum is in fact to provide the microbial flora to the digester and limit organic matter load. Total methane production of the inoculum should be below 20% of the production of the substrate. If the inoculum has too high organic load, a pre-incubation period could be requested to bring it down. Other possible pretreatments of the inoculum can be sieving and dilution with nitrogen flushed deionized water. For sieving, usually a mesh of 1–5 mm is used. Dilution is performed in the case the volatile solid concentration reaches very high values, such as higher than 100 g L^{-1}. Storage of inoculums should be performed at ambient temperature for a maximum of 4–5 days.

10.3.2.2 Substrate

The sampled substrate has to be representative of the final feedstock which will be digested on an industrial scale. The preparation of sample to be used in the tests has to be well documented, e.g., describing the sampling process and technique in brief details and by taking pictures. In the German guideline VDI, a complete explanation of sampling processes is provided. Usually, sample preparation has to be reduced to leave its properties and its digestibility similar to those of the original substrate. These are the most followed procedures:

- Coarse materials and inert materials (e.g., plastics, gravel, and sand) have to be removed.
- If the particles in the organic fraction are too large, grinding or shredding might be required. To avoid heating too much the sample during grinding, freeze grinding or cryogenic grinding can be performed. Optimal diameter of particles is lower than 10 mm. To obtain this length, the sample can be filtered; in this way the big particles will be separated and then milled.
- All the pretreatments have to be noted carefully in the laboratory book by the staff.
- The storage of the substrate can be performed for a maximum og 2–5 days at 4 °C. If longer storage periods are required, the samples can be frozen at −20 °C. Drying of the sample for storage purposes has to be avoided (Kreuger et al. 2011).
- Before performing BMP tests, the complete characterization of the substrate has to be performed. The most important analyses are represented by volatile solids, total solids, pH, Kjeldahl nitrogen, alkalinity, ammonium, and volatile fatty acids. Another key parameter is represented by chemical oxygen demand (COD), Raposo (Raposo et al. 2009); Raposo (Raposo et al. 2008); Noguerol-Arias (Noguerol-Arias et al. 2012), together with elemental composition (CNHX) and total organic carbon.

10.3.2.3 Test Setup

In this section, the conventional setup most used for BMP tests it is described; accordingly, other previous setups can be found in the German guideline VDI and in the work of Guwy (2004).

The volume of the vessels used to perform the BMP tests depends on three factors:

1. The degree of homogeneity of the substrate
2. The volume of biogas which will be produced
3. The precision of the techniques used for gas measurement

For homogenous substrates, smaller volumes (≈100 mL) can be used, whereas for heterogeneous substrates, larger volumes are preferred, in the rage of 500 mL to 2000 mL.

Usually, the conventionally employed volume is comprised between 400 mL and 500 mL. This gives good results also in terms of reproducibility. In the case of volumetric and manometric measurement of the gas, the volume of the vessels can be also higher and comprised between 500 mL and 1000 mL, respectively.

The vessels have to be locked with butyl rubber or silicon septs to obtain gastight conditions. The abovementioned septs, in the case of manometric measurement of the produced biogas, need to have enough dense to be pierced many times with a needle to allow the biogas to evacuate from the bottle and be sampled. If the biogas volume and composition is measured with a device which is connected to the vessel, the piping has to grant that no leakage and no air infiltration should happen. To avoid any leakage, tests should be carried out before starting the incubation test. When composing the mixture to be inserted in the vessel to perform the BMP tests, usually the quantity of inoculum is higher than that of the substrate; for this reason, the inoculum in this case not only represents the origin of microbia but also provides micro and macro-elements, vitamins, and nutrients to the same, together with an important buffering capacity. If not sufficient, micro and macro-elements can also be supplied or integrated (Angelidaki et al. 2009). If the measurement of the alkalinity is below 3 $gCaCO_3\ L^{-1}$, then it has to be controlled by adding sodium bicarbonate to the inoculum, to reach at least 3 $gCaCO3\ L^{-1}$. During the test preparation, to avoid changes in the carbonate balance (Koch et al. 2015) considering the quantity of carbon dioxide that dissolves in water, flushing has to be performed using a gas mixture of N_2 and CO_2, in which the volumetric concentration of CO_2 resembles that expected for the concentration of CO_2 in the biogas (e.g., 20–40v% CO_2; remaining concentration represented by N_2). When preparing the mixture to be digested, a concentration of volatile solids of about 20 to 60 $gVS\ L^{-1}$ is recommended. In all the vessels, the volatile solids added through the inoculum should be the same. If the sample is very dry, this can be diluted using deionized water. The inoculum to substrate ration (ISR) is an important parameter to be considered in the BMP test; this is calculated as the ration of the volatile solids contained in the inoculum to the volatile solids contained in the substrate. To avoid acidification and inhibition processes, it is recommended that the concentration of volatile solids in the inoculum should be higher than that in the substrate (e.g., ISRs of 2 and 4 are optimal for easily degradable substrates, to avoid accumulation of VFA). An ISR less than or equal to one can be applied for less degradable substrates, such as lignocellulosic organic matter. Anaerobic digestion can be performed both at thermophilic and mesophilic conditions. Usually, the same conditions of the anaerobic digestion which has provided the inoculum should be adopted. During incubation, mixing is needed. If the substrate is mixed with continuous mechanical movement, this should be performed at moderate intensity. In most of the cases, manual mixing can be performed once a day to avoid the formation of scum layers.

10.3.3 Limitations of the BMP Tests

Current limitations to the BMP practices are correctly identified in the works of Hafner; see (Koch et al. 2019, 2020b; Justesen et al. 2019; Hafner and Astals 2019; Hafner et al. 2018b). Among them, leakages, the difficulty to describe correctly co-digestion mixtures, and the toxicity of substances added to the digested mixture, make it not possible to extend the results obtained in batch mode to industrial reactors working in continuous way.

Also in (Koch et al. 2019), a nice graphical approach on how to understand mistakes in experimental procedures from the curves showing the methane yield trend is proposed.

Usually, a normal BMP curve can be recognized based on four basic aspects:

1. The increase of methane production has to be monotonic and the slope should not be negative
2. The increase had to be quite steep in the first part with a reduced initial lag; then after the first increase, a steady state should be reached and an almost constant value of CH_4 production
3. The slope has not undulate
4. The progression of the curve has to be of first order (Brulé et al. 2014) or based on the equation by Monod (Koch and Drewes 2014), but not the one describing the Gompertz (Gompertz 1825; Zwietering et al. 1990) model. If the curve resembles that of the Gompertz model, this can indicate probably the probable existence of design and/or execution errors.

The main three errors which are taken into account in (Koch et al. 2019) (see also Fig. 10.6) are as follows:

– Incorrect inoculum storage
– Incorrect inoculum dilution
– Errors in the choice of inoculum to substrate ration (ISR)

Inoculum storage plays a fundamental role in permitting the activation of the used inoculum when inserted in the BMP reactor or bottle (De Vrieze et al. 2015; Koch et al. 2017). The most important parameter to perform a good storage procedure is temperature and not time (Li et al. 2014; Hagen et al. 2015; Wang et al. 2016).

In fact, storage at low temperatures can induce a lag phase in the experiment, while storage at ambient temperature is recommended (Li et al. 2014; Hagen et al. 2015).

Dealing with inoculum dilution with deionized water, some studies suggest that this can have a beneficial effect and contribute to avoid inhibition from unknown substances contained in the substrate (Holliger et al. 2016; Angelidaki et al. 2009). The volatile solid concentration in the original inoculum has to be around 10–30gVS/L (Raposo et al. 2011, 2012). These values are also very similar to the values that the final mixture should have. On the other hand, an excessive dilution of the inoculum can also have drawbacks and underestimate the final production of

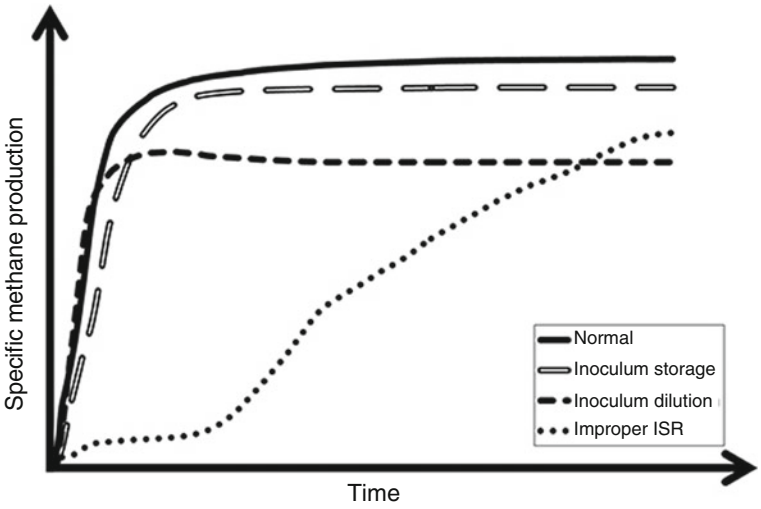

Fig. 10.6 How three typical errors in BMP experiments can affect the trend of the final CH4 production curve (Koch et al. 2019)

biogas and methane, according to (Wang et al. 2015; Reilly et al. 2016). With dilution, we can have two main disadvantages:

- An impairment of the buffer capacity exerted by alkali components contained in the inoculum and in the substrate, which will be more diluted in water
- A decrease in the concentration of microbia, where methanogens have a lower growth rate (Pagliano et al. 2018) with respect to other bacteria which perform the first reaction of the anaerobic digestion process (this will bring again to an acidification of the substrate being acids and alcohols formed without the existence of a sufficient quantity of bacteria which is necessary to transform them in methane). This can be also verified with kinetic modeling of the bacterial consortium (Eastman and Ferguson 1981).

Dealing with the inoculum to substrate ratio (ISR), this has not to be too low; in fact in that case the quantity of inoculum will be reduced, with respect to the quantity of the substrate, and so there would not be a balance between the microbia and the substrate (Hashimoto 1989; Raposo et al. 2006; Kafle et al. 2014; Polizzi et al. 2017). The optimal ISR usually is about 2, as reported in (Holliger et al. 2016).

10.4 Gas Yield Quantification

Methane production is the final result of the BMP tests; this can be measured with different methods, among them are as follows:

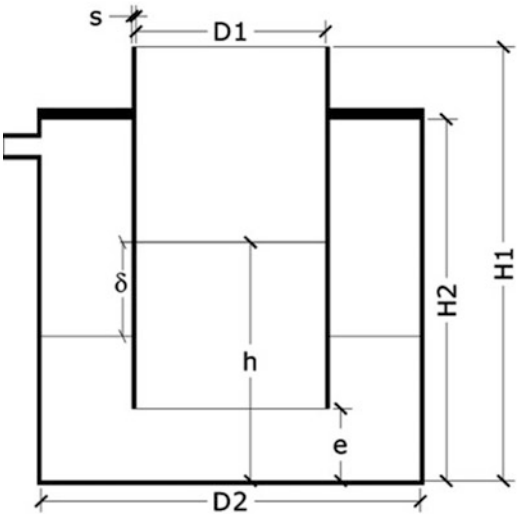

Fig. 10.7 Biogas yield monitoring system (Fantozzi and Buratti 2009)

1. Manometric method
2. Volumetric method
3. Gas chromatography

In the volumetric methods, CO_2 is adsorbed in an alkaline solution and so separated from methane; in this way only the volume of CH_4 is measured by volumetric displacement. In the manometric method, the production of biogas is calculated using a regression based on the increase of the pressure values inside the vessels. To avoid the too much CO_2 dissolving inside the substrate and also the risk of explosion, the pressure in the vessels must not exceed 300 kPa. Once it has been estimated or measured, the gas volume has to be converted to dry gas, referring at standard conditions (273.15 K, 101.33 kPa) following the procedure reported in Strömberg et al. (Strömberg et al. 2014). For this sake, the ambient temperature and pressure needs to be always registered. For the manometric measurement and the gas chromatographic measurement, the GC should be calibrated with mixtures of standard gases (e.g., a mixture of CH_4 and CO_2; 50%/50%; v/v). The volume of the produced biogas can also be measured using a so-called gas meter. As reported in (Fantozzi and Buratti 2009), this can be done with a storage vessel, which is filled with water, and by measuring the height of the water in an inner column, the volume of the produced biogas can be estimated.

The gasometer presented in Fig. 10.7 can be linked to an instrumented and automatized anaerobic digestion batch plant, as shown in Fig. 10.8 (Fantozzi and Buratti 2011).

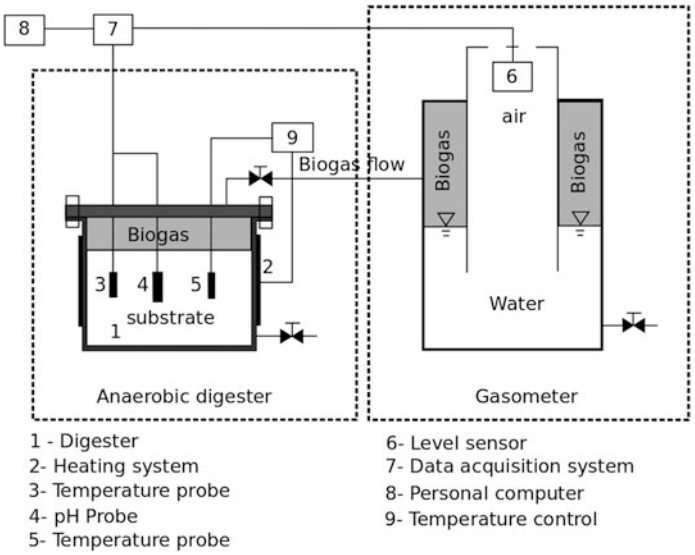

Fig. 10.8 Batch anaerobic digestion plant coupled to a gasometer (Fantozzi and Buratti 2011)

10.5 Biogas Composition Analysis

Gas composition analysis mainly aims at determining the concentration of the two main components of biogas: CO_2 and CH_4 (Mahmoodi et al. 2018). This can be easily done in discontinuous and continuous analysis performed with gas-chromatography. Often TCD sensors are used to this aim. Inside the gas are also trace components, like ammonia (Strik et al. 2006) and siloxanes (Hayes et al. 2003) which also have to be measured.

10.6 Data Analysis and Reporting

As already said, the main result of the BMP test is the volume of dry methane which is produced. This has to be referred to normal conditions of pressure and temperature (273.15 K, 101.33 kPa). In the German guideline VDI, a very useful comprehensive explanation of the data analysis process is available.

Good data reporting has to be the more detailed as possible, as reported in Angelidaki et al. (2009). In the final report, BMP test must contain the following:

- Precise description and characterization of the inoculum
- Description and characterization of the substrate
- Description of test conditions and of the setup
- Description of the results obtained with positive controls and blanks

– The graphs of gross production of methane in the different tests
– The net methane production obtained subtracting the blanks
– The final methane yield expressed as $NLCH_4\ kgVS^{-1}$

10.7 Microbiome Analysis

It is very important to understand the anaerobic digestion process to have a clear idea of the evolution of the microbia inside the reactor. This can be traced with different methods which have undergone an important improvement in the last decade; the most important are as follows:

– Culture-independent methods
– Imaging
– Isotope labeling
– Chemical analyses

With the term "culture independent methods," we identify the methods that are not based on microbial cultivation to study their ecosystem. These methods permit us to have a good idea of the composition and of the main metabolic functions of bacterial population inside the anaerobic digester. Special attention is focused on the methanogenesis reaction.

These methods allow also the researcher to understand better what are the interactions between the feedstock, reactor configuration, operational conditions, and microbial community.

In this way, the structure, dynamics, performance efficiency, and stability of the microbia are also analyzed (Werner et al. 2010; Talbot et al. 2008; Regueiro et al. 2012; Pervin et al. 2013; Ho et al. 2013; Nelson et al. 2011; Sundberg et al. 2013; Lee et al. 2012).

The use of culture independent methods has revealed that anaerobic digesters (Nelson et al. 2011; Sundberg et al. 2013) host a multitude of previously uncharacterized microorganisms, of which the interaction mechanisms are not known. To understand the anaerobic digestion process, it is in fact required to know how the bacterial metabolism is working the functional redundancy inside the bacterial community and the interactions between different species.

10.7.1 PCR (Polymerase Chain Reaction)

The composition of the microbia populating the anaerobic digester can be determined through PCR amplification and the analysis of genes markers (see Fig. 10.9). The most widely adopted is the 16S rRNA gene. It is also the one with the most extensive databases of references (Talbot et al. 2008; Su et al. 2012; Musat et al. 2011).

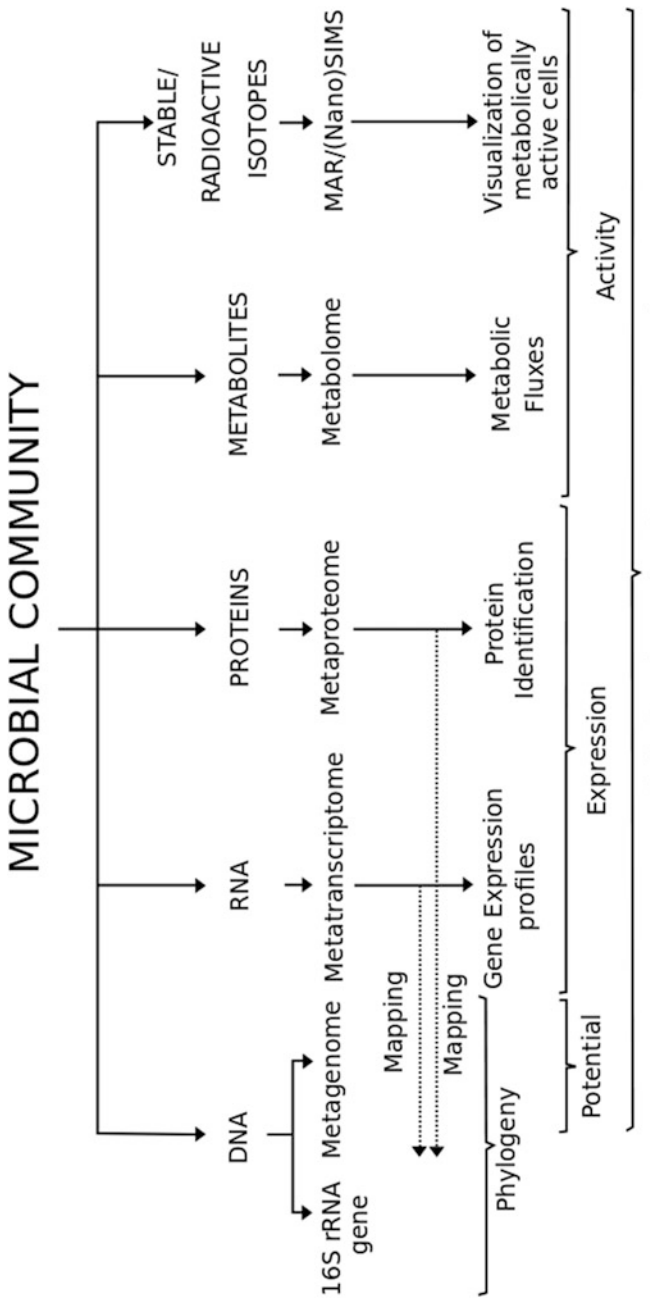

Fig. 10.9 Different methods to determine the functional diversity of the anaerobic digestion microbia (Vanwonterghem et al. 2014)

Through the 16S rRNA gene, the following traditional molecular fingerprinting methods can be applied to characterize the microflora in the anaerobic digesters (Talbot et al. 2008):

- Denaturing/temperature gradient electrophoresis;
- Single-strand-conformation polymorphism,
- (Terminal) restriction fragment length polymorphism
- Sanger sequencing of clone libraries.

New applications in research are represented by the use of high-throughput sequencing technologies as the Roche 454 and the Illumina platforms for sequencing and the 16S rRNA gene amplicon sequencing. These have increased the resolution which is available for the analysis of the microbial populations (Werner et al. 2010; Lee et al. 2012). The results of these analyses have to be correlated with the operative parameters of the digester to understand really how those can influence the microbial community structure and metabolic paths (Sundberg et al. 2013; Ziganshin et al. 2013; Zhang et al. 2009).

Metagenomics is the random sequencing of genomic DNA, which has been extracted directly from the microbial community populating the biogas reactor. Metagenomics gives different information with respect to the 16S rRNA gene-based analysis because it provides data on the physiology of the anaerobic digestion microbia and their single components (Su et al. 2012; Shakya et al. 2013) (Fig. 10.9).

Metagenomes also can be sequenced and provide information about microbial genomic diversity and their physiological complexity (Temperton and Giovannoni 2012). The metagenomics has the final goal to reconstruct large genome parts or complete genomes of all the community members (Tyson et al. 2004; Wrighton et al. 2012).

10.7.2 Metatranscriptomics

Metatranscriptomics is used to sequence the reverse transcribed mRNA which has been extracted from the bacteria (Su et al. 2012). This can enable the measurement of the expression of genes in situ (Fig. 10.9). This method can reduce the complexity level which has been detected with the metagenomics so that only the members of the community which are metabolically active can be analyzed (Su et al. 2012; Carvalhais et al. 2012).

10.7.3 Metaproteonomics

This is the analysis of the protein complement of a microbial community at a specific time (Su et al. 2012) (Fig. 10.9). Basically, the metaproteonomics integrates the data provided by the metatranscriptomics, because genes expression and activity, have to

be integrated with the cellular regulation which occurs at the protein level (Langley et al. 2013).

10.7.4 Metabolomics

Metabolomics is the analysis of the qualitative and quantitative aspects of all the molecules with low molecular weight which are involved in the microbial metabolism. Those molecules are required for the maintenance, the growth, and the normal function of the microbial population (Dunn and Ellis 2005; Goodacre et al. 2004; Lin et al. 2006; Hettich et al. 2013; De Kok et al. 2013) (see Fig. 10.9).

10.7.5 MAR

Microautoradiography (MAR) uses radioactive isotopes to study the in situ uptake of specific substrates (Talbot et al. 2008; Okabe et al. 2004; Ito et al. 2011, 2012). It is an approach that allows to visualize and measure the uptake of the substrate by the specific microbial populations and to visualize the organization of the community in the space (Fig. 10.9).

Once the radioactively labeled substrate has been assumed by the microbia, the researcher can monitor and visualize those bacteria and count them (Musat et al. 2011). Besides this if MAR is coupled to the fluorescence in situ hybridization (FISH), this can permit to identify also the metabolically active microbial cells (Musat et al. 2011).

10.8 Conclusions

This work describes in detail laboratory analytical methods to characterize the inoculum and the substrate to be used in anaerobic digestion tests and also to perform biomethanation potential tests and to measure biogas yields and methane yields. The main analysis to fully characterize the inoculum and the substrate are the following: sampling, storage, pH measurement, measurement of total solids and volatile solids, measurement of chemical oxygen demand, measurement of nitrogen content, measurement of total organic carbon, measurement of trace elements and analysis of phosphorous and sulfur content, measurement of alkalinity and volatile fatty acid (VFA) concentrations, and measurement of fats, carbohydrates, and proteins. The biomethanation potential tests have been highly discussed and standardized in the literature. We present how to optimize the procedure, focusing on inoculum management, substrate management, and the test procedure itself. When performing BMP tests, it is recommended to keep in mind what are the limitations of this kind of

analysis and when it can be applied and give reliable data. Data analysis and reporting is also described. Finally, the latest advances on the characterization of the microbial population in anaerobic digestion reactors are presented, to understand the composition, the physiology, and the metabolism of the bacteria which perform the anaerobic digestion process.

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