BIOCONVERSION OF LIGNOCELLULOSIC AGRO-RESIDUES TO FUEL ALCOHOL - A REVIEW

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ABSTRACT

Over the past few decades considerable research and development is directed towards understanding and commercialization of renewable energy sources. A process for conversion of lignocellulosic biomass into fuel alcohol involving pretreatment of the raw material, cellulase production, enzymatic hydrolysis and fermentation of the hydrolysates has been developed. Research efforts directed towards understanding and manipulating the bioconversion process suggest that greater yields of ethanol can be achieved through process optimization. These studies have yielded considerable insights into the role that cellulase plays in the bioconversion process. Better understanding of the various pretreatments and molecular mechanisms at work in hydrolysis will make it possible to improve the conversion efficiency and economics of the bioconversion process.

The natural energy resources such as petroleum and coal are being utilized at a rapid rate and these resources have been estimated to last over a few years. Due to dwindling of fossil fuel, microbial production of biofuel from organic byproducts has acquired significance in recent years. Biomass has been shown to have considerable promise as a raw material for gaseous fuels, liquid fuels and certain petrochemical intermediates (Correa et al., 1999; Yamada and Ono, 1999). Currently, the concern over energy and food shortages is increasing. Much research effort has been spent on the recovery and reuse of abundant waste lignocellulosic biomass (Fan et al., 1982; Buchert et al., 1988; Kanotra and Mathur, 1994; Ahring et al., 1999).

The production of biofuel and chemicals form indigenous lignocellulosic biomass will stimulate new markets for the agricultural sector, thereby increasing domestic employment while reducing the waste management problems. Hence, enzymatic hydrolysis of lignocellulosic biomass shows promising potential in areas such as transportation, fuel production and solid waste disposal (Coughlan, 1992). The lignocellulosic agro residues mainly comprise of cellulose, hemicellulose and lignin (Kaur et al., 1998). The carbohydrate portion of the agro residues is approximately 60% by weight and is considered as a feedstock for conversion to sugars, which may be fermented to ethanol (Vlasenko et al., 1997).

In lignocellulose, the cellulose and hemicellulose are intimately associated with lignin (Cowling and Kirk, 1976; Okeke and Obi, 1994). The lignin component acts as a physical barrier (Lynch, 1992) and must be removed to make the carbohydrates available for further transformation. Thus, to utilize lignocellulose, it must be first pretreated to increase the surface area, bulk density and decrease the crystallinity of the cellulose, so as to make it accessible for hydrolysis (Gharpuray et al., 1983; Rao and Setta, 1983; Gould, 1984; David et al., 1985; Wood and Saddler, 1988; Viesturs et al., 1996; Nikolov et al., 2000). The pretreated lignocellulose, with free cellulose, is susceptible to enzymatic hydrolysis (Nahmad et al., 1994; Wyk van, 1999). Biomass conversion processes through enzymatic hydrolysis of pretreated lignocellulosics yield mono- and oligomeric sugar solutions, which can further be converted into fuels or chemicals, ethanol being one of them (Nguyen and

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Availability and disposal of agro residues

The collectible agricultural residues have been estimated to total 300 million tons annually (Clausn and Gaddy, 1989). The large availability of lignocellulosic agricultural residue make it possible to use these materials for energy applications and as chemical feedstock (Philippidis et al., 1993; Gregg and Saddler, 1996; Oslo and Hahn-Hagerdal, 1996). Among the easily available agricultural waste, rice and wheat straw are most abundant. Straw generated from crop production is approximately double the amount of yield (Kanotra and Mathur, 1994). Wheat straw has been used as an animal feed. Rice straw could be used as an animal feed; however, it has not been popular, major limitations being low digestibility, low protein content and lignin and silica content (Kahlon and Dass, 1987).

Disposal of agricultural residues presents an environmental problem. The surplus agro residues have traditionally been removed from field by the practice of open field burning (Kanotra and Mathur, 1994). This practice clears the field for new plantings but has caused widespread environmental concerns. Open field burning has an adverse effect on the air quality (Wyman, 1994). Besides causing air pollution this practice could potentially reduce crop yields, increase foliar disease, and degrade soil conditions by killing the microbial population of top soil (Vlasenko et al., 1997). In certain parts of the world, the legislation mandates farmers to phase down burning of agricultural residues. If such mandate were given throughout the world then the disposal of agricultural residues would pose serious concerns. Thus it is realistic to consider this surplus agricultural residue as a renewable resource for energy generation (Zhang and Zhang, 1999).

Ethanol as an alternative fuel

Enhanced interest has been devoted to alternative fuel, such as ethanol, especially from non-food sources such as straw or wood chips. These waste products could be an environmentally friendly alternative (Wyman and Goodman, 1993; Ahring et al., 1996). Hence it is possible to use these agro residues for energy applications. Production of ethanol from renewable sources of lignocellulosic biomass can improve energy security, decrease urban air pollution and reduce accumulation of carbon dioxide in the atmosphere (Lynd et al., 1991). Ethanol, being an excellent transportation fuel can be used as a blend with gasoline, 10% and 22% blends are being used in the US and Brazil respectively (Wyman, 1994). It may be used directly, 95% ethanol and 5% water, as a fuel. Such nearly pure ethanol fuel provide a number of environment benefits, due to their low vapour pressure and reduced emission of ethanol into the atmosphere, plus their clean burning characteristics (Lynd et al., 1991). In addition, ethanol has a low atmospheric photochemical reactivity, further reducing its impact on ozone (Lynd et al., 1991). Ethanol blended with gasoline oxygenates it thereby reducing the formation of carbon monoxide and ozone, which is desirable for the implementation of the Clean Air Act Amendments (Wyman, 1994).

Structure of lignocellulose

Lignocellulosic biomass represents a major fraction of most plant matter. Lignocellulose is composed of cellulose, hemicellulose, lignin, protein and various extraneous materials (Chahal and Overend, 1983). The carbohydrate portion of lignocellulose (cellulose and hemicellulose) can be hydrolyzed into their component sugars (Bergeron et al., 1989).

Cellulose is the largest fraction typically of the order of 35-50%. Cellulose is a linear polymer of D-glucose (a six-carbon sugar). It occurs as long slender bundles composed of
long chains of β-D-glucopyranose residues linked by (1→4) - glycosidic linkage (Chahal and Overend, 1983). Cellulose fibers are arranged in bundles of parallel chains in which adjacent chains are held together by hydrogen bonding between hydroxyl groups and hydrogen atoms, forming a crystalline material with high mechanical strength and high chemical stability. Since the bond between the glucose units is the weak link in the chain, the polymer can be hydrolyzed into its component sugars (Bergeron et al., 1989). Hemicellulose is the next largest fraction of the order of 15-35%. Hemicellulose is a polymer composed primarily of five carbon sugars (xylans), six carbon sugars and organic acids. Hemicellulose is not crystalline and its five carbon sugars cannot be fermented (Bergeron et al., 1989). The third largest fraction, of the order of 15 to about 25%, is typically lignin. Lignin is a phenyl - propene polymer of complex composition that cannot be broken down to form sugars (Bergeron et al., 1989).

The hemicellulose-lignin sheath surrounds the crystalline cellulose, thereby disrupting any enzymatic action on it (Wright, 1989). The cellulose in the lignocellulose is not directly available for bioconversion because of its intimate association with lignin (Binder et al., 1980). To effectively hydrolyze cellulose, pretreatment of the raw material is necessary to make cellulose more accessible to enzymatic attack.

Structural parameters influencing lignocellulose hydrolysis

The non-uniform distribution of lignin in lignocellulosic residue brings about physical barrier to homogeneous reactions. Another problem arises from the polar nature of lignin due to phenolic and alcoholic hydroxyl groups resulting in the formation of intermolecular hydrogen bonds (Ghosh et al., 1983). Enzymatic hydrolysis of native cellulose is difficult and slow. Hence, specific pretreatment processes are necessary in order to enhance the enzymatic susceptibility. Untreated lignocellulose is slowly degraded by microorganisms due to its compact and stringent structure of β(1,4) linkages in the cellulose and due to the close association of lignin with cellulose and hemicellulose, leaving very few reactive sites for enzyme attachment (Bjerre et al., 1996).

The key role that lignin plays in restricting enzyme accessibility to the lignocellulosic substrate is indicated by the dramatic increase that delignification play in enhancing enzyme adsorption (Mooney et al., 1998). Lignin has a three dimensional structure embedded and immobilized on the crystalline carbohydrate chains, thus greatly reducing enzyme accessibility to cellulose. Lignin blocks the access of cellulases to cellulose and binds irreversibly to the enzyme. The fibre integrity and structural rigidity of lignocellulose is disrupted on delignification, which facilitates more sites for enzyme attachment to cellulose (Mooney et al., 1998). It has also been suggested that lignin restricts fibre swelling and thus on lignin removal enzyme adsorption increases (Ramos et al., 1992). Even increasing enzyme loading to a very high level cannot completely hydrolyse lignified cellulose (Mooney et al., 1998). There are evidences the enzymes are adsorbed on both isolated lignin and lignaceous residues (Lee et al., 1982; Deshpande and Eriksson, 1984; Ooshima et al., 1990) and the rate of cellulose hydrolysis decreases when lignin is added to the hydrolysis reaction (Chernogla zov et al., 1988).

Cellulose has many defining characteristics. Factors such as crystallinity, degree of polymerization, particle size and surface area influence enzymatic hydrolysis to some extent (Fan et al., 1980). The amorphous portion of cellulose is hydrolyzed more easily than the crystalline portion. Fan et al. (1980) showed that in relatively pure cellulose there
was no good correlation between crystallinity and rate of hydrolysis. However, with lignocellulosic substrate this relationship is not clear-cut, due to the more heterogeneous nature of this material and the contribution of other components such as lignin (Fan et al., 1980; Mooney et al., 1998). The high rate of hydrolysis in the initial stages is due to amorphous regions of cellulose; as the reaction proceeds, and the amorphous regions are exhausted, the overall rate slows to the value of crystalline region (Desai and Converse, 1997). However several investigators have found no significant changes in substrate crystallinity as the hydrolysis proceeds (Ohmine et al., 1983; Puis and Wood, 1991).

The susceptibility of cellulose to hydrolysis is determined largely by accessibility of cellulose surface to cellulolytic enzymes. Thus direct physical contact between the enzyme molecules and the substrate cellulose is a prerequisite to hydrolysis. Since the enzymatic conversion of cellulose to soluble sugars is a heterogeneous reaction, the surface area available for enzyme-substrate interaction is the main factor influencing the reaction rate. Pretreatment processes increase the available surface area by removing the hemicellulose and lignin that cover the cellulose and by creating a greater pore volume accessible to enzyme systems (Fan et al., 1980; Puls and Wood, 1991).

Pretreatment of lignocellulose

Numerous pretreatment methods have been developed in searching for ways to remove the lignin barrier and to enhance the accessibility of cellulose to hydrolytic degradation (Gharpuray et al., 1983; Rao and Setta 1983; Gould, 1984; Gregg and Saddler, 1996). The pretreatments may be subdivided into physical, chemical and biological.

Physical Pretreatments: Physical pretreatments may be divided into two general categories: mechanical and non-mechanical. In mechanical pretreatment physical forces are applied which are able to subdivide lignocellulosic material into fine particles. The shearing and compressive forces cause a reduction in particle size, decrease in crystallinity, an increase in bulk density and a decrease in mean degree of polymerization (Fan et al., 1980). Subsequently, the material is highly susceptible to hydrolysis due to the larger surface to volume ratio. Hammer milling, physical grinding, wet milling and dry milling all have the aim of enhancing surface of cellulose fibers resulting in an increase in enzymatic hydrolysis (Fan et al., 1980; Gharpuray et al., 1983; Ladisch and Svarczkopf, 1991).

Non mechanical physical pretreatments cause decomposition of lignocellulosics by exposing them to harsh external forces such as irradiation (Xin and Kumakura, 1992), high pressure steaming (Tanaka et al., 1988; Wright, 1989; Bjerre et al., 1996; Palmqvist et al., 1996; Ahring et al., 1999), pyrolysis and microwave treatment. Treatment like irradiation of pure cellulose results in oxidative degradation of the molecules, dehydrogenation, destruction of anhydroglucose units to yield carbon dioxide, and cellulotic chain cleavage, all this enhance the digestibility of cellulose.

Among all the physical pretreatments high pressure steaming is most extensively studied. Biomass may be treated with water or steam alone, or with small amounts of acids, it is referred to as prehydrolysis or autohydrolysis steaming; when high pressure oxygen or air is present, the reaction is called wet oxidation (Bjerre et al., 1996). The steam explosion process is conducted with high pressure and temperature, followed with rapid depressurization. This pretreatment causes a decrease of molecular weight of lignin and hemicellulose, renders cellulose porous and
causes decrease of degree of crystallization of cellulose (Tanaka et al., 1988). Ahring et al. (1999) showed that steam pretreatment gave a hexose rich cellulose fraction and lignin was degraded to lower molecular weight compounds.

An important advantage of autohydrolysis process is that it breaks the lignin into relatively small fragments that can either be solubilised in base or organic solvents and extracted (Puls and Dietrichs, 1980; Wright, 1989). This cellulose becomes highly accessible for enzymatic degradation inspite of the presence of some lignin. Steam pretreatment with the addition of inorganic acids, such as sulfuric acid and phosphoric acid, greatly enhanced the sugar yields after saccharification. But the use of inorganic acids cause corrosion the reactors thereby steam explosion without acid addition may be used (Ropars et al., 1992).

Schmidt and Thomsen (1998) observed that about 50-70% of the lignin was degraded by alkaline wet oxidation, but above 185°C the cellulose recovery decreased due to increased degradation. The major disadvantage of high-pressure steam pretreatment is the production of inhibitors. The water soluble inhibitors such as acetic acid, sugar derived byproducts and non volatile lignin derivatives have a pronounced effect on enzymatic hydrolysis (Bjerre et al., 1996; Palmqvist et al., 1996).

Chemical pretreatment: Various chemical pretreatments have been used extensively for the removal of lignin and disruption of the crystalline cellulose structure. The chemical processes are severe and the chemicals used act as swelling and oxidizing agents causing a structural modification of the cellulose (Ladisch and Svarczkopf, 1991). The swelling leads to an increase in internal surface area, decrease in crystallinity and decrease in degree of polymerization. The various chemical pretreatments are acid (Detroy et al., 1980; Roberts et al., 1980; Thompson et al., 1992), alkali (Andreoni et al., 1980; Tanaka et al., 1988; Nikolov et al., 2000), alkaline peroxide (Gould, 1984; Thompson et al., 1992), organic solvents such as ethylene diamine (Detroy et al., 1980; Thompson et al., 1992), ethanol and butanol (Ghosh et al., 1983), phenol (Zacchi et al., 1998), hydrogen fluoride (Lamport et al., 1980) and ammonia (Detroy et al., 1980). All these pretreatments facilitate hydrolysis and were found to be very effective.

Dilute acid hydrolysis (0.5 – 2%) under mild conditions (120 – 160°C) is an effective pretreatment for enzymatic hydrolysis of cellulose (Ingram et al., 1998). Grohman et al. (1986) and Vlasenko et al. (1997) reported that dilute acid pretreatment removes the hemicellulose fraction of lignocellulosic biomass. Pretreatment with sulfuric acid and phosphoric acid solubilize cellulose and make it amorphous. However, complete removal of lignin from lignocelluloses with a high yield of cellulose is impossible by the above methods of pretreatments (Tanaka et al., 1988). The degree of polymerization after pretreatment is assessed after enzymatic hydrolysis. Phosphoric acid provided a higher degree of subsequent enzymatic degradation by cellulase complex (Nikolov et al., 2000). The major challenge in using acids as pretreatment agent is to recover acids for reuse to make the process more cost effective.

Moderate delignification is observed when alkali is used as pretreatment agent (Andreoni et al., 1980; Detroy et al., 1980). Contradictory to this Ghosh et al. (1983) obtained high degree of delignification with alkali pretreatment. Alkaline wet oxidation pretreatment (water, sodium carbonate, oxygen, high pressure and temperature) of wheat straw as performed by Klinke et al. (2002) resulted in high cellulose recovery (96%) and high convertibility to glucose on enzymatic hydrolysis (67%). Anionima pretreatment as
applied by Detroy et al. (1980) to wheat straw resulted in moderate delignification. The same workers showed that ammonia pretreatment is highly temperature dependent. Delignification increased considerably with slight increase in temperature. Ammonia fiber explosion is another effective pretreatment process. In this method the cellulose containing material reacts with liquid ammonia at temperature from 20-90°C under 10-20 atmospheric pressure, with treatment times less than 20 minutes (Vlasenko et al., 1997). When the reaction is complete, the pressure is explosively released. The combined chemical (lignin alteration, acetate removal from hemicellulose, cellulose decrystallization) and physical (increased surface area) effects substantially enhance the enzymatic hydrolysis of the material (Holtzapple et al., 1990).

In the 'organosolv' pretreatment an organic solvent is added to the pretreatment reaction, to dissolve and remove the lignin fraction (Goncalves and Benar, 2001). In this pretreatment the internal lignin and hemicellulose bonds are broken and both fractions are solubilized, while the cellulose remains as solid (Wright, 1989). The organic fraction is removed by evaporation and recycled. Lignin precipitates without the organic fraction and can be removed by filtration or centrifugation. Thus this process separates the solid cellulose residue, a solid lignin and liquid stream containing solubilized hemicellulose in the form of xylan (Wright, 1989). Organic solvents, such as ethanol, butanol (Ghosh et al., 1983), methanol and acetone (Paszner and Cho, 1989) also bring delignification to a substantial extent. Ethylene diamine is found to be a very strong pretreatment agent (Detroy et al., 1980). Thompson et al. (1992) also carried out pretreatment with ethylene diamine, which resulted in moderate lignin and hemicellulose removal while swelling the matrix markedly.

Considerable increase in the free cellulose component is observed when lignocellulose is pretreated with phenol (Zacchi et al., 1998). Hydrogen fluoride when used as a pretreating agent attacks the polysaccharides including cellulose at circumambient temperatures to yield glycosyl fluorides which can be hydrolysed to free sugars (Lamport et al., 1980).

Biological pretreatment: Biological pretreatments utilize microorganisms that can degrade lignin. These are classified into three categories of fungi - brown rots, white rots and red rots (Ladisch and Svarczkopf, 1991). The former mainly attacks cellulose, while the white and red rots attack both lignin and cellulose. White rot fungi has been studied for delignification of lignocellulosic materials in order to improve the digestibility of wood or straw for animal feed (Valmaseda et al., 1991). Pleurotus eryngii also causes delignification of wheat straw (Valmaseda et al., 1991). The white rot fungus Phlebia radiata can be used for delignification on different lignocellulosic substrates (Vares et al., 1995). Akin et al. (1995) reported delignification of Bermuda grass by white rot fungi. The white rot fungus Phanerochaete chrysosporium produces lignin degrading enzymes (Waldner et al., 1988). The prerequisite of biological pretreatment are mild environmental conditions. Low energy requirement is the advantage of biological pretreatment. Thus the possibility of removing lignin by microbial means is a matter of consideration, but the slow rate of the process may not be compatible with large-scale industrial processes.

Hydrolysis of pretreated lignocellulosic biomass

The pretreated lignocellulosic biomass may be broken down into glucose monomers which may be further used for the production of chemicals and fuel ethanol. Hydrolysis may be done either by acids or by enzymes.
Enzymatic hydrolysis of cellulose is being given more consideration in recent years (Ballerini et al., 1994; Gregg et al., 1998).

Acid hydrolysis of pretreated lignocellulosic biomass: Dilute or concentrated acids break down the cellulose and hemicellulose polymers in the lignocellulosic biomass to form individual sugar molecules, which can be further fermented to ethanol (Chahal and Overend, 1983). Dilute acid catalysis requires a temperature of the order of 200-240°C; these were severe enough to hydrolyze lignocellulose to sugars, but the glucose is degraded into hydroxymethyl furfural, which in turn is degraded to form tars and other undesirable products (Wyman, 1994). Under the similar conditions hemicellulose fraction is converted to xylose, which in turn is degraded to form furfurals, tars and undesirable compounds. The formation of substantial quantities of furfural, tars and other byproducts cannot be avoided by known conditions required for dilute acid hydrolysis, and these degradation co-products must be sold to achieve favorable economics (Abasaeed and Mansour, 1992).

Strong acids, such as concentrated sulfuric acid and halogen acids, hydrolyse cellulose and hemicellulose at moderate temperatures with little sugar degradation (Goldstein et al., 1983). As a result, concentrated acid processes achieve the high yields of ethanol critical to economic success. On the other hand because of large quantities of sulfuric acid required and the relatively high cost of halogen acids, a substantial fraction of these acids must be recovered to achieve economic operation (Abasaeed and Mansour, 1992; Gregg et al., 1998). The main drawbacks of acid hydrolysis were found to be formation of undesirable byproducts, sugar degradation, the cost of acid and its recovery cost (Wyman, 1994).

Enzymatic hydrolysis of pretreated lignocellulosic biomass: Enzymes known as cellulases catalyze the breakdown of cellulose to glucose. As the enzymes are highly specific for the reaction that they catalyze, formation of byproducts as evidenced in acid hydrolysis are avoided, and waste treatment costs are reduced (Ballerini et al., 1994; Gregg et al., 1998). Furthermore, enzymatic reactions take place at mild conditions and achieve high yields with relatively low amounts of catalyst. Enzymes have a further advantage that they are naturally occurring compounds which are biodegradable and environmentally benign. Advances in enzyme based technology for hydrolysis for sugar production has been substantial over the years and as a result, hydrolysis costs are reduced considerably (Walker and Wilson, 1991).

Enzymatic hydrolysis is generally considered to consist of three steps; the adsorption of cellulase enzymes onto the surface of cellulose, the subsequent breakdown of cellulose to fermentable sugars through the synergistic action of the cellulase enzymes, and the desorption of the cellulase enzyme from the lignocellulosic residue into the supernatant (Ghosh and Bisaria, 1979; Lee and Fan, 1983; Ryu et al., 1984).

Sources of cellulases: A number of fungal cultures have been reported for the production of cellulolytic enzymes that can degrade cellulose to constituent sugars. Number of fungal cultures have been screened for cellulase activity (Pyc et al., 1977). The most common are Aspergillus wentii, Trichoderma sp. QM 9414, Trichoderma sp. QM 9123, Trichoderma sp. 6a, Trichoderma sp. M 4643, Trichoderma sp. M493, Trichoderma pilulifera M 7240, Trichoderma ramata M4604, Aspergillus wentii M 2852, Chaetomium globosum M 4802, Stachybotrys cylindrospora M 456, Hypocrea sp. M 2500. More cellulolytic activity is observed with shaken
cultures than with still cultures. Production of enzymes was strongly affected by wall growth obtained in fungal cultivation. Lack of homogeneity in the mycelial distribution resulted in the production of large clumps of low enzyme activity (Pyc et al., 1977). Culture supernants are taken by the same workers to assess the release of reducing sugars from cellulosic biomass.

Most of the cellulase producing fungi under investigation are related to Trichoderma reesei (viride) (Warzywoda et al., 1992; Ramos et al., 1993; Nikolov et al., 2000). Many mutants of Trichoderma reesei have been produced to enhance enzymatic production by it (Dien et al., 2000). The advantages of this strain are that it is hyperproducing (produces amounts of cellulase greater than that needed for culture growth) and is catabolite repression resistant (Warzywoda et al., 1992). Some other fungi have also been reported to be producers of cellulases, Chrysosporium pruinosum, Penicillium pusillum, Pestalotiopsis westerdijkii and Myrothecium verrucaria (Bjerre et al., 1996; Wyk van, 1999). Penicillium funiculosum is another extensively studied fungal strain for cellulase production (Mishra et al., 1984). Aspergillus niger is also studied for cellulase production (Kona et al., 2001; Sharma et al., 2001).

Cellulases enzyme system and mechanism of hydrolysis: Cellulase is a multi enzyme system that depolymerizes cellulose to give soluble sugars. Cellulases enzyme system comprises of endo-β-glucanases, exo-β-glucanases or cellobiohydralases and β-glucosidases (Walker and Wilson, 1991). Hydrolysis occurs by the synergistic action of these three enzymes.

Several mechanisms have been proposed for the hydrolysis of cellulose to glucose. According to Fan et al. (1980), there are two mechanisms for hydrolysis. One is serial mechanism, in which crystalline cellulose is first transformed into amorphous cellulose and the amorphous cellulose is converted into reducing sugars. In the parallel mechanism the crystalline and amorphous cellulose are separately converted to reducing sugars by different components of cellulase. Since the crystallinity index is steadily increased through hydrolysis so parallel mechanism is a more plausible mechanism than the serial mechanism. According to Wright (1989) the endo-β-glucanases attacks the interior of the cellulose polymer in a random fashion, exposing chain ends. As this enzyme catalyzes a solid phase reaction, it adsorbs strongly but reversibly to the crystalline cellulose. The strength of the adsorption is greater at lower temperature. This enzyme is necessary for the hydrolysis of crystalline substrates. Exo-β-glucanases removes cellobiose units (two glucose units) from the non-reducing ends of the cellulose chains. This is also a solid phase reaction and the exoglucanases absorb more strongly on both crystalline and amorphous substrates. As these enzymes continue to split off cellobiose units, the concentration of cellobiose in the solution may increase. The action of exoglucanases may be severely inhibited or stopped by the accumulation of cellobiose. The cellobiose is hydrolyzed to glucose by the action of β-glucosidases. This is a liquid phase reaction and β-glucosidases absorbs either weakly or not at all on cellulosic substrates. The action of the β-glucosidases can be slowed or halted by the accumulation of glucose in the solution. The hydrolysis of cellulosic material depends on the presence of the proper amounts of all three enzymes.

Endoglucanases introduces nicks into the linear backbone of β-1,4-linked glucose, cellobiohydralases cleaves the disaccharide units (cellobiose) from the ends, and β-glucosidases hydrolysis cellobiose and short chain soluble products (5-glucosyl residues or shorter) into monomeric glucose (Ingram et al., 1998).
Catalytic rates for these enzymes on crystalline cellulose are quite low.

Factors affecting enzymatic hydrolysis: The susceptibility of cellulosic substrates to enzymatic hydrolysis is thought to be dependent upon a number of substrate structural features including cellulose crystallinity (Sasaki et al., 1979), degree of cellulose polymerization (Puri, 1984), the lignin content (Ryu et al., 1984) and the surface area accessible to cellulases (Thompson et al., 1992).

During the course of enzymatic degradation, the hydrolysis rate drops substantially due to the increase in crystallinity index. It has been shown that the amount of enzyme adsorbed on the substrate increases drastically (four to five fold) with increasing pretreatment severity (Ooshima et al., 1990). This is due to an increase in available surface area caused by pretreatment, which would allow much more enzyme to be adsorbed. Lee and Fan (1982) also reported that the extent of enzyme adsorption increased approximately linearly as the specific surface area was increased while the effectiveness of the adsorbed enzymes in producing reducing sugars was strongly dependent on the initial crystallinity index.

Lignin remaining in the substrate after pretreatment decreases the rate of hydrolysis by cellulases and prevents hydrolysis when it accumulates as residues in the hydrolysis tank (Tanaka et al., 1988). Large amounts of cellulases are required to hydrolyse insoluble cellulose associated with lignin. Tanaka et al. (1988) devised a continuous hydrolysis process in which lignin was coagulated with chitosan in the hydrolysis tank. The coarse lignin was removed from the reactor on settlement. Whereas, the fine lignin having very slow settling velocity as compared to substrate cannot be effectively flocculated with chitosan. The separation of the coagulating agent was not easy (Tanaka et al., 1988). The enzyme was recirculated in this process after complete hydrolysis.

There are several factors, which can contribute to the low degree of cellulose conversion at high substrate concentration and low enzyme loading. These factors may include the decrease in the reactivity of cellulosic material in the course of hydrolysis (Lee and Fan, 1983), different kinds of enzyme inactivation (Gusakov et al., 1992), non specific adsorption of cellulolytic enzymes onto lignin (Chernoglazov et al., 1988) and end product inhibition (Holtzapple et al., 1990; Ramos et al., 1993; Desai and Converse, 1997).

Ramos et al. (1993) found that more the enzyme loading, the lesser the time required to completely hydrolyze the cellulose. They also analyzed the hydrolysates from the reaction, glucose was found to be the predominant sugar, with only low level of cellobiose. Zacchi et al. (1998) found that by varying the enzyme substrate ratio, hydrolysis rate could also be varied. The lower the enzyme concentration, the lower the productivity of sugar per unit time from cellulose. At lower substrate concentration complete hydrolysis of the substrate could release most of the original cellulose components back into the solution (Ramos et al., 1993).

The hydrolysis rates increase with decrease in temperature (Wright, 1989). The optimum temperature for fungal enzymatic hydrolysis was found to be 35-45°C. Desai and Converse (1997) evaluated that considerable sugar was made during the first hour of hydrolysis, from which the initial rate of hydrolysis may be determined. A number of kinetic studies have been conducted to evaluate the various factors affecting enzymatic hydrolysis (Nidetzky et al., 1993; Zilliox and Debeire, 1998).

The rate of hydrolysis starts decreasing after some time period. This decrease is
attributed to a surface deactivating mechanism or an enzyme deactivating mechanism, such as enzyme inhibition (Fan et al., 1980). Cellulase adsorption on cellulose is partially responsible for this inhibition. Cellulase activity is inhibited by cellobiose and glucose (Vlasenko et al., 1997). The supplementation of β-glucosidases during hydrolysis can reduce enzyme inhibition and increase ethanol yield (Krishna et al., 2001). The enzyme inhibition may also occur due to thermal, mechanical and chemical actions. Enzyme inhibition may also be controlled by periodic removal of product sugars. Simultaneous saccharification and fermentation of the substrate also improves enzymatic hydrolysis since it removes glucose by fermentation to ethanol, thus preventing accumulation of sugars and hence enzyme inhibition (Hinman et al., 1992). An end product inhibition was found to greatly influence the initial rate of hydrolysis, while other factors, such as increasing substrate recalcitrance, reduced the overall rate and yield of hydrolysis (Ramos et al., 1993). Several methods have been proposed to overcome the end product inhibition resulting from the rapid accumulation of sugars during hydrolysis. These include use of high concentrations of enzymes (Ishihara et al., 1991) and elimination of sugars from the hydrolysate by ultrafiltration (Tan et al., 1986; Ishihara et al., 1991).

Strategies for cost reduction of enzymatic hydrolysis of lignocellulose

Enzymatic hydrolysis of pretreated lignocellulose by using commercial cellulases is commonly recognised as a major (50 to 60% of total price) component in the cost of producing bio-fuels from biomass (Easyterbauer et al., 1992; Srinivas et al., 1995). Various strategies have been used to reduce the cost of overall process of hydrolysis. One very successful approach has been to increase the productivity of cellulase production by mutation of cellulolytic fungi and optimizing culture conditions (Srinivas et al., 1995). Two other strategies suggested as a way of decreasing the cost of the hydrolysis step involve increasing the specific activity of the cellulase enzymes or reusing the enzymes various times (Tanaka et al., 1988; Ramos et al., 1993; Gregg and Saddler, 1996). Since the enzyme production is expensive, the amount of enzyme is extremely important. If lower enzyme loading can be used and the specific activity of enzyme is unchanged, then lower hydrolysis enzyme usage would translate directly into cost reduction in enzyme production. According to Nguyen and Saddler (1991) the cost of enzymes used for saccharification of cellulosic residues is still the most expensive part of the overall bioconversion process based on enzymatic hydrolysis. Most of the workers have been using commercial cellulases, which adds to the cost of enzymatic hydrolysis (Palmqvist et al., 1996).

Another mean of reducing the cost of enzyme to the process is to recycle the enzyme. Work on cellulase recycle has shown that benefits such as the reuse of cellulases, shorter incubation times, and reduced end product inhibition can all increase the efficiency and decrease the costs associated with the cellulose hydrolysis (Tanaka et al., 1988; Nguyen and Saddler, 1991; Ramos et al., 1993). As the hydrolysis proceeds part of the adsorbed enzymes are gradually released into the reaction supernatant (Wright, 1989; Gregg and Saddler, 1996; Ingram et al., 1998) and the cellulases become distributed between the substrate and supernatant. Cellulases can be recovered either from the liquid supernatant and or solid substrate (Gregg and Saddler, 1996). The enzyme recycling strategies use enzymes free in the supernatant where hydrolysis is allowed to proceed to completion resulting in the release of the adsorbed enzyme into solution. Ramos et al. (1993) found that using this type of recycle strategy and a substrate with low lignin content, the same enzyme preparation could be recycled.
Fermentation of reducing sugars obtained after enzymatic hydrolysis of lignocellulosic biomass

Fermentation of reducing sugars obtained after enzymatic hydrolysis of lignocellulosic biomass can be fermented to ethanol. Various fungi and bacteria can be used for the fermentation process. The most commonly used yeast in the process are *Saccharomyces cerevisiae*, *Pichia stipitis* and *Candida shehatae* (Delegens et al., 1996). *Zymomonas mobilis*, a gram-negative bacterium, is considered as an alternative organism in large-scale fuel ethanol production (Gunasekaran and Raj, 1999). *Saccharomyces cerevisiae* and *Zymomonas mobilis* have better ethanol yields and productivity from hexose sugars (Srinivas et al., 1995). *Zymomonas mobilis* produced 0.4% ethanol from 4% lactose in 40 h (Gunasekaran and Raj, 1999). The only disadvantage of *Zymomonas mobilis* is its low salt tolerance. *Pichia stipitis* and *Candida shehatae* can ferment pentose as well as hexose sugars (Delegens et al., 1996; Gunasekaran and Raj, 1999). Kadam and Schmidt (1997) found that *Candida acidothermophillum* produced 80% of theoretical ethanol yield at 40°C using poplar as substrate. These microorganisms utilize the sugars to carry out their metabolic processes and give ethanol as a byproduct of metabolism. Fermentation of reducing sugars to ethanol gave a yield of 45.1% as reported by Ballerini et al. (1994). *Saccharomyces cerevisiae* is significantly inhibited by the presence of toxic compounds such as furaldehyde and hydroxymethylfurufural (Delegens et al., 1996). According to Eklund and Zacchi (1995) it is possible to attain over 85% of the theoretical ethanol yield with simultaneous saccharification and fermentation in 3 days with *Saccharomyces cerevisiae* at 37°C. Several intergeneric fusants of these species have been developed (Gunasekaran and Raj, 1999; Meinander et al., 1999). The fermentation process may be carried out in batch as well as continuous mode with appropriate control on the process (Buttlar et al., 1994). *Saccharomyces cerevisiae*-ethanol process produced 0.128 g/1 ethanol in batch. In contrast, *Zymomonas mobilis* produced 0.4% ethanol from 4% lactose in 40 h (Gunasekaran and Raj, 1999). *Kluveromyces fragilis* gave a better ethanol yield (2.5-3.5% w/v) than *Saccharomyces cerevisiae* (2.0-2.5% w/v).

CONCLUSIONS

In recent years, attention has been focussed on effective utilization of agro-byproducts to produce fuel ethanol. There is an array of pretreatment processes available that can significantly increase the rate and extent of hydrolysis. All the chemical pretreatments involve recycling or environmental costs associated with it. Process optimization of the pretreatments is required. Since enzyme cost is the major impediment to commercialization of enzymatic lignocellulose hydrolysis, the major challenge is to significantly increase the activity of cellulase. Chemical modifications of cellulase and site directed mutagenesis would contribute much to the effort to improve cellulase activity. In addition, detailed data on biomass compositions and
yields upon hydrolysis are required. Pretreatment costs, the ability to ferment sugars to ethanol and identification of markets and marketing approaches that can generate by-products may be the key determinants in the economies of obtaining ethanol from biomass.

REFERENCES