Biochemical Conversion of Biomass to Fuels

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Contents

Abstract

Biomass can provide both hydrocarbon fuels and chemical compounds such as alcohols, gums, sugars, lipid-based products, etc. Biomass-derived fuels have acquired a lot of attention during recent years because of the abundance of supply of resources and lower green house gas emissions. Grasses, agricultural residues, animal residues and waste, used oils, etc., can be used as starting materials in the production of biofuels. Ethanol and biodiesel have found greatest application and contribute significantly to fuels. However, there is growing interest in other alternatives: hydrogen, methane, butanol, renewable diesel, and petroleum compatible fuels from advanced catalytic biotech processes. Characteristics of various feedstocks and fuels, processes for conversion of biomass to biofuels,

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the physical, chemical factors, and limitations affecting the conversion of biomass to fuels are discussed in this chapter. Process parameters include pH, temperature, and residence time. Additionally, chemical parameters include carbon source, nutrients, acid and alkaline hydrolysis agents, and phenolic inhibitors and sugars generated within the process. Several limitations to bioconversion of biomass are described such as size reduction, crystallinity, byproduct inhibition to fermentation, deactivation of cellulases, ethanol tolerance by yeast, and cofermentation of various sugars. Recent innovations and future developments in recombinant DNA technology and protein engineering are aimed at overcoming limitations to bioconversion. Understanding the limitations and applying suitable biotechnological applications will support future developments for producing biofuels.

Introduction

With increasing demands for transportation fuel, renewable sources of energy content, have gained importance in the recent years. Important fuel parameters are energy content, combustion quality such as octane or cetane number, volatility, freezing point, toxicity, and its adaptability to current combustion engines (Lee et al. 2008c). Biofuels such as hydrogen, methane, ethanol, butanol, and biodiesel are of current interest in replacing (in partial or complete) gasoline to mitigate greenhouse gas emissions.

Table 1 presents comparative data for various fuels against gasoline and can be produced from biochemical conversion of biomass. Current working status of these fuels is also mentioned in the Table 1. Among the fuels mentioned in the table, butanol and biodiesel (biodiesel from pure vegetable oils) can be used in existing gasoline and diesel engines respectively with a little modification. For others, engine modification is required. For ethanol, lower blends in gasoline do not require engine modification. Use in higher blends requires engine modification. Engine modification is required for some nongasoline fuels due to difference in their air-fuel ratio, latent heat of evaporation, and corrosiveness. Air-fuel ratio of gasoline is 14.6 kg air for 1 kg of fuel. However, 10 % v/v ethanol blend of gasoline has 3.5 % w/w oxygen in the fuel which influences the air-fuel ratio at which the engine performs. Engine management systems in modern vehicles adjust the air-fuel ratio to maintain the stoichiometric oxygen in the fuel. Absence of engine management system or use of higher blend gasolines/biodiesel alters the air-fuel ratio, therefore requiring engine modification. Ethanol and biodiesel have higher latent heat of evaporation compared to gasoline, which may present difficulties with starting in cold conditions. To avoid cold start difficulties, vehicles require a small tank fitted to accommodate gasoline to initiate combustion. Moreover, viscosity of biodiesel increases during cold conditions requiring alternative starting methods for vehicles using higher blends of biodiesel. Higher blends of ethanol are known to be corrosive on fuel lines and tanks; therefore, vehicles using 20 % v/v ethanol blend gasoline require to have nickel-plated steel fuel lines and tank.

Table 1 Properties of various biofuels (Adapted from sources Lee et al. (2008c) and http://en.wikipedia.org/wiki/Energy_density)

	Hydrogen	Methane	Ethanol	Butanol	Biodiesel	Gasoline
Heat of vaporization (KJ/Kg)	451.9	760	920	430	2,639.9	360
Energy density (MJ/L)	10.1 (liq)	0.0378	19.6	29.2	37.3	32.0
Research octane number	>130	135	129	96	>25	97–98
Air to fuel ratio	34	17.2	9.0	11.2	13.5	14.6
Freezing point (°F)	−435	−296.5	−173.2	−128.7	26–66	−40
Flash point, closed cup (°F)	−423	−306.4	55	84	212–338	−45
Solubility in water, volume%	–	–	100	9	Negligible	Negligible
Technology	Microbial	Microbial	Microbial	Microbial	Chemical	Chemical
				Chemical	Enzymatic	Physical
Status	Laboratory	Industrial	Industrial	Laboratory	Industrial	Industrial
					Laboratory	
Engine application	Blend	Blend	Blend	Blend	Blend	N/A
	Pure	Pure	Pure	Pure	Pure	
Current engine modification	Required	Required	Required for higher blends	Not required	Not required[a]	N/A

[a]Not required for lower blending

Various sources such as agricultural residues, municipal waste, animal waste, perennial grasses, etc., are used for conversion to biofuels. In this chapter, processes of production of biofuels, hydrogen, methane, ethanol, butanol, and biodiesel are described with recent progress. Applications of recombinant DNA technology and bioengineering to overcome production bottlenecks and enhance fuel production are discussed.

Sources

Biomass represents all materials derived from plant, animal, and microbial origins. Classification of biomass used in conversion to biofuels may be based on the origin (plant/animal), carbon source (woody/herbaceous), and physical and chemical characteristics. However, biomass from plant origin is considered highly desirable for its abundance and potential to mitigate emission of greenhouse gases. Carbohydrate monomers in plants are formed through photosynthesis, in which the atmospheric carbon dioxide is converted by sunlight to chemical energy. Moreover, the same amount of carbon dioxide is released, when biomass-derived fuels for energy are used, as taken up during the plant growth using sustainable means, therefore, production of more biomass, consequently mitigates and not add up to the atmospheric carbon dioxide (McKendry 2002).

Biomass can be majorly divided into woody plants, herbaceous plants or grasses, aquatic plants, and manures. Among these, some herbaceous plants, aquatic plants, and manures contain high moisture content and are suitable for wet processing or biochemical processing. Aqueous processing or wet processing is generally initiated through enzyme action. This method is suitable for high moisture content biomass because of challenged efficiency of overall energy retrieval, compared to the energy required for drying involved in dry processing. Moisture content, carbon source, and cellulose to lignin ratio are the most important factors affecting the wet process. Biomass with low moisture content is subjected to dry process or thermal treatment such as gasification, pyrolysis, and combustion. Factors that influence the dry processes are ash content, alkali, and trace components as they adversely affect the thermal conversion processes (McKendry 2002).

The products of wet processes are ethanol, butanol, and biogas. Ethanol and butanol products majorly depend on the plant composition – cellulose, hemicellulose, and lignin. Cellulose, hemicellulose, and lignin are the three main components of any plant material. Cellulose is a polymer of glucose with linear chains of (1,4)-D-glucopyranose units in β-configuration with an average molecular weight of around 100,000. Another polymer of glucose with linear chains of (1,4)-D-glucopyranose units in α-configuration, called amylose constitute about 20 % of starch. Starch also includes amylopectin, a branched polymer chain of D-glucose molecules called α-1,6 glycosidic linkage (Shuler and Kargi 2008). Starch can be more easily digested to sugars compared to cellulose due to the beta configuration and high crystallinity offered by cellulose linear structure. Starch can be obtained

from any of the food storage units of plants, while cellulose constitutes all the other parts of the plant.

Hemicellulose is a heterogeneous polymer of pentoses (xylose and arabinose) primarily xylose, hexoses (mannose, glucose, and galactose), and sugar acids. Although it is not covalently bonded, it is tightly bonded to the surface of each cellulose microfibril. Cellulose digestibility to sugars partially depends on the hemicellulose content.

After cellulose and hemicellulose, lignin is the third most abundant biopolymer, consisting primarily of phenylpropane units most commonly linked by ether bonds. It provides structural support , through its hydrophobic nature impermeability, and resistance to microbial and oxidative attack (Feldman 1985; Chang and Holtzapple 2000). Additionally, woody plants have higher lignin than herbaceous plants, thus imparting lesser strength in the latter due to loosely bound fibers (Saha 2004). Lignin also inhibits the conversion of carbohydrates to ethanol making it imperative to maximize the elimination of lignin in biomass. However, woody plants having higher lignin proportions resist moderately severe treatments, unlike herbaceous biomass. Some herbaceous plants like switch grass and miscanthus (*Miscanthus*) require less severe treatments for lignin removal. Since lignin alone causes inhibition to conversion of sugars and to ethanol, cellulose to lignin ratio is an important factor effecting conversion. Removed lignin can be used for combustion in boilers for energy generation.

For dedicated energy crops, cultivation of herbaceous plants is greatly encouraged for biochemical conversion to fuels compared to the woody biomass for several reasons such as, lesser harvest time, ease of harvesting, usage of surplus land, less intensive agricultural practices, less lignin content, and less severe treatment for conversion. Selection of plants for energy production depends on the climatic conditions, geographical location, and availability and type of treatment employed (either thermal or biochemical).

In the UK, a perennial crop, *miscanthus*, has attained a lot of attention for energy production through biochemical conversion due to the ease in growing, harvesting, and good annual yield. This thin-stemmed crop has been considered a good energy crop due to its annual harvest and low mineral content and is grown in ten countries in Europe. In the USA, another thin-stemmed crop, switch grass, is a model crop for the Oak Ridge National Laboratory, as it yields high ethanol from fermentation with the existing technologies. Its low ash and alkali content allow it to be used for combustion. Brazil, one of the pioneers for the production of ethanol, for fuel uses sugarcane as the source (McKendry 2002). Sources of biomass other than herbaceous plants include agricultural residues such as wheat straw, rice straw, corn fiber, corn stover, baggase, etc. Animal residues such as pig slurry (Murphy and McCarthy 2005), cattle dung, horse dung (Kalia and Singh 1998), etc., are used for biogas production, which upon upgrading to >97 % methane, can be used as transport fuel. Marine algae have gained importance as potential sources for biofuel production, both as substrates for fermentation to hydrogen, ethanol, and butanol, and as oil-rich sources for biodiesel production. Due to their less energy and water requirement, higher carbon dioxide capture and negligible lignin, they are considered as superior

to terrestrial biomass (Tran et al. 2010; Jung et al. 2011). However, several factors including availability, moisture content, and cellulose/lignin ratio impact the biochemical production of biofuels.

Process Overview

Major processes involved in the biochemical production of biofuels are biomass handling, biomass pretreatment, hydrolysis, and fermentation. However, depending on the source of biomass, the route of conversion to biofuel and the type of biofuel, the series of processes can alter. Figure 1 shows a schematic representation of some common unit operations and processes for the biofuels mentioned in section "Biofuels."

Handling

Biomass, either grown or obtained from various sources, needs to be transported to the production sites for biochemical conversion to fuels. Postharvest it is prepared as bales, pellets, and briquettes for which the biomass has to be size reduced. Size reduction is an important mechanical preprocessing step to increase the bulk density and flowability of particles for transportation. Biomass is generally ground to 3–8 mm particles to compact it into pellets or briquettes of higher density. Important parameters in evaluating the efficiency of size reduction are particle size, particle size distribution, shape, surface area, density, and energy efficiency of mill used (Miao et al. 2011). Due to the unavailability of a continuous supply of biomass feedstocks, storage of biomass becomes important to ensure uninterrupted supply for continuous production of biofuels. Although outdoor storing of wood chunks is a commonly practiced method, studies show that terpenes are emitted from wood due to the exposure of direct heat from sunlight (Rupar and Sanati 2005). Large silos and specially constructed facilities are used for biomass storage

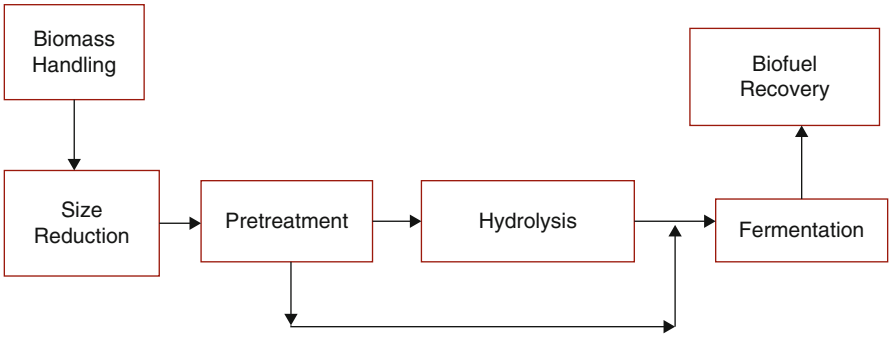

Fig. 1 Schematic representation of processes in biochemical conversion of biomass to fuels

to protect feedstock from the effects of weather, rodents, and microbial growth. Microbial growth during storage causes loss of substrate and also has the potential to result in self-ignition due to exothermic reactions. Therefore, it is required to maintain dry conditions to allow little microbial activity in the biomass during storage. Field drying postharvest is a common method for drying in sunny regions. However, thermal or mechanical drying techniques using drum driers are available for drying biomass after harvest and before storage in colder regions (Venturi et al. 1999).

Pretreatment

Pretreatment plays an important role in the biochemical conversion yields of biofuels. Complex structures in biomass are broken down into oligomeric subunits through pretreatment. These oligomers are further broken down into monomeric units during hydrolysis and fermentation. Pretreatment enhances the product yields by disrupting and solubilizing the hemicelluloses and lignin structures in biomass. Key properties affecting the conversion of lignocellulose are the crystallinity of cellulose, degree of polymerization, moisture content, available surface area, and lignin content (Chang and Holtzapple 2000). The aim of pretreatment is to disrupt the lignocellulosic structure by (1) removing hemicellulose, increasing mean pore size, and facilitating the entrance of enzymes and hydrolysis; (2) removing or redistributing lignin to reduce its "shielding" effect (Alvira et al. 2010).

Pretreatment processes will ideally achieve the following (Yang and Wyman 2008):

- High yields for multiple crops, sites ages, and harvesting times
- Highly digestible pretreated solid
- Minimum amount of toxic compounds
- Biomass size reduction not required
- Operation in reasonable size and moderate cost reactors
- Nonproduction of solid-waste residues
- Effective at low moisture content
- Obtains high sugar concentration (from hydrolysis)
- Fermentation compatibility (minimal production of inhibitors)
- Lignin recovery
- Minimum heat and power requirements

Main Classes of Pretreatment

The main classes of pretreatment covered in this chapter are mechanical, chemical, physiochemical, and biological. Mechanical pretreatment is discussed at this point as it applies to most process trains for biomass conversion. Chemical, physiochemical, and biological pretreatments are described in section "Pretreatment," as they pertain most closely to bioethanol production. At that point, characteristics making acid and alkali pretreatments suitable for methane production are also discussed.

Mechanical

Milling uses grinding to reduce particle size and crystallinity. Specific surface area is increased and degree of polymerization gets decreased. Numerous milling systems can be employed: ball, hammer, roller, colloid, and vibro energy milling (Alvira et al. 2010; Taherzadeh and Karimi 2008). Coupled with other pretreatment, milling can increase hydrolysis yield for lignocellulose by 5–25 % and reduces digestion time by 23–59 % (Delgenes et al. 2003; Hartmann and Ahring 2000). There are limits to effectiveness. Size reduction below #40 mesh does not improve hydrolysis yield or rate (Chang and Holtzapple 2000). Power requirements are large, which will limit economic feasibility (Hendriks and Zeeman 2009).

- Chemical (section "Pretreatment")
  - Acid pretreatment – concentrated and dilute
  - Alkali pretreatment – NaOH, $Ca(OH)_2$, or ammonia
- Physiochemical (section "Pretreatment")
  - Thermal processes include liquid hot water (LHW) and steam pretreatment
  - Steam explosion
  - Ammonia explosion (and CO_2 explosion)
  - Other physiochemical methods include organosolv and wet oxidation
- Biological pretreatment – brown and white soft-rot fungi (section "Pretreatment")

Alvira et al. conclude that chemical and thermochemical methods are the most effective and promising technologies for industrial applications (Alvira et al. 2010). They suggest combination of different pretreatments should be considered for optimal fractionation of components and high yields. They also stress the need for additional fundamental research plant cells to better understand the reactions induced by pretreatment.

Taherzadeh and Karimi (2008) concluded that concentrated acids, wet oxidation, solvents, and metal complexes are effective, but too expensive (Fan et al. 1987; Mosier et al. 2005a). They concluded that steam pretreatment, lime pretreatment, LHW systems, and ammonia-based pretreatments have a high potential. Eggeman and Elander (2005) presented an economic evaluation showing only small differences in cost for five different pretreatment technologies (dilute acid, hot water, ammonia fiber explosion (AFEX), ammonia recycle percolation (ARP), and lime). This analysis appears in the special issue "Coordinated development of leading biomass pretreatment technologies" (Wyman et al. 2005). Optimizing enzyme blends and hydrolysate conditioning may better differentiate process economics.

Hydrolysis and Fermentation

During hydrolysis, breaking down of polymeric and oligomeric cellulosic structure, to simpler molecules such as glucose, cellobiose, xylose, galactose, arabinose, and mannose, takes place. It is done by the action of either chemical or enzymatic agents. Enzymatic hydrolysis is a complex process that takes place at the solid/liquid

interphase. Several processes such as chemical and physical changes in the solid biomass, primary hydrolysis of soluble intermediates from the surface, and secondary hydrolysis to ultimately simpler molecules such as glucose take place simultaneously (Balat 2007). More discussion about enzymes used in hydrolysis is provided in section "Hydrolysis."

Conversion of simpler carbohydrates to alcohol through action of microbes is called as fermentation. Fermentation is both substrate and microbe specific, more details about fermentation are mentioned in section "Biofuels" for each biofuel, hydrogen, methane, ethanol, butanol, and biodiesel.

A combination of hydrolysis and fermentation is another process where simultaneous breaking down of complex carbohydrates to simpler ones and converting to alcohol takes place. This process is commonly called as simultaneous saccharification and fermentation (SSF). Product yields from SSF are higher than separate hydrolysis and fermentation (SHF), as the end product inhibition during hydrolysis of higher carbohydrates to glucose and cellobiose, is relieved by simultaneous fermentation of glucose to ethanol (Balat 2007).

Hydrolysis and fermentation are carried out in both batch and continuous modes. Batch reactors require higher reactor volume compared to the continuous reactors to achieve similar product yields. Two basic types of continuous reactors used in biochemical reactions are continuously stirred tank reactor (CSTR) and plug flow reactor (PFR). Most commonly, CSTR is used for hydrolysis and fermentation during the biochemical production of biofuels. Studies show usage of a packed bed reactor (PBR) in comparison with upflow anaerobic sludge bed (UASB) for the production of hydrogen from organic fraction of municipal solid waste, where the PBR was packed with municipal solid waste. The retention times of 50 and 24 h gave maximum hydrogen yields of 23 % v/v and 30 % v/v (based on volume of waste) for PBR and UASB, respectively (Alzate-Gaviria et al. 2007). Another study investigated combined or sequential two-stage processes involving coproduction of hydrogen and methane since hydrogen is an intermediate byproduct of methane production (Park et al. 2010; Zhu et al. 2008; Koutrouli et al. 2009). Dissolved oxygen and heat transfer are known to be limited by reactor volume. Fermentation for hydrogen, methane, ethanol, and butanol production is anaerobic, and the reactor volume is not limited by the dissolved oxygen and heat transfer when run in continuous mode. Therefore, CSTR fermentation systems with recycling of cell mass are sufficient to overcome solvent toxicity and limited cell growth (García et al. 2011).

Biofuels

Hydrogen

Biohydrogen is considered as a potential biofuel for the future, it is produced from biomass through different routes and their combinations. Gasification of biomass is one of the routes; refer to the chapters on thermal conversion of biomass, integrated

gasification for combined cycle (IGCC), and conversion of syngas to fuels in this handbook for more details about the gasification process. Hydrogen is a natural byproduct of many microbial processes under anaerobic conditions. Certain microbes release hydrogen from water in the presence of sunlight and/or carbon dioxide. Microbes that derive carbon from carbohydrates and need sunlight as a source of energy to release hydrogen are called phototrophic or photosynthetic organism (e.g., *Rhodobacter*) and those that derive their carbon from carbon dioxide and energy from sunlight are called photoautotrophic organisms (e.g., green microalgae and cyanobacteria) (Wukovits et al. 2009). Different fermentative processes, based on different sources of energy and their combinations, are anaerobic fermentation, dark fermentation, photo fermentation, direct photolysis, indirect biophotolysis, and fermentative water-gas shift reaction. The majority of these processes combine microbiological routes led by several microbes.

Anaerobic fermentation is a four-stage process carried out by a consortium of microbes. In the first stage, the complex organic components are converted to simpler components (e.g., sugars) by hydrolysis. In the second stage, the products of hydrolysis are further broken down to short-chain fatty acids by acidogenic bacteria. During the third stage, acidogenesis, the products of second stage are converted to acetic acid, hydrogen, and carbon dioxide. In the final stage, methanogenesis, the products from the third stage are used by the methanogenic bacteria to produce methane. Thus, hydrogen in this process is an intermediate product and its production can be increased by increasing the substrate content in the raw material used.

Figure 2 represents three different two-stage routes that are under active investigation. In the first stage, optimized technologies of above-mentioned conventional methods are used to convert biomass to organic acids and hydrogen. In the second stage, additional energy such as light, electricity, and methane and hydrogen from the first stage are used for achieving stoichiometric conversions. Although this combination of two stages produces a mixture of methane and hydrogen, the process can be developed to achieve hydrogen stream. Dark fermentation is carried out by the anaerobes that convert biomass substrate to hydrogen under the absence of light and is shown in Fig. 2. This process is similar to the first three stages of anaerobic fermentation where the initial raw substrate is simpler carbohydrate. For a complex substrate, hydrolysis such as a chemical/physical pretreatment of biomass is required to break down the complex polymeric biomass substrate to simpler monomeric and oligomeric carbohydrates, which can be later converted to organic acid, carbon dioxide, and hydrogen by anaerobes during dark fermentation. Reaction (1) represents a general formula for hydrogen metabolism from glucose. It is evident that in the presence of hydrogenase enzyme, 4 moles of hydrogen are released for every 1 mole of glucose. Thermophilic bacteria, that grow at high temperatures (above 60 °C) ferment biomass, produce hydrogen at higher rates than the mesophilic bacteria that grow at moderate temperatures (below 50 °C), due to aseptic conditions maintained at high temperatures. Additionally, hydrogen production depends on the other byproduct organic acids present in the effluent. Acetic acid and other organic acids have an inhibitory effect on the growth of microbes, consequently influencing

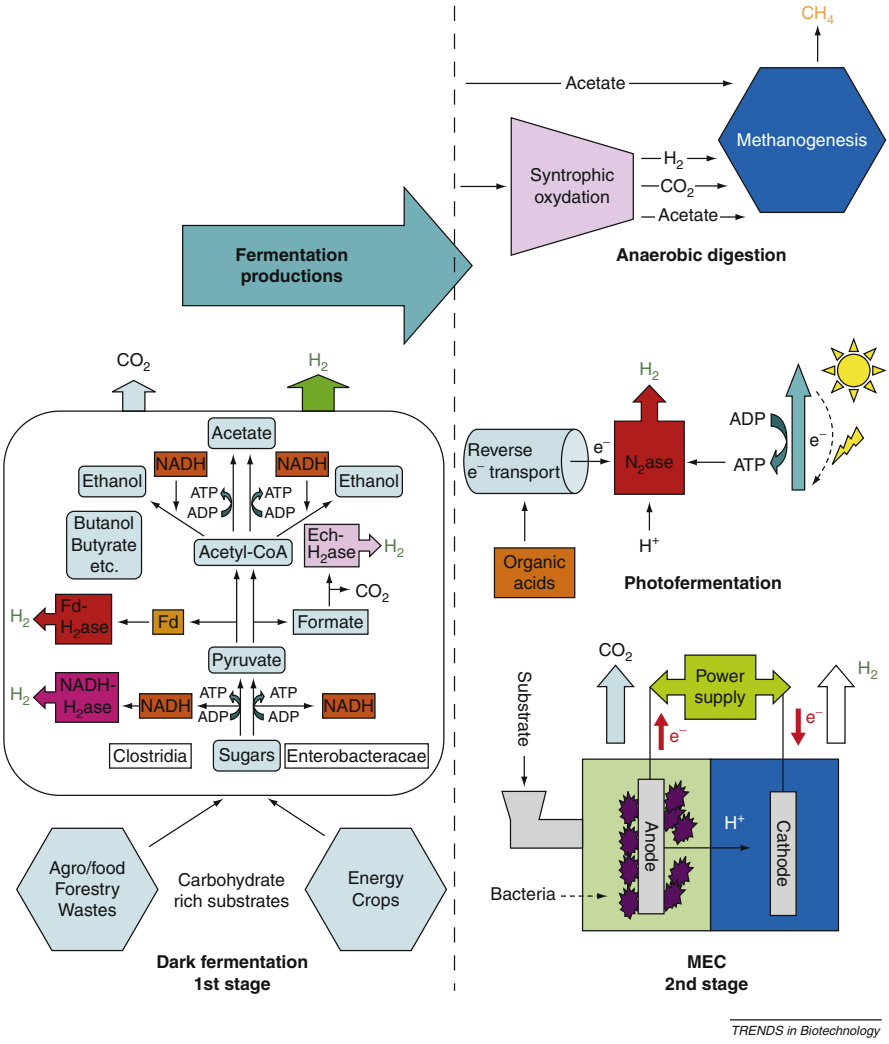

Fig. 2 Different two-stage routes for conversion to hydrogen and methane (Hallenbeck and Ghosh 2009)

hydrogen yield. Besides its inhibitory effect, acetic acid influences the pH of the system, thus affecting the activity of hydrogenase enzyme responsible for the production of hydrogen.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (1)$$

Photo fermentation involves a series of biochemical reactions such as anaerobic digestion. However, unlike dark fermentation, it requires light for energy during the process of hydrogen production. Simple, short-chain fatty acids are converted to

carbon dioxide and hydrogen catalyzed by nitrogenase enzyme in the absence of nitrogen by purple nonsulfur bacteria or green micro algae. Reaction (2) describes the conversion process. Theoretically, 4 moles of hydrogen are produced for every mole of acetic acid but, in practice, part of the acetic acid is used for the production of cells. Moreover, large surface area is required to capture the necessary light energy, making it practically challenging in terms of bioreactor design. Transparent tubular reactors and flat panel reactors consisting of transparent rectangular boxes are under investigation (Wukovits et al. 2009).

$$CH_3COOH + 2H_2O + \text{light energy} \rightarrow 4H_2 + 2CO_2 \quad (2)$$

Combination of the above-mentioned fermentations enhances the yield of hydrogen production. One such combination is dark fermentation and anaerobic digestion in which the monomeric components of the polymeric biomass are converted to biohydrogen. Dark fermentation and photo fermentation is another combination process that theoretically yields 12 moles of hydrogen for every mole of hexose sugar. This approach, called "Hyvolution," would allow complete digestion of biomass, enhancing small-scale, cost-effective production of hydrogen, which otherwise is limited by thermodynamic considerations (Wukovits et al. 2009).

Another approach mentioned in the second stage (lower right of Fig. 2) employs microbial electrohydrogenesis cells (MECs). In this method, electricity is applied to a microbial fuel cell that provides the necessary energy to convert the byproducts (typically organic acids) of the first stage into hydrogen (Hallenbeck and Ghosh 2009).

Several raw materials such as kitchen waste, animal waste, agricultural residues, etc., are used as substrates for biohydrogen production. Fermentation of kitchen waste devoid of plastic and bones was used to produce hydrogen with a maximum efficiency of 4.77 LH_2/(L reactor day) in a continuous stirred tank reactor (Shi et al. 2009). Use of second-generation feedstocks that are of cellulose origin such as corn stalks, wheat straw, switch grass, and *miscanthus* further enhance economical production of hydrogen. Pretreated lipid extracted microalgal biomass residue (LMBR) showed threefold hydrogen yields compared to the untreated LMBR (Yang et al. 2010). However, noncellulosic components such as xylose require conversion by a fermentative organism. High-thermophilic mixed culture was developed for xylose fermenting to biohydrogen at 1.36 ± 0.03 mol H_2/mol xylose consumed (Kongjan et al. 2009).

Organisms belonging to genus *Clostridium* such as *Clostridium butyricum*, *C. acetobutylicum*, *C. saccharoperbutylacetonicum*, and *C. pasteurianum* are often used in the anaerobic production of hydrogen. Anaerobic thermophilic bacterial fermentation to hydrogen is the most suitable option due to increasing chemical and enzymatic reaction rates at high temperatures. Additionally, thermophilic processes yield lesser undesirable products as compared to mesophilic processes (Koskinen et al. 2008). An optimized fermentation of hydrolysate obtained from treating sugarcane bagasse with 0.5 % H_2SO_4 under 121 °C and 1.5 kg/cm^2 in autoclave for 60 min was obtained at initial pH 5.5 and initial total sugar

concentration of 20 g/L at 37 °C (Pattra et al. 2008). Thus, initial pH and total sugar concentration are important factors for an optimal hydrogen yield. However, an increase in hydrolysate (sugar) concentrations from 25 % (v/v) to 30 % (v/v) led to no hydrogen production. Further, an increase in lag time was observed from 11 to 38 h for an increase in hydrolysate concentrations from 20 % (v/v) to 25 % (v/v) for a mixed thermophilic dark fermentation process (Kongjan et al. 2010). Supplemental glucose and xylose with a ratio of 2:3 along with suitable pH control and inoculum concentration are realized to be the key factors for enhanced hydrogen production (Prakasham et al. 2009).

Finally, biophotolysis is a low productivity method for hydrogen gas production. It involves dissociation of water by solar energy using green micro algae. The process takes place in two ways, direct biophotolysis and indirect biophotolysis. In direct biophotolysis, the microbes split the water into oxygen and hydrogen using sunlight by releasing two photons, which can either reduce carbon dioxide or form hydrogen in the presence of hydrogenase enzyme. However the released oxygen has an inhibitory effect on the hydrogenase enzyme which can be overcome by indirect biophotolysis. Indirect biophotolysis is carried out by cyanobacteria, in which water and carbon dioxide form carbohydrates and oxygen via photosynthesis. The second stage involves either dark fermentation or a combination of dark and photo fermentation to produce hydrogen. Fermentative water-gas shift reaction is another biological route in which carbon monoxide in the presence of water is converted to carbon dioxide and hydrogen (Wukovits et al. 2009).

Methane

Methane is the main component of natural gas which is used as an energy carrier and raw material all over the world (Seiffert et al. 2009). Biogas produced from anaerobic digestion of biomass contains methane which can be used for energy purposes. The biochemical conversion of manure and other biomass to methane involves three stages.

In the first stage, hydrolysis, enzymes produced by strict anaerobes such as *Clostridia*, *Bactericides*, and *Streptococci*, break up the complex molecules such as lipids, polysaccharides, proteins, fats, nucleic acids, etc., to simpler molecules such as monosaccharides, amino acids, fatty acids, etc. In the second stage, acidogenesis, a group of bacteria ferment the byproducts of hydrolysis to acetic acid, propionic acid, and butyric acid. In the third stage, methanogenesis, methanogens convert the acetic acid, hydrogen, and carbon dioxide into methane and carbon dioxide.

Figure 3 shows a block diagram of biogas production from manure. Biogas production is greatly affected by temperature. Anaerobic fermentation is effective mostly at mesophilic (15–40°C) and thermophilic (50–60°C) temperature ranges. Therefore, the reactors are coated with biomass residues such as charcoal and even constructed in a sun-facing direction to avoid cold winds and make maximum use of heat available from nature (Anand and Singh 1993). Reactors have been designed to have a polythene sheet covering the top of it to utilize the energy from sun to heat up the reactor contents even during winter (Bansal 1988).

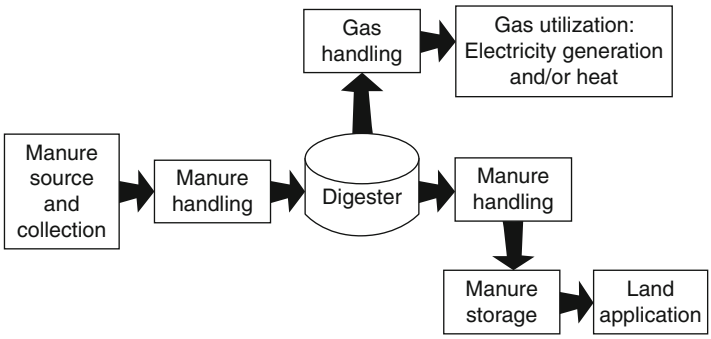

Fig. 3 Block diagram of biogas production from manure (Source: http://pubs.ext.vt.edu/442/442-881/442-881.html)

As acetic acid and hydrogen produced during the process decrease the pH of the system, pH maintenance is another important parameter affecting the methane production, the desired pH being 6.8–7.2. Several techniques are involved in enhancing the production of biogas, such as addition of organic and inorganic additives, microbial strains, recycling of digested slurry, and maintaining C:N ratio. Additives, such as powdered green leaves, allow adsorption of substrate to increase localized concentration and enhance microbial growth. Addition of Ca and Mg salts act as microbial energy supplements and avoid foaming. Recycling of slurry avoids loss of active culture which otherwise occurs through the effluent stream. As the microbes tend to utilize carbon 25–30 times faster than nitrogen for the production of methane, maintaining C:N ratio is another critical factor in efficient production of biogas (Yadvika et al. 2004).

Biomethane can be distributed into the natural gas grid. In the case of existing pipelines in UK, Italy, and Germany, this concept is called the "green gas concept" (Åhman 2010). However, to employ biogas as a transportation fuel, concentration of biogas to 97 $\pm$ 1 % of methane by removing the carbon dioxide is required (Power and Murphy 2009). About 30–60% of the wet biomass can be converted to methane by anaerobic digestion, while the remaining residue can be used as biofertilizer (Åhman 2010). Coproduction of methane and hydrogen using a two-stage anaerobic digestion process is another way to optimize simultaneous production of methane and hydrogen (Zhu et al. 2008).

An energy input approximating 22 % of the fuel value is utilized in the production of biomethane, compared to approximately 57 % in the production of bioethanol (Power and Murphy 2009). The majority of the difference arises from the thermal energy consumption involved in the distillation of ethanol and drying of the residue obtained from fermentation. Thus methane's gaseous nature has an added advantage over liquid biofuels. However, biomethane losses during digestion and upgrading constitute about 7.41 % of total biogas produced. Minimizing these losses and improving infrastructure efficiency for biomethane is needed to enhance the utility of methane relative to ethanol (Power and Murphy 2009).

Ethanol

Ethanol is the most extensively studied biofuel to date and has gained great attention as sustainable biofuel. Bioethanol production and utilization is estimated to reduce green house gas emissions, improve agricultural economy, enhance rural employment, and increase national security (Mabee and Saddler 2009). Bioethanol has higher octane number, broader flammability limits, higher flame speeds, and higher heats of vaporization than gasoline, which allow for higher compression ratio, shorter burn time, and leaner burn engine. A major problem with ethanol is its water solubility and azeotropic mixture formation with water, limiting separation during distillation, consequently intensifying the cost of the separation process. Other major disadvantages include lower energy density than gasoline, low vapor pressure (making cold starts difficult), and toxicity to ecosystems (Balat 2007). However, ethanol is a 35 % oxygenated fuel and reduces particulate and NO_x emissions. It increases combustion efficiency as it provides a reasonable antiknocking value. It can be blended with gasoline in various amounts, ranging from 5 % to 85–100 %, for use in the existing internal combustion engines, where 85 % (E85, meaning 85 % ethanol in gasoline) blends are used in flexible fuel vehicles (FFVs). Table 2 shows various blends of ethanol in gasoline used in different countries worldwide. In pure ethanol cars, sulfur emissions have totally disappeared; gasoline-driven cars with ethanol replacing lead have negligible carbon monoxide emissions (Goldemberg et al. 2008).

Substrates used for the production of bioethanol vary with the availability of feedstock and geographical location. The USA and Brazil are the two major bioethanol producers in the world. Sugarcane and cane molasses are the substrates for the ethanol production in Brazil as is cornstarch in the USA (Almeida et al. 2007). Other substrates used are cassava, sugar beet, wheat, etc. However, use of food products like corn and cassava for ethanol production has an inflating effect on the prices of these staple crops and an effect on their supply. Additionally, storage of high concentration sugar substrates is liable to microbial contamination

Table 2 Common gasoline ethanol blends available in various countries (Balat 2007)

	Common vehicles	Flexible fuel vehicles (FFVs)
USA	E10	E85
Canada	E10	E85
Sweden	E5	E85
India	E5	–
Australia	E10	–
Thailand	E10	–
China	E10	–
Columbia	E10	–
Peru	E10	–
Paraguay	E7	–
Brazil	E20, E25	Any blend available

and requires sophisticated storage methods, such as refrigeration, which in turn requires energy use over long periods (Dodic et al. 2009). Work by Dodic et al. suggests the use of intermediate products such as thick juice in sugar beet production as substrates for ethanol production, in order to reduce storage volume and microbial contamination. Use of lignocellulosic materials such as switch grass, *miscanthus*, sorghum, and corn stover is highly encouraged due to high substrate availability, economic feasibility of production, and storage, and due to other reasons mentioned in section "Sources" of this chapter. Waste mushroom logs have been studied for their potential as substrates for ethanol production where 12 g/L ethanol concentration was obtained as against 8 g/L concentration for normal logs (Lee et al. 2008b). Mahua flowers were investigated for their potential as substrates for ethanol fermentation, with ethanol productivity of 3.13 g/kg flower/h at 77.1 % efficiency (Mohanty et al. 2009).

Lignocellulosic biomass consists of majorly cellulose, hemicelluloses, and lignin of which cellulose is the most desired component for ethanol production. Ethanol is produced from the sugars that are present in the cellulose in polymeric form. Biomass is initially preprocessed, such as size reduced and washed for ease of handling and removal of soil. As shown in Fig. 4, the first major stage requires release of sugars from the cellulose-hemicellulose-lignin matrix; the second major stage involves the hydrolysis of higher sugars and fermentation of the monomeric sugars to ethanol; and the third stage involves the separation of ethanol from the fermentation broth.

Pretreatment

Pretreatments for bioethanol production may be performed using chemicals such as sulfuric acid, sodium hydroxide, ammonium hydroxide, supercritical ammonia, and supercritical carbon dioxide at both high and low temperature and pressure conditions to separate undesirable components such as lignin from biomass. Pretreatment disrupts the biomass structure and increases the surface area to enhance enzyme

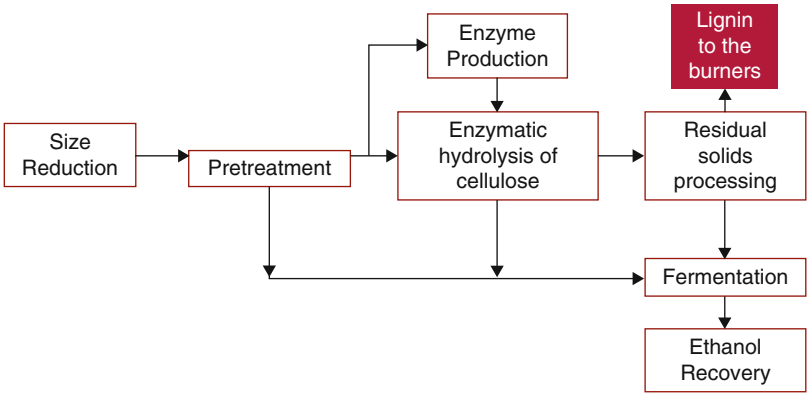

Fig. 4 Cellulosic ethanol "sugar platform"

access during the hydrolysis stage. Several pretreatment methods such as hot water treatment, steam explosion, dilute sulfuric acid treatment, and ammonia fiber expansion can be employed to remove lignin and/or depolymerize lignocelluloses structure in biomass.

Thermal processes include liquid hot water (LHW) and steam pretreatment. At temperatures above 150–180°C, hemicellulose and then lignin begin to dissolve (Bobleter 1994a; Garrote et al. 1999). Hot water pretreatment primarily dissolves hemicellulose to increase access for enzyme hydrolysis and to limit formation of inhibitors (Mosier et al. 2005a). Liquid hot water has removed up to 80 % of the hemicellulose to improve enzymatic hydrolysis by increasing the accessible surface area of the cellulose (Mosier et al. 2005a; Laser et al. 2002). pH should be kept between 4 and 7 to maintain hemicellulosic sugars in oligomeric, reducing formation of degradation products and thus inhibitors (Mosier et al. 2005a). Hemicellulose can be hydrolyzed to form acids which further hydrolyze the hemicelluloses (Gregg and Saddler 1996). The main advantages for LHW are recovery of pentoses, minimization of inhibitors, compared to steam explosions and minimal need for chemical and neutralization as compared to dilute acid pretreatment (Taherzadeh and Karimi 2008). Hot water pretreatment of lignocellulosic biomass has three types of reactor configurations, cocurrent, counter current, and flow through. In cocurrent pretreatment, biomass and water are heated to a desired temperature and held in the reactor for a controlled residence time before cooling. In counter current flow system, biomass slurry and water are allowed to flow in opposite directions into the reactor. In flow through configuration, hot water is allowed to flow through a stationary bed of biomass (Mosier et al. 2005b). Therefore, pretreatment technologies have been developed to be carried out in both batch and continuous flow reactor configurations.

Steam explosion has been widely tested in lab and pilot-scale systems. Biomass is pressurized with steam at 160–260°C for several seconds to minutes and pressure is rapidly released. Mechanical forces separate fibers and the high temperature promotes conversion of acetyl groups to acetic acid (Alvira et al. 2010; Taherzadeh and Karimi 2008). The main action of the acetic acid is probably to catalyze the hydrolysis of soluble hemicellulose oligomers (Bobleter 1994b). Lignin is redistributed and some removed (Pan et al. 2005). Removing hemicellulose increases accessibility of enzymes to the cellulose (Alvira et al. 2010). The advantages of steam explosion include use of larger chip size, reduced need for acid catalyst, high sugar recovery, and feasibility for industrial-scale use (Alvira et al. 2010). The primary disadvantages include partial hemicellulose degradation and generation of inhibitory compounds (Oliva et al. 2003). Steam explosion can be combined with addition of sulfur dioxide and sulfuric acid to enhance recovery of cellulose and hemicellulose. It improves the solubilization of hemicelluloses, lowers optimal treatment temperatures, and partially hydrolyzes cellulose (Brownell et al. 1986; Tengborg et al. 1998). Acid addition is particularly effective with softwoods, which have a low content of acetyl groups (Sun and Cheng 2002).

Acid pretreatment removes hemicellulose to make cellulose more accessible. It can also hydrolyze fermentable sugars. Acid pretreatment can be practiced using

high concentrations of acid (generally sulfuric) at low temperatures or low concentrations at high temperatures (Taherzadeh and Karimi 2008). Use of concentrated acid requires corrosion resistant process equipment. Recovery of the acid is energy intensive and produces degradation products inhibitory to fermentation (Alvira et al. 2010; Taherzadeh and Karimi 2008; Chisti 1996). Use of dilute acid is more promising, for example at 0.1–1 % sulfuric acid at 140–190°C. This achieves almost total hemicellulose removal and high cellulose conversion (Taherzadeh and Karimi 2008). Production of inhibitory compounds is lessened (Hendriks and Zeeman 2009). Addition of nitric acid greatly improves solubilization of lignin in newspaper (Xiao and Clarkson 1997). The use of acid pretreatment for methane production is more forgiving because methanogens can tolerate the inhibitory compounds (Xiao and Clarkson 1997; Benjamin et al. 1984).

Alkali pretreatment uses NaOH, $Ca(OH)_2$, or ammonia. Lime is very effective (Hendriks and Zeeman 2009). It removes acetyl groups and has lower cost and less safety concerns. Solvation and saponification reactions (Hendriks and Zeeman 2009) lead to swelling. The swelling increases internal surface area of cellulose, decreases polymerization and crystallinity, and disrupts lignin structure and removes some lignin and hemicellulose (Taherzadeh and Karimi 2008), increasing accessibility to enzymes enhancing saccharification (Kassim and El-Shahed 1986). Processing can be done at low (ambient) temperature (Xu et al. 2007) for long time periods (24 h) or at elevated (120–130°C) levels for minutes to hours (Silverstein et al. 2007). Production of inhibitory compounds is significantly less (Taherzadeh and Karimi 2008). But, solubilization and redistribution of lignin and modifications in crystalline state of lignin can counteract the benefits of the method (Gregg and Saddler 1996). Addition of hydrogen peroxide to alkaline pretreatment enhances lignin removal and improves enzymatic hydrolysis (Carvalheiro et al. 2008). Alkaline pretreatment, as with acid, is more forgiving for production of methane versus ethanol (Pavlostathis and Gossett 1985).

Ammonia fiber explosion or "expansion" (AFEX) is analogous to the steam expansion method. Anhydrous ammonia is added to biomass at approximately 1 kg NH_3: 1 kg dry and held at temperatures of approximately 100–120°C for several minutes. Pressure is rapidly released, swelling and disrupting the lignocellulose structure (Alvira et al. 2010; Taherzadeh and Karimi 2008). Only a solid residue is produced and a little hemicellulose and lignin are removed (Wyman et al. 2005). Enzyme hydrolysis yields and ethanol production are increased (Alizadeh et al. 2005). AFEX does not produce inhibitors, although some lignin may remain on the biomass surface (Alvira et al. 2010). It is more effective on lower-lignin crop residues and herbaceous crops than woody material (Wyman et al. 2005).

CO_2 explosion uses CO_2 at high pressure to penetrate the pores of lignocellulose. Explosive depressurization disrupts the cellulose and hemicellulose structure and improves enzymatic hydrolysis. Supercritical conditions at 35 °C and 73 bar remove lignin and increase digestibility more effectively (Alvira et al. 2010). However, pretreatment with appropriate conditions is a highly desirable step for lignocellulosic biomass to improve its digestibility.

Other physiochemical methods include organosolv and wet oxidation. Organosolv uses organic solvents to dissolve lignin. Solvent recovery is essential, and inexpensive, low molecular weight alcohols are favored. The recovery of low molecular weight lignin as a coproduct is potentially a significant advantage (Pan et al. 2005). Wet oxidation uses water and oxygen under elevated pressure and temperature (Taherzadeh and Karimi 2008). Hydrogen peroxide can be used at ambient temperature can also be used to enhance enzymatic hydrolysis (Azzam 1989). Batch treatment of corn stover using $FeCl_3$ in tubular reactors resulted in the hydrolysis yield of 98 % compared to 22.8 % yield for the untreated corn stover (Liu et al. 2009).

Biological pretreatment primarily uses brown and white soft rot fungi that degrade lignin and hemicelluloses (Taherzadeh and Karimi 2008). White rot fungi in particular have been evaluated and several shown to have high delignification efficiency (Kumar et al. 2009). Increase in total sugar yields during hydrolysis has been reported for switch grass preprocessed with *Phanerochaete chrysosporium* for 7 days (Mahalaxmi et al. 2010). Advantages include low energy and chemical requirements and ambient conditions. However, hydrolysis rates after biological pretreatment are low, and more research is needed (Alvira et al. 2010).

Hydrolysis

Hydrolysis of the pretreated biomass can be performed both chemically and biochemically. Chemical hydrolysis uses a continuous two-step dilute sulfuric acid process. The first step involves low temperature treatment and the second step, a high temperature treatment, as hemicellulose depolymerizes at lower temperature than the cellulose polymer. In the first step, the hemicellulosic fraction is removed, followed by the second step in which hexose release occurs. A batch process, using concentrated sulfuric acid, is also used for biomass hydrolysis; however, the use of concentrated acid requires high capital investment due to the requirement of corrosive resistant process equipment. Additionally, it requires acid recycling and recovery for economic viability of the process (Balat 2007).

Biochemical hydrolysis is the most sought out process in recent years and is commonly called as saccharification. It is initiated by enzymes that cleave the cellulose-lignin matrix into various monomeric, dimeric, and oligomeric sugars. Most common enzymes that act synergistically for cellulose hydrolysis, called cellulases, are endoglucanases or endo-1,4-β-glucanases (EG), exoglucanases or cellobiohydrolases (CBH), and β-glucosidases (BGL). While endoglucanases cleave the intramolecular bonds of the cellulose polymer, CBH and BGL catalyze the release of cellobiose and glucose from oligomeric ends and glucose from cellobiose respectively as shown in the Fig. 5. A synergistic effect of an enzyme component system consisting of at least endo-β-glucanases, exo-β-glucanases, and β-glucosidases results in hydrolytic efficiency (Sun and Cheng 2002; Maeda et al. 2011).

Enzymes related to hemicellulose hydrolysis, hemicellulases, are majorly endo-1,4- β-xylanase, β-xylosidase, α-glucuronidase, α-L-arabinofuranosidase, and acetylylan esterase as shown in Fig. 6. Therefore, the hydrolysate contains both

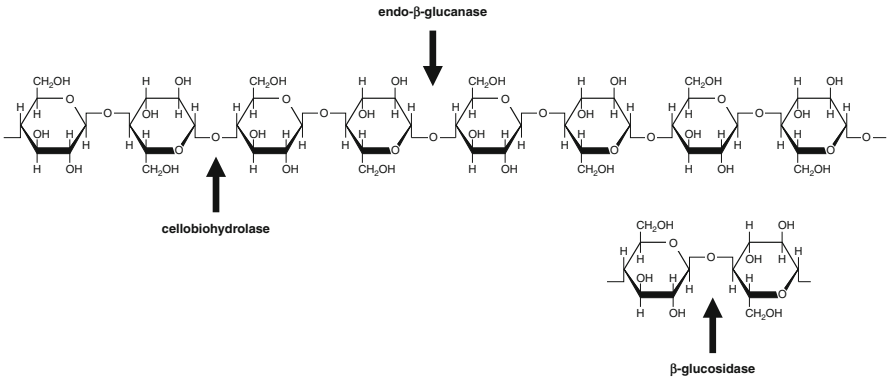

Fig. 5 Molecular structure of cellulose and site of action of endoglucanase, cellobiohydrolase, and β-glucosidase (Kumar et al. 2008)

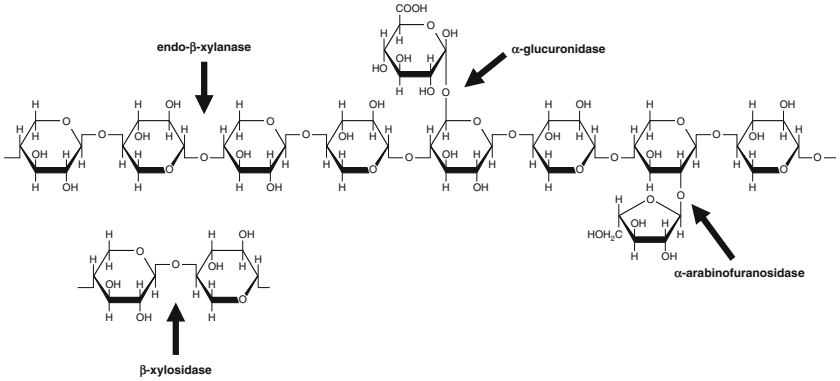

Fig. 6 Polymeric chemical structure of hemicellulose and targets of hydrolytic enzymes involved in hemicellulosic polymer degradation (Kumar et al. 2008)

hexoses and pentoses and their oligomeric forms depending on the treatment (Kumar et al. 2008).

Various bacteria such as *Clostridium*, *Cellulomonas*, *Bacillus*, *Thermomonospora*, *Ruminococcus*, *Bacteriodes*, *Erwinia*, *Acetovibrio*, *Microbispora*, and *Streptomyces* produce these enzymes to hydrolyze lignocelluloses. Fungi such as *Trichoderma*, *Ceriporiopsis*, *Aspergillus*, and *Sporotrichum* also possess the cellulolytic abilities to hydrolyze lignocellulosic biomass. Therefore, enzyme extracts from these cultures are used for hydrolyzing biomass and recent developments in enzyme technology have reduced their price of production significantly.

The factors that influence the enzymatic hydrolysis are mainly temperature, pH, and substrate concentration. At low substrate concentration, increase in substrate concentration increases the yield and the reaction rate of hydrolysis. However, at

high substrate concentration, yield and reaction rate decrease due to substrate inhibition of enzymes (Sun and Cheng 2002; Chisti 1996). Temperature and pH for enzyme activity varies by the microbe source from which it is derived. However, most commonly used industrial cellulases are derived from wild and modified strains of *Trichoderma reesei* and have an optimum temperature between 45 °C and 50 °C. Hydrolysis yields are also increased by addition of surfactants such as Tween-20. It is reported that the addition of Tween-20 resulted in 8 % increase in ethanol and 50 % reduction in cellulases dosage, increase in enzyme activity and the hydrolysis rate (Sánchez and Cardona 2008).

Consolidated microbial treatment of biomass is another method of saccharification of biomass. Loss of sugars during the process is inevitable, due to the consumption by microbes, which makes the use of enzyme extracts advantageous for hydrolysis. Enzyme hydrolysis is limited byproduct inhibition, which requires continuous removal of hydrolysis products apart from the use of BGL for subsequent conversion of the generated cellobiose to glucose. Therefore, simultaneous saccharification and fermentation (SSF) is a potential solution for product inhibition, where release of glucose using enzyme hydrolysis and its subsequent fermentation to ethanol by yeast take place in the same system (Balat 2007).

Fermentation

Fermentation of biomass to ethanol is commonly carried out using yeast such as *Saccharomyces* and *Pichia*, bacteria such as *Zymomonas* and *Escherichia*, and fungi such as *Aspergillus.* Products of hydrolysis and sugars are converted to ethanol producing carbon dioxide as byproduct and energy for cell growth. The most commonly used microbe *Saccharomyces cerevisiae* ferments sugars to ethanol at almost anaerobic conditions, although it requires a certain amount of oxygen for essential polyunsaturated fats and lipids. Figure 7 depicts the ethanol fermentation pathway of *Saccharomyces* from glucose. It briefly describes the conversion of glucose to ethanol through intermediate biochemical reactions involving NAD^+ and NADH (Nicotinamide adenine dinucleotide – oxidized and reduced forms, respectively). Since lignocellulosic biomass consists of several components such as pentoses, hexoses, and acids (acetic acid), degradation products derived from the pretreatment stage could inhibit the fermentation process. Chemical, physical, and biological methods have been developed to overcome the inhibition effect of these compounds by detoxification. *Trichoderma reesei* has been reported to degrade the inhibitors present in willow hydrolysate after steam pretreatment. Overnight extraction of spruce hydrolysate with diethyl ether at pH 2 showed detoxification effects with ethanol yields comparable to the reference fermentation. Detoxification by alkali treatment at pH 9 using $Ca(OH)_2$ and readjustment of pH to 5.5 allowed better fermentability due to precipitation of toxic compounds (Palmqvist and Hahn-Hägerdal 2000).

Usually, the temperature of operation is in the mesophilic range (15–40°C) for most of the species mentioned above. Increases in temperature beyond the optimum condition result in a decrease in ethanol yield and eventually in cell death. Another important factor in maintaining good cell growth is pH, generally a pH range of 6.5–7.5 (Aminifarshidmehr 1996) is suitable for ethanol fermentation for most of the

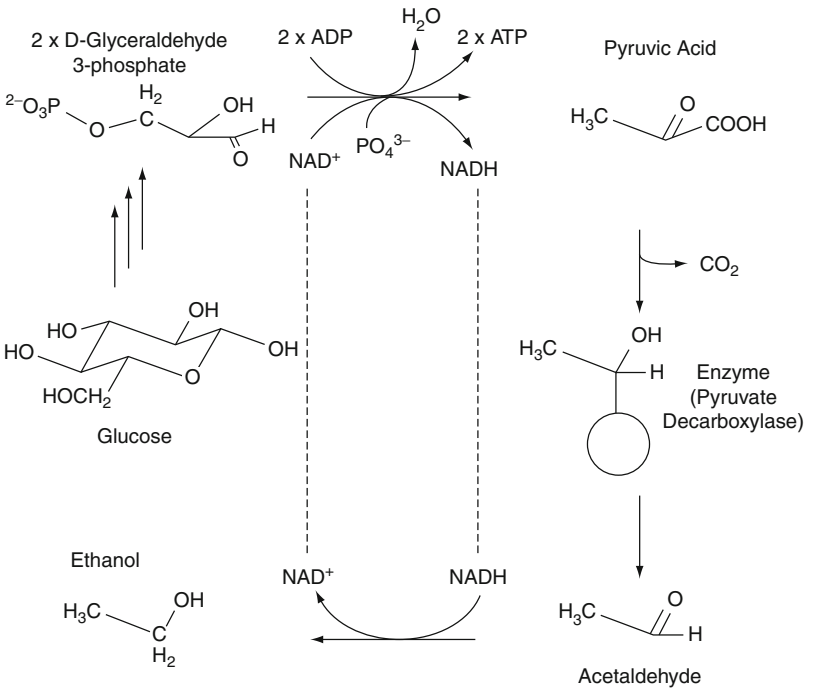

Fig. 7 Ethanol fermentation pathway of *Saccharomyces*

strains, although, yeast and fungal strains can tolerate up to 3.5–5.0. pH below 4.0 reduces the potential of bacterial contamination thus alleviating the requirement of severe aseptic techniques (Balat 2007).

Fermentation of biomass is affected by several other factors such as ethanol tolerance, substrate concentration, and byproduct inhibition. Ethanol tolerance is one of the factors which determine the maximum ethanol concentration that can be reached during fermentation, as most of the microbes responsible for fermentation cannot tolerate high concentrations of ethanol, eventually leading to cell death. *Zymomonas* has higher ethanol tolerance and achieves 5 % higher ethanol yields, as compared to the other yeast strains (Mohagheghi et al. 2002). Increase in substrate concentration decreased the ethanol yield. However, batchwise charging of substrate reduces this kind of inhibition. Therefore, fed-batch reactors are more suitable for industrial applications. Byproduct inhibition is overcome by chemical, mechanical, or biological detoxification as mentioned above (Balat 2007).

Butanol

Butanol is a colorless liquid which causes a narcotic effect at high concentrations. It is used as a solvent in biopharmaceutical, chemical, and cosmetic applications because of its high solubility in organic solvents and low water miscibility. Its physical properties very closely resemble those of gasoline, making it a potential

additive in partial or complete to transportation fuel (Lee et al. 2008c). Butanol can also be used as a replacement fuel to gasoline-driven engines with minimum or no changes; it can also be blended with gasoline at much higher composition than ethanol as butanol has similar energy content as that of gasoline. It can be added to gasoline at the refinery and distributed through existing gasoline pipeline unlike ethanol, as butanol is less corrosive and does not absorb water (Dürre 2008).

Butanol, a four carbon primary alcohol, can be synthesized both chemically and biochemically; chemical synthesis of butanol is conducted majorly by three methods, namely, Oxo synthesis, Reppe synthesis, and crotonaldehyde hydrogenation. However, the discussion of this chapter is limited to biochemical conversion of biomass to butanol.

In biochemical route, butanol is a fermentation product of anaerobic bacteria *Clostridium acetobutyliticum*, *Clostridium butyricum*, etc. Industrial production of butanol dates back to 1914 during World War I, as a byproduct in the production of acetone (which was used in war ammunition) by fermentation using *C. acetobutyliticum*. Although there was no immediate application of butanol during that time, later in 1920s in the USA, it was used to replace amyl acetate, a product from amyl alcohol, a solvent for lacquers in the automobile industry. By the 1950s, 66 % of the butanol used in the world was produced biochemically. However, due to increased biomass cost and low crude oil prices, crude oil replaced butanol as a transportation fuel (Dürre 2007). Substrates used for butanol production can be of both starch and cellulose origin such as molasses, corn fiber, wheat straw, etc. However, the conflict of using food substrates for fuel production regulates the usage of starch-based substrates. Figure 4, which depicts the flow of processes for ethanol, can also be applied for butanol. However, fermentation of biomass is carried out by butanol producing bacteria.

The biochemical routes involved in butanol formation are given in Fig. 8 (Lee et al. 2008c). Butanol formation takes place through the glucose-pyruvate-butyraldehyde route. Butanol fermentation is a biphasic transformation consisting of an acidogenic phase which occurs during exponential growth phase and solventogenic phase. During the acidogenic phase, acid-forming pathways are activated, and acetate, butyrate, hydrogen, and carbon dioxide are produced as major products. Acetone, butanol, and ethanol/propanol are the products of solventogenic phase which occurs after the exponential growth phase (Lee et al. 2008c). Both acidogenic and solventogenic phases can be seen in the Fig. 8 based on the final products produced in the two phases. The solventogenic phase is a response to the increased acid production after acidogenic phase, which if not initiated, would lead to a decrease in the extracellular pH, and finally to cell death due to increasing proton gradient between inner and outer cellular environments (Dürre 2008). Therefore, pH control has a very crucial effect on butanol production, and it requires being in the acidic range for the solventogenic phase.

Solvent toxicity is another major concern that causes cell death, due to cell wall weakening in the presence of acetone, ethanol, and butanol (the most toxic compound), leading to low product concentrations and productivity (Lee et al. 2008c). Solvent toxicity can be overcome by continuous removal of the solvents through

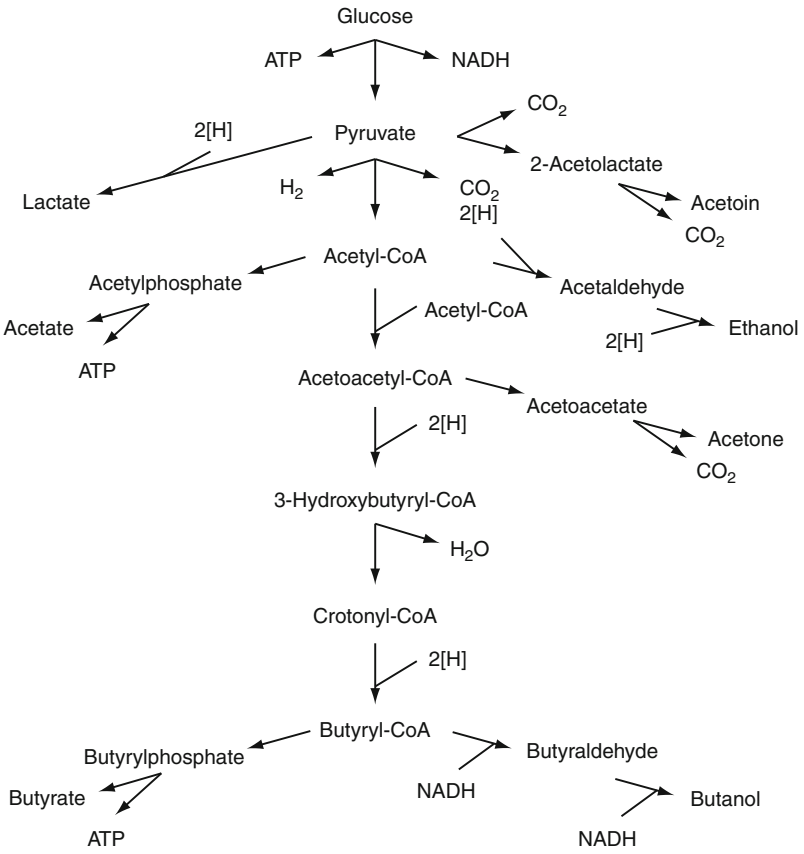

Fig. 8 Butanol fermentation pathway of *Clostridium acetobutylicum* (Dürre 2008)

various unit operations. Traditionally, butanol formed is separated by distillation which is a cost-intensive operation due to its high boiling point. Alternative methods for butanol separation are adsorption, gas stripping, liquid-liquid extraction, perstraption, pervaporation, and reverse osmosis (Dürre 2007). Each of these processes has certain limitations, among which, gas stripping is simple and successful in spite of low selectivity, as it can be used in a continuous operation for removing butanol. Liquid-liquid extraction requires use of a solvent that is noninhibitory to the microbes. In pervaporation, butanol is selectively diffused through a membrane and evaporated without removing the medium components necessary for the microbial growth (Qureshi et al. 1999). However, it is limited by fouling of membranes by the particles present in the fermentation broth.

Biodiesel

Biodiesel is a biofuel derived from transesterification of fats and oils with properties similar to the petroleum diesel. It can be blended with diesel or used directly in the

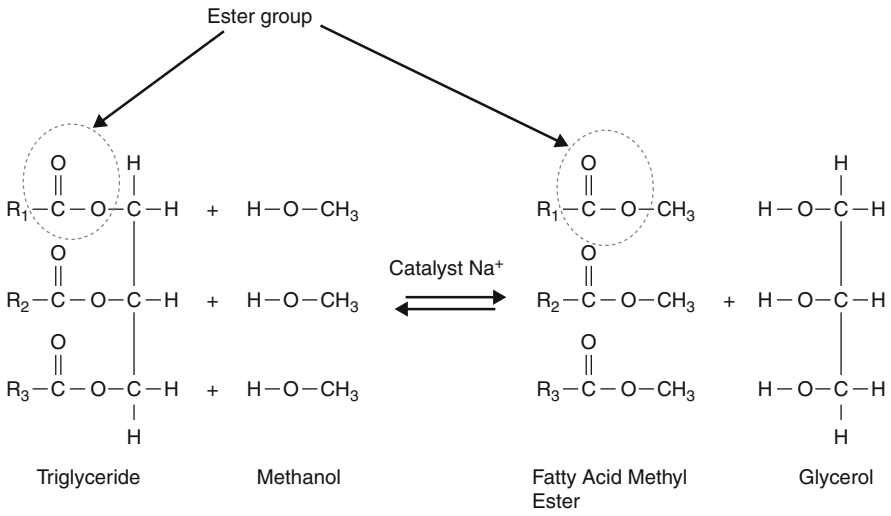

Fig. 9 Formation of biodiesel (Fatty acid methyl ester)

existing diesel engines without significant modifications. The main advantage of biodiesel is that, as a biomass-derived fuel, it produces 78 % less (net) carbon dioxide emissions, compared with that for petroleum-derived diesel fuel. Because its structure is nonaromatic, it combusts more efficiently, producing 46.7 % less carbon monoxide emissions, 66.7 % less particulate emissions, and 45.2 % less unburned hydrocarbons compared to conventional diesel. Therefore, it can be used in highly sensitive environments such as marine and mining environments (Helwani et al. 2009). Additionally, its high boiling point (about 150 °C) and presence of fatty acids impart lesser volatility and higher lubricating effect respectively, on engines, eventually reducing wear and tear and enhancing longer service life (Al-Zuhair 2007).

Biodiesel is conventionally produced from transesterification of oil (triglycerides) with alcohol (methanol) in the presence of an acid, base, or enzyme catalyst with glycerin as byproduct as shown in Fig. 9.

The sources of oil include oil seed plants such as palm, rapeseed, soybean, castor, and jatropha, used oils, lard, animal fat residue, etc. Palm oil having the highest yield of around 4,000 kg of oil per hectare is considered to be the best source of oil for biodiesel production (Al-Zuhair 2007). However, the majority of the cost involved in biodiesel production arises from the cost of the feedstock oil. Further, with the increasing edible oil consumption, it is more economical and environmentally sustainable to employ used oils and nonedible oils for biodiesel production. The major differences between the fresh and used oils are the moisture and free fatty acid (FFA) content, with used oils having high moisture and FFA content, which affect the acid- and alkaline-catalyzed transesterification, respectively. Alternatively, animal fats from waste residues are a useful source of oils. However, the heat at their high melting points denatures the enzymes used during enzyme-catalyzed

transesterification. Other sources of oil are oleaginous yeasts and filamentous fungi which on their outer surface secrete oil (Miao and Wu 2006).

As mentioned earlier, biodiesel production process can be alkali, acid, or enzyme catalyzed depending on the amount of FFAs and moisture present in the oil feedstock. The stoichiometry from Fig. 9 suggests oil to methanol ratio to be 1:3. However, for equilibrium to proceed toward the formation of biodiesel, use of excess alcohol is suggested.

During an alkali-catalyzed reaction, the oils in the presence of excess methanol are converted to fatty acid methyl esters and glycerin (Fig. 10). Alternately, during an acid-catalyzed reaction the triglycerides are esterified followed by a transesterification process (Fig. 11) (Schuchardt et al. 1998). Low FFA-containing feedstock is more suitable for alkali-catalyzed transesterification and high FFA-containing ones for acid-catalyzed reaction. FFAs present in oils during base-catalyzed reaction react with the oils to form soap and emulsions that hinder the purification processes of biodiesel apart from base consumption (Basu and Norris 1996). Alkaline methoxides are high biodiesel yielding base catalysts with short reaction times, even at very low (0.5 mol%) concentrations. However, they are more expensive than metal hydroxides (KOH and NaOH) (Helwani et al. 2009). On the other hand, acid-catalyzed reactions are 400 times slower than the alkali-catalyzed transesterification (Al-Zuhair 2007) and less sensitive to FFA content. The presence of water greatly inhibits the conversion due to catalyst deactivation.

The major reaction parameters affecting the biodiesel conversion are temperature, oil/methanol ratio, FFA, and moisture contents. An increase in temperature will increase the conversion the most appropriate range being 60–70°C, the alcohol boiling range at atmospheric pressure.

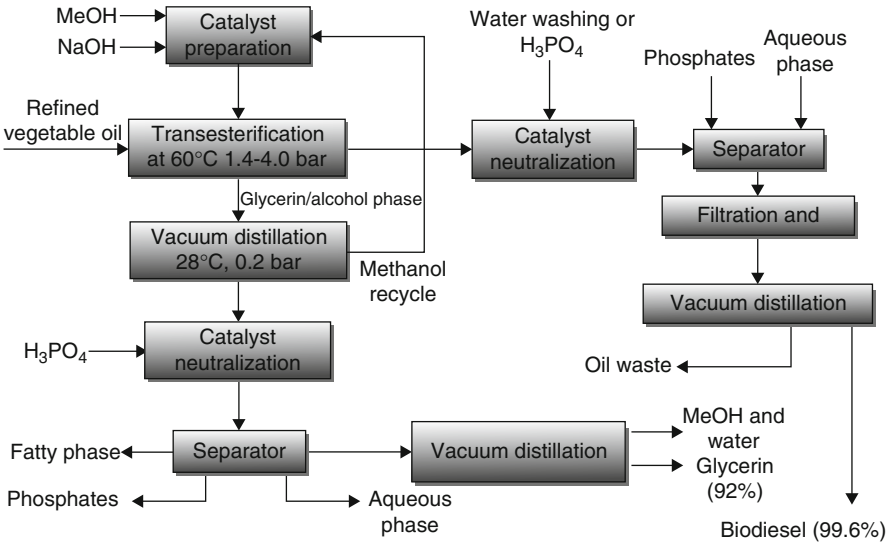

Fig. 10 Block diagram for base-catalyzed production of biodiesel (Helwani et al. 2009)

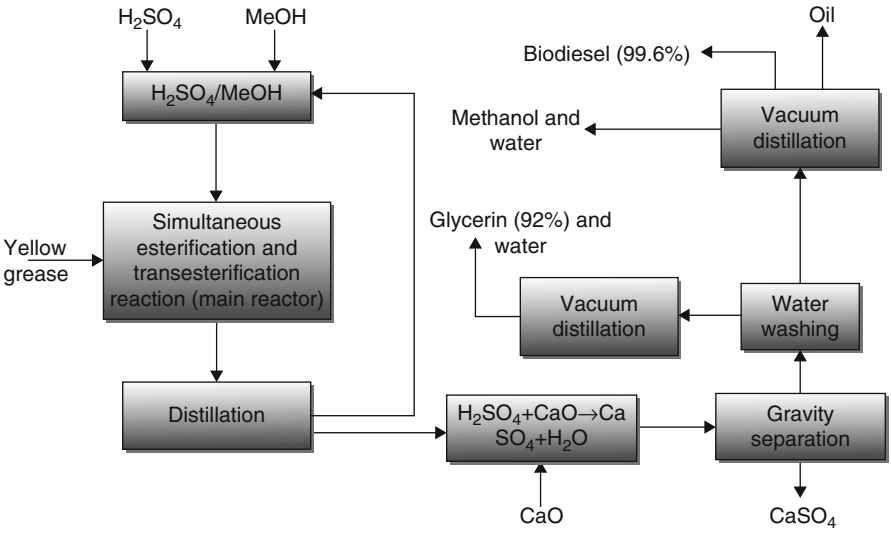

Fig. 11 Block diagram for acid-catalyzed production of biodiesel (Helwani et al. 2009)

Enzyme-catalyzed transesterification is achieved using lipases obtained from organisms such as *Candida rugosa*, *Pseudomonas fluorescens*, *Rhizopus oryzae*, *Burkholderia cepacia*, *Aspergillus niger*, *Thermomyces lanuginosa*, and *Rhizomucor miehei* (Al-Zuhair 2007). Enzymes are more compatible in terms of usage of a wide range of feedstocks, fewer processing steps, and fewer separation steps. Enzymes do not form soaps with the FFAs present in the feedstock, which allows the use of spent oils and animal fats for biodiesel production. They can convert both FFAs and triglycerides (TAG) simultaneously without another pretreatment step for converting FFAs to TAG (Fjerbaek et al. 2009). An increase in temperature increases the enzymatic conversion of biodiesel due to increased rate constants and lesser mass transfer limitations (Al-Zuhair et al. 2003). Additionally, optimal water content increases the biodiesel conversion as lipase acts as an interface between the aqueous and organic phases which allow its activation by rendering suitable conformation for transesterification (Panalotov and Verger 2000). However, they are currently facing challenges related to lower reaction rate, high cost, and loss of activity.

Methanol is the most widely used alcohol for biodiesel production due to its availability from syngas. However, it is required to use an alcohol produced from a renewable source, such as ethanol, to make biodiesel production a completely green process. Additionally, methanol is toxic and renders lipases inactive at high concentrations. Therefore, methyl acetate can be used as a methyl acceptor in place of methanol, as it still has no negative effects on Novozyme 435, the only commercial lipase known, used for biodiesel production from soybean oil (Du et al. 2004). Immobilization of lipases is considered an economical process to overcome the

limitations of using a batch process and employing a continuous process to enable glycerol separation for higher conversion rates (Watanabe et al. 2002).

Genetic Engineering Approaches

With the above background of conversion of biomass to fuels, it is evident that several factors such as biomass composition, pH, temperature, by-products, etc., have a potential impact on the biofuel production. Process factors such as pH and temperature can be maintained using appropriate reactor and process conditions. Intrinsic factors such as biomass composition, product tolerance such as ethanol and butanol tolerance, specific binding of enzymes, and byproduct inhibition will remain potential challenges without recombinant DNA technology.

Recombinant DNA technology is comprised of five general procedures (Nelson and Cox 2008):

1. A desired segment of the microbe DNA of interest is cut using sequence-specific endonucleases which are nucleotide cleaving enzymes, otherwise called restriction endonucleases. These endonucleases act as molecular scissors to obtain the required nucleotide sequence.
2. A small molecule of DNA capable of self-replication is selected. These molecules, called cloning vectors, are generally plasmids or viral DNA which can be coupled with the nucleotide sequence obtained from the previous step.
3. The two segments are incubated in the presence of DNA ligase to obtain a recombinant DNA.
4. Recombinant DNA is introduced into the host cell for replication. The most common host cell used is *E. coli* for its well-understood DNA metabolism and its well-characterized bacteriophages (viruses that live on bacteria) and plasmids.
5. After cell replication, the host cells with recombinant DNA are identified and used for expression.

The most commonly used host cells for metabolic engineering are *Escherichia coli*, *Zymomonas mobilis*, and *Saccharomyces cerevisiae* as their genetic maps are the most well studied (Banerjee et al. 2010). They are facultative anaerobes with fast growth rates and viability (Lee et al. 2008a). Incorporation and expression of pyruvate decarboxylase and alcohol dehydrogenase II genes from *Z. mobilis* into *E. coli* has resulted in high yields of ethanol from the utilization of both pentoses and hexoses, as against only hexoses (Banerjee et al. 2010). Although the recombinant strains are helpful in exploring the solutions for pathway-related problems, their industrial sustenance is limited due to the lack of robustness. Recombinant *E. coli* can produce isopropanol, n-butanol, and fatty acid ethyl esters through various engineered pathways (Atsumi and Liao 2008).

Modification of enzymes used in hydrolysis of biomass to produce sugars is generating immense interest. However, it is noticed that the enzymes belonging to the same class have different amino acid sequences conferring low level of

homogeneity, for example CBH1 (*T. reesei*) has <65 % amino acid identity in the nonredundant database. Additionally, cellulases from fungal origin have different optimized conditions than those from the prokaryotic origin and to predict a better choice between them is a challenge. Prediction of biochemical activity of an enzyme from its amino acid sequence is unreliable, and the only better way to evaluate it is to employ the extracted cellulase for hydrolysis and test it on various substrates (Banerjee et al. 2010). Alternatively, manipulation of plant species is directed toward altering its components such as lignin. Rastogi and Dwivedi discussed altering the lignin of woody species by introducing *omt* or *f5h* gene to introduce syringyl units in lignin to increase the paper pulping capability (Rastogi and Dwivedi 2008). Modification of lignin can be a potential area of research to increase the enzymatic digestibility and hydrolysis for enriching animal feed and enhancing biofuel production respectively.

Another area where genetic engineering principles can be applied is biomass cultivation. It is understood that by increasing the light interception efficiency and solar energy conversion to biomass, the productivity and yield of cultivation can be increased. Genetic engineering techniques can be applied to identify and manipulate the genes related to light reception and energy conversion to biomass in plants for dual interest, mitigation of elevated levels of atmospheric carbon dioxide, and increasing crop yield (Jansson et al. 2010).

Recombinant DNA technology is also used as a means to identify various enzymatic activities present in an organism. Two β-glucosidases were identified from *Pichia etchellsii* by cloning and expression of the corresponding genes in *E. coli* (Wallecha and Mishra 2003).

Advanced Fuels from Biochemical Conversion

Algal biofuels are of growing interest, where microalgae are grown in either closed or open photobioreactors to produce fatty acids. Although, algae use the sunlight with the same photosynthetic efficiency as that of land plants, the productivity of algal systems is much higher than plant biomass, as they do not produce roots, stems, and other structures. However, the extraction and processing costs during oil extraction from algae are challenging and require being minimized (Kaparaju et al. 2009). In an alternative use of algae, macro algae cells are used to tap the sunlight, and the tapped energy is converted to electricity. The excited electrons in the chloroplasts of the algae are intercepted through gold electrodes to create a tiny electrical current (Alternative energy).

The University of California, Los Angeles, is conducting research on microbes producing fuels from proteins rather than utilizing them for their growth. The microbes are induced to produce certain kind of proteins, which can be converted to fuels (Alternative energy).

Modified cyanobacteria utilize atmospheric carbon dioxide and sunlight to produce isobutanol which can be used as biofuel. Bacteria-powered battery is a very recent development, contrary to the belief that bacteria cannot produce electricity.

The byproducts of bacterial metabolism produce ions which pass through an ion filtering membrane, and then through an external circuit, converting the chemical energy provided by the bacteria to electrical energy (Alternative energy).

Isobutene, a fuel additive is produced through natural enzyme, rather than from the traditional petroleum-based route, by Prof. Thomas Bobik and his doctoral student David Gogerty. They have identified a new natural enzyme which can convert glucose found in plants to isobutene and is called as Bobik's enzyme. A limitation with the enzyme is the time taken to convert glucose to isobutene. However, efforts are in progress to reduce the conversion time for industrial feasibility (Alternative energy).

Qteros and Applied Clean Tech are working together to produce ethanol from wastewater and have developed technologies to handle the sludge remaining from the wastewater plant. Tobacco oil is another promising biofuel that is being studied by the researchers in Biotechnology Foundation Labs at Thomas Jefferson University. Current research is being conducted on improving the oil production from the tobacco leaves. Tobacco is a very attractive source for biofuel as it is not used in food production. Two genes in the plant have been modified to result in 6.8 % of oil per dry weight in leaves (Alternative energy).

Future Directions

It is evident from the above discussion that biofuels have high potential for meeting the needs of the transportation sector and supporting fuel independence and greener living. Choice of biomass and its properties are critical for optimum production of biofuel. Biomass selection depends on its availability, type of pretreatment employed, and the biofuel desired for production and their economics. Exploring various sources of biomass and improving the system of biomass collection and handling can improve the availability of biomass. Chemical, physical, or microbial techniques that can enrich the biomass composition are very desirable. Improvements in reactor design, process conditions, and other engineering aspects help optimize the biofuel production process. Adaptation and modifications of engine systems to biofuel use and innovation of newer ones is another important area of interest. Application of biotechnological tools such as recombinant DNA technology and genetic engineering can be used to overcome the bottlenecks of biochemical conversion and microbial robustness.

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