Investigating biomass saccharification for the production of cellulosic ethanol

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ABSTRACT

The production of second generation biofuels -- cellulosic ethanol from renewable lignocellulosic biomass has the potential to lead the bioindustrial revolution necessary to the transition from a fossil fuel-based economy to a sustainable carbohydrate economy. Effective release of fermentable sugars through biomass pretreatment followed by enzymatic hydrolysis is among the most costly steps for emerging cellulosic ethanol biorefineries.

In this project, two pretreatment methods (dilute acid, DA, and cellulose solvent- and organic solvent-lignocellulose fractionation, COSLIF) for corn stover were compared. It was found that glucan digestibility of the corn stover pretreated by COSLIF was much higher, along with faster hydrolysis rate, than that by DA- pretreated. This difference was more significant at a low enzyme loading. Quantitative measurements of total substrate accessibility to cellulase (TSAC), cellulose accessibility to cellulase (CAC), and non-cellulose accessibility to cellulase (NCAC) based on adsorption of a non-hydrolytic recombinant protein TGC were established to find out the cause. The COSLIF-pretreated corn stover had a CAC nearly twice that of the DA-pretreated biomass. Further supported by qualitative scanning electron microscopy images, these results suggested that COSLIF treatment disrupted microfibrillar structures within biomass while DA treatment mainly removed hemicelluloses, resulting in a much less substrate accessibility of the latter than of the former. It also concluded that enhancing substrate accessibility was the key to an efficient bioconversion of lignocellulose.

A simple method for determining the adsorbed cellulase on cellulosic materials or pretreated lignocellulose was established for better understanding of cellulase adsorption and desorption. This method involved hydrolysis of adsorbed cellulase in the presence of

10 M of NaOH at 121^{o}C for 20 min, followed by the ninhydrin assay for the amino acids released from the hydrolyzed cellulase. The major lignocellulosic components (i.e. cellulose, hemicellulose, and lignin) did not interfere with the ninhydrin assay. A number of cellulase desorption methods were investigated, including pH adjustment, detergents, high salt solution, and polyhydric alcohols. The pH adjustment to 13.0 and the elution by 72% ethylene glycol at a neutral pH were among the most efficient approaches for desorbing the adsorbed cellulase. For the recycling of active cellulase, a modest pH adjustment to 10.0 may be a low-cost method to desorb active cellulase. More than 90% of cellulase for hydrolysis of the pretreated corn stover could be recycled by washing at pH 10.0.

This study provided an in-depth understanding of biomass saccharification for the production of cellulosic ethanol for cellulose hydrolysis and cellulase adsorption and desorption. It will be of great importance for developing better lignocellulose pretreatment technologies and improving cellulose hydrolysis by engineered cellulases.

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1. Background and Goals

1.1 Introduction of biofuels

Soaring oil prices associated with concerns of climate change and national energy security are driving us to utilize sustainable alternative energy sources, such as solar energy, nuclear energy, wind energy, hydropower, tidal energy, and so on (Lynd et al. 2003). In the USA, transportation accounts for 30% of the total energy consumption. Liquid biofuel (bioethanol or biodiesel) is providing a significant fraction of transportation fuels because they are compatible with current infrastructure (The White House and National Economic Council 2006). Although a few novel advanced biofuels are proposed to be produced by genetically modified microorganisms (Atsumi et al. 2008; Fortman et al. 2008; Mukhopadhyay et al. 2008), bioethanol is, currently, the most intensively-studied and widely-accepted alternative transportation biofuel (Gray et al. 2006; National Research Council 2000). It offers a great amount of advantages over liquid fossil fuels, such as gasoline, and has been integrated into the transportation system (Moxley 2007).

So far, most bioethanol is made by yeast fermentation based on soluble sugars that come from sugarcane in Brazil and corn kernels in U.S.A. (Ballerini et al. 1994). Ethanol derived from grain starch has many limitations. Most debates focus on the soaring price of food, which has a great impact on the whole chain of agricultural products and further leads to food crisis. As a result, widely available lignocellulosic biomass (cellulosic biomass) for the production of a second generation biofuels is receiving more attentions.

Lignocellulosic biomass includes: agricultural residues [e.g., corn stover (cob and stalk), rice straw, bagasse, cotton gin trash, etc.], forestry wastes (e.g., wood chips, and sawdust) bioenergy crops (sweet sorghum, switchgrass and common reeds), industrial wastes (e.g., paper sludge, recycled newspaper), municipal solid wastes. Unlike food-based (starch-derived) biomass, it embraces a series of advantages, such as, low cost, abundant supplies, non-competition with grain as food (Sathisuksanoh et al. 2009).

Lignocellulose, a natural complicated composite, primarily consists of cellulose, hemicellulose, and lignin. Figure 1-1 describes a vivid structure of lignocellulose. Cellulose and hemicellulose are tangled together and wrapped by lignin outside (de Vries and Visser 2001). Depending on sources and cell types, the dry weight typically makes up of around 35 to 50% cellulose, 20 to 35% hemicellulose, and 10 to 25% lignin (Demirbas 2005).

Cellulose, the most abundant natural carbon bioresource on the earth, is a homopolysaccharide of anhydroglucopyranose linked by ß-1, 4-glycosidic linkages (McMillan 1997). Adjacent cellulose chains are coupled via orderly hydrogen bonds and Van der Waal's forces, resulting in a parallel alignment and a crystalline structure (Zhang et al. 2007a). Several elementary fibrils gether, forming much larger microfibrils, which are further bundled into larger macrofibrils, leading to the rigidity and strength of cell walls. Efficient conversion of cellulose into glucose has been a centre topic for long.

Hemicellulose, the second main polysaccharide, is a polymer containing primarily pentoses (xylose and arabinose) with hexoses (glucose and mannose), which are dispersed throughout and forming a short-chain polymer that intertwines with cellulose and lignin like a glue (Wilkie 1979).

Lignin is a polymer consisting of various aromatic groups. It can be converted to numerous chemical products that are made from fossil-based chemical industry, including coal, oils and natural gas.

The production of cellulosic ethanol holds promises for an improved strategic national security, job creation, strengthened rural economies, improved environmental quality, nearly zero net greenhouse gas emissions, and sustainable local resource supplies (Demain et al. 2005; Lynd et al. 1991; Lynd et al. 2002; Lynd et al. 1999; Zhang 2008). So far, more companies are working on decreasing costs of cellulosic ethanol production, such as Iogen Corporation, Abengoa Bioenergy, Dupont, British Petroleum, Mascoma (Biotechnology Industry Organization 2008). The price of E85 is still as high as $1.81 per gallon (DOE 2009). More efforts are still urgently

needed for achieving the goal of lowering the production cost to less than $2, which is projected to be the price-competitive with current corn-based ethanol.

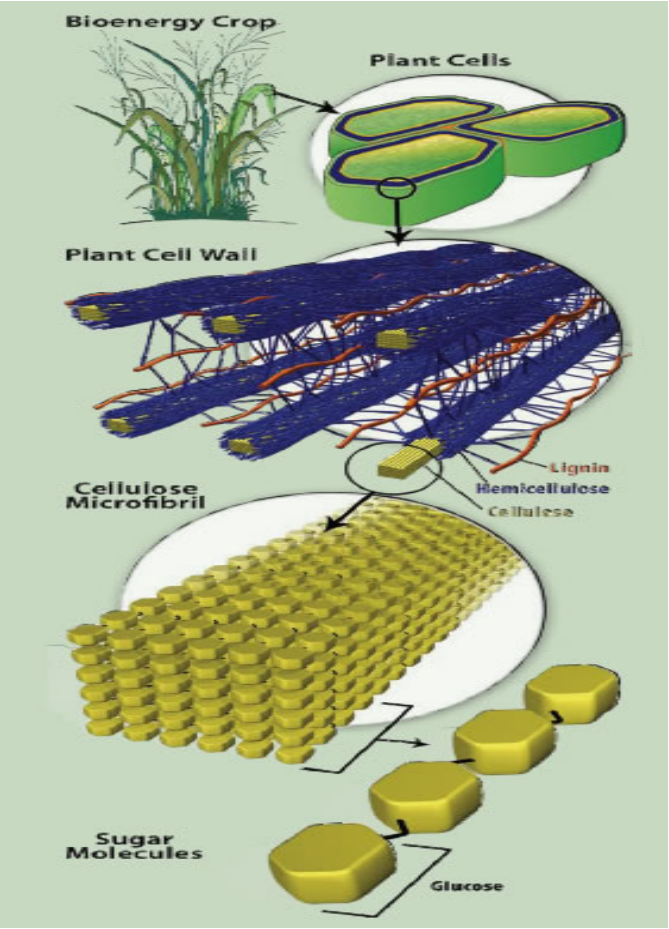


Figure 1-1. Basic structure of lignocellulose (http://www.lbl.gov/Publications/YOS/Feb/)

1.2 Lignocellulose pretreatment

1.2.1 Bioconversion of lignocellulose and pretreatment

Lignocellulosic biomass can be converted into soluble sugars via a large number of approaches. Different from the case of bioconversion of starch or any other soluble sugars into fuels and chemicals, this conversion for lignocellulose is much more complicated and difficult, considering their complex and recalcitrant structures (Jorgensen et al. 2007; Yang and Wyman 2008).

The bioconversion of lignocellulose basically contains three major processes (Figure 1-2): biomass pretreatment, enzymatic hydrolysis, and fermentation (Mielenz 2001). The purpose of pretreatment is to produce reactive cellulosic materials from the lignin matrix and open or partially break up recalcitrant structure while minimizing chemical degradation of fermentable sugars by some additional physical, chemical or biological technologies. Then, biomass can be more readily hydrolyzed by mixed cellulase after a pretreatment, and converted into monosaccharides, including mainly glucose and xylose. Microbes utilize these sugars, called fermentation, for producing cellulosic ethanol. The final product needs to be separated by distillation followed by dehydration.

Cost-efficient liberation of fermentable sugars from recalcitrant biomass is the most challenging technical and economic barrier to biorefineries (Fortman et al. 2008; Lynd et al. 2008; Zhang 2008). In other words, saccharification of lignocellulose, including pretreatment followed by enzymatic hydrolysis, is the central task of the whole bioconversion process.

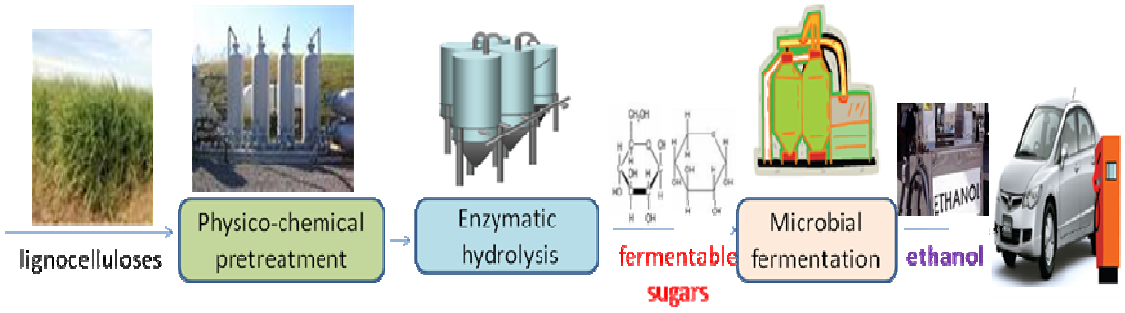


Figure 1-2. General flowchart of conversion from lignocellulose into ethanol.

Pretreatment is among the most costly steps in biochemical conversion of lignocellulosic biomass (Eggeman and Elander 2005), accounting for up to 40% of the total processing cost (Lynd 1996). Moreover, it strongly affects the downstream costs of operations including enzymatic hydrolysis and fermentation. For example, costs of detoxification if inhibitors are generated, limited enzymatic hydrolysis rate, high enzyme loading, low product concentration,

complicated product purification, waste treatment demands, and other process variables are all involved if a pretreatment is not efficient (Wyman et al. 2005b).

Numerous lignocellulose pretreatment approaches have been developed, which are reviewed by many researchers (Chandra et al. 2007b; Dale 1985; Eggeman and Elander 2005; Himmel et al. 2007; Ladisch et al. 1992; Lin et al. 1981; Lynd et al. 2008; McMillan 1994; Mosier et al. 2005; Office of Energy Efficiency and Renewable Energy and Office of Science 2006; Ragauskas et al. 2006; Sun and Cheng 2002; Vertès et al. 2006; Wyman 2007; Wyman et al. 2005a; Wyman et al. 2005b). Most notably, a collaborative team called consortium for applied fundamentals and innovation (CAFI) funded by the Department of Energy and Department of Agriculture has formed and focused on several "leading pretreatment technologies", including dilute (sulfuric) acid pretreatment, flow-through pretreatment, ammonia fiber expansion (AFEX), ammonia recycle percolation (ARP), and lime pretreatment for the past several years (Moxley 2007).

1.2.2 Various pretreatment technologies

Focusing on different components in lignocellulose, many researchers have developed hemicellulose-targeted, cellulose-targeted, and lignin-targeted pretreatment approaches (Dale et al. 1984). Lignocellulose pretreatment methods can also generally be divided into three categories: physical method, chemical method, and biological method. The combinations of these approaches are also popular and have been used for long, for example, physic-chemical pretreatment, such as ammonia fiber expansion (Dale et al. 1996; Ferrer et al. 2000; Gollapalli et al. 2002; Teymouri et al. 2005) or supercritical fluid extraction (Kim and Hong 2001; McMillan 1994). Recently, more "non-mainstream" pretreatment technologies have been developed, such as ozone pretreatment (Garcia-Cubero et al. 2009), alkali plus peracetic acid pretreatment (Zhao et al. 2009c), microwave irradiation (Zheng et al. 2007) and sulfite pretreatment for soft wood (Zhu et al. 2009a). But there are still many problems remained, such as, use of toxic chemical reagents, high reaction temperature, low digestibility, high cost of chemicals, poor chemicals recovery efficiency and difficult scale up.

Using solvent to fractionate lignocellulose has been proposed as a promising pretreatment approach. Solvent-based pretreatment includes cellulose solvent-based, organic solvent-based, or their combination. Cellulose solvent pretreatment has a long history (Ladisch et al. 1978). Cadoxen was used as a potential solvent for dissolving lignocellulose. However, its corrosivity, toxicity and high inhibition to subsequent enzymes in the hydrolysis step limited its further application.

Ionic liquid (IL) was found to possess a great potential in dissolving cellulose (Swatloski et al. 2002). A few ILs were developed to dissolve cellulose with various cellulose solubilities. Their performances could be improved by heating, microwave, or sonication (ElSeoud et al. 2007; Zhu et al. 2006). It was shown that lignocellulose could be pretreated by ILs, generating more digestible and less recalcitrant materials to the enzymes (Dreyer and Kragl 2008; Kilpeläinen et al. 2007; Zhao et al. 2009a; Zhao et al. 2009b; Zhu 2008). ILs have been of interest because of its ion-volatility. However, the presence of only 1 wt.% water in ILs was reported to result in a significant decrease of cellulose solubility through competitive hydrogen bonding to the cellulose microfibrils (Dadi et al. 2006; Kilpeläinen et al. 2007). Although ILs demonstrated a great potential as cellulose solvents as both salts and anions can be tailored, there are many stumbling barriers ahead before converting this process into reality. For instance, the high price of ILs, lack of physic-chemical data as well as toxicological and inhibitory results all suggest that more research should be done to understand its mechanisms and conduct solvent recycling. In addition, how to remove hemicellulose and lignin fractions from ionic liquids remains a big challenge.

Organic solvent (also can be referred as organosolv) is a relatively traditional technology which has been tested in wood or pulping industry for long (Aziz and Sarkanen 1989; Hergert 1998). The main purpose of organosolv is to delignify lignocellulose (McDonough 1993). It was normally conducted in the presence of dilute acid (Pan et al. 2005; Pan et al. 2007) or alkali pretreatment (Mutje et al. 2005) in that it removed lignin so that broke up the linkages between cellulose and hemicellulose. Additionally, low solvent recycle efficiency and large amount of usage are always the two largest shortcomings.

1.2.3 Lignocellulose fractionation

The main goal of pretreatment is to unlock sugars from its protective shells of lignin and increase enzymatic susceptibility of cellulose (and hemicellulose). There are several criteria for an effective pretreatment technology as following.

1) Generic (feedstock-independent) pretreatment is required to accommodate different biomass properties and diverse biomass sources.
2) It must promote an effective release of fermentable sugars from the recalcitrant biomass matrix such that sugar yield is maximized while degradation products are minimized. Since the degradation usually happens at harsh pretreatment conditions (i.e., high temperature), it would be better to pretreat lignocellulose at modest conditions.
3) Low enzyme loadings could be used after a pretreatment so that nonspecific binding of enzymes to lignin and other components of pretreated biomass can be avoided.
4) Efficient chemical recovery could be easily conducted.
5) All co-products are usable and marketable. Considering biofuels themselves are low-price commodities, a good strategy to earn more profits is through various co-products from bioconversion process. Therefore, the separation of different components in lignocellulose via pretreatment plays a vital role.

A new cellulose solvent- and organic solvent-based lignocellulose fractionation (COSLIF) has been demonstrated for separating lignocellulose components under modest reaction conditions (e.g., 50^{o}C and atmospheric pressure) by using a cellulose solvent, an organic solvent, and water (Zhang et al. 2007a). The key ideas of COSLIF are (1) removal of partial lignin and hemicellulose from cellulose (i.e., fewer substrate obstacles to enzyme so that cellulase can access the substrate more efficiently), (2) break up of orderly hydrogen bonds in crystalline cellulose (i.e., more cellulose accessibility so that cellulase can work on the substrate more efficiently) and (3) modest reaction conditions (i.e., avoid sugar degradation, less inhibitor formation, lower utility consumption, and less capital investment) (McMillan 1994; Moxley et al. 2008), (4) fractionation of lignocellulose components (i.e., increase in revenues by co-product utilization, de-toxification).

Figure 1-3 describes the overall process of lignocellulose fractionation technology, by using a cellulose solvent (concentrated phosphoric acid) and an organic solvent (i.e., acetone or ethanol) with their recycling (Zhang et al. 2009). The mechanisms for each unit operation are:

1) In the digestion tank, concentrated H_3PO_4 (> 83%) is mixed with milled lignocellulose at 50^{o}C for ~30-60 minutes. The cellulose solvent can disrupt the recalcitrant structure, dissolve cellulose fibrils and hemicellulose so as to break up orderly hydrogen bonds among sugar chains, weakly hydrolyze cellulose and hemicellulose so as to modestly reduce their degree of polymerization (DP), and de-acetyl groups from hemicellulose in an acidic condition.
2) In the precipitation tank, an organic solvent (e.g., acetone or ethanol) is added to precipitate the dissolved cellulose and hemicellulose and to dissolve partial lignin in the organic solvent.
3) In the washer-1 (solid/liquid separator), the organic solvent washes out ~99.5% of phosphoric acid from the precipitated solids and to further remove (leach) the lignin.
4) In the washer-2 (solid/liquid separator), water is used to wash out the organic solvent in the solids and to remove water-soluble short-DP hemicellulose fragments from the solid cellulose.
5) In the hydrolysis reactors, nearly pure amorphous cellulose is hydrolyzed fast at 50^{o}C by using the Trichoderma cellulase;
6) In the distiller, the black liquor containing phosphoric acid, acetone, acetone-soluble lignin, and acetic acid can be separated. Highly volatile acetone and modestly volatile acetic acid are separated by fractionation distillation; after removal of the organic solvent, the precipitated lignin can be separated from concentrated phosphoric acid at the bottom of the column by a solid/liquid separator.
7) In the flash tank, the light liquor containing acetone, a tiny amount of phosphoric acid and water-soluble hemicellulose can be separated. Acetone can be recovered by flashing. Addition of $CaCO_3$ can neutralize trace phosphoric acid and form a precipitate of $Ca_3(PO4)_2$; the precipitated $Ca_3(PO_4)_2$ can be regenerated to concentrated phosphoric acid by adding concentrated sulfuric acid. Water soluble hemicellulose remains in the liquid phase.

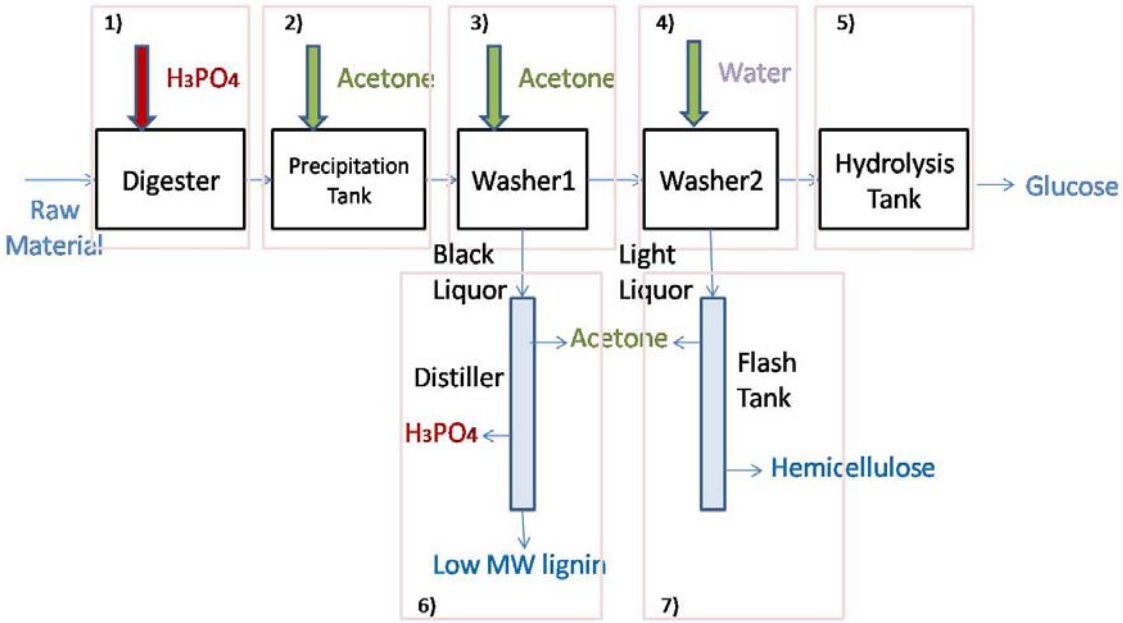


Figure 1-3. Conceptual flowchart of cellulose solvent- and organic solvent-based lignocellulose fractionation with recycling of concentrated phosphoric acid and acetone.

To sum up, this technology can fractionate lignocellulose into amorphous cellulose (glucose mainly after hydrolysis), lignin, hemicellulose, and acetic acid at modest reaction conditions with easy recycling of the organic solvent and phosphoric acid. This new technology isolates lignocellulosic components based on their solubilities and volatilities in different solvents, and each unit operation for the separation is easy, e.g., solid/liquid separation.

1.3 Cellulose hydrolysis

1.3.1 Cellulose hydrolysis overview

Pretreated lignocellulose is subjected to be hydrolyzed into fermentable sugars by enzymes before conversion to fuels. Cellulose hydrolysis has been the dominant topic in the area of biofuels.

Enzymatic hydrolysis of cellulose is a very slow and complicated biological process, much slower than degradation of any other natural polysaccharide such as starch or hemicellulose. It is

implemented by two different cellulase systems: a non-complexed cellulase system and a complexed cellulase system (cellulosome) (Lynd et al. 2002; Tomme et al. 1995). Both systems involve multiple enzymatic components, including endoglucanase (EC 3.2.1.4), exoglucanase or cellobiohydrolase (EC 3.2.1.91), and ß-glucosidase (EC 3.2.1.21). Generally speaking, accessible intramolecular β-glucosidic bonds of cellulose chains are randomly cut by endoglucanases to generate new chain ends; exoglucanases release soluble cellobiose in a processive manner from both ends of cellulose chains; β-glucosidases hydrolyze cellobiose (and other soluble oligoglucan) into glucose (Teeri 1997; Warren 1996; Zhang and Lynd 2004).

Cellulases from aerobic fungi have been intensively investigated for a long time (Reese 1956; Reese et al. 1950). For example, current industrial cellulase are mainly made from fungi (Cherry and Fidantsef 2003; Kirk et al. 2002). Figure 1-4 is a generally-accepted mechanism for fungal enzymatic cellulose hydrolysis which considers synergistic actions among endoglucanase, exoglucanase, and β-glucosidase (Lynd et al. 2002; Zhang and Lynd 2004).

1.3.2 Substrate properties

Cellulose hydrolysis is a heterogeneous process, including insoluble substrates and soluble enzymes. Therefore, characteristics of substrates impacts on this process. Cellulose is made up of repeating anhydrocellobiose units. Coupling of adjacent cellulose molecules by orderly hydrogen bonds and van der Waal's forces results in a parallel alignment and a crystalline structure, producing a straight, stable supramolecular structure and low accessibility (Zhang and Lynd 2004; Zhbankov 1992). Three most investigated structural characteristics of cellulose are considered the most important factors limiting the rate of cellulose hydrolysis. They are crystallinity, degree of polymerization, and accessibility (including particle size, pore volume and accessibility to certain molecules).

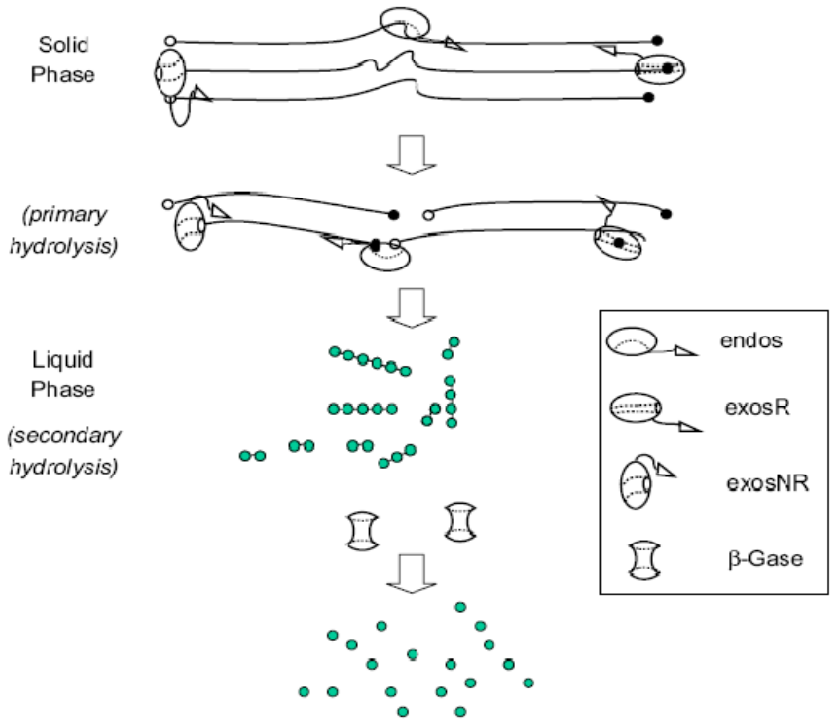


Figure 1-4. Mechanistic scheme of enzymatic cellulose hydrolysis by Trichoderma non-complexed cellulase system (Zhang et al. 2006b).

Crystallinity of cellulose has been proposed as an indicator of substrate reactivity since 1975 (Wood 1975). The crystallinity of dried cellulose samples can be quantitatively measured from the wide-range X-ray diffraction pattern (Krassig 1993). Based on crystallinity, cellulose can be generally classified into crystalline cellulose and amorphous cellulose. Cellulose hydrolysis rates mediated by fungal cellulases are typically 3 to 30 times faster for amorphous fraction than for crystalline one (Zhang and Lynd 2004). Due to the preferential hydrolysis on amorphous cellulose (Ooshima et al. 1983), crystallinity of cellulose is supposed to increase during hydrolysis process. However, opposite conclusions were found against this hypothesis (Ohmine et al. 1983; Puls and Wood 1991). A lot of conflicting results on the changes in crystallinity during hydrolysis (maybe due to a unreliable crystallinity measurement) suggest that crystallinity may not be a key substrate characteristic (Mansfield et al. 1999; Zhang and Lynd 2004).

The degree of polymerization (DP) of cellulosic substrates determines the relative abundance of terminal ß-glucosidic bonds and of substrates for exo-acting (Zhang and Lynd 2004). It changes over the period of cellulose hydrolysis and the change is affected by the relative proportion of exo- and endo-glucanases as well as substrate properties (Zhang et al. 2006a), because exoglucanases attach to the end of cellulose chain, leading a slow decrease in DP (Divne et al. 1993; Kleman-Leyer et al. 1996), while endoglucanases act on the middle of accessible cellulose chains with a result of a dramatic decrease in DP (Srisodsuk et al. 1998; Whitaker 1957; Wood 1975). Various cellulosic materials have different DPs (Zhang and Lynd 2004). Different DPs also result in different cellulase preferences because of various catalytic domains of different cellulases (Kleywegt et al. 1997; Teeri 1997).

Substrate accessibility is of great importance to a heterogeneous hydrolysis, in which most of ß-glucosidic bonds are not accessible to cellulase. The geometric sizes and configurations of cellulosic substrates vary, leaving their different accessibilities to enzymes. Cellulose particles contain both external and internal surfaces. The internal surface area of cellulose is generally 1-2 orders of magnitude higher than the external surface area (Chang et al. 1981), except for bacterial cellulose. There are many techniques available for measuring the internal surface area of cellulose, such as size exclusion, small angle X-ray scattering (SAXS), and water vapor adsorption (Caulfield and Moore 1974; Grethlein 1985; Neuman and Walker 1992; Stone et al. 1969). However, these approaches do not reflect the real reaction surface (110 face) accessibility to cellulase, resulting in an over-estimation of it. Also, these internal surface measurement techniques do not give an estimation of the external area (Converse 1993). The external surface area of cellulose is closely related to the shape and particle size of cellulosic samples, and can be estimated by microscopy (Henrissat et al. 1988; Lehtio et al. 2003; White and Brown 1981). Gross cellulose accessibility is generally measured by the molecule adsorption method, such as using nitrogen, argon, water, alkali, or organic liquids (Zhang and Lynd 2004). The most widely used procedure for determination of the gross surface area is the Brunauer-Emmett-Teller (BET) method based on nitrogen adsorption. However, due to variations in experimental conditions such as adsorption time, vacuum time, vacuum pressure, sample preparation, and sample origin (Marshall and Sixsmith 1974), a wide range of gross area values has been reported in literatures

even for the same substrate. Moreover, since molecules such as nitrogen or water are much smaller than real cellulase, they can access many pores and cavities on the fiber surface that cellulase cannot enter, therefore causing a misleading of accessibility.

1.3.3 Cellulase adsorption and desorption

Cellulase adsorption on cellulose is a prerequisite to hydrolysis. Adsorption of cellulase usually reaches the equilibrium state within a short time. This adsorption is commonly described using Langmuir isotherm (Hong et al. 2007; Zhang and Lynd 2004), which assumes that single layer adsorption, balance between rates of adsorption and desorption, and constant binding affinity and capacity. T he Langmuir equation provides a good data fitting in most cases. Actually, more complicated situation exists, such as partially irreversible adsorption or entrapment by substrate matrix (Lee et al. 1983; Palonen et al. 1999), interaction among several cellulase components (Jeoh et al. 2002), multi-layer adsorption (Carrard and Linder 1999), and multi-cellulase competitive adsorption with various affinities (Beldman et al. 1987). A large amount of values of parameters in Langmuir equation are available, whereas these data have a wide variation, due to different measurements taken and different experimental conditions. Considering the complexity of cellulase system and various properties of different substrates, numerous studies have been conducted and several different conclusions or hypothesis co-exist (Zhang and Lynd 2004). For example, some cellulases can bind completely reversible to some cellulosic materials while others bind partially irreversible (Kyriacou et al. 1989; Palonen et al. 2004). Different cellulase components may competitively adsorb on the same site in substrate (Jeoh et al. 2002; Medve et al. 1997; Pinto et al. 2004), and have different binding preferences (Boraston 2005; Ding et al. 2006; Lehtio et al. 2003). From a structural point of view, adsorption of cellulase is through a specific protein domain called cellulose binding domain (or more generally, based on the recent study, carbohydrate binding module) (Carrard et al. 2000; Creagh et al. 1996; Divne et al. 1994; Linder and Teeri 1996). Several papers have reviewed the roles and function of these binding domains (Linder and Teeri 1997; Shoseyov et al. 2006; Teeri 1997).

Desorption of cellulase is linked with the potential of cellulase recycling and re-utilization for saving cellulase cost. In the hydrolysis of pure cellulose, theoretically all the cellulase should be

reversible bound so that they can release and move onto the new intact substrate after they finish the job on the old one. However, sometimes this is not the case (Jung et al. 2003; Kyriacou et al. 1989). For lignocellulosic substrate, it is more complicated due to the presences of lignin, hemicellulose and other components such as protein or hydrolysis inhibitors. These fractions could unproductively adsorb most cellulase, therefore hindering the hydrolysis process (Converse 1993; Lee et al. 1995; Selig et al. 2007; Tu et al. 2007) and causing the cellulase difficult to remove or recycle (Desai and Converse 1997; Gregg and Saddler 1996; Mansfield et al. 1999). To investigate substrate reactivity during the course of hydrolysis, it is also needed to develop an efficient approach to desorb cellulase from hydrolysis residues without influencing substrate reactivity. Previous several studies on substrate reactivity have controversial conclusions mainly due to the un-efficient cellulase removal by certain methods (Yang et al. 2006).

In all, cellulose (lignocellulose) hydrolysis incorporates not only knowledge of enzymes but also deep understanding of various substrates. Undoubtedly, the hydrolysis of lignocellulose (normally after pretreatment) is much more complicated than one of pure cellulose in that the former incorporates more variables that significantly influence performances of enzymes and characteristics of substrates themselves (Chandra et al. 2007b). To find out more relationship between hydrolysis and pretreatment, a deeper understanding of lignocellulose saccharification is needed.

1.4 Goals and Significances

This study contains two parts:

1. To investigate the most important substrate characteristic influenced by pretreatment and affecting enzymatic cellulose hydrolysis by quantifying cellulose and non-cellulose fractions' accessibility to cellulase in a comparative study of two pretreatments (Chapter 2);
2. To develop a new method which can measure the adsorbed cellulase directly on lignocellulosic residues for potential application to cellulase recycling (Chapter 3).

Two different pretreatment methods were compared. The key cause for their different hydrolysis performance was quantified -- substrate accessibility. In addition, a method was developed that can directly measure adsorbed cellulase on lignocellulosic samples. Using this method, several potential approaches for cellulase recycling and removal were examined.

This study would provide more information to compare lignocellulose pretreatment technologies, study enzymatic hydrolysis mechanisms, and investigate potential cellulase recycling for low-cost production of cellulosic ethanol.

2. Comparative study of corn stover pretreated by dilute acid and cellulose solvent and organic solvent lignocellulose fractionation

2.1 Abstract

Liberation of fermentable sugars from recalcitrant biomass is among the most costly steps for emerging cellulosic ethanol production. Here we compared two pretreatment methods (dilute acid, DA, and cellulose solvent and organic solvent lignocellulose fractionation, COSLIF) for corn stover. At a high cellulase loading [15 filter paper units (FPUs) or 12.3 mg cellulase per gram of glucan], glucan digestibilities of the corn stover pretreated by DA and COSLIF were 84% at hour 72 and 97% at hour 24, respectively. At a low cellulase loading (5 FPUs per gram of glucan), digestibility remained as high as 93% at hour 24 for the COSLIF-pretreated corn stover but only reached ~60% for the DA-pretreated biomass. Quantitative determinations of total substrate accessibility to cellulase (TSAC), cellulose accessibility to cellulase (CAC), and non-cellulose accessibility to cellulase (NCAC) based on adsorption of a non-hydrolytic recombinant protein TGC were measured for the first time. The COSLIF-pretreated corn stover had a CAC of 11.57 m^2/g, nearly twice that of the DA-pretreated biomass (5.89 m^2/g). These results, along with scanning electron microscopy images showing dramatic structural differences between the DA- and COSLIF-pretreated samples, suggest that COSLIF treatment disrupts microfibrillar structures within biomass while DA treatment mainly removes hemicellulose. Under the tested conditions COSLIF treatment breaks down lignocellulose structure more extensively than DA treatment, producing a more enzymatically reactive material exhibiting a higher CAC accompanied by faster hydrolysis rates and higher enzymatic digestibility.

2.2 Introduction

Lignoellulosic biomass is a natural complex composite primarily consisting of three biopolymers: cellulose, hemicellulose, and lignin (Fengel and Wegener 1984; Himmel et al. 2007; Lynd et al. 2002; Zhang 2008). Efficient, cost-competitive production of fermentable sugars from

recalcitrant biomass remains the largest obstacle to emerging cellulosic ethanol biorefineries (Lynd et al. 2008; Wyman 2007; Zhang 2008). Biomass saccharification via biological conversion involves two steps – lignocellulose pretreatment or fractionation followed by enzymatic cellulose (and perhaps hemicellulose) hydrolysis. Dilute acid pretreatment (DA), typically using dilute sulfuric acid, is the most investigated pretreatment method (Bernardez et al. 1993; Grethlein 1985; Grethlein and Converse 1991; Lloyd and Wyman 2005; Ooshima et al. 1990; Schell et al. 2003; Thompson et al. 1992). Conducted at relatively high temperatures (150-200°C) and pressures (120-200 psia), DA pretreatment solublizes acid-labile hemicellulose and thereby disrupts linkages between cellulose, hemicellulose, and lignin (Burns et al. 1989; Lloyd and Wyman 2005; Ooshima et al. 1990). As a result, the condensed lignin remains on the surface of crystalline cellulose following DA, potentially hindering subsequent enzymatic hydrolysis (Bernardez et al. 1993; Jeoh et al. 2007; Liu and Wyman 2003; Lloyd and Wyman 2005). In cellulose solvent and organic solvent lignocellulose fractionation (COSLIF), a cellulose solvent (e.g., concentrated phosphoric acid or ionic liquid) enables the crystalline structure of cellulose to be disrupted. This type of pretreatment can also be carried out at low temperature (~50^{o}C) and the atmospheric pressure where sugar degradation is minimized (Moxley et al. 2008; Zhang et al. 2007a). Subsequent washing steps are used to fractionate biomass; a first washing with an organic solvent to remove lignin; and a second washing with water to remove fragments of partially-hydrolyzed hemicellulose. The COSLIF approach produces highly reactive amorphous cellulose, which can be enzymatically hydrolyzed quickly with high glucan digestibility yield (Moxley et al. 2008; Zhang et al. 2007a).

The root causes of biomass recalcitrance could be attributed to a number of factors, such as substrate accessibility to cellulase, cellulose degree of polymerization (DP), cellulose crystallinity, lignin content and structure, and hemicellulose content (Chandra et al. 2007a; Himmel et al. 2007; Kim and Holtzapple 2005; Zhang et al. 2006b; Zhang and Lynd 2004). A functionally-based mathematical model of fungal enzyme-based enzymatic cellulose hydrolysis has been developed, accounting for cellulose characteristics (degree of polymerization and substrate accessibility) and different modes of action for endoglucanase and cellobiohydrolase enzyme system components (Zhang and Lynd 2006). This model not only correlates disparate

phenomena reported in the literature but also clearly suggests that low cellulose accessibility is the most important substrate characteristic limiting enzymatic hydrolysis rates (Zhang and Lynd 2006). More recently, a quantitative assay for determining cellulose accessibility to cellulase (CAC) has been established based on adsorption of a non-hydrolytic fusion protein (TGC) containing a cellulose-binding module and a green fluorescence protein (Hong et al. 2007). This new approach more accurately assesses substrate characteristic related to enzymatic cellulose hydrolysis than traditional methods such as nitrogen adsorption-based Brunauer-Emmett-Teller (BET), size exclusion, and small angle X-ray scattering (Hong et al. 2007; Zhang and Lynd 2004). Regenerated amorphous cellulose (RAC) that is prepared from microcrystalline cellulose (Avicel) has ~20 fold higher CAC (Hong et al. 2008b; Hong et al. 2007) and exhibits much faster enzymatic hydrolysis rates than microcrystalline cellulose (Zhang et al. 2006a), which is in agreement with model predictions that increasing CAC is more important for increasing hydrolysis rates than decreasing DP (Zhang and Lynd 2006). The CAC data of Avicel (m^2 per gram of Avicel) based on the TGC adsorption is only one tenth of that based on the BET method (Marshall and Sixsmith 1974), implying that about 90% gross surface area measure based on nitrogen adsorption cannot be accessible to large-size cellulase protein molecules, at least initially. Traditional size exclusion techniques are labor intensive and cannot distinguish the real cellulase binding area (110 face) or ignore the external surface area.

Enzymatic hydrolysis of pretreated lignocellulose is more challenging than enzymatic hydrolysis of pure cellulose because any remaining lignin and residual hemicellulose could adsorb cellulase components and thereby block or impede cellulose hydrolysis (Berlin et al. 2005; Bernardez et al. 1993; Converse et al. 1990; Grethlein and Converse 1991; Kurabi et al. 2005b; Ooshima et al. 1990; Wyman 2007). The total substrate accessibility has been measured previously by using cellulase-size molecule exclusion (Burns et al. 1989; Esteghlalian et al. 2001; Grethlein 1985; Thompson et al. 1992), low-temperature cellulase adsorption (Gerber et al. 1997; Kumar and Wyman 2008b; Lee et al. 1994; Lu et al. 2002; Mooney et al. 1998) or labeled cellulase (Jeoh et al. 2007; Palonen et al. 2004). However, in pretreated lignocellulose materials it remains relatively challenging to quantitatively differentiate between enzyme accessibility to cellulose and non-cellulose fractions.

In this study, we compared the enzymatic hydrolysis behaviors (enzymatic cellulose hydrolysis rates and yields) of corn stover pretreated by DA and COSLIF pretreatment approaches. We also used scanning electron microscopy (SEM) to examine the supramolecular structures of DA- and COSLIF-pretreated corn stover samples. Additionally, we developed and applied new quantitative assays for substrate accessibility by distinguishing cellulose and non-cellulose fractions of pretreated lignocellulose.

2.3 Materials and methods

2.3.1 Chemicals and microorganisms

All chemicals were reagent grade, purchased from Sigma (St. Louis, MO, USA) and Fisher Scientific (Pittsburgh, PA, USA), unless otherwise noted. Fungal cellulase Spezyme CP was gifted from Genencor (Palo Alto, CA, USA). Novozymes 188 ß-glucosidase was purchased from Sigma. Microcrystalline cellulose (Avicel PH105) was purchased from FMC (Philadelphia, PA, USA). Regenerated amorphous cellulose (RAC) was prepared through Avicel dissolution in concentrated phosphoric acid followed by regeneration in water (Zhang et al. 2006a). Corn stover was obtained from the National Renewable Energy Laboratory (NREL, Golden, CO). Corn was grown from biomass AgriProducts (Harlan, IA). The tub-ground materials was approximately 9 month old (harvested in fall 2006). The recombinant thioredoxin-green fluorescent protein-cellulose binding module (TGC) fusion protein was produced in Escherichia coli BL 21 (pNT02) (Hong et al. 2007) and purified by affinity adsorption on RAC followed by modest desorption using ethyl glycol (EG) (Hong et al. 2008b). The EG was removed by using membrane dialysis in a 50 mM sodium citrate buffer (pH 6.0). The TGC protein solution was re-concentrated using a 10,000 Dalton molecular weight cut-off centrifugal ultrafilter columns (Millipore Co., Billerica, MA).

2.3.2 Pretreated corn stovers

Dilute sulfuric acid pretreated corn stover was produced in a pilot-scale continuous vertical reactor at 190ºC, 0.048 g acid/g dry biomass, 1 min residence time, and a 30% (w/w) total solid

loading by using procedures discussed elsewhere (Schell et al. 2003). The acidic slurry was stored at 4 ^{o}C prior to use. Before experiments, the slurry was dewatered and the pretreated solids were washed with deionized water until the pH of the washed water reached pH ~6.

Cellulose solvent and organic solvent lignocellulose fractionation (COLSIF) was conducted as described previously (Moxley et al. 2008; Zhang et al. 2007a). One gram of dry corn stover in a 50-mL plastic centrifuge tube was mixed with 8 mL of 84% phosphoric acid using a glass rod, and the mixed slurry was then incubated at a 50°C water bath for 20 or 45 min. The reaction was then stopped by an ice-water bath. Forty milliliters of acetone was then added to precipitate dissolved cellulose and hemicellulose. The slurry was spun down for 20 min at room temperature using a swinging bucket centrifuge operating at 3,600 rpm. The pellets were re-suspended and washed in 40 mL of acetone twice more. After 3 acetone washes, the pellets were washed 2 more times with water. The residual amorphous solid pellet was neutralized to pH 5-7 using 2 M sodium carbonate.

The amounts (dry weight percentages) of sugars and lignins in the DA- and COSLIF-pretreated corn stover were measured using a modified quantitative saccharification method, which can determine acid-labile hemicellulose composition more accurately (Moxley and Zhang 2007).

2.3.3 Enzymatic hydrolysis of pretreated corn stover

The pretreated corn stover samples were diluted to 10 g glucan per liter in a 50 mM sodium citrate buffer (pH 4.8) supplemented with 0.5 g/L sodium azide for enzymatic hydrolysis. All hydrolysis experiments were carried out in a rotary shaker at 250 rpm and 50°C. Two enzyme loadings were used: (1) 15 FPUs cellulase and 30 units Novozyme 188 ß-glucosidase per gram of glucan (12.3 mg cellulase and 9.4 mg ß-glucosidase per gram of glucan) as well as (2) 5 FPUs cellulase and 30 units ß-glucosidase per gram of glucan (4.1 mg cellulase and 9.4 mg ß-glucosidase per gram of glucan). Eight hundred micro-liters of evenly-mixing hydrolysate were taken followed by immediate centrifugation at 13,000 rpm for 5 min. Exactly 500 μL of the supernatant was transferred to another microtube and held at room temperature for 30 min, ensuring conversion of nearly all cellobiose to glucose by free ß-glucosidase. The supernatant

was acidified by adding 30 μL of 10% (w/w) sulfuric acid and then frozen overnight. The freshly-thawed liquid samples were mixed well and then centrifuged at 13,000 rpm for 5 min to remove any solid sediments. Glucose concentration in the clear supernatants was measured by HPLC using a Bio-Rad HPX-87H column operating at 65°C with a mobile phase of 0.005 M sulfuric acid at a flow rate of 0.6 mL/min (Zhang and Lynd 2003a; Zhang and Lynd 2005a). After 72-hour hydrolysis, the remaining hydrolysis slurries were transferred to 50-mL centrifuge tubes and centrifuged at 3600 rpm for 20 min. After decanting the supernatant, the pellets were re-suspended in 20 mL of water and then centrifuged to remove soluble sugars. After centrifugation, the remaining sugars and lignin in the lyophilized pellets were measured by quantitative saccharification. The soluble glucose and xylose was measured by HPLC as described above.

Glucan digestibility (X_G) at the end of hydrolysis (hour 72) was calculated using the ratio of soluble glucose (G_{sol}) in the supernatant to the sum of G_{sol} and the residual glucan expressed in terms of glucose equivalents (G_{res}) in the solid phase (Eq. 2-1) (Moxley et al. 2008; Zhang et al. 2007b).

$$X_G = \frac{G_{sol}}{G_{sol} + G_{res}} \times 100\% \qquad [2\text{-}1]$$

2.3.4 Scanning electron microscopy

Supramolecular structures of the intact and pretreated corn stover samples were examined by scanning electron microscopes, as described elsewhere (Selig et al. 2007; Zhang et al. 2006a).

2.3.5 Protein mass concentration assays

The mass concentrations of the non-adsorbed proteins – bovine serum albumin (BSA), ß-glucosidase, and cellulase–were measured by the BioRad Bradford protein kit (Richmond, CA) with BSA as the protein standard. Mass concentration of the non-adsorbed TGC protein was measured based on fluorescence reading using a BioTek multi-detection microplate reader, as described elsewhere (Hong et al. 2008a; Hong et al. 2007).

2.3.6 Protein adsorption

Adsorption of cellulase, ß-glucosidase, and BSA on pure cellulose samples or birch xylan was conducted at room temperature for 1 hour in a 50 mM sodium citrate buffer using Avicel and RAC and birch xylan at various concentrations (0.5-100 g Avicel/L, 0.1-10 g RAC/L and 0.5-50 g). After centrifugation at 13,000 g for 5 min, the protein concentrations in the supernatant were measured by the Bradford method, as described previously (Zhang and Lynd 2003b; Zhang and Lynd 2005b).

The maximum TGC adsorption capacity was calculated based on the Langmuir isotherm (i.e., a fixed amount of adsorbent in terms of various concentrations of TGC). Eight hundred microliters of pretreated corn stover slurry solutions containing 1 g glucan/L and a final TGC concentration from 0.05 to 0.3 g/L was well mixed in a 50 mM sodium citrate buffer (pH 6.0) at room temperature for 1 hour. After centrifugation, the free TGC concentrations were measured by the BioTek multidetection microplate reader. $A_{max,TGC}$ was calculated based on the maximum TGC adsorption capacity of the pretreated samples. $A_{max,BSA/TGC}$ was calculated based on the maximum TGC adsorption capacity of the pretreated samples that had been blocked by adding an excess of BSA (5 g/L, final) for 1 hour before adding TGC. The BAS blocking was conducted at a pH 4.80. After BSA blocking, the pH was adjusted back to 6.00 by adding sodium carbonate, and TGC adsorption was assessed by fluorescence measurement as described above.

2.3.7 Quantitative substrate accessibility determination

Cellulase adsorption on the surface of cellulose can be described by the Langmuir equation:

$$E_a = \frac{W_{max} K_p E_f}{1 + K_p E_f} \qquad [2\text{-}2]$$

in which E_a is adsorbed protein (μmole/L), W_{max} is the maximum protein adsorption per L (μmole/L), E_f is free cellulase (μmole/L), and K_P is the dissociation constant ($K_P = E_a/(E_f S)$) in terms of L/g cellulose. The W_{max} and K_p values in Equation 2-2 can be calculated by a number of mathematical data fitting methods.

Cellulose accessibility to cellulase (CAC, m^2/g cellulose) has been defined previously (Hong et al. 2007; Zhang and Lynd 2004; Zhang and Lynd 2006)

$$CAC = \alpha * A_{max} * N_A * A_{G2} \quad [2\text{-}3]$$

where

α is 21.2 cellobiose lattices occupied by a TGC molecule (Hong et al. 2007),

A_{max} = maximum cellulase adsorption capacity (mole cellulase/g cellulose),

$A_{max} = W_{max}/(10^6 * S)$,

S = cellulose concentration (g cellulose/L),

N_A = Avogadro's constant (6.023×10^{23} molecules/mol), and

A_{G2} = area of the cellobiose lattice in the 110 face (0.53×1.04 nm = 5.512×10^{-19} m^2).

Total (biomass) substrate accessibility to cellulase (TSAC), including CAC and NCAC, represents cellulase adsorption capacity for the entire pretreated biomass. For pure cellulosic samples, TSAC equals CAC since NCAC equals zero.

For pretreated lignocellulosic biomass, TSAC (m^2/g biomass) can be estimated from direct adsorption of the TGC protein,

$$TSAC = \alpha * A_{max,TGC} * N_A * A_{G2} \quad [2\text{-}4]$$

where

$A_{max,TGC}$ = maximum TGC adsorption capacity of the biomass (μmole TGC/g biomass).

CAC (m^2/g biomass) can be measured based on the maximal TGC adsorption capacity after first blocking adsorption by using a large amount of BSA (e.g., 5 g/L) that can non-specifically bind on the surface of lignin (Berlin et al. 2005; Yang and Wyman 2006)

$$CAC = \alpha * A_{max,BSA/TGC} * N_A * A_{G2} \quad [2\text{-}5]$$

where

$A_{max,BSA/TGC}$ = maximum TGC adsorption capacity of biomass after BSA blocking (μmole TGC/g biomass).

Therefore, NCAC (m^2/g biomass) can be calculated as

$$NCAC = TSAC - CAC \quad [2\text{-}6]$$

2.4 Results

For COSLIF pretreatment, we have previously found that (1) phosphoric acid at concentrations beyond the critical value (> ~83%) acts as a cellulose solvent, (2) reaction time should be sufficient to dissolve biomass but be short enough to prevent complete hydrolysis, and (3) reaction temperature is set below 60^{o}C for no detectable xylose degradation (Zhang et al. 2007a). The optimal reaction condition for corn stover is 84% phosphoric acid, 50^{o}C, and 45 min. Although the DA pretreatment conditions used in this study are known to produce enzymatically digestible material, they have not been optimized. Table 2-1 shows glucan, hemicellulose, and lignin contents of the COSLIF-pretreated and DA-pretreated corn stover. COSLIF pretreatment removes more lignin than DA pretreatment, producing a material with lower levels of residual lignin (19.7% versus 30.3%, respectively). On the other hand, COSLIF pretreatment removes less hemicellulose than DA pretreatment; residual levels of hemicellulose are 6.2% and 3.4%, respectively.

Table 2-1. Composition of corn stover samples

method	cellulose	hemicellulose	Total lignin	Acid-soluble lignin	Klason lignin	ashes	protein
Non-pretreated	38.9 ± 1.9 %	28.3 ± 3.0 %	18.3 ± 0.9 %	7.0 ± 0.5 %	11.3 ± 0.5 %	6.6 ± 0.2 %	5.9 ± 0.5 %
COSLIF	58.2 ± 2.5 %	6.2 ± 0.3 %	19.7 ± 0.3 %	0.7 ± 0.0 %	18.9 ± 0.3 %	7.1 ± 0.1 %	7.5 ± 0.8 %
DA	53.7 ± 1.5 %	3.4 ± 0.2 %	30.3 ± 0.7 %	1.2 ± 0.1 %	29.2 ± 0.7 %	3.4 ± 0.2 %	4.3 ± 0.4 %

2.4.1 Enzymatic hydrolysis

Figure 2-1 shows the glucan digestibility profiles for the corn stover pretreated by DA and COSLIF at two different enzyme loadings (A, 15 FPUs cellulase per gram of glucan; and B, 5 FPUs cellulase per gram of glucan). At the high enzyme loading, glucan digestibility of the COSLIF-pretreated corn stover (45 min) was greater than 90% at hour 12 and reached 97% at hour 24. When COSLIF pretreatment time was decreased to 20 min, hydrolysis rates were slower and digestibility was lower. But a long COSLIF reaction time was not recommended because it resulted in low solid glucan retention. If concentrated phosphoric acid completely hydrolyzed cellulose to soluble sugars, cost- effective separation of soluble sugars and soluble acid would be challenging, similar to what occurs using concentrated sulfuric acid for cellulose saccharification (Fengel and Wegener 1984; Zhang et al. 2007a). In contrast, DA-pretreated corn stover exhibited considerably slower enzymatic hydrolysis rates, with glucan digestibility reaching 84% at hour 72. At a low enzyme loading (5 FPUs per gram of glucan), final glucan digestibility of the COSLIF-pretreated biomass was 93% within 24 hours, while digestibility of DA-pretreated biomass only reached 60% at hour 72. The significant difference in observed enzymatic hydrolysis behaviors between the COSLIF-pretreated and DA-pretreated biomass samples motivated additional studies to develop a better understanding of the causes. at enzyme loadings of (A) 15 FPUs cellulase + 30 units /g glucan β-glucosidase per gram of glucan and (B) 5 FPUs cellulase + 30 units /g glucan β-glycosidase per gram of glucan.

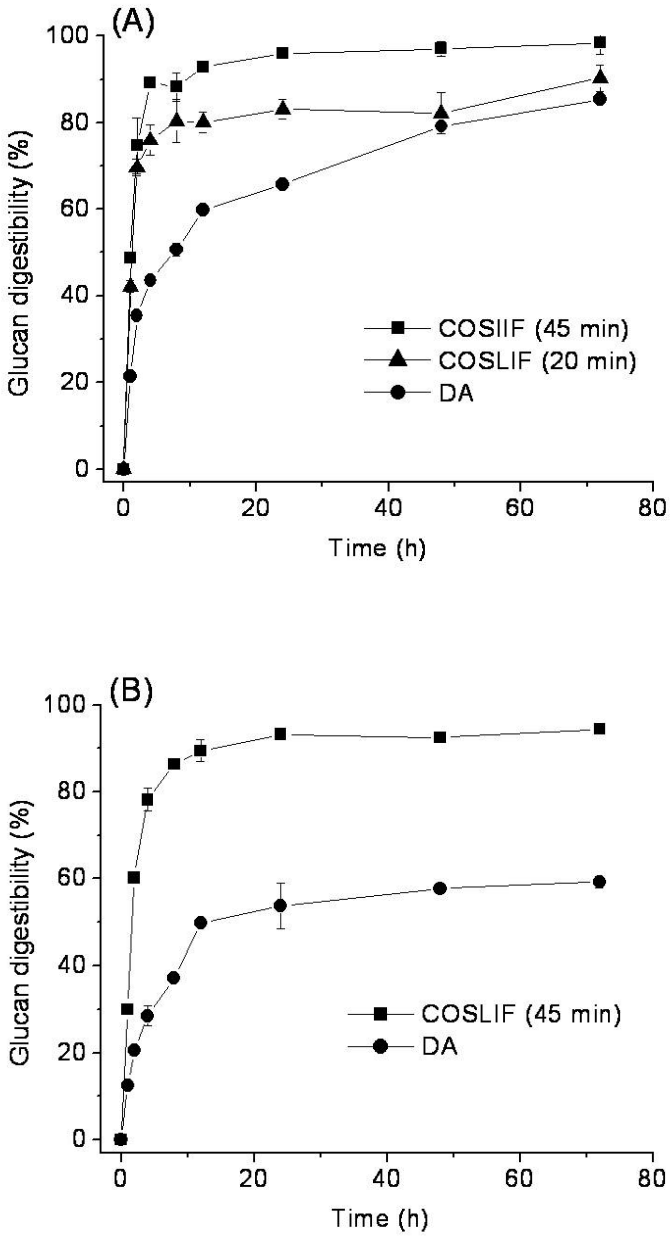


Figure 2-1. Enzymatic hydrolysis profiles of corn stover pretreated by COSLIF (84% H_3PO_4, 50°C, and 45 min or 20 min) and DA (4.8 % sulfuric acid, 190ºC, and 1 minutes).(A) 15 FPUs cellulase / g glucan, (B) 5 FPUs cellulase / g glucan.

2.4.2 Supramolecular structures

The supramolecular structure changes in corn stover before and after the different pretreatments are shown by using SEM. The intact plant cell wall structure of corn stover shows evidence of plant cell wall vascular bundles and a highly fibrillar structure (Fig. 2-2A). Dilute acid pretreatment disrupts the linkages among cellulose, hemicellulose, and lignin by mainly dissolving hemicellulose. As a result, major microfibrous cellulose structures remain (Fig. 2-2B) and some lignin or lignin-carbohydrate complexes may be condensed on the surface of the cellulose fibers. Treatment with concentrated H_3PO_4 significantly alters the fibrillar structure. A well-pretreated lignocellulose (corn stover) COSLIF sample (84.0% H_3PO_4, 50^oC and 45 min) shows no clear fibrous structure (Fig. 2-2C). These qualitative images are consistent with the observations that faster hydrolysis rates and higher glucan digestibilities are obtained for COSLIF-pretreated biomass than for DA-pretreated biomass.

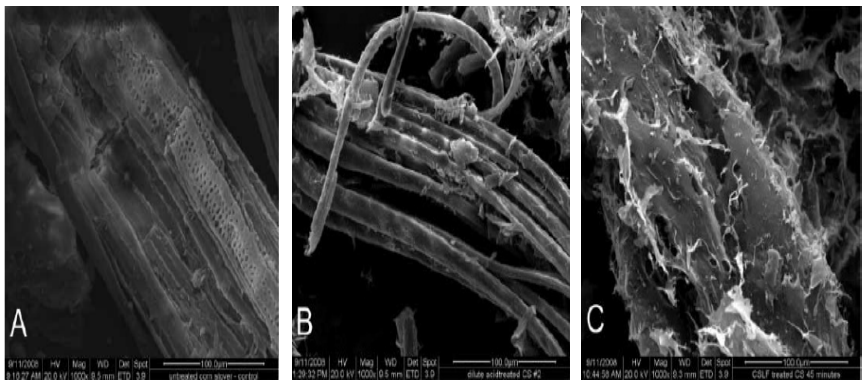


Figure 2-2. SEM images of corn stover before pretreatment (A) and pretreated by DA (B), and 45-min COSLIF (C).

2.4.3 Protein adsorption on pure cellulosic substrates

Adsorption of three proteins (cellulase, ß-glucosidase, and BSA) was conducted on pure crystalline cellulose – Avicel (Fig. 2-3). The free cellulase concentration decreased with increasing cellulose concentrations. In contrast, there was no adsorption of ß-glucosidase and BSA because they do not contain cellulose-binding modules. Similarly, cellulase was adsorbed by regenerated amorphous cellulose (RAC) but neither were ß-glucosidase and BSA (data not shown). Because significant cellulose hydrolysis occurs (especially for amorphous cellulose

fraction) during the active cellulase adsorption process, accompanied by a change in substrate accessibility (Steiner et al. 1988), we have proposed to determine cellulose accessibility to cellulase based on adsorption of a non-hydrolysis fusion protein, TGC, containing a cellulose-binding module (CBM) and a green fluorescent protein (GFP) (Hong et al. 2007).

Figure 2-4 shows the adsorption equilibrium curves of the TGC protein on Avicel and RAC. The maximum protein adsorption capabilities (A_{max}) after data fitting were determined to be 7.38 ± 0.13 μmol TGC per gram of RAC and 0.32 ± 0.01 μmol TGC per gram of Avicel. The CAC value of RAC (51.94 ± 0.91 m^2 per gram of RAC) was greater than 20 fold higher than that of Avicel (2.25 ± 0.07 m^2 per gram of Avicel). The effects of adsorption temperature and substrate concentration on maximum adsorption capacity were also investigated. No significant change was observed in A_{max} over ranges

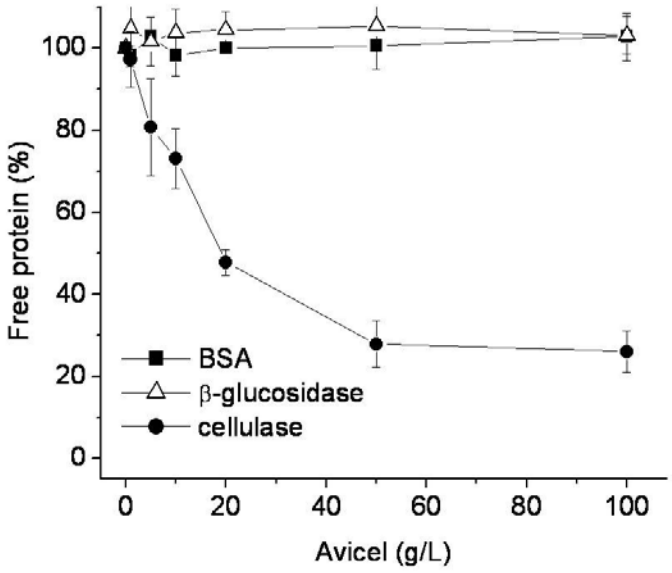


Figure 2-3. Adsorption of BSA, β-glucosidase, and cellulase on Avicel.

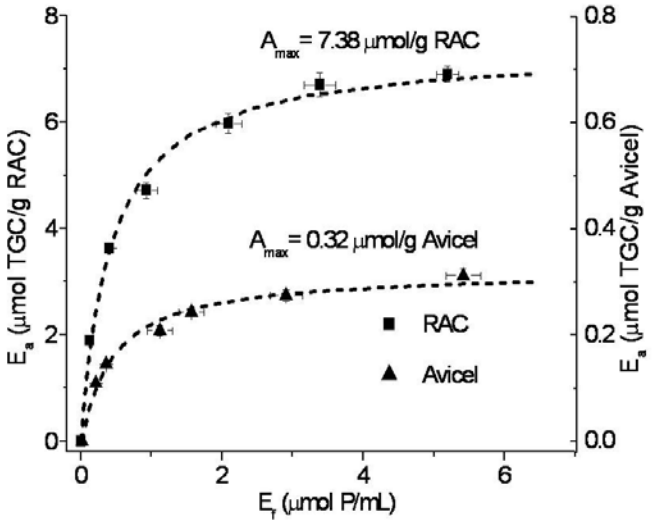


Figure 2-4. Adsorption of TGC fluorescence-tagged fusion protein on 1 g RAC/L and 20 g Avicel/L. The dashed curves were fitted by the Langmuir equations.

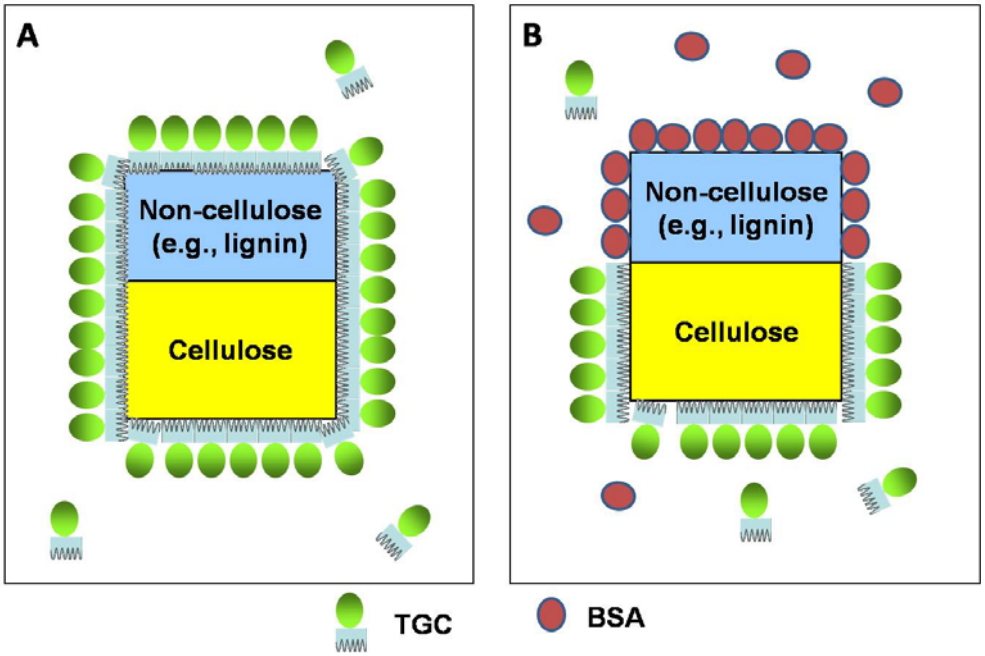


Figure 2-5. Scheme for quantitative determination of TSAC and CAC for the pretreated biomass. (A) Direct TGC adsorption for determining TSAC, including the cellulose and non-cellulose

(lignin) fractions, and (B) Second TGC adsorption for determining CAC after BSA blocking for the lignin fraction.

of 1-5 g RAC/L and 2-50 g Avicel/L at both room temperature and 50^{o}C (data not shown). In addition, the TGC maximum binding capacity on hemicellulose was determined to be 0.17 μmol TGC per gram of birch xylan.

2.4.4 Lignocellulosic substrate accessibilities

In order to quantitatively determine pretreated lignocellulosic substrate accessibility to cellulase in the presence of residual lignin and hemicellulose (Table 2-1), it is important to distinguish substrate accessibility for cellulose and non-cellulose (lignin-rich) fractions. Here we have applied a new scheme for quantitatively determining CAC and NCAC for pretreated lignocellulosic substrates (Fig. 2-5), based on the facts that (i) BSA can irreversibly bind with the accessible lignin fraction of lignocellulosic biomass (Berlin et al. 2005; Yang and Wyman 2006) and (ii) BSA cannot bind with cellulose (Fig. 2-3). Similarly to several substrate accessibility assays, total substrate accessibility to cellulase can be determined based on one-enzyme adsorption, where in this case TGC is used rather than a hydrolytic cellulase. For CAC measurement, a high concentration of BSA (5 g/L, final) was first mixed with the pretreated biomass for blocking accessible lignin, where 5 g BSA/L is much larger than $A_{max,lignin}$ × lignin content × 1 g biomass/L prior to the TGC adsorption. The TGC protein was then added to assess the maximum adsorption capacity of the lignin-blocked pretreated biomass, and the maximum TGC adsorption capacity of the BSA-blocked biomass was used to represent cellulose accessibility to cellulase (CAC). The difference between TSAC and CAC was NCAC that represented the accessibility of the non-cellulose (lignin-rich) fraction. Figure 2-6 shows TGC adsorption equilibrium curves obtained using corn stover pretreated by either DA or COSLIF with or without BSA blocking. The $A_{max,TGC}$ and $A_{max,BSA/TGC}$ are 2.05 ± 0.15 and 1.64 ± 0.13 μmol per gram of COSLIF-pretreated biomass and 1.09 ± 0.08 and 0.84 ± 0.05 μmol per gram of DA-pretreated biomass, respectively. The impact of remaining hemicellulose on CAC was very low because of low hemicellulose contents in the COSLIF- and DA-pretreated samples

relative to cellulose contents and low TGC binding capacity of hemicellulose compared to the A_{max} of pretreated cellulose.

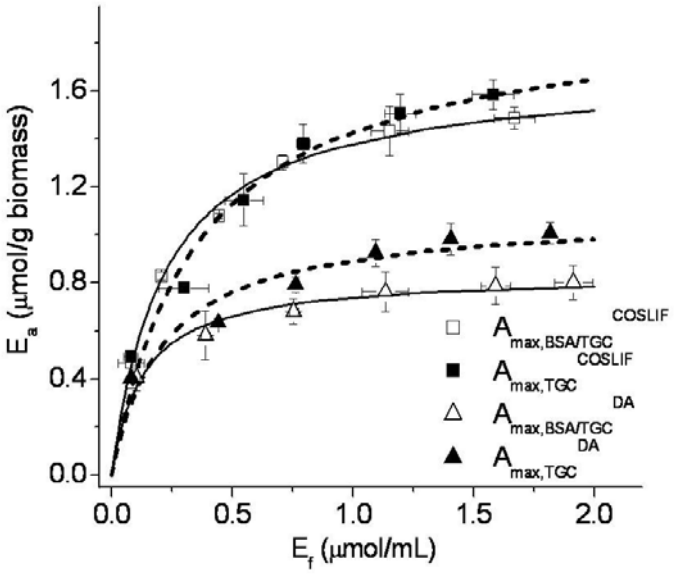


Figure 2-6. Adsorption of TGC protein on the DA- and COSLIF-pretreated corn stover with and without BSA blocking. The dashed curves were fitted by the Langmuir equations.

Table 2-2 shows the TSAC, CAC and NCAC values of the pretreated corn stover. For COSLIF-pretreated sample, TSAC was 14.44 ± 1.09 m^2 per gram of biomass, where CAC and NCAC were 11.57 ± 0.90 and 2.88 ± 0.20 m^2 per gram of biomass, respectively. The TSAC, CAC and NCAC of the DA-pretreated sample were 7.66 ± 0.55, 5.89 ± 0.34, and 1.78 ± 0.09 m^2 per gram of biomass, respectively. The normalized CAC of the COSLIF-pretreated and DA-pretreated biomass were 19.94 ± 1.53 and 10.90 ± 0.63 m^2 per gram of cellulose, respectively. The normalized NCAC of the COSLIF-pretreated and DA-pretreated biomass were 14.38 ± 0.70 and 5.93 ± 0.63 m^2 per gram of non-cellulose, respectively, suggesting that non-cellulose fraction of the COSLIF-pretreated biomass has higher substrate accessibility based on mass weight than that of the dilute acid-pretreated biomass.

Table 2-2. Total substrate accessibility to cellulase (TSAC), cellulose accessibility to cellulase (CAC), and non-cellulose accessibility to cellulase (NCAC) values of corn stover samples before pretreatment and after COSLIF or DA pretreatment.

	TSAC		CAC			NCAC		
method	μmol/g biomass	m^2/g biomass	μmol/g biomass	m^2/g biomass	m^2/g cellulose	μmol/g biomass	m^2/g biomass	m^2/g non-cellulose
Non-pretreated	0.16 ± 0.008	1.13 ± 0.006	0.06 ± 0.001	0.42 ± 0.007	1.07 ± 0.18	0.10 ± 0.005	0.70 ± 0.05	1.15 ± 0.06
COSLIF	2.05 ± 0.15	14.44 ± 1.09	1.64 ± 0.13	11.57 ± 0.90	19.94 ± 1.53	0.41 ± 0.02	2.88 ± 0.20	6.89 ± 0.45
DA	1.09 ± 0.08	7.66 ± 0.55	0.84 ± 0.05	5.89 ± 0.34	10.90 ± 0.63	0.25 ± 0.03	1.78 ± 0.09	3.84 ± 0.19

2.5 Discussion

Hydrolysis results clearly show that soluble sugars are released faster, and to a greater extent, in the COSLIF-pretreated corn stover than in the DA-pretreated corn stover studied here. Such differences (93% digestibility for COSLIF samples achieved within 24 hours vs. 60% for DA smaples in 72 hours) were more significant at a low enzyme loading of 5 filter paper units. Although intensive efforts have been made to increase specific cellulase activity and decrease cellulase production costs (Zhang et al. 2006b), additional reductions in enzyme usage costs are important to promote the economy of biorefineries.

Beyond having different enzymatic hydrolysis characteristics, these substrates exhibited significant differences in their supramolecular structures and substrate accessibilities. Qualitative SEM images clearly indicate that the cellulose solvent (concentrated phosphoric acid) treatment conducted at a low temperature substantially disrupts the biomass fibrillar structure (Fig. 2C), whereas dilute acid pretreatment at a higher temperature does not (Fig. 2B). The CAC of the COSLIF-pretreated corn stover was nearly double that of DA-pretreated biomass, consistent with

the hypothesis that CAC is one of the most important (rate-limiting) factors influencing enzymatic hydrolysis (Zhang and Lynd 2006). However, while changes in CAC appear to be a major causative factor for the changes in hydrolysis rates (especially initial rates), additional factors involving feedstock structure and composition undoubtedly contribute to the increased glucan digestibility (enzymatic hydrolysis yields) observed in COSLIF-pretreated as compared to DA-pretreated samples.

Quantitative determination of lignocellulosic substrate accessibility to enzymes (or chemicals) is important to understand the mechanism of cellulose hydrolysis and be able to accurately model hydrolysis kinetics (Zhang and Lynd 2004; Zhang and Lynd 2006). Such information may help improve the methods for evaluating pretreatment efficiency. As discussed in the introduction, total substrate accessibility has been measured previously using several approaches. The TGC-based CAC method used here has been previously applied to determine the CAC of pure cellulosic samples (Hong et al. 2007). This work found that a transition from substrate excess to substrate limitation occurs over the course of the process of enzymatically hydrolyzing crystalline cellulose (Hong et al. 2007).

Although it is well-known that cellulase adsorption on cellulose often can be empirically described using a Langmuir isotherm model (Lynd et al. 2002; Zhang and Lynd 2004), Jeoh et al. (Jeoh et al. 2007) attempted to estimate total substrate accessibility based on the linear range of cellulase adsorption. This estimation can be good only when a very low concentration cellulase is used for adsorption experiments because in the first order equation approximation of the Langmuir equation any small variations in free protein measurement may result in large deviations in calculated A_{max} values. Such deviation could be large especially for the easily-hydrolyzed pretreated biomass studied here (COSLIF samples) because some hydrolysis occurs during the active stage of cellulase adsorption, even when experiments are carried out at a decreased temperature (Beldman et al. 1987; Ooshima et al. 1983; Steiner et al. 1988).

But not all of the accessible substrate in pretreated lignocellulosic biomass can be hydrolyzed by cellulase, as reflected here by the measurement of NCAC. Previous efforts reported in the

literature to assay lignin accessibility to enzymes (lignin being the dominant component in the non-cellulose fraction) can be divided into two classes: (1) extracting lignin from lignocellulosic materials using chemicals (Bernardez et al. 1993; Converse et al. 1990; Gerber et al. 1997; Lee et al. 1994; Mooney et al. 1998; Palonen et al. 2004) and (2) leaving primarily only lignin remaining by hydrolyzing (dissolving away) the hemicellulose and cellulose fractions (Bernardez et al. 1993; Converse et al. 1990; Ooshima et al. 1990; Palonen et al. 2004). Both classes of methods may suffer from the large changes in lignin (or non-cellulose) substrate properties that occur during extraction or hydrolysis.

Here we attempted to distinguish cellulose accessibility and non-cellulose (lignin-rich) fraction accessibility without substrate hydrolysis or lignin extraction. We blocked accessible lignin by using 5 g BSA/L prior to the TGC adsorption because BSA can non-specifically irreversibly bind with lignin (Berlin et al. 2005) and cannot bind with cellulose (Fig. 3). The results shown in Table II suggest that TSAC overestimated CAC by 24.8% for the COSLIF-pretreated biomass (14.44 vs. 11.57 m^2/g) and by 30.0% for the DA-pretreated biomass (7.66 vs. 5.89 m^2/g).

DA pretreatment efficiency is often correlated with the extent of hemicellulose and/or lignin removal (Chang and Holtzapple 2000a; Zhu et al. 2008). But this comparative study of DA- and COSLIF-pretreated corn stover suggests that hemicellulose removal efficiency is not a primary determinant of the rate or extent of glucan enzymatic digestibility. In particular, the COSLIF-pretreated corn stover has nearly 2-fold higher hemicellulose composition (6.2%) than has DA-pretreated corn stover (3.4%) but the former has much higher digestibility and faster hydrolysis rates. A common belief is that lignin removal promotes faster and more efficient enzymatic cellulose hydrolysis (Berlin et al. 2005; Chang and Holtzapple 2000b; Esteghlalian et al. 2001; Kurabi et al. 2005a; Lee et al. 1994; Mooney et al. 1998; Ohgren et al. 2007; Zhu et al. 2008), but the data presented here suggests that increasing cellulose accessibility to cellulase is more important for achieving fast hydrolysis rates and high glucan digestibility because NCAC of the COSLIF-pretreated biomass (2.88 m^2/g biomass) is higher than that of the DA-pretreated biomass (1.78 m^2/g biomass).

The results improve the understanding of how DA- and COSLIF-pretreatments have different mechanisms of reducing biomass recalcitrance to enzymatic hydrolysis. DA pretreatment substantially removes hemicellulose and thereby disrupts the linkages among cellulose, hemicellulose, and lignin. COSLIF pretreatment partially removes lignin and hemicellulose but also substantially disrupts the fibrillar structure of biomass. The resulting faster hydrolysis rates and higher glucan enzymatic digestibility of COSLIF-pretreated corn stover as compared to DA pretreated corn stover are in a good agreement with (i) more efficient biomass structure destruction qualitatively shown by SEM images and (ii) the almost two-fold higher cellulose accessibility to cellulase levels measured by quantitative TGC adsorption. COSLIF pretreatment produces more highly digestible material than DA pretreatment, but similar to DA pretreatment it is not yet commercially proven and its economic viability for use in large scale biorefining remains to be demonstrated. The COSLIF technology remains at an earlier stage of development than DA pretreatment technology and more detailed economic analysis based on rigorous Aspen-plus models are needed to understand its potential for practical applications.

3. Direct quantitative determination of adsorbed cellulase on lignocellulosic biomass with its application to study cellulase desorption for potential recycling

3.1 Abstract

Effective hydrolysis of pretreated lignocellulose mediated by cellulase requires an in-depth understanding of cellulase adsorption and desorption. Here we developed a simple method for determining the adsorbed cellulase on cellulosic materials or pretreated lignocellulose, which involves (i) hydrolysis of adsorbed cellulase in the presence of 10 M of NaOH at 121^{o}C for 20 min, and (ii) the ninhydrin assay for the amino acids released from the hydrolyzed cellulase. The major lignocellulosic components (i.e. cellulose, hemicellulose, and lignin) did not interfere with the ninhydrin assay. A number of cellulase desorption methods were investigated: pH change as well as the use of detergents, high salt solution, and polyhydric alcohols. The pH adjustment to 13.0 and the elution by 72% ethylene glycol at neutral pH were among the most efficient approaches for desorbing the adsorbed cellulase. For the recycling of active cellulase, a modest pH adjustment to 10.0 may be a low-cost viable method to desorb active cellulase. It was found that more than 90% of cellulase for hydrolysis of the pretreated corn stover could be recycled by washing at pH 10.0.

3.2 Introduction

Cost-effective liberation of fermentable soluble sugars from non-food lignocellulosic biomass through biomass pretreatment/fractionation followed by enzymatic hydrolysis is still the largest obstacle to large-scale implementation of biorefineries (Lynd et al. 2008; Zhang 2008). Significant advances in a ~20-30 fold cost reduction of cellulase have been made through production process optimization and cellulase engineering (Himmel et al. 2007; Zhang et al. 2006b). Cellulase cost, which could ranges from ~30 to more than 100 (US) cents per gallon of cellulosic ethanol, is still far more expensive than that of starch-hydrolyzing enzymes for corn

kernel based ethanol biorefineries (e.g., ~2-5 cents per gallon of starchy ethanol). A cost reduction in cellulase utilization is one of the central tasks for production of low-cost cellulosic ethanol. Several approaches can be conducted for decreasing cellulase costs: (i) decreasing cellulase loading (e.g., gram of cellulase used per gram of glucan) by increasing substrate reactivity of pretreated biomass (Kumar and Wyman 2008a; Sathitsuksanoh et al. 2009; Zhang et al. 2007a) and/or recycling costly cellulase (Gregg and Saddler 1996; Lu et al. 2002; Tan et al. 1986; Tu et al. 2007), (ii) increasing cellulase performance (unit per gram of cellulase) by using cellulase engineering, and (iii) decreasing cellulase production costs ($ per gram of cellulase) (Taylor et al. 2008; Zhang et al. 2006b).

Study of cellulase adsorption and desorption is of great importance for understanding cellulose hydrolysis mechanism and evaluating the potential of cellulase recycling. Different from common enzyme recycling in aqueous homogeneous reactions via enzyme immobilization, cellulase in heterogeneous hydrolysis can be easily recycled by re-adsorbing free cellulase in the aqueous phase onto newly-added insoluble substrates (Gregg and Saddler 1996; Lu et al. 2002; Tan et al. 1986; Tu et al. 2007). Also, desorption of cellulase can be conducted through the addition of reagents, such as Tween, urea, alkali, glycerol, and Triton X-100 (Otter et al. 1989).

Adsorption of cellulase on the surface of cellulose is a prerequisite of cellulose hydrolysis. After hydrolysis, significant amounts of adsorbed cellulase are released to the aqueous phase (Hong et al. 2007; Zhang and Lynd 2003b; Zhang and Lynd 2005b). Adsorption of cellulase on the surface of cellulose and pretreated biomass are often described by the Langmuir equation based on the reversible adsorption assumption (Hong et al. 2007; Lynd et al. 2002; Shao et al. 2008; Zhang and Lynd 2004). In fact, adsorption of cellulase components is not strictly reversible. For example, binding of all *Thermobifida fusca* cellulase components to bacterial microcrystalline cellulose is irreversible (Jung et al. 2002a; Jung et al. 2002b). On bacterial microcrystalline cellulose, approximately 10% of the bound *Trichoderma* cellobiohydrolase I and ~30-40% of the bound *Trichoderma* cellobiohydrolase II are irreversible, respectively (Palonen et al. 1999). For pretreated biomass, competitive cellulase adsorption by lignin along with cellulose makes

cellulase adsorption/desorption more complicated (Berlin et al. 2005; Palonen et al. 2004; Zhu et al. 2009b).

Table 3-1. Summary of various approaches for adsorbed cellulase assay

Cellulase assay		Disadvantages
Indirect methods (bound = total - free)	Bradford	Supernatant varies and interferes with reagent, and too dilute to measure
	BCA	
	Lowry	
	UV 280	
	Protein precipitation	
	Radioactive labeling-based	Pure enzyme, special instrument and labor intensive
Direct methods	Total nitrogen-based	All the nitrogen is included, special instrument and labor intensive
	Bradford	Highly interfered by lignin or other complex in biomass

The adsorbed cellulase is often calculated based on the mass difference of initial cellulase and free cellulase (Hong et al. 2007; Kumar and Wyman 2008b; Zhu et al. 2009b) since most protein assays, such as UV, Bradford, Lowry, and BCA, cannot be applied directly for determining adsorbed cellulase on the surface of cellulose or lignocellulosic materials. Desorbed or free cellulase has been measured based on their activity (Otter et al. 1989; Zhang and Lynd 2005a; Zhang and Lynd 2005b), but cellulase activity assays are subject to changes in cellulase composition (Zhang et al. 2006b; Zhang and Lynd 2006). When reversibly adsorbed cellulase is washed by a large volume of solution, very low concentrations of free cellulase result in a challenge for accurate assays of protein mass concentration and/or enzyme activity. Therefore, radio-labeled cellulases have been used to study reversibility of their adsorption/desorption (Palonen et al. 1999; Palonen et al. 2004). However, this technology requires protein purification, protein labeling, and a costly radioactivity detection instrument. Recently, a direct method for measuring adsorbed cellulase has been developed based on nitrogen element analysis (Kumar

and Wyman 2008b). However, this method is only a rough measurement because it detects all nitrogen-containing compounds, such as alkaloids, ammonia, protein in plant samples, and a relatively costly analytical instrument is required.

In this study, desorption of cellulase under different elution conditions, such as pH, buffer, salt, and detergents, was studied. The adsorbed cellulase was measured by complete hydrolysis to amino acids followed by the ninhydrin assay.

3.3 Materials and Methods

3.3.1 Chemicals and materials

All chemicals were reagent grade and purchased from Sigma Aldrich (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA), unless otherwise noted. Microcrystalline cellulose (Avicel PH105) was purchased from FMC (Philadelphia, PA). Regenerated amorphous cellulose (RAC) was prepared through Avicel dissolution in concentrated phosphoric acid followed by regeneration in water (Zhang et al. 2006a). Birchwood xylan was purchased from Sigma Aldrich. Lignin was isolated from bagasse through the Kraft pulping and NaOH treatment at 170˚C, gifted from Dr. Scott Renneckar at Virginia Tech (Blacksburg, VA). The fungal cellulase Spezyme CP was a gift from Genencor (Palo Alto, CA). Corn was grown from Biomass AgriProducts (Harlan, IA). The tub-ground materials for corn stover were approximately nine months old. Dilute sulfuric acid (DA) pretreated corn stover was produced in a pilot-scale continuous vertical reactor at 190ºC, 0.048 g acid/g dry biomass, 1 min residence time, and a 30% (w/w) total solid loading (Zhu et al. 2009b). Cellulose solvent- and organic solvent-based lignocellulose fractionation (COSLIF) was conducted by using 85% H_3PO_4 at 50^{o}C, 1 atm, and 45 min, as described elsewhere (Zhang et al. 2007a; Zhu et al. 2009b). Lyophilized cellulase powder, as a standard protein for the adsorbed cellulase assay, was prepared through total protein precipitation by trichloroacetic acid (TCA) for soluble sugar removal (Zhang et al. 2007b), and then washing by ice-cold acetone for TCA removal.

3.3.2 Protein assays

Protein mass concentration was measured by using the Bradford, ninhydrin, or UV280 assays. Dry bovine serum albumin (BSA) and lyophilized cellulase powder were used as reference standards. The ninhydrin assay can be described as follows: one hundred μL of the protein solutions containing up to 100 μg of protein (protein concentrations of up to 1 mg/mL) were mixed with 300 μL of 13.5 M NaOH and autoclaved at 121°C for 20 min for complete protein hydrolysis. After cooling down to room temperature, the solutions were neutralized by adding 500 μL of 100% acetic acid, followed by adding 500 μL of 2% ninhydrin reagent while mixing well. After boiling for 10 min and cooling down to room temperature, the samples were diluted by three volumes of 95% ethanol. After centrifugation for removing solids, 200 μL of the colored supernatant was added into a 96-well microplate. The absorbance of the supernatants was read by the BioTek multi-detection microplate reader at a 570 nm wavelength. The readings from the microplate reader were normalized to 1-cm length of light path. The inferences from Avicel, RAC, xylan, lignin or pretreated corn stover samples on the protein assay were determined according to the ninhydrin assay.

3.3.3 Cellulase adsorption and desorption

Adsorption of cellulase was conducted at a total volume of 1000 μL of a 50 mM sodium citrate buffer (pH 4.8), containing various amounts of Avicel or dilute acid (DA)- pretreated corn stover at 4˚C. Final cellulase concentrations used were 0.35 mg/mL for Avicel and 0.15 mg/mL for DA corn stover. After 10-min adsorption followed by a centrifugation at 13,000 g for 5 min, the samples were washed in 200 μL of distilled water. The adsorbed cellulase was suspended in 100 μL of distilled water, transferred to glass tubes, and then measured by the ninhydrin assay. An excessive amount of water (200 μL each, 4 times) was also tested. Other washing conditions, including 200 μL of 1 M sodium chloride, 80% ethylene glycol, 50% glycerol, 0.01% Tween 80, 0.01% Triton X-100, 1.1% sodium dodecyl sulfate, 50 mM citrate solution with pH adjusted to 8, 9 and 10, and 0.135 M sodium hydroxide (pH 14), followed by 200 μL of water washing, were tested at the substrate loading of 5 mg of Avicel or 1 mg of DA corn stover.

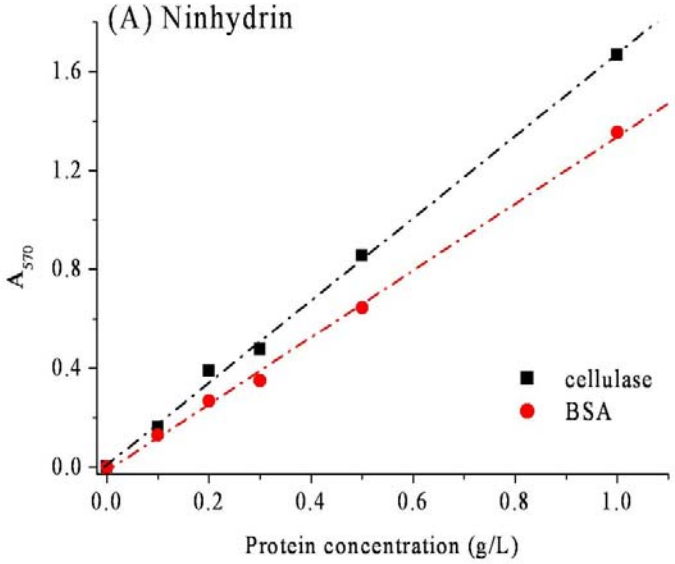
Total cellulase (P_{total}, mg/mL) was measured by the Bradford method. The adsorbed protein (P_{ads}, mg/mL) was calculated based on a difference between P_{total} and P_{free}, or measured by the ninhydrin assay directly.

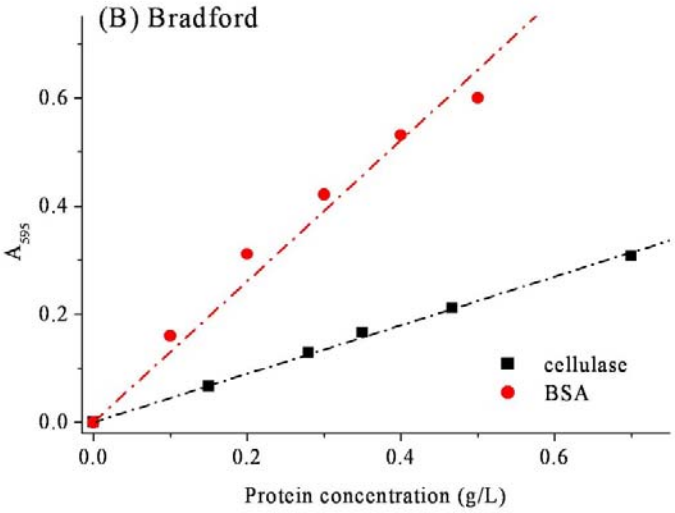
3.3.4 Enzymatic hydrolysis of pretreated biomass

The pretreated corn stover samples were hydrolyzed at 10 g of glucan per L in a 50 mM sodium citrate buffer, pH 4.8, and 0.5 g/L sodium azide with an enzyme loading of 15 filter paper units (FPUs) of cellulase and 30 units of Novozyme 188 ß-glucosidase per gram of glucan (12.3 mg cellulase and 9.4 mg ß-glucosidase per gram of glucan). The hydrolysis was conducted at 50˚C with a shaking rate of 250 rpm. The samples were taken for product and protein assays during the hydrolysis. Soluble sugar was measured by HPLC and free protein was measured by the Bradford assay (Bradford 1976). After 72-hour hydrolysis followed by centrifugation, biomass residues were washed in an excessive amount of the washing solvent (pH 10.0 citrate solution). The non-washable (irreversibly-bound) cellulase in the biomass residuals was measured by the ninhydrin assay.

3.4 Results

Although mass concentration of soluble proteins can be measured by a number of assays (Lovrien and Matulis 2005), most of them cannot be applied to measure adsorbed proteins on the surface of solid materials or in the prescene of solid particles. Previously, we measured the adsorbed cellulase on pure cellulose, by using the Lowry assay after SDS desorption (Zhang and Lynd 2005a; Zhang and Lynd 2005b). But this method is not applicable to pretreated biomass, containing hemicellulose and lignin, due to the interference from these lignocellulose components.


(A) Ninhydrin
1.6
1.2
0.8
0.4
0.0
A_{570}
0.0
0.2
0.4
0.6
0.8
1.0
Protein concentration (g/L)
cellulase
BSA


(B) Bradford
0.6
0.4
0.2
0.0
A_{595}
0.0
0.2
0.4
0.6
Protein concentration (g/L)
cellulase
BSA

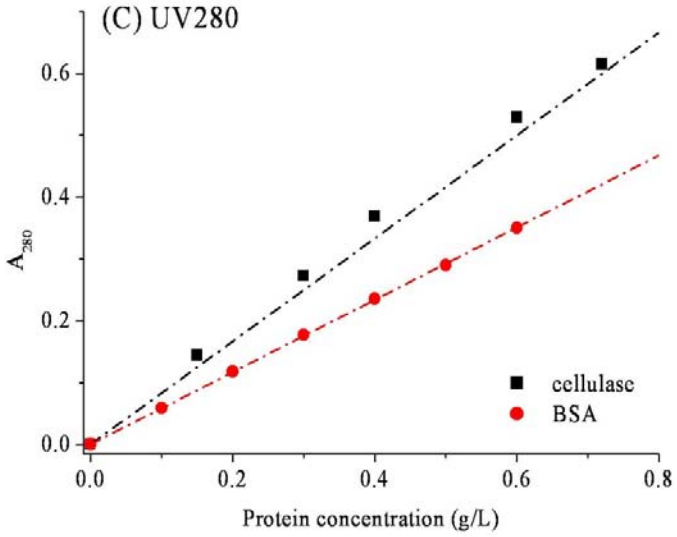


Figure 3-1. Standard curves of different free protein assays with BSA and cellulase as references.

The ninhydrin assay has been used for quantifying the total amount of animo acids and analyzing amino acid components (Friedman 2004; Lovrien and Matulis 2005). Protein contents in plant samples have been measured by the ninhydrin assay in the prescence of tanin (Makkar 1989a; Makkar et al. 1987; Marks et al. 1987; Marks et al. 1985). Both tannin and lignin are polyphenols with similar structures and chemical properties (Makkar 1989b). In addition, prior to the ninhydrin assay, protein samples must be hydrolyzed to amino acids in the presence of alkali. This information suggested that the bound cellulase on the surface of lignocellulosic materials could be measued by the ninhydrin assay because bound cellulase was hydrolyzed to free amino acids and the presence of lignin could not interfere with the ninhydrin assay.

Figure 3-1 shows that two free proteins (BSA and cellulase) have different slopes in terms of protein mass concentration by the ninhydrin (A), Bradford (B), and UV280 (C) assays due to their differences in amino acid compositions. Since assays of mass protein concentrations are protein-composition dependent, it is important to choose the right protein as a reference for these assays. For example, the cellulase assay was conducted based on cellulase as a reference or BSA

as a reference with an adjustment coefficient (i.e., 1 g BSA = 0.8 g cellulase for the ninhydrin method).

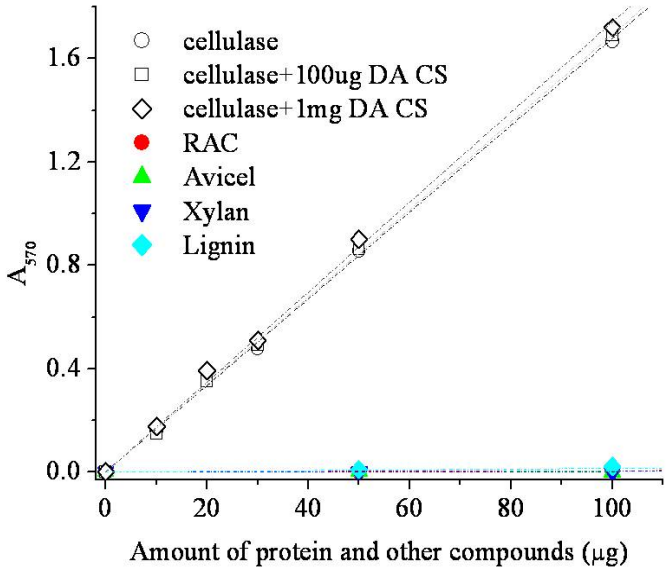


Figure 3-2. Effects of various lignocellulose components (cellulose, hemicellulose, and lignin) on the ninhydrin-based cellulase assay.

The effects of lignocellulosic biomass components were investigated on the ninhydrin-based adsorbed cellulase assay. They included pure cellulosic samples (Avicel and RAC), hemicellulose (birchwood xylan), lignin, monosaccharides (glucose and xylose), as well as DA-pretreated corn stover, and COSLIF-pretreated corn stover. Figure 3-2 shows nearly horizontal curves for all tested lignocellulosic components, suggesting that they did not interfere with the ninhydrin assay. Similar slopes without any significant difference were obtained for the cellulase samples in the absence and presence of dilute acid pretreated corn stover from 100 μg to 1 mg per sample. These results validated the feasibility of determining the adsorbed cellulase by using ninhydrin in the presence of cellulose, lignin, hemicellulose, and lignocellulose, when their masses are less than one mg per sample.

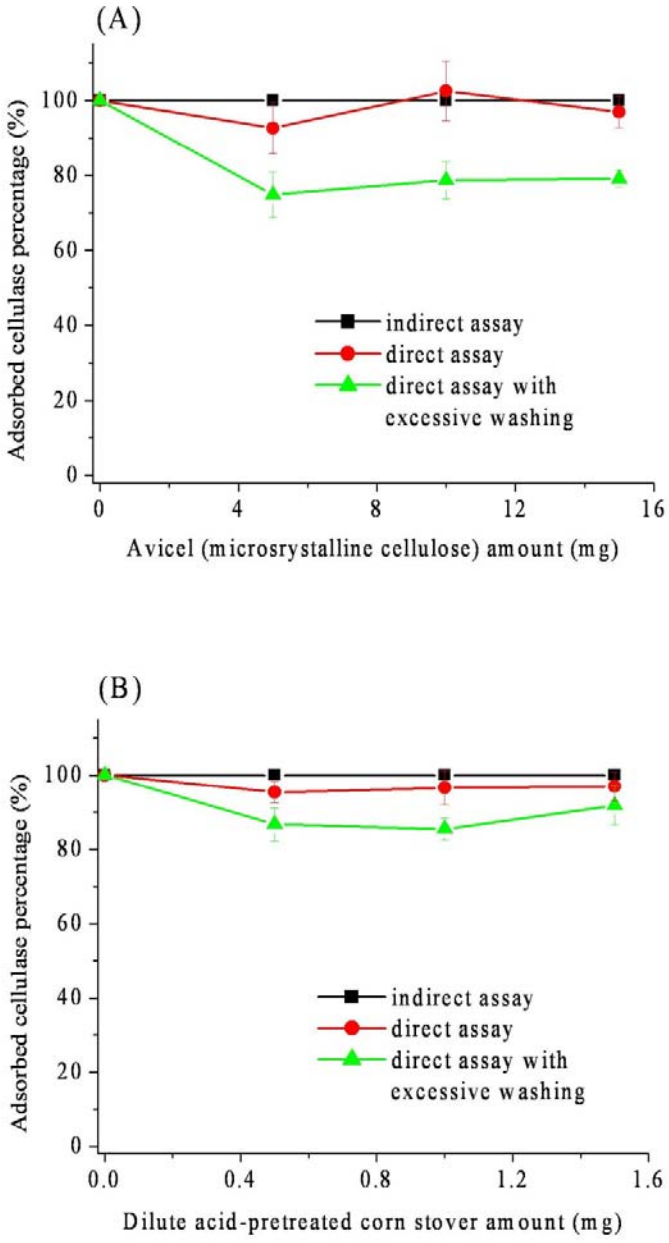


Figure 3-3. Comparison of Ninhydrin assay (direct assay), with Bradford (indirect assay, difference = total – free) for Avicel (A) and DA corn stover (B). Substrate amounts at different mass concentration had the same binding capability.

The total cellulase (P_{total}) during enzymatic cellulose hydrolysis includes - free (unbound) cellulase (P_{free}), reversibly bound cellulase (P_{rev}), and irreversibly bound cellulase (P_{irr}) as below,

$$P_{total} = P_{free} + P_{rev} + P_{irr} \quad [3\text{-}1]$$

Determination of P_{irr} is important to know the potential of cellulase recycling, and the desorption efficiency depends on the conditions of the washing solvents. Figure 3-3 shows that the amounts of adsorbed cellulase ($P_{rev} + P_{irr}$), measured by a direct ninhydrin assay, are close to those measured by the indirect method ($P_{total} - P_{free}$) in the presence of Avicel and DA-pretreated corn stover. After excessive water washing, more bound cellulase was removed from Avicel than from pretreated corn stover, suggesting that pretreated biomass can bind cellulase more tightly. The results also indicated that pure water washing was not efficient to remove adsorbed cellulase.

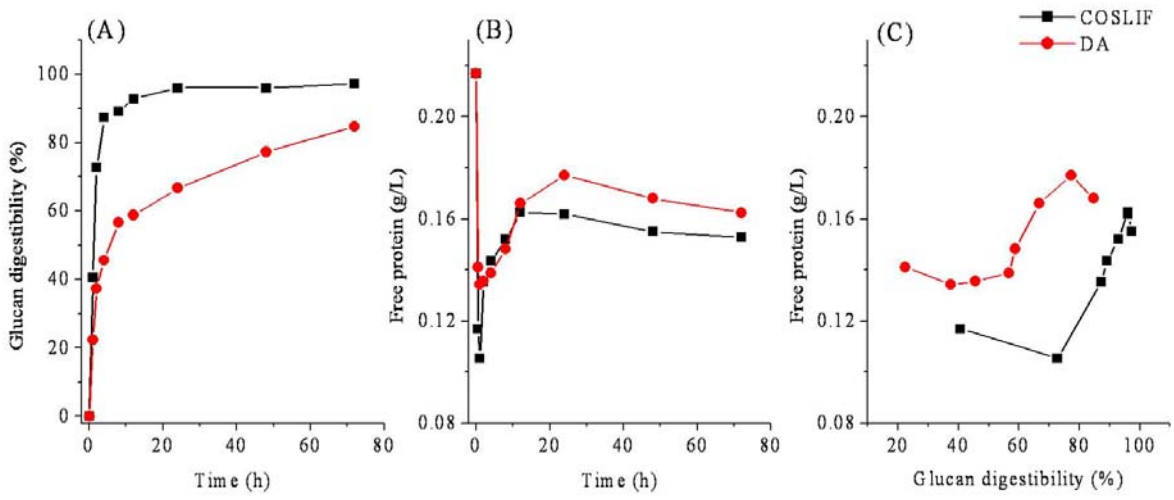


Figure 3-4. Hydrolysis profiles (A) and protein concentration changes in the supernatant (B) for pretreated corn stover at 50 °C, 250 rpm, 10 g glucan/L with the enzyme loading of 15 FPUs of cellulase and 30 units of β-glucosidase per gram of glucan.

Figure 3-4 shows profiles of glucan digestibility and free protein concentration for corn stover pretreated by DA and COSLIF, at a typical enzyme loading of 15 FPUs of cellulase and 30 units of β-glucosidase per gram of glucan. Glucan digestibility of the COSLIF-pretreated corn stover

reached 97% at hour 24, while the DA-pretreated corn stover exhibited considerably slower enzymatic hydrolysis rates with a final glucan digestibility of 84% at hour 72 (Fig. 3-4A). The free protein concentrations in both cases were decreased after the first 2 hours, indicating a rapid cellulase adsorption required for cellulose hydrolysis. At the beginning of hydrolysis, more cellulase was adsorbed by the COSLIF-pretreated corn stover than the DA-pretreated corn stover, consistent with faster hydrolysis rates and larger substrate accessibility to cellulase. Later, the concentration of free cellulase rose, mainly due to bound cellulase release accompanying substrate consumption (Hong et al. 2007). Similar dynamic trends of free cellulase were reported previously (Lu et al. 2002; Tu et al. 2007). Notably, the protein concentration in the supernatant decreased slightly after ~20 h, which might be because some newly-exposed lignin after cellulose hydrolysis adsorbed free cellulase at the end of hydrolysis (Fig. 3-4B). Moreover, it was found that more cellulase was adsorbed by COSLIF-pretreated corn stover than DA-pretreated corn stover at the same glucan digestibility (Fig. 3-4C), because the former had more total substrate accessibility (Zhu et al. 2009b). At the end of hydrolysis, more cellulase was adsorbed on the COSLIF-pretreated corn stover due to its higher non-cellulose accessibility to cellulase than DA-pretreated corn stover (Zhu et al. 2009b).

3.5 Discussion

The ninhydrin assay was shown as a simple, fast and low-cost approach in determining the adsorbed cellulase on cellulosic materials and pretreated lignocellulosic biomass. Protein hydrolysis releases the adsorbed cellulase, and lignocellulose components (cellulose, lignin, and hemicellulose) did not interfere with the protein assay.

Table 3-2. Cellulase removal efficiency from Avicel and DA corn stover after following various washing steps.

Washing conditions		Cellulase desorption efficiency (%)	
		Avicel	DA CS
Salt	1M NaCl	10 ± 0.5	6 ± 0.4
Polyhydric alcohols	72% EG	81 ± 5.5	76 ± 4.5
	45% Glycerol	77 ± 3.3	74 ± 2.9
Detergents	0.01% Tween80	38 ± 1.1	28 ± 0.8
	0.01% Triton X-100	42 ± 1.5	39 ± 4.7
	1.0% SDS	46 ± 4.0	42 ±3.5
pH	pH 8	55 ± 4.6	35 ± 1.1
	pH 9	57 ± 1.5	48 ± 4.8
	pH 10	61 ± 3.9	56 ±1.6
	pH 13	85 ± 3.7	94 ± 4.1

200 μL of these solvents were used followed by 200 μL of DI water washing to remove residual solvent.

Table 3-2 shows the effects of desorption conditions on desorption efficiency of cellulase for Avicel and dilute acid pretreated corn stover. Desorption efficiency was associated with several experimental factors (e.g., solvent type, ratio of solvent to adsorbent, adsorbent type, etc.). Since the ultimate goal was to economically recycle active desorbed cellulase, the use of a large volume of solvent was not practical. Polyhydric alcohols, such as ethylene glycol and glycerol, are more efficient for removing adsorbed cellulase than mild detergents (e.g., Tween 80, and Tritrin X-100) or strong detergents (e.g., SDS). Similar results have been reported by Otter and co-workers (Otter et al. 1989). It was found that 75% ethylene glycol (EG) was the most effective, removing 81 ± 5.5 % and 76 ± 4.5 % of adsorbed cellulase from pure cellulose and pretreated biomass, respectively. EG was also previously used for desorbing the cellulose-

binding module tagged protein for protein purification (Hong et al. 2007). It was found that a pH increase, from 5 to 8, 9, and 10, increased desorption efficiency by 61 ± 3.9% compared to those observed prior to cellulase deactivation at a higher pH range. At pH 13, desorption efficiencies were 86% and 94% for Avicel and dilute acid pretreated corn stover, respectively. But cellulase was deactivated under these conditions. Although high concentration salt (1 M NaCl) was used to desorb cellulase (Nutor and Converse 1991; Ooshima et al. 1990), it was found to be inefficient at the tested condition. From a cost-effective point of view, adjustment of the solution pH was more operative for desorbing the bound cellulase compared to the addition of other chemicals.

Table 3-3. Cellulase desorption from the enzymatic hydrolysis residues of the corn stover pretreated by DA and COSLIF after washing at pH = 10.

	DA	COSLIF
Adsorbed enzymes on residue (% of initial protein)	28.0 ± 3.2	31.9 ± 2.1
Washable enzyme (% of adsorbed protein)	71.8 ± 3.0	81.2 ± 2.3
Overall recovery potential (% of initial protein)	92.1 ± 3.9	94.0 ± 2.7

The hydrolysis was conducted at 10 g of glucan per L in a 50 mM sodium citrate buffer (pH 4.8) with an enzyme loading of 15 FPUs of cellulase and 30 units of Novozyme 188 glucosidase per gram of glucan (12.3 mg cellulase and 9.4 mg glucosidase per gram of glucan).

The remaining adsorbed cellulase was quantified by the ninhydrin assay after 72-hour hydrolysis (Table 3-3). Although COSLIF-pretreated biomass adsorbed more cellulase than DA-pretreated biomass, the bound cellulase on the former was washed away more easily than the latter. The removal efficiencies were 81% and 72% on COSLIF- and DA-pretreated biomass, respectively. The irreversibly bound cellulase (i.e., net cellulase loss) for the DA and COSLIF-pretreated biomass, which had no significant difference, were ~8% and 6% of initially added total cellulase, suggesting a great potential for cellulase recycling. If cellulase stability can be enhanced greatly, cellulase recycling will greatly decrease enzyme costs.

Two major factors preventing practical cellulase recycling are (i) inefficient cellulase release from pretreated biomass, particularly the irreversible adsorption on lignin, which can be addressed by a pH switch (shown here), addition of surfactant, or a more efficient lignin removal during biomass pretreatment, and (ii) cellulase denaturation over time, which can be overcome by cellulase engineering for better thermostability (Heinzelman et al. 2009; Liu et al. 2009; Zhang et al. 2006b). More work should be done to study cellulase desorption and recycling. To sum up, ninhydrin assay would be a good method in evaluating cellulase removal or recycle efficiency.

4. Conclusions and Perspectives

4.1 Conclusions

Never before has the reality of cellulosic ethanol production been so close to us. This work, presented in this thesis, leads to a better understanding of biomass pretreatment and cellulose hydrolysis, especially providing quantitative information that substrate accessibility is of great importance for efficient cellulose hydrolysis and providing a direct approach to study cellulase adsorption and desorption.

The results from comparative study of two pretreatment technologies suggest that faster hydrolysis rates and higher glucan enzymatic digestibility of COSLIF-pretreated corn stover as compared to DA-pretreated one are due to nearly two-fold higher cellulose accessibility to cellulase levels of the former quantified by TGC adsorption. This finding also leads to the conclusion that enhanced cellulose accessibility to cellulase is more important than removal of hemicellulose or lignin during pretreatment. The work of a direct adsorbed cellulase assay indicates that the ninhydrin assay is a good way to measure adsorbed cellulase on lignocellulosic substrates. Major components in lignocellulose (cellulose, lignin, and hemicellulose) do not affect the assay, which can be successfully applied to study cellulase removal or recycle. A modest adjustment of pH to 10.0 would be a cost-effective way to desorb active cellulase for its recycling.

4.2 Perspectives

Although COSLIF pretreatment produces a lot highly digestible material and it has many attractive advantages, it has not yet commercially scaled up. More diverse feedstocks are being tested and more comparative studies on its mechanistic advantages are under way. This technology remains at its early stage of development and more detailed economic analysis should be conducted to understand its potential for practical applications.

Using the adsorbed cellulase measurement by ninhydrin assay, more desorption conditions will be tested and the most effective way will be found to remove adsorbed cellulase efficiently from lignocellulose. Further study for substrate reactivity and accessibility during the course of hydrolysis will be conducted. With the development of more cellulose binding module – based recombinant protein tools, in-depth insights into hydrolysis of pure cellulose and pretreated biomass will be acquired in the future.

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APPENDIX

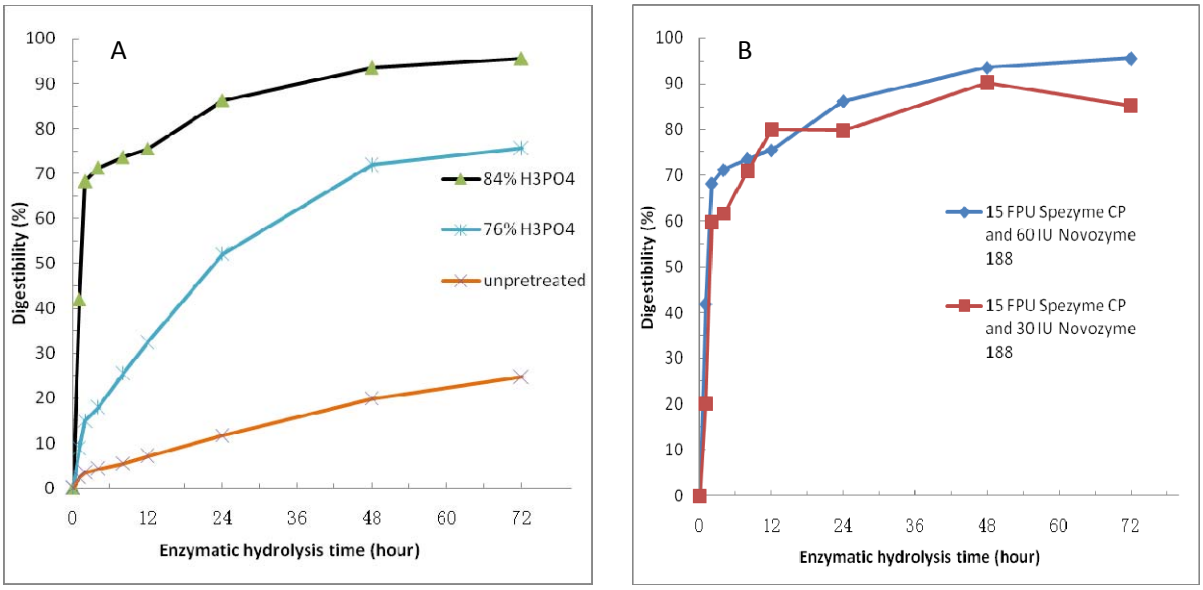


Figure A1. Hydrolysis profiles of COSLIF pretreated industrial hemp hurds. (A) Effect of phosphoric acid concentration on cellulose digestibility of the pretreated lignocelluloses, (B) Effect of ß-glucosidase loading on cellulose digestibility of the pretreated lignocellulose

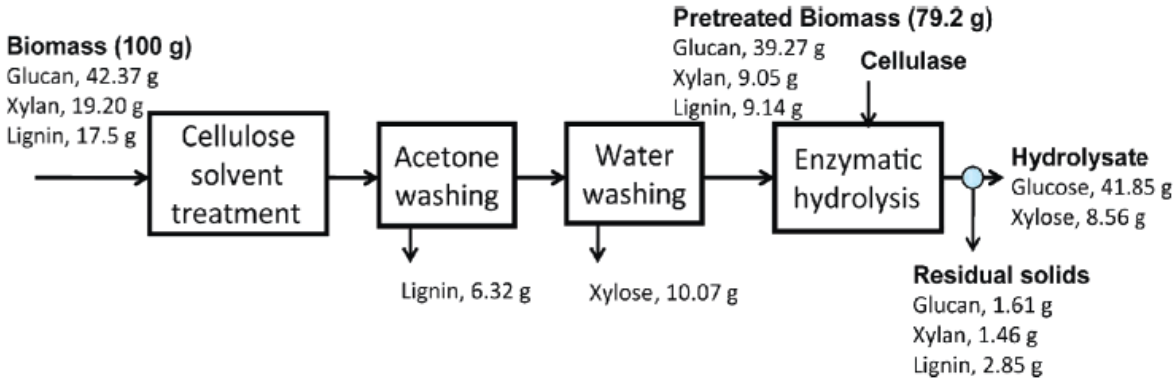


Figure A2. Mass balance of industrial hemp hurds after lignocellulose fractionation pretreatment and enzymatic hydrolysis. The pretreatment reaction condition was 84.0% H_3PO_4 at 50 °C for 1h.

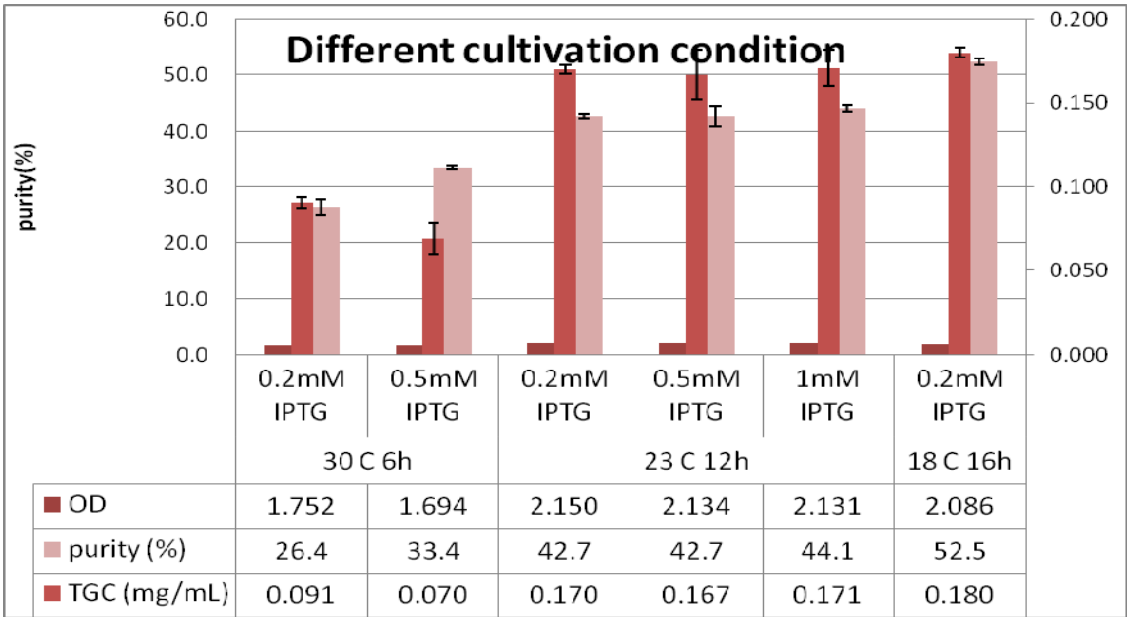


Figure A3. Optimization of the TGC expression in *E coli*. BL21. IPTG was added when OD reached 0.7. The harvested cells were dismembraned through freezing / thawing followed by sonification.

Table A1. The TGC protein with a his-tag purified by a Ni-column.

	Cell Lysate (diluted /5)	**Recovery after $(NH_4)_2SO_4$ precipitate (/10)**	**After binding with resin (/5)**	**After washing (/1)**	**After elution (/10)**	**After dialysis (/5)**
Fluorescence	277	254	43	166	211	389
Concentration by Bradford (mg/mL)	0.564	0.598	0.415	0.337	0.34	0.585
Volume (mL)	80	20	20	25	17	15
Yield	100%	53%	18.4%	3.7%	25.6%	19.4%

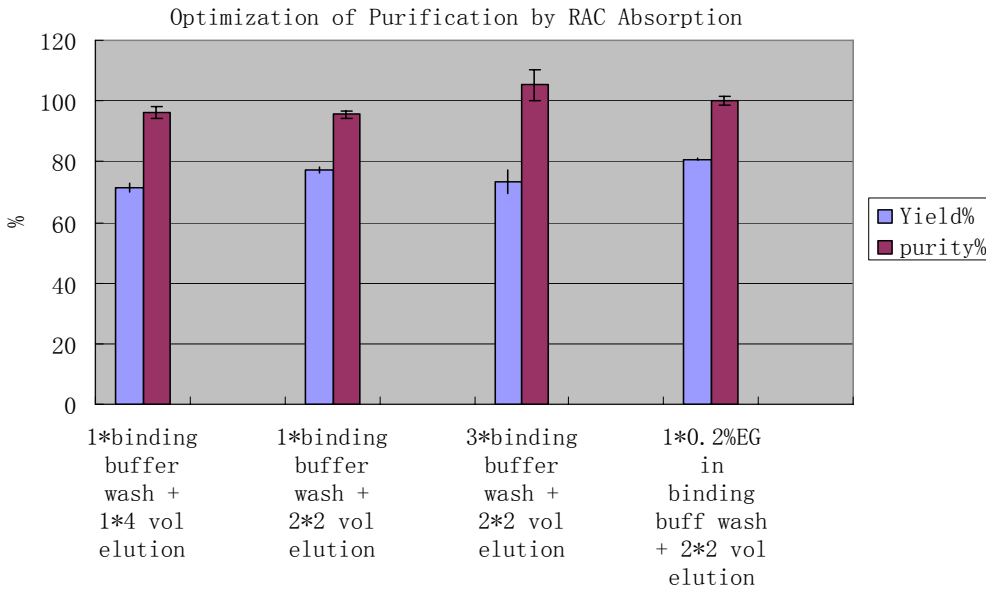


Figure A4. Optimization of TGC purification by RAC adsorption. Four volumes of RAC was used for adsorption and the final yield could achieve around 70-80% (higher than His-tag purification).

Table A2. The CAC and NCAC values of various feedstocks. The method was described in the main text. CAC/NCAC ratio shows that it increases significant after pretreatment.

feedstock	**Amax w/o BSA (umol P/g biomass)**	**Amax w/BSA (umol P/g biomass)**	**TSAC (m^2/g biomass)**	**CAC (m^2/g biomass)**	**NCAC (m^2/g biomass)**	**CAC/NCAC**
COSLIF Corn Stover	2.05 (0.15)	1.64 (0.13)	14.44 (1.09)	11.57 (0.89)	2.87 (0.20)	**4.02**
DA Corn Stover	1.09 (0.08)	0.84 (0.05)	7.67 (0.54)	5.88 (0.34)	1.78 (1.62)	**3.30**
COSLIF Switch grass	1.37 (0.09)	1.14 (0.16)	9.64 (0.63)	8.02 (1.13)	1.62 (0.11)	**4.96**
COSLIF Common reed	2.29 (0.19)	1.86 (0.15)	16.12 (1.34)	13.09 (1.06)	3.03 (0.23)	**4.33**
Untreated Corn Stover	0.16 (0.008)	0.06 (0.001)	1.13 (0.056)	0.42 (0.007)	0.70 (0.049)	*0.60*
Untreated Switch grass	0.18 (0.011)	0.07 (0.007)	1.27 (0.077)	0.49 (0.049)	0.77 (0.028)	*0.64*
Untreated Common reed	0.05 (0.008)	0.02 (0.005)	0.35 (0.056)	0.14 (0.035)	0.21 (0.021)	*0.67*

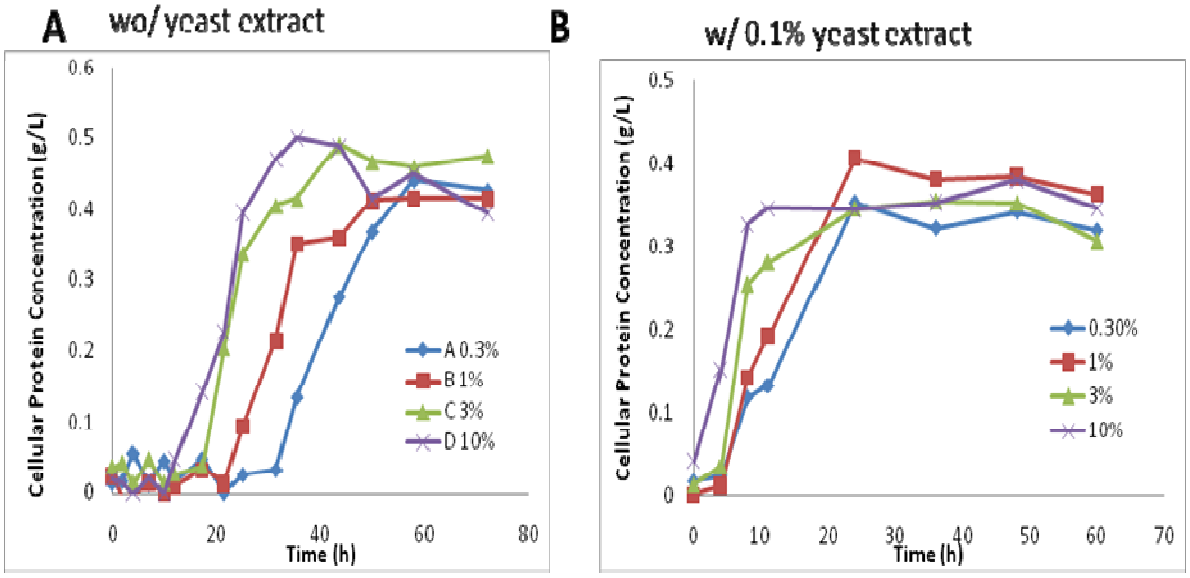
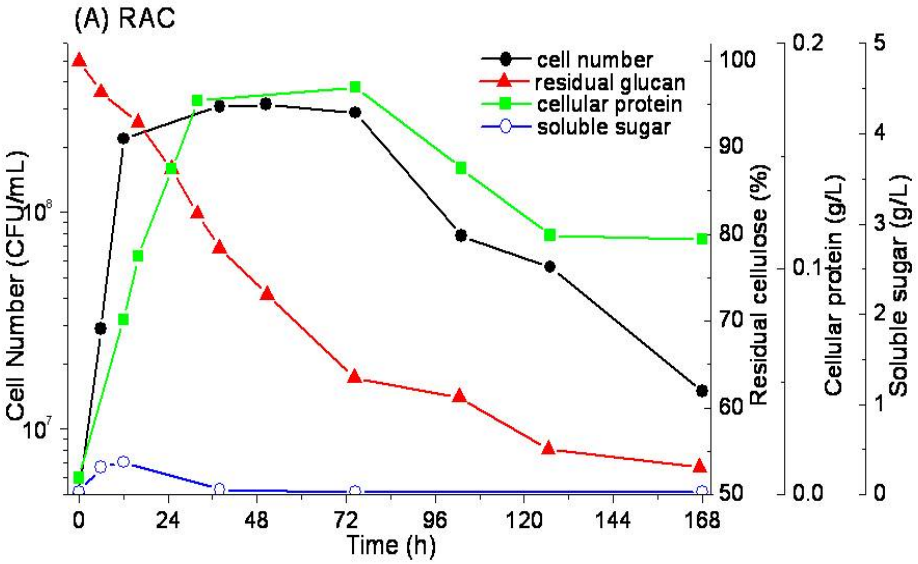


Figure A5. The growth profiles of different inoculation sizes (v/v%) of *B. subtilis* WB800 on the M9 medium containing RAC as a sole carbon source. (A) without yeast extract, (B) with 0.1% yeast extract.



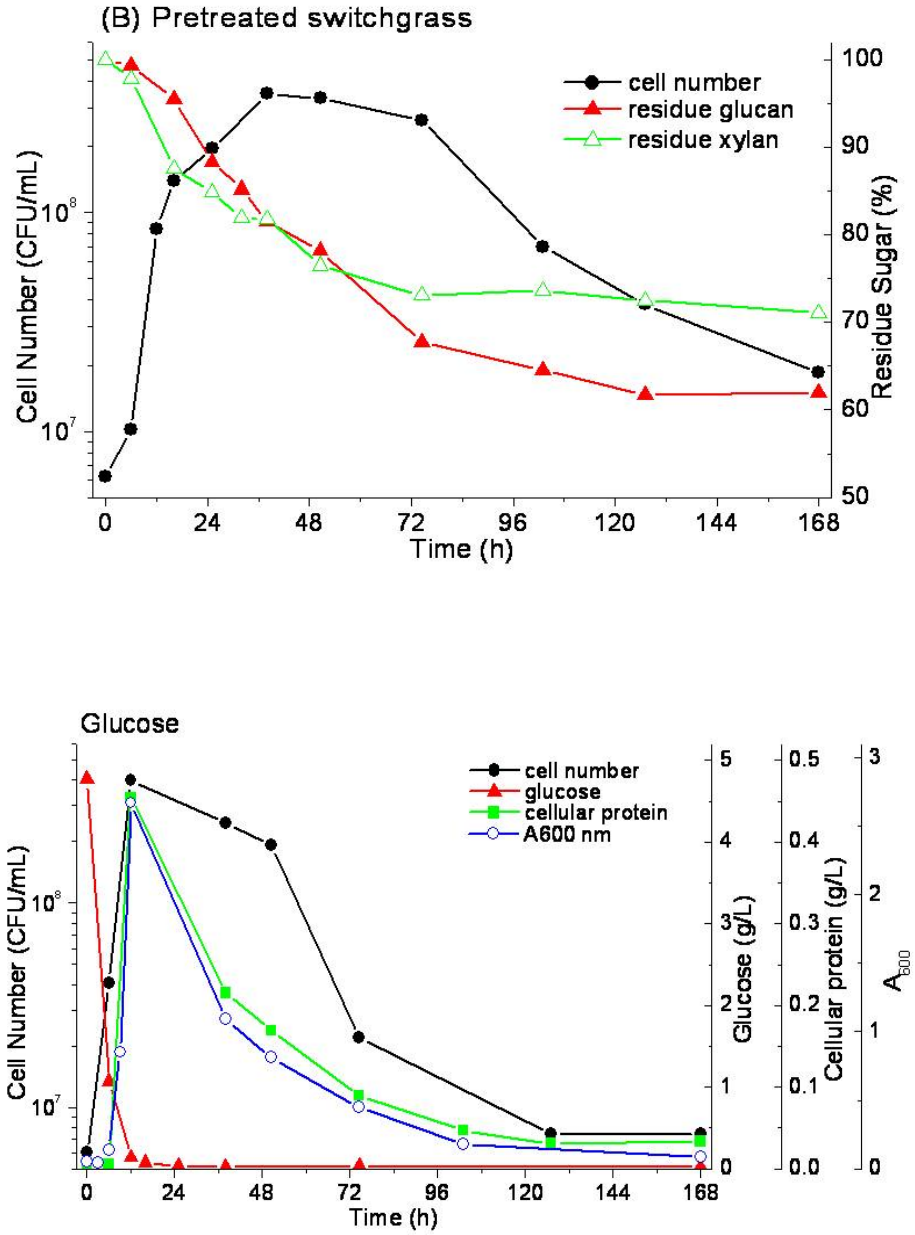


Figure A6. The growth profiles of *B. subtilis* IH6140(pP43N-Bscel5) on the M9 medium containing RAC, COSLIF-switchgrass or glucose as a sole carbon source.

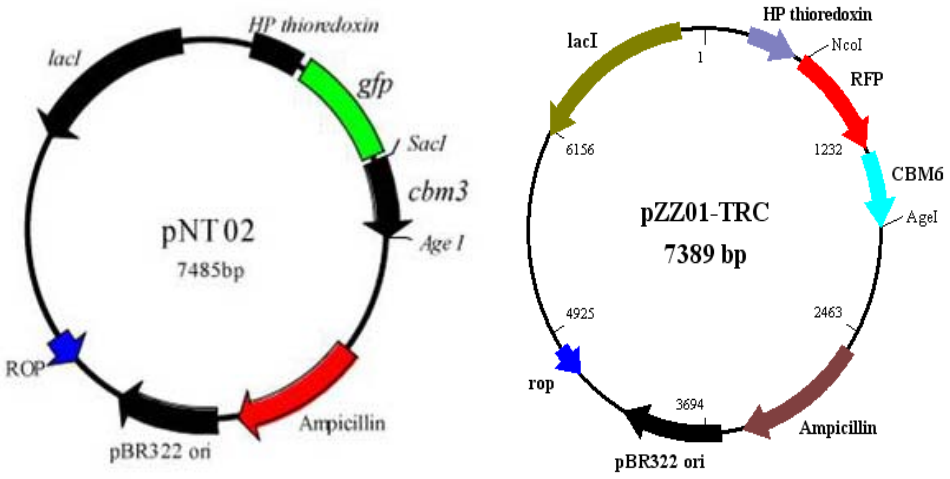


Figure A7. Maps of TGC protein (in this thesis) and Thiodorexin-RFP-CBM6 (TRC, for future work) protein.