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Advances in Biofeedstocks and Biofuels

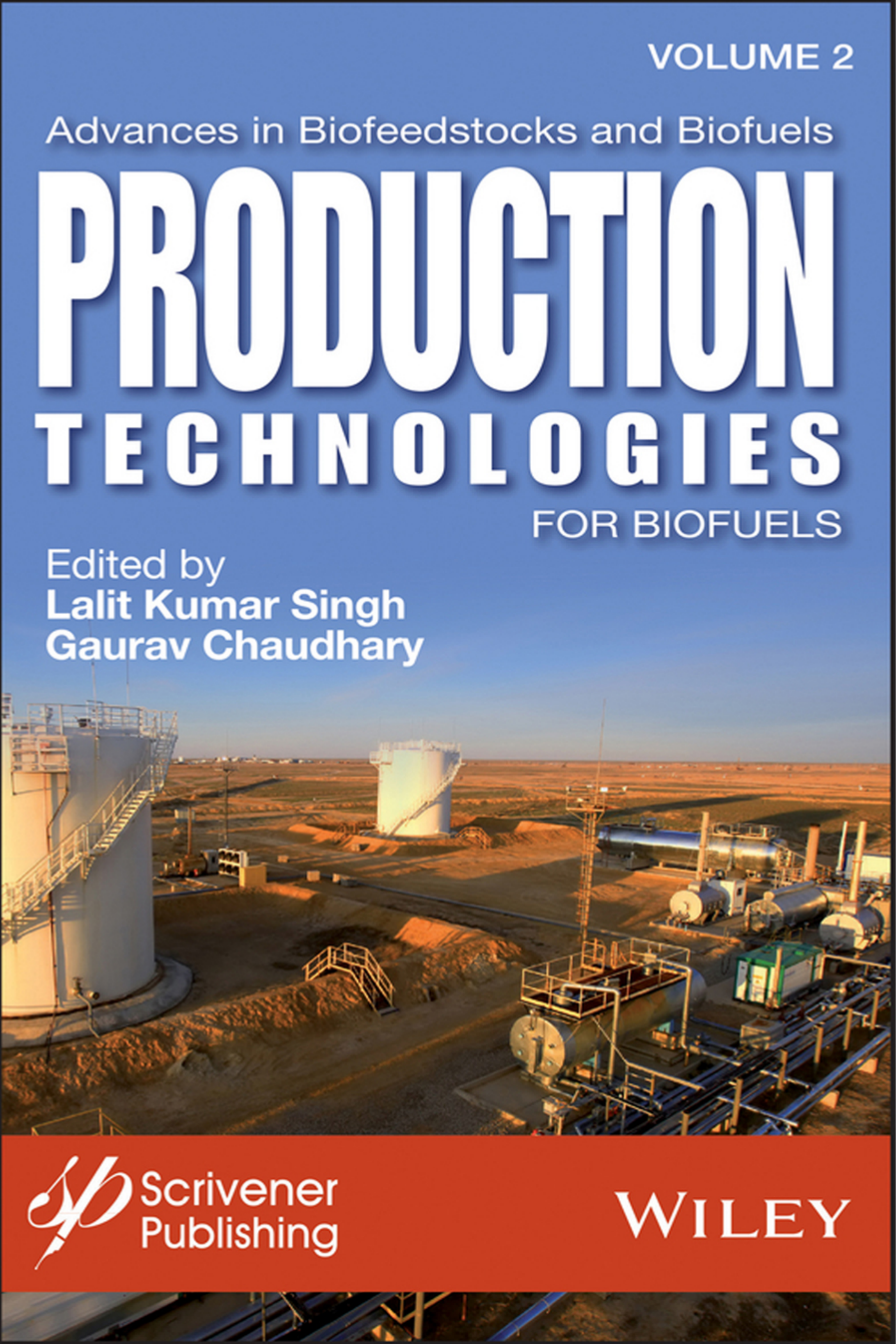
PRODUCTION TECHNOLOGIES

FOR BIOFUELS

Edited by

Lalit Kumar Singh

Gaurav Chaudhary



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1

Processing of Bioethanol from Lignocellulosic Biomass

Rebecca Gunn and Pattanathu K.S.M. Rahman*

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Abstract

Increasing population and urbanisation combined with depleting fossil fuel reserves have resulted in the need for the development of an alternative transport fuel source. Additionally, climate change associated with fossil fuels has resulted in the need for a greener energy source. Biofuels are fuels that are derived from biological sources to be used alone as transport fuel or as part of a fuel blend. Biofuels may provide a solution to the current fuel crisis and their need and potential is well-recognised. Bioethanol is a biofuel produced via the fermentation of sugars. Second generation bioethanol is produced from lignocellulosic biomass found in abundance in agricultural wastes. The complex structure of lignocellulose results in the necessity of a multi-step process encompassing: pretreatment, saccharification, fermentation, and distillation. Process integration is currently the most promising prospect in second-generation technologies, and efforts should now focus more on the optimisation of such integrated processes.

***Keywords*:** Bioethanol, biofuel, lignocellulose, biomass, second generation, pretreatment, distillation, fermentation, saccharification, process integration

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1.1 Introduction

The demand for and consumption of energy has never been higher due to substantial increases in population and urbanisation. Mass increases in energy are required by transportation, industrial, and agricultural sectors; the use of fossil fuels has long played a primary role in providing for this need. However, in recent decades, it has been made apparent that this is a limited resource and reserves are incapable of sustaining the present rising demand. Major concerns are also raised regarding the environmental impact of the emissions of 'green house gases,' such as CO_2, when burning fossil fuels. These issues have given rise to a vital need to develop new greener sources of energy [1].

Biomass fuels (biofuels) are obtained from currently harvested biological sources and are not by any means a recent discovery. Burning plants for light and the use of solid biofuel for cooking was a common post 20th Century practice. More recently, the fuel crisis has seen an increased drive on the development of modern biofuels as an efficient, clean, and sustainable transport fuel alternative [2, 3]. Interest was renewed in the mid-1970s when production of ethanol from sugarcane and corn began in Brazil and the USA. Their need and potential has since been recognised and is supported by government policies, with over 50 countries setting biofuel blending targets and quotas [4, 5].

One of the most predominant biofuels presently being utilised and developed is bioethanol, C_2H_6O, which is structurally identical to ethanol (see Figure 1.1b). First

(1st) generation bioethanol is produced from the fermentation of edible sugars and starch sourced from crops grown primarily for the production of biofuels. Unfortunately, issues arise in the production of 1st generation bioethanol as the growth of crops for energy, as opposed to food, results in reduced food production and, consequently, increased food prices. Second (2nd) generation bioethanol offers a potential solution as it utilises non-edible lignocellulosic biomass found in abundance in readily available agricultural wastes such as corn stover, sugarcane bagasse, straw, and woodchips [2, 6]. Lignocellulosic biomass is principally composed of cellulose, hemicellulose, and lignin. The complex structure of lignocellulose results in the necessity of a multi-step process to produce bioethanol; an overview of the process is shown in Figure 1.1a and Figure 1.1b [7].

1.2 Method

1.2.1 Pretreatment

The structure of lignocellulose consists of polymers of cellulose and hemicellulose encased in lignin; ratios of these components may vary largely, depending on the source of the biomass. Pretreatment of the lignocellulosic biomass promotes the depolymerisation and removal of the lignin outer layer. This structural deformation results in the exposure of cellulose and hemicellulose, and an increase in biomass surface area which is essential for optimal hydrolysis [8].

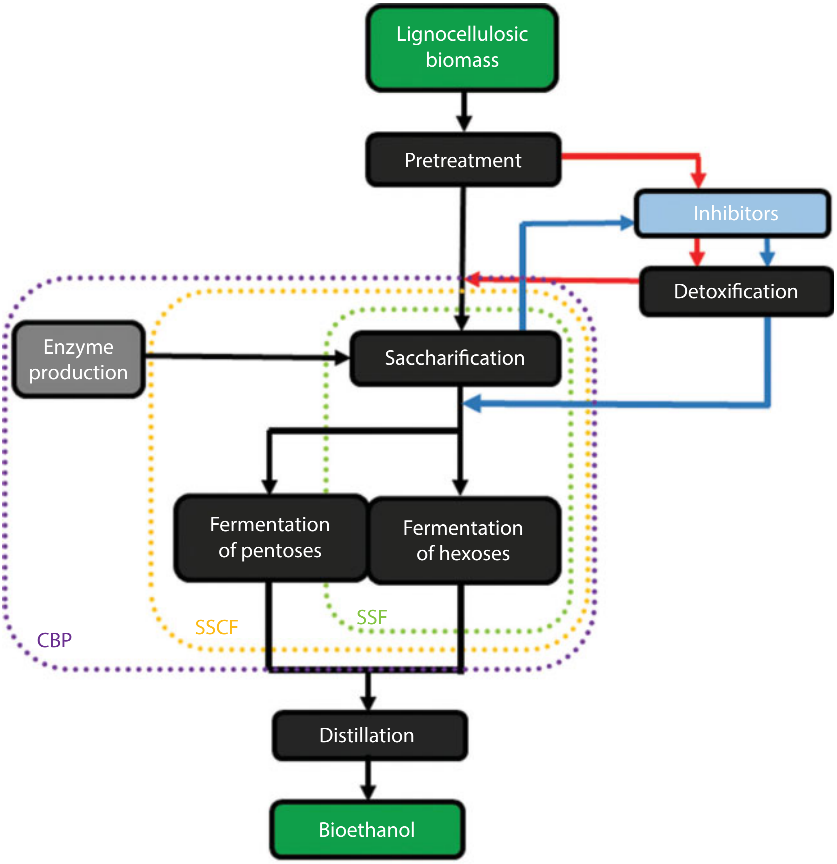
Lignocellulosic biomass
Pretreatment
Inhibitors
Detoxification
Enzyme production
Saccharification
Fermentation of pentoses
Fermentation of hexoses
CBP
SSCF
SSF
Distillation
Bioethanol

Figure 1.1a A process flow diagram depicting the key process steps required in the production of bioethanol from lignocellulosic biomass. SSF: Simultaneous saccharification and fermentation; SSCF: Simultaneous saccharification and co-fermentation; CBP: Consolidated bioprocessing.

Pretreatment methods range through physical, chemical, and biological (see Table 1.1); in some cases, a combination of methods may be employed to give optimum cellulose/hemicellulose exposure. The method of pretreatment exploited depends fundamentally on the biomass source. An optimum pretreatment method is characterised by the following criteria; minimum degradation of cellulose and hemicellulose fractions,

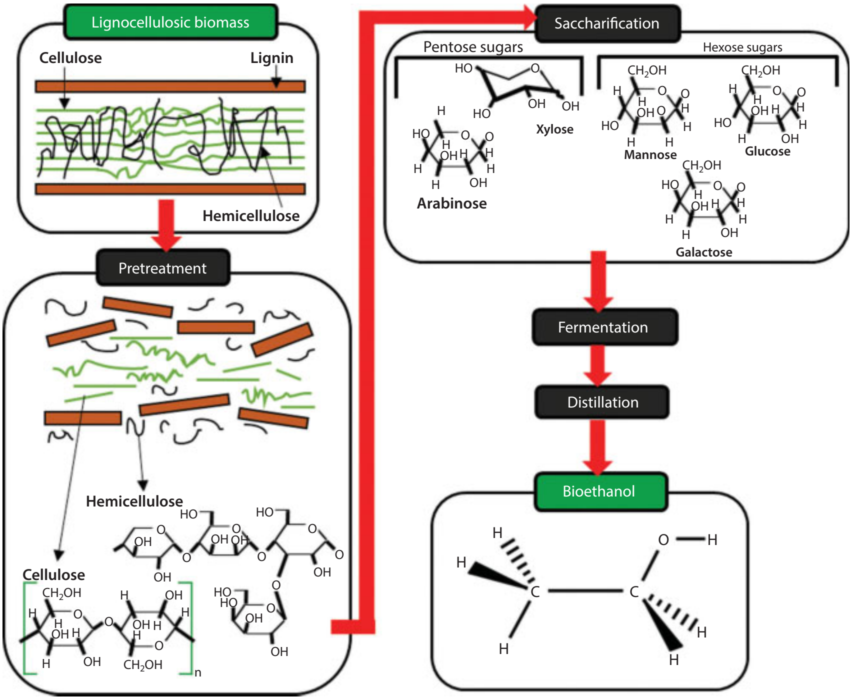
Lignocellulosic biomass
Cellulose
Lignin
Hemicellulose
Pretreatment
Hemicellulose
Cellulose
Saccharification
Pentose sugars
Xylose
Arabinose
Hexose sugars
Mannose
Glucose
Galactose
Fermentation
Distillation
Bioethanol

Figure 1.1b Overview of the key modifications and products attained at various stages of lignocellulosic biomass to bioethanol processing. Lignocellulosic biomass consists of cellulose and hemicellulose bound by lignin. Pretreatment of lignocellulose depolymerises the lignin exposing the cellulose and hemicellulose. Saccharification aims to hydrolyses cellulose and hemicellulose into their constituent sugar monomers; glucose, galactose, mannose, xylose, and arabinose. Fermentation of the sugars followed by distillation allows the formation and subsequent recovery of bioethanol. Adapted from [12, 30].

limitation of inhibitor formation, biomass size conservation, minimal energy input, and cost-efficiency. At present, there is no single pretreatment method which is thought to encompass all of these traits as each bears individual advantages and disadvantages including, but not limited to, those shown in Table 1.1. The initial pretreatment step may also influence the selection of other methods in the remaining process [8, 9].

Table 1.1 A selection of pretreatments suitable for lignocellulosic biomass [8, 9, 11, 12, 21, 35–37].

Pretreatment		**Method**	**Advantages**	**Disadvantages**
Physical	Mechanical (Milling)	Biomass particle size is physically reduced increasing accessible surface area	No inhibitors produced	High energy requirement High equipment cost
Chemical	Concentrated Acid	Concentrated biomass (10–40%) is submerged in concentrated acid at <160 °C	Simultaneously promotes cellulose and hemicellulose hydrolysis Minimal sugar degradation	Corrosion of equipment High inhibitor formation Slurry requires neutralisation Acid must be recovered
	Dilute Acid	Low concentration biomass (5–10%) is combined with a low concentration dilute acid at a ~220 °C	Low acid usage Cost efficient	Corrosion of materials Slurry requires neutralisation

	Alkali	Biomass is incubated with alkaline bases, such as sodium and potassium hydroxide resulting in the degradation of ester and glyosidic side chains	Does not require complex reactors Low inhibitor formation Removes hemicellulose making cellulose more accessible	Long residence time Slurry requires neutralisation Irrecoverable salts formed May cause chemical swelling of fibrous cellulose
	Ozonolysis	Ozone gas is utilised as powerful oxidant to break down bonds in lignin and hemicellulose	No toxic residues produced Reduces lignin content	Large volume of ozone required–high cost
Biological	White-, brown-, soft-rot fungi	Wood degrading microorganisms are added to the biomass and incubated at conditions suitable for the chosen microorganism	Environmentally sound Re-usable Particularly suitable for biomass with high lignin content	Long residence time Low yields of fermentable sugars

(*Continued*)

Table 1.1 Cont.

Pretreatment		**Method**	**Advantages**	**Disadvantages**
Physico-chemical	Liquid hot water	Biomass is subject to cooking by water at high temperature and pressure.	Removes hemicellulose making cellulose more accessible	Long residence time Small amounts of lignin removed
	CO_2 explosion	Biomass is subjected to CO_2 at high temperature and pressure before the pressure is suddenly reduced resulting in explosive decompression of materials	Low inhibitor formation Cellulose de-crystallised Cost efficient	High equipment cost Lignin unmodified

	Steam explosion	Biomass is subjected to high pressures (0.69–4.83 MPa) and temperatures (160–260 °C) before being suddenly reduced to atmospheric pressure resulting in explosive decompression of materials	High fermentable sugar yield	Incomplete depolymerisation of lignin High inhibitor formation
	Ammonia fibre expansion	Biomass is subjected to liquid ammonia at high pressure at a temperature of 90 °C before the pressure is suddenly reduced resulting in explosive decompression of materials	Removes lignin and hemicellulose making cellulose more accessible Low residence time (30 min) Ammonia can be reused	Inefficient for biomass with high lignin content

1.2.2 Saccharification

Saccharification is the process in which polymers of cellulose and hemicellulose are hydrolysed into their constituent fermentable reducing sugars. The most prevalent sugar monomers produced are the hexose sugars: glucose, galactose, and mannose; and the pentose sugars: xylose and arabinose (as shown in Figure 1.1b). Saccharification is commonly achieved via chemical and enzymatic methods [10].

Chemical methods include concentrated acid hydrolysis (CAH) and dilute acid hydrolysis (DAH); these methods are also considered as effective pretreatments (see Table 1.1) to be used in conjunction with other saccharification procedures. CAH entails the addition of concentrated acid, such as sulphuric and hydrochloric acid, to high concentration dried biomass (10–40% substrate) in a batch process at a temperature of below 160 °C, promoting complete hydrolysis of cellulose and hemicellulose. CAH is considered effective in that it gives a high yield of fermentable sugars; however, the corrosive nature of concentrated acids is detrimental to the reactors, and in order to be cost effective, the acids must be recovered and recycled [11, 12]. The development of DAH is more economically feasible. DAH involves the addition of a dilute acid, such as a 4% concentration sulphuric acid solution, to low concentration dried biomass (5–10% substrate) in a continuous process. Higher temperatures have been found to be favourable for DAH, with glucose yields of approximately 70% given at 260 °C. However, increased temperatures produce additional by-products, such as furfural, a fermentation inhibitor, and therefore, DAH is typically undertaken at temperatures of around 220 °C, giving a fermentable sugar yield of 50–60% [12, 13].

Enzymatic approaches to the saccharification of lignocellulose are more environmentally assured, operate under milder conditions (40–50 °C), and encompass less corrosion issues. Cellulolytic multienzymatic complexes, synonymously known as cellulosomes, are comprised primarily of endoglucanases, exoglucanases, cellobiohydrolases, and β-glucosidases; all of which catalyse the hydrolysis of cellulose to glucose via the cleavage of β-1, 4 glucosidic bonds. Cellulases are naturally synthesised by a wide range of fungi, bacteria, and plants, the most extensively documented and industrially utilised of these being the fungus *Trichoderma reesei* [8]. Hemicellulase is a collective term for an array of enzymes which can be categorised into two main groups; depolymerising enzymes responsible for backbone cleavage and enzymes responsible for the removal of substituents causing hindrances to depolymerising catalytic proteins. These include enzymes such as β-xylanase, β-mannosidase, α–galactosidase and ferulic acid esterase [14]. Ultimately, cellulases and hemicellulases catalyse the degradation of cellulose and hemicellulose into both hexose and pentose sugars (shown in Figure 1.1b).

1.2.3 Detoxification

Often harsh conditions of pretreatment and saccharification of lignocellulose, particularly through the use of acids, leads to the formation of by-products which act as inhibitors during fermentation, resulting in reduced productivity. Inhibitors generally fall into three categories: weak acids, furans, and phenolic compounds, such acetic acid, furfural, and vanillin respectively. The nature and quantity of inhibitory compounds present in the hydrolysate is strongly dependant on the pretreatment/saccharification methods employed [15].

Processes of detoxification (biological, chemical, and physical) may be employed to reduce the quantity of inhibitors in the hydrolysate, enabling maximum potential bioethanol yields. Overliming is a chemical method which consists of the addition of the alkali $Ca(OH)_2$ to adjust the hydrolysate pH to 9–10 reducing inhibitor concentration. This technique is identified as one of the most effective detoxification methods; however, concerns arise regarding the undesirable consequence of sugar degradation [16, 17]. Other recognised methods for reducing inhibitors include the addition of enzymes, such as laccase, and microbial treatment, such as the addition of *Coniochata ligiania*. The efficiency of detoxification methodologies is difficult to directly compare due to variance in hydrolysate compositions and differences in the tolerance of subsequent chosen fermentation microorganisms [1].

1.2.4 Organism Selection

During fermentation, sugars in the hydrolysate are harnessed for use as cellular energy by a microorganism which consequently excretes ethanol as a metabolic waste product. The primary aspects to be considered in the selection of a suitable microorganism include: the ability to utilise hexoses and pentoses, ethanol yield and production rate, and inhibitor tolerance. These factors may also be influenced by the prior methodology and the biomass feedstock (see Table 1.2) The yeast *Saccharomyces cerevisiae* is able to effectively utilise hexoses and is the traditional choice in the integrated process of simultaneous saccharification and fermentation (SSF) (see Figure 1.1a), as SSF is often designed to favour the generation of hexose sugars [18].

However, as technologies advance, industries require systems that are able to ferment both hexose and pentose sugars derived from lignocellulosic biomass (shown in Figure 1.1b) in order to optimise ethanol yield. Simultaneous saccharification and co-fermentation (SSCF) (see Figure 1.1a) is a process that achieves this either through the use of genetically modified organisms and/or co-cultures *S. cerevisiae* and *Zymomonas mobilis* (a gram-negative bacterium) are commonly employed to undergo genetic modification as they already possess the ability to effectively ferment hexoses, and so the addition of pentose fermentation ability is a complementary trait [19, 20]. *Escherichia coli* is also a favourable candidate for genetic modification due to extensive genome knowledge, making genetic manipulation of *E. coli* relatively straightforward in comparison to other less researched microorganisms [18]. One of the most common modifications is the insertion of genes allowing the utilisation of xylose, such as xylose reductase and xylitol dehydrogenase

Table 1.2 A selection of methods employed in the production of bioethanol from various lignocellulosic biomass sources and their resultant ethanol yields.

Biomass source	**Pretreatment**	**Saccharification**	**Fermentation organism**	**Ethanol yield**	**Reference**
Rice hulls	Alkali–calcium hydroxide ($Ca(OH)_2$)	Enzymatic–cellulase, β-glucoidase, and hemicellulase	*Escherichia coli* FBR5	9.8 ± 0.5 gl^{-1}	[38]
Sugarcane bagasse	Alkali–sodium hydroxide (NaOH)	Enzymatic - cellulase	*Saccharomyces cereviceae*	11.8 gl^{-1}	[39]
Sugarcane leaf litter	Dilute sulphuric acid (H_2SO_4)		*Saccharomyces cereviceae* 765	3.35 gl^{-1}	[40]
Wheat straw	Dilute sulphuric acid (H_2SO_4)	Enzymatic–cellulase, β-glucoidase, xylanase, and esterase	*Escherichia coli* FBR5	19 ± 1 gl^{-1}	[41]
Paper sludge	Not specified	Enzymatic - cellulase	*Zymomonas mobilis* NBRC 13756	18 ± 1 gl^{-1}	[42]

Cedar	Steam explosion	Enzymatic - cellulase	*Saccharomyces cereviceae* BA11	15.5 gl^{-1}	[43]
Sweet sorghum bagasse	Steam explosion	Enzymatic - cellulase	*Kluyveromyces marxianus* CECT 10875	16.2 gl^{-1}	[44]
Forage sorghum	Ammonia fibre expansion	Enzymatic–cellulase and xylanase	*Saccharomyces cereviceae* 424A	30.9 gl^{-1}	[45]

acquired from *Pichia stipites*, as it is the second most abundant sugar in lignocellulosic hydrolysate [21].

1.2.5 Media Composition and Operating Parameters

The medium should satisfy the fundamental nutrient and oxygen requirements of the fermenting organism, and therefore, the composition of the media depends primarily on the microorganism chosen to perform the fermentation whilst also taking into account other process variables, such as the use of batch or fed-batch or the use of immobilised cells [19]. Countless optimised media compositions can be found in literature for innumerable variations of methodology. Chankeng, Qinh and Peipei [22] describe an optimum medium consisting of 300 g/L initial glucose, 3.3 mmol/L Mg^{2+}, 5.0 mmol/L Ca^{2+}, 15.0 g/L peptone, and 21.5 g/L yeast extract. This media composition, however, is only optimal for the specific methodology, which employs a fusant of *Schizosaccharomyces pombe* and *S. cerevisiae* as a fermenting organism, and a very high gravity fermentation technique.

The pH and temperature also depends predominantly on the optimum reaction conditions of the chosen microorganism. *Z. mobilis* exhibits an optimum fermentation pH of around 4.5 and temperature of 25–30 °C [23]; *S.cerevisiae* exhibits an optimum pH range of 4.0–5.0 and temperature of 30 °C [24]; and *E.coli* exhibits a more neutral optimum pH range of 6.0–8.0 and temperature of 37 °C [25, 26]. In a process in which saccharification and fermentation are undertaken separately, optimum conditions can be provided for, however, in processes which combine saccharification and fermentation in a single process, such as SSF, SSCF and CBP (see Figure 1.1a), this is more problematic. For example, the optimum temperature for saccharification by cellulases is 45–50 °C whereas many fermentation microorganisms perform greatest at 28–37 °C. To allow for this discrepancy, a compromised temperature of ~37 °C is commonly applied [27]. Efforts have recently been made in the screening and selection of strains of microorganisms that encompass an increased optimum temperature to reduce the effect that this compromise may assert on the efficiencies of both processes [26].

1.2.6 Ethanol Recovery

The broth recovered from fermentation is a solution composed of water and ethanol, however, ethanol composes only 5–12 wt% and so product purification is an essential process to increase ethanol concentration to a wt% acceptable for use as a biofuel. The differing boiling points of water (100 °C) and ethanol (78.37 °C) allows distillation to be utilised as a means of refinement as when the fermented broth is heated in a distillation column the substances take their gaseous forms. Ethanol and water form an aezotropic solution causing co-distillation at 95.6 wt% ethanol at 78.15 °C and so cannot be separated sufficiently by a simple conventional distillation. A three-step process is therefore required for adequate ethanol purification involving distillation, rectification, and dehydration [28].

Distillation and rectification produce a solution with an ethanol concentration of ~92.4 wt%. This solution then undergoes dehydration, often by azeotropic distillation, extractive distillation, liquid-liquid extraction, adsorption, or membrane pervaporation. The final ethanol product has an ethanol concentration of 95–96 wt%, limited by the formation of the water-ethanol azeotrope [29, 30].

1.3 Discussion

2nd generation ethanol production efficiency varies profusely due to the large selection of methods available at each process stage and the differences in initial compositions of biomass from different sources. Each protocol selection, in turn, also influences the subsequent production stage causing even more variation in the production results, as shown in Table 1.2. Optimising ethanol production is therefore particularly challenging as methods cannot be directly compared due to the substantial number of possible combinations [27].

In regards to process optimisation, efforts should largely be focused on technologies, allowing more cost-efficient process integrations to increase the economic viability of 2nd generation production on an industrial scale. Selection and engineering of more inhibitor tolerant, and both hexose and pentose utilising strains of fermenting microorganisms is a promising practice for improved SSF and SSCF.

Consolidated bioprocessing (CBP) (see Figure 1.1a) is a process in which one organism produces saccharifying enzymes and ferments the resulting sugars into ethanol in a single vessel. At present, no organism is known to perform this efficiently, however, development of candidates, such the thermophilic bacteria *Clostridium thermocellum,* are proving hopeful [31]. Development of a viable CBP process would dramatically reduce the presently large production costs of bioethanol due to an eight fold reduction in the cost of biological conversion [8].

Recently, investigation of third (3rd) generation bioethanol, bioethanol from algal sources, has arisen as a more sustainable and viable approach. Concerns regarding potential overshadowing by more feasible 3rd generation technologies may call into question the future prospects of 2nd generation production. Presently, however, 3rd generation bioethanol is still in preliminary stages of investigation and optimisation of 2nd generation production would be of great benefit for the current rising demand for fuel as lignocellulosic materials available from agricultural waste possess a maximum potential ethanol yield of 10.84 billion litres per year [32, 33]. As well as a stand-alone technology, 2nd generation bioethanol also displays potential as an integrated process utilising lignocellulosic wastes from 1st generation production; this would particularly benefit countries such as Brazil, where the production of 1st generation has an already well-established platform [34].

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2

A Perspective on Current Technologies Used for Bioethanol Production from Lignocellulosics

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Abstract

Traditional fossil fuels such as coal, oil, and natural gases are main sources of energy in the present era. However, their depletion is anticipated within the next 50 years. Bioethanol has shown its potential as one of the most promising alternatives for gasoline as transportation fuel. Currently, ethanol has been recognized as a fuel for direct ethanol fuel cells. The so-called 2nd generation bioethanol produced from lignocellulosic material is more economic, energetic, and environmentally advantageous than the traditional sugarcane and corn as feedstock. At bench scale, significant advances have been made towards bioethanol production from lignocellulosic biomasses. However, various economical and technological hurdles make it unsuccessful at industrial scale. This chapter reviews the current status of various bioethanol production technologies along with their associated economic and environmental viability. Understanding this review will help achieve an integrated and efficient lignocellulosic biomass-based conversion process to ethanol and develop a comprehensive bioenergy development process.

***Keywords*:** Fossil fuel, second generation bioethanol, lignocellulosic biomass

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2.1 Introduction

With the increase in global energy demand, which is expected to increase by 50% till 2025, energy shortage has become a global problem. Population growth and industrial development have contributed majorly to it in recent years. During the year 2000's energy crisis, the world saw the price of oil as high as $148 per barrel in 2008 (Ragauskas *et al*. 2006). There are many causes of energy crisis, among them: overconsumption, overpopulation, and unexplored renewable energy options. A possible solution might be moving towards renewable energy resources.

In the long run, bioethanol as an energy source would be more than just supplementing for wind, solar, and other periodic renewable energy sources (Lin and Tanaka 2006). The current situation for ethanol-petrol mixture used in various countries are shown in Table 2.1, which depicts the importance of ethanol as a gasoline substitute in petroleum used for transportation. Various technology advancements for ethanol production have been developed during the last two decades, which points out that large scale ethanol production can be a reality within next few years (Yu and Zhang 2004; Moiser *et al*. 2006).

2.2 Biothanol Production from Various Feedstocks

Usually bioethanol is produced from feedstocks that contain sugar. Feedstocks can be categorized as first

Table 2.1 Common ethanol-petrol mixture (Kang *et al.* 2014; Baeyens *et al.* 2015).

Code	Countries	Comments
E5 (Maximum 5% ethanol with petrol)	Western Europe, India	For regular cars
E10 (Maximum 10% ethanol with petrol)	USA, Europe	
E15 (Maximum 15% ethanol with petrol)	USA	
E25 (Maximum 25% ethanol with petrol)	Brazil	
E85 (Maximum 85% ethanol with petrol)	USA, Europe	Flex-fuel vehicles
E100 (Hydrous ethanol having 5.3 wt% water)	Brazil	

generation feedstocks, which include starch and second generation feedstocks such as lignocellulosic biomass. Sugars can be fermented directly using suitable microorganisms to produce ethanol. If molasses and sugarcane juice are used in fermentation directly, then prior steps (milling, pretreatment, hydrolysis, and detoxification) are not required (Vohra *et al.* 2014). For fermentable sugar production from starchy materials, milling, liquefaction, and saccharification are required; otherwise, lignocellulosic feedstock processes like milling, pretreatment, and hydrolysis are used. The detoxification unit is considered only when toxic compounds are fed to the bioreactors. The step used to obtain fermentable sugars is the main distinction between production processes from starch, simple sugar, or lignocellulosic biomass.

2.2.1 Bioethanol Production from Sucrose Based Feedstocks

Examples include sugarcane, molasses, sweet sorghum etc. Raw material is processed within 24–72 h of harvesting, and sugar is extracted by specialized rollers to separate the juice. Released sugars can be directly fermented to ethanol (Vohra *et al*. 2014).

2.2.2 Bioethanol Production from 1st Generation Feedstocks (Starch)

Ethanol production from starch involves grains (wheat, corn, or barley) which contain 60–70% starch. To produce ethanol, it is necessary to break down the starch (long chain of carbohydrates) stored in grains to obtain the glucose syrup which produces ethanol after fermentation produce (Vohra *et al*. 2014). There are two methods for starch processing:

a. Dry milling: Dry mills are usually smaller in size and produce ethanol only.
b. Wet milling: Many value added co-products (high fructose corn syrup, dextrose syrup, etc.) are produced along with ethanol in corn refineries.

The main disadvantage of the feedstocks above (sucrose and starch-based) are their seasonal availability and competition with human food crops for land (Vohra *et al*. 2014).

2.2.3 Bioethanol Production from 2nd Generation Feedstocks (Lignocellulosic Biomass)

Production includes agricultural residues, municipal wastes, grasses, and wood residues. Basically, lignocellulosic biomasses are composed of holocellulose (hemicellulose and cellulose), lignin, and extractives. Cellulose, which is a linear crystalline β-D-glucose polymer and very difficult to break, is major component (Chesson and Forsberg 1988). The cellulosic fraction of the biomass is converted into a glucose monomer by a chemical or enzymatic method for saccharification (Mosier *et al*. 2005). Hemicellulose is a heteropolymer that is composed of both linear and branched chain of D-glucose, D-xylose, D-mannose, D-galactose, and L-arabinose. Since its structure is not crystalline, it is therefore easier to hydrolyse comparatively (Chang and Holtzapple 2000). Lignin is the most rigid 3-D polymeric component of a plant cell wall, and it consists of three different phenyl propane precursors as its monomeric unit, which are non-biodegradable (Palonen 2004). Composition of some important lignocellulosic biomass is presented in Table 2.2.

Bioethanol production from lignocellulosic biomasses typically involves three steps:

1. Hydrolysis step to obtain fermentable sugars from biomass

Table 2.2 Important lignocellulosic feedstocks and their composition.

Lignocellulosic biomass	Cellulose (% dry weight)	Hemicellulose (% dry weight)	Lignin (% dry weight)
Agricultural residue	37–50	25–50	5–15
Hardwood	45–47	25–40	20–25
Softwood	40–45	25–29	30–60
Grasses	25–40	35–50	Not present
Switch grass	40–45	30–35	12
Waste papers (from chemical pulps)	50–70	12–20	6–10
Newspaper	40–55	25–40	18–30

2. Fermentation for sugar conversion into ethanol
3. Separation and purification by distillation, rectification, and dehydration usually.

The fermentation process involves conversion of any sugar-containing material to ethanol. One or many steps can be combined depending on the type of feedstock used and conversion technology involved. After reaching the ethanol plant, the biomass is stored in a warehouse to prevent contamination and early fermentation; then it goes through conditioning. Pretreatment is done to extract the carbohydrates present in the biomass to make it more accessible for further extraction. During this process, the amount of sugar released depends on the biomass used and pretreatment method used (Vohra *et al.* 2014). A large portion of holocellulosic fibres become available for conversion into monomeric sugars through hydrolysis techniques. In the batch mode of operation, at the beginning of fermentation, microbes and other ingredients are added to the hydrolysate and nutrients. As fermentation progresses, one or many ingredients can be added in the fed batch operation. In continuous operation, ingredients can be constantly added and products can be removed from the reactor. By recycling or immobilization of microbe, cell density can be made high to improve their activity and make the process more efficient (Wyman *et al.* 2004; Vohra *et al.* 2014). Usually fermentation temperature is kept between 25 °C and 30 °C, and time varies from 6 h to 72 h depending on the hydrolysate composition, physiological activity, cell density, and microorganism used. On a volume basis, broth contains 8–14% ethanol usually. Above this concentration, microbial inhibition may occur which reduces their activity. The distillation step results in an azeotropic mixture (95.5% alcohol + 4.5% water), which is hydrous ethanol and needs to be dehydrated to obtain alcohol up to 99.6%. Remains flow from the distillation column (vinasse or stillage) and can be volatilized to form co-products. These products include animal products and other valuable byproducts, steam, and electricity (Gnansounou 2009; Vohra *et al.* 2014). In addition, moderate concentration stillage can be used as fertilizer. Schematic representation of ethanol production processes from all three feedstocks is shown in Figure 2.1.

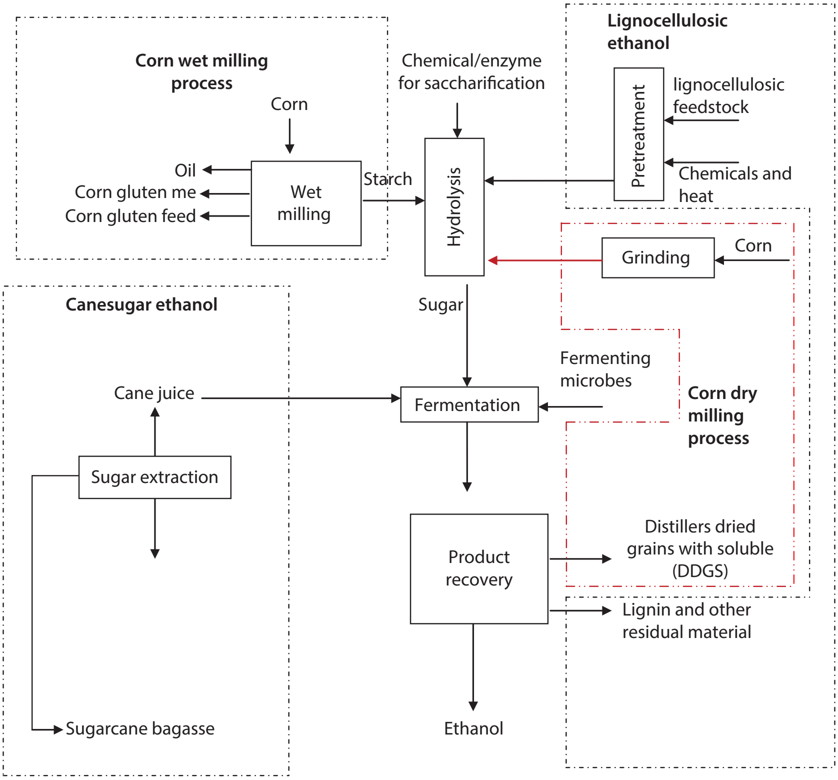
Corn wet milling process
Corn
Oil
Corn gluten me
Corn gluten feed
Wet milling
Starch
Chemical/enzyme for saccharification
Hydrolysis
Lignocellulosic ethanol
Pretreatment
lignocellulosic feedstock
Chemicals and heat
Grinding
Corn
Canesugar ethanol
Sugar
Fermenting microbes
Corn dry milling process
Cane juice
Fermentation
Sugar extraction
Product recovery
Distillers dried grains with soluble (DDGS)
Lignin and other residual material
Sugarcane bagasse
Ethanol

Figure 2.1 Schematic representation of production of ethanol from corn (corn wet milling, corn dry milling), cane sugar and lignocellulosic biomass.

2.3 Various Conversion Paths or Technology Routes from Lignocellulosic Biomass to Ethanol

There are two main routes for lignocellulosics to bioethanol conversion.

1. **Biochemical route:** it has been further subcategorized into
   a. Separate hydrolysis and fermentation (SHF)

   b. Integrated technologies:
      - Simultaneous saccharification and fermentation (SSF)
      - Simultaneous saccharification and co-fermentation (SSCF)
      - Consolidated bioprocessing (CBP) or direct microbial conversion (DMC)
2. **Thermochemical route or syngas platform**: divided further into
   a. Syngas catalytic conversion
   b. Biological path or gasification syngas fermentation

True diagrammatic representations of steps involving both the routes for ethanol production from lignocellulosic biomass are given in Figure 2.1.

2.3.1 Seperate Hydrolysis and Fermentation (SHF)

Chemical or enzymatic hydrolysis performed separately from fermentation step in SHF (Sree NK *et al.* 2000; Wingren *et al.* 2003; Chandel *et al.* 2007). To produce cellulosic ethanol on a pilot scale, typically it involves treatment of milled or grinded biomass with hot acid resulting into hydrolysis of cellulose, hemicellulose, and other polysaccharides which cause disruption of the association of lignin with the carbohydrate (Menon and Rao 2012; Vohra *et al.* 2014). The hydrolysate is then subjected to neutralization and separated from the insolubles and solid fraction. It is then fermented to produce alcohol. The insoluble fraction is then kept for treatment with glycosidase

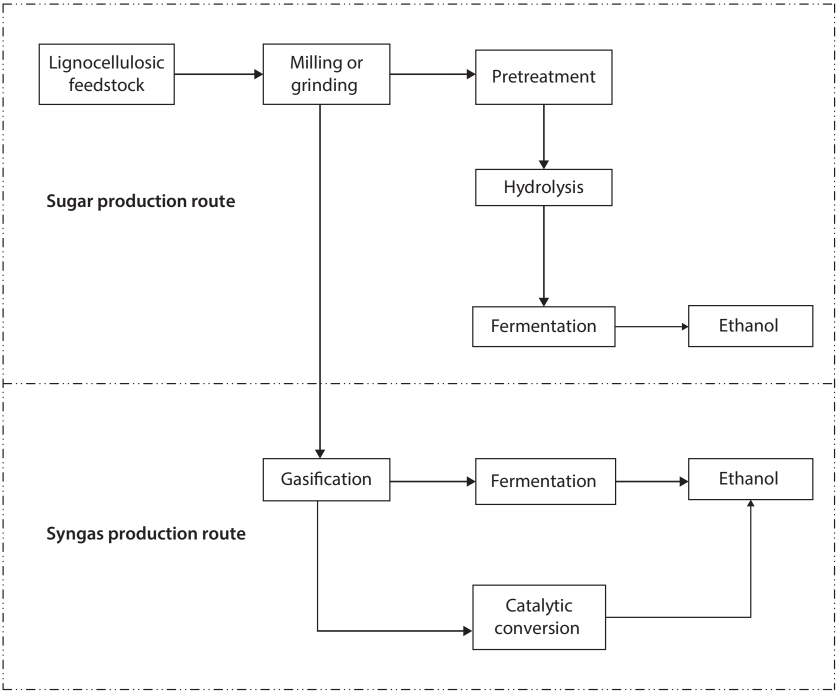
Lignocellulosic feedstock
Milling or grinding
Pretreatment
Hydrolysis
Sugar production route
Fermentation
Ethanol
Gasification
Fermentation
Ethanol
Syngas production route
Catalytic conversion

Figure 2.2 Different routes for bioethanol production from lignocellulosics.

and cellulase to release glucose sugar which is again fermented for ethanol production. Lignin, in the form of residual insoluble material, is burnt for energy generation for the overall process (Huber and Iborra 2006; Vohra *et al.* 2014). Some development of plants are in the process to modify lignin which can be readily hydrolyzed, or chemical catalysts or enzymes improvement for lignin hydrolysis can result in lignin use as a plastic component or as a liquid fuel fermentation feedstock production. Typically, the fermentation process generates a nutrient-enriched microbial cell mass which can be used as fertilizer after inactivation, and mineral nutrients can be recycled to the land

(Somerville 2011; Vohra *et al.* 2014). SHF is the most extensively tested configuration. Pentose fermentation is carried out in an independent unit. In SHF, joint liquids that flow from both reactors after sugar release first enters into the glucose fermentation bioreactor. Leaving the unconverted xylose behind, the mixture is then distilled to remove the pure ethanol. In the second reactor, xylose fermentation takes place and the same procedure follows.

Each step can be carried out at optimum condition which is main advantage of SHF (Cardona and Sánchez 2007; Balat 2011; Vohra *et al.* 2014), but it has proved to be very costly. Therefore, several integrated technologies or routes have been developed based on the different technology combinations adopted at the all three stages i.e. pretreatment, hydrolysis, and fermentation for ethanol production which is discussed below.

2.3.2 Simultaneous Saccharification and Fermentation (SSF)

Saccharification and fermentation are both carried out in a single reactor simultaneously which saves overall costs, reduces inhibitor formation, and increases the hydrolysis rate of the process (Foust *et al.* 2009; Vohra *et al.* 2014). However, the process conditions for optimization of enzymes used for saccharification and the microorganisms for fermentation at the same time is the most critical issue of this method (Chiaramonti 2007; Vohra *et al.* 2014). The key point which should be considered for this process is that the sugar should be converted rapidly into ethanol after its formation following saccharification so that its accumulation is diminished. Considering that sugars are more inhibitive than ethanol for the conversion process, compared to SHF, SSF can reach a higher ethanol formation rate and yield (Brethauer and Wyman 2010; Vohra *et al.* 2014). As no separate hydrolysis reactors are needed, SSF offers an easy operation and requires less instruments than SHF. In addition, the ethanol presence in both leads to less vulnerability of the action of undesired microorganisms to the reaction mixture. Yet, SSF has the disadvantage of difficulty in controlling process parameters as optimum conditions for saccharification and fermentation are different. Furthermore, a very high amount of exogenous enzymes are needed for this process (Taylor *et al.* 2009; Vohra *et al.* 2014). The most well-suited temperature for hydrolysis using cellulolytic enzymes is around 50 °C, whereas most of the fermenting microorganisms have an optimum temperature between 28 °C and 37 °C for ethanol fermentation. Even through protein engineering, it is difficult to reduce the optimum temperature of cellulases. High-temperature fermentation is highly desired for SSF due to which thermotolerant yeast strains have been screened for alcohol fermentation (Hasunama and Kondo 2012; Vohra *et al.* 2014). *Kluyveromyces marxianus* has been found most promising among these, as various strains of this yeast can grow well at high temperatures (45–52 °C) and produce ethanol efficiently at a temperature range of 38 °C–45 °C. In addition, *Kluyveromyces marxianus* has the additional advantage of a high growth rate and its ability to utilize various sugar substrates like galactose, xylose arabinose, and mannose at high temperatures (Cardona and Sánchez 2007; Hasunama and Kondo 2012; Vohra *et al*. 2014).

2.3.3 Simultaneous Saccharification and Co-Fermentation (SSCF)

SSCF is subjected to the complete assimilation of all the sugars which are released during the pretreatment and hydrolysis of lignocellulosic biomass (Figure 7). Using mixed culture of yeasts which can ferment both pentose and hexose sugars has been proposed, but hexose utilizing microbes grow faster compared to pentose utilizing microbes; therefore, the conversion of hexose to ethanol is more elevated (Sánchez and Cardona 2008; Vohra *et al*. 2014).

A single microbe is capable of assimilating both pentose and hexose sugars in an optimal way and can also be used to produce a high sugar conversion and ethanol yield (Banerjee *et al*. 2010). Although these microbes exist, high conversion can only be reached through the genetic modification of these organisms which are already adapted to the ethanolic fermentation (Cardona and Sánchez 2007; Vohra *et al*. 2014).

2.3.4 Consolidated Bioprocessing (CBP) or Direct Microbial Conversion (DMC)

Ethanol and all the enzymes required for its production are formed in a single bioreactor by a single microbial community (Carere *et al*. 2008; Vohra *et al*. 2014). Reaction-reaction integration for the biomass transformation into ethanol is the consolidated bioprocessing (CBP) or direct microbial conversion (DMC) (Figure 2.7). The only difference between CBP and other technologies like SSF for ethanol production is that a single microbial community is used to carry out both cellulases production and fermentation. All three steps i.e. cellulase enzyme production, hydrolysis of cellulose, and fermentation are carried out in a single reactor and a single step. Zero capital or operation costs are required for enzyme production, which is an additional advantage (Lynd *et al*. 2005; Vohra *et al*. 2014). Also, part of the substrate does not diverge for cellulase production. Additionally, the enzymatic and fermentation processes are fully compatible (Cardona and Sánchez 2007; Vohra *et al*. 2014). Thermophilic cellulolytic bacteria which are anaerobic have been examined extensively as potential ethanol producers. Some popular strains of these bacteria are *Clostridium thermosaccharolyticum, Clostridium thermohydrosulfuricum, Thermoanaerobium brockii, Thermoanaerobacter ethanolicus,* and *Thermoanaerobacter mathranii*. They can directly use a variety of inexpensive feedstocks and can withstand extreme temperatures, which makes it more beneficial. However, low alcohol tolerance (<2%, v/v) is a major limitation to their industrial application for ethanol production (Balat 2011; Careen *et al*. 2008; Vohra *et al*. 2014). Cell surface engineering has been tested on *Kluyveromyces marxianus,* a thermotolerant strain of the yeast for cellulolytic enzymes displayed on the cell surface. The recombinant *Kluyveromyces marxianus* strain, which co-displays β-glucosidase and endoglucanase on the cell surface, can grow well at high temperatures up to 48 °C. At this temperature, from the cellulosic material *β*-glucan, 0.47 g ethanol/g of consumed carbohydrate was produced which gives support to CBP yeast development for an efficient bioethanol production (Hasunama and Kondo 2012; Vohra *et al*. 2014). One more approach is possible with mixed culture utilization so that both hydrolysis and fermentation of lignocellulosic feedstock can be be carried out simultaneously (Cardona and Sánchez 2007; Vohra *et al*. 2014). Singh and Kumar (1991) have worked on *Fusarium oxysporum* and found that these strains have the potential to convert both cellulose and D-xylose to ethanol in a single step. The advantage of using *Fusarium oxysporum* for ethanol production includes *in situ* cellulase production, both cellulose and pentose sugar fermentation simultaneously as well as its sugars and ethanol tolerance, but its sugars to ethanol conversion rate is too slow as compared to yeast.

Procurement or production of cellulase enzyme contributes significantly to the enzymatic hydrolysis process overall cost. DMC cannot be considered the leading potential process alternative because of the non availability of a robust organism to produce cellulases or some other cell wall degrading enzyme with high yield ethanol.

A generic block diagram for bioethanol production from lignocellulosic biomass showing possibilities of various reaction- reaction integrations (SHF, SSF, SSCF and DMC) is presented in Figure 2.3.

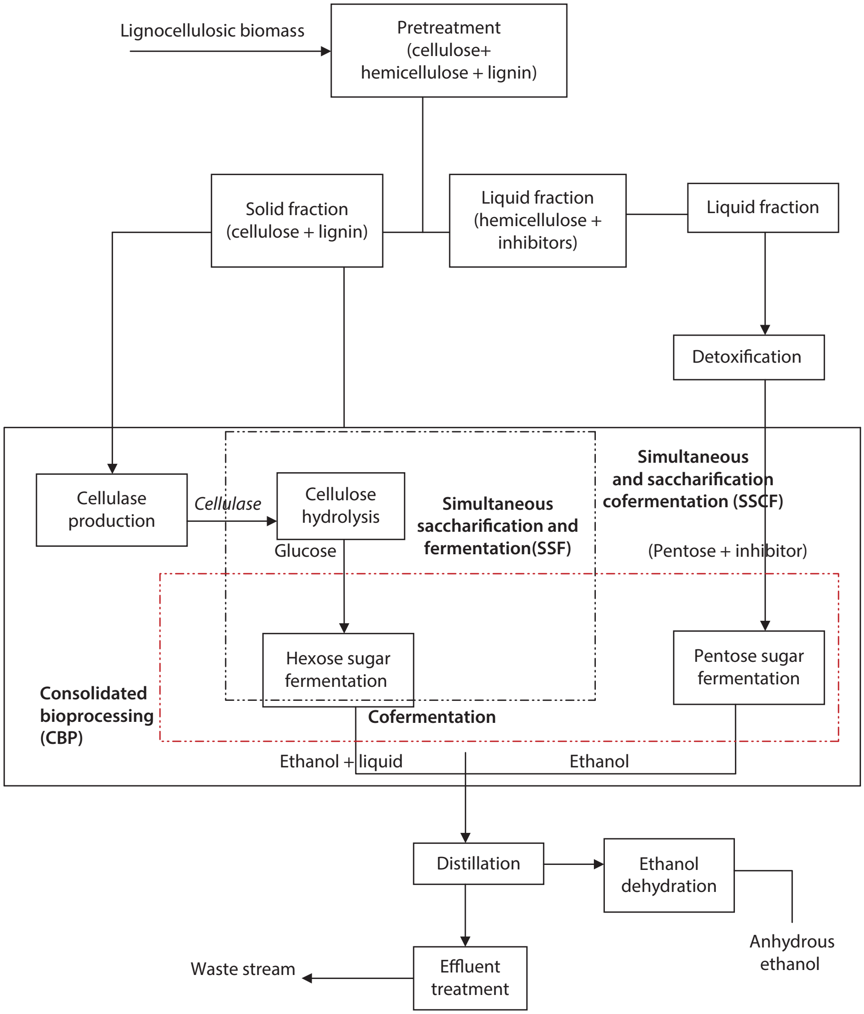
Lignocellulosic biomass
Pretreatment (cellulose+ hemicellulose + lignin)
Solid fraction (cellulose + lignin)
Liquid fraction (hemicellulose + inhibitors)
Liquid fraction
Detoxification
Cellulase production
Cellulase
Cellulose hydrolysis
Glucose
Simultaneous saccharification and fermentation(SSF)
Simultaneous and saccharification cofermentation (SSCF)
(Pentose + inhibitor)
Hexose sugar fermentation
Pentose sugar fermentation
Consolidated bioprocessing (CBP)
Cofermentation
Ethanol + liquid
Ethanol
Distillation
Ethanol dehydration
Anhydrous ethanol
Effluent treatment
Waste stream

Figure 2.3 Generic block diagram of bioethanol production from lignocellulosic biomass showing possibilities of various reaction- reaction integrations.

2.3.5 Thermochemical Conversion Processes or Syngas Platform

Bioethanol production from syngas is an emerging technology which can utilize a wide variety of lignocellulosic feedstocks. The major advantantage of syngas platform is the utilization of whole biomass including lignin which is difficult to break. First, the biomass is converted to syngas by a process called gasification. Gasification is a process through which liquid or solid carbonaceous material (like biomass, coal, or oil) that react with oxygen and/or steam for the production of a gas product called a producer gas or syngas. Syngas contains carbon monoxide (CO), hydrogen, carbon dioxide (CO_2), methane (CH_4) and nitrogen (N_2) in different proportions (Huber and Iborra 2006; Vohra *et al.* 2014). The biomass-syngas composition can be converted into various biofuels (ethanol, methanol etc.) via biocatalytic or metal-catalytic methods (Cardona and Sánchez 2007; Vohra *et al.* 2014).

2.3.5.1 Syngas Catalytic Conversion

At very high temperatures (750–800 °C), the lignocellulosic feedstocks gasification process produces a gas mixture ($CO + CO_2 + H_2 + N_2 + CH_4$). It also produces some other higher hydrocarbons which are commonly called producer gas (Cardona and Sánchez 2007; Vohra *et al.* 2014). This overall process of gasification is endothermic (requires heat energy to drive the process). The producer gas composition depends on gasifier and biomass types as well as the gasification conditions. The synthesis gas commonly known as syngas contains H_2 and CO predominantly. The syngas mixture passes through a series of filters after gasification to remove undesirable pollutants (some solid particles and tar) (Datal *et al.* 2004; Vohra *et al.* 2014). After that, to create a variety of liquid fuels, syngas is passed through the Fischer Tropsch process. These liquids are suitable for marine applications and aviation as well as some chemicals including ethanol. Gas is heated to 300 °C at pressure of 69 bar before entering into the chamber. It is also mixed with methanol and water to improve high alcohol yield. The mixture is then passed through the synthetic catalyst to obtain shorter chain alcohols (methanol and ethanol) as well as higher linear alcohols (pentanol), methane, water, and small amounts of some other hydrocarbon byproducts (Dwivedi *et al*. 2009; Vohra *et al*. 2014). The process's reaction rate is very high and is over within seconds to minutes. During the process, up to 60% of carbon monoxide converts into ethanol. After that, the gas is allowed to cool, which results in alcohol condensation and separation from the syngas which is unconverted. Further liquid ethanol is refined by alcohol separation and purification methods (Subramani and Gangwal 2008; Vohra *et al*. 2014). Cobalt, molybdenum, rhodium, and some other multi-component catalysts have been used as a catalyst historically. Rhodium is found to be the most suitable catalytic metal for the conversion of natural gas into ethanol because of its ability to perform all the four catalytic specific functions. These functions include: a. on catalysts, adsorption, and dissociation properties of oxygen and carbon monoxide molecules; b. adsorbed carbon hydrogenation to methyl species; c. non –dissociated carbon monoxide insertion into the methyl species which results in the formation of adsorbed acyl species; and, d. hydrogenation of the formed acyl species for ethanol formation (Vohra *et al.* 2014).

2.3.5.2 Biological Path or Syngas Fermentation Route

One more possible option for bioethanol production is syngas fermentation using mixed thermo-chemical-biological methods. There are several advantages for using microbial catalysts for syngas as lower temperature and pressure conditions are required for this process (atmospheric conditions usually), less susceptibility to feed gas compositions variation. The specificity of chemical catalysts is lower, and they are less susceptible to contamination in comparison to microbial processes, although the microbial route usually has a slower conversion time (Köpke *et al.* 2011; Vohra *et al.* 2014). The initial stage is biomass gasification, same as with the syngas catalyst technology. Then the cleaned gas is cooled down to the normal ambient temperature and further stored at a high pressure. The cleaned and cooled gas is fed into the ethanol conversion chamber where microorganisms converts gas into acetic acid and ethanol. The liquid is distilled to separate ethanol and other products after fermentation. The produced ethanol is then dehydrated, which produces fuel-quality ethanol (Dwivedi *et al.* 2009; Vohra *et al.* 2014). The microbial cell mass can be recycled back to the gasifier, in case it is not approved for animal feed (Cardona and Sánchez 2007; Vohra *et al.* 2014). Various bacterial strains have been isolated which have the ability to ferment producer gas components (carbon monoxide, carbon dioxide, and hydrogen) to acetic acid, ethanol, and some other valuable liquid products. *Clostridium ijungdahlii* is recognized as the first organism which can ferment ethanol from producer gas components (Henstra *et al.* 2007; Vohra *et al.* 2014). At high pH levels (5–7), this organism favors acetate production, while at pH 4–4.5, ethanol is the dominant product. *Clostridium acetogen,* which has been isolated recently, has been shown to produce ethanol from producer gas that is generated from biomass. Some other microorganisms which have shown similar capability include *Butyribacterium methylotrophicum* and *Clostridium autoethanogenum,* although ethanol is not a major product in these cases (Abubacker *et al.* 2011; Vohra *et al.* 2014). Producer gas fermentation is not commercially viable because of its low productivity of ethanol in the bioreactor. Several problems should be addressed to make this process economically feasible, including: a. low cell density; b. lack of metabolic pathways regulation to yield desired product only; c. biological catalysts inhibition by substrate and products; and, d. low gas–liquid mass transfer (Ungerman and Heindel 2007; Vohra *et al.* 2014). The rate limiting step in most of the fermentation processes is gas to bulk liquid transport through the liquid film, which is formed around gas bubbles involving sparingly soluble gaseous substrates. On a mass basis, carbon monoxide and hydrogen have aqueous solubilities of 60% and 4% respectively at mild temperature, compared to oxygen.

Lower concentration gradients occur due to these low solubilities leading to low mass transfer rates. This problem can be overcome by the use of an agitator system to achieve higher mass transfer rates. It can also be increased by increasing the operating pressure of producer gas fermentation. At low power consumption, microbubble dispersions (bubbles having diameters of 50–100 mm) have been also used to provide a larger gas transport area (Lewis *et al*. 2006; Vohra *et al*. 2014). Both routes of syngas platform for ethanol production are shown in Figure 2.4 below.

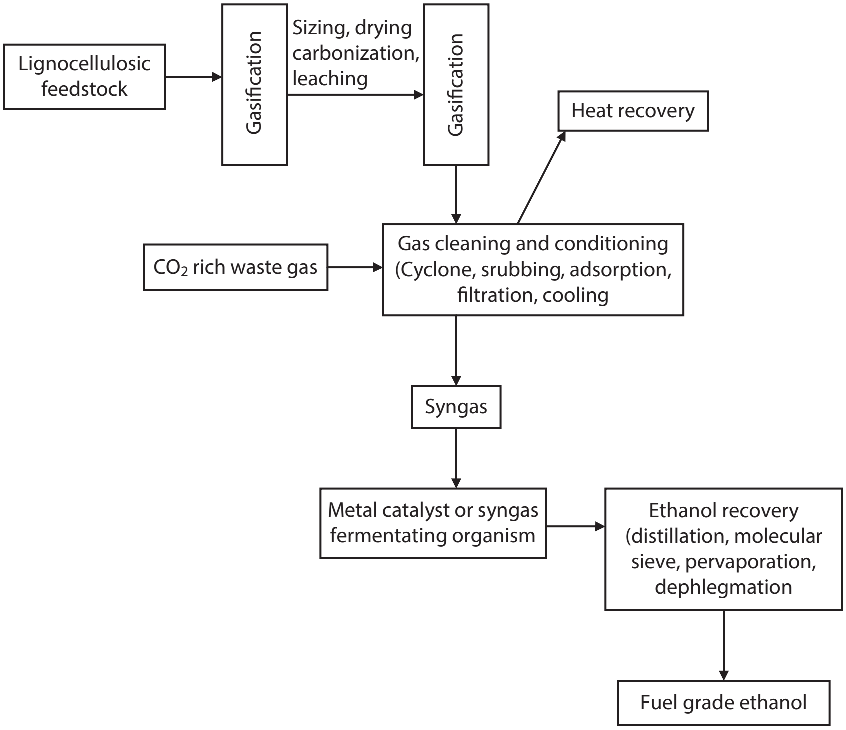
Lignocellulosic feedstock
Gasification
Sizing, drying carbonization, leaching
Gasification
Heat recovery
CO_2 rich waste gas
Gas cleaning and conditioning (Cyclone, srubbing, adsorption, filtration, cooling
Syngas
Metal catalyst or syngas fermentating organism
Ethanol recovery (distillation, molecular sieve, pervaporation, dephlegmation
Fuel grade ethanol

Figure 2.4 Bioethanol production from lignocellulosics via catalytic and biological syngas route.

2.4 Bioethanol Production Technologies Based on Different Fermentation Modes

The sugar solution obtained after hydrolysis is utilized for fermentation to produce ethanol. Microorganisms that have the ability to ferment both hexose and pentose sugar are not widespread (Toivolla *et al.* 1984; Chandel *et al.* 2007). *Saccharomyces cereviseae* is capable of converting hexose sugars only to ethanol. Promising yeasts that can ferment pentose sugars are *Candida shehatae, Pichia stipitis* and *Pachysolan tannophilus*. Currently, commercial bioethanol production from sugars which are derived from sucrose and starch are fermented by *Saccharomyces cereviseae* species predominantly (Lin and Tanaka 2006; Chandel *et al.* 2007). At the industrial scale, thermotolerant yeasts could be more suitable for bioethanol production from lignocellulosics. By reducing cooling costs, energy savings can be achieved in high temperature process. This approach was utilized by Sree *et al.* (1999); they developed a solid state fermentation system for sweet sorghum and potato as a raw material for ethanol production using a heat tolerant *Saccharomyces Cereviseae* strain. Focus is now shifting towards developing recombinant yeasts by researchers for metabolization of all available sugars, improving ethanol production and reducing operation costs. Two approaches have been taken to achieve it: A. genetic modification of yeast and other natural ethanol fermenting organisms for additional pentose utilizing metabolic pathways; and, B. genetic engineering of ethalogens having the ability to ferment both pentose and hexose sugar simultaneously to produce ethanol (Jeffries and Jin, 2000; Dien *et al.*, 2003; Chandel *et al.* 2007).

Using mutagenesis and adaptive evolution, strain selection through quantitative metabolism models are helpful in increasing ethanol production rates and the overall productivity of the process (Jeffries and Jin 2004). Recent developments in bioinformatics and comparative genomics to explain high ethanol production mechanism from *Saccharomyces* species have also been shown (Chandel *et al.* 2007). Although various new technologies have improved the bioethanol production process significantly, there are lot of problems that need to be solved. These problems include: a. maintaining the stable performance of genetically modified yeast for commercial scale fermentation operations; (Ho *et al.* 1998, 1999; Chandel *et al.* 2007) b. for lignocellulosic biomass, development of more effective and efficient pretreatment technologies; and, c. integration of optimized components into commercial ethanol production processes (Dien *et al.* 2000; Chandel *et al.* 2007). UV radiation treatments can improve yeast isolates thermo tolerance (Sridhar *et al.* 2002; Chandel *et al.* 2007). Depending on the microbial kinetics and lignocellulosic feedstock type, batch, fed batch, or continuous fermentation process can be selected as the most suitable process.

2.4.1 Batch Fermentation

Batch fermentation refers to a closed system in which substrates and other required materials are loaded onto the bioreactor, sterilized before the process starts up, fermenting microbes are added, and then the product is removed at the end. Only pH control solutions and gas exchange can be added and removed during the process. Conventionally, batch-based fermentation has been used to produce biethanol. Nearly the entire fermentation industry at present uses the batch mode.

Microorganisms work in initial high substrate concentration, and high product concentration is received at the end of a batch operation (Olsson and Han-Hagerdal 1996; Chandel *et al*. 2007). It is a multi-vessel process that allows easy control and flexible operation over the process. Batch fermentation is characterized by intensive labour and low productivity (Shama 1988; Chandel *et al*. 2007). Elaborate preparatory procedures are required for batch operation, and high labour costs are needed because of the interrupted start up and shut down reactor operations. This inherent disadvantage along with low productivity has led many commercial operators to consider the fermentation methods other than batch mode.

2.4.2 Fed Batch or Semi-Batch Fermentation

Fed batch, also knowns as semi-batch fermentation, is an operational technique in which one or more nutrients (substrates) are supplied to the reactor, and the product remains present in the reactor until the end of the run during cultivation. Here, microbes work at low substrate concentration with an increasing alcohol concentration during the course of fermentation. For the microbial metabolites production, fed batch cultures provide better productivity and yield than batch cultures. It has therefore replaced some batch operations for practical reasons (Schugerl 1987; Chandel *et al.* 2007). The toxic effect of furfural, 5-hydroxymethyl furfural and phenolics (fermentation inhibitors) to the yeast can be reduced by keeping the substrate feed rate low if high concentrations of these substance are present in the substrate solution. In fed batch mode, complete fermentation of an acid hydrolysate of spruce (lignocellulosic biomass) has been achieved without any detoxification, which was strongly inhibited in batch mode (Taherzadeh 1999; Chandel *et al.* 2007). Fed batch fermentation productivity is limited by the feed rate which is limited by the concentration of cell mass. Specific ethanol productivity is decreased with an increase in cell mass concentration (Lee and Chang 1987; Palmqvist *et al.* 1996; Chandel *et al.* 2007). To obtain maximum ethanol yield and productivity, cell density should be kept at an ideal level.

2.4.3 Continuous Fermentation

In continuous fermentation or chemostat (i.e. the chemical environment is static), to keep culture volume constant, fresh medium is added continuously while the product, with its fermenting organisms and culture liquid (which contains left over nutrients), is removed continuously. Single or series stirred tank reactors and plug flow reactors can be used for continuous fermentation. The highest productivity is achieved at low dilution rates and often gives a higher productivity than batch fermentation. In steady state continuous culture of *Candida shehatae*, the effect of temperature shift and aeration was studied for ethanol effect determination on xylose metabolism (Alexender *et al.* 1989; Chandel *et al.* 2007). Ethanol accumulation caused a delayed inhibitory effect on the specific substrate utilization rate. Continuous mode of fermentation is less labour intensive and offers ease of control compared to batch operation, although contamination is major problem in this operation. As the process is interrupted, equipment must be cleaned, and the operation must be started again with new inoculum growth. Continuous operation eliminates most of the unproductive time which is associated with cleaning and recharging of the reactor, sterilization, and adjustment of media. High cell density culture in the continuous mode of fermentation is locked in the exponential phase of microbial growth, resulting in high ethanol productivity and shortening the processing time to 4–6 h compared to the traditional batch fermentation mode (24–60 h). This results in substantial labour savings and minimizes investment costs by achieving the required production level with a significantly smaller plant.

2.4.4 Fermentation Using Immobilized Cells

A major limitation of continuous fermentation culture is the difficulty in maintaining high cell concentration in the reactor. Immobilized cell use overcomes this difficulty. Cell immobilization is done by adhering the cells to a covalent or electrostatic surface, then entrapping these cells in polymeric matrices or membranes; retention has been reported successful for ethanol production from hexose sugars (Godia *et al*. 1987; Chandel *et al*. 2007). Immobilized cell application is a remarkable advancement in bioethanol production technology. These cells offer accelerated fermentation rates with high alcohol productivity. Direct intact cell immobilization helps in retaining it during broth transfer into the collecting vessel during continuous fermentation. Additionally, intracellular enzyme activity loss can also be minimized by avoiding cell removal from downstream products (Najafpour 1990; Chandel *et al*. 2007). Fermentation microbial cell immobilization has been developed to remove high substrate and product inhibition in order to enhance ethanol yield and productivity. It is observed that sugar consumption rate by immobilized *Candida shehatae* cells was slightly less than the free cells of yeast which have led to higher ethanol production (Abbi *et al*. 1996). When microbes are attached to solid supports, the viscosity of media is lower, which results in better mixing and mass transfer in the reactor. The study of ethanol production in an immobilized cell reactor has shown that it doubled when using *Zymomonas mobilis* culture. 46.7 g/l ethanol was produced from 150 g/l of liquefied cassava starch using co-immobilized *Saccharomyces diastaticus* and *Zymomonas mobilis* cells (Amutha and Gunasekaran 2001). Recombinant *Zymomonas mobilis* cells were immobilized successfully resulting in high sugar concentration (12–15%). The significant role of increased biomass concentration was also observed for increased ethanol production (Yamada *et al.* 2002). Osmotolerant *Saccharomyces cereviseae* immobilized cells were used for ethanol production in a repeated batch fermentation system resulting in an ethanol concentration of 93 g/l using 200 g/l of glucose (Sree *et al.* 2000; Chandel *et al.* 2007). A 42.8 g/l/h ethanol production rate has been reported from pineapple canary biomass derived sugar fermentation by *Saccharomyces cereviseae* ATCC 24553 (Nigam 2000).

2.4.5 Fermentation Using Process Stream Recycling

Fresh water use, wastewater amount, and energy consumption must be at a minimum level in an environmentally sustainable process. Water consumption can be decreased by process streams recirculation for use in the hydrolysis step and washing (Palmqvist and Hahn- Hagerdal 2000; Chandel *et al.* 2007). The dilute ethanol stream (i.e., the recirculating part of the reactor) can improve the feed ethanol concentration to the distillation stage. One disadvantage in using recirculation streams is the accumulation of non-volatile inhibitory compounds which is shown by computer simulations (Galbe and Zacchi 1992; Palmqvist *et al.* 1996; Chandel *et al.* 2007). Cell recycling has been employed to increase ethanol productivity and retain batch mode simplicity by several researchers (Fein *et al.* 1984; Maleszka *et al.* 1981; Chandel *et al.* 2007). Cell recycling results in the reduction of fermentation time by 60–70%, but does not increase sugar consumption or ethanol productivity. Reduction in ethanol production has been observed after the 3rd cell cycle due to oxygen and sugar limitations as a result of cell density increases (Schneider 1989).

2.5 Conclusion and Preferred Technology Route

Although there are bioethanol production success stories at laboratory scale, the production of fuel ethanol at the industrial scale still remains a challenge. A positive solution to this problem will bring economic advantages for the fuel and power industries as well as benefit environmental rehabilitation problems and balance issues. Bioethanol policy implementation can be helpful in environmental improvement and rural economic development through employing sustainable agricultural practices.

Iogen Corporation in Canada is the only company worldwide which produces bioethanol at commercial scale using lignocellulosic feedstocks (wheat straw and corn stover) while in India, there are no commercial ethanol production plants that employ lignocellulosic biomasses, despite its plentiful availability. Some key factors that will make bioethanol production successful at commercial scale include: a. advancement in pretreatment technology by acid catalyzed hydrolysis of hemicelluloses; b. emphasizing various integrated approaches in the form of consolidated bioprocessing; c. application of novel mixtures of enzymes for cellulose breakdown; and, d. genetically engineered microorganisms that can ferment all available sugars in biomass to ethanol resulting in high yield and productivity. Enhancing biomass feedstock-conscious usage will also be helpful for the bioethanol industry. Coordinated actions for improvement in bioethanol production from lignocellulosic biomass are shown in Figure 2.5.

Even after many years of research and development of near commercial demonstration, currently there is not any clear technical or commercial advantage

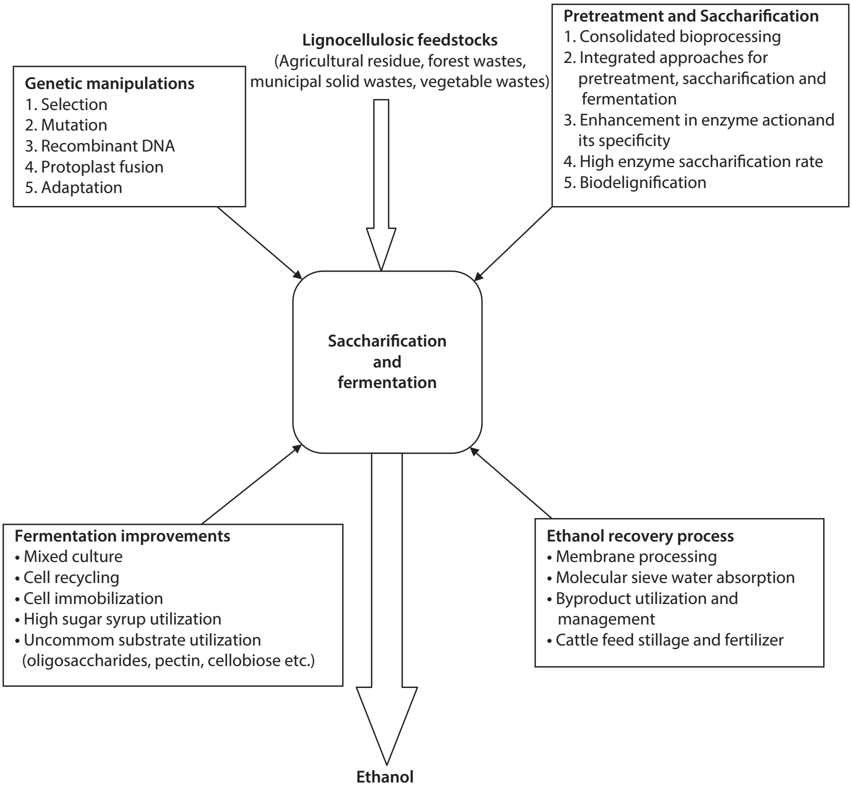
Lignocellulosic feedstocks
(Agricultural residue, forest wastes, municipal solid wastes, vegetable wastes)
Genetic manipulations
1. Selection
2. Mutation
3. Recombinant DNA
4. Protoplast fusion
5. Adaptation
Pretreatment and Saccharification
1. Consolidated bioprocessing
2. Integrated approaches for pretreatment, saccharification and fermentation
3. Enhancement in enzyme actionand its specificity
4. High enzyme saccharification rate
5. Biodelignification
Saccharification and fermentation
Fermentation improvements
• Mixed culture
• Cell recycling
• Cell immobilization
• High sugar syrup utilization
• Uncommom substrate utilization (oligosaccharides, pectin, cellobiose etc.)
Ethanol recovery process
• Membrane processing
• Molecular sieve water absorption
• Byproduct utilization and management
• Cattle feed stillage and fertilizer
Ethanol

Figure 2.5 Coordinated actions for improvement in bioethanol production from lignocellulosic biomass.

between the thermochemical and biochemical pathways. Both routes of technologies remain unproven at the industrial scale. Several significant technical and environmental barriers are yet to be overcome; however, process is under continuous development and evaluation. For the biochemical route technology, several factors need to be improved, including: a. feedstock characteristics; b. cost reduction by pretreatment technology perfection; c. improving the enzyme efficacy; d. lowering enzyme production costs; and, e. improvement in overall process integration (Tao and Aden 2009; Vohra *et al*. 2014). As some cost reduction strategies have proven successful to date, the advantage of the biochemical route is that it could provide cheaper biofuels than the thermochemical route. However, much of the technology is already proven for thermochemical routes and therefore, has less technical hurdles (Foust *et al*. 2009; Vohra *et al*. 2014). The major difference between the thermochemical and biochemical route is that, for the thermochemical route, the lignin component can be used for heat and power generation as it is a residue of the enzymatic hydrolysis process, whereas in the thermochemical process, the whole biomass is converted into synthesis gas [64, 65]. The second difference is that only ethanol is produced via the biochemical pathway whereas the thermochemical path results in various longer chain hydrocarbons along with alcohol from the syngas. These biofuels can be utilized for aviation and marine purposes also. The net environmental impact is the most important factor when selecting bioethanol production technology from lignocellulosic biomass among all other factors.

The life cycle assessment (LCA) is the most commonly used method for assessing the environmental profile of engineered products or conducting processes evaluation. The LCA follows a holistic approach and provides a thorough view of the environmental impacts over the complete life cycle of a product or process, hence, it presents the most specific and detailed scenario of real environmental trade-offs (Mu *et al*. 2010; Vohra *et al*. 2014). Environmental impacts that are associated with process inputs for the thermochemical route can be ignored, while in the case of biochemical conversion, lime, sulfuric acid, and other nutrients consumed contribute to fossil fuel, water consumption, and emission of green house gases (GHG) (Mu *et al*. 2010). In other words, the biochemical conversion pathway has shown better performance regarding the emission of green house gases and fossil fuel consumption, mainly due to credits from electricity exported. It is concluded from the study that the overall economics of both processes are very similar. When energy credits for all of the coproducts are taken into account, both processes are similar on an energy efficiency basis. Emission profiles are also very similar for both the conversion routes (Foust *et al*. 2009; Vohra *et al*. 2014). Therefore, only time will tell which conversion technology for ethanol production will be preferred.

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3

Immobilized Enzyme Technology for Biodiesel Production

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Abstract

Due to their short reaction time and stability, chemical catalysis is currently the primary vehicle for biodiesel production. Production of biodiesel using immobilized enzymes, however, offers many advantages over chemically catalyzed biodiesel production such as being less energy intensive and more selective, having reduced waste and by-products, operating under milder operating conditions (temperature and pH), and having fewer complications with enzyme and product recovery. This chapter represents a review of the current state-of-the-art methods with respect to biodiesel production using immobilized enzyme technology. Results of a comprehensive literature review are presented and discussed in relation to the use of immobilized enzymes for biodiesel production. Different enzyme immobilization methods applied to biodiesel production are reviewed. Kinetics of enzymatic transesterification reactions are described and various configurations of reactors employed for biodiesel production are outlined. In addition, the practical problems and challenges associated with the use of immobilized enzymes are discussed from an engineering perspective.

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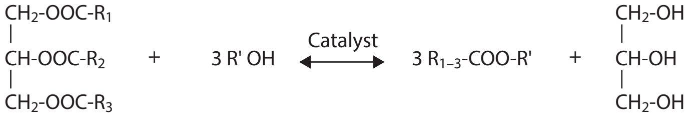
3.1 Introduction

Biodiesel is an environmentally-friendly fuel that emits low exhaust emissions and can be used either on its own or blended with petroleum diesel for use in unmodified engines (Tan *et al.*, 2010). It is a mixture of fatty acid alkyl esters that can be produced via the transesterification of triglycerides. Transesterification is a catalytic reaction that converts triglycerides into fatty acid alkyl esters and glycerol in the presence of an alcohol as shown in Figure 3.1. Since biodiesel can be produced entirely from vegetable oils or animal fats, it is both renewable and biodegradable (Vasudevan and Briggs, 2008), and it is considered an alternative energy source (Jaeger and Eggert, 2002). The majority of biodiesel is produced using alkaline catalysts (sodium or potassium hydroxide) due to the short reaction time required, but this process is energy intensive; glycerol recovery is difficult; removal of the catalyst from the product is complicated; wastewater is produced and must be treated; and, free fatty acids and water inhibit the reaction (Fukuda *et al.*, 2001; Meher *et al.*, 2006; Ranganathan



$$\begin{array}{l} CH_2\text{-}OOC\text{-}R_1 \\ | \\ CH\text{-}OOC\text{-}R_2 \\ | \\ CH_2\text{-}OOC\text{-}R_3 \end{array} + 3\ R'OH \xleftrightarrow{\text{Catalyst}} 3\ R_{1-3}\text{-}COO\text{-}R' + \begin{array}{l} CH_2\text{-}OH \\ | \\ CH\text{-}OH \\ | \\ CH_2\text{-}OH \end{array}$$

Figure 3.1 Transesterification of a triglyceride with an alcohol to produce fatty acid alkyl esters and glycerol.

et al., 2008; Vasudevan and Briggs, 2008). Acid catalysts (sulfuric, hydrochloric, or sulfonic acid) are also used to produce biodiesel, but these reactions typically have low reaction rates; the acidic environment is a challenge for reactor design; and high alcohol to oil ratios are necessary (Vasudevan and Briggs, 2008).

Therefore, alternative catalysts that overcome the disadvantages of acidic and alkaline catalysts are desirable. Transesterification can also be catalyzed by the enzyme lipase, so there is significant interest in enzymatic production of biodiesel. Some of the benefits of enzymatic biodiesel production over chemically catalyzed biodiesel production are that it is less energy intensive and more efficient; it produces less waste and by-products; it involves milder operating conditions (temperature and pH); enzyme and product recovery are less complicated; and, there is no soap (fatty acid salt) formation (Akoh *et al*., 2007; Fjerbaek *et al*., 2009; Fukuda *et al*., 2001; Marchetti *et al*., 2007; Vasudevan and Briggs, 2008).

The challenges of lipase catalyzed biodiesel production include the potential for product and substrate inactivation of the enzyme and the high cost of enzymes (Akoh *et al*., 2007; Fukuda *et al*., 2001; Ganesan *et al*., 2009; Marchetti *et al*., 2007; Robles-Medina *et al*., 2009; Vasudevan and Briggs, 2008). There is currently considerable interest in immobilizing lipase for biodiesel production because immobilization improves the biocatalyst thermal and chemical stability, and facilitates recovery and reuse of the enzyme which in turn decreases the costs associated with the enzyme (Akoh *et al*., 2007; Bajaj *et al*., 2010; Fukuda *et al*., 2001; Jaeger and Eggert, 2002; Macario *et al*., 2009; Vasudevan and Briggs, 2008). Biodiesel production using immobilized lipase is known to be less costly than that using free lipase, and the costs associated with enzymatic biodiesel production are expected to be competitive with those of alkaline biodiesel production if the immobilized lipase is recovered and reused more than five times or the cost associated with the enzyme is reduced significantly (Jegannathan *et al*., 2011).

3.2 Production of Biodiesel

Biodiesel production is a very active area of research with numerous recent reviews (Abbaszaadeh *et al*., 2012; Adewale *et al*., 2015; Aransiola *et al*., 2014; Atadashi *et al*., 2013; Bhuiya *et al*., 2016; Kuss *et al*., 2015; Lourinhoand Brito, 2014; Ruhul *et al*., 2015;Soltani *et al*., 2015). Oils from different feedstocks have been examined as raw material for biodiesel production (Canakci *et al*., 2008). Edible and non-edible vegetable oils (Patil *et al*., 2009), animal fats (Encinar *et al*., 2011), and lipids harvested from cultivated microalgae (Mata *et al*., 2010; Ebrahimian *et al*., 2014) are amongst these materials. Attempts to produce biodiesel from frying oil waste has been successful and are well-established (Atapour *et al*., 2014; Felizardo *et al*., 2006).

Biodiesel production can be implemented using various catalysts that can be categorized as acid, alkaline, heterogeneous or enzymatic (Gog *et al*., 2012). The production of biodiesel using lipase has advantages over chemically (acid/base) catalyzed processes because it is less energy intensive, more efficient, and highly selective; in addition, enzymatic biodiesel production involves mild operating conditions, has little downstream processing, produces less waste and by-products, and does not involve soap formation (Akoh *et al.*, 2007; Marchetti *et al.*, 2007; Vasudevan and Briggs, 2008). However, some challenges associated with enzymatic biodiesel production are the potential for enzyme inhibition by the alcohol substrate, inhibition by the glycerol product, and the high cost of enzymes (Akoh *et al.*, 2007; Fukuda *et al.*, 2001; Vasudevan and Briggs, 2008). Immobilized enzymes are undergoing significant research for biodiesel production and have the potential to be competitive with chemical catalysts for commercial biodiesel production (Zhang *et al.*, 2012).

3.3 Immobilized Lipase for Biodiesel Production

Lipase (E.C. 3.1.1.3), the enzyme used for transesterification reactions, is a triacylglycerol hydrolase which catalyzes the hydrolysis of ester bonds of triglycerides at lipid/water interfaces. At hydrophobic interfaces, lipase undergoes a conformational change called interfacial activation. Prior to contact with a hydrophobic interface, the enzyme has limited catalytic activity; at hydrophobic interfaces, the movement of an α-helical loop (or lid) uncovers the enzyme's active site, and the enzymatic activity is dramatically increased (Sarda and Desnuelle, 1958). Due to the interfacial activation phenomenon exhibited by lipases, it is crucial to maintain a hydrophobic interface during lipase-catalyzed reactions. The presence of water during enzymatic transesterification and interesterification reactions is necessary due to the formation of a liquid-liquid interface involving water and the oily substrate where the enzymatic reaction occurs. The addition of a small amount of a water-miscible solvent, such as ethanol or methanol, may also provide the interface necessary for the reaction in addition to acting as a reaction substrate. However, as the concentration of the water/solvent is increased sufficiently, there is an inhibitory effect on the enzymatic activity since water hinders the interaction between the enzyme and substrate (Al-Zuhair, 2005; Fukuda *et al.*, 2001; Hsu *et al.*, 2001a; Robles-Medina *et al.*, 2009; Samukawa *et al.*, 2000; Shimada *et al.*, 2002; Watanabe *et al.*, 2001). Therefore, the activity of lipase is strongly influenced by the nature of the interface, the interfacial properties, and the interfacial area (Akoh *et al.*, 2007).

Lipase can be used in the form of free enzyme, immobilized enzyme, or whole cells. In the former two cases, lipase is produced through fermentation and recovered and purified prior to utilization. Different microorganisms such as *Rhizopus oryzae*, *Candida antarctica*, *Mucor miehei*, and *Pseudomonas cepacia* have been employed for lipase production. Lipase in soluble form as a free enzyme has the advantage of low cost due to a relatively simple preparation procedure. However, free enzyme can be used only once because of inactivation

(Al-Zuhair *et al.*, 2003; Ranganathan *et al.*, 2008). Using whole cells that synthesize lipase intracellularly as biocatalysts can be employed resulting in lower cost. Filamentous fungi such as *Aspergillus* sp. and *Rhizopus* sp. have been widely studied (Ghaderinezhad *et al.*, 2014; Torres *et al.*, 2003; Xu *et al.*, 2002). However, issues with mass transfer of the substrates and products has limited further advances in the application of whole cells. In contrast, there is currently considerable interest in immobilizing lipase for biodiesel production because immobilization improves the biocatalyst's thermal and chemical stability and facilitates recovery and reuse of the enzyme, which in turn decreases the costs associated with the enzyme (Akoh *et al.*, 2007; Bajaj *et al.*, 2010; Fukuda *et al.*, 2001; Jaeger and Eggert, 2002; Macario *et al.*, 2009; Vasudevan and Briggs, 2008).

3.3.1 Enzyme Selection

One of the challenges that has hampered the development of enzymatic biodiesel production at the industrial scale is the high cost of lipase (Jaeger and Eggert, 2002). To avoid cost constraints, the activity of the lipase must be both enhanced and prolonged, and the lipase must be tolerant to the desired solvents and substrates (Fukuda *et al.*, 2001). To address these concerns, several studies have screened lipases based on their ability to produce biodiesel, and then immobilized the lipase in or on support materials so that it can be more easily reused. Table 3.1 presents a summary of various lipase screening studies. Comparing these

Table 3.1 Some lipase screening studies for enzymatic biodiesel production.

Lipases considered	**Results**	**Reference**
Rhizopus delemar, *Aspergillus niger*, *Fusarium heterosporum*, Novozym® 435 and Lipozyme® IM 6	Novozym® 435 was the most effective lipase for methanolysis.	(Shimada *et al.*, 1999)
Candida rugosa, *Penicillium camembertii*, *Penicillium roqueforti*, *Pseudomonas cepacia*, *Pseudomonas fluorescens*, *Candida lypolytica* and *Klebsiella oxytoca*	*Candida rugosa*, *Pseudomonas fluorescens* and *Pseudomonas cepacia* had high catalytic activity. *Pseudomonas cepacia* was the most methanol resistant and was less dependent on the amount of water present.	(Kaieda *et al.*, 2001)
Pseudomonas fluorescens, *Pseudomonas cepacia*, *Mucor javanicus*, *Candida rugosa* and *Rhizopus niveus* (free and immobilized)	*Pseudomonas fluorescens* had the highest activity. Immobilized enzymes had improved activity over free enzymes.	(Iso *et al.*, 2001)
Free: *Rhizopus oryzae*, *Candida rugosa*, *Penicillium camembertii*, *Pseudomonas cepacia* and *Pseudomonas fluorescens*. Immobilized: Lipozyme® RM IM, Lipozyme® TL IM, *Candida antarctica* A and B and *Rhizomucor miehei*	The lipase with the highest conversion was *Pseudomonas fluorescens*. Lipozyme® RM IM also achieved high conversion.	(Soumanou and Bornscheuer, 2003a)

Chromobacterium viscosum (free and immobilized), *Candida rugosa* and porcine pancreatic lipase	*Chromobacterium viscosum* was the only lipase to have appreciable biodiesel yield. Immobilizing *Chromobacterium viscosum* increased the yield by 10%.	(Shah *et al.*, 2004)
Novozym® 435 and Lipozyme® IM 60	Novozym® 435 was most active.	(Lai *et al.*, 2005)
Novozym® 435, Lipozyme® RM IM, PS-C and PS-D	PS-D (immobilized on diatomaceous earth) was the most active for biodiesel production.	(Salis *et al.*, 2005)
Thermomyces lanuginosus, *Pseudomonas fluorescens*, *Burkholderia cepacia*, *Penicillium camembertii*, and porcine pancreatic lipase	*Pseudomonas fluorescens* had the highest activity.	(Moreira *et al.*, 2007)
Lipozyme® TL IM and Novozym® 435	Novozym® 435 was the most robust catalyst.	(Hernández-Martín and Otero, 2008)
Novozym® 435 and Lipozyme® TM IM	Novozym® 435 achieved the highest conversions, but a mixture of 60% Novozym® 435 and 40% Lipozyme® TM IM was optimal.	(Huang *et al.*, 2010)

(*Continued*)

Table 3.1 Cont.

Lipases considered	**Results**	**Reference**
Candida antarctica, *Pseudomonas cepacia*, and *T. lanugunosus* (immobilized)	*Pseudomonas cepacia* and *T. lanugunosus* had the highest reaction rates in alkane solvents	(Gagnon and Vasudevan, 2011)
Novozym® 435, Lipozyme TL-IM and Lipozyme RM-IM	A mixture of 1/3 Novozym® 435 and 2/3 Lipozyme RM-IM (by weight) had the optimal performance.	(Yücel and Demir, 2012)
Pseudomonas aeruginosa 42A2 (free and immobilized)	Cold-adapted lipase from *Pseudomonas* sp. and its thermostable variant cloned in another *Pseudomonas* sp. and tested for biodiesel production.	(Cesarini *at al.*, 2014)
Candida rugosa	Recombinant *Candida rugose* lipase isozyme employed for biodiesel production.	(Kuo *et al.*, 2015)
Rhizopus oryzae and Novozym® 435	Synergic catalytic effect was observed when *Rhizopus oryzae* lipase and Novozym® 435 used together.	(Su *et al.*, 2015)

studies, the best lipase for biodiesel production is quite specific to the nature of the reaction being performed, depending on the alcohol and oil substrates used and the amount of water present in the system. The commercially immobilized lipases considered in these studies typically have high activities in comparison to free lipases. The demonstrated advantages of immobilized lipase include improved reusability and stability.

Many non-commercial immobilized lipases have also been considered. For example, a porous kaolinite immobilization medium improves lipase activity (Iso *et al.*, 2001), and cotton membranes are good immobilization supports (Nie *et al.*, 2006). Kumari *et al.* compared a variety of free lipases, commercially immobilized lipase on anion exchange resins, lipase immobilized on a microporous resin, cross-linked enzyme aggregates, and protein-coated microcrystals (Kumari *et al.*, 2007). From this study, it was observed that the protein-coated microcrystals had the highest percent conversion but involved a very complicated formation procedure.

Finally, several groups have considered sol-gel immobilized lipase. Hsu *et al.* achieved high conversions and considered process optimization with phyllosilicate clay sol-gels from cetyltrimethyl ammonium chloride and tetramethylorthosilicate. The immobilized lipase reacted more slowly, but it achieved higher conversion, and was more reusable, more thermally stable, and not affected by methanol inhibition, in comparison to the free lipase (Hsu *et al.*, 2001a; Hsu *et al.*, 2001b; Hsu *et al.*, 2003; Hsu *et al.,* 2004). Similarly, lipase immobilized in a TMOS (tetramethylorthosilicate) and iso-BTMS (iso-butyltrimethoxysilane) sol-gel had good methanol resistance, good reusability, and more activity than free lipase (Noureddini *et al.*, 2005). Orçaire *et al.* considered silica aerogels reinforced with silica quartz fibre felt and dried with supercritical carbon dioxide (Orçaire *et al.*, 2006). As in the other studies, sol-gel immobilized lipase exhibited improved reusability and higher activity than free lipase, but at high substrate concentrations, a severe diffusion limitation was noted due to the plugging of the aerogel pores (Orçaire *et al.*, 2006). Moreira *et al.* also used sol-gels to immobilize lipase – tetraethoxysilane (TEOS) and poly (vinyl alcohol) (PVA) sol-gels had high activity at elevated temperatures (50 °C) and high ethanol to oil molar ratios (18:1). The sol-gels were much more active than free lipase, and the viscosity of the biodiesel produced was comparable to that of commercial diesel (Moreira *et al.*, 2007). Meunier and Legge (2010) examined the application of diatomaceous earth (Celite®) as a support for lipase immobilized in sol-gels. Long-term stability and functionality (over 1.5 years) for the immobilized lipase in Celite® supported lipase sol–gels was confirmed. Over 90% conversion of oleic acid to methyl oleate was observed in a 6h reaction period (Meunier and Legge, 2012). Accumulation of water as a product of the transesterification reaction influenced the equilibrium and was observed to hinder the reaction rate. Celite® sol-gel supports, in addition to possessing a capacity for high enzyme loading, displayed the lowest water content compared to other enzyme supports studied including anion-exchange resin AG3-X4 and Quartzel® felt (Meunier and Legge, 2013).

3.3.2 Enzyme Immobilization Methods

A number of methods have been well-described for the immobilization of lipase that have been recently reviewed (DiCosimo *et al.*, 2013; Zhang *et al.*, 2012). These immobilization methods can be categorized into binding to a solid support, crosslinking, and entrapment. Binding is easy to perform at low cost. Lipase attachment to the carrier is weak and observed to have a limited adsorption capacity. Novozym® 435, the most commonly used commercial immobilized lipase, is produced by adsorption of lipase onto an acrylic resin (Franssen *et al.*, 2013). Crosslinking is a method that provides a strong interaction between the carrier and the lipase. Crosslinking reagents, such as glutaraldehyde, are used and promote the crosslinking of enzymes to each other. To avoid enzyme activity loss, crosslinking is accompanied by other immobilization methods such as adsorption (Hilal *et al.*, 2004). Entrapment is a technique in which the enzyme is captured in a polymeric network. Mass transfer limitations of the reactants is dominant compared to other methods of immobilization. A successful example of lipase immobilization by entrapment is the application of sol-gel matrices (Meunier and Legge, 2012).

3.3.3 Reaction Conditions

Along with screening for the best source and preparation of lipase for biodiesel production, considerable current research focuses on optimizing the reaction conditions, such as the substrate molar ratio, solvent presence and type, temperature, water content, substrate flow rate, and type of acyl acceptor, to achieve the highest percent conversion and reusability of the enzyme (Akoh *et al.*, 2007). Table 3.2 provides a review of the primary results gathered from some current studies on the optimization of reaction parameters for biodiesel production.

Based on these results, the best operating conditions are dependent on the type of lipase, alcohol, and oil used for the reaction. Immobilized lipase at approximately 40 °C with an oil to methanol molar ratio of 1:3 (the stoichiometric ratio) are consistently successful reaction conditions.

Chang *et al.* used a statistical approach to determine the optimal reaction parameters employing response surface methodology with a 5-level 5-factor central composite rotatable design (Chang *et al.*, 2005). The optimal reaction parameters for the reaction of canola oil with methanol using Novozym® 435 in a hexane reaction medium were 38 °C, 12.4 hour reaction time, 42.3 wt% enzyme, 1:3.5 oil to methanol ratio, and 7.2 wt% water. The predicted conversion was 99.4%, while the actual achieved conversion for this reaction was 97.9%.

The presence of glycerol in the reaction medium may cause enzyme inhibition (Samukawa *et al.*, 2000; Vasudevan and Briggs, 2008). Removal of glycerol by dialysis using an ultrafiltration flat sheet membrane for continuous methanolysis was very successful, and

Table 3.2 Enzymatic biodiesel production reaction optimization literature results.

Lipase	**Parameters**	**Results**	**Reference**
Rhizopus oryzae	Enzyme amount; water content	Reaction rate increased with increasing enzyme amount until 25 IU/mL. Without water, lipase was inactive and insufficient water caused irreversible inactivation.	(Kaieda *et al.*, 1999)
Porous kaolinite immobilized	Solvent; temperature; water content	Methanol and ethanol required solvent, but 1-propanol and 1-butanol did not. 1-propanol had the highest solvent-free activity. Optimal conditions: 50 °C and 0.3 wt% water.	(Iso *et al.*, 2001)
Phyllosilicate sol-gel immobilized	Alcohol; enzyme amount	Methanol, ethanol and n-butanol had the highest conversion, and the optimal enzyme amount was 150 mg/mL.	(Hsu *et al.*, 2001b)
Novozym® 435	Enzyme amount; substrate ratio; temperature; time	Optimal conditions using refined cotton seed oil and methanol were 30% enzyme, 1:4 oil:alcohol, 30 °C, and 7 h.	(Köse *et al.*, 2002)

(*Continued*)

Table 3.2 Cont.

Lipase	**Parameters**	**Results**	**Reference**
Phyllosilicate sol-gel immobilized	Temperature; solvent	Optimal reaction temperature: free lipase: 40 °C and immobilized: 40 °C – 70 °C. Hexane had higher yield than solvent-free.	(Hsu *et al.*, 2003)
Commercial free and immobilized	Temperature; solvent	Optimal temperature: 40 °C – 50 °C, hexane was a good solvent, and solvent-free reactions were successful.	(Soumanou and Bornscheuer, 2003a)
Commercial free and immobilized	Oil; alcohol	The highest conversion occurred with palm olein and methanol was the best alcohol.	(Soumanou and Bornscheuer, 2003b)
Commercial immobilized lipases	Substrate ratio; temperature; time	Optimal conditions: 40 °C, 10 h, and 1:12 oil:methyl acetate.	(Xu *et al.*, 2003)
Phyllosilicate sol-gel immobilized	Temperature; flow rates; time	Optimal conditions using a recirculating packed column reactor were 50 °C, 30 mL/min flow rate, and 48 h.	(Hsu *et al.*, 2004)
Lipozyme® TL IM	Temperature; enzyme amount; alcohol	Optimal conditions with three-step alcohol addition of 1 molar each: 40 °C, 10 wt% enzyme, and methanol.	(Xu *et al.*, 2004)

Sol-gel immobilized	Temperature; substrate ratio; water content; enzyme amount; alcohol	Optimal conditions: 35 °C, 1:15.2 oil:ethanol, 1:7.5 oil:methanol, 0.5 g water (methanol) or 0.3 g water (ethanol), and 475 mg lipase (methanol). Methanol reduced the reusability of the enzyme.	(Noureddini *et al.*, 2005)
Commercial immobilized	Temperature; water; substrate ratio; alcohol	Optimal conditions: 40 °C, 0.4 – 0.6 water activity, 1:3 and 1:6 oil:alcohol. Butanol was the most efficient alcohol.	(Salis *et al.*, 2005)
Lipozyme® RM IM	Temperature; enzyme amount; substrate ratio	Optimal conditions in a closed batch reactor: 67 °C, 4.5 wt% enzyme, and 1:2 palmitic acid:ethanol.	(Vieira *et al.*, 2006)
Sol-gel immobilized	Substrate ratio; temperature	Optimal conditions using palm oil and ethanol were 1:18 oil:alcohol and 58 °C.	(Moreira *et al.*, 2007)
Novozym® 435	Temperature; enzyme amount; substrate ratio; water addition; acyl acceptor	Optimal conditions: 45 °C, 3 wt% enzyme, 3:1 methanol:oil, and no water. Methyl acetate improved the lipase half-life while maintaining high yield.	(Ognjanovic *et al.*, 2009)

(*Continued*)

Table 3.2 Cont.

Lipase	**Parameters**	**Results**	**Reference**
Novozym® 435	Oil; substrate ratio; enzyme amount; temperature	Optimal conditions: 3.8:1methanol:oil, 100% waste frying oil, 15 wt% enzyme, and 44.5 °C.	(Azócar *et al.*, 2010)
Novozym® 435 and Lipozym® TM IM mixture	Enzyme amount; enzyme; solvent amount; substrate amount; time	Optimal conditions: 4 wt% enzyme:oil, 49% Novozym® 435, 55 vol% *tert*-butanol to oil, 5.12:1 methanol:oil, and 20 h.	(Huang *et al.*, 2010)
Lipoprime 50T	Solvent; temperature; substrate ratio; time	Methanolysis in *n*-hexane was slow while in *t*-butanol had high reaction rates. Optimal conditions: 40 °C, oil:alcohol = 1:6 – 1:8, and 1.5 h.	(Bendikiene *et al.*, 2011)
Immobead 150 immobilized	Solvent	Alkane solvents had high yields and rates. Isooctane and *n*-hexane were optimal.	(Gagnon and Vasudevan, 2011)
Calcium alginate immobilized *Rhizopus oryzae*	Alcohol; enzyme amount; time; temperature; substrate ratio	Optimal conditions: methanol, 30 °C, 1:3 (oil:alcohol), 24 h, and 10 wt% enzyme:oil. Pure enzyme had higher conversion than the whole cell immobilized biocatalyst.	(Balasubramaniam *et al.*, 2012)

Novozym® 435, Lipozyme TL-IM, and Lipozyme RM-IM	Time; temperature; enzyme amount; substrate ratio	Optimal conditions vary depending on lipase used: 3.9–6.2 h; 39.5–55.8 °C; 4.4–21.2% enzyme; 1.6:1–4.1:1 oil:alcohol.	(Yücel and Demir, 2012)
Candida antarctica lipase immobilized on styrene-divinylbenzene beads	Substrate ratio; enzyme amount; water amount	Optimal conditions: 5.6:1 methanol:oil; 25% enzyme; 5.44% of added water.	(Poppe *et* al., 2013)
Candida rugosa lipase covalently bound to magnetic polymer-coated microspheres	Bond lipase; water:oil ratio;	Optimal conditions: 50% bond lipase; 0.1% water:oil ratio.	(Xie *et al.*, 2014)
Pseudomonas fluorescens lipase	Enzyme amount; temperature; water content; methanol: oil ratio	Optimal conditions: 10% enzyme amount; 35 °C; 2.5% water content; 3:1methanol:oil.	(Guldhe *et al.*, 2015)

(*Continued*)

Table 3.2 Cont.

Lipase	Parameters	Results	Reference
Mixtures of three immobilized lipases including Novozym® 435 (CALB), Lipozyme TL-IM (TLL) and Lipozyme RM-IM (RML)	Amount of enzymes combined	Optimal conditions: For olive oil, 29.0% TLL, 12.5% RML, and 58.5% CALB; For palm oil, 52.5% TLL and 47.5% RML.	(Poppe *et al.*, 2015)

increasing the removal of glycerol improved the conversion of the reaction significantly – 20% conversion without glycerol compared to 10% conversion with 5.0 g glycerol in the reaction medium. Using a membrane separation approach, as opposed to the typical glycerol removal via settling, is much more practical for a continuous biodiesel production process (Bélafi-Bakó *et al.*, 2002).

Silica beds have also been used to adsorb glycerol in biodiesel streams (Mazzieri *et al.*, 2008; Samukawa *et al.*, 2000; Yori *et al.*, 2007). One complication of this approach is that one of the reaction substrates, methanol, causes the glycerol saturation capacity of the silica gel to be reduced (Mazzieri *et al.*, 2008) and glycerol to desorb from the silica gel (Yori *et al.*, 2007). Also, silica gel adsorbs methanol, which can also lead to a reduced biodiesel yield (Wang *et al.*, 2006).

To minimize lipase inhibition by methanol and ethanol substrates, several procedures have been considered including lipase pre-treatment, alternative acyl acceptors to replace methanol, and step-wise addition of methanol (Bélafi-Bakó *et al.*, 2002; Chen and Wu, 2003; Du *et al.*, 2004; Ruzich and Bassi, 2010a; Ruzich and Bassi, 2010b; Ruzich and Bassi, 2011; Samukawa *et al.*, 2000; Shimada *et al.*, 1999; Shimada *et al.*, 2002; Watanabe *et al.*, 2000; Watanabe *et al.*, 2001; Watanabe *et al.*, 2002; Xu *et al.*, 2003; Xu *et al.*, 2004).

Lipase pre-treatment by incubation with methyl oleate and soybean oil for twelve hours helped prevent deactivation of Novozym® 435 by methanol, increased the initial reaction rate, reduced the effect of water on the reaction rate, and helped prevent activity loss even after twenty uses (Samukawa *et al.*, 2000). Another method that helped reduce Novozym® 435 inhibition by methanol and ethanol was a pre-treatment by immersion with *tert*-butanol which increased the fatty acid methyl ester yield from 2.5% to 24.5%. In addition, periodic regeneration of the enzyme with a 2-butanol or *tert*-butanol wash allowed continuous use for seventy days while maintaining the conversion above 70%. The conclusion of this study was that the alcohol is adsorbed onto the immobilized enzyme support thereby blocking the oil substrate from reaching the reaction site and, consequently, preventing the reaction from progressing (Chen and Wu, 2003).

Another approach to preventing alcohol deactivation of lipase involves using methyl acetate as the acyl acceptor as opposed to methanol or ethanol. Du *et al.* (2004) showed that an oil to methyl acetate molar ratio of 1:12 can be used with Novozym® 435 without deactivating the enzyme for both crude and refined soybean oil, and there is no activity loss in a 0.5 L bioreactor after 100 cycles. In a similar study, methyl acetate as an acyl acceptor gave a higher methyl ester yield and minimal activity loss (Xu *et al.*, 2003).

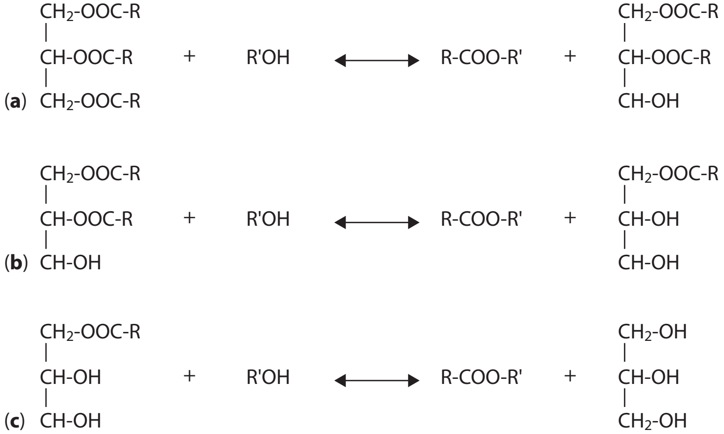
The most common approach for preventing methanol inactivation of lipase in biodiesel production is three-step methanolysis. In these studies, methanol deactivation was prevented by adding methanol in three steps of one mole of methanol per mole of oil each to achieve the stoichiometric ratio of three moles of methanol per mole of oil. In one study, continuous methanol addition had the best conversion of 97% (Bélafi-Bakó *et al.*, 2002). Several studies have shown that both three-step methanolysis with one mole ratio of methanol in each step (Shimada *et al.*, 1999; Watanabe *et al.*, 2000; Watanabe *et al.*, 2001; Watanabe *et al.*, 2002; Xu *et al.*, 2004) and a two-step process with one mole ratio of methanol in the first step and two mole ratios of methanol in the second step (Shimada *et al.*, 2002) both help to prevent methanol inhibition.

3.4 Reaction Kinetics

The kinetics of enzymatic biodiesel production have not been widely studied. Al-Zuhair considered the kinetics and developed a mathematical model based on the reaction mechanism with vegetable oil as a substrate. The model was compared to experimental results from an ion-exchange resin immobilized lipase and silica gel immobilized lipase with reasonable agreement with the initial reaction rate (Al-Zuhair, 2005). A ping-pong mechanism was used with Michaelis Menten kinetics, and, in contrast to many other studies, both the substrates (oil and alcohol) could be studied independently since an organic solvent was used to keep the bulk volume constant. The inhibition effects of the oil and alcohol were dependent on the immobilization support and as the oil concentration increased, the inhibition effect of the alcohol decreased (Al-Zuhair, 2005). A subsequent study using free lipase rather than immobilized lipase found that the model underestimated the inhibition effects of both substrates, and that the reaction was more inhibited by the alcohol than the oil (Al-Zuhair *et al.*, 2007). Comparing solvent-free and *n*-hexane-based reaction media with lipase immobilized on ceramic beads using ping-pong bi-bi kinetics and competitive inhibition by both substrates found that a higher yield could be achieved without solvent, and that the rate determining step was the surface reaction rather than mass transfer (Al-Zuhair *et al.*, 2009).

Based on a kinetics study of biodiesel production with methyl acetate as the acyl acceptor and immobilized lipase Novozym® 435, three consecutive second-order reversible reactions describe the interesterification of triglycerides and methyl acetate, and a kinetic model with a ping-pong bi-bi mechanism with substrate competitive inhibition was developed (Xu *et al.*, 2005). The three reactions are: triglycerides to diglycerides, diglycerides to monoglycerides, and monoglycerides to triacetylglycerol. From the kinetic constants, the first reaction step — triglycerides to diglycerides— was the rate limiting step for the overall interesterification reaction (Xu *et al.*, 2005). Similarly, three reversible reactions could be elucidated for the transesterification of triglycerides to fatty acid alkyl esters and glycerol using an alcohol rather than methyl acetate (Figure 3.2).

In-depth studies of the kinetic mechanism of enzyme catalyzed reactions by Cleland are commonly used as starting points for the kinetic studies described in the literature, including a series of ping-pong bi-bi mechanisms with substrate and product inhibition



$$\text{(a)}\quad \begin{array}{l} CH_2\text{-OOC-R} \\ | \\ CH\text{-OOC-R} \\ | \\ CH_2\text{-OOC-R} \end{array} + R'OH \longleftrightarrow R\text{-COO-R'} + \begin{array}{l} CH_2\text{-OOC-R} \\ | \\ CH\text{-OOC-R} \\ | \\ CH\text{-OH} \end{array}$$

$$\text{(b)}\quad \begin{array}{l} CH_2\text{-OOC-R} \\ | \\ CH\text{-OOC-R} \\ | \\ CH\text{-OH} \end{array} + R'OH \longleftrightarrow R\text{-COO-R'} + \begin{array}{l} CH_2\text{-OOC-R} \\ | \\ CH\text{-OH} \\ | \\ CH\text{-OH} \end{array}$$

$$\text{(c)}\quad \begin{array}{l} CH_2\text{-OOC-R} \\ | \\ CH\text{-OH} \\ | \\ CH\text{-OH} \end{array} + R'OH \longleftrightarrow R\text{-COO-R'} + \begin{array}{l} CH_2\text{-OH} \\ | \\ CH\text{-OH} \\ | \\ CH_2\text{-OH} \end{array}$$

Figure 3.2 Intermediate reactions for the transesterification of triglycerides with alcohol to produce fatty acid alkyl esters and glycerol: (a) triglycerides to diglycerides, (b) diglycerides to monoglycerides, and (c) monoglycerides to glycerol.

(Cleland, 1963a; Cleland, 1963b; Cleland, 1963c). Lipase catalyzed transesterification has been successfully described by a ping-pong bi-bi mechanism (Al-Zuhair 2005; Al-Zuhair *et al.*, 2007; Al-Zuhair *et al.*, 2009; Cheirsilp *et al.*, 2008; Dossat *et al.*, 2002; Xu *et al.*, 2005). In such a kinetic mechanism, the first substrate (ester) binds to the enzyme, forms an enzyme intermediate and the first product (alcohol) is released before the second substrate (alcohol) can bind to the enzyme to form an intermediate and release the second product (ester) (Al-Zuhair *et al.*, 2007; Cheirsilp *et al.*, 2008; Rizzi *et al.*, 1992; Yadav and Devi 2004). Figure 3.3 shows this reaction mechanism when applied to the production of biodiesel via transesterification.

The mechanistic steps are as follows: the free enzyme (E) reacts with the triglyceride (T) to produce an enzyme-triglyceride complex (E.T) from which the

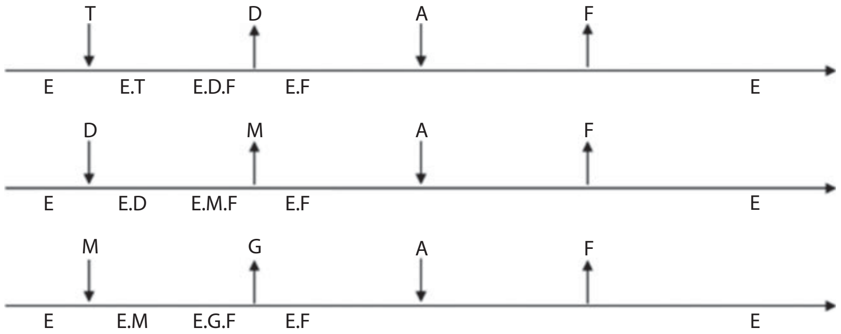


Figure 3.3 Schematic representation of the ping-pong bi-bi kinetic mechanism for the transesterification of triglycerides to produce biodiesel. Adapted from Cheirsilp *et al.* (2008).

second complex (E.D.F) releases the diglyceride (D). The third complex (E.F) reacts with the alcohol (A) and releases the fatty acid alkyl ester (F). Similar mechanisms exist for the diglyceride (D) to monoglyceride (M) reaction and the monoglyceride (M) to glycerol (G) reaction as shown in Figure 3.3. This is consistent with the results obtained using TLC analysis of the reaction intermediates by Kaieda *et al.*, supporting the notion that each ester bond of the triglyceride undergoes a two-step mechanism: first, the ester bond is hydrolyzed to produce partial glycerides and free fatty acids, followed by esterification of the free fatty acids with the alcohol to produce the fatty acid alkyl ester (Kaieda *et al.*, 1999).

The initial rate equation with inhibition of one substrate (Equation 3.1) is often used to model the kinetics of lipase-catalyzed transesterification for the production of biodiesel (Al-Zuhair, 2005; Dossat *et al.*, 2002; Xu *et al.*, 2005).

$$v_i = \frac{V_{maxF}[A][T]}{K_{mT}[A](1+[A]/K_{IA})+K_{mA}[T]+[A][T]} \tag{3.1}$$

where v_i is the initial reaction velocity, V_{maxF} is the maximum initial reaction velocity, $[A]$ is the alcohol concentration, $[T]$ is the triglyceride concentration, K_{mT} is the apparent Michaelis Menten constant for the triglyceride, K_{IA} is the inhibition constant for the alcohol, K_{mA} is the apparent Michaelis Menten constant for the alcohol.

Al-Zuhair *et al.* considered the initial inhibition effects of both substrates by involving a solvent so that the initial oil and alcohol concentrations could be independently varied and studied (Al-Zuhair *et al.*, 2007; Al-Zuhair *et al.*, 2009). The equation used for the kinetic modeling was similar to Equation 3.1 with the addition of a K_{IT} term to take into account the effect of triglyceride inhibition. These studies show comparable inhibition constants for the triglyceride and alcohol, which were quite large (approximately 3000–4500), indicating that the effect of inhibition was low (the inhibition constant represents the dissociation of the inhibitor from the enzyme-inhibitor complex so smaller numbers indicate higher inhibitory effects).

Alternatively, a complete rate equation (Equation 3.2) for a ping-pong bi-bi mechanism with inhibition of both substrates and both products has been developed and can be used as a more sophisticated model for the entire reaction rather than solely the initial conditions (Rizzi *et al.*, 1992; Yadav and Devi, 2004).

$$v = \frac{V_{maxR} V_{maxF} \left([A][B] - \frac{[P][Q]}{K_{eq}} \right)}{V_{maxR} R + V_{maxF} F} \tag{3.2}$$

with

$$\begin{gathered} R = [A][B] + K_{mB}[A]\left(1 + \frac{[A]}{K_{IA}}\right) + K_{mA}[B]\left(1 + \frac{[B]}{K_{IB}}\right) + \\ \frac{K_{mA}}{K_{IQ}}[A][Q] + \frac{K_{mA}}{K_{BP}}[P][B] + \frac{K_{mB}}{K_{AQ}}[A][Q] \end{gathered} \tag{3.3}$$

$$\begin{gathered} F = \frac{K_{mP}}{K_{eq}}[Q]\left(1 + \frac{[Q]}{K_{IQ}}\right) + \frac{K_{mQ}}{K_{eq}}[P]\left(1 + \frac{[P]}{K_{IP}}\right) + \\ \frac{1}{K_{eq}}[P][Q] + \frac{K_{mQ}}{K_{eq} K_{IA}}[A][P] \end{gathered} \tag{3.4}$$

$$\frac{1}{K_{BP}} = \frac{1}{K_{IP}} + \frac{K_B K_{IA}}{K_A K_{IP} K_{IB}} \tag{3.5}$$

$$\frac{1}{K_{AQ}} = \frac{1}{K_{IQ}} + \frac{K_A K_{IB}}{K_B K_{IQ} K_{IA}} \tag{3.6}$$

The reaction for this model is: A + B ↔ P + Q; v is the reaction velocity; V_{maxR} and V_{maxF} are the maximum velocities of the reverse and forward reactions; $[A]$, $[B]$, $[P]$, and $[Q]$ represent the concentrations of components A, B, P, and Q; K_{eq} is the equilibrium constant; K_{mA}, K_{mB}, K_{mP}, and K_{mQ} are the Michaelis constants for A, B, P, and Q; and K_{IA}, K_{IB}, K_{IP}, and K_{IQ} are the inhibition constants for A, B, P, and Q. Dependency of the parameters on temperature in the ping-pong mechanism has been studied and presented in terms of the Arrhenius expression for reaction temperatures in the range of 30 to 50 °C (Zarejoshaghani *et al.*, 2015).

A kinetic model for oleic acid conversion to methyl oleate was developed for immobilized lipase on Celite® with consideration of the inhibitory effects of both glycerol and methanol. According to the magnitude of the estimated kinetic parameters, methanol shows less of an inhibition effect than glycerol. The maximum velocity of the forward reaction was about 25% faster than that of the backward reaction (Meunier *et al.*, 2014).

3.5 Bioreactor Configurations

Some common immobilized biocatalyst continuous reactors are stirred tank reactors, fluidized bed reactors, and packed bed reactors (Hartmeier, 1988; Messing, 1975). Stirred reactors are simple and inexpensive, but are more common for aerobic fermentations than immobilized biocatalysis because the intensive stirring introduces unnecessary shear forces on the immobilized biocatalysts that could disrupt the enzyme carrier (Hartmeier, 1988). Fluidized bed reactors have beds that are loosely filled with catalyst particles, and the substrate is forced upwards through the bed. Although fluidized beds are advantageous for immobilized biocatalysts, the retention of biocatalyst particles is challenging if the viscosity of the substrate is high

(Hartmeier, 1988). A packed bed reactor is a column filled with biocatalyst particles that remain stationary. Reactants flow continuously through the column, and no separation of the biocatalyst from the reaction product is necessary. Packed bed reactors can accommodate the highest density of catalyst particles, and therefore, the highest possible substrate conversion is attainable (Hartmeier, 1988). In addition, packed bed reactors help minimize diffusion limitations of immobilized enzymes because the reactor ensures proper mixing between the immobilized catalyst and the reaction medium thereby improving external mass transfer (Messing, 1975). Packed bed reactors have the simplest reactor design to achieve a high degree of contact between the solid catalyst particles and the liquid substrates (Thoenes, 1994). Despite the ease of application of stirred tank reactors, by using a packed bed reactor, immobilized enzymes can easily be reused and continuous processing is feasible. However, one challenge of a packed bed reactor is based on the lifetime of the biocatalyst – it is impractical to shut down the reactor to change the catalyst particles frequently (Thoenes, 1994). Care must be taken to ensure the catalyst has a long lifetime within the reactor so it can run continuously with little maintenance.

Several packed bed reactor studies have been completed using the commercially immobilized Novozym® 435. Hama *et al.* developed a bench scale solvent-free packed bed reactor with a glycerol separating tank and achieved final fatty acid methyl ester contents above 96% using either 10 passes on a single reactor bed (Hama *et al.*, 2011a) or 550 h continuous production using five reactors in series (Hama *et al.*, 2011b). Similarly, Shimada *et al.* and Watanabe *et al.* developed a continuous, three-step, fixed bed reactor with Novozym® 435 that achieved 98% (Shimada *et al.*, 1999) and 96% (Watanabe *et al.*, 2000; Watanabe *et al.*, 2001) conversion with good enzyme reusability and without enzyme deactivation. Other packed bed reactor studies using Novozym® 435 also have promising results: 76% molar conversion with no activity loss after 7 days (Chang *et al.*, 2009); 83% conversion and 30 days continuous production without conversion decrease (Chen *et al.*, 2011); and 75.2% conversion using a *tert*-butanol co-solvent (Shaw *et al.*, 2008).

Considering alternative immobilized lipases, Hsu *et al.* (2004) successfully developed a recirculating packed column reactor with lipase immobilized on packages of commercial paper coffee filters. Nie *et al.* (2006) developed a continuous reactor that achieved 92% conversion by immobilizing lipase on a cotton membrane for use in a three-step methanolysis continuous fixed bed reactor with nine columns packed with immobilized lipase. Wang *et al.* (2011) developed a lipase-Fe_3O_4 immobilized on cotton for use in both a single bed reactor and four packed beds in series. A textile cloth immobilized lipase was used in a three-step packed bed reactor with a hexane solvent and gravity driven glycerol separation, achieving a 91% fatty acid methyl ester product (Chen *et al.*, 2009). After three hours in a single packed bed reactor using an immobilized lipase of mixed sources and stepwise methanol addition, 98% conversion was achieved dropping to 90% after 108 h due to glycerol accumulation in the reactor (Lee *et al.*, 2010).

A study using Lipozyme® IM-20 compared the performance of batch reactors and tubular reactors for biodiesel production. The tubular reactor required the addition of glass beads to increase the void space in the reactor, and had higher reactor rates, caused less stress on the lipase, and was more flexible in terms of recycle rates (Mukesh *et al.*, 1993).

Celite® supported sol-gel lipase was successfully used in a packed bed bioreactor for biodiesel production (Meunier *et al.*, 2015). When immobilized *Burkholderia cepacia* lipase was used in the packed bed, the biocatalyst showed stable enzymatic activity over a five-day period.

Despite the advantages of immobilized lipases compared to chemical catalysts, industrial realization of this technology for biodiesel production has not occurred appreciably. Two companies in China have established enzymatic biodiesel production using stirred tank bioreactors (Tan *et al.*, 2010). Hunan Rivers Bioengineering Co. (20,000 t per year) and Lvming and Environmental Protection Technology Co. Ltd. (10,000 t per year) using Novozym® 435 and lipase from a *Candida* sp., respectively (Tan *et al.*, 2010). The US company Blue Sun announced the operation of its large scale plant (30 million gallons per year) in 2014 (Scherer, 2014). It uses an enzyme that has been developed by Novozymes and applied in the BioFAME® process which includes enzyme reuse in stirred tank reactors (Nielsen, 2014).

3.6 Conclusions

Biodiesel production using immobilized lipase-catalyzed reactions has attracted considerable attention over the past decade. The enzyme-based approach offers the advantages of mild reaction conditions accompanied by simplified separation and reduction of waste. This production method has great potential for commercialization if the issues surrounding production cost can be resolved. Efforts to find enzymes with higher catalytic activity and stability are in progress. Various methods for lipase immobilization have been developed to provide biocarriers with durable/reusable functionality at low cost. Operational parameters such as loading on carriers, oil to alcohol ratio, water concentration, and temperature have been studied and their optima well-established at the laboratory scale. Transesterification kinetics for immobilized lipase have been studied and the inhibitory effects of alcohol or glycerol on the enzyme activity have been modeled. Bioreactors suitable for the production of biodiesel in batch and continuous modes have been examined, and stirred tank and packed bed reactors have exhibited the most promising results at the laboratory scale. Few industrial-scale facilities have been built based on enzymatic catalysis for biodiesel production. Based on the large amount of information and experimental data available, it is anticipated that enzymatic biodiesel production technology will continue to advance leading to more industrial-scale installations.

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4

Oleaginous Yeast- A Promising Candidatea for High Quality Biodiesel Production

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Abstract

Current research scenario has been shifted to biomass based biofuels due to increasing global energy crisis and greenhouse effects. Biodiesel is renewable and alternative to the petroleum diesel, well-defined as a blend of fatty acid alkyl esters. It is biologically decomposable, non-toxic, non-flammable, expedient and free from aromatic contents. Sustainability of biodiesel is major concerned regarding its availability and inherent properties that make it cleaner fuel for polluted cities. It is produced by a chemical reaction known as transesterification in which fatty acids regardless of its origin (oil derived from plants, animals, and waste cooking oil) react with methanol in the presence of suitable catalysts (homogeneous/heterogeneous). Using vegetable oils for biodiesel production is no more economical and sustainable due to direct competition with human food sources. Microbial oils are noble substitutes, which have similar fatty acid profiles with vegetable oils. Among various microorganisms, oleaginous yeast is considered as microscopic bio-factory for oil generation that can be used as feedstock for biodiesel production. The utilization of oleaginous yeast for biodiesel production has many advantages over other non-conventional renewable sources like higher lipid productivity in terms of g/l/day than the algae and plants, easier scale-up

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of upstream and downstream processing, less affected by the season and climatic variation to grow.

***Keywords*:** Oleaginous yeast, triacylglycerols (TAGs), fatty acid methyl esters, biodiesel, low-cost substrates

4.1 Introduction

The World is facing the problem of energy crisis and related environmental deterioration. India as being the second most populous country after China and fourth largest fossil fuel consumer after USA, China, and Japan, the problems associated with energy supply are more vital. It is crucial to maintaining our economical and sustainable growth with the utilization of domestic and renewable sources of energy so that import of oil from foreign can be reduced. Biomass-based biofuels are getting much attention now these days among other sources of renewable energy [1]. Biodiesel production from renewable sources such as vegetable oils, waste cooking oils, animal fats and neutral lipids (TAGs) derived from oleaginous microbes is gaining much attention. These oils are transesterified to form biodiesel which can be directly used in conventional diesel engines. A simple transesterification reaction requires a short chain alcohol (methanol or ethanol) and a catalyst (acid or base) to boost up the reaction (Figure 4.1). Amongst the renewable sources, microbial oils have several benefits over other sources including high lipid yield in terms of g/l/day, devoid of climatic and seasonal variations and easily scale up of upstream/downstream processes [2]. In addition, the lipid profile of oleaginous yeast resembles vegetable oils having a composition of oleic acid ($C_{18:1}$) > palmitic acid ($C_{16:0}$) > linolenic acid

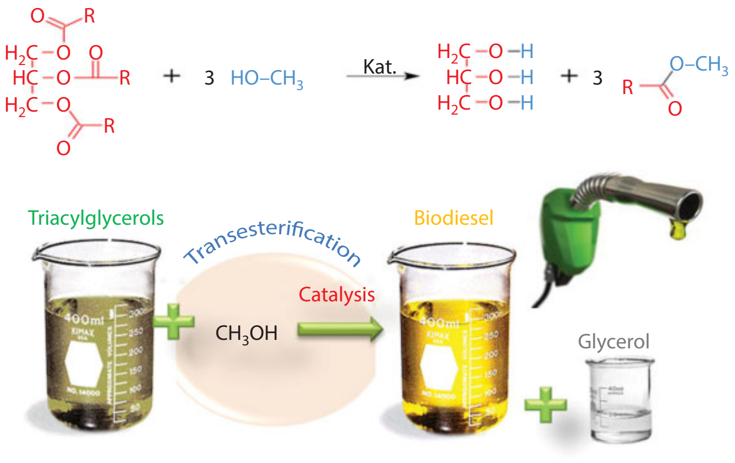
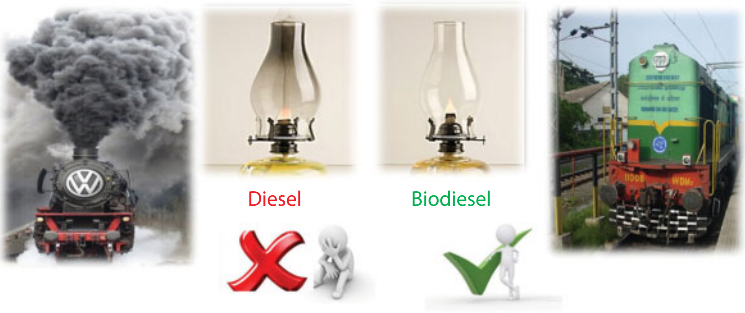


Figure 4.1 Conversion of triacylglycerol (TAGs) into fatty acid methyl esters (FAMEs) by transesterification reaction.

($C_{18:2}$) = stearic acid ($C_{18:0}$). Bacteria, yeasts, fungi and algae are currently being explored for their oils as they can utilize organic carbon for accumulating neutral lipids in their cellular compartment [5–8]. In this regard, oleaginous yeasts are suitable candidates as they can utilize a large number of low-cost renewable substrates for their growth and lipid accumulation. The growth period of oleaginous yeast is short as compared to algae and plants, taking only 6–7 days to achieve lipid accumulation phase [11–15]. *Rhodosporidium, Rhodotorula, Yarrowia, Cryptococcus, Candida, Lipomyces, and Trichosporon* are major oleaginous yeast genera that can accumulate >65% lipid of their dry cell weight (w/w). However, high cost associated with organic carbon sources required for the growth of oleaginous yeast is the major bottleneck for its commercialization. Exploration of non-edible lignocellulosic materials and industrial wastes as feedstock for oleaginous yeast can reduce the production cost thereby aiding its large scale production.

4.2 Advantages of Biodiesel as Vehicular Fuel [19, 21]

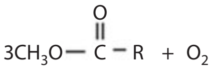
- Biodiesel is renewable, nontoxic, biodegradable, sustainable, non-flammable and eco-friendly.
- Biodiesel is free of sulfur, aromatic contents, and particulate matters, it reduces smoke due to free from soot that makes it an ideal fuel for polluted metro cities.
- Biodiesel has high combustion property due to carrying enough quantity of oxygen (10–11%). It can directly use in conventional diesel engines without any modification (Figure 4.2).
- Its usage increases engine efficiency due to having high lubricity, so no need to add extra lubricant for engines.
- Biodiesel exhaust has a less dangerous influence on human health than diesel fuel. Its emissions have reduced levels of hydrocarbons and nitrited compounds that have been known as latent cancer-causing compounds.
- Biodiesel helps to improve the economy of rural areas to generate employment.
- Biodiesel production didn't generate waste products to harm the environment. Glycerol is the main byproduct of biodiesel that may be utilized by cosmetic or pharmaceutical industries.
- Utilization of biodiesel helps diminish reliance on limited fossil fuel reserves.



Complete combustion of diesel

$$C_{12}H_{24} + 36O_2 \longrightarrow 12CO_2 + 12H_2O + \text{energy}$$

Complete combustion of biodiesel



$$3CH_3O-\overset{\overset{\displaystyle O}{\|}}{C}-R + O_2 \longrightarrow CO_2 + H_2O + \text{energy}$$

E. sters **R- the hydrocarbon chain length*

Incomplete combustion of fuel

$$\text{Fuel} + \text{low } O_2 \longrightarrow CO_2 + H_2O + \text{energy} + \text{soot} + CO$$

Figure 4.2 Demonstration of diesel (from fuel station) and biodiesel (from oleaginous yeast) combustion characteristics. Combustion includes a sequence of chemical reactions between a fuel and oxygen. The major combustion difference between diesel and biodiesel is the presence of oxygen molecule in biodiesel that helps in clean burning.

- It can be propelled, stored and burned just like petroleum diesel fuel, and can be used pure, or in blends with diesel fuel in any proportion.

4.3 Technical Aspects of Biodiesel Production Using Oleaginous Yeast [24] (Figure 4.3)

The first step towards sustainable biodiesel production from oleaginous yeast is the screening of the yeast species that can accumulate more than 20% (w/w) lipid in their

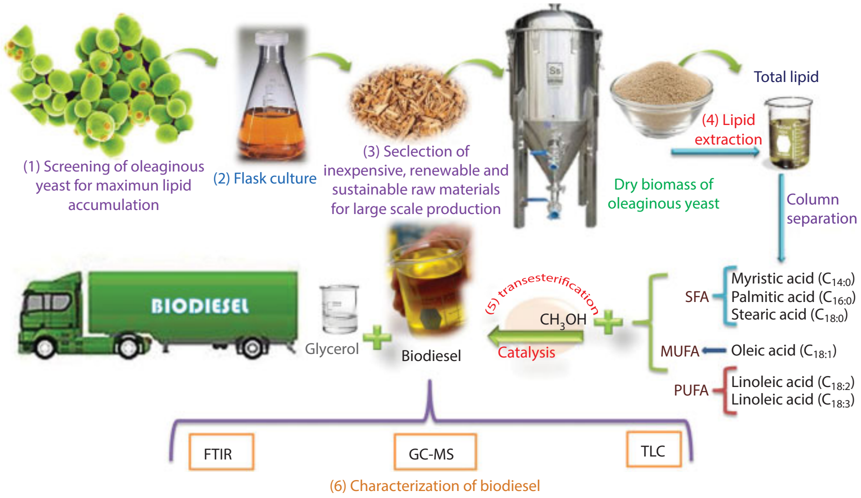

(1) Screening of oleaginous yeast for maximun lipid accumulation
(2) Flask culture
(3) Seclection of inexpensive, renewable and sustainable raw materials for large scale production
Dry biomass of oleaginous yeast
(4) Lipid extraction
Total lipid
Column separation
SFA
Myristic acid ($C_{14:0}$)
Palmitic acid ($C_{16:0}$)
Stearic acid ($C_{18:0}$)
MUFA
Oleic acid ($C_{18:1}$)
PUFA
Linoleic acid ($C_{18:2}$)
Linoleic acid ($C_{18:3}$)
(5) transesterification
CH_3OH
Catalysis
BIODIESEL
Glycerol
Biodiesel
FTIR
GC-MS
TLC
(6) Characterization of biodiesel

Figure 4.3 Large scale biodiesel productions from oleaginous yeast.

cellular compartment [28]. The yeasts are part of microbiota in all natural ecosystems including soils, freshwaters, marine waters, ocean surface and deep sea [29]. They can also colonize in more extreme environments such as low temperatures, low oxygen availabilities, and deep hypersaline oceanic waters. In order to identify high lipid accumulating oleaginous yeast among an extensive variety of samples obtained from diverse niches, there is compulsive need to develop simple, reliable and rapid screening methods [28–30]. Apart from high lipid yields, selected oleaginous yeast must be able to grow on a wide range of inexpensive substrates. Selection of suitable yeast candidates followed by designing of cost-effective feedstocks for their cultivation which includes optimization of physiological parameters such as media components, pH, aeration rate, temperature etc. After media optimization at lab scale, a large fermenter (500–1000 L) is required for pilot scale up. Consequently, after cultivation, the cells need to be harvested at their lipid accumulating phase (96 h to 144 h). This step is crucial as in the late stationary phase or late lipid accumulation phase there is a catabolic breakdown of lipids decreasing overall lipid yield [9, 31]. Harvesting of samples can be done either by centrifugation or by settling the samples. The next step afterward harvesting is the total lipid extraction from the dry biomass which can be completed by diverse methods such as Bligh and Dyer method, ultrasonication, microwave, acid catalyzed hot water treatment, H_2O_2 with $FeSO_4$, and osmotic shock etc. [32–38]. However, oleaginous yeast imposes limitation regarding their cell disruption and lipid extraction which are the major hold up for commercial scale production. Conventional methods for lipids extraction consist of hexane extraction and vacuum distillation, which employ flammable or toxic solvents, cause adverse health and environmental effects [39, 40]. Lipid extraction step followed by conversion of fatty acids (TAGs) into fatty acid methyl esters (FAMEs) that can be carried out by different techniques such as microemulsions (solvent blending), thermal cracking (pyrolysis) and transesterification (alcoholysis). Among these techniques, transesterification is the most suitable method as it reduces the viscosity associated problems with microbial lipids [41, 42]. Moreover, it has an additional advantage which includes mild reaction conditions, eco-friendly and suitable for various feedstocks. This reaction is classified into two types, catalyzed and non-catalyzed. Catalyzed transesterification process can be achieved by homogeneous, heterogeneous or enzymatic catalysts [43, 44]. The most significant method for biodiesel production is using homogenous acid/base catalysts. Sodium and potassium hydroxides as a base catalyst have been used to convert the microbial oil into FAME. However, usage of the base catalyst has many critical issues such as saponification that causes the problem in separation and purification of the end product. Homogeneous catalysts are also very sensitive towards free fatty acids (FFA) and water contents present in the oil. High FFA content in the feedstocks responsible for soap formation in the presence of NaOH/KOH. In view of limitations associated with the homogeneous catalysts, solid heterogeneous catalysts for transesterification reaction is preferable due to their eco-friendly nature and the potential for producing high purity biodiesel. In view of the many challenges involved, a possible workaround in the production of biodiesel is the improvement of the biodiesel production process through *in-situ* transesterification. *In-situ* process in the context of transesterification refers to the direct use of the lipid-rich biomass without prior extraction of the lipids and allowing the transesterification reaction to take place within the solid matrix [1, 43].

4.4 Selection of Low-cost Feedstock for Biodiesel Production

The raw materials required for the production of biodiesel from oleaginous yeast raises its production cost and make it unreasonable. For biodiesel to be economically competitive, the production cost must be reduced. The feedstock used by oleaginous yeast accounts for 60–85% of the total cost of biodiesel production [45, 46]. Finding techniques to reduce the high cost of biodiesel is of much interest in recent literature (Figure 4.4). Various inexpensive materials used by oleaginous yeast for lipid production are listed in Table 4.1. Sugarcane molasses (SCM) obtained as the waste product from sugar industry and is extensively used as a low-cost raw material for oleaginous yeast. It is rich in a diverse type of sugars which can be utilized

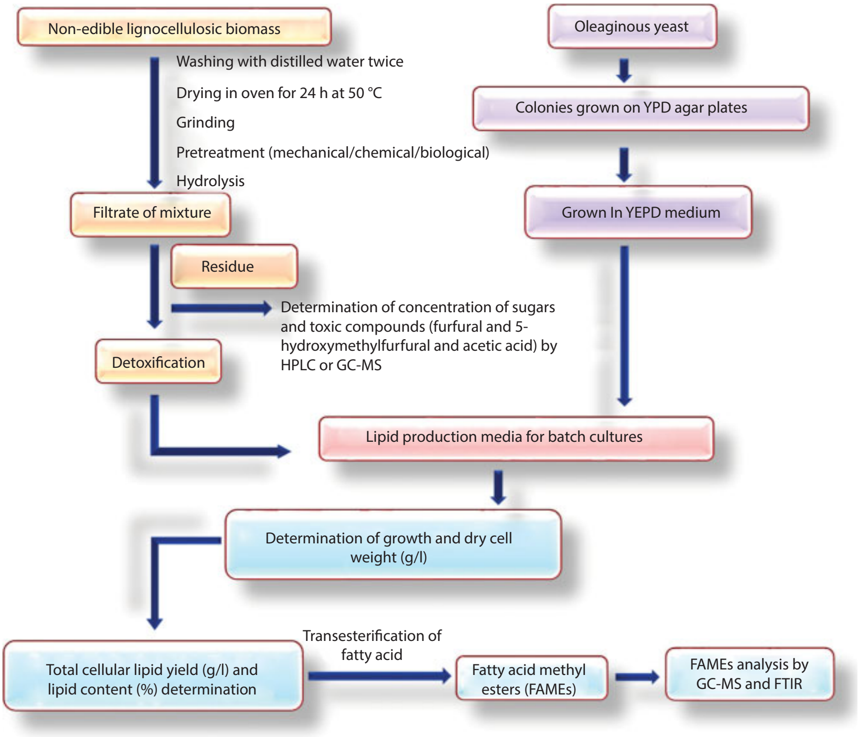

Non-edible lignocellulosic biomass
Washing with distilled water twice
Drying in oven for 24 h at 50 °C
Grinding
Pretreatment (mechanical/chemical/biological)
Hydrolysis
Filtrate of mixture
Residue
Determination of concentration of sugars and toxic compounds (furfural and 5-hydroxymethylfurfural and acetic acid) by HPLC or GC-MS
Detoxification
Oleaginous yeast
Colonies grown on YPD agar plates
Grown In YEPD medium
Lipid production media for batch cultures
Determination of growth and dry cell weight (g/l)
Total cellular lipid yield (g/l) and lipid content (%) determination
Transesterification of fatty acid
Fatty acid methyl esters (FAMEs)
FAMEs analysis by GC-MS and FTIR

Figure 4.4 Schematic diagram of biodiesel production by oleaginous yeast utilizing non-edible lignocellulosic biomasses as feedstock.

Table 4.1 Low cost substrates used for the production of lipids from oleaginous yeasts.

S. No.	Oleaginous yeast	Low cost substrates	Lipid content (%); w/w	References
1	*Cryptococcus curvatus* ATCC 20509	Sweet sorghum bagasse	73.26	[72]
2	*Cryptococcus sp.*	Corncob hydrolysate	61.3	[73]
3	*Cryptococcus curvatus*	Wheat straw acid hydrolysate	33.5	[48]
4	*Cryptococcus curvatus* ATCC 20509	Detoxified liquid wheat straw hydrolysate	27.1	[48]
5	*Cryptococcus curvatus* ATCC 20509	Non-detoxified liquid wheat straw hydrolysate	33.5	[48]
6	*Rhodotorula glutinis* ATCC 204091	Detoxified liquid wheat straw hydrolysate	20.7	[48]
7	*Rhodotorula glutinis* ATCC 204091	Non-detoxified liquid wheat straw hydrolysate	25.0	[48]
8	*Yarrowia lipolytica* (ATCC 20460)	Detoxified liquid wheat straw hydrolysate	4.4	[48]
9	*Yarrowia lipolytica* (ATCC 20460)	Non-detoxified liquid wheat straw hydrolysate	4.6	[48]
10	*Rhodosporidium toruloides* Y4	Jerusalem artichoke hydrolysates	56.5	[55]
11	*Rhodosporidium toruloides* ATCC10788	Wheat straw hydrolysate	24.6	[48]
12	*Rhodosporidium toruloides* 21167	Cassava starch	64.9	[74]
13	*Rhodosporidium toruloides* Y4	Crude glycerol	48.9	[75]
14	*Rhodosporidium toruloides*21167	Cassava starch	63.2	[76]
15	*Rhodosporidium toruloides* Y2	Bioethanol wastewater	53.8	[77]

by yeast for its growth [47]. Researchers have used non-food crops such as Cassava and Jerusalem artichoke for the cultivation of *Rhodosporidium toruloides* that synthesized 63.2% and 56.5% of lipid content (w/w) respectively in respect to their cell dry biomass [48]. Furthermore, oleaginous yeast like *Cryptococcus curvatus*, *Rhodosporidium toruloides and Yarrowia lipolytica* were utilized hydrolysates of non-edible lignocellulosic biomass as a carbon source [49–51]. Wheat straw is another waste lignocellulosic biomass which is utilized by these microorganisms. More feedstocks include sugarcane bagasse, sugar cane husk, wheat and rice straws and corn stover that are being used in the U.S.A., Asia and Europe respectively [52–55].

4.5 Triacylglycerols (TAGs) Accumulation in Oleaginous Yeasts

It is interesting to know how oleaginous microorganisms accumulate lipids in their cellular compartment and act in a different way from non-oleaginous microorganisms with respect of fatty acid metabolism [56]. Oleaginous yeast converts the excess carbon source present in the medium into fatty acids and stores it in the form of lipid droplets (LDs). However, non-oleaginous yeast such as *Saccharomyces cerevisiae* and food yeast (*Candida ultilis*) cannot accumulate lipid content more than 10% of their total cell dry biomass [57], but when they grown in nitrogen-limited medium with excess carbon source, increased amount of mannans and glucans was reported [58]. While oleaginous yeast cultivated under nitrogen limited condition, adenosine monophosphate deaminase gets activated and catalyze the conversion of AMP to inosine 5′-monophosphate (IMP) and ammonium [59]. It has been reported that the adenosine monophosphate deaminase enzyme is present only in oleaginous yeast but no such absolute dependency in non-oleaginous yeast [60]. Furthermore, isocitrate dehydrogenase gets inactivated when the concentration of AMP decreases that further hampering the synthesis of isocitrate via tricarboxylic acid cycle [61]. In the cytosol of oleaginous organisms, ATP: citrate lyases (ACL) cleaved citrate and citrate translocate from mitochondria to the cytosol via Malate/citrate translocase system while ACL is inactive in non-oleaginous microorganisms which is responsible for the synthesis of triacylglycerols (Figure 4.5) [58]. Acetyl-CoA carboxylase enzyme converts the acetyl-CoA into malonyl-Co A. The *de-novo* synthesis of lipids involves both acetyl-Co A and malonyl-CoA adding carbon to form 14 and 16 long fatty acid chains. The fatty acids profile of oleaginous yeast depends on the provided culture condition [62, 63]. It has been reported that under nitrogen-limited condition oleaginous yeast accumulates more lipid (>70% of their CDW) than the condition with access nitrogen [64, 65]. Gill *et al.*, 1977 earlier reported that oleaginous yeasts *Candid*a 107 under phosphate-limited conditions (high C/P molar ratio) accumulate high quantity of lipids [66]. Granger *et al.*, 1993 studied the effect of various nutrient limitations (nitrogen (N), phosphorus (P), zinc (Zn) or iron/Fe) on fatty acid production by *Rhodotorula glutinis*

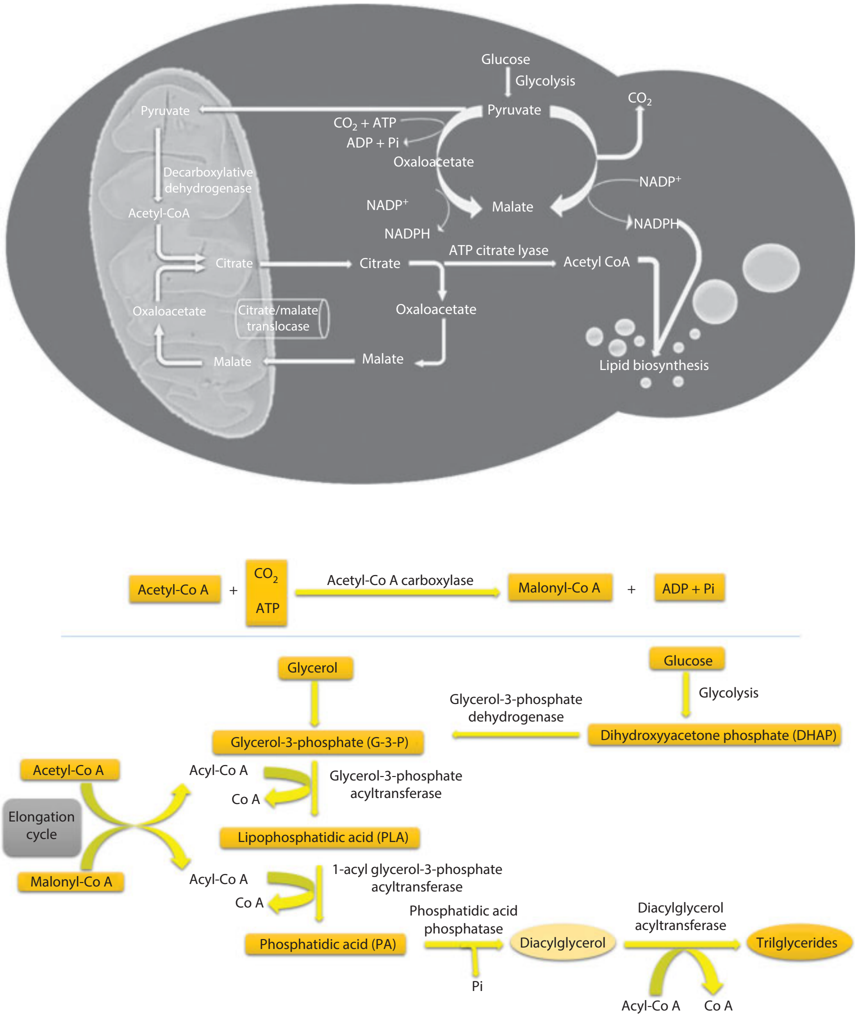

Glucose
Glycolysis
Pyruvate
CO$_2$
Pyruvate
CO$_2$ + ATP
ADP + Pi
Oxaloacetate
Decarboxylative dehydrogenase
NADP$^+$
Acetyl-CoA
NADP$^+$
Malate
NADPH
NADPH
ATP citrate lyase
Citrate
Citrate
Acetyl CoA
Oxaloacetate
Citrate/malate translocase
Oxaloacetate
Malate
Malate
Lipid biosynthesis
Acetyl-Co A
+
CO$_2$
ATP
Acetyl-Co A carboxylase
Malonyl-Co A
+
ADP + Pi
Glycerol
Glucose
Glycerol-3-phosphate dehydrogenase
Glycolysis
Glycerol-3-phosphate (G-3-P)
Dihydroxyacetone phosphate (DHAP)
Acetyl-Co A
Acyl-Co A
Glycerol-3-phosphate acyltransferase
Co A
Elongation cycle
Lipophosphatidic acid (PLA)
Malonyl-Co A
Acyl-Co A
1-acyl glycerol-3-phosphate acyltransferase
Co A
Phosphatidic acid phosphatase
Diacylglycerol acyltransferase
Phosphatidic acid (PA)
Diacylglycerol
Trilglycerides
Pi
Acyl-Co A
Co A

Figure 4.5 Lipids synthesis in oleaginous yeast via flow of citrate and malate as precursors of acetyl-CoA and NADPH (Adapted from Ratledge, 2004) [8].

and demonstrated that P limitation resulted in the best production of fatty acids [67]. *Rhodosporidium toruloides* Y4 also produced higher amount lipid under P-limited condition in growth medium [68]. Under the phosphate-limited condition, *C. utilis* synthesized more intracellular non-polar lipid content but polar lipid remained relatively constant [69]. It has been suggested that under the N-limited condition, the batch culture of *R. glutinis* showed reduced growth rate and stopped when N was exhausted [70]. But the condition altered under phosphate deprivation with cells showing an increase in growth rate and lipid-free biomass. Other factors which affect the lipid accumulation include temperature, pH, presence of trace elements, aeration, and dissolve oxygen [43, 58, 71].

4.6 Conclusion

According to current research, for developing nations like India, oleaginous yeast seems to be most favorable non-conventional source of energy. As compared with the other plants, yield of oleaginous yeast oil is much higher and this proves that oleaginous yeast has great potential than other biofuel crops. Oleaginous yeasts have ability to replace petroleum diesel completely. Oleaginous yeast does not contest with other crops for land requirement. However, despite the historical and recently renewed interest in yeast-based fuels, our understanding of regulatory mechanisms governing oleaginous yeast lipid metabolism, particularly the regulation of fatty acid and TAG accumulation, remains inadequate. Characterization of key regulators of genes, proteins, and metabolites prompting lipid synthesis opens the door for genetic and metabolic engineering strategies targeting increased rates and absolute quantity of lipid accumulation. As such, molecular examination of yeast lipid accumulation mechanisms has recently strengthened. Moreover, biodiesel production from oily yeast represents one of the most favorable ways to combat the current scenario of food security and energy crisis. The utilization of various non-edible lignocellulosic biomass for the growth and lipid accumulation by oleaginous yeast shows cost effective way for production of biodiesel. Furthermore, nutrient stress conditions can also provide a new means to regulate lipid accumulation enabling more viable production of oleaginous yeast neutral lipids. The nitrogen (N) and phosphorus (P) starved condition is perhaps the best characterized inducer of lipid accumulation in oleaginous yeast.

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5

Current Status of Biodiesel Production from Microalgae in India

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Abstract

Microalgal lipids that are similar to vegetable oils serve as a prominent source for biodiesel production. Microalgae have the potential to produce 100 times more oil per acre of land than any other plants. They have a short lifecycle with high growth rates. Microalgae also permit diversified cultivation conditions by using waste and sea waters, which does not impair the global food supply. However, large-scale production of microalgal biofuels encounters a number of technical challenges including growth and harvest of algal biomass to compete with that of petroleum-based conventional fuels. The yield of lipids depends mostly on the culture conditions such as light, salinity, pH, stress conditions, etc. This chapter aims to give a mini-review on the production of high dense algal biomass with high lipid content through engineering modifications. In the concluding sections, there

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is a brief discussion on the status of microalgal biodiesel production in India using the SWOT analysis approach.

***Keywords*:** Micro algae, biodiesel, stress conditions, harvesting, swot analysis

5.1 Introduction

Microalgae denote a maintainable energy source because of their high biomass and lipid production rate. Initiatives have been laid down to demonstrate the current status of production of biofuel underlying the advantages associated with it. Efforts have also been taken to elucidate cost-effective technologies for harvesting and processing. The first exploration of algae as a fuel alternative was under the supervision of President Jimmy Carter in 1978, when gas prices had skyrocketed, and there were often delays in filling due to the long stretch on filling stations. These problems led to the burgeoning of biofuel across the globe. The Aquatic Species Program (1978), run by the National Renewable Energy Laboratory, researched high oil-output algae for biofuel. In 1942, scientists such as Harder and von Witsch proposed the cultivation of microalgae as a source of lipids for livelihood [1]. Research was fostered in the US [2, 3] after World War II, along with Israel [4], Japan [5], England [6], and Germany [7] to find out itinerary for the culturing of microalgae using species of the genus Chlorella on a large scale. This esoteric production of fuel was used as a substitute for transportation fuel, although its production declined at this time due to the use of microalgae as a source of food and for wastewater treatment. Biofuels are generally categorized as biodiesel and bioalcohol. To craft bioalcohol, primarily ethanol, scientists from various R&D commercially utilized modified bacteria and yeast to break down the hydrocarbon moiety in various plants like corn, sweet sorghum, sugarcane, etc. (http://www.praj.net/ethanol-plant.html). To produce biodiesel, refineries utilized oil already existing in crops such as *Jatropha curcas* (http://bulkagro.com/products/jatropha-curcas/). However, there are some drawbacks of these biofuels that cannot be ignored. The cultivation of the crop involves large hectares for area, which has certain constraints relating to deforestation and cost. The US government developed its research base on the production of biofuels from photosynthetic microorganisms to overcome the oncoming fuel crisis. In mid-2010, the US Department of Energy encouraged research groups to commercialize algae-based biofuels and invested up to $24 million in three research groups (http://energy.gov/eere/bioenergy/algal-biofuels). Solazyme and Sapphire Energy started commercialization of algal based biofuel in 2012 and 2013, respectively, and Algenol tends to produce commercially. Indian Oil Corporation was the first national oil company to determinedly take the lead in algal biofuel by signing an MOU with Petro Algae in order to license technology for the large-scale biofuel production utilizing microalgae. Bengal has also entered the algal fuel race with its pilot project at the Kolaghat thermal power plant to start oil production. Sustainable production that takes into consideration environmental hazards serves as the basic constraints of the biotechnology-based industry in India [8].

5.2 Algal Species for Oil Production

Microalgae are easier to culture in a bioreactor due to their simpler morphologic features, short doubling time, and suitability for maintenance of easier sterilization methods. Moreover, algae consume carbon dioxide to grow in number and produce various other valuable products such as proteins, fatty acids, fertilizers, and biomass for energy production. Microalgae also serve as a potent source for wastewater treatment by removing heavy metals from water.

Above all, they are present in almost all water resources of every climatic condition and geographical region. Presently, there are many evidenced microalgal strains that possess a limited capacity to produce biofuel, which are known to support small-scale production units (Table 5.1). However, the research can only be acknowledged if the strains are viable for the commercial-scale production of biofuel. Thus, researchers are working on modifying algal strains through metabolic engineering in order to overcome their deficiencies. These modifications are streamlined to boost their cultivation with increased lipid content to produce huge quantities of biodiesel [9].

5.3 Engineering Modifications

Research is more lucrative when the research activities remove considerable shortcomings and produce a highly definitive product that targets its audience and

Table 5.1 List of algal species.

Name of species	References
Nannochloropsis gaditana, Tetraselmis chuii,	[10–12]
Tetraselmis Suecica, Phaeodactylum tricornutum	[13, 14]
Scenedesmus obliquus,	[15–17]
Schizochytrium sp., *Spirogyra* sp	[18–21]
Spirulina Maxima, Spirulina platensis, Synechoccus sp	[17, 22–24]
Chlorella minutissima, Tetraselmis maculate	[25–27]
Chlorella protothecoides, Chlorella pyrenoidosa,Chlorella vulgaris	[17, 28, 29]
Crypthecodinium cohnii, Cylindrotheca sp	[30, 31]
Dunaliella bioculata, Dunaliella primolecta, Dunaliella salina, Dunaliella tertiolecta	[27, 32–35]
Euglena gracilis, Hormidium sp, *Isochrysis* sp	[28, 36–38]

possesses a competitive position in the marketplace. In order to produce significant amounts of biofuels, the ventures emphasize two important parameters: (1) production of high-density cultivated microalgae and (2) improvisation of high lipid content in the intrinsic part of the algal cells [39].

5.3.1 Production of High Density Cultivated Microalgae

It is very important that the proliferating organism is high in density and possesses high metabolic activity and short generation time. The design of the bioreactor should be such that it supports good multiplication rate of the organism in optimum culture conditions of temperature, pH, salt, and elucidators. Broadly, two approaches have been undertaken for this purpose: (1) cultivation systems and (2) manipulation of the metabolic pathways [40].

5.3.1.1 Cultivation Conditions

Culture conditions in production assemblies (bioreactor) directly depict the feasibility of the scale of production as well as its scope. The conventional photoautotrophic system of production has many deficiencies, and thus cannot be sustainably used for large-scale production of biofuels. Thus, the need for the emergence of a cost-effective method is very important. Cultivation of microalgae generally involves two methods: an open or closed system. An open system needs less investment, but is highly susceptible to contamination. On the other hand, a closed bioreactor is safe, free of contamination, but demands high investment. An open bioreactor for microalgae is generally considered a photo bioreactor such as a tubular photobioreactor, plate photobioreactor, wall panel photobioreactor, and bubble column bioreactor. In 1996, Feng Chen employed heterotrophic strategies for high cell density of microalgae for commercial production. Some studies have revealed high production of microalgae in a tubular photobioreactor with high dissolved oxygen (DO) levels [41, 42]. A.H. Scargg [43] grew *Chlorella vulgaris* and *C.emersonii* in 260 L in a pumped tubular photobioreactor in Watanabe's medium, and the biomass productivity was 40 mg dry wt. $L^{-1}d^{-1}$. Bryan McCarty, Solix Biofuels' vice president of engineering designed the improved bioreactors with less shear sensitivity to microalgae [44]. McCarty [45] described the cultivation technology as an outdoor system in a controlled environment. They made closed panels with a blend of algae, growth media, nutrients, and salts.

5.3.1.2 To Get High Lipid Content

The potential for modifying lipid content is believed to increase the content of storage lipid during logarithmic growth. Various expression methods, such as the lipid catabolism method, are used extensively in R&D pertaining to oleaginous industries. This can be done by genetic modification, nutrient management, and culture conditions. The most important factor is selecting a strain that is capable of producing oil and has the tendency of undergoing mutations [46].

5.3.1.2.1 Genetic Modification

The engineering of lipid biosynthetic pathways in microalgae is of high importance because lipid content directly correlates to the quantity of biodiesel produced. The lipid biosynthesis pathway is determined by the availability of fatty acids, and that the production of fatty acids is facilitated by acetyl CoA carboxylase (ACCase) [47]. Increasing the activity of ACCase would change the flux of deposited malonyl-CoA into the lipid biosynthesis pathway. *Chlamydomonas reinhardtii* was amongst those algae on which genetic modifications proved to be fortuitous in accumulating lipid in their intracellular spaces. Under the Aquatic Species Program, *Cyclotella cryptic* was engineered with an extra copy of the ACCase gene, enhancing the activity by three-fold. This resulted in the steady increase of lipid accumulation due to the absence of "ate" nuclear transformation system [48]. Gene silencing experiments have also been undertaken to increase fatty acid content and change the fatty acid chain length, Emily *et al.* [49] produced a genetically engineered strain of *Thalassiosira pseudomonas* (diatom) by knocking down certain genes coding for acyltransferase, phospholipase, and lipase, which increase the lipid content. A variant form *Chlorella pyrenoidosa* has also been shown to have a high content of polyunsaturated fatty acid [50]. Researchers overexpressed two genes coding for the enzyme ketoacyl ACP synthase (KAS) and thioesterase from *C. hookeriana*, which elevated the fatty acid content by approximately 40% [51].

5.3.1.2.2 Nutrient Management

Jia Yanga *et al.* [52] highlighted that 0.33 kg nitrogen, 3726 kg water, and 0.71 kg phosphate are necessary to produce 1 kg of biodiesel. The researchers also emphasized the recycling of used water to reduce water usage by 84% and nutrient usage by 55%. Usage of seawater in this experiment dwindled the water requirement by

90%, and only phosphate was required in the nutrients along with it [52]. *Chlorella zofingiensis* was detected in the concentration of nutrients present in different forms of available water resources. Pollutants in piggery wastewater were effectively utilized by *Chlorella zofingiensis*. The initial concentration of nutrients effected the lipid accumulation with biomass productivity showing a tremendous increase from 106.28 to 296.16 mg L^{-1} day^{-1}. Biodiesel productivity has previously ranged from 11.85 to 30.14 mg L^{-1} day^{-1}. Piggery wastewater with a considerable COD of about 1900 mg L^{-1} was considered optimal for the growth of algae [53].

5.3.1.2.3 Culture Conditions

The carbon source in the bioprocess constitutes approximately 60% of its costs, and microalgae helps to minimize this cost by fixing carbon dioxide. It has been reported by Tsukahara and Sawayama [54] that microalgal species can fix 183 tons of CO_2 by every 100 tons of microalgae. Various other factors that affect culture conditions are intensity of light, nutrient concentration, pH, temperature [55], salinity, toxic chemicals, osmotic stress, amount of gases, biotic factors, and operational factors [56, 57] such as shear stress [58].

5.4 Production of Biodiesel

5.4.1 Culturing of Microalgae

Open systems for microalgae propagation are usually achieved in open ponds utilizing sunlight as the immediate source of solar energy, whereas photo bioreactors or fermenters are considered closed systems. Many companies have conducted research to manage huge masses of algae in diversified photobioreactors in order to harvest filamentous and non-filamentous algae as a useful nutraceutical product in a septic environment, producing a minimum amount of wastage. Microalgae productivity can be affected when cultivated in open raceway ponds which have negligible disadvantages including evaporation, and contaminants (bacteria protozoa and other microalgae) [59]. Photobioreactors are continuous culture systems which can achieve concentration of microalgae up to 6.7 g/L [60–62] in fresh or sea water. Flat plate, tubular, airlift, bubble column, and stirred tank [28] are different models of photobioreactors that have been developed. Even if a closed photobioreactor has a higher harvesting efficiency and a good control on culture parameters such as temperature, pH, CO_2 concentration, etc. [63], its capital costs remain higher (around 10 times) than those of open ponds [64]. However, the combination of ponds and photobioreactors can be profitable because microalgae can be grown in open ponds while reducing contamination by undesired species [65]. In this culture process, the first step of microalgae production is conducted in a controlled temperature, e.g. by a sea water bath (16–18 °C) photobioreactor.

5.4.2 Harvesting

There is not yet a single protocol that proves to be best in harvesting microalgae. Microalgae found in open ponds are highly diluted with a concentration around

0.5 g/L. The harvesting costs account for 20 to 30% of the industrial microalgae biomass production cost of \$2.95 and \$3.80 US dollars/kg for biomass in photobioreactors and raceway, respectively [62, 66]. The harvesting method depends on the nature of the microalgae, its shear sensitivity, growth medium, purity of the end product, and cost involved [67]. Y. Chisti [62] discussed the effect of various factors such as harvesting methods, light intensity, and microbial metabolism on the quantity of the oil produced. E.J. Middlebrooks *et al.*, 1974 [68] classified several harvesting methods appropriate for wastewater and categorized filtration of the media as the best harvesting methods for maximum yield. There are always constraints associated with cost due to poor volumetric efficiencies. The various chemicals studied as algal flocculants can be broadly divided into two groups: polymeric organic flocculants or inorganic agents, including polyvalent metal ions as Al^{+3} and Fe^{+3} which form polyhydroxy complexes at the appropriate pH. Dispersed air flotation, dissolved air flotation (DAF), and electrolytic flotation are a few of the flotation techniques that have been adopted in the algal industry [67]. Dissolved air flotation involves the formation of small bubbles generally between 10–100 μm used for microalgal biomass production [69]. Addition of chemical agents to algal suspensions in order to induce algal flocculation is the innovative approach in various separation technologies i.e. centrifugation [70, 71], sedimentation [72, 73], flotation [71], and filtration [73]. Algae harvesting generally employs flotation technologies when low-density algae is under supersaturated oxygen conditions (Figure 5.1).

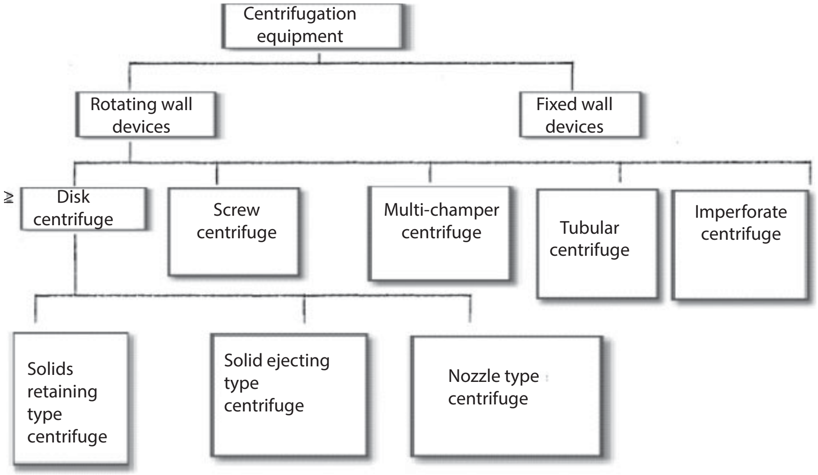
Centrifugation equipment
Rotating wall devices
Fixed wall devices
Disk centrifuge
Screw centrifuge
Multi-champer centrifuge
Tubular centrifuge
Imperforate centrifuge
Solids retaining type centrifuge
Solid ejecting type centrifuge
Nozzle type centrifuge

Figure 5.1 Classification of centrifugation equipment [79].

Filtration techniques like macro-filtration are used generally for larger microalgae (>70 μm) like Coelastrum and Spirulina [74]. Microalgae possessing negatively charged surfaces and small wall size (5–50 μm) with perfused mobility, thereby slowing recovery [75]. Ultra-filtration and micro-filtration are widely used. Ultra-filtration is often not used due to its high cost and tedious maintenance, yet it is extremely beneficial due to its low cross-flow velocity and low transmembrane conditions [76, 77]. For small-scale production, chemical coagulation followed by sedimentation or dissolved air flotation (DAF) is also commonly used when there are lacks of big channelized machinery [72]. On certain circumstances, DAF is considered to be more promising than sedimentation to harvest algae [78]. Solid-liquid separation processes include flotation and sedimentation [79]. While sedimentation follows Stokes Law, the same is not applicable for non-flocculating cell structures.

To develop algal harvesting, the U.S. Department of Energy Advanced Research Projects Agency-Energy (DOE-ARPA-E) funded a research project for Algae Venture Systems (AVS) that studied dewatering and drying technologies based on the principles of capillary action and liquid adhesion for removal of water for forming concentrated algal suspensions. Precipitation of algal cells will take place by the selective adsorption of medium ionic components each other with the aid of inorganic functional groups which reveals the exposing of charged ends of algal cells to each other. Mechanical extraction by expression involves various types of presses available in screw, piston, and expeller configurations which are selected on the basis of physical and morphological characteristics of algae [80]. To enhance the amount of the extraction, a chemical method may be undertaken in conjunction to help extract 80% of the oil from algae. [http://www.virtuosobiofuels.com/aboutus.html]. The oil extracted from green photosynthetic organisms through biotechnological tools is referred to as "green crude," but its potency remains suppressed until this biofuel is esterified by a mechanism called as transesterification. The blending ratio is significant for using this green crude in automobiles (Figure 5.2).

H.F. Mohn, 1980 [73] researched various kinds of filtration techniques for algal harvesting which classified chamber filter press as the most reliable method

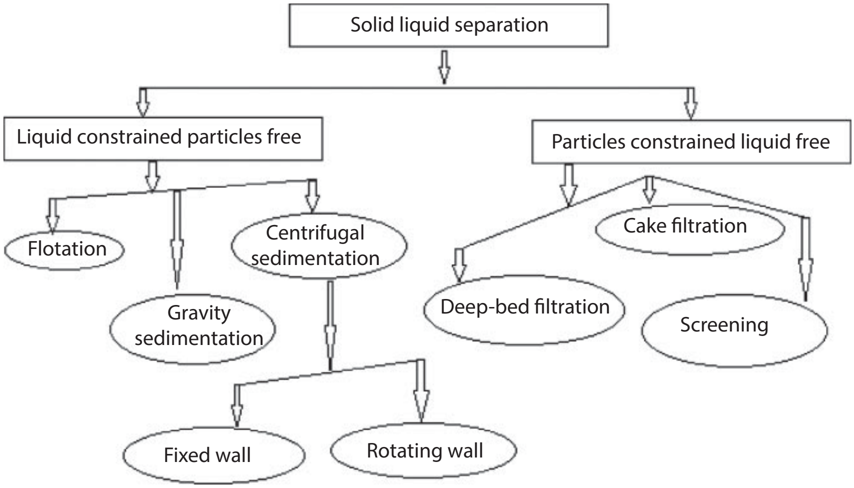
Solid liquid separation
Liquid constrained particles free
Particles constrained liquid free
Flotation
Gravity sedimentation
Centrifugal sedimentation
Fixed wall
Rotating wall
Cake filtration
Deep-bed filtration
Screening

Figure 5.2 Different types of solid-liquid separation techniques.

of extraction for algal *Ceolastrum* (power: 0.88 kWh per metric cube), and filter basket to be the least reliable method. Dryer compartment studies were conducted on Wet Spirulina cultures (55–60% moisture) dried at 62 °C for an interval of 14 hours. Also several algal drying methods were evaluated at CFTRI, Mysore, India [81].

5.5 Current Status of Biodiesel Production in India and Abroad

India constitutes only 1% of the global biofuel production with 80 million litres of ethanol fuel and 45 million litres of biodiesel although it is the fifth largest consumer of energy in the world. Initially, The Centre of Jatropha Promotion and Biodiesel (CJP) worked intensively towards the commercialization of crop-based biodiesel in India, but remained unsuccessful due to its harvesting methods; the amount of oil obtained was inadequate (approximately 40% less), and cultivation needed large hectares of land. Eventually, this gave birth to the use of naturally occuring renewable resources for commercial exploitation of biofuel. Algae were considered a potent source for biodiesel production around the world. The cost of the commercially available diesel and petrol demand had been alarming for the last 17–20 years, but during 2013–2014, the fuel demand declined due to the exhaustion of resources and high cost of filling [82]. The year 2013 witnessed an increase in gasoline consumption by 8.8 percent and a decline in vehicle transportation by 2.6 percent in 2013/14. At the time, India was importing about 3.81 million barrels per day (bpd) of crude oil. Imports declined 1 percent to 3.5 million bpd in March 2014, according to the Petroleum Planning and Analysis Cell (PPAC) of the oil ministry (http://ppac.org.in/content/212_1_ImportExport.aspx). Both import and export of oil products declined drastically by 4.6 percent in March 2014. The rapid demand of energy resources in India, along with these internal and external constraints, led to the emergence of the use of renewable sources for producing biofuel. Biodiesel would also help to reduce the dependence of the Indian nation on fossil fuels [82].

To date, commercial exploitation of algal as biomass is approximately 10,000 tonnes, which is mainly directed towards low-volume, high-value nutraceutical products. In the US, Cyanotech Corp. in Hawaii and Earthrise Nutritionals, LLC in California have employed open systems for algal harvesting. Another US-based company, Martek Co. in Maryland, produces algal bioproducts by fermentation. Hutt Lagoon (520 hectares) and Whyalla (440 hectares) of Cognis Australia Pty Ltd are among the largest algal production systems in the world for pond surface area acquisition for algal-based projects [83].

According to India's 12th Five-Year Plan (2012–2017), a sum of $44.6 billion has been allotted by the Indian government to spend on various missions related to renewable resources. The oil yield from algae biomass (3800–50,800 litres/per hectares/year) is much larger compared to crop-based oil production (1890 litres/per hectares/year). To boost the Indian algal industry, algal projects have been sponsored by several international and national companies, amounting to several millions of dollars in funding. Reuters reports that a US-Israeli company, World Health Energy Holdings (WHEN.PK), had two projects in India, which targeted over $200 million in sales in 2013 and $150 million in sales of biodiesel and food for commercial fish farms from 250 acres of algae. A 70% equity stake in the project is held by India-based company Prime, which provides transportation services to the oil industry. The state of Maharashtra, India is set to get its first biodiesel pump by the end of the year. My Eco Energy set up the first Indian biodiesel pump in Pimpri-Chinchwad based on treating organic waste materials, investing around Rs 250 crore. Maharashtra is in line to join other states such as Karnataka and West Bengal, which are leading the way in biodiesel consumption. The National

Biodiesel Mission venture is estimated to replace traditional diesel usage by 20%. In India, algal research activities have been carried out for the production of transesterified non-edible oil and its use in biodiesel by the Indian Institute of Science, Bengaluru and Tamil Nadu. Blended diesel fuel extracted from the Jatropha plant has been employed by Indian Railways to power its diesel engines with great success ("National Policy on Biofuels," *Jatropha World*, September 28, 2011). Diesel locomotives run from Thanjavur to Nagore section and Tiruchirapalli to Lalgudi, Dindigul and Karur sections all of which are operated on a blended diesel oil [84, 85].

Four biodiesel plants costing about Rs 120 crore form the Ethanol Plant, which is the biggest initiative laid by Indian Railways (Ethanol India 2009). Two of the biodiesel plants have been planned to be commissioned at Raipur and Chennai in the next two years while the rest are due in the coming years (http://gain.fas.usda.gov/Recent%20GAIN%20Publications/Biofuels%20Annual_New%20Delhi_ India_7-1-2011.pdf). Each plant, estimated to cost around Rs 30 crore, will produce 30 tons of biodiesel per day, accounting for more than 9,000 tons a year (*The Economic Times*, February 6, 2011: http://articles.economictimes.indiatimes.com/2011-02-06/news/28423691_1_biodiesel-locomotives-alternative-fuels) (Table 5.2).

Analysis on biofuel patents was done through patents related to biofuels, which helped to project relationships and visualize trends within the patent landscape. The number of biofuel patents is phenomenal in the

Table 5.2 Showing estimated demand of diesel and biofuel blending requirements (Indian Biofuel Scenario and Planning Commission, Government of India).

Year	Diesel demand in mt	Diesel blending requirement in mt		
		@5%	@10%	@20%
2006–2007	52.32	2.62	5.23	10.46
2011–2012	66.91	3.35	6.69	13.38
2016–2017	83.98	4.18	8.36	16.72

USA and Japan, followed by Germany and Canada with the number of patents published and granted being 24436, 25282, 4847, and 5104, respectively. China has the highest number of biofuel patents, followed by Brazil and Russia. Although this number is very small when compared to the developed nations, it has paved the way for biofuel's early development. In India, the maximum number of patents is filed by the Council of Scientific & Industrial Research (CSIR) (approximately 13 patents published and filed) (Figure 5.3).

There has been an increase in biofuel patents from when it began in 1997 to the year 2007. The decrease in patent filing after 2007 can be attributed to the shift in researchers' focus on the progressing domains, i.e third and fourth generation biofuels, which are tapered domains. The reason behind the decrease in cases of patent filing is the lack of innovative activities in this branch as it involves high technology and experimentation. The recent evolution in the biofuels sector has been characterized by strong price volatility and a mismatch

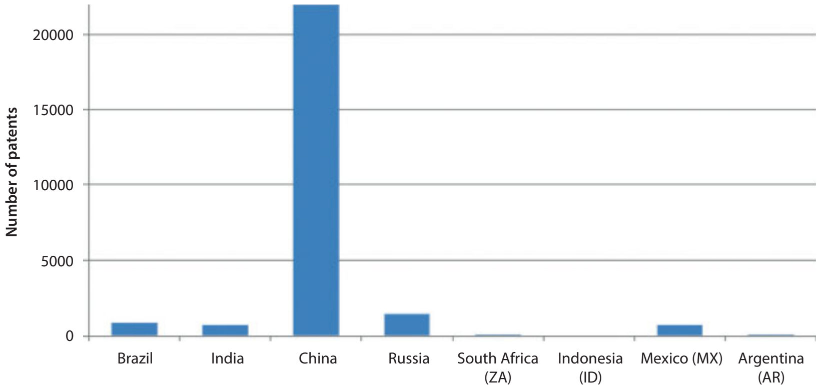

Figure 5.3 Biofuel patenting trends in developing countries (2008 to 2012).

between demand and supply. The conflict created by governmental policies due to lack of knowledge of the biofuels production system is partly responsible for the current situation (Planning Commission, 2007).

5.6 SWOT Analysis of Biofuels in India

A structured planning method used to appraise the strengths, weaknesses, opportunities, and threats involved in a project or in a business venture are referred to as SWOT analysis.

Strengths: Biodiesel will strengthen energy security in India in a very lucrative and effective way and support sustainable development. This will also reduce greenhouse gas (GHG) emissions, thus helping to reduce global warming. It would involve the use of renewable energy source, which would reduce the stress on exhaustive fossil resources. This is bringing to life the use of alternate energy [82].

Weakness: The market is still in the dormant stage of accepting biodiesel. Automobiles are also required to operate on blended diesel because the current machinery fails completely. There is an abundant need for wastelands. Initial set up and operational cost is high [84].

Opportunity: It would increase R&D in biofuel field, thus providing employment and pose a competitive position in the marketplace. It will also proliferate National policy on biofuels. Interest areas are primarily coming from private companies [85].

Threat: The involvement of cost in R&D is very high, so it may cause a rise in the value of the biodiesel sold to the retailers. The cost of extracting the raw material is high because the algae are diluted in ponds [85].

5.7 Challenges

The major drawback of using algae as the renewable source of biofuel needs huge biomass of microalgae for industrialization. Isolation of algae from the water resources in itself is a problem when algae is diluted in high ratios generally 600 mg/l [86] and possesses extremely small size typically 3 to 30 μm [87]. To overcome this limitation, the scientists around the globe developed genetically modified strains of the algal species which did not prove fortuitous in all experiments. Unfortunately, some water ponds and lagoons in the USA are devoid of fruitful algal species [88]. The initial harvesting cost has skyrocketed in few decades further complicating the downstream processing.

5.8 Conclusions

A number of enthusiastic start-up algae fuel companies are making wildly optimistic predictions about the volumes of algae biofuel that could be produced at competitive prices in the coming eras. A long-awaited dream of entrepreneurs is turning algae into a beneficial bio-based oil to run in conventional refineries alongside crude. Algae can certainly make an influence on many industrial companies. Algal-based biofuel technology is pollution-free and hence has the potential to substantially affect companies' policies, wherein it makes various ventures more profitable. Companies having problems with environmental control can surely benefit from such technology as algae will allow the capture and recycling of CO_2 from smokestacks. Thus, the algal-based biofuel industry presents significant opportunities to Indian entrepreneurs both small and large.

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6

Biobutanol: An Alternative Biofuel

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Abstract

With the depletion of fossil fuels reserves and rising cost of gasoline, alternative sources of energy are required to power internal combustion engines. Biobutanol has emerged as a better alternative to gasoline. The biochemical method for its production has reduced the cost of production while the efficiency of product has been increased. Various substrates ranging from grains, biomass feedstocks, molasses, and cellulosic and lignocellulosic materials have been used for the production of butanol. *Clostridium acetobutylicum* is one of the widely used and established strains for butanol fermentation. Genetic manipulation and metabolic engineering of microbial strains have been carried out to increase the production yield of butanol from the mixture of acetone-butanol-ethanol (ABE) fermentations. Distillation has been the most commonly used method for alcoholic separations. However, pervaporation, gas stripping, and membrane-based methods are showing promise for cost effective recovery of butanol from fermentation broth.

***Keywords*:** Biobutanol, biofuel, ABE fermentation, clostridium acetobutylicum, biomass feedstocks

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6.1 Introduction

Fossil fuels are extensively used and reserves are rapidly declining, which is leading to a considerable fluctuation in its prices. Extensive research is being done to come up with alternative fuels to power engines alone or as a blend with fossil fuels. In the Indian scenario, we are still dependent on foreign countries for about 67% of the crude oil demand, which is imported. Bioethanol and biodiesel have been considered as an alternative resource for fossil fuels [1]. However, certain disadvantages such as a blending limit of 20%, separation at low temperature, and reduced energy (heat) content has limited its efficiency as a blend for fossil fuels. **Biobutanol** or **biogasoline** is a colourless, flammable alcohol which comes as a safer and more competitive alternative to bioethanol or biodiesel. The physico-chemical properties of butanol (n-Butyl alcohol) are presented in Table 6.1 [2]. It is produced using biomass feedstock and can alternatively be used as a gasoline additive/fuel blend in an internal combustion engine [1]. It also acts as an industrial solvent.

Biobutanol, in comparison with ethanol, is chemically more analogous to regular gasoline with about 90% of its energy content, whereas the energy content of ethanol to that of regular gasoline is only 50%. During the 1960's, limited production was achieved owing to the high cost of bioproduction. Research for alternative fuel production was necessary, and one had a choice between ethanol and butanol for an subsidized alternative fuel. However, it was then felt that the production

Table 6.1 Physico-chemical properties of butanol (Adopted from OECD, SIDS initial assessment report on n-butyl alcohol).

Serial no.	Physico-chemical property	Value
1	Physical Form of Marketed Product	Neat Liquid
2	Melting point	–89.9 °C
3	Boiling point	117.6 °C
4	Relative density	0.809 – 0.811 g/cm^3
5	Vapour pressure	0.56 kPa at 20 °C
6	Water solubility	77 g/l at 20 °C
7	Partition coefficient n-octanol/water (log value)	0.88
8	Odor Threshold	15 ppm (average)
9	Conversion Factor	1ppm = 3.03 mg/ m^3 at 25 °C
10	Flashpoint	98 °F (37 °C)

of butanol via fermentation was too expensive along with the difficulty in recovering butanol (Table 6.2). So, ethanol was then selected and subsidized. However, with recent technological developments in biobutanol production and the rising cost of petroleum products, biobutanol has become more competitive. It is safer to use and is environmentally friendly with 85% less carbon emissions than regular gasoline [3]. Thus, biobutanol is emerging as a safer and more competitive alternative to gasoline and bioethanol.

Table 6.2 Points considered during the 1970's for selection of ethanol over butanol.

Particulars	Ethanol only	Abe fermentation			Butanol only
		Acetone	Butanol	Ethanol	
Final End product concentration	10–15 %	0.3 %	0.6 %	0.1 %	N/A*
Yield (gallons/ bushel)	2.5[2]	0.70	1.3	0.36	2.5
Boiling Point	78.5 °C	56.5 °C	117 °C	78.5 °C	117 °C#

*The final concentration cannot be measured as butanol needs to be continuously extracted.

#Owing to higher boiling point as compared to water, the process becomes expensive as too much of water is needed to be boiled off to recover 1–2 % of final batch concentration.

6.1.1 Advantages of Biobutanol

Biobutanol is a better alternative biofuel for internal combustion engines. It is a promising new generation alternative biofuel that offers a range of advantages as a liquid fuel for transportation. It is a 4-carbon alcoholic biofuel which has been commercially used as an industrial solvent and as a precursor for the synthesis of different types of organic chemicals. These organic chemicals have been used for manufacturing products such as thinners, paints, plastics, resins, adhesives, emulsifiers, elastomers, adsorbents, flocculents and brakes, hydraulics, dicing and cleaning fluids. These have been further used for producing different cosmetics, perfumes, and in the industrial manufacturing of leather, pesticides, safety glass, textiles, and paper [3].

The advantages of biobutanol as an alternative fuel are described below:

High Energy: The energy density of biobutanol is approximately 10% to 20% lower than gasoline. Still, its energy content is much higher when compared to other alternative biofuels, e.g. ethanol has 60% less energy content than gasoline.

Lower Heat of Vaporization: Biobutanol has a much lower heat of vaporization than bioethanol, which results in lower volatility and less evaporative emissions.

Economic Aspects: It can be produced from a variety of low cost feedstocks, grains, starches, and algal biomass on a small scale resulting in an increase in employment opportunities.

Environmentally friendly: Growing feedstocks such as algal biomass can capture CO_2 emissions and thus, may reduce the overall emission of greenhouse gases, which can balance out CO_2 emissions from burning biobutanol as a fuel.

6.2 Biobutanol as Alternative Fuel

Various parameters such as energy density, air-to-fuel ratio, specific energy, heat of vaporization, research octane number, and motor octane number have been used to evaluate the suitability of fuels for internal combustion engines [4]. In order to replace gasoline or to blend an alternative fuel source with gasoline, the properties of the alternative fuel should match closely with those of gasoline. Biobutanol matches more closely than any other alternative biofuels (Table 6.3).

Table 6.3 Comparative assessment of biobutanol as a biofuel.

Fuel	Energy density (MJ/L^{-1})	Air to fuel ratio	Specific energy (MJ/L^{-1})	Heat of vaporization (MJ/L^{-1})	Research octane number	Motor octane number
Gasoline	**32**	**14.6**	**2.9**	**0.36**	**91–99**	**81–89**
Methanol CH_3OH	16	6.5	3.1	1.2	136	104
Butanol C_4H_9OH	**29.2**	**11.2**	**3.2**	**0.43**	**96**	**78**
Ethanol C_2H_5OH	19.6	9.0	3.0	0.92	129	102

Source: Wikipedia, the free encyclopedia.
[Butanol url: http://en.wikipedia.org/wiki/Butanol-fuel]

However, biobutanol will lead to a consumption penalty of around 10% [5].

Biobutanol demonstrates increased tolerance to water contamination, an acceptable research octane number, and a comparable heat of vaporization and motor octane number. Thus, a 20% blend with fossil fuels does not require any changes in the current engine system. However, for biobutanol to completely replace fossil fuel, the air-to-fuel ratio needs to be increased despite the value for biobutanol being within the acceptable limits of variation that could be permitted in existing engines. Furthermore, lower vapour pressure and less corrosion of pipelines results in an efficient and easier transportation of the biofuel. Because the heat of vaporization of butanol is so close with gasoline, the cold start problem is avoided which is often the case with bioethanol [3].

6.3 Biobutanol Production

Biobutanol is obtained from fermentation of biomass feedstocks using specific strains of *Clostridium acetobutylicum* [1]. A flow diagram for its prodcution using acetone-butanol-ethanol (ABE) fermentation is given in Figure 6.1. The biomass feedstock contains carbohydrates (sugars) that are broken down and converted to various alcohols including acetone, butanol, and ethanol. However, alcohol production is limited owing to the toxic effects of these alcohols at increased concentrations. The microbial strains initially used for alcoholic production were susceptible to increases in

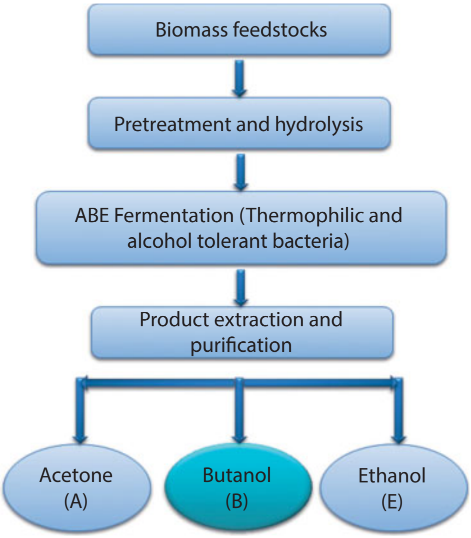
Biomass feedstocks
Pretreatment and hydrolysis
ABE Fermentation (Thermophilic and alcohol tolerant bacteria)
Product extraction and purification
Acetone (A)
Butanol (B)
Ethanol (E)

Figure 6.1 A general schematic of biobutanol production via ABE (Acetone-Butanol-Ethanol) fermentation.

alcohol concentration. Hence, the economics of biobutanol production limited its feasibility for competitive production when compared to the cost of petroleum in the 1960's. Technological developments such as 'pet mutants' and the application of rDNA technology has resulted in isolation, improvement, and selection of efficient microbial strains that are tolerant to high concentrations of alcohol. Furthermore, newer modifications, genes, and enzymes are constantly researched which can improve the bioproduction of butanol.

Considering the advantages of biobutanol over bioethanol [6], a recent trend is emerging where many bioethanol companies are being purchased by biobutanol producers. These companies are converting the bioethanol fermentation plants to biobutanol production plants by updating them with advanced separation units.

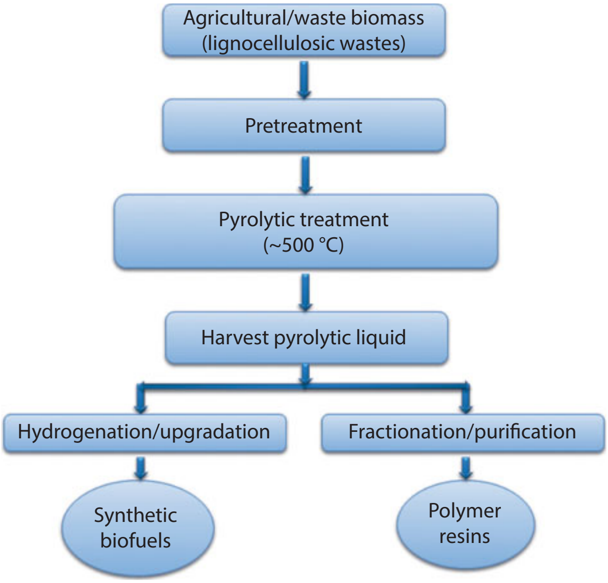
Agricultural/waste biomass (lignocellulosic wastes)
Pretreatment
Pyrolytic treatment (~500 °C)
Harvest pyrolytic liquid
Hydrogenation/upgradation
Fractionation/purification
Synthetic biofuels
Polymer resins

Figure 6.2 Pyrolytic technique for biobutanol production.

However, alcoholic fermentation is now establishing itself as a viable alternative to petroleum, and several companies are pursuing pyrolytic technique for biobutanol production (Figure 6.2). These companies aim to convert agricultural or waste biomass to biobutanol production. The agricultural biomass mostly consists of cellulosic and lignocellulosic residues. To prepare the hydrolysate, various pre-treatment methodologies are used to digest the lignocellulosic material, which is followed by pyrolysis at around 500 °C. The pyrolytic liquid is then finally converted to synthetic biofuels.

6.3.1 Steps to Biobutanol Production

Any production process in the bioprocess industry typically constitutes of three components, namely upstream, fermentation, and downstream processes.

The considerations and challenges for enabling biobutanol as a commercially viable alternative to depleting fossil fuels is summarized below:

Optimization of Upstream Components:

1. Need for alternate substrates other than molasses and grains
2. Genetic modification of *C. acetobutylicum* for increasing the selectivity of ABE (Acetone-Butanol-Ethanol) fermentation towards butanol

Optimization of Fermentation:

1. Effective pre-treatment for conversion of cellulosic biomass to hydrolysate and its fermentation
2. Enhancement of product yield
3. Optimization of reactor conditions for anaerobic bioconversion

Optimization of Downstream Processing for Effective Commercialization:

1. To reduce the cost of bioproduction
2. To overcome end product inhibition
3. To prevent phage infection and yield loss during production

6.3.2 Directed ABE Fermentation to Butanol

In the ABE process (Figure 6.3), all carbon is not converted to butanol, but much of it is utilized to produce undesired by-products such as acetic acid, lactic acid,

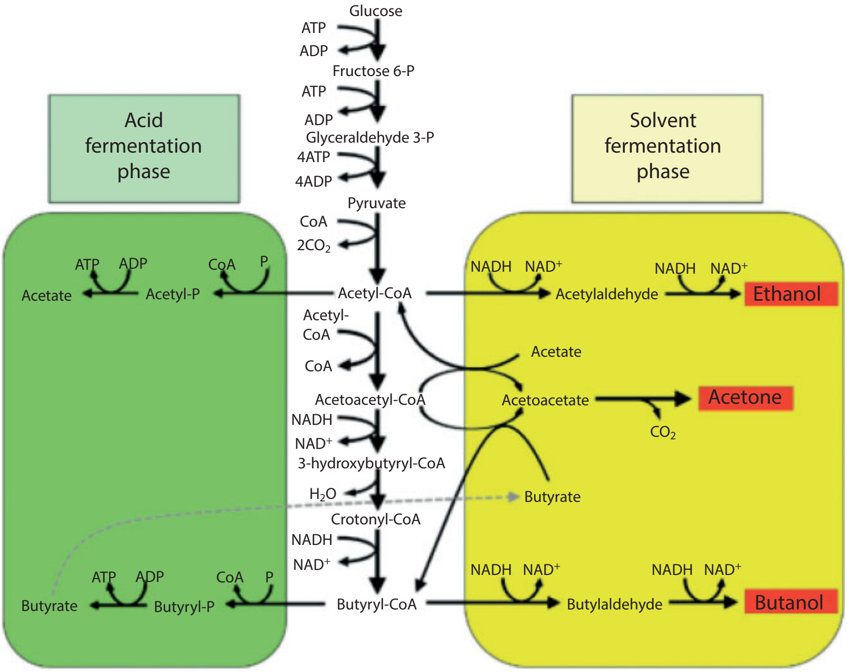
Acid fermentation phase
Solvent fermentation phase
Glucose
ATP
ADP
Fructose 6-P
ATP
ADP
Glyceraldehyde 3-P
4ATP
4ADP
Pyruvate
CoA
$2CO_2$
Acetyl-CoA
ATP
ADP
CoA
P
Acetate
Acetyl-P
NADH
NAD+
Acetylaldehyde
NADH
NAD+
Ethanol
Acetyl-CoA
CoA
Acetate
Acetoacetyl-CoA
Acetoacetate
Acetone
CO_2
NADH
NAD+
3-hydroxybutyryl-CoA
H_2O
Butyrate
Crotonyl-CoA
NADH
NAD+
ATP
ADP
CoA
P
Butyrate
Butyryl-P
Butyryl-CoA
NADH
NAD+
Butylaldehyde
NADH
NAD+
Butanol

Figure 6.3 ABE fermentation pathway.
Source: Wikipedia (https://en.wikipedia.org/wiki/Acetone%E2%80%93butanol%E2%80%93ethanol_fermentation)

propionic acid, and butyric acid [1, 7]. However, at reduced pH, the bacteria changes its morphology and starts the bioconversion of the feedstock to acetone, butanol, and ethanol. The interesting thing is that the butyric acid production enables butanol production, so is it possible to enhance butanol productivity directly. Reports show that the conversion of butyric acid to butanol can be used for the same. One study revealed that a 3% addition of butyric acid resulted in reduced production of undesired by products with double the yield of butanol [5].

6.3.3 Substrates Used for Biobutanol Production

A variety of substrates have been used for biobutanol production, including molasses and starch grains such as wheat, maize, corn, rye, etc. [8–13]. Biomass feedstock obtained from these crops has also been successfully used as substrates for biobutanol production. However, these substrates have alternatively been used to produce animal feed and other important metabolites; thus, extensive research has been carried out to utilize cheaper substrates. The cost of the substrate used turns out to be a major factor in deciding the economics of biobutanol production and its large-scale applicability. Wheat straw [11], corn fiber and liquefied starch [12, 13], whey [9], Jerusalem artichokes [8], and apple pomace [10] have emerged as cheaper alternative substrates for butanol fermentation [8]. Algal biomass has a high lipid content, which could act as an important substrate for biobutanol production. The manipulation of different process conditions also assists in increasing the yield of alcoholic fermentation, including:

1. Fed batch operation
2. Aeration of biomass without agitation
3. Hydrolysis and simultaneous fermentation with agitation and gas stripping
4. Gas stripping of solvents
5. Pervaporation technique with different membranes for solvent recovery to reduce product inhibition

Pre-treatment methods are required for increasing the availability of substrates for fermentation reaction. Lignocellulosic and hemicellulosic substrates need to be pre-processed to produce pentose sugars and cellulose hydrolysates. Short oligomeric fructans are the carbohydrates with inulinic structure that are present in Jerusulum artichokes, which need to be pre-treated with acid or alkali to hydrolyse the carbohydrate polymers [8]. Similarly, pre-treatment of cheese whey for precipitation and the removal of casein hydrolysate is required before the fermentation reaction can be carried out [9]. Cheese whey also contains lactose in low percentages, and the yield of fermentation is limited due to toxicity caused by the end product. However, the solvent yield of 15 g/L is observed with cheese whey whereas the observed yield with Jersulum artichokes is generally 24 g/L. Apple pomace has a carbohydrate content of 10% w/w, which consists of 6% fructose and 23% sucrose; its fermentation typically yields 2.2% butanol [10]. Algal biomass has emerged as the most promising source of biofuels. Algal biomass is rich in lipid content but pre-treatment is essential to degrade the tough algal cell wall consisting of the trilaminar structure of algaenan. A 4% glycerol supplementation of algal biomass has resulted in the yield of 16 g/L of butanol.

Many other substrates including liquefied corn starch, wheat straws, and hydrolysate of corn fibers (pre-treated with H_2SO_4) have also been recently tried with different culture conditions leading to yields of 81.3 g/L, 12 g/L, and 9g/L of ABE solvents respectively [11–13].

6.3.4 Microbial Strains for Biobutanol Production

The selection of microbial strains for an exacting process not only depends upon the fermentation substrate, but also on the ratio of the end products produced. *Clostridium acetobutylicum* is the most extensively used and successful microbial strain for ABE fermentation and biobutanol production. Further, *C. aurantibutyricum* [14] and *C. Tetanomorphum* [15] have been successfully utilized to increase the yield of biobutanol using biomass feedstocks. Various other strains have been used to enhance product yield and overcome end product inhibition. Different microbial strains that are used with alternative substrates are given below:

1. *C. pasteurianum* has been used to ferment algal biomass.
2. Lignocellulosic and hemicellulosic biomass have been fermented using *C. acetobutylicum*, Mixed microbial cultures have also been used along with enzymes capable of producing cellulose and hemicelluloses hydrolysates to enhance the production of Biobutanol.
3. Wheat straw has been fermented with *C. acetobutylicum,* which was co-cultured with cellulolytic fungi, *T. reesei*.
4. A mutant strain of *C. beijerinckii* developed by University of Illinois [16] gives an enhanced yield.

6.3.5 Purification of Biobutanol

Adsorption, extraction, membrane pertraction, reverse osmosis, pervaporation, and gas stripping are among the methods now being explored for cost and energy efficient recovery of butanol [17–20]. Pervaporation shows promise for simultaneous extraction purification and concentration of the alcohol [17]. Different methods for butanol recovery are outlined below:

6.3.5.1 Adsorption for Butanol Recovery

Adsorption had been evaluated for butanol recovery from the fermentation broth [17]. However, its application has been met with limited success due to the low capacity of adsorbents, which limits its application at industrial or pilot scale. Among the various adsorbents analyzed for the recovery process, silicate is the most extensively used adsorbent. This form of silica has a zeolite-like structure with hydrophobic properties. It has the ability to adsorb C1–C5 organic molecules from dilute aqueous solutions. Butanol, a C4 alcohol, is generally around at 3% concentration; hence, silicate is most suitable for its recovery. This separation from the fermentation broth has, however, been achieved at lab-scale only.

6.3.5.2 Membrane Processes for Recovery of Butanol

In order to reduce the energy and cost of separating biomass from the fermentation broth, the immobilization of microbial cells has been successfully applied

[18, 19]. Alternatively, membrane reactors, such as hollow fibre membrane reactor systems with capillary membranes, have been successfully used for the same. However, there are certain disadvantages associated with membrane reactors, including:

1. Poor mechanical strength
2. Increased resistance to mass transfer
3. Leakage of cells from immobilized membranes has been observed frequently.

6.3.5.3 Pervaporation for Recovery of Butanol

Pervaporation has emerged as one the most capable techniques for the separation of butanol (ABE), which is toxic to *Clostridium acetobutylicum* [19]. This method is based on the principle of selective transport of metabolites through a given membrane via diffusion while a vaccum is applied on the permeate side. After permeation, the vapours then condense on the side with lower pressure. A hydrophobic membrane is preferred to facilitate the transfer of organic compounds that are present in the fermentation broth. Silicon rubber and polydimethylsiloxane membranes have been commonly used for the same. The disadvantage of the technique is that the method becomes expensive as constant low pressure needs to be exerted and maintained.

6.3.5.4 Gas Stripping for Recovery of Butanol

Among the different techniques used for recovery of biobutanol, gas stripping is one the most promising after pervaporation. Gas stripping does not need any costly apparatus and is also harmless for the microbial producer culture. The nutrients and reaction intermediates concentration are also not affected by the method, and the product toxicity is reduced. By the application of heat or inert gases at low pressure values, volatile compounds can be easily separated from the concentrated sugar solutions. However, this technique has mostly been applied in combination with other separation methods [20].

6.4 Advancements in Biobutanol Production

Biobutanol has received increased popularity due to its associated advantages. The selection of microbial strain and fermentation substrates dictate the yield percentage and the speed of bioproduction. Continuous research is being done extensively to advance the field of optimum production of butanol. Microbial strains have been manipulated and improved for enhanced fermentation. Another important step in increasing the productivity is the downstream processing for product recovery and purification. Membrane-based techniques for separation of solvents are currently being evaluated to balance the economics of biobutanol production by reducing the cost by 40 to 50%. Genetic manipulation of microbial strains has resulted in culture conditions that do not require exhaustive labour and elevated costs. Many microalgal strains contain considerably high amount of carbohydrates and lipids in dry matter; *Chlorella* strains, for example, consist of 30 to 40%

carbohydrates, which greatly enhances its applicability in the production of biobutanol. Genetic modification has been successfully carried out on bacterial strains *C. acetobutylicum* and *C. beijerinckii* to amplify the resistance against the end product butanol.

Systematic removal of butanol using pervaporization and membrane-based separation techniques eliminates the end product toxicity on the producer strains in the fermentation broth. Traditionally, distillation has been the method of choice for recovery of alcohols. However, the boiling point of butanol is higher than that of the water, and so a considerable amount of energy is required for the process. Recovery by way of distillation becomes expensive and less feasible energetically, resulting in a low concentration of butanol, which necessitates alternative methodologies for its recovery.

Summary

With the depletion of fossil fuels reserves and rising cost of gasoline, alternative sources of energy are required to power internal combustion engines. Butanol has emerged as one such viable option that is safe to use and closely matches the properties of gasoline. Biochemical methods for producing biobutanol have reduced the cost of production, while the efficiency of product yield has increased. Various substrates ranging from grains, biomass feedstocks, molasses, and cellulosic and lignocellulosic materials have been used to produce butanol. *Clostridium acetobutylicum* is one of the widely used and established strains for butanol fermentation. However, dilute solution of biobutanol in fermentation broth and the end product toxicity of the alcohol increases the cost of production. The genetic manipulation of microbial strains has been carried out to increase the production yield of butanol from the mixture of acetone-butanol-ethanol (ABE) fermentations. Metabolic engineering of various *Clostridium* species for optimized butanol production in ABE fermentation is the most promising strategy for butanol bioproduction. Distillation has been the most commonly used method for alcoholic separations. However, pervaporation, gas stripping, and membrane-based methods are showing promise for cost-effective recovery of butanol from the fermentation broth. Thus, the biobutanol industry is environmentally friendly and causes a net positive effect on ecology. It has emerged as a better alternative for fuel additives in comparison with other alcohols, and its application will lead to reductions in greenhouse gas emissions.

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7

The Production of Biomethane from the Anaerobic Digestion of Microalgae

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Abstract
The current research addressing the use of microalgal biomass as a substrate for the generation of biogas was reviewed. It was recognised to have potential to partially replace fast depleting and environmentally harmful fossil fuels due to its ease of cultivation, ability to fix CO_2 during growth, low lignin content, and high growth rates. However, the technology is not currently feasible due to prohibitively high water consumption, inhibition phenomena during digestion, and seasonal variation in chemical composition. If the technology is to be developed to commercial scale, then these factors must be addressed.

***Keywords*:** Anaerobic, digestion, biogas, biomethane, biotrickling, purification, bioscrubbing, pre-treatment, microalgae, H_2S

7.1 Introduction

As the global demand for energy increases, greater attention is being paid to sources of ecologically sustainable alternatives to fossil fuels. First generation biofuels have shown initial potential, but have disadvantages that have

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undermined their suitability. They have been shown to lead to the production of more greenhouse gases than the use of fossil fuels and can directly compete with land that could be used for food crops (Fargione *et al.*, 2008; Searchinger *et al.*, 2008; Johansson and Azar, 2007). The high lignocellulosic content of second generation sources is recalcitrant to enzymatic action within fermenters and requires extensive pre-treatment leading to a lack of economic viability on a large scale (Yang *et al.*, 2015). Although originally lumped in with second generation biofuels, algae are now being considered a third generation fuel in their own right due to much higher yields, zero food source competition, and lower resource input. Microalgae also has a much higher theoretical biogas yield (Mentrez, 2012). Before biogas can be used in generators or added to national gas networks, all hydrogen sulphide and other trace gases must be removed to facilitate compression, raise calorific value, and prevent corrosion of metal components. Although this can be completed via chemical scrubbing protocols (Krischan, Makaruk and Harasek, 2012; Xu *et al.*, 2015), the systems involved are large and hard to scale down and can produce highly toxic by-products. More recently, environmentally sustainable biological methods such as biotrickling and bioscrubbing have been developed.

If the technology is to be scaled up to commercial level, then aspects such as integration into wastewater treatment plants to reduce clean water consumption and reduction of inhibition phenomena must be addressed.

7.2 The Process

The process is similar to many other anaerobic digestion (AD) processes. Extra elements such as pre-treatment, lipid extraction, and upgrading of the biogas to biomethane, however, add extra complexity, which has thus far hindered large-scale development (Figure 7.1).

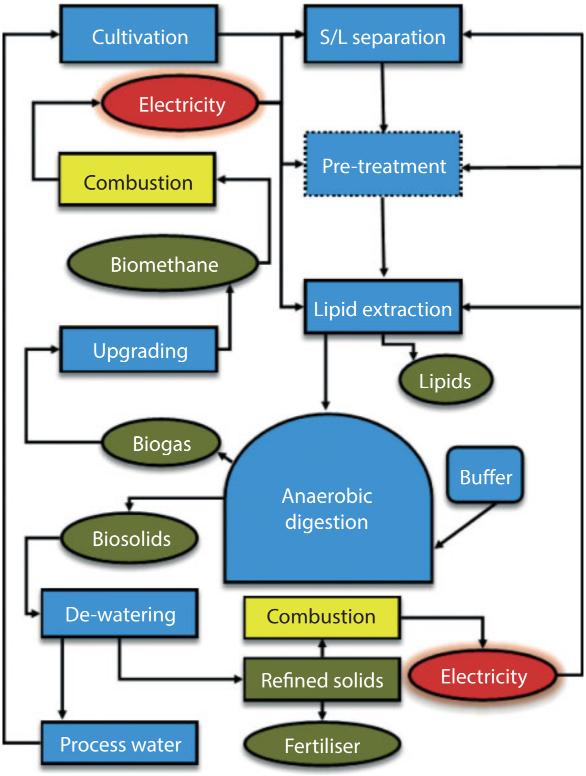
Cultivation
S/L separation
Electricity
Combustion
Pre-treatment
Biomethane
Lipid extraction
Upgrading
Lipids
Biogas
Anaerobic digestion
Buffer
Biosolids
De-watering
Combustion
Refined solids
Electricity
Process water
Fertiliser

Figure 7.1 Process overview. Although many aspects of the process have existed for a long time (solid/liquid separation using centrifugation, AD, etc.), others, such as pre-treatment and cultivation, are relatively new.

7.2.1 Selection and Cultivation of Microalgae

A large variety of microalgal species are available from culture collection, although great care should be taken as some species have lost some of their original properties due to being cultivated for many decades (Zhu, Rong and Zhong, 2013). Many factors should be taken into account when selecting individual species including salinity of water source, temperature, cultivation method, and nutrient requirements. Also microalgae can be either autotrophic or heterotrophic, and others, such as the *Chlorella* genus, which has become very popular for biofuel production, are mixotrophic and can adapt to almost any water type (Chu *et al.*, 2015). Cultivation of microalgae has been widespread for many years for the pharmaceutical industry and is predominantly carried out in two ways: in closed photobioreactors and in raceway ponds (Figure 7.2(a) and 7.2(b) and Table 7.1) (Collett *et al.*, 2011).

Despite having the benefit of being able to sequester waste carbon from industrial processes, large-scale microalgae production has been inhibited by water requirements, and electricity input into centrifugation, liquid transport, and heating. To take large-scale biodiesel production as an example, it has been shown that 3726 kg of fresh water is required to produce just one kilo of biodiesel (Yang *et al.*, 2011; Collett *et al.*, 2011). Although poor quality water supplies can be utilised, in some cases there remains a need for other nutrients, particularly nitrogen and phosphorus, that increases costs and potentially draws resources away from food crops (Chisti, 2007). Recycling of water is effective in

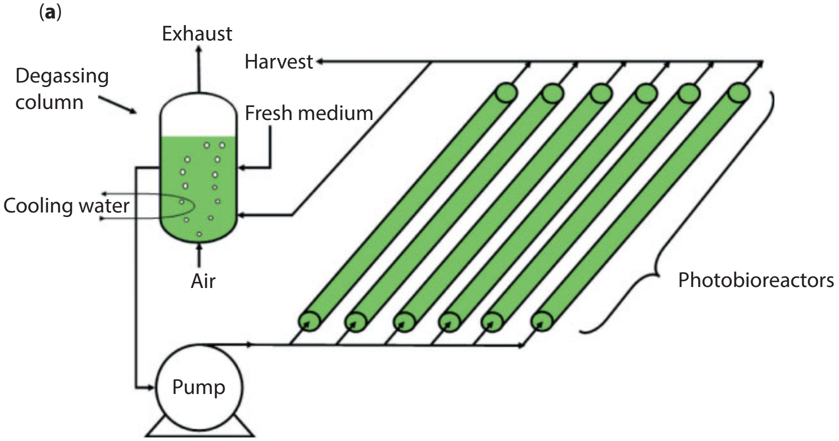

Figure 7.2 (a) Photobioreactor (closed system). Can take the form of tubular (illustrated) or plate design. They have the advantage of having a uniform light penetration throughout and a large illumination surface area. They are also easy to sterilise and relatively cheap to set up at a small scale. However, they are prone to blockages and fluctuations in air temperature. Due to the amount of glass and area usage, they are difficult to scale up (Collet *et al.*, 2011; Zhu, Rong and Zhong, 2013).

reducing water and energy consumption and improving growth of algae, but the use of chemical flocculants during harvest and essential pre-treatments prior to reintroduction into the process make it commercially unviable (Farooq *et al.*, 2015).

7.2.2 Pre-Treatment

Theoretical biogas production of microalgae after AD is 0.48–0.80 L CH_4/g volatile solids (VS) (Sialve, Bernet, and Bernard, 2009) using stoichiometric conversions. In practice, achieved results have been much lower at 0.05–0.31 L CH_4/g VS (González-Fernández *et al.*, 2012). Variability is attributed to macromolecular composition and cell wall characteristics. Cell walls are

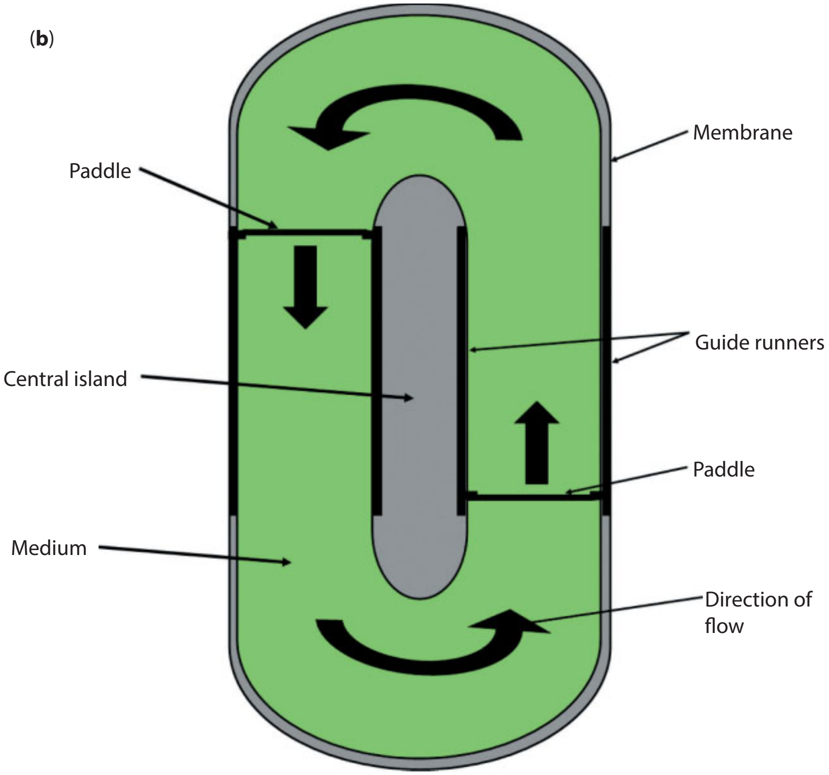

Figure 7.2 (b) Raceway pond (open system). The main advantages of open systems are that they are relatively cheap and easy to set up using simple engineering methods and that they can be built on agricultural lands. Although they are easy to maintain and clean, they produce low amounts of biomass per m^2 compared to photobioreactors and are only suitable for a small number of species (Collet *et al.*, 2011; Zhu, Rong and Zhong, 2013; Ali *et al.*, 2015).

composed of organic compounds and usually take the form of cellulose or hemicellulose, both of which have limited bioavailability. This hinders methane generation, as much of the organic matter contained within the cytoplasm remains inaccessible to methanogens. Four pre-treatment methods, to solubilise algal biomass prior to digestion, are available (Figure 7.3).

Table 7.1 Relative advantages/disadvantages of open and closed cultivation systems (Yang *et al*., 2011; Zhu, Rong and Zhong, 2013; Olivieri, Salatino, and Marzocchella, 2014; Ali *et al*., 2015).

Method	Advantages	Disadvantages
Raceway ponds	Low construction costs. Low energy requirements Simple to operate	Low production rates (0.1–0.5 g/L) for amount of area required High harvest costs High contamination risks Difficult to control biomass quality due to influence of weather which leads to difficulties with repeatability High water and CO_2 losses Long culture periods (6–8 weeks)
Closed systems	High production rates Very low water footprint Low area footprint Much easier to control quality of biomass due to limited effect of weather and therefore much higher level of repeatability Short culture period (2–4 weeks)	High construction costs Skilled operators required

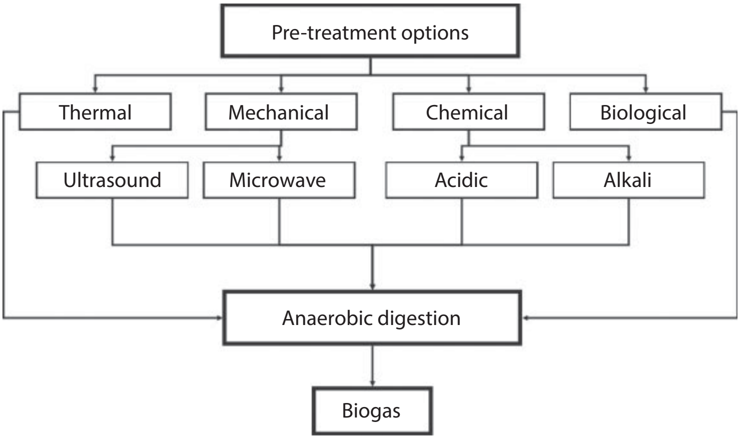
Pre-treatment options
Thermal
Mechanical
Chemical
Biological
Ultrasound
Microwave
Acidic
Alkali
Anaerobic digestion
Biogas

Figure 7.3 Many pre-treatment options exist to carry out cell disruption and facilitate the hydrolysis stage of digestion.

7.2.2.1 Thermal Pre-Treatment

Algal biomass does not respond well to low temperature thermal treatments (<100 °C), even during extended exposure times (>10 hours) (Weil, 1994). Alzate *et al.* (2012) found that a pre-treatment of 50–70 °C had little effect on methane production, but with a >100 °C treatment, a 60-220% increase was observed (species dependent).

Thermal treatment with steam explosion has been in use for a long time in wastewater treatment plants where it has been shown to improve biogas yields by <100 °C (Kepp *et al.*, 2000). Biomass is held at high pressure in a vessel under high temperature before having the pressure suddenly released to disrupt the cells. It has been found at laboratory scale by Mendez *et al* (2014) that an increase in methane yield of <80 °C was possible using this method on microalgae.

7.2.2.2 Mechanical Pre-Treatment

Mechanical pre-treatments involve the direct disruption of the algal cells using physical forces. Ultrasound and microwaves have shown promise although they both require large amounts energy and therefore may not be lend themselves well to larger processes (Passos *et al.*, 2014).

7.2.2.3 Chemical Pre-Treatment

Chemical treatments have not been widely used due to the potential for interference of chemicals with the digestion process, particularly the formation of toxic by-products for methanogens, although it has been suggested by Passos *et al* (2014) that a small increase in pH may help the acidogenesis stage of digestion.

7.2.2.4 Biological Pre-Treatment

Enzymic pre-treatment is perhaps the most promising of all due to the small amount of energy required to carry it out. Cell lysis has been achieved after a 180-minute treatment of *Chlorella sorokiniana* with endo-b-1,4-glucanase (Fu *et al.*, 2010). Ehimen *et al.* (2013) also noticed a 20% increase in methane yield after a combined blending of *Rhizoclonium* sp. with five enzymes, and suggested that chain hydrolysis may be the key to a successful treatment.

7.2.3 Lipid Extraction

Lipids can be extracted from algal biomass prior to digestion and can then be converted into biodiesel (description of the biodiesel production process is beyond the scope of this paper, although Halim, Danquah and Webley (2012) is an excellent source). High lipid content is thought to produce inhibitory metabolites during digestion and its extraction – particularly using $SCCO_2$ – can significantly improve methane yield (Hernández *et al.*, 2014).

7.2.4 Digestion

Extensive research into the AD process has been going on since the 1930s (Mao *et al.*, 2015). Many good reviews have been published regarding the different aspects of the process (Kythreotou, Florides and Tassou, 2014; Mao *et al.*, 2015; Astals *et al.*, 2015); essentially there are four microbial processes that run contiguously within the digester – hydrolysis, acidogenesis, acetogenesis, and methanogenesis – that break down large organic molecules into constituent parts (Figure 7.4).

During hydrolysis, proteins, cellulose, and lipids are broken down by a consortium of bacteria – using extracellular enzymes – into amino acids, monosaccharides, glycerols and fatty acids (Batstone and Jensen 2011). Acidogenesis involves the fermentation and β-oxidation of the above products into organic acids, ketones, alcohols, acetate, $CO_{2,}$ and hydrogen. Acetogenesis is the conversion of fatty acids and alcohols to acetate, hydrogen and CO_2 by syntrophic acetogens. These products are then utilised by methanogenic archaea and prokaryotes to produce CH_4, CO_2 and H_2S (Appels *et al.*, 2010).

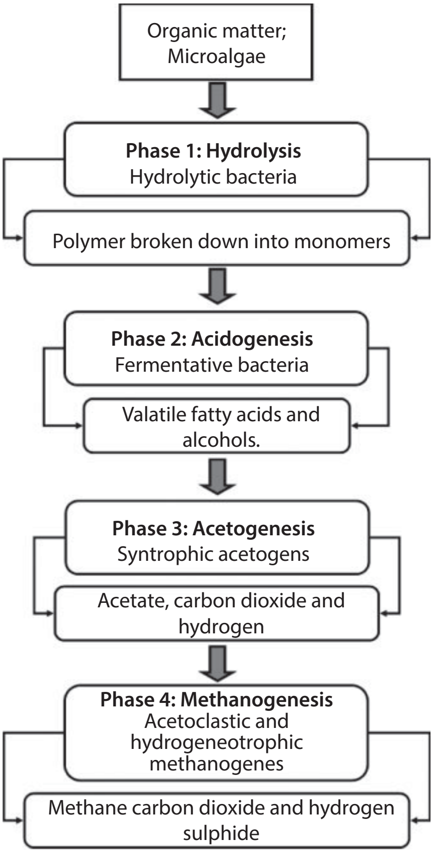
Organic matter;
Microalgae
Phase 1: Hydrolysis
Hydrolytic bacteria
Polymer broken down into monomers
Phase 2: Acidogenesis
Fermentative bacteria
Valatile fatty acids and alcohols.
Phase 3: Acetogenesis
Syntrophic acetogens
Acetate, carbon dioxide and hydrogen
Phase 4: Methanogenesis
Acetoclastic and hydrogeneotrophic methanogenes
Methane carbon dioxide and hydrogen sulphide

Figure 7.4 The AD process is long existing and well-documented. More research is still required, however, particularly into the biodiversity that exists within the digester and its plasticity during the four stages illustrated above (Vanwonterghem *et al.*, 2014; Jiménez *et al.*, 2014: Carballa, Regueiro and Lema, 2015). The residual biomass that is left over at the end of the digestion process is high in elemental value but is currently underutilised due to public perception of waste and legislative barriers. The potential benefit of digestate as a soil conditioner is well-reported, with high levels of available nitrogen and stable carbon (Baugnom *et al.*, 2012). However, at the current time, most goes to landfill at the cost of the plant operator. **N.B**. Great care should be taken when operating anaerobic digesters of any kind, particularly when gaining entry to the vessel itself to carry out maintenance/ inspections. H_2S produced during the latter stages of the process is highly toxic and is potentially fatal to humans at concentrations of >300 ppm (Berge, Zwart and Appelman, 1986).

Hydrolysis is recognised as the primary rate-limiting step due to the slow degradation of insoluble polymers, although with appropriate pre-treatment, this can be mitigated (see section 7.2.2).

7.2.4.1 Inhibition of the Digestion Process

Inhibition of AD by a multitude of toxicants is seen as one of the largest obstacles to integration of the technology into commercial biogas operations.

7.2.4.2 Ammonia

Although NH_4^+ <1,500 mg L^{-1} does not inhibit methanogenesis, concentrations of as little as 80 mg L^{-1} of NH_3 have been shown to have a deleterious effect (Chen, Cheng and Creamer, 2008). NH_3 content increases with temperature and pH. Substrates with a high nitrogen fraction (such as microalgae) have also been shown to increase NH_3 production (González-Fernández *et al.*, 2011).

7.2.4.3 Volatile Fatty Acids

Production of VFAs at >6,000 mg L^{-1} is thought to inhibit methanogenic activity at the bottom of the reactor and appears to be closely linked to loading rate (Jegede, 2012).

7.2.4.4 Hydrogen Sulphide

The presence of H_2S slows methanogenesis and reduces overall production due to competition for available hydrogen at concentrations of >100 mg L^{-1} (Chen *et al.*, 2014).

7.3 Downstream Processing and Use of Gaseous Products

7.3.1 Purification

Before biogas can be utilised in combustion engines or integrated into national grids, it must first be upgraded to biomethane by reducing CO_2 and H_2S content to 65–330 ppm (Peu *et al*., 2012). Usually this has involved physical and chemical processes such as pressure swing adsorption, pressured water scrubbing (PWS), chemical scrubbing, and membrane separation (Kapdi *et al*., 2005). These do not lend themselves to smaller scale processes and can produce environmentally harmful by-products (Starr *et al*., 2012). The need to limit the environmental impact of energy production has led to the development of less damaging and more compact biological methods specifically for use within AD processes. These involve exposing contaminated gases to bacteria capable of degrading the toxic components. Once all toxic components have been removed from the biogas, it can be compressed to the same pressure as distribution networks (<13.8 bar) and introduced to the grid (Kapdi *et al*., 2005).

7.3.1.1 Bioscrubbing

Bioscrubbing involves bringing the raw gas stream into high pressure contact with a wash water stream to bring some components into a liquid phase. The wash water is then passed through a biological reactor where the contaminants are oxidised by aerobic microorganisms into less harmful substances such as sulphates and biomass (Figure 1.5). Bio scrubbing has the advantage that it can remove up to 98% of H_2S even at high flow rates and is not sensitive to fluctuations in volatile organic compounds (VOC) (Potivichayanon,

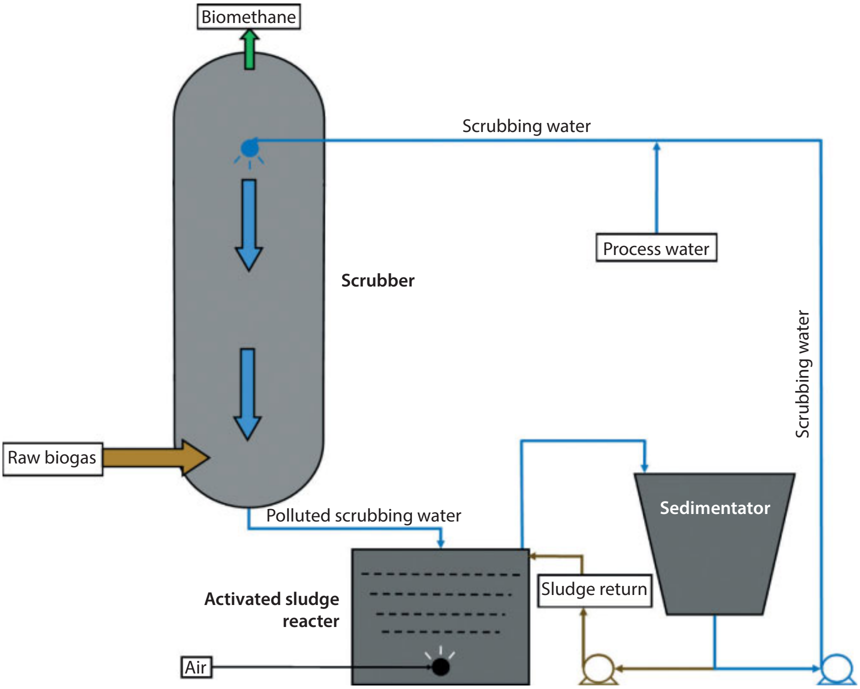
Biomethane
Scrubbing water
Process water
Scrubber
Scrubbing water
Raw biogas
Sedimentator
Polluted scrubbing water
Sludge return
Activated sludge reacter
Air

Figure 7.5 Bioscrubbing. Pollutants are absorbed into the wash water inside the scrubber. Wash water is then transferred to the reactor where pollutants are degraded by aerobic bacteria. Clean wash water is recycled into the scrubber where it can reabsorb more pollutants. To avoid biological degradation, salt content must be controlled via frequent draining and addition of fresh wash water. A constant source of carbon and thiosulphate-rich nutrients must be supplied to the organisms in the reactor along with oxygen to facilitate the breakdown of pollutants (Diaz *et al.*, 2010). Although adjustment of microorganisms to reactor conditions and composition of gases can take up to a few weeks, <98% reduction of H_2S levels have been reported using bioscrubbers simultaneously inoculated with *Acinetobacter* sp. MU1 03 and *Alcaligenes faecalis* MU2 03 (Potivichayanon, Pokethitiyook and Kruatrachue, 2006).

Pokethitiyook, and Kruatrachue, 2006). However, the system does produce sludge waste and wash water that must be further treated before discharge.

7.3.1.2 Biotrickling

Biotrickling, although similar to bioscrubbing, does not require a second bioreactor and therefore lends itself well to smaller scale processes (Figure 7.6). Bacteria are immobilised on a filter material, usually synthetic foam or structured plastic, which then has the scrubbing water continuously trickled over it. Although capable of running at low pH and highly effective in H_2S removal (<80–90%), the system is more prone

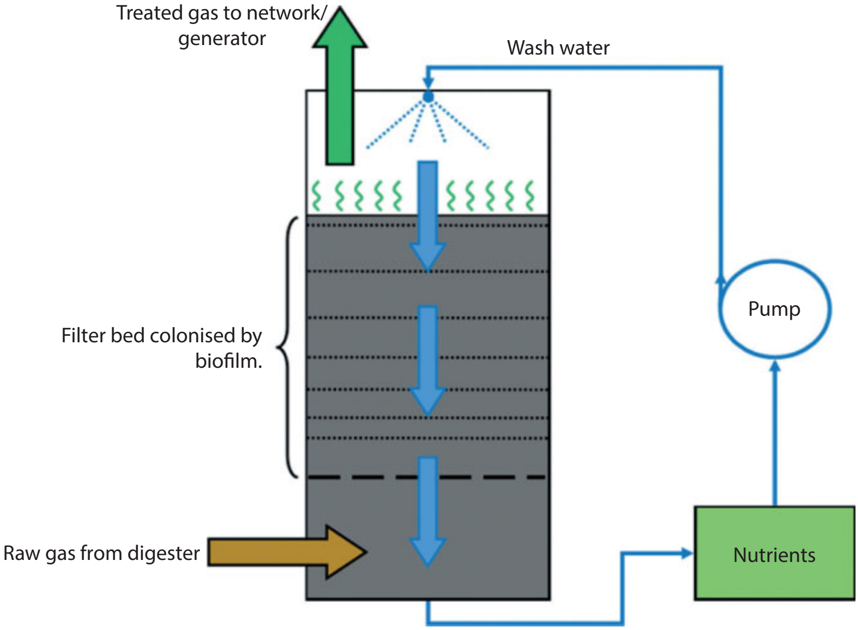
Treated gas to network/ generator
Wash water
Pump
Filter bed colonised by biofilm.
Raw gas from digester
Nutrients

Figure 7.6 Biotrickling has a number of advantages such as suitability to small-scale processes and ease of operation.

to failure than bioscrubbing (Fortuny *et al*., 2009). Strong growth of the biofilm can lead to preferred flows through the packing causing sudden drops in efficiency and the need to remove and replace packing. Although little research has been carried out into the species inhabiting the biofilm (most inoculations are taken from sewage treatment sludge), it has been found by Montebello *et al*. (2013) that pH fluctuations can have a negative effect on the diversity of bacterial genera inhabiting the biofilm with *Acidithiobacillus* and *Acidophilum* sp. Dominating, although this did not have a significant effect on the removal of reduced sulphur components. In contrast, sharp rises in pH have been found to have a negative effect on function. Despite these limitations biotrickling is still a viable option for small-scale operations due to its small size, lower operating costs and its insensitivity to gas supply shutdowns (Ramirez *et al*., 2009).

7.3.2 Product Use: Current and Potential

No large-scale biogas production operations utilising microalgal biomass currently exist. However, a large proportion of biogas is used at the site of production in combined heat and power (CHP) engines. The transition from this use to larger scale processing that can clean and compress large and continuous quantities of gas, which can then be used for powering vehicles or introduced to national grids, is hindered by the current decentralised nature of production (Lantz, 2012; Olugasa, Odesola and Oyewola, 2014). Due to the potential of algal biomass to produce biomethane continually,

Table 7.2 Comparison of the benefits and disadvantages of the two most common biological modes of biogas purification.

Purification method	Advantages	Disadvantages
Bioscrubbing	• Not sensitive to fluctuations in in supply emissions • Can cope with larger volumes • Cheap investment and exploitation • Low electricity and heat requirements • No extra chemicals or equipment required • Simple operation and maintenance	• Production of sludge, which must be disposed of • Discharge water requires further treatment • Excessive air input results in explosive mixture • More space required than biotrickling operations
Biotrickling	• Acid waste waters can be used to neutralise alkaline wastes • Uses little energy (< 1 kWh/1 000 Nm^3) (Jiang and Tay, 2010) • Can be operated at ambient conditions (atmospheric pressure and 30–35 °C optimum temperature) (Jiang, Yang and Tay, 2009) • Cheap investment and exploitation • Low electricity and heat requirements • No extra chemicals or equipment required • Simple operation and maintenance	• Must have relatively consistent supply of emissions • Overgrowth of biofilm can lead to dysfunction and the need to replace packing • Excessive air input results in explosive mixture

and of uniform quality, it lends itself to the future development of larger centralised operations. Also, 1.5 billion people in the developing world currently do not have access to electricity and 3 billion burn biomass as their primary energy source (Surendra *et al.*, 2014). To facilitate sustainable development, access to renewable and sustainable energy is imperative. Improvement of biomass technologies represents an opportunity to generate this access while simultaneously preserving the local and global environment.

7.4 Conclusions

The AD of microalgae for the production of biogas has great potential due to its ease of cultivation and low lignin content. However, many obstacles remain in the way of commercial scale up such as prohibitively high water consumption, variable quality/quantity of gaseous products and inhibition phenomena. If these problems are to be overcome, then more research is necessary into the diversity and function of microbial life within the digester and the development of a more robust approach to cultivation, such as integration into sewage treatment processes. As the process is new, certain opportunities exist to develop it in a sustainable way, such as the centralisation of operations at their inception to allow for proper integration into existing natural gas networks instead of more scattered individual projects.

As research into alternatives to fossil fuels advances, it is becoming apparent that a single panacea to completely replace them may never be found, and that the combined use of a multitude of green technologies may be the only real alternative.

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8

Electrohydrogenesis: Energy Efficient and Economical Technology for Biohydrogen Production

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Abstract

For a long time, petroleum-based products such as petrol, diesel, and gasoline constituted a major share of our energy sources. However, exhausting stocks of fossil fuels and environmental concerns owing to increasing CO_2 concentration due to their combustion have made it obligatory for us to develop alternative sources of energy. Hydrogen has been identified as an ideal replacement for fossil fuels. It has the highest specific energy content with 142 MJ/kg, much higher than gasoline (46 MJ/kg) or coal (24MJ/kg), and has a clean combustion profile, i.e. emits no greenhouse gasses after burning, as water vapor is the only combustion by-product. These features make hydrogen a fitting alternative to conventional petroleum-based fuels and has been widely accepted as an environmentally safe and renewable energy source which does not contribute to greenhouse gasses (Das, 2009).

Most of the present day hydrogen (about 95%) is produced from fossil fuel sources (Balat and Kırtay, 2010) by various thermo catalytic and gasification methods such as hydrocarbon reforming, plasma reforming, ammonia reforming, and other methods like pyrolysis etc. These methods are highly energy intensive and operate at high temperature and pressure conditions. Therefore, these industrial

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methods are not economic in their approach towards hydrogen production. Electrohydrogenesis is a recently discovered electrolytic method for biohydrogen production, wherein biodegradable material is converted into hydrogen by specific exoelectrogenic bacteria in modified microbial fuel cells (MFCs) called microbial electrolysis cells (MECs) (Cheng & Hamelers, 2008; Rozendal *et al.*, 2006). Electrohydrogenesis, as a method for biohydrogen production, enjoys several apparent advantages such as versatility in substrate use, high yield, and energy efficiency of hydrogen production, etc. These advantages make electrohydrogenesis an ideal alternative for biohydrogen production.

The present book chapter discusses the basic biochemistry and working principle of hydrogen production process by electrohydrogenesis, its design, components, and the setup of a basic microbial electrolytic cell reactor. In the end, the basic mathematical calculations associated with determination of energy efficiency of the process are also included.

Keywords: Biohydrogen, electrohydrogenesis, microbial fuel cell, microbial electrolytic cell, energy efficiency

8.1 Introduction

8.1.1 The Present Energy Scenario

Ever since the industrial revolution, the availability of better fuels and energy sources have been a catalytic factor in deciding the pace of mankind's development. Fuels, should not only satisfy energy requirements, but should also be easy for production, operation, and storage. For a long time, petroleum-based products such as petrol, diesel, and gasoline constituted a major share of our energy sources. However, exhausting stocks of fossil fuels and environmental concerns owing to increasing CO_2 concentration due to their combustion have made it obligatory for us to develop alternative sources of energy. Hydrogen has been identified as an ideal replacement for fossil fuels. It has the highest specific energy content with 142 MJ/kg, much higher than gasoline (46 MJ/kg) or coal (24 MJ/kg). It also has a clean combustion profile, i.e. emits no greenhouse gasses after burning, as water vapor is the only combustion by-product, and since it can be produced from biomass, it has an easy availability of substrates. These features make hydrogen a fitting alternative to conventional petroleum-based fuels and has been widely accepted as an environmentally safe and renewable energy source which does not contribute to greenhouse gasses [1].

8.1.2 Biohydrogen: The Current Status

A number of technologies are available and known for hydrogen production. Most of the present day hydrogen (about 95%) is produced from fossil fuel sources [2] by various thermo catalytic and gasification methods such as hydrocarbon reforming, plasma reforming, ammonia reforming and other methods like pyrolysis etc.

As can be seen from Figure 8.1, biomass-based hydrogen production contributes just 1% of the total hydrogen production of world, while fossil fuel based process accounts up to 95% of total hydrogen production. However, these methods, although have been used most extensively for commercial hydrogen production suffer from various drawbacks. Since these

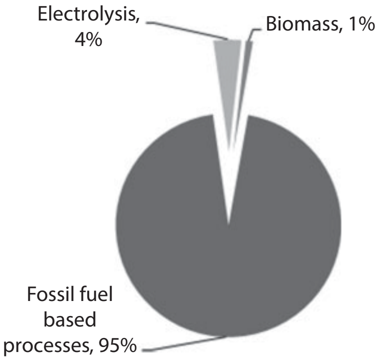

Figure 8.1 The current status of hydrogen production processes. Fossil fuel based processes account for 95% of world's hydrogen production, while electrolysis and biomass based methods contribute to only 4% and 1% respectively.

methods produce hydrogen from fossil fuel, they release carbon dioxide in the atmosphere which is the main contributor to the greenhouse effect. Also, these methods are highly energy intensive and operate at high temperature and pressure conditions. Therefore, these industrial methods are not economic in their approach towards hydrogen production. To make the process more sustainable and economic, it is therefore necessary to develop biological routes of hydrogen production. Biological hydrogen or bio-hydrogen production is advantageous over industrial methods as they can be operated at ambient conditions of temperature and pressure and uses biomass as substrate which is abundant and virtually inexhaustible [1]. Therefore biohydrogen production has gained considerable attention in the last few years as it promises hydrogen production with sustainable development and waste minimization [3].

8.1.3 Electrohydrogenesis: Need of the Hour

The major bioprocess technologies that have been used for biohydrogen production include biophotolysis of water by algae and cyanobacteria, water-gas shift reaction, and dark or photo fermentation reaction by anaerobic bacteria. Of these methods, the most intensively studied methods are fermentation methods, i.e. dark or photofermentation. However, considering the low rate and yield of hydrogen (in the case of dark fermentation) and high costs of production (in the case of photofermentation), these methods are again not fruitful for commercial-scale economic biohydrogen production.

Another potential method of biohydrogen production that has recently been discovered is electrohydrogenesis. It is essentially an electrolytic method, wherein biodegradable material is converted into hydrogen by specific exoelectrogenic bacteria in modified microbial fuel cells (MFCs) called microbial electrolysis cells (MECs) [4, 5]. In microbial fuel cells, exoelectrogenic bacteria consume organic matter in an anodic chamber and release electrons and protons outside the cell. The electrons travel to cathode from anode via an external circuit. The protons either travel through a membrane or reach the cathode due to potential difference. At the cathode, electrons and protons are captured by oxygen to form water (vapor). The electron flow generates an electric current. In the MEC, the MFC is modified such that the cathode is made anaerobic, and a small external voltage is applied across the electrodes of the cell. The external voltage is required because otherwise, in a MEC where the cathode is anaerobic, the movement of electrons from anode to cathode is non-spontaneous due to thermodynamic limitations. Therefore, the external voltage pulls the electrons from anode to cathode where they reduce the protons to form hydrogen. Electrohydrogenesis, as a method for biohydrogen production, enjoys several apparent advantages. Foremost, any biodegradable organic material can be used as a substrate by either pure or mixed bacterial consortia for hydrogen production via electrohydrogenesis. Carbohydrates, proteins [6], organic acids [7, 8], fermenter effluents [9], and waste waters [10–13] are some of the substrates that have been reported to be used in MECs. Also, MECs produce hydrogen at much higher yield and rate and energy efficiency than any other biological process. Cheng and Logan [8] have reported electrohydrogenesis with an energy efficiency in the range of 68–91% using various substrates like cellulose, glucose, acetate, and butyrate. Additionally, the reactor fabrication cost for MECs is much less as the reactor can be assembled using cheaply available materials such as polyacrylates and polycarbonates. Such materials confer low internal resistance to the cell, are robust, and easy to scale up. These advantages make electrohydrogenesis an ideal alternative for biohydrogen production.

8.2 Microbial Electroytic Cell

8.2.1 Working Principle

The process of electrohydrogenesis is carried out in microbial electrolysis cells or MECs as they are commonly called. The MEC reactors have also been called by different names such as ***biocatalysed electrolysis cells (BECs)*** as the process involves biocatalysed electrolysis of organic matter, or ***bio-electrochemically assisted microbial reactor (BEAMR)*** as an external additional voltage must be applied to carry out the process. How the biological and electrical assistance together help in producing hydrogen can be explained by looking at an example. Consider a MEC inoculated with exoelectrogens and acetate as substrate. Acetate at anode is oxidized by the exoelectrogens and is broken down as given in Equation (8.1):

$$CH_3COO^- + 4H_2O \longrightarrow 2HCO3^- + 9H^+ + 8e^- \quad (8.1)$$

The above equation depicts the anodic half-cell reaction of the MEC. The 8 electrons released reduce the protons at cathode as in Equation (8.2):

$$8H^+ + 8e^- \longrightarrow 4H_2 \quad (8.2)$$

Equation (8.2) depicts the cathodic half-cell reaction of the MEC. Nernst equation calculations as shown by [14] reveal that maximum potential of the anodic half-cell reaction, as compared against a Standard Hydrogen Electrode (SHE), could be –0.3 V and that for cathodic half-cell could be –0.414 V. Thus, the net *EMF* of the cell to produce hydrogen at cathode would be:

$$E_{EMF} = E_{cat} - E_{an} = (-0.414\ V) - (0.3\ V) = -0.114\ V.$$

The negative net cell voltage depicts that the process is non-spontaneous. Therefore, an external voltage supply of more than 0.114 V must be supplied across the cell to overcome the thermodynamic barrier and enable transfer of electrons from anode to cathode. Thus, the whole process of hydrogen production in MEC is carried out together by microbial oxidation of substrate at anode followed by proton reduction at cathode by electrical assistance. Although, in theory, an external voltage of more than 0.11 V is sufficient for electrons to move to cathode from anode, but in practice, a minimum of 0.2 V is required for hydrogen production. This is because of development of electrode overpotentials at cathode and anode [4, 10, 15, 16].

8.2.2 Design

Microbial electrolytic cells, or MECs, are designed by slight modifications of microbial fuel cells, or MFCs. These include making the cathodic chamber completely anaerobic and attaching an additional external power source (0.2–0.8 V). MECs can either be single chambered or double chambered. In single chambered MECs, there is no partition between the anodic and cathodic chamber, while in a double chambered MECs, an ion exchange membrane separates the MEC into anodic and cathodic chambers [5, 10, 16, 17]. Use of an ion exchange membrane prevents hydrogen from diffusing back to anode from cathode, where it might be reconsumed by methanogenic bacteria which fix hydrogen into methane. Also, the presence of the membrane prevents hydrogen from mixing up with other gaseous metabolic by-products such as carbon dioxide etc., thereby maintaining its purity. However, use of an ion exchange membrane increases the internal resistance of MEC [18] leading to low current densities and coulombic efficiencies of the cell. Additionally, an ion exchange membrane leads to the development of a pH gradient across the cell leading in loss of potential and electron transfer across the cell. An ion exchange membrane also significantly increases the overall setup cost of the reactor, thereby making the process expensive [15, 16, 19]. Other than that, membrane fouling and fabrication complications are other disadvantages associated with a double chambered MEC. Figure 8.2 depicts a detailed line diagram of a single and double chambered MEC and shows how hydrogen is produced in both.

The MEC reactor for hydrogen production can be fabricated using different materials including glass or plastics such as polyacrylates or polycarbonates. Where polyacrylates are brittle and cheaper, polycarbonates are durable, stronger, resistant to scratches, and highly transparent to light. Plastics as material for reactor fabrication are the obvious choice over glass as they are robust, offer low electrical resistance as compared to glass, and can be easily scaled up. MEC reactors also have been reported in different geometrical shapes. While the most common shape is cuboidal, cylindrical [15], bottle shaped [20] or tube-shaped [21] MEC reactors have also been reported.

8.2.3 Setting up the Reactor

The MEC reactor, as can be seen in Figure 8.2 can either be a single chambered or double chambered.

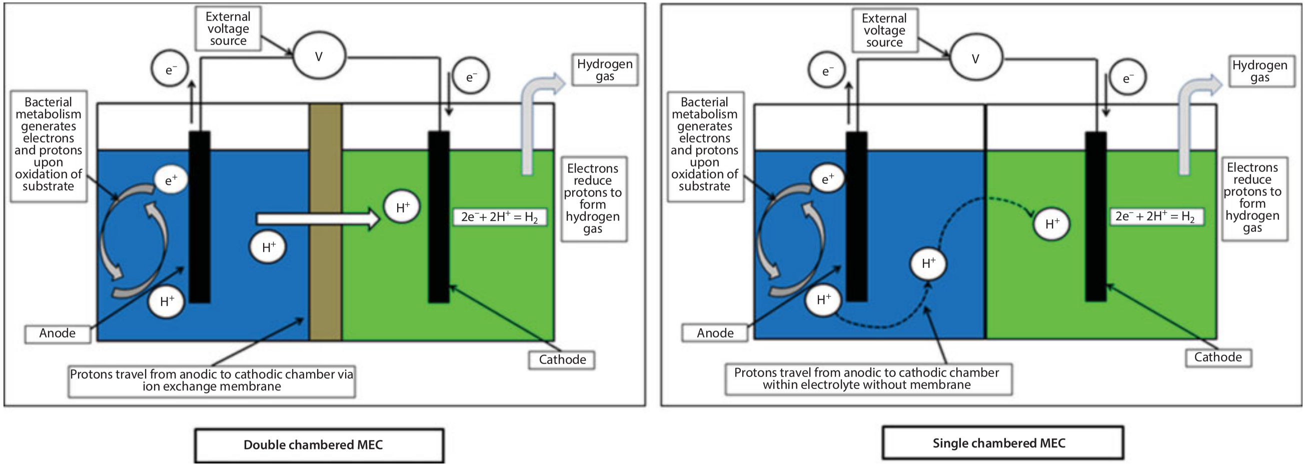
External voltage source
V
e⁻
e⁻
Hydrogen gas
Bacterial metabolism generates electrons and protons upon oxidation of substrate
e⁺
H⁺
H⁺
H⁺
2e⁻+ 2H⁺ = H₂
Electrons reduce protons to form hydrogen gas
Anode
Cathode
Protons travel from anodic to cathodic chamber via ion exchange membrane
Double chambered MEC
External voltage source
V
e⁻
e⁻
Hydrogen gas
Bacterial metabolism generates electrons and protons upon oxidation of substrate
e⁺
H⁺
H⁺
H⁺
2e⁻ + 2H⁺ = H₂
Electrons reduce protons to form hydrogen gas
Anode
Cathode
Protons travel from anodic to cathodic chamber within electrolyte without membrane
Single chambered MEC

Figure 8.2 Electrohydrogenesis is a key technology for energy efficient and economic biohydrogen production. The process is carried out in either single or double chambered MEC reactors. In double chambered MEC, an ion exchange membrane separates the anodic and cathodic chamber, while in single chambered MEC, no such separation is present. The key players of electrohydrogenesis are exoelectrogenic bacteria which metabolize a range of biodegradable substrate and release electron and protons in anodic chamber which further combine in cathodic chamber to produce biohydrogen.

A double chambered reactor has an anion exchange membrane that separates the anodic and cathodic chambers. Initially before setting up the MEC reactor, the electrodes are placed at their respective positions, and the chamber is fully flushed with ultra-high pure nitrogen or argon gas. This is done to replace the oxygen with an inert gas, since the presence of oxygen would be detrimental for hydrogen production. The reactor is then properly sealed, filled with the desired media and inoculated with exoelectrogenic bacteria. All the steps after are carried out aseptically to inhibit contamination. The electrodes are connected to an external voltage supply that provides the necessary potential for hydrogen production. The anode of the MEC is connected to a variable resistor which is in turn connected to the positive terminal of the voltage. The cathode is connected directly to the negative terminal of the voltage source. The current produced in the MEC is measured across the external resistor at various short time intervals and is used to evaluate the energy efficiency of the system, as is explained later. The gas port is connected near the cathode terminal where hydrogen gas is collected in gas bags or other collecting apparatuses. To characterise the gas for the presence of hydrogen, gas chromatography is done, and the retention time is matched to that of pure hydrogen. After a cycle is complete, the reactor is exposed to oxygen to inhibit the growth of methanogens and is again flushed with any inert gas and sealed for the next cycle. Figure 8.3 shows a line diagram of MEC reactor assembly.

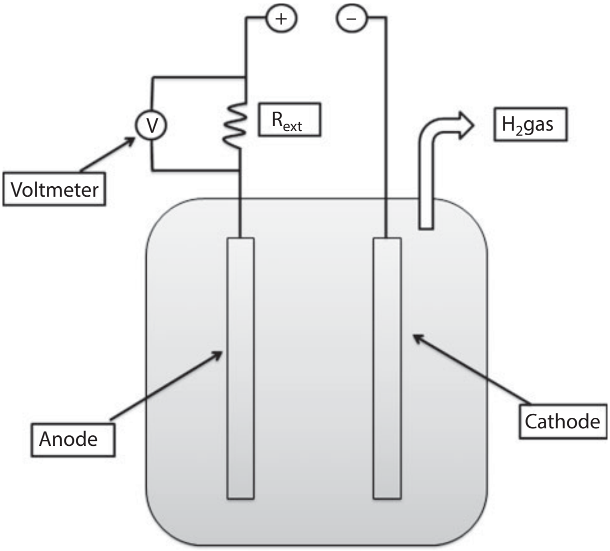

Figure 8.3 The figure depicts the assembly of an MEC. The anode of the reactor is connected to the positive end of the external power source through a variable resistor, and the cathode to the negative terminal. The voltage (**V**) through the resistor is measured by a voltmeter attached in parallel to the circuit. The current through the circuit (**I**) is calculated using Ohm's law ($\mathbf{V = IR_{ext}}$).

8.2.4 Fuelling the MEC Reactor: Substrates

The economic efficiency of any process is largely determined by the input cost of the process. In the case of hydrogen production, the nature and availability of substrates is an important factor that ascertains the competence of the process. This is primarily the reason why electrohydrogenesis is considered more economical and energy efficient than fermentation reactions, as it can effectively utilize the end products of both dark and photofermentation, and produce hydrogen from the otherwise so called dead-end fermentation products such as acetate and butyrate. Furthermore, the diversity of anode inhabiting microbial communities makes it feasible to feed the MEC with a variety of substrates that can be oxidized by the exoelectrogens releasing electrons outside the cell, which ultimately leads to production of hydrogen. A wide range of substrates have been reported to be used for MEC including pure substrates such as glucose, xylose, and glycerol, industrial wastewaters, agricultural wastes such as husk and straw from rice, wheat or maize, as well as complex polymers such as cellulose

Use of industrial wastewaters or effluents as substrates is what makes biohydrogen production from MEC highly energy efficient and cost-effective. Industrial effluents mostly carry oxidized wastes which are produced as byproducts in industrial processes and hardly have any economic value. Their utilization as substrates in MEC serves the dual purpose of hydrogen production and wastewater treatment. Using such a cheap starting material greatly reduces the input costs and makes the overall economics of the process technically and commercially viable. However, low exoelectrogenic activity cultures with wastewaters and high ohmic losses are a few issues related to the use of wastewaters as a substrate for hydrogen production in MEC. The exoelectrogenic capacity of many bacterial cultures is reported to be low with wastewaters as substrate since their composition is far more complex than pure, easily biodegradable substrates such as acetate, butyrate, or lactate. Furthermore, wastewaters have low conductivity and therefore, their use as substrate leads to high ohmic losses. Consequently, exoelectrogens which are acclimatized to grow in industrial wastewaters should be used as inoculum, and the ionic strength of wastewater should be adjusted so as to reduce ohmic losses and to make the process energy efficient and economical in operation.

8.2.5 Powering the MEC Reactor: Exoelectrogens

Exoelectrogens are those anaerobic bacteria which have the ability of transferring the terminal electron of their electron transport chain directly outside the cell. This ability of exoelectrogens is exploited in MFCs for current generation and in MECs for hydrogen production. These bacteria are known to transfer electrons across the cell membrane via these major mechanisms. The first mechanism is via nanowires or conductive appendages, which were first reported in *Geobacter* and *Shewanella* [22]. Another mechanism of electron transfer is via cell surface electron transfer. Such bacteria grow surface protrusions even in the presence of nanowires that act as conductive points of contact [14]. Another mechanism by which exoelectrogens transfer electrons outside the cell is via mediators, which could either be endogenous (produced by bacteria itself) or exogenous (added into the medium externally). Among the bacterial species that produce the endogenous mediator, the most prominent is *Pseudomonas aeruginosa*, which produces the pigment pyocyanin [23]. Another bacteria that has been reported to produce pigments is *Geothrix fermentans* [14]. Other bacteria such as *E. coli,* which do not have any mechanism of electron transfer, have been reported to produce current in MFC with the help of mediators added externally into the medium. Many such chemicals have been used as mediators for shuttling electrons from the cell to outside in MFCs. A few examples include neutral red, potassium ferricyanide, methyl viologen, thionin etc. [14].

For electrohydrogenesis in an MEC, a mixed consortia of exoelectrogens, mostly habitating in sludge or sewage wastewaters have been preferred [24, 25] Heterogeneous populations of bacteria are more tolerant to environmental changes and have low risk of contamination (Liu *et al*., 2010). Additionally, interspecies interactions among the various species of the consortia may give rise to higher current densities in the MEC, which otherwise might not be possible with pure cultures. However, using mixed culture populations in an MEC reactor also increases the chances of methanogenesis as certain bacteria may use up the hydrogen produced in the cell and start producing methane. Thus, a careful monitoring of the process variables such as pH, temperature, and hydraulic retention time (HRT) is vital for efficient hydrogen production in an MEC with mixed bacteria consortia.

8.3 Components of a Microbial Electroytic Cell

The most important components of any MEC reactor are the anode and cathode electrodes that aid in the transport of electrons, which ultimately leads to the production of hydrogen. Other than those, the ion exchange membranes (in the case of the double chambered MEC reactors) and gas collection unit also form an integral part of an MEC reactor.

8.3.1 Electrodes: Anode and Cathode

The shape, design, and material of electrodes play an important role in competent electron transfer from anode to cathode and subsequent hydrogen production. Since their major role is effective transport of electrons, they must obviously be highly conductive with low to negligible internal electrical resistance. This ensures that the electrons have hassle-free transport from media to anode, from anode to cathode through the external circuit, and from cathode to protons for hydrogen production. Apart from being highly conductive, the electrodes must be non-corrosive so that they have a longer life span, should be easily and cheaply available so that the input costs are minimum, and most importantly, should be easily scalable so that lab scale optimization can be effortlessly scaled up to pilot scale. The most common electrodes that have been used in MECs are metals such as copper and aluminium, or inert materials such as graphite. However, since metallic electrodes are costly and also corrode over time, graphite electrodes are preferred. They are cheap, easily available, durable, and non-corrosive. Electrodes made of stainless steel have also been reported to be highly efficient for hydrogen production in MECs.

Graphite electrodes have high conductivity, low overpotential, are biocompatible, and do not corrode with time. They are available in various shapes and sizes including sheets, felts, brushes, rods, mesh, cloth, and granules. These features make them an ideal choice as the anode in MECs. Many strategies have been adapted to increase the anodic performance for electron transport in MECs. One such strategy is ammonia treatment of the graphite anode [15, 18, 27, 28]. Ammonia treatment enhances the bacterial cell adhesion to the anode, thereby increasing the electron transfer efficiency. This leads to a shorter acclimatization period and a higher power density of the electrolytic system. Other strategies to increase anodic performance involve reducing electrode distance, addition of exogenous mediators, and increasing the ionic strength of electrolyte to enhance electron transfer.

All such approaches have resulted in better performance of the anodic electrode of the MEC. However, the most crucial step of the biohydrogen production is the final transfer of electrons from the cathode to reduce protons into hydrogen. Therefore, the development of cathodes with higher catalytic efficiency is indispensable for realising effective electrohydrogenesis. The use of ***platinated*** cathodes is the most common approach being used for enhanced hydrogen production. Platinum catalyses a higher rate of electron transfer by reducing cathodic overpotentials and thus makes the rate of reaction more favourable [29]. However, platinum also has certain disadvantages as a catalyst in the cathode. First, its high cost increases the overall cost of the MEC reactor setup. This seriously affects the process economics of electrohydrogenesis. Secondly, as postulated by Rozendal *et al.,* [4], certain constituents of wastewater such as sulphite may cause platinum poisoning thereby reducing its catalytic efficiency. Thus, it is imperative to develop alternative catalysts to platinum that are cheaper but similar in performance so as to reduce the total input costs. Many non-precious alternatives to platinum have been reported to be used as cathode catalysts with comparable performance to platinated cathodes. A few examples include cobalt and iron cobalt tetramethylphenylporphyrin (CoTMPP and FeCoTMPP) [30], nickel powder and nickel based alloys [21, 31, 32], stainless steel [29, 32, 33] and tungsten carbide [34]. A detailed comparison of the performance of such catalysts with platinated cathodes is presented in Table 8.1.

Although the performances of the alternative catalysts seen in Table 8.1 are not better than platinum, they are still comparable. Different metal alloys and stainless steel can be effectively used as cathodes to replace platinum as the metal catalyst for hydrogen production by electrohydrogenesis. Process optimization and dedicated research in areas like the development of nanomaterial based catalysts may further enhance the performance of cathodes and significantly cut down the higher input costs as a result of using platinum.

8.3.2 Gas Collection Units

The gas produced in MEC reactors can be collected via various methods, the most common being gas bags. An outlet from the reactor carries the gas produced into the gas bags, which can be sealed and removed. Gas can be

Table 8.1 A comparative assessment of different alternative cathode catalysts with platinated cathodes.

Catalyst class	Cathode catalyst	Hydrogen production rate	Remark	References
Biocathode	Microbial biocathode	0.63 m^3–H_2/m^3–d	First report to describe the development of a microbial biocathode	(Rozendal, Jeremiasse, Hamelers, & Buisman, 2008)
Stainless steel	Stainless steel brush cathodes	1.7 ± 0.1 m^3–H_2/m^3–d	First report to show that comparable rate of hydrogen production using cheaper cathode catalysts than to expensive platinated cathode catalysts is possible	(Call, Merrill, & Logan, 2009)
	Stainless steel alloy and Nickel oxide alloy	• **SS A286** – 1.50 ± 0.04 m^3–H_2/m^3/d • **Ni 625** – 0.79 ± 0.27 m^3–H_2/m^3/d • **Pt** – 0.68 ± 0.06 m^3–H_2/m^3/d	SS alloys were made by cutting from a SS plate. Nickel oxides were electrodeposited on a sheet metal support. **First report to show SS cathodes performed better than PT or nickel oxide cathode.**	(Selembo, Merrill, & Logan, 2009)

(*Continued*)

Table 8.1 Cont.

Catalyst class	**Cathode catalyst**	**Hydrogen production rate**	**Remark**	**References**
	Stainless steel	• **SS** – 4.9 L/m^2/h	5 mm stainless steel disk electrode embedded in teflon	(Munoz *et al.* 2010)
Metal based catalysts	Cobalt tetra-methylphenyl-porphyrin (CoTMPP) and Iron Cobalt tetra-methylphenyl-porphyrin (FeCoTMPP)	• **Control** – 51.6 mA/m^2 V • **CoTMPP** – 113.3 – 121.9 mA/m^2 V • **FeCoTMPP** – 98.4 mA/m^2 V • **Pt** – 166.4 mA/m^2 V	Catalyst efficiency was evaluated on the basis on cyclic voltamograms. Better the slope (V_h), better the catalyst.	(Cheng & Logan, 2008)
	NiMo and NiW electrodeposited on carbon fibre cloth	• **NiMo** – 2.0 m^3–H_2/m^3/d • **NiW** – 1.5 m^3–H_2/m^3/d • **Pt** – 2.3 m^3–H_2/m^3/d	NiMo catalysts performed better than NiW and were quite comparable with Pt catalysts.	(Hu, Fan, & Liu, 2009)

	Nickel powder catalyst, Nickel oxide (NiO_x) catalyst	• **Ni powder** – 1.3 ± 0.3 m^3–H_2/m^3/d • **NiO_x** – 0.9 ± 0.1 m^3–H_2/m^3/d • **Pt** – 1.6 m^3–H_2/m^3/d	Ni powder catalyst was prepared by mixing it with Nafion binder, Nickel oxide was electrodeposited on carbon cloth.	(Selembo, Merrill, & Logan, 2010)

sampled from the bags for characterisation by gas chromatography. Another common method of measuring the gas produced is by respirometer. These respirometers are connected directly to the reactor's gas outlet and quantify the gas produced. Water displacement units are another way of measuring gas production. The gas outlet is inserted into an inverted chamber filled with water. Hydrogen gas being lighter than water gets collected on top of the inverted chamber by downward displacement of water. For characterisation, the most common technique is gas chromatography. The area of peak obtained by injecting a known amount of gas is compared with the area of peak of a known volume of pure hydrogen gas. From the area of peak and total volumetric gas production, the number of moles of hydrogen gas produced can be calculated by the universal gas law.

8.4 Mathematical Expressions and Calculations

To evaluate the efficiency of electrohydrogenesis, many parameters have been used. The most important factors among them are yield and rate of hydrogen production. Current density, coulombic efficiency, and energy efficiency are the other parameters that describe the process efficacy of electrohydrogenesis.

8.4.1 Hydrogen Yield (Y_{H_2})

The yield of hydrogen corresponds to the unit amount of hydrogen produced per unit consumption of substrate. The most common unit to measure hydrogen is moles (mol) or milimole (mmol). However, no such generalised unit for substrate consumption is used. This is primarily because the substrates used for hydrogen production in electrohydrogenesis vary in their nature and composition. For example, if glucose is used as the substrate, then yield may be represented as moles or milimoles of hydrogen produced per mole of glucose or glucose equivalents (in case of polymeric sugars). In other cases, where complex substrates are used, hydrogen yield is represented in terms of change in COD (chemical oxygen demand) of the substrate after a batch cycle. This is common when wastewaters are used as substrates because their composition is not defined. Representing hydrogen yield in terms of COD change also gives a measure of efficacy of process for wastewater treatment.

Hydrogen yield in terms of COD removal is given by:

$$Y_{H2} = \frac{n_{H2} M_{H2}}{v_L \Delta COD}$$

Here, n_{H2} is the number of moles of hydrogen gas produced which is calculated from volumetric hydrogen production using the ideal gas law. M_{H2} is the molecular weight of hydrogen, i.e. 2, v_L is the reactor volume, and ΔCOD is the change in COD of reactor media before and after every batch cycle. For the substrates with defined compositions, ΔCOD is replaced with Δc_s, i.e. change in mass concentration of substrate.

8.4.2 Hydrogen Recovery

Hydrogen recovery represents a measure of efficacy of the electrohydrogenesis system. It is measured by calculating the coulombic efficiency of the system and cathodic hydrogen recovery.

For a substrate with a defined composition, the maximum number of moles of hydrogen produced is given by:

$$n_{th} = \frac{x_{H2/\mathbf{s}} v_L \Delta c_s}{M_s}$$

Where $x_{H2/s}$ is the stoichiometric moles of hydrogen produced per mole of substrate consumed, and M_s is the molecular weight of the substrate. For complex substrates, where Δc_s and M_s can't be calculated, the theoretical stoichiometric limit based on COD removal is represented as:

$$n_{th} = Y_{th}\, v_L\, \Delta COD$$

The ***coulombic efficiency*** (or ***coulombic hydrogen recovery***) (n_{CE}) of the system is defined as the ratio of the number of moles of hydrogen that can be recovered from the current that is produced in MEC over one batch cycle to the number of moles produced theoretically.

The number of moles of hydrogen that can be recovered from the current produced in MEC is given as:

$$n_{CE} = \frac{\int_{t=0}^{t} I\, dt}{2F}$$

Here, I is the current measured across the variable resistor using Ohm's law, dt is the time interval at which the currents are measured, and F is the Faraday's constant (96485 C mol^{-1}).

The coulombic efficiency is now represented as:

$$r_{CE} = \frac{n_{CE}}{n_{th}}$$

Now, ***cathodic hydrogen recovery*** (r_{cat}) is defined as the ratio of moles of hydrogen produced to the moles of hydrogen that can be recovered from the current measured (***coulombic efficiency***). It is therefore a measure of hydrogen that is actually recovered from what could be theoretically recovered based on the current produced. It is mathematically represented as:

$$r_{cat} = \frac{n_{H2}}{n_{CE}}$$

The ***overall hydrogen recovery*** (r_{H2}) cab now be defined as the ratio of total recovered moles of hydrogen to that which is theoretically possible. It is depicted as:

$$r_{H2} = r_{cat} r_{CE} = \frac{n_{H2}}{n_{th}}$$

In ideal conditions where there is no loss of hydrogen, n_{H2} and n_{th} are equal and thus, r_{H2} equals to 1. However, that is never the case, and there is always some loss of hydrogen due coulombic losses, concentration losses, and losses due to diffusion of hydrogen into water or membrane (in double chambered MECs).

8.4.3 Energy Efficiency

Energy efficiency or recovery corresponds to the efficiency of the system to recover the energy input into the system from the power source and substrate in the form of hydrogen. The amount of energy added into the system by the external power source can be shown as:

$$W_{ps} = \int_{t=0}^{t} IE_{ap} dt$$

Where I is the current measured across the resistor using Ohm's law across a variable external resistor (R_{ext}). The power loss across the resistor can be given as:

$$W_R = \int_{t=0}^{t} \left(I^2 R_{ext}\right) dt$$

The net energy input from the power source now can be written as:

$$W_P = W_{ps} - W_R = \int_{t=0}^{t} (IE_{ap} - I^2 R_{ext}) dt$$

The amount of energy input as substrate can be expressed as:

$$W_s = -\Delta H_s * n_s$$

Where ΔH_s is the specific heat of combustion of substrate, and n_s is the number of moles of substrate consumed in single batch cycle based on COD removal. Thus, the total input energy becomes:

$$W_{IN} = W_p + W_s$$

The output energy, i.e. the amount of energy recovered in form of hydrogen, is:

$$W_{H2} = \Delta H_{H2} * n_{H2}$$

Where ΔH_{H2} is the molar heat of combustion of hydrogen, and n_{H2} is the number of moles of hydrogen formed.

Therefore, the total energy efficiency of the process (η_E), i.e. the ratio of output energy to the total input energy becomes:

$$\eta_E = \frac{W_{H2}}{W_{IN}}$$

8.5 Challenges and Future Prospects

The fact that the world is facing the grave danger of running out of its reserves of fossil fuels has forced the scientific world to develop solutions for the looming problem. Just reducing the usage of fossil fuels will only delay their extinction, but not permanently resolve the issue. It has, therefore, become inevitable to develop and utilize alternative, non-conventional sources of energy that perform on par with oil-based energy sources, but at the same time, are easy to procure, process, and most important, are environmentally friendly upon combustion. Of the various options available, among them biodiesel, bioalcohols and few others, biomass-based hydrogen or biohydrogen are the most fitting to replace conventional fossil fuels. This is because of their high calorific value and clean combustion profile. Much of the research recently has been targeted in developing systems that can produce biohydrogen efficiently and economically. The most efficient of all approaches is the method of electrohydrogenesis, which utilizes exoelectrogenic bacteria and an electrolytic cell (MEC) with a small external voltage (0.2–0.8 V) for hydrogen production.

Although electrohydrogenesis is a fast, efficient, and competent method of hydrogen production, it also faces drawbacks that need to be addressed before it can be a full-fledged industrial-scale method for biohydrogen production. First, methanogenesis is a serious issue that hampers the total yield of hydrogen in membrane-less MECs. The problem is more so obvious with MECs running on wastewater as substrates or inoculum sources because the consortia are not defined, and the composition of wastewaters favours the development of methanogens. One way of tackling this problem is to use a membrane to separate cathode from anode, but that increases the input cost of reactor fabrication. Other solutions are to use a pure culture or inoculum source, and in the case of when a mixed consortium is used, reducing HRT suitably so as to prevent the growth of methanogens.

Another problem is energy losses during reactor operation. Ohmic and coulombic losses affect the energy efficiency of the process as a whole. Ohmic losses occur when internal resistance of the cell or electrode alter the movement of electrons. Coulombic losses occur when the substrate used for hydrogen production gets used up for biomass accumulation by exoelectrogens rather than current production [35]. To overcome the energy losses, the MEC reactor must be fabricated with precision, and the operation conditions must be optimized meticulously to derive maximum energy output from the process. Each and every parameter of the reactor that affects the energy output such as reactor fabrication material, its shape and design (single or double chambered), inoculum size and age, electrode material and geometry, as well as electrolyte strength and conductivity must be taken into account and optimized.

A third issue is to develop better exoelectrogenic strains for enhanced electron release into the electrolyte. Many metabolic engineering approaches are being put to use in order to develop strains that have higher current-producing capabilities so that maximum efficiency can be achieved.

With these issues properly addressed and resolved, electrohydrogenesis would be the most potent option available for biohydrogen production. Currently, parallel research is already in progress for developing equipment for the storage and transport of hydrogen as a fuel and to develop machinery that harnesses hydrogen fuel as energy source. Significant developments in such areas have already begun pouring in, and it is accurate to say that the age of hydrogen fuel is not a distant dream, but a close reality.

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