ELSEVIER

Bioresource Technology 83 (2002) 1–11

BIORESOURCE TECHNOLOGY

Review paper

Hydrolysis of lignocellulosic materials for ethanol production: a review ☆

Ye Sun, Jiayang Cheng *

Department of Biological and Agricultural Engineering, North Carolina State University, Raleigh, NC 27695-7625, USA

Received 6 November 2000; received in revised form 30 October 2001; accepted 31 October 2001

Abstract

Lignocellulosic biomass can be utilized to produce ethanol, a promising alternative energy source for the limited crude oil. There are mainly two processes involved in the conversion: hydrolysis of cellulose in the lignocellulosic biomass to produce reducing sugars, and fermentation of the sugars to ethanol. The cost of ethanol production from lignocellulosic materials is relatively high based on current technologies, and the main challenges are the low yield and high cost of the hydrolysis process. Considerable research efforts have been made to improve the hydrolysis of lignocellulosic materials. Pretreatment of lignocellulosic materials to remove lignin and hemicellulose can significantly enhance the hydrolysis of cellulose. Optimization of the cellulase enzymes and the enzyme loading can also improve the hydrolysis. Simultaneous saccharification and fermentation effectively removes glucose, which is an inhibitor to cellulase activity, thus increasing the yield and rate of cellulose hydrolysis.

Keywords: Cellulase; Cellulose; Ethanol; Fermentation; Hydrolysis; Lignocellulosic biomass; Pretreatment

1. Introduction

Energy consumption has increased steadily over the last century as the world population has grown and more countries have become industrialized. Crude oil has been the major resource to meet the increased energy demand. Campbell and Laherrere (1998) used several different techniques to estimate the current known crude oil reserves and the reserves as yet undiscovered and concluded that the decline in worldwide crude oil production will begin before 2010. They also predicted that annual global oil production would decline from the current 25 billion barrels to approximately 5 billion barrels in 2050. Because the economy in the US and many other nations depends on oil, the consequences of inadequate oil availability could be severe. Therefore, there is a great interest in exploring alternative energy sources.

Unlike fossil fuels, ethanol is a renewable energy source produced through fermentation of sugars. Ethanol is widely used as a partial gasoline replacement in the US. Fuel ethanol that is produced from corn has been used in gasohol or oxygenated fuels since the 1980s. These gasoline fuels contain up to 10% ethanol by volume. As a result, the US transportation sector now consumes about 4540 million liters of ethanol annually, about 1% of the total consumption of gasoline (Wang et al., 1999). Recently, US automobile manufacturers have announced plans to produce significant numbers of flexible-fueled vehicles that can use an ethanol blend – E85 (85% ethanol and 15% gasoline by volume) – alone or in combination with gasoline. Using ethanol-blended fuel for automobiles can significantly reduce petroleum use and exhaust greenhouse gas emission (Wang et al., 1999). Ethanol is also a safer alternative to methyl tertiary butyl ether (MTBE), the most common additive to gasoline used to provide cleaner combustion (McCarthy and Tiemann, 1998). MTBE is a toxic chemical compound and has been found to contaminate groundwater. The US Environmental Protection Agency recently announced the beginning of regulatory action to eliminate MTBE in gasoline (Browner, 2000). However, the cost of ethanol as an energy source is relatively high compared to fossil fuels. A dramatic increase in ethanol

☆ Paper No. BAE 2000-08 of the Journal Series of the Department of Biological and Agricultural Engineering, North Carolina State University, Raleigh, NC 27695-7625, USA.

* Corresponding author. Tel.: +1-919-515-6733; fax: +1-919-515-7760.

E-mail address: jay_cheng@ncsu.edu (J. Cheng).

0960-8524/02/$ - see front matter
PII: S0960-8524(01)00212-7

Table 1
The contents of cellulose, hemicellulose, and lignin in common agricultural residues and wastes[a]

Lignocellulosic materials	Cellullose (%)	Hemicellulose (%)	Lignin (%)
Hardwoods stems	40–55	24–40	18–25
Softwood stems	45–50	25–35	25–35
Nut shells	25–30	25–30	30–40
Corn cobs	45	35	15
Grasses	25–40	35–50	10–30
Paper	85–99	0	0–15
Wheat straw	30	50	15
Sorted refuse	60	20	20
Leaves	15–20	80–85	0
Cotton seed hairs	80–95	5–20	0
Newspaper	40–55	25–40	18–30
Waste papers from chemical pulps	60–70	10–20	5–10
Primary wastewater solids	8–15	NA[b]	24–29
Swine waste	6.0	28	NA[b]
Solid cattle manure	1.6–4.7	1.4–3.3	2.7–5.7
Coastal Bermuda grass	25	35.7	6.4
Switch grass	45	31.4	12.0

[a] Source: Reshamwala et al. (1995), Cheung and Anderson (1997), Boopathy (1998) and Dewes and Hünsche (1998).
[b] NA – not available.

production using the current cornstarch-based technology may not be practical because corn production for ethanol will compete for the limited agricultural land needed for food and feed production. A potential source for low-cost ethanol production is to utilize lignocellulosic materials such as crop residues, grasses, sawdust, wood chips, and solid animal waste.

Extensive research has been completed on conversion of lignocellulosic materials to ethanol in the last two decades (Dale et al., 1984; Wright, 1998; Azzam, 1989; Cadoche and López, 1989; Reshamwala et al., 1995; Bjerre et al., 1996; Duff and Murray, 1996). The conversion includes two processes: hydrolysis of cellulose in the lignocellulosic materials to fermentable reducing sugars, and fermentation of the sugars to ethanol. The hydrolysis is usually catalyzed by cellulase enzymes, and the fermentation is carried out by yeasts or bacteria. The factors that have been identified to affect the hydrolysis of cellulose include porosity (accessible surface area) of the waste materials, cellulose fiber crystallinity, and lignin and hemicellulose content (McMillan, 1994). The presence of lignin and hemicellulose makes the access of cellulase enzymes to cellulose difficult, thus reducing the efficiency of the hydrolysis. The contents of cellulose, hemicellulose, and lignin in common agricultural residues are listed in Table 1. Removal of lignin and hemicellulose, reduction of cellulose crystallinity, and increase of porosity in pretreatment processes can significantly improve the hydrolysis (McMillan, 1994).

2. Pretreatment of lignocellulosic materials

The effect of pretreatment of lignocellulosic materials has been recognized for a long time (McMillan, 1994). The purpose of the pretreatment is to remove lignin and hemicellulose, reduce cellulose crystallinity, and increase the porosity of the materials. Pretreatment must meet the following requirements: (1) improve the formation of sugars or the ability to subsequently form sugars by enzymatic hydrolysis; (2) avoid the degradation or loss of carbohydrate; (3) avoid the formation of byproducts inhibitory to the subsequent hydrolysis and fermentation processes; and (4) be cost-effective. Physical, physico-chemical, chemical, and biological processes have been used for pretreatment of lignocellulosic materials.

2.1. Physical pretreatment

2.1.1. Mechanical comminution

Waste materials can be comminuted by a combination of chipping, grinding and milling to reduce cellulose crystallinity. The size of the materials is usually 10–30 mm after chipping and 0.2–2 mm after milling or grinding. Vibratory ball milling has been found to be more effective in breaking down the cellulose crystallinity of spruce and aspen chips and improving the digestibility of the biomass than ordinary ball milling (Millet et al., 1976). The power requirement of mechanical comminution of agricultural materials depends on the final particle size and the waste biomass characteristics (Cadoche and López, 1989). A comparison is shown in Table 2.

2.1.2. Pyrolysis

Pyrolysis has also been used for pretreatment of lignocellulosic materials. When the materials are treated at temperatures greater than 300 °C, cellulose rapidly decomposes to produce gaseous products and residual char (Kilzer and Broido, 1965; Shafizadeh and Brad-

Table 2
Energy requirement of mechanical comminution of agricultural lignocellulosic materials with different size reduction (Cadoche and López, 1989)

Lignocellulosic materials	Final size (mm)	Energy consumption (kWh/ton)	
		Knife mill	Hammer mill
Hardwood	1.60	130	130
	2.54	80	120
	3.2	50	115
	6.35	25	95
Straw	1.60	7.5	42
	2.54	6.4	29
Corn stover	1.60	NA[a]	14
	3.20	20	9.6
	6.35	15	NA[a]
	9.5	3.2	NA[a]

[a] NA – not available.

bury, 1979). The decomposition is much slower and less volatile products are formed at lower temperatures. Mild acid hydrolysis (1 N H_2SO_4, 97 °C, 2.5 h) of the residues from pyrolysis pretreatment has resulted in 80–85% conversion of cellulose to reducing sugars with more than 50% glucose (Fan et al., 1987). The process can be enhanced with the presence of oxygen (Shafizadeh and Bradbury, 1979). When zinc chloride or sodium carbonate is added as a catalyst, the decomposition of pure cellulose can occur at a lower temperature (Shafizadeh and Lai, 1975).

2.2. Physico-chemical pretreatment

2.2.1. Steam explosion (autohydrolysis):

Steam explosion is the most commonly used method for pretreatment of lignocellulosic materials (McMillan, 1994). In this method, chipped biomass is treated with high-pressure saturated steam and then the pressure is swiftly reduced, which makes the materials undergo an explosive decompression. Steam explosion is typically initiated at a temperature of 160–260 °C (corresponding pressure 0.69–4.83 MPa) for several seconds to a few minutes before the material is exposed to atmospheric pressure. The process causes hemicellulose degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis. Ninety percent efficiency of enzymatic hydrolysis has been achieved in 24 h for poplar chips pretreated by steam explosion, compared to only 15% hydrolysis of untreated chips (Grous et al., 1986). The factors that affect steam explosion pretreatment are residence time, temperature, chip size and moisture content (Duff and Murray, 1996). Optimal hemicellulose solubilization and hydrolysis can be achieved by either high temperature and short residence time (270 °C, 1 min) or lower temperature and longer residence time (190 °C, 10 min) (Duff and Murray, 1996). Recent studies indicate that lower temperature and longer residence time are more favorable (Wright, 1998).

Addition of H_2SO_4 (or SO_2) or CO_2 in steam explosion can effectively improve enzymatic hydrolysis, decrease the production of inhibitory compounds, and lead to more complete removal of hemicellulose (Morjanoff and Gray, 1987). The optimal conditions of steam explosion pretreatment of sugarcane bagasse have been found to be as following: 220 °C; 30 s residence time; water-to-solids ratio, 2; and 1% H_2SO_4 (Morjanoff and Gray, 1987). Sugar production was 65.1 g sugar/100 g starting bagasse after steam explosion pretreatment.

The advantages of steam explosion pretreatment include the low energy requirement compared to mechanical comminution and no recycling or environmental costs. The conventional mechanical methods require 70% more energy than steam explosion to achieve the same size reduction (Holtzapple et al., 1989). Steam explosion is recognized as one of the most cost-effective pretreatment processes for hardwoods and agricultural residues, but it is less effective for softwoods (Clark and Mackie, 1987). Limitations of steam explosion include destruction of a portion of the xylan fraction, incomplete disruption of the lignin–carbohydrate matrix, and generation of compounds that may be inhibitory to microorganisms used in downstream processes (Mackie et al., 1985). Because of the formation of degradation products that are inhibitory to microbial growth, enzymatic hydrolysis, and fermentation, pretreated biomass needs to be washed by water to remove the inhibitory materials along with water-soluble hemicellulose (McMillan, 1994). The water wash decreases the overall saccharification yields due to the removal of soluble sugars, such as those generated by hydrolysis of hemicellulose. Typically, 20–25% of the initial dry matter is removed by water wash (Mes-Hartree et al., 1988).

2.2.2. Ammonia fiber explosion (AFEX)

AFEX is another type of physico-chemical pretreatment in which lignocellulosic materials are exposed to liquid ammonia at high temperature and pressure for a

period of time, and then the pressure is swiftly reduced. The concept of AFEX is similar to steam explosion. In a typical AFEX process, the dosage of liquid ammonia is 1–2 kg ammonia/kg dry biomass, temperature 90 °C, and residence time 30 min. AFEX pretreatment can significantly improve the saccharification rates of various herbaceous crops and grasses. It can be used for the pretreatment of many lignocellulosic materials including alfalfa, wheat straw, wheat chaff (Mes-Hartree et al., 1988), barley straw, corn stover, rice straw (Vlasenko et al., 1997), municipal solid waste, softwood newspaper, kenaf newspaper (Holtzapple et al., 1992a), coastal Bermuda grass, switchgrass (Reshamwala et al., 1995), aspen chips (Tengerdy and Nagy, 1988), and bagasse (Holtzapple et al., 1991). The AFEX pretreatment does not significantly solubilize hemicellulose compared to acid pretreatment (to be discussed in the following section) and acid-catalyzed steam explosion (Mes-Hartree et al., 1988; Vlasenko et al., 1997). Mes-Hartree et al. (1988) compared the steam and ammonia pretreatment for enzymatic hydrolysis of aspenwood, wheat straw, wheat chaff, and alfalfa stems, and found that steam explosion solubilized the hemicellulose, while AFEX did not. The composition of the materials after AFEX pretreatment was essentially the same as the original materials. Over 90% hydrolysis of cellulose and hemicellulose has been obtained after AFEX pretreatment of Bermuda grass (approximately 5% lignin) and bagasse (15% lignin) (Holtzapple et al., 1991). However, the AFEX process was not very effective for the biomass with high lignin content such as newspaper (18–30% lignin) and aspen chips (25% lignin). Hydrolysis yield of AFEX-pretreated newspaper and aspen chips was reported as only 40% and below 50%, respectively (McMillan, 1994).

To reduce the cost and protect the environment, ammonia must be recycled after the pretreatment. In an ammonia recovery process, a superheated ammonia vapor with a temperature up to 200 °C was used to vaporize and strip the residual ammonia in the pretreated biomass and the evaporated ammonia was then withdrawn from the system by a pressure controller for recovery (Holtzapple et al., 1992b). The ammonia pretreatment does not produce inhibitors for the downstream biological processes, so water wash is not necessary (Dale et al., 1984; Mes-Hartree et al., 1988). AFEX pretreatment does not require small particle size for efficacy (Holtzapple et al., 1990).

2.2.3. CO_2 explosion

Similar to steam and ammonia explosion pretreatment, CO_2 explosion is also used for pretreatment of lignocellulosic materials. It was hypothesized that CO_2 would form carbonic acid and increase the hydrolysis rate. Dale and Moreira (1982) used this method to pretreat alfalfa (4 kg CO_2/kg fiber at the pressure of 5.62 MPa) and obtained 75% of the theoretical glucose released during 24 h of the enzymatic hydrolysis. The yields were relatively low compared to steam or ammonia explosion pretreatment, but high compared to the enzymatic hydrolysis without pretreatment. Zheng et al. (1998) compared CO_2 explosion with steam and ammonia explosion for pretreatment of recycled paper mix, sugarcane bagasse, and repulping waste of recycled paper, and found that CO_2 explosion was more cost-effective than ammonia explosion and did not cause the formation of inhibitory compounds that could occur in steam explosion.

2.3. Chemical pretreatment

2.3.1. Ozonolysis:

Ozone can be used to degrade lignin and hemicellulose in many lignocellulosic materials such as wheat straw (Ben-Ghedalia and Miron, 1981), bagasse, green hay, peanut, pine (Neely, 1984), cotton straw (Ben-Ghedalia and Shefet, 1983), and poplar sawdust (Vidal and Molinier, 1988). The degradation was essentially limited to lignin and hemicellulose was slightly attacked, but cellulose was hardly affected. The rate of enzymatic hydrolysis increased by a factor of 5 following 60% removal of the lignin from wheat straw in ozone pretreatment (Vidal and Molinier, 1988). Enzymatic hydrolysis yield increased from 0% to 57% as the percentage of lignin decreased from 29% to 8% after ozonolysis pretreatment of poplar sawdust (Vidal and Molinier, 1988). Ozonolysis pretreatment has the following advantages: (1) it effectively removes lignin; (2) it does not produce toxic residues for the downstream processes; and (3) the reactions are carried out at room temperature and pressure (Vidal and Molinier, 1988). However, a large amount of ozone is required, making the process expensive.

2.3.2. Acid hydrolysis

Concentrated acids such as H_2SO_4 and HCl have been used to treat lignocellulosic materials. Although they are powerful agents for cellulose hydrolysis, concentrated acids are toxic, corrosive and hazardous and require reactors that are resistant to corrosion. In addition, the concentrated acid must be recovered after hydrolysis to make the process economically feasible (Sivers and Zacchi, 1995).

Dilute acid hydrolysis has been successfully developed for pretreatment of lignocellulosic materials. The dilute sulfuric acid pretreatment can achieve high reaction rates and significantly improve cellulose hydrolysis (Esteghlalian et al., 1997). At moderate temperature, direct saccharification suffered from low yields because of sugar decomposition. High temperature in dilute acid treatment is favorable for cellulose hydrolysis (McMillan, 1994). Recently developed dilute acid hydrolysis

processes use less severe conditions and achieve high xylan to xylose conversion yields. Achieving high xylan to xylose conversion yields is necessary to achieve favorable overall process economics because xylan accounts for up to a third of the total carbohydrate in many lignocellulosic materials (Hinman et al., 1992). There are primarily two types of dilute acid pretreatment processes: high temperature (T greater than 160 °C), continuous-flow process for low solids loading (5–10% [weight of substrate/weight of reaction mixture]) (Brennan et al., 1986; Converse et al., 1989), and low temperature (T less than 160 °C), batch process for high solids loading (10–40%) (Cahela et al., 1983; Esteghlalian et al., 1997). Although dilute acid pretreatment can significantly improve the cellulose hydrolysis, its cost is usually higher than some physico-chemical pretreatment processes such as steam explosion or AFEX. A neutralization of pH is necessary for the downstream enzymatic hydrolysis or fermentation processes.

2.3.3. Alkaline hydrolysis

Some bases can also be used for pretreatment of lignocellulosic materials and the effect of alkaline pretreatment depends on the lignin content of the materials (Fan et al., 1987; McMillan, 1994). The mechanism of alkaline hydrolysis is believed to be saponification of intermolecular ester bonds crosslinking xylan hemicelluloses and other components, for example, lignin and other hemicellulose. The porosity of the lignocellulosic materials increases with the removal of the crosslinks (Tarkow and Feist, 1969). Dilute NaOH treatment of lignocellulosic materials caused swelling, leading to an increase in internal surface area, a decrease in the degree of polymerization, a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure (Fan et al., 1987). The digestibility of NaOH-treated hardwood increased from 14% to 55% with the decrease of lignin content from 24–55% to 20%. However, no effect of dilute NaOH pretreatment was observed for softwoods with lignin content greater than 26% (Millet et al., 1976). Dilute NaOH pretreatment was also effective for the hydrolysis of straws with relatively low lignin content of 10–18% (Bjerre et al., 1996). Chosdu et al. (1993) used the combination of irradiation and 2% NaOH for pretreatment of corn stalk, cassava bark and peanut husk. The glucose yield of corn stalk was 20% in untreated samples compared to 43% after treatment with electron beam irradiation at the dose of 500 kGy and 2% NaOH, but the glucose yields of cassava bark and peanut husk were only 3.5% and 2.5%, respectively.

Ammonia was also used for the pretreatment to remove lignin. Iyer et al. (1996) described an ammonia recycled percolation process (temperature, 170 °C; ammonia concentration, 2.5–20%; reaction time, 1 h) for the pretreatment of corn cobs/stover mixture and switchgrass. The efficiency of delignification was 60–80% for corn cobs and 65–85% for switchgrass.

2.3.4. Oxidative delignification

Lignin biodegradation could be catalyzed by the peroxidase enzyme with the presence of H_2O_2 (Azzam, 1989). The pretreatment of cane bagasse with hydrogen peroxide greatly enhanced its susceptibility to enzymatic hydrolysis. About 50% lignin and most hemicellulose were solubilized by 2% H_2O_2 at 30 °C within 8 h, and 95% efficiency of glucose production from cellulose was achieved in the subsequent saccharification by cellulase at 45 °C for 24 h (Azzam, 1989). Bjerre et al. (1996) used wet oxidation and alkaline hydrolysis of wheat straw (20 g straw/l, 170 °C, 5–10 min), and achieved 85% conversion yield of cellulose to glucose.

2.3.5. Organosolv process

In the organosolv process, an organic or aqueous organic solvent mixture with inorganic acid catalysts (HCl or H_2SO_4) is used to break the internal lignin and hemicellulose bonds. The organic solvents used in the process include methanol, ethanol, acetone, ethylene glycol, triethylene glycol and tetrahydrofurfuryl alcohol (Chum et al., 1988; Thring et al., 1990). Organic acids such as oxalic, acetylsalicylic and salicylic acid can also be used as catalysts in the organosolv process (Sarkanen, 1980). At high temperatures (above 185 °C), the addition of catalyst was unnecessary for satisfactory delignification (Sarkanen, 1980; Aziz and Sarkanen, 1989). Usually, a high yield of xylose can be obtained with the addition of acid. Solvents used in the process need to be drained from the reactor, evaporated, condensed and recycled to reduce the cost. Removal of solvents from the system is necessary because the solvents may be inhibitory to the growth of organisms, enzymatic hydrolysis, and fermentation.

2.4. Biological pretreatment

In biological pretreatment processes, microorganisms such as brown-, white- and soft-rot fungi are used to degrade lignin and hemicellulose in waste materials (Schurz, 1978). Brown rots mainly attack cellulose, while white and soft rots attack both cellulose and lignin. White-rot fungi are the most effective basidiomycetes for biological pretreatment of lignocellulosic materials (Fan et al., 1987). Hatakka (1983) studied the pretreatment of wheat straw by 19 white-rot fungi and found that 35% of the straw was converted to reducing sugars by *Pleurotus ostreatus* in five weeks. Similar conversion was obtained in the pretreatment by *Phanerochaete sordida* 37 and *Pycnoporus cinnabarinus* 115 in four weeks. In order to prevent the loss of cellulose, a cellulase-less mutant of *Sporotrichum pulverulentum* was developed for the degradation of lignin in wood chips

(Ander and Eriksson, 1977). Akin et al. (1995) also reported the delignification of Bermuda grass by white-rot fungi. The biodegradation of Bermuda grass stems was improved by 29–32% using *Ceriporiopsis subvermispora* and 63–77% using *Cyathus stercoreus* after 6 weeks.

The white-rot fungus *P. chrysosporium* produces lignin-degrading enzymes, lignin peroxidases and manganese-dependent peroxidases, during secondary metabolism in response to carbon or nitrogen limitation (Boominathan and Reddy, 1992). Both enzymes have been found in the extracellular filtrates of many white-rot fungi for the degradation of wood cell walls (Kirk and Farrell, 1987; Waldner et al., 1988). Other enzymes including polyphenol oxidases, laccases, H_2O_2 producing enzymes and quinone-reducing enzymes can also degrade lignin (Blanchette, 1991). The advantages of biological pretreatment include low energy requirement and mild environmental conditions. However, the rate of hydrolysis in most biological pretreatment processes is very low.

3. Enzymatic hydrolysis of cellulose

Enzymatic hydrolysis of cellulose is carried out by cellulase enzymes which are highly specific (Béguin and Aubert, 1994). The products of the hydrolysis are usually reducing sugars including glucose. Utility cost of enzymatic hydrolysis is low compared to acid or alkaline hydrolysis because enzyme hydrolysis is usually conducted at mild conditions (pH 4.8 and temperature 45–50 °C) and does not have a corrosion problem (Duff and Murray, 1996). Both bacteria and fungi can produce cellulases for the hydrolysis of lignocellulosic materials. These microorganisms can be aerobic or anaerobic, mesophilic or thermophilic. Bacteria belonging to *Clostridium*, *Cellulomonas*, *Bacillus*, *Thermomonospora*, *Ruminococcus*, *Bacteriodes*, *Erwinia*, *Acetovibrio*, *Microbispora*, and *Streptomyces* can produce cellulases (Bisaria, 1991). *Cellulomonas fimi* and *Thermomonospora fusca* have been extensively studied for cellulase production. Although many cellulolytic bacteria, particularly the cellulolytic anaerobes such as *Clostridium thermocellum* and *Bacteroides cellulosolvens* produce cellulases with high specific activity, they do not produce high enzyme titres (Duff and Murray, 1996). Because the anaerobes have a very low growth rate and require anaerobic growth conditions, most research for commercial cellulase production has focused on fungi (Duff and Murray, 1996).

Fungi that have been reported to produce cellulases include *Sclerotium rolfsii*, *P. chrysosporium* and species of *Trichoderma*, *Aspergillus*, *Schizophyllum* and *Penicillium* (Sternberg, 1976; Fan et al., 1987; Duff and Murray, 1996). Of all these fungal genera, *Trichoderma* has been most extensively studied for cellulase production (Sternberg, 1976).

Cellulases are usually a mixture of several enzymes. At least three major groups of cellulases are involved in the hydrolysis process: (1) endoglucanase (EG, endo-1,4-D-glucanohydrolase, or EC 3.2.1.4.) which attacks regions of low crystallinity in the cellulose fiber, creating free chain-ends; (2) exoglucanase or cellobiohydrolase (CBH, 1,4-β-D-glucan cellobiohydrolase, or EC 3.2.1.91.) which degrades the molecule further by removing cellobiose units from the free chain-ends; (3) β-glucosidase (EC 3.2.1.21) which hydrolyzes cellobiose to produce glucose (Coughlan and Ljungdahl, 1988). In addition to the three major groups of cellulase enzymes, there are also a number of ancillary enzymes that attack hemicellulose, such as glucuronidase, acetylesterase, xylanase, β-xylosidase, galactomannanase and glucomannanase (Duff and Murray, 1996). During the enzymatic hydrolysis, cellulose is degraded by the cellulases to reducing sugars that can be fermented by yeasts or bacteria to ethanol.

4. Improving enzymatic hydrolysis

The factors that affect the enzymatic hydrolysis of cellulose include substrates, cellulase activity, and reaction conditions (temperature, pH, as well as other parameters). To improve the yield and rate of the enzymatic hydrolysis, research has focused on optimizing the hydrolysis process and enhancing cellulase activity (Cantwell et al., 1988; Durand et al., 1988; Orpin, 1988).

4.1. Substrates

Substrate concentration is one of the main factors that affects the yield and initial rate of enzymatic hydrolysis of cellulose. At low substrate levels, an increase of substrate concentration normally results in an increase of the yield and reaction rate of the hydrolysis (Cheung and Anderson, 1997). However, high substrate concentration can cause substrate inhibition, which substantially lowers the rate of the hydrolysis, and the extent of substrate inhibition depends on the ratio of total substrate to total enzyme (Huang and Penner, 1991; Penner and Liaw, 1994). Huang and Penner (1991) found that the substrate inhibition occurred when the ratio of the microcrystalline substrate Avicel pH 101 to the cellulase from *Trichoderma reesei* (grams of cellulose/FPU [filter paper unit, defined as a micromole of reducing sugar as glucose produced by 1 ml of enzyme per minute] of enzyme) was greater than 5. Penner and Liaw (1994) reported that the optimum substrate to enzyme ratio was 1.25 g of the microcrystalline substrate Avicel pH 105 per FPU of the cellulase from *T. reesei*. The susceptibility of cellulosic substrates to cellulases depends on the structural features of the substrate in-

cluding cellulose crystallinity, degree of cellulose polymerization, surface area, and content of lignin. Lignin interferes with hydrolysis by blocking access of cellulases to cellulose and by irreversibly binding hydrolytic enzymes. Therefore, removal of lignin can dramatically increase the hydrolysis rate (McMillan, 1994).

4.2. Cellulase

Increasing the dosage of cellulases in the process, to a certain extent, can enhance the yield and rate of the hydrolysis, but would significantly increase the cost of the process. Cellulase dosage of 10 FPU/g cellulose is often used in laboratory studies because it provides a hydrolysis profile with high levels of glucose yield in a reasonable time (48–72 h) at a reasonable enzyme cost (Gregg and Saddler, 1996). Cellulase enzyme loadings in hydrolysis vary from 7 to 33 FPU/g substrate, depending on the type and concentration of substrates.

Enzymatic hydrolysis of cellulose consists of three steps: adsorption of cellulase enzymes onto the surface of the cellulose, the biodegradation of cellulose to fermentable sugars, and desorption of cellulase. Cellulase activity decreases during the hydrolysis. The irreversible adsorption of cellulase on cellulose is partially responsible for this deactivation (Converse et al., 1988). Addition of surfactants during hydrolysis is capable of modifying the cellulose surface property and minimizing the irreversible binding of cellulase on cellulose. The surfactants used in the enzymatic hydrolysis include nonionic Tween 20, 80 (Wu and Ju, 1998), polyoxyethylene glycol (Park et al., 1992), Tween 81, Emulgen 147, amphoteric Anhitole 20BS, cationic Q-86W (Ooshima et al., 1986), sophorolipid, rhamnolipid, and bacitracin (Helle et al., 1993). Inhibitory effects have been observed with cationic Q-86W at high concentration and anionic surfactant Neopelex F-25 (Ooshima et al., 1986). Nonionic surfactants are therefore believed to be more suitable for enhancing the cellulose hydrolysis. The rate of enzymatic hydrolysis was improved by 33% using Tween 80 as a surfactant in the hydrolysis of newspaper (Castanon and Wilke, 1981). Wu and Ju (1998) tested Pluronic F68 and F88 (BASF) and Tween 20 and 80 for enhancing the enzymatic hydrolysis of pretreated newsprint (Table 3). The cellulose conversion with 2% (w/v) F68 and 2 g/l cellulase reached 52%, compared to 48% conversion with 10 g/l cellulase in a surfactant-free system. However, Tween 20 was highly inhibitory to *D. clausenii* even at a low concentration of 0.1%.

Use of a cellulase mixture from different microorganisms or a mixture of cellulases and other enzymes in the hydrolysis of cellulosic materials has been extensively studied (Beldman et al., 1988; Excoffier et al., 1991; Xin et al., 1993). The addition of β-glucosidases into the *T. reesei* cellulases system achieved better saccharification than the system without β-glucosidases (Excoffier et al., 1991; Xin et al., 1993). β-Glucosidases hydrolyze the cellobiose which is an inhibitor of cellulase activity. A mixture of hemicellulases or pectinases with cellulases exhibited a significant increase in the extent of cellulose conversion (Ghose and Bisaria, 1979; Beldman et al., 1984). A cellulose conversion yield of 90% was achieved in the enzymatic saccharification of 8% alkali-treated sugar-cane bagasse when a mixture of cellulases (dose, 1.0 FPU/g substrate) from *A. ustus* and *T. viride* was used (Mononmani and Sreekantiah, 1987). A nearly complete saccharification of steam-explosion pretreated *Eucalyptus viminalis* chips (substrate concentration of 6% and enzyme loading of 10 FPU/g cellulose) was obtained using a cellulase mixture of commercial Celluclast and Novozym preparations (Ramos et al., 1993). Baker et al. (1994) found a new thermostable endoglucanase, *Acidothermus cellulolyticus* E1, and another bacterial endoglucanase, *T. fusca* E5 that exhibited striking synergism with *T. reesei* CBH1 in the saccharification of microcrystalline cellulose.

Cellulases can be recovered from the liquid supernatant or the solid residues and most recycled cellulases are from the liquid supernatant. Enzyme recycling can effectively increase the rate and yield of the hydrolysis and lower the enzyme cost (Mes-Hartree et al., 1987). Ramos et al. (1993) reported that the enzyme mixture of the

Table 3
Effects of different surfactants on hydrolysis of cellulose newsprint (Wu and Ju, 1998)[a]

Surfactants		Cellulose conversion (%)			
Type	Concentration (%)	10 h	15 h	44.5 h	123.5 h
Control	0	11.9	17.5	20.7	27.5
Tween 20	0.5	14.1	21.6	27.2	43.6
	2.0	16.0	24.7	32.1	46.8
Tween 80	0.5	14.5	22.0	28.0	43.1
	2.0	14.2	24.7	29.6	43.6
F68	0.5	17.3	26.7	34.4	51.0
	2.0	16.6	27.5	34.0	56.5
F88	0.5	15.4	24.7	32.8	47.8
	2.0	14.5	24.6	33.9	51.2

[a] Enzyme loading: 2 g/l; solid substrate concentration: 10%.

commercial Celluclast and Novozym preparation was successfully recycled for five consecutive steps with an elapsed time of 48 h between each recycling step. The efficiency of cellulose hydrolysis decreased gradually with each recycling step.

4.3. End-product inhibition of cellulase activity

Cellulase activity is inhibited by cellobiose and to a lesser extent by glucose. Several methods have been developed to reduce the inhibition, including the use of high concentrations of enzymes, the supplementation of β-glucosidases during hydrolysis, and the removal of sugars during hydrolysis by ultrafiltration or simultaneous saccharification and fermentation (SSF).

The SSF process has been extensively studied to reduce the inhibition of end products of hydrolysis (Takagi et al., 1977; Blotkamp et al., 1978; Szczodrak and Targonski, 1989; Saxena et al., 1992; Philippidis et al., 1993; Zheng et al., 1998). In the process, reducing sugars produced in cellulose hydrolysis or saccharification are simultaneously fermented to ethanol, which greatly reduces the product inhibition to the hydrolysis.

The microorganisms used in the SSF are usually the fungus *T. reesei* and yeast *S. cerevisiae*. The optimal temperature for SSF is around 38 °C, which is a compromise between the optimal temperatures for hydrolysis (45–50 °C) and fermentation (30 °C) (Philippidis, 1996). Hydrolysis is usually the rate-limiting process in SSF (Philippidis and Smith, 1995). Thermotolerant yeasts and bacteria have been used in the SSF to raise the temperature close to the optimal hydrolysis temperature. Ballesteros et al. (1991) have identified *Kluyveromyces marxianus* and *K. fragilis* that have the highest ethanol productivity at 42 °C from 27 yeast strains. *K. marxianus* has an ethanol yield of 0.5 g/g cellulose in 78 h using Solka Floc 200 as substrate at 42 °C. Kadam and Schmidt (1997) found that a thermotolerant yeast, *Candida acidothermophilum*, produced 80% of the theoretical ethanol yield at 40 °C using dilute acid pretreated poplar as substrate. *Kluyveromyces* strains have been found to be more thermotolerant than *Candida* and *Saccharomyces* strains (Hacking et al., 1984).

Compared to the two-stage hydrolysis–fermentation process, SSF has the following advantages: (1) increase of hydrolysis rate by conversion of sugars that inhibit the cellulase activity; (2) lower enzyme requirement; (3) higher product yields; (4) lower requirements for sterile conditions since glucose is removed immediately and ethanol is produced; (5) shorter process time; and (6) less reactor volume because a single reactor is used. However, ethanol may also exhibit inhibition to the cellulase activity in the SSF process. Wu and Lee (1997) found that cellulase lost 9%, 36% and 64% of its original activity at ethanol concentrations of 9, 35 and 60 g/l, respectively, at 38 °C during SSF process. The disadvantages which need to be considered for SSF include: (1) incompatible temperature of hydrolysis and fermentation; (2) ethanol tolerance of microbes; and (3) inhibition of enzymes by ethanol.

5. Future prospects

The US fuel ethanol industry produced more than 6.2 billion liters of ethanol in 2000, most of which was produced from corn (MacDonald et al., 2001). However, an increase of ethanol production from corn will compete for the limited land against corn-based food and feed production. The price of corn was estimated to increase by $1.20–2.00/ton for every 2.5 million tonnes of corn used to make ethanol (Elander and Putsche, 1996). On the other hand, there is a huge amount of low-value or waste lignocellulosic materials that are currently burned or wasted. Utilization of lignocellulosic materials can replace the equivalent of 40% of the gasoline in the US market (Wheals et al., 1999). Using lignocellulosic materials such as agricultural residues, grasses, forestry wastes and other low-cost biomass can significantly reduce the cost of raw materials (compared to corn) for ethanol production.

A reduction of the cost of ethanol production can be achieved by reducing the cost of either the raw materials or the cellulase enzymes. It was predicted that the use of genetically engineered raw materials with higher carbohydrate content combined with the improvement of conversion technology could reduce the cost of ethanol by $0.11 per liter over the next 10 years (Wooley et al., 1999). Reducing the cost of cellulase enzyme production is a key issue in the enzymatic hydrolysis of lignocellulosic materials. Genetic techniques have been used to clone the cellulase coding sequences into bacteria, yeasts, fungi and plants to create new cellulase production systems with possible improvement of enzyme production and activity. Wood et al. (1997) reported the expression of recombinant endoglucanase genes from *Erwinia chrysanthemi* P86021 in *Escherichia coli* KO11 and the recombinant system produced 3200 IU endoglucanase/l fermentation broth (IU, international unit, defined as a micromole of reducing sugar as glucose released per minute using carboxymethyl cellulose as substrate). The thermostable endoglucanase E1 from *Acidothermus cellulolyticus* was expressed in *Arabidopsis thaliana* leaves (Ziegler et al., 2000), potato (Dai et al., 2000), and tobacco (Hooker et al., 2001).

Using genetically engineered microorganisms that can convert xylose and/or pentose to ethanol can greatly improve ethanol production efficiency and reduce the cost of the production. The constructed operons encoding xylose assimilation and pentose phosphate pathway enzymes were transformed into the bacterium

Zymomonas mobilis for the effective fermentation of xylose to produce ethanol (Zhang et al., 1995). The recombinant strain of *E. coli* with the genes from *Z. mobilis* for the conversion of pyruvate into ethanol has been reported by Dien et al. (2000). The recombinant plasmids with xylose reductase and xylitol dehydrogenase genes from *Pichia stipitis* and xylulokinase gene from *Saccharomyces cerevisiae* have been transformed into *Saccharomyce spp.* for the co-fermentation of glucose and xylose (Ho et al., 1998).

Although bioethanol production has been greatly improved by new technologies, there are still challenges that need further investigations. These challenges include maintaining a stable performance of the genetically engineered yeasts in commercial scale fermentation operations (Dipardo, 2000), developing more efficient pretreatment technologies for lignocellulosic biomass, and integrating the optimal components into economic ethanol production systems.

References

Akin, D.E., Rigsby, L.L., Sethuraman, A., Morrison, W.H.-III., Gamble, G.R., Eriksson, K.E.L., 1995. Alterations in structure, chemistry, and biodegradability of grass lignocellulose treated with the white rot fungi Ceriporiopsis subvermispora ad Cyathus stercoreus. Appl. Environ. Microbiol. 61, 1591–1598.

Ander, P., Eriksson, K.-E., 1977. Selective degradation of wood components by white-rot fungi. Physiol. Plant. 41, 239–248.

Aziz, S., Sarkanen, K., 1989. Organosolv pulping – a review. Tappi. J. 72, 169–175.

Azzam, A.M., 1989. Pretreatment of cane bagasse with alkaline hydrogen peroxide for enzymatic hydrolysis of cellulose and ethanol fermentation. J. Environ. Sci. Health. B. 24 (4), 421–433.

Baker, J.O., Adney, W.S., Nieves, R.A., 1994. A new thermostable endoglucanase, *Acidothermus cellulolyticus* E1: synergism with *Trichoderma reesei* CBH1 and comparison to *Thermomonospora fusca* E5. Appl. Biochem. Biotechnol. 45/46, 245–256.

Ballesteros, I., Ballesteros, M., Cabanas, A., Carrasco, J., Martin, C., Negro, M.J., Saez, F., Saez, R., 1991. Selection of thermotolerant yeasts for simultaneous saccharification and fermentation (SSF) of cellulose to ethanol. Appl. Biochem. Biotechnol. 28/29, 307–315.

Béguin, P., Aubert, J.-P., 1994. The biological degradation of cellulose. FEMS Microbiol. Rev. 13, 25–58.

Beldman, G., Rombouts, F.M., Voragen, A.G.J., Pilnik, W., 1984. Application of cellulase and pectinase from fungal origin for the liquefaction and saccharification of biomass. Enzyme Microb. Technol. 6, 503–507.

Beldman, G., Voragen, A.G.J., Rombouts, F.M., Pilnik, W., 1988. Synergism in cellulose hydrolysis by endoglucanases and exoglucanases purified from *Trichoderma viride*. Biotechnol. Bioeng. 31, 173–178.

Ben-Ghedalia, D., Miron, J., 1981. The effect of combined chemical and enzyme treatment on the saccharification and in vitro digestion rate of wheat straw. Biotechnol. Bioeng. 23, 823–831.

Ben-Ghedalia, D., Shefet, G., 1983. Chemical treatments for increasing the digestibility of cotton straw. J. Agric. Sci. 100, 393–400.

Bisaria, V.S., 1991. Bioprocessing of Agro-residues to glucose and chemicals. In: Martin, A.M. (Ed.), Bioconversion of Waste Materials to Industrial Products. Elsevier, London, pp. 210–213.

Bjerre, A.B., Olesen, A.B., Fernqvist, T., 1996. Pretreatment of wheat straw using combined wet oxidation and alkaline hydrolysis resulting in convertible cellulose and hemicellulose. Biotechnol. Bioeng. 49, 568–577.

Blanchette, R.A., 1991. Delignification by wood-decay fungi. Annu. Rev. Phytopathol. 29, 381–398.

Blotkamp, P.J., Takagi, M., Pemberton, M.S., 1978. Enzymatic hydrolysis of cellulose and simultaneous fermentation to alcohol. Biochemical engineering: renewable sources of energy and chemical feedstocks. AIChE Symp. Series 74 (181), 85–90.

Boominathan, K., Reddy, C.A., 1992. cAMP-mediated differential regulation of lignin peroxidase and manganese-dependent peroxidase production in the white-rot basidiomycete *Phanerochaete chrysosporium*. Proc. Natl. Acad. Sci. (USA) 89 (12), 5586–5590.

Boopathy, R., 1998. Biological treatment of swine waste using anaerobic baffled reactors. Bioresour. Technol. 64, 1–6.

Brennan, A.H., Hoagland, W., Schell, D.J., 1986. High temperature acid hydrolysis of biomass using an engineering-scale plug flow reactor: result of low solids testing. Biotechnol. Bioeng. Symp. 17, 53–70.

Browner, C., 2000. Remarks as prepared for delivery to press conference on March 20, 2000. Available from http://www.epa.gov/otaq/consumer/fuels/mtbe/press34b.pdf.

Cadoche, L., López, G.D., 1989. Assessment of size reduction as a preliminary step in the production of ethanol from lignocellulosic wastes. Biol. Wastes 30, 153–157.

Cahela, D.R., Lee, Y.Y., Chambers, R.P., 1983. Modeling of percolation process in hemicellulose hydrolysis. Biotechnol. Bioeng. 25, 3–17.

Campbell, C.J., Laherrere, J.H., 1998. The end of cheap oil. Sci. Am. 3, 78–83.

Cantwell, B.A., Sharp, P.M., Gormley, E., Mcconnell, D.J., 1988. Molecular cloning of bacillus β-glucanases. In: Aubert, J.P., Beguin, P., Millet, J. (Eds.), Biochemistry and Genetics of Cellulose Degradation. Academic Press, San Diego, CA, pp. 181–201.

Castanon, M., Wilke, C.R., 1981. Effects of the surfactant Tween 80 on enzymatic hydrolysis of newspaper. Biotechnol. Bioeng. 23, 1365–1372.

Cheung, S.W., Anderson, B.C., 1997. Laboratory investigation of ethanol production from municipal primary wastewater. Bioresour. Technol. 59, 81–96.

Chosdu, R., Hilmy, N., Erizal, Erlinda, T.B., Abbas, B., 1993. Radiation and chemical pretreatment of cellulosic waste. Radiat. Phys. Chem. 42, 695–698.

Chum, H.L., Johnsoon, D.K., Black, S., 1988. Organosolv pretreatment for enzymatic hydrolysis of poplars: 1. enzyme hydrolysis of cellulosic residues. Biotechnol. Bioeng. 31, 643–649.

Clark, T.A., Mackie, K.L., 1987. Steam explosion of the soft-wood *Pinus radiata* with sulphur dioxide addition. I. Process optimization. J. Wood Chem. Technol. 7, 373–403.

Converse, A.O., Matsuno, R., Tanaka, M., Taniguchi, M., 1988. A model for enzyme adsorption and hydrolysis of microcrystalline cellulose with slow deactivation of the adsorbed enzyme. Biotechnol. Bioeng. 32, 38–45.

Converse, A.O., Kwarteng, I.K., Grethlein, H.E., Ooshima, H., 1989. Kinetics of thermochemical pretreatment of lignocellulosic materials. Appl. Biochem. Biotechnol. 20/21, 63–78.

Coughlan, M.P., Ljungdahl, L.G., 1988. Comparative biochemistry of fungal and bacterial cellulolytic enzyme system. In: Aubert, J.-P., Beguin, P., Millet, J. (Eds.), Biochemistry and Genetics of Cellulose Degradation, pp. 11–30.

Dai, Z.Y., Hooker, B.S., Anderson, D.B., Thomas, S.R., 2000. Improved plant-based production of E1 endoglucanase using potato: expression optimization and tissue targeting. Mol. Breed. 6, 277–285.

Dale, B.E., Moreira, M.J., 1982. A freeze-explosion technique for increasing cellulose hydrolysis. Biotechnol. Bioeng. Symp. 12, 31–43.

Dale, B.E., Henk, L.L., Shiang, M., 1984. Fermentation of lignocellulosic materials treated by ammonia freeze-explosion. Dev. Ind. Microbiol. 26, 223–233.

Dewes, T., Hünsche, E., 1998. Composition and Microbial degradability in the soil of farmyard manure from ecologically-managed farms. Biol. Agric. Hortic. 16, 251–268.

Dien, B.S., Nochols, N.N., O'Bryan, P.J., Bothast, R.J., 2000. Development of new ethanologenic *Escherichia coli* strains for fermentation of lignocellulosic biomass. Appl. Biochem. Biotechnol. 84/86, 181–196.

Dipardo, J., 2000. Outlook for biomass ethanol production. Energy information administration. Available from http://www.eia.doe.gov/oiaf/analysispaper/biomass.html.

Duff, S.J.B., Murray, W.D., 1996. Bioconversion of forest products industry waste cellulosics to fuel ethanol: a review. Bioresour. Technol. 55, 1–33.

Durand, H., Baron, M., Calmels, T., Tiraby, G., 1988. Classical and molecular genetics applied to *Trichoderma reesei* for the selection of improved cellulolytic industrial strains. In: Aubert, J.P., Beguin, P., Millet, J. (Eds.), Biochemistry and Genetics of Cellulose Degradation. Academic Press, San Diego, CA, pp. 135–151.

Elander, R.T., Putsche, V.L., 1996. Ethanol from corn: technology and economics. In: Wyman, C.E. (Ed.), Handbook on Bioethanol: Production and Utilization. Taylor & Francis, Washington, DC, pp. 329–349.

Esteghlalian, A., Hashimoto, A.G., Fenske, J.J., Penner, M.H., 1997. Modeling and optimization of the dilute-sulfuric-acid pretreatment of corn stover, poplar and switchhgrass. Bioresour. Technol. 59, 129–136.

Excoffier, G., Toussaint, B., Vignon, M.R., 1991. Saccharification of steam-exploded poplar wood. Biotechnol. Bioeng. 38, 1308–1317.

Fan, L.T., Gharpuray, M.M., Lee, Y.-H., 1987. In: Cellulose Hydrolysis Biotechnology Monographs. Springer, Berlin, p. 57.

Ghose, T.K., Bisaria, V.S., 1979. Studies on mechanism of enzymatic hydrolysis of cellulosic substances. Biotechnol. Bioeng. 21, 131–146.

Gregg, D.J., Saddler, J.N., 1996. Factors affecting cellulose hydrolysis and the potential of enzyme recycle to enhance the efficiency of an integrated wood to ethanol process. Biotechnol. Bioeng. 51, 375–383.

Grous, W.R., Converse, A.O., Grethlein, H.E., 1986. Effect of steam explosion pretreatment on pore size and enzymatic hydrolysis of poplar. Enzyme Microb. Technol. 8, 274–280.

Hacking, A.J., Taylor, I.W.F., Hanas, C.M., 1984. Selection of yeasts able to produce ethanol from glucose at 40 °C. Appl. Microbiol. Biotechnol. 19, 361–363.

Hatakka, A.I., 1983. Pretreatment of wheat straw by white-rot fungi for enzymatic saccharification of cellulose. Appl. Microbiol. Biotechnol. 18, 350–357.

Helle, S.S., Duff, S.J.B., Cooper, D.G., 1993. Effect of surfactants on cellulose hydrolysis. Biotechnol. Bioeng. 42, 611–617.

Hinman, N.D., Schell, D.J., Riley, C.J., Bergeron, P.W., Walter, P.J., 1992. Preliminary estimate of the cost of ethanol production for SSF technology. Appl. Biochem. Biotechnol. 34/35, 639–649.

Ho, N.W.Y., Chen, Z., Brainard, A.P., 1998. Genetically engineered Saccharomyces yeast capable of effective cofermentation of glucose and xylose. Appl. Environ. Microbiol. 64 (5), 1852–1859.

Holtzapple, M.T., Davison, R.R., Stuart, E.D., 1992b. Biomass refining process, US patent 5, 171, 592.

Holtzapple, M.T., Humphrey, A.E., Taylor, J.D., 1989. Energy requirements for the size reduction of poplar and aspen wood. Biotechnol. Bioeng. 33, 207–210.

Holtzapple, M.T., Jun, J-H., Ashok, G., Patibandla, S.L., Dale, B.E., 1990. Ammonia fiber explosion (AFEX) pretreatment of lignocellulosic wastes. American Institute of Chemical Engineers National Meeting, Chicago, IL.

Holtzapple, M.T., Jun, J-H., Ashok, G., Patibandla, S.L., Dale, B.E., 1991. The ammonia freeze explosion (AFEX) process: a practical lignocellulose pretreatment. Appl. Biochem. Biotechnol. 28/29, 59–74.

Holtzapple, M.T., Lundeen, J.E., Sturgis, R., 1992a. Pretreatment of lignocellulosic municipal solid waste by ammonia fiber explosion (AFEX). Appl. Biochem. Biotechnol. 34/35, 5–21.

Hooker, B.S., Dai, Z., Anderson, D.B., Quesenberry, R.D., Ruth, M.F., Thomas, S.R., 2001. Production of microbial cellulases in transgenic crop plants. In: Himmel, M.E., Baker, J.O., Saddler, J.N. (Eds.), Glycosyl Hydrolases for Biomass Conversion. American Chemical Society, Washington, DC, pp. 55–90.

Huang, X.L., Penner, M.H., 1991. Apparent substrate inhibition of the *Trichoderma reesei* cellulase system. J. Agric. Food Chem. 39, 2096–2100.

Iyer, P.V., Wu, Z.-W., Kim, S.B., Lee, Y.Y., 1996. Ammonia recycled percolation process for pretreatment of herbaceous biomass. Appl. Biochem. Biotechnol. 57/58, 121–132.

Kadam, K.L., Schmidt, S.L., 1997. Evaluation of *Candida acidothermophilum* in ethanol production from lignocellulosic biomass. Appl. Microbiol. Biotechnol. 48, 709–713.

Kilzer, F.J., Broido, A., 1965. Speculations on the nature of cellulose pyrolysis. Pyrodynamics 2, 151–163.

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

Mackie, K.L., Brownell, H.H., West, K.L., Saddler, J.N., 1985. Effect of sulphur dioxide and sulphuric acid on steam explosion of aspenwood. J. Wood Chem. Technol. 5, 405–425.

McCarthy, J.E., Tiemann, M., 1998. CRS report for congress. MTBE in gasoline: clean air and drinking water issues. Available from http://www.epa.gov/otaq/consumer/fuels/mtbe/crs-mtbe.pdf.

MacDonald, T., Yowell, G., McCormack, M., 2001. Staff report. US ethanol industry production capacity outlook. California energy commission. Available from http://www.energy.ca.gov/reports/2001-08-29_600-01-017.PDF.

McMillan, J.D., 1994. Pretreatment of lignocellulosic biomass. In: Himmel, M.E., Baker, J.O., Overend, R.P. (Eds.), Enzymatic Conversion of Biomass for Fuels Production. American Chemical Society, Washington, DC, pp. 292–324.

Mes-Hartree, M., Hogan, C.M., Saddler, J.N., 1987. Recycle of enzymes and substrate following enzymatic hydrolysis of steam pretreated aspenwood. Biotechnol. Bioeng. 30, 558–564.

Mes-Hartree, M., Dale, B.E., Craig, W.K., 1988. Comparison of steam and ammonia pretreatment for enzymatic hydrolysis of cellulose. Appl. Microbiol. Biotechnol. 29, 462–468.

Millet, M.A., Baker, A.J., Scatter, L.D., 1976. Physical and chemical pretreatment for enhancing cellulose saccharification. Biotech. Bioeng. Symp. 6, 125–153.

Mononmani, H.K., Sreekantiah, K.R., 1987. Saccharification of sugar-cane bagasse with enzymes from *Aspergillus ustus* and *Trichoderma viride*. Enzyme Microb. Technol. 9, 484–488.

Morjanoff, P.J., Gray, P.P., 1987. Optimization of steam explosion as method for increasing susceptibility of sugarcane bagasse to enzymatic saccharification. Biotechnol. Bioeng. 29, 733–741.

Neely, W.C., 1984. Factors affecting the pretreatment of biomass with gaseous ozone. Biotechnol. Bioeng. 20, 59–65.

Ooshima, H., Sakata, M., Harano, Y., 1986. Enhancement of enzymatic hydrolysis of cellulose by surfactant. Biotechnol. Bioeng. 28, 1727–1734.

Orpin, C.G., 1988. Genetic approaches to the improvement of lignocellulose degradation in the rumen. In: Aubert, J.P., Beguin, P., Millet, J. (Eds.), Biochemistry and Genetics of Cellulose Degradation. Academic Press, London, pp. 171–179.

Park, J.W., Takahata, Y., Kajiuchi, T., Akehata, T., 1992. Effects of nonionic surfactant on enzymatic hydrolysis of used newspaper. Biotechnol. Bioeng. 39, 117–120.

Penner, M.H., Liaw, E.-T., 1994. Kinetic consequences of high ratios of substrate to enzyme saccharification systems based on *Trichoderma* cellulase. In: Himmel, M.E., Baker, J.O., Overend, R.P. (Eds.), Enzymatic Conversion of Biomass for Fuels Production. American Chemical Society, Washington, DC, pp. 363–371.

Philippidis, G.P., 1996. Cellulose bioconversion technology. In: Wyman, C.E. (Ed.), Handbook on Bioethanol: Production and Utilization. Taylor & Francis, Washington, DC, pp. 253–285.

Philippidis, G.P., Smith, T.K., 1995. Limiting factors in the simultaneous saccharification and fermentation process for conversion of cellulosic biomass to fuel ethanol. Appl. Biochem. Biotechnol. 51/52, 117–124.

Philippidis, G.P., Smith, T.K., Wyman, C.E., 1993. Study of the enzymatic hydrolysis of cellulose for production of fuel ethanol by the simultaneous saccharification and fermentation process. Biotechnol. Bioeng. 41, 846–853.

Ramos, J.P., Breuil, C., Saddler, J.N., 1993. The use of enzyme recycling and the influence of sugar accumulation on cellulose hydrolysis by *Trichoderma* cellulases. Enzyme Microb. Technol. 15, 19–25.

Reshamwala, S., Shawky, B.T., Dale, B.E., 1995. Ethanol production from enzymatic hydrolysates of AFEX-treated coastal Bermuda grass and switchgrass. Appl. Biochem. Biotechnol. 51/52, 43–55.

Sarkanen, K.V., 1980. Acid-catalyzed delignification of lignocellulosics in organic solvents. Prog. Biomass Convers. 2, 127–144.

Saxena, A., Garg, S.K., Verma, J., 1992. Simultaneous saccharification and fermentation of waste newspaper to ethanol. Bioresour. Technol. 39, 13–15.

Schurz, J., 1978. In: Ghose, T.K. (Ed.), Bioconversion of Cellulosic Substances into Energy Chemicals and Microbial Protein Symposium Proceedings, IIT, New Delhi, pp. 37.

Shafizadeh, F., Lai, Y.-Z., 1975. Thermal degradation of 2-deoxy-D-arabino-hexonic acid and 3-deoxy-D-ribo-hexono-1,4-lactone. Carbohyd. Res. 42, 39–53.

Shafizadeh, F., Bradbury, A.G.W., 1979. Thermal degradation of cellulose in air and nitrogen at low temperatures. J. Appl. Poly. Sci. 23, 1431–1442.

Sivers, M.V., Zacchi, G., 1995. A techno-economical comparison of three processes for the production of ethanol from pine. Bioresour. Technol. 51, 43–52.

Szczodrak, J., Targonski, Z., 1989. Simultaneous saccharification and fermentation of cellulose: effect of ethanol and cellulases on particular stages. Acta Biotechnol. 6, 555–564.

Sternberg, D., 1976. Production of cellulase by *Trichoderma*. Biotechnol. Bioeng. Symp., 35–53.

Takagi, M., Abe, S., Suzuki, S., Emert, G.H., Yata, N., 1977. A method for production of alcohol directly from cellulose using cellulase and yeast. In: Ghose, T.K. (Ed.), Proceedings of Bioconversion of Cellulosic Substances into Energy, Chemicals and Microbial Protein, IIT, New Delhi, pp. 551–571.

Tarkow, H., Feist, W.C., 1969. In: A Mechanism for Improving the Digestibility of Lignocellulosic Materials with Dilute Alkali and Liquid NH_3 Advance Chemistry Series 95. American Chemical Society, Washington, DC, pp. 197–218.

Tengerdy, R.P., Nagy, J.G., 1988. Increasing the feed value of forestry waste by ammonia freeze explosion treatment. Biol. Wastes 25, 149–153.

Thring, R.W., Chorent, E., Overend, R., 1990. Recovery of a solvolytic lignin: effects of spent liquor/acid volume ration, acid concentration and temperature. Biomass 23, 289–305.

Vidal, P.F., Molinier, J., 1988. Ozonolysis of lignin – improvement of in vitro digestibility of poplar sawdust. Biomass 16, 1–17.

Vlasenko, E.Y., Ding, H., Labavitch, J.M., Shoemaker, S.P., 1997. Enzymatic hydrolysis of pretreated rice straw. Bioresour. Technol. 59, 109–119.

Waldner, R., Leisola, M.S.A., Fiechter, A., 1988. Comparison of ligninolytic activities of selected fungi. Appl. Microbiol. Biotechnol. 29, 400–407.

Wang, M., Saricks, C., Santini, D., 1999. Effects of Fuel Ethanol use on Fuel-Cycle Energy and Greenhouse Gas Emissions. Argonne National Laboratory, Argonne, IL.

Wheals, A.E., Basso, L.C., Alves, D.M.G., Amorim, H.V., 1999. Fuel ethanol after 25 years. Trends Biotechnol. 17 (12), 482–487.

Wood, B.E., Beall, D.S., Ingram, L.O., 1997. Production of recombinant bacterial endoglucanase as a co-product with ethanol during fermentation using derivatives of *Escherichia coli* KO11. Biotechnol. Bioeng. 55 (3), 547–555.

Wooley, R., Ruth, M., Glassner, D., Sheehan, J., 1999. Process design and costing of bioethanol technology: a tool for determining the status and direction of research and development. Biotechnol. Prog. 15, 794–803.

Wright, J.D., 1998. Ethanol from biomass by enzymatic hydrolysis. Chem. Eng. Prog. 84 (8), 62–74.

Wu, Z., Lee, Y.Y., 1997. Inhibition of the enzymatic hydrolysis of cellulose by ethanol. Biotech. Lett. 19, 977–979.

Wu, J., Ju, L.K., 1998. Enhancing enzymatic saccharification of waste newsprint by surfactant addition. Biotechnol. Prog. 14, 649–652.

Xin, Z., Yinbo, Q., Peiji, G., 1993. Acceleration of ethanol production from paper mill waste fiber by supplementation with β-glucosidase. Enzyme Microb. Technol. 15, 62–65.

Zhang, M., Eddy, C., Deanda, K., Finkelstein, M., Picataggio, S., 1995. Metabolic engineering of a pentose metabolism pathway in ethanologenic *Zymomonas mobilis*. Science 267, 240–243.

Zheng, Y.Z., Lin, H.M., Tsao, G.T., 1998. Pretreatment for cellulose hydrolysis by carbon dioxide explosion. Biotechnol. Prog. 14, 890–896.

Ziegler, M.T., Thomas, S.R., Danna, K.J., 2000. Accumulation of a thermostable endo-1,4-β-D-glucanase in the apoplast of *Arabidopsis thaliana* leaves. Mol. Breed. 6, 37–46.