Biotechnology and Molecular Biology Review Vol. 6(8), pp. 172-182, November 2011
Available online at http://www.academicjournals.org/BMBR
ISSN 1538-2273 ©2011 Academic Journals

Review

Lignocellulosic ethanol production: Current practices and recent developments

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Accepted 2 August, 2011

Production of renewable fuels, especially bio-ethanol from lignocellulosic biomass, holds remarkable potential to meet the current energy demand as well as to mitigate greenhouse gas emissions for a sustainable environment. Present technologies to produce bioethanol largely depend on sugarcane and/or starch based grains and tubers (mainly corn, potatoes). This is partly due to ease of substrate handling and processing. On the other hand, use of sugarcane and food grains to produce bio-ethanol has caused significant stress on food prices and food security. Accordingly, the recent focus has been on lignocellulosic materials as a source for bio-ethanol. In fact, many countries are moving towards developing or have already developed technologies to exploit the potential of lignocellulosic materials for the production of bioethanol. This process of ethanol production generally involves hydrolysis of lignocellulosic biomass to fermentable sugars followed by fermentation of such sugars to ethanol. Achieving fermentable levels of sugars from lignocellulosic biomass requires relatively harsh pretreatment processes. The pretreatment process has pervasive impact on the overall operation because the process depends on the choice of lignocellulosic source, the size reduction via grinding, chemical treatment, acid hydrolysis, neutralization and fermentation. Recent advances in the process technologies have made it possible to use simultaneous saccharification and fermentation. In this process cellulase enzyme is the critical reagent as well as the cost determining factor. The advances in biotechnology as related to bioethanol have focused on engineering organisms that are capable of producing ethanol from cellulose, hemicellulose and lignocellulose. Such organisms are expected to be capable of not only degrading cellulose, hemicellulose and lignocellulose to fermentable sugars, but also are able to utilize both pentose and hexose sugars to produce ethanol at a relatively high yield. More recent and emerging approaches in bioethanol production are focused on reducing production costs. This approach uses consolidated bioprocessing schemes in which cellulase production, substrate hydrolysis, and fermentation are all accomplished in a single step. Countries, such as Nepal, that totally depend on the import of fossil fuels cannot ignore the potential of bioethanol derived from lignocellulosic biomass. Nepal is rich in biodiversity and posses variety of energy crops. Accordingly, developing policies and mechanisms that promote bioethanol will go a long-way in reducing the fuel crises in the countries lacking oil resources.

Key words: Lignocellulosic biomass, bioethanol, saccharification and fermentation (SSF), consolidated bioprocessing (CBP).

INTRODUCTION

Ethanol is an oxygenated fuel with high octane value like that of petroleum fuels. Ethanol is known to run combustion engines at higher compression ratios and thus provides superior performance (Wheals et.al., 1999). The blending of ethanol into petroleum-based automobile fuels can significantly decrease petroleum use and release of greenhouse gas emissions. Further, ethanol can be a safer alternative to the common additive, methyl tertiary butyl ether (MTBE), in gasoline. MTBE is toxic and is a known contaminant in ground water (Wang et al.,

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1999). Thus, ethanol can be a substitute to mitigate the problems associated with the rising energy demands across the world as well as a way to reduce green house gas emissions to an extent of 85% (Perlack et al., 2005).

Ethanol may be produced either from petroleum products or from biomass. Today, most of the ethanol produced comes from renewable resources. Although currently most of the ethanol produced from renewable resources comes from sugarcane and starchy grains, significant efforts are being made to produce ethanol from lignocellulosic biomass (almost 50% of all biomass in the biosphere) such as agriculture residues (Bothast and Saha, 1997). The technological advances in recent years are promising to produce ethanol at low cost from lignocellulosic biomass.

The world population is estimated to increases from 6.7 billion to 8 billion by 2030 (USCB, 2008). On the other hand, global oil production is expected to decline from 25 billion barrels to 5 billion barrels by 2050 (Campbell and Laherree, 1998). Thus the energy demands of the future is likely to play a key role in geo-political economics. Given this reality, nations around the world are investing in alternative sources of energy, including bioethanol. The leading nations in bioethanol production are Brazil and the USA (Table 1), and USA is the world's largest producer of bioethanol (Carere et al., 2008). Asian countries altogether account for about 14% of world's bioethanol production.

Bioethanol production from sugarcane and starch rich feed stocks such as corn, potato, etc., is considered first generation process and it has already been developed. The long-term viability of this process is in question because it will require significantly increased amounts of cultivatable land and significant hike in food prices that will ultimately lead to food insecurity (Mitchell, 2008). Estimates clearly point to the fact that first generation ethanol production process can not sufficiently meet the global energy needs. Therefore, second generation processes to produce bioethanol are gaining momentum. The second generation processes will use lignocellulosic materials for this purpose and biosphere clearly has sufficient supplies of lignocellulosic materials. The production of ethanol from lignocellulosic biomass [corn stover, wheat straw, sugarcane bagasse, rice straw, rice hull, corn cob, oat hull, corn fiber, woodchips and cotton stalk; energy crops such as switch grass and Alfa Alfa, and various weeds such as *Saccharum spontaneum*, *Lantana camara*, *Eichhornia crassipes* (water hyacinth), etc.] has become one of the best alternatives, because these sources have widespread abundance and the cost of their procurement is relatively cheap.

Even though the lignocellulosic biomass is abundant, the commercialization of the process to produce bioethanol from it is limited due to insufficient research, especially the research related to minimization of production cost. Bioethanol production from lignocellulosic materials relies on technologies that will efficiently hydrolyze cellulosic biomass to fermentable sugars. The hydrolysis process produces byproducts that are toxic to yeast cells, thus interfere with fermentation process (Palmqvist, 2000a). Although several detoxification methods, such as activated charcoal adsorption and lime treatment process, have been devised, an appropriate strategy for efficient hydrolysis of cellulose to fermentable sugars is still lacking (Kaya et al., 2000; Aden et al., 2002).

CELLULOSIC BIOMASS AND THEIR SUGAR COMPOSITION

Lignocellulosic biomass consists of lignin, cellulose, hemicellulose, pectin and other components (Table 2).

Cellulose is the principle component of lignocellulosic biomass and its concentration ranges from 40 to 50% of dry weight. Cellulose is a homopolysaccharide composed of repeating β-D-glucopyranose units. The degree of polymerization and crystallinity of cellulose varies from species to species and this is shown to have a significant impact on hydrolytic process (acidic and enzymatic) (Zhang et al., 2004).

Hemicellulose is less complex, its concentration in lignocellulosic biomass is 25 to 35% and it is easily hydrolysable to fermentable sugars (Saha et al., 2007). Hemicellulose is a heteropoly saccharide composed of pentoses (D-xylose and D-arabinose), hexoses (D-mannose, D-glucose and D-galactose) and sugar acids. Softwood hemicellulose mainly contains mannose as a major constituent whereas hardwoods mainly contain Xylans (Balan et al., 2009).

Lignin is the third major component of lignocellulosic biomass and its concentration ranges for 20 to 35%. It is a complex polymer of phenyl propane (*p*-coumaryl, coniferyl and sinapyl alcohol). Lignin acts as cementing agent and an impermeable barrier for enzymatic attack (Howard et al., 2003). Lignin provides plants with the structural support and impermeability they need as well as resistance against microbial attack and oxidative stress. These properties of lignin may be attributed to its amorphous nature, water insolubility and optical inactivity. The later properties also make it tough to degrade it (Fengel and Wegener, 1984).

OVERVIEW OF LIGNOCELLULOSIC FERMENTATION

The lignin-hemicellulose-pectin complex forms one of the most stringent seals around cellulose. The first step in the overall process of lignocellulosic fermentation is breaking this barrier (pretreatment). This is the most important and rate limiting step in the overall process. Further steps involve isolation and hydrolysis of cellulose and hemicellulose to generate fermentable sugars (saccharification) followed by fermentation and distillation (Figure 1). The pretreatment processes involve the use of acids, alkalis and/or organic solvents. The aim of this

Table 1. Leading bioethanol producers in the world.

Country/group of countries	Ethanol produced in:	
	Million liters	MTOE
Brazil	19,000	10.44
Canada	1,000	0.55
China	1,840	1.01
India	400	0.22
USA	26,500	14.55
European Union	2,253	1.24
Others	1,017	0.56
World (Total)	52,000	28.57

*Source: Data from OECD-FAOA glink-Casimo database (2007). MTOE: Million tons of oil equivalents.

Table 2. Chemical composition of common lignocellulosic biomass.

Constituents	Hardwood (%)	Softwood (%)
Cellulose	40 to 50	40 to 50
Hemicellulose	25 to 35	25 to 30
Lignin	20 to 25	25 to 35
Pectin	1 to 2	1 to 2
Starch	Trace	Trace

*Source: Miller (1999).

process is to separate lignin, cellulose, hemicellulose and pectin from lignocellulosic biomass. Post pretreatment, the recalcitrant lignocellulosic biomass becomes susceptible to acid and/or enzymatic hydrolysis as the cellulosic microfibrils are exposed and/or accessible to hydrolyzing agents. In the pretreatment process, small amounts of cellulose and most of hemicellulose is hydrolyzed to sugar monomers; mainly D-xylose and D-arabinose. The pretreated biomass is then subjected to filtration to separate liquids (hemicellulose hydrolysate) and solid (lignin + cellulose). The liquid is sent to a xylose (pentose) fermentation column for ethanol production. Solids are subjected to hydrolysis (also called second stage hydrolysis). This process is mainly accomplished by enzymatic methods using cellulases. Mild acid hydrolysis using sulfuric and hydrochloric acids is an alternative procedure. The hydrolyzed sugars such as D-glucose, D-galactose, and D-mannose, can be readily fermented to ethanol using various strains of *Saccharomyces cerevisae.* The pentoses (D-xylose and D-arabinose) from hemicellulose hydrolysis are not easily utilized by saccharomyces strains; therefore, genetically modified strains of *Pichia stipitis, Zymomonas mobilis*, are used for their fermentation. *Candida shehatae* is capable of co-fermenting both pentoses and hexoses to ethanol and other value-added products at high yields.

PRETREATMENT OF LIGNOCELLULOSIC BIOMASS

Some of the important reasons for the pretreatment step are to (i) break the lignin-hemicellulose-pectin complex, (ii) disrupt/loosen-up the crystalline structure of cellulose and (iii) increase the porosity of the biomass. These changes in lignocellulosic materials make it easier for enzymatic saccharification (hydrolysis), results in higher fermentable sugars levels and will have a significant impact on the overall process (Mosier et al., 2005a; Sun and Cheng, 2007; Yang and Wyman, 2008). An ideal pretreatment process should yield high levels of pentoses, the hydrolysates will not have any inhibitory substances and the process is cost effective (Lynd, 1996). The current methods in practice include physical, chemical and biological processes. Physical pretreatment methods include combination of stem explosion and hydrothermolysis. Chemical pretreatment processes employ acids, alkali and organic solvents (Carillo et al., 2005). More recent processes have also employed pretreatment with hydrogen peroxide (Saha and Cotta, 2007), sulfite (Kuhad et al., 1999), ammonia (Teymouri et al., 2005) and sodium chlorite (Gupta et al., 2009). Biological pre treatments employ brown-, white- and soft-rot fungi (Basidiomycetes) to degrade lignin and hemicelluloses (Cardona et al., 2007).

Acid pretreatment

The common pretreatment practice in the last two decades has been to incubate lignocellulosic materials in the medium of dilute sulfuric acid (0.5 to 1.5% H_2SO_4) at 100 to 150°C (Wingren et al., 2003). In this process the

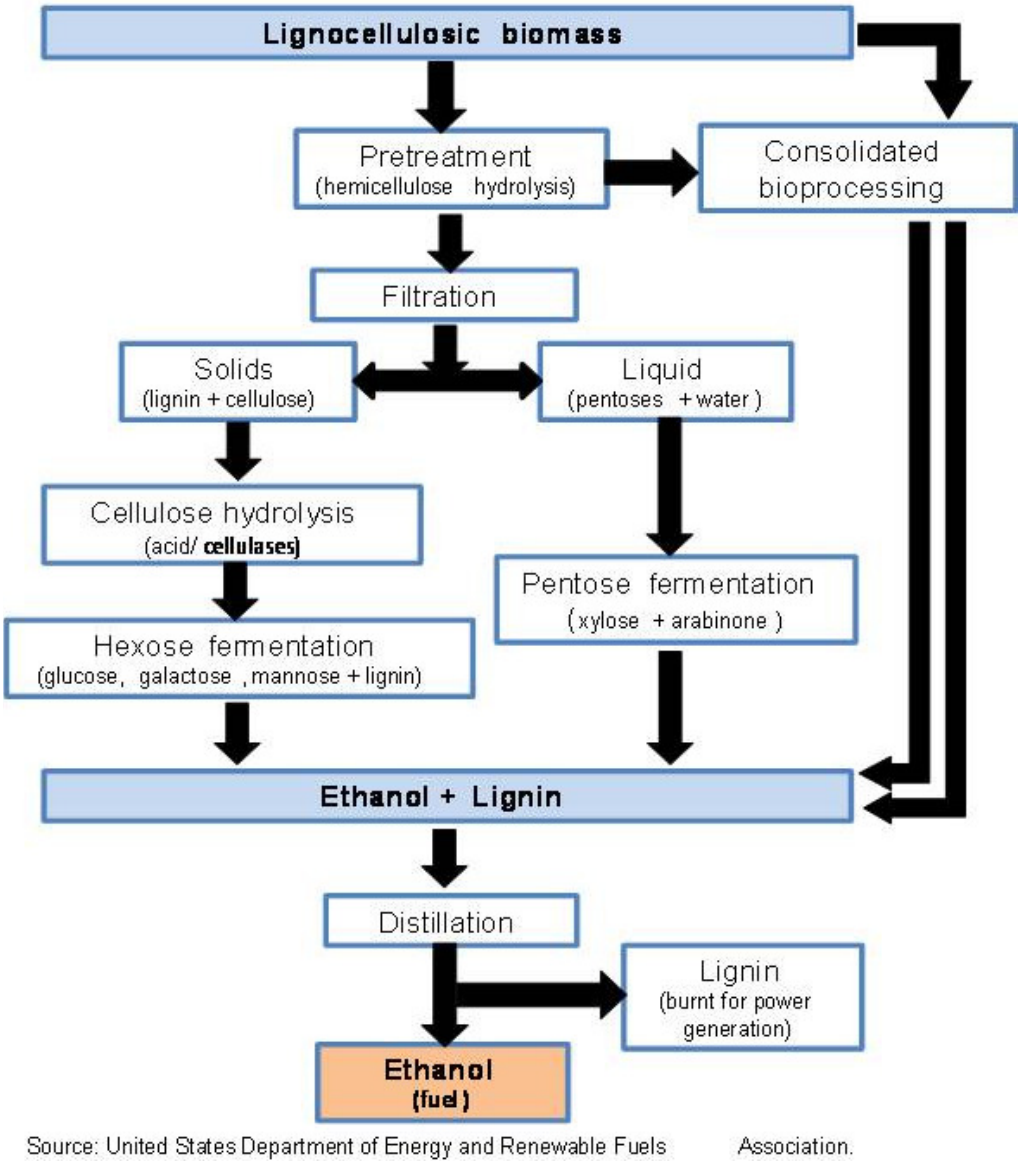

Figure 1. Overview of the process of ethanol production from lignocellulosic biomass.

hemicellulose is hydrolyzed into sugar monomers which are recovered in the liquid fraction after filtration. The residual solids will contain cellulose and lignin which will be further subjected to hydrolysis by cellulases (Taherzadeh and Karimi, 2008). This process has been shown to result in byproducts such as furfural (pentose sugars byproduct), and hydroxyl methyl furfural (HMF; hexose sugar byproduct), phenolic acid (lignin byproduct) and acetate (diacetylation product of hemicellulose). Each of these byproducts at concentration 5 mM or above have been shown to have significant inhibitory effect on the fermentation process (Martinez et al., 2000; Palmqvist et al., 2000b; Klinke et al., 2004). Use of higher concentrations of acid and higher temperatures yield higher reducing sugar concentrations, however, under such conditions reducing sugars are degraded at a much higher level (Zhu et al., 2009). Use of concentrated acid alone has been shown to (i) yield very high levels of sugar (90%), (ii) can handle diverse feedstock, (iii) is relatively rapid (10 to 12 hours), and (iv) causes less degradation. The latter requires more expensive corrosion resistant equipment (Hamelinck et al., 2005) and the hydrolysate have to be neutralized by the addition of lime and contaminants removed by treatment with activated charcoal (Gupta et al., 2009; Shleser,1994). In the later process, over-liming has been shown to catalyze condensation of lignin-derived compounds. The advantage of these reactions is that they detoxify the hydrolysate and these reactions occur at a much higher rate at higher pH. On the other hand, at

higher pH, D-glucose is converted to HMF, for example, at pH 11 as much as 30% of glucose is converted to HMF. Additionally, over-liming the hydrolysate at an industrial scale and accurately controlling pH in fermenters pose a major challenge. Steam treatment of H_2SO_4 impregnated lignocellulose is saccharified at a much higher rate. The most favorable conditions for this process require that the samples be treated with 0.5% H_2SO_4 and hold the sample at 200°C for 4 to 8 min (Sassner et al., 2008). Yet another pretreatment method used a mixture of H_2SO_4 and acetic acid with an efficiency of 90% saccharification (DeMoraes-Racha et al., 2010).

Alkali pretreatment

Most commonly used alkali in the alkali pretreatment processes are NaOH and $Ca(OH)_2$. This process results in (i) the removal of all lignin and part of hemicellulose, and (ii) increased reactivity of cellulose in further hydrolysis steps (Hamelinck et al., 2005), especially, enzymatic hydrolysis. Effective removal of lignin minimizes adsorption of enzyme onto lignin and thus allows for effective interactions with cellulose (Aswathy et al., 2010). NaOH causes swelling of the fibers and with increase in internal surface area. This also increases the degree of polymerization and crystallinity of cellulose (Taherzadeh and Karimi, 2008). Pretreatment with NaOH increases the digestibility cellulose from 14 to 55% while decreasing the lignin content from 25 to 20 % (Kumar et al., 2009). Alkali pretreatment process show decreased sugar degradation and are more effective on agriculture residues as compared to wood materials (Kumar et al., 2009). Between NaOH and $Ca(OH)_2$, pretreatment with $Ca(OH)_2$ is preferable because it is less expensive, more safer as compared to NaOH and it can be easily recovered from the hydrolysate by reaction with CO_2 (Mosier et al., 2005b).

Pretreatment with oxidizing agents

In these methods, lignocellulosic materials were treated with powerful oxidizing agents such as per-acetic acid or hydrogen peroxide (Gould, 1984). For example, Teixeira and associates observed ethanol yields of 98% when the lignocellulosic biomass was pretreated with 21% per-acetic acid (Teixeira et al., 1999). Sugar cane bagasse was pretreated with 50% per-acetic acid solution in a liquid to solid ratio of 6:1 at 80°C for 2 h, 80% of the cellulose recovered was converted to glucose by cellulose (Teixeira et al., 1999). Additionally, pre-soaking lignocellulosic biomass with NaOH required significantly smaller concentrations of per-acetic acid to achieve the same results. The latter is likely due to partial removal of lignin prior to per-acetic acid treatment (Teixeira et al., 1999; Zhao et al., 2009).

Organic-solvent pretreatment

The organic solvents such as methanol, ethanol, acetone, ethyleneglycol, triethylene glycol, and tetrahydrofurfuryl alcohol have been used to pretreat lignocellulosic biomass. These solvents are especially effective because they solubilize lignin and some of hemicellulose. Typical pretreatment conditions require that the lignocellulosic biomass be treated with 40 to 60% organic solvent, at 160 to 190°C, for 30 to 60 min (Pan et al., 2005). Pretreatment of lignocellulosic materials with acidified organic solvents (mixture of 80% ethylene glycol 19.5% water and 0.5% HCl at 178°C for 90 min) has also been successfully used (Yamashita et al., 2010). The advantages of these methods include recovery and recycling of organic solvents as they can be easily distilled out. Further the recovery process has been shown to isolate lignin as a solid and carbohydrates as syrup (Lora and Aziz, 1985; Johansson et al., 1987; Aziz and Sarkanen, 1989). The disadvantages are that the process requires expensive high pressure equipment.

HYDROLYSIS

The goal of this process is to generate fermentable monomeric sugars from hemicellulose and cellulose content of lignocellulosic biomass. This can be accomplished by two different processes, namely, acid hydrolysis and enzymatic hydrolysis.

Acid hydrolysis

Mineral acids such as sulfuric acid, hydrochloric acid, hydrofluoric acid and nitric acid are widely employed for the hydrolysis of lignocellulosic biomass. Among these, the oldest and best understood process utilizes sulfuric acid. The sulfuric acid-based hydrolysis process is operated under two different conditions; (í) a process that uses high sulfuric acid concentration that operates at a lower temperature and, (íí) a process that uses low sulfuric acid concentration and operates at a higher temperature. Among the two, the latter is most commonly used. This process produces relatively large number of undesirable byproducts as compared to the concentrated acid process. On the other hand, the acid recovery and recycling process is more complicated and cost intensive. Use of hydrochloric acid, although not commonly used, has technical advantages over the sulfuric acid process, given it is relatively volatile and can be recovered by vacuum stripping methods. Following pretreatment, the acid hydrolysis process is applied to lignocellulosic biomass in a two-step (stage) process

because the pentose sugars degrade/decompose more rapidly than hexose sugars. Accordingly, in the first stage hemicellulose is hydrolyzed (Figure 1) with dilute acid under ambient conditions and the more resistant cellulose is hydrolyzed at higher temperatures (213°C) and relatively higher concentration of acid (0.4%) in the second stage. Under the latter conditions, the recovery yields are 89% for mannose, 82% for galactose and 50% for glucose (Graf and Koehler, 2000; USDOE, 2003). In contrast, the concentrated acid methods result in high sugar recovery yields (90%), can handle diverse feed-stocks containing lignocellulose and the process is relatively rapid (10 to12 h) (Graf and Koehler, 2000; USDOE, 2003).

Enzymatic hydrolysis

Three major groups of enzymes are involved in the hydrolysis of cellulose, namely, 1,4-β-D-glucanglucanohydrolase (EC 3.2.1.3), 1,4-β-D-glucancellobiohydrolyase (EC 3.2.1.91) and β-D-glucosidase (EC 3.2.1.21) (Ladisch et al., 1983; Wright et al., 1988). These enzymes are commonly referred to as endoglucanase, exoglucanase and cellobiase, respectively. The endoglucanases attack randomly and cleave the cellulose chains to form glucose, cellobiose and cellotriose. The exoglucanases attack the non-reducing end of cellulose to form the cellobiose units. Finally, cellobiase converts cellobiose into D-glucose.

The factors affecting activity of cellulases include enzyme source and the concentration of enzyme. An effective concentration of enzyme for cellulose hydrolysis has been determined to be 10 to 60 FPU (filter paper units) per gram of dry cellulose or glucan- glucanase- β-D-glucosidase ratio of 1-75-2 IU (Kim et al., 2005). The yield of fermentable sugar levels obtained from pretreated biomass increases as the enzyme load increases and cellulose load decreases (Yang et al., 2002). The use of surfactants is known to block lignin and thus enhance enzymatic saccharification of cellulose (Tu et al., 2009). Addition of bovine serum albumin, polyethylene glycol (PEG) or Tween 20 have been shown to increase enzymatic saccharification and reduces the adsorption of cellulases on lignin and consequently improves the efficiency of cellulose hydrolysis (Pan et al., 2005; Yasmashita et al., 2010).

Both bacteria and fungi can produce glucanases (cellulases) that hydrolyze of lignocellulosic materials. These microorganisms can be aerobic or anaerobic and mesophilic or thermophilic. Bacteria belonging to genera of *Clostridium, Cellulomonas, Bacillus, Thermomonospora, Ruminococcus, Bacteriodes, Erwinia, Acetovibrio, Microbispora*, and *Streptomyces* are known to produce Cellulase (Bisaria, 1998). Anaerobic bacterial species such as *Clostridium phytofermentans, Clostridium thermocellum, Clostridium hungatei, and Clostridium papyrosolvens* produces cellulases with high specific activity (Duff and Murray, 1996; Bisaria, 1998). Most commercial glucanases (cellulases) are produced by *Trichoderma ressei* and β-D-glucosidase is produced from *Aspergillus niger* (Kaur et al., 2007). Fungi known to produce cellulases include *Sclerotium rolfsii, Phanerochaete chrysosporium* and various species of *Trichoderma, Aspergillus, Schizophyllum and Penicillium* (Sternberg, 1976; Fan et al., 1987; Duff and Murray, 1996). Among the fungi, *Trichoderma* species have been extensively studied for cellulase production (Sternberg, 1976).

FERMENTATION

During this process, both pentose and hexose sugars are fermented to ethanol under anaerobic/aerobic conditions. *Saccharomyces cerevisiae* is the most favored organism for ethanol production from hexoses. *P. stipitis* and *Candida shehatae* are capable of fermenting both hexose (glucose) and pentose (xylose) sugars to ethanol (Parekh et al., 1986).

An optimal process for fermentation uses a broth containing *saccharomyces cerevisiae* supplemented with 22% (w/v) sugar, 1% (w/v) of each of ammonium sulfate and potassium dihydrogen phosphate, and fermented at pH 5.0 and 30°C (Junior et al., 2009). Under such conditions a typical strain of *S. cerevisiae* is capable of producing 46.1 g ethanol/L broth (Maziar, et al., 2010).

Cane molasses conditioned with EDTA, ferrocyanide or zeolites, and fermented under similar conditions has been show to enhance ethanol production (Ergun et al., 1997). Further, addition of minimal concentrations of hops acids to the fermentation broth has been shown to prevent bacterial growth and thus enhances ethanol yields (Maye, 2006). Fermentation using immobilized yeast and broth supplemented with Mg, Zn, Cu or Ca-pantothenate has also been shown to increase fermentation efficiency by almost 20% (Nikolic, et al., 2009).

Among the pentose fermenting organisms, *P. stipitis* has been shown to have most promise for industrial applications (Agbogbo et al., 2006). For example, the hemicellulosic hydrolysates of *Prosopis juliflora* (18.24 g sugar/L broth) when fermented with *P. stipitis* produced 7.13 g/L ethanol (Gupta et al., 2009). Detoxified xylose rich hydrolysate of *L. camara* when fermented with *P. stipitis* 3498 at pH 5 and 30°C for 36 h resulted 0.33 g alcohol/g lignocellulose used (Kuhad et al., 2010). In yet another example, the detoxified water hyacinth hemicellulose acid hydrolysate (rich in pentose sugars) fermented with *P. stipitis* NCIM-3497 at pH 6.0 and 30°C resulted 0.425 g ethanol/g lignocellulose. *Candida tropicalis* is also capable of fermenting xylose (pentoses) under oxygen limited conditions in presence of increasing concentrations of polyethylene glycol (Hagerdal et al.,

1985). Genetically engineered strains of *Escherichia coli, S. cerevisiae,* and *Z. mobilis* have been developed to ferment xylose (Kim et al., 2005). Xylose fermentation has also been achieved by expressing xylose reductase and xylitol dehydrogenase together with overexpression of the endogenous xylulokinase and xylose isomerase in *P. stipitis* and other bacterial or fungal species (Eliasson et al., 2000; Kuyper et al., 2003; Karhumaa et al., 2005).

INTEGRATED FERMENTATION TECHNOLOGIES

Simultaneous saccharification and fermentation (SSF)

In simultaneous saccharification and fermentation (SSF) process both cellulose hydrolysis and fermentation of glucose are carried out in presence of fermentative microorganisms in a single step and the process optimally operates at 37 to 38°C. This technique reduces the number of steps in the process, and is a promising way for converting cellulose to ethanol (Lynd et al., 2005; Demain et al., 2005). In SSF process, the lignocellulosic biomass is first pretreated with a dilute acid (1.1% sulfuric acid at 160°C for 10 min) to breakdown lignin-hemicellulose-pectin complex. The resulting broth is filtered to drain the liquid from the system. The drained liquid containing pentose sugars is neutralized with lime and processed via xylose fermentation process (Figure 1). Remaining solids containing cellulose and lignin is then hydrolyzed and fermented simultaneously using by cellulase enzymes and yeast. The cellulase enzymes hydrolyze cellulose to D-glucose, which in turn is fermented to ethanol by yeast (Krishna et al., 2001). This combined process improves both kinetics of fermentation as well as economics of biomass processing via minimization of accumulation of hydrolysis product (glucose) that is inhibitory to cellulases. The disadvantages of using SSF process include (i) the operating temperature should be around 37 to 38°C and (ii) much of the sugar released by cellulose hydrolysis is used for the growth of yeast necessary to ensure good ethanol production.

Simultaneous saccharification and co-fermentation (SSCF)

In simultaneous saccharification and co-fermentation (SSCF), the pretreated lignocellulosic biomass is neutralized and directly exposed to different enzymes and microorganisms that are capable of hydrolyzing cellulose and hemicelluloses to fermentable sugars as well as ferment sugars to ethanol. The process is carried out using genetically engineered microorganisms. This technology is superior to SSF technology in terms of cost effectiveness, better yields, and shorter processing time (Wright, 1988; Lynd et al., 2005; Chandel et al., 2007).

Consolidated bioprocessing (CBP)

Consolidated bioprocessing (CBP) is an alternative processing strategy in which cellulase production, substrate hydrolysis, and fermentation are accomplished in a single step and in one reactor (Lynd et al., 2005; Demain et al., 2005; Vanzyl et al., 2007; Cardona and Sánchez, 2007). It is important to note that, in CBP, only one microbial consortium is employed for both the production of cellulase and fermentation. CBP offers the potential of lower production costs due to simpler lignocellulosic material processing, lower energy inputs and higher conversion efficiencies than SSF or SSCF based processes. CBP is an economically attractive near-term goal for processes involving “third generation” biofuel production (Lynd et al., 2005; Demain et al., 2005). There are two strategies for enabling consolidated bioprocessing; (i) engineering naturally occurring cellulolytic microorganisms to improve product-related properties such as yield and titer, and (ii) engineering non-cellulolytic organisms that exhibit high product yields and titers to express a heterologous cellulase system enabling cellulose utilization (Lynd et al., 2005). CBP is gaining increasing recognition as a potential breakthrough for low-cost biomass processing. The potential is limited by the fact that natural microorganisms exhibiting all the desired features for CBP are not readily available, although, a number of microorganisms, both bacteria and fungi, that possess some of the desirable properties have been identified (Vanzyl et al., 2007).

At present, there is no ideal CBP microorganism that can degrade lignocellulosic biomass proficiently and at the same time consume all the sugars released from biomass to yield ethanol. However, efforts are being made to engineer ethanol producing yeast (*S. cerevisiae*) capable of producing cellulases/hemicellulases and lignocellulose degrading bacteria (*C. thermocellum*) to be an efficient ethanol producer (Lynd et al., 2005). *S. cerevisiae* strains currently used in fermentation processes are incapabe of fermenting xylose to ethanol. The efforts refered to above via biotechnological engineering of yeast genome and protoplast fusion techniques have developed strains that show some promise (Demain et al., 2005). The genome of *C. phytofermentans* (ATCC 700394) encodes for the highest number of enzymes for degradation of lignocellulosic biomass among sequenced clostridial genomes (Weber et al., 2010). In one of our laboratories, this species has been used to ferment ligocellulosic biomass with and with-out pretreatment and observed production of ethanol under laboratory conditions (Mandal and Sreerama, 2011 unpublished observations). Above all, this species can ferment lignocellulosic biomass directly without any pretreatment procedures applied and the efficiency of

ethanol production remains the same under either conditions. Thus, it has become a promising native anaerobic CBP microorganism. Recently published work shows that it secretes individual enzymes which not only degrade cellulose and hemicellulose to fermentable sugars but also can consume almost all the sugars present in lignocellulosic biomass and produce ethanol and acetate as the major products (Warnick et al., 2002; Weber et al., 2010).

GENETIC ENGINEERING PROSPECTS FOR BIOETHANOL PRODUCTION

Non-cellulolytic microorganisms with desired product formation properties, for example high yield and titer, can be starting points for CBP organism development. The primary objective of such developments will be to engineer a heterologous cellulase system that enables growth and fermentation on pretreated lignocellulosic biomass. Metabolic engineering via application of recombinant DNA techniques to direct the production of bioethanol is an emerging field. This can be achieved by improving cellular function through the modulation of enzymatic, transport, or other regulatory functions of the cell. Metabolic engineering often involves the introduction of heterologous genes or regulatory elements that are employed to confer novel metabolic configurations.

The cloning and expression of heterologous genes can serve several purposes, for example, (i) extending existing pathway to produce novel products, (ii) engineering arrays of enzymatic activities that synthesize novel structures, (iii) shifting metabolic flux towards synthesis of desired end-products, and (iv) accelerating a rate determining step (Bailey et al., 1991). The genes encoding alcohol dehydrogenase II and pyruvate decarboxylase from *Z. mobilis* have been inserted into *E. coli* under the control of a common promoter. This has resulted in high levels of expression of both enzymes in *E. coli* and resulted in increased cell growth and the production of ethanol (Ingram et al., 1987). To accomplish this, the coding sequences of pyruvate decarboxylase and alcohol dehydrogenase II from *Z. mobilis* were first cloned into the shuttle vector, pCB4, and then transformed into the *Cyanobacterium synechococcus* strain (PCC 7942) (Deng et al., 1999). It has been shown that the ability of *C. phytofermentans* to degrade cellulose relies upon a single Cphy3367 gene which encodes the sole family nine glycoside hydrolases (GH9). This was accomplished by interspecies conjugation with *E. coli* that allows for disruption in Cphy3367 gene (Tolonen et al., 2009). Heterologous gene transfer of pyruvate decarboxylase and alcohol dehydrogenase II from *Z. mobilis* and their expression in *C. cellulolyticum* shows an increase in growth by 2-fold, 1.5 fold increases in cellulose consumption, 93% increase in acetate formation, and a 53% increase in ethanol production as compared to wild type strains. This engineered bacterium also showed 75% increase in H_2 yields (Guedon et al., 1999; Guedon et al., 2002). Antisense RNA (as RNA) strategies allow for down-regulation of specific genes coding for native proteins and thus redirect metabolism (Desai et al., 1999). The feasibility of such technologies in a CBP organism could be extremely useful. This is further supported by models that combine fermentation bioenergetics and the kinetics of pretreated substrate hydrolysis with declining reactivity over the course of the reaction (Van Walsum et al., 1998). High cell density suspensions of the recombinant strains have also been shown to ferment amorphous cellulose, raw starch, and birch wood xylon to ethanol with relatively high yields (Hong et al., 2003). Given the above examples, genetic engineering clearly holds the future prospects of providing technologies that will not only lead to production of bioethanol efficiently, but also holds the potential to reshape bioethanol industry.

LIQUID BIOFUELS IN NEPAL: DEVELOPMENT, STATUS AND PROSPECTS

Nepal is a land-locked country with rich biodiversity and renewable resources. These resources have never been utilized to their full potential given the social and economic challenges the country faces. In Nepal, the traditional biomass such as firewood, cow dung and agricultural waste continues to supply virtually all energy demands. The rate at which the traditional biomass is consumed exceeds its replenishment. The increasing consumption pattern coupled with rising population and increasing per capita demand for energy has placed an unsustainable burden on the environment in Nepal. About 87% of the total energy consumption in Nepal is from non-monetized biomass such as firewood, cow dung and agricultural waste. The imported fossil fuels contribute to about 11% of all energy demands and Nepal invests 40% of its foreign exchange earnings (Pokharel, 2006) to import fossil fuels. Energy consumption in Nepal by fuel type includes 87% traditional fuel and 13% commercial fuel. The commercial fuels include petroleum products (9%), coal (2%), electricity (1.5%) and renewable fuels (0.54%) (WECS, 2006). For fossil fuel imports Nepal is entirely dependent on oil-rich countries and these are imported from middle-eastern countries via India. The price of petroleum products in Nepal are sky-rocketing, the number of motor vehicles are increasing and the demand for petroleum products is growing at a rate of 12% each year.

Although, Nepal is a resource rich country, the investments in renewable energy development is almost non-existent. On the other hand, the potential for the development of biofuels in Nepal is very high. It has been estimated that 30% of Nepal's land is climatically favorable for the cultivation of Jatropha (locally called

Sajiyon or Sajiba). Apart from Jatropha, there are many other non-edible oil seed-bearing plants such as soap nut (*Sapindus mukorossi*), Dhaka (*Aregmone Mexicana*), and Nageswhor (*Mesua ferrea*). In addition, the pine resin (turpentine) is another source of biofuels. The ethanol industry in Nepal is thriving however most of the alcohol is produced from grains and starch commodities. The government of Nepal has a policy on hand to blend 10% ethanol into gasoline (petrol). This has never been implemented due to unsettled disputes over ethanol prices and other vested interests. Studies have shown that bioethanol production from molasses and lignocellulosic materials in Nepal are financially feasible, commercially and economically viable, and environmentally friendly. The raw materials need to establish bioethanol industry using both first generation crops (sugarcane, maize) and second generation lignocellulosic biomass (corn stover, sugarcane bagasse, vegetable wastes, terrestrial weeds such *S. spontaneum, L. camara* (red sage), *Euphatorium adenophorum* (banmara), *Parthenium hysterophorus*, and *E. crassipes* (water hyacinth) are in large supply. However, currently there are no visible commercial applications of biodiesel or bioethanol in Nepal of any significance although credible adaptive research and development, and pilot/demonstration activities have been carried out since 1980's. Nepal has made headways in biotechnology education. Slowly but surely the biotechnology industry is taking a foot-hold.

CONCLUSIONS AND FUTURE PROSPECTS

Demand for transportation fuels across the globe is increasing. This demand is abnormally affecting developing counties in particular. The demand for transportation fuel may be mitigated by bioethanol in the scenario of shrinking energy resources. Lignocellulosic materials are in abundance and they are renewable. Hence, bioethanol production from lignocellulosic biomass holds tremendous potential in terms of meeting energy needs, and providing environmental benefits. Apart from bioethanol, a wide range of chemicals (olefins, plastics, and organic solvents) and value added products such as fermentable sugars, solvents and drink softeners can be produced from the lignocellulosic biomass. The latter needs technical knowledge and capabilities to deal with complexity of biomass. The improvements in pretreatment processes, improvement in efficacy of enzymatic hydrolysis via the development more efficient enzymes, development of efficient fermentation processes via the development of CBP microorganisms, efficient technologies to recover ethanol and removal of toxic byproducts will decrease the operating and capital costs. The reduction in cost of ethanol production can be achieved by reducing the cost of raw material and co-fermenting hexose and pentose sugars in the same tanks. Continued research to isolate enzymes from the natural resources such as termite gut, rumen and anaerobic soils in conjunction with developing more efficient enzymes using biotechnological tools is essential. CBP systems that will use lignocellulosic materials with little or no pretreatment to produce ethanol in a single step are currently evolving. In this regard, studies leading to metabolic engineering of specific strains of microorganisms that simultaneously utilize glucose and xylose have already yielded positive results. Commercialization of these technologies is forthcoming. Combination of strategies that include (meta) genomics, biodiversity studies, systems biology and metabolic engineering hold promising potential to improve biofuel yields and the establishment of renewable, non-polluting energy sources in the future.

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