CHAPTER

5

Biofuels and Bioproducts Produced through Microbial Conversion of Biomass

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LIGNOCELLULOSIC BIOMASS AND ITS PRETREATMENT

Lignocellulose is the primary building block of plant cell walls and is composed mainly of cellulose, hemicelluloses, lignin and small quantities of pectin, proteins, extractives and ash. The cellulose, hemicelluloses and lignin are present in varying amounts in the different parts of the plant and are intimately associated to form the complex structural framework of the plant cell wall where cellulose and hemicellulose are bound together with lignin and other components to form a tight matrix. The composition of lignocellulose depends on plant species as well as growth conditions and age.

Lignocellulose biomass is a renewable, sustainable, abundant and cheap resource for producing renewable biofuels and bioproducts. However, their conversion into fermentable sugar before fermentation is a major hurdle due to its complex structure and recalcitrant nature. While hydrolysis of cellulose and hemicellulose yields fermentable sugars, they are not easily accessible due to the crystalline structure of cellulose and interference by the phenyl-propanoid polymer, lignin.

Bioconversion of carbohydrates from lignocellulosic feedstocks into fermentable sugars is a key challenge in the biorefinery process. Efficient, cost-effective and environmentally benign pretreatment and hydrolysis methods are required. The primary purpose of pretreatment is to change the architecture of the cell wall by delignification and disrupting the cellulose structure and making the lignocellulosic biomass accessible and reactive to allow high rates and yields on enzymatic hydrolysis. Pretreatment has been considered as one of the most expensive processing steps in biomass to fermentable sugar conversion (Mosier et al., 2005).

This article focuses mainly on biological conversion of biomass with microorganisms. However, nonbiological pretreatments, as well as the most frequently studied and applied procedures, will also be discussed.

Nonbiological Pretreatment

A variety of nonbiological pretreatment methods have been extensively reviewed. These include physical, chemical, physicochemical and other combinations of procedures (Alvira et al., 2010; Chandra et al., 2007; da Costa Sousa et al., 2009; Hendriks and Zeeman, 2009; Sun and Cheng, 2002; Taherzadeh and Karimi, 2008). Based on their effects on biomass structure, pretreatments can be divided into different categories: those that increase enzyme accessibility to crystalline cellulose by decreasing the fiber's degree of polymerization or by facilitating hemicellulose and/or lignin removal to create pores in the cellulose fibrils. Since hemicellulose and lignin are the two main protective coats surrounding cellulose, they have to be removed or altered in order to achieve fast enzymatic hydrolysis of the biomass. However, to obtain high sugar yield for both hexoses and pentoses, an ideal pretreatment procedure should efficiently remove or modify lignin and also hydrolyze hemicellulose, but not degrade these hemicellulose sugars (Ohgren et al., 2007). Some of the most widely investigated procedures are briefly described.

Physical Pretreatments

These include mechanical methods to chip, grind and mill the biomass to reduce particle size and, potentially, the crystallinity and degree of polymerization of lignocellulose in order to maximize the downstream enzyme hydrolysis process (Tassinari et al., 1980). Recently, a novel extrusion method was developed where the biomass materials are subjected to heating, mixing and shearing to cause both physical and chemical modifications to the material in order to increase cellulose accessibility (Karunanithy and Muthukumarappan, 2010a,b; Karunanithy et al., 2012).

Chemical Pretreatments

These are mainly alkali and acid pretreatments. Alkali pretreatments increase cellulose digestibility by enhancing lignin solubilization and decreasing cellulose crystallinity. This method is more effective on agricultural biomass than on wood material (Kumar et al., 2009; Playne, 1984). Acid pretreatment, mostly diluted acid pretreatments, increase cellulose accessibility mainly by solubilizing hemicellulose. It can be used as either a pretreatment or a direct hydrolysis process but leads to toxic degradation products that inhibit downstream fermentation (Alvira et al., 2010). On the contrary, ozonolysis uses the powerful oxidant ozone to delignify lignocellulosic materials at room temperature and does not form inhibitory compounds, yet it is economically unviable due to large amounts of ozone consumed (Sun and Cheng, 2002). On the other hand, organosolv process can efficiently remove lignin and result in minimal cellulose loss. This is a promising process if economic solvents are available at commercial scales (Wood and Saddler, 1988; Zhao et al., 2009).

Physicochemical Pretreatments

Steam explosion is the most studied and commonly used physicochemical method and extensively reviewed (Hsu, 1996; McMillan, 1994; Saddler et al., 1993). During this hydrothermal procedure, biomass is subjected to pressurized steam for a short time and then suddenly depressurized. The process leads to hemicellulose degradation and lignin transformation and as a result, increases pore volumes in the pretreated biomass, leading to enhanced enzymatic accessibility (Grous et al., 1986). It is recognized as one of the most cost-effective

processes for hardwoods and agricultural residues, but less effective for softwoods (Sun and Cheng, 2002). Another disadvantage is the production of inhibitory compounds. Addition of diluted acids can decrease pretreatment time and temperature thus reducing the production of inhibitory compounds and also enhancing softwood pretreatment efficiency (Ballesteros et al., 2006; Duff and Murray, 1996; Jørgensen, 2007; Kumar et al., 2009; Stenberg et al., 1998). As a relatively energy and environmentally friendly procedure, steam explosion had been scaled up and used in pilot-scale production at Iogen (Canada) and is to be used in many of the planned commercial size facilities worldwide.

Other physicochemical methods explored include ammonia fiber explosion (AFEX) (Alizadeh et al., 2005; Teymouri et al., 2004, 2005), carbon dioxide explosion (Zheng et al., 1995, 1998), liquid hot water (LHW) pretreatment (Kim et al., 2009; Mosier et al., 2005), ultrasound pretreatment (Gonzalez-Fernandez et al., 2012; Sasmal et al., 2012), and microwave pretreatment (Azuma et al., 1984; Ma et al., 2009; Ooshima et al., 1984).

For practical application, different pretreatment methods have to be tested for each specific biomass to determine the best procedure that is compatible with the downstream hydrolytic enzyme cocktail. For example, in a recent report describing switchgrass hydrolysis, different pretreatment methods were tested including ammonia fiber expansion (AFEX), dilute acid (DA), LHW, lime, lime + ball milling, soaking in aqueous ammonia, and sulfur dioxide (SO_2). It was demonstrated that lime + ball milling lead to the highest overall sugar yield (98.3%) from pretreated biomass with xylanase addition (Falls et al., 2011).

Biological Pretreatment with Microorganisms

Potential Advantages over Nonbiological Pretreatment

Microbial pretreatment by solid state cultivation (SSC) has the potential to be a low-cost, environmentally friendly alternative to chemical approaches. Existing nonbiological pretreatment methods as described above have largely been developed on the basis of physicochemical technologies such as steam explosion, microwave radiation, ionizing radiation, dilute acid, alkali, and oxidation or various combinations of these methodologies (Mosier et al., 2005). Most of these methods require expensive, complicated, high-pressure and corrosion-resistant equipment and may consume large amounts of energy and water. Furthermore, chemical pretreatments can be detrimental to subsequent enzymatic hydrolysis and microbial fermentation in addition to producing acidic or alkaline waste water, which requires predisposal treatment to ensure environmental safety (Keller et al., 2003). Due to its low energy and material costs, mild reaction conditions with simple equipment, and environmental benefits, microbial/biological pretreatment has received increased attention as an alternative to physicochemical or thermochemical pretreatments (Kumar and Wyman, 2009; Rabinovich et al., 2004; Sanchez, 2009; Saritha et al., 2012a; Shi et al., 2008; Sun and Cheng, 2002; Zeng et al., 2011).

Biological Degradation of Lignin

Lignin is a complex, heterogeneous phenylpropanoid polymer that is linked to both hemicelluloses and cellulose to form an impenetrable physical and chemical barrier for biodegradative systems (Sanchez, 2009; Blanchette, 1991). Unless lignin is modified or removed, hydrolytic enzymes cannot penetrate and effectively degrade woody substrates. In addition to producing the extracellular polysaccharide degradative enzymes, such as cellulases, xylanases, and mannanases, saprophytic fungi have a unique oxidative and extracellular lignolytic system called Fenton's reagents to degrade lignin and open phenyl rings (Green and Highley, 1997; Jensen et al., 2001; Arantes et al., 2012; Contreras et al., 2007; Irbe et al., 2011; Kramer et al., 2004; Ray et al., 2010; Suzuki et al., 2006; Yanase et al., 2010b). In addition to cellulase and hemicellulases, lignolytic enzymes have also been detected in some strains. Particularly, species among the Basidiomycotina fungi that cause white rots of wood may simultaneously degrade lignin and cell wall carbohydrates (Sanchez, 2009). Furthermore, a small number of the white-rot fungi preferentially degrade lignin leading to little to no loss of cellulose (Blanchette, 1991). For practical applications, these species that can selectively remove lignin without extensive cellulose degradation are of special interest. The most widely studied white-rot fungus, *Phanerochaete chrysosporium*, can significantly degrade lignin and simultaneously degrade a small fraction of cellulose and hemicellulose, whereas others such as *Ceriporiopsis subvermispora* tend to remove lignin in advance of cellulose and hemicellulose (Blanchette et al., 1992; Hatakka, 1994; Sanchez, 2009).

COMMONLY USED MICROORGANISMS FOR BIOLOGICAL PRETREATMENT

Microbial pretreatment makes use of microorganisms and their enzyme systems to breakdown lignin and/or hemicellulose present in lignocellulosic biomass. So far, the isolated and identified lignocellulolytic microorganisms mainly include fungi and a few bacterial strains. Fungi including brown-, white-, and soft-rot fungi are the predominant organisms responsible for lignocellulose degradation, and among the fungi, the Basidiomycetes that cause both white and brown rots

are the most rapid degraders (Bennet et al., 2002; Loguercio-Leite et al., 2008; Rabinovich et al., 2004; Sanchez, 2009; ten Have and Teunissen, 2001). Several Basidiomycetes such as *P. chrysosporium*, *C. subvermispora*, *Phlebia subserialis*, *Pleurotus ostreatus*, and *Irpex lacteus* have been shown to efficiently degrade lignin in different lignocellulosic materials (Hatakka and Usi-Rauva, 1983; Keller et al., 2003; Sawada et al., 1995; Taniguchi et al., 2005; Zeng et al., 2011).

Natural Microorganisms and Practical Applications in Bioconversion

Application of White-Rot Fungus in Treatment of Different Biomasses

CORN STOVER

When corn stover is pretreated with *C. subvermispora* for downstream bioethanol production, lignin is selectively degraded up to 31.59% with a limited cellulose loss of less than 6% during an 18-day pretreatment. Longer pretreatment time was found to increase lignin removal, resulting in correspondingly higher glucose yields from enzymatic hydrolysis. The highest overall ethanol yield of 57.80% was obtained with 35-day-pretreated corn stover (Wan and Li, 2010).

In a later study, the effectiveness of *C. subvermispora* pretreatment on different types of feedstocks, including corn stover, wheat straw, soybean straw, switchgrass, and hardwood was tested. After an 18-day pretreatment, corn stover, switchgrass, and hardwood were effectively delignified, leading to a two- to threefold increase in glucose yield over those of the untreated raw materials. In contrast, wheat straw and soybean straw did not show glucose yield increase after undergoing the same pretreatment, suggesting the importance of using a specific strain for pretreatment of specific biomass (Wan and Li, 2011).

Pretreatments of corn stover with the white-rot fungus *I. lacteus* CD2 also resulted in significant lignin degradation with limited cellulose loss (Zeng et al., 2011). Pretreatment of corn stover with *Cyathus stercoreus* led to a three- to fivefold improvement in enzymatic cellulose digestibility (Keller et al., 2003). Pretreatment of corn stover with a newly isolated white-rot fungus, *Trametes hirsuta* yj9, led to selective lignin degradation up to 71.49% and a significant increase in enzymatic digestibility of 73.99% after a 42-day pretreatment (Sun et al., 2011). Pretreatment of corn stover fractions (leaves, cobs, and stalks) with the white-rot fungus *C. subvermispora* showed that the leaves were the least recalcitrant to fungal pretreatment with a 45% lignin degradation as well as higher carbohydrate degradation after 30 days of pretreatment. However, corn cobs produced the highest sugar yield after fungal pretreatment (Cui et al., 2012).

SOFTWOOD

The effect of pretreatment on the softwood *Pinus densiflora* by three white-rot fungi, *Ceriporia lacerata*, *Stereum hirsutum*, and *Polyporus brumalis,* has been investigated. Among the three white-rot fungi tested, *S. hirsutum* selectively degraded the lignin rather than the holocellulose component. Consistently, extracellular enzymes from *S. hirsutum* showed higher activity of ligninase and lower activity of cellulase than those from the other white-rot fungi. In addition, the available pore size and surface area in the pretreated wood were increased, possibly due to degradation of lignin and a small portion of hemicellulose by the secreted enzymes. Sugar yield of the *S. hirsutum* pretreated wood also greatly increased compared to a nonpretreated sample, indicating *S. hirsutum* might be a potentially effective fungus for use in biological pretreatment of woody biomass (Lee et al., 2007).

COTTON STALKS

Conditions for pretreatment of cotton stalks using *P. chrysosporium* by SSC have also been explored. While substrate moisture content significantly affects lignin degradation, supplementation with modified salts did not affect the reaction process. Over a period of 14 days, SSCat 75% moisture content without salts resulted in 27.6% lignin degradation, 71.1% solids recovery and 41.6% availability of carbohydrates, suggesting that microbial pretreatment by SSC has the potential to be a low-cost, environmentally friendly alternative to chemical approaches (Shi et al., 2008).

RICE STRAW

Fungal pretreatment of rice straw for improved enzymatic saccharification has been reported. Yamagishi et al. (2011) tested 17 *C. stercoreus* isolates for their ability to treat rice straw for improved enzymatic hydrolysis. A negative correlation was found between cellulase and xylanase activity in these isolates and enzymatic saccharification yields in the pretreated straw. A 25-day pretreatment with the strain *C. stercoreus* TY-2 led to a more than fivefold increase in enzymatic saccharification yield compared to untreated control samples, suggesting this isolate has the potential for biological pretreatment of rice straw under conditions of low energy input. A 15-day pretreatment of rice straw with *P. chrysosporium* in an optimized media resulted in a treated biomass with an enzymatic digestibility of 64.9% of the theoretical maximum glucose yield. When the fungal-pretreated rice straw was used as a substrate in simultaneous saccharification and fermentation (SSF), a 9.49 g/l ethanol concentration, 58.2% of the theoretical maximum production yield, and 0.40 g/l/h productivity were achieved after 24 h and a 62.7% of the theoretical maximum ethanol yield was expected after 96 h (Bak et al., 2009).

When rice straw was pretreated with the wood-rot fungus, *Dichomitus squalens,* for 15 days, an enzymatic digestibility of 58.1% of theoretical glucose yield was reached for the treated biomass. When the pretreated rice straw was used as a substrate for ethanol production in SSF, the ethanol production yield and productivity were 54.2% of the theoretical maximum and 0.39 g/l/h, respectively, after 24 h (Bak et al., 2009). Taniguchia et al. (Taniguchi et al., 2005) reported the effect on rice straw composition and susceptibility to enzymatic hydrolysis after pretreatment with four white-rot fungi (*P. chrysosporium*, *Trametes versicolor*, *C. subvermispora*, and *P. ostreatus*). Among the four strains, *P. ostreatus* selectively degraded the lignin fraction of rice straw rather than the cellulose component. A 60-day pretreatment of rice straw with *P. ostreatus* led to a total weight loss of 25% and 41% lignin degradation, but only a 17% loss of cellulose and a 48% loss of hemicellulose. A 48-h enzymatic hydrolysis lead to 52% holocellulose and 44% cellulose solubilization in the pretreated rice straw corresponding to a net sugar yield of 33% from holocellulose and 32% from cellulose.

PADDY STRAW

A recent report of a study on the pretreatment of paddy straw with the white-rot fungus *T. hirsuta* (Microbial Type Culture Collection) MTCC 136 showed high ligninase and low cellulase activities. It showed that within 10 days of solid state fermentation, the carbohydrate content was enhanced by 11.1% and a much higher yield of sugars was obtained after enzymatic hydrolysis. Saccharification efficiency of the biologically pretreated paddy straw with the commercial enzyme Accellerase®1500 reached 52.69% within 72 h suggesting the delignification potential of *T. hirsuta* for pretreatment of lignocellulosic substrate and facilitating efficient enzymatic digestibility of cellulose (Saritha et al., 2012b).

White-Rot Fungus Pretreatment of Biomass for Animal Feed

Pretreatment of lignocellulosic biomass with the white-rot fungi increases biodegradability and leads to high-quality ruminant feed. For example, white-rot fungi-treated cedar wood shows significant improvement for rumen digestibility (Okano et al., 2005). When high-lignin forages such as grass, oat straw and alfalfa stems were treated with various white-rot fungi, substantial improvements in digestibilities have also been obtained (Akin et al., 1995, 1993; Jung et al., 1992).

White-Rot Fungus Pretreatment in Biological Pulping

White-rot fungi have also been used in biological pulping (biopulping) to reduce the utilization of chemicals in the pulping industry and decrease the environmental hazard caused by the traditional pulping process (Singh et al., 2010). Biopulping process removes not only lignin and hemicellulose but also some of the wood extractives. It can also improve paper quality and significantly reduce the electrical energy and cooking time required for pulping wood chips (Ali and Sreekrishnan, 2001; Hunt et al., 2004; Singh et al., 2010). When *C. subvermispora* was used for biopulping of agricultural residues including rice, wheat and barley straw samples, the tensile strength and burst factor of hand sheets produced from the biopulping process improved significantly compared to the chemical process (Yaghoubi et al., 2008). Blanchette et al. (Blanchette et al., 1992) evaluated the potential application in biopulping of 19 strains of *P. chrysosporium* and 9 strains of *C. subvermispora*. For the *P. chrysosporium* isolates, only a few strains preferentially removed large amounts of lignin from wood while the majority of the isolates removed all cell wall components nonselectively. In contrast, all nine isolates of *C. subvermispora* led to moderate weight losses and preferential degradation of lignin in aspen, birch and loblolly pine wood.

White-Rot Fungus Pretreatment of Biomass for Biofiber

Microbial pretreatment can also improve the feature of the fiber in biomass for biocomposite production. For example, corn stalk pretreated with the white-rot fungus *Trametes hirsuta* has been used to produce fiberboard by hot pressing without adhesive. The corn stalk-based fiberboard made of the pretreated biomass has an increase of 3.40- and 8.87-fold in moduli of rupture and elasticity, respectively, over the fiberboard made from untreated corn stalk. Further analyses showed that the increase in the mechanical properties of the fiberboard resulted from the pretreated biomass possessing more than twice the number of hydroxyl groups, an 18% higher crystallinity, and twice the polysaccharide content of untreated corn stalk (Wu et al., 2011).

Brown-Rot Fungi

Brown-rot fungi are Basidiomycete fungi that, unlike white-rot fungi, selectively modify and then completely hydrolyze lignocellulose polysaccharides, typically without secreting an exoacting glucanase and without removing lignin (Schilling et al., 2009; Tewalt and Schilling, 2010). The wood decay resulting from the action of brown-rot fungi leads to an increased volume of pores in the wood cell wall and decreased degree of polymerization of holocellulose along with a dramatic weight loss (Flournoy et al., 1991). Depolymerization of holocellulose occurs rapidly during the early decay process leading to an extensive degradation of holocellulose in wood (Blanchette, 1995; Irbe et al., 2011; Kumar et al., 2009) and as high as 75% wood strength loss even when only 1% weight loss has occurred (Green and Highley, 1997; Richards, 1954; Wilcox, 1978).

The exact mechanism for brown-rot decay is still unclear. For the selective removal of polysaccharides, a two-step procedure has been proposed: a nonenzymatic radical-based modification of the wood cell wall through small molecules, followed by secretion of enzymes to catalyze the breakdown of polysaccharides into their sugar monomers (Green and Highley, 1997; Tewalt and Schilling, 2010). However, cellulose and hemicellulose removal by brown-rot fungi does not open up cell walls to facilitate enzyme penetration (Flournoy et al., 1991). Primarily because enzymes are too large to penetrate the decayed wood, attack by cellulolytic enzymes may only be limited to a localized, superficial area (Baldrian and Valaskova, 2008; Flournoy et al., 1991). It has been proposed that Fenton's reagents and not enzymes are responsible for rapid wood decomposition early in brown-rot decay (Green and Highley, 1997; Jensen et al., 2001; Ray et al., 2010). Other study results also support that hydroxyl radicals (HO˙) generated through Fenton chemistry (H2O2−Fe(II)) initiate lignocellulose breakdown (Arantes et al., 2012; Contreras et al., 2007; Hammel et al., 2002; Kaneko et al., 2005; Kramer et al., 2004; Suzuki et al., 2006). Consequently, this suggests that reactive oxygen species play an important role in the early stages of wood degradation by brown-rot fungi (Irbe et al., 2011). In brown-rot wood decay, hemicellulose is removed considerably faster than cellulose (Curling et al., 2002; Highley, 1987; Monrroy et al., 2011). Consistently, the total secretome hemicellulase expression and activity for brown-rot fungi peak prior to cellulase activity (Lyr, 1960; Martinez et al., 2009).

Hemicellulose is embedded in cellulose microfibrils and its prior removal may facilitate cellulose degradation and removal (Green and Highley, 1997). Continual degradation of holocellulose by brown-rot fungi leads to gradually increased weight loss but the percent crystallinity in decayed wood increases apparently at an early stage, peaks between 2 and 4 weeks and then decreases implying structural changes of cellulose chains during fungal attack (Howell et al., 2009). Towards the end of brown-rot decay, nearly 100% of carbohydrates can be removed; however, most of the lignin remains (Eriksson et al., 1990). Only a small fraction of the lignin is oxidized, demethylated and depolymerized, often leading to lignin-derived volatile components (Ewen et al., 2004; Irbe et al., 2011; Schilling et al., 2012).

Recently, the potential application of brown-rot fungi for the pretreatment of biomass to increase downstream enzymatic hydrolysis has been explored. When spruce and pine woods were treated with one of two brown-rot fungi, *Gloeophyllum trabeum* or *Fomitopsis pinicola*, saccharification efficiency was increased significantly even though total sugar yield was low, probably due to low enzyme loading (Schilling et al., 2009). In another effort, *G. trabeum*-treated pine wood block only led to a maximum 22% glucose release even though 60 FPU Celluclast was loaded, suggesting brown-rot fungus *G. trabeum* modification of pine wood may not be sufficient to increase cellulose accessibility (Tewalt and Schilling, 2010). Similarly, when the brown-rot fungi *G. trabeum* and *Laetoporeus sulphureus* were used for the pretreatment of the wood *Pinus radiate* and *Eucalyptus globules*, the highest glucose yield was 14% after 8 weeks of biodegradation (Monrroy et al., 2011). On the other hand, when *G. trabeum* was used to pretreat different biomass including aspen, spruce, or corn stover, sugar yield was significantly increased up to threefold. In the best case, a 2-week pretreatment of aspen by *G. trabeum* led to a 72% cellulose-to-glucose yield corresponding to 51% yield relative to original glucan. For corn stover, a weak colonization with minor degradation by another tested brown-rot fungus, *Postia placenta,* resulted in more than a twofold increase in sugar yield (Schilling et al., 2012). Similar to wood biomass, when corn stover is pretreated with the brown-rot fungus *Fomitopsis* sp. IMER2, the amorphous regions of the cellulose are preferentially degraded in contrast to the significant lignin degradation by the white-rot fungus *I. lacteus* CD2 (Zeng et al., 2011). In another successful case, simple pretreatment of Scots pine (*Pinus sylvestris*) with the brown rot fungus *Coniophora puteana* for 15 days permitted recovery of greater than 70% of the glucose present in the biomass, with a total wood mass loss of 9%, suggesting great potential for use of this specific group of fungi in lignocellulosic biomass pretreatment (Ray et al., 2010). Brown-rot fungi therefore hold significant potential for practical application in biological pretreatment.

Soft-Rot Fungi

Even though the process of wood decay by many common white- and rot fungi has been well characterized, other types of decay caused by soft-rot fungi or bacteria are still not well understood (Blanchette et al., 2002, 2004). Soft rot is caused by fungi taxonomically classified in the phylum *Ascomycota*, including related asexual taxa. The term soft rot is used because it was first identified from soft, decayed wood surfaces in contact with excessive moisture (Findlay, 1984). Soft rot can also occur in dry environments (Blanchette, 2000) and seems to predominate in extreme environments such as excessively wet or dry sites, where white- and brown-rot fungi growth is inhibited, and in substrates that do not favor the growth and development of other types of fungi (Blanchette, 1995; Blanchette et al., 2004). Soft-rot fungi attack the lignocellulose matrix in wood by formation of cavities (type I) or cell wall erosion (type II). Cellulases and hemicellulases, but not ligninases, are involved in soft-rot attack leading to extensive loss of the carbohydrate polymers; high amounts of lignin remain even in advanced stages of

soft rot (Blanchette, 1995; Eriksson et al., 1990; Nilsson et al, 1989). The most studied and applied soft-rot fungus, *Trichoderma reesei*, and its mutants, are mainly used for large-scale commercial production of cellulases and hemicellulases (Durand et al., 1988; Esterbauer et al., 1991; Tomme et al., 1988).

Bacteria

Bacteria degrade plant cell walls through three main morphological forms: tunneling, erosion, and cavitation (Blanchette, 1995; Daniel et al., 1987; Singh and Butcher, 1991, 1985; Singh et al., 1990). An early study has confirmed that the Gram-positive filamentous bacterium *Streptomyces viridosporus* degrades softwood lignin into low molecular weight fragments (Crawford et al., 1982). Furthermore, enzymes similar to the fungal system such as peroxidases, ligninases and manganese peroxidases have been implicated in bacterial biomass delignification (Glenn and Gold, 1983; Kirk et al., 1986). Interestingly, some bacteria can attack high lignin-containing hard wood that is considered durable and resistant to fungal decay (Nilsson et al., 1992; Singh and Butcher, 1991). However, compared to fungi, bacteria are not as efficient for lignocellulosic biomass pretreatment, as shown by a recent work comparing eight microorganisms including fungi and bacteria, for pretreatment of sugarcane waste (Singh et al., 2008).

Genetically Modified Microorganisms for Biomass Conversion

Since the 1990s, bacteria, fungi and yeasts have been genetically engineered for the industrial production of biofuels and bioproducts. More conventionally, the improvement of microorganisms for biomass conversion has been done using classical chemical mutagenesis, a random approach followed by the screening and selection of a desired trait. Nevertheless, with advancements in molecular biology and biotechnology approaches, the improvement of microorganisms via rational engineering of proteins and metabolic engineering of pathways has become more prevalent (Strohl, 2001). This is due to the economic needs of the industry, which demands the development of strains that produce greater yields and a different variety of products. Specifically, in the bioconversion of biomass, researchers face challenges related to the substrate such as appropriate enzymes for conversion and microorganisms that produce them, fermentation of nonglucose sugars (i.e. xylose), and "consolidated bioprocessing", where the production of enzymes for biomass conversion (i.e. cellulose production), hydrolysis or modification of the biomass (i.e. cellulose hydrolysis), and fermentation of solubilized carbohydrates occur in a single step (Lynd et al., 1999). Therefore, prior to engineering microorganisms for biomass conversion it is important to select host organisms with desired characteristics; with emphasis on strains that can utilize low-cost substrates, have high product yield, competitive fitness, and are more robust to environmental stresses (Lynd et al., 1999). Once a good host has been selected based on targeted physiological characteristics and functionalities, one can identify the additionally desirable characteristic that will then be engineered into the host, whether targeting proteins such as enzymes through rational engineering or changing the metabolism and/or metabolic flux through metabolic engineering (Zhang et al., 2009).

Rational Engineering

Generally speaking, rational engineering refers to planned biochemical changes to a protein through the use of protein sequence and structure information, which in theory corresponds to a physiological or functional change in the proteins behavior. The engineered changes are usually predicted using computational biology and protein sequence data. However, there is limited structural information available for enzymes, for example, in structure—function relationship—so predictions on behavioral changes after rational engineering still remain in a trial-like state (Maki et al., 2009). Nonetheless, with increasing knowledge of biomass substrates and a rigorous test of our knowledge about enzyme interactions with plant-based biomass, rational engineering can be a valuable tool in the economical production of biofuels and value-added by-products.

Briefly, rational design of proteins can be summed up in three simple steps: (1) a suitable enzyme is chosen based on desired characteristics, (2) using computational biology or a high resolution crystallographic structure, the amino acid sites to be changed are identified, and (3) mutants produced from rationally engineered proteins are characterized (Percival Zhang et al., 2006).

Moreover, rational modifications to enzymes often include amino acids substitutions using site-directed mutagenesis, which can be used to increase the stability of enzymes (i.e. thermostability), substrate specificity, cofactor specificity, and the elucidation of enzymatic mechanisms (Bornscheuer and Pohl, 2001). In the field of biomass conversion to biofuels and bioproducts, the use of rational design has pioneering examples as outlined here.

For the most part, there are numerous reviews that summarize studies that revealed the mechanism of cellulase and other biomass-converting genes through the use of site-directed mutagenesis (Schulein, 2000; Wilson, 2004; Wither, 2001). On the contrary, very few researchers have reported increasing cellulase and other biomass-converting activities or enhancing properties through site-directed mutagenesis. However, Baker et al. were able to improve the activity of endoglucanase

Cel5A of *Acidothermus celluloyticus* toward microcrystalline cellulose by 20% (Baker et al., 2005). This was accomplished utilizing a high-resolution crystallographic structure (Sakon et al., 1996) to determine a series of mutations designed to alter the active cleft through a change in chemistry of the product-leaving side. As a result, structural information allowed end-product inhibition to be alleviated by a substitution of a nonaromatic residue at site 245; a Y245G mutant increased the K_I of cellobiose by 15-fold.

In a similar study, site-directed mutagenesis was used to improve the catalytic activity of endo/exocellulase Cel9A in *Thermobifida fusca* by 40% with soluble and amorphous cellulose, such as carboxymethyl cellulose (CMC) and swollen cellulose. Through the use of computer modeling, the conserved phenylalanine residue F476 (one of three residues) was found at the end of the carbohydrate binding module and appeared to play an important role in the initial binding of the cellulase to substrate. Also, computer modeling was used to predict that a new hydrogen bond could be created as a result of mutating the conserved phenylalanine residue F476 to a tyrosine. Therefore, the observed increase in catalytic activity of mutant F476Y is thought to be attributed to better binding properties, which are key for placing the soluble and amorphous cellulose chains in the carbohydrate binding domain (Escovar-Kousen et al., 2004).

Rational engineering of enzymes can also be used to improve characteristics such as thermostability and alkalinity in addition to specific activity. The roles of highly conserved residues (Asp 60, Tyr 35 and Glu 141), near the catalytic site, were investigated in the pH-dependent activity of xylanase XYL1p from *Scytalidium acidophilum* using site-directed mutagenesis. In doing so, three single mutants, D60N, Y35W and E141A, were created and the activities of three combined xylanase mutants DN/YW, DN/EA and YW/EA were evaluated at different pHs and temperatures. An increased pH optimum of 0.5−1.5 pH units and lower specific activities were observed in all the mutants except one. Mutant E141A exhibited a 50% increase in specific activity at pH 4.0 and had an overall higher catalytic efficiency than wild-type enzyme (Al Balaa et al., 2009). This work presents some important knowledge in acidophilic adaptation and, at the same time, is a prime example of how rational engineering can lead to the development of enzymes more suitable for the bioconversion industry environment, with competitive catalytic efficiency maintained.

Finally, the possibility of using rational engineering to improve the pH optimum and catalytic efficiency of laccase enzymes, involved in the oxidation of lignin, has been increasing as several researchers explore important residues conserved in laccases from fungi (Rogers et al., 2009). In one compelling example, researchers replaced an Asp residue in position 206 with an Asn residue in a laccase from *T. versicolor*, using site-directed mutagenesis. Upon expression of mutants in the yeast *Yarrowia lipolytica*, it was noted that catalytic activity was significantly affected as the pH optimum was raised by 1.4 pH units (Madzak et al., 2006), highlighting the interaction between the reducing substrate and the binding pocket of laccase. This study, like those discussed previously, pave the way for future development of efficient biomass-converting enzymes.

Metabolic Engineering of Microbial Pathways for Enhanced Bioproduct Production

Contrary to rational engineering, partial and/or additional metabolic pathways of microorganisms can be engineered to enhance bioproduct production. The term "metabolic engineering" was first coined by Bailey and was described as a vast variety of manipulations and experimental procedures to improve the productivity of a desired metabolite by an organism (Bailey, 1991). More specifically, examples of metabolic engineering can include increased productivity and/or yield, improvement of substrate uptake, widening the scope of substrate range for an organism, modification of metabolic flux, and elimination of unnecessary or competing metabolic pathways (Stephanopoulos, 1999).

Metabolic engineering, similar to rational engineering, requires the selection of a good host/microorganism as a candidate for the production of biofuels and/or bioproducts from biomass. This could include engineering desired pathways into well-studied host microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae*; these microorganisms have been used for industrial-scale production for several years. However, some experts suggest that engineering desired pathways into microorganisms that already possess industrial properties may be more successful. This is due to the potential for metabolic burden to the cell; new metabolic pathways require amino acids, redox cofactors, and energy for synthesis and function of its enzymes (Lee et al., 2008a).

Furthermore, metabolic engineering poses several general challenges for researchers including the development of recombinant DNA technologies for selected host microorganisms, development of quantitative tools, methods to understand flux modification in complex biological systems, and the development of quantitative techniques to determine changes in fluxes or metabolite concentrations (Cameron and Tong, 1993). A few successful examples of metabolic engineering to improve general host and select host microorganisms metabolism for the digestion and conversion of biomass are outlined below.

Recently, the development of genome-scale modeling permits the prediction of how new metabolic pathways

may impact growth and product production using metabolic models. These models result in a more rational approach to metabolic engineering (Patil et al., 2004). Moreover, stoichiometric models can be defined by established equations through the use of metabolic flux analysis (MFA); this is established by measuring exchange fluxes experimentally (Lee et al., 2008b). For example, the native metabolism of *E. coli* under different growth conditions (Kayser et al., 2005) and during recombinant protein production (Ozkan et al., 2005) has been determined using MFA. For efficient application in biofuel and bioproduct production, genome-scale models should be developed with constraints to optimize flux in desired pathways, while balancing important cofactors and energy metabolites (Lee et al., 2008b).

Host microorganisms such as *E. coli and S. cerevisae* have been improved time and again for the fermentation of sugars to ethanol. In particular, due to the broad range of carbohydrates metabolized by *E. coli*, it has been a potential candidate for the expression of ethanologenic pathways in some studies. For example, a portable cassette called the production of ethanol operon (PET operon) was used to genetically engineer the homoethanologenic pathway from *Zymomonas mobilis* into *E. coli*, which included the pyruvate decarboxylase and alcohol dehydrogenase B genes. Using the PET system, these genes were integrated into the chromosome of *E.coli* at the *pfl* locus. Meanwhile the fumarate reductase (*frd*) gene was deleted to eliminate succinate production, therefore preventing carbon loss. These metabolic changes resulted in the recombinant strain KO11, which produced ethanol yields as high as 95% in complex medium (Jarboe et al., 2007; Ohta et al., 1991). However, host strains such as *E.coli* may encounter metabolic burdens and are often not naturally adapted to the toxicity of end products like ethanol. Thus, there have also been some attempts to metabolically engineer known biomass-converting bacteria or fungal strains.

Typically, bacteria produce more desirable end products through facultative and anaerobic digestion, as is the case for bacteria belonging to the class *Clostridia*. Much of the metabolic engineering in these species focuses on product formation, which may include the elimination of undesirable products such as in the case of an engineering project conducted on *Clostridium acetobutylicum*—a well-known ethanogenic strain studied often for the production of butanol. In brief, the acetoacetate decarboxylase gene (*adc*) was disrupted in the hyperbutanol-producing strain *C. acetobutylicum* EA 2018 using TargeTron technology (Sigma Aldrich) (Jiang et al., 2009). TargeTron is a group II intron developed for rapid and site-specific gene disruption in prokaryotes. The disruption of *adc* led to an increase in butanol ratio from 70% to 80.05%, with a simultaneous reduction in acetone of 0.21 g/l (Jiang et al., 2009).

In contrast, one can implement metabolic engineering to improve native metabolism in microorganisms by engineering entirely novel pathways for desired product formation, which is more practically done in hosts able to hydrolyze biomass, such as the example with *Clostridium cellulolyticum*. Recently, Higashide et al. demonstrated the production of isobutanol from crystalline cellulose in *C. cellulolyticum* (Higashide et al., 2011). In this study, the development of valine biosynthesis pathway required the expression of five genes, alsS, ilvC, ilvD, kivD, and ahdA, to convert pyruvate into isobutanol. Consequently, only the expression and function of kivD (2-keto-acid decarboxylase) and alsS (alpha-acetolactate synthase) were confirmed; nonetheless modified *C. cellulolyticum* produced up to 660 mg/l of isobutanol over a 7- to 9-day growth period (Higashide et al., 2011).

These examples of engineering and modeling to improve the metabolic capabilities of strains helped lay the foundation for future development of biomass-converting microorganisms. Combined with the ability to rationally design enzymes with greater stability and/or increased specific activity the modification of microorganisms in industrial production of biofuels and bioproducts looks promising.

STRATEGIES OF USING MICROBIAL PRETREATMENT TO ENHANCE SUGAR RELEASE FOR BIOFUEL AND BIOPRODUCT PRODUCTION

The advantages of biological pretreatment include minimum facility cost, low energy requirement and mild environmental conditions. However, for practical application, there are two major disadvantages associated with this process. First, fungi growth consumes holocellulose as an energy source leading to significant carbohydrate loss; second, most biological pretreatments are long processes due to slow microbial growth and delignification reaction rates. Since lignin breakdown in the biomass would lead to enzyme access to cellulose and hemicellulose, selective lignin degradation by white-rot fungi hold some promise for real application in biomass pretreatment if the procedure can be cut shorter and sugar consumption can be controlled to an insignificantly low level. However, not even white-rot fungi can use lignin as a sole carbon and energy source; fungi growth inevitably results in carbohydrate loss (Fan et al., 2012; Sanchez, 2009). Strategies taken to shorten biological pretreatment time and decrease carbohydrate consumption include (1) selection for naturally occurring white-rot fungi that preferentially attack lignin (Ander Eriksson, 1977; Kirk and Moore, 1972; Lee et al., 2007; Muller and Trosch, 1986; Salvachua

et al., 2011), (2) selection of cellulase-deficient mutants (Akin et al., 1993; Eriksson et al., 1980; Ruel et al., 1981), or (3) repression of cellulase and hemicellulase expression (Yang et al., 1980). As an example of strain selection, among 22 screened *Basidiomycetes*, mostly the white-rot fungi *Pleurotus* sp. "*florida*" preferentially attacks lignin in wheat straw to increase cellulose accessibility. After 90 days pretreatment with *Pleurotus* sp. "*florida*", the resulting biomass can release the same amount of glucose as Avicel, the lignin-free cellulose (Muller and Trosch, 1986). However, pretreatment using this strain is still time consuming.

Furthermore, there are many limitations to the strategies for strain improvement. First, carbohydrate consumption is needed for microbial growth; therefore, strains can only be selected for increased delignification and decreased sugar loss and not for minimal sugar loss. In addition, decreasing the secretion of carbohydrate hydrolysis enzymes would lower the reaction rate and lead to even longer pretreatment time. Genetic modification of white-rot fungi to improve the required features may help resolve some of the drawbacks, but the technical process is quite challenging (Fan et al., 2012).

Another way to improve the biological pretreatment process is through optimization of nutrients, temperature, and preprocessing time to reach a balance between maximum sugar release and minimum sugar loss within the shortest possible time. Based on the enzymatic activity profile obtained in a 28-day pretreatment analysis, switchgrass is pretreated with *P. chrysosporium* for 7 days. The pretreatment of switchgrass led to higher glucan, xylan, and total sugar yields than the unpretreated sample, suggesting enzyme profile assays may be utilized for initial estimation of pretreatment time in order to enhance sugar yields and reduce sugar loss (Mahalaxmi et al., 2010). By monitoring compositional changes during biological pretreatment, a 15-day pretreatment time was selected for the pretreatment of the woody biomasses *Prosopis juliflora* and *Lantana camara* with the white-rot fungus *Pycnoporus cinnabarimus* (Gupta et al., 2011). This 15-day pretreatment resulted in a relatively small weight loss in the pretreated feedstocks with decreased lignin and increased holocellulose contents. Enzymatic hydrolysis of the pretreated biomass led to sugar releases of 389 and 402 mg per gram of dried solid.

Alternatively, as a compromise, preliminary microbial pretreatment of biomass can be used in combination with downstream thermochemical, chemical or other pretreatment. This procedure would reduce, for example, the amount of acid needed combined with lower temperature and shorter time, thus reducing energy and chemical costs. In addition, there would be less biomass degradation and inhibitor production compared to conventional thermochemical pretreatment. Preliminary tests showed that after corn stover pretreatment with *P. chrysosporium*, the shear forces needed to obtain the same shear rates of 3.2−7 rev/s were reduced 10- to 100-fold, respectively. The digestibility of *C. stercoreus*-pretreated corn stover showed a three- to fivefold improvement in enzymatic cellulose digestibility (Keller et al., 2003). Sawada et al. reported that combination of fungal pretreatment with less severe steam explosion maximizes enzymatic saccharification of beech wood meal (Sawada et al., 1995). Compared to steam explosion alone, combined pretreatments improve saccharification by 20−100% of the polysaccharide in the wood. However, 17% of the holocellulose was degraded during fungal pretreatment, and there was an unspecified holocellulose loss during steam explosion at optimum 215 °C for 6.5 min (Sawada et al., 1995). Pretreatment of wheat straw with *P. juliflora* followed by acid hydrolysis led to a reduction in acid load and an increase in sugar release as well as ethanol yield (Kuhar et al., 2008).

Interestingly, a recent study showed that by simply changing the pretreatment sequence, i.e. when the wood *Pimus radiata* biomass was treated first with steam explosion followed by fungi pretreatment, a 10-fold increase in glucose yield was achieved after enzymatic hydrolysis (Vaidya and Singh, 2012). A combination of selected fungal pretreatment with a mild alkali treatment of wheat straw led to a maximum of 69% glucose yield and an ethanol yield of 62% with no inhibitor formation during the pretreatment (Salvachua et al., 2011). Also, a combination of the white-rot fungus *Lenzites betulina* C5617 pretreatment with LHW treatment enhanced the enzymatic hydrolysis of the poplar wood *Populus tomentosa* led to the highest hemicellulose removal of 92.33%, which was almost two times higher than that of LHW treatment alone and a 2.66-fold increase in glucose yield (Wang et al., 2012).

Application of Microbial Pretreatment for Biogas Production

A promising application for microbial pretreatment of lignocellulosic materials is for increasing biogas yield in the anaerobic fermentation process. Anaerobic digestion of organic waste and residues not only provides a good solution for the sustainable processing and treatment of large amounts of biomaterials, but also leads to value-added renewable energy production. Natural lignocellulosic materials can only be converted to biogas at a very low efficiency due to their resistance to anaerobic digestion. The low biogas conversion rate results from the resistance to enzymatic attack by the biomass due to the tight association of lignin, cellulose, and hemicellulose. Under anaerobic conditions, cellulose and hemicellulose can be degraded during biogas production but not lignin (Fernandes et al., 2009). Pretreatment procedures to increase the accessibility of holocellulose are necessary

to increase biogas production. Different pretreatment methods, including physical and chemical pretreatments, effectively enhance anaerobic digestion, but these procedures have disadvantages as described beforehand. A microbial pretreatment followed by another step of biological process seems very promising and close to practical application as shown by some following examples.

Pretreatment of wheat straw with *Pleurotus* sp. *"florida"* doubles both cellulase digestibility of the treated biomass and the resulting biogas yield, compared with untreated wheat straw (Muller and Trosch, 1986). Pretreatment of softwood in the presence of wheat bran with the white-rot fungus *C. subvermispora*, which can effectively degrade the lignin component, enhanced methane fermentation of softwood to 35% of the theoretical yield, based on holocellulose content of the biomass. In contrast, pretreatment with *Pleurocybella porrigens*, which has a lower ability to decompose lignin, led to no significant changes (Amirta et al., 2006).

Application of a lignocellulose degrading composite microbial system with high xylanase activity (XDC-2), instead of a pure culture of microorganisms for biomass pretreatment has also been tested. XDC-2 is composed of 26 different clones from three phyla: Clostridiales, Proteobacteria, and Bacteriodetes. However, these degrade mainly carbohydrate but not lignin. After a 5-day pretreatment with XDC-2, corn stalk was efficiently degraded by nearly 45%, and the cellulose and hemicellulose contents were decreased by 22.7% and 74.1%, respectively. Biodegradability of the pretreated biomass is improved resulting from changes in chemical structure due to decreased holocellulose content. Compared with untreated corn stalks, total biogas production and methane yield were increased by 68.3% and 87.9%, respectively, and the technical digestion time (T80) was shortened by 35.7% (Yuan et al., 2011).

Effectiveness of biological pretreatments in enhancing corn straw biogas production has also been reported with complex microbial agents including yeast (*S. cerevisiae*, *Coccidioides immitis*, and *Hansenula anomala*), cellulolytic bacteria (*Bacillus licheniformis*, *Pseudomonas* sp., *Bacillus subtilis*, and *Pleurotus florida*), and the lactic acid bacteria *Lactobacillus deiliehii*. A 15-day pretreatment of corn straw at ambient temperature led to reduced contents of total lignin, cellulose, and hemicellulose, and increased content of hot-water extractives. Anaerobic digestion of the pretreated material resulted in 33.07% more biogas yield, 75.57% more methane yield, and 34.6% shorter technical digestion time compared with the untreated sample (Zhong et al., 2011).

In conclusion, under proper conditions, microbial/biological pretreatment can be an effective method for improving biodegradability and enhancing downstream biological conversion efficiency of biomass into bioenergy and other value-added bioproducts.

Application of Microbial Pretreatment for Biomass Conversion

Strategies for Microorganism Application in Biomass

Most naturally occurring microorganisms cannot utilize untreated lignocellulose efficiently for the production of biofuel or bioproducts due to the inaccessibility of the carbohydrate polymers, even though many of them secrete a variety of hydrolytic enzymes. For efficient utilization, biomass must first be pretreated to open up the cell wall and then hydrolyzed by acidic or enzymatic processes to fermentable sugar monomers. In addition to monomeric sugars, the pretreatment and acidic hydrolysis processes may also produce low molecular weight organic acids like acetic acid, furfural, hydroxymethylfurfural and various lignin-degradation products that are potent inhibitors of microbial metabolism (Larsson et al., 1999; Palmqvist and Hahn-Hägerdal, 2000).

For an economically viable manufacturing process from lignocellulosic biomass, both hexose and pentose sugars produced during hydrolysis of both cellulose and hemicelluloses need to be utilized efficiently. In the course of cellulosic biomass conversion into biofuels and bioproducts, four biologically mediated processes are involved: (1) saccharolytic enzyme production, (2) enzymatic hydrolysis of biomass, (3) fermentation of hexose sugars, and (4) fermentation of pentose sugars (Lynd et al., 2005, 2002). For an industrially viable process, each of the four steps must be rapid and efficient. As suggested by a recent calculation, an economically competitive fermentation process for industrial application needs to approach an anaerobic yield of ~95% of the theoretical yield, produce around 100 g/l of end product with a productivity of more than 2 g/l/h (Sheridan, 2009).

DIFFERENT PROCESSES OF MICROORGANISM-MEDIATED BIOMASS CONVERSION

For enzymatic hydrolysis and fermentation, different strategies have been explored including separate hydrolysis and fermentation (SHF), SSF nonisothermal simultaneous saccharification and fermentation (NSSF), simultaneous saccharification and cofermentation (SSCF), or consolidated bioprocessing (CBP) (Lynd et al., 2002; Taherzadeh and Karimi, 2007). Each process has advantages and disadvantages.

For SHF, the main advantage is the possibility to separately optimize hydrolysis and fermentation steps and the main drawback is the inhibition of cellulase activity by the released sugars, mainly cellobiose and glucose (Taherzadeh and Karimi, 2007). SSF, different from SHF, combines the enzymatic hydrolysis and fermentation in one step, thus minimizing the product inhibition of cellulase enzymes as the released sugars are immediately

consumed by the microorganism. In addition, cellulase production and fermentation of hemicellulose hydrolysis products occur in two additional, discrete process steps. This process has many advantages over SHF such as increased ethanol yield, decreased enzyme loading, decreased contamination, and lower capital cost. The disadvantages are differences between optimum temperatures for enzyme hydrolysis and fermentation and inhibition of cellulase by the produced ethanol (Lynd et al., 2002; Olofsson et al., 2008).

To solve the issue of temperature difference, the NSSF process was proposed (Wu and Lee, 1998) in which saccharification and fermentation occur simultaneously but in two separate reactors, each operated at its own optimum temperature. Compared to SSF, NSSF increased ethanol yield and productivity with a reduced overall enzyme loading of 30−40%. The disadvantage is increased capital cost for extra equipment.

In SSCF, enzymatic biomass hydrolysis and fermentation of both cellulose and hemicellulose hydrolysis products all occur in a single bioreactor with a single microorganism (Teixeira et al., 2000). It is considered an improved process compared to SSF, which requires two bioreactors with two different microorganisms and two different biomass production setups (Hamelinck et al., 2005; McMillan, 1997; McMillan et al., 1999). However, SSCF usually requires a metabolically engineered microorganism that can robustly coferment both glucose and xylose (Teixeira et al., 2000) without synthesis of side products. For example, when a naturally occurring strain, *Lactobacillus pentosus* (American Type Culture Collection, ATCC 8041), was used in an SSCF process using pretreated corn stover as substrate and the commercial cellulase Spezyme-CP for hydrolysis, the maximum yield of lactic acid was >90% of the theoretical maximum on the basis of all available fermentable sugars. However, acetic acid was also produced through a different metabolic pathway that assimilates pentoses (xylose and arabinose). Another drawback of the process is the difficulty in improving lactic acid concentration due to end-product inhibition of the nonengineered strain (Zhu et al., 2007).

All the above-mentioned processes require a separate enzyme production step or an external supply of enzymes for biomass hydrolysis. In CBP, enzyme production, biomass hydrolysis, and fermentation of pentoses and hexoses are accomplished in a single reactor by mono- or cocultures of microorganisms (Lynd et al., 2002). The obvious advantages of CBP are decreased capital costs and no extra cost for enzyme production or purchasing (Hamelinck et al., 2005; Lynd et al., 2005). However, since naturally occurring microorganisms cannot simultaneously synthesize enough of the necessary saccharolytic enzymes and convert released sugars into the desired end products, the CBP configuration requires the development of engineered microorganisms (Hasunuma and Kondo, 2012a; Xu et al., 2009). Such "superbugs" need to not only secrete high titer, robust enzymes, but also efficiently produce ethanol and other bioproducts at high yields under harsh environments containing toxic compounds. CBP is gaining increasing recognition as a potential breakthrough for low-cost biomass processing (Hasunuma and Kondo, 2012a; van Zyl et al., 2007). The company Mascoma Corporation claims to have successfully engineered microorganisms for industrial CBP application (http://www.mascoma.com/).

Commonly Used Microorganisms in Biomass Conversion and Some Application Examples

A large number of microorganisms are capable of degrading plant cell walls including bacteria and fungi. With few exceptions, two distinct cellulolytic strategies have been adapted by the aerobic and anaerobic groups. While aerobic bacteria and fungi produce numerous individual, extracellular enzymes with many of them acting in synergy for effective hydrolysis, anaerobic bacteria and fungi possess a unique extracellular multienzyme complex, termed the cellulosome, that can efficiently hydrolyze crystalline cellulose (Bayer et al., 2004, 1998; Doi and Kosugi, 2004; Fontes and Gilbert, 2010; Lamed et al., 1983; Lynd et al., 2002; Schwarz, 2001; Shoham et al., 1999; Steenbakkers et al., 2003). Metabolic utilization of the monomeric sugars from hydrolyzed biomass leads to the natural production of biofuels and bioproducts, mostly as side products by different microorganisms. For ethanol fermentation of lignocellulosic biomass, most frequently considered microorganisms include the bacteria *E. coli*, *Z. mobilis* and *Clostridium phytofermentans;* themophilic bacteria such as *Clostridium thermocellum;* yeasts such as *S. cerevisiae*and *Pichia stipitis*; and filamentous fungi (Amore and Faraco, 2012; Hahn-Hagerdal et al., 2007; Weber et al., 2010; Xu et al., 2009).

Like ethanol, the majority of other potential biofuels and bioproducts are naturally produced by various microorganisms as side products. The viability of a fermentation process for industrial application depends on its cost-competitiveness. As listed in Table 5.1, most microorganisms cannot use polymeric carbohydrates directly as fermentation substrates; therefore, biomass has to be broken down into monomeric sugars to be used as fermentation substrates. For an economically viable manufacturing process of biofuels from lignocellulosic biomass, pentose utilization is essential. Therefore, an optimal microorganism should be able to simultaneously ferment both hexose and pentose sugars and give rise to high productivities and yields. In addition, it should have high tolerance to fermentation inhibitors and end products and resist microbial

TABLE 5.1 Typical Features of Representative Microorganisms for Biofuel Production

Strain	Pros	Cons	References
E. coli	Pentose utilization	Not resistant to environmental stress, low ethanol and butanol tolerance	(Jeffries, 1983; Knoshaug and Zhang, 2009; Shin et al., 2010; Trinh and Srienc, 2009; Yomano et al., 1998, 2008)
Z. mobilis	High ethanol yield and productivity; high ethanol tolerance	Cannot metabolize pentose sugars	(Rogers et al., 1982; Weber et al., 2010)
Clostridium phytofermentans (ethanol), *Clostridium acetobutylicum* (butanol)	Saccarify cellulose and hemicellulose, ferment hexose and pentose sugars	Slow growth rate, low productivity, sensitive to bacteriophage infection	(Jones et al., 2000; Lee et al., 2008a,b; Maki et al., 2009; Warnick et al., 2002)
S. cerevisiae	High robustness, highly resistant to toxic inhibitors and end products	Cannot naturally ferment pentose sugars	(Olofsson et al., 2008; Yanase et al., 2010a,b)
P. stipitis	Naturally ferment xylose	Lower sugar consumption rate than *S. cerevisiae*; sequential fermentation of glucose and xylose	(Agbogbo and Coward-Kelly, 2008; Jeffries, 1983; Jeffries et al., 2007; Parekh and Wayman, 1986)
Kluyveromyces marxianus	Thermotolerance allowing higher fermentation temperature, optimum SSF process at lower enzyme loading, lower operation cost, potential application in CBP	Poor xylose fermentation, undesirable side product	(Babiker et al., 2010; Banat et al., 1992; Hasunuma and Kondo, 2012a,b; Yanase et al., 2010a,b)
Clostridium thermocellum	Thermophilic anaerobe that grows fast on crystalline cellulose, both cellulolytic and ethanologenic, hydrolyze homocellulose and directly ferment hexose sugars to ethanol and organic acids, no need for external enzyme addition	No pentose fermentation, branched fermentation pathways lead to acetate and lactate by-products, low ethanol production efficiency, low ethanol tolerance	(Demain et al., 2005; Lynd et al., 2005; Ng et al., 1981; Raman et al., 2011; Roberts et al., 2010; Zhang and Lynd, 2005)
T. reesei	Hyper producer of cellulolytic enzymes, extensive knowledge and tools for genetic manipulation and practical application	Extensive efforts needed for strain development, low ethanol yield and productivity, low ethanol tolerance	(Amore and Faraco, 2012; Xu et al., 2009)

contamination, e.g. bacteriophage infections (Weber et al., 2010).

No naturally occurring microorganism has all the required features. Promising means to develop a microorganism for sustainable bioethanol/bioproduct production include breeding technologies, genetic engineering and the search for undiscovered species (Weber et al., 2010). For production of a particular product from a specific biomass, native organisms can be selected from a group of different species of microbes based on their fermentation performance, such as substrate utilization efficiency, inhibitor resistance, and productivity (Rumbold et al., 2010, 2009). The yeast *S. cerevisiae* is by far the most widely used organism in the existing fermentation industry. To improve its application in bioethanol fermentation from biomass, targeted evolution strategy has been applied to obtain inhibitor-tolerant *S. cerevisiae* that can resist individual or multiple inhibitors (Ding et al., 2012; Heer and Sauer, 2008; Liu, 2006). When adaptation and selection processes were applied to the parental fungus *Rhizopus oryzae*, a new strain was obtained that exhibited significantly improved efficiency of substrate utilization and enhanced production of L-(+)-lactic acid from corncob hydrolysate. The final product concentration, yield, and volumetric productivity more than doubled compared with its parental strain (Bai et al., 2008).

Applications of thermotolerant mesophilic microorganisms in the fermentation process have considerable potential for cost-effective ethanol and other bioproduct production. The thermotolerant yeast *Kluyveromyces marxianus* grows well at temperatures as high as 45–52 °C and can efficiently ferment ethanol at temperatures of between 38 and 45 °C. A 5 °C increase in the fermentation temperature can greatly decrease fuel

ethanol production costs (Babiker et al., 2010). Results from solid state fermentation of sweet sorghum stalk to ethanol with the thermotolerant yeast strain *Issatchenkia orientalis* IPE 100A showed great potential for its practical application in large-scale, deep-bed solid state fermentation (Kwon et al., 2011).

The thermotolerant *Bacillus coagulans* strain 36D1 can ferment both hexoses and pentoses from enzymatically hydrolyzed biomass at 50–55 °C and pH 5.0 producing L (+)-lactic acid as the primary fermentation product. Since such conditions are closer to the optimum fungal enzyme functioning requirements, the amount of enzyme required for cellulose conversion is significantly reduced in comparison with yeast or lactic acid bacteria currently used by the industry as microbial biocatalysts. In addition, both biomass conversion efficiency and product yield are greatly increased with a dramatically decreased fermentation time, thus reducing the cost of both the process and final product (Ou et al., 2009).

The anaerobic mesophilic bacterium *C. phytofermentans* (ATCC 700,394) is a promising native microorganism for biomass conversion since its genome encodes the highest number of enzymes for degradation of lignocellulosic material among sequenced *Clostridial* genomes (Warnick et al., 2002; Weber et al., 2010). It secretes noncomplex, individual enzymes to hydrolyze both cellulose and hemicelluloses to both hexose and pentose sugars, which are mostly directly consumed, producing ethanol and acetate as the major products (Warnick et al., 2002; Weber et al., 2010). When used in the CBP process with pretreated corn stover as substrate, at optimal conditions with low solid loading (0.5% w/w), *C. phytofermentans* hydrolyzed 76% of glucan and 88.6% of xylan in 10 days. These values reach 87% and 102% of those obtained by SSCF process using commercial enzymes and *S. cerevisiae* 424A with an ethanol titer of 2.8 g/l corresponding to 71.8% of that yielded by SSCF (3.9 g/l) (Jin et al., 2011a). However, using a similar process with high solid loading (4% w/w), the side product acetate became a major product (Jin et al., 2012).

Even though *C. thermocellum* seems a good candidate for ethanol fermentation from cellulosic biomass, there are a few disadvantages as listed in Table 5.1. Despite its ability to degrade lignocellulosic waste to both hexose and pentose sugars, it can only utilize hexose sugars from cellulose and not the pentose sugars derived from hemicellulose (Lynd et al., 2002; Taylor et al., 2009). This drawback could be solved by the use of mixed cultures for the degradation and fermentation of all sugars derived from lignocellulosic materials. For example, the anaerobic thermophile *Thermoanaerobacterium saccharolyticum,* which can ferment xylan and almost all soluble biomass sugars, would be a good candidate for coculture with *C. thermocellum*. A twofold reduction of the bioethanol production cost from lignocellulose could be achieved when using thermophilic anaerobic mixed cultures (Demain et al., 2005; Lynd et al., 2002). Since there is currently no perfect CBP microbe that can degrade lignocellulosic biomass efficiently and at the same time utilize all the sugars released from biomass to produce mostly ethanol, coculture or community/mixed fermentation may be a suitable option (Barnard et al., 2010; Demain, 2009; Jin et al., 2011a). Chen reviewed 35 coculture systems for ethanol production by cofermentation of glucose and xylose and concluded that even though still in its infancy, this strategy is promising as it can increase ethanol yield and productivity, shorten fermentation time, and reduce process costs (Chen, 2011).

FUTURE PERSPECTIVES

For a particular product made from lignocellulosic biomass fermentation, it will be difficult to predict which particular microorganism should be finally used in commercial production. For different processes, it is possible that different species may be required. For bioethanol production, *S. cerevisiae* has some advantages since it is already widely used in large-scale, first-generation bioethanol production with well-established processes and technology. An ideal biomass sugar fermentation process needs to reach high product yield by fermenting all biomass sugars including glucose, xylose, arabinose, mannose, and galactose with an optimal microorganism that is resistant to toxic materials/chemicals in biomass hydrolysates such as acids, phenolics, salts, and sugar oligomers. In addition, the microorganism should be robust, resistant to contamination and environmental stresses, with minimal metabolic by-product production. To achieve these goals, metabolic engineering, or extensive physiological reprogramming of the producing organisms may provide solutions.

Other Bioproducts Produced by Microbial Conversion of Biomass: Introduction

The use of microorganisms in conversion processes to produce usable material from biomass sources has been ongoing for several decades. Most of the reports in the literature discuss the development of bioprocesses that are involved in the production of simple sugars, which are then used to produce bioethanol or related compounds for use as biofuels. However, there are new trends emerging for the use of biomass conversion by microbes, as shown in Table 5.2. Biomass conversion processes may eventually be implemented to produce a much greater array of useful bioproducts, in addition to biofuels.

TABLE 5.2 List of Bioproducts Produced by Different Microorganisms

Bioproduct	Organism	Conversion	References
Biofuel	*Clostridium thermosaccharolyticum*	Xylose to ethanol	(Mistry and Cooney, 1989)
	Engineered *Escherichia coli*	Cell wall sugars to biofuel	(Doran-Peterson et al., 2008)
	Lactobacillus buchneri NRRL B-30929	Xylose and glucose to ethanol and chemicals	(Liu et al., 2009)
	Saccharomyces cerevisiae	Heptanal to heptanol	(Verma et al., 2010)
	Saccharomyces cerevisiae AM12	Spent shiitake mushroom medium (using Meicelase) into ethanol	(Asada et al., 2011)
	Pichia stiptis NCIM3498 and *Saccharomyces cerevisiae*-VS3	Hemicellulosic hydrolysate to ethanol	(Chandel et al., 2011)
	Methanosarcinales and *Methanomicrobiales*	Coal to methane	(Wawrik et al., 2012)
	Saccharomyces cerevisiae daughter strains	Pretreated pine to ethanol	(Hawkins and Doran-Peterson, 2011)
	Trichoderma reesei xylanase	Wheat biomass to bioethanol	(Juodeikiene et al., 2012)
	Saccharomyces cerevisiae	Lignocellulose-derived sugars to ethanol	(Madhavan et al., 2012)
	Clostridium saccharoperbutylacetonicum	n-butyrate to n-butanol	(Richter et al., 2012)
	Burkholderia sp. C20	Microalgal oil to biodiesel	(Tran et al., 2012)
Pretreated/delignified biomass	*Cyathus stercoreus* and *Ceriporiopsis subversmispora*	Grass stem pretreatment	(Akin et al., 1995)
	Ceriporia lacerata, Stereum hirsutum, and *Polyporus brumalis*	Softwood pretreatment	(Lee et al., 2007)
	Ceriporiopsis subvermispora	Corn stover pretreatment for enzymatic hydrolysis and ethanol production	(Wan and Li, 2010)
	Trametes versicolor	Canola straw pretreatment for biofuel production	(Canam et al., 2011)
	Pleurotus ostreatus	Wood degradation	(Piskur et al., 2011)
	Irpex lacteus	Straw saccharification	(Pinto et al., 2012)
	Tramete hirsuta	Paddy straw pretreatment for improved enzymatic saccharification	(Saritha et al., 2012b)
	Phanerochaete chrysosporium	Pretreatment of cornstalk to enhance enzymatic saccharification and hydrogen production	(Zhao et al., 2012)
Simple sugars	*Aureobasidium pullulans* (yeastlike mold strain)	Glucose to gluconic acid	(Anastassiadis et al., 2003)
	Enterobacter aerogenes 230S	L-Psicose to L-tagatose	(Rao et al., 2008)
	Debaryomyces hansenii	D-xylose and sugarcane bagasse hemicellulose to xylitol	(Prakash et al., 2011)
	Agromyces sp. C42 and *Stenotrophomonas* sp. A10b (from yellow mealworm gut)	Lignocellulose to reducing sugars	(Qi et al., 2011)
	Ustilago maydis	Fungal lignocellulosic biomass to glucose and other sugars	(Couturier et al., 2012)

(*Continued*)

TABLE 5.2 List of Bioproducts Produced by Different Microorganisms—cont'd

Bioproduct	Organism	Conversion	References
	Debaryomyces hansenii NRRL Y-7426	Distilled grape marc hemicellulosic hydrolysates to xylitol	(Salgado et al., 2012)
	Candida athensensis SB18	D-xylose and horticultural waste hemicellulosic hydrolysate to xylitol	(Zhang et al., 2012a)
	Acidotermus celluloyticus endoglucanase	Cellulose to glucose	(Zhang et al., 2012b)
Lipids	Cellulolytic fungus of *Aspergillus oryzae* A-4	Wheat straw to lipid	(Lin et al., 2010)
	Engineered *Escherichia coli*	Simple sugars to fatty esters, fatty alcohols and waxes	(Steen et al., 2010)
	Ustilago maydis	Crude glycerol to glycolipids	(Liu et al., 2011)
	Cryptococcus curvatus	Crude glycerol to oleic acid, palmitic acid, stearic acid and linoleic acid	(Thiru et al., 2011)
	Trichosporon coremiiforme	Organic acids and residual sugars (following butanol fermentation) to oil	(Chen et al., 2012a)
	Trichosporon cutaneum	Corncob acid hydrolysate to oil	(Chen et al., 2012b)
	Lipomyces starkeyi	Cellobiose and xylose into intracellular lipids	(Gong et al., 2012)
	Rhodococcus opacus DSM 1069 and PD630	Lignin model compounds to triglycerides	(Kosa and Ragauskas, 2012)
Organic chemicals	*Clostridium lentocellum* SG6	Cellulose to acetic acid	(Tammali et al., 2003)
	Saccharomyces uvarum SW-58	Ethyl 4,4,4-trifluoroacetoacetate to ethyl (R)-4,4,4-trifluoro-3-hydroxybutanoate [(R)-2]	(He et al., 2007)
	Engineered *E. coli*	Glucose to glucuronic and glucaric acid	(Moon et al., 2009)
	Phanerochaete chrysosporium	Rice straw biodelignification in the presence of dirhamnolipid biosurfactant	(Liang et al., 2010)
	Schizophyllum commune	Cinnamic acid derivatives to phenols	(Nimura et al., 2010)
	Aspergillus parasiticus speare BGB	Glycyrrhizinic acid in liquorice to 18-beta glycyrrhetinic acid	(Wang et al., 2010)
	Gliocladium spp. and *E. coli*	Cellulosic biomass to hydrocarbons	(Ahamed and Ahring, 2011)
	Actinobacillus succinogenes	Sugarcane bagasse hemicellulose hydrolysate to succinic acid	(Borges and Pereira, 2011)
	Engineered *Thermobifida fusca*	Untreated lignocellulosic biomass to 1-propanol	(Deng and Fong, 2011)
	Plasticicumulans acidivorans/*Thauera selenatis* mixed culture	Lactate, lactate/acetate mix to poly-3-hydroxy butyrate	(Jiang et al., 2011)
	Klebsiella pneumoniae	Glycerol and xylose cofermentation to 1,3-propanediol	(Jin et al., 2011b)
	Clostridium ragsdalei	Acetone to isopropanol	(Ramachandriya et al., 2011)
Other	*Pseudonocardia carboxydivorans*	Compactin to pravastatin	(Lin et al., 2011)
	Ganoderma sp. rckk02	Wheat straw to nutritive ruminant feed	(Shrivastava et al., 2012)
	Brevundimonas sp. SGJ	L-Tyrosine to L-dihydroxyphenylalanine	(Surwase et al., 2012)
	Lactobacillus brevis TCCCC13007	Monosodium glutamate to gamma-aminobutyric acid	(Zhang et al., 2012c)

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