ENZYME-BASED HYDROLYSIS PROCESSES FOR ETHANOL FROM LIGNOCELLULOSIC MATERIALS: A REVIEW

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This article reviews developments in the technology for ethanol production from lignocellulosic materials by "enzymatic" processes. Several methods of pretreatment of lignocelluloses are discussed, where the crystalline structure of lignocelluloses is opened up, making them more accessible to the cellulase enzymes. The characteristics of these enzymes and important factors in enzymatic hydrolysis of the cellulose and hemicellulose to cellobiose, glucose, and other sugars are discussed. Different strategies are then described for enzymatic hydrolysis and fermentation, including separate enzymatic hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), non-isothermal simultaneous saccharification and fermentation (NSSF), simultaneous saccharification and co-fermentation (SSCF), and consolidated bioprocessing (CBP). Furthermore, the by-products in ethanol from lignocellulosic materials, wastewater treatment, commercial status, and energy production and integration are reviewed.

Keywords: Lignocellulosic materials, Enzymatic hydrolysis, Ethanol, Fermentation

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INTRODUCTION

Ethanol is the most important product of biotechnology in terms of volume and market values. The current raw materials are sugar substances, such as sugarcane juice and molasses, as well as starch-based materials such as wheat and corn. However, intensive research and developments in the last decades on lignocelluloses will most likely make them important raw material for ethanol production in the future.

Lignocelluloses are composed of cellulose, hemicellulose, lignin, extractives, and several inorganic materials (Sjöström 1993). Cellulose or β-1-4-glucan is a polymer of glucose made of cellobiose units with about 2,000 to 27,000 glucose residues (Delmer and Amor 1995; Morohoshi 1991). These chains are packed by hydrogen bonds in so-called 'elementary fibrils' originally considered to be 3 to 4 nm wide and contain about 36 chains, although larger crystalline fibrils up to 16 nm were also discovered (Ha et al. 1998). These elementary fibrils are then packed in so-called microfibrils, where the elementary fibrils are attached to each other by hemicelluloses, amorphous polymers of different sugars as well as other polymers such as pectin and covered by lignin. The microfibrils are often associated in the form of bundles or macrofibrils (Delmer and Amor 1995).

In order to produce ethanol from lignocellulosic materials, we should (a) open the bundles of lignocelluloses in order to access the polymer chains of cellulose and hemicellulose by a process of so-called pretreatment, (b) hydrolyze the polymers in order to achieve monomer sugar solutions, (c) ferment the sugars to ethanol solution (mash) by microorganisms, and (d) purify ethanol from mash by e.g. distillation and dehydration (Fig. 1). By-product recovery, utilities (steam and electricity generation and cooling water), wastewater treatment, and eventually enzyme production are the other units which are demanded in ethanol production from lignocellulosic materials.

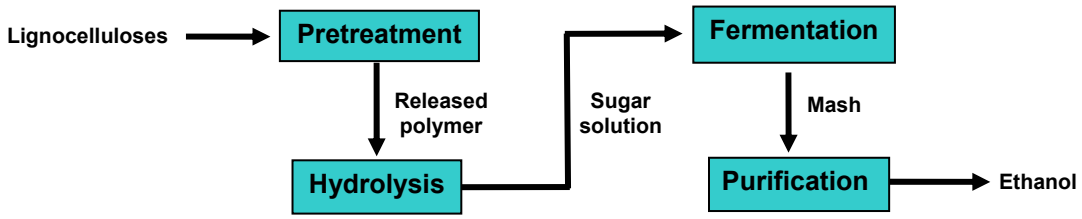

Fig. 1. Different units in the main line of ethanol production from lignocellulosic materials

The hydrolysis of cellulose and hemicellulose in this process can be carried out chemically by e.g. dilute sulfuric acid or enzymatically. We have recently reviewed the acid-based processes (Taherzadeh and Karimi 2007), and the present work is dedicated to enzymatic processes of ethanol production from lignocellulosic materials. The enzymatic hydrolysis is catalyzed by cellulolytic enzymes. Without any pretreatment, the conversion of native cellulose to sugar is extremely slow, since cellulose is well protected by the matrix of lignin and hemicellulose in macrofibrils. Therefore, pretreatment of these materials is necessary to increase the rate of hydrolysis of cellulose to fermentable sugars (Galbe and Zacchi 2002).

There are several advantages and disadvantages of dilute-acid and enzymatic hydrolyses, which are listed in Table 1. Enzymatic hydrolysis is carried out under mild conditions, whereas acid hydrolysis requires high temperature and low pH, which results in corrosive conditions. While it is possible to obtain cellulose hydrolysis of close to 100% by enzymatic hydrolysis (Ogier et al. 1999), it is difficult to achieve such high yield with the acid hydrolyses. Furthermore, several inhibitory compounds are formed during acid hydrolysis, whereas this problem is not so severe for enzymatic hydrolysis (Lee et al. 1999; Taherzadeh 1999; Wyman 1996).

Table 1. Comparison between Dilute-acid and Enzymatic Hydrolyses

Comparing variable	*Dilute-acid hydrolysis*	*Enzymatic hydrolysis*
Mild hydrolysis conditions	No	Yes
High yields of hydrolysis	No	Yes
Product inhibition during hydrolysis	No	Yes
Formation of inhibitory by-products	Yes	No
Low cost of catalyst	Yes	No
Short time of hydrolysis	Yes	No

On the other hand, enzymatic hydrolysis has its own problems compared to dilute-acid hydrolysis. A hydrolysis time of several days is necessary for enzymatic hydrolysis (Tengborg et al. 2001), whereas a few minutes is enough for the acid hydrolysis (Taherzadeh et al. 1997). The prices of the enzymes are much higher than e.g. sulfuric acid that is used in acid hydrolysis (Sheehan and Himmel 2001), although some breakthrough in cutting the prices by e.g. the Danish Novozyme company has recently been reported. In acid hydrolysis, the final products, e.g. released sugars, do not inhibit the hydrolysis. However, in enzymatic hydrolysis, the sugars released inhibit the hydrolysis reaction (Eklund and Zacchi 1995; Hari Krishna and Chowdary 2000; Kádár et al. 2004; Linde et al. 2007). In order to overcome this problem, simultaneous saccharification and fermentation (SSF) was developed, in which the sugars released from the hydrolysis are directly consumed by the present microorganisms (Wyman 1996). However, since fermentation and hydrolysis usually have different optimum temperatures, separate enzymatic hydrolysis and fermentation (SHF) is still considered as a choice.

PRETREATMENT OF LIGNOCELLULOSIC MATERIALS

Pretreatment of lignocelluloses is intended to disorganize the crystalline structure of macro- and microfibrils, in order to release the polymer chains of cellulose and hemicellulose, and/or modify the pores in the material to allow the enzymes to penetrate into the fibers to render them amenable to enzymatic hydrolysis (Galbe and Zacchi 2002). Pretreatment should be effective to achieve this goal, avoid degradation or loss of carbohydrate, and avoid formation of inhibitory by-products for the subsequent hydrolysis and fermentation; obviously, it must be cost-effective (Sun and Cheng 2002). There are several methods introduced for pretreatment of lignocellulosic materials, which are summarized in Table 2.

The pretreatment methods may be classified into "Physical pretreatment" such as mechanical comminution, pyrolysis, and irradiation (McMillan 1994; Wyman 1996), "Physico-chemical pretreatment" such as steam explosion or autohydrolysis, ammonia fiber explosion (AFEX), CO_2 explosion and SO_2 explosion (Alizadeh et al. 2005; Ballesteros et al. 2000; Boussaid et al. 1999; Dale et al. 1996; Eklund et al. 1995; Holtzapple et al. 1991; Ogier et al. 1999; Ohgren et al. 2005; Sassner et al. 2005; Stenberg et al. 1998a; Tengborg et al. 1998; Vlasenko et al. 1997), "Chemical pretreatment" including ozonolysis, dilute-acid hydrolysis, alkaline hydrolysis, oxidative delignification, and organosolv processes (Arato et al. 2005; Barl et al. 1991; Berlin et al. 2006; Karimi et al. 2006a; Karimi et al. 2006b; Lee et al. 1999; Nguyen et al. 2000; Sanchez et al. 2004; Schell et al. 2003; Sidiras and Koukios 2004; Tucker et al. 2003), and "Biological pretreatment" (Fan et al. 1982; Wyman 1996). However, not all of these methods have yet developed enough to be feasible technically or economically for large-scale processes. In some cases, a method is used to increase the efficiency of another method. For instance, milling could be applied to create a better steam explosion by reducing the chip size. Furthermore, it should be noticed that the selection of pretreatment method should be compatible with the selection of hydrolysis. For example,

if acid hydrolysis is to be applied, a pretreatment with alkali may not be beneficial (Taherzadeh and Niklasson 2004). The pretreatment methods were reviewed by McMillan (1994) , Wyman (1996), Sun and Cheng (2002), and Mosier et al. (2005b).

Table 2. Pretreatment Methods of Lignocellulosic for Enzymatic Hydrolysis

Method	Processes	Mechanism of changes on biomass
Physical pretreatments	- *Ball-milling* - *Two-roll milling* - *Hammer milling* - *Colloid milling* - *Vibro energy milling* - *Hydrothermal* - *High pressure steaming* - *Extrusion* - *Expansion* - *Pyrolysis* - *Gamma-ray irradiation* - *Electron-beam irradiation* - *Microwave irradiation*	- Increase in accessible surface area and size of pores - Decrease of the cellulose crystallinity and its degrees of polymerization - Partial hydrolysis of hemicelluloses - Partial depolymerization of lignin
Physicochemical & chemical pretreatments	Explosion: - *Steam explosion* - *Ammonia fiber explosion (AFEX)* - *CO_2 explosion* - *SO_2 explosion* Alkali: - *Sodium hydroxide* - *Ammonia* - *Ammonium Sulfite* Gas: - *Chlorine dioxide* - *Nitrogen dioxide* Acid: - *Sulfuric acid* - *Hydrochloric acid* - *Phosphoric acid* - *Sulfur dioxide* Oxidizing agents: - *Hydrogen peroxide* - *Wet oxidation* - *Ozone* Cellulose solvents: - *Cadoxen* - *CMCS* Solvent extraction of lignin: - *Ethanol-water extraction* - *Benzene-water extraction* - *Ethylene glycol extraction* - *Butanol-water extraction* - *Swelling agents*	- Delignification - Decrease of the cellulose crystallinity and its degrees of polymerization - Partial or complete hydrolysis of hemicelluloses
Biological pretreatments	- *Actinomycetes* - *Fungi*	- Delignification - Reduction in degree of polymerization of hemicellulose and cellulose

Dilute-acid hydrolysis is probably the most commonly applied method among the chemical hydrolysis methods. It is a method that can be used either as a pretreatment preceding enzymatic hydrolysis, or as the actual method of hydrolyzing lignocellulose to the sugars. Different types of reactors such as batch, plug flow, percolation, countercurrent, and shrinking-bed reactors for either pretreatment or hydrolysis of lignocellulosic materials by the dilute acid processes have been applied so far. Most of the commercial programs underway are using dilute acid pretreatment (Taherzadeh and Karimi 2007). The dilute-acid pretreatment can achieve high reaction rates and significantly improve cellulose hydrolysis. Different aspects of dilute-acid hydrolysis have recently been reviewed (Taherzadeh and Karimi 2007). One of the main advantages of dilute acid hydrolysis is achieving high xylan to xylose conversion yields, which is necessary to achieve favorable overall process economics in ethanol production from lignocellulose (Sun and Cheng 2002). On the other hand, a main disadvantage of this pretreatment method is the necessity of neutralization of pH for the downstream enzymatic hydrolysis. Furthermore, different chemical inhibitors might be produced during the acid pretreatment which reduce cellulase activity, and therefore, water wash is necessary for the pretreated biomass before enzymatic hydrolysis (Mes-Hartree and Saddler 1983; Sun and Cheng 2002). The main advantage of this method is the possibility to recover a high portion (e.g. 90%) of the hemicellulose sugars. The hemicellulose, mainly xylan or mannan, accounts for up to a third of the total carbohydrate in many lignocellulosic materials. Thus, hemicellulose recovery can have a highly positive effect on the overall process economics of ethanol production from lignocellulosic material.

Steaming with or without explosion (autohydrolysis) is one of the popular pretreatment methods of lignocellulosic materials. Steam pretreatment removes the major part of the hemicellulose from the solid material and makes the cellulose more susceptible to enzymatic digestion. In this method the biomass is treated with high-pressure steam. The pressure is then swiftly reduced, in steam explosion, which makes the materials undergo an explosive decompression. Steam explosion is typically initiated at a temperature of 160 to 260°C for several seconds to a few minutes before the material is exposed to atmospheric pressure (Cullis et al. 2004; Kurabi et al. 2005; Ruiz et al. 2006; Sun and Cheng 2002; Varga et al. 2004b; Wyman 1996). Negro et al. (2003) evaluated steam explosion to enhance ethanol production from poplar (*Populus nigra*) biomass and compared the results with hydrothermal pretreatment. The best results were obtained in steam explosion pretreatment at 210 °C and 4 min, taking into account cellulose recovery above 95%, enzymatic hydrolysis yield of about 60%, SSF yield of 60% of theoretical, and 41% xylose recovery in the liquid fraction. The results also showed that large particles can be used for poplar biomass in both pretreatments, since no significant effect of particle size on enzymatic hydrolysis and SSF was obtained. Ballesteros et al. (2004) used steam explosion for ethanol production from several lignocellulosic materials with *Kluyveromyces marxianus*. They treated poplar and eucalyptus biomass at 210 °C for 4 min; wheat straw at 190 °C for 8 min; sweet sorghum bagasse at 210 °C for 2 min, and *Brassica carinata* residue at 210 °C at 8 min. These conditions were selected with regard to the maximum glucose recovery after 72 h of enzymatic hydrolysis. Hemicellulose sugars were extensively solubilized during steam explosion and xylose content decreased by about 75–90%, depending on the substrate.

Steaming and mechanical treatment might be combined to effectively disrupt the cellulosic structure. Several technologies for this combination have been developed (Mason 1926; Katzen et al. 1995; Chum et al. 1985). Generally, steam explosion is the basic pretreatment of lignocellulosic substrates because the process is so well documented, was tested at several levels and at various institutions, and satisfies all the requirements of the pretreatment process. Its energy costs are relatively moderate, and the general process has been demonstrated on a commercial scale at the Masonite plants (Chum et al. 1985).

AFEX, or ammonia fiber explosion, is one of the physicochemical pretreatment methods in which lignocellulosic materials are exposed to liquid ammonia at high temperature (e.g. 90-100°C) for a period of time (such as 30 min), and then the pressure is swiftly reduced. There are many adjustable parameters in the AFEX process: ammonia loading, water loading, temperature, time, blowdown pressure, and number of treatments (Holtzapple et al. 1991). AFEX, with a concept similar to steam explosion, can significantly improve the enzymatic hydrolysis. The optimal conditions for pretreatment of switchgrass with AFEX were reported to be about 100°C, ammonia loading of 1:1 kg of ammonia per kg of dry matter, and 5 min retention time (Alizadeh et al. 2005). Enzymatic hydrolysis of AFEX-treated and untreated samples showed 93% vs. 16% glucan conversion, respectively. An advantage of AFEX pretreatment is no formation of some types of inhibitory by-products, which are produced during the other pretreatment methods, such as furans in dilute-acid pretreatment. However, cleaved lignin phenolic fragments and other cell wall extractives may remain on the biomass surface, which can easily be removed by washing with water (Chundawat et al. 2007). Although AFEX enhances hydrolysis of (hemi)cellulose from grass, the effect on biomass that contains more lignin (soft and hardwood) is meager. Furthermore, the AFEX pretreatment does not significantly solubilize hemicellulose, compared to dilute-acid pretreatment. On the other hand, to reduce the cost and protect the environment, ammonia must be recycled after the pretreatment (Eggeman and Elander 2005; Sun and Cheng 2002; Wyman 1996). SunOpta BioProcess Group claimed to have developed the first continuous process in the world to pretreat cellulosic materials with the AFEX process (www.sunopta.com/).

Hydrothermal pretreatment or cooking of lignocellulosic materials in liquid hot water (LHW) is one of the old methods applied for pretreatment of cellulosic materials. Autohydrolysis plays an important role in this process, where no chemical is added. It results in dissolution of hemicelluloses mostly as liquid-soluble oligosaccharides and separates them from insoluble cellulosic fractions. The pH, processing temperature, and time should be controlled in LHW pretreatment in order to optimize the enzymatic digestibility of lignocellulosic materials (Mosier et al. 2005a; Mosier et al. 2005c; Wyman 1996). LHW pretreatment of corn fiber at 160 °C and a pH above 4.0 dissolved 50% of the fiber in 20 min (Mosier et al. 2005c). The results showed that the pretreatment enabled the subsequent complete enzymatic hydrolysis of the remaining polysaccharides to the corresponding monomers. The carbohydrates dissolved by the LHW pretreatment were 80% soluble oligosaccharides and 20% monosaccharides with less than 1% of the carbohydrates lost to degradation products. LHW causes ultrastructural changes and formation of micron-sized pores that enlarge accessible and susceptible surface area and make the cellulose more accessible to hydrolytic enzymes (Zeng et al. 2007). Without

any pretreatment, corn stover with sizes of 53-75 μm was 1.5 times more susceptible to enzymatic hydrolysis than the larger stover particles of 425-710 μm. However, this difference was eliminated when the stover was pretreated with liquid hot water at 190 °C for 15 min, at a pH between 4.3 and 6.2 (Zeng et al. 2007). Laser et al. (2002) compared the performance of LHW and steam pretreatments of sugarcane bagasse in production of ethanol by SSF. They used a 25-l reactor, temperature 170-230 °C, residence time 1-46 min and 1% to 8% solids concentration. Both of the methods generated reactive fibers, but LHW resulted in much better xylan recovery than steam pretreatment. It was concluded that LHW pretreatment produces results comparable with dilute-acid pretreatment processes.

Organosolv may be used to provide treated cellulose suitable for enzyme hydrolysis, using solvents to remove lignin (Itoh et al. 2003; Pan et al. 2006). The process involves mixing of an organic liquid and water together in various portions and adding them to the lignocellulose. This mixture is heated to dissolve the lignin and some of the hemicellulose and leave a reactive cellulose cake. In addition, a catalyst is sometimes added either to reduce the operating temperature or to enhance the delignification process. Most of these processes produce similar results and for that reason are grouped here as a single class (Chum et al. 1985). Delignification of lignocellulosic materials has been known to occur in a large number of organic or aqueous-organic solvent systems with or without added catalysts at temperatures of 150-200°C. Among the solvents tested, those with low boiling points (ethanol and methanol) have been used as well as a variety of alcohols with higher boiling points (ethylene glycol, tetrahydro furfuryl alcohol) and other classes of organic compounds such as dimethylsulfoxide, phenols, and ethers (Chum et al. 1985). In these methods, the solvent action is accompanied with e.g. acetic acid released from acetyl groups developed by hydrolysis of hemicelluloses. The main advantage of the use of solvents over chemical pretreatment is that relatively pure, low-molecular-weight lignin can be recovered as a by-product (Katzen et al. 1995; Sun and Cheng 2002). Organic acids such as oxalic, salicylic, and acetylsalicylic acid can be used as catalysts in the organosolv process. Usually, a high yield of xylose can be obtained with the addition of the acids. However, addition of the catalysts is unnecessary for satisfactory delignification at high temperatures (above 185 °C). Solvents used in the process need to be drained from the reactor, evaporated, condensed, and recycled to reduce the operational costs. Removal of solvents from the system is usually necessary because the solvents may be inhibitory to the growth of organisms, enzymatic hydrolysis, and fermentation (Sun and Cheng 2002). The delignification is accompanied by solvolysis and dissolution of lignin and hemicellulosic fractions, depending on the process conditions (solvent system, type of lignocellulose, temperature, reactor design [batch versus continuous processes]), as well as by solvolysis of the cellulosic fraction to a smaller extent (Chum et al. 1985).

Wet oxidation is the process of treating lignocellulosic materials with water and air or oxygen at temperatures above 120 °C (e.g. 148-200 °C) for a period of time of e.g. 30 min (Garrote et al. 1999; Palonen et al. 2004; Varga et al. 2004a). Oxygen participates in the degradation reactions, enhancing the generation of organic acids and allowing operation at comparatively reduced temperatures. The fast reaction rates and heat generation by reaction make the control of reactor temperature critical. Wet oxidation is

among the simplest process in terms of equipment, energy, and chemicals required for operation (Chum et al. 1985). Bjerre et al. (1996) combined wet oxidation and alkaline hydrolysis for pretreatment of wheat straw. The process resulted in convertible cellulose (85% conversion yield of cellulose to glucose) and hemicellulose. However, this method is suitable for materials with low lignin content, since the yield decreases with increased lignin content, and also a large fraction of the lignin is oxidized and solubilized. As with many other delignification methods, the lignin produced by wet oxidation cannot be used as a fuel, which considerably reduces the income from by-products in industrial-scale ethanol production from lignocellulose (Galbe and Zacchi 2002).

ENZYMATIC HYDROLYSIS

Enzymatic hydrolysis of cellulose to glucose is carried out by cellulase enzymes that are highly specific catalysts. The hydrolysis is performed under mild conditions (e.g. pH 4.5-5.0 and temperature 40–50°C). Therefore, one may expect low corrosion problems, low utility consumption, and low toxicity of the hydrolyzates as the main advantages of this process.

Cellulolytic Enzymes

Enzymatic hydrolysis of cellulose and hemicellulose can be carried out by highly specific cellulase and hemicellulase enzymes (glycosylhydrolases). This group includes at least 15 protein families and some subfamilies (Rabinovich et al. 2002). Enzymatic hydrolysis of cellulose consists of the cellulase adsorption onto the surface of the cellulose, the biodegradation of cellulose to fermentable sugars, and desorption of the cellulase. Enzymatic degradation of cellulose to glucose is generally accomplished by synergistic action of at least three major classes of enzymes: endo-glucanases, exo-glucanases, and ß-glucosidases. These enzymes are usually called together cellulase or cellulolytic enzymes (Wyman 1996).

The endoglucanases attack the low-crystallinity regions of the cellulose fiber and create free chain-ends. The exoglucanases further degrade the sugar chain by removing cellobiose units (dimers of glucose) from the free chain-ends. The produced cellobiose is then cleaved to glucose by β-glucosidase (Fig. 2). This enzyme is not a cellulase, but its action is very important to complete depolymerization of cellulose to glucose. Since hemicellulose contains different sugar units, the hemicellulytic enzymes are more complex and involve at least endo-1,4-β-D-xylanases, exo-1,4-β-D-xylosidases, endo-1,4-β-D-mannanases, β-mannosidases, acetyl xylan esterases, α-glucuronidases, α-L-arabinofuranosidases, and α-galactosidases (Jorgensen et al. 2003). Several species of bacteria such as *Clostridium, Cellumonas, Thermomonospora, Bacillus, Bacteriodes, Ruminococcus, Erwinia, Acetovibrio, Microbispora,* and *Streptomyces*, and fungi such as *Tricoderma, Penicillium, Fusarium, Phanerochaete, Humicola,* and *Schizophillum spp.*, are able to produce cellulases and hemicellulases (Rabinovich et al. 2002; Sun and Cheng 2002). Among the cellulases produced by different microorganisms, cellulases of *Trichoderma reesei* or *T. viride* have been the most broadly studied and best characterized. A full complement production of cellulase, stability under the enzymatic

hydrolysis conditions, and resistance of the enzyme to chemical inhibitors are the advantages of the cellulase produced by *Trichoderma.* The main disadvantages of *Trichoderma* cellulase are the suboptimal levels and low activity of ß-glucosidases. On the other hand, *Aspergilli* are very efficient ß-glucosidase producers. In several studies, *Trichoderma* cellulase was supplemented with extra ß-glucosidases and showed good improvement (Hari Krishna et al. 2001; Itoh et al. 2003; Ortega et al. 2001; Tengborg et al. 2001; Wyman 1996).

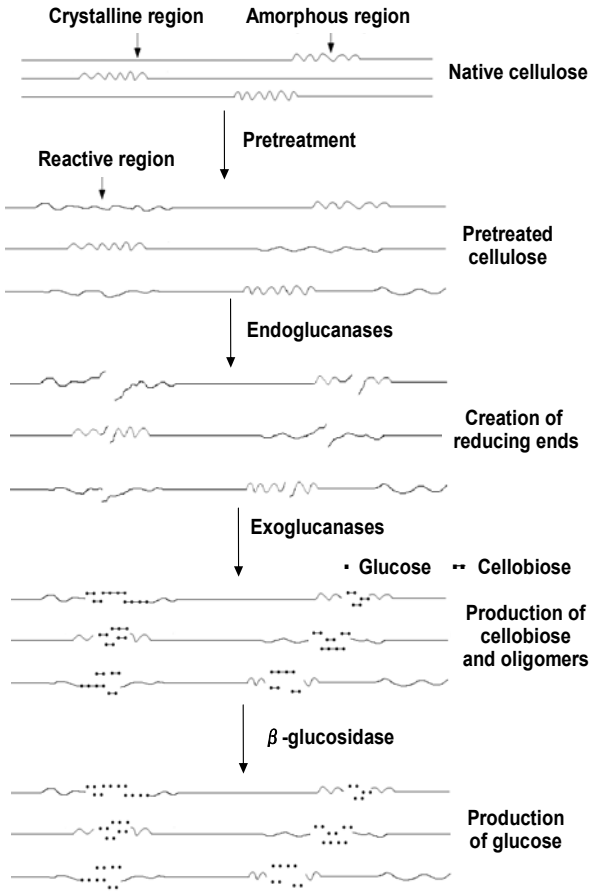

Fig. 2. Schematic presentation of hydrolysis of cellulose to glucose by cellulolytic enzymes.

Production and application of cellulase by *Trichoderma* has some difficulties. The enzyme is produced in the late stage of fermentation and needs a well-controlled pH, and its activity is reduced by adsorption to cellulose and lignin. Furthermore, it has problems in scaling-up of the enzyme production process due to oxygen transfer into mycelial broth; lower cell-bound enzyme activity; and poor mixing due to shear sensitivity of the fungus (Lee 1997; Wyman 1996). However, in spite of these deficiencies, the soft-rot fungus *T. reesei* is currently among the best vehicles for cellulase production (Xia and Shen 2004; Wyman 1996). Most commercial cellulases are produced from *Trichoderma spp.*, with a few also produced by *Aspergillus niger.*

IMPORTANT FACTORS IN ENZYMATIC HYDROLYSIS

Substrate concentration and quality, applied pretreatment method, cellulase activity, and hydrolysis conditions such as temperature, pH, and mixing are the main factors in enzymatic hydrolysis of lignocellulosic materials. The optimum temperature and pH are functions of the raw material, the enzyme source, and hydrolysis duration. The optimum temperatures and pH of different cellulases are usually reported to be in the range of 40 to 50 °C and pH 4 to 5 (Olsson and Hahn-Hägerdal 1996). However, the optimum residence time and pH might affect each other. Tengborg et al. (2001) showed an optimal temperature of 38 °C and pH 4.9 within 144 h residence time for cellulase (Commercial enzyme solutions, Celluclast 2 L, Novo Nordisk A/S, Bagsværd, Denmark).

One of the main factors that affect the yield and initial rate of enzymatic hydrolysis is substrate (cellulose and/or hemicellulose) concentration in the slurry solution. High substrate concentration can cause substrate inhibition, which substantially lowers the hydrolysis rate. The extent of the inhibition depends on the ratio of total enzyme to total substrate (Sun and Cheng 2002). Problems in mixing and mass transfer also arise in working with high substrate concentration. The ratio of enzyme to substrate used is another factor in enzymatic hydrolysis. Obviously application of more cellulase, up to a certain level, increases the rate and yield of hydrolysis. However, increase in cellulase level would significantly increase the cost of the process. Cellulase loading is usually in the range of 5 to 35 FPU per gram of substrate.

Addition of surfactants during hydrolysis can modify the cellulose surface properties. An important effect of surfactant addition in a process for lignocellulose conversion is the possibility to lower the enzyme loading. A number of surfactants have been examined for their ability to improve enzymatic hydrolysis. Non-ionic surfactants were found to be the most effective. Fatty acid esters of sorbitan polyethoxylates (Tween® 20 and 80), and polyethylene glycol, are among the most effective surfactants reported for enzymatic hydrolysis (Alkasrawi et al. 2003; Börjesson et al. 2007; Kim et al. 2006a). Addition of polyethylene glycol to lignocellulose substrates increased the enzymatic conversion from 42% to 78% in 16 h (Börjesson et al. 2007). One reason for this effect might be adsorption of surfactants to lignin, which prevents unproductive binding of enzymes to lignin and results in higher productivity of the enzymes (Eriksson et al. 2002). However, the surfactant should be selected carefully, since it may have negative impact on the fermentation of the hydrolyzate. For instance, addition of 2.5 g/l Tween 20 helped to reduce enzyme loading by 50%, while retaining cellulose conversion (Eriksson et al. 2002). However, this surfactant is an inhibitor to *D. clausenii* even at low concentration of 1.0 g/l (Wu and Ju 1998).

The recycling of cellulase enzymes is one potential strategy for reducing the cost of the enzymatic hydrolysis during the bioconversion of lignocelluloses to ethanol (Tu et al. 2007). However, presence of solid residuals (mainly lignin) and dissolution of the enzymes in the hydrolyzates make the enzymes difficult to separate. Immobilization is an alternative to retain the enzymes in the reactor, but steric hindrance, freedom of movement and gradual reduction of the cellulases' activity must be considered. In this regard, it should be kept in mind that endoglucanase and exoglucanase should diffuse into lignocelluloses and be adsorbed to the surface of the particles in order to initiate

hydrolysis and convert the cellulose to cellobiose. However, cellobiose is in the aqueous phase, where it is converted to glucose by ß-glucosidase. Therefore, immobilization of ß-glucosidase might theoretically be possible and effective (Tu et al. 2006). It is also possible to co-immobilize ß-glucosidase and a fermenting microorganism in order to improve the overall conversion of cellulose to ethanol (Lee and Woodward 1983). One of the major problems in immobilization is to separate the immobilized support from the residual solid of the reactor. One possible solution could be immobilization of the enzymes in magnetic particles, such as magnetic agarose composite microspheres (Qiu and Li 2000; Qiu and Li 2001), or magnetic chitosan microspheres (Feng et al. 2006).

HYDROLYSIS & FERMENTATION STRATEGIES

Separate Enzymatic Hydrolysis and Fermentation (SHF)

In this process, pretreated lignocelluloses are hydrolyzed to glucose and subsequently fermented to ethanol in separate units (Fig. 3). The major advantage of this method is that it is possible to carry out the cellulose hydrolysis and fermentation at their own optimum conditions. The optimum temperature for cellulase is usually between 45 and 50 °C, depending on the cellulose-producing microorganism (Olsson et al. 2006; Saha et al. 2005; Söderström et al. 2003; Wingren et al. 2003). However, the optimum temperature for most of the ethanol-producing microorganisms is between 30 and 37°C.

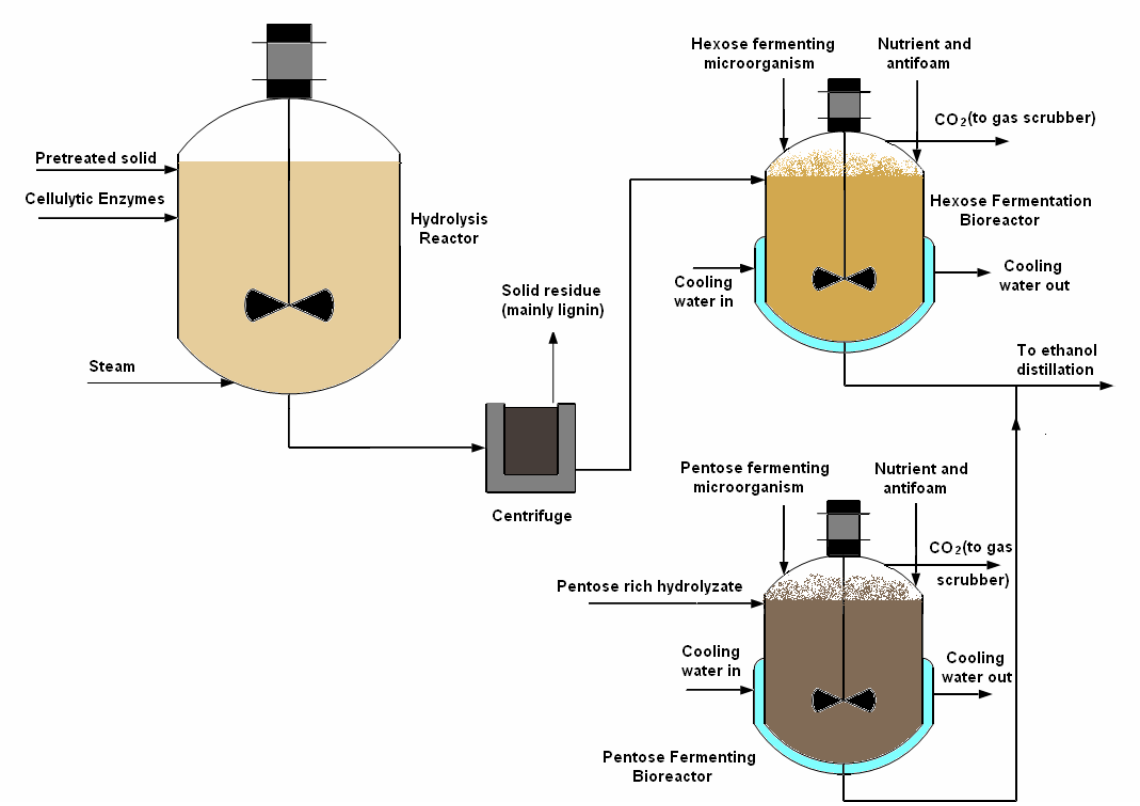

Fig. 3. Simplified process flow diagram for separate enzymatic hydrolysis and fermentation (SHF)

Inhibition of cellulase activity by the released sugars, mainly cellobiose and glucose, is the main drawback of SHF. At a cellobiose concentration as low as 6 g/l, the activity of cellulase is reduced by 60%. Although glucose decreases the cellulase activity as well, the inhibitory effect of this sugar is lower than that of cellobiose. On the other hand, glucose is a strong inhibitor for ß-glucosidase. At a level of 3 g/l of glucose, the activity of ß-glucosidase is reduced by 75% (Philippidis and Smith 1995; Philippidis et al. 1993). Another possible problem in SHF is that of contaminations. The hydrolysis process is rather long, e.g. one to four days, and a dilute solution of sugar always has a risk of microbial contaminations, even at rather high temperature such as 45-50 °C. A possible source of contamination could be the enzymes. In practice, it is difficult to sterilize the cellulase in large scale, since it should be filtered because of its deactivation in an autoclave.

Softwood hemicellulose is mainly composed of mannose, which can be separated during the pretreatment by e.g. dilute-acid pretreatment and fermented in a separate bioreactor (as indicated in Fig. 3) or possibly fermented together with the pretreated cellulose in the SHF bioreactor. However, the dominant sugar in hemicellulose derived from hardwood and crop residues is usually pentose, which can be converted to ethanol in a separate pentose-fermenting bioreactor (Fig. 3).

Simultaneous Saccharification and Fermentation (SSF)

One of the most successful methods for ethanol production from lignocellulosic materials is combination of the enzymatic hydrolysis of pretreated lignocelluloses and fermentation in one step, termed SSF (Fig. 4).

In this process, the glucose produced by the hydrolyzing enzymes is consumed immediately by the fermenting microorganism present in the culture. This is a great advantage for SSF compared to SHF, since the inhibition effects of cellobiose and glucose to the enzymes are minimized by keeping a low concentration of these sugars in the media. SSF gives higher reported ethanol yields from cellulose than SHF and requires lower amounts of enzyme (Eklund and Zacchi 1995; Karimi et al. 2006a; McMillan et al. 1999; Sun and Cheng 2002). The risk of contamination in SSF is lower than in the SHF process, since the presence of ethanol reduces the possibility of contamination. Furthermore, the number of vessels required for SSF is reduced in comparison to SHF, resulting in lower capital cost of the process.

An important strategy in SSF is to have the optimum conditions for the enzymatic hydrolysis and fermentation as close as possible, particularly with respect to pH and temperature. However, the difference between optimum temperatures of the hydrolyzing enzymes and fermenting microorganisms is still a drawback of SSF. The optimum temperature for cellulases is usually between 45 and 50 °C, whereas *S. cerevisiae* has an optimum temperature between 30 and 35 °C and is practically inactive at more than 40 °C. The optimum temperature for SSF by using *T. reesei* cellulase and *S. cerevisiae* was reported to be around 38 °C, which is a compromise between the optimal temperatures for hydrolysis and fermentation (Tengborg 2000). Hydrolysis is usually the rate-limiting step in SSF (Philippidis and Smith 1995). Several thermotolerant bacteria and yeasts, e.g. *Candida acidothermophilum* and *Kluyveromyces marxianus* have been proposed for use in SSF to raise the temperature close to the optimal temperature of hydrolysis

(Ballesteros et al. 2004; Golias et al. 2002; Hari Krishna et al. 2001; Hong et al. 2007; Kadam and Schmidt 1997).

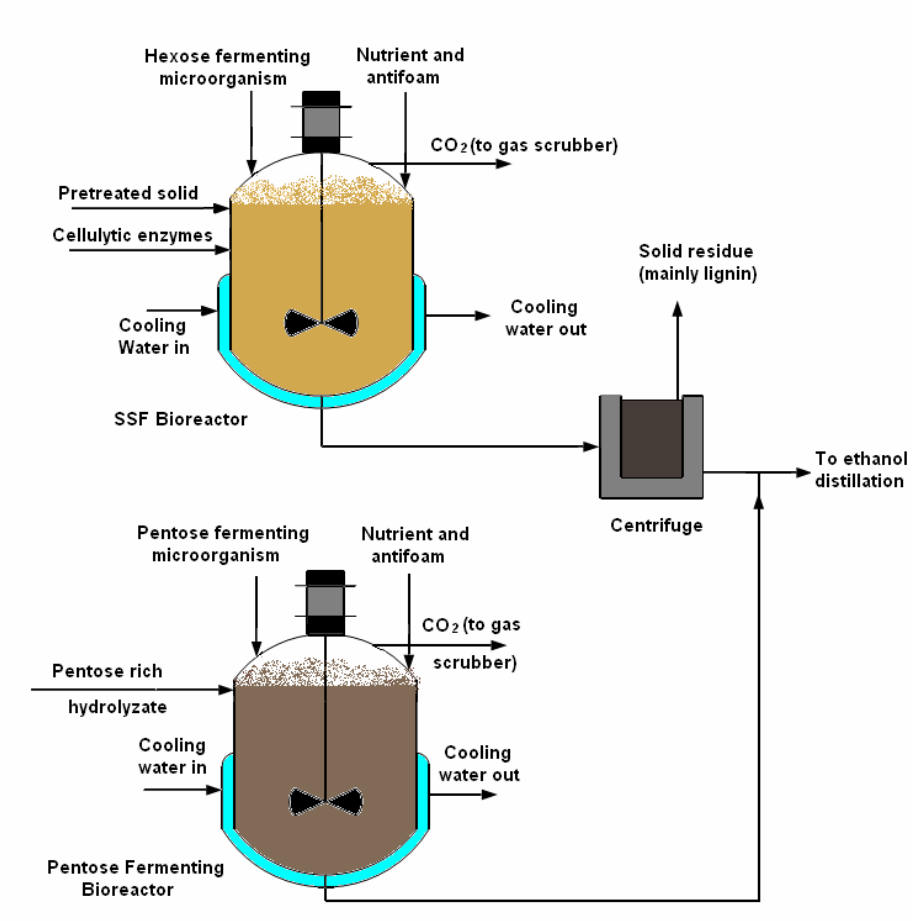

Fig. 4. Simplified process flow diagram for simultaneous saccharification and fermentation

Inhibition of cellulase by produced ethanol might be also a problem in SSF. It was reported that 30 g/l ethanol reduces the enzyme activity by 25% (Wyman 1996). Ethanol inhibition may be a limiting factor in producing high ethanol concentration. However, there has been less attention to ethanol inhibition of cellulase, since practically it is not possible to work with very high substrate concentration in SSF because of the problem with mechanical mixing and insufficient mass transfer. Despite the mentioned problems, SSF is the preferred method in many laboratory studies and pilot scale studies for ethanol production.

In the case of ethanol production from hardwood and agriculture residues, the hemicellulose mainly contains pentoses. If the pentose is separated during the pretreatment, the pentose-rich hydrolyzate (hemicellulosic hydrolyzate) can be converted to ethanol in a separate pentose-fermenting bioreactor (Fig. 4).

Nonisothermal Simultaneous Saccharification and Fermentation (NSSF)

The enzymatic hydrolysis reaction in the SSF process is operated at a temperature lower than the optimum level of enzymatic hydrolysis. This forces the enzyme activity to be far below its potential, which results in raising the enzyme requirement. In order to overcome this problem, a nonisothermal simultaneous saccharification and fermentation process (NSSF) was suggested (Wu and Lee 1998). In this process, saccharification and fermentation occur simultaneously but in two separate reactors at different temperatures (Fig. 5). The lignocellulose is retained inside a hydrolysis reactor and hydrolyzed at the optimum temperature for the enzymatic reactions (e.g. 50 °C). The effluent from the reactor is recirculated through a fermentor, which runs at its optimum temperature (e.g. 30 °C). The cellulase activity is increased 2-3 times when the hydrolysis temperature is raised from 30 to 50 °C.

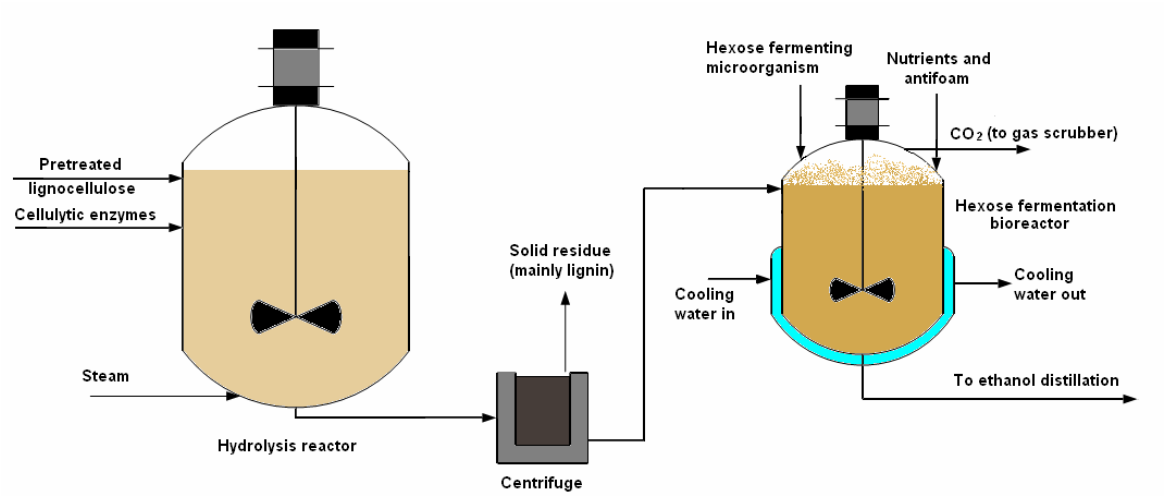

Fig. 5. Simplified process flow diagram for nonisothermal simultaneous saccharification and fermentation process (NSSF)

The NSSF process has improved the kinetic enzymatic reaction compared to SSF, resulting in reduction of the overall enzyme requirement by 30-40%. It is suggested that the effect of temperature on ß-glucosidase activity is the most significant among the individual cellulase enzymes. Higher ethanol yield and productivity have been observed in the NSSF compared to SSF at an enzyme loading as low as 5 IFPU/g glucan. With 10 IFPU/g glucan, improvement in productivity was clearly observed for the NSSF. Besides, the overall time in NSSF was significantly lower than SSF. The terminal yield, which has been obtained in 4 days with the SSF, was obtained in 40 h with the NSSF (Wu and Lee 1998).

Varga et al. (2004a) suggested another form of NSSF for production of ethanol from pretreated corn stover. In the first step, small amounts of cellulases were added at 50 °C, the optimal temperature of enzymes, in order to obtain better mixing conditions due to some liquefaction. To maximize the solid concentration, the prehydrolysis step was carried out in fed-batch manner to obtain better mixing conditions by some liquefaction of the cellulase containing substrate. In the second step, more cellulases were added in combination with the fermenting organism, *S. cerevisiae*, at 30 °C. This method

made it possible to carry out the SSF at a higher dry matter content, and is referred to as nonisothermal SSF. This process can be compared with a similar suggestion made by Kádár et al. (2004). They proposed 24 h prehydrolysis at 50 °C prior to inoculation with *S. cerevisiae* or *Kluyveromyces marxianus*. After the prehydrolysis, the media was inoculated with yeast cells and incubated at 30 °C. They compared the results of the NSSF method with traditional SSF at 40 °C for both microorganisms. Their results showed that the NSSF operation did not increase the ethanol yield at all, and slightly lower values were obtained compared to SSF with both microorganisms. However, they did not check the NSSF at higher temperature in the latter stage. Although 30 °C is suitable for fermentation, the activity of cellulase is very low at this temperature, which could result in incomplete hydrolysis of cellulose.

Simultaneous Saccharification and Cofermentation (SSCF)

Another mode of operation is simultaneous saccharification and cofermentation (SSCF), in which cofermentation refers to the fermentation of both five-carbon and six-carbon sugars to ethanol. The hydrolyzed hemicellulose during pretreatment and the solid cellulose are not separated after pretreatment, allowing the hemicellulose sugars to be converted to ethanol together with SSF of the cellulose (Teixeira et al. 2000).

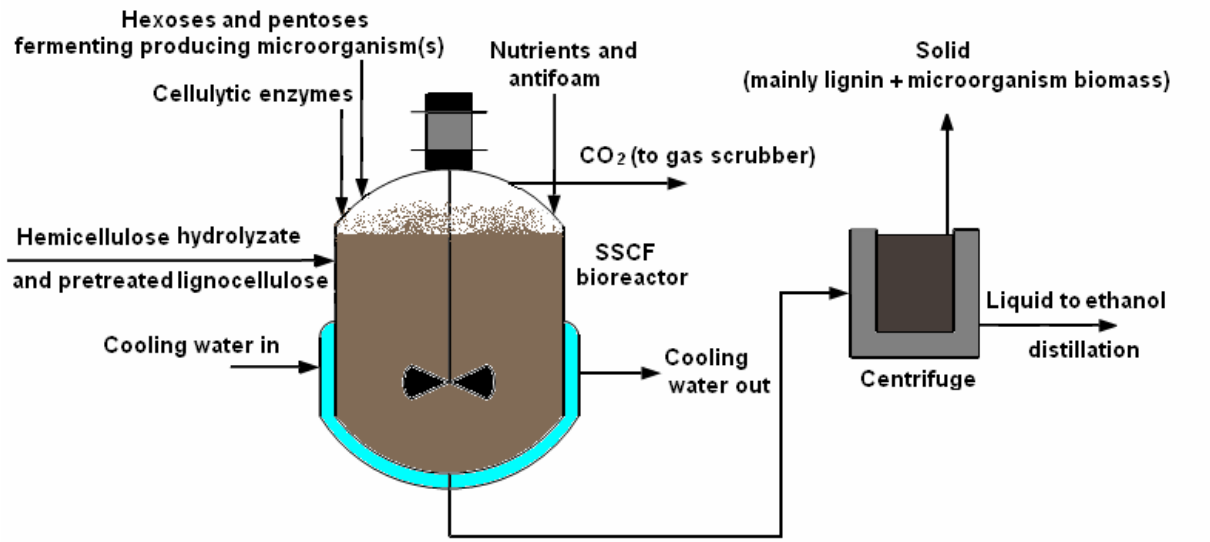

Fig. 6. Simplified process flow diagram for simultaneous saccharification and cofermentation (SSCF)

The SSCF process is considered to be an improvement to SSF (Hamelinck et al. 2005) and is meanwhile being tested at pilot scale by the U.S. Department of Energy. In SSF bioreactor, only hexoses are converted to ethanol, and pentoses can be fermented in another bioreactor with different microorganism. Therefore, two bioreactors and two biomass production setup is required in SSF. In SSCF process, it is suggested to ferment both hexoses and pentoses in a single bioreactor with a single microorganism. Therefore, only a single fermentation step is required to process hydrolyzed and solid fractions of the pretreated lignocellulose (McMillan 1997).

Lawford and Rousseau (1998) used a metabolically engineered strain of *Zymomonas mobilis* that can coferment glucose and xylose, developed in the National Renewable Energy Laboratory (NREL) for ethanol production by SSCF from a synthetic hardwood prehydrolyzate and glucose. McMillan et al. (1999) used an adapted variant of the NREL xylose-fermenting *Z. mobilis* for ethanol production from dilute-acid-pretreated yellow poplar by SSCF. The integrated system produced more than 30 g/l ethanol and achieved 54% conversion of all potentially available sugars in the biomass (total sugars) entering SSCF. Kim et al. (2006b) used a recombinant *E. coli* in the SSCF of corn stover, which was pretreated by ammonia. Both the xylan and glucan in the solid were effectively utilized, giving an overall ethanol yield of 109% of the theoretical maximum based on glucan, a clear indication that at least some of the xylan content was being converted into ethanol. Teixeira et al. (2000) used a recombinant strain of *Z. mobilis* for ethanol production from hybrid poplar wood and sugarcane bagasse. The biomasses were pretreated by peracetic acid combined with an alkaline pre-pretreatment. The SSCF with the recombinant strain resulted in ethanol yields of 92.8 and 91.9% of theoretical from pretreated hybrid poplar wood and sugarcane bagasse, respectively.

A complete process design and its economic evaluation for production of ethanol from corn stover is being analyzed by Aden et al. (2002) in the National Renewable Energy Laboratory (NREL). The process applies dilute acid process for pretreatment and SSCF process for conversion of glucose and xylose to ethanol. The process design also includes feedstock handling and storage, product purification, wastewater treatment, lignin combustion, product storage, and all other required utilities.

Consolidated Bioprocessing (CBP)

In all of the processes considered up to this point, a separate enzyme production unit operation is required, or the enzymes should be provided externally. In consolidated bioprocessing (CBP), ethanol together with all of the required enzymes is produced in a single bioreactor by a single microorganism's community (Fig. 7). The process is also known as direct microbial conversion (DMC). It is based on utilization of mono- or co-cultures of microorganisms which ferment cellulose to ethanol. CBP seems to be an alternative approach with outstanding potential and the logical endpoint in the evolution of ethanol production from lignocellulosic materials. Application of CBP entails no operating costs or capital investment for purchasing enzyme or its production (Hamelinck et al. 2005; Lynd et al. 2005).

Two potential paths have been identified for obtaining organisms for use in CBP. The first path involves modification of excellent ethanol producers, so that they also become efficient cellulase producers, while the second path involves modification of excellent cellulase producers, so that they also become efficient ethanol producers (Lynd et al. 2005). Cellulase production, ethanol tolerance, and ethanol selectivity are considered for both Path 1 and Path 2 organisms (Hogsett et al. 1992). In the past, several cellulolytic anaerobes have been isolated and characterized for potential technology development for fuel or chemical production by CBP of lignocellulosic materials (Lee 1997). This type of activity is shown by various anaerobic thermophilic bacteria, such as *Clostridium thermocellum*, as well as by some filamentous fungi, including *Neurospora*

crassa, *Monilia SP.*, and *Paecilomyces SP.* However, the fermentation process utilizing these microorganisms was very slow (e.g. 3-12 days), and resulted in poor yield of ethanol (Szczodrak and Fiedurek 1996). Furthermore, several by-products, primarily lactic and acetic acids, are produced.

In order to obtain high ethanol yield from the lignocellulosic materials by CBP, intensive research for new thermophilic strains has been carried out. Microorganisms have been sought that do not produce organic acids and are more resistant to higher ethanol concentrations (Szczodrak and Fiedurek 1996; Wyman 1996). South et al. (1993) compared the conversion efficiencies of pretreated hardwood to ethanol in SSF process by *T. reesei* cellulase and *S. cerevisiae* and in a CBP process by *C. thermocellum* through continuous experiments. The SSF system achieved substrate conversions varying from 31% in 9 h retention time to 86% at 48 h retention time. At comparable substrate concentrations (4-5 g/l) and residence times (12-14 h), substrate conversion in the CBP system (77%) was significantly higher than that in the SSF system (31%).

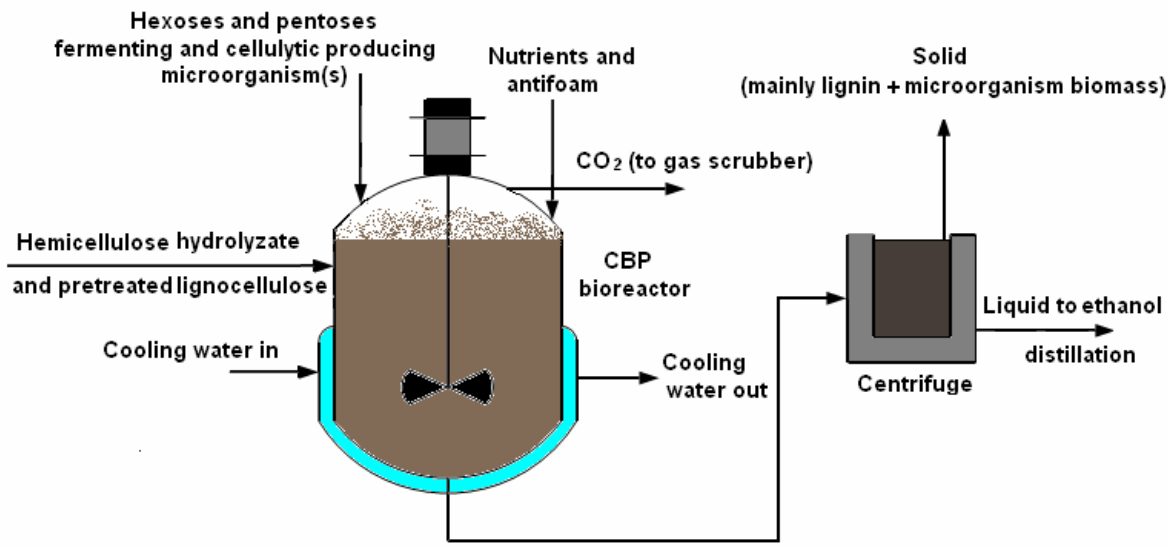

Fig. 7. Simplified process flow diagram for consolidated bioprocessing (CBP)

There are as yet no organisms or compatible combinations of microorganisms available that produce cellulase and other enzymes at the required high levels and also produce ethanol at the required high yields and concentrations, although various organisms already combine multiple functions (Hamelinck et al. 2005). Fujita et al. (2004) constructed a whole-cell biocatalyst with the ability to induce synergistic and sequential cellulose hydrolysis reaction through codisplay of three types of the cellulolytic enzyme on the cell surface of *S. cerevisiae*. Efficient direct fermentation of cellulose to ethanol was achieved by developing the yeast strain. A yield (in grams of ethanol produced per gram of carbohydrate consumed) as high as 0.45 g/g was obtained, which corresponds to 88.5% of the theoretical yield. This indicates that simultaneous and synergistic saccharification and fermentation of the cellulose to ethanol can be efficiently accomplished by using an ethanol-producing yeast strain codisplaying the three cellulolytic enzymes.

Den Haan et al. (2007) developed a recombinant strain of *S. cerevisiae* which can be used for CBP. Two cellulose-encoding genes, an endoglucanase of *T. reesei*, and the β-glucosidase of *Saccharomycopsis fibuligera*, in combination, were expressed in *S. cerevisiae*. The resulting strain was able to grow on cellulose by simultaneous production of sufficient extracellular endoglucanase and β-glucosidase. They demonstrated the construction of a yeast strain capable of growing and of converting cellulose to ethanol in one step, representing significant progress towards realization of one-step processing of lignocellulose in a CBP configuration.

BY-PRODUCTS IN ETHANOL FROM LIGNOCELLULOSIC MATERIALS

The fermented broth or "mash" should be further processed toward pure ethanol. Downstream processing of the produced ethanol depends on the method of ethanol production and the fermentation broth composition. In addition to water and ethanol, the mash contains a number of other materials that we can classify into microbial biomass, fusel oil, volatile components, and stillage. In the SSF, NSSF, SSCF, and CBP processes, residual lignin is also available in the mash.

The main by-product of the process of ethanol production from lignocellulosic materials is lignin. Lignocelluloses contain typically 10-30% lignin; however, its amount and quality in the solid residue differ with feedstock and the applied processes. Part of the lignin might be solubilized during the pretreatment process (e.g. during dilute-acid hydrolysis) or possibly degraded by lignin-degrading enzymes, which are usually present in commercial cellulases. Lignin-degrading enzymes have been found in the extracellular filtrates of many white-rot fungi (Sun and Cheng 2002).

Production of co-products from lignin is important in order to reduce the environmental effects of the ethanol process and increase its competitiveness. Lignin can be gasified into several chemicals and fuels (Osada et al. 2004; Yoshida and Matsumura 2001). NREL has developed a conceptual design for a process that converts lignin into a hydrocarbon that can be used as a high-octane automobile fuel additive. In the first stage of the NREL process, alkali catalyzes depolymerization and breaks the lignin polymers into phenolic intermediates that can be hydroprocessed into the final product. The depolymerized lignin is a mixture of alkoxyphenols, alkylated phenols, and other hydrocarbons. In the second stage, the depolymerized lignin is subjected to a two-step hydroprocessing reaction to produce a reformulated hydrocarbon gasoline product (Montague 2003). Lignin also can replace phenol in the widely used phenol-formaldehyde resins (Pérez et al. 2007). However, both costs of production and market value of these products are complex (Hamelinck et al. 2005). The residual solids of the process (lignin, residual cellulose and hemicellulose) can be efficiently used for heating, cooling, and electricity generation, which can be partly used within the process and partly as final products to the market.

Fusel oil is another by-product of the process. This oil needs to be separated only in order to produce a potable and pharmaceutical grade of ethanol, but not for production of fuel ethanol. The dominant components in fusel oil are found to be a mixture of primary methyl propanol and methyl butanol, formed from α-ketoacids, derived from or

leading to amino acids. Depending on the resources used, the important components of fusel oil might be isoamyl alcohol, n-propyl alcohol, n-butyl alcohol, isobutyl alcohol, sec-butyl alcohol, active amyl alcohol, isoamyl alcohol, and n-amyl alcohol. The amount of fusel oil in the fermented broth depends strongly on the pH of the fermentation. Furthermore, acetaldehyde and trace amounts of other aldehydes and volatile esters are usually produced during fermentation (Kosaric et al. 1983; Maiorella 1983).

The biomass of the fermenting microorganisms is a by-product of the ethanol production processes. It is not possible to avoid formation of the biomass during fermentation. In the ethanol production process, it is desirable to recirculate the produced cell mass to various degrees depending on the conditions in the fermentation (Brandberg 2005; Brandberg et al. 2007). Filtration, immobilization, encapsulation, and sedimentation are possible methods for separation of cells from the media and its recirculation (Purwadi 2006; Talebnia and Taherzadeh 2006). However, in the SSF, NSSF, SSCF, and CBP processes it is not easy to separate the cells from the solid residue. The possibility of cell recirculation is one of the advantages of the SHF process.

WASTEWATER TREATMENT

The remaining liquid after distillation of alcohol is the major part of the plant's wastewater, which contains non- or low-volatile fractions of materials. Its composition depends greatly on the type of feedstock. It generally contains residual sugars (e.g. non-fermentable sugars), traces of ethanol, other metabolites produced during fermentation such as glycerol, inhibitors produced during hydrolysis, waxes, fats, and mineral salts (Kosaric et al. 1983). In the actual process, the liquid streams must be partly recirculated to minimize the requirement for fresh water and the production of wastewater. However, the consequence of recirculation is an accumulation of inert materials and of compounds inhibitory to the cellulase enzymes and/or fermenting microorganisms in the process. The degree of recirculation depends on the process conditions. As a rule of thumb, 40-75% of the streams might be safe to recirculate to the process (Alkasrawi et al. 2002; Stenberg et al. 1998b).

Since there is no large-scale process based on the enzymatic hydrolysis for ethanol from lignocellulosic materials, we should still rely on the results from labs and pilot plants while discussing the wastewater. Generally speaking, the characteristics of stillage from cellulosic materials might be comparable with those of conventional feedstocks and, therefore, methods of stillage treatment and utilization applied to conventional feedstocks might also be applicable to cellulosic feedstocks. Two possible exceptions to the similarity of cellulosic and conventional stillage characteristics deserve attention: (a) the potential for higher levels of heavy metals from the acid pretreatment equipment, and (b) the presence of inhibitors, such as hardwood extractives, associated with phenolic compounds present in the feedstock (Wilkie et al. 2000). The non-circulated stillage and other wastewater in the plant, such as condensed pretreatment flash vapor, cooling tower blowdown, boiler blowdown, and CIP wastewater, could be concentrated by evaporation. The concentrated wastewater could be incinerated or neutralized with alkali, followed by incorporation into some special application such as

road-building materials (Davies 1946). Similar to other substrate stillage, the stillage of lignocellulosic materials may be used for production of potentially viable biological products including enzymes (Morimura et al. 1994; Morimura et al. 1991), chitosan (Yokoi et al. 1998), astaxanthin (Fontana et al. 1997), and single cell protein (Cabib et al. 1983; Kujala et al. 1976). Anaerobic digestion can be used as an effective process for removing COD from stillage and converting it to biogas, which is a readily usable fuel for the ethanol facilities (Wilkie et al. 2000).

COMMERCIAL STATUS

Several companies and government-funded laboratories have already engineered enzymes and microorganisms to optimize lignocellulose hydrolysis and help turn it into fuel (Service 2007). Pilot plant and commercial-scale facilities for converting lignocellulosic biomass to ethanol have existed since the mid-1900s. However, all these early plants used acids for hydrolysis of cellulose to ethanol, while enzymatic conversion technologies are on the agenda of the new plants (Nguyen et al. 1996).

Several cellulosic-based ethanol production companies are to be built in the near future. The U.S. Department of Energy (DOE) announced awards of $385 million for six commercial-scale cellulosic-ethanol biorefineries that are expected to produce more than 500 million liters of ethanol per year. Poet Company (formerly Broin) is probably the largest U.S. dry-mill ethanol producer, with 18 ethanol plants and more than 3.8 billion liters of ethanol annually. The company will expand one of the existing corn-grain ethanol plants in Emmetsburg, Iowa, to produce approximately 100 million liters of ethanol per year from corncobs and other cellulosic feedstock. The facility in Emmetsburg is expected to be operational by 2009. Recently, DOE awarded Poet an $80 million grant to fund its new cellulosic ethanol facility. Named Project LIBERTY, the biorefinery is part of a $200-million expansion of Poet's two-year-old Voyager ethanol plant in Emmetsburg. Furthermore, "BlueFire Ethanol" from waste wood, "Alico" from wood and agriculture wastes, "Abengoa Bioenergy" from corn stover, wheat straw, etc., "Iogen Biorefinery" from agricultural wastes, and "Range Fuels" from waste wood and energy crops are among the companies which have expected to start cellulosic-based ethanol plants in the next 2-5 years and received the awards from DOE (Service 2007).

A large pilot plant is run by Iogen (Ottawa, ON, Canada), which is one of the enzyme manufacturers. Iogen's cellulose ethanol process is designed to prove the feasibility of the ethanol process by validating equipment performance and identifying and overcoming production problems prior to the construction of larger plants. The plant is claimed to be able to handle all functions involved in the production of ethanol from lignocellulose, including receipt and pretreatment of up to 40 tonnes per day of wheat, barley and oat straw; cellulose conversion to glucose; fermentation; and distillation. The plant is designed to produce up to 3 million liters of ethanol per year. The yield of cellulose ethanol is claimed to be more than 340 l/ton of the feedstocks.

Another pilot plant for ethanol production from lignocellulosic materials is the NREL bioethanol pilot plant in Golden, Colorado, USA, at a scale of about 900 kilograms per day of dry biomass. This Mini-Pilot Plant is ideal for preliminary testing of

the process at small scale. The pilot equipment includes four 9000-liter, two 1450-liter, and two 160-liter fermentors for enzymatic hydrolysis and fermentation (Nguyen et al. 1996). Since opening in 1994, the bioethanol pilot plant has already been used for a number of cooperative projects to help developing bioprocessing technologies for bioethanol production, and several of them are now going into commercial ethanol production from biomass.

Another pilot plant is SEKAB in Sweden, which has a capacity of 500 liters of ethanol per day. In order to manufacture this quantity, approximately 2 tonnes (dry weight) of wood chips are used. The technology is based on both dilute-acid and enzymatic hydrolysis of cellulose and hemicellulose, whereupon the sugar is fermented to ethanol and purified by distillation. In dilute-acid hydrolysis or pretreatment, sulfuric acid or sulfur dioxide is used as a catalyst at temperatures of around 200 ºC within a two-stage continuous hydrolysis unit. In enzymatic hydrolysis, the material is first treated with dilute acid at mild conditions, after which enzymes hydrolyze the remaining cellulose in a third stage. Both the dilute-acid and enzymatic processes are being evaluated at the plant. In the four fermentors, it is possible to perform the fermentation with fed-batch or continuous technology (www.sekab.com).

SunOpta built the first cellulosic ethanol plant 20 years ago in France. There are four cellulosic ethanol projects that are or will be operational using SunOpta's technology and equipment to produce ethanol from lignocellulose. The company has provided the technology to (a) China Resources Alcohol Corporation in September 2006, and the plant began production of ethanol from local corn stover in October 2006, (b) Spain for the start-up of the Abengoa wheat straw to an ethanol facility located in Salamanca in the summer of 2007, (c) the Celunol facility being built in Jennings, Louisiana, to produce ethanol from wood and sugarcane bagasse, and (d) GreenField Ethanol Inc., Canada's largest producer of ethanol (www.sunopta.com)

Xethanol recently announced aggressive plans for its new BlueRidge facility. The Xethanol company was to begin producing lignocellulosic ethanol in Spring Hope, NC, by February 2007 using acid hydrolysis. It announced plans to construct a 190-million liters per year lignocellulosic ethanol plant in Augusta, GA, which was supposed to begin producing ethanol by mid-2007 (www.xethanol.com). In Soustons, France, there is a pilot plant for steam-explosion pretreatment of lignocellulose with large fermentors (e.g. 30-50 m^3). A pilot plant at the Voest-Alpine Biomass Technology center used a 3000-liter steam digester either for pretreatment and produced cellulase or for performing saccharification in 15 m^3 fermentors (Nguyen et al. 1996).

Researchers are also looking for improvements in different parts of the process, e.g. development of biocatalysts and process design. A final target for many researchers lies inside plants themselves, whereas some companies and academic groups are working to re-engineer resources such as poplar trees, corn, and switchgrass to boost their yields and make them easier to turn into fuel. Development of engineered poplar trees with 50% less lignin and more cellulose content than conventional varieties is among the efforts in this area (Service 2007).

Cellulase prices have a high impact on ethanol production from lignocellulosic materials. Iogen uses its own proprietary cellulases and is laying plans for a 30-million-gallon-per-year facility with partners such as Royal Dutch / Shell Oil (London and The

Hague) and PetroCanada (Calgary, AB, Canada). Two other enzyme-producing companies, supported by large grants from the DOE, have brought down the costs of the enzymes (Aden et al. 2002). Genencor International (Palo Alto, CA, USA) and the Danish company Novozymes (Bagsvaerd, Denmark) have significantly reduced the cost of cellulases, about 20-fold, to about 4-5 cents per liter. However, the price of cellulase is still high compared to amylases, the enzymes that break down corn starch for fermentation (Schubert 2006).

ENERGY PRODUCTION AND INTEGRATION

Running processes for ethanol production from lignocellulosic source material requires electricity and heat, mainly used for steam generation and for cooling water. It would be ideal if a process can produce the necessary required energy. In ethanol production from lignocelluloses, this can be achieved by using the solid residue, which mainly contains lignin, and the concentrated stillage residue from the evaporation plant. Combustor, boiler, and turbogenerator subsystems can be used to burn various by-product streams for electricity and steam generation (Aden et al. 2002). However, the lignin is a valuable by-product, which could be essential for the process economics. Therefore, it is necessary to minimize the energy demand in the process and thereby increase the fraction of lignin that can be sold as a by-product (Galbe et al. 2007; Galbe and Zacchi 2002). This can be achieved by energy integration. A technology for design of heat exchanger networks, such as pinch technology, can be used for heat integration (Cardona and Sanchez 2007). Grisales et al. (2005) studied heat integration of fermentation and recovery steps for fuel ethanol production from lignocellulosic materials by using the software ASPEN PLUS for the preliminary balances of mass and energy. Pinch technology was employed for optimal design of heat exchanger networks. Application of a proper heat exchange between cold and hot streams can reduce the use of utilities (cooling water and steam). There are diverse possibilities in distillation of the produced ethanol. Heat exchange between the stripping and rectifying distillation columns can be used to reduce the utility consumption in distillation. However, in order to provide a temperature difference that favors heat exchange, the rectifying section should operate at higher pressures than the stripping section (Batista et al. 1998; Cardona and Sanchez 2007). Various process configurations to reduce the energy demand, such as running the distillation integrated with a multiple-effect evaporation unit, were suggested by Larsson et al. (1997). Running the process at higher solids consistency or to recirculated process streams, in order to maintain a high concentration of ethanol and dissolved solids, are other ways to reduce the energy consumption. Higher ethanol concentration would reduce the energy requirements in the distillation, especially in azeotropic distillation, and also in evaporation units (Cardona and Sanchez 2006; Galbe et al. 2007; Galbe and Zacchi 2002).

The remaining wastewater can be considered as another source of energy. Anaerobic digestion of the wastewater results in biogas, which contain 50-70% methane. The biogas can be sold as a by-product or be burned to generate steam and electricity, allowing the plant to be self sufficient in energy. This approach results also in reduction

of disposal costs of the wastes and generates additional revenue through sales of excess electricity.

CONCLUDING REMARKS

During recent years much valuable work has been performed on different aspects of ethanol production from lignocelluloses based on enzymatic hydrolysis, and great achievements have been attained. Starting with a simple SHF process, there are now several advanced alternatives for the process. Numerous commercial plants are in the commissioning stage. With the start of these plants, abundant real data will be obtained and also many questions and problems will arise. Struggling with the problems will open some new areas in ethanol production from lignocelluloses. Process integration and optimization will also improve the energy consumption of the process. Scientists' efforts in reduction of prices of cellulase, as well as progress in optimization of the enzymatic hydrolysis process, will help to improve the economy of the process. Generally, these strivings in basic and applied sciences are essential to lead the world toward clean and reliable sources of energy.

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Article submitted: August 21, 2007; First round of reviewing completed: Sept. 26, 2007; Revised version received: Oct. 11, 2007; Accepted: Oct. 15, 2007; Published Nov. 2, 2007.