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.