# Chapter 13
# Biofilms for Biofuel Production


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**Abstract** The syntropic consortium of microorganisms with the self-secreted exopolysaccharide (EPS) layer is usually known as a biofilm. The presence of EPS develops the antibiotic resistivity by inhibiting penetration of the antibiotic through this layer, leading to several negative aspects on the environment as well as the human being. Apart from this negativity, some bacterial biofilm that is found to be beneficial to society can be used in wastewater treatment, in polyethylene degradation, in bioremediation, and also in the food industry. Another positive aspect of biofilm technology is biofuel production which needs to be further explored. The conversion of lignocellulose materials to biofuel through pretreatment, saccharification, and product recovery using current technologies is cost-effective. Biofilm has the potency which can improve the efficiency of the product recovery processes, and also a condensation of hydrolytic enzymes, which are analogous to the cells and present at the biofilm-substrate interface, can increase the reaction rate. Biofilm is a microbial syntropy where multiple species are involved in the conversion of complex substrates and fermentation of both hexose and pentose to hydrolysates which disperse outward. Also, both the bacterial and fungal symbiosis allows simultaneous delignification and saccharification. The intercellular gene and signal exchange between the cells get enhanced due to the microenvironment of the biofilm. The separation of biofuel from its producer gets simpler due to the immobilization

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property of biofilm, and it assists in the retention of biomass, to continue reaction within the fermenter. Thus, the use of biofilm has the added advantages to biofuel production using solid-state fermentation (SSF). Biofilm technology is capable to spur significant innovations to optimize biofuel production.

**Keywords** Biofilms · Biofuel · Saccharification · Fermenter

## 13.1 Introduction

Man-made transformation of dead-decaying or organic-biological materials results in the development of biofuels, whereas the fuels that are derived from long-dead material by natural processes over the millions of years are considered as fossil fuels. The production of biofuels involves low-cost feedstock like lignocellulose materials, and their importance is increasing rapidly due to the increased finite reserve, unstable supplies of fossil fuels, and the adverse environmental impacts. The enhancement of global energy consumption and increased demand for energy resulted in the advent of alternative energy sources substituting the use of fossil fuels. Moreover, the rapid decrease in the number of fossil fuels and various climatic changes associated with the use of fossil fuels provided a potent indicator for the alternate source of energy (Lan et al. 2013; Al-Shuhoomi et al., 2021). The production of fossil fuels accounts for its biodegradable and environmentally friendly characteristics that showed significant substitution for fossil fuels (Pandey et al. 2019). Lignocellulosic biomass acts as a source of renewable source of energy and is calculated as a potent substrate for the production of biofuel (Ben-Iwo et al. 2016). It has been observed that the production of biofuels from the lignocellulose feedstock proved to be environmentally friendly and is successfully coming up as an alternative to the applications of fossil fuels (Adegboye et al. 2018). The mechanism of the production of biofuels is delignification, where nonspecific physical and chemical methods are involved to liberate cellulose and hemicellulose from the intricate structure of lignin (Hahn-Hagerdal et al. 2006). This step is also responsible for the production of phenolic compounds, furan derivatives, and weak acids. The second step of production utilizes various types of cellulosic and hemicellulosic components that get converted into simple carbohydrate products comprising of five to six carbon groups after being exposed to purified microbial enzymes. The third step involves the utility of very specific microbial species, and the final step involves the mechanism of separation that involves the products, including a substrate, microorganisms, and culture broth, all being separated from the producing system. Ethanol is one of the biofuels which is produced from lignocellulose. Ethanol is used to get concentrated 4% to 4.5% in the separation step, and distillation is usually used to separate biofuels from liquid media (Stephanopoulos 2007). The combined second and third step in a single step termed as simultaneous saccharification and fermentation has been proposed to avert product inhibition by hydrolytic enzymes (Gauss et al. 1976).

Thirty years from the invention of solid-state fermentation (SSF), which is considered as simpler equipment and more efficient in operation, separate hydrolysis and fermentation (SHF) is common practice in the industry. In both the processes of SSF and SHF, cell-free purified enzymes are used for a large amount of ethanol production (Cardona and Sanchez 2007). In a consolidated bioprocess (CBP), the use of cell-associated enzymes has been proposed, where one cell is considered to undertake both hydrolysis and co-fermentation, which can reduce the cost, wherever there is no organism known which can perform this operation (Lynd et al. 2005). The probability of utilizing hydrolytic product by the cells gets increased if the enzyme is present in closer proximity to the cell surface (Wetzel 1991). It has been observed that a compact enzyme-substrate-microbe structure of the biofilm is required for microbial biofuel production to receive the maximum energetic return (Fan et al. 2005). The extracellular polymer matrix of the biofilm provides a special microenvironment to the cells, and within which enzyme activity is concentrated. Though biofilm technology has been used in wastewater treatment to mineralize soluble organic matter, the ability to convert insoluble lignocellulose into biofuel has not been fully recognized. This chapter would focus on the use of biofilm in the mechanism of the production of biofuel.

## 13.2 Quorum Sensing

Quorum sensing is crucial for microbial interaction as it provides a novel approach to consortia control (Miller and Bassler 2001). Quorum sensing is also responsible for the development of biofilms by controlling a specific ratio of microbes (Nag et al. 2021) and has a wide application in bioprocess. In biofuel production, another extent of physicochemical response in the manifestation of self-regulating activators or inhibitors is provided by quorum sensing and also can be manipulated through genetic engineering strategies, which can be observed through microbial cross-talk.

## 13.3 Biofilm Formation

Microbial consortia with extracellular polymeric substances (DNA, protein, carbohydrate), adhering to the solid surface, is known as a biofilm (Costerton 1995, Dutta et al. 2021). Secretion of the antimicrobial peptide can inhibit the corrosion-causing organism (*D. vulgaris*) which is an example of engineered and controlled biofilm formation (Jayaraman et al. 1999). Interaction between the biofilm members through quorum sensing is important within the biofuel consortia. Along with the protective role, biofilm decreases diffusion requirement for metabolites as well as activator or inhibitor control elements (Wang and Chen 2009). Within the biofilm, the cell surface proximity gets increased, which is considered as an important aspect for systematic cellulose hydrolysis, which is also advantageous over non-adherent

microbes (Lu et al. 2006). It was observed that biofilm overall can improve the fitness and robustness of the environmental conditions to produce toxic fuel molecules. To maintain high titer production, cell immobilization is required to retain the biomass concentration in a bioreactor (Rosche et al. 2009). Biofilm provides additional support to the biofuel consortia, and spatial arrangement within the biofilm regulates the interactions of multiple species and generation of chemical and metabolite gradients. Appropriate lignocellulose degradation and biofuel production are regulated by an engineered model of both the natural and synthetic spatial organizations (Jasu et al. 2021). Organic substrate-bound biofilms drive the sequential utilization in most of the common consortia models. The fermentative organisms initiate the energetic cascade to produce alcohols and fatty acids, which are being used by secondary fermenting bacteria for the production of $CO_2$ and $H_2$ and acetate, which are also subsequently utilized for methane production by methanogenic bacteria (Davey and O'toole 2000). The combined transformation of substrate and product and combined control over the environment and activator/inhibitor regulates a biofuel system. An environmental zone is provided by the spatial organization to stabilize the biofilm consortia. The spatial organization gets beneficial through the biofilm model with the minimal loss of fuel energy content. Metabolic capabilities of symbiotic partners as well as the advancement of stable, scalable techniques are required for the formation of biofuel consortia by establishing symbiosis through bioreactor scale-up principles. A mass transfer limitation is being introduced in consortia-based bioprocessing while issues related to bioreactor design, control, and scale-up are also considered.

## 13.4 Biofuel Production by Biofilm Optimization

Biofilm showed a marked utility in various industries but has a potent utility in the process of wastewater treatment. Still, various studies are being performed to understand the wide applications of biofilm. The mechanism of solid-state fermentation (SSF) is dependent on surface adhesion reaction that is mainly associated with the production of biofuel (Vega et al. 1988). The microenvironment and layered structure of the microbial biofilm and the specific gradients of substrates and products associated with SSF is the main reason for utilizing SSF over submerged fermentation (SF) (Rahardjo et al. 2006). SSF is categorized as surface adhesion fermentation, and the growth of the microorganism on a solid substrate having an adequate amount of moisture for maintaining the microbial growth and metabolism can define SSF (Gutierrez-Correa and Villena 2003, Ishida et al. 2000). SSF is a simple technology with high volumetric yield and low downstream processing in the production of glucoamylase B (de Vrije et al. 2001) and fungal spores which is required for biocontrol (Rahardjo et al. 2006). Along with the product concentration, SSF provides added advantage in the mechanism of biofilm formation, which is being supported by a solid surface. It has been reported that mixed culture SSF is

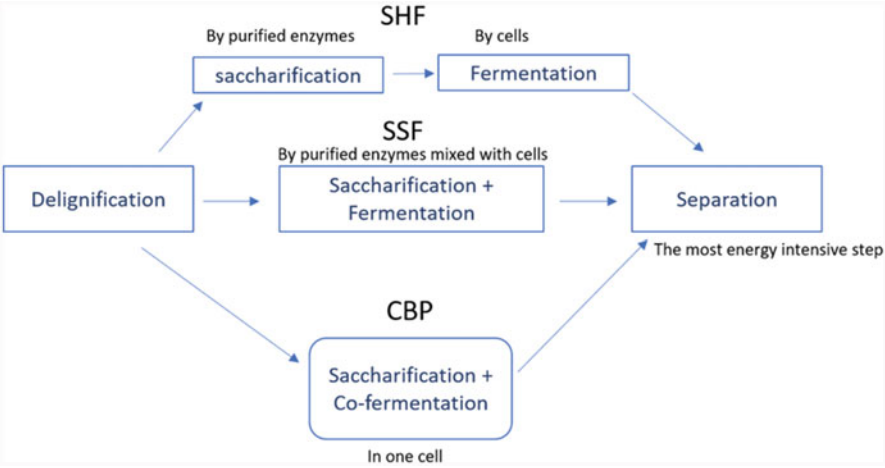


**Fig. 13.1** Flow diagram of conversion processes from lignocellulose to biofuel

more efficient in cellulase production and lignocellulose degradation compared to isolated culture (Gutierrez-Correa and Tengerdy 1997).

For biofuel production, breakdown of the lignocellulose structure is a prerequisite. Lignocellulolytic fungi are used in SSF to produce lignocellulolytic enzyme complexes having cellulases, hemicellulases, ligninases, and pectinases (Tengerdy and Szakacs 2003) (Fig. 13.1). Due to the growing demand for sustainable energy, subsequent fuel production by breaking down of lignocellulosic material utility of living organisms has shown a tremendous increase. Processes like integration and intensification get modified and condensed to consolidated bioprocessing where cellulolytic and ethanologenic microbes are involved in simultaneous hydrolysis and fermentation (Lynd 1996). This process is ideal for mixed consortia, comprising of organisms that are selected and engineered in a way to meet the required bioconversion goal within a single bioreactor system. The interactive grouping between the microorganisms within the consortia enhances the transformation of cellulose and other sugar mixtures to alcohol over the monoculture system (Lynd et al. 2002).

In an industrial wastewater treatment system, the consortia-mediated bioprocessing is mediated through multi-organism cooperation. The critical relationship for biofuel production has been defined by the consortia referred to as symbiosis. The biological process in biofuel production stops the electron transfer cascade to capture this energy in fuel molecules. Biocatalytic capabilities of the simple and multi-species biofilms are important features in the community design of biofuel production (Rosche et al. 2009). Ideal microorganism for biofuel production has been categorized into two alternatives: the first one is an industry-friendly model hosts like *Escherichia coli* and *Saccharomyces cerevisiae*, and the second one is a novel host with enhanced functional elements, which is mainly required for substrate degradation and fuel production (Wang and Chen 2009; Ingale et al. 2019;

Al-Shuhoomi et al. 2021). Natural consortia with the connotation of genetic engineering have been defined as engineered consortia which are commonly used in wastewater treatment. Mutualistic consortia and their applicability are considered in the formulation of the symbiotic biofuel production model.

### *13.4.1 Natural Consortia*

The production of lignocellulosic biofuel is dependent on the access of various types of hydrolytic enzymes capable of performing the degradation of hemicellulose and lignin. Various termite species have different types of consortia in their gut, which makes them capable of utilizing lignocellulose that further helps in the production of biofuel (Chaffron and von Mering 2007). Studies are intervening on the symbiotic interaction between the microbes and termites. It has been observed through metagenomic analysis that, in higher *Nasutitermes* species and *Fibrobacter* species, both are liable for utilization of cellulose and hemicellulose. Both the fungal and bacterial lignin degraders are the members of consortia that are required for consolidated bioprocessing including lignin solubilization, whereas the complex, aerobic nature of lignin degradation is amalgamated with anaerobic fermentation. Some intermediate steps like degradation of an aromatic acid, fatty acid, and primary alcohol intermediates oxygen deficiency in the ecosystems can be used for biofuel production. The organic material breakdown is regulated by symbiotic interactions between anaerobic bacteria and methanogenic or sulfate-reducing bacteria which consume hydrogen for survival (Schink 1997). Breakdown of cadaverine, an aliphatic amine intermediate that is produced due to degradation of proteinaceous organic matter is an example of symbiosis.

The important aspect of natural consortia belongs to the sturdy performance under a great extent to the environmental conditions. In a wastewater treatment plant, a process called completely autotrophic nitrogen removal over nitrite (CANON) has been used which includes three specific functions like (1) aerobic ammonia oxidizers where ammonia gets partially converted to nitrite followed by (2) conversion of ammonium to $N_2$ by utilizing nitrite by anaerobic ammonia-oxidizing bacteria and lastly (3) aerobic nitrite-oxidizing bacteria that can provide acceptable micro-aerobic conditions. Oxygen introduction gets incremented when these three members of the mutualistic community from the marine sample get assembled (Yan et al. 2010). Self-assembled consortia and $H_2$-consuming bacteria provide control over the bioreactor microenvironment that serves an aerobic condition for lignin degradation and anaerobic condition for biofuel cumulation during lignocellulosic biofuel production. Except for the methane high levels of biofuel, molecules do not get accumulated in the natural consortia system. There may be a controversy between genetically engineered consortia and recombination of natural capabilities to get more accumulation of biofuel molecules (Table 13.1).

**Table 13.1** Different microbial consortia in lignocellulose degradation

| Substrate | Source and composition of consortia | Conclusion | References |
|---|---|---|---|
| Bagasse, corn Stover, and rice straw | Isolated from high-temperature compost having bagasse and sugarcane (*Clostridium* sp., *Rhodocyclaceae* sp., *Thermoanaerobacterium* sp.) | Stable consortia with a multi-species enzyme system and around 75% of rice straw, 70% of corn Stover, and 60% bagasse can be degraded in 7 days at 50 °C | Wongwilaiwalin et al. (2010) |
| Bermuda grass | Enriched form of lignocellulosic substrates (*bacillus* sp., *microbacterium oxydans, Ochrobactrum anthropi*, *Pseudoxanthomonas byssovorax,* and *Klebsiella trevisanii*) | Consortia with an assorted assemblage of cellulolytic/xylanolytic enzymes having characterized activity | Okeke and Lu (2011) |
| Cotton, filter paper, and rice straw | 9 different species isolated from mature compost | 99% of filter paper, 81% of rice straw, and 77% of cotton get degraded after 3 days at 50 °C without considering product formation | Wang et al. (2011) |
| Cotton, filter paper, printing paper, and rice straw | Enriched form of cellulose/feces mixtures (*Betaproteobacterium*, *Brevibacillus* sp., *clostridium* sp., and *Pseudoxanthomonas* sp.) | Stable consortia degraded 60% of rice straw, 88% of cotton, and 79% of filter paper after the incubation of 4 days at 50 °C with getting ethanol as a major product | Haruta et al. (2002) |
| Pretreated sugarcane leaves | Isolated from various lignocellulosic materials (*Bacillus subtilis, Cellulomonas* sp., *Streptomyces* sp.) | Consortia of 4 isolated strains degraded 90% compared to 60% degradation by the mixture of all 9 strains within minimal salts media | Guevara and Zambrano (2006) |
| Raw corn Stover powder (RCSP) and filter paper | Isolated from enriched soil (*Ralstonia* sp., *clostridium* sp., *Propionibacterium acnes*, uncultured *Firmicutes, Betaproteobacterium*, and *Pantoea* sp.) | 51% of RCSP and 81% of filter paper get degraded in 8 days under facultative anoxic conditions at 40 °C providing major product acetate | Feng et al. (2011) |
| Sugarcane bagasse (SCB) and filter paper | Unknown composition mainly isolated from compost, dung, and soil | 77% of alkali treated SCB get degraded in 6 days and 85% of filter paper in 4 days at 50 °C is possible along with substrate-bound cellulases | Lv et al. (2008) |

### *13.4.2 Genetically Engineered Consortia*

Genetically engineered consortia are designed in such a way that they become capable of performing the production of biofuel. Studies showed that genetically engineered two strains of *E. coli* are able to utilize xylan, in which one engineered stain was responsible to co-express two hemicellulases that possess the ability to bring about hydrolysis of xylan into xylooligosaccharides and another stain carries xylooligosaccharides to produce ethanol (Shin et al. 2010). The yield of ethanol was approximately around 55% of the theoretical yield by this co-culture on purified xylan. The addition of three purified hemicellulases along with engineered *E. coli* strain can increase the yield up to 71%. The differences in the growth rate of two different stains were estimated by cultivating them separately. It was demonstrated that two engineered *S. cerevisiae* strains can exchange metabolites reciprocally, in which adenine is required by one strain to overproduce lysine and the other one requires lysine to overproduce adenine (Shou et al. 2007). The mutualistic relationship between these two stains provides sustainability of the dual culture system, where adenine is released when senescence is approached synchronously to support the growth of the partner which provides the lysine, required by the first strain. Other studies showed that combined genetic engineering and natural capabilities can establish a cooperative dual culture that can convert cellulose to methyl halides (Bayer et al. 2009). *Actinotalea fermentans*, a cellulolytic bacterium, gets inhibited by alcohol and organic acids which are usually produced due to hydrolysis and fermentation of cellulose. Alleviation of feedback inhibition on *A. fermentans* hydrolysis is possible by engineered *S. cerevisiae* which can produce methyl halides in co-culture. It was observed that genetic engineering can create symbiotic cooperation between microorganisms for chemical production and a combination of natural consortia and genetic engineering can develop efficient biofuel producing consortia.

## 13.5 Modelling and Regulating Biofuel Consortia

A large variety of microorganisms are involved in the mixed culture of the consortia that are chosen depending upon some factors like desired product, environmental conditions, symbiosis mechanism, and also the genetic manipulation. Physicochemical requirements and metabolic properties of the organisms are the main criteria for designing fuel-producing consortia. Stable symbiosis is coming from a mutualism that is required to provide an environment to the industrial process.

### 13.5.1 Sequential Utilization

Sequential utilization introduces control over the product inhibition. In the case of cellulose fermentation, the final products like alcohol, hydrogen, and organic acids can be shifted to methane by methanogenic bacteria (Nakashimada et al. 2000). Co-culture of an anaerobic, syntropic bacterium and a *Desulfovibrio* species produce acetate that sequentially brings about the degradation of benzoate (Warikoo et al. 1996). Introduction of an acetate-utilizing bacterium removes acetate and completes benzoate degradation. The rate and yield of the biofuel product can be boosted up by simple sequential utilization and, apart from the addition of activator or lack of control of inhibitor, can affect the product yield.

### 13.5.2 Co-Utilization of Symbiosis

To establish a stable symbiosis exchange of both the activator and inhibitor is required even though co-utilization of electron donor becomes competitive. Also, some essential nutrients get exchanges between species naturally (Paerl and Pinckney 1996). In genetically engineered consortia, metabolite exchange is defined as synthetic mutualism, whereas mutual benefits get provided through reciprocal metabolite exchange, and it is also effective for biofuel production.

### 13.5.3 Substrate Transformation

Substrate transformation is one of the main steps in biofuel production as it is aimed to synthesize fuel from complex, inexpensive substrates to extract electrons through specialized biological activity. Biological pretreatment of the lignocellulose to convert partially degraded lignin barrier is the basic step for substrate transformation. This process may require either microenvironment manipulation or sequential treatment to achieve an integrated aerobic and anaerobic process. An engineered substrate transformation occurs between cellulolytic and non-cellulolytic organisms to release carbohydrates from lignin. Due to the mutualism, the non-cellulolytic organism makes use of hydrolysis products to provide benefits in return (Kato et al. 2004). In fuel production, logical symbiosis is required where cellulolytic organisms (M1) get benefitted from the second organism. It was observed that non-cellulolytic *Klebsiella* provides vitamins with added mutualistic tether of nitrogen fixation to cellulolytic *Clostridium papyrosolvens* C7, which supports *Klebsiella* growth (Cavedon and Canale-Parole 1992). Due to the low nitrogen content of lignocellulose, this approach has been used in biofuel consortia. The beneficial exchange of metabolites is accomplished by engineering the physiological environment. The canon process is one of the engineered processes where the culture

oxygen tension is controlled by one partner to protect anaerobic metabolism (Pearland Pinckney1996). Organic acid has been produced from this bacteria/fungus mutualism within the consortia which are responsible for fuel production. A cellulolytic organism is used to get respiratory protection from a facultative anaerobic ethanologenic in return for soluble sugars which are released due to cellulose hydrolysis. Controlled oxygen level has an impact on biofuel production along with decreasing alcohol toxicity and increasing glycerol production (Franzén 2003).

### *13.5.4 Transformation of Products*

Product transformation is a phenomenon when fuel products without being used as consequential electron sources or acceptors get converted into alternative fuel molecules. Due to the toxicity of the fuel molecules towards microbes, this transformation is beneficial for sequential utilization of fuel value and yield without any dramatic degradation. It was observed that a single culture system is responsible for the conversion of alcohols and fatty acids into fatty acid ethyl esters (FAEEs) through in vivo esterification (Kalscheuer et al. 2006). A dual culture system is designed in such a way that M1 converts cellulosic feedstock to ethanol and that can be used eventually to biosynthesize FAEEs by M2. Mutual benefits rely on relief of alcohol toxicity by M2, and supply of soluble substrate from cellulose by M1, along with additional control inhibitor and activator.

## 13.6 Enhanced Biofuel Production in Algal Biofilm Bioreactor

In the past few decades, microalgal-based biodiesel has become an immensely favorable renewable resource with much more attention. Lack of well-grounded and economical methods are the main barrier to biofuel production from microalgal biomass. Incorporation of algal biomass production into the wastewater treatment has been suggested in order to attain the requirement of fertilizer and freshwater. The algal biofilm culture system not only facilitates harvesting but also immobilizes the algal cells within an algal biofilm after getting separated from liquid culture media. Hence it leads to the increase of the microalgal biomass/biofuel content level. The attachment cultivation of the biofuel production system is involved in the separation of hydraulic retention time (HRT) and biofuel/biomass retention time (BRT). This separation is favorable for wastewater treatment with the requirement of a high ratio of BRT:HRT. Rotating algal biofilm (RAB) is one of the novel biofilm systems which have been developed to harvest algal biomass and producing biofuel by-products. RAB is a submerged system that works in submerged conditions by rotating in between the air phase and liquid media. Various RAB systems were

designed in order to maximize biofuel/biomass production. In the RAB system, a cylinder is wrapped by the ropes, and this structural arrangement makes a route between the air phase and liquid phase, and the rope is passed through an adjustable diameter scraper to harvest algal biomass (Christenson et al. 2012). Another rocker algal biofilm system has been developed with a chamber within a rocking shaker. At the bottom of the chamber, attachment material is placed and alternatively gets submerged in culture media for algal growth to get exposed to gleam for algal photosynthesis (Johnson and Wen 2010). Microalgal cells attach and grow after being supported by an artificial material that is vertically oriented in a vertical plate-attached algal biofilm system (Liu et al. 2013). The development of an inexpensive, scalable algal culture system for algal cultivation is required. Hence these various algal biofilm systems exhibited a great promising role in producing algal biomass and reducing harvesting costs.

### *13.6.1 Biohydrogen Production*

One of the most promising ways to create renewable energy is the generation of molecular hydrogen by photosynthetic microbial consortia. This process happens at ideal temperatures, with adequate sunshine, water supplementation, and the smallest quantities of macro- and micronutrients. Production of hydrogen using photosynthesis can be anticipated as future efforts to lead the eco-friendly engineering principle for industrial production of renewable energy with the null emission of greenhouse gases and other environmental pollutants (Seibert 2009; Chowdhary et al. 2018; Chowdhary and Raj 2020; Chowdhary et al. 2020). Biohydrogen directly can be used in internal combustion engines and can also be used to power fuel cells for electricity.

The creation of biohydrogen involves two primary techniques, the first of which is an indirect process that uses photosynthetic capacity for biomass production. Through the processes of fermentation and photo-fermentation, the whole biomass including carbs is transformed into biohydrogen. This process has two discrete stages, which are separated by reactions happening in two different bioreactors or by the alternating of photosynthesis and fermentation periods. The second method is essential since it tries to use photosynthesis to break water down into hydrogen and oxygen via direct or indirect water biophotolysis processes. The evolution of $H_2$ in the light biophotolysis by green alga *Scenedesmus obliquus* is related to the R&D sector for the past few decades (Gaffron and Rubin 1942). Simultaneous production of $H_2$ and evolution of $O_2$ were manifested by filamentous cyanobacteria *Anabaena cylindrica*l after being exposed to argon (Ar) atmosphere.

Two steps are involved in direct water biophotolysis of green algae and cyanobacteria:

$$H_2O + 2Fd_{ox} \rightarrow 2H^+ + 1/2O_2 + 2Fd_{red} \quad (13.1)$$

$$2H^+ + 2Fdred \leftrightarrow H_2 + 2Fd_{ox} \quad (13.2)$$

The first reaction is universal to all oxygenic phototrophs, but the second reaction requires either anaerobic or microaerobic conditions. The H2 generation process is catalyzed by the bidirectional hydrogenase enzyme. Two types of hydrogenase enzymes exist: one is an algae bidirectional hydrogenase ([FeeFe] enzyme) and the other is a cyanobacterial hydrogenase ([NieFe]-hydrogenase) (Ghirardi et al. 2009). It was observed that, usually after a dark anaerobic adaptation period when the cultures are exposed to the light, a direct water biophotolysis process occurs. The initial rate of H2 generation is considerable, reaching 300 mmolH2/(Michael*h). Because bidirectional hydrogenase, particularly [FeeFe] enzyme, is more susceptible to oxygen, direct biophotolysis lasts just a few seconds to minutes. Indirect biophotolysis is used by microalgae and cyanobacteria to generate hydrogen from stored carbohydrates, glycogen, and starch.

With contrast to the direct biophotolysis, in a two-staged indirect biophotolysis the presence of light carbohydrates, the synthesis at early stage in photo-fermentation to produce hydrogen:

$$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 12H_2 \quad (13.3)$$

The partially degraded carbohydrate is accompanied by the accumulation of fermentation end products, and spatially or temporally, stages like $O_2$ evolution and $H_2$ production are separated from each other. Vegetative cells carry out oxygen evolution in the case of filamentous heterocystous cyanobacteria, whereas in specialized cells, heterocysts are responsible for $H_2$ photoproduction, which is navigated by the nitrogenase system. It was observed that production rate of $H_2$ is quite high in the absence of nitrogen due to catalysis of reduction of $H^+$ to $H_2$ by nitrogenase. In contrast to the inception of the dark period, a microoxic intracellular environment has been created due to high rates of respiration, which also facilitates fixation of $N_2$ and production of $H_2$. It was observed that in wild-type *Cyanothece*, the rate of $H_2$ production is 465 mmol ($H_2$)/(mg Chl*h) (Bandyopadhyay et al. 2010).

### *13.6.2 Dark Fermentation in Biohydrogen Production*

Dark fermentation is usually referred to as the production of $H_2$ by bacteria anaerobically in the absence of light. The term "dark fermentation" is collectively used in the industrial fermentative biohydrogen production using anaerobic fungi. $H_2$ is produced by anaerobic fungi in membrane-bound organelles called hydrogenosomes, where protons are used as electron acceptors in mixed acid fermentation of monomeric sugars (mainly glucose and xylose) to generate ATP.

Parameters like feedstock type, feedstock concentration, pH, temperature, and species all significantly influence the yield of $H_2$ from dark fermentation. Use of biologically converted soluble fermentation end product increases yield of biofuels in dark fermentation.

A wide range of organic acids in addition to $H_2$ and $CO_2$ gases, which are released from mixed acid pathways of anaerobic fungi, have the potential in converting biofuels at the downstream of biological processes. It was observed that acetic and formic acids are co-products of fungal $H_2$ production. Formation of these alternative end products alters the hydrogenosome metabolic pathway and also reduces 100% efficiency in the conversion of carbohydrate H atoms to $H_2$ gas. 4 mol-$H_2$ mol-hexose$^{-}$1 maximum theoretical yield of $H_2$ has been reported from dark fermentation (Hawkes et al. 2007):

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (13.1)$$

Equation 1 represents the overall dark fermentation reaction. Integration of microorganisms and utilization of organic acids add more value to the dark fermentation process by anaerobic fungi in order to produce additional biofuel in the form of $CH_4$ and $H_2$.

## 13.7 Biodiesel Production

In the past few decades, biodiesel is considered as an alternative fossil fuel with an increased worldwide attention. Usually, renewable biological materials are used to produce biodiesel which can replace petroleum diesel fuels (Geetha et al. 2020). Transesterification of various animal fats and vegetable oils with the help of ethanol or methanol is the main step of biodiesel production (Naik et al. 2010; Geetha et al. 2020). Natural characteristics of the feedstock used for the biodiesel production determine its quality. Sustainable and renewable, inexpensive with low toxic waste biodiesel has the great importance to the rural area. Production of biodiesel from plant tissue or any kind of vegetable is considered as first and second generation of biofuel production with various disadvantages which have been overcome in the third generation of biofuel production from microalgae (Li et al. 2008). Nowadays, photosynthetic microalgae emerge as the best candidate to meet the global energy demand. An estimation showed that microalgae have an enhanced capacity to produce biodiesel which is 200 times more efficient compared to traditional crops. Compared to land plants, harvest of the microalgae is a bit easier and faster process for biofuel production. Light energy has been used by microalgae to convert carbon dioxide into organic compounds, and they are being considered as a superior source for biofuel production. *C. protothecoides*, a microalgae cultivated heterotrophically under nitrogen restriction, has 55% lipid (Xu et al. 2006). *B. braunii* 765, a green colonial microalgae, generates biodiesel, hydrocarbons, and biocrude oil at a temperature of 25 °C (Ge et al. 2011). Due to the presence of C16 and C18,

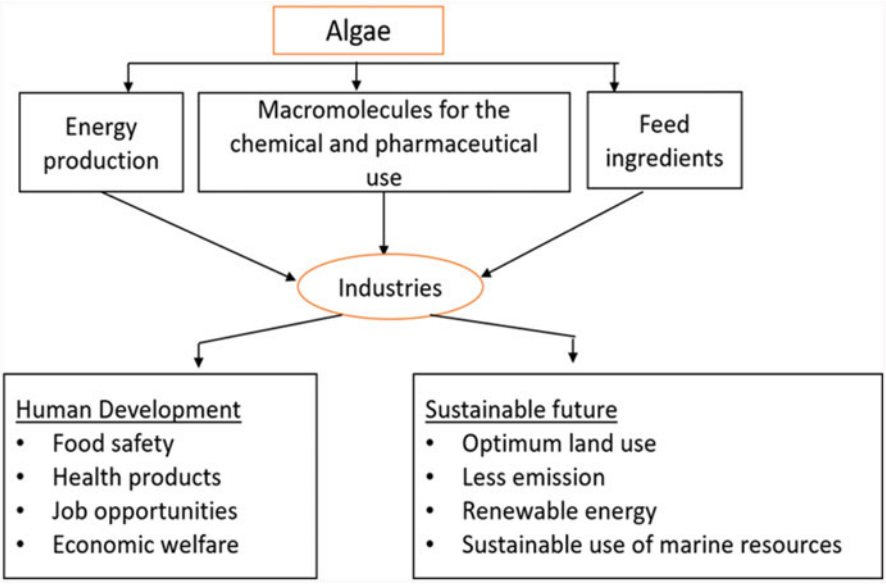


**Fig. 13.2** Various applications of algae in different aspects

*C. minutissima* UTEX2341 became a significant source of biodiesel (Li et al. 2011). Biodiesel is made by a series of procedures that include cultivation, harvesting, drying, cell disruption, and lipid extraction, whereas bioethanol is made through transesterification followed by hydrolysis and fermentation distillation. *Cyanobacteria* is one of five types of microalgae: 1. blue-green algae (*Cyanobacteria*), 2. green algae, 3. diatoms, 4. red algae, and 5. brown algae, which appear as the most presiding for biofuel production. The biodiesel which is derived from microalgae has appeared to be similar to petroleum diesel based on viscosity and density (Schenk et al. 2008) (Figs. 13.2 and 13.3).

## 13.8 Conclusion

Biofilms being a polyspecific association have the potential to ameliorate the process of biofuel production from lignocellulose residues. Multiple species of organisms may sequentially convert complex substrates with the help of enzymes present at biofilm-substrate interface. The increased rate of reaction is attributed by the compact microenvironment of biofilm matrix. A biofilm forming mixed consortium usually present the highest biomass productivity. Biodiesel can be produced from various biofilm forming algae including cyanobacteria. Microbial biofuel cells are the latest technology by which biofilm engineering can be applied in biofuel production.

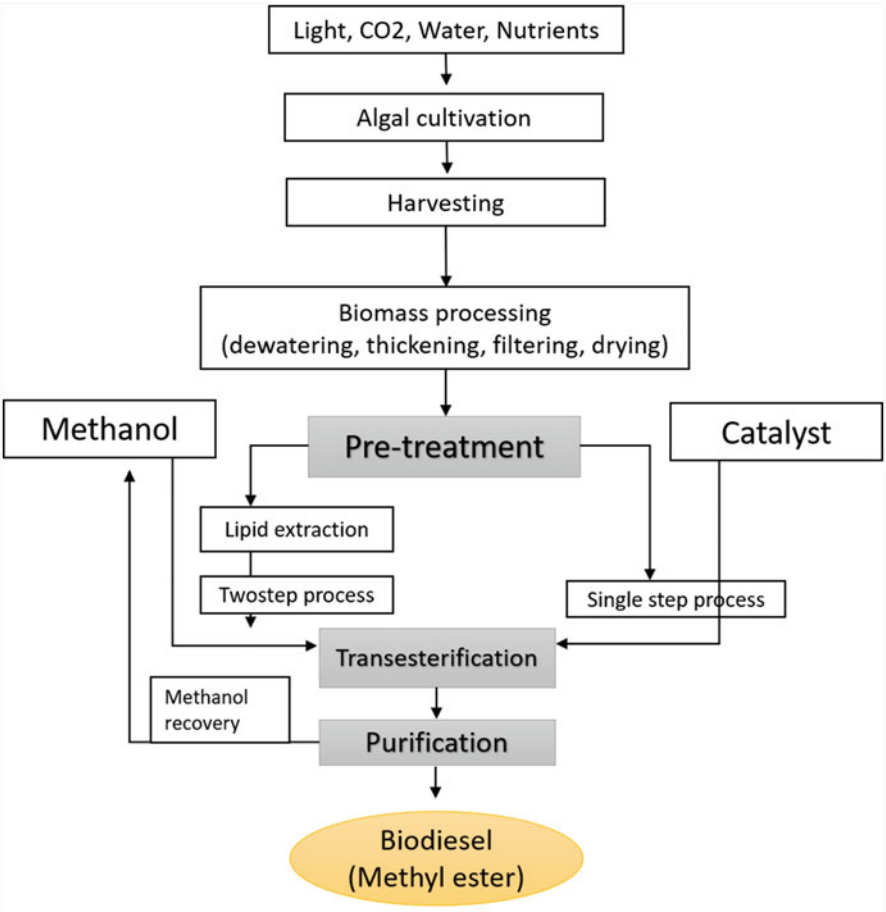


**Fig. 13.3** Production of biodiesel through algal cultivation

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