Bio Technology

Bioethanol Production from Lignocellulosic Feedstocks Based on Enzymatic Hydrolysis: Current Status and Recent Developments

[1]Noura El-Ahmady El-Naggar, [2]Sahar Deraz and [2]Ashraf Khalil
[1]Department of Bioprocess Development, Genetic Engineering and Biotechnology Research Institute,
City of Scientific Research and Technological Applications, Alexandria,
New Borg El-Arab City, Egypt
[2]Department of Protein Research, Genetic Engineering and Biotechnology Research Institute,
City of Scientific Research and Technological Applications, Alexandria,
New Borg El-Arab City, Egypt

Abstract: All over the world, research on bioethanol production has grown with increasing of energy needs and it has become a research area of great interest to many governments, academic groups and companies. Ethanol which is presently the most common renewable fuel, can be produced biologically from a variety of feedstocks and wastes. Due to advances in agriculture and biotechnology one can envision inexpensive production of ethanol based on lignocellulosic biomass. This review summarizes various processes involved in lignocellulosic-derived biofuel bioconversion. Several methods of pretreatment of lignocelluloses are discussed. Characteristics of enzymes and important factors in enzymatic hydrolysis of the cellulose and hemicellulose are reviewed. Different strategies are then described and illustrated in a simpler form for enzymatic hydrolysis and fermentation, including separate enzymatic hydrolysis and fermentation, simultaneous saccharification and fermentation, simultaneous saccharification and co-fermentation and consolidated bioprocessing. Furthermore, recent trends, major challenges and perspective of future development are highlighted.

Key words: Lignocellulosic biomass, bioethanol, bioconversion, fermentation, hydrolysis

INTRODUCTION

One of the biggest problems facing the world is the energy crisis. The price of oil dramatically escalated in 1973, creating an oil crisis. Energy consumption has increased steadily as the world population has grown and more countries have become industrialized. The fossil fuels, including crude oil, coal and natural gas are the major resources to meet the increased energy demands. As widely believed, the fossil energies will be exhausted in the near future (Wyman, 2001). The world currently consumes 30 billion barrels of oil per year; Colin (2003) estimates that oil reserves will become scarce by the 2050s. Because petroleum is a nonrenewable resource, there is an urgent need to seek alternative energy sources that are inexhaustible.

In recent decades, an international awareness of the increasing CO_2 concentration levels in the atmosphere and concern for global warming led to the formulation of the Kyoto Protocol in 1997 which has led many countries to make the commitment to decrease the emission of CO_2.

One way of decreasing CO_2 emissions could be substitution of fossil fuels with renewable energy sources.

The development of alternative fuel and energy sources has thus become a worldwide research priority in recent years. Biofuels are fuels produced from biomass. These fuels are usually in the form of bioalcohols, biodiesel, biogas and other chemicals produced from biomass. The two main biofuels are biodiesel and bioethanol. Among those, bioethanol produced by bioconversion of lignocellosic biomass is being seen as one of the most promising alternative biofuels. The choice of lignocellulose to bioethanol conversion should be decided on the basis of overall economics (lowest cost), environment (lowest pollutants) and energy (higher efficiency), i.e., comprehensive process development and optimization are still required to make the process economically viable. The increasing petroleum price and negative impact of fossil fuels on the environment are encouraging the use of lignocellulosic materials to help meet energy needs (Di Nasso *et al.*, 2011).

Corresponding Author: Noura El-Ahmady El-Naggar, Department of Bioprocess Development,
Genetic Engineering and Biotechnology Research Institute,
City of Scientific Research and Technological Applications, Alexandria, New Borg Al-Arab City, Egypt

There are many advantages of biofuels over fossil fuels that make the alternative fuel source an attractive option now and in the future. The main advantage of biofuels is that they are considered 'carbon neutral' by some people. This is because the carbon dioxide released during the combustion of biofuels is equal to the amount that assimilated during photosynthesis (Kheshgi *et al.*, 2000) resulting in no net increase to CO_2 levels. Therefore, they don't contribute to global warming. Consumption of biofuels releases no sulfur and has much lower particulate and toxic emissions, particularly when compared with other liquid transportation fuels (Scott and Wyman, 2004). Bioethanol production can provide an attractive route to dispose of problematic lignocellosic wastes such as stalks, stovers and leaves of agricultural crops. Biofuels are renewable energy source (made from organic materials and even organic waste, there is practically an infinite amount of biofuels available), inexpensive to produce and reduce dependence on foreign oils.

Ethanol is one of the most promising biofuels that can be used to replace gasoline for tomorrow's transportation vehicles. Fuel ethanol is mainly used as an oxygenated fuel additive. The higher octane number of the fuel mixture, when it contains ethanol, reduces the need for toxic, octane-enhancing additives such as methyl tertiary butyl ether. Due to the oxygen in ethanol molecules, there is also a reduction of carbon monoxide emission and non-combusted hydrocarbons (Hu *et al.*, 2008).

Feedstocks for bioethanol production

Generations of bioethanol: There are three "generations" of bioethanol: First-generation or conventional bioethanol is bioethanol produced from sugar substances, such as sugarcane juice, sugar beets and molasses, as well as starch-based materials such as wheat, corn, potatoes or cassava. Sucrose can be fermented directly to ethanol, but starch must be hydrolyzed to glucose before it can be fermented by yeast, generally by *Saccharomyces cerevisiae* (Sameera *et al.*, 2011). Even though the production of ethanol from starch biomass materials represents the most convenient and technically advanced option for bioenergy, such practices would result in severe competition between energy and food supplies. The raw materials account for 40-70% of the total ethanol production costs based on current sugar- or starch-containing feedstocks. Reduction of the production cost would increase the competitiveness of ethanol with fossil fuels. Lignocellulosic biomass is believed to be less expensive and more plentiful than either starch- or sucrose-containing feedstocks (Hu *et al.*, 2008).

Second generation bioethanol is bioethanol produced from sustainable feedstock that cannot be used directly for food production. Examples of wastes that could potentially be converted into bioethanol include perennial grasses, agricultural wastes and forest residues. The conversion of these potential sources of feedstock for bioethanol offers the double benefit of a reduction in global waste and the generation of valuable transportation fuels (Kaylen *et al.*, 2000).

Third generation bioethanol is produced via., direct cellulose fermentation (cellulase production, substrate hydrolysis and fermentation are accomplished in a single process step by cellulolytic microorganisms).

Lignocelluloses: Approximately 2×10^{11} tons of lignocellulosic biomass is produced every year and organic agricultural and forestry by-products are annually renewable, available in abundance and of limited value (Chen *et al.*, 2007). By-products produced from the cultivation of corn (corn stover), wheat (wheat straw), rice (rice straw), sorghum (sorghum stalks), barley (barley straw) and sugarcane (sugarcane bagasse) are the major sources of agro-based lignocellulosics which contain high contents of cellulose and hemicellulose, as described in Table 1 (Sun and Cheng, 2002). However, the excess lignocellulosic waste is often disposed of by biomass burning, causing an environmental pollution problem through smoke and development of CO_2 gas which is not restricted to developing countries, but is considered a global phenomenon (Levine, 1996). Burning also causes almost complete losses of soil N, P, K and S (Dobermann and Fairhurst, 2002). At the same time, the cellulose and hemicellulose could provide sugar for bioconversion to biofuels (Malherbe and Cloete, 2003).

It is an important issue to deal with the agricultural waste both for the comprehensive utilization of lignocellulosic resources and for the prevention of environmental pollution. One of the different approaches to overcome this problem is the biological conversion of agricultural waste to biofuels. Production of ethanol from renewable lignocellulosic sources is a promising means to

Table 1: Composition of some common agricultural and forestry residues

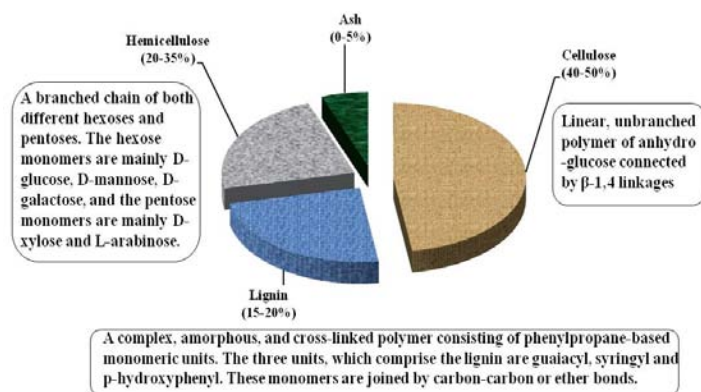| Source	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood	40-55	24-40	18-25
Softwood	45-50	25-35	25-35
Corn stover	38-40	28	7-21
Corn cobs	45	35	15
Bagasse	32-48	19-24	23-32
Wheat straw	33-38	26-32	17-19
Rice straw	28-36	23-28	12-14
Sorghum stalks	27	25	11
Barley straw	31-35	27-38	14-19
Leaves	15-20	80-85	0

Adapted from Kumar *et al.* (2009)

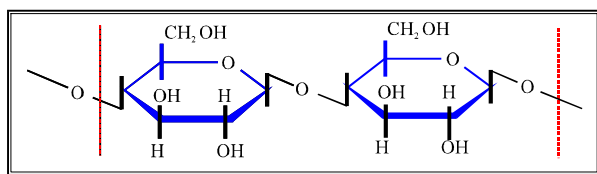Fig. 1: Constituents of lignocellulosic biomass

Fig. 2: Cellulose molecular structure

decrease the accumulation of greenhouse gas and alleviate pressure on fossil fuel shortage (Galbe and Zacchi, 2002).

The main constituents of lignocellulosic biomass are cellulose, hemicellulose and lignin. Minor components include ash and extractives such as waxes. On a dry weight basis, lignocelluloses contain 40-50% cellulose, 20-35% hemicellulose and 15-20% lignin (Fig. 1). Together, these components represent approximately 90% of the dry weight of most plant material (Brown, 2003).

The cellulose chain is a linear, unbranched polymer of glucose monomers linked by β-1,4-glucosidic bonds (Fig. 2). The conformation of cellulose, as well as its close association with lignin and hemicellulose makes cellulose very resistant to hydrolysis.

Lignocelluloses-to-bioethanol processes: There are two basic approaches to degrading biomass to sugars: Enzymatic hydrolysis and dilute acid hydrolysis. Compared to dilute acid hydrolysis, enzymatic approach is promising because it can achieve high sugar yields and eliminate the need for large quantities of chemicals and the formation of inhibitory by-products during dilute acid hydrolysis (Tran and Chambers, 1986).

The production of ethanol from lignocellulosic material requires the release of monomeric sugar units constituting cellulose and hemicellulose. Based on the specific lignocellulosic structure, the bioconversions of lignocellulosic materials to bioethanol normally require multi-step processes. There are five stages to produce

ethanol using a biological approach (Zhu *et al.*, 2009) (1) A "pretreatment" phase, to make the lignocellulosic material such as wood or straw amenable to hydrolysis, (2) Cellulose hydrolysis, to break down the molecules into sugars, (3) Separation of the sugar solution from the residual materials, notably lignin, (4) Microbial fermentation of the sugar solution and (5) Distillation to produce roughly 95% pure alcohol. An optional further step would be dehydration by molecular sieves to bring the ethanol concentration to over 99.5%.

Pretreatment of lignocellulose for enzymatic hydrolysis: The combination of hemicellulose and lignin provide a protective sheath around cellulose which must be modified or removed before efficient hydrolysis of cellulose can occur. Additionally, the higher order crystalline structure of cellulose is an obstacle to hydrolysis (Eriksson *et al.*, 1993). To make cellulose and hemicellulose accessible for enzymatic digestibility, a number of pretreatments should be carried out. These pretreatments are summarized in Table 2. Pretreatment is the first step required to fractionate lignocellulosic materials into the major plant components of lignin, cellulose and hemicellulose and/or modify the pores in the material to allow the enzymes to penetrate into the fibers to render them amenable to enzymatic hydrolysis (Galbe and Zacchi, 2002; Mosier *et al.*, 2005). The rice straw before and after pretreatment is depicted in Fig. 3.

The mechanisms by which pretreatments improve the digestibility of lignocellulose are however, not well

Table 2: Pretreatment methods of lignocellulosic materials for enzymatic hydrolysis

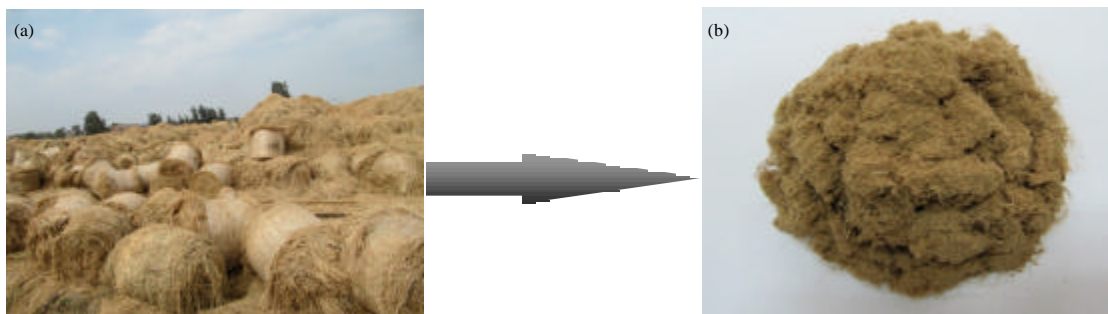| Pretreatment method	Processes	Advantage	Disadvantage	References
Physical pretreatments	Milling	Intensive decrystallization / Increase in accessible surface area and pore size	Energy-intensive	Chang and Holtzapple (2000)
Physicochemical and chemical pretreatments	Ammonia fiber explosion (AFEX)	Increases accessible surface area / Removes lignin and hemicellulose to an extent / Does not produce inhibitors	Not efficient for biomass with high lignin content / Does not significantly solubilize hemicellulose compared to other pretreatment processes	Esterbabur *et al.* (1991)
	Dilute acid: Sulfuric acid, Hydrochloric acid	Mild condition / High xylose yields / Increases the surface area and the pore volume by removing hemicellulose	Equipment corrosion / Formation of toxic substances / Relatively expensive	Grethlein and Converse (1991)
	Sodium hydroxide	Effective ester removal / Increase surface area and the porosity	Expensive reagent / Alkali recovery	Fan *et al.* (1987)
	Ammonia	Effective delignification	Alkali recovery / Relatively expensive	Kim *et al.* (2003)
	Lime	Effective lignin and acetyl removal / Inexpensive	Less effective due to poor solubility of lime	Chang *et al.* (1997)
	Ozonolysis	Effectively removes lignin / Does not produce toxic residues / The reactions are carried out at room temperature and pressure	A large amount of ozone is required, making the process expensive	Vidal and Molinier (1988)
Biological pretreatments	Fungi, Actinomycetes	Degrades lignin and hemicelluloses / Mild environmental conditions	Cellulose loss / Rate of hydrolysis is relatively slow	Kirk and Farrell (1987)

Fig. 3(a-b): Rice straw before and after pretreatment (a) Rice straw and (b) Pretreated rice straw

understood (Brown, 2003). A successful pretreatment must meet the following requirements: (1) Improve formation of sugars or the ability to subsequently form sugars by hydrolysis, (2) Avoid the degradation or loss of the pentose (hemicellulose) fractions to completely use the carbohydrates, (3) Avoid the formation of byproducts inhibitory to the subsequent hydrolysis and fermentation processes and (4) Be cost effective (Sun and Cheng, 2005).

Several methods have been introduced for pretreatment of lignocellulosic materials prior to enzymatic hydrolysis. The pretreatment methods can be classified into "Physical pretreatment" such as milling and irradiation (Wyman, 1996), "Physico-chemical pretreatment" such as steam explosion or autohydrolysis, ammonia fiber explosion (AFEX) (Ohgren *et al.*, 2005), "Chemical pretreatment" including ozonolysis, dilute-acid pretreatment and alkaline pretreatment (Berlin *et al.*, 2006) and "Biological pretreatment" (Fan *et al.*, 1982; Wyman, 1996).

Physical pretreatment: Physical pretreatment can increase the accessible surface area and size of pores and decrease the crystallinity and degrees of polymerization of cellulose. Different types of physical processes such as milling and irradiation can be used to improve the enzymatic hydrolysis of lignocellulosic waste materials.

Milling: Milling (cutting the lignocellulosic biomass into smaller pieces) can be employed to alter the inherent ultrastructure of lignocelluloses and degree of crystallinity and consequently make it more amenable to cellulase. Milling and size reduction have been applied prior to enzymatic hydrolysis, or even other pretreatment processes with dilute acid, steam or ammonia, on several lignocellulosic waste materials (Mais *et al.*, 2002). Milling can improve enzymatic degradation of lignocellulosic

materials toward ethanol by reducing the size of the materials (Chang and Holtzapple, 2000; Zeng *et al.*, 2007), the reduction in particle size leads to an increase of available specific surface and a reduction of the degree of polymerization (Palmowski and Muller, 1999). Although, decrystallization has been reported to be less important than lignin removal on sugar yields, decreasing crystallinity significantly increased the initial hydrolysis rate and to some extent, the ultimate biomass conversion (Chang and Holtzapple, 2000).

Irradiation: Irradiation by e.g., gamma rays and microwaves can improve enzymatic hydrolysis of lignocelluloses. The combination of the irradiation and other methods such as acid treatment can further accelerate enzymatic hydrolysis (Mamar and Hadjadj, 1990). Irradiation has enhanced enzymatic degradation of cellulose into glucose. Kumakura and Kaetsu (1983) studied the effect of irradiation for pretreatment of bagasse prior to its enzymatic hydrolysis. The pretreated bagasse resulted in doubling the yield of glucose by the hydrolysis compared to the untreated one. The cellulose component of the lignocellulose materials can be degraded by irradiation to fragile fibers and low molecular weight oligosaccharides and even cellobiose (Kumakura and Kaetsu, 1983).

Chemical pretreatment
Dilute acid pretreatment: Treatment of lignocellulosic materials with acid at a high temperature can efficiently improve the enzymatic hydrolysis. Sulfuric acid is the most applied acid while other acids such as HCl and nitric acid also have been reported (Taherzadeh and Karimi, 2007a). The acid pretreatment can operate either under a high temperature (e.g., 180°C for 5 min) and low acid concentration (dilute-acid pretreatment) or under a low temperature (e.g., 120°C for 30-90 min) and high acid

concentration (concentrated-acid pretreatment). Dilute-acid hydrolysis is probably the most commonly applied method among the chemical pretreatment methods. It can be used either as a pretreatment of lignocellulose for enzymatic hydrolysis, or as the actual method of hydrolyzing to fermentable sugars. Dilute acid pretreatment increases the surface area and the pore volume by removing hemicellulose. Actually, chemical treatments have a dual effect on biomass crystallinity: (1) They remove amorphous lignin and hemicellulose components to increase biomass crystallinity and (2) They loosen the highly packed crystalline structure through swelling to decrease crystallinity (Gharpuray *et al.*, 1983).

The main advantage of dilute-acid pretreatment is the possibility to recover a high portion (e.g., 90%) of the hemicellulose sugars. The hemicellulose, mainly xylan or mannan, accounts for up to a third of the total carbohydrate in many lignocellulosic materials. Thus, hemicellulose recovery (e.g., achieving high xylan to xylose conversion yields) can have a highly positive effect on the overall process economics of ethanol production from lignocellulosic material (Sun and Cheng, 2002). On the other hand, a main disadvantage of this pretreatment method is the necessity of neutralization of pH for the downstream enzymatic hydrolysis. Furthermore, different chemical inhibitors might be produced during the acid pretreatment which reduces cellulase activity and therefore, water wash is necessary for the pretreated biomass before enzymatic hydrolysis (Sun and Cheng, 2002). Dilute-acid pretreatment is not effective in dissolving lignin, but it can disrupt lignin and increases the cellulose's susceptibility to enzymatic hydrolysis (Yang and Wyman, 2004).

Alkaline pretreatment: Alkaline pretreatment refers to the application of alkaline solutions such as NaOH, Ca(OH)$_2$ (lime) or ammonia to remove lignin and a part of the hemicellulose and efficiently increase the accessibility of enzyme to the cellulose. Pretreatment can be performed at low temperatures but with a relatively long time and high concentration of the base. The mechanism of alkali pretreatment is believed to be saponification (breaking) of intermolecular ester bonds between lignin, hemicellulose and cellulose and avoiding fragmentation of the hemicellulose polymers (Gaspar *et al.*, 2007). After alkaline pretreatment, the porosity of the material is increased due to the extensive swelling facilitated by removal of the crosslinks (Tarkow and Feist, 1969). Dilute NaOH pretreatment causes swelling, leading to an increase in internal surface area and the porosity of the material is increased, a decrease in the degree of polymerization and

crystallinity and disruption of lignin structure (Fan *et al.*, 1987). In comparison with dilute acid pretreatment, the high cost of caustic soda is the main obstacle to its large scale implementation. Therefore, aqueous ammonia and lime (calcium hydroxide) pretreatments have been developed to reduce the cost of chemicals. These two kinds of alkali are effective in removing lignin and acetyl groups in biomass. Lime is a promising chemical because it is inexpensive, safe and can be recovered by carbonating wash water (Chang *et al.*, 1997).

Ozonolysis pretreatment: Pretreatment of lignocellulosic materials can be performed by treatment with ozone, referred to as "ozonolysis" pretreatment. This method can effectively degrade lignin and part of hemicellulose. The pretreatment is usually carried out at room temperature and does not lead to inhibitory compounds (Vidal and Molinier, 1988). However, ozonolysis might be expensive since a large amount of ozone is required (Sun and Cheng, 2002). The main parameters in ozonolysis pretreatment are moisture content of the sample, particle size and ozone concentration in the gas flow. Among these parameters, an essential factor is the percentage of water in the feed and it has the most significant effect on the solubilization. The optimum water content was found to be around 30%, corresponding to the saturation point of the fibers. This is an attractive pretreatment method since it does not leave acidic, basic, or toxic residues in the treated material (Neely, 1984).

Physico-chemical pretreatment: Pretreatments that combine both chemical and physical processes are referred to as physicochemical processes (Chandra *et al.*, 2007).

Ammonia fiber explosion (AFEX): AFEX is one of the alkaline physico-chemical pretreatment processes. Here the biomass is exposed to liquid ammonia at relatively high temperature (e.g., 90-100°C) for a period of e.g., 30 min, followed by immediate reduction of pressure. The effective parameters in the AFEX process are ammonia loading, temperature, water loading, blowdown pressure and time (Holtzapple *et al.*, 1991). The AFEX process can either modify or effectively reduce the lignin fraction of the lignocellulosic materials while the hemicellulose and cellulose fractions may remain intact. One of the major advantages of AFEX pretreatment is no formation of some types of inhibitory by-products which are produced during the other pretreatment methods, such as furans in dilute-acid and steam explosion pretreatment. However, part of phenolic fragments of lignin and other cell wall extractives may remain on the cellulosic surface.

Therefore, washing with water might be necessary to remove part of these inhibitory components, although increasing the amount of wastewater from the process (Chundawat *et al.*, 2007). However, there are some disadvantages in using the AFEX process compared to some other processes. AFEX is more effective on the biomass that contains less lignin and the AFEX pretreatment does not significantly solubilize hemicellulose compared to other pretreatment processes such as dilute-acid pretreatment.

Biological pretreatment: Microorganisms can also be used to treat the lignocelluloses and enhance enzymatic hydrolysis. The applied microorganisms usually degrade lignin and hemicellulose but very little part of cellulose, since cellulose is more resistance than the other parts of lignocelluloses to the biological attack. White-rot fungi are among the most effective microorganisms for biological pretreatment of lignocelluloses (Sun and Cheng, 2002). Some bacteria such as *Bacillus circulans* (Kurakake *et al.*, 2007) can also be used for biological pretreatment of lignocellulosic materials. Low energy requirement and mild environmental conditions are the main advantages of biological pretreatment. However, this method is relatively slow (Hahn-Hagerdal *et al.*, 2006).

Detoxification of lignocellulose-derived inhibitor compounds: The major drawback of some pretreatment methods is the formation of different types of potential microbial inhibitors (Chandel *et al.*, 2009, 2010). The amounts and types of inhibitory compounds vary strongly between different raw materials and also depend on the pretreatment method. These inhibitors can be divided into three major groups, (1) Organic acids (acetic, formic and levulinic acids), (2) Furan derivatives [furfural and 5-hydroxymethylfurfural (5-HMF)] and (3) Phenolic compounds (Chandel *et al.*, 2010). These inhibitors may not affect the enzymatic hydrolyses, but they usually inhibit the microbial growth and fermentation which results in less yield and productivity of ethanol (Taherzadeh and Karimi, 2007a). The ethanologenic microorganisms have ability to degrade some of the inhibitors. Progress has been made to achieve higher levels of sugars by diminishing the overall impact of fermentative inhibitors which in-turn improves the fermentability of lignocellulosic hydrolysates (Alriksson *et al.*, 2011; Parawira and Tekere, 2011). The ion exchange resins, active charcoal, enzymatic detoxification using laccase and overliming with calcium hydroxide are among selective detoxification (i.e., removal of inhibitory compounds) strategies which have been investigated in the past (Jurado *et al.*, 2009). Treatment

with the soft-rot fungus *Trichoderma reesei* and other microorganisms to degrade inhibitors in a hemicellulase hydrolysate has also been proposed (Fonseca *et al.*, 2011; Yu *et al.*, 2011). Biodetoxification methods provides high yields, uses limited waste water and has low energy costs, but its slow rate prohibits its practical use.

Enzymatic hydrolysis: Enzymatic hydrolysis has attracted increasing attention as an alternative to concentrated acid hydrolysis because the process is highly specific, can be performed under mild reaction conditions (pH around 5 and temperature less than 50°C) with lower energy consumption and lower environmental impact. In addition, it does not present corrosion problems and gives high yield of pure glucose with low formation of by-products that are favorable for the subsequent hydrolysate use in fermentation processes. The cellulolytic enzymes are either secreted into the substrate or attached to the cell wall of the microorganism. The former system is called noncomplexed and the latter complexed cellulase system. Noncomplexed cellulase systems are mostly found in filamentous fungi and actinomycete bacteria, because they can penetrate the lignocellulosic material with their hyphal extensions. The enzymes of noncomplexed cellulase systems are then just released into the substrate and the free enzymes start hydrolyzing the cellulose. The glucose and cellodextrins of a length of maximal four glucose molecules are taken up by the microorganism and either used directly or cleaved further via intracellular hydrolases. Organisms that produce noncomplexed cellulase systems are most often used in the industrial production of cellulolytic enzymes, because the secreted enzymes can easily be harvested (Lynd *et al.*, 2002). Complexed cellulase systems are mainly found in anaerobic bacteria and anaerobic fungi that cannot penetrate lignocellulosic material. The different cellulolytic enzymes of these bacteria form a stable "complex", called the cellulosome.

Enzymatic degradation of cellulose to glucose is generally accomplished by synergistic action of at least three major classes of enzymes: Endo-glucanases, exoglucanases and β-glucosidases. These enzymes are usually called together cellulase or cellulolytic enzymes (Wyman, 1996).

Cellulases: Cellulases responsible for the hydrolysis of cellulose are composed of three major enzymes (Rabinovich *et al.*, 2002): Endoglucanases or carboxymethyl cellulases (endo-1,4-β-glucanase; EC 3.2.1.4), are proposed to initiate attack randomly at multiple internal sites in the amorphous regions of cellulose fiber opening-up sites and create free chain-ends

for subsequent attack by the cellobiohydrolases (Wood, 1991). Exoglucanases or cellobiohydrolases (Exoglucanase; EC 3.2.1.91), is the major component of cellulase system accounting for 40 to 70% of the total cellulase proteins and can hydrolyze highly crystalline cellulose (Esterbabur *et al.*, 1991). Exoglucanases act in a processive manner on the reducing or nonreducing ends of cellulose polysaccharide chains, liberating either glucose or cellobiose as major products. Exoglucanases can also act on microcrystalline cellulose, presumably peeling cellulose chains from the microcrystalline structure. β-glucosidases (EC 3.2.1.21), splits cellobiose into two glucose units (Lynd *et al.*, 2002). This enzyme is not a cellulase, but its action is very important to complete depolymerization of cellulose to glucose.

Synergism in cellulose hydrolysis: The application of cellulolytic enzymes in degrading lignocellulosic materials needs synergistic actions of by endoglucanase, exoglucanase, or cellobiohydrolase and β-glucosidase (Rabinovich *et al.*, 2002; Zhang *et al.*, 2006). In enzymatic reactions, synergism occurs when the combined activity of two enzymes exceeds the sum of the individual enzyme activities. In the hydrolysis of filter study, a one to one mixture of exoglucanase and endoglucanase from *T. reesei* resulted in 23% (w/w) conversion, whereas the individual conversion was 6 and 8% using exoglucanase and endoglucanase, respectively (Gama *et al.*, 1998).

Hemicellulases: Hemicelluloses are degraded more easily than cellulose because of a lower degree of polymerization and their amorphous nature. Since, hemicellulose contains different sugar units, the hemicellulolytic enzymes are more complex and include endo-1,4-β-D-xylanases, exo-1,4-β-D-xylosidases, endo-1,4-β-D-mannanases, β-mannosidases, galactanases, α-glucuronidases, endo-arabinases, α-L-arabinofuranosidases and α-galactosidases (Jorgensen *et al.*, 2003). Hemicellulases also include the accessory enzymes, such as ferulic acid esterases, coumaric acid esterases and acetylxylan esterase which are necessary for hydrolyzing various substituted xylans (Saha, 2003). Xylanases are one of the major hemicellulases which hydrolyse the β-1,4 bond in the xylan backbone yielding short xylooligomers which are further hydrolysed into single xylose units by β-xylosidase. β-mannanases hydrolyse mannan-based hemicellulose and liberate short β-1, 4-manno-oligomers which can further be hydrolysed to mannose by β-mannosidases. Hemicellulolytic esterases include acetyl esterases which hydrolyse the acetyl substitutions on xylose moieties and feruloyl esterase which hydrolyse the ester bond between the arabinose substitutions and ferulic acid. Feruloyl esterase aids the release of hemicellulose from lignin and renders the free polysaccharide product more amenable to degradation by the other hemicellulases (Prates *et al.*, 2001).

Ligninases: Lignin is very hard to be degraded compared with other celluloses and hemicelluloses in most agriculture wastes. The breakdown of lignin is not well understood; however, most literatures proved correlation between the ability to degrade lignin and the production of extracellular phenolases, such as laccase, peroxidase and tyrosinase which oxidize phenolic compounds, e.g., gallic and tannic acids. Fungi breakdown lignin aerobically through the use of family of extracellular enzymes collectively termed lignases. Phenol oxidase (laccase), lignin peroxidase and manganese peroxidase are widely considered to play a key role in lignin degradation (Krause *et al.*, 2003; Malherbe and Cloete, 2003).

Enzyme cost: Cellulase production was the most expensive step during ethanol production from cellulosic biomass, in that it accounted for approximately 50% of the total hydrolysis process cost. Significant cost reduction is required in order to enhance the commercial viability of cellulase production technology (Howard *et al.*, 2004). However the cost of enzymes has decreased over the last twenty years but is still considered to be very high. Much research efforts have been focused on lowering the cost of enzymes.

The following strategies can be applied for reducing the cellulase production cost: (1) Strain improvement of existing industrial organisms (by mutagenesis, by protoplast fusion technology, by genetic engineering and recombinant DNA technology), (2) Screening for overproducing strains with novel enzymes, (3) Using mixed cultures or co cultures of organisms. In order to enhance cellulase activity, mixed cultures are generally used. Application of mixed cultures is an alternative tool to overcome feedback inhibition and catabolite repression, (4) Using cheap, renewable and easily available cellulosic wastes. Due to the high production cost of pure cellulose such as Avicel, the various lignocellulosic raw materials is proved to be an best alternative for cellulase production such as wheat straw, rice straw, corn stover etc., (5) By using fed batch system and (6) Applying recent trends in solid state fermentation, solid state fermentation gives a concentrated product which is advantageous in cellulase use for biomass hydrolysis.

Microorganisms and their cellulolytic enzymes: Several species of bacteria such as *Cellumonas, Bacillus, Thermomonospora, Erwinia, Microbispora* and Streptomyces (El-Naggar *et al.*, 2011; El-Naggar and

Abdelwahed, 2012) and fungi such as *Tricoderma, Penicillium, Fusarium, Humicola* spp. and *Aspergillus fumigates* (Sherief *et al.*, 2010) are able to produce cellulases and hemicellulases. Among the cellulases produced by different microorganisms, cellulases of *Trichoderma reesei* or *T. viride* and its mutants have been the most broadly studied and best characterized (Esterbabur *et al.*, 1991). A full complement production of cellulase, stability under the enzymatic hydrolysis conditions and resistance of the enzyme to chemical inhibitors are the advantages of the cellulase produced by *Trichoderma*. The main disadvantage of *Trichoderma* cellulase is low activity of β-glucosidases. On the other hand, *Aspergilli* are very efficient β-glucosidase producers. In several studies, *Trichoderma* cellulase was supplemented with extra β-glucosidases and showed good improvement (Krishna *et al.*, 2001; Itoh *et al.*, 2003).

Factors affecting the enzymatic hydrolysis of lignocellulosic materials: The enzymatic hydrolysis of lignocellulose is affected by many factors. The limiting factors have been traditionally divided into two groups: Those related to lignocellulose structural features and those related to the mechanism and interactions of the cellulolytic enzymes. Generally, structural features can be categorized into two groups: Physical and chemical. Physical structural features include cellulose crystallinity, degree of cellulose polymerization, pore volume, accessible surface area and particle size. Chemical structural features include the contents of lignin, hemicellulose and acetyl groups. Table 3 summarizes the relationship between structural features and biomass digestibility.

Substrate-related factors
Lignin content: Lignin plays a significant role in the rate and extent of lignocellulose hydrolysis. Literature results have all shown that cellulose digestibility enhances with increasing lignin removal (Draude *et al.*, 2001). The major

Table 3: Summary of relationship between structural features and digestibility (Zhu *et al.*, 2008)

Structural features	Relationship between structural features and digestibility	References
Chemical		
Lignin	Negative	Draude *et al.* (2001)
Hemicellulose	Negative	Kim *et al.* (2003)
Acetyl group	Negative	Kong *et al.* (1992)
Physical		
Surface area	Positive	Sinitsyn *et al.* (1991)
Crystallinity	Negative	Fan *et al.* (1981)
	No correlation	Grethlein (1985)
Degree of polymerization	Negative	Puri (1984)
	No correlation	Sinitsyn *et al.* (1991)
Particle size	No correlation	Draude *et al.* (2001)

inhibitory role of lignin has been attributed to nonspecific adsorption of enzyme to lignin (Sewalt *et al.*, 1997) thus preventing their reaction with substrates. The removal of lignin leaves the cellulose more accessible and more open to swelling on contact with cellulase (Mooney *et al.*, 1998). The degree that lignin adsorption of enzymes is decreased depends on the severity of pretreatment and the resulting decrease in lignin content (Ooshima *et al.*, 1991). Addition of surfactants during hydrolysis can modify the cellulose surface properties. An important effect of surfactant addition in a process for lignocellulose conversion is the possibility to lower the enzyme loading. One reason for this effect might be adsorption of surfactants to lignin which prevents unproductive binding of enzymes to lignin and results in higher productivity of the enzymes (Eriksson *et al.*, 2002).

Hemicellulose content: Because hemicellulose and lignin are covalently linked, acid hydrolysis of hemicellulose can open biomass structure as well. Moderate hemicellulose removal (>50%) is required to significantly increase cellulose digestibility (Mosier *et al.*, 2005).

Acetyl content: In hemicellulose, xylan backbones are extensively acetylated (CH_3COO-) (Holtzapple, 1993). Several studies showed that the removal of acetyl groups from hemicellulose reduced the steric hindrance of enzymes and greatly enhanced cellulose and xylan digestibility (Kong *et al.*, 1992). The acetate groups interfere with enzyme recognition thereby slowing the hydrolysis rate.

Accessible surface area: Particle size associated with accessible surface area has a significant impact on the saccharification of lignocellulose by cellulolytic enzymes and is thought to be a controlling factor for conversion rates and yield (Zeng *et al.*, 2007). Since, enzyme adsorption is a prerequisite step in the hydrolytic process, it seems that specific surface area would have an effect on hydrolysis rates since a higher surface area-to-weight ratio should mean more available adsorption sites per mass of substrate (Mansfield *et al.*, 1999). It was hypothesized that initial rate of hydrolysis is a function of cellulose's accessible surface area. Small particle sizes of untreated cellulosic substrate are more readily hydrolyzed than large ones because of higher specific surface area (Laivins and Scallan, 1996). Pretreatment increases accessible and susceptible surface area leading to enhanced enzymatic cellulose hydrolysis.

Cellulose crystallinity: Cellulose crystallinity presents a unified front that resists enzymatic attack. The rate of

hydrolysis of amorphous cellulose is 3-30 times faster than that of high crystalline cellulose (Lynd *et al.*, 2002). Several studies have shown that crystallinity prevents the rapid access of enzymes (Zhu *et al.*, 2008); however, there are conflicting views about this (Fan *et al.*, 1980). The conflict likely results from not distinguishing between the crystallinity of cellulose and biomass. For example, a pretreatment that selectively removes lignin enhances enzymatic digestibility, but would actually increase biomass crystallinity because lignin is amorphous. To properly assess the role of crystallinity, it would be necessary to know how the cellulose crystallinity, not the biomass crystallinity, is affected by the pretreatment. This is difficult to determine because measuring the crystallinity of cellulose that is blended with other biomass components is difficult.

Degree of polymerization: The degree of polymerization (DP, number of glucosyl residues per cellulose chain) of cellulosic substrates varies greatly, depending on substrate origin and preparation. It is still unclear if the DP of cellulose is a contributing limiting factor that influences the efficiency of enzymatic hydrolysis, because different conclusions have been drawn. Some of the results show that the DP of wood-derived cellulose fragments decreased with increasing enzyme hydrolysis time (Ramos *et al.*, 1993) while others showed that the molecular weight of residual material remains unchanged after hydrolysis which indicates that the DP is relatively unimportant (Sinitsyn *et al.*, 1991).

Enzyme-related factors
End-product inhibition: End-product inhibition is a major enzymatic factor that limits cellulase hydrolysis (Xiao *et al.*, 2004). β-glucosidase, hydrolyzes the glucose dimer cellobiose to glucose. With a shortage of β-glucosidase, cellobiose accumulates, thereby inhibiting the action of endoglucanases and cellobiohydrolases in hydrolyzing cellulose. Adding extra β-glucosidase which hydrolyzes cellobiose to glucose, thereby preventing inhibition of cellobiohydrolases by cellobiose (Breuil *et al.*, 1992). Increasing cellulase loading, removing sugars during hydrolysis by filtration (Gan *et al.*, 2005) or using Simultaneous Saccharification and Fermentation (SSF) (Vinzant *et al.*, 1999) are strategies designed to resolve this problem.

Enzyme synergism: Synergism seems to be particularly important for crystalline cellulose hydrolysis. Amorphous cellulose can be hydrolyzed by both endoglucanases and cellobiohydrolases while crystalline cellulose is largely hydrolyzed by cellobiohydrolases. Thus, crystallinity

probably influences hydrolysis when synergism is lacking due to an incomplete cellulase system or an insufficient enzyme loading (Mansfield *et al.*, 1999).

Ethanol fermentation: The lignocellulose hydrolysis yields D-glucose from cellulose and a mixture of hexoses (D-glucose, D-mannose, D-galactose, L-rhamanose), pentoses (D-xylose, L-arabinose) and uronic acids from hemicelluloses. As a result, the ability of the fermenting microorganisms to use the whole range of sugars available from the hydrolysate is vital to increase the economic competitiveness of cellulosic ethanol. The general requirements on an organism to be used in ethanol production is that it should give a high ethanol yield, a high productivity and be able to withstand high ethanol concentrations in order to keep distillation costs low (Von Sivers and Zacchi, 1996). In addition to these general requirements, inhibitor tolerance, temperature tolerance and the ability to utilize multiple sugars are essential. Tolerance towards low pH-values will minimize the risk of contamination.

Baker's yeast (*Saccharomyces cerevisiae*) has long been employed for the industrial production of ethanol from hexose sugars (Kuyper *et al.*, 2005). *S. cerevisiae* has many positive attributes which makes it suitable for industrial ethanol production such as a high rate of ethanol production from glucose. However this yeast species also has the limitation of not being able to ferment pentoses such as xylose or arabinose. Even though certain types of yeast, such as *Pachysolen tannophilus*, *Pichia stipitis*, or *Candida shehatae*, are xylose-fermenting, they have poor ethanol yields and low ethanol tolerance compared with the common glucose-fermenting yeasts, such as *S. cerevisiae*.

Thermophilic anaerobes have several advantages that make them suitable for an industrial conversion of lignocellulosic biomass to ethanol. First, there are organisms, such as *Clostridium thermocellum* that already have the ability to convert lignocellulosic biomass directly into ethanol. Second, the fermenters do not need aeration, what is an expensive part of industrial fermentation, because the organisms are anaerobes. Third, the optimal growth temperature of e.g., *C. thermocellum* is about 60°C; a high temperature simplifies the removal and recovery of ethanol by evaporation and distillation, renders expensive cooling systems unnecessary and furthermore it lessens problems with contaminations. Fourth, anaerobes have in general a low cell yield, so more of the substrate is converted to the endproduct (Demain *et al.*, 2005). Despite the advantages of these organisms, one of the major drawbacks is that the achieved ethanol yields are still rather low compared to

good ethanol producers like *S. cerevisiae*. This is to some part because during the fermentation of glucose not only ethanol is produced but also other compounds such as lactate and acetate. Another problem is the relative low tolerance of these organisms to ethanol. The tolerance to inhibitors that can be produced during the pretreatment can also be a problem. However, *C. thermocellum* lacks the ability to convert pentose sugars. Therefore the use of a coculture (or mixed culture) of *C. thermocellum* with another thermophilic anaerobe that is capable of producing ethanol out of pentoses is of great interest.
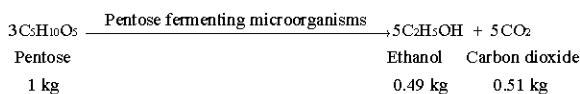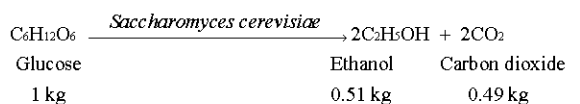
Zymomonas mobilis, an obligately anaerobic bacterium which gives a lower biomass yield and a higher ethanol yield on glucose compared to *S. cerevisiae* (Rogers *et al.*, 1979). However, wild-type *Z. mobilis* lacks the ability to ferment pentose sugars and a major drawback is furthermore that it is not a very robust organism. In general, bacteria appear to be less tolerant to lignocellulose-derived inhibitors (Hahn-Hagerdal *et al.*, 2007) and a detoxification step may be needed prior to the fermentation.

The inability of *S. cerevisiae* and *Z. mobilis* to utilize pentose sugars necessitates the search for pentose utilizing strains. Two groups of micro-organisms, i.e., enteric bacteria (*E. coli* and *Klebsiella oxytoca*) and some yeasts, are able to ferment pentoses, but with low ethanol yields. Furthermore, xylose fermenting yeasts (*Pachysolen tannophilus, Candida shehatae* and *Pichia stipitis*) are sensitive to high concentrations of ethanol (≥ 40 g L^{-1}), require micro-aerophilic conditions, are highly sensitive to inhibitors and are not capable of fermenting xylose at low pH (Zaldivar *et al.*, 2001).

As no naturally occurring organism can satisfy all necessary specifications (e.g., high ethanol yield, temperature tolerance, wide-substrate range, ethanol tolerance, tolerance to inhibitors and tolerance towards low pH-values), this has to come by the utilization of genetic engineering techniques for the construction of organisms that have most of the desirable characteristics for bioethanol production. Metabolic engineering for microorganisms used in fuel ethanol production has generated recombinant organisms displaying attractive features for the bioconversion of lignocellulose to ethanol (Jeffries and Jin, 2004). The three most promising microbial species that have been developed by metabolic engineering in the last two decades are *S. cerevisiae*, *Z. mobilis* and *Escherichia coli*. By genetic engineering it is possible to transfer cellulase genes from *Trichoderma* to *Saccharomyces cerevisiae* to produce "superstrains" via genetic enginnering capable of hydrolyzing cellulose and xylan along with fermentation of glucose and xylose into ethanol.

Due to the very attractive properties of *S. cerevisiae* in industrial fermentations, there have been significant efforts made to design recombinant *S. cerevisiae* strains to co-ferment the pentose sugars xylose and arabinose (Karhumaa *et al.*, 2006). Furthermore, strains of *S. cerevisiae* were engineered to co-ferment mixtures of xylose and cellobiose, using a xylose fermenting strain and an intracellular β-glucosidase to hydrolyse cellobiose (Ha *et al.*, 2011). It was shown that intracellular hydrolysis of cellobiose minimised glucose repression of xylose fermentation allowing co-consumption of cellobiose and xylose that improved ethanol yields.

According to the reactions, the theoretical maximum yield is 0.51 and 0.49 kg of ethanol kg^{-1} of C6 or C5 sugar (Qin, 2010):

$$C_6H_{12}O_6 \xrightarrow{\text{Saccharomyces cerevisiae}} 2C_2H_5OH + 2CO_2$$

Glucose	Ethanol	Carbon dioxide
1 kg	0.51 kg	0.49 kg

$$3C_5H_{10}O_5 \xrightarrow{\text{Pentose fermenting microorganisms}} 5C_2H_5OH + 5CO_2$$

Pentose	Ethanol	Carbon dioxide
1 kg	0.49 kg	0.51 kg

Hydrolysis and fermentation strategies: In the process of ethanol production out of lignocellulosic biomass 4 steps are driven by biological processes. (1) The production of the cellulase enzymes, (2) The hydrolysis of the cellulosic material, (3) The fermentation of hexose sugars and (4) The fermentation of pentose sugars.

Separate enzymatic hydrolysis and fermentation (SHF): In this process, pretreated lignocelluloses are hydrolyzed to glucose and subsequently fermented to ethanol in separate units (Fig. 4). The major advantage of this method is that it is possible to carry out the cellulose hydrolysis and fermentation at their own optimum conditions. The optimum temperature for cellulase is usually between 45 and 50°C, depending on the cellulose-producing microorganism (Saha *et al.*, 2005; Olsson *et al.*, 2006). However, the optimum temperature for most of the ethanol-producing microorganisms is between 30 and 37°C. Inhibition of cellulase activity by the released sugars, mainly cellobiose and glucose, is the main drawback of SHF. At a cellobiose concentration as low as 6 g L^{-1}, the activity of cellulase is reduced by 60%. Although glucose decreases the cellulase activity as well, the inhibitory effect of this sugar is lower than that of cellobiose. On the other hand, glucose is a strong inhibitor for β-glucosidase. At a level of 3 g L^{-1} of glucose, the activity of β-glucosidase is reduced by 75% (Philippidis and Smith, 1995). Another possible problem in
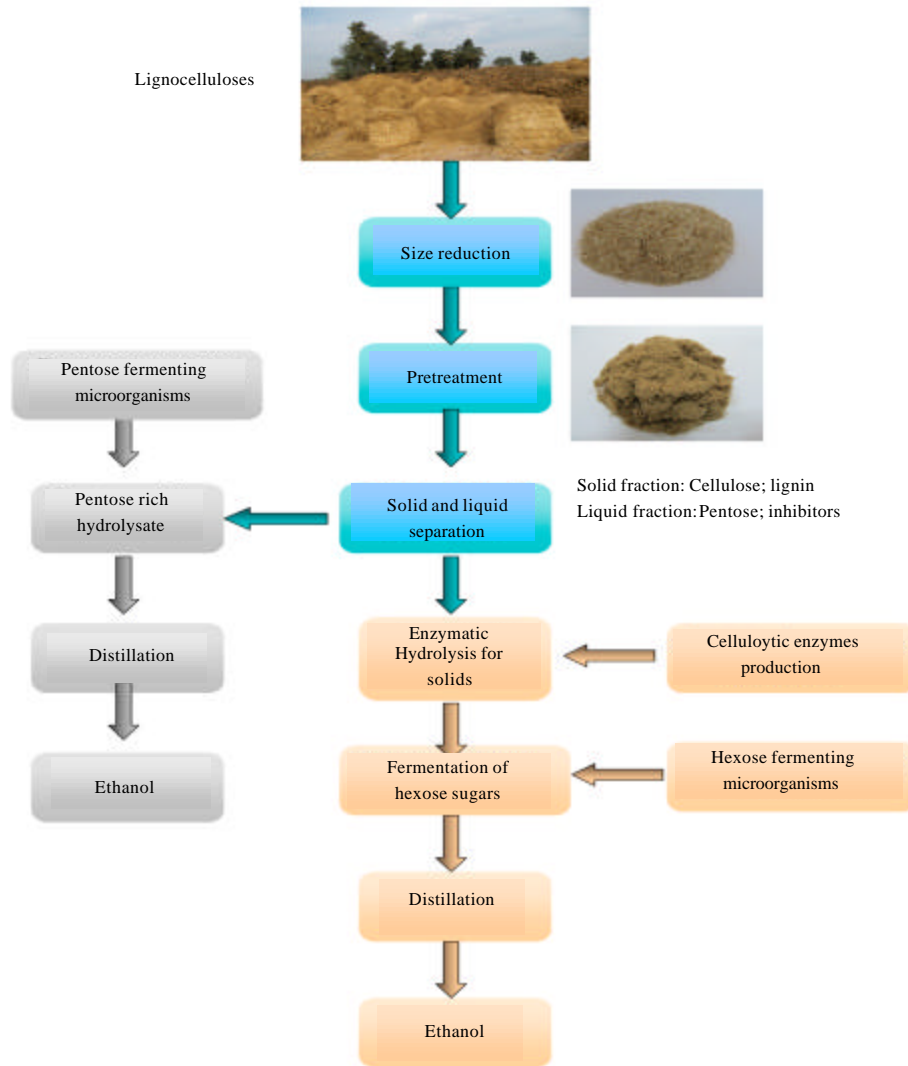
Lignocelluloses

Size reduction

Pentose fermenting microorganisms

Pretreatment

Pentose rich hydrolysate

Solid and liquid separation

Solid fraction: Cellulose; lignin
Liquid fraction: Pentose; inhibitors

Distillation

Enzymatic Hydrolysis for solids

Celluloytic enzymes production

Ethanol

Fermentation of hexose sugars

Hexose fermenting microorganisms

Distillation

Ethanol

Fig. 4: Schematic demonstration of bioethanol production using separate enzymatic hydrolysis and fermentation (SHF)

SHF is that of contaminations. The hydrolysis process is rather long, e.g., 1-4 days and a dilute solution of sugar always has a risk of microbial contaminations, even at rather high temperature such as 45-50°C. A possible source of contamination could be the enzymes. In practice, it is difficult to sterilize the cellulase in large scale, since it should be filtered because of its deactivation in an autoclave (Taherzadeh and Karimi, 2007b).

Simultaneous saccharification and fermentation (SSF):
The Simultaneous Saccharification and Fermentation (SSF) combines the hydrolysis of pretreated lignocelluloses and the hexose fermentation in one step (Fig. 5). This configuration has the advantage that the

sugars do not inhibit the cellulases during the hydrolysis because they are immediately used up by the fermenting organisms. 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 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. Remaining solids containing cellulose and lignin is then hydrolyzed and fermented simultaneously by using cellulase enzymes and yeast.

The glucose produced by the hydrolyzing enzymes is consumed immediately by the fermenting microorganism present in the culture (Krishna *et al.*, 2001). This is a great advantage for SSF compared to SHF, since
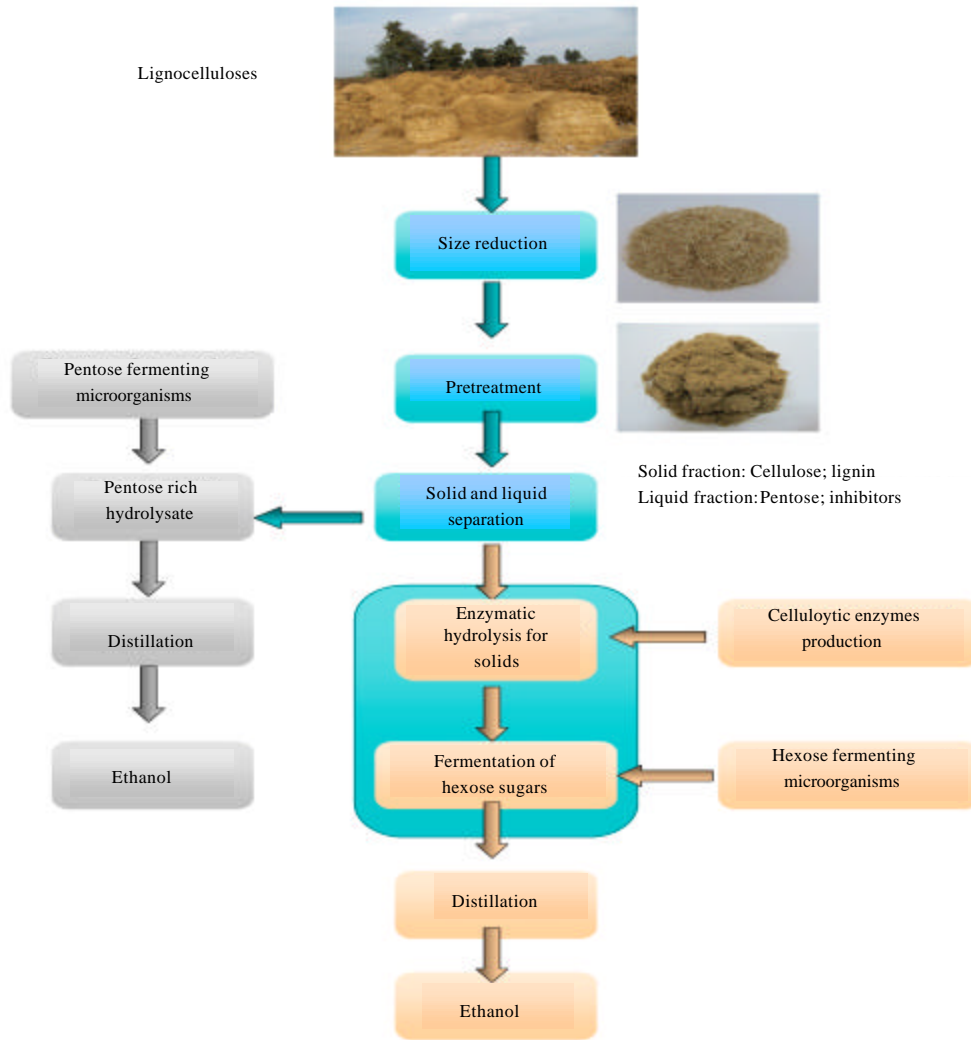
Fig. 5: Schematic demonstration of bioethanol production using simultaneous saccharification and fermentation (SSF)

the inhibition effects of cellobiose and glucose to the enzymes are minimized by keeping a low concentration of these sugars in the media. SSF gives higher reported ethanol yields from cellulose than SHF and requires lower amounts of enzyme (Sun and Cheng, 2002; Demain *et al.*, 2005; Karimi *et al.*, 2006). The risk of contamination in SSF is lower than in the SHF process, since the presence of ethanol reduces the possibility of contamination. Furthermore, the number of vessels required for SSF is reduced in comparison to SHF, resulting in lower capital cost of the process (Table 4).

An important strategy in SSF is to have the optimum conditions for the enzymatic hydrolysis and fermentation as close as possible, particularly with respect to pH and temperature. However, the difference between optimum

temperatures of the hydrolyzing enzymes and fermenting microorganisms is still a drawback of SSF. The optimum temperature for cellulases is usually between 45 and 50°C, whereas *S. cerevisiae* has an optimum temperature between 30 and 35°C and is practically inactive at more than 40°C. The optimum temperature for SSF by using *T. reesei* cellulase and *S. cerevisiae* was reported to be around 38°C which is a compromise between the optimal temperatures for hydrolysis and fermentation (Tengborg, 2000). Several thermotolerant bacteria and yeasts, e.g., *Candida acidothermophilum* and *Kluyveromyces marxianus* have been proposed for use in SSF to raise the temperature close to the optimal temperature of hydrolysis (Golias *et al.*, 2002; Ballesteros *et al.*, 2004; Hong *et al.*, 2007). The currently

Table 4: Comparison of the different fermentation process configurations

| Process	Concept	Advantages	Disadvantages
Separate enzymatic hydrolysis and fermentation (SHF)	Pretreated lignocelluloses are hydrolyzed to glucose and subsequently fermented to ethanol in separate units	Hydrolysis and fermentation take place at their own optimum conditions	Inhibition of cellulase activity by the released sugars, mainly cellobiose and glucose The hydrolysis process is long and a dilute solution of sugar always has a risk of microbial contaminations
Simultaneous saccharification and fermentation (SSF)	Cellulose is hydrolyzed and the glucose produced is fermented simultaneously by using cellulase enzymes and yeast Pentose rich hydrolysate processed via xylose fermentation process	Requires lower amounts of enzymes High ethanol yield than SHF Lower cost The inhibition effects of cellobiose and glucose to the enzymes are minimized The presence of ethanol reduces the risk of contamination	Inhibition of cellulase by produced ethanol Incompatible temperature of hydrolysis and fermentation
Simultaneous saccharification and cofermentation (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 coferment glucose and xylose to ethanol	Shorter process time High bioethanol yield Less contamination risk Cost effective	High enzyme load Either hydrolysis or fermentation can be perform under optimal conditions
Consolidated bioprocessing (CBP)	Cellulase production, substrate hydrolysis and fermentation are accomplished in a single step. only one microorganism is employed for both the production of cellulase and fermentation	Lower production costs Lower energy inputs Higher conversion efficiencies than SSF or SSCF processes	Lack of suitable organisms that produce cellulase and other enzymes and also produce ethanol at the required high yields Difficulty in process control
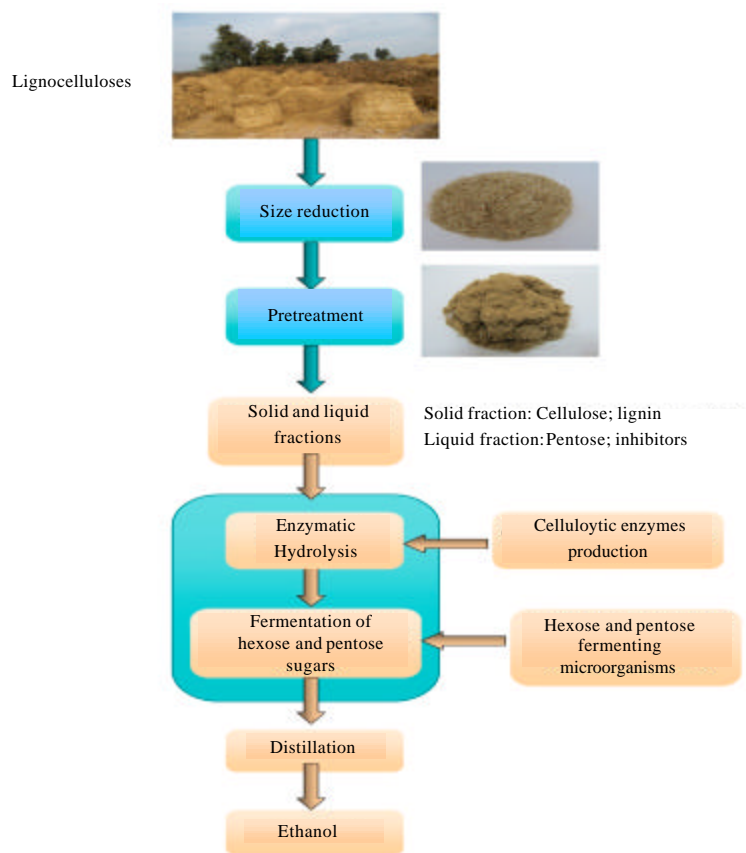
Fig. 6: Schematic demonstration of bioethanol production using simultaneous saccharification and cofermentation (SSCF)

most promising ethanologenic bacteria for industrial exploitation are *E. coli*, *Klebsiella oxytoca* and *Z. mobilis*. Genetic engineering made it possible to transfer cellulose genes from *Trichoderma* to *S. cerevisiae* (Shoemaker, 1984). However, the cellulases were produced at a concentration too low to be useful.

Inhibition of cellulase by produced ethanol might be also a problem in SSF. It was reported that 30 g L^{-1} ethanol reduces the enzyme activity by 25% (Wyman, 1996). Ethanol inhibition may be a limiting factor in producing high ethanol concentration. Despite the mentioned problems, SSF is the preferred method in many laboratory studies and pilot scale studies for ethanol production. In the case of ethanol production from hardwood and agriculture residues, the hemicellulose mainly contains pentoses. If the pentose is separated during the pretreatment, the pentose-rich hydrolyzate (hemicellulosic hydrolyzate) can be converted to ethanol in a separate pentose-fermenting bioreactor (Fig. 5).

Simultaneous saccharification and cofermentation (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 hexoses and pentoses in one step to ethanol (Teixeira *et al.*, 2000) (Fig. 6). One of the main problems of this approach is that the pentose utilizing organisms also prefer hexoses as substrate. So, if they are together with an organism as e.g., *S. cerevisiae* there is a competition between them, what usually results in lower ethanol yields. To overcome this effect sequential fermentation of hexoses and pentoses has been proposed, where the pentose fermenting organisms are added to the substrate after the hexose fermentation is completed, but in the same device. However ethanol yields of sequential fermentation are still low.

The process is carried out using genetically engineered microorganisms. Lawford and Rousseau (1998) used a metabolically engineered strain of *Zymomonas mobilis* that can coferment glucose and xylose. McMillan *et al.* (1999) used an adapted variant of the NREL xylose-fermenting *Z. mobilis* for ethanol
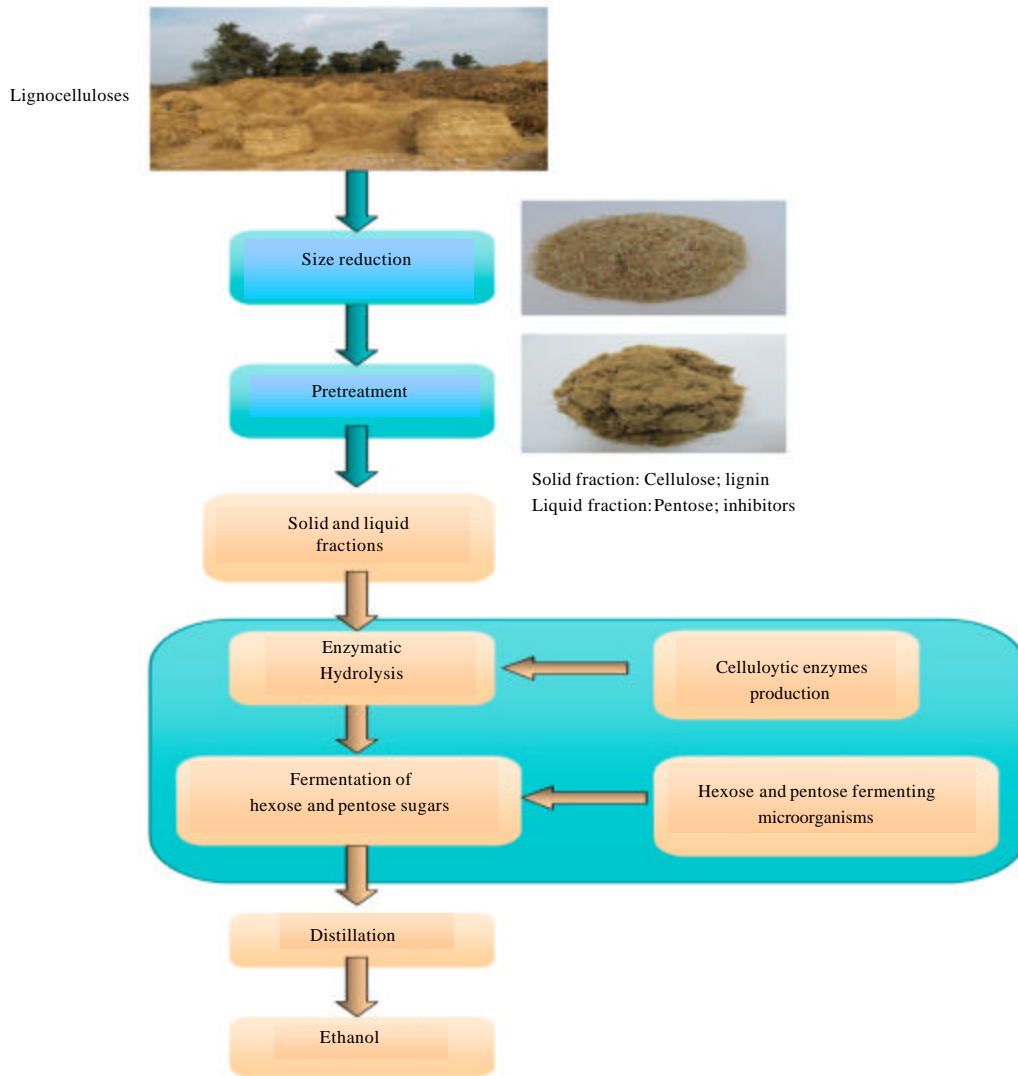
Fig. 7: Schematic demonstration of bioethanol production using consolidated bioprocessing (CBP)

production from dilute-acid- pretreated yellow poplar by SSCF. The integrated system produced more than 30 g L^{-1} ethanol and achieved 54% conversion of all potentially available sugars in the biomass (total sugars) entering SSCF. This technology is superior to SSF technology in terms of cost effectiveness, better yields and shorter processing time (Table 4) (Lynd *et al.*, 2005).

Consolidated bioprocessing (CBP): Consolidated bioprocessing (CBP) is a system in which cellulase production, substrate hydrolysis and fermentation are accomplished in a single step by cellulolytic microorganisms in microbial community and in one reactor (Fig. 7) (Lynd *et al.*, 2005; Cardona and Sanchez, 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 (Table 4). CBP is an economically attractive near-term goal for processes involving "third generation" biofuel production (Demain *et al.*, 2005; Hamelinck *et al.*, 2005). There are two strategies for enabling consolidated bioprocessing; (1) Modification of excellent ethanol producers, so that they also become efficient cellulase producers and (2) Modification of excellent cellulase producers, so that they also become efficient ethanol producers (Lynd *et al.*, 2005).

There are as yet no organisms or compatible combinations of microorganisms available that produce cellulase and other enzymes at the required high levels and also produce ethanol at the required high yields and concentrations, although various organisms already combine multiple functions (Hamelinck *et al.*, 2005). Den Haan *et al.* (2007) developed a recombinant strain of *S. cerevisiae* which can be used for CBP. Two cellulose-encoding genes, an endoglucanase of *T. reesei* and the β-glucosidase of *Saccharomycopsis fibuligera*, in combination, were expressed in *S. cerevisiae*. The resulting strain was able to grow on cellulose by simultaneous production of sufficient extracellular endoglucanase and β-glucosidase. They demonstrated the construction of a yeast strain capable of growing and of converting cellulose to ethanol in one step, representing significant progress towards realization of one-step processing of lignocellulose in a CBP configuration.

REFERENCES

Alriksson, B., A. Cavka and L.J. Jonsson, 2011. Improving the fermentability of enzymatic hydrolysates of lignocellulose through chemical *in-situ* detoxification with reducing agents. Bioresour. Technol., 102: 1254-1263.

Ballesteros, M., J.M. Oliva, M.J. Negro, P. Manzanares and I. Ballesteros, 2004. Ethanol from lignocellulosic materials by a simultaneous saccharification and fermentation process (SFS) with *Kluyveromyces marxianus* CECT 10875. Process Biochem., 39: 1843-1848.

Berlin, A., M. Balakshin, N. Gilkes, J. Kadla, V. Maximenko, S. Kubo and J. Saddler, 2006. Inhibition of cellulase, xylanase and β-glucosidase activities by softwood lignin preparations. J. Biotechnol., 125: 198-209.

Breuil, C., M. Chan, M. Gilbert and J.N. Saddler, 1992. Influence of β-glucosidase on the filter paper activity and hydrolysis of lignocellulosic substrates. Bioresour. Technol., 39: 139-142.

Brown, R.C., 2003. Biorenewable Resources: Engineering New Products from Agriculture. Iowa State Press, Ames, IA., ISBN: 978-0813822631, Pages: 286.

Cardona, C.A. and O.J. Sanchez, 2007. Fuel ethanol production: Process design trends and integration opportunities. Bioresour. Technol., 98: 2415-2457.

Chandel, A.K., M.L. Narasu, R. Rudravaram, R. Pogaku and L.V. Rao, 2009. Bioconversion of De-Oiled Rice Bran (DORB) hemicellulosic hydrolysate into ethanol by *Pichia stipitis* NCM3499 under optimized conditions. Int. J. Food Eng., Vol. 5. 10.2202/1556-3758.1453

Chandel, A.K., O.V. Singh and L.V. Rao, 2010. Biotechnological Applications of Hemicellulosic Derived Sugars: State-of-the-Art. In: Sustainable Biotechnology: Renewable Resources and New Perspectives, Singh, O.V. and S.P. Harvey (Eds.). Springer, Netherland, ISBN-13: 978-90-481-3294-2, pp: 63-81.

Chandra, R.P., R. Bura, W.E. Mabee, A. Berlin, X. Pan and J.N. Saddler, 2007. Substrate pretreatment the key to effective enzymatic hydrolysis of lignocellulosics. Adv. Biochem. Eng. Biotechnol., 108: 67-93.

Chang, V.S., B. Burr and M.T. Holtzapple, 1997. Lime Pretreatment of Switchgrass. Proceedings of the 18th Symposium on Biotechnology for Fuels and Chemicals, May 5-9, 1996, Gatlinburg, Tennessee, pp: 3-19.

Chang, V.S. and M. T. Holtzapple, 2000. Fundamental factors affecting biomass enzymatic reactivity. Applied Biochem. Biotechnol., 84-86: 5-37.

Chen, M., L. Xia and P. Xue, 2007. Enzymatic hydrolysis of corncob and ethanol production from cellulosic hydrolysate. Int. Biodeterior. Biodegrad., 59: 85-89.

Chundawat, S.P.S., B. Venkatesh and B.E. Dale, 2007. Effect of particle size based separation of milled corn stover on AFEX pretreatment and enzymatic digestibility. Biotechnol. Bioeng., 96: 219-231.

Colin, C., 2003. The Coming Oil Crisis. Multi-Science Publishing Co., New York.

Demain, A.L., M. Newcomb and J.D. Wu, 2005. Cellulase, clostridia and ethanol. Microbiol. Mol. Biol. Rev., 69: 124-154.

Den Haan, R., S.H. Rose, L.R. Lynd and W.H. van Zyl, 2007. Hydrolysis and fermentation of amorphous cellulose by recombinant *Saccharomyces cerevisiae*. Metab. Eng., 9: 87-94.

Di Nasso, N.O.N., N. Roncucci, F. Triana, C.Tozzini and E. Bonari, 2011. Seasonal nutrient dynamics and biomass quality of giant reed (*Arundo donax* L.) and miscanthus (*Miscanthus x giganteus* Greef et Deuter) as energy crops. Ital. J. Agron., 6: 152-158.

Dobermann, A. and T.H. Fairhurst, 2002. Rice straw management. Better Crops Int., 16: 7-11.

Draude, K.M., C.B. Kurniawan and S.J.B. Duff, 2001. Effect of oxygen delignification on the rate and extent of enzymatic hydrolysis of lignocellulosic material. Bioresour. Technol., 79: 113-120.

El-Naggar, N.E.A., A.A. Sherief and S.S. Hamza, 2011. Bioconversion process of rice straw by thermotolerant cellulolytic *Streptomyces viridiochromogenes* for production of bioethanol under SSF conditions for bioethanol production. Afr. J. Biotechnol., 10: 11998-12011.

El-Naggar, N.E.A. and N.A.M. Abdelwahed, 2012. Optimization of process parameters for the production of Alkali-tolerant carboxymethyl cellulase by newly isolated *Streptomyces* sp. strain NEAE-D. Afr. J. Biotechnol., 11: 1185-1196.

Eriksson, K.E.L., N. Habu and M. Samejima, 1993. Recent advances in fungal cellobiose oxidoreductases. Enzyme Microbial Technol., 15: 1002-1008.

Eriksson, T., J. Borjesson and F. Tjerneld, 2002. Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. Enzyme Microbial Technol., 31: 353-364.

Esterbabur, H., W. Steiner and I. Labudova, 1991. Production of *Trichoderma* cellulose in laboratory and pilot scale. Bioresour. Technol., 36: 51-65.

Fan, L.T., Y.H. Lee and D.H. Beardmore, 1980. Mechanism of the enzymatic hydrolysis of cellulose: Effects of major structural features of cellulose on enzymatic hydrolysis. Biotechnol. Bioeng., 22: 177-199.

Fan, L.T., Y.H. Lee and D.R. Beardmore, 1981. The influence of major structural features of cellulose on rate of enzymatic hydrolysis. Biotechnol. Bioeng., 23: 419-424.

Fan, L.T., Y.H. Lee and M.M. Gharpuray, 1982. The nature of lignocellulosics and their pretreatments for enzymatic hydrolysis. Adv. Biochem. Eng., 23: 157-187.

Fan, L.T., M.M. Gharpury and Y.H. Lee, 1987. Nature of Cellulose Materials and Enzymatic Hydrolysis. In: Cellulose Hydrolysis, Fan, L.T., M.M. Gharpury and Y.H. Lee (Eds.). Springer-Verlag, Berlin, pp: 5-69.

Fonseca, B.G., R. de Oliveira Moutta, F. de Oliveira Ferraz, E.R. Vieira and A.S. Nogueira *et al.*, 2011. Biological detoxification of different hemicellulosic hydrolysates using *Issatchenkia occidentalis* CCTCC M 206097 yeast. J. Ind. Microbiol. Biotechnol., 38: 199-207.

Galbe, M. and G. Zacchi, 2002. A review of the production of ethanol from softwood. Applied Microbiol. Biotechnol., 59: 618-628.

Gama, F.M., M. Vilanova and M. Mota, 1998. Exo-and Endo-glucanolytic activity of cellulases purified from *Trichoderma reesei*. Biotechnol. Tech., 12: 677-681.

Gan, Q., S. Allen and G. Taylor, 2005. Analysis of process integration and intensification of enzymatic cellulose hydrolysis in a membrane bioreactor. J. Chem. Technol. Biotechnol., 80: 688-698.

Gaspar, M., G. Kalman and K. Reczey, 2007. Corn fiber as a raw material for hemicellulose and ethanol production. Process Biochem., 42: 1135-1139.

Gharpuray, M.M., Y.H. Lee and L.T. Fan, 1983. Structural modification of lignocellulosics by treatment to enhance enzymatic hydrolysis. Biotechnol. Bioeng., 25: 157-172.

Golias, H., G.J. Dumsday, G.A. Stanley and N.B. Pamment, 2002. Evaluation of a recombinant *Klebsiella oxytoca* strain for ethanol production from cellulose by simultaneous saccharification and fermentation: Comparison with native Cellobiose-utilising yeast strains and performance in co-culture with thermotolerant yeast and *Zymomonas mobilis*. J. Biotechnol., 96: 155-168.

Grethlein, H.E. and A.O. Converse, 1991. Common aspects of acid prehydrolysis and steam explosion for pretreating wood. Bioresour. Technol., 36: 77-82.

Grethlein, H.E., 1985. The effect of pore size distribution on the rate of enzymatic hydrolysis of cellulosic substrates. Nature Biotechnol., 3: 155-160.

Ha, S.J., J.M. Galazka, S.R. Kim, J.H. Choi and X. Yang *et al.*, 2011. Engineered *Saccharomyces cerevisiae* capable of simultaneous cellobiose and xylose fermentation. Proc. Natl. Acad. Sci., 108: 504-509.

Hahn-Hagerdal, B., M. Galbe, M.F. Gorwa-Grauslund, G. Liden and G. Zacchi, 2006. Bio-ethanol: The fuel of tomorrow from the residues of today. Trends Biotechnol., 24: 549-556.

Hahn-Hagerdal, B., K. Karhumaa, C. Fonseca, I. Spencer-Martins and M.F. Gorwa-Grauslund, 2007. Towards industrial Pentose-fermenting yeast strains. Applied Microbiol. Biotechnol., 74: 937-953.

Hamelinck, C.N., G.V. Hooijdonk and A.P. Faaij, 2005. Ethanol from lignocellulosic biomass: Techno-economic performance in Short-, middle-and Long-term. Biomass Bioenergy, 28: 384-410.

Holtzapple, M.T., 1993. Hemicellulose. In: Encyclopedia of Food Science, Food Technology and Nutrition, Macrae, R., R.K. Robinson and M.J. Sadler (Eds.). Vol. 2, Academic Press, London, pp: 2324-2334.

Holtzapple, M.T., J.H. Jun, G. Ashok, S.L. Patibandla and B.E. Dale, 1991. The ammonia freeze explosion (AFEX) process. Applied Biochem. Biotechnol., 28: 59-74.

Hong, J., Y. Wang, H. Kumagai and H. Tamaki, 2007. Construction of thermotolerant yeast expressing thermostable cellulase genes. J. Biotechnol., 130: 114-123.

Howard, R.L., E. Abotsi, E.J. van Rensburg and S. Howard, 2004. Lignocellulose biotechnology: Issues of bioconversion and enzyme production. Afr. J. Biotechnol., 2: 602-619.

Hu, G., J.A. Heitmann and O.J. Rojas, 2008. Feedstock pretreatment strategies for producing ethanol from wood, bark and forest residues. BioResources, 3: 270-294.

Itoh, H., M. Wada, Y. Honda, M. Kuwahara and T. Watanabe, 2003. Bioorganosolve pretreatments for simultaneous saccharification and fermentation of beech wood by ethanolysis and white rot fungi. J. Biotechnol., 103: 273-280.

Jeffries, T.W. and Y.S. Jin, 2004. Metabolic engineering for improved fermentation of pentoses by yeasts. Applied Microbiol. Biotechnol., 63: 495-509.

Jorgensen, H., T. Eriksson, J. Borjesson, F. Tjerneld and L. Olsson, 2003. Purification and characterization of five cellulases and one xylanase from *Penicillium brasilianum* IBT 20888. Enzyme Microbial Technol., 32: 851-861.

Jurado, M., A. Prieto, A. Martinez-Alcala, A.T. Martinez and M.J. Martinez, 2009. Laccase detoxification of Steam-exploded wheat straw for second generation bioethanol. Bioresour. Technol., 100: 6378-6384.

Karhumaa, K., B. Wiedemann, B. Hahn-Hagerdal, E. Boles and M.F. Gorwa-Grauslund, 2006. Co-utilization of L-arabinose and D-xylose by laboratory and industrial *Saccharomyces cerevisiae* strains. Microbial Cell Factories, Vol. 5. 10.1186/1475-2859-5-18

Karimi, K., G. Emtiazi and M.J. Taherzadeh, 2006. Ethanol production from dilute-acid pretreated rice straw by simultaneous saccharification and fermentation with *Mucor indicus, Rhizopus oryzae* and *S. cerevisiae*. Enzyme Microbial. Technol., 40: 138-144.

Kaylen, M., D.L. van Dyne, Y.S. Choi and M. Blase, 2000. Economic feasibility of producing ethanol from lignocellulosic feedstocks. Bioresour. Technol., 72: 19-32.

Kheshgi, H.S., R.C. Prince and G. Marland, 2000. The potential of biomass fuels in the context of global climate change: Focus on transportation fuels. Ann. Rev. Energy Environ., 25: 199-244.

Kim, T.H., J.S. Kim, C. Sunwoo and Y.Y. Lee, 2003. Pretreatment of corn stover by aqueous ammonia. Bioresour. Technol., 90: 39-47.

Kirk, T.K. and R.L. Farrell, 1987. Enzymatic combustion: The microbial degradation of lignin. Annu. Rev. Microbiol., 41: 465-505.

Kong, F., C.R. Engler and E.J. Soltes, 1992. Effects of cell-wall acetate, xylan backbone and lignin on enzymatic hydrolysis of aspen wood. Applied Biochem. Biotechnol., 34-35: 23-35.

Krause, D.O., S.E. Denman, R.I. Mackie, M. Morrison, A.L. Rae, G.T. Attwood and C.S. McSweeney, 2003. Opportunities to improve fiber degradation in the rumen: Microbiology, ecology and genomics. FEMS Microbiol. Rev., 27: 663-693.

Krishna, S.H., T.J. Reddy and G.V. Chowdary, 2001. Simultaneous saccharification and fermentation of lignocellulosic wastes to ethanol using a thermotolerant yeast. Bioresour. Technol., 77: 193-196.

Kumakura, M. and I. Kaetsu, 1983. Effect of radiation pretreatment of bagasse on enzymatic and acid hydrolysis. Biomass, 3: 199-208.

Kumar, P., D.M. Barrett, M.J. Delwiche and P. Stroeve, 2009. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind. Eng. Chem. Res., 48: 3713-3729.

Kurakake, M., N. Ide and T. Komaki, 2007. Biological pretreatment with two bacterial strains for enzymatic hydrolysis of office paper. Current Microbiol., 54: 424-428.

Kuyper, M., M.M. Hartog, M.J. Toirkens, M.J. Almering, A.A. Winkler, J.P. Dijken and J.T. Pronk, 2005. Metabolic engineering of a xylose-isomerase-expressing *Saccharomyces cerevisiae* strain for rapid anaerobic xylose fermentation. FEMS Yeast Res., 5: 399-409.

Laivins, G.V. and A.M. Scallan, 1996. The influence of drying and beating on the swelling of fines. J. Pulp Paper Sci., 22: 178-184.

Lawford, H.G. and J.D. Rousseau, 1998. Improving fermentation performance of recombinant *zymomonas* in acetic acid-containing media. Applied Biochem. Biotechnol., 70: 161-172.

Levine, J.S., 1996. Biomass Burning and Global Changes. In: Remote Sensing and Inventory Development and Biomass Burning in Africa, Levine, J.S. (Ed.). Vol. 1, MIT Press, Cambridge, MA., USA., pp: 35.

Lynd, L.R., P.J. Weimer, W.H. van Zyl and I.S. Pretorius, 2002. Microbial cellulose utilization: Fundamentals and biotechnology. Microbiol. Mol. Biol. Rev., 66: 506-577.

Lynd, L.R., W.H.V. Zyl, J.E. McBride and M. Laser, 2005. Consolidated bioprocessing of cellulosic biomass: An update. Curr. Opin. Biotechnol., 16: 577-583.

Mais, U., A.R. Esteghlalian, J.N. Saddler and S.D. Mansfield, 2002. Enhancing the enzymatic hydrolysis of cellulosic materials using simultaneous ball milling. Applied Biochem. Biotechnol., 98: 815-832.

Malherbe, S. and T.E. Cloete, 2003. Lignocellulose biodegradation: Fundamentals and applications. Rev. Environ. Sci. Biotechnol., 1: 105-114.

Mamar, S. and A. Hadjadj, 1990. Radiation pretreatments of cellulose materials for the enhancement of enzymatic hydrolysis. Int. J. Radiat. Appl. Instrum. C, 35: 451-455.

Mansfield, S.D., D.J. Swanson, N. Roberts, J.A. Olson and J.N. Saddler, 1999. Enhancing douglas-fir pulp properties with a combination of enzyme treatments and fiber fractionation. Tappi, 82: 152-158.

McMillan, J.D., M.M. Newman, D.W. Templeton and A. Mohagheghi, 1999. Simultaneous saccharification and cofermentation of dilute-acid pretreated yellow poplar hardwood to ethanol using xylose-fermenting *Zymomonas mobilis*. Proceedings of the 20th Symposium on Biotechnology for Fuels and Chemicals, May 3-7, 1998, Gatlinburg, Tennessee, pp: 649-665.

Mooney, C.A., S.D. Mansfield, M.G. Touhy and J.N. Saddler, 1998. The effect of initial pore volume and lignin content on the enzymatic hydrolysis of softwoods. Bioresour. Technol., 64: 113-119.

Mosier, N., C. Wyman, B. Dale, R. Elander, Y.Y. Lee, M. Holtzapple and L. Michael, 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour. Technol., 96: 673-686.

Neely, W.C., 1984. Factors affecting the pretreatment of biomass with gaseous ozone. Biotechnol. Bioeng., 26: 59-65.

Ohgren, K., M. Galbe and G. Zacchi, 2005. Optimization of steam pretreatment of SO_2-impregnated corn stover for fuel ethanol production. Applied Biochem. Biotechnol., 124: 1055-1067.

Olsson, L., H.R. Soerensen, B.P. Dam, H. Christensen, K.M. Krogh and A.S. Meyer, 2006. Separate and simultaneous enzymatic hydrolysis and fermentation of wheat hemicellulose with recombinant xylose utilizing *Saccharomyces cerevisiae*. Applied Biochem. Biotechnol., 129: 117-129.

Ooshima, H., M. Kurakake, J. Kato and Y. Harano, 1991. Enzymatic activity of cellulase adsorbed on cellulose and its change during hydrolysis. Applied Biochem. Biotechnol., 31: 253-266.

Palmowski, L. and J. Muller, 1999. Influence of the size reduction of organic waste on their anaerobic digestion. Proceedings of the 2nd International Symposium on Anaerobic Digestion of Solid Waste, June 15-17, 1999, Barcelona, Spain, pp: 137-144.

Parawira, W. and M. Tekere, 2011. Biotechnological strategies to overcome inhibitors in lignocellulose hydrolysates for ethanol production: Review. Crit. Rev. Biotechnol., 31: 20-31.

Philippidis, G.P. and T.K. Smith, 1995. Limiting factors in the simultaneous saccharification and fermentation process for conversion of cellulosic biomass to fuel ethanol. Applied Biochem. Biotechnol., 51: 117-124.

Prates, J.A., N. Tarbouriech, S.J. Charnock, C.M. Fontes, L. Ferreira and G.J. Davies, 2001. The structure of the feruloyl esterase module of xylanase 10B from *Clostridium thermocellum* provides insights into substrate recognition. Structure, 9: 1183-1190.

Puri, V.P., 1984. Effect of crystallinity and degree of polymerization of cellulose on enzymatic saccharification. Biotechnol. Bioeng., 26: 1219-1222.

Qin, W., 2010. High consistency enzymatic hydrolysis of lignocellulose. M.Sc. Thesis, University of British Columbia, Faculty of Forestry, Vancouver, Canada.

Rabinovich, M.L., M.S. Melnick and A.V. Bolobova, 2002. The structure and mechanism of action of cellulolytic enzymes. Biochemistry (Moscow), 67: 850-871.

Ramos, L.P., M.M. Nazhad and J.N. Saddler, 1993. Effect of enzymatic hydrolysis on the morphology and fine structure of pretreated cellulosic residues. Enzyme Microb. Technol., 15: 821-831.

Rogers, P.L., K.J. Lee and D.E. Tribe, 1979. Kinetics of alcohol production by *Zymomonas mobilis* at high sugar concentrations. Biotechnol. Lett., 1: 165-170.

Saha, B.C., 2003. Hemicellulose bioconversion. J. Ind. Microbiol. Biotechnol., 30: 279-291.

Saha, B.C., L.B. Iten, M.A. Cotta and Y.V. Wu, 2005. Dilute acid pretreatment, enzymatic saccharification and fermentation of rice hulls to ethanol. Biotechnol. Prog., 21: 816-822.

Sameera, V., C. Sameera and Y. Ravi Teja, 2011. Current strategies involved in biofuel production from plants and algae. J. Microbial. Biochem. Technol.

Scott, C.D. and C.E. Wyman, 2004. Biotechnology for fuels and Chemicals-past, present and future. Proceedings of the 25th Symposium on Biotechnology for Fuels and Chemicals, May 4-7, 2003, Breckenridge, Co., pp: 761-763.

Sewalt, V.J.H., W.G. Glasser and K.A. Beauchemin, 1997. Lignin impact on fiber degradation. 3. Reversal inhibition of enzymatic hydrolysis by chemical modification of lignin and by additives. J. Agric. Food Chem., 45: 1823-1828.

Sherief, A.A., N.E.A. El-Naggar and S.S. Hamza, 2010. Bioprocessing of lignocellulosic biomass for production of bioethanol using thermotolerant *Aspergillus fumigates* under solid state fermentation conditions. Biotechnology, 9: 513-522.

Shoemaker, S.P., 1984. The cellulase system of *Trichoderma reesei*: *Trichoderma strain* improvement and expression of *Trichoderma cellulases* in yeast. World Biotech. Rep., 2: 593-600.

Sinitsyn, A.P., A.V. Gusakov and E.Y. Vlasenko, 1991. Effect of structural and Physico-chemical features of cellulosic substrates on the efficiency of enzymatic hydrolysis. Applied Biochem. Biotechnol., 30: 43-59.

Sun, Y. and J. Cheng, 2002. Hydrolysis of lignocellulosic materials for ethanol production: A review. Bioresour. Technol., 83: 1-11.

Sun, Y. and J.J. Cheng, 2005. Dilute acid pretreatment of rye straw and bermudagrass for ethanol production. Bioresour. Technol., 96: 1599-1606.

Taherzadeh, M.J. and K. Karimi, 2007a. Acid-based hydrolysis processes for ethanol from lignocellulosic materials: A review. BioResources, 2: 472-499.

Taherzadeh, M.J. and K. Karimi, 2007b. Enzymatic-based hydrolysis processes for ethanol from lignocellulosic materials: A review. Bioresources, 2: 707-738.

Tarkow, H. and W.C. Feist, 1969. A Mechanism for Improving the Digestibility of Lignocellulosic Materials with Dilute Alkali and Liquid Ammonia. In: Cellulases and Their Applications, Hajny, G.J. and E.T. Reese (Eds.). American Chemical Society, Washington, DC., ISBN-13: 9780841200951, pp: 197-218.

Teixeira, L.C., J.C. Linden and H.A. Schroeder, 2000. Simultaneous saccharification and cofermentation of peracetic Acid-pretreated biomass. Applied Biochem. Biotechnol., 84: 111-127.

Tengborg, C., 2000. Bioethanol production: Pretreatment and enzymatic hydrolysis of softwood. Ph.D. Thesis, Lund University, Sweden.

Tran, A.V. and R.P. Chambers, 1986. Ethanol fermentation of red oak acid prehydrolysate by the yeast *Pichia stipitis* CBS 5776. Enzyme Microbial Technol., 8: 439-444.

Vidal, P.F. and J. Molinier, 1988. Ozonolysis of lignin-Improvement of *in vitro* digestibility of poplar sawdust. Biomass, 16: 1-17.

Vinzant, T.B., L. Ponfick, N.J. Nagle, C.I. Ehrman, J.B. Reynolds and M.E. Himmel, 1999. SSF comparison of selected woods from Southern Sawmills. Applied Biochem. Biotechnol., 45-46: 611-626.

Von Sivers, M. and G. Zacchi, 1996. Ethanol from lignocellulosics: A review of the economy. Bioresour. Technol., 56: 131-140.

Wood, T.M., 1991. Fungal Cellulases. In: Biosynthesis and Biodegradation of Cellulose, Haigler, C.H. and P.J. Weimer (Eds.). Macel Dekker Inc., New York, pp: 491-534.

Wyman, C.E., 1996. Handbook on Bioethanol: Production and Utilization. Taylor and Francis, Washington, DC., USA.

Wyman, C.E., 2001. Twenty years of trials, tribulations and research progress in bioethanol technology: Selected key events along the way. Applied Biochem. Biotechnol., 91-93: 5-21.

Xiao, Z., X. Zhang, D.J. Gregg and J.N. Saddler, 2004. Effects of sugar inhibition on cellulases and β-glucosidase during enzymatic hydrolysis of softwood substrates. Applied Biochem. Biotechnol., 115: 1115-1126.

Yang, B. and C.E. Wyman, 2004. Effect of xylan and lignin removal by batch and flowthrough pretreatment on the enzymatic digestibility of corn stover cellulose. Biotechnol. Bioeng., 86: 88-98.

Yu, Y., Y. Feng, C. Xu, J. Liu and D. Li, 2011. Onsite Bio-detoxification of Steam-exploded corn stover for cellulosic ethanol production. Bioresour. Technol., 102: 5123-5128.

Zaldivar, J., J. Nielsen and L. Olsson, 2001. Fuel ethanol production from lignocellulose: A challenge for metabolic engineering and process integration. Applied Microbiol. Biotechnol., 56: 17-34.

Zeng, M., N.S. Mosier, C.P. Huang, D.M. Sherman and M.R. Ladisch, 2007. Microscopic examination of changes of plant cell structure in corn stover due to hot water pretreatment and enzymatic hydrolysis. Biotechnol. Bioeng., 97: 265-278.

Zhang, Y.H.P., M.E. Himmel and J.R. Mielenz, 2006. Outlook for cellulase improvement: Screening and selection strategies. Biotechnol. Adv., 24: 452-481.

Zhu, L., J.P. O'Dwyer, V.S. Chang, C.B. Granda and M.T. Holtzapple, 2008. Structural features affecting biomass enzymatic digestibility. Bioresour. Technol., 99: 3817-3828.

Zhu, J.Y., X.J. Pan, G.S. Wang and R. Gleisner, 2009. Sulfite pretreatment (SPORL) for robust enzymatic saccharification of spruce and red pine. Bioresour. Technol., 100: 2411-2418.