University of Alberta

CELLULASE IMMOBILIZATIONS WITH HIGHLY RETAINED ENZYMATIC ACTIVITIES

by

YUKO IKEDA

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Bioresource and Food Engineering

Department of Agricultural, Food and Nutritional Science

Fall 2013

Edmonton, Alberta

ABSTRACT

Immobilization has the potential to make enzyme utilization more cost efficient, but reduced enzymatic activity makes it unfeasible. The purpose of this study was to develop immobilized cellulase systems on silica supports that retain high enzymatic activity. Two of the four systems created resulted in better-than-expected productivity during hydrolysis and make good candidates for further research.

The immobilized systems on fumed non-porous silica retained enzyme activity equivalent to free cellulases when hydrolyzing crystalline cellulose. However, sugar composition analysis indicated that immobilization decreased cellobiose conversion to glucose, resulting in substantial cellobiose production. More research is required to understand the mechanism at work. The immobilized cellulases obtained wider pH stability, but displayed decreased thermal and ionic stability. Storage stability was unchanged. One major finding was increased enzyme reusability, with activity remaining at 30% after nine uses.

When lignocellulose biomass was used for hydrolysis, production was substantially lower than when using free cellulases. It is hypothesized that the immobilized cellulases had difficulty hydrolyzing the amorphous regions of the lignocellulose. Although further optimization is necessary, the immobilized cellulase systems developed in this study contribute greatly to increasing the commercial viability of hydrolysis and lignocellulose pretreatment.

ACKNOWLEDGEMENTS

I would like to thank Alberta Advanced Education and Technology, the Alberta Agricultural Research Institute, and Alberta Innovates Bio Solutions, all of whom funded this project. I am grateful for the personal financial support I received from the following programs and agencies: the Natural Sciences and Engineering Research Council of Canada (NSERC) for the Alexander Graham Bell Canadian Graduate Scholarship (2008-2011) and Alberta Innovates Technology Futures for the Alberta Ingenuity Graduate Scholarship in Nanotechnology (2009-2011). For their generosity, I thank the University of Alberta for the Queen Elizabeth II Graduate Scholarship- Doctoral level (2011), Profiling Alberta's Graduate Students Award (2011), the President's Doctoral Prize of Distinction (2008-2010), and GRAF tuition scholarships (2007 and 2008).

I am especially thankful to Dr. David Curtis Bressler, my dissertation supervisor, for his great mentorship and understanding. Without his advice and support, I would not have been able to complete my degree. Also, I highly appreciate Dr. Lynn McMullen and Dr. William McCaffrey who gave me excellent advice and support for my research project and personal development, both one-on-one and as committee members. I also thank Dr. Jack Saddler, a professor in the Department of Wood Science, University of British Columbia, who gave me wonderful advice on my experiment and allowed me to visit to his lab to learn an advanced analytical technique. Also, I appreciate Dr. Dominic

Sauvageau to be an examining committee member and to give me great advice for my dissertation. In addition, I thank Dr. Laki Goonewardene who supported my statistical analysis. I thank the Biological Sciences Microscopy Unit, University of Alberta, for help with SEM.

Next, I offer a big thanks to my labmates. Their suggestions and advice on my research project helped me, and their friendship motivated me to keep working.

Finally, I owe my greatest thanks to my husband, Neill Lindsey Walker, and my son, Sho Liam Walker, for their deep understanding, patience, and unending support for my study.

Table of Contents

List of Figures

Lists of Tables

1. Introduction

1.1. Lignocellulose

Lignocellulose is an attractive resource for biotechnology, bioenergy, and chemical industries because it can be degraded into sugar solutions and used as a building block for the production of value-added materials including platform chemicals and bioethanol (Menon and Rao, 2012). In this research, various forms of lignocellulose were used as substrates for hydrolysis. Therefore, it is important to understand the structure and components of the lignocellulose, specifically cellulose, hemicellulose and lignin, to fully understand its hydrolysis.

Lignocellulose is a mixture of polymers that comprise the structural elements of the cell wall in plants. As shown in Figure 1.1, its main components are cellulose, hemicellulose, and lignin. Cellulose is a targeted primary resource for the production of sugar solutions, and it exists in microfibril form in a plant cell walls. Hemicellulose is a heteropolysaccharide, intermixed with cellulose fibers. The cellulose-hemicellulose complex is embedded in the lignin fraction, which is a complex aromatic polymer that acts as a binder with cross-linking. Each of these three polymers will be discussed in the following sections.

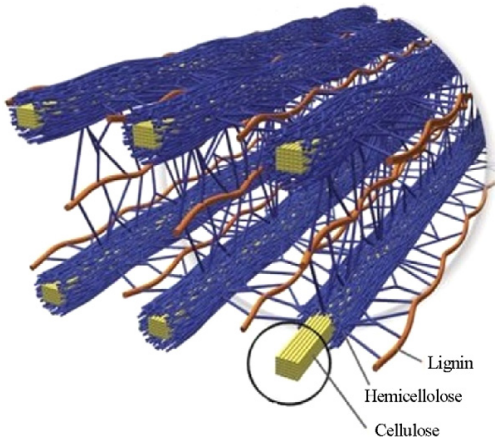


Figure 1.1. The structure of lignocellulose.
(Reproduced with permission, Doherty, Mousavioun, and Fellows, 2011)

1.1.1. Cellulose

Cellulose is the most abundant polymer in nature (Agbor *et al.,* 2011). It is the main constituent of plant cell walls that provides structural support. It is a linear polysaccharide, consisting of D-anhydroglucopyranose linked together by β -1,4,-glycoside bonds. It is comprised of 45% carbon, 6.5% hydrogen, and 48.5% oxygen (Zugenmaier, 2010). Cellulose does not have a fixed molecular weight, but the degree of polymerization (DP) is generally from 100 to 20,000 DP (Zhang and Lynd, 2004). A cellulose chain, which is usually 20-300 DP, groups together and forms a microfibril through hydrogen bonds and van der Waals forces (Agbor *et al.,* 2011). Cellulose contains two regions: crystalline and amorphous. In a crystalline region, cellulose chains and sheets are tightly packed and form straight and stable supra-molecular fibers with great tensile strength and

low accessibility (Zugenmaier, 2010). This cohesive structure limits access of water-soluble compounds and enzymes. Cellulose chains in an amorphous region are loosely packed and less ordered, allowing enzymes and other water-soluble compounds easy access to the region (Park *et al.,* 2010). In addition, amorphous cellulose can be artificially prepared by converting the crystalline fraction of cellulose to the amorphous form by mechanical or chemical methods (Zhang *et al.,* 2006). Mechanically made amorphous cellulose is often prepared by ball milling or severe blending. Phosphoric acid-swollen cellulose and regenerated cellulose (e.g. viscose rayon and cellophane paper) are commonly used as examples of chemically converted amorphous cellulose. The former is made by swelling dry cellulose powder using a high concentration of phosphoric acid. The latter is made by converting insoluble cellulose to a soluble form using solvents, then restoring it to a physically insoluble form (Zugenmaier, 2010; Zhang *et al.,* 2006).

The ratio of amorphous to crystalline regions varies depending on the plant source and is measured by the crystallinity index (CrI), which shows the ratio of crystalline to total cellulose. The crystalline index can be calculated by comparing the intensity of the crystalline fraction to the noncrystalline fraction using X-ray scattered reflections (Thygesen *et al*. 2005). Cotton, bacterial cellulose and algal cellulose are examples of highly crystalline cellulose with a

crystallinity index of approximately 80, 65-79, and 56-65%, respectively (Zugenmaier, 2010). A Whatman No.1 filter is an example of relatively low crystalline cellulose with a crystallinity index of approximately 45% (Dong *et al*., 1998). By contrast, the crystallinity index of regenerated cellulose is very close to zero (Zugenmaier, 2010).

Crystalline cellulose is categorized into 7 types, depending on its packing arrangements: I α , I β , II, III_1, III_2, IV_1, and IV_2 (Zugenmaier, 2010). Native cellulose is referred to cellulose I. Cellulose I α is dominant in bacterial and algal cellulose, whereas I β is dominant in higher plants such as cotton, wood, and agricultural grains. Cellulose II is made from Cellulose I β by regeneration; their chain arrangement is in antiparallel fashion in a two-chain unit. This paper deals with types Cellulose I β and cellulose II only, so the rest will not be described here.

1.1.2. Hemicellulose

Hemicellulose is the second most abundant polymer in lignocellulosic material. It is a non-crystalline heteropolysaccharide existing in the primary and secondary cell walls of plants (Agbor *et al.*, 2011). It is defined as an alkali soluble fraction in cell wall components after the removal of pectin (Aspinall, 1959). It forms hydrogen bonds with cellulose, covalent bonds with lignin, and

ester linkages with acetyl units and hydroxycinnamic acids. Hemicellulose does not have a characteristic molecular weight, has wide polydispersity, and its degree of polymerization ranges from approximately 80 to 200 molecules (Sun *et al.,* 2004).

In general, hemicellulose consists of a main polysaccharide chain backbone, with a substitution of the sugar monomers and branching at specific positions. Monosaccharides present in hemicelluloses are mainly xylose, glucose, mannose, arabinose, galactose, and fructose. Also, glucuronic acid is sometimes contained in a side chain (Timell, 1965; Timell, 1964). The composition of hemicellulose depends on the original source. Hemicelluloses in agricultural biomass including straw and grasses are mainly composed of xylan; while softwood hemicellulose mainly contains glucomannan (Agbor *et al*. 2011).

Depending on the main chain and substitution types, hemicelluloses are categorized into five major types: xylans, glucomannans, arabinans, galactans, and glucans. The most abundant hemicellulose in nature is xylan, which is present in all terrestrial plants and comprises up to 30% of the cell wall materials of annual plants, 15-30% of hardwood, and 5-10% of softwood (Wilkie, 1979).

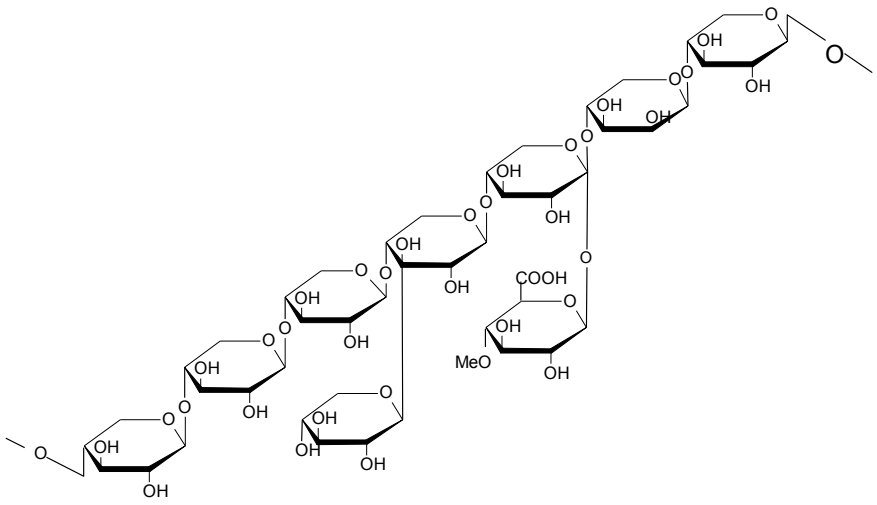


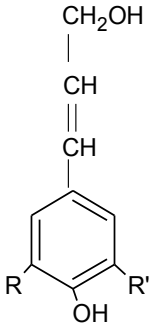
Figure 1.2. A simple representative chemical structure of wheat straw hemicellulose.

1.1.3. Lignin

Lignin is the third most abundant natural polymer in nature. It is present in plant cell walls and forms a rigid and impermeable matrix (Agbor *et al.,* 2011). The main function of lignin in plant cell walls is to create a barrier to prevent evaporation and to help send water to critical cells. Several studies have suggested that lignin may possess antimicrobial and antifungal activities, antioxidant properties, UV radiation absorption characteristics, and flame-retardant properties (Ugartondo, Mitjans, & Vinardell*,* 2008; Cruz *et al*., 2001). The lignin content of plant material varies depending on the original source. For instance, lignin content can range from 20% (w/w) for hard woods to 28% (w/w total dry solid) for softwoods (Dimmel, 2010). Higher lignin content may contribute to plants being

more resistant to environmental stresses such as direct sunlight and frost (Doherty *et al.*, 2011).

Lignin is a complex polymer that is composed of cross-linked amorphous heteropolymers, made up of many different phenylpropanoid monomer units (Hendricks and Zeeman, 2009). It also has wide polydispersity so that it does not have a characteristic molecular weight (Figure 1.3). The main building blocks of lignin are ρ -coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Dimmel, 2010; Figure 1.3).



Substituents	Name	Location
R = R' = H	ρ - Coumarylalcohol	Compression wood, grasses
R = H, and R' = OCH_3	Coniferyl alcohol	Hardwoods and softwoods
R = R' = OCH_3	Sinapyl alcohol	Hardwoods

Figure 1.3. Lignin monomeric building blocks.

Natural lignin is formed through the polymerization of monomeric alcohols via oxidative coupling with each other or with a growing polymer end, which is generally initiated by oxidases or peroxidases (Doherty *et al.*, 2011). Although the precise mechanism is not yet determined, radical-radical combination of free radicals generated by enzymatic dehydrogenation is the key

reaction (Davin *et al,* 2008; Ralph *et al.,* 2004). Oxidation generally produces a phenolic radical with unpaired electron density delocalized to the positions O-4, C-1, C-3, C-5, and C- β . The phenoxyl β -O-4 linkage is the most abundant in lignin polymerization. In fact, NMR studies showed that 30-40% of total cross-linkings in a softwood lignin were this type and also accounted for 40-50% of cross-linkings in hardwood lignin (Jiang and Argyropoulos, 1994; Argyropoulos, 1994). The second major type of bonding is biphenyl-linkage 19% of polymeralization linkages in softwood lignin are this type. The third bonding type is phenoxyl β -C5 linkage, accounting for 10% of the polymerization linkages in spruce lignin.

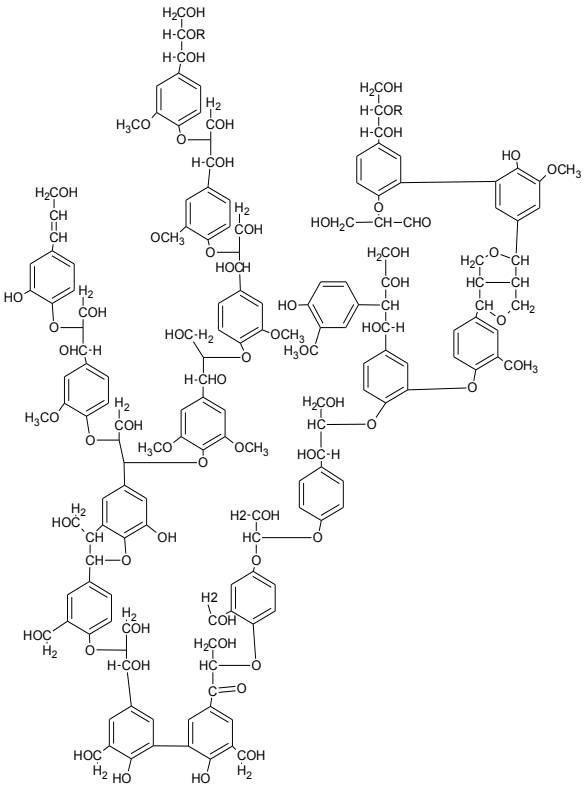


Figure 1.4. An example of lignin structure

1.2. Lignocellulose Decomposing Enzymes

As is mentioned in the previous section, lignocellulose is a complex substrate that can degrade into sugar solutions in the presence of "lignocellulose-degrading enzyme cocktails". Such cocktails are composed of mainly cellulases and a certain amount of hemicellulases, secreted by microorganisms. The former hydrolyze cellulose fibers and the latter hydrolyze the hemicellulose fraction. In this thesis, the cellulase cocktails were immobilized on silica to hydrolyze lignocellulose substrates. Thus, it is important to know the general compositions

of the cocktails and how each enzyme component decomposes lignocellulose materials in order to understand the immobilized enzyme systems. This section will explore the structure and mechanisms of each enzyme and how they synergistically and efficiently hydrolyze lignocellulose. Microorganisms that produce enzyme cocktails will also be introduced.

1.2.1. Cellulases

Cellulases are a mixture of individual enzymes that can decompose cellulose fibers, such as exoglucanases, cellobiohydrolases, endoglucanases, and β-glucosidase/cellobiose. Exoglucanases cleave the cellulose chain from the chain-end to produce glucose; cellobiohydrolases cleave cellulose from the chain end to produce cellobiose; endoglucanases intramolecularly cleave cellulose to produce oligosaccharide or more chain ends for the former two cellulases; and β-glucosidases along with cellobiase, which acts on cellobiose, cleave oligosaccharides to produce glucose. In addition, some *Trichodermas* ssp., a major cellulase producing aerobic fungi, can produce non-specific glucanases. They are not only able to hydrolyze cellulose, cellobiose, and cellooligomers, but they are also able to attack the hemicellulose fraction (Lynd *et al*., 2002). This raises the question as to what produces cellulases, how cellulases cleave lignocellulose, and what the structures of cellulases are like. The answers to these questions will be discussed in the next sections which provide an overview of

cellulase producing microorganisms, catalytic mechanisms of cellulases, and the structure of cellulases.

1.2.1.1. Cellulases Producing Microorganisms

Cellulolytic fungi and aerobic and anaerobic microorganisms have been isolated from various habitats (Tamaru *et al*., 2010; Wilson, 2009, Doi, 2008; Beukens and Pletschke, 2006; Pothiraj, Balaji, & Eyini, 2006; Doi& Tamaru, 2001; Gielkens *et al*., 1999; Gal *et al.,* 1997; Gerbi *et al.,* 1996; Kuhls *et al*., 1996; Schlochtermeier *et al,* 1992; Li and Calza, 1991; Kluepfel *et al*., 1986). In particular, aerobic fungi play a major role in the degradation of plant material. They are often found in decomposing wood and plants, in soil, and on agricultural wastes. They secrete a set of cellulases from their cells that work together synergistically to efficiently degrade lignocellulose. For example, *Trichoderma reesei* is known to produce several endo-glucanases and cellobiohydrolases, and their purified cellulases are commercialized worldwide (Liu, Glenn, & Buckley, 2008).

Both aerobic and anaerobic bacteria are also important producers of lignocellulose-degrading enzymes. Aerobic bacteria are usually found in soil, in water, on plant materials, in animal feces, and in sugar cane fields. Anaerobic bacteria are found in natural habitats such as soil and decaying plant materials. Additionally, some are enriched by human activities, such as in compost piles, in

sewage plants and in wood processing plants. Like anaerobic fungi, they are found in the rumen, where bacteria process plant materials for the host's nutrition. Unlike aerobic fungi, both aerobic and anaerobic bacteria produce large multienzyme complexes called cellulosome on their outer surface.

1.2.1.1. Catalytic Mechanisms of Cellulases

Cellulases are a mixture of cellulose decomposing enzymes, and they specifically hydrolyze β-(1,4) glycosidic bonds. There are many types of individual cellulases, but the general reaction mechanism involves cleaving glycosidic linkages in oligosaccharides and polysaccharides based on the general acid-base reaction (Davies and Henrissat, 1995). In an acid-base reaction, an acidic residue on the enzyme is required to protonate the glycosidic oxygen atom as a proton donor, whereas the base extracts a proton from the nucleophilic molecules that attach the anomeric carbon atom (White and Rose, 1997). In the case of active sites on cellulases, one amino acid residue acts as a catalytic proton donor and a second residue acts as a catalytic nucleophile. These amino acid residues may be two aspartate residues, two glutamate residues, or one of each (Reilly, 2007).

Cellulases employ two main types of catalytic mechanisms: inverting and retaining reactions (Reilley, 2007; Schülein, 2000). Inverting mechanisms produce a reducing-end glycosyl residue in an opposite configuration to that prior

to the cleaving of its glycosidic bond. This is called a single displacement reaction (Figure 1.5). In this reaction, the base deprotonates water molecules, resulting in the protonation of the base that turns to act as a general acid in next reaction. The deprotonated water molecule attacks the anomeric centre to form a new bond and the enzyme-substrate complex (in this case, the remaining cellulose chain) is cleaved, assisted by the general acid, causing the deprotonation of the acid. The deprotonated acid then becomes the general base in subsequent reactions (White and Rose, 1997).

In the retaining mechanism, the new reducing-end glycosyl residue retains its original configuration through a double-displacement reaction (Figure 1.6). The first reaction is glycosylation: the acid protonates the glycosidic linkage of the cellulose chain. At the same time, the base residue acts as a nucleophile attacking the anomeric carbon and forming a covalent glycosyl ester intermediate, resulting in the displacement of the enzyme-substrate complex. In the presence of water, the deprotonated acid behaves as a base to deprotonate the water molecule, which then attacks the carbon atom of the anomeric center. The reaction releases the hydrolysed product and regenerates the nucleophile at the active site (White and Rose, 1997).

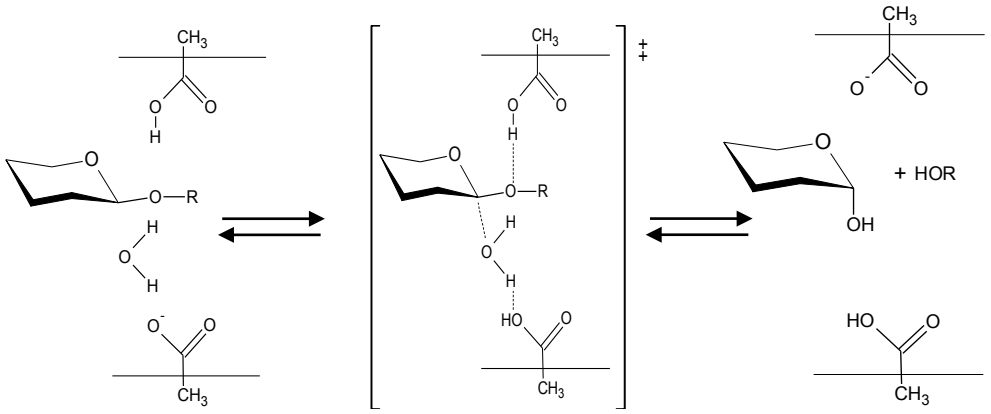


Figure 1.5. The mechanisms of inverting reaction.
(Reproduced with permission, Schülein, 2000)

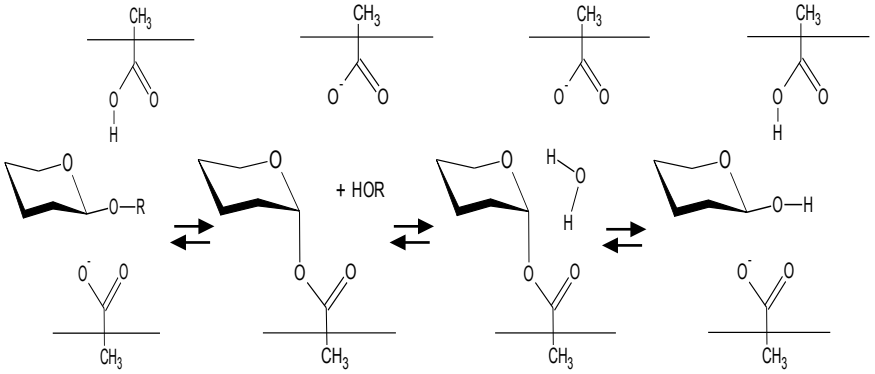


Figure 1.6. The mechanisms of retaining reaction.
(Reproduced with permission, Schülein, 2000)

1.2.1.3. Structures of Cellulases

Cellulases are composed of three domains: catalytic, carbohydrate binding modules (CBM), and linker peptides (Shoseyov, Shani, & Levy, 2006). The catalytic domain contains active sites for substrate hydrolysis. Cellulase catalytic domains are classified into a glycoside hydrolase/ transglycosidase classification. More than 100 families are categorized according to the carbohydrate-active enzymes (CAZy) database which reflects active site structures, catalytic mechanisms, and substrate specificity (Coutinho and Henrissat, 2011). Individual families are defined by the amino acid sequences of their protein members, and enzymes in the same family are often derived from the same ancestors (Reilly, 2007; Coutinho and Henrissat, 2011).

Carbohydrate binding modules (CBMs) are the second largest regions in cellulases. One of their roles is to bind the enzyme to the cellulose so that the catalytic domain spends less time away from the substrate. It also gives the catalytic domain time to move the chain into its active site before the enzyme diffuses away from the cellulose particle (Wilson, 2011). Their sizes range from 4-20 kDa (33 to 180 amino acid residues), and they are located at different positions including at the N-terminal, C-terminal, and along the peptide chain (Tomme *et al.*, 1998). More than 180 sequences of CBMs have been defined and categorized into 13 families with distinctly different properties. CBMs assist the

catalytic domain by increasing the accessibility of crystalline cellulose. This is done by penetrating and anchoring to the surface (Tomme *et al.*, 1998). Penetration may cause exfoliation of the surface fibers (Din *et al.*, 1991). In addition, once a cellulosic single chain interacts with aromatic rings of CBM, polar residues stabilize the binding structure through hydrogen bonds and Van der Waals interactions (Tomme *et al.* 1996). This unique functionality with cellulose may point towards future applications including bioseparations and enzyme immobilization (Tomme *et al*., 1998).

The third component is a linker. Short chain linker peptides (6-59 amino acid residues) connect the catalytic domain and cellulose binding domain (CBD). They possess an O-glycosylated interdomain and are rich in proline and hydroxyl amino acid residues, although the specific content may vary (Srisodsuk *et al*., 1993). The linker peptide has critical influences on catalytics in three ways. First, it facilitates independent functions of two adjacent domains by means of a flexible hinge. This hinge-like characteristic may be due to the peptide's composition of repeating units, allowing it to attach to the crystalline cellulose chain. Second, it functions as a spacer to provide the necessary distance between the catalytic domain and CBD and to fold the structure in the correct orientation (Srisodsuk *et al*., 1993; George and Heringa, 2002). These functions allow the CBD to attach to crystalline cellulose and maintain catalytic activities. Third, it protects the enzyme

from proteolytic degradation by using glycosylated proline and threonine rich sequences (Langsford *et al*., 1987).

Cellulases are fundamentally composed of the domains mentioned above: a large catalytic domain containing active sites and small substrate binding domain, which are connected by linker peptides.

1.2.2. Hemicellulases

Lignocellulose contains hemicellulose, in particular, xylan in plant lignocellulose resources, which interacts with cellulose fibers hindering cellulases from accessing cellulose fibers. Thus, hemicellulose has to be removed before cellulose hydrolysis is initiated. The enzyme cocktails which degrades the hemicellulose fraction are comprised of hemicellulases (Kabel *et al.*, 2007). The cocktails may contain several types of enzymes, including endoxylanases, exoxylanases, and accessory enzymes. Endoxylanase and exoxylanase decompose xylan backbones. Specifically, the former hydrolyze internal β -1,4 glycosidic bonds in the backbone and produce a set of different oligosaccharides. The latter hydrolyze terminal 1,4- glycosidic bond and liberate mono- or oligo-saccharides from the polymer backbones (Tenkanen *et al.,* 2003). Enzymes which remove side chains are called accessory enzymes, such as α -arabinosidase, α -glucuronidase, and acetyl xylan esterase (Kabel *et al*., 2007). They usually synergistically function with endo- or exo-xylanases and have the highest activity towards oligomeric substrates. Also, some of them may cooperate with each other to prevent different side groups from blocking accessibility to one another. Finally, enzymes that can cleave oligosaccharides

and produce monomers are β-xylosidases, which act on the non-reducing end of oligosaccharides and release xylose.

1.2.3. Three Strategies of Microorganisms for Lignocellulose Degradation

Cellulases are very unique enzymes because they can degrade an insoluble substrate. In general, in the case of enzymes hydrolyzing soluble substrate, the substrates diffuse to the enzymes and bind into the active site. On the other hand, to hydrolyze insoluble substrates, cellulases themselves have to diffuse onto the substrate and move a segment of a cellulose molecule into its active site (Wilson, 2011). There are three strategies that microorganisms employ to degrade lignocellulosic resources: a free cellulase mechanism, a cellulosomal mechanism, and the relatively new mechanism for *Cytophaga hutchinsonii* and *Fibrobacter succinogenes*, which secrete neither free cellulases nor cellulosomes (Wilson, 2008).

1.2.3.1. Mechanisms of Free Cellulases

Many aerobic microorganisms use the free cellulase mechanism in which they secrete a set of individual cellulases. The individual enzymes work together synergistically and efficiently degrade crystalline cellulose, as well as other fractions that are relatively easy to degrade (Wilson, 2011). There are five functionally distinct types of cellulases: endoglucanases, processive

endoglucanases, exoglucanases, endo-type exoglucanases, and β -glucosidase (Wilson, 2009).

In general, endoglucanases possess an open active site allowing them to attack the interior of amorphous cellulose regions, resulting in the generation of new chain ends or soluble oligosaccharides. Exoglucanases have their active sites in a tunnel-like structure to conduct a processive cleaving of cellobiose units from the chain ends of crystalline regions. In other words, endoglucanases randomly attack cellulose surfaces and produce new chain ends, and the generation of the new ends accelerates the hydrolysis for exoglucanases to cleave cellobiose. Therefore, endoglucanases and exoglucanases synergistically decompose cellulose fibres in an efficient manner.

Additionally, some endoglucanases have partially covered active sites working in a similar manner to exocellulases, called processive endoglucanases (Parsiegla *et al*., 1998). The enzymes show synergism with ordinary endoglucanases as well as exoglucanases and cellobiohydrolases. Also, some exo-acting glucanases behave like an endo-glucanases. For example, cellobiohydrolase II derived from *T. reesei* acts like an endoglucanase and synergistically hydrolyze cellulose fibers with other exoglucanases which processively decompose cellulose surfaces (Parsiegla *et al*., 1998). In addition, there are two classes of exoglucanases: one cleaves cellulose from non-reducing

ends and other attacks reducing ends. The model of synergistic degradation mechanisms of cellulases is shown in Figure 1.7 - Figure 1.8.

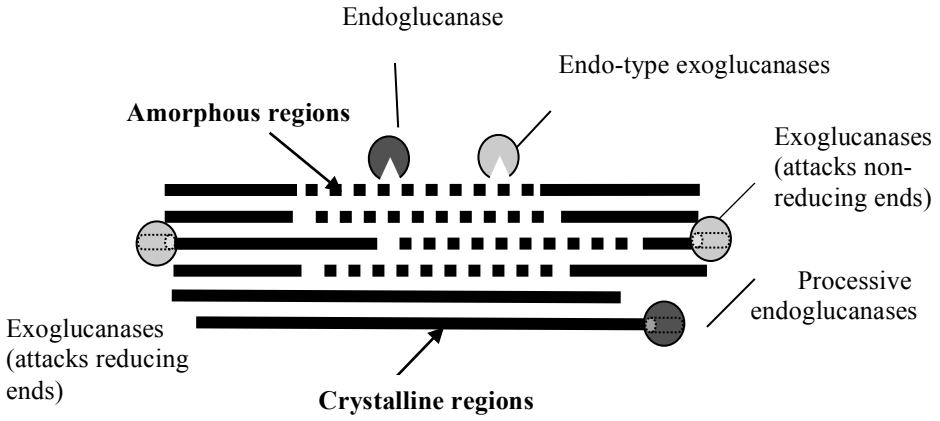


Figure 1.7. The model of synergistic degradation of cellulose fibres. Endoglucanases and endo-type exoglucanases attack amorphous regions, whereas exoglucanases and processive endo-glucanases attack chain ends of crystalline regions.

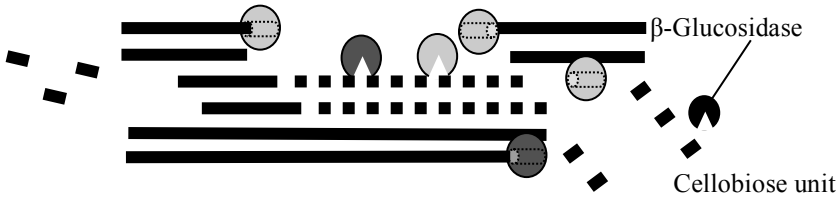


Figure 1.8. The model of synergistic degradation of cellulose fibres. Degradation is processing. Endoglucanase and endo-type exoglucanase cleave deeper/other amorphous regions of cellulose fibre, whereas exoglucanases and processive endoglucanases generate cellobiose units and glucose. β-glucosidases attack cellobiose.

1.2.3.2. Cellulosomal Mechanism

Some anaerobic microorganisms express cellulases in the form of cellulosomes in protuberances on the cell surface to efficiently hydrolyze lignocellulose (Blair and Anderson, 1999; Lamed *et al*., 1987). Cellulosomes are large multienzyme complexes that have a multimillion molecular weight (Ding *et al.,* 2008). In general, cellulosomes are composed of multi-domain scaffolding units containing multiple cohesin domains linked to cellulose-specific carbohydrate-binding modules (CBM). The cohesin domains interact with dockerin modules which are naturally present in hydrolytic enzymes such as cellulases, hemicellulases, pectinases, chitonases, glycosidases, and esterases (Schoffelen and van Hest, 2012). The specific recognition of a dockerin and cohesin causes the attachment of the enzymes to the scaffolding proteins (Salamitou *et al.*, 1994; Tokatlidis, Dhurjati, & Beguin, 1993). Also, CBMs are the major binding sources for cellulosomes to specifically recognize a cellulose substrate. The CBMs and catalytic subunits with dockerins interact through cohesions that attach on the cell surface (Figure 1.9; Wilson, 2011; Tamaru *et al*., 2010). Once the various cellulosomal subunits have been secreted at the bacterial cell surface, the molecular mechanisms assemble them into final mature conformations (Figure 1.9). Microorganisms usually allow multiple individual cellulosomes to form polycellulosomal protuberances (Desvaux, 2005). In addition, the combinations of enzymes on cellulosomes are diverse depending on

the original strain. In general, microorganisms choose the combinations that maximize the cellulosic activity to crystalline cellulose and preferable soluble sugars such as cellobiose, though the exact mechanisms behind such a synergistic property remain speculative and require further research (Desvaux, 2005).

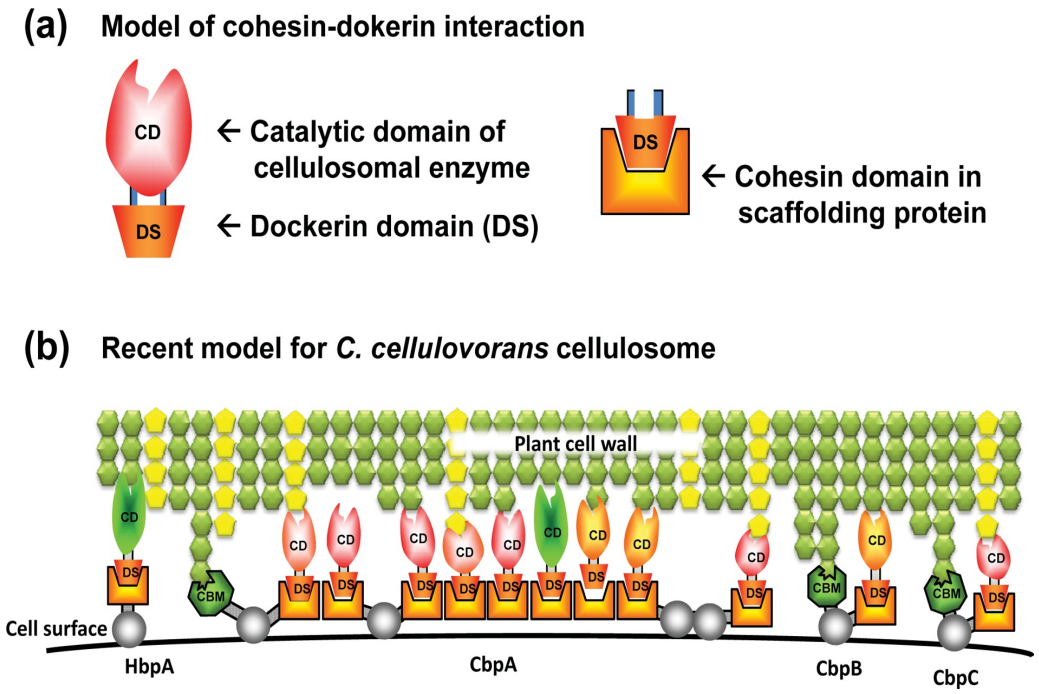


Figure 1.9. Models of cellulosomes.
(a) Model of cohesion-dockerin interaction, (b) Recent model of interaction of cellulosomes with substrate and cell surface. Gray round balls refer to scaffolding proteins (Tamaru *et al.,* 2010. Reproduced with permission from Taylor & Francis.).

1.2.3.3. The Mechanisms for Cytophaga Hutchnsonii and Fibrobacter Succunogenes

There is a relatively new discovery in cellulose degradation. Genomic sequencing research found that two types of cellulolytic bacteria did not follow either free cellulase mechanism or cellulosome systems: *Cytophaga hutchinsonii*, an aerobic soil bacterium, and *Fibrobacter succinogenes*, an anaerobic rumen bacterium (Wilson, 2008; Xie *et al.,* 2007). Their genome sequences did not contain processive cellulases, lignin degrading enzymes, nor scaffolding proteins. Their gene sequences encoded several endoglucanases and only certain number of cellulose binding domains. Both organisms tightly bind to cellulose during their growth, and they effectively degrade cellulose fiber (Wilson, 2008). Additionally, *F. succinogenes* grows faster on cellulose than most other studied microorganisms (Warnecke *et al.,* 2007). From these findings, Wilson (2008) proposed a third mechanism of cellulose degradation (Figure 1.10). The membrane on the outer surface of the outer membrane of *C. hutchinsonii* removed individual cellulose chains from the cellulose surface and transported the chain through the outer membrane into the periplasmic space, where endoglucanases were present. Extracellular endoglucanase might produce cellulose ends for binding to the outer membrane through a receptor (Wilson, 2008)

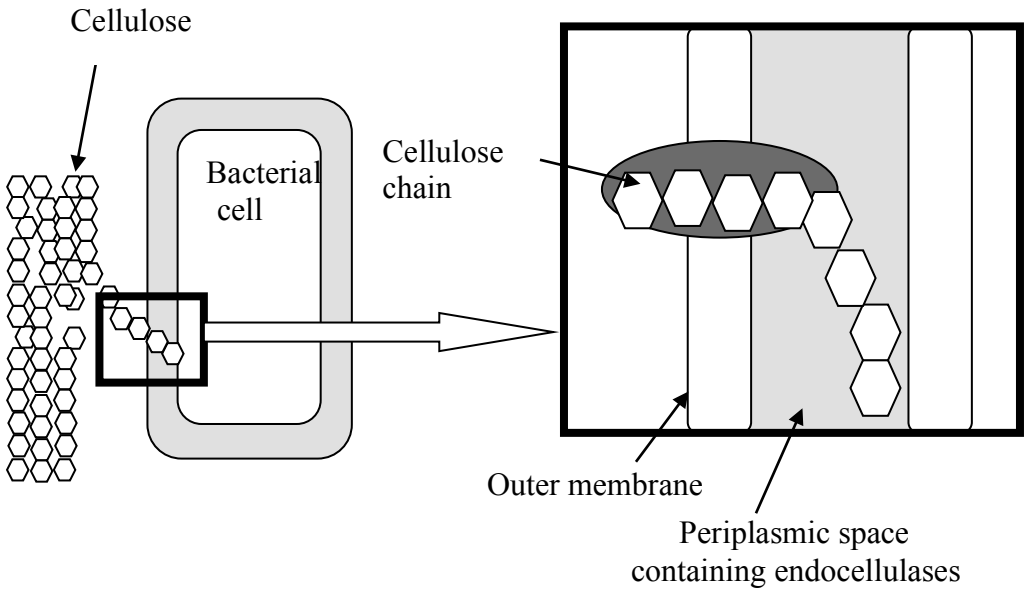


Figure 1.10. The proposed mechanism of cellulose degradation by Cytophaga Hutchinsonii and Fibrobacter succinogenes.

1.3. Current Trends and Challenges in Hydrolysis of Lignocellulose, Efforts to Improve Hydrolysis Steps

Lignocellulose is heterogeneous and has a complex structure, thus enzymes have difficulty accessing cellulose, resulting in a decrease in hydrolysis. A large quantity of enzymes are required to overcome the low hydrolysis efficiency, which is not economically feasible. In addition, the high cost of cellulases hampers their application for industry. Thus, research to demonstrate a reduction in cost for cellulases is of great benefit. Currently, tremendous efforts have been undertaken to make cellulase application more economically viable. In lignocellulose-based bioethanol production, such efforts included pretreatment, enzyme recycling, high-solid operation, simultaneous saccharification and

hydrolysis (SSF), multi-enzyme system, design of novel bioreactors, and strain improvements (Schoffelsn and Van Hest, 2012; Huang *et al*., 2011). This dissertation proposes an alternate approach with improved efficiency for the hydrolysis of lignocellulose by means of enzyme immobilization. Therefore, it is important to review recent trends in enzyme hydrolysis optimization to elucidate the merit of this study. In this section, some of the efforts will be discussed from the viewpoint of their advantages to enhance hydrolysis efficiency and challenges that necessitate further studies.

1.3.1. Pretreatments

1.3.1.1. Advantages of Pretreatments

There are efforts to increase enzyme accessibility to lignocellulose substrates (Saddler, 2012; Agbor *et al*., 2011; Kumar *et al*. 2009a; Chandra *et al*., 2007). To achieve higher enzyme performance, the lignocellulose structure can be altered by removing some fractions through a pretreatment process.

An optimal pretreatment process will:

(1) modify the substrate structure to improve sugar formation for the subsequent hydrolysis process,

(2) avoid degrading or losing carbohydrates, especially cellulose microfibrils,

(3) avoid forming by-products that generally inhibit the hydrolysis process or microbial fermentation,

(4) be cost-effective (Kumar *et al*., 2009b), and

(5) recover all components derived from the substrate in useable form (Chandra *et al.,* 2007).

As shown in Figure 1.1, the hemicelluloses within plant cell walls coat the cellulose-fibrils. It has been proposed that at least 50% of hemicellulose should be removed or modified to significantly increase cellulose digestibility (Agbor *et al.*, 2011). Hemicellulose is least resistant to chemical and thermal degradation (Liu *et al.,* 2012), and most hemicellulose in plant cell walls can be removed through solvating into a liquid fraction with thermochemical treatment.

Additionally, lignin removal or modification strongly affects the substrates, susceptibility towards cellulase attacks in a positive manner. For example, Chang and Holtzapple (2000) obtained 78% lignin removal from poplar wood through an oxidative lime pretreatment and demonstrated that biomass digestibility was enhanced with increasing lignin removal. Removal of lignin resulted in (1) elimination of a physical barrier that allows enzymes to access the cellulose fraction, (2) reduction of non-specific adsorption of hydrolytic enzymes to lignin or lignin-carbohydrate complexes, and (3) removal of lignin derivatives that are toxic to enzymes or microorganism (Chang and Holtzapple, 2000).

During pretreatment processes, the structure of lignin is often altered. It melts and coalesces on cooling, and then, is subsequently precipitated (Lynd *et al*., 2002; Brownell and Saddler, 1987). Donohoe *et al*. (2008) successfully visualized modified lignin using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The modified lignin appeared as droplets on the cell wall surface and are aptly called lignin droplets (Figure 1.11). Also, lignin droplets accumulated at specific regions including pits, cell corners, and delamination zones after chemical pretreatment (0.8% diluted H_2SO_4 at 150°C for 20 min). From these results, a model of delignification and lignin modification was proposed (Figure 1.12. According to this model, native lignin coalesces under thermochemical treatment and forms droplets in cell walls, followed by migration to specific areas within cell walls, and then extrusion to the cell surface via thermal expansion. Delignification causes biomass swelling, disruption of lignin structure, increases in internal surface area, and increased accessibility of enzymes to cellulose fibers (Agbor *et al.,* 2011).

Researchers have shown that removing hemicellulose and lignin or modifying lignin can enhance cellulase accessibility and suggests the importance of a pretreatment step to effectively degrade lignocellulose. A number of pretreatment approaches have shown promise and will be discussed, along with their challenges, in the next section.

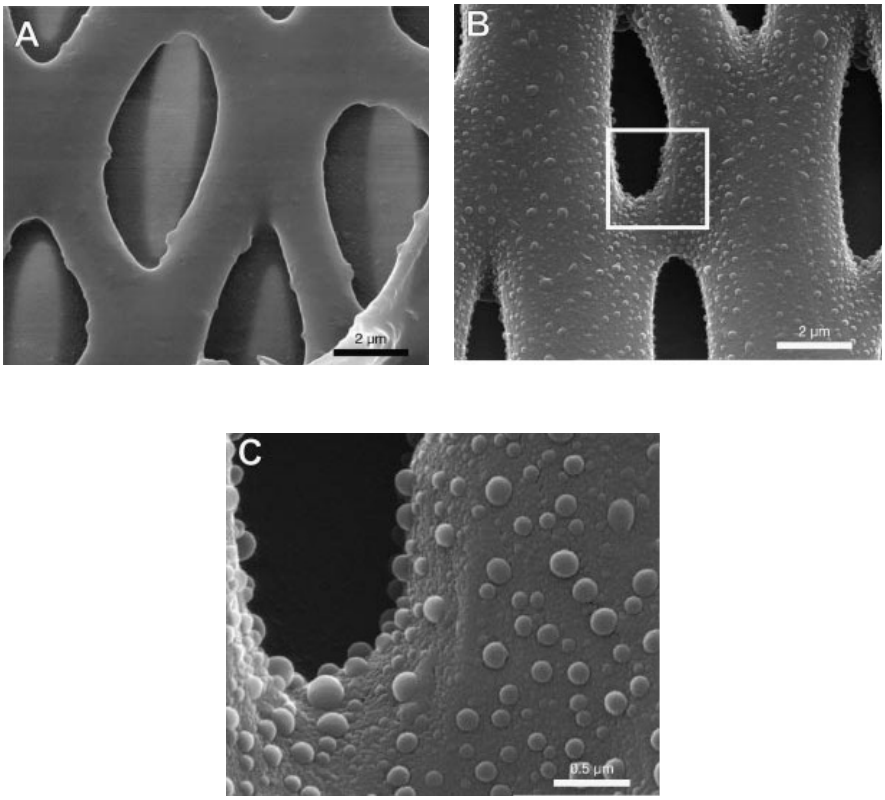


Figure 1.11. SEM micrographs of the cell surface of corn stover.
A) Untreated, B) 0.8% H_2SO_4 at 150°C for 20 min. C) Higher magnification of the region boxed in B) (Donohoe *et al*., 2008. Reproduced with permission from John Wiley and Sons)

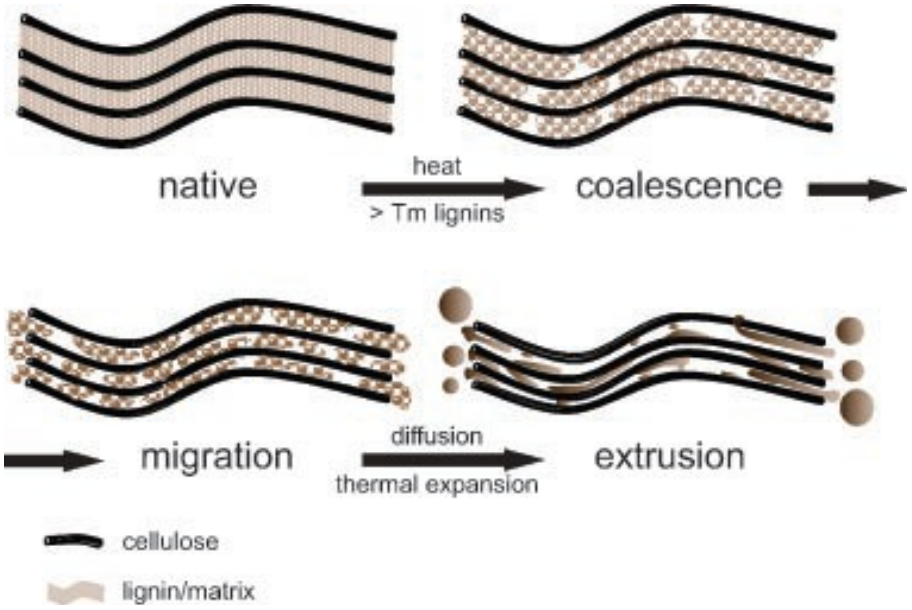


Figure 1.12. Model of a proposed mechanism for delignification and modification. (Donohoe *et al.,* 2008. Reproduced with permission from John Wiley and Sons.)

1.3.1.1.1. Pretreatment Methods

A number of pretreatment methods have been well established, including:

(1) Physical treatment, such as mechanical comminution;

(2) Physicochemical treatment, such as steam explosion, ammonia fiber expansion, liquid hot water pretreatment, carbon dioxide explosion, and microwave explosion;

(3) Chemical treatment, such as acid hydrolysis, alkaline hydrolysis, oxidative delignification, ozonolysis and ionized liquid treatment, organosolv;

(4) Biological treatment using white-rot fungi and brown-rot fungi or enzyme (Agbor *et al.,* 2011; Kumar *et al*., 2009b).

1.3.1.1.2. Physical Pretreatment

Comminution is a mechanical size reduction method including chipping, shredding, grinding and milling (Palmowski and Muller, 1999). It can reduce the size of biomass, cellulose crystallinity, and degree of polymerization, resulting in an increase in the surface area of the biomass (Agbor *et al*., 2011). This process is often used as an initial step of pretreatment and then combined with one of the three other methods mentioned above. The final particle size and biomass characteristics determine the power requirement for mechanical comminution of agricultural materials (Cadoche and Lopez, 1989). Even though taking energy consumption into consideration, comminution is still economically feasible in an industrial scale (Hendricks and Zeeman, 2009).

1.3.1.1.3. Physicochemical Pretreatment

Physicochemical pretreatments include steam explosion, liquid hot water pretreatment, ammonia fiber explosion, ammonia recycle percolation, and carbon dioxide explosion. In this section, steam explosion will be introduced because it is the most common method for the pretreatment of lignocellulosic materials and has been extensively studied (Qiu ad Chen, 2012; Kumar *et al.,* 2012; Bura and Saddler*,* 2004; Bura *et al.,* 2002; Shevchenko *et al.,* 2001; Wu *et al*., 1999; Wu *et*

al., 1998; McMillan, 1994; Brownell *et al.,* 1986). In the process of steam explosion, physically pretreated biomass is exposed to saturated steam under high pressure range of approximately 160-240°C and between 0.7 and 4.8 MPa (Agbor *et al.,* 2011; Sun and Cheng, 2002). The pressure is held for several seconds to a few minutes and then quickly reduced to atmospheric pressure, causing the materials to undergo explosive decomposition (Kumar *et al.,* 2009a). During this process, the lignin fraction is transformed and modified as a result of the high temperature. As a consequence, cellulose becomes more accessible for enzymatic attacks. Additionally, hemicellulose hydrolysis is attributed to the acetic acid which is generated from acetyl groups in hemicellulose and other acids released during pretreatment (Mosier *et al.,* 2005; Weil *et al.,* 1997). Water itself also acts as an acid at high temperature. The heated water cleaves *O*-acetyl groups and uronic acid substitutions in hemicellulose, thus the released acids accelerate further catalysis (Palmqvist and Hahn-Hägerdal, 2000; Weil *et al.,* 1997; Baugh *et al*., 1988). Steam efficiently transfers heat to biomass and increases the target temperature without excessive dilution of the sugar products. The addition of acid also effectively enhances the process of steam explosion (Agbor *et al.*, 2011). The use of acid decreases the recovery of hemicellulose sugars and the production of inhibitory compounds, and improves the enzymatic hydrolysis on the solid residue in subsequent hydrolysis (Mosier et al., 2005; Sun and Cheng, 2002). Steam explosion is strongly effective at removing hemicellulose but its lignin removal

ability is low. Therefore, this pretreatment is suitable for hardwoods and agricultural residues, which possess high hemicellulose and low lignin contents, but less effective for softwood which possesses a much higher lignin content. To increase process efficiency, the use of an acid catalyst is commonly used for softwood pretreatment (Agbor *et al.*, 2011).

1.3.1.1.4. Chemical Pretreatment

Chemical pretreatments include acid, alkaline, oxidative delignification, ozonolysis, organosolv, and ionic liquid treatment. In this section, acid pretreatment and alkaline pretreatment will be introduced. Acid pretreatments have been extensively studied and are the subject of reviews by Carvalheiro *et al*. (2008) and Yang and Wyman (2008). The concentrated inorganic acids such as H_2SO_4 and HCl are used to treat biomass; however, they are toxic, corrosive, and hazardous. Therefore, dilute acid hydrolysis is often applied to treat biomass (Kumar *et al*., 2009a). Typical acids used are sulfuric, nitric, hydrochloric, and phosphoric acids at a concentration of approximately 4% (w/w biomass) (Nguyen, 2000; Torget *et al*., 2000; Torget *et al.,* 1990). Dilute acids are mixed or contacted with biomass, and the mixture is held at 160-220 °C for periods ranging from minutes to seconds (Mosier *et al.*, 2005). Researchers have effectively hydrolyzed hemicelluloses to monomeric units and increased cellulose accessibility, resulting

in enhancing the hydrolysis process (Agbor *et al*., 2011; Mosier *et al.*, 2005; Lloyd and Wyman, 2005).

Alkaline hydrolysis involves the use of bases such as sodium hydroxide, calcium hydroxide, potassium hydroxide, and ammonium hydroxide. They swell lignocellulosic biomass, increase the internal surface area of the biomass, and decrease both cellulose crystallinity and degree of polymerization. Also, alkaline pretreatment disrupts the lignin structure and breaks the linkage between lignin and the other carbohydrate fractions. Acetyl and other uronic acid substitutions on hemicellulose, which decrease the accessibility of enzymes to cellulose surfaces, are also removed, resulting in higher accessibility of cellulose (Agbor *et al.,* 2011; Chandra *et al.,* 2007; Moseir *et al.,* 2005). The process of alkaline treatment is relatively mild, using lower temperature and pressure compared to other pretreatment technologies (0.5 g $Ca(OH)_2$/ g biomass and temperature at less than 55°C) (Kim and Holtzapple, 2006; Mosier *et al.,* 2005). Alkaline pretreatment is most effective with low lignin content biomass such as agricultural residues (Agbor *et al.,* 2011). The advantages of alkaline pretreatment are low reagent cost, and safety.

1.3.1.1.5 Biological Pretreatment

Biological pretreatment incorporates the use of microorganisms that produce enzymes to degrade lignin, hemicellulose, and polyphenols (Agbor *et al.,*

2011). Three types of microorganisms are most often used for this type of treatment: brown rot fungi, white rot fungi, and soft rot fungi (Galbe and Zacchi, 2007). Brown rot fungi mainly attack cellulose, whereas white- and soft- rot fungi can decompose both lignin and cellulose, producing enzymes such as lignin peroxidases, manganese peroxidase polyphenol oxidases, and laccases (Lee *et al*., 2007; Sun and Cheng, 2002).

Biological delignification is very effective and selective without negative impacts on cellulose fractions (Qiu and Chen, 2012; Hatakka, 1994). These methods do not require high energy for lignin removal, and are considered safe and environmentally friendly, compared to other pre-treatment processes (Kumar *et al*., 2009a; Yang and Wyman, 2007). Also, it generates less hazardous or inhibitory by-products (Qiu and Chen, 2012).

1.3.1.2. Challenges of Pretreatment

As is mentioned above, pretreatments are effective in altering the structure of lignocellulose and making it more susceptible to enzymatic attacks (Agbor *et al*., 2011; Kumar *et al*., 2009a; Yang and Wyman, 2008). However, barriers to the widespread adoption of pretreatment processes include high energy input and cost, environmental concerns, substrate dependency, and inhibitor generation.

First, energy input should always be taken into account. All treatments require a large amount of energy so balancing pretreatment efficiencies and associated costs is important. For example, mechanical comminution is a very efficient primary treatment for size reduction, but it requires high energy input (Cadoche and López, 1989). Some pretreatments may require additional energy in downsteam treatments, including neutralization, water removal, treated-substrate wash, or chemical removal (Agbor *et al*., 2011). For example acid or alkali treatments use a large volume of water during their process, and they also require a high volume of water for neutralization later in the process. Thus, techno-economical analysis will be required when pretreatment processes are designed in industrial scale. In addition, several reaction conditions cause environmental or occupational concerns such as waste disposal, corrosion of equipment or risks for operators (Von Sivers and Zacchi, 1995). For example, diluter or concentrated acid pretreatment can easily damage equipment and pose a danger for operators (Agbor *et al*., 2011). Moreover, pretreatment efficiency depends on substrates. For example, some delignification methods are effective for substrates with lower lignin content, such as agricultural residues, but are not very effective for substrates with high lignin content including softwoods (Agbor *et al*., 2011; Chang *et al*., 2001; McMillan, 1994). Therefore, careful considerations are required for each substrate. Finally, one of the most problematic issues related to pretreatment is the generation of inhibitory compounds for enzyme hydrolysis or

further microbial fermentation (Hendricks and Zeeman, 2009; Almeida *et al.,* 2007; Palmqvist and Hahn-Hägerdal, 2000).

The inhibitory compounds from lignocellulose are divided into three categories: furan derivatives [2-furaldehyde and 5-hydroxymethyl-2-furaladehyde (HMF)], weak acids (acetic acid, formic acid, and levulinic acid), and phenolic compounds. Furan derivatives, furfural and HMF, are formed by dehydration of hexose and pentose sugars (Reilly, 2006). They inhibit growth of microorganisms, particularly *Saccharomyces cerevisiae,* giving a longer lag phase, and decrease in ethanol productivity (Almedia *et al.,* 2007). They inhibit alcohol dehydrogenase, pyruvate dehydrogenase, aldehyde dehydrogenase, and glycolytic enzymes (eg. hexokinase and glyceraldehyde-3-phosphate dehydrogenase) (Modig, Liden & Taherzadeh, 2002; Banerjee, Bhatnagar, & Viswantathan, 1981). Also, furfural affects glycolytic and TCA fluxes which are involved in energy metabolism (Horváth *et al*., 2003). Furthermore, the reduction of furans by yeast also results in NAD(P)H depletion so that yeast has to redirect energy for fixing the damage caused by furans and by reduced intracellulase ATP and NAD(P)H levels, either by enzymatic inhibition or by consumption and regeneration of cofactors (Almedia *et al.,* 2007). Additionally, acetic, formic, and levulinic acids are the most common weak acids generated from lignocellulose pretreatment. Acetic acid is formed by de-acetylation of hemicellulose fraction, whereas formic and

levulinic acids are the degradation products of HMF or furfural (Ulbricht, Northup, & Thomas, 1984). They slow the growth of microorganism and reduce ethanol productivity, which is caused by the accumulation of intracellulase anion (Larsson *et al*., 1999; Russell, 1992). Also, weak acids can diffuse from the fermentation medium across the plasma membrane (Verduyn *et al*., 1992; Verduyn, 1991). The resultant decrease in intracellular pH is compensated by the plasma membrane ATPase, which pumps protons out of the cell at the expense of ATP hydrolysis (Verduyn *et al*., 1992). As a consequence, less ATP is available for biomass formation. In addition, weak acids reduce the uptake of aromatic amino acids from the medium, probably because of a strong inhibition of Tat2p amino acid permease, which regulates the utilization of the amino acids (Bauer *et al*., 2003). Finally, lignin breakdown and carbohydrate degradation under acidic conditions derive a variety of phenolic compounds (Popoff and Theander, 1972; Popoff and Theander, 1970). They decrease the microbial growth and product-productivity. Researchers suspect that they act on biological membranes, causing loss of integrity (Heipieper *et al*., 1994). Also, phenolic compounds with weakly acidic in nature may destroy the electrochemical gradient by transporting the protons back across the mitochondrial membranes (Terada, 1990).

From the discussion above, lignocellulose pretreatment technology is beneficial in increasing enzyme accessibility to cellulose fibers. However, they

also have disadvantages that significantly affect subsequent hydrolysis and fermentation. Therefore, the pretreatment method and its conditions must be carefully selected to maximize the accessibility and minimize inhibitory productions, with careful consideration to balancing the cost requirements (Agbor *et al*., 2011).

1.3.2. High-Solid Hydrolysis

1.3.2.1. Advantages of High-Solid Hydrolysis

Hydrolysis of lignocellulose in aqueous solution has traditionally been examined with low solid concentration. However, this method generates a large amount of by-products that dissolve into the solution, and it produces a great mass of wastewater, which requires the post-treatment to be more complex and more expensive (Ran *et al.,* 2012). High solid hydrolysis is on strategy to address these problems. High-solid enzymatic hydrolysis is defined as the hydrolysis with high solid concentration without significant amounts of free liquid water present at the initial phase of the hydrolysis (Hodge *et al*., 2009). Operating with a high solid concentration increases both the product concentration and plant productivity. In addition, lower water content allows for less energy for heating and cooling in a plant with a large system capacity. It also generates less wastewater due to reduced equipment size, resulting in a decrease of capital and operational costs (Ran *et al.,* 2012; Kristensen, Felby, & Jørgensen, 2009; Um and Hanley, 2008;

Jøgensen *et al*., 2007). For example, the ethanol concentration in the fermentation broth before distillation process should be above 4% (w/w) in order to make the process economical in a bioethanol plant using starch as a substrate, (Fan *et al*., 2003; Wingren *et al.,* 2003). To achieve 4% (w/w) final ethanol concentration in a lignocellulose-based ethanol process requires a solid level above 15-20% (w/w) for most types of lignocellulose materials (Jøgensen *et al*., 2007). For example, Hodge *et al*. (2009) hydrolyzed dilute acid-pretreated corn stover using a fed-batch stirred tank reactor and achieved a final cellulose conversion of 80% with 25% of solid content. Furthermore, Jøgensen *et al*. (2007) designed a gravimetric mixing reactor, equipped with a horizontally placed drum with a horizontal rotating shaft mounted with paddlers for mixing, and obtained 86 g/kg glucose with 40% solid loading resulting in 48 g/kg ethanol.

1.3.2.2. Challenges of High-Solid Hydrolysis

High-solid hydrolysis is an economically viable option as it substantially reduces both capital and operational costs. However, many studies reported that increasing solid content resulted in a corresponding linear decrease in cellulose and hemicellulose conversions, as well as a decrease in ethanol yield (Wang *et al*., 2011; Kristensen *et al*., 2008; Cara *et al.,* 2007; Jøgensen *et al*., 2007; Sørensen Pedersen, & Meyer, 2006; Rudolf *et al.,* 2005; Varga *et al*., 2004). Although the exact reason and mechanism that decrease the conversions and ethanol

productions have not yet been elucidated, several potential factors related to the solid effects have been studied: mechanical mixing deficiency and insufficient mass transfer, viscosity, product inhibition, inhibitors derived from pretreatment, and cellulase adsorption inhibition.

In general, mass transfer is critical factor in enzyme reactions. Mechanical mixing in high solid hydrolysis is difficult (Xue *et al*., 2012b). This might decrease the mass transfer between enzymes and substrate and ultimately decrease cellulose conversion (Palmqvist and Lidén, 2012). On the contrary, Ingesson *et al.* (2001) examined the effects of mixing conditions and concluded that mixing did not have a substantial effect. In fact, they reported that an increase in shaking from 20 to 150 rpm increased the hydrolysis rate whereas the final cellulose conversion was only slightly affected by any change in shaking rate. Also, intermittent stirring, which possessed periods of low or no stirring, was almost as efficient as constant stirring. Um and Hanley (2008) also showed that the rotation speed of 120 rpm and 180 rpm did not affect the glucose yield, due to a substantial decrease in the viscosity of the reaction mixture and better interaction between the enzymes and the remaining substrate. However, the glucose yield at 60 rpm was significantly lower than that at 120 or 180 rpm. The authors mentioned that a threshold line existed for the mixing speed to produce glucose.

Additionally, viscosity might be involved in the decreased hydrolysis yields. The viscosity at initial hydrolysis phase rises with the increasing solid loading. Therefore, the high viscosity negatively causes initial glucose production at the initial hydrolysis phase, which is likely due to low mass transfer (Lu *et al*., 2010). However, a rapid decrease of viscosity occurs after initial phase (Jøgensen *et al*., 2007; Fan *et al.* 2003). While viscosity decreases, the structure of substrate dramatically changes from solid to paste or liquid form. This change may be a result of the combined effects of separation of the cell wall matrix and gradual reduction of the average chain lengths of cellulose and hemicellulose by endoglucanase activity in enzyme cocktails (Olsen *et al*., 2011; Jøgensen *et al*., 2007). From these observations, high viscosity derived from high solid loading affected initial hydrolysis rates and did not greatly affect overall hydrolysis performance.

Product inhibition is well known to reduce enzyme performance. High-solid loading may cause product inhibition in enzyme hydrolysis. In order to examine the effect of product inhibition on high-solid hydrolysis, Kristensen *et al.* (2008) hydrolyzed filter paper using Celluclast 1.5 L and Novozyme 188 (75 FPU/g). They added 50 g/L glucose on filter paper hydrolysis with 5% and 20% solid loadings. Although glucose addition decreased the initial hydrolysis rate up to 4 h, it did not affect the hydrolysis performance. The group concluded that

either stronger factor inhibited the hydrolysis after 4 h and masked the product inhibition, or the 50 g/L of glucose was below threshold to affect the hydrolysis. In addition, some product could inhibit the adsorption of cellulases on cellulose fiber, particularly cellobiose (Kumar and Wyman, 2008). There is a strong correlation between decreasing glucose yields and cellulase adsorption (Kristensen *et al.,* 2008). Therefore, increasing the concentration of sugars in high-solid hydrolysis results in inhibition of adsorption of the enzymes.

Additionally, pretreated lignocellulosic material contains toxic by-products such as acetic acid, furfural, and lignin derived phenol as mentioned in the previous section. The toxic compounds also are increased as solid content increases; thus, inhibition may become more detrimental to enzyme hydrolysis and microorganism growth (Lu *et al*., 2010; Jøgensen *et al*., 2007; Klinke, Thomsen, & Ahring, 2004; Palmqvist *et al*., 1999). Indeed, Lu *et al*. (2010) compared hydrolysis performance and ethanol production using washed and unwashed steam-exploded corn stover with 30% (w/w) solid loading. Most of the harmful components were water soluble, so that washed substrate did not contain the compounds. The glucose concentration, cellulose conversion, and ethanol production of washed substrate were 103.3 g/L, 72.5%, and 49.5 g/L, respectively. Using unwashed substrate, glucose yield was less than 85 g/L, and microorganisms did not ferment the hydrolysate at all. In other words, water-

soluble components inhibit the glucose and ethanol productions, and the washing process may prevent the effect of inhibition on hydrolysis and fermentation (Lu *et al.,* 2010).

1.3.3. Enzyme Recycling

1.3.3.1. Advantages of Enzyme Recycling

One tactic to increase hydrolysis efficiency is to recover and recycle the enzymes in free form (Xue *et al.,* 2012a; Qi *et al.*, 2011; Tu and Saddler, 2010; Tu *et al*., 2009; Tu *et al.,* 2007 ab; Gregg and Saddler, 1996; Lee *et al.,* 1995; Ramos *et al*., 1994; Ramos and Saddler, 1993). After a hydrolysis reaction, some cellulases remain free in solution, whereas others are bound to the residual substrate (both on cellulose and lignin) (Tu *et al,* 2009). Cellulases from both liquid and solid fractions can be recovered and recycled back through the system (Tu *et al*., 2007b; Gregg and Saddler, 1995).

Tu *et al.* (2007b) found that 90, 65, and 51% of cellulases remained in liquid phase when Avicel (2% w/v), pretreated pulp (3% w/w lignin content), and ethanol pretreated mixed softwood (6% w/w lignin content) were used as substrates, respectively. Free cellulases in a reaction supernatant can be recovered by the addition of fresh substrate. Cellulases have a high affinity to cellulose; thus, cellulases in liquid fraction can be effectively adsorbed and recovered onto

fresh substrate (Qi *et al*., 2011). Once fresh substrate was added into the liquid phase, free cellulases quickly adsorbed onto them within the first 20 min, thereafter adsorption rate slowed during the next 30 min after which it plateaued (Tu *et al*., 2007b). Tu *et al.* (2007b) was able to recover 76% of the free cellulases from liquid phase using Avicel compared to 51% using an ethanol-pretreated mixed softwood substrate.

In addition, ultrafiltration can be employed to recover free cellulases from the reaction supernatant (Qi *et al.,* 2011; Lu *et al*., 2002; Ramos and Saddler, 1994). Ultrafiltration can separate all cellulases, including β-glucosidases, from hydrolysis products. For example, Lu *et al.* (2002) recycled cellulases 3 rounds with 20 FPU/g cellulose enzymatic strength using steam-exploded Douglas-fir which was further extracted by hot alkali peroxide. The cellulose conversion rates of 1^{st}, 2^{nd}, and 3^{rd} cycles were 97.2, 92.6, and 73.6%, respectively. Ultrafiltration has also been applied to column cellulose hydrolysis reactors (Tan *et al.,* 1986 ab; Tan *et al.,* 1987). Tan *et al.* (1986 a) built a column cellulose hydrolysis reactor equipped with an ultrafiltration apparatus [1000 molecular weight cut off (MWCO)] which allowed for continuous hydrolysis and enzyme recycling. The ultrafiltration apparatus selectively removed all cellulase components, including β-glucosidase, and enzymes were returned to the reactor. As a result, they

achieved rapid production of monomeric sugars in the product stream without the addition of fresh cellulases.

As well as reaction supernatants, a large amount of cellulase remains on hydrolysis residues, in particular residues containing a large amount of lignin (Tu *et al.,* 2007b). One method to recover bound cellulases from a solid phase is by collecting the hydrolysis residues containing bound enzymes and re-hydrolyzing it with fresh substrate (Lee *et al.*, 1995). This is a simple enzyme desorption method which allows cellulases to desorb from the residue and to re-adsorb onto fresh substrate (Lee *et al.*, 1995; Girard and Converse, 1993; Ramos and Saddler, 1994; Ramos and Saddler, 1993). Lee *et al.* (1995) proved that bound cellulase on lignin-rich residue still had activity after its desorption, and the cellulases quickly partitioned themselves between both residual substrate and fresh substrate. The other method to recycle enzymes that attach on solid residues is by extracting cellulases from the residues (Jackson *et al.,* 1996). Various methods to recover cellulases from hydrolysis residues have been studied, including surfactants, alkali, glycerol, urea, and phosphate or acetate buffers with varying pH (Otter *et al*., 1989; Clesceri *et al.*, 1985; Deshpande and Eriksson, 1984). Tu *et al*. (2009) attempted to recycle cellulases using a surfactant, such as Tween 80, as well as recovering enzymes from the reaction supernatant. Using a response surface methodology, they found that temperature, pH, and surfactant concentration were

the major factors to regulate cellulase desorption. This method was effective towards substrates containing a larger amount of lignin because surfactant competes with cellulases for hydrophobic adsorption sites on lignin-rich residue. Consequently, surfactants reduce non-productive binding of cellulase to substrate and allow cellulases to more effectively desorp from the residue (Eriksson, Borjesson, & Tjerneld, 2002; Tu *et al.,* 2007ab). Additionally, Qi *et al*. (2011) extracted bound cellulases from lignin-rich residues altering the pH values in buffer from 4.8 to 7.0, as well as recovering enzymes from a reaction supernatant. When the buffer pH is increased to neutral range, cellulases are more negatively charged. Since the overall charges of the polysaccharide and phenolic groups on the lignocellulose substrate are also negative, the binding between cellulases and substrate become weaker at neutral pH (Clesceri *et al.*, 1985). As a consequence, cellulases are effectively desorbed from lignin-rich residues by simple pH alteration.

1.3.3.2. Challenges of Enzyme Recycling

Enzyme recycling can reduce enzyme usage and enhance economic feasibility of lignocellulose hydrolysis. However, this strategy also faces challenges. First of all, readsorbing cellulases on fresh substrate alters enzyme compositions. Tu *et al.* (2007a) examined reabsorbed cellulase distribution on freshly added substrate. They found that the recovered cellulases were CBHI,

CBHII, EGI, and EGII; β-glucosidase was not readsorbed on the fresh substrate. This group also conducted isoelectric focusing analysis in the hydrolysis supernatant and found that the band for β-glucosidase remained free of charge. Cellulose and cellulases bind via electrostatic interactions, thus uncharged β-glucosidase was not adsorbed onto the fresh substrate. This finding indicated that β-glucosidases are not recycled and need to be added each recycling round, which is not economically feasible. Cellulase recovery by ultrafiltration can overcome this problem and recycle all cellulases. However, it is capital intensive, and optimizations are required to prevent protein folding (Baker, 2010).

A large amount of cellulases in liquid fraction were not recovered. This was observed more frequently when the substrate contained a large amount of lignin. For example, 76% of the free cellulases from liquid phase were recovered by the addition of fresh substrate when Avicel was used, whereas only 51% of the cellulases were recovered when an ethanol-pretreated mixed softwood substrate was used, the lignin content of which was 3% (Tu *et al.*, 2007b). This observation showed the significant effect of lignin on cellulase desorption. Regarding cellulase desorption from solid residues using surfactants, the addition of Tween 80 only recovered 50% of protein bound on the residue (Tu *et al*., 2007b).

The collected solid residues for a subsequent hydrolysis with fresh cellulases were hydrolyzed much more slowly than the fresh substrate. As a

consequence, it is likely that the hydrolysis performance decreased due to the accumulation of lignin with each every repeating cycle (Lu *et al,* 2002; Lee *et al.*, 1995; Girard and Converse, 1993). In fact, Lee *et al.* (1995) examined protein recyclability and enzymatic activities using water-washed steam-exploded birch. The group successfully recovered 90% (w/w) of proteins; however, the activity was only 16% of its original activity. Also, this method was negatively affected by the distribution of lignin in the substrate. This was likely because lignin served as a barrier limiting the accessibility of the cellulose fraction, as well as nonproductive bindings on cellulases (Qi *et al.,* 2011; Pejic *et al.,* 2008). Also, cellulases exhibit a higher affinity to lignin than cellulose based on hydrophobic interactions. Moreover, cellulases may also have lignin binding sites on the catalytic domain, which may increase the amount of nonproductive binding (Berlin *et al*., 2005). The high affinity of cellulases for lignin is problematic for cellulase extraction. It restricts cellulase desorption from a solid residue into the liquid phase (Lu *et al,* 2002). Indeed, alkaline pretreated wheat straw, which contains a low amount of lignin (3.5% (w/w)), showed more efficient recycling than acid pretreated wheat straw, which contains a higher amount of lignin (25% (w/w)) (Qi *et al.*, 2011). Additionally, cellulase extraction by the pH alteration method greatly decreased enzyme activity. This is probably because the change in pH disrupted the native structure of the cellulases, resulting in the inactivation of the cellulases (Qi *et al*., 2011).

1.3.4. Simultaneous Saccharification and Fermentation (SSF)

1.3.4.1. Advantages of SSF

Product inhibition is one factor that significantly affects cellulase hydrolysis in a negative manner (Xiao *et al.,* 2004). In order to overcome this problem, end-products or intermediate products need to be constantly removed from the hydrolysis reaction mixture. One potential method is ultrafiltration (Sun and Cheng, 2002). Tan *et al*. (1986) built a column cellulose hydrolysis reactor equipped with ultrafiltration apparatus, continuously removing sugar products and recycling cellulases. The researchers mentioned that the ultrafiltration successfully minimized the negative influence of product inhibition on cellulase hydrolysis; however, the capital cost for this apparatus was relatively expensive.

An alternative method is simultaneous saccharification and fermentation (hereby referred as SSF) (Sun and Cheng, 2002). SSF combines enzymatic hydrolysis and fermentation to keep the concentration of glucose low; that is, SSF simultaneously conducts enzymatic hydrolysis, cell growth, and product production (Kumar *et al*., 2009b; Zhang, Jin, & Kelly, 2007). Enzymes decompose lignocellulosic substrates and generate sugars, and then microorganisms immediately consume the sugars to produce end-products. Consequently, the product inhibition on enzymatic hydrolysis can be avoided.

Compared to the two-stage hydrolysis-fermentation process, which is a separate hydrolysis and fermentation processes, SSF has several benefits:

(1) to decrease product inhibition caused by glucose or cellobiose accumulation;

(2) to decrease inhibitor concentration on enzyme hydrolysis, which are produced during the pretreatment process, including acetic acid, furfural, and hydroxymethyl furfural. Microorganisms convert them to less inhibitory compounds. (Kumar *et al*., 2009b; Taherzadeh *et al*., 1997);

(3) to reduce required enzyme amounts;

(4) to lower the requirement for sterile conditions since glucose is removed immediately and ethanol is produced;

(5) to shorten process time; and

(6) to require a smaller reactor volume because a single reactor is used, resulting in lower capital investment (Sun and Cheng, 2002).

As a consequence, SSF may increases saccharification rates and productivity (Zhang *et al*., 2007). In fact, several researchers reported that SSF is a better process configuration than separate hydrolysis and fermentation (SHF) under their

enzymes and conditions (Öhgren *et al.,* 2007; Wingren *et al*., 2003; Wright *et al*., 1998).

1.3.4.2. Challenges of SSF

SSF has advantages over traditional separate hydrolysis and fermentation. However, the process also has disadvantages, including the temperature compromise, microorganism and enzyme recycle, and ethanol tolerance for microorganisms and enzymes (Zhang *et al.,* 2007; Sun and Cheng, 2002). One of its main demerits is the incompatibility of temperatures between hydrolysis and fermentation. The optimum temperature of enzymatic hydrolysis is typically higher than that of microbial fermentation. The temperature for hydrolysis is approximately 50°C and that for fermentation is approximately 30°C. In the two-stage hydrolysis-fermentation process, the optimum temperatures can be independently optimized to maximize the performance of both hydrolysis and fermentation, whereas SSF requires a temperature compromise (Olofsson, Bertilsson, & Lidén, 2008). A temperature of 37°C was often used for SSF experiments because the temperature was regarded as a suitable compromise at the high end of what *S. cervisiae* can tolerate (Sassner, Galbe, & Zacchi, 2006; Rudolf *et al*., 2005; Eklund and Zacchi, 1995; Wyman, Spindler, & Grohmann, 1992). Thus, utilizing thermotolerant microorganisms is important to allow for a fermentation temperature close to the optimal temperature for the enzyme activity,

such as *Fabospora fragilis, Saccharomyces uvarum, Candida brassicae, C. lusitaniae,* and *Kluyveromyces marxianus* (Ballesteros *et al.,* 2004; Hari Krishna, Janardhan Reddy, & Chowdary, 2001; Ballesteros *et al.,* 1991). However, some studies report an alternative option that the lower temperature positively affects xylose uptake in the co-fermentation for hexose and pentose. For example, Rudolf *et al.* (2008) reported that *S. cervisiae* TMB3400 consumed more xylose at 32 °C than at 37 °C during the SSF for a sugar cane bagasse to produce ethanol from a hemicellulose-rich lignocellulose. Olofsson, Rudolf, and Lidén (2008) also found that a temperature of 34°C was preferable for SSF of wheat straw. These are likely the result of mechanisms of a hexose transporter in a xylose fermenting strains of *S. cervisiae.* Hexose transporters transport xylose into cells, as well as glucose, but the affinity for xylose is approximately 200-fold lower than that for glucose (Meinander and Hahn-Hägerdal, 1997; Kötter and Ciriacy, 1993; Kilian and Uden, 1988). As a result, when less hydrolysis occurred at a lower temperature in SSF resulting from the lower glucose release, the condition was favorable for consuming xylose and increasing xylose fermentation yield (Lee *et al*., 2003).

Moreover, recycling of enzymes and microorganisms are difficult because separating enzymes and microorganisms from lignin-rich residues after fermentation is very problematic (Wingren *et al*., 2003). Also, enzyme and yeast

concentrations must be carefully balanced with their performances. Also, characterizing the microbial and biochemical kinetics of enzymes and determining optimal process conditions are essential to enhance SSF, particularly, the substrate loading, decreasing enzyme and yeast concentration, and varying temperature and pH (Da Silveira Dos Santos *et al*., 2010; Ko *et al*., 2009; Li *et al*., 2009; Chen, Han, & Xu, 2008; Olofsson, Rudolf, and Lidén, 2008; Stenberg *et al*., 2000).

1.3.5. Designer Cellulosomes

1.3.5.1. Advantages of Designer Cellulosomes

One of strategies to improve hydrolysis efficiency is construct a multi-enzyme macromolecular complex (Schoffelen and van Hest, 2012). The strategy brings biocatalyst together in an artificial way, which has major advantages.

(1) The active sites of the enzymes are brought in close proximity of each other,
(2) The intermediates are transferred from one catalytic site to another without or with less diffusion into the solution.

For example, the cellulases assembled on cellulosome configurations increased cellulose degradation and the concentration of fermentable sugar, resulting in the increase of final fermentation end-products (Desvaux, 2005). Moraïs *et al.* (2010) amplified cohesion A domain from the scaffoldin C region of

Acetovibrio cellulolyticus, cohesin B domain from the scaffoldin B region of *Bacteroides cellulosolvens*, cohesin F domain from the scaffolding B region of *Ruminococcus flurefraciens* strain 17, CBM-T domain from the cohesin 3 and cellulose binding module regions from *Clostridium thermocellum* YS; and then, all modules were assembled in linearized pET28a plasmid. Further, the group combined the plasmid with Cel 48 exoglucanase, Cel 5A endoglucanase, and Xyl 10 B and Xyl 11A endoxylanases that originated from *Thermobifida fusca.* The hydrolysis yield of wheat straw using the designer cellulosome was 2.4 times higher than that of free enzyme system. Mitsuzawa *et al.* (2009) genetically created a synthetic cellulosome containing four different cellulase assembled on an 18-subunit protein complex called a rosettasome, and a cohesin module was fused to each 60 kDa rosettasome subunit to induce binding cellulases. The bound enzymes have increased cellulose degrading activity compared to that of the free forms in solution.

1.3.5.2. Challenges of Designer Cellulosomes

Constructing designer cellulosomes is beneficial for lignocellulose hydrolysis and study of cellulases reactions. However, the hydrolysis yield of the designer cellulosome was much lower than that of naturally produced cellulosome from typical cellulosic bacteria. Therefore, the combination of scaffolding

domains and enzymes in the recombinant DNA needs to be improved (Moraïs *et al.*, 2010).

1.4. Alternate Approach --- Enzyme Immobilization

1.4.1. Advantages of Enzyme Immobilization

In addition to the previously mentioned efforts to enhance hydrolysis performance, cellulase immobilization is an alternative method to enhance hydrolysis performance. Immobilized enzymes are physically confined enzymes that retain their catalytic activity and can be used repeatedly and continuously (Worsfold, 1995). Also, immobilization allows enzymes to form a macromolecular complex. This strategy brings biocatalysts together in an artificial way which the actives sites of enzymes are brought in close proximity of each other and which the intermediates are transferred to subsequent enzymes with less diffusion into the solution (Schoffelen and van Hest, 2012).

The primary advantage of immobilization is its reusability, which offers significant cost benefits for industrial applications (Worsfold, 1995). One such approach to enzyme recycling is immobilization of cellulases on magnetic particles. The distinct advantage of magnetic particles is their ease of separation from reaction mixtures simply by the application of a magnetic field (Khoshnevisan *et al*., 2011). Additionally, the recycling of the magnetic particles

also reduces capital and operational costs (Feng *et al.,* 2006). Magnetic particles have been extensively studied in pharmaceutical, medical, and biological industries due to their unique magnetic properties and potential wide range of applications including in molecular detection and biosensors, drug delivery, hyperthermia, magnetic resonance imaging, enzyme immobilization and protein purification (Colombo *et al.,* 2012; Cho *et al*., 2012; Jordan *et al*., 2011; Woo *et al.,* 2010).

Magnetic ferrites are commonly-used magnetic materials. They are biocompatible magnetic materials with low toxicity and strong magnetic properties (Huang, Liao. & Chen, 2003). They are synthesized by hydrolytic synthesis with co-precipitation techniques, from which magnetite is obtained by alkaline co-precipitation of stoichiometric amounts of ferrous and ferric salt in aqueous solution (Colombo *et al.,* 2012; Massart, 1981). Hydrothermal conditions can also be applied to improve the magnetic properties (Ge *et al*., 2009). The co-precipitation method has a wide range of advantages, including the use of inexpensive chemicals, mild reaction conditions, the possibility to perform direct synthesis in water, ease of scale- up, and production of highly concentrated ferrofluids. Furthermore, for biological applications, magnetic nanoparticles must be stabilized and functionalized because nanoparticles themselves tend to flocculate, attributed to van der Waals forces (Berry *et al*., 2003). It is possible to

synthesize the particles with a stabilizing agent such as dextran; however, they still require further functionalization and stabilization to increase stability on the surface of the target enzymes. To achieve the required functionalization and stabilization, coating strategies can be applied. Coating materials for biological applications include organic molecules (eg. methylene diphosphoric acid, citrate), polymers (eg. dextrin, poly(ethylene glycol)), surfactants, and inorganic molecules (eg. silica) (Colombo *et al*., 2012). In particular, inorganic materials have been largely used to produce multifunctional materials, as well as giving stability. In addition, silica is a suitable material for immobilization because of its large surface area, chemical and mechanical stability, narrow pore size distribution, and relatively low cost (Hartono *et al*., 2010). In general, silica shows exceptional adsorptive affinity for various organic molecules in aqueous solution, such as proteins and polymers (Gun'ko *et al*., 2006; Rugal *et al.,* 2006). Adsorption is attributed by the formation of non-covalent interaction between the available groups on the surface of the silica and the carbonyl or amino groups on enzyme surface (Gun'ko *et al*., 2003). Non-covalent processes are very economic and simple, and enzyme activities tend to be retained during immobilization (Huang *et al.,* 2011). There are some reports that successfully immobilized enzymes on magnetic nanoparticles coated with silica. For example, Woo *et al.* (2010) immobilized lipase on magnetic nanoparticles coated with silica and achieved 70% of initial activity after three uses. Also, Huang *et al.* (2011) applied

glucose oxidase on Fe_3O_4/SiO_2 magnetic nanoparticles using glutaraldehyde. The immobilized enzyme retained 80% of its initial activity after 6 h at 45 °C, while free enzyme retained only 20% activity. The immobilized enzymes maintained 60% of initial activity after 6 cycles.

In the case of the cellulase immobilization, magnetic particles have been recently used as well. For instance, Cho *et al.* (2012) immobilized three types of cysteine-tagged cellulases, including endoglucanse I, cellobiohydrolase II, and β-glucosidase, on gold-dropped magnetic silica nanoparticles. They achieved 99% loading efficiency with 20 μg of enzyme, and the immobilized enzymes were reused seven times, retaining 90% of initial activity as hydrolyzing ρ-nitrophenyl β-$_D$-glucopyranoside. Alahakoon *et al*. (2012) immobilized cellulases originated from *T.reesei* on amine and aldehyde functionalized magnetic nanoparticles, hydrolyzing CMC. They reported that their retained enzyme activities ranged from 48.4 to 64.3% depending on the loading density, that were from 0.035 to 0.054 mg immobilized enzymes/mg nanoparticles. Moreover, Xu *et al.* (2011a) covalently immobilized cellulase on magnetic nanoparticle. The retained enzyme activity, measured by the specific enzyme activity using CMC as a substrate, decreased to 32.9%. However, the immobilized cellulases gained thermal stability, pH stability and storage stability. Furthermore, Liao *et al.* (2010) immobilized Cellulase R-10 on polyvinyl alcohol/ Fe_2O_3 magnetic nanoparticle and obtained

91% of retained enzyme activity using the specific enzyme activity method. Also, they examined the reusability, and the immobilized cellulases retained 40% of the initial activity at 4^{th} cycle, hydrolyzing microcrystalline cellulose.

In addition, there are successful reports recently using not only magnetic particles but also polymers and inorganics. For example, Liang and Cao (2012) covalently immobilized cellulases (50-300 mg protein) on pH sensitive polyacrylate amphiphilic copolymer (P_{MDB}) and gained 63.3% of retained enzyme activity, hydrolyzing filter paper. The immobilized cellulases also obtained thermal stability and reusability (Retained 83.1% at 5^{th} cycle). Also, Yu *et al.* (2012) covalently and non-covalently immobilized cellulases Suhong B989N on water-soluble-insoluble reversible polymer (Eudragit S-100). The retained enzyme activities of covalent and non-covalent methods were 81.08 and 56.83%, hydrolyzing CMC. The covalently immobilized cellulases obtained pH stability and thermal stability, specifically at high pH and high temperature. Moreover, Zhang *et al.* (2012) immobilized the crude cellulases from *T.viride* using carbodiimide and obtained 88.76% of the retained enzyme activity, hydrolyzing wheat straw. Also, Chang, Jing, and Wu (2011) immobilized cellulases on mesoporous silica with small pores and large pores by adsorption, and they also chemically bind cellulases on (3-triethoxysilylpropyl)succinic acid anhydride (TESP-SA)-functionalized large pore mesoporous silica. The retained activities of

three immobilized cellulases were 33.3%, 77.8%, and 83.8%, hydrolyzing cellulose powder (Sigma-Aldrich). In particular, TESP-SA-functionalized silica showed better storage stability than free cellulases at room temperature for 23 days. Xu *et al.* (2011b) adsorbed cellulases on reversibly soluble polymer, Eudragit L-100; the immobilized enzyme gained 88.76% of the retained enzyme activity, hydrolyzing filter papers. Furthermore, Zhou (2010) immobilized cellulase on N-succinyl-chitosan using adsorption method. The immobilized cellulases had 48.8% of retained activity, hydrolyzing CMC, and thermal stability at the high temperature. Also, Tébéka *et al.* (2009) immobilized cellulase C8546 on silica wafers by adsorption and obtained 80% of retained activity, hydrolyzing microcrystalline cellulose. Finally, Afsahi *et al.* (2007) covalently immobilized semi-purified *T.reesei* PTCC 5142 cultivation solution on non-porous ultrafine silica. The retained enzyme activity reduced to 35%, but it obtained thermal stability. Table 1.1 shows a summary of cellulase immobilization. Although these studies were successful, there is no report that the immobilized cellulases retained the equivalent enzyme activities as free cellulases and none of them showed the sugar compositions in the hydrolysates.

Table 1.1. Examples of cellulase immobilization

Enzyme (Source)	Support	Immobili-zation method	Sbstrate	Retained activity	Characteristics	References
Cellulases cocktail (NOVOZYMES, USA)	P_{MDB} polymer	Covalent	**Filter paper**	**63.3%**	Thermal stability, Reusability (Retained 83.1%at5th cycle)	Liang and Cao (2012)
Cellulases Suhong B989N (NOVOZYMES, USA)	Eudragit S-100	Covalent	**CMC**	**45%**	pH stability, Thermal stability,	Yu *et al.,* (2012)
Cellulases cocktail (Sigma-Aldrich Co. USA)	Magnetic nano-particle	Covalent	**CMC**	**48.4-64.3%**	Reusability (75.7% at 10th cycle)	Alahakoon *et al.*, (2012)
Crude cellulases (*T.viride*, Shanghai Bio Life Science & Technology. Co.Ltd)	Eudragit L-100	Covalent	**Wheat straw**	**88.76%**	N/A	Zhang *et al.,* (2012)
Enzyme complex NS 50013, (Novozymes investment, China)	Eudragit L-100	Adsorption	**Filter paper**	**75%**	N/A	Xu *et al.,* (2011b)
Cellulase cocktail (Shanghai Bio Life Science and Technology Co., Ltd., China)	Magnetic nano-particles	Covalent	**Steam-exploded corn stalk, Bagass**	**32.9%**	Thermal stability, pH stability, Storage stability	Xu *et al*., (2011a)
Cellulase cocktail (Sinopharm Chemical Reagent Co., Ltd., China)	N-succinyl-chitosan	Adsorption	**CMC**	**48.8%**	Thermal stability	Zhou (2010)
Cellulase R-10 (Dingguo Biologic Technique Company, China)	PVA/Fe_2O_3 Magnetic nano-particles	Adsorption	**Micro-crystalline cellulose**	**91%**	Reusability (Retained 40% at 4th cycle)	Liao *et* al. (2010)
Semi-purified *Trichoderma reesei* PTCC 5142 culture	Non-porous ultrafine silica	Covalent	**CMC**	**35%**	Thermal stability	Afsahi *et al*. (2007)
Cellulase C8546 (Sigma-Aldrich Co. USA)	Silica wafers	Adsorption	**Micro-crystalline cellulose**	**80%**	Reusability (Retained 100% at 6th cycles),	Tébéka *et al*. (2006)

1.4.2. Challenges of Enzyme Immobilization

Magnetic particles have a very efficient separation and recoverability; however, they share a common problem with most immobilized enzymes – the potential loss of enzymatic activity post-immobilization. The loss is often attributed to conformational changes as a result of interactions between the enzymes and supports (Cao, 2005). For example, Afsahi *et al.* (2007) immobilized cellulases originating from *Trichoderma reesei* on non-porous ultrafine silica with physical adsorption and with covalent binding using glutaraldehyde. The retained enzymatic activities of both immobilized cellulases greatly decreased to approximately 30% of free cellulases. Furthermore, Hartono *et al.* (2010) immobilized cellulases on silica with or without organic molecule functionalization. Both conditions reduced the retained activities. The activities of vinyltrimethoxysilane-functionalized and nonfunctionalized immobilized cellulases were 80.28 and 76.76% compared to that of free cellulases, respectively. The examples above show that the loss of enzymatic activities is commonplace in immobilized enzyme studies. However, the proper selection of both enzymes and support can occasionally retain high enzymatic activities (Cao, 2005). Thus, it is crucial to find the specific combination of enzymes and support which can retain high enzymatic activity.

Another disadvantage is a weak interaction between the support and the enzymes (Huang and Juang, 2011). The interaction between silica surfaces and amino acid residues on enzymes are generally electrostatic and hydrogen bonds (Huang and Juang, 2011). The stability of the immobilized enzyme depends on the strength of those interactions. The interaction is highly dependent on temperature, concentration, ionic strength and pH (Cao, 2005). Thus, it is essential to investigate how those microenvironmental conditions for enzymes influence cellulase adsorption on silica support and catalytic activity.

Enzyme activity measured under standard conditions may not correlate with the enzymatic performance towards real substrates containing cellulose-hemicellulose complexes. This means it is important to examine the enzymatic efficiency for not only the model substrate, but also the actual lignocellulose feedstock, such as wood samples (Kabel *et al*, 2006). Kabel *et al*. (2006) generated starch-removed and cellulose-rich wheat bran and grass samples, and hydrolyzed them using 14 different commercial cellulases. They found that the degradation of wheat bran was much better than that of grass, despite the use of standardized enzyme activities. For instance, the ratio of degraded glucan to glucose for wheat bran was approximately 60% using GC220 (DuPont™ Genencor® Science), whereas that for grass was less than 5%. This phenomenon was probably due to a less rigid lignin-hemicellulose-cellulose network in wheat

bran than in grass. Moreover, the ratio for grass using Cellulyve 50L (LYVEN-ZAC Normandial, Colombelles, France) was approximately 65%, whereas the same enzyme did not hydrolyze the wheat bran sample at all. The authors stated that the substrate characteristics are vital for cellulase efficiency (Kabel *et al.*, 2006). Therefore, it is critical to investigate the hydrolysis performance towards actual lignocellulose samples in this study.

Finally, there are many academic studies of immobilized enzymes; however, they are rarely applied in industry. For example, more than 140 research papers about immobilized amylase, one of the starch degrading enzyme cocktails, have been published within the last five years (Scopus, 2012); however, industries often use amylase in a free form, most likely due to its relatively high enzymatic activity, low price, and simpler substrate structure. In fact, starch is mostly glucose polymer in polymeric form with α 1-4 linkages (amylose and amylopectin). Thus, starch is relatively easily decomposed to produce sugar solutions. These factors make the starch hydrolysis process economically feasible for industry without using immobilization techniques. However, sugar production from more complex and recalcitrant substrates, such as plant lignocellulose, require higher dosage of enzyme cocktails. Lignocellulose is a highly heterogeneous substrate for an enzymatic degradation (Ran et al., 2012). In particular, the enzymatic conversion of cellulose-hemicellulose association is a complex process involving

the synergistic action not only with exoglucanases, endglucanases, cellobiohydrolases, and β-glucosidase, but also with hemicellulases, such as endo and exoxylanases, arabinosidase, acetylesterase, and glucuronosidases, to remove the hemicellulose fraction and its hydrolysis intermediates (Qing, Yang, &Wyman, 2010; Kabel *et al*., 2006; Tenkanen *et al.,* 2003). Therefore, the lignocellulose degrading enzyme cocktails require highly complex compositions and high dosage to obtain efficient hydrolysis activity, causing the process to be economically unviable. In this case, enzyme recovery and recycling is useful, with the potential to reduce capital and operational costs to make the process more economically feasible.

1.5. Hypothesis of the Study

To understand the hydrolysis system developed in this dissertation, the components and structure of lignocellulose, the structure and hydrolysis mechanisms of enzyme cocktails, and recent trends in improving hydrolysis steps and their challenges were reviewed in this introduction section. In this dissertation, the interactions between silica and cellulases will be discussed from the viewpoint of the future application for magnetic particles to achieve complete enzyme recovery. In particular, the main hypothesis of this dissertation is that the specific combination of immobilized cellulases on silica particles can retain enzymatic activity as high as that of free cellulases. To prove this hypothesis, many

immobilization methods and enzyme-support combinations were attempted to find the proper combination. Also, because enzyme-support interactions are highly dependent on microenvironmental conditions for enzymes, the effect of various conditions such as temperature, ionic strength, and pH cellulase adsorption and hydrolysis were studied; the characteristics of the immobilized cellulases was also examined. Also, various industrial lignocellulose substrates were applied on the immobilized cellulases, and the behavior of the enzymes on the actual biomass was investigated. Finally, β-glucosidase was supplemented, and its effects on sugar compositions were examined.

2. Materials & Methods

2.1. Materials

2.1.2. Enzymes

Cellulase 1, originating from *Trichoderma reesei* ATCC 29621, was purchased from Sigma-Aldrich Co.. (MO, USA; hereafter it is referred to as C1). A commercial enzyme cocktail, Cellulases 2, was kindly donated from Novozymes North America Inc. (NY, USA; hereafter referred to as C2). C2 is composed of two enzyme cocktails: NS50013 and NS50010. They were mixed at the ratio of 9:1 according to the manufacture's protocol. In general, the NS50013 is a cocktail of cellulases primarily used to reduce viscosity and increase extraction yield of various products from plant biomass. The major reaction products of cellulose hydrolysis using NS50013 are cellobiose and glucose. Measured in filter paper units (FPU), its cellulases, xylanase, polygalacturonase, and endoglucanse activities are 57 FPU/mL, 443 IU/mL, 3497 unit/mL, and 700 IU/g, respectively (Brandon *et al.,* 2008; Novozymes North America Inc.). NS50010 is β-glucosidase which hydrolyzes cellobiose to glucose and is often used to supplement NS50013 to maximize fermentable sugars. It originates from *Aspergillus niger* and its cellobiose activity is 250 IU/g (Novozymes North America Inc.).

2.1.2. Supports

Amberlite IRC-50, calcium chloride, and aluminum chloride, were purchased from Sigma-Aldrich Co. Three types of platy silicas were kindly provided by Dr. Steven Kuznicki's lab (Chemical & Materials Engineering, University of Alberta): ETS-2, ETS-4, and Chabazite. ETS-4 and ETS-2 are synthetic analogues of zorite ($Na_6Ti_5(Si_{12}O_{34})(O\ OH)_5\ 11H_2O$) and chivruaite ($(Ca_3Ti_5[(Si_6O_{17})_2O(OH)_4]14H_2O$) which are titanium silicate. Their molecular weights are 1538.53 g. The major contents are Na, 8.97%; Ti, 15.56%; silicon, 21.91%; H, 1.57%; O, 52%. Chabazite [(Ca, Na_2, K_2, Mg) $Al_2Si_4O_{12}\ 6H_2O$] is a natural mineral which is in tectosilicate mineral of the zeolite group.

Fumed silica S5130, or Silica 1, was purchased from Sigma-Aldrich Co. (hereafter referred to as S1), and Davisil chromatographic silica 633N, or Silica 2, was purchased from Thermo Fisher Scientific Inc. (MA, USA; hereafter referred to as S2). According to manufacture catalogue, S1 is non-porous in nature with a particle diameter of 7 nm and surface area of 390 ± 40 m^2/g. In solution S1 forms highly branched three-dimensional molten matrices consisting of chains 10-30 units long. On the other hand, S2 is porous in nature (pore diameter: 60 Å and pore volume: 0.8 mL/g) with a particle diameter of 47-60 μm and surface area of 500 m^2/g. In solution S2 remains as insoluble individual particles.

2.1.3. Chemicals

Sodium phosphate monobasic dihydrate, chitosan powder, tripolyphosphate, epichlorihydrin, polyvinyl alcohol, sodium hydroxide, sodium potassium tartrate, sodium dodecyl sulfate, hydrogen chloride, and andtrizma hydrochloride were purchased from ThermoFisher Scientific Inc. (CA, USA).

2.1.4. Substrates

Seven different substrates were used in this experiment. Microcrystalline cellulose and xylan were purchased from Sigma-Aldrich Co., and crystalline cellulose II and PASC were generated from microcrystalline cellulose (Percival Zhang, 2006). Cellophane paper was purchased from Thermo Fisher Scientific Inc., and it was shredded by an office shredder to the size of 4 x 26 mm. Steam-exploded poplar was kindly provided by Mascoma Canada Inc. (ON, Canada). It contained 42.1% cellulose, 25.4% hemicellulose, and 32.5% lignin, according to the provider. The pretreated poplar samples in wet condition were stored at –20°C and thawed at room temperature prior to use. Our laboratory provided the waste office automation (OA) papers, which were originally purchased from Grand & Toy® (ON, Canada; Premium copy paper, # 99115), and shredded by an office shredder to the size of 4 x 26 mm. OA paper generally contains 80% cellulose, 10.4% hemicellulose, and lignin 0.9% (Sosulski, 1993).

2.2. Methods

2.2.1. Determination of Immobilization Methods and Supports

2.2.1.1. Cellulase Immobilization on Polyvinyl Alcohol Modified Chitosan Beads

The polyvinyl alcohol modified chitosan beads were produced as previously described by Dinçer and Telefoncu (2007). Chitosan powder (1.0 g) was dissolved in 50 mL of 2% acetic acid (v/v). The solution was added drop-wise into 2% tripolyphosphate solution (w/v) to form beads which were left at room temperature for 4 h to harden. The beads were filtered using Whatman No.1 filter paper, and 5 g of filtered beads were added to 12.5 mL 0.04 M epichlorohydrin (pH 10). They were agitated at 70 rpm at 50°C for 5 hours to form cross linkages, and then they were washed three times to remove excess chemical. Meanwhile, polyvinyl alcohol (PVA) was modified with maleic anhydride. PVA (2 g) was dissolved in 20 mL distilled water. Maleic anhydride (1 g) was slowly added while pH was maintained at 9.0 using NaOH. The final volume was adjusted to 50 mL with distilled water. The washed beads were added to the maleic acid modified PVA solution and incubated for 1 hour at 40 °C. The beads were filtered and added to 0.015 M epichlorohydrin (pH 10.0). They were agitated at 70 rpm for 2 hours at 40°C. After incubation, they were washed three

times with 0.1 M phosphate buffer (pH 5.0) and added to a 10 mL cellulase solution containing 15 mg C1 enzyme dissolved in 0.1 M phosphate buffer (pH 5.0). The mixture was incubated at 4°C for 3 h at 70 rpm and washed three times with 0.1 M phosphate buffer to remove excess cellulases. As a positive control, 15 mg C1 enzymes were dissolved in 10 mL 0.1 M phosphate buffer (pH 5.0) and stored at 4°C. All reactions were performed in triplicate.

Microcrystalline cellulose (300 mg) was distributed into each tube of test samples and positive controls. The hydrolysis reactions for immobilized and free cellulases were conducted in a water bath shaker (50°C) with 150 spm agitation. Each reaction was terminated at 24 h by heating in boiling water for 5 min and their sugar content and total protein were analyzed.

2.2.1.2. Cellulase Immobilization on Glutaraldehyde Coated Amberlite

The glutaraldehyde coated amberlite was produced as previously described by Obón *et al.* (2000). Four grams of amberlite resin was weighed in a 50 mL centrifuge tube and washed with distilled water twice. Ten mL of 10% polyethylenimine was added and agitated at 100 rpm for 2 h at room temperature. After agitation, the resin was washed three times with distilled water, and 10 mL of 2.5% glutaraldehyde was added. The mixture was agitated at 100 rpm for 30 min and washed with 50 mM phosphate buffer (pH 7.0). Glutaraldehyde cross-

linked amberlite resin was then added into 10 mL of the cellulase solution, containing 15 mg of C1 in 50 mM phosphate buffer (pH 5.0), and agitated for 2 h at room temperature. The immobilized enzyme on amberlite was washed three times with the phosphate buffer (pH 5.0). They were stored in 10 mL of 0.1 M phosphate buffer at 4 °C until required for hydrolysis. As a positive control, 15 mg of C1 was dissolved in 10 mL 0.1 M phosphate buffer (pH 5.0) and stored at 4°C. All reactions were performed in triplicate.

At the time of hydrolysis, the immobilized and positive control cellulases were distributed into a 50 mL plastic tube, containing 10 mL of 0.1 M phosphate buffer (pH 5.0) with 300 mg microcrystalline cellulose. Each hydrolysis reaction for both immobilized and free cellulases was conducted in a water bath shaker (Model number 228, Fisher Scientific,) at 50°C with 150 rpm agitation. Each reaction was terminated at 24 h by heating in boiling water for 5 min and their sugar content and total protein were analyzed.

2.2.1.3. Chloride Coated Fumed Silica, Chloride and Aluminum Coated Silica/ Chloride Coated Amberlite, Chloride and Aluminum Coated Amberlite

The chloride coated silica and both chloride and aluminum coated silica were produced as previously described by Sinegani *et al.* (2005). Fumed silica S5130 (S1) or amberlite was treated with three methods: (1) no treatment

(negative control), (2) coated with calcium chloride, and (3) coated with calcium chloride and aluminum chloride.

For the first treatment, 1.5 g of S1 or amberlite was distributed into individual 50 mL plastic tubes. The supports were extensively washed with 0.1 M phosphate buffer (pH 5.0) for negative control or distilled water for chemically modified silica treatments. The negative control supports were stored in the refrigerator. For the second treatment, 10 mL of 1 N calcium chloride was added to each of the washed silica or amberlite supports and shaken at 200 rpm and 37°C for 1 h. They were washed three times with 0.1 M phosphate buffer to remove excess chemicals. S1 or amberlite coated with calcium chloride were stored at 4°C until used for hydrolysis. For the third treatment, 10 mL 1 M ammonium chloride (pH 7.0) was added to the 1.5 g of calcium chloride coated silica or amberlite and agitated for 1 h at 200 rpm and 37 °C. Unbound aluminum chloride was washed three times using 0.1 M phosphate buffer (pH = 5.0). All prepared silica or amberlite were added into 10 mL cellulase solution, containing 15 mg C1 enzyme dissolved in 0.1 M sterile phosphate buffer (pH 5.0), and shaken at 200 rpm and 37 °C for 1 h. In all treatment, after incubation, the immobilized cellulases were washed three times with 0.1 M phosphate buffer to remove excess cellulase. For a positive control, 15 mg C1 enzymes were

dissolved in 20 mL 0.1 M phosphate buffer (pH 5.0) and stored at 4 °C. All reactions were performed in triplicate.

Microcrystalline cellulose (300 mg) was distributed into each tube of test samples and positive controls. The hydrolysis reactions for immobilized and free cellulases were conducted in a water bath shaker (model number 228, Fisher Scientific) at 50°C with 150 spm agitation. Each reaction was terminated at 24 h by heat treatment in boiling water and their sugar content and total protein were analyzed.

2.2.1.4. ETS-2, ETS-4, Chabazite

ETS-2, ETS-4, and chabazite were neutralized, washed, and air-dried in Dr. Steven Kuznicki's lab. Each silica (200 mg) was distributed into 15 mL centrifuge tubes. Then, 3 mL of cellulase solution was added, containing 2 FPU of C2 enzymes dissolved in 0.3 M phosphate buffer (pH 5.0). They were well mixed using a vortex and stored at 4°C overnight prior for immobilization. After the immobilization, they were washed three times with 0.1 M phosphate buffer (pH 5.0) and stored at 4°C until used for hydrolysis reaction. The supernatants and washes were analyzed for total protein content to calculate loading efficiency. For a positive control, 17 mg C1 enzymes were dissolved in 5 mL 0.1 M phosphate buffer (pH 5.0) and stored at 4°C. All reactions were performed in triplicate.

The immobilized cellulases on ETS-2, ETS-4, or chabazite were added into a 15 mL centrifuge tube, containing 3 mL 0.1 M phosphate buffer (pH 5.0) with 100 mg microcrystalline cellulose. The hydrolysis reactions for immobilized and free cellulases were conducted in a water bath at 50°C with 150 spm agitation. Samples were periodically obtained and analyzed for sugar and protein content. The reaction was terminated at 24 h by heat treatment in a boiling water.

2.2.1.5. Hydrolysis in microcentrifuge Tubes Using Non-Porous Silica (S1) & Porous Silica (S2)

Fumed silica S5130 (S1; non-porous) or Davisil chromatographic silica 633N (S2; porous) were used for immobilization. Two types of cellulase preparations were immobilized on S1 and S2. The enzyme strength of C1 and C2 were unified to 2 FPU, the actual protein amounts of which were 1.8 mg and 10 mg, respectively. The appropriate amounts of enzymes and supports were mixed with 1 mL of phosphate buffer and added into 1.5 mL microcentrifuge tubes. Negative controls were prepared in a similar manner except that cellulases were not added. Test samples and negative controls were individually mixed by repeated pipetting and placed at 4ºC for about 2 h to facilitate enzyme immobilization (Dinçer *et al*., 2007). All samples were subsequently vortexed and centrifuged at 8087 x *g* for 30 sec to separate supernatants. The pellets were washed (by centrifugation at 8087 x *g* for 30 sec) twice with the respective

phosphate buffer (pH 5.0) used for immobilization, and finally with 0.1 M phosphate buffer (pH 5.0). The respective supernatants and post-wash solutions were placed in a boiling water bath for 5 min and stored at 4 ºC until further analysis.

Four combinations of immobilized cellulases were generated: Cellulases 1 x Silica 1 (C1S1), Cellulases 1 x Silica 2 (C1S2), Cellulases 2 x Silica 1 (C2S1), and Cellulases 2 x Silica 2 (C2S2). Additionally, the corresponding free cellulase solutions, C1 and C2, were prepared under optimal conditions of pH and ionic strength recommended by the manufacturer and stored at 4ºC. The detailed immobilization conditions including pH, ionic strength, support amounts were determined in later section (The effects affecting loading efficiency). Their loading efficiencies were maintained at over 90% in all immobilized cellulases.

All samples including test samples (C1S1, C1S2, C2S1, and C2S2), negative controls, and positive controls were pre-incubated at 50°C in a water bath for 3-5 min, and the hydrolysis reaction was initiated by the addition of 35 mg microcrystalline cellulose. Cellulase activity of immobilized enzyme and free enzyme were unified at 2 FPU in each reaction mixture. The hydrolysates were taken out of the water bath at various time points, placed in a boiling water bath for 5 min, and centrifuged at 8087 x *g* for 30 sec. The respective supernatant and

solid fractions were stored at 4°C until further analysis. All reactions were performed in triplicate.

2.2.2. Determination of Reaction Scale and Thermal Stability of the Immobilized Cellulases

2.2.2.1. Hydrolysis in 250 mL Flasks

The hydrolysis reactions for immobilized C1 on S1, and modified chitosan beads were conducted in this experimental design with a positive control. Microcrystalline cellulose (600 mg) was added to 250 mL Erlenmeyer flasks containing 20 mL 0.1 M phosphate buffer (pH=5.0). Immobilized cellulases on S1 of 1 g and chitosan beads of 5 g were added, and they were incubated at 20, 37, 50, or 65°C at 150 rpm for 24 h. Cellulase activity of immobilized enzyme and free enzymes were unified at 5 FPU in each reaction mixture (See filter paper unit assay in Section 2.2.8.1.). The reaction was terminated at 24 h by heat treatment in boiling water. The test samples were centrifuged at 1000 rpm for 5 min and respective supernatant and solid fractions were stored at 4°C until further analysis. All reactions were performed in triplicate.

2.2.2.2. Hydrolysis in 5 L Bioreactor

The hydrolysis reactions for immobilized C1 on S1, and modified chitosan beads were conducted in this experimental design with a positive control. Microcrystalline cellulose (20 g) was added into pre-incubated 5 L bioreactor (Minifors AG CH-4103, Infors HT, Switzerland), containing either 70 mL of immobilized C1 enzymes on S1 (C1S1) or 150 g of modified chitosan beads in 0.1 M phosphate buffer (pH 5.0) (approximately 220 FPU). Hydrolysis was conducted at various temperatures between 40 and 70°C. C1S1 was mixed at 350 rpm, whereas C1 on modified chitosan beads was mixed at 100 rpm because the beads were weak against mechanical mixing. Sampling of 10 mL was conducted every 30 seconds for C1S1 and free enzymes. Sugar production was plotted in a scatter plot and trend lines were drawn, then the slopes were calculated as a specific enzyme activity. Values were compared to that of free enzyme.

2.2.3. Determination of Parameters Affecting Loading Efficiency

The parameters affecting loading efficiency in cellulase immobilization, ionic strength, pH and the amount of silica support, were examined. For examination of ionic strength, cellulases C1 or C2 (2 FPU) were mixed with 1 mL phosphate buffer (pH 5.0) of ionic strength 0.1, 0.2, 0.3, or 0.4 M, and added into 1.5 mL microcentrifuge tubes containing 40 mg S1 or S2. Negative controls were

prepared in a similar manner without the addition of cellulases. Test samples and negative controls were individually mixed by repeated pipetting and held at 4ºC for approximately 2 h to facilitate enzyme immobilization (Dinçer *et al*., 2007; Li *et al*., 2007). All samples were subsequently vortexed and centrifuged at 8087 × *g* for 30 sec, and the supernatant was removed. The pellets were then washed with the phosphate buffer (pH 5.0) used for immobilization and centrifuged at 8087 × *g* for 30 sec) twice and finally with 0.1 M phosphate buffer (pH 5.0). The respective supernatants and post-wash solutions were placed in a boiling water bath for 5 min and stored at 4ºC until further analysis. For examination of pH, phosphate buffer of pH 4.5, 5.0, 6.0, or 7.0 was used and washing and immobilization was done as described in section 2.2.1.5. For examination of enzyme-support ratios, 10-40 mg of S1 or 40-160 mg of S2 was used and immobilization was done as described in section 2.2.1.5. All experiments were done independently in triplicate. The parameters that gave the highest loading efficiencies were used for the further experiments as optimum immobilized conditions.

2.2.4. Examination of Enzyme Activities (Hydrolysis yields & Hydrolysate Compositions)

C1 and C2 were immobilized on S1 and S2 under conditions that maintained a loading efficiency over 90-95%. The enzyme activities of C1 and C2

were unified at 0.5, 1.0, and 2 FPU to investigate the effect of enzyme strength. The microcrystalline cellulose of 35 mg was added into the immobilized cellulases which prepared under the optimum condition and free cellulases, described in section 2.2.1.5 and 2.2.2. Hydrolysis was conducted at 50°C for 24 h in a water bath. After the reaction, the test samples and positive controls were taken, and the reaction was terminated by heat treatment in boiling water. Then, the hydrolysate supernatants were retained for sugar analysis.

2.2.5. Protein Desorption

Protein desorption was investigated after immobilization and hydrolysis. After immobilization, supernatants and post-wash solutions were examined for the presence of unbound protein by Bradford protein assay (Bradford, 1976), and protein loading efficiencies were calculated (See section 2.2.8.3.). In the case of hydrolysis, samples were taken at 0 h and 24 h, placed in boiling water for 5 min to termination the reaction and examined for the presence of unbound protein by Bradford protein assay (Bradford, 1976). The amount of dissociated protein was calculated by subtracting the amount of protein in the 0 h samples from that of the 24 h post-reaction samples. All samples were analyzed in triplicate.

2.2.6. Examination of the Parameters Affecting Hydrolysis

To investigate effects on stability properties and product composition, various conditions were applied for the hydrolysis using the immobilized and free cellulases including temperature, pH, ionic strength, storage duration, and recycle stages. To examine the effects of temperature on hydrolysis, all samples (C1S1, C1S2, C2S1, and C2S2; See section 2.2.1.5.), including immobilized enzymes, negative controls, and positive controls, were pre-incubated at 40, 50, 60, or 65°C in a water bath for 3-5 min. Microcrystalline cellulose (35 mg) was added to each tube to initiate the reaction. The hydrolyses were conducted in a 1 mL 0.1 M phosphate buffer (pH 5.0) under varied temperature conditions ranging from 40, 50, 60, or 65°C. Hydrolysis reactions were terminated after 24 h by placing samples in boiling water for 5 min. The hydrolysates were centrifuged at 8087 × *g* for 30 sec. The supernatants were stored at 4°C until analysis.

To determine the effect of ionic strength on hydrolysis, samples were treated under the optimum condition determined in section 2.2.2, except the values of ionic strength were adjusted to 0.05, 0.1, 0.2, 0.3, or 0.4 M. C1 and C2 of 2 FPU were added to 1.5 mL microcentrifuge tubes containing 1 mL phosphate buffer (pH 5.0) of varying ionic strength in the range of 0.05 - 0.4 M. All samples were pre-incubated at 50°C for 3-5 min. The hydrolysis conditions were

maintained at 50°C and pH 5.0. The initiation and termination of hydrolysis were conducted in the same manner as described in section 2.2.1.5.

To test the effects of pH, the immobilized enzymes and negative controls were prepared under the optimum conditions determined in section 2.2.2,with the pH values adjusted to 4.5, 5.0, 6.0, 7.0, or 8.0, the range which was determined by the previous experiment (data not shown). C1 and C2 were adjusted to 2 FPU/mL in 1.5 mL microcentrifuge tubes containing 1 mL of 0.1 M phosphate buffer, the pH of which was also altered in the range of 4.5 to 8.0. Reaction mixtures were incubated at 50°C for 24 h. Initiation and termination of the hydrolysis were conducted in the same manner as that used to assess the effects of temperature.

To examine the effect of storage on enzyme activity, numbers of individual immobilized enzymes, negative controls (buffer and silica), and positive controls (buffer and enzyme) were prepared at the same time under the optimum immobilization conditions determined in the section 2.2.2. and stored at 4°C. A sample was removed every week for 3 weeks and applied to a hydrolysis reaction at 50°C for 24 h in 0.1 M phosphate buffer (pH=5.0). Initiation and termination of the hydrolysis were conducted in the same manner as that used to assess the effects of temperature.

To determine if immobilized enzymes could be recycled, immobilized enzymes and negative controls were adjusted to the optimum conditions, and

hydrolysis was conducted in 0.1 M phosphate buffer (pH 5.0) at 50°C. One hydrolysis cycle was set for 24 h. All samples were removed from the water bath after every cycle and centrifuged at 8087 × *g* for 30 sec to separate supernatants from solid fractions. The supernatants were placed in boiling water for 5 min and stored at 4°C. The solids were washed three times with 0.1 M phosphate buffer (pH = 5.0) to exclude remaining cellulose and reaction products. Then, 1 mL fresh buffer and 35 mg microcrystalline cellulose were added to the tubes for the next cycle. This process was repeated 9 times until enzyme activity became very low. The recycle ability of four immobilized cellulases were compared each other.

2.2.7. The Effects of Substrates

For the hydrolysis reaction, prepared immobilized cellulases (C1S1, C1S2, C2S1, and C2S2) under optimal conditions were tested using various substrates: microcrystalline cellulose, crystalline cellulose II, cellophane paper, PASC, CMC, xylan, poplar, and waste paper. The free cellulase solutions (C1 and C2) were prepared in 0.1 M phosphate buffer (pH=5.0). All samples were pre-incubated at 50°C in a water bath for 3-5 min, and the hydrolysis reaction was initiated by adding 35 mg (dry weight) substrate. The hydrolysis reactions were terminated after a 24 h reaction by placing the samples in a boiling water bath for 5 min followed by centrifugation at 8087 × *g* for 30 sec. The respective supernatant and

solid fractions were stored at 4°C until further analysis. All reactions were performed in triplicate.

2.2.8. Methods of Analysis

2.2.8.1. Filter Paper Unit Assay

Filter paper unit (FPU) assay (IUPAC; Decker *et al.,* 2003; Ghose, 1987) was done to determine total enzyme activity of the two types of cellulase cocktails. Both enzyme solutions were diluted 3-4 fold using 0.1 M phosphate buffer (pH 5.0), and 100 µL of the diluted enzymes were added to 1.5 mL microcentrifuge tubes containing 200 mL of 0.1 M phosphate buffer and 1 cm x 1.2 cm Whatman No.1 filter paper strips. The mixtures were incubated for 60 min at 50°C. Six hundred µL of dinitrosalycilic acid (DNS) dye was added to each tube. The tubes were placed in a boiling water bath for 5 min; 400 µL distilled water was added, and the tubes were vortexed briefly to mix the reaction mixture. After fiber debris had settled at the bottom, 200 µL of supernatant from each tube was transferred into a 96-well plate and the absorbance was measured at 540 nm using a microplate reader (Synergy MX, BioTek Instrument Inc., VT, USA) with Gen 5^{th} 1.09 software. The FPU was calculated following the standard protocol (IUPAC; Decker *et al.,* 2003; Ghose, 1987).

2.2.8.2. Sugar Concentration

The total reducing sugar concentration in the sample supernatant was measured by the Dinitrosalicylic acid method (DNS; Miller, 1959). Briefly, 10 µL of each sample supernatant was individually added into 1.5 mL microcentrifuge tubes containing 60 µL DNS dye and 20 µL distilled water. The tubes were placed in a boiling water bath for 5 min; 400 µL of distilled water was added, and the tubes were vortexed briefly. Supernatant (200 µL) from each tube was transferred to a 96-well plate and the absorbance was measured at 540 nm. The concentration of sugar in the samples was expressed as hydrolysis yield (Zhu *et al*., 2008) as defined in Equation 1.

Equation 1. Hydrolysis yield

Hydrolysis yield (%) = sugar production / theoretical sugar production

= $(G_f V_f - G_i V_i)$ / (B × glucan content × 0.90 +B × xylan content × 0.88)

Glucan conversion factor

= MW of glucose (in cellulose) / MW of glucose

= 162/180 = 0.90

Xylan conversion factor

= MW of xylose (in xylan) / MW of xylose

= 132/150 = 0.88

where G_i is the initial concentration of sugar; V_i is the initial volume of enzyme mixture; G_f and V_f are the sugar concentration in the hydrolysate and its volume, respectively; B is the dry weight of biomass and MW is molecular weight. The value of 0.90 is a conversion factor of glucose to equivalent glucan, and that of 0.8 is the factor of xylose to the equivalent xylan. The glucan content in microcrystalline cellulose, crystalline cellulose II, cellophane paper, Phosphoric acid swollen cellulose (PASC) is 1; the glucan and xylan contents of poplar wood were 0.48 and 0.25 (Mascoma Canada Inc., 2011); and those of waste shredded paper were 0.80 and 0.10 (Sosulski, 1993).

Hydrolysis yields were also converted to retained enzyme activities in order to compare results with those of other studies (Equation 2).

Equation 2. Retained enzyme activity

Retained enzyme activity (%)

= sugar production of immobilized enzyme

/sugar production of the corresponding free enzyme × 100

Individual sugars were measured by high performance liquid chromatography (HPLC; 1200 series, Agilent Technologies, CA, USA) controlled by Chem station for LC system Rev. 04.01, SP1 (Agilent Technologies) using a refractive index (RI) detector (G1362A, Agilent Technologies). Two columns

were used: Aminex HPX-87H column (Bio-Rad Laboratories Inc.) and an RCX-30 anion exchange column (Hamilton Company, USA). For the former column, sulfuric acid-water mixture (0.005M) was applied as the mobile phase at 0.6 mL/min at 60°C; for the latter column, sodium hydroxide (0.2 M) was applied as the mobile phase at 2.0 mL/min at 35°C. Also, external standards were used to calculate sugar concentration. The concentrations of individual sugars measured by HPLC were converted to carbon concentration using Equation 3 and 4.

Equation 3. The calculation of carbon mass from cellobiose

Cellobiose (µmol/mL) x 12 mol carbon/ substrate (mg)
= Carbon (µmol/mg)

Equation 4. The calculation of carbon mass from glucose

Glucose (µmol/mL) x 6 mol carbon /substrate (mg)
= Carbon (µmol/mg)

Carbon mass in total sugar, glucose, and cellobiose was further converted to corresponding sugar production rates in order to quantify the difference in sugar production using Equation 5.

Equation 5. Production rates

Production rates (mmol/mL h)
= (carbon mass at time point x – carbon mass at time point y)/ (y-x)

Also, specific enzymatic activity was based on the International Unit (IU; Ghose, 1987; Equation 6).

Equation 6. Specific enzyme activity

1 IU = 1 μmol min^{-1} of glucose formed during the hydrolysis reaction

2.2.8.3. Total Protein Concentration

Total protein concentration was measured by the Bradford method (Bradford, 1976) using a protein assay kit (Bio-Rad Laboratories Inc.). Known amounts of the actual cellulases (C1 and C2) were used as standards since Coomassie Brilliant Blue did not react with the enzyme solutions with the same intensity as that of bovine serum albumin (BSA). The amount of protein was measured using a microplate reader and calculated using a standard curve. The amount of unbound or dissociated protein after immobilization and washing was converted to enzyme loading efficiency in order to evaluate the immobilization efficiency (Equation 7).

Equation 7. Loading efficiency

$$\text{Loading efficiency (\%)} = \{C_iV_i - (CsVs + C_{w1}V_{w1} + C_{w2}V_{w2} + C_{w3}V_{w3})\} / C_iV_i \times 100$$

where, C_i is the initial protein concentration; V_i is the initial volume of the enzyme solution (1 mL); V_s, V_{w1}, V_{w2}, V_{w3} are volume of the washing solution in supernatant, 1st wash, 2nd wash and 3rd wash of each sample; and C_s, C_{w1}, C_{w2}, C_{w3} are protein concentration in supernatant, 1st wash, 2nd wash and 3rd wash of each sample .

The amount of protein in hydrolysates was measured to detect the presence of protein leaks in the hydrolysis reaction (Equation 8).

Equation 8. Amount of dissociated protein

Protein (%)

= Protein amount of post-reaction supernatant / total protein amount initially added x 100

2.2.8.4. Scanning Electron Microscopy (SEM)

Solid fractions of each hydrolysate were mounted on the surface of an aluminum stub using double sided tape, air-dried, and sputter-coated with gold for 165 sec at 15 mA (Hummer 6.2 sputter coater; Anatech Ltd.). The samples were viewed at 20 kV using a scanning electron microscope (Philips XL30, EFI company, CA, USA) equipped with Scadium software (FEI Electron optics, EFI company, CA, USA).

2.2.8.5. Statistical Analysis

All experiments were performed in triplicate. Two-way ANOVA was conducted and Tukey's test was used for multiple comparisons. All statistical analysis was done using TIBCO spotfire S+ 8.2 software for Windows (TIBCO Spotfire, MA, USA) and SAS 9.3 (SAS Institute, NC, USA).

3. Results

3.1. Determination of Immobilization Methods and Supports

3.1.1. Cellulase Immobilization on Polyvinyl Alcohol Modified Chitosan Beads

Several immobilization methods and enzyme supports were tested to discover the combination resulting in the highest enzyme activity of immobilized cellulases. Dinçer and Telefoncu (2007) reported that immobilization of cellulase enzymes (*Aspergillus niger*) on chitosan beads was found to result in highly retained enzymatic activity. Based upon this understanding, chitosan beads were chosen for the support in initial experimental trials. A commercially prepared cellulase cocktail was immobilized on polyvinyl alcohol-modified chitosan beads, and tested for its ability to hydrolyze microcrystalline cellulose hydrolysis as reported in Table 3.1. Total sugar production from cellulases immobilized on chitosan beads was significantly lower than production by free enzyme systems ($p<0.05$).Even though the value was significantly lower, the retained hydrolysis yield was over 40%, which was relatively higher. Thus, this method was selected for use in further experiments.

Table 3.1. Sugar production and retained enzyme activity of immobilized cellulases on chitosan beads. Hydrolysis reactions were conducted at 50°C for 24 h in 0.1 M phosphate buffer (pH=5.0). n = 12, Mean ± Standard Error, a, b: $p < 0.05$.

	Immobilized cellulases on chitosan beads	Free cellulases
Sugar production (mg/mL)	2.3 (± 0.36)$_a$	7.5 (± 0.85)$_b$
Retained enzyme activity (%)	30	100

3.1.2. Covalent Immobilization of Cellulase on Amberlite with Glutaraldehyde

A commonly used method for immobilization is covalent bonding of enzymes to a rigid support. To compare the efficiency of this type of immobilization, cellulase cocktails were cross-linked with glutaraldehyde to amberlite residues and used for the hydrolysis of microcrystalline cellulose as shown in Table 3.2. The activity of the immobilized cellulases were significantly lower than that of the free cellulases. The retained enzyme activity of the immobilized enzymes compared to the free cellulases was ~40% which was higher than with the chitosan beads method. However, the observed sugar production (2.2 mg/mL) from the free cellulases was much lower than the previous experiment (7.5 mg/mL, Section 3.1.1.) with unknown reasons, demonstrating a degree of non-reproducibility.

Table 3.2. Sugar production and retained enzyme activity of immobilized cellulases on glutaraldehyde coated amberlite and free cellulases. Hydrolysis reactions were conducted at 50°C for 24 h in 0.1 M phosphate buffer (pH=5.0). n = 3, Mean ± Standard Error, a, b: $p < 0.05$.

	Immobilized cellulases on glutaraldehyde coated amberlite	Free cellulases	Free cellulases (chitosan experiment)
Sugar production (mg/mL)	0.9 $_a$	2.2 (± 0.16)$_b$	7.5 (± 0.85)
Retained enzyme activity (%)	40	100	

3.1.3 Fumed Silica, Calcium Chloride Coated Fumed Silica, and Calcium Chloride-Aluminum Chloride Coated Silica

Sinegani et al. (2005) found that sorption of cellulases are improved on calcium chloride coated silica and dramatically increased on calcium and aluminum chloride coated silica. The hypothesis here is that the higher amounts of enzymes are adsorbed on silica using the method, the higher enzyme activity will be obtained. Thus, physical adsorption and ionic interaction methods were tested. Cellulases were immobilized on fumed silica (S1, physical adsorption), calcium chloride coated S1 (ionic interaction), and calcium chloride and aluminum coated S1 (ionic interaction). Once immobilized, hydrolysis of microcrystalline cellulose was performed.

All immobilized cellulases showed significantly lower values compared to that for free cellulases ($p<0.05$) (Table 3.3.). Sugar production for the immobilized cellulase on S1 and that on calcium chloride coated S1 were not significantly different from each other, and they were higher than that for chloride and aluminum coated S1. Based on these results and the overall simplicity of the method, the physical adsorption method was selected for use in further experiments. The results obtained using S1 were found to be sufficiently good and the method was selected for use in further experiments.

Table 3.3. Sugar production and retained enzyme activity of immobilized cellulases on Fumed silica, calcium chloride coated silica, and calcium and aluminum coated silica. Hydrolysis reactions were conducted at 50°C for 24 h in 0.1 M phosphate buffer (pH=5.0). n = 3, Mean ± Standard Error, a, b, c: $p < 0.05$.

	Immobilized cellulases			Free cellulases
	Fumed silica	Calcium chloride coated silica	Calcium and aluminum chloride coated silica	
Sugar production (mg/mL)	2.4 (± 0.22)$_b$	2.1 (± 0.36)$_b$	0.6 (± 0.22)$_c$	5.3 (± 0.73)$_a$
Retained enzyme activity (%)	44	40	11	100

3.1.4. Amberlite, Calcium Chloride Coated Amberlite, and Calcium Chloride – Aluminum Chloride Coated Amberlite

Based on the successful results using S1, the same procedures described in Section 3.1.3. were applied to amberlite, which is an ion exchange resin, to see if stronger ionic binding improves the enzyme adsorption and increases enzyme activity. The results can be seen in the Table 3.4. below.

Table 3.4. Sugar production and retained enzyme activity of immobilized cellulases on amberlite, calcium chloride coated amberlite, and calcium and aluminum coated amberlite. Hydrolysis reactions were conducted at 50°C for 24 h in 0.1 M phosphate buffer (pH=5.0). n = 3, Mean ± Standard Error, a, b, c: $p < 0.05$.

	Immobilized cellulases			Free cellulases
	Amberlite	Calcium chloride coated amberlite	Calcium and aluminum chloride coated amberlite	
Sugar production (mg/mL)	0.7 (± 0.06)$_b$	0.7 (± 0.07)$_b$	0.3 (± 0.08)$_c$	7.5 (± 0.11)$_a$
Retained enzyme activity (%)	9	9	3	100

The sugar production of all immobilized cellulsases was significantly lower than that of the free cellulases. To find out if proteins were adsorbed onto the resins, the protein content of the immobilization supernatants and wash solutions were determined. The immobilized cellulases were washed three times with 0.1 M phosphate buffer (pH=5.0), and 20.8 mg of dissolved protein was detected. This experiment showed that most protein did not attach to the resins,

which appear to be an unsuitable support for cellulase immobilization because they are unable to sufficiently bind the enzymes. Due to the low enzyme activity, strong ionic binding with the supports chosen might not be suitable for cellulase immobilization, and it was not used further.

3.1.5. ETS-2, ETS-4, CHABAZITE

Because the adsorption on S1 showed a relatively high-retained enzyme activity (Section 3.1.3), different types of silica were evaluated under the same conditions. It was hypothesized that the larger surface would adsorb a larger amount of enzymes resulting in the higher enzyme activity. As shown in Figure 3.1, unexpectedly, none of the silica supports resulted in sugar production. Figure 3.2 shows the protein amounts obtained from the supernatant and wash solutions of the immobilized cellulases on ETS-2, ETS-4, and chabazite. Most cellulases did not attach to the ETS-2 or chabazite supports, whereas protein was not detected in either the supernatant or wash solutions of ETS-4. ETS-2, ETS-4, and chabazite retain a positive charge. This positive charge may have been responsible for repulsing the cellulases, preventing immobilization. Also, the positive charge on ETS-4 might react with the catalytic site of the enzymes and disrupt the protein conformation. Thus, again, ionic interaction seems to be unsuitable for cellulase immobilization and ETS-2, ETS-4, and chabazite were not used in further experiments.

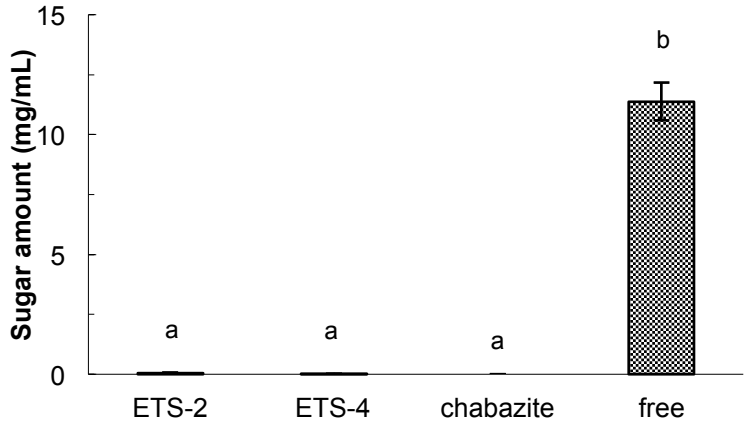


Figure 3.1. Sugar production for immobilized cellulases on ETS-2, ETS-4, and chabazite, compared to free cellulases.
Hydrolysis reactions were conducted at 50°C for 24 h in 0.1 M phosphate buffer (pH=5.0). n = 3, Mean ± Standard Error, a, b: $p < 0.05$.

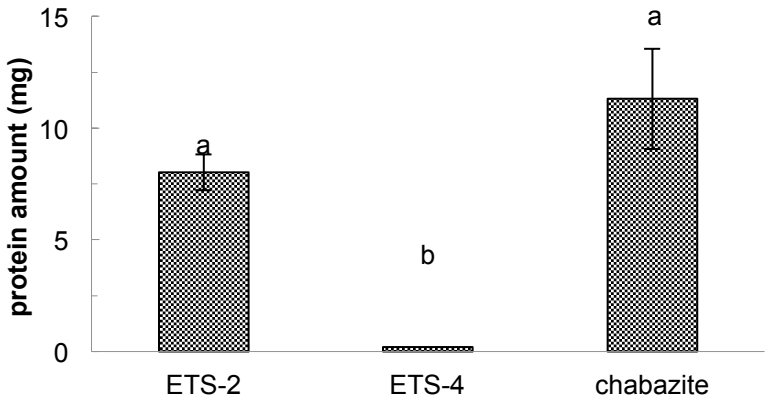


Figure 3.2. Total protein amount obtained from the supernatant and wash solutions of the immobilized cellulases on ETS-2, ETS-4, and chabazite.
Immobilization was conducted at 4°C for 2.5 h, and the immobilized cellulases were washed with 0.1 M phosphate buffer (pH=5.0). n = 3, Mean ± Standard Errors, a, b: $p < 0.05$.

3.1.6. Porous Silica (Silica 2)

Based on the successful results in Section 3.1.3 (adsorption on S1 demonstrating relatively high retained enzyme activity), porous silica (S2) was also evaluated under the same conditions. The immobilized cellulases on S2 were compared to both S1 and the free cellulases in 1 mL systems as shown in Figure 3.3. S2 retained similar enzyme activity as S1, meaning that S2 appears to be a suitable support for cellulase immobilization with performance on par with S1. The results obtained using S2 were found to be promising and the method was selected for use in further experiments.

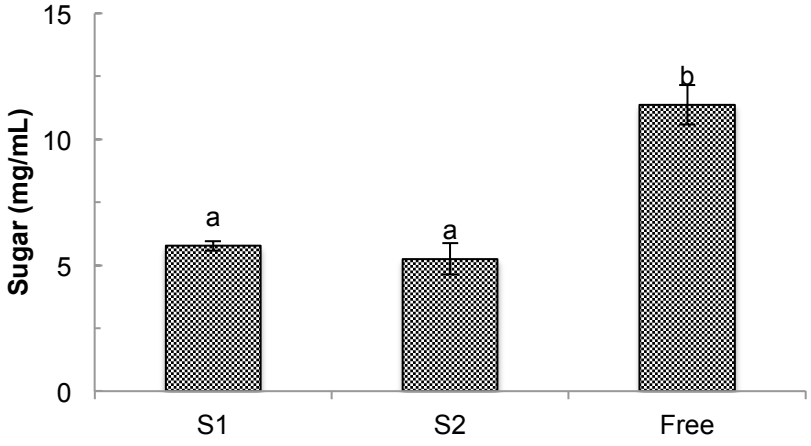


Figure 3.3. Sugar production for immobilized cellulases on S1 and S2, and free cellulases.
Hydrolysis reactions were conducted at 50°C for 24 h in 0.1 M phosphate buffer (pH=5.0). n = 3, Mean ± Standard Errors, a, b: $p < 0.05$.

3.2. Determination of Reaction Scale and Thermal Stability of the Immobilized Cellulases

Based on results from the previous experiments, two immobilization methods were selected for further study: adsorption of cellulases on silica (S1 or S2) and adsorption on polyvinyl modified chitosan beads. The scale of the reactions in previous experiments was relatively small: 1 mL to 10 mL, most often using 1.5 mL microcentrifuge tubes to conveniently deal with the large number of samples. In this section, experiments were scaled up to 250 mL Erlenmeyer flask and 5 L bioreactor volumes. One of the commonly known advantages for immobilization is heat resistance. Therefore, thermal stabilities for two types of the immobilized cellulases were examined: immobilized cellulases (C1) on S1 as a representative of silica support and immobilized cellulases (C1) on polyvinyl modified chitosan beads.

3.2.1. Thermal Stability of the Immobilized Cellulases on S1 and the Polyvinyl Modified Chitosan Beads in 250 mL Flasks

The experiments were scaled up to 250 mL flask volumes. Table 3.5 shows the results of thermo-stability testing of the immobilized cellulases on S1, those on chitosan beads, and free cellulases. For free cellulases, the highest sugar production was obtained at 50°C with significantly lower sugar production at

other temperatures. Sugar production for immobilized cellulases on S1 were not significant different across the temperature range. Immobilized cellulases on chitosan beads produced the most sugar at a higher temperature than all other supports, although the values between 37 to 65°C were not statistically different.

Table 3.5. Sugar production of free cellulases and immobilized cellulases on S1 and chitosan beads in 250 mL flask. n = 2, 3, Mean ± Standard Errors, Means within the same column with different subscripts are not sign different (p>0.05).

	Sugar production (mg/mL)		
Temperature (°C)/ Test matrix	Immobilized cellulases S1	Chitosan	Free cellulases
20	0.5 (±0.10) $_{a}$	0.3 (±0.08) $_{A}$	0.7 (±0.03) $_{p}$
37	0.8 (±0.08) $_{a}$	2.0 (±0.33) $_{B}$	1.9 (±0.17) $_{q}$
50	1.2 (±0.10) $_{a}$	2.6 (±0.49) $_{B}$	3.0 (±0.73) $_{r}$
65	0.6 (±0.31) $_{a}$	3.0 $_{B}$	1.8 $_{pq}$

The enzyme reactions at lower temperatures, such as 20°C and at 37°C, were conducted without much variation. In contrast, the standard errors at the high temperatures were large, even though the sugar values were high. Indeed, some reactions had difficulty proceeding, specifically at 65°C. To sum up, temperature control for successful incubation was difficult at the 250 mL flask scale. Due to the difficulty of temperature control, the 250 mL flask scale was not used for further experiments.

3.2.2. Thermal Stability of Immobilized Cellulases on S1 and Polyvinyl Modified Chitosan Beads in 5 L Bioreactor

To seek better temperature control, enzyme reactions were scaled up to 5 L bioreactors with temperature control systems. Because enzyme activity stopped at temperatures above 63°C in previous experiments at the 5 L scale (data not shown), testing was conducted at temperatures of 40, 50, 60 and 63°C. Enzyme activity was shown by the specific enzyme activity. The results are shown in Figure3.4. The maximum activity was obtained at 60°C for both the free cellulases and the immobilized cellulases on S1. Interestingly, the free cellulases lost enzymatic activity at 63°C; however, the immobilized cellulases retained 46% of enzymatic activity compared to that at 50°C. This means that the immobilized cellulases gained thermal stability.

Only advantage using 5 L scale was to improve temperature control. However, it was inconvenient for handling a large number of samples at once because of their large size. The inconvenience outweighed the advantage, Thus, all further experiments were carried out using 1.7 mL microcentrifuge tubes.

The polyvinyl modified chitosan beads were also utilized for the enzyme reaction in a 5 L bioreactor. However, they were physically destroyed by the mechanical mixing (data not shown). This method is therefore not suitable for

industrial practice, which would require better physical strength of the supports for stable production.

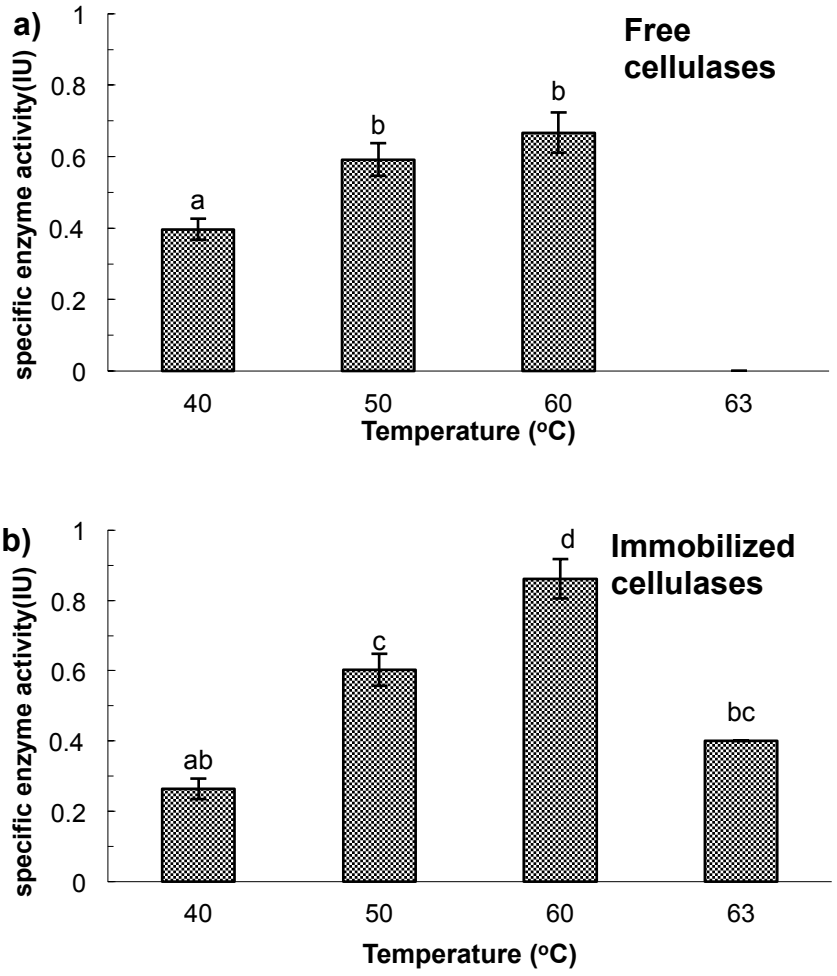


Figure. 3.4. Specific enzyme activities at 40, 50, 60, 63°C for a) the free cellulases and b) the immobilized cellulases on S1.
Hydrolysis reactions were conducted for 5 min in 0.1 M phosphate buffer (pH=5.0). n = 3, Mean ± Standard Error, a, b, c, d: $p < 0.05$.

3.3. Effect of Immobilization Conditions on Loading Efficiency

Based on the successful results from the previous experiments in section 3.1.3 and 3.1.6, two types of cellulases, C1 and C2, were immobilized on two types of supports, S1 and S2, creating four immobilized systems in 1.7 mL microcentrifuge tubes: C1S1, C1S2, C2S1, and C2S2. The characteristics of these systems are the focus of the following sections, beginning with the effects of immobilization conditions on loading efficiency, which will be examined here.

The environment of the enzymes and supports substantially affects immobilization (Cao, 2005). Thus, examining immobilization conditions, such as pH and ionic strength, on enzyme loading efficiency is important. The enzyme to support ratio is also critical to achieve a high loading efficiency. In this experiment, pH, ionic strength, and enzyme-support ratios were varied to investigate how they affect the enzyme loading efficiency. The most efficient values were chosen for further experiments to maximize loading efficiency of the four systems.

3.3.1. Effect of pH on Loading Efficiency

The effects of pH on loading efficiency were examined. A pH range of 4.5 - 7.0 was chosen because the cellulases did not effectively associate with the silica support outside of this range in previous experiments (data not shown). The

loading efficiencies obtained at these different pH values are shown in Figure 3.5. Because pH 5.0 demonstrated high loading efficiency and was also, conveniently, the same pH as the hydrolysis condition, pH 5.0 was selected for C1S1 and C1S2, and C2S1. For C2S2, the highest loading efficiency of C2S2 was observed at pH 6.0, which was significantly higher than any other treatment.

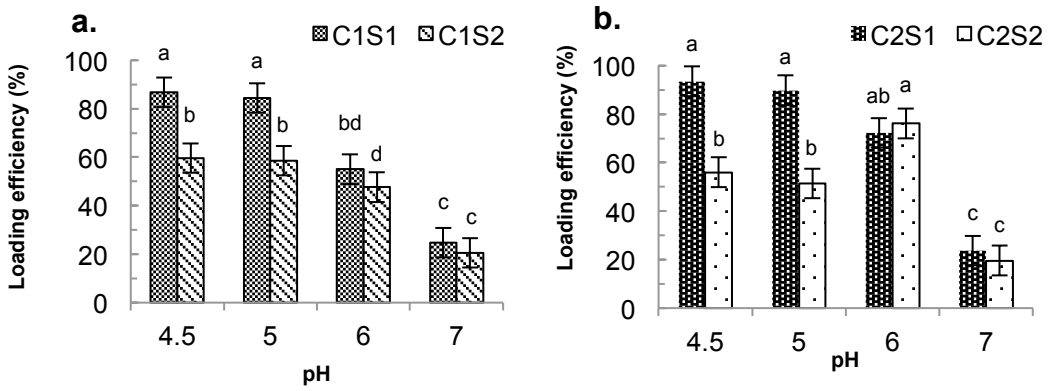


Figure. 3.5. Loading efficiency as affected by various pH conditions for four systems of immobilized cellulases.
(a) C1S1, C1S2, (b) C2S2, C2S2. The loading efficiency was indirectly calculated from the dissolved protein concentrations in the supernatant, wash 1, wash 2, and wash 3 after the heat treatment. n = 3, Mean ± standard error, a, b, c, d: $p < 0.05$.

3.3.2. Effect of Ionic Strength on Loading Efficiency

The effects of ionic strength on loading efficiency were examined in the four systems. Ionic strengths ranging from 0.1 - 0.4 M of phosphate buffer were chosen based on results from previous experiments (data not shown). The loading efficiencies obtained at different ionic strengths are shown in Figure 3.6. The maximum loading efficiencies were observed at 0.3 M for C1S1 and C1S2,

although C1S1 was not significantly different from any other values. The values at 0.2 M for C2S1 and C2S2 gained the highest although C2S2 was not statistically different from 0.3 and 0.4 M. Thus, for C2S1 and C2S2, ionic strength of 0.2 M was selected for further experiments.

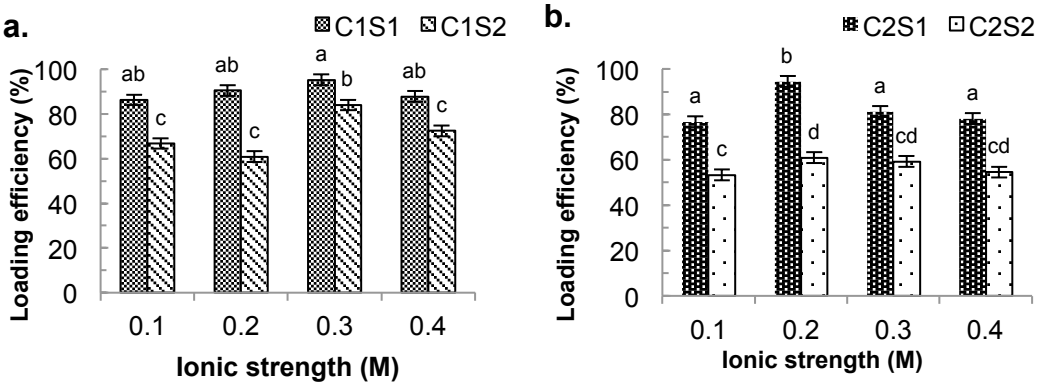


Fig. 3.6. The loading efficiency as affected by ionic strength conditions for four systems of immobilized cellulases: a) C1S1, C1S2, b) C2S1, and C2S2. The loading efficiency was indirectly calculated from the dissolved protein concentrations in the supernatant, wash 1, wash 2, and wash 3 after the heat treatment. n = 3, Mean ± standard error, a, b, c, d: $p < 0.05$.

3.3.3. Effect of Enzyme-Support Ratios on Loading Efficiency

Finally, the effects of enzyme-support ratios on loading efficiency were examined to maximize the loading efficiency. Silica support amounts ranging from 10 – 40 mg (5-8 mg/FPU) for S1 silica and 40 – 160 mg (20-80 mg/FPU) for S2 silica were tested based on results from previous experiments (data not

shown). The loading efficiencies obtained using different support amounts for S1 are shown in Figure 3.7.a.

The enzyme support of 30 mg for C1S1 and C2S1 showed sufficiently high loading efficiency. 30 mg was standardized for the further experiments. Further, the loading efficiencies obtained using different support amounts for S2 are shown in Figure 3.7.b.

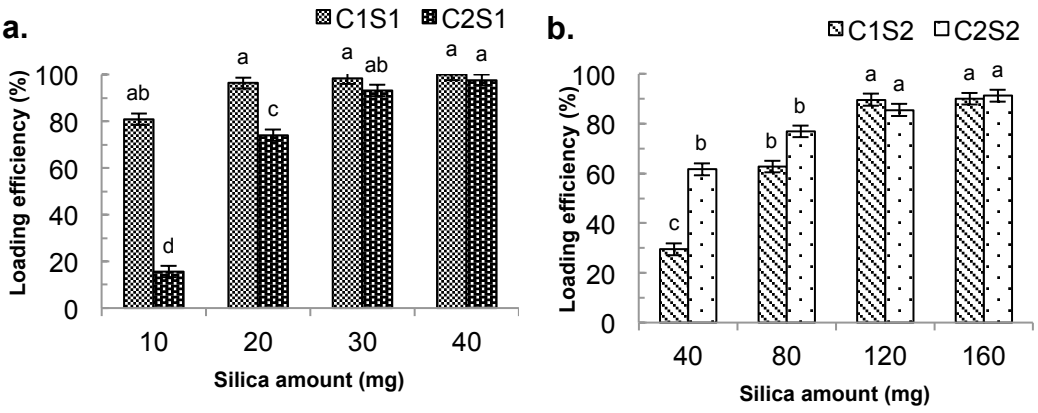


Fig.3.7. The loading efficiency as affected by various enzyme-support ratios for four sytems of immobilized cellulases.
a) C1S1, C2S1, b) C1S2, and C2S2. The loading efficiency was indirectly calculated from the dissolved protein concentrations in the supernatant, wash 1, wash 2, and wash 3 after the heat treatment. n = 3, Mean ± standard error, a, b, c, d: $p < 0.05$.

For C1S2 and C2S2, the highest loading efficiencies were at 160 mg, which were not statistically different from that at 120 mg for C1S2 or at 80 mg and 120 mg for C1S2 ($p>0.05$). For C1S2 and C2S2, of 120 mg of enzyme

support was selected for further experiments, to maximize cost performance. Based on the observation in Section 3.3.1-3.3.3, the parameters outlined in Table 3.6. were selected for the further experiments.

Table 3.6. The immobilization conditions used in further experimentation.

The immobilized cellulases	pH	Ionic strength (M)	Support amount (mg)
C1S1	5.0	0.3	30
C1S2	5.0	0.3	120
C2S1	5.0	0.2	30
C2S2	6.0	0.2	120

3.4. Enzyme Activity

3.4.1. Retained Enzyme Activity

After determining the optimum immobilization conditions, the hydrolysis performance of the four systems was examined. The act of immobilization often lowers catalytic activity. Thus, examining catalytic activity to determine whether immobilized cellulases retain hydrolytic activity is critical. Catalytic activity is expressed by hydrolysis yield in this experiment because higher amounts of final sugar production are desirable for subsequent bioconversions in order to produce value-added products in a biorefining processes. Hydrolysis yields for immobilized and free cellulases are shown in Figure 3.8. The hydrolysis

conditions were set at 50°C in 0.1 M phosphate buffer (pH 5.0), hydrolyzing microcrystalline cellulose (35 mg). The four systems of the immobilized cellulases (C1S1, C1S2, C2S1, C2S2) were compared to their corresponding free cellulases (C1 and C2). C1S1 and C2S1 retained enzyme activity equivalent to the free cellulose systems. This result shows that immobilized cellulases on silica supports were able to retain high activity on microcrystalline cellulose. This is a novel result in this dissertation. A similar trend to that shown in Figure 3.8 was observed at higher enzyme loadings of 1.0 and 2.0 Filter paper unit (FPU).

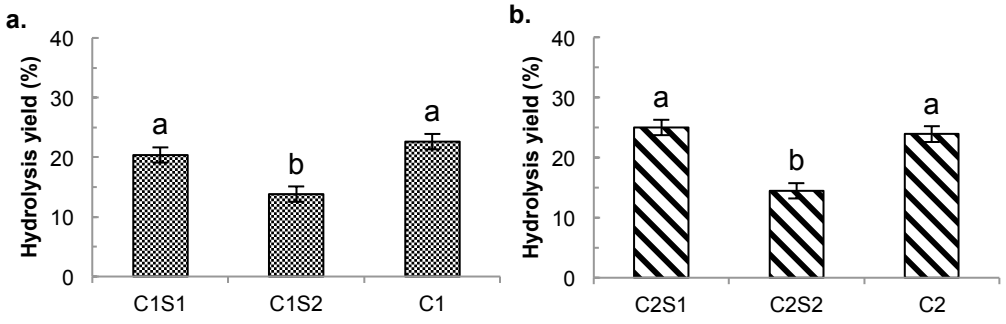


Fig. 3.8. Comparison of hydrolysis yields for four systems of immobilized cellulases (C1S1, C1S2, C2S2, and C2S2) and their corresponding free forms (C1 and C2) with the enzyme strength of 0.5 FPU. a) C1S1, C1S2, and C1, b) C2S1, C2S2, and C2. Hydrolysis reaction was conducted for 24 h in 0.1 M phosphate buffer (pH=5.0) at 50°C using a water bath. n = 3, Mean ± standard error, a, b: $p < 0.05$.

3.4.2. Effect of Duration of Hydrolysis

To gain further insight into the mechanism of hydrolysis, hydrolysate samples, which were made from hydrolysis of microcrystalline cellulose (35 mg/mL), were taken at various time points and individual sugars were profiled and expressed in terms of carbon mass (refer to Figure 3.9.a-c for C1S1, C1S2 and C1 and Figure 3.9.d-f for C2S1, C2S2, and C2). Only two types of individual sugars were detected, which included glucose and cellobiose. At most time points, total carbon mass values of C1S1 and C1 were not significantly different ($p>0.05$). In terms of glucose production, carbon mass for the immobilized cellulases on S1 was not significantly different ($p>0.05$) from C1 at most time points, while carbon mass for the immobilized cellulases on S2 was statistically lower ($p<0.05$) than C1 (Figure 3.9.b). Interesting differences were observed in the case of cellobiose production: cellobiose accumulated slightly in the hydrolysate without conversion to glucose. It is possible that immobilization might impact cellobiose conversion. In summation, the individual sugar profiles, specifically the cellobiose profile, showed a difference between immobilized cellulases on S1 and S2.

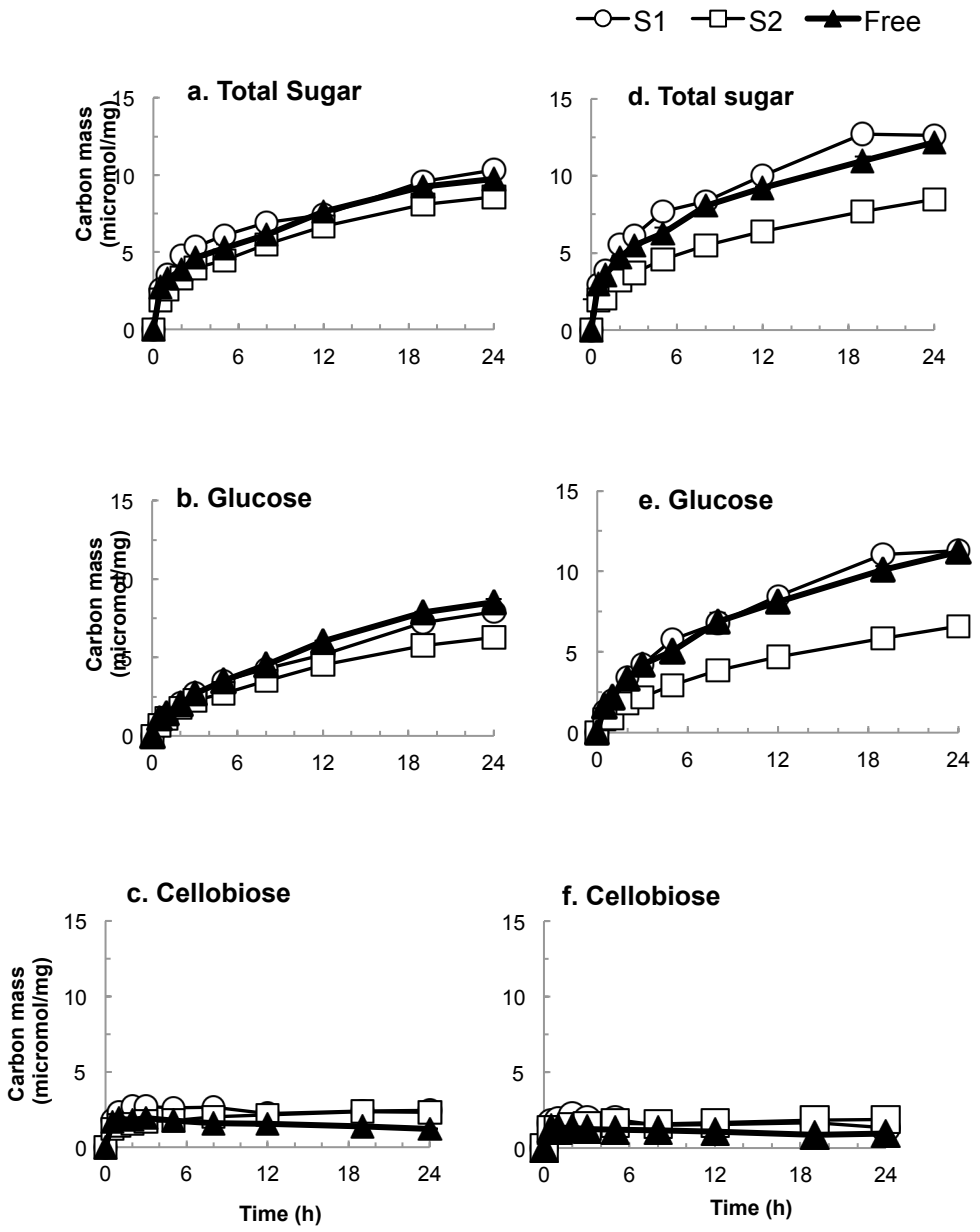


Figure 3.9. Individual sugar profiles of C1S1, C1S2, C2S1, and C2S2 with an enzyme strength of 2 FPU.
(Graphs a, b, and c are for enzymes systems C1S1, C1S2, and C1, while d, e and f, are for enzyme systems C2S1, C2S2, and C2. Open circles: immobilized cellulase on S1; open squares: immobilized cellulase on S2;

closed triangles: free cellulases. Hydrolysis reaction was conducted for 24 h in 0.1 M phosphate buffer (pH=5.0) at 50°C using a water bath. n = 3

3.5. Scanning Electron Microscopy

To visually assess the physical characteristics of the silica support binding the immobilized cellulases and to examine the interaction of cellulose with silica, scanning electron microscopy (SEM) was performed. Figure 3.10 shows solid fractions of the hydrolysates obtained using C1and C2 immobilized on S1 and S2. The interaction of microcrystalline cellulose particles (long rectangular crystal; indicated by black arrow) with S1 (fluffy clusters; indicated by white arrow) was clearly visible (Figure 3.10.c and e). The cellulose particle is fully covered with the three-dimensional matrix of S1. The interaction of microcrystalline cellulose particles (long rectangular crystal; indicated by black arrow) with S2 (large crystal; indicated by white arrow) was clearly visible (Fig 3.10.d and f). Despite the large size of the S2 particles compared to S1, its surface was only partially bound by cellulose.

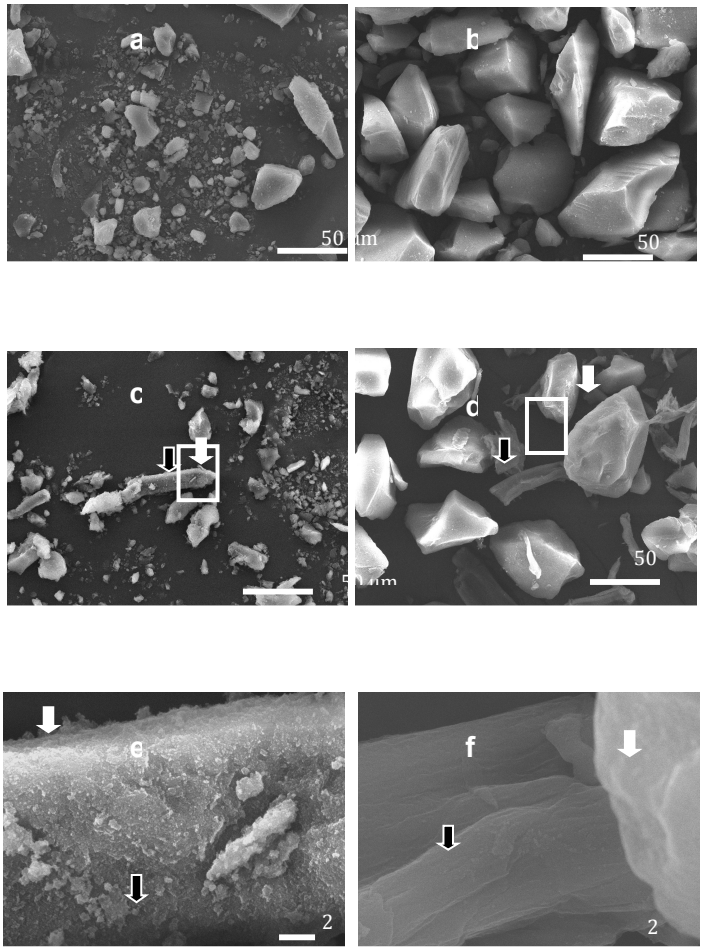


Fig. 3.10. SEM images of hydrolysates for two systems of immobilized cellulases (C2S1 and C2S2) and negative controls (S1 and S2) after a 24 h hydrolysis reaction. Hydrolysis reactions were conducted in 0.1 M phosphate buffer (pH=5.0) at 50°C for 24 h using a water bath. Negative controls refer to the mixture of each support and 0.1 M phosphate buffer (pH 5.0) without cellulases. (a) S1 at 800x magnification (b) S2 at 800 x magnification. (c) Microcrystalline cellulose (indicated by black arrow) bound to S1 (indicated by white arrow) with immobilized C2 at 800x magnification. (d) Microcrystalline cellulose (indicated by black arrow) bound to S2 (indicated by white arrow) with immobilized C2 at 800x magnification. (e) Magnified image of the inset shown in (c) at 20000x. (f.) Magnified image of the inset shown in (d) at 20000x.

3.6. Protein Desorption

To examine the strength of enzyme-support interaction after batch operation of the hydrolysis process, the desorbed protein amounts in the reaction supernatants were measured using the Bradford assay (Figure 3.11). Post-hydrolysis protein losses for C1S1, C1S2, C2S1 and C2S2 were not significantly different ($p>0.05$) compared to that of their respective negative controls (silica without cellulases), meaning that most proteins did not desorb from the support.

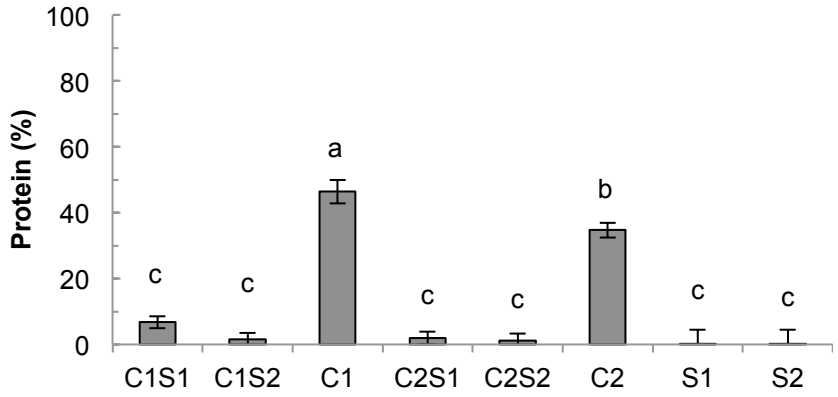


Fig. 3.11. Comparison of desorbed protein percentages in four systems of immobilized cellulases C1S1, C1S2, C2S1, and C2S2.
The dissolved proteins in the supernatant of hydrolysate were measured after 24 h hydrolysis. Negative controls refer to the mixture of each support and 0.1 M phosphate buffer (pH 5.0) without cellulases. Hydrolysis reaction was conducted in 0.1 M phosphate buffer (pH=5.0) at 50°C for 24 h using a water bath. All samples were heat treated before the analysis. n = 3, Mean ± Standard Error, a, b, c: $p<0.05$.

3.7. Influence of Reaction Conditions on Stability Properties and Hydrolysis Products

3.7.1. Effects of Temperature

After the optimization of enzyme immobilization and the examinations of activity, tests were conducted to investigate the effects of various parameters on the hydrolysis of microcrystalline cellulose by four immobilized systems. First, thermal stability was examined because it is an important property in industrial practice to maintain reaction rates. To determine how immobilization affects thermal stability, various incubation temperatures were applied during hydrolysis.

Figure 3.12 shows the retained yields and individual sugar profiles of the C1S1 hydrolysates of C1S1 (Figure 3.12.a), C1S2 (Figure 3.12.b), C1 (Figure 3.12.c), C2S1 (Figure 3.12.d), C2S2 (Figure 3.12.e), and C2 (Figure 3.12.f) at each incubation temperature. The optimum temperature of the immobilized cellulases was 50°C, whereas the optimum temperature of the free cellulases was 60°C. Free cellulases also showed better thermal stability in both C1 and C2. Interestingly, the stability experiments also revealed that the reaction conditions affected the sugar components of the hydrolysate. The sugar composition in hydrolysate is important because some microorganisms can only consume glucose. Also, oligosaccharides can alter microorganisms' metabolic pathways and cause the production of byproducts that could decrease production rates and make the

purification processes more complex. To examine the influence of temperature on individual sugar production, the detailed sugar types and concentrations in the hydrolysates of C1S1, C1S2, and C1 were investigated (Figure 3.12.a-c). Overall, the immobilized cellulases produced higher quantities of cellobiose, which corresponds with the findings in Section 3.4.2. Also, higher temperatures produced less cellobiose, meaning the activity of β-glucosidases might be differentially accelerated at high temperatures.

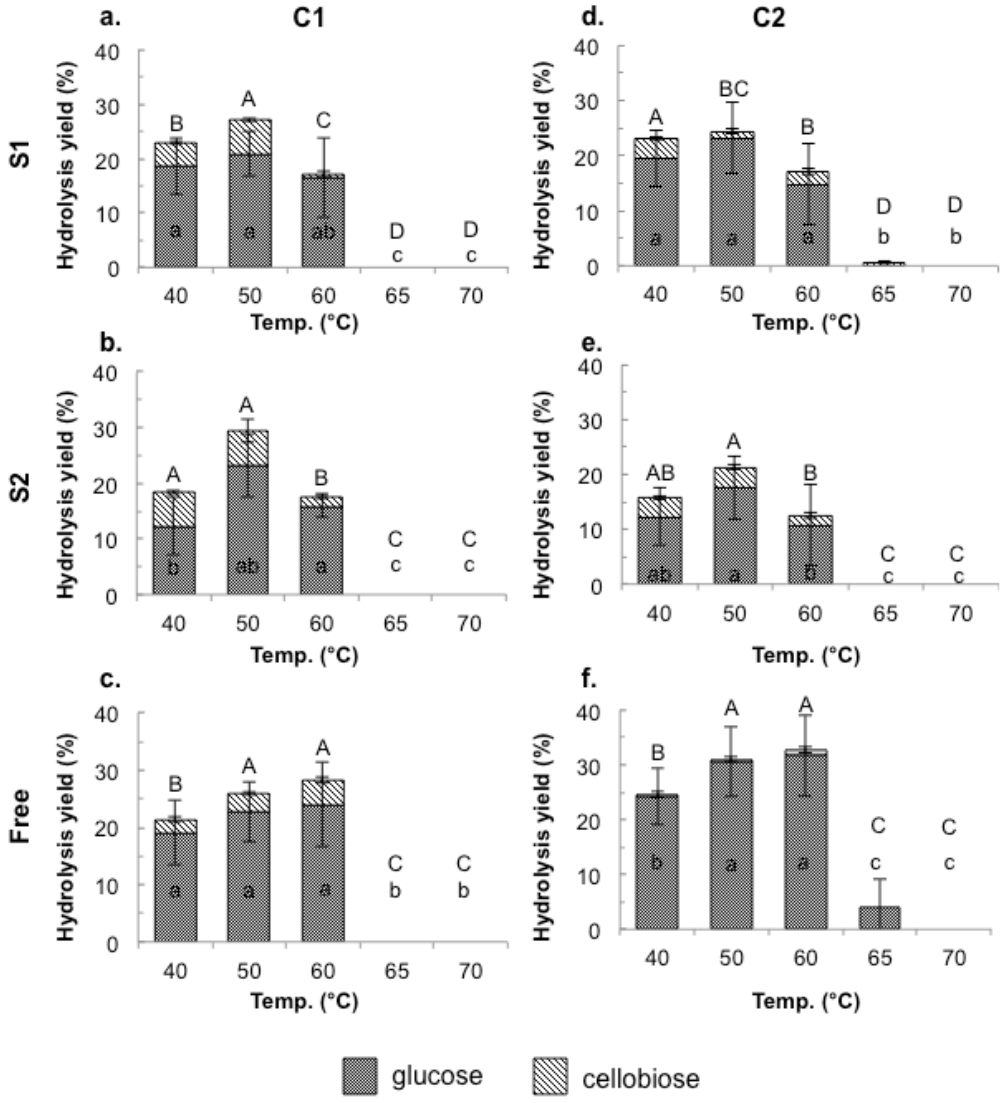


Fig. 3.12. Comparison of hydrolysis yields as affected by various temperatures, hydrolyzing microcrystalline cellulose (35 mg/mL).
(a.) C1S1, (b.) C1S2 and (c.) C1, (d.) C2S1, (e.) C2S2, and (f.) C2. Hydrolysis reactions were conducted in 0.1 M phosphate buffer (pH=5.0) at various temperatures for 24 h using a water bath. n = 3, Mean ± Standard Error, Glucose hydrolysis yield, a, b, c: $p<0.05$, Cellobiose hydrolysis yield, A, B, C, D: $p<0.05$. The result of S1/S2 was compared to that of free cellulases.

3.7.2. Effects of pH

To investigate the pH stability and the effect of pH on sugar composition, the pH conditions were varied to 4.5, 5.0, 6.0, 7.0, and 8.0. Below pH 4.5, immobilization was not successful (data not shown). Figure 3.13 shows the hydrolysis yields and individual sugar profiles in the hydrolysates of C1S1, C1S2, and C1 (Figure 3.13.a-c) and C2S1, C2S2, and C2 (Figure 3.13.d-f) at various pH conditions. For both immobilized cellulases and free cellulases, the optimum pH was between 4.5-5.0. Although immobilization did not affect the optimal pH, enzyme activity was observed at pH 8.0 in C1S1 (Figure 3.13.a), demonstrating that immobilization may enhance pH stability. For the C2 system, pH 8.0 resulted in no sugar production from either the immobilized or the free C2 enzymes.

To investigate the influence of pH on sugar composition, individual sugar yields were measured. In the case of C1 enzymes, the higher pH resulted in a higher ratio of cellobiose in C1S2, suggesting that β-glucosidase might be inhibited at higher pH. Figure 3.13.d-f show the effect of pH on the hydrolysis products of C2S1, C2S2, and C2. Although pH 4.5 and 5.0 obtained similar total sugar production in C2S1 (Figure 3.13.d), the glucose contents were substantially different. The immobilization negatively affected sugar production outside of the optimum pH. For C2S2 and C2 (Figure 3.13.e-f), most of the sugar products were glucose.

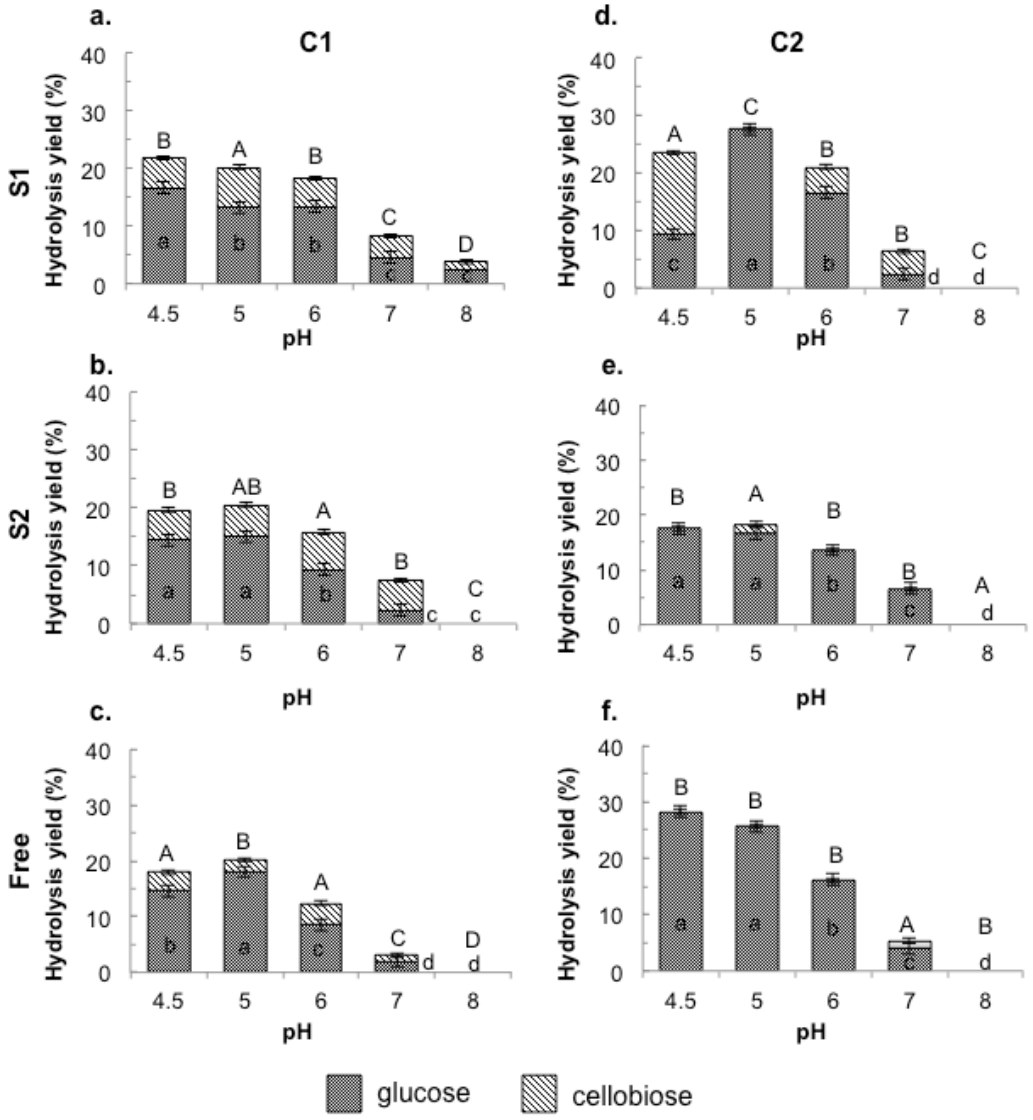


Fig. 3.13. Comparison of hydrolysis yields affected by pH, hydrolyzing microcrystalline cellulose (35 mg/mL).
(a) C1S1, (b) C1S2, (c) C1, (d) C2S1, (e) C2S2, and (f) C2. Hydrolysis reactions were conducted in 0.1 M phosphate buffer at 50 °C at various temperatures for 24 h using a water bath. n = 3, Mean ± Standard Error, Glucose hydrolysis yield, a, b, c, d: $p<0.05$, Cellobiose hydrolysis yield, A, B, C, D: $p<0.05$. The result of S1/S2 was compared to that of free cellulases.

3.7.3. Effects of Ionic Strength

To examine the effects of ionic strength, hydrolysis reactions were carried out under various strengths of phosphate buffers. Figure 3.14 shows the hydrolysis yields and individual sugar profiles in the hydrolysates of C1S1, C1S2, and C1 (Figure 3.14.a-c) and C2S1, C2S2, and C2 (Figure 3.14.d-f) under different ionic strength conditions.

For C1S1, the value at 0.4 M was statistically lower than those obtained for other conditions ($p<0.05$), suggesting that C1S1 had reduced ionic stability at higher ionic strength. In the cases of C1S2 and C1 (Figure 3.14.b), no significant differences were observed ($p>0.05$), proposing that these systems maintained high ionic stability from 0.05 M to 0.4 M. Therefore, immobilization decreased ionic strength stability in C1S1 but did not affect C1S2. In C2S1 (Figure 3.14.d), the value at 0.4 M was significantly lower than those obtained at the other ionic strengths ($p<0.05$). This system reduced ionic stability at higher ionic strength. When C2S2 was used (Figure 3.14.e), the hydrolysis yields were significantly lower at 0.05 M and 0.4 M, meaning that C2S2 appeared to lose ionic stability at high and low ionic strength and displayed the lowest ionic stability of all the systems. Finally, the hydrolysis yields of C2 at 0.4 M was statistically lower than those obtained for other conditions ($p<0.05$). C2 demonstrated reduced ionic

stability at high ionic strength much like C2S1 did. Thus, immobilization did not affect ionic strength stability in C2S1, but lowered the stability in C2S2.

To examine the influence of ionic strength on the components of hydrolysis products, the yield of individual sugars was measured. In the case of C1S1 and C1S2 (Figure 3.14.a.b.), the glucose values decreased when the ionic strength increased while the oligosaccharides yields increased at higher ionic strengths. C1 (Figure 3.14.c) maintained relatively constant individual sugar compositions. Figure 3.14.d-f show the results of using C2 enzymes for hydrolysis. In C2S1 (Figure 3.14.d), the yields of glucose were relatively stable except at 0.4 M, and the yields of cellobiose increased as the ionic strength increased. With respect to C2S2 (Figure 3.14.e), higher ionic strength resulted in lower cellobiose yields. In the case of C2 (Figure 3.14.f), the lone product was glucose; cellobiose was not detected. The effects of immobilization on sugar compositions appeared to be system dependent without any overarching trends observed.

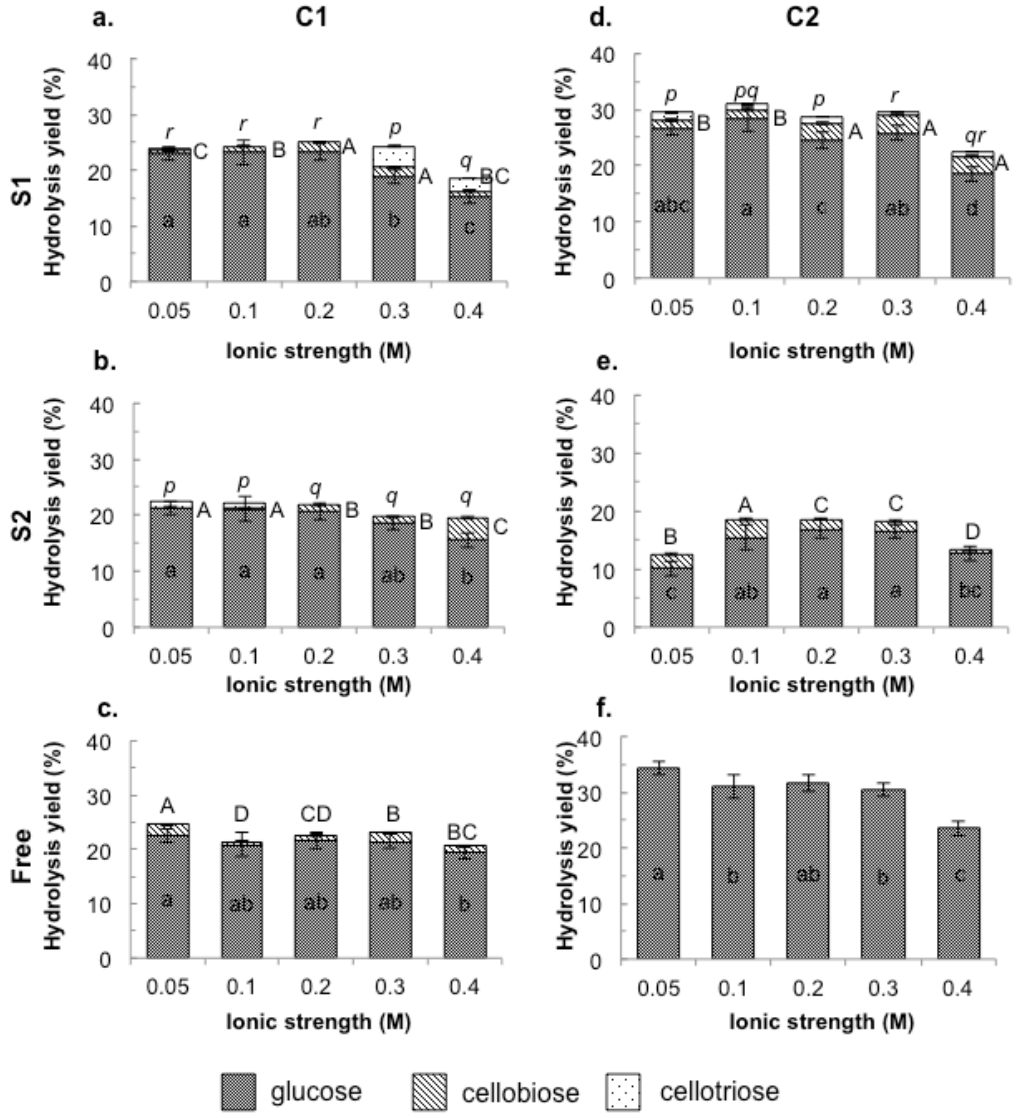


Figure. 3.14. Comparison of hydrolysis yields affected by ionic strength, hydrolyzing microcrystalline cellulose (35 mg/mL).
(a) C1S1, (b) C1S2, (c) C1, (d) C2S1, (e) C2S2, and (f) C2. Hydrolysis reactions were conducted in phosphate buffer (pH=5.0) at 50°C at various ionic strength for 24 h using a water bath. n = 3, Mean ± Standard Error, Glucose hydrolysis yield, a, b, c, d: $p<0.05$, Cellobiose hydrolysis yield, A, B, C, D: $p<0.05$. Cellotriose, *p, q, r*: $p<0.05$. The result of S1/S2 was compared to that of free cellulases.

3.7.4. Effects of Storage Duration

Next, to investigate the influence of storage duration on the hydrolysis yield and the resulting sugar compositions, six systems (C1S1, C1S2, C1, C2S1, C2S2, and C2) were refrigerated for 0, 1, 2, and 3 weeks before use in hydrolysis reactions. Figure 3.15 shows the results of hydrolysis by C1 (Figure 3.15.a-c) and C2 enzymes (Figure 3.15.d-f) after storage. All systems maintained storage stability for the duration of testing in terms of hydrolysis yields and sugar composition.

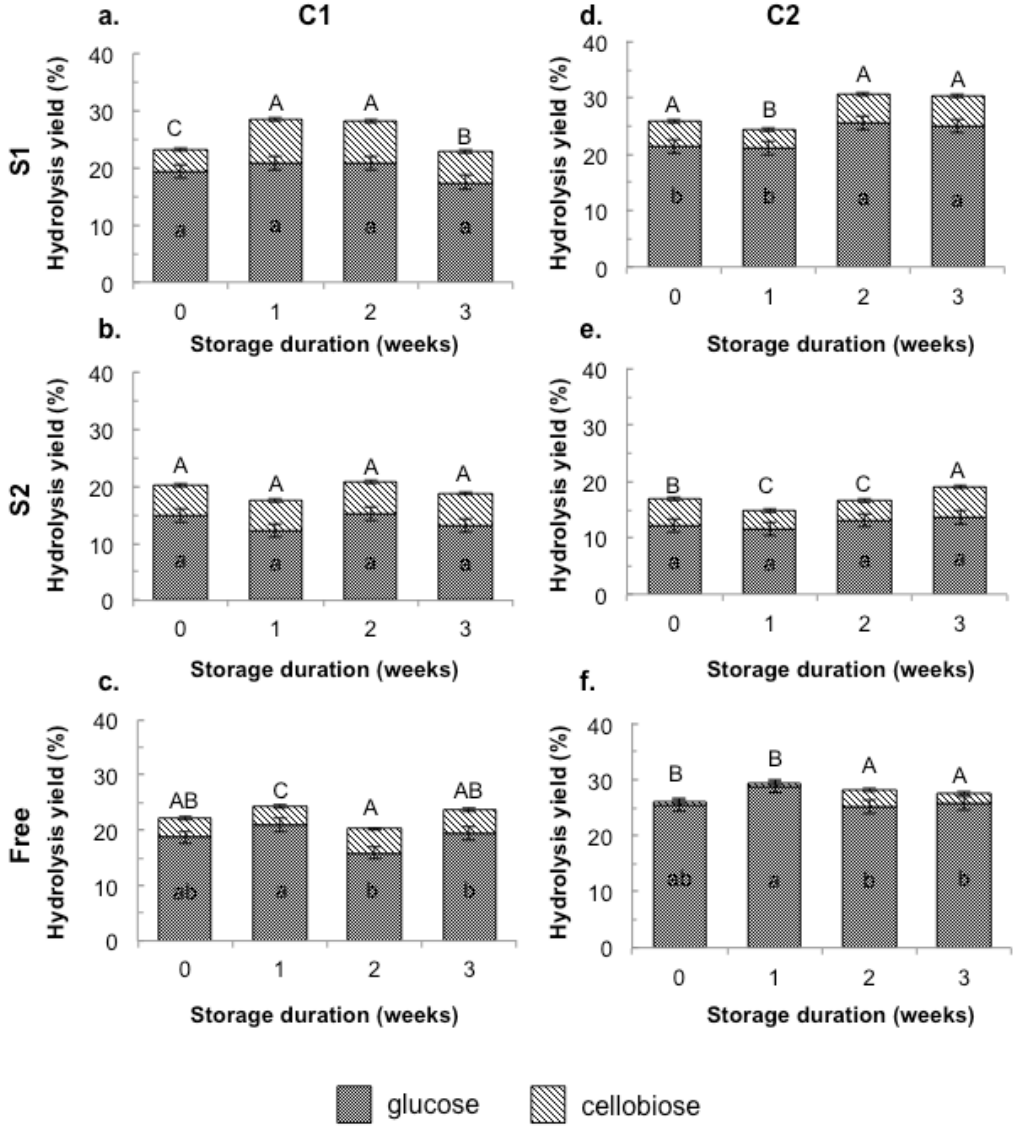


Fig. 3.15. Comparison of hydrolysis yields affected storage, hydrolyzing microcrystalline cellulose (35 mg/mL).
(a) C1S1, (b) C1S2, (c) C1, (d) C2S1, (e) C2S2, and (f) C2. Hydrolysis reactions were conducted in 0.1 M phosphate buffer (pH=5.0) at 50°C for 24 h using a water bath. n = 3, Mean ± Standard Error, Glucose hydrolysis yield, a, b: $p<0.05$, Cellobiose hydrolysis yield, A, B, C: $p<0.05$. The result of S1/S2 was compared to that of free cellulases.

3.7.5. Effects of Immobilized Biocatalyst Reuse

The ability to recycle enzymes is the most desirable reason for immobilization, and is something that is often impossible with free enzymes. To examine the effect of recycling on hydrolysis yields and sugar production, the immobilized cellulases were recycled every 24 hours. Table 3.7 shows the decline rates of the immobilized cellulases and Figure 3.16 describes the hydrolysis yields and individual sugar profiles in the hydrolysates of C1S1 (Figure 3.16.a), C1S2 (Figure 3.16.b), C2S1 (Figure 3.16.c), and C2S2 (Figure 3.16.d) in various recycling stages.

Table 3.7. The decline rates of the immobilized cellulases of C1S1, C1S2, C2S1, and C2S2

Test materials	C1S1	C1S2	C2S1	C2S2
Decline rate (% hydrolysis yields/cycle)	-1.96 ($\pm$ 0.06)$_a$	-2.21 ($\pm$ 0.20)$_a$	-2.41 ($\pm$ 0.16)$_a$	-2.34 ($\pm$ 0.03)$_a$

All immobilized systems demonstrated recyclability. The activity decline rates of the immobilized cellulases were calculated from their hydrolysis yield values (i.e., hydrolysis yields were plotted on scatter plots and the slopes of the trend lines were calculated as a relative decline rate). The slowest decrease was observed in C1S1, which retained 30% of its initial activity even at the 9th cycle, though this value was not significantly different from the others ($p>0.05$).

Additionally, individual sugar yields of the hydrolysis products were measured to examine how recycling immobilized cellulases affects product compositions, with interesting results: the immobilized cellulases on S1 retained consistent sugar composition through 9 cycles; for the immobilized cellulases on S2, cellobiose was regularly detected until the 4^{th} cycle, after which all resultant sugar products were glucose.

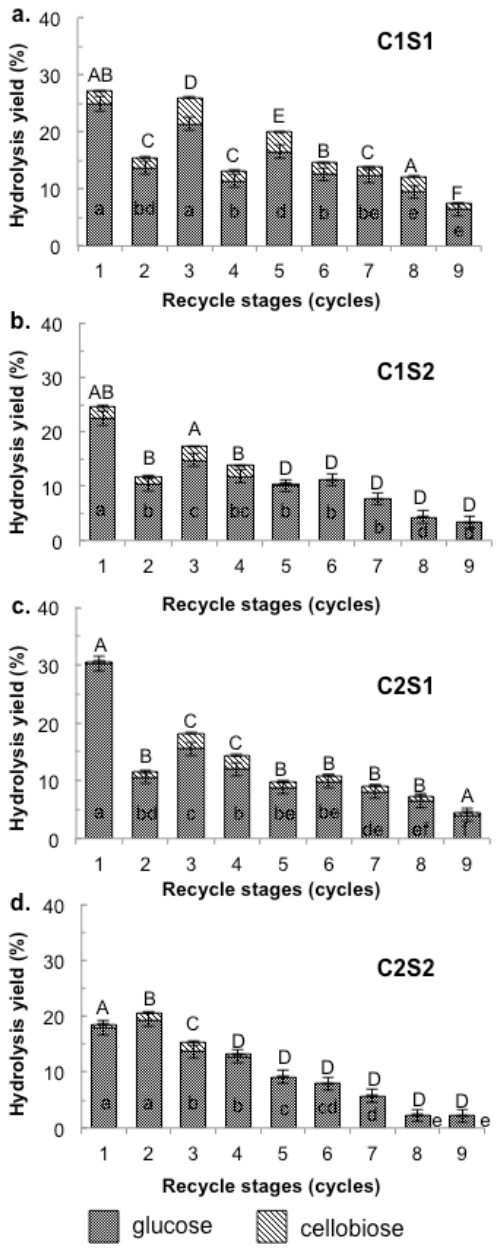


Fig. 3.16. Comparison of hydrolysis yields affected by recycling stages, hydrolyzing microrystalline cellulose (35 mg/mL).
(a) C1S1, (b) C1S2, (c) C2S1, (d) C2S2. Hydrolysis reactions were conducted in 0.1 M phosphate buffer (pH=5.0) at 50°C for 24 h using a water bath. n = 3, Mean ± Standard Error, Glucose hydrolysis yield, a, b, c, d, e, f: $p<0.05$, Cellobiose hydrolysis yield, A, B, C, D, E, F: $p<0.05$.

3.8. Influence of Substrates on Hydrolysis Yields and Products Using Immobilized Cellulases with Highly Retained Enzymatic Activity

Hydrolytic activity differs depending on the substrate configuration even when enzyme strength is equivalent (Kabel *et al.,* 2006). Thus, in this section, to find the effects of different substrates on the immobilized cellulases, seven different substrates were hydrolyzed using the immobilized cellulases. The substrates used in this section were microcrystalline cellulose and crystalline cellulose II to represent the crystalline regions of cellulose fiber; commercial cellophane paper and phosphoric acid swollen cellulose (PASC) to represent its amorphous regions; xylan as its hemicellulose fraction; and wood and waste office automation (OA) paper for a crude lignocellulose biomass. To examine the effects of different substrates, the hydrolysis yield and the sugar compositions were examined.

3.8.1. Hydrolysis of Crystalline Cellulose

Crystalline structure of cellulose is one of the major fractions in lignocellulosic biomass and is very difficult to degrade. It is thus an excellent substrate to evaluate the effectiveness of immobilized cellulases. Table 3.8 and Figure 17 show the total hydrolysis yields and individual sugar compositions of microcrystalline cellulose hydrolysis in the six systems tested.

Table 3.8. Total hydrolysis yields affected by microcrystalline cellulose hydrolysis. Hydrolysis reactions were conducted in 0.1 M phosphate buffer (pH=5.0) at 50°C for 24 h using a water bath. n = 3, Mean ± Standard Error, C1enzymes, a: $p<0.05$, C2 enzymes, A, B, C: $p<0.05$.

Test materials	C1S1	C1S2	C1	C2S1	C2S2	C2
Total hydrolysis yields (%)	34.4 $(\pm 0.73)_a$	30.6 $(\pm 0.36)_a$	31.3 $(\pm 0.21)_a$	46.1 $(\pm 1.67)_A$	30.0 $(\pm 0.88)_B$	40.0 $(\pm 0.19)_C$

The trend of the hydrolysis was the similar to the previous experiments: C1S1 and C2S1 yields were statistically equivalent to their corresponding free forms and the yields of C1S2 and C2S2 were lower than those of C2. Additionally, the hydrolysate compositions of microcrystalline cellulose were examined (Figure 3.17). The glucose production of immobilized C1 and C2 on S1 did not differ significantly from those of C1 and C2 ($p>0.05$), whereas those on S2 were statistically lower ($p<0.05$). The amounts of cellobiose produced with C1S1, C1S2, C2S1, and C2S2 were significantly higher than that of C1 and C2 ($p<0.05$).

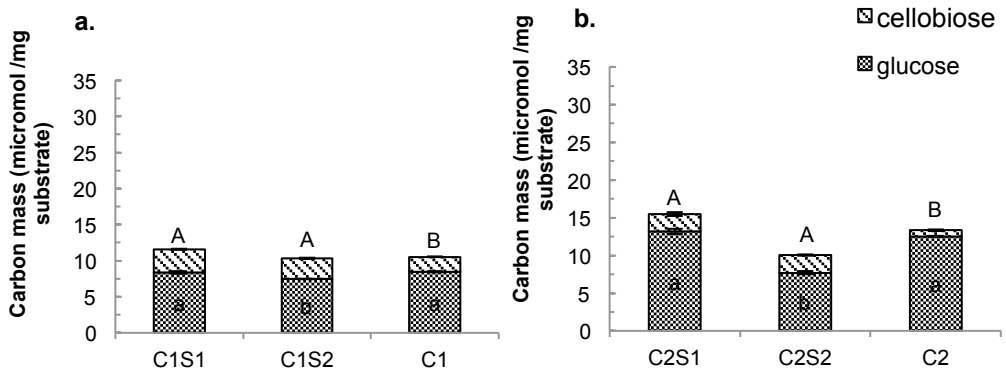


Fig. 3.17. Comparison of carbon production by hydrolysis of microcrystalline cellulose (35 mg/mL).
(a) C1S1, C1S2, C1 (b) C2S1, C2S2, C2,. Hydrolysis reactions were conducted in 0.1 M phosphate buffer (pH=5.0) at 50°C for 24 h using a water bath. n = 3, Mean ± Standard Error, Glucose carbon mass, a, b: $p<0.05$, Cellobiose carbon mass, A, B: $p<0.05$.

Table 3.9 and Figure 3.18 show the results of crystalline cellulose type II hydrolysis. The hydrolysis of cellulose II displays the same tendencies as hydrolysis of microcrystalline cellulose: C1S1 and C2S1 were statistically equivalent with their corresponding free forms, and the C1S2 and C2S2 was lower than their corresponding free forms. Overall, two types of crystalline cellulose, cellulose II and microcrystalline cellulose, showed relatively similar results. The immobilized cellulases on S1 hydrolyzed crystalline cellulose as productively as free cellulases.

Table 3.9. Total hydrolysis yields affected by crystalline cellulose II hydrolysis. Hydrolysis reactions were conducted in 0.1 M phosphate buffer (pH=5.0) at 50°C for 24 h using a water bath. n = 3, Mean ± Standard Error, C1enzymes, a, b, c: $p<0.05$, C2 enzymes, A, B: $p<0.05$.

Test materials	C1S1	C1S2	C1	C2S1	C2S2	C2
Total hydrolysis yields (%)	43.7 $(\pm 0.43)_a$	31.7 $(\pm 0.30)_b$	41.8 $(\pm 7.31)_a$	45.2 $(\pm 1.15)_A$	31.0 $(\pm 0.69)_B$	42.1 $(\pm 3.74)_A$

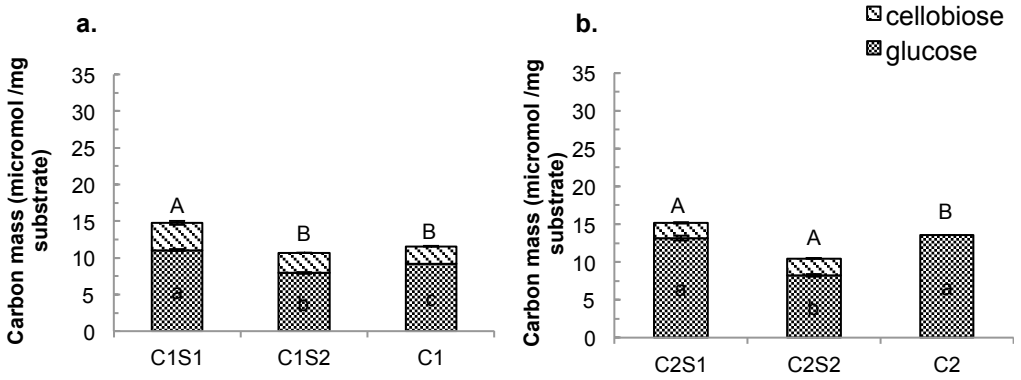


Fig. 3.18. Comparison of carbon production by hydrolysis of crystalline cellulose II (35 mg/mL).
(a) C1S1, C1S2, C1 (b) C2S1, C2S2, C2. Hydrolysis reactions were conducted in 0.1 M phosphate buffer (pH=5.0) at 50°C for 24 h using a water bath. n = 3, Mean ± Standard Error, Glucose carbon mass, a, b, c: $p<0.05$, Cellobiose carbon mass, A, B: $p<0.05$.

3.8.2. Hydrolysis of Amorphous Cellulose

The structure of cellulose contains amorphous regions that are easier for free cellulases to hydrolyze than crystalline regions. The hypothesis here is that the immobilized cellulases, which hydrolyzed crystalline cellulose well, should also hydrolyze amorphous cellulose well due to the characteristics of easier

degradation of amorphous region. Table 3.10 and Figure 3.19 show the results of efforts to hydrolyze amorphous cellulose in the form of cellophane paper.

Table 3.10. Total hydrolysis yields affected by cellophane paper hydrolysis. Hydrolysis reactions were conducted in 0.1 M phosphate buffer (pH=5.0) at 50°C for 24 h using a water bath. n = 3, Mean ± Standard Error, C1enzymes, a, b: $p<0.05$, C2 enzymes, A, B, C: $p<0.05$.

Test materials	C1S1	C1S2	C1	C2S1	C2S2	C2
Total hydrolysis yields (%)	61.0 $(\pm 7.07)_a$	55.6 $(\pm 1.52)_a$	93.4 $(\pm 0.60)_b$	72.9 $(\pm 2.23)_A$	56.8 $(\pm 1.35)_B$	93.4 $(\pm 1.15)_C$

The yields obtained from immobilized cellulases were significantly lower than that of their corresponding free cellulases. The immobilized cellulases did not hydrolyze amorphous cellulose as efficiently as the free cellulases. The hydrolysis products were also measured, and the immobilized cellulases had a higher ratio of cellobiose. Cellobiose conversion was reduced in the case of the immobilized cellulases.

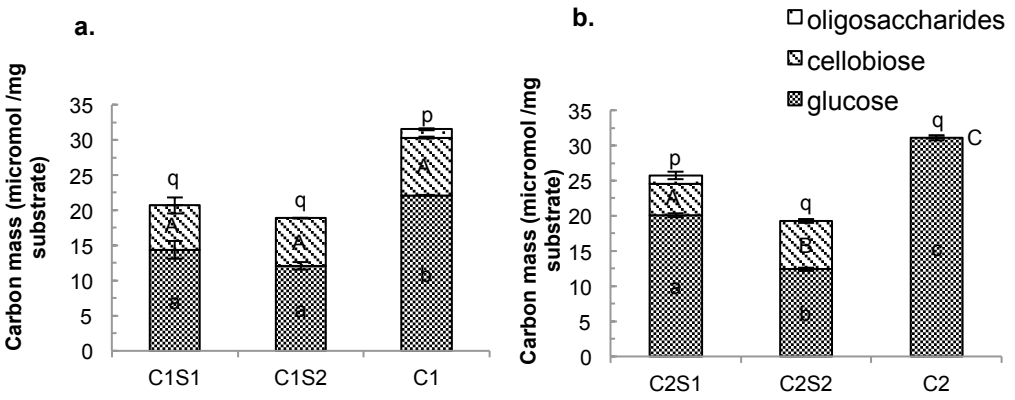


Fig. 3.19. Comparison of carbon production by hydrolysis of cellophane paper (35 mg/mL).
(a) C1S1, C1S2, C1 (b) C2S1, C2S2, C2.. Hydrolysis reactions were conducted in 0.1 M phosphate buffer (pH=5.0) at 50°C for 24 h using a water bath. n = 3, Mean ± Standard Error, Glucose carbon mass, a, b, c: $p<0.05$, Cellobiose carbon mass, A, B, C: $p<0.05$, Cellotriose carbon mass, p, q:$p<0.05$.

Table 3.11 and Figure 3.20 show the results of another amorphous cellulose hydrolysis; phosphoric acid swollen cellulose (PASC). In this system, the immobilized cellulases did not hydrolyze PSAC as efficiently as the free cellulases, a result that corresponds with that of the cellophane paper hydrolysis. Again, the ratio of cellobiose was higher in the immobilized cellulases, likely because immobilization inhibited β-glucosidase or the enzyme was depleted from the support . The overall results in this section were disappointing because it is likely that the hydrolysis of lignocellulose biomass would be negatively affected by immobilization as it contains amorphous cellulose as well as crystalline cellulose.

Table 3.11. Total hydrolysis yields affected by PASC hydrolysis. Hydrolysis reactions were conducted in 0.1 M phosphate buffer (pH=5.0) at 50°C for 24 h using a water bath. n = 3, Mean ± Standard Error, C1enzymes, a, b: $p<0.05$, C2 enzymes, A, B: $p<0.05$.

Test materials	C1S1	C1S2	C1	C2S1	C2S2	C2
Total hydrolysis yields (%)	52.0 $(\pm 1.28)_a$	52.6 $(\pm 0.34)_a$	72.7 $(\pm 1.12)_b$	56.9 $(\pm 0.48)_A$	58.2 $(\pm 0.61)_A$	72.1 $(\pm 3.74)_B$

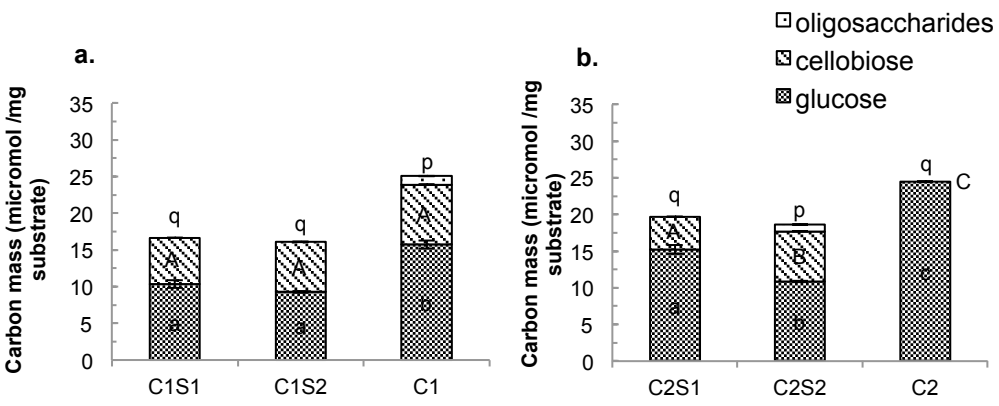


Fig. 3.20. Comparison of carbon production by hydrolysis of phosphoric acid swollen cellulose (35 mg/mL).
(a) C1S1, C1S2, C1 (b) C2S1, C2S2, C2. Hydrolysis reactions were conducted in 0.1 M phosphate buffer (pH=5.0) at 50°C for 24 h using a water bath. n = 3, Mean ± Standard Error, Glucose carbon mass, a, b, c,: $p<0.05$, Cellobiose carbon mass, A, B, C: $p<0.05$, Oligosaccharides carbon mass: p, q: $p<0.05$.

3.8.3. Hydrolysis of Hemicellulose

Lignocellulose substrates contain hemicellulose which interferes with hydrolysis of the cellulose fraction (Ikeda, *et al*., 2007). To examine the effect of

immobilization on the enzyme systems ability to hydrolyze the hemicellulose fraction, xylan as a representative of hemicellulose fraction was hydrolyzed and the hydrolysis yields were examined (Table 3.12 and Figure 3.21).

Table 3.12. Total hydrolysis yields affected by xylan hydrolysis. Hydrolysis reactions were conducted in 0.1 M phosphate buffer (pH=5.0) at 50°C for 24 h using a water bath. n = 3, Mean ± Standard Error, C1enzymes, a, b: $p<0.05$, C2 enzymes, A, B: $p<0.05$.

Test materials	C1S1	C1S2	C1	C2S1	C2S2	C2
Total hydrolysis yields (%)	41.8 $(\pm 1.24)_a$	30.8 $(\pm 0.71)_a$	49.7 $(\pm 2.65)_b$	35.4 $(\pm 4.38)_A$	34.9 $(\pm 0.68)_A$	50.5 $(\pm 3.16)_B$

Because the total hydrolysis yields of the immobilized cellulases were lower than those of free cellulases, the immobilized cellulose system did not hydrolyze xylan as efficiently as free cellulases. Hemicellulose has an amorphous structure, and the results corresponded with those from the amorphous cellulose hydrolysis. The hydrolysate compositions were also examined (Figure 3.21). The main product of xylan hydrolysates was xylose. The xylose content of the C1S1 system was not significantly different from that of C1 ($p>0.05$) and that of C1S2 was statistically lower than that of C1 ($p<0.05$). The xylotriose produced with C1 was statistically equivalent to that of C1S1 ($p>0.05$) and significantly higher than that of C1S2 ($p<0.05$). Small amounts of xylobiosee were detected in C1S1 and C1, but not in C1S2. As for the C2 enzymes, xylose and xylobiose production in the immobilized C2 were lower than those in C2, suggesting that the immobilized

cellulases did not hydrolyze xylose and cellobiose fractions as efficiently as free cellulases.

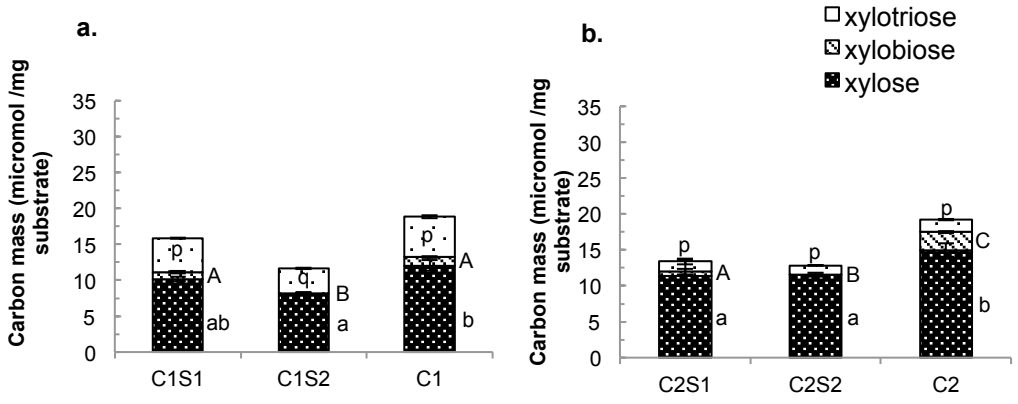


Fig. 3.21. Comparison of carbon production by hydrolysis of phosphoric acid swollen Cellulose (35 mg/mL).
(a) C1S1, C1S2, C1 (b) C2S1, C2S2, C2. Hydrolysis reactions were conducted in 0.1 M phosphate buffer (pH=5.0) at 50°C for 24 h using a water bath. n = 3, Mean ± Standard Error, Xylose carbon mass, a, b: $p<0.05$, Xylobiose carbon mass, A, B, C: $p<0.05$, Xylotriose carbon mass, p, q: $p>0.05$,

3.8.4. Hydrolysis of Lignocellulose Biomass

Finally, actual industrial lignocellulose biomass, steam-exploded poplar wood and shredded waste OA paper were used in hydrolysis trials. Table 3.13 and Figure 3.22 show the results of steam-exploded poplar hydrolysis as an example of wood hydrolysis. The results show that the immobilized cellulases did not hydrolyze the wood sample as efficiently as the free cellulases, but still retained substantial activity on this complex substrate. The retained enzyme activities of C1S2, C1S2, C2S1, and C2S2 were 62%, 41%, 62%, and 57%, respectively.

Table 3.13. Total hydrolysis yields affected by steam-exploded poplar hydrolysis. Hydrolysis reactions were conducted in 0.1 M phosphate buffer (pH=5.0) at 50°C for 24 h using a water bath. n = 3, Mean ± Standard Error, C1enzymes, a, b, c: $p<0.05$, C2 enzymes, A, B: $p<0.05$.

Test materials	C1S1	C1S2	C1	C2S1	C2S2	C2
Total hydrolysis yields (%)	34.5 $(\pm 0.59)_a$	22.85 $(\pm 0.12)_b$	55.7 $(\pm 1.07)_c$	45.4 $(\pm 3.03)_A$	42.2 $(\pm 1.08)_A$	72.9 $(\pm 1.01)_B$

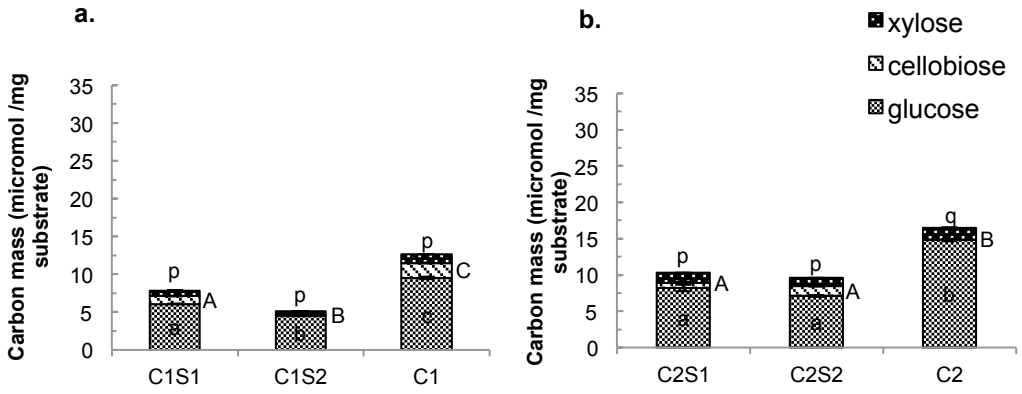


Fig. 3.22. Comparison of carbon production by hydrolysis of phosphoric acid swollen cellulose (35 mg/mL).
(a) C1S1, C1S2, C1 (b) C2S1, C2S2, C2. Hydrolysis reactions were conducted in 0.1 M phosphate buffer (pH=5.0) at 50°C for 24 h using a water bath. n = 3, Mean ± Standard Error, Glucose carbon mass, a, b, c: $p<0.05$, Cellobiose carbon mass, A, B, C: $p<0.05$, Xylose carbon mass, p, q: $p>0.05$.

Finally, the effects of waste OA paper on the immobilized cellulases were examined. Table 3.14 and Figure 3.23 show the results of waste OA paper hydrolysis.

Table 3.14. Total hydrolysis yields affected by OA paper hydrolysis. Hydrolysis reactions were conducted in 0.1 M phosphate buffer (pH=5.0) at 50°C for 24 h using a water bath. n = 3, Mean ± Standard Error, C1enzymes, a, b: $p<0.05$, C2 enzymes, A, B: $p<0.05$.

Test materials	C1S1	C1S2	C1	C2S1	C2S2	C2
Total hydrolysis yields (%)	12.4 $(\pm 1.71)_a$	15.6 $(\pm 0.33)_a$	36.1 $(\pm 0.44)_b$	21.5 $(\pm 0.82)_A$	18.3 $(\pm 0.41)_A$	44.5 $(\pm 0.53)_B$

Likewise, the immobilized cellulases had reduced rates of hydrolysis on waste OA paper based on their lower yields of glucose, cellobiose, and xylose compared to the free cellulases. The retained enzyme activities of C1S1, C1S2, C2S1, and C2S2 were 34%, 43%, 48%, and 41%, respectively.

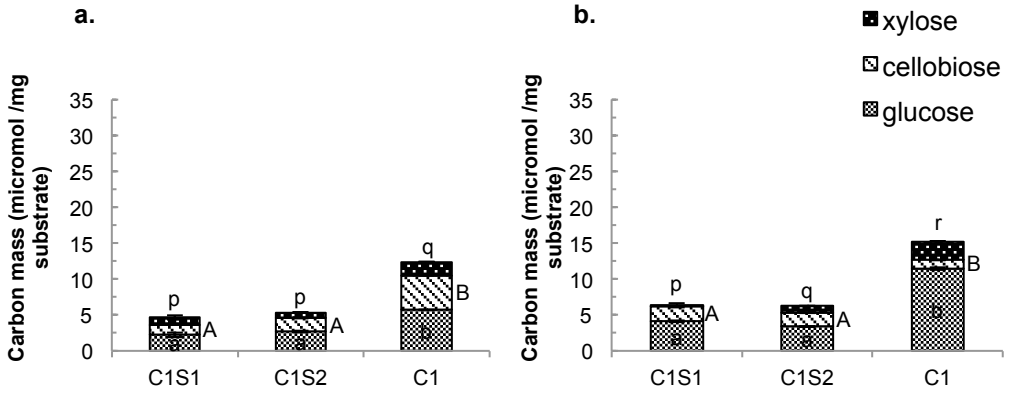


Fig. 3.23. Comparison of carbon production affected by waste OA paper hydrolysis (35 mg/mL).
(a) C1S1, C1S2, C1 (b) C2S1, C2S2, C2. Hydrolysis reactions were conducted in 0.1 M phosphate buffer (pH=5.0) at 50°C for 24 h using a water bath. n = 3, Mean ± Standard Error, Glucose carbon mass, a, b: $p<0.05$, cellobiose carbon mass, A, B: $p<0.05$, Xylose carbon mass, p, q, r: $p>0.05$.

4. Discussion

4.1. Determination of Immobilization Methods and Supports

Cellulose is the most abundant polymer in nature, and its use is therefore critical for emerging bioindustries. The enzymes that decompose cellulose are cellulases. They are relatively expensive and have, to date, limited the commercialization of technologies that utilize lignocellulose as substrates. Thus, the development of a way to reduce the amount of cellulases used in cellulose decomposition is essential. One approach is enzyme immobilization, which makes it possible to reuse enzymes and thus reduce enzyme cost. However, immobilized cellulases typically exhibit reduced enzyme activity because the interaction with the support may have impacts on the enzyme structure. Therefore, developing a support by which enzymes can retain their activity levels is desirable for industry. Highly retained enzymatic activities in immobilized systems are occasionally obtained depending on methods and combinations of support-enzymes (Cao, 2005).To find a suitable support-enzyme combination for hydrolysis of microcrystalline cellulose, the following methods of cellulose immobilization were tested based on a thorough literature review:

1. Polyvinyl alcohol modified chitosan beads
2. Glutaraldehyde coated amberlite

3. Calcium chloride coated Silica and calcium-aluminum coated fumed silica
4. Calcium chloride coated amberlite and calcium-aluminum coated amberlite
5. ETS-2, ETS-4, and chabazite
6. Porous silica

4.1.1. Cellulase Immobilization on Polyvinyl Alcohol Modified Chitosan Beads

First, chitosan beads were applied on the cellulase immobilization. Chitosan is a copolymer of β-(1,4)linked 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-d-glucopyranose. It is often produced by the alkaline deacetylation from chitin (Dinçer and Telefoncu, 2007). Chitosan is an attractive biomaterial for immobilization as it is the second most widely available polymer in nature (El-Ghaffar *et al.*, 2010). In this dissertation, the polyvinyl modified chitosan beads resulted in highly retained enzyme activity, and the immobilized cellulases on chitosan beads retained 30% of enzyme activity. Thus, this method was determined to be a viable option, and was used for the further experiments..

Chitosan beads have been shown to effectively retain high enzyme activities. For instance, Xie *et al*. (2012) cross-linked cellulases on chitosan magnetic particles and retained 50.6% activity, using glutaraldehyde. El-Ghaffar *et al.* (2010) also cross-linked cellulases (oiriginated from *Aspergillus niger*) on

chitosan, chitosan modified by glutamic acid, and chitosan modified by 4-aminobutyric acid, and achieved enzyme activities of 65.5%, 85.3%, and 63.1%,compared to free cellulases. Furthermore, Dinçer and Telefoncu (2006) immobilized cellulases (originating from *Aspergillus niger*) on polyvinyl alcohol modified chitosan beads and retained 87% activity. Based on the results from the literature and from observations in this study, chitosan beads may be a suitable support for cellulase immobilization as well.

The immobilized cellulases in literatures (Dinçer and Telefoncu, 2007;Table 1.1.) obtained higher retained enzyme activity than in this study because different cellulases were used. In the literature, *Aspergillus niger*, which produces a large amount of β-glucosidase, was used. The cellulases used in this research originated from *Trichoderma ressei*, and include a large amount of cellobiohydrolases (Ikeda *et al*., 2007) which were desirable for this study. Thus the efficiency obtained here is not directly comparable to results found in the literature.

4.1.2. Covalent Immobilization of Cellulases on Amberlite with Glutaraldehyde

Second, covalent immobilization was applied using glutaraldehyde. Glutaraldehyde is often used as an immobilization reagent because it generates intense multi-point cross-links between enzymes and supports, and between

enzymes themselves. Glutaraldehyde also causes chemical reactions between amino acid residues on enzyme surfaces and modifies protein conformations (El-Aassar *et al*., 2013). The method used in this research was based on the report from Obón *et al*. (2000), who immobilized β-galactosidase using glutaraldehyde, and obtained high enzyme activity from the immobilized enzymes. However, in this report, the sugar production for the immobilized cellulases was statistically lower than that for free cellulases, and the differences in sugar production were substantial. Glutaraldehyde therefore reduced cellulase activity. due to protein conformational change. Because the catalytic sites of cellulases require flexibility to cleave cellulose, the inflexible immobilization caused by covalent binding was not suitable for cellulase immobilization. Glutaraldehyde cross-linking is a very delicate method, easily affected by the storage conditions of glutaraldehyde, the concentration of original glutaraldehyde, and the detailed processes to create cross-linkings. Thus, small differences between processes, which were not written in detail in Obón *et al*. (2000) likely reduced the retained enzyme activity in this research, not to mention the different enzymes used.

There are several reports that demonstrate that glutaraldehyde lowers enzyme activities, supporting the results of the current research. For example, Feng *et al.* (2012) immobilized lipase on a woolen cloth with glutaraldehyde and polyethylenimine, and the retained enzyme activity decreased to 30.2%. Also, Xu

et al. (2011a) immobilized cellulases on magnetic particles with glutaraldehyde and retained only 40% of the enzyme activity. The covalent immobilization method was not selected for the further experiments in this dissertation.

4.1.3. Fumed Silica, Calcium Chloride Coated Fumed Silica, Calcium- Aluminum Chloride Coated Silica

Next, physical adsorption and ionic interactions were used to immobilize cellulase. To increase sorption capacity, Sinegani *et al*. (2005) coated silica with calcium and aluminum ions, and reported that the sorption capacity of the support increased with calcium ions and greatly increased with both calcium and aluminum ions. Thus, the same procedure was applied for fumed non-porous silica (S1) in this experiment. The physical adsorption method was used as a control in this experiment. The retained enzyme activity for the immobilized cellulases on S1 was 44%, which was relatively high compared to other procedures in this dissertation. Unfortunately, Sinegani *et al*. (2005) did not report retained enzyme activity, so comparison is impossible in that regard. Because the sugar values for the immobilized cellulases on S1 and those on S1 with calcium ion were not significantly different, the effects of calcium ions were not observed.. Due to the relatively high retention of enzyme activity and ease of immobilization, S1 was selected for further research.

By contrast, aluminum ions greatly decreased enzymatic activity in this experiment. The negative effects of aluminum ions on cellulases have also been recently reported in the literature. For example, Antunes *et al.* (2011) found a significant and negative correlation in interactions between soil enzymes (cellulases) and metallic elements including Al, Ne, Cu, and U. Likewise, Bin *et al*. (2010) reported that aluminum ions decreased β-glucosidase activity to 8.1%. Supports with strong charges tend to react more strongly with enzymes often with greater affect on the catalytic site of cellulases and reduce enzymatic activity. Takimoto *et al.* (2008) immobilized cellulases on amine-functionalized mesoporous silica and found a significant decrease in enzymatic activity. They explained that this was due to the interaction between the amine group of the support and the carboxyl groups of cellulase's catalytic site, inhibiting cellulase activity. Although the sorption capacity may have increased as in Sinegani *et al*. (2005), the same or similar reaction may have caused the decreased enzymatic activity observed here as well. Thus, S1 coated with aluminum chloride was deemed inadequate for the immobilization of cellulases.

4.1.4. Amberlite, Calcium Chloride Coated Amberlite, Calcium and Aluminum Chloride Coated Amberlite

The simplicity of the adsorption procedure used in immobilization on silica particles (3.1.3) made it attractive for use in further experiments. Thus, a different

support, amberlite, was tested using the same adsorption method. However, the sugar production of immobilized cellulases on amberlite, calcium chloride coated amberlite, and calcium chloride and aluminum chloride coated amberlite was significantly lower than that of the free cellulases, with retained enzyme activities of less than 10%. Examination of the amount of protein showed that most of enzymes did not attach to the support, possibly because the charge on the support might repulse cellulases. Because of the low loading efficiency and resulting low retained activity, amberlite was not used in further experiments.

4.1.5. ETS-2, ETS-4, CHABAZITE

Because the adsorption on S1 showed highly retained enzyme activity, other types of silica were tested for immobilization: platy silica, including ETS-2, ETS-4, and chabazite. These have often been used for leaching and sorption studies, such as adsorbing heavy metals, hydrogen sulfide, and radioactive components, because of their large surface area (Cappelletti *et al.,* 2012; Popa and Pavel, 2012; Sabereh Rezaei *et al.,* 2012). The hypothesis in this experiment was that the silica might have a high loading efficiency due to its large surface area. As a result, they might enable highly retained enzyme activities. However, none produced sugars, indicating very low retained enzyme activities. The protein assay showed that most of protein did not attach to the silica in ETS-2 and chabazite. ETS-2, ETS-4, and chabazite possess a positive charge on their

surfaces; it appears that ETS-2 and chabazite repulsed the cellulases. In the case of ETS-4, it appeared that the proteins did attach to the surface of the silica but did not produce sugar. ETS-4 contains aluminum ions in its structure; the ions may have reacted with the catalytic site of the cellulases and decreased the enzymatic activity, similar to the results discussed in 4.1.3..From these observations, platy silica was deemed unsuitable as a support.

4.1.6. Porous Silica (Silica 2)

Based on the successful result of the cellulase adsorption on S1, a different type of silica was applied for the immobilization: porous silica (S2). Porous silica is often used for enzyme immobilization. For example, Yu *et al.* (2012) immobilized carbonic anhydrase on the carboxylic acid group functionalized mesoporous silica to convert carbon dioxide to bicarbonate. Ahn *et al*. (2011) immobilized lipase on mesoporous and microporous silica in methanolysis of soybean oil to produce biodiesel. Also, Nwagu *et al.* (2011) immobilized amylase on porous silica gel and obtained 75% of the retained enzyme activity.

The hydrolysis reaction was conducted with the cellulases immobilized on S1 as a positive control. This experiment was performed in 1.7 mL microcentrifuge tubes in a water bath. It was a simple method for immobilization and enabled handling of a large numbers of samples with adequate heat control.

The sugar production and retained enzyme activity for the immobilized cellulases on S2 were very similar to those for the immobilized cellulases on S1. Thus, S2 was selected as a potential support for the immobilization of cellulases and was used for further experiments. S1 and S2 do not have a charge on their surfaces. Thus, the immobilization force must be primary hydrogen bonds. This interaction is likely ideal for cellulase immobilization using C1 because ionic interaction and covalent bonding demonstrably lowered enzyme activities in the previous sections.

The S1 immobilization method and reaction conditions were similar to that described in Tébéka *et al.*(2006; Table 1.1.), who immobilized cellulases (originating from *Aspergillus niger*) on silica wafers using the adsorption method. The immobilized cellulases retained 80% enzyme activity. The retained enzyme activity obtained in this research was 44%. The differences were likely because the immobilization conditions of S1 were not optimized at this stage, and also because the origins of the cellulases were different.

4.2. Determination of Reaction Scale

The enzyme reactions in the previous experiments were conducted on a relatively small scale (1.7 mL microcentrifuge tubes). To determine the best scale at which to carry out the experiments, 250 mL flask and 5 L bioreactor were

tested. The criteria were as follows: ease of experimentation, temperature control, and ease of handling a large number of samples.

Hydrolysis reactions were scaled up to 250 mL flasks and were incubated in a rotary shaker. Because the sugar productions at 65°C were highest in the immobilized cellulases on chitosan beads, the immobilized cellulases gained thermal stability over free cellulases. Thermal inactivation often occurs due to the unfolding of three-dimensional protein structure (Dinçer and Telefoncu, 2007), thus, immobilization might prevent protein deformation. Other researchers have also reported on thermal stability of immobilized cellulases. For example, Zhou (2010), who immobilized cellulases on N-succinil-chitosan using adsorption method, and Dinçer and Telefoncu (2007), who immobilized cellulases on polyvinyl modified chitosan beads, observed higher temperature stability at 40°C and 65°C compared to that of free cellulases (Table 1.1.).

In this experiment, there was large variability in the data obtained when samples were hydrolyzed at 50°C. Also, some reactions did not proceed, specifically at 65°C. These results indicate that heat may not have been conducted evenly or that heat transfer in the reaction mixtures might not have been uniform. Thus, the temperature control at the 250 mL scale was inadequate, and this scale was not used for further experiments.

The hydrolysis reactions were scaled up to 5 L bioreactors for better heat transfer control. The immobilization on S1 particles showed a higher retained enzyme activity at 50°C and still exhibited enzyme activity at 63°C, compared to those for free cellulases, which showed no activity at high temperatures. Enzymes denature via a variety of conditions including temperature, pH, ionic strength, denaturing reagents, pressure, and mechanical mixing (Dinçer and Telefoncu, 2007). It was hypothesized that immobilization on S1 might protect the enzymes from conformational change, specifically from heat denaturation. However, the immobilized cellulases on chitosan beads demonstrated weakness to mechanical stirring. Although polyvinyl modified chitosan beads were shown to be a suitable support and demonstrated relatively high retained enzyme activity and thermal stability in the previous experiments, they were not used in further experiments.

Although the enzyme reaction in the 5 L bioreactor enabled better heat control than that in flasks, it required a large amount of substrate and enzyme. Additionally, the large volume of water and large equipment was not practical for handling large numbers of samples, so this experimental scale was not used for further experiments in this dissertation. All further experiments were carried out at the microcentrifuge tube scale.

4.3. Effects of Immobilization Condition on Loading Efficiency

From the previous experiments, two suitable supports using the adsorption method were selected, and four immobilized systems were created (C1S1, C1S2 C2S1, and C2S2). Their characteristics will be examined here and in the following sections. First, the effects of immobilization conditions on loading efficiency were examined. Microenviroments between supports and enzymes substantially affect the immobilization (Cao, 2005). Thus, several variables were tested for the immobilization process, including pH and ionic strength, to examine their influence on enzyme loading efficiencies. Also, various enzyme-support ratios were evaluated in an attempt to maximize loading efficiencies.

. The interaction forces in adsorption immobilization are non-physical associations and could be a combination of hydrogen bonds, hydrophilicity, electrostatic interactions and van der Waals force (Cao, 2005). The primary interaction is most likely the hydrogen bonds between hydroxyl groups on silica particles and hydrogen groups on enzymes since the adsorption energy of cellulases on silica materials is closely related to that of hydrogen bond generation (Tébéka *et al*. 2009). From these observations, pH 5.0 was chosen for C1S1 C1S2, and C2S1, and pH 6.0 was chosen for C2S2 for the further experiments.

C1S1 loading efficiency was not substantially affected by changes to ionic strength; C1S2 obtained the highest efficiency at 0.3 M; C2S1 obtained the highest efficiency at 0.2 M; and C2S2 showed the highest efficiency at 0.2 M, though the influence of ionic strength was not great. Based on these results, ionic strengths were set at 0.3 M for C1 enzymes and 0.2 M for C2 enzymes in further experiments. This experiment also indicated that C1 tends to require a higher ionic strength than C2 irrespective of the type of silica. In this experiment, loading efficiencies were increased over the results of the pH experiment as follows: C1S1 increased from 85% to 95%; C1S2 increased from 59% to 84%; C2S1 increased from 90% to 95%. C2S2 decreased from 76% to 61% with unknown reason.

In the experiments to determine the enzyme-support ratios, 30 mg for S1 and 120 mg for S2 were selected and standardized for further experiments irrespective of the type of cellulases due to the achieved high loading efficiencies and for cost-performance considerations. The difference between S1 and S2 requirements were made obvious in this experiment: S2 required approximately four times the amount of support to obtain an equivalent amount of loading efficiency as S1, suggesting that non-porous S1 absorbs more protein than porous S2. This presumably is due to differences in the characteristics of silica, especially the actual surface area. Although according to the manufacturer both types of

silica have comparable surface areas (390 ± 40 m^2/g for S1 and 500 m^2/g for S2), S2 may actually have a much smaller available surface area than S1 because of its pore sphere characteristics. S1 forms a three-dimensional network that likely traps cellulases and water molecules resulting in a large available surface area, while S2 binds cellulases on its outer and inner surfaces. The average pore diameter of S2 is approximately 60 Å, which is close to the size of cellulase as reported by other researchers: 60 x 50 x 40 Å (Henriksson *et al*., 1996), 5.2 x 7.6 x 11.3 nm (Takimoto *et al*., 2008) and 13 x 79 Å to 42 x 252 Å (Hartono *et al*., 2010). Therefore, cellulase may block the silica's pores, reducing the actual available surface area. This experiment resulted in increased loading efficiencies over the ionic strength experiment as follows: C1S1 increased from 95% to 98%; C1S2 increased from 84% to 90%; C2S1 increased 95% to 98% compared to the effect of ionic strength experiments. C2S2 increased from 76% to 91% compared to the effect of pH experiment.

4.4. Enzyme Activity

Next, the characteristics of enzyme activity were examined. Conventional wisdom would predict that an insoluble substrate would be unfavourable for an immobilization system. Also, the act of immobilization is known to cause loss of enzyme activity. Several methods are available to compare retained enzymatic activity such as specific enzyme activity measurement, the filter paper unit (FPU)

assay, hydrolysis product measurement, and individual cellulase assay. Of these methods, the hydrolysis product measurement was chosen for practicality: the measurement of fermentable sugar yield was also necessary for the downstream fermentation process.

Surprisingly, the hydrolysis yields for C1S1 and C2S1 were not significantly different from those for their corresponding free cellulases, meaning that they retained activity. This higher-than-expected activity is most likely due to the immobilization method used, which involved adsorption of cellulases on non-porous and porous silica. Less rigid bonds are formed during adsorption as opposed to chemical cross-linking, resulting in increased conformational flexibility and thus retention of enzyme activity. Another critical factor for retention of enzyme activity is the physiochemical property of the support surface (Cao, 2005); S1 and S2 must have suitable surface properties that allow for retention of activity of the immobilized cellulases. Furthermore, considering this result and those in Table 1.1, it appears that the type of substrates used for hydrolysis seem to be an important factor in retaining high enzyme activities. All results that obtained 60% or higher retained enzyme activity used insoluble substrates such as filter paper, wheat straw, and microcrystalline cellulose (Table 1.1; Liang and Cao, 2012; Zhang *et al*., 2012; Xu *et al*., 2011b; Liao *et al*., 2010; and Tébéka *et al*., 2006). The sole exception was Xu *et al*.'s (2011a; Tabel 1.1.),

who immobilized cellulases on the magnetic nanoparticles by covalent method, using stem-exploded corn stalk and bagasse, resulting in retained enzyme activity was only 32%. The researchers immobilized cellulases directly on the magnetic nanoparticles without a coating, which may explain their relatively lower enzyme activity. In the interactions between insoluble substrates and immobilized cellulases, it is possible that the immobilized cellulase molecules behave like a cellulosome on the silica particles, resulting in synergistic enzymatic activity. Cellulosomes consist of non-enzymatic scaffolding proteins that are associated with various enzyme subunits and act in concert to degrade lignocellulosic materials (Doi *et al*., 2003). They enhance the efficiency of hydrolysis by preventing diffusion of enzyme molecules into the medium, and thereby facilitate controlled and synergistic hydrolysis (Bayer *et al*., 2000). They also protect the enzyme from product intermediates and feedback regulation, and enable transfer of enzyme complexes to other fractions of cellulose. In the current study, it is possible that the silica particles behaved like scaffolding proteins, providing a cellulosome-like environment that contributed to high hydrolysis yields.

The results of the sugar composition analysis of the hydrolysates showed that the total sugar and glucose production for C1S1 and C2S1 was similar to that of the corresponding free cellulases, but C1S2 and C2S2 produced less sugar, compared to free cellulases. Higher cellobiose accumulation in the immobilized

systems was found, compared to the free system, presumably due to possible inhibition of β-glucosidase activity in the cellulase cocktails or lack of immobilization of β-glucosidase.

The type of support significantly affected hydrolysis yield; this may be due to differences in substrate accessibility and the available surface area of S1 and S2. S1 features a three-dimensional network that increases available surface area; S2's two-dimensional structure, by contrast, may limit enzyme accessibility, as shown in Figure 4.1 below.

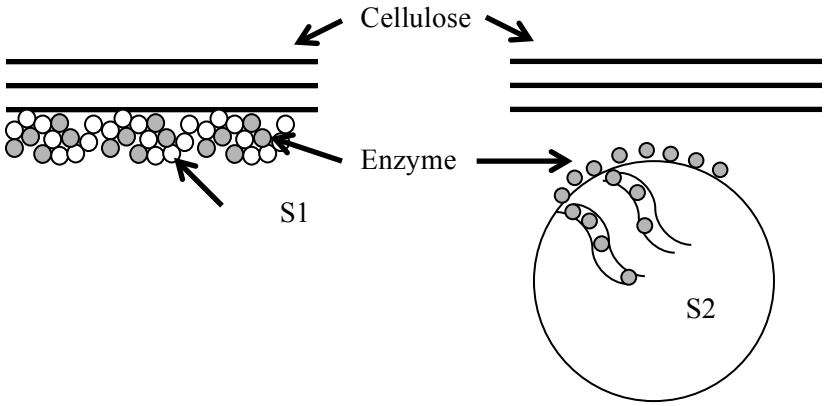


Fig. 4.1. Model of substrate interaction of immobilized cellulases on S1 and S2.

The size of the support has also been reported to affect the activity of immobilized enzymes; enzymes immobilized on smaller-sized supports retained high activity. Vertegel *et al.* (2004) found that lysozyme immobilized on 4 nm

particles obtained higher activity than on 100 nm particles because it formed a monolayer conformation that increased available surface area; on larger particles it formed a multilayer arrangement which caused protein aggregation. Likewise, Park *et al*. (2006) immobilized lipase on twelve different types of silica particles and found that higher activity was retained using smaller supports. This could be due to the fact that the surface of the smaller particles is more curved than that of the larger particles, allowing for conformational flexibility of the immobilized enzyme and preventing protein aggregation. The size of S1 is 7 nm and that of S2 is 60 μm, suggesting that the S1's smaller particle size may have contributed to its higher enzyme activity. In order to determine why the results from S1 and S2 differed, scanning electron microscopy (SEM) was performed.

4.5. Scanning Electron Microscopy

To visualize the difference in substrate accessibility between S1 and S2, SEM images were taken. The visuals revealed that the cellulose particles were completely surrounded by the three-dimensional matrix of S1, making them fully accessible to the cellulases. Furthermore, the silica network appears to be irregular and soft, which likely allows increased conformational flexibility of the immobilized cellulases. The high hydrolysis yields obtained with S1 can therefore be explained to be due to increased substrate accessibility and enzyme flexibility. By contrast, the surface of S2 was partially attached to the cellulose; the lower

hydrolysis yields obtained with S2 therefore can be explained to be due to decreased substrate accessibility. As expected, the immobilized cellulases were too small to be visualized by SEM; however, the SEM images clearly showed that cellulose interaction differed substantially between S1 and S2.

4.6. Protein Desorption

Although the immobilized cellulases using the adsorption method retained high enzyme activity, there was the risk that some enzymes might have escaped from the support during hydrolysis. Therefore, post-hydrolysis protein amounts were measured and compared to the negative controls to determine the amount of cellulase lost. The amounts were found to not be significantly different from the controls, meaning that proteins likely did not desorb during hydrolysis reactions. This is not unusual; other studies have shown that immobilization of enzymes on silica is robust. For example, Tébéka *et al*. (2009) studied the simple adsorption behavior of cellulases when immobilized on silica wafers and found that the interaction was strong. The adsorption energy of the interaction was determined to be 24.2 kJ/mol, which is comparable to the energy required for hydrogen bonding (20 kJ/mol) but not sufficient to disrupt the bond. Further, Wahlgren *et al.* (1995) studied adsorption kinetics of lysozyme immobilized on silica and concluded that the interaction between the enzyme and silica was strong.

4.7. Influence of Reaction Conditions on Stability Properties and Hydrolysis Products

Immobilizing enzymes on supports is beneficial; it not only extends enzymes' life spans and thereby reduces cost, but can also bring desirable changes to enzyme characteristics. For example, enzyme stability is enhanced because the native protein structure changes during immobilization (Afsahi *et al*., 2007; Dincér and Telefoncu, 2007; Cao, 2005). Stability properties depend on the carrier-enzyme interactions such as their binding nature, the chemical and physical structures of carriers, the microenvironments that the supports provide, and the freedom for enzyme conformational changes in the matrix (Cao, 2005). However, to achieve stability, a certain degree of enzymatic activity is lost. For example, Zhou *et al.* (2010) immobilized cellulases on N-succinyl-chitosan and achieved increased thermal stability, but at the expense of total enzyme activity, which decreased to 48.8%, compared to free cellulases. Occasionally, under appropriate conditions, suitable combinations between carriers and enzymes achieve stability while also retaining high enzyme activity (Cao, 2005). Cao (2005) stressed that it is critical to develop appropriate immobilization methods and find suitable combinations to obtain desirable properties. The current dissertation successfully developed two immobilized cellulase systems that obtain hydrolysis yields equivalent to a free cellulase system. In the following sections, the stability of the immobilized cellulases systems will be examined.

The stability experiments also revealed that the reaction conditions affected the sugar components of the hydrolysate. The substrate for cellulases is lignocellulose, which is heterogeneous in nature. It makes the hydrolysate composition more complex, potentially decreasing production rates and increasing operational costs in subsequent fermentation and purification stages in industrial settings. For example, sugar solutions from biomass contain cellobiose, xylose, and various oligosaccharides from cellulose and hemicellulose fractions in addition to glucose (Park, *et al*., 2004). The presence of other oligosaccharides can cause a feedback inhibition that reduce can reduce production yields in a hydrolysis process (Yue *et al*., 2004). Additionally, bacteria and yeasts often ignore oligosaccharides in a hydrolysate without utilizing them because they lack the metabolic capability to use these carbon sources. Some microorganisms, including fungi, can utilize oligosaccharides and pentose sugars, but digestion of oligosaccharides presumably reduces production rates, and pentose sugars may alter the microorganisms' metabolic pathways, reducing the production of desirable products and generating byproducts which complicate down-stream purification processes (Park *et al.,* 2004). Therefore, sugar compositions of hydrolysates have to be carefully monitored before fermentation, and their suitability to the target microorganisms should be taken into account. For these reasons, individual sugar content in hydrolysates must be examined to maximize subsequent value-added chemical production. Cao (2005) described

microenvironments in which exposure to immobilized enzymes affected enzyme properties; in other words, hydrolysis conditions, including reaction conditions and enzyme conditions, may change sugar profiles in hydrolysates.

In the following sections, hydrolysis conditions including temperature, pH, ionic strength, storage duration, and recycling stages are examined. The objectives are to examine the stability properties that the immobilized cellulases obtained and to investigate how reaction conditions influenced their product compositions.

Both free C1 and C2 were able to efficiently hydrolyze cellulose at a wide range of temperatures, even at 60°C; however, immobilized cellulases' hydrolysis activities at 60°C were reduced, meaning that immobilization reduced their heat resistance capacity. Thermal stability is controlled by non-catalytic parts of the protein (Cao, 2005); the absorption process on the silica particles' surface might damage the protein fractions that regulate thermal stability. On the contrary, many researchers reported that immobilization increased thermal stability (Table 1.1; Liang and Cao, 2012; Yu *et al.*, 2012; Xu *et al*., 2011a; Zhou, 2010; Afsahi *et al*., 2007). This is likely because their immobilization forces were covalent bonds. Covalent bonds create rigid bindings between support and enzyme (Cao, 2005); these bindings may protect the enzymes from heat denaturation. In this study, the bonds were generated by the adsorption method and may not have been sufficiently strong to maintain the protein structure under the effect of heat. In

addition, the sugar composition analysis in this study showed that thermal stability depended on a support-enzyme combination. Higher temperature resulted in less cellobiose produced, meaning that the activity of β-glucosidases might be accelerated at high temperature.

The pH condition directly correlates with the required amounts of acid or base, which is ultimately reflected in product cost. Also, the usage of harsh chemicals increases environmental burden. In this section, the influence of immobilization on pH stability and product composition will be discussed.

Immobilization did not affect the optimum pH of the cellulases; it remained identical to that of the free cellulases. Sandwick and Schray (1988) mentioned that if the enzyme's conformation in the enzyme-support conjugate resembles that of native enzymes, the support likely stabilizes the enzyme without altering the properties. However, C1S1 retained activity as high as pH 8.0, meaning its pH stability was enhanced. Guisán (1988) stated that tightening the enzyme conformation as a result of multipoint attachment sometimes enhanced stability; therefore, the bindings between C1 and S1 might protect the enzyme conformation from denaturation in the neutral pH, resulting in the improved pH stability.

Increased pH stability due to immobilization generally comes at the cost of reduced enzyme activity (Cao, 2005). For instance, Dinçer and Telefoncu (2007;

Table 1.1.) immobilized cellulases on chitosan beads coated with modified polyvinyl alcohol using an entrapment method. The pH optima shifted from 4.0 to 7.0, although enzymatic activity fell to 87% of free enzymes. The current project, however, has demonstrated that C1S1 retained high activity while gaining pH stability. Improved pH stability could be greatly beneficial in a production environment where careful pH balance is difficult or costly to maintain. However, not all results were positive. Cellobiose production by C2S1 increased when pH increased outside of the optima, suggesting that the immobilization inhibited β-glucosidase or that β-glucosidase did not bind to silica.

In industrial settings, substrates might contain a high amount of salts from the pretreatment processes. Therefore, it is important to know the extent to which immobilized cellulases can resist salt solutions. C1S2 possesses a higher resistance against high ionic strength than C1S1; its ionic strength stability was not affected by immobilization. Immobilization on S1, however, resulted in a decrease in sugar yield at high salt concentrations, possibly due to modification of C1's protein fractions that regulate ionic strength resistance. C2, by contrast, naturally loses activity at the high ionic strength, and the same tendency was observed in C2S1, meaning that immobilization did not affect the stability of ionic strength. C2S2 performed the worst, with stability loss even at low ionic strengths; total hydrolysis values decreased at 0.05 M and 0.4 M. As shown in

Table 1.1, none of researchers reported positive results of ionic stability. Thus, stability might be relatively difficult to maintain.

In the sugar composition analysis, free C1 relatively maintained constant individual sugar production, but immobilized C1 on S1 and S2 showed higher oligosaccharide and cellobiose values as ionic strength increased. When protein configurations at non-catalytic sites of enzymes are modified, stability is often disrupted (Cao, 2005). Thus, the interaction between the silica and the individual enzymes, which decompose oligosaccharides to monosaccharides, might have altered the protein structures which control salt resistance of those enzymes. In the case of C2 enzymes, free C2 only produced glucose, but the immobilized cellulases produced glucose and cellobiose. Thus, the immobilization might disrupt the enzymes that convert cellobiose to glucose as well.

Industrial application requires that pre-prepared immobilized cellulases maintain activity during storage to achieve high production. Other studies have demonstrated that immobilized cellulases retain activity levels longer than free cellulases. For example, Andriani *et al.* (2012) immobilized cellulases from *Bacillus subtillis* TD6 on calcium alginate beads. They found that the immobilized cellulases could be stored at 4°C for 12 days without losing enzyme activity. Ince *et al.* (2012) compared stored free cellulases with immobilized cellulases which were on polyanilline coated poly(styrene-divinylbenzene) grafted

polysterene for 8 weeks. The free cellulases completely lost enzyme activity while the immobilized cellulases maintained 47% of the original enzyme activity. Xu *et al.* (2011a) directly immobilized cellulases on magnetic nanoparticles and retained 70% enzyme activity for 21 days (Table 1.1.). Likewise, the results of the storage experiments here showed that both C1 and C2 enzymes maintained consistent hydrolysis yields and sugar compositions throughout the whole storage duration period in this experiment, with no significant change in hydrolysis yield after being stored for 3 weeks at 4°C, which is a relatively favorable result compared to other studies. A future experiment that discovers the absolute storage limit of immobilized enzymes at room temperature would be extremely useful for industrial applications, which can benefit greatly from mass-prepared, long-term storable reagents.

Finally, the greatest advantage of immobilization is that it enables enzymes to be reused, which is a feature that free enzymes do not normally provide. Immobilization reduces the total quantity of enzymes that have to be used, extends their life span, and ultimately reduces production cost. All immobilized cellulases tested here improved reusability. C1S1 had the lowest loss of activity per recycle of the four systems and retained approximately 28% of initial activity level even at the 9^{th} cycle, which is a desirable characteristic for continuous operations of a long duration. These results are in line with several

reports that have looked at enzyme reusability. Wu *et al*. (2005) examined immobilized cellulases on a PVA membrane treated with glutaraldehyde vapour. The enzymes retained 36% of the initial activity at the 6th cycle. Liao *et al*. (2010; Table 1.1.) immobilized cellulase R-10 on PVA/Fe_2O_3 magnetic nanoparticles, and the enzyme retained 40% of its initial activity level at the 4th cycle. Compared to those studies, enzyme reusability obtained in this research was favourable. Although several studies reported higher reusability, the experimental conditions were substantially different from this study. For example, Tébéka *et al*., (2009; Table 1.1.) found that immobilized cellulases originating from *Trichoderma viride* immobilized on silica wafers maintained 100% of the initial activity for 6 cycles; however, the reaction period was 0.5 hours, which differed substantially from the reaction period in this study. Alahakoon *et al*. (2012; Table 1.1.) immobilized the cellulase cocktail on magnetic particles and maintained approximately 76% of the initial activity for 10 cycles; however, their cycles for enzyme reuse were at 4 °C for 24 h without substrate. Because the conditions were so different, direct comparison is impossible.

Important to note is that the sugar compositions of the hydrolysis changed during recycling with the enzymes immobilized on S2. Therefore, the hydrolysis product composition analysis was essential. Sugar compositions did not change with S1.

4.8. Influence of Substrates on Hydrolysis Yields and Hydrolysis Products Using Immobilized Cellulases with Highly Retained Enzymatic Activity

There are many studies that have reported on immobilized cellulases, but the majority used pure substrates such as microcrystalline cellulose or carboxymethyl (CMC) cellulose to evaluate the retained enzymatic activities of immobilize cellulases. However, it is well known that the hydrolysis abilities of cellulases depend on the characteristics of the substrate (Kabel *et al*., 2006). Indeed, Mandali and Mandali (2010) reported that their immobilized cellulases on glass beads showed different retained activities on various substrates. For example, the retained activities of the immobilized cellulases on Avicel were 43-61%. However, those on xylan and corn stover were 8.0-19% and 7-14%, respectively.

In this experiment, five pure substrates and two types of crude lignocellulose biomass were used: microcrystalline cellulose and crystalline cellulose II to represent the crystalline regions of cellulose fiber; commercial cellophane paper and phosphoric acid swollen cellulose (PASC) for that of amorphous regions; xylan for that of the hemicellulose fraction; and wood and waste office automation (OA) paper for a crude lignocellulose biomass. The reason why wood was selected as an example of lignocellulose was because

Canada is one of the world's largest forestry product producers. The net profit for exports of wood products exceeds $17 billion each year, second only to oil and gas exports (2012, Forest Products Association, Ottawa, ON, Canada). Harvesting and processing wood generates a significant amount of available cellulose resources, including sawdust and sustainably harvested wood (Kumar *et al*., 2009a).

Paper represents another potential lignocellulose biomass because the pulp and paper industry is also one of Canada's important industries. According to the Forest Products Association, the annual export of pulp was $7.5 billion and paper was $9.8 billion (Ottawa, ON, Canada, 2012). Producing pulp and paper generates a significant amount of paper sludge waste (Lou *et al*., 2012). Paper sludge contains approximately 25% cellulose fiber, 10% clay, and 65% water (Prasetyo, Kato, and Park, 2010). It is traditionally landfilled for its disposal. However, as public awareness of environmental issues increases, landfill becomes problematic. Therefore, researchers have started to seek an alternative use for paper sludge. For example, some researchers focused on the high cellulose content of the paper sludge, which can be a suitable material for biorefineries (Prasetyo, Kato, and Park, 2010; Kerstetter *et al*., 1997).

The objectives of this study were to examine the hydrolysis of the immobilized cellulases on different substrates using seven different materials:

microcrystalline cellulose, crystalline cellulose, commercial cellophane paper, phosphoric acid swollen cellulose (PASC), xylan, steam-exploded wood, and waste office automation paper.

Crystalline cellulose was examined as a control for the rest of the experiments. In microcrystalline cellulose and crystalline cellulose II hydrolysis, C1S1 and C2S1 retained either higher or equivalent yields levels compared with their respective free forms (C1 or C2). This result corresponded with the results of the previous section (Figure 3.4.1), meaning that its reproducibility was high. These results determined that the immobilized cellulases were suitable for crystalline cellulose hydrolysis. Because immobilization processes often compromise the enzymatic activities (Zhou, 2010; Afsahi *et al*., 2007), the discovery that immobilized cellulases retain the full efficiency of their free forms is especially noteworthy.

The hydrolysis product analysis from microcrystalline cellulose showed that the support type affected glucose production: immobilized cellulases on S1 tended to produce higher amounts of glucose than those on S2. This is likely based on the different characteristics of the silica particles between S1 (non-porous small particles forming a 3D matrix) and S2 (large particles with porous surfaces), which directly influence substrate accessibility. In both crystalline cellulose hydrolyses, the immobilized cellulases produced relatively higher

amounts of cellobiose, indicating that the immobilization might have negatively affected the cellobiose conversion (detailed discussion in 4.5.).

Because the immobilized cellulases could hydrolyze crystalline cellulose well, which is more difficult to be digested than amorphous cellulose, I expected that the immobilized cellulases could also hydrolyze amorphous cellulose well. However, the immobilized cellulases did not hydrolyze the amorphous celluloses as efficiently as the free cellulases. This is because of the characteristics of amorphous cellulose's structure: compared to crystalline cellulose, cellulose chains in amorphous regions are loosely packed and have fewer hydrogen bonds. The immobilized cellulases might have had difficulty to move around on the loosely packed chains. Another possibility is that the immobilization may have modified the protein structure of the enzymes that cleave the amorphous region.

Another result was that the cellobiose conversions to glucose tended to be lower with the immobilized cellulases; immobilization may disrupt the enzymes that convert cellobiose to glucose, or the enzyme desorbed from the support. Whatever the reason, the immobilization systems or the reaction conditions might have to be modified and optimized when amorphous cellulose is used as a substrate. Future experiments determining the reason behind the poor digestion of amorphous cellulose and the reduced cellobiose conversion would be greatly beneficial.

Next, the effects of hemicellulose on the immobilized cellulases were examined, using xylan as a model substrate of hemicellulose. Xylan is one of the major hemicellulose compounds, existing along with and penetrating cellulose microfibers. Steam-exploded poplar and shredded waste OA paper contain approximately 25.44% and 10.4% hemicellulose fractions (Mascoma Canada Inc., ON, Canada; Sosulski, 1993).

Hemicellulose must normally be removed before cellulose degradation to allow cellulases to attack cellulose fibers. Thus, examining hemicellulose hydrolysis is important to investigate to properly evaluate the ability of the immobilized cellulases.

Both immobilized C1 and C2 demonstrated lower xylan hydrolysis yields than their free forms, except C1S1. Immobilization may modify the hemicellulases activity to cleave xylan. Hemicellulose also has an amorphous structure, which was shown above to be difficult for the immobilized cellulases to cleave (Section 4.8.2.). Some reports in the literature have reported xylan hydrolysis using immobilized lignocellulose decomposing enzyme cocktails with similar results to those shown here. For example, Xu *et al.* (2011b) reported that the immobilized cellulase cocktail (NS50013, NOVOZYMES) on Eudragit L-100 (Evonik Degussa Investment) retained 59% of xylanase activity. Also, Sardar *et al*. (2000) immobilized xylanases originating from *Aspergillus niger* on Eudragit

(TM) L-100 and hydrolyzed xylan. The retained specific enzyme activity of the xylanase was 60%. An ideal enzyme cocktail, which efficiently hydrolyzes both cellulose and hemicellulose, must be developed.

Finally, the influence of actual lignocellulose biomass was examined. Wood and paper were selected as target lignocellulose biomasses for industrial purposes. Steam-exploded poplar samples and shredded office automation (OA) paper were also applied as a substrate to represent wood and paper samples.

In the steam-exploded poplar and waste OA paper hydrolyses, the yields from all the immobilized cellulase systems were significantly lower than from the free cellulases. The poplar sample contained both amorphous and crystalline cellulose fractions; Section 4.8.1. and 4.8.2 discussed how the immobilized cellulases efficiently hydrolyzed crystalline regions, but not amorphous regions. Therefore, the lower hydrolysis ability of immobilized C1 and C2 in this experiment might be a result of inefficient hydrolysis of the amorphous fraction. However, the retained enzyme activities were higher, compared to Xu *et al*.'s (2011a) (32.9%; Table 1.1.). That group used steam-exploded corn stalks and bagasse; the different substrates may explain differences in retained enzyme activity. In addition, the hydrolysis product composition analysis indicates that all immobilized cellulase systems hydrolyzed the hemicellulose fraction as efficiently as their corresponding free cellulases, a result that conflicts with the result from the

hemicellulose hydrolysis experiment. This might be because the amount of hemicellulose in the wood samples was much lower than that in xylan hydrolysis experiment, or because the chain length of hemicellulose fractions in lignocellulose biomass may be much shorter than that of raw xylan. In any case, the results show that the hydrolysis for the lignocellulose has to be optimized, with more attention to successfully hydrolyzing amorphous cellulose.

5. Conclusion

Cellulase immobilization, particularly immobilization on magnetic particles, can greatly enhance the economic feasibility for industrial bio-applications by allowing the recycling of expensive enzymes, enabling the ability to mass-produce enzyme supports and store them until needed, reducing costs. However, the interactions of enzymes on support surfaces typically result in the loss of enzymatic activity, making immobilization impractical. The goals of this dissertation were to develop improved immobilization methods and enzyme-support combinations that retain high enzymatic activities for future applications. Understanding the characteristics of the immobilized cellulases, including production stability, yields, and sugar compositions under varied hydrolysis conditions was another feature of this study. The results add a lot to our knowledge of hydrolysis using immobilized cellulases.

Twelve combinations of enzymes & supports were tested with various immobilization methods to discover the best combination. The results showed that weak interactions between the enzymes and supports were preferable because strong cross-linking did not allow the active site of the enzymes to be flexible. Supports with strong charges were shown to be detrimental, repulsing the enzymes or interfering with their catalytic sites. From these observations, four suitable systems were selected for the further experiments using the adsorption method: C1S1 (cellulase cocktail 1 immobilized on fumed non-porous silica), C1S2 (cellulase cocktail 1 on porous silica, C2S1(cellulase cocktail 2 on fumed non-porous silica.) and C2S2 (cellulase cocktail 2 on porous silica).

Next, the characteristics of the immobilized cellulases under the following conditions were carefully examined: pH, ionic strength, and, once the optimal conditions were found, optimal enzyme-support ratios were determined. After extensive experimentation, the four immobilized cellulases achieved approximately 90% loading efficiency. Two of the systems, C1S1 and C2S1, achieved levels of hydrolysis as high as their corresponding free cellulase controls, which has never been achieved in other studies, proving that cellulases on a silica support can retain high enzymatic activity. This is extremely promising for future applications, since magnetic particles are often coated with silica.

Stability of immobilized enzyme systems is a challenge for researchers. Additional stability was achieved in one enzyme system, C1S1, which gained a wider range of pH stability. The immobilized cellulases lost certain ranges of thermal stability and ionic stability but appropriate support-enzyme combinations mitigated the loss. Immobilization did not affect storage stability. The most impressive result of the stability tests was that all the immobilized systems gained reusability. The best result was with C1S1, which still possessed 30% of its original activity even after the 9^{th} cycle. This result is extremely promising for future industrial applications, where enzyme cost could be greatly reduced by reusing enzymes. One very important finding of the stability experiments is that cellulase-support combinations can greatly affect enzyme activity, which changes the sugar compositions produced during hydrolysis. Most studies tend to measure total sugar production; this study also carefully measured the types of sugar produced and found that under some conditions, significant levels of non-glucose sugars were produced. Because these sugars could require an extra processing step in a production environment, it is critical that further research carefully consider not only the total sugar produced, but the quality of the sugar products as well.

Because hydrolysis abilities depend on the characteristics of the substrate (Kabel *et al*., 2006), the effects of substrate on the immobilized cellulases were

examined. Most research has measured immobilized enzyme activity on a model substrate; this paper took a different approach and tested real-world substrates. The results showed that C1S1and C2S1 hydrolyzed crystalline cellulose as efficiently as their corresponding free cellulases. However, when tested on substrates that contained amorphous cellulose or lignocellulose, hydrolysis activity dropped. While the results were promising, it is clear that optimization will be necessary to deal with amorphous cellulose and lignocellulose, which is likely in a real-world scenario. The immobilized cellulases that were developed during the process of writing this thesis have advanced the potential to make hydrolysis and pretreatment of lignocellulose more commercially viable.

6. Future Plans

The observations of hydrolysis product compositions in this dissertation suggested that the immobilization altered individual enzyme activities, with the result that glucose conversion was reduced. One future project I would like to undertake is to compare the actual individual cellulose activities of free and immobilized cellulases to explain these observations. Such a comparison would reveal exactly which individual enzymes were affected by the immobilization process and how their enzymatic activities were altered and which binding would be better.

This research resulted in successfully creating two types of immobilized cellulase suitable for use in hydrolysis reactions. A good next step would be to apply this immobilization method on silica-coated magnetic particles that could be recovered, cleaned, and reused for further reactions, greatly improving the reusability of the enzymes and moving the process a step closer to commercial viability.

7. References

Afsahi, B., Kazemi, A., Kheirolomoom, A., Nejati, S., 2007. Immobilization of cellulase on non-porous ultrafine silica particles. Scientia Iranica. 14(4), 379-383.

Agbor, V. B., Cicek, N., Sparling, R., Berlin, A., Levin, D. B., 2011. Biomass pretreatment: Fundamentals toward application. Biotechnology Advances. 29(6), 675-685.

Ahn, K. W., Ye, S. H., Chun, W. H., Rah, H., Kim, S. G., 2011. Yield and component distribution of biodiesel by methanolysis of soybean oil with lipase-immobilized mesoporous silica. Microporous and Mesoporous Materials. 142(1), 37-44.

Alahakoon, T., Koh, J. W., Chong, X. W. C., Lim, W. T. L., 2012. Immobilization of cellulases on amine and aldehyde functionalized Fe_2O_3 magnetic nanoparticles. Preparative Biochemistry and Biotechnology. 42(3), 234-248.

Almeida, J. R. M., Modig, T., Petersson, A., Hähn-Hägerdal, B., Lidén, G., Gorwa-Grauslund, M. F., 2007. Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *saccharomyces cerevisiae*. Journal of Chemical Technology and Biotechnology. 82(4), 340-349.

Andriani, D., Sunwoo, C., Ryu, H. W., Prasetya, B., Park, D. H., 2011. Immobilization of cellulase from newly isolated strain bacillus subtilis TD6 using calcium alginate as a support material. Bioprocess and Biosystems Engineering., 35(1-2), 29-33.

Antunes, S. C., Pereira, R., Marques, S. M., Castro, B. B., Gonçalves, F., 2011. Impaired microbial activity caused by metal pollution: A field study in a deactivated uranium mining area. Science of the Total Environment. 410-411, 87-95.

Aspinall, G. O., 1959. Structural chemistry of the hemicelluloses. Advances in Carbohydrate Chemistry.14, C, 429-468.

Baker, R. W., 2010. Research needs in the membrane separation industry: Looking back, looking forward. Journal of Membrane Science. 362(1-2), 134-136.

Ballesteros, I., Ballesteros, M., CabaÑas, A., Carrasco, J., MartÍn, C., Negro, M. J., Saez, F., Saez, R., 1991. Selection of thermotolerant yeasts for simultaneous saccharification and fermentation (SSF) of cellulose to ethanol. Applied Biochemistry and Biotechnology. 28-29(1), 307-315.

Ballesteros, M., Oliva, J. M., Negro, M. J., Manzanares, P., Ballesteros, I., 2004. Ethanol from lignocellulosic materials by a simultaneous saccharification and fermentation process (SFS) with *Kluyveromyces marxianus* CECT 10875. Process Biochemistry. 39(12), 1843-1848.

Banerjee, N., Bhatnagar, R., Viswanathan, L., 1981. Inhibition of glycolysis by furfural in saccharomyces cerevisiae. European Journal of Applied Microbiology and Biotechnology. 11(4), 226-228.

Barr, B. K., Hsieh, Y. L., Ganem, B., Wilson, D. B., 1996. Identification of two functionally different classes of exocellulases. Biochemistry. 35(2), 586-592.

Bauer, B. E., Rossington, D., Mollapour, M., Mamnun, Y., Kuchler, K., Piper, P. W., 2003. Weak organic acid stress inhibits aromatic amino acid uptake by

yeast, causing a strong influence of amino acid auxotrophies on the phenotypes of membrane transporter mutants. European Journal of Biochemistry. 270(15), 3189-3195.

Baugh, K. D., Levy, J. A., McCarty, P. L., 1988. Thermochemical pretreatment of lignocellulose to enhance methane fermentation: II. Evaluation and pretreatment model. Biotechnology and Bioengineering. 31(1), 62-70.

Bayer, E. A., Shoham, Y., Lamed, R., 2000. The cellulosome, and exocellular organelle for degrading plan cell wall polysaccharides, Glycomicrobiology. Kluwer Academic/Plenum Publishers. NY, USA, pp. 387-440.

Becker, D., Braet, C., Brumer III, H., Claeyssens, M., Divne, C., Fagerström, B. R., Harris, M., Jones, T. A., Kleywegt, G. J., Koivula, A., 2001. Engineering of a glycosidase family 7 cellobiohydrolase to more alkaline pH optimum: The pH behaviour of trichoderma reesei Cel7A and its E223S/A224H/L225V/T226A/D262G mutant. Biochemical Journal. 356(1), 19-30.

Berlin, A., Gilkes, N., Kurabi, A., Bura, R., Tu, M., Kilburn, D., Saddler, J., 2005. Weak lignin-binding enzymes: A novel approach to improve activity of cellulases for hydrolysis of lignocellulosics. Applied Biochemistry and

Biotechnology - Part A Enzyme Engineering and Biotechnology. 121(1-3), 163-170.

Berry, C. C., Wells, S., Charles, S., Curtis, A. S. G., 2003. Potential drug-cell delivery routes using magnetic nanoparticles. European Cells and Materials. 6(SUPPL. 2), 29.

Beukes, N., Pletschke, B. I., 2006. Effect of sulfur-containing compounds on bacillus cellulosome-associated 'CMCase' and 'avicelase' activities. FEMS Microbiology Letters. 264(2), 226-231.

Bin, Y., Hongzhang, C., 2010. Effect of the ash on enzymatic hydrolysis of steam-exploded rice straw. Bioresource Technology. 101(23), 9114-9119.

Blair, B. G., Anderson, K. L., 1999. Regulation of cellulose-inducible structures of *clostridium cellulovorans*. Canadian Journal of Microbiology. 45(3), 242-249.

Brownell, H. H., Saddler, J. N., 1987. Steam pretreatment of lignocellulosic material for enhanced enzymatic hydrolysis. Biotechnology and Bioengineering. 29(2), 228-235.

Brownell, H. H., Yu, E. K. C., Saddler, J. N., 1986. Steam-explosion pretreatment of wood: Effect of chip size, acid, moisture content and pressure drop. Biotechnology and Bioengineering. 28(6), 792-801.

Bura, R., Mansfield, S. D., Saddler, J. N., Bothast, R. J., 2002. SO_2-catalyzed steam explosion of corn fiber for ethanol production. Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology. 98-100, 59-72.

Bura, R., Saddler Sr., J. N. (2004). Process modifications of SO_2-catalysed steam explosion of corn fibre for ethanol production. ACS National Meeting Book of Abstracts*, 227*(1) CELL-40.

Cadoche, L., López, G. D., 1989. Assessment of size reduction as a preliminary step in the production of ethanol from lignocellulosic wastes. Biological Wastes. 30(2), 153-157.

Cao, L., 2005. Adsorption-based immobilization, Carrier-bound immobilized enzymes. 1st ed., WILEY-VCH Verlag GmbH & Co.KGaA, Weinhein. Strauss GmbH, Morlenbach, Germany, pp. 53.

Cappelletti, P., Rapisardo, G., De Gennaro, B., Colella, A., Langella, A., Graziano, S. F., Bish, D. L., De Gennaro, M., 2011. Immobilization of Cs

and Sr in aluminosilicate matrices derived from natural zeolites. Journal of Nuclear Materials. 414(3), 451-457.

Cara, C., Moya, M., Ballesteros, I., Negro, M. J., González, A., Ruiz, E., 2007. Influence of solid loading on enzymatic hydrolysis of steam exploded or liquid hot water pretreated olive tree biomass. Process Biochemistry. 42(6), 1003-1009.

Carvalheiro, F., Duarte, L. C., Gírio, F. M., 2008. Hemicellulose biorefineries: A review on biomass pretreatments. Journal of Scientific and Industrial Research. 67(11), 849-864.

Chandra, R. P., Bura, R., Mabee, W. E., Berlin, A., Pan, X., Saddler, J. N., 2007. Substrate pretreatment: The key to effective enzymatic hydrolysis of lignocellulosics? Advances in Biochemical Engineering/Biotechnology, 108, 67-93.

Chang, V. S., Holtzapple, M. T., 2000. Fundamental factors affecting biomass enzymatic reactivity. Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology. 84-86, 5-37.

Chang, V. S., Nagwani, M., Kim, C. H., Holtzapple, M. T., 2001. Oxidative lime pretreatment of high-lignin biomass: Poplar wood and newspaper. Applied

Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology. 94(1), 1-28.

Chen, H., Han, Y., Xu, J., 2008. Simultaneous saccharification and fermentation of steam exploded wheat straw pretreated with alkaline peroxide. Process Biochemistry. 43(12), 1462-1466.

Cho, E. J., Jung, S., Kim, H. J., Lee, Y. G., Nam, K. C., Lee, H. J., Bae, H. J., 2012. Co-immobilization of three cellulases on au-doped magnetic silica nanoparticles for the degradation of cellulose. Chemical Communications. 48(6), 886-888.

Clesceri, L. S., Sinitsyn, A. P., Saunders, A. M., Bungay, H. R., 1985. Recycle of the cellulase- enzyme complex after hydrolysis of steam-exploded wood. Applied Biochemistry and Biotechnology. 11(6), 433-443.

Colombo, M., Carregal-Romero, S., Casula, M. F., Gutiérrez, L., Morales, M. P., Böhm, I. B., Heverhagen, J. T., Prosperi, D., Parak, W. J., 2012. Biological applications of magnetic nanoparticles. Chemical Society Reviews. 41(11), 4306-4334.

Coutinho, P. M., & Henrissat, B. (2011). Carbohydrate-active enZymes server. Retrieved 06/07, 2011, from http://www.cazy.org/

Cruz, J. M., Domínguez, J. M., Domínguez, H., Parajó, J. C., 2001. Antioxidant and antimicrobial effects of extracts from hydrolysates of lignocellulosic materials. Journal of Agricultural and Food Chemistry. 49(5), 2459-2464.

Da Silveira Dos Santos, D., Camelo, A. C., Rodrigues, K. C. P., Carlos, L. C., Pereira Jr., N., 2010. Ethanol production from sugarcane bagasse by *Zymomonas mobilis* using simultaneous saccharification and fermentation (SSF) process. Applied Biochemistry and Biotechnology. 161(1-8), 93-105.

Davies, G., Henrissat, B., 1995. Structures and mechanisms of glycosyl hydrolases. Structure. 3(9), 853-859.

Davin, L. B., Jourdes, M., Patten, A. M., Kim, K. W., Vassão, D. G., Lewis, N. G., 2008. Dissection of lignin macromolecular configuration and assembly: Comparison to related biochemical processes in allyl/propenyl phenol and lignan biosynthesis. Natural Product Reports. 25(6), 1015-1090.

Deshpande, M. V., Eriksson, K. E., 1984. Reutilization of enzymes for saccharification of lignocellulosic materials. Enzyme and Microbial Technology. 6(8), 338-340.

Desvaux, M. 2005. The cellulosome of *Clostridium cellulolyticum*. Enzyme and Microbial Technology. 37(4), 373-385.

Dimmel, D., 2010. Overview, in: Heitner, C., Dimmel, D.Schmidt, J. A. (Eds.), Lignin and lignans, advances in chemistry. 1st ed., CRP Press, Taylor & Francis Group. FL. USA, pp. 1-10.

Din, N., Gilkes, N. R., Tekant, B., Miller Jr., R. C., Anthony, R., Warren, J., Kilburn, D. G., 1991. Non-hydrolytic disruption of cellulose fibres by the binding domain of a bacterial cellulase. Nature Biotechnology. 9(11), 1096-1099.

Dinçer, A., Telefoncu, A., 2007. Improving the stability of cellulase by immobilization on modified polyvinyl alcohol coated chitosan beads. Journal of Molecular Catalysis B: Enzymatic. 45(1-2), 10-14.

Ding, S. Y., Xu, Q., Crowley, M., Zeng, Y., Nimlos, M., Lamed, R., Bayer, E. A., Himmel, M. E., 2008. A biophysical perspective on the cellulosome: New opportunities for biomass conversion. Current Opinion in Biotechnology. 19(3), 218-227.

Divne, C., Ståhlberg, J., Reinikainen, T., Ruohonen, L., Pettersson, G., Knowles, J. K. C., Teeri, T. T., Jones, T. A., 1994. The three-dimensional crystal structure of the catalytic core of cellobiohydrolase I from *Trichoderma reesei*. Science. 265(5171), 524-528.

Divne, C., Ståhlberg, J., Teeri, T. T., Jones, T. A., 1998. High-resolution crystal structures reveal how a cellulose chain is bound in the 50 Å long tunnel of cellobiohydrolase I from *Trichoderma reesei*. Journal of Molecular Biology. 275(2), 309-325.

Doherty, W. O. S., Mousavioun, P., Fellows, C. M., 2011. Value-adding to cellulosic ethanol: Lignin polymers. Industrial Crops and Products. 33(2), 259-276.

Doi, R. H., 2008. Cellulases of mesophilic microorganisms: Cellulosome and noncellulosome producers. Annals of the New York Academy of Sciences, 1125, 267-279.

Doi, R. H., Kosugi, A., Murashima, K., Tamaru, Y., Han, S. O., 2003. Cellulosomes from mesophilic bacteria. Journal of Bacteriology. 185(20), 5907-5914.

Doi, R. H., Tamaru, Y., 2001. The *Clostridium cellulovorans* cellulosome: An enzyme complex with plant cell wall degrading activity. Chemical Records. 1(1), 24-32.

Dong, X. M., Revol, J. F., Gray, D. G., 1998. Effect of microcrystallite preparation conditions on the formation of colloid crystals of cellulose. Cellulose. 5(1), 19-32.

Donohoe, B. S., Decker, S. R., Tucker, M. P., Himmel, M. E., Vinzant, T. B., 2008. Visualizing lignin coalescence and migration through maize cell walls following thermochemical pretreatment. Biotechnology and Bioengineering. 101(5), 913-925.

Eklund, R., Zacchi, G., 1995. Simultaneous saccharification and fermentation of steam-pretreated willow. Enzyme and Microbial Technology. 17(3), 255-259.

El-Aassar, M. R. 2013. Functionalized electrospun nanofibers from poly (AN-co-MMA) for enzyme immobilization. Journal of Molecular Catalysis B: Enzymatic. 85-86, 140-148.

El-Ghaffar, M. A. A., Hashem, M. S., 2010. Chitosan and its amino acids condensation adducts as reactive natural polymer supports for cellulase immobilization. Carbohydrate Polymers. 81(3), 507-516.

Eriksson, T., Börjesson, J., Tjerneld, F., 2002. Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. Enzyme and Microbial Technology. 31(3), 353-364.

Fan, G., Xu, Y., Zhang, X., Lei, S., Yang, S., Pan, S., 2011. Characteristics of immobilized β-glucosidase and its effect on bound volatile compounds in orange juice. International Journal of Food Science and Technology. 46(11), 2312-2320.

Fan, Z., South, C., Lyford, K., Munsie, J., Van Walsum, P., Lynd, L. R., 2003. Conversion of paper sludge to ethanol in a semicontinuous solids-fed reactor. Bioprocess and Biosystems Engineering. 26(2), 93-101.

Feng, T., Du, Y., Yang, J., Li, J., Shi, X., 2006. Immobilization of a nonspecific chitosan hydrolytic enzyme for application in preparation of water-soluble low-molecular-weight chitosan. Journal of Applied Polymer Science. 101(3), 1334-1339.

Feng, X., Patterson, D. A., Balaban, M., Emanuelsson, E. A. C., 2012. Enabling the utilization of wool as an enzyme support: Enhancing the activity and stability of lipase immobilized onto woolen cloth. Colloids and Surfaces B: Biointerfaces. 102, 526-533.

Forest Products Association of Canada, 2012. The forest products industry in Canada. Retrieved Feb/15, 2012, accessed June 2013. http://www.fpac.ca.login.ezproxy.library.ualberta.ca/index.php/en/

Gal, L., Pages, S., Gaudin, C., Belaich, A., Reverbel-Leroy, C., Tardif, C., Belaich, J. P., 1997. Characterization of the cellulolytic complex (cellulosome) produced by *Clostridium cellulolyticum*. Applied and Environmental Microbiology. 63(3), 903-909.

Galbe, M., & Zacchi, G., 2007. Pretreatment of lignocellulosic materials for efficient bioethanol production, Advances in Biochemical Engineering/Biotechnology, 108(3), 41-65.

Ge, S., Shi, X., Sun, K., Li, C., Uher, C., Baker Jr., J. R., Banaszak Holl, M. M., Orr, B. G., 2009. Facile hydrothermal synthesis of iron oxide nanoparticles with tunable magnetic properties. Journal of Physical Chemistry C. 113(31), 13593-13599.

George, R. A., Heringa, J., 2002. An analysis of protein domain linkers: Their classification and role in protein folding. Protein Engineering. 15(11), 871-879.

Gerbi, C., Bata, J., Breton, A., Prensier, G., 1996. Glycoside and polysaccharide hydrolase activity of the rumen anaerobic fungus caecomyces communis (sphaeromonas communis SENSU ORPIN) at early and final stages of the developmental cycle. Current Microbiology. 32(5), 256-259.

Ghose, T. K. 1987. Measurement of cellulase activities. Pure and Applied Chemistry, 59(2), 257-268.

Gielkens, M. M. C., Dekkers, E., Visser, J., De Graaff, L. H., 1999. Two cellobiohydrolase-encoding genes from aspergillus niger require D-xylose and the xylanolytic transcriptional activator XlnR for their expression. Applied and Environmental Microbiology. 65(10), 4340-4345.

Girard, D. J., Converse, A. O., 1993. Recovery of cellulase from lignaceous hydrolysis residue. Applied Biochemistry and Biotechnology. 39-40(1), 521-533.

Gregg, D. J., Saddler, J. N., 1996. Factors affecting cellulose hydrolysis and the potential of enzyme recycle to enhance the efficiency of an integrated wood to ethanol process. Biotechnology and Bioengineering. 51(4), 375-383.

Guimarães, B. G., Souchon, H., Lytle, B. L., David Wu, J. H., Alzari, P. M., 2002. The crystal structure and catalytic mechanism of cellobiohydrolase CelS, the major enzymatic component of the clostridium thermocellum cellulosome. Journal of Molecular Biology. 320(3), 587-596.

Guisán, J. M. 1988. Aldehyde-agarose gels as activated supports for immobilization-stabilization of enzymes. Enzyme and Microbial Technology. 10(6), 375-382.

Gun'ko, V. M., Mikhailova, I. V., Zarko, V. I., Gerashchenko, I. I., Guzenko, N. V., Janusz, W., Leboda, R., Chibowski, S., 2003. Study of interaction of proteins with fumed silica in aqueous suspensions by adsorption and photon correlation spectroscopy methods. Journal of Colloid and Interface Science. 260(1), 56-69.

Gun'ko, V. M., Zarko, V. I., Voronin, E. F., Goncharuk, E. V., Andriyko, L. S., Guzenko, N. V., Nosach, L. V., Janusz, W., 2006. Successive interaction of pairs of soluble organics with nanosilica in aqueous media. Journal of Colloid and Interface Science. 300(1), 20-32.

Gupta, R., Lee, Y. Y., 2009. Mechanism of cellulase reaction on pure cellulosic substrates. Biotechnology and Bioengineering. 102(6), 1570-1581.

Hari Krishna, S., Janardhan Reddy, T., Chowdary, G. V., 2001. Simultaneous saccharification and fermentation of lignocellulosic wastes to ethanol using a thermotolerant yeast. Bioresource Technology. 77(2), 193-196.

Harjunpää, V., Teleman, A., Koivula, A., Ruohonen, L., Teeri, T. T., Teleman, O., Drakenberg, T., 1996. Cello-oligosaccharide hydrolysis by cellobiohydrolase TT from *Trichoderma reesei*. Association and rate constants derived from an analysis of progress curves. European Journal of Biochemistry. 240(3), 584-591.

Hartono, S. B., Qiao, S. Z., Liu, J., Jack, K., Ladewig, B. P., Hao, Z., Lu, G. Q. M., 2010. Functionalized mesoporous silica with very large pores for cellulase immobilization. Journal of Physical Chemistry C. 114(18), 8353-8362.

Hatakka, A. 1994. Lignin-modifying enzymes from selected white-rot fungi: Production and role in lignin degradation. FEMS Microbiology Reviews. 13(2-3), 125-135.

Heipieper, H. J., Weber, F. J., Sikkema, J., Keweloh, H., De Bont, J. A. M., 1994. Mechanisms of resistance of whole cells to toxic organic solvents. Trends in Biotechnology. 12(10), 409-415.

Hendriks, A. T. W. M., Zeeman, G., 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresource Technology. 100(1), 10-18.

Henriksson, H., Ståhlberg, J., Isaksson, R., Pettersson, G., 1996. The active sites of cellulases are involved in chiral recognition: A comparison of cellobiohydrolase 1 and endoglucanase 1. FEBS Letters. 390(3), 339-344.

Hirvonen, M., Papageorgiou, A. C., 2003. Crystal structure of a family 45 endoglucanase from melanocarpus albomyces: Mechanistic implications based on the free and cellobiose-bound forms. Journal of Molecular Biology. 329(3), 403-410.

Hodge, D. B., Karim, M. N., Schell, D. J., McMillan, J. D., 2009. Model-based fed-batch for high-solids enzymatic cellulose hydrolysis. Applied Biochemistry and Biotechnology. 152(1), 88-107.

Horváth, I. S., Franzén, C. J., Taherzadeh, M. J., Niklasson, C., Lidén, G., 2003. Effects of furfural on the respiratory metabolism of *Saccharomyces cerevisiae* in glucose-limited chemostats. Applied and Environmental Microbiology. 69(7), 4076-4086.

Huang, R., Su, R., Qi, W., He, Z., 2011. Bioconversion of lignocellulose into bioethanol: Process intensification and mechanism research. Bioenergy Research. 4(4), 225-245.

Huang, S. H., Juang, R. S., 2011. Biochemical and biomedical applications of multifunctional magnetic nanoparticles: A review. Journal of Nanoparticle Research. 13(10), 4411-4430.

Huang, S. H., Liao, M. H., Chen, D. H., 2003. Direct binding and characterization of lipase onto magnetic nanoparticles. Biotechnology Progress. 19(3), 1095-1100.

Ikeda, Y., Hayashi, H., Okuda, N., Park, E. Y., 2007. Efficient cellulase production by the filamentous fungus *Acremonium cellulolyticus*. Biotechnology Progress. 23(2), 333-338.

Iler, R. K., 1979. Polymerization of silica, The chemistry of silica: Solubility, polymerization, colloid and surface properties and biochemistry. A Wiley-Interscience Publication/ John Wiley & Sons. pp. 174.

Ince, A., Bayramoglu, G., Karagoz, B., Altintas, B., Bicak, N., Arica, M. Y., 2012. A method for fabrication of polyaniline coated polymer microspheres and its application for cellulase immobilization. Chemical Engineering Journal. 189-190, 404-412.

Ingesson, H., Zacchi, G., Yang, B., Esteghlalian, A. R., Saddler, J. N., 2001. The effect of shaking regime on the rate and extent of enzymatic hydrolysis of cellulose. Journal of Biotechnology. 88(2), 177-182.

Jackson, L. S., Joyce, T. W., Heitmann, J. A., Giesbrecht, F. G., 1996. Enzyme activity recovery from secondary fiber treated with cellulase and xylanase. Journal of Biotechnology. 45(1), 33-44.

Jordan, J., Kumar, C. S. S. R., Theegala, C., 2011. Preparation and characterization of cellulase-bound magnetite nanoparticles. Journal of Molecular Catalysis B: Enzymatic. 68(2), 139-146.

Jørgensen, H., Vibe-Pedersen, J., Larsen, J., Felby, C., 2007. Liquefaction of lignocellulose at high-solids concentrations. Biotechnology and Bioengineering. 96(5), 862-870.

Kabel, M. A., Bos, G., Zeevalking, J., Voragen, A. G. J., Schols, H. A., 2007. Effect of pretreatment severity on xylan solubility and enzymatic breakdown of the remaining cellulose from wheat straw. Bioresource Technology. 98(10), 2034-2042.

Kabel, M. A., Van Der Maarel, M. J. E. C., Klip, G., Voragen, A. G. J., Schols, H. A., 2006. Standard assays do not predict the efficiency of commercial

cellulase preparations towards plant materials. Biotechnology and Bioengineering. 93(1), 56-63.

Kalantari, M., Kazemeini, M., Tabandeh, F., Arpanaei, A., 2012. Lipase immobilisation on magnetic silica nanocomposite particles: Effects of the silica structure on properties of the immobilised enzyme. Journal of Materials Chemistry. 22(17), 8385-8393.

Kerstetter, J. D., Lynd, L., Lyford, K., & South, C. (1997). Assessment of potential for conversion of pulp and paper sludge to ethanol fuel in the pacific northwest. No. ACG-6-15177-01) National Renewable Energy Laboratory.

Khoshnevisan, K., Bordbar, A. -., Zare, D., Davoodi, D., Noruzi, M., Barkhi, M., Tabatabaei, M., 2011. Immobilization of cellulase enzyme on superparamagnetic nanoparticles and determination of its activity and stability. Chemical Engineering Journal. 171(2), 669-673.

Kilian, S. G., van Uden, N., 1988. Transport of xylose and glucose in the xylose-fermenting yeast *Pichia stipitis*. Applied Microbiology and Biotechnology. 27(5-6), 545-548.

Kim, S., Holtzapple, M. T., 2006. Effect of structural features on enzyme digestibility of corn stover. Bioresource Technology. 97(4), 583-591.

Kleywegt, G. J., Zou, J. Y., Divne, C., Davies, G. J., Sinning, I., Ståhlberg, J., Reinikainen, T., Srisodsuk, M., Teeri, T. T., Jones, T. A., 1997. The crystal structure of the catalytic core domain of endoglucanase I from *Trichoderma reesei* at 3.6 Å resolution, and a comparison with related enzymes. Journal of Molecular Biology. 272(3), 383-397.

Klinke, H. B., Thomsen, A. B., Ahring, B. K., 2004. Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Applied Microbiology and Biotechnology. 66(1), 10-26.

Kluepfel, D., Shareck, F., Mondou, F., Morosoli, R., 1986. Characterization of cellulase and xylanase activities of *Streptomyces lividans*. Applied Microbiology and Biotechnology. 24(3), 230-234.

Ko, J. K., Bak, J. S., Jung, M. W., Lee, H. J., Choi, I. G., Kim, T. H., Kim, K. H., 2009. Ethanol production from rice straw using optimized aqueous-ammonia soaking pretreatment and simultaneous saccharification and fermentation processes. Bioresource Technology. 100(19), 4374-4380.

Koivula, A., Ruohonen, L., Wohlfahrt, G., Reinikainen, T., Teeri, T. T., Piens, K., Claeyssens, M., Weber, M., Vasella, A., Becker, D., 2002. The active site of cellobiohydrolase Cel6A from *Trichoderma reesei*: The roles of aspartic acids D221 and D175. Journal of the American Chemical Society. 124(34), 10015-10024.

Kotter, P., Ciriacy, M., 1993. Xylose fermentation by *Saccharomyces cerevisiae*. Applied Microbiology and Biotechnology. 38(6), 776-783.

Krengel, U., Dijkstra, B. W., 1996. Three-dimensional structure of endo-1,C4-β-xylanase I from *Aspergillus niger*: Molecular basis for its low pH optimum. Journal of Molecular Biology. 263(1), 70-78.

Kristensen, J. B., Felby, C., Jørgensen, H., 2009. Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. Biotechnology for Biofuels. 2:11.

Kuhls, K., Lieckfeldt, E., Samuels, G. J., Kovacs, W., Meyer, W., Petrini, O., Gams, W., Börner, T., Kubicek, C. P., 1996. Molecular evidence that the asexual industrial fungus *Trichoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jecorina*. Proceedings of the National Academy of Sciences of the United States of America. 93(15), 7755-7760.

Kumar, P., Barrett, D. M., Delwiche, M. J., Stroeve, P., 2009a. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Industrial and Engineering Chemistry Research. 48(8), 3713-3729.

Kumar, R., Wyman, C. E., 2008. An improved method to directly estimate cellulase adsorption on biomass solids. Enzyme and Microbial Technology. 42(5), 426-433.

Kumar, S., Singh, S. P., Mishra, I. M., Adhikari, D. K., 2009b. Recent advances in production of bioethanol from lignocellulosic biomass. Chemical Engineering and Technology. 32(4), 517-526.

Lamed, R., Naimark, J., Morgenstern, E., Bayer, E. A., 1987. Scanning electron microscopic delineation of bacterial surface topology using cationized ferritin. Journal of Microbiological Methods. 7(4-5), 233-240.

Langsford, M. L., Gilkes, N. R., Singh, B., Moser, B., Miller Jr., R. C., Warren, R. A. J., Kilburn, D. G., 1987. Glycosylation of bacterial cellulases prevents proteolytic cleavage between functional domains. FEBS Letters. 225(1-2), 163-167.

Larsson, S., Palmqvist, E., Hahn-Hägerdal, B., Tengborg, C., Stenberg, K., Zacchi, G., Nilvebrant, N. O., 1999. The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. Enzyme and Microbial Technology. 24(3-4), 151-159.

Lee, D., Yu, A. H. C., Saddler, J. N., 1995. Evaluation of cellulase recycling strategies for the hydrolysis of lignocellulosic substrates. Biotechnology and Bioengineering. 45(4), 328-336.

Lee, J. W., Gwak, K. S., Park, J.Y., Park, M. J., Choi, D. H., Kwon, M., Choi, I. G., 2007. Biological pretreatment of softwood *Pinus densiflora* by three white rot fungi. Journal of Microbiology. 45(6), 485-491.

Lee, W. J., Kim, M. D., Ryu, Y. W., Bisson, L., Seo, J. H., 2003. Kinetic studies on glucose and xylose transport in *Saccharomyces cerevisiae*. Applied Microbiology and Biotechnology. 60(1-2), 186-191.

Li, C., Yoshimoto, M., Fukunaga, K., Nakao, K., 2007. Characterization and immobilization of liposome-bound cellulase for hydrolysis of insoluble cellulose. Bioresource Technology. 98(7), 1366-1372.

Li, H., Kim, N. J., Jiang, M., Kang, J. W., Chang, H. N., 2009. Simultaneous saccharification and fermentation of lignocellulosic residues pretreated with

phosphoric acid-acetone for bioethanol production. Bioresource Technology. 100(13), 3245-3251.

Li, X., Calza, R. E., 1991. Fractionation of cellulases from the ruminal fungus *Neocallimastix frontalis* EB188. Applied and Environmental Microbiology. 57(11), 3331-3336.

Liang, W., Cao, X., 2012. Preparation of a pH-sensitive polyacrylate amphiphilic copolymer and its application in cellulase immobilization. Bioresource Technology. 116, 140-146.

Liao, H., Chen, D., Yuan, L., Zheng, M., Zhu, Y., Liu, X., 2010. Immobilized cellulase by polyvinyl alcohol/Fe_2O_3 magnetic nanoparticle to degrade microcrystalline cellulose. Carbohydrate Polymers. 82(3), 600-604.

Liu, B., Glenn, D., Buckley, K., 2008. *Trichoderma* communities in soils from organic, sustainable, and conventional farms, and their relation with southern blight of tomato. Soil Biology and Biochemistry. 40(5), 1124-1136.

Liu, Y. S., Baker, J. O., Zeng, Y., Himmel, M. E., Haas, T., Ding, S. Y., 2011. Cellobiohydrolase hydrolyzes crystalline cellulose on hydrophobic faces. Journal of Biological Chemistry. 286(13), 11195-11201.

Lloyd, T. A., Wyman, C. E., 2005. Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids. Bioresource Technology. 96(18 SPEC. ISS.), 1967-1977.

Lou, R., Wu, S., Lv, G., Yang, Q., 2012. Energy and resource utilization of deinking sludge pyrolysis. Applied Energy. 90(1), 46-50.

Lu, Y., Wang, Y., Xu, G., Chu, J., Zhuang, Y., Zhang, S., 2010. Influence of high solid concentration on enzymatic hydrolysis and fermentation of steam-exploded corn stover biomass. Applied Biochemistry and Biotechnology. 160(2), 360-369.

Lu, Y., Yang, B., Gregg, D., Saddler, J. N., Mansfield, S. D., 2002. Cellulase adsorption and an evaluation of enzyme recycle during hydrolysis of steam-exploded softwood residues. Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology. 98-100, 641-654.

Lynd, L. R., Weimer, P. J., Van Zyl, W. H., Pretorius, I. S., 2002. Microbial cellulose utilization: Fundamentals and biotechnology. Microbiology and Molecular Biology Reviews. 66(3), 506-577.

Mandali, P., Dalaly, B. K., 2011. Immobilization of cellulase and hemicellulases on porous glass beads. ASTM Special Technical Publication. 1477, 549-565.

Mark Lawther, J., Sun, R., Banks, W. B., 1995. Extraction, fractionation, and characterization of structural polysaccharides from wheat straw. Journal of Agricultural and Food Chemistry. 43(3), 667-675.

Massart, R. 1981. Preparation of aqueous magnetic liquids in alkaline and acidic media. IEEE Transactions on Magnetics. MAG-17(2), 1247-1248.

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

Meinander, N. Q., Hahn-Hägerdal, B., 1997. Influence of cosubstrate concentration on xylose conversion by recombinant, XYL1-expressing *Saccharomyces cerevisiae*: A comparison of different sugars and ethanol as cosubstrates. Applied and Environmental Microbiology. 63(5), 1959-1964.

Menon, V., Rao, M., 2012. Trends in bioconversion of lignocellulose: Biofuels, platform chemicals & biorefinery concept. Progress in Energy and Combustion Science. 38(4), 522-550.

Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry. 31(3), 426-428.

Mitsuzawa, S., Kagawa, H., Li, Y., Chan, S. L., Paavola, C. D., Trent, J. D., 2009. The rosettazyme: A synthetic cellulosome. Journal of Biotechnology. 143(2), 139-144.

Modig, T., Lidén, G., Taherzadeh, M. J., 2002. Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase. Biochemical Journal. 363(3), 769-776.

Moraïs, S., Barak, Y., Caspi, J., Hadar, Y., Lamed, R., Shoham, Y., Wilson, D. B., Bayer, E. A., 2010. Cellulase-xylanase synergy in designer cellulosomes for enhanced degradation of a complex cellulosic substrate. MBio. 1(5) e00285-10.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M., Ladisch, M., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technology. 96(6), 673-686.

Mueller-Harvey, I., Hartley, R. D., Harris, P. J., Curzon, E. H., 1986. Linkage of p-coumaroyl and feruloyl groups to cell-wall polysaccharides of barley straw. Carbohydrate Research. 148(1), 71-85.

Murashima, K., Ksugi, A., Doi, R. H., 2002. Syneffects on crystalline cellulose degradation between cellulosomal cellulases from *Clostridum cellulororans*. Journal of Bacteriology. 184(18), 5088-5095.

Nguyen, Q. A., Tucker, M. P., Keller, F. A., Eddy, F. P., 2000. Two-stage dilute-acid pretreatment of softwoods. Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology. 84-86, 561-576.

Nijikken, Y., Tsukada, T., Igarashi, K., Samejima, M., Wakagi, T., Shoun, H., Fushinobu, S., 2007. Crystal structure of intracellular family 1 β-glucosidase BGL1A from the basidiomycete *Phanerochaete chrysosporium*. FEBS Letters. 581(7), 1514-1520.

Nwagu, T. N., Okolo, B. N., Aoyagi, H., 2011. Immobilization of raw starch digesting amylase on silica gel: A comparative study. African Journal of Biotechnology. 10(71), 15989-15997.

Obón, J. M., Castellar, M. R., Iborra, J. L., Manjón, A., 2000. β-Galactosidase immobilization for milk lactose hydrolysis: A simple experimental and modelling study of batch and continuous reactors. Biochemical Education. 28(3), 164-168.

Öhgren, K., Bura, R., Lesnicki, G., Saddler, J., Zacchi, G., 2007. A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover. Process Biochemistry. 42(5), 834-839.

Olofsson, K., Bertilsson, M., Lidén, G., 2008. A short review on SSF - an interesting process option for ethanol production from lignocellulosic feedstocks. Biotechnology for Biofuels. 1:7

Olofsson, K., Rudolf, A., Lidén, G., 2008. Designing simultaneous saccharification and fermentation for improved xylose conversion by a recombinant strain of saccharomyces cerevisiae. Journal of Biotechnology. 134(1-2), 112-120.

Olsen, S. N., Lumby, E., McFarland, K., Borch, K., Westh, P., 2011. Kinetics of enzymatic high-solid hydrolysis of lignocellulosic biomass studied by calorimetry. Applied Biochemistry and Biotechnology. 163(5), 626-635.

Otter, D. E., Munro, P. A., Scott, G. K., Geddes, R., 1989. Desorption of *Trichoderma reesei* cellulase from cellulose by a range of desorbents. Biotechnology and Bioengineering. 34(3), 291-298.

Palmowski, L. M., & Müller, J. A. 2000. Influence of the size reduction of organic waste on their anaerobic digestion. Water Science and Technology. 41(3), 155-162.

Palmqvist, B., Lidén, G., 2012. Torque measurements reveal large process differences between materials during high solid enzymatic hydrolysis of pretreated lignocellulose. Biotechnology for Biofuels. 5:57.

Palmqvist, E., Grage, H., Meinander, N. Q., Hahn-Hägerdal, B., 1999. Main and interaction effects of acetic acid, furfural, and p- hydroxybenzoic acid on growth and ethanol productivity of yeasts. Biotechnology and Bioengineering. 63(1), 46-55.

Palmqvist, E., Hahn-Hägerdal, B., 2000. Fermentation of lignocellulosic hydrolysates. I: Inhibition and detoxification. Bioresource Technology. 74(1), 17-24.

Park, E. Y., Anh, P. N., Okuda, N., 2004. Bioconversion of waste office paper to L(+)-lactic acid by the filamentous fungus *Rhizopus oryzae*. Bioresource Technology. 93(1), 77-83.

Park, E. Y., Sato, M., Kojima, S., 2006. Fatty acid methyl ester production using lipase-immobilizing silica particles with different particle sizes and different specific surface areas. Enzyme and Microbial Technology. 39(4), 889-896.

Park, S., Baker, J. O., Himmel, M. E., Parilla, P. A., Johnson, D. K., 2010. Cellulose crystallinity index: Measurement techniques and their impact on interpreting cellulase performance. Biotechnology for Biofuels. 3:10

Parsiegla, G., Juy, M., Reverbel-Leroy, C., Tardif, C., Belaïch, J. P., Driguez, H., Haser, R., 1998. The crystal structure of the processive endocellulase CelF of clostridium cellulolyticum in complex with a thiooligosaccharide inhibitor at 2.0 Å resolution. EMBO Journal. 17(19), 5551-5562.

Pejic, B. M., Kostic, M. M., Skundric, P. D., Praskalo, J. Z., 2008. The effects of hemicelluloses and lignin removal on water uptake behavior of hemp fibers. Bioresource Technology. 99(15), 7152-7159.

Perez-Pons, J. A., Padros, E., Querol, E., 1995. Prediction and fourier-transform infrared-spectroscopy estimation of the secondary structure of a recombinant β-glucosidase from *Streptomyces sp*. (ATCC 11238). Biochemical Journal. 308(3), 791-794.

Popa, K., Pavel, C. C., 2012. Radioactive wastewaters purification using titanosilicates materials: State of the art and perspectives. Desalination. 293, 78-86.

Popoff, T., Theander, O., 1970. Formation of aromatic compounds from D-glucuronic acid and D-xylose under slightly acidic conditions. Journal of the Chemical Society D: Chemical Communications. 22(1), 135-149

Popoff, T., Theander, O., 1972. Formation of aromatic compounds from carbohydrates. part 1. reaction of d-glucuronic acid, d-glacturonic acid, d-xylose, and l-arabinose in slightly acidic, aqueous solution. Carbohydrate Research. 22(1), 135-149.

Pothiraj, C., Balaji, P., Eyini, M., 2006. Enhanced production of cellulases by various fungal cultures in solid state fermentation of cassava waste. African Journal of Biotechnology. 5(20), 1882-1885.

Prasetyo, J., Kato, T., Park, E. Y., 2010. Efficient cellulase-catalyzed saccharification of untreated paper sludge targeting for biorefinery. Biomass and Bioenergy. 34(12), 1906-1913.

Qi, B., Chen, X., Su, Y., Wan, Y., 2011. Enzyme adsorption and recycling during hydrolysis of wheat straw lignocellulose. Bioresource Technology. 102(3), 2881-2889.

Qing, Q., Yang, B., Wyman, C. E., 2010. Xylooligomers are strong inhibitors of cellulose hydrolysis by enzymes. Bioresource Technology. 101(24), 9624-9630.

Ralph, J., Lundquist, K., Brunow, G., Lu, F., Kim, H., Schatz, P. F., Marita, J. M., Hatfield, R. D., Ralph, S. A., Christensen, J. H., 2004. Lignins: Natural polymers from oxidative coupling of 4-hydroxyphenyl- propanoids. Phytochemistry Reviews. 3(1-2), 29-60.

Ramos, L. P., Breuil, C., Saddler, J. N., 1993. The use of enzyme recycling and the influence of sugar accumulation on cellulose hydrolysis by *Trichoderma* cellulases. Enzyme and Microbial Technology. 15(1), 19-25.

Ramos, L. P., Saddler, J. N., 1994. Enzyme recycling during fed-batch hydrolysis of cellulose derived from steam-exploded *Eucalyptus viminalis*. Applied Biochemistry and Biotechnology. 45-46(1), 193-207.

Ran, Y., Wang, Y. Z., Liao, Q., Zhu, X., Chen, R., Lee, D. J., Wang, Y. M., 2012. Effects of operation conditions on enzymatic hydrolysis of high-solid rice straw. International Journal of Hydrogen Energy. 37(18), 13660-13666.

Reilly, P., 2006. Amylase and cellulase structure and function, in: Yang, S. T. (Ed.) Bioprocessing for Value-Added Products from Renewable Resources. 1st ed., Elsevier. Berin, pp. 119-129.

Reshmi, R., Sugunan, S., 2013. Improved biochemical characteristics of crosslinked β-glucosidase on nanoporous silica foams. Journal of Molecular Catalysis B: Enzymatic. 85-86, 111-118.

Rouvinen, J., Bergfors, T., Teeri, T., Knowles, J. K. C., Jones, T. A., 1990. Three-dimensional structure of cellobiohydrolase II from *Trichoderma reesei*. Science. 249(4967), 380-386.

Rudolf, A., Alkasrawi, M., Zacchi, G., Lidén, G., 2005. A comparison between batch and fed-batch simultaneous saccharification and fermentation of steam pretreated spruce. Enzyme and Microbial Technology. 37(2), 195-204.

Rugal, A. A., Gun'ko, V. M., Barvinchenko, V. N., Turov, V. V., Semeshkina, T. V., Zarko, V. I., 2006. Interaction of fibrinogen with nanosilica. Central European Journal of Chemistry. 5(1), 32-54.

Russell, J. B. 1992. Another explanation for the toxicity of fermentation acids at low pH: Anion accumulation versus uncoupling. Journal of Applied Bacteriology. 73(5), 363-370.

Sabereh Rezaei, Tavana, A., Sawada, J. A., Wu, L., Junaid, A. S. M., Kuznicki, S. M., 2012. Novel copper-exchanged titanosilicate adsorbent for low temperature H 2S removal. Industrial and Engineering Chemistry Research. 51(38), 12430-12434.

Salamitou, S., Raynaud, O., Lemaire, M., Coughlan, M., Beguin, P., Aubert, J. P., 1994. Recognition specificity of the duplicated segments present in *Clostridium thermocellum* endoglucanase CelD and in the cellulosome-integrating protein CipA. Journal of Bacteriology. 176(10), 2822-2827.

Sandwick, R. K., Schray, K. J., 1988. Conformational states of enzymes bound to surfaces. Journal of Colloid and Interface Science. 121(1), 1-12.

Sardar, M., Roy, I., Gupta, M. N., 2000. Simultaneous purification and immobilization of *Aspergillus niger* xylanase on the reversibly soluble polymer eudragit(TM) L-100. Enzyme and Microbial Technology. 27(9), 672-679.

Sassner, P., Galbe, M., Zacchi, G., 2006. Bioethanol production based on simultaneous saccharification and fermentation of steam-pretreated salix at high dry-matter content. Enzyme and Microbial Technology. 39(4), 756-762.

Schlochtermeier, A., Walter, S., Schroder, J., Moorman, M., Schrempf, H., 1992. The gene encoding the cellulase (avicelase) Cel1 from *Streptomyces reticuli* and analysis of protein domains. Molecular Microbiology. 6(23), 3611-3621.

Schmid, G., Biselli, M., Wandrey, C., 1988. Preparation of cellodextrins and isolation of oligomeric side components and their characterization. Analytical Biochemistry. 175(2), 573-583.

Schoffelen, S., Van Hest, J. C. M., 2012. Multi-enzyme systems: Bringing enzymes together in vitro. Soft Matter. 8(6), 1736-1746.

Schülein, M. 2000. Protein engineering of cellulases. Biochimica Et Biophysica Acta - Protein Structure and Molecular Enzymology. 1543(2), 239-252.

Shevchenko, S. M., Paszner, L., Saddler, J. N., 2001. Treatment of organosolv and steam explosion lignins with molecular hydrogen iodide: A structural probe. Cellulose Chemistry and Technology. 35(3-4), 295-318.

Shoseyov, O., Shani, Z., Levy, I., 2006. Carbohydrate binding modules: Biochemical properties and novel applications. Microbiology and Molecular Biology Reviews. 70(2), 283-295.

Safari Sinegani, A. A., Emtiazi, G., Shariatmadari, H., 2005. Sorption and immobilization of cellulase on silicate clay minerals. Journal of Colloid and Interface Science. 290(1), 39-44.

Sørensen, H. R., Pedersen, S., Meyer, A. S., 2006. Optimization of reaction conditions for enzymatic viscosity reduction and hydrolysis of wheat arabinoxylan in an industrial ethanol fermentation residue. Biotechnology Progress. 22(2), 505-513.

Sosulski, K., Swerhone, B., 1993. Waste paper and pulpsludge as feedstock for ethanol production. 2, 1032. US DOE Report NREL-CP-200-5768.

Spezio, M., Wilson, D. B., Karplus, P. A., 1993. Crystal structure of the catalytic domain of a thermophilic endocellulase. Biochemistry. 32(38), 9906-9916.

Srisodsuk, M., Reinikainen, T., Penttila, M., Teeri, T. T., 1993. Role of the interdomain linker peptide of *Trichoderma reesei* cellobiohydrolase I in its

interaction with crystalline cellulose. Journal of Biological Chemistry. 268(28), 20756-20761.

Stenberg, K., Bollók, M., Réczey, K., Galbe, M., Zacchi, G., 2000. Effect of substrate and cellulase concentration on simultaneous saccharification and fermentation of steam-pretreated softwood for ethanol production. Biotechnology and Bioengineering. 68(2), 204-210.

Sun, R., Sun, X. F., Tomkinson, J., 2004. Hemicelluloses and their derivatives, in: Gatenholm, P.,Tenkanen, M. (Eds.), Hemicelluloses: Science and Technology. 1st ed., American Chemical Society. pp. 2-22.

Sun, R., Lawther, J. M., Banks, W. B., 1996. Fractional and structural characterization of wheat straw hemicelluloses. Carbohydrate Polymers. 29(4), 325-331.

Sun, Y., Cheng, J., 2002. Hydrolysis of lignocellulosic materials for ethanol production: A review. Bioresource Technology. 83(1), 1-11.

Taherzadeh, M. J., Eklund, R., Gustafsson, L., Niklasson, C., Lidén, G., 1997. Characterization and fermentation of dilute-acid hydrolyzates from wood. Industrial and Engineering Chemistry Research. 36(11), 4659-4665.

Takimoto, A., Shiomi, T., Ino, K., Tsunoda, T., Kawai, A., Mizukami, F., Sakaguchi, K., 2008. Encapsulation of cellulase with mesoporous silica (SBA-15). Microporous and Mesoporous Materials. 116(1-3), 601-606.

Tamaru, Y., Miyake, H., Kuroda, K., Ueda, M., Doi, R. H., 2010. Comparative genomics of the mesophilic cellulosome-producing *Clostridium cellulovorans* and its application to biofuel production via consolidated bioprocessing. Environmental Technology. 31(8-9), 889-903.

Tan, L. U. L., Yu, E. K. C., Mayers, P., Saddler, J. N., 1986. Column cellulose hydrolysis reactor: Cellulase adsorption profile. Applied Microbiology and Biotechnology. 25(3), 256-261.

Tan, L. U. L., Yu, E. K. C., Mayers, P., Saddler, J. N., 1987. Column cellulose hydrolysis reactor: The effect of retention time, temperature, cellulase concentration and exogenously added cellobiase on the overall process. Applied Microbiology and Biotechnology. 26(1), 21-27.

Tébéka, I. R. M., Silva, A. G. L., Petti, D. F. S., 2009. Hydrolytic activity of free and immobilized cellulase. Langmuir. 25(3), 1582-1587.

Tenkanen, M., Kontkanen, H., Isoniemi, R., Spetz, P., Holmbom, B., 2003. Hydrolysis of steryl esters by a lipase (lip 3) from *Candida rugosa*. Applied Microbiology and Biotechnology. 60(1-2), 120-127.

Terada, H. 1990. Uncouplers of oxidative phosphorylation. Environmental Health Perspectives. 87, 213-218.

Thygesen, A., Oddershede, J., Lilholt, H., Thomsen, A. B., Ståhl, K., 2005. On the determination of crystallinity and cellulose content in plant fibres. Cellulose. 12(6), 563-576.

Timell, T. E., 1964. Wood hemicelluloses: Part I. Advances in Carbohydrate Chemistry, 19C, 247-302.

Timell, T. E., 1965. Wood hemicelluloses: Part II. Advances in Carbohydrate Chemistry, 20C, 409-483.

Tokatlidis, K., Dhurjati, P., Beguin, P., 1993. Properties conferred on *Clostridium thermocellum* endoglucanase CelC by grafting the duplicated segment of endoglucanase CelD. Protein Engineering. 6(8), 947-952.

Tomme, P., Boraston, A., McLean, B., Kormos, J., Creagh, A. L., Sturch, K., Gilkes, N. R., Haynes, C. A., Warren, R. A. J., Kilburn, D. G., 1998.

Characterization and affinity applications of cellulose-binding domains. Journal of Chromatography B: Biomedical Applications. 715(1), 283-296.

Tomme, P., Creagh, A. L., Kilburn, D. G., Haynes, C. A., 1996. Interaction of polysaccharides with the N-terminal cellulose-binding domain of *Cellulomonas fimi* CenC. 1. Binding specificity and calorimetric analysis. Biochemistry. 35(44), 13885-13894.

Torget, R., Werdene, P., Himmel, M., Grohmann, K., 1990. Dilute acid pretreatment of short rotation woody and herbaceous crops. Applied Biochemistry and Biotechnology. 24-25, 115-126.

Torget, R. W., Kim, J. S., Lee, Y. Y., 2000. Fundamental aspects of dilute acid hydrolysis/fractionation kinetics of hardwood carbohydrates. 1. Cellulose hydrolysis. Industrial and Engineering Chemistry Research. 39(8), 2817-2825.

Tu, M., Chandra, R. P., Saddler, J. N., 2007a. Evaluating the distribution of cellulases and the recycling of free cellulases during the hydrolysis of lignocellulosic substrates. Biotechnology Progress. 23(2), 398-406.

Tu, M., Chandra, R. P., Saddler, J. N., 2007b. Recycling cellulases during the hydrolysis of steam exploded and ethanol pretreated lodgepole pine. Biotechnology Progress. 23(5), 1130-1137.

Tu, M., Zhang, X., Paice, M., MacFarlane, P., Saddler, J. N., 2009. The potential of enzyme recycling during the hydrolysis of a mixed softwood feedstock. Bioresource Technology. 100(24), 6407-6415.

Ugartondo, V., Mitjans, M., Vinardell, M. P., 2008. Comparative antioxidant and cytotoxic effects of lignins from different sources. Bioresource Technology. 99(14), 6683-6687.

Ulbricht, R. J., Northup, S. J., Thomas, J. A., 1984. A review of 5-hydroxymethylfurfural (HMF) in parenteral solutions. Fundamental and Applied Toxicology. 4(5), 843-853.

Um, B. H., Hanley, T. R., 2008. High-solid enzymatic hydrolysis and fermentation of solka floc into ethanol. Journal of Microbiology and Biotechnology. 18(7), 1257-1265.

Varga, E., Klinke, H. B., Réczey, K., Thomsen, A. B., 2004. High solid simultaneous saccharification and fermentation of wet oxidized corn stover to ethanol. Biotechnology and Bioengineering. 88(5), 567-574.

Verduyn, C. 1991. Physiology of yeasts in relation to biomass yields. Antonie Van Leeuwenhoek, International Journal of General and Molecular Microbiology. 60(3-4), 325-353.

Verduyn, C., Postma, E., Scheffers, W. A., Van Dijken, J. P., 1992. Effect of benzoic acid on metabolic fluxes in yeasts: A continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast. 8(7), 501-517.

Vertegel, A. A., Siegel, R. W., Dordick, J. S., 2004. Silica nanoparticle size influences the structure and enzymatic activity of adsorbed lysozyme. Langmuir. 20(16), 6800-6807.

Voloch, M., Ladisch, M. R., Cantarella, M., Tsao, G. T., 1984. Preparation of cellodextrins using sulfuric acid. Biotechnology and Bioengineering. 26(5), 557-559.

Von Sivers, M., Zacchi, G., 1995. A techno-economiical comparison of three processes for the production of ethanol from pine. Bioresource Technology. 51(1), 43-52.

Wahlgren, M., Arnebrant, T., Lundström, I., 1995. The adsorption of lysozyme to hydrophilic silicon oxide surfaces: Comparison between experimental data

and models for adsorption kinetics. Journal of Colloid and Interface Science. 175(2), 506-514.

Wang, W., Kang, L., Wei, H., Arora, R., Lee, Y. Y., 2011. Study on the decreased sugar yield in enzymatic hydrolysis of cellulosic substrate at high solid loading. Applied Biochemistry and Biotechnology. 164(7), 1139-1149.

Warnecke, F., Luginbühl, P., Ivanova, N., Ghassemian, M., Richardson, T. H., Stege, J. T., Cayouette, M., McHardy, A. C., Djordjevic, G., Aboushadi, N., 2007. Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. Nature. 450(7169), 560-565.

Weil, J., Sarikaya, A., Rau, S. L., Goetz, J., Ladisch, C. M., Brewer, M., Hendrickson, R., Ladisch, M. R., 1997. Pretreatment of yellow poplar sawdust by pressure cooking in water. Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology. 68(1-2), 21-40.

Weil, J., Westgate, P., Kohlmann, K., Ladisch, M. R., 1994. Cellulose pretreatments of lignocellulosic substrates. Enzyme and Microbial Technology. 16(11), 1002-1004.

White, A., Rose, D. R., 1997. Mechanism of catalysis by retaining β-glycosyl hydrolases. Current Opinion in Structural Biology. 7(5), 645-651.

Wilkie, K. C. B., 1979. The hemicelluloses of grasses and cereals. Advances in Carbohydrate Chemistry and Biochemistry, 36C, 215-264.

Wilson, D. B., 2008. Three microbial strategies for plant cell wall degradation. Annals of the New York Academy of Sciences, 1125, 289-297.

Wilson, D. B. 2009. Evidence for a novel mechanism of microbial cellulose degradation. Cellulose. 16(4), 723-727.

Wilson, D. B. 2011. Microbial diversity of cellulose hydrolysis. Current Opinion in Microbiology. 14(3), 259-263.

Wingren, A., Galbe, M., Zacchi, G., 2003. Techno-economic evaluation of producing ethanol from softwood: Comparison of SSF and SHF and identification of bottlenecks. Biotechnology Progress. 19(4), 1109-1117.

Woo, E., Ponvel, K. M., Ahn, I. S., Lee, C. H., 2010. Synthesis of magnetic/silica nanoparticles with a core of magnetic clusters and their application for the immobilization of his-tagged enzymes. Journal of Materials Chemistry. 20(8), 1511-1515.

Worsfold, P. J. 1995. Classification and chemical characteristics of immobilized enzymes (Technical report). Pure and Applied Chemistry. 67(4), 597-600.

Wright, J. D., Wyman, C. E., Grohmann, K., 1988. Simultaneous saccharification and fermentation of lignocellulose - process evaluation. Applied Biochemistry and Biotechnology. 18(1), 75-90.

Wu, L., Yuan, X., Sheng, J., 2005. Immobilization of cellulase in nanofibrous PVA membranes by electrospinning. Journal of Membrane Science. 250(1-2), 167-173.

Wu, M. M., Chang, K., Gregg, D. J., Boussaid, A., Beatson, R. P., Saddler, J. N., 1999. Optimization of steam explosion to enhance hemicellulose recovery and enzymatic hydrolysis of cellulose in softwoods. Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology. 77-79, 47-54.

Wu, M. M., Chang, K., Gregg, D. J., Boussaid, A., Beatson, R. P., & Saddler, J. N., 1998. Optimization of steam explosion to enhance hemicellulose recovery and enzymatic hydrolysis of cellulose in softwoods. Proceedings of the International Conference on Biotechnology in the Pulp and Paper Industry, 3C, 249-C252.

Wyman, C. E., Dale, B. E., Elander, R. T., Holtzapple, M., Ladisch, M. R., Lee, Y. Y., 2005. Coordinated development of leading biomass pretreatment technologies. Bioresource Technology. 96(18 SPEC. ISS.), 1959-1966.

Wyman, C. E., Spindler, D. D., Grohmann, K., 1992. Simultaneous saccharification and fermentation of several lignocellulosic feedstocks to fuel ethanol. Biomass and Bioenergy. 3(5), 301-307.

Xiao, Z., Zhang, X., Gregg, D. J., Saddler, J. N., 2004. Effects of sugar inhibition on cellulases and β-glucosidase during enzymatic hydrolysis of softwood substrates. Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology. 115(1-3), 1115-1126.

Xie, G., Bruce, D. C., Challacombe, J. F., Chertkov, O., Detter, J. C., Gilna, P., Han, C. S., Lucas, S., Misra, M., Myers, G. L., 2007. Genome sequence of the cellulolytic gliding bacterium *Cytophaga hutchinsonii*. Applied and Environmental Microbiology. 73(11), 3536-3546.

Xie, X., Li, B., Wu, Z., Dong, S., Li, L., 2012. Preparation of cross-linked cellulase aggregates onto magnetic chitosan microspheres. Advanced Materials Research, 550-553, 1566-1571.

Xu, J., Huo, S., Yuan, Z., Zhang, Y., Xu, H., Guo, Y., Liang, C., Zhuang, X., 2011a. Characterization of direct cellulase immobilization with superparamagnetic nanoparticles. Biocatalysis and Biotransformation. 29(2-3), 71-76.

Xu, Z., Miao, Y., Chen, J. Y., Jiang, X., Lin, L., Ouyang, P., 2011b. Co-immobilization mechanism of cellulase and xylanase on a reversibly soluble polymer. Applied Biochemistry and Biotechnology. 163(1), 153-161.

Xue, Y., Jameel, H., Park, S., 2012a. Strategies to recycle enzymes and their impact on enzymatic hydrolysis for bioethanol production. BioResources. 7(1), 602-615.

Xue, Y., Jameel, H., Phillips, R., Chang, H. M., 2012b. Split addition of enzymes in enzymatic hydrolysis at high solids concentration to increase sugar concentration for bioethanol production. Journal of Industrial and Engineering Chemistry. 18(2), 707-714.

Yang, B., Wyman, C. E., 2008. Pretreatment: The key to unlocking low-cost cellulosic ethanol. Biofuels, Bioproducts and Biorefining. 2(1), 26-40.

Yoo, C. G., Kuo, M., Kim, T. H., 2012. Ethanol and furfural production from corn stover using a hybrid fractionation process with zinc chloride and

simultaneous saccharification and fermentation (SSF). Process Biochemistry. 47(2), 319-326.

Yu, A., Yuan, J., Wang, Q., Fan, X., Wang, P., 2012. Covalent immobilization of cellulases onto a water-soluble-insoluble reversible polymer. Applied Biochemistry and Biotechnology. 166(6), 1433-1441.

Yue, Z., Bin, W., Baixu, Y., Peiji, G., 2004. Mechanism of cellobiose inhibition in cellulose hydrolysis by cellobiohydrolase. Science in China.Series C, Life Sciences / Chinese Academy of Sciences. 47(1), 18-24.

Zacchi, G., Axelsson, A., 1989. Economic evaluation of preconcentration in production of ethanol from dilute sugar solution. Biotechnology and Bioengineering. 34(2), 223-233.

Zhang, Y., Xu, J. L., Yuan, Z. H., Qi, W., Liu, Y. Y., He, M. C., 2012. Artificial intelligence techniques to optimize the EDC/NHS-mediated immobilization of cellulase on eudragit L-100. International Journal of Molecular Sciences. 13(7), 7952-7962.

Zhang, Y. -. P., Lynd, L. R., 2003. Cellodextrin preparation by mixed-acid hydrolysis and chromatographic separation. Analytical Biochemistry. 322(2), 225-232.

Zhang, Y. -. P., Lynd, L. R., 2004. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: Noncomplexed cellulase systems. Biotechnology and Bioengineering. 88(7), 797-824.

Zhang, Y. H. P., Lynd, L. R., 2006. A functionally based model for hydrolysis of cellulose by fungal cellulase. Biotechnology and Bioengineering. 94(5), 888-898.

Zhou, J. 2010. Immobilization of cellulase on a reversibly soluble-insoluble support: Properties and application. Journal of Agricultural and Food Chemistry. 58(11), 6741-6746.

Zhu, L., O'Dwyer, J. P., Chang, V. S., Granda, C. B., Holtzapple, M. T., 2008. Structural features affecting biomass enzymatic digestibility. Bioresource Technology. 99(9), 3817-3828.

Zugenmaier, P., 2010. in: Timell, T. E.,Wimmer, R. (Eds.), Crystalline Cellulose and Cellulose Derivatives, Characterization and Structures. 1st ed., Springer-Verlag. Berlin Heidelberg, Germany, pp. 7-208.