EFFICACY AND RECOVERY OF CELLULASES IMMOBILIZED ON POLYMER

BRUSHES GRAFTED ON SILICA NANOPARTICLES

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Ashani Rangana Samaratunga

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Ashani Rangana Samaratunga

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SUPERVISORY COMMITTEE:

Dr. Scott Pryor
Chair

Dr. Dennis Wiesenborn

Dr. Andriy Voronov

Approved:

01/28/2014	Dr. Sreekala Bajwa
Date	Department Chair

ABSTRACT

Cellulosic biofuels can be more economical if cellulases are recovered and reused. Cellulase and β-glucosidase were immobilized on poly(acrylic acid) brush particles. Impact of brush enzyme density on efficacy and recovery was tested. Use of free enzymes led to higher sugar concentrations than the attached for both the enzymes. Increasing cellulase density on the brushes did not impact efficacy. Higher proportions of cellobiose in hydrolyzates suggest differential attachment or efficacy of attached enzymes. Higher β-glucosidase density on brushes led to increased glucose concentrations. Density on brushes did not impact β-glucosidase recovery and ≈66% was recovered. Effect of pH and temperature on hydrolysis rates and enzyme recovery was modeled. Free β-glucosidase was more stable with temperature than attached. Optimal pH for attached cellulase and β-glucosidase was 4.98 and 4.39, respectively. Recovery of β-glucosidase decreased with increasing pH and was not impacted by temperature.

ACKNOWLEDGEMENTS

DEDICATION

This thesis is dedicated to my parents, Shiranee and Palitha Samaratunga for everything they have given me in my life. I also dedicate this thesis to my late grandparents: Louisa-nona and Anthony Samaratunga, and Nanda and Saliya Kumarasinghe.

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1. BACKGROUND

Growing demand for fuel, uncertainty of petroleum reserves, and environmental considerations have led to the search for alternative fuels that could reduce or replace the use of petroleum-based fuels.

The majority of petroleum fuel is currently consumed in the transportation sector. According to the US Energy Information Administration, the transportation sector consumed 13.5 million barrels of petroleum per day in 2010 (EIA, 2011), representing 71% of the total US petroleum consumption. The alternative fuel to petroleum should have the capability to replace it as a transportation fuel with the least amount of modifications to the existing infrastructure.

Cellulosic biofuels are emerging as potential replacements to petroleum-based fuels. These fuels are derived from lignocellulosic biomass such as agricultural residue, forest thinnings, furniture industry waste, or dedicated energy crops such as switchgrass. CO_2 emissions from cellulosic biofuels are those that were sequestered from the atmosphere relatively recently when compared to petroleum, which contains carbon that has been sequestered for hundreds of million years. Furthermore, the reliability of feedstocks for producing cellulosic biofuel is a potential advantage over the non-renewable supply of petroleum which is also dependent on international politics. The Energy Independence and Security Act of 2007 mandated that 36 billion gallons of biofuels be produced in the USA by 2022 (Holtberg, 2007). Of these, 16 billion gallons must be derived from cellulosic biomass.

One method of producing cellulosic biofuels is via the sugar platform, where cellulosic biomass is first hydrolyzed to soluble sugars. Enzymes used in this step are cellulases, which are a mixture containing endoglucanases, exoglucanases and β-glucosidases. These sugars can be

used to produce cellulosic biofuels such as ethanol, butanol, or hydrogen (García et al., 2011; Huber et al., 2006).

The current production cost of cellulosic ethanol is high and must be lowered for it to be economically competitive with fossil fuels. In 2007 the National Renewable Energy Laboratory (NREL) modeled the 2012 production cost of cellulosic ethanol from corn stover with dilute acid pretreatment to be $2.15 per gallon (2007$), confirming its potential to compete with petroleum-based fuels (Humbrid et al., 2011).

Increasing yields and reducing capital and operating costs have been identified as methods for making cellulosic ethanol competitive with gasoline in terms of cost (Wyman, 2007). Processing costs could account for up to 67% of total costs. Lowering the use of chemicals, nutrients and other additives, including enzymes, used in cellulosic ethanol production will have a significant impact on operating costs.

Enzymatic hydrolysis of biomass was identified as the second most expensive unit operation after pretreatment, with cellulase enzymes being a major component of those costs. Though many studies have been carried out to reduce the cost of producing cellulases, they are still considered expensive and a significant cost that raises the overall production cost of ethanol.

Wyman (1994) suggested that recovering enzymes during conversion of cellulose to sugars is a possible method of reducing the cellulosic ethanol production costs. Other cost reducing options included increasing enzyme activity, extending shelf life of enzymes, optimizing the milling process for feedstock size reduction, utilizing hemicellulose for ethanol production in addition to cellulose, using improved and efficient pretreatment technologies, and using enhanced fermenting organisms.

2. LITERATURE REVIEW

2.1. Cellulosic biomass

The cell walls of lignocellulosic biomass contain cellulose, hemicellulose, and lignin. Cellulose, which is composed of bundles of linear chains containing hundreds to tens of thousands of glucose monomers, exists in a crystalline form within cell walls and is bound to hemicellulose and lignin. To separate cellulose from lignin and hemicellulose, chemical or thermochemical pretreatment of biomass is necessary. This makes cellulose more accessible for enzymes to reach during enzymatic hydrolysis of lignocellulosic biomass, where cellulose is converted to glucose.

2.2. Enzymes

Enzymes are biological catalysts that increase reaction rates by lowering the activation energy for respective reactions. They create pocket-like active sites where the reactions take place by binding to reactant molecules called substrates. Nearly all enzymes are proteins except for some catalytic RNA molecules. The catalytic behavior of each enzyme depends on its amino acid sequence and its resultant three-dimensional conformation. Changes in the environment such as pH and temperature change this conformation and may cause the protein to break down into amino acid residues which would not allow the enzyme to perform its desired catalytic task.

2.2.1. Enzymatic hydrolysis

Hydrolysis is cleavage of chemical bonds while a water molecule is added. During this process, long chains of cellulose are cleaved to form glucose monomers. This reaction is catalyzed by cellulase enzymes.

2.2.2. Cellulases

Commercial cellulases are mixtures of endoglucanase (endocellulase), exoglucanase (exocellulase) and β-glucosidase (cellobiase). Endoglucanases break cellulose chains at random, internal, amorphous regions, to give shorter chains with more chain ends. Exoglucanases cut these chains from ends to give glucose dimers (cellobiose). Then β-glucosidases further break these glucose dimers to glucose monomers. These cleaving mechanisms on cellulose chains and glucose dimers are shown in figure 2.1.
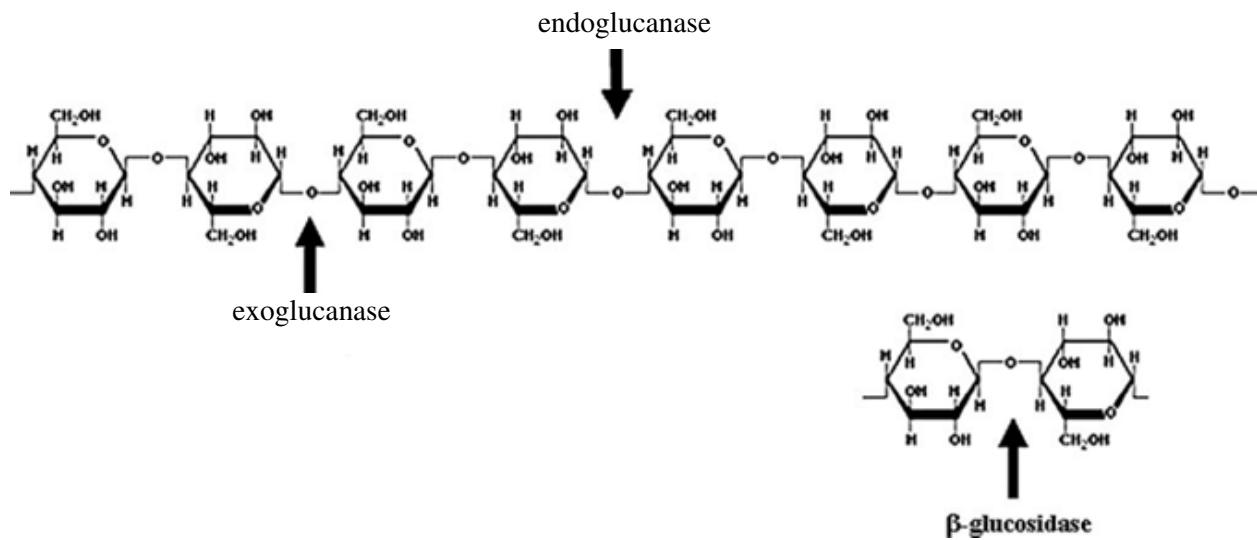
Figure 2.1: Molecular structure of cellulose and the sites on which enzymes cleave during hydrolysis (Kumar et al., 2008).

Activity of cellulase is measured in filter paper units (FPU) and cellulase is often applied as FPU per gram of substrate (FPU/g) (Ghose, 1987). FPU is determined based on concentration of enzyme needed to release 2 mg of glucose. The optimum pH range for cellulase activity is 4.2 to 5.8, with 4.8 considered ideal (Pardo and Forchiassin, 1999b; Vatanparast et al., 2012). The most commonly used temperature range for hydrolysis with cellulase is from 50° to 55°C (Andreaus et al., 1999; Kumar et al., 2008; Sternberg et al., 1977). On the other hand, unfavorable conditions could deactivate cellulase enzymes. Hydrolysis reactions are often

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terminated by immersing hydrolyzate samples in boiling water for 5 to 10 minutes (Al-Zuhair, 2008; Park et al., 2002).

2.2.2.1. β-glucosidase

The enzyme subclass β-glucosidase, found in cellulase mixtures, is commercially available. The operational pH range for β-glucosidase activity is from 3 to 7, with 4.3 considered optimal (Sternberg et al., 1977; Woodward and Wiseman, 1982). The operating temperature is from 23° to 55^oC with 45^oC as the ideal (Pardo and Forchiassin, 1999a; Woodward and Wiseman, 1982). The activity of β-glucosidase is measured in cellobiose units (CBU) and is used as CBU per gram of substrate (CBU/g) (Ghose, 1987).

Temperatures close to 100^oC assure deactivation of β-glucosidase. Hydrolysis experiments carried out with β-glucosidase can be terminated by immersing hydrolyzates in boiling water baths for 3 to 5 minutes (Abdel-Naby, 1998; Messner and Kubicek, 1990). Exposing β-glucosidase to ultrasonic frequencies has also been shown to reduce enzyme activity (Özbek and Ülgen, 2000). Therefore, if sonication is used for dispersing a mixture of particles, it must be done before enzyme is added to the mixture.

2.2.3. Reusing cellulase

An early attempt at recovery and reuse of cellulase was tested by immobilizing cellulase on polysaccharides (Woodward and Zachry, 1982). This was achieved by adsorption of cellulase to the carbohydrate-binding protein, concanavalin A, which was immobilized on Sepharose 4B, a cross-linked and beaded polysaccharide. Initial recovery of cellulase using this method was only 6%. However, this method of immobilization showed positive results in terms of high retention of enzyme activity. This indicates that the active sites of the enzymes were accessible for cellulose substrates. Furthermore, these immobilized enzymes showed relatively higher

efficiency than free cellulase for converting cellulosic biomass to glucose during enzymatic hydrolysis. Woodward and Zachary (1982) suggest that immobilized enzymes could be less affected than free enzymes to some or all of the factors: product inhibition, thermal inactivation, and inactivation at the air-liquid interface of the reaction mixture.

Cellulase has also been recycled by reusing unhydrolyzed residue from a previous hydrolysis cycle that would have enzymes attached to them (Gregg and Saddler, 1996). Recycling of cellulase absorbed on these residues also reduced hydrolysis time as the rate of hydrolysis was increased. This method of reusing enzymes has led to consecutive cycles of hydrolysis by recovering cellulase after each hydrolysis step and reusing them in the next. Five such cycles of recovery and reuse were achieved for hardwood substrate while two cycles were achieved for softwood. The cycles of reuse were limited due to the presence of lignin in residue that was affecting hydrolysis efficiency. Furthermore, β-glucosidase still needed to be added at each cycle as sufficient amounts of β-glucosidase was not recovered with residues.

Cellulase has also been recovered by filtration of supernatant (Lu et al., 2002). The substrates used were steam-exploded Douglas fir, with and without SO_2. The first filtration was carried out using glass filter paper, and the second filtration was accomplished via ultrafiltration, where a hydrostatic pressure is applied to achieve filtration by forcing through the semipermeable membrane. This retentate from filtration was reused up to three consecutive cycles of hydrolysis and recovery.

Tu et al (2007a) studied a cellulase recycling method where the free cellulase remaining after hydrolysis was reabsorbed into the fresh substrate. The solid substrate was then used in subsequent hydrolysis cycles. Substrates used in this study were organosolv-pretreated Douglas fir, Avicel, and ethanol-pretreated mixed wood. This study also modeled an 82% cellulase

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recovery for ethanol-pretreated mixed softwood, and it found that free cellulase is recoverable up to 85% in terms of cellulase activity and up to 88% in terms of protein content.

Surfactants (Tween80) have also been used to enhance recycling of cellulase (Tu et al., 2007b). The substrates used were steam-exploded lodge pole pine and ethanol-pretreated lodge pole pine. After hydrolysis, cellulase was recovered by filtration through a glass microfiber membrane. The filtrate was re-suspended and fresh substrate was used to readsorb free cellulase from this suspension. This re-suspended filtrate was used in consecutive hydrolysis cycles after further filtration. Using this method, cellulase was reused in 5 consecutive cycles resulting in savings of up to 60% of the total enzyme cost required to hydrolyze ethanol-pretreated lodge pole pine (Tu et al., 2009).

2.2.4. Conditions for enzymatic hydrolysis with cellulases

Hydrolysis conditions determine the effectiveness of an enzyme catalyzed reaction. Changes in pH cause changes in ionizations of the amino acids of the proteins and the ionization of the substrates. pH also affects the physical structure of the protein altering its activity. At temperatures below the optimum range, enzyme activity will be slower due reduced kinetic energy in protein molecules. At temperatures higher than the optimum range, thermal denaturation will occur due to high internal energy of the protein molecule causing some of its bonds to break leading to changes in physical structure.

For studies performed to reuse cellulases, the temperature at which hydrolysis is performed ranged from $45^{o}C$ to $50^{o}C$ (Singh et al., 1991; Tu et al., 2007b; Woodward and Zachry, 1982) in water baths with stirring ranging from 120 to 180 rpm (Qi et al., 2011; Singh et al., 1991; Tu and Saddler, 2010). The operating pH used for hydrolysis was typically 4.8 (Tu et al., 2007a; Tu et al., 2009; Woodward and Zachry, 1982) and this was achieved by using buffer

solutions of 50 mM sodium citrate or sodium acetate (Singh et al., 1991; Tu et al., 2007a). The hydrolysis time allowed for the conversion of substrate to glucose varied from 24 to 120 h (Gregg and Saddler, 1996; Tu and Saddler, 2010; Tu et al., 2009).

The amount of β-glucosidase present in commercial cellulase mixtures is not sufficient to achieve complete hydrolysis of cellobiose. Therefore, further β-glucosidase are typically added for hydrolysis at cellulase to β-glucosidase ratios ranging from 1:1.75 to 1:2 for FPU: CBU (Lu et al., 2002; Tu et al., 2009), where FPU indicating the activity of cellulase and CBU indicates the activity of β-glucosidase.

In order to determine the most suitable enzyme loading for each case, various enzyme loadings have been attempted. The range of enzyme loading was very high in some instances such as 7.5 to 120 FPU/g for Douglas-fir substrate and 10 to 80 FPU/g for Avicel (Lu et al., 2002); relatively smaller ranges were seen in other instances such as 5 to 20 FPU/g for various peroxide treated wood, and 9 to 10 FPU/g for filter paper substrate (Gregg and Saddler, 1996; Ramos et al., 1993). Therefore, it is necessary to perform preliminary experiments to determine the most suitable enzyme loading within these ranges for the substrates that will be used. Substrate loadings for enzymatic hydrolysis in literature range from 2 to 10% (Gregg and Saddler, 1996; Lu et al., 2002; Ramos et al., 1993). These substrate loadings provide sufficient sugar yields and enzyme efficacy can be compared if this loading is kept constant for experiments.

The most common enzyme loading for cellulase among studies with a fixed enzyme loading was 20 FPU/g of substrate, while it was 40 CBU/g of substrate for β-glucosidase (Qi et al., 2011; Tu et al., 2007b; Tu and Saddler, 2010). Furthermore, these values confirm that β-

glucosidase was added based on the ratio of 1:2 for FPU:CBU, which is within the range of ratios indicated above.

2.3. Immobilized enzymes

Immobilization of enzymes can be done by attaching enzymes to solid supports limiting or controlling the free motion of otherwise soluble enzymes. This technique keeps enzymes localized during a desired reaction and will enable separation of the immobilized enzymes from reactants, products, and reaction media.

Enzymes have been attached to chitin and nylon supports (Vaillant et al., 2000), and pumice particles (Pazarlioglu et al., 2005), and immobilized enzymes can be used for biological applications such as drug delivery (Kost and Langer, 2012), food processing (Ge et al., 2012), creating biosensors (Teles and Fonseca, 2008).

Immobilization or attachment to supports may change the properties and behavior of the attached enzyme. Immobilization limits changes to the three dimensional structure of enzymes, restricting folding and unfolding. Furthermore, interactions of the enzymes' functional groups with the support will alter their interactions with substrates. When the enzyme invertase was immobilized on carboxymethylcellulose acid chloride (CMC-Cl), the attached enzyme showed lower enzyme activity than the free enzyme for varying pH values (Simionescu et al., 1984). On another instance where the enzyme trypsin was immobilized on xanthane (Biozan-R), the attached enzyme showed higher reaction rates than the free enzyme for various substrate loadings (Simionescu et al., 1986). Therefore the properties of the attached enzyme may vary for different enzyme and support combinations.

2.3.1. Polymer brushes

Polymer brushes are made of polymer chains that have been end-tethered or grafted on solid surfaces, nanoparticles, or inside walls of micro-channels (Brittain and Minko, 2007). They can be made from polymer chains with the same chemical formula and configuration, or from a mixture of several types of polymer chains. The polymers of the brush are attached at a high grafting density so that the surface is crowded with them. This crowding causes the grafted polymer chains to stretch or extend to lessen interactions with adjacent chains.

Polymer brushes have been used to manipulate various biological systems including proteins. Intentional folding and unfolding caused by varying the concentration of a denaturing agent, was observed for the protein RNase H while it was attached to polymer brush made of Poly(ethylene oxide) (Groll et al., 2004). This indicates that the functionality of the protein was retained while it was immobilized.

A study by Kawai et al. (2003) shows protein binding to polymer brushes made from two polymers, Poly(glycidyl methacrylate) (PGMA) and Polyvinyl acetate (PVAc) that were grafted on a microporous hollow-fiber membrane. These functionalized brushes were used for protein separation and purification by utilizing differences in electrostatic (ion-exchange), hydrophobic, and affinity interaction properties of the two types of brushes involved. These brushes were tuned to attach to the desired protein by altering pH and salt concentrations.

However, polymer brushes have not yet been used to immobilize cellulases so that they can be recovered and reused in consecutive hydrolysis cycles.

2.3.1.1. Poly(acrylic acid) brush

Poly acrylic acid (PAA) chains can be grafted on nano scale particles or a surface to create polymer brushes. PAA brushes are polyelectrolytes since the PAA chains of a brush are charged

(Wang et al., 2010). This allows protein molecules to attach or detach to brushes depending on the charge of the chains of a brush. Furthermore, polyelectrolyte brushes respond to ionic strength and pH of its surrounding: the protein bovine serum albumin showed strong binding to both spherical and planer PAA brushes at low ionic strength (Czeslik et al., 2004a), and changes in pH resulted in polyelectrolyte brushes changing physical state by stretching or shrinking (Minko, 2006). PAA brushes are more negatively charged at pH higher than 7, while they are less negatively charged at pH less than 4. The stretching is caused by higher negative charge on the polymer chains repelling each other. Therefore the brushes are stretched at pH 7 and shrunk at pH 4, while their physical structure could be between the stretched and shrunk stage within this pH range.

PAA brushes have been successfully used to attach several proteins including bovine serum albumin (Czeslik et al., 2004a; Dai et al., 2006; de Vos et al., 2008), lipase (Chen and Hsieh, 2005), and hen egg-white lysozyme (Hollmann and Czeslik, 2006).

2.4. Separation and recovery

2.4.1. Centrifugation

Centrifugation is a separation technique that utilizes centrifugal force to separate components utilizing earth's gravitational acceleration and the rotational velocity used.

$$\text{Relative centrifugal force, RCF} = \frac{r\omega^2}{g}$$

(2.1)

where r = rotational radius (cm)

ω = angular velocity (rad/s)

g = gravitational acceleration

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Hence for each centrifuge, the relative centrifugal force can be calculated as follows when angular velocity in rad/s is converted to revolutions per minute:

$$\text{RCF x } g = r \text{ x } \left(\frac{N \text{ x } 2\pi}{60}\right)^2$$

(2.2)

where r = maximum rotational radius (cm)

N = rotational speed (revolutions/minute)

Centrifugal force makes high density components migrate away from the axis of the centrifuge, leaving low density components closer to the axis. This causes sedimentation of high density components. Therefore a solution containing a mixture of components could be separated as high density products settle as a pellet and the low density products remain in the supernatant. However, the rate of sedimentation depends on particle size, particle density, density of the medium, viscosity of the medium, and the gravitational acceleration (Burgi and Hershey, 1963; Gill et al., 1992).

2.4.1.1. Centrifugation for recovering nanoparticles

Centrifugation has been used to separate various nanoparticles such as gold (Demers et al., 2000), silver (Pal et al., 2007), protein (Weber et al., 2000), polymer coated gold (Gittins and Caruso, 2001), and protein bound copolymer (Cedervall et al., 2007) nanoparticles. The diameters of the nanoparticles recovered via centrifugation varies from 1 to 358 nm (Gittins and Caruso, 2001; Mafuné et al., 2001; Weber et al., 2000). The centrifugal forces used varies from 84×g to 20,000×g (Pal et al., 2007; Sharma et al., 2009; Weber et al., 2000), while the duration of centrifugation ranges from 10 to 40 min (Cedervall et al., 2007; Pal et al., 2007; Sharma et al., 2009). Furthermore, the centrifugation force used for separation has been altered for various

particle sizes. For silver nanoparticles, 84×g was used for the largest diameter particles while 2,100×g was used for the smallest diameter particles (Mafuné et al., 2000).

2.4.2. Magnetic separation

Magnetic separation can be utilized to recover magnetic particles from a solution that will not be magnetized. This may be achieved by using a permanent magnet or from an electromagnet.

Immobilizing enzymes on magnetic particles has been attempted before since it enables particles smaller than other residues in solution to be recovered. Robinson et al. (1973) tested activity and settling properties of α-chymotrypsin and β-galactosidase immobilized on iron oxide-cellulase particles with diameters ranging from 53 to 63 µM. For these attached enzymes, particles at lower shear rates had higher particle settling compared to particles at high shear rates such as in a continuously stirred reactor (CSTR). Therefore, samples with magnetic particles must be left idle with minimal stirring for more efficient magnetic recovery.

2.4.2.1. Magnetic separation of nanoparticles

Recovery of magnetic nanoparticles has been successfully achieved via magnetic separation Fe_3O_4-Ag heterodimer nanoparticles (Jiang et al., 2008), mixtures of Fe_3O_4 and Fe_2O_4 particles (Berry and Curtis, 2003), Ni and NiO particles bound to histadine-tagged proteins (Lee et al., 2006), and quantum dots and magnetic nanoparticles encapsulated in silica shells (Yi et al., 2005). The diameters of magnetic particles used range from 4 nm to 1,000 nm (Berry and Curtis, 2003; Ebner et al., 1997; Yavuz et al., 2006), while the magnetic force used ranged from 0.1 T to 8 T (Ebner et al., 1997; Yavuz et al., 2006).

2.5. Quantification of recovery

2.5.1. Protein quantification

For studies that involved cellulase recycling using methods such as recovery substrate-bound enzymes, protein quantification was used to evaluate the efficacy of enzyme recovery. Protein quantification tests used for enzymes include Bradford assay (Qi et al., 2011; Tu et al., 2007a; Tu et al., 2009), Ninhydrin assay (Tu et al., 2007b; Tu et al., 2009), Bio-Rad assay (Lu et al., 2002), RC DC assay (Tu et al., 2009), and isoelectric focusing (Tu et al., 2007a).

2.5.1.1. Micro-Bradford assay

Bradford assay utilizes the binding of proteins to the dye, Coomassie brilliant blue G-250, to quantify the protein content (Bradford, 1976). This dye changes its color when binding to proteins and the maximum absorbance shifts from 465 nm to 595 nm. Therefore, the amount of protein present in a sample is directly proportional to its absorbance when measured via a spectrophotometer. The absorbance values are measured at 595 nm wavelength and protein concentrations for each sample are obtained by comparing absorbance values of standards to known concentrations of a sample protein, typically bovine serum albumin (BSA).

The standard Bradford assay is used to test protein concentrations of 0.1 to 1.4 mg/ml and the micro-assay is used to test protein concentrations of 1 to 10 µg/ml. Therefore, micro-Bradford assay can be successfully used when relatively low concentrations of protein are present which would otherwise be undetectable by other protein quantifying methods. Low protein concentrations were successfully quantified with micro-Bradford assay for DNA binding protein (Dempsey et al., 1998), soluble guanylate cyclase (Zhao and Marletta, 1997), and allergenic protein in sunflower oil (Zitouni et al., 2000).

The maximum absorbances for various color stages of Coomassie brilliant blue dye has been identified as 470 nm for red, 590 nm for blue, and 650 nm for green, when tested for a range of wavelengths with 10 nm increments (Chial et al., 1993). Due to the reduction of the free dye concentration as protein is added, there is an overlap in the spectrum of the colors red and blue (Zor and Seliger, 1996). This affects the linearity of the standard curve for Bradford assay. Therefore to increase the linearity of the standard curve, Zor and Selinger (1996) used absorbance values at two wavelengths: 590nm and 450nm. They obtained more linear standard curves by plotting for the absorbance ratio 590 nm over 450 nm (A_{590}/A_{450}) than plotting for absorbances at 590 nm only. This linear range has been observed for the concentration region 0 to 20 µg/ml of the BSA standards. Furthermore, the use of A_{590}/A_{450} ratio improved the sensitivity of this assay by 10-fold.

When standard curves from the Bradford assay for the proteins BSA, cytochrome, lysosome, egg albumin, and ribonuclease were compared, each protein gave different curves with BSA being the most linear (Van Kley and Hale, 1977). This indicates that different proteins such as two different types of enzymes must not be compared against each other quantitatively; but rather the same type of protein at different situations can be compared.

3. PROBLEM STATEMENT AND OBJECTIVES

3.1. Problem statement

Growing demand for fuel, uncertainty of petroleum reserves, and environmental considerations has led to the search for alternative fuels that could reduce or replace the use of petroleum-based fuels. Cellulosic biofuel is an alternative fuel derived from an abundant source which emits less net carbon dioxide and can be used with existing transportation fuel infrastructure. Sugars obtained from the enzymatic hydrolysis of lignocellulosic biomass can be fermented to produce cellulosic biofuels such as ethanol, butanol or hydrogen.

Cellulosic biomass contains cellulose, hemicellulose and lignin, bound together within the plant cell walls. Of these, cellulose is composed of linear chains of hundreds to tens of thousands of glucose monomers. These chains can be broken down to glucose via enzymatic hydrolysis using cellulases. Cellulases are a mixture of enzymes containing endoglucanase (endocellulase), exoglucanase (exocellulase) and β-glucosidase (cellobiase). Endoglucanases break cellulose chains at random, internal, amorphous regions to give shorter chains with more chain ends. Exoglucanases cut these chains from ends to give glucose dimers (cellobiose). Then β-glucosidases further break these glucose dimers to glucose monomers. This glucose can be fermented to obtain fuel or chemicals.

One method of reducing enzyme cost is recovering and reusing enzymes after every cycle of hydrolysis. A possible method of recovering and reusing cellulases is by keeping the enzymes stationary and attached to a solid, recoverable substance so that it can be separated from solution after hydrolysis.

Enzymes have been attached to supports such as chitin, nylon, aluminium oxide beads, and pumice particles, and have been immobilized for various biological applications.

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Furthermore, cellulases have been immobilized on substances such as unhydrolyzed residue for reusing cellulase in consecutive cycles for hydrolysis of cellulosic substrates. However, immobilizing cellulases by attaching to polymer brushes for consecutive cycles of recovery and reuse in enzymatic hydrolysis of cellulosic substrates has not yet been attempted.

Polymer brushes are chains of polymers grafted or tethered on to solid surfaces or nano scale particles that give the physical appearance of a brush. The properties of these polymer brushes depend on the properties of the polymers used for creating the brush. Therefore, polymer brushes made from poly(acrylic acid) (PAA) are polyelectrolytes since the PAA chains of the brushes are charged. The negative charge of PAA brushes increase with increasing pH. This increased negative charge makes the brushes repel each other and stretch out. Therefore the PAA brushes are stretched at pH values higher than 7 and shrunk at pH values less than 4, while their structure is in between these two physical stages at pH between 7 and 4.

Enzymes are attached to PAA polymer brushes for recovery and reuse after initial hydrolysis. These brushes are made by grafting PAA polymers on nano-scale silica particles that contain magnetizable (Fe_3O_4) cores. This allows the particles with attached enzymes to be recovered using centrifugation or magnetic separation.

Once the enzymes are attached to the polymer brushes, the accessibility of enzymes to substrates may depend on the manner in which enzymes are attached. They could be: (1) attached to the silica surface rather than the brushes with brushes covering them since the brushes shrink and overlap at pH ideal for attachment, (2) several layers of enzymes could overlap leaving the bottom most enzymes inaccessible to substrates, or (3) the active sites of some enzymes could be inaccessible to substrates depending on how it is attached to the polymer brush.

When recovering attached enzymes after each cycle of hydrolysis, it is also important to quantify the amount of recovered enzymes. Protein quantification is necessary to evaluate the recovered enzyme content. When the assay used to quantify protein utilizes spectrophotometry, only the supernatants in each case should be tested since un-hydrolyzed residue and polymer brushes could be present in samples. Furthermore, the protein quantification method used must be capable of detecting low concentrations of protein since the amounts of enzyme loss to supernatant is usually below 10 µg/mL in hydrolysis experiments with enzymes attached to polymer brushes.

Furthermore, it is also necessary to evaluate the reusability of attached enzymes in consecutive cycles of enzymatic hydrolysis. Assessing the glucose yields at each cycle will lead to a clear understanding of how the recovered enzymes will function.

In this study, cellulase and β-glucosidase will be attached to PAA brushes grafted on silica nanoparticles. Hydrolysis experiments will be carried out with filter paper and Solka-Floc as substrates for cellulase and with cellobiose as substrate for β-glucosidase. First, the impact of the enzyme density on the brush particles will be studied to determine its effect on efficacy and recovery of attached enzymes. Then the effect of pH and temperature on hydrolysis with immobilized cellulases will be modeled using response surface methodology.

3.2. Project objectives

1. Determine the impact of polymer brush enzyme density on efficacy and recovery of immobilized cellulase and β-glucosidase.
2. Model effect of temperature and pH on sugar yields and enzyme recovery for enzymatic hydrolysis using attached (immobilized) β-glucosidase and cellulase.

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3.3. Hypotheses

1. Attachment to polymer brushes will cause only minor reduction in the activity of β-glucosidase and cellulase such that the glucose yields will be within 90% or higher when compared to free β-glucosidase and cellulase.

2. PAA brush particles have a saturation limit for attachment to cellulase and β-glucosidase.

3. Varying enzyme densities on polymer brush particles will result in different sugar yields for cellulase and β-glucosidase due to inaccessibility of enzymes to substrates as the enzyme density increase.

4. Varying enzyme densities on polymer brush particles will result in varying recovery results due to decreasing strength of attachment as the enzyme density increases on the brushes.

5. The ideal operating conditions (temperature and pH) for sugar yields and recovery of attached enzymes (β-glucosidase and cellulase) are different than for the free enzymes.

4. IMPACT OF ENZYME LOADING ON ACTIVITY AND RECOVERY OF CELLULASES IMMOBILIZED ON POLYMER BRUSHES

4.1. Abstract

The production cost of cellulosic biofuels can be reduced if the enzymes used in the enzymatic hydrolysis of biomass can be recovered and reused. Cellulases were attached to polymer brushes grafted on nano-scale silica particles in order to recover them. Enzyme densities on polymer brush particles were varied to determine the impact on hydrolysis and recovery. Hydrolysis was carried out with filter paper and powdered cellulose (Solka-Floc) as the substrates for attached cellulase (NS50013), and with cellobiose as the substrate for attached β-glucosidase (Novozyme188). For cellulase hydrolysis sugar concentrations with attached enzymes were 45% and 53% of those with free enzymes for filter paper and Solka-Floc, respectively. For the best case of attached β-glucosidases, hydrolysis glucose concentration was 38% of that with free enzymes. Increasing cellulase density on the brush particles had no impact on net enzyme efficacy. For attached cellulase, different proportions of cellobiose and glucose in hydrolyzates suggest differential attachment or efficacy of endoglucanases, exoglucanases, and β-glucosidases. For β-glucosidase, higher enzyme density on the brushes led to increased glucose concentrations. Enzyme density on the brushes did not impact β-glucosidase recovery. Approximately 66% of β-glucosidase remained attached to brushes and was recovered.

4.2. Introduction

Sugars obtained via the enzymatic hydrolysis of cellulosic biomass can be used to produce biofuels such as ethanol, butanol, or hydrogen (García et al., 2011; Huber et al., 2006). Enzymatic hydrolysis was identified as the second most expensive unit operation for producing cellulosic ethanol after pretreatment of biomass (Wyman, 2007). This is primarily due to the cost

of cellulase enzymes used in enzymatic hydrolysis. Wyman (1994) also suggested that recovering enzymes during conversion of cellulose to sugars is a possible method of reducing the cellulosic ethanol production costs. Immobilization of cellulases on a solid and recoverable substance will enable separation, recovery, and reuse.

Polymer brushes are polymers chains grafted on to solid surfaces or nano-scale particles giving the physical appearance of a brush (Brittain and Minko, 2007). They can be used as supports for immobilizing enzymes. Polymer brushes made from poly(acrylic acid) (PAA) are polyelectrolytes and their negative charge increases with increasing pH (Minko, 2006; Wang et al., 2010). Proteins can be attached to PAA brushes by electrostatic interactions. Bovine serum albumin (Czeslik et al., 2004b; Dai et al., 2006; de Vos et al., 2008), lipase (Chen and Hsieh, 2005), and hen egg-white lysozyme (Hollmann and Czeslik, 2006) are some examples of proteins which have all been successfully immobilized on PAA brushes. However, immobilization may also change the properties and activity of the attached enzymes. It could hinder (Gianfreda and Bollag, 1994; Simionescu et al., 1984) or enhance (Iso et al., 2001) enzyme activity.

Cellulase and β-glucosidase are often loaded based on activity for hydrolysis in filter paper units (FPU) or cellobiose units (CBU), respectively. Immobilized enzymes are loaded to match the protein content of the volume of free enzyme needed at the desired activity in FPU or CBU. The amount of enzyme immobilized per unit mass of brush particles can be altered. Various enzyme densities on brush particles can be obtained by varying the initial amount of enzyme that will be added to brush particle dispersion during the attachment process. The brushes could be under-loaded or overcrowded with enzymes as illustrated in figure 4.1. Therefore, the usage of brush particles can be reduced if they have a higher density of

immobilized enzymes. However, the enzyme density on the brush matrix could also have an impact on net enzyme efficacy. The enzymes could be either attached on the surface of PAA chains as a monolayer, or as overlapping layers of enzymes on the brush surface (Srere and Ovadi, 1990). Accessibility of enzymes to substrates may be limited if several layers of enzymes are overlapping. Therefore, it is necessary to find the most suitable enzyme to brush particle ratio that will facilitate the highest enzyme density on the polymer brushes without hindering net enzyme efficacy.
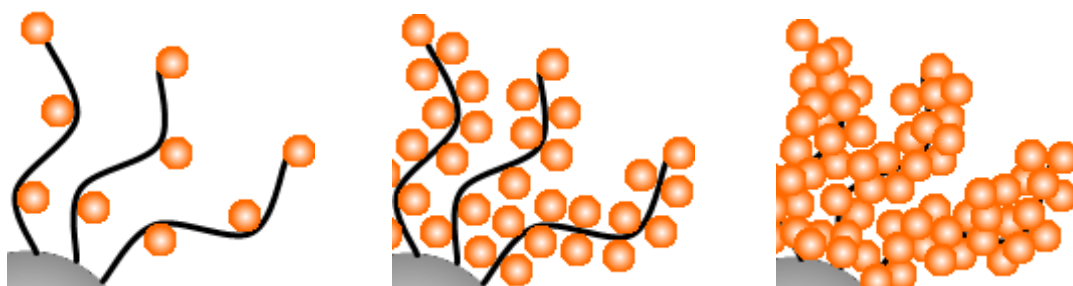
Figure 4.1: Illustration of increasing enzyme density on brush particles, from left to right.

The objective of this study is to determine the impact of enzyme to brush particle ratio on efficacy and recovery of commercial enzymes: cellulase (NS50013) and β-glucosidase (Novozyme188). Enzyme densities on polymer brush particles were varied to determine the impact on hydrolysis and recovery. Efficacy of cellulase was tested by hydrolysis of filter paper and Solka-Floc, while β-glucosidase was evaluated using cellobiose as substrate. PAA brushes with attached enzymes were recovered by centrifugation after hydrolysis.

4.3. Materials and methods

4.3.1. Enzymes

Cellulase (NS50013) from *Trichoderma reesei* and β-glucosidase (Novozyme188) from *Aspergillus niger* were provided by Novozyme (Franklinton, NC). Activities of cellulase and β-

glucosidase were found to be 77 FPU/mL and 500 CBU/mL, respectively, according to IUPAC

activity assays (Ghose, 1987).

4.3.2. Polymer brush particles

Polymer brush particles for immobilizing cellulases were synthesized at Clarkson

University (Potsdam, NY) (Ionov et al., 2009; Minko, 2006). They consisted of poly(acrylic

acid) (PAA) polymer brushes (≈25 nm brush length) grafted on silica particles (≈100 nm in

diameter). Brush particles were obtained as a suspension with deionized water. Brush particles

were dried for storage by freezing the suspension and freeze drying for 48 h in FreeZone 4.5 L

benchtop freeze dry system (LABCONCO; Kansas City, MO).

4.3.3. Enzyme attachment to polymer brushes

Dried polymer brush particles were resuspended in 50 mM sodium citrate buffer (pH

6.1), by the ratio of 0.02 g of brush particles per 1 mL of buffer. The suspension was mixed by

two alternating cycles of vortexing for 2 min at 3200 rpm and sonicating for 20 min at a

frequency of 40 kHz. The pH of the dispersion was slowly adjusted to 4.5 with 1 M HCl before

adding enzymes at the desired enzyme loadings. Varying amounts of cellulase or β-glucosidase

were added to the polymer brush particle suspensions to obtain attached enzymes stocks with

various enzyme to brush ratios.

After enzyme addition, each solution was left for 24 h with magnetic stirring for enzymes

to attach to the brushes. Attached enzymes were recovered from the solution as pellets via

centrifugation at 16,000×g for 30 minutes. The supernatants were tested for protein content using

micro-Bradford assay (Bradford, 1976). The assay was carried out with Bovine Serum Albumin

(BSA) as protein standards and Brilliant Blue G in phosphoric acid and methanol as the reagent.

Both the standards and the reagents were from Sigma-Aldrich (St. Louis, MO). For improved

accuracy, the method of linearization of micro-Bradford assay (Zor and Seliger, 1996) was used by measuring abosrbances at 590 and 450 nm. Enzyme attachment was determined from the protein content of enzyme added during attachment and protein content remaining in supernatant. The recovered pellets of attached enzymes were re-suspended in 50 mM sodium citrate buffer at pH 4.8 to use in hydrolysis and recovery experiments.

4.3.4. Determining enzyme saturation on brushes

Attached enzyme stocks were prepared with increasing volumes of enzyme loadings for the same amount of brush particles to obtain brush particles loaded with varying enzyme densities. The amount of enzyme added was quantified based on protein content. At each enzyme loading, a protein balance was carried out to determine the amount of protein attached based on the protein amounts added and that remaining in supernatant after separation. The resulting amount of attached protein was plotted against the amount of protein added to determine enzyme density saturation point.

4.3.5. Hydrolysis

Enzymatic hydrolysis was carried out at 50°C in pH 4.8 sodium citrate buffer mixed at 150 rpm. Total volume of hydrolysis solution was 10 mL in each 50-mL Erlenmeyer flask. Hydrolysis with cellulase was carried out for two separate substrates: Whatman number 1 filter paper and Solka-Floc (200 NF) powdered cellulose (International Fiber Corporation, Urbana, Ohio). Substrate used for β-glucosidase was cellobiose powder. Substrate loading for all cases was 1% (0.1 g of substrate per 10 mL of hydrolysis solution). Free cellulase was added at 10 FPU/g of filter paper and 5 FPU/g of Solka-Floc as controls. Attached cellulase with different enzyme densities were added based on protein content to match 10 and 5 FPU/g of substrate loading of free enzyme for both cases. Similarly, free β-glucosidase was loaded based on 10

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CBU/g of substrate as the control. Attached β-glucosidase at different enzyme densities were added based on protein content to match 10 CBU/g of substrate of free enzyme. Each hydrolysis treatment was carried out in triplicate.

4.3.6. Quantification of sugars

Sampling for quantifying sugar concentrations after hydrolysis with cellulase was carried out after 24 h of hydrolysis for cellulose substrates. 24 h of hydrolysis was used to compare the initial hydrolysis rates for free and attached enzymes, rather than allowing them to reach complete hydrolysis. Samples for cellobiose hydrolysis with β-glucosidase were taken after 6 h. All samples were immersed in boiling water for 10 min to terminate the enzyme catalyzed reactions. Cellulase hydrolyzate samples were centrifuged at 16,000×g for 10 min to remove unhydrolyzed residue. All hydrolyzate samples were filtered with Acrodisc Sterile Syringe Filters with 0.2 µm nylon membranes (Pall Life Sciences; Port Washington, NY). Glucose and cellobiose concentrations were quantified using high-performance liquid chromatography (HPLC) (Waters Corporation; Milford, MA), with an Aminex HPX-87P resin column by Bio-Rad (Hercules, CA) operated at 50°C. The refractive index detector (model 2414) (Waters, Milford, MA) was operated at 85°C. Flow rate of the mobile phase, 18 mΩ water, was 0.6 mL/min.

4.3.7. Enzyme recovery after hydrolysis

Sampling for enzyme recovery was carried out after 72 h of hydrolysis for cellulase. The 72 h hydrolysis duration for cellulase was chosen to allow near complete hydrolysis of the insoluble substrates: filter paper and Solka-Floc, so that the unhydrolyzed residue of these insoluble substrates would not interfere with recovery. Sampling for enzyme recovery for β-

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glucosidase was carried out after 6 h of hydrolysis. Hydrolysis duration of 6 h for β-glucosidase was not increased since the soluble cellobiose will not interfere with recovery.

Quantification of attached enzyme recovery after hydrolysis was carried out similarly to the initial quantification of enzyme attachment to brushes. The samples were centrifuged at 16,000×g for 30 minutes followed by testing of the supernatants with modified micro-Bradford assay (Bradford, 1976; Zor and Seliger, 1996) after recovering attached enzymes as pellets. The percentage of protein recovered was calculated based on the protein content of attached enzyme added for hydrolysis and the protein content in supernatant after separation.

4.3.8. Statistical analysis

One way analysis of variance (ANOVA) was carried out for some results using Microsoft Excel (2010) with Analysis ToolPak (Microsoft; Redmond, WA). Post hoc comparisons using Tukey-Kramer multiple comparison test was carried out using multcompare function in the MATLAB Statistics Toolbox (2013b) (The Mathworks Inc.; Natick, MA).

4.4. Results and discussion

4.4.1. Cellulase

Figure 4.2 shows a steady increase in protein attachment as enzyme loading increases up to the attachment density of 104 μg protein /mg brush (point C). Above the protein loading of point C, no increase in attached enzyme density was seen, indicating that the brushes were saturated with enzymes. Like any other enzyme immobilization surface, there is a saturation limit to immobilizing proteins on polymer brushes (Gautrot et al., 2009; Jain et al., 2007; Sun et al., 2006).
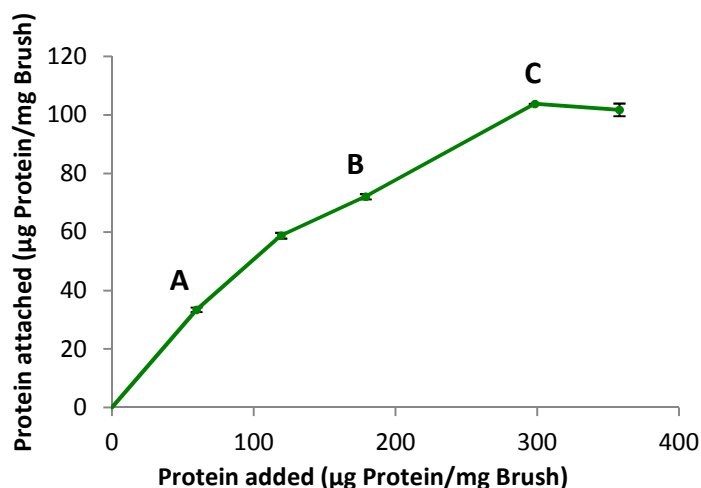
Figure 4.2: Cellulase attachment for increasing amounts of initial enzyme added for a fixed amount of polymer brush particles. Error bars represent standard deviations in protein testing.

Attached enzyme stocks containing 33, 72, and 104 µg protein /mg brush, marked A, B, and C, respectively, in figure 4.2 were used for hydrolysis experiments. These three attached enzyme stocks were chosen to determine the effect of enzyme density on brush particles on hydrolysis rates at a constant enzyme loading based on activity. The respective soluble sugar concentrations are presented in Figure 4.3.
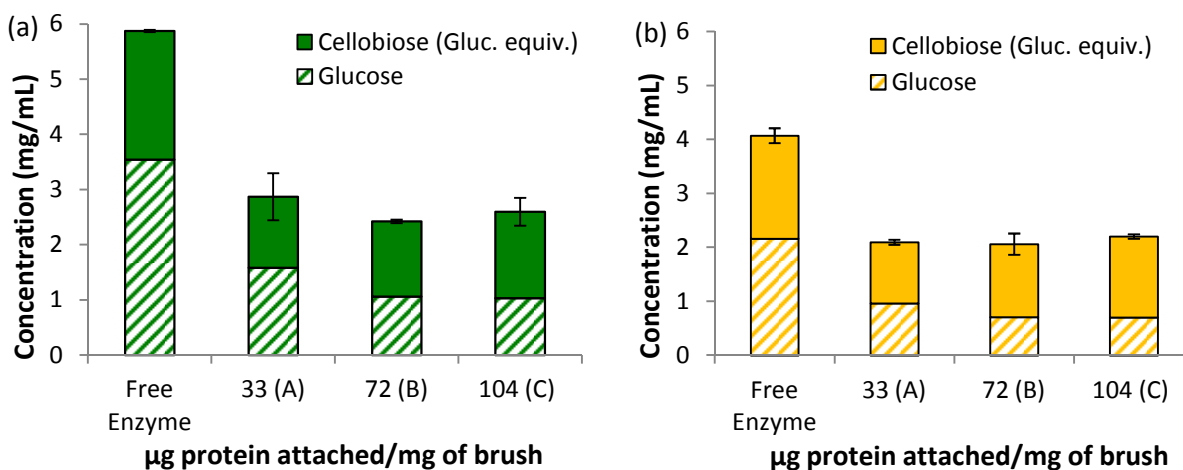
Figure 4.3: 24 h soluble sugar concentrations for hydrolysis of (a) filter paper (10 FPU/g) and (b) Solka-Floc (5 FPU/g) with free cellulases, and attached cellulases with increasing enzyme densities. Error bars represent standard deviations of the sum of glucose and glucose equivalents of cellobiose.

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Hydrolysis with attached cellulase resulted in soluble sugar concentrations 45% and 53% of that with free enzyme for filter paper and Solka-Floc, respectively. Reduced hydrolysis rates could be caused by (1) reduced activity of attached enzymes or (2) physical limitations of substrates reaching the enzymes attached on the brushes. The electrostatic properties of enzymes could change when they are attached to the brushes leading to changes in the physical structure of the protein and its affinity towards the substrate. Secondly, enzymes could be overlapping on the brush surface or buried within the brush matrix, leading to physical limitations for the interactions between enzymes and substrates. However, the loading of attached enzymes for hydrolysis can be increased to get higher sugar rates since a larger proportion of attached enzymes can be recovered.

The attached cellulase samples, with different enzyme densities on brush particles, resulted in statistically similar sugar concentrations for filter paper and Solka-Floc. Analysis of variance (ANOVA) (table 4.1) was carried out for both the substrates to compare total soluble sugars (sum of glucose and glucose equivalence of cellobiose) obtained by the three enzyme densities. Total reducing sugars was not significantly different for filter paper ($p>0.05$) across the three enzyme densities. Total reducing sugars for Solka-Floc was also not significantly different for filter paper ($p>0.05$) across the three enzyme densities. Therefore, cellulase density on the brushes did not have an effect on hydrolysis. The attached enzymes were observed to be moving throughout the brush surface rather than being static (Kudina et al., 2013). This movement of enzymes throughout the brush matrix could allow attached enzymes to interact with substrates, irrespective of the level of enzyme crowding on the brushes. It may even lead to better association of enzymes to insoluble substrates. Therefore, use of high enzyme density brush

particles (similar to stock C) will reduce the amount of brush particles required without

negatively impacting hydrolysis rates.

Table 4.1: ANOVA for the comparison of total soluble sugars obtained during hydrolysis of filter paper and Solka-Floc with three cellulase densities on the brushes.

Source of Variation	Filter paper				Solka-Floc			
	df[a]	MS[b]	F	p-value	df[a]	MS[b]	F	p-value
Between groups	2	0.150	1.84	0.239	2	0.016	1.45	0.336
Within groups	6	0.082			4	0.011		
Total	8				6			

[a] df = degrees of freedom
[b] MS = mean square

The proportion of cellobiose in hydrolyzates differed for free and attached enzymes.

Cellobiose (glucose equivalence) to glucose ratios were 1:1.5 and 1:1.1 with free enzymes for

filter paper and Solka-Floc respectively. Cellobiose to glucose ratios with free enzymes were

1:0.8 and 1:0.6 for filter paper and Solka-Floc, respectively. This could be due to (1) β-

glucosidase having less affinity for polymer brushes compared to endoglucanases and

exoglucanases leading to differential attachment, (2) reduced activity in attached β-glucosidase,

or (3) reduced accessibility of cellobiose substrate to attached β-glucosidase. Protein attachment

to polymer brushes is selective and is depended on the charge of the brushes and the protein at

that particular environment (Gautrot et al., 2009; Minko, 2006). When considering isoelectric

points of cellulases from *Trichoderma reesei*, exoglucanases range from 4.7 to 5.1,

endoglucanases range from 4.2 to 7.6, and β-glucosidase range from 5.3 to 6.4 (Cantarel et al.,

2009). These isoelectric points also differ depending on the organism from which they were

extracted and the commercial preparation. Isoelectric points of endoglucanases from

Trichoderma viride and *Trichoderma harzianum* were 4.32 and 5.0, respectively, while the

isoelectric points of a cellulase mixture from *Trichoderma harzianum* ranged from 6.4 to 7.6

(Huang et al., 2010; Thrane et al., 1997). Even though some of these ranges overlap, the

differences in the isoelectric points of individual endoglucanases, exoglucanases, and β-glucosidase at the attachment pH of 4.5 could cause differential attachment.

Attached β-glucosidase may also have reduced accessibility to substrates due to mass transfer limitations. Substrates for endoglucanases and exoglucanases are insoluble cellulose chains. Endoglucanases and exoglucanases attached to the same brush particle could cleave multiple sections of the same cellulose chain since the enzymes and the substrate will be immobilized together while enzymes are cleaving. Substrates for β-glucosidase, however, are soluble cellobiose. These soluble dimers are randomly dispersed in the hydrolysis solution and will not be localized like the insoluble cellulose chains. Therefore, attached β-glucosidase could be less effective than attached endoglucanases and exoglucanases because enzymes are concentrated on brushes while substrate is uniformly distributed through solution.

Results for the recovery of attached cellulase after hydrolysis, quantified based on protein content, is presented in figure 4.4. Almost all of the cellulase appears to be recovered from hydrolysis of both filter paper and Solka-Floc. However, complete hydrolysis of substrates was not achieved by 72 h. Therefore, insoluble unhydrolyzed residue was also present in hydrolyzate and was also pelleted with the attached enzymes during centrifugation. Any free enzymes that were detached from the brushes could be pelleted along with these residues when they are attached to the substrates. Enzyme attachment to unhydrolyzed residue has been documented in literature (Gregg and Saddler, 1996). Therefore, in addition to the actual recovery on polymer brush particles, free enzymes might have also attached to unhydrolyzed residue resulting in higher recoveries than would be seen at loadings where more complete hydrolysis would occur. Actual recovery efficiency for cellulases requires additional experimentation at higher enzyme loadings so that complete hydrolysis can be observed.
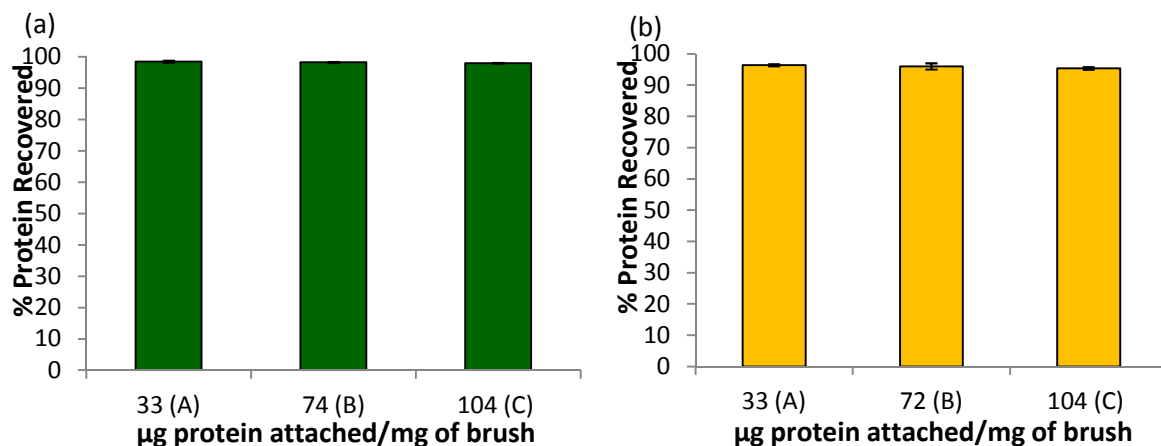
Figure 4.4: Apparent protein recovery after 72 h of hydrolysis with attached cellulases with increasing enzyme densities on the brushes for (a) filter paper (10 FPU/g) and (b) Solka-Floc (5 FPU/g). Error bars represent standard deviations in protein recovery.

4.4.2. β-glucosidase

Figure 4.5 shows the concentration of attached β-glucosidase with increasing enzyme loadings during attachment. As in the case of cellulase, the enzyme attachment curve shows a steady increase in protein attachment as enzyme addition increases up to attachment density of 111 µg protein /mg brush. Enzyme attachment did not increase beyond 113 µg protein/mg brush onwards (point C), indicating that the brushes were saturated with β-glucosidase. Attached enzyme stocks with 31, 66, and 113 µg protein /mg brush, marked A, B, and C respectively in figure 4.5 were used for hydrolysis experiments. The glucose concentrations for these three attached enzyme stocks are presented in Figure 4.6.
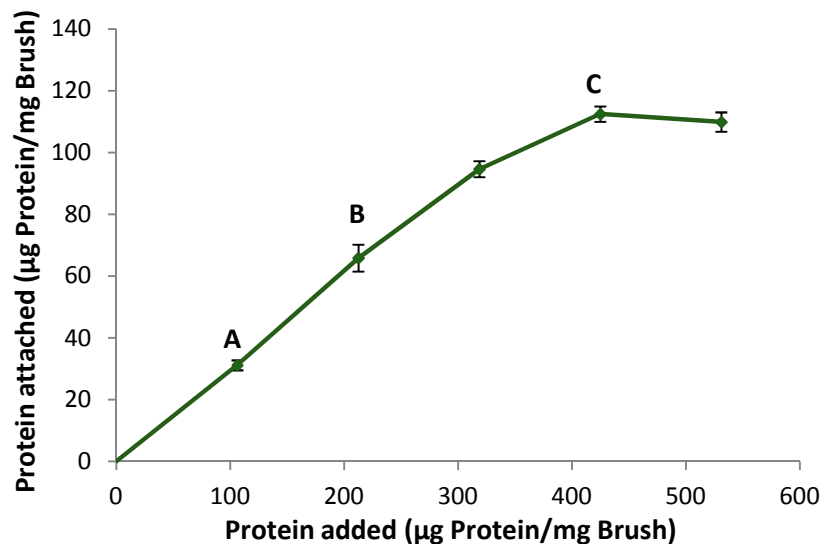
Figure 4.5: β-glucosidase attachment levels with varying amounts of initial enzyme added per fixed amount of polymer brush particles. Error bars represent standard deviations in protein testing.
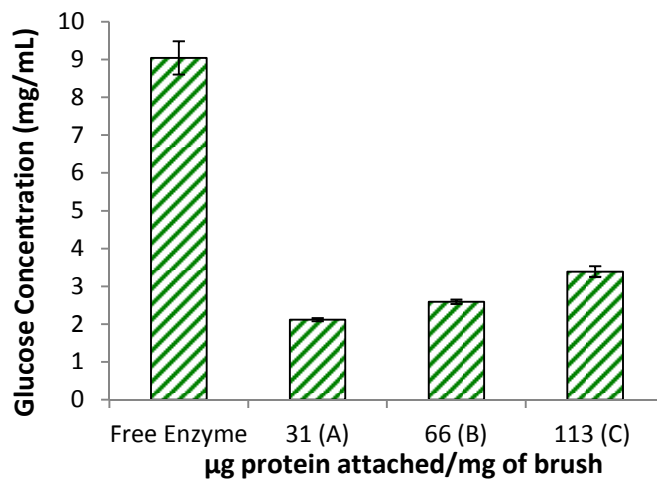
Figure 4.6: Glucose concentrations by free and attached β-glucosidase at an enzyme loading equivalent to 10 CBU/g of cellobiose at 6 h. Error bars represent standard deviations in glucose concentrations.

Similar to cellulase, higher glucose concentrations were obtained for free β-glucosidase than the attached, indicating reduced activity of the attached enzymes. Hydrolysis glucose concentrations with attached β-glucosidase as percentages of that with free β-glucosidase were between 23% to 38%. Percentage soluble sugars from hydrolysis with attached cellulase were

approximately 49% of that of free. Endogulcanases and exoglucanases attached on a brush particle could simultaneously work on one cellulose fibril that is within close proximity to the brush. However, the attached β-glucosidase will be localized on brush particles while the soluble cellobiose substrates will be in random motion throughout the hydrolysis solution. This could lead to relatively lower efficacy of attached β-glucosidase compared to attached cellulase.

Furthermore, contrary to results with attached cellulase, treatments with higher β-glucosidase density showed increased glucose concentrations. Increasing enzyme densities on the brushes from 31, 66, and 113 µg protein /mg brush had decreasing amounts of brush particles per unit volume of hydrolysis solution since attached enzymes were loaded based on protein concentrations. This observation could be due to a physical change in the brushes caused by the presence of β-glucosidase since PAA brushes have shown swelling of brushes, increasing the thickness of the brush layer, in the presence of enzymes (Kudina et al., 2013).

Recovery results in Figure 4.7 were not impacted by unhydrolyzed residue since celloboiose is soluble and therefore not separable via centrifugation. Higher enzyme density on the brush particles did not impact enzyme recovery (figure 4.7). Analysis of variance (ANOVA) was carried out to compare protein recovery for the three β-glucosidase densities on the brushes and is presented in table 4.2. Protein recovery was significantly different ($p = 0.004$) for the three enzyme densities. According to the Tukey-Kramer multiple comparison test (table 4.3), protein recovery for attached enzymes at densities 66 and 113 µg protein /mg brush (B and C) were not significantly different. However, the recovery at enzyme density 31 µg protein/mg brush (A) was significantly different from the remaining two higher enzyme densities. Approximately 66% of attached enzymes were recovered via centrifugation.
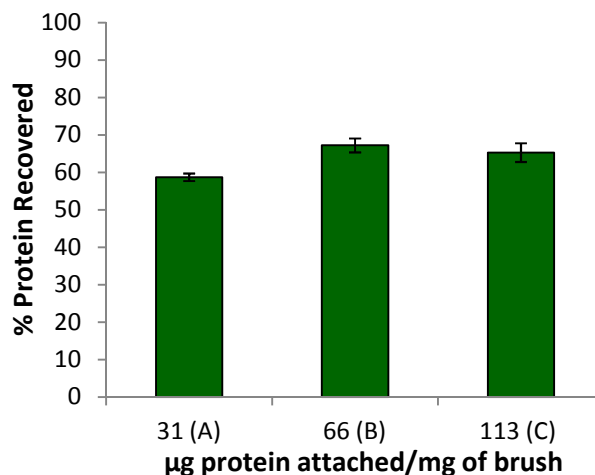
Figure 4.7: Protein recovery after 6 h of cellobiose hydrolysis with attached β-glucosidase with varying enzyme densities on the brushes loaded at an equivalence of 10 CBU/g of cellobiose. Error bars represent standard deviations in protein recovery.

Table 4.2: ANOVA for the comparison of protein recovery after hydrolysis of cellobiose with the three β-glucosidase densities on the brushes.

Source of Variation	Df	MS	F	p-value
Between Groups	2	59.63	16.7	0.004
Within Groups	6	3.57		
Total	8			

[a] df = degrees of freedom
[b] MS = mean square

Table 4.3: Analysis by Tukey-Kramer multiple comparison test for protein recovery after hydrolysis of cellobiose with the three β-glucosidase densities on the brushes.

Enzyme density on the brushes (μg protein attached/mg brush)	Protein recovery (%)*
31 (A)	58.72[a]
66 (B)	67.22[b]
113 (C)	65.31[b]

* Means sharing same superscripted letter are not significantly different at p<0.05 according to Tukey-Kramer multiple comparison test.

4.5. Conclusions

For both the cellulase and β-glucosidases, sugar concentrations by hydrolysis with free enzymes were higher than that with attached enzymes at the selected sampling times. Increasing

cellulase densities on the brush particles did not affect hydrolysis rates. Therefore, a smaller amount of brush particles with a higher enzyme density can be used. However, the proportion of cellobiose in hydrolyzates were higher for attached enzymes than for free enzymes. This could be due less affinity of β-glucosidase to polymer brushes compared to endoglucanases and exoglucanases, reduced activity in attached β-glucosidase, or reduced accessibility of substrates to attached β-glucosidase.

Increasing β-glucosidase densities on the brushes gave increasing glucose concentrations. This was contrary to results with attached cellulase, and these increasing enzyme densities on the brushes had decreasing amounts of brush particles per unit volume of hydrolysis solution since attached enzymes were loaded based on protein concentrations. This could be due to a physical change in the brushes caused by the presence of β-glucosidase.

The high recovery of attached cellulases seen was affected by the presence of unhydrolyzed residue. High enzyme density on the brushes did not impact the recovery of attached β-glucosidase. Approximately 66% of attached β-glucosidase was recovered.

5. MODELING THE EFFECT OF PH AND TEMPERATURE FOR CELLULASES IMMOBILIZED ON POLYMER BRUSHES

5.1. Abstract

Production cost of cellulosic biofuels can be lowered if cellulases are recovered and reused. Cellulase (NS50013) and β-glucosidase (Novozyme188) were immobilized on polymer brushes consisting poly(acrylic acid) chains grafted on silica nanoparticles. Response surface methodology was used to model the effects of pH and temperature on hydrolysis and recovery. Highest hydrolysis sugar concentrations with attached cellulase and β-glucosidase were 71% and 54% of that with free enzymes, respectively. Attached enzyme loading can be increased to overcome this lower efficacy since they can be recovered and reused. Ideal temperature for hydrolysis with both the attached and free cellulase and β-glucosidase was 50ºC. The optimal pH for attached and free cellulase were 5 and 4.4 respectively, while it was 4.4 for both attached and free β-glucosidase. Free β-glucosidase was more stable with temperature range tested than attached enzymes. Recovery of attached β-glucosidase decreased with increasing temperature and was not affected by temperature.

5.2. Introduction

Sugars from lignocellulosic biomass can be used to produce biofuels such as ethanol, butanol, or hydrogen (García et al., 2011; Huber et al., 2006). As production costs are lowered, these fuels will become more competitive with petroleum-based fuels. Recovering and reusing enzymes has been identified as one method of lowering production costs of cellulosic biofuel (Wyman, 1994). Immobilizing cellulases, the enzymes used for the hydrolysis of cellulosic biomass on recoverable supports will enable these enzymes to be reused in consecutive cycles of hydrolysis.

Polymer brushes can be used as a support for immobilizing enzymes. They are made of polymer chains grafted on flat surfaces, nanoparticles, or inside walls of micro-channels to give the physical appearance of a brush (Brittain and Minko, 2007). Poly(acrylic acid) (PAA) is a polyelectrolyte and therefore polymer brushes made from PAA chains are charged (Wang et al., 2010). This allows protein molecules to attach or detach to brushes depending on the brush charge. Polyelectrolyte brushes respond to the ionic strength and pH of the surrounding; strong binding of protein to PAA brushes occurs at low ionic strength (Czeslik et al., 2004b) and changes in pH causes the brushes to physically stretch or shrink (Minko, 2006). The negative charges of the PAA brushes increase with increasing pH, making the brushes repel each other and stretch out. Therefore, the PAA brushes are stretched at pH higher than 7 and shrunk at pH less than 4, while their physical structure is in between these two stages for pH between 7 and 4.

Immobilization or attachment to supports may change the properties and behavior of the attached enzyme. Immobilization could hinder (Gianfreda and Bollag, 1994; Simionescu et al., 1984) or enhance (Iso et al., 2001) the efficacy of enzymes, depending on the enzyme and immobilization support combination. Immobilization restricts folding and unfolding of the enzymes leading to changes in how they behave at different pH and temperature compared to free enzymes. Also, interactions with the immobilization support could alter the enzymes' affinity towards the substrates. The active sites could also be inaccessible to substrates due to immobilization. Therefore the properties and behavior of the attached enzyme may vary for different enzyme and support configurations.

The ideal pH range for the activity of commercially available free cellulase is from 4.2 to 5.8, with 4.8 being the most commonly used pH (Pardo and Forchiassin, 1999b; Vatanparast et al., 2012). The most commonly used temperature range is from 50° to 55^oC (Andreaus et al.,

1999; Kumar et al., 2008; Sternberg et al., 1977). The active pH range for free β-glucosidase activity is from 3 to 7, with 4.3 being the most commonly used (Sternberg et al., 1977; Woodward and Wiseman, 1982); the active temperature is from 23° to 55^oC with 45^oC considered optimum (Pardo and Forchiassin, 1999b; Woodward and Wiseman, 1982). However, the optimal conditions for immobilized cellulase and β-glucosidase may differ from those of free.

The objective of this study is to model the effect of pH and temperature on sugar hydrolysis rates and protein recovery after hydrolysis of cellulosic substrates with attached cellulase (NS50013) and β-glucosidase (Novozyme188). Response surface methodology was used for modeling and the effects of using enzymes attached on polymer brush particles were compared against the effects of using free enzymes.

5.3. Materials and methods

5.3.1. Enzymes

Novozyme (Franklinton, NC) provided cellulase (NS50013) from *Trichoderma reesei* and β-glucosidase (Novozyme188) from *Aspergillus niger*. Enzyme activity of cellulase was 77 FPU/mL, while that of β-glucosidase was 500 CBU/mL, according to IUPAC activity assays by Ghose (1987).

5.3.2. Polymer brush particles

The supports used for immobilizing the enzymes were polymer brush particles synthesized at Clarkson University (Potsdam, NY) (Ionov et al., 2009; Minko, 2006). They consisted of silica cores (≈100 nm diameter) on which poly(acrylic acid) (PAA) polymer brushes (≈25 nm length) were grafted. These particles were received as a suspension in water, and freeze

dried for 48 h with FreeZone 4.5 L benchtop freeze dry system by LABCONCO (Kansas City, MO) to obtain dried brush particles for storage.

5.3.3. Immobilization of cellulases on polymer brushes

Brush particle dispersions were made by resuspending dried polymer brush particles at a ratio of 0.02 g of particles per 1 mL of 50 mM sodium citrate buffer (pH 6.1). The resuspension process was expedited by two alternating cycles of vortexing at 3200 rpm for 2 min and sonicating at a frequency of 40 kHz in Branson 5510 sonic bath (Branson Ultrasonics; Danbury, CT). After the dispersion process, the pH was slowly adjusted to 4.5 with drop wise addition of 1M HCl under stirring. Enzymes (cellulase or β-glucosidase) were added and left for 24 h with magnetic stirring to allow attachment. To recover the attached cellulase, the enzyme-brush suspension was centrifuged at 16,000×g for 30 minutes. The pelleted attached enzymes were recovered and resuspended in 50 mM sodium citrate buffer at the desired pH for use in hydrolysis experiments. Protein contents of the supernatants were tested to quantify enzyme attachment as a protein balance of protein initially added for attachment and protein lost to supernatant.

5.3.4. Experimental design and statistical analysis

Response surface methodology (RSM) was used to model the effect of pH and temperature on hydrolysis in terms of sugar concentrations or protein recovery. A face centered design was used for the two factors with three levels per factor and five replicates at the center point. The experimental design and coded levels are presented in figure 5.1 and table 5.1. The second order equations for modeling temperature and pH by multiple regression were in the form:

$$Y = \beta_0 + \beta_T T + \beta_P P + \beta_{TP} TP + \beta_{TT} T^2 + \beta_{PP} P^2 \tag{5.1}$$

Y represents the response variable (glucose concentration, total reducing sugars, or protein recovery) while T and P represent the uncoded independent variables temperature and pH respectively. β_0 is the constant while the regression coefficients are represented by β_T and β_P for linear, β_{PP} and β_{TT} for quadratic, and β_{TP} for interaction, coefficients for the model.

Table 5.1: Coded levels for modeling temperature and pH.

Variable	Coded Variable	Level		
		-1	0	1
Temperature (°C)	T	37	43.5	50
pH	P	4	4.7	5.4
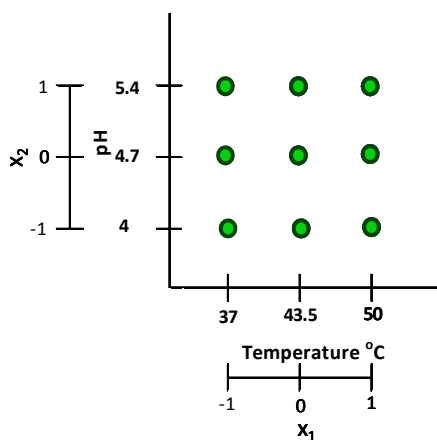
Figure 5.1: Experimental design and coded levels for modeling temperature and pH.

Analysis of variance (ANOVA) and the regression analysis were carried out using MINITAB statistical software (Minitab Inc.; State College, PA). Statistically insignificant (p>0.05) terms were not included in the final models.

5.3.5. Hydrolysis

Temperature and pH for enzymatic hydrolysis with free and attached cellulase and β-glucosidase were used according to the experimental design. Each run was performed in 50 mL Erlenmeyer flasks with 0.1 g of substrate in 10 mL of hydrolysis solution (1% substrate loading). Substrates used were Solka-Floc powdered cellulose (200 NF) by International Fiber Corporation (Urbana, Ohio) for cellulase and cellobiose powder for β-glucosidase. Free cellulase

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was added at an enzyme loading of 25 FPU/g of substrate and attached cellulase was added based on protein content to match the concentration of free enzyme. Similarly, free β-glucosidase was added at a loading of 30 CBU/g of substrate while attached β-glucosidase was added based on protein content to match the concentration of free enzyme. The pH of each flask was set at the desired value by using 50 mM sodium citrate buffer prepared at each pH value. All runs were continuously stirred in water baths at 150 rpm.

5.3.6. Sampling and enzyme recovery

Hydrolysis flasks with cellulase were sampled after 18 h of hydrolysis to test for glucose and total reducing sugar concentrations. This time point was used to compare the initial hydrolysis rates for free and attached enzymes. Further sampling was carried out at 120 h to test enzyme recovery after more complete hydrolysis. Experiments using β-glucosidase were sampled after 6 h of hydrolysis to test glucose concentration and enzyme recovery. Samples were immersed in boiling water for 10 min to terminate further catalytic reactions before filtration with 13 mm Acrodisc syringe filters with 0.2 μm nylon membranes (Pall Life Sciences; Port Washington, NY). Samples retrieved to quantify enzyme recovery were centrifuged at 16,000×g for 30 min and supernatants were tested for protein content to determine recovery efficiency.

5.3.7. Quantification of sugars

High-performance liquid chromatography (HPLC) by Waters Corporation (Milford, MA) was used for determining glucose and cellobiose concentrations in each sample. They were quantified using an Aminex HPX-87P resin column by Bio-Rad (Hercules, CA) operated at 50°C, and a refractive index detector (model 2414) by Waters (Milford, MA) operated at 85°C. The flow rate of the mobile phase (18 mΩ water) through the column was 0.6 mL/min.

Dinitrosalicylic acid (DNS) assay was used to quantify total reducing sugars of the hydrolysate samples by free and attached cellulase (Ghose, 1987). DNS reagent was made with 3,5-dinitrosalycilic acid, sodium hydroxide (NaOH), deionized water, Na-K tartrate, phenol, and sodium metabisulphate ($Na_2S_2O_5$). D-glucose dissolved in deionized water was used as a standard and the analysis was carried out at 540 nm.

5.3.8. Quantification of protein

Protein content was tested using micro-Bradford assay (Bradford, 1976) with Bovine Serum Albumin (BSA) as a protein standard and Brilliant Blue G in phosphoric acid and methanol as reagents; all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Accuracy of this quantification method was improved by using the linearization method by Zor and Selinger (1996) with the absorbance readings at wavelengths 590 and 450 nm.

5.4. Results and discussion

5.4.1. Efficacy of attached cellulase

Table 5.2 contains the experimental data and the model-predicted values for glucose concentrations and total reducing sugars using free and attached cellulase. Analysis of variance (ANOVA) for glucose and reducing sugar concentrations obtained by hydrolysis with cellulase are shown in table 5.3. Terms with a p-value higher than 0.05 were considered insignificant.

The model for glucose concentrations achieved by hydrolysis with free cellulase is presented by equation 5.2, with the response indicated by the term G_{FC}. The quadratic term for temperature was statistically not significant ($p>0.05$) (table 5.3) and was therefore removed from the model.

$$G_{FC} = -27.1 + 0.205T + 10.7P - 1.10P^2 - 0.0192TP \tag{5.2}$$

Table 5.2: Treatment conditions, data, and predicted values for glucose and reducing sugar concentrations after hydrolysis of Solka-Floc using free and attached cellulase.

Run	Uncoded Levels		Glucose (g/L)				Reducing Sugars (g/L)			
	$T^{[a]}$	$P^{[b]}$	Free		Attached		Free		Attached	
			$Exp^{[c]}$	$Pre^{[d]}$	$Exp^{[c]}$	$Pre^{[d]}$	$Exp^{[c]}$	$Pre^{[d]}$	$Exp^{[c]}$	$Pre^{[d]}$
1	37	4	2.78	2.77	0.40	0.28	4.48	4.50	1.95	1.90
2	37	4.7	3.01	3.06	1.72	2.01	4.51	4.57	2.83	3.12
3	37	5.4	2.28	2.27	1.41	1.31	3.78	3.80	2.47	2.38
4	43.5	4	3.61	3.61	0.36	0.38	5.33	5.24	2.20	2.09
5	43.5	4.7	3.78	3.81	2.35	2.34	5.38	5.31	3.64	3.58
6	43.5	4.7	3.79	3.81	2.37	2.34	5.35	5.31	3.61	3.58
7	43.5	4.7	3.81	3.81	2.37	2.34	5.33	5.31	3.61	3.58
8	43.5	4.7	3.83	3.81	2.38	2.34	5.22	5.31	3.66	3.58
9	43.5	4.7	3.79	3.81	2.39	2.34	5.30	5.31	3.63	3.58
10	43.5	5.4	2.92	2.93	1.89	1.88	4.61	4.54	3.14	3.11
11	50	4	4.41	4.44	0.37	0.47	5.90	5.98	2.14	2.28
12	50	4.7	4.58	4.56	2.82	2.68	6.08	6.05	4.14	4.04
13	50	5.4	3.56	3.59	2.35	2.46	5.23	5.28	3.74	3.84

[a] T = temperature (°C)
[b] P = pH
[c] Exp = experimental results
[d] Pre = predicted values by model

The experimental results of glucose concentrations by hydrolysis with free cellulase are within 1% of the model predicted values for all experimental conditions. The coefficient of determination, R^2 for these results is 0.999, while the adjusted R^2 is 0.998.

The model for glucose concentration (G_{AC}) with attached cellulase is presented in equation 5.3. Similar to the model for free cellulase, the quadratic term for temperature was not significant (p>0.05) and removed as indicated in table 5.3.

$$G_{AC} = -48.7 - 0.199T + 22.0P - 2.48P^2 + 0.0533TP \tag{5.3}$$

For this model, 77% of the experimental glucose concentration results obtained were within 5% of the model predicted values. The R^2 for the glucose results is 0.983 and the adjusted R^2 is 0.975. The contour plots for the glucose concentrations by hydrolysis with attached and free cellulase, sampled at 18 h is presented in figure 5.2.

Table 5.3: ANOVA for glucose and reducing sugar concentrations after hydrolysis of Solka-Floc with free and attached cellulase.

	Degrees of freedom	Sequential sum of squares	Adjusted sum of squares	F-value	p-value
Glucose by free cellulase					
Regression	4	5.006	5.006	1616.52	<0.001
T	1	3.345	0.156	201.01	<0.001
P	1	0.694	0.832	1075.06	<0.001
P^2	1	0.937	0.937	1210.34	<0.001
T*P	1	0.031	0.031	39.55	<0.001
Residual	8	0.006	0.001		
Lack of fit	4	0.005	0.005	2.87	0.166
Pure error	4	0.002	0.000		
Total	12	5.013			
Glucose by attached cellulase					
Regression	4	9.066	9.066	117.06	<0.001
T	1	0.673	0.146	7.55	0.025
P	1	3.405	3.538	182.74	<0.001
P^2	1	4.753	4.753	245.45	<0.001
T*P	1	0.235	0.235	12.15	0.008
Residual	8	0.155	0.155		
Lack of fit	4	0.154	0.154	12.15	0.008
Pure error	4	0.001	0.000		
Total	12	9.221			
Reducing sugars by free cellulase					
Regression	3	4.588	4.588	325.65	<0.001
T	1	3.286	3.286	699.62	<0.001
P	1	0.728	0.504	107.41	<0.001
P^2	1	0.574	0.574	122.32	<0.001
Residual	9	0.042	0.042		
Lack of fit	5	0.027	0.027	1.47	0.366
Pure error	4	0.015	0.004		
Total	12	4.630			
Reducing sugars by attached cellulase					
Regression	4	6.246	6.246	77.68	<0.001
T	1	1.279	0.160	7.94	0.023
P	1	1.561	3.115	154.97	<0.001
P^2	1	3.115	3.115	154.97	<0.001
T*P	1	0.292	0.292	14.51	0.005
Residual	8	0.161	0.161		
Lack of fit	4	0.159	0.040	88.33	<0.001
Pure error	4	0.159	0.002		
Total	12	6.407			
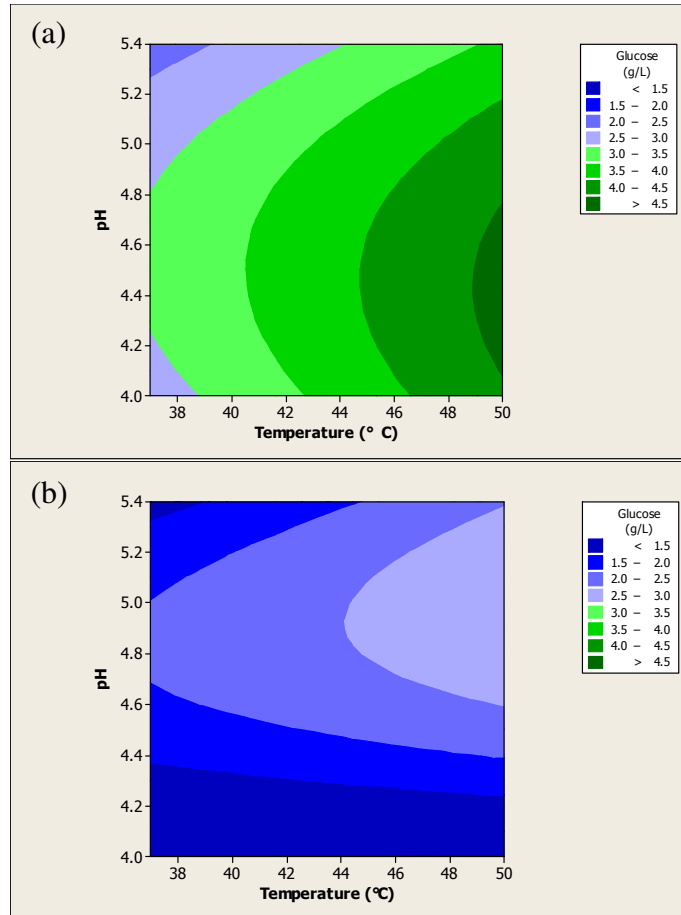
Figure 5.2: Glucose production by (a) free and (b) attached cellulase, modeled by response surface methodology for pH and temperature.

According to figure 5.2, the highest glucose concentration from hydrolysis with free cellulase is two folds that by attached cellulase. The reduced hydrolysis rates with attached cellulase relative to free could be due to reduced activity of attached enzymes or physical limitations to interactions between substrates and enzymes. The interactions with brushes could affect amino acid residues in, leading to changes in the physical structure of the protein and its affinity towards the substrates. Alternatively, enzymes could be overlapping on the brush surface or buried within the brush matrix, physically limiting the interactions between enzymes and substrates. These relatively low hydrolysis rates could be overcome by increasing the loading of

attached cellulase since a larger portion can be recovered and reused due to being immobilized on the brush particles.

Figure 5.2 also shows that the most effective pH, in terms of glucose concentrations, for hydrolysis with attached cellulase is 4.98. This is higher than the most effective pH for free cellulase which is 4.4. One possible explanation is that the physical structure of PAA brushes changes with pH of the solution; brushes are completely stretched at pH higher than 7, and they are shrunk as much as possible at pH less than 4 (Minko, 2006). Therefore the attached cellulases would be more spread out on the surface of the brush particles as the pH increases from 4, making the insoluble substrates more accessible to the attached enzymes. Furthermore, enzymes are attached most strongly to PAA brushes at pH 4.5 and the strength of attachment decreases with increasing pH, until completely detaching at pH above 7 (Kudina et al., 2013). Also, Kudina et al. (2013) have shown that cellulase attachment to the brushes is not static and that the enzymes move fluidly throughout the brush matrix. Therefore, with increasing pH, the loosely-attached enzymes are more fluid within the surface of the brush. This could enable the endoglucanases, exoglucanases and β-glucosidase to fluidly move within the brush surface and cleave chains of cellulose substrates more efficiently than at a lower pH. For industrial applications, this would mean that a higher operating pH would be beneficial when using cellulases immobilized on polymer brush particles.

Although glucose is the expected end product from hydrolysis, the total efficacy of endoglucanases, exoglucanases, and β-glucosidase is best modeled by the amount of reducing ends created. Therefore, reducing sugar concentrations from hydrolysis with free and attached cellulase (table 5.2) were also modeled for pH and temperature.

The quadratic term for temperature and interaction term for temperature and pH were not significant (p>0.05) and were removed from the regression analysis. The model for total reducing sugars with free cellulase (S_{FC}) is presented by equation 5.4.

$$S_{FC} = -16.3 + 0.114T + 7.59P - 0.861P^2 \qquad (5.4)$$

The experimental results of reducing sugars from hydrolysis with free cellulase were also within 1% of the model predicted values. The R^2 for these results is 0.991, while the adjusted R^2 is 0.988.

For attached cellulase, the quadratic term for temperature was not significant (p>0.05) and removed from the model due to not being (table 5.3). The equation of the model is:

$$S_{AC} = -35.1 - 0.208T + 17.0P - 2.00P^2 + 0.0593TP \qquad (5.5)$$

Experimental results of reducing sugars from hydrolysis with attached cellulase (S_{AC}) were within 7% of the model predicted values. The R^2 for the reducing sugar results is 0.975 and the adjusted R^2 is 0.962. The contour plots for the total reducing sugars by hydrolysis with free and attached cellulase are portrayed in figure 5.3.

The models for total reducing sugars (figure 5.3) show that the most effective pH for attached cellulase is higher than that of the free cellulase. This observation is similar to the higher effective pH seen with attached cellulase than with free when modeling of glucose concentrations (figure 5.2). If concentrations of reducing sugars were different than glucose concentrations, it would suggest differential attachment or differential activity of attached enzymes.
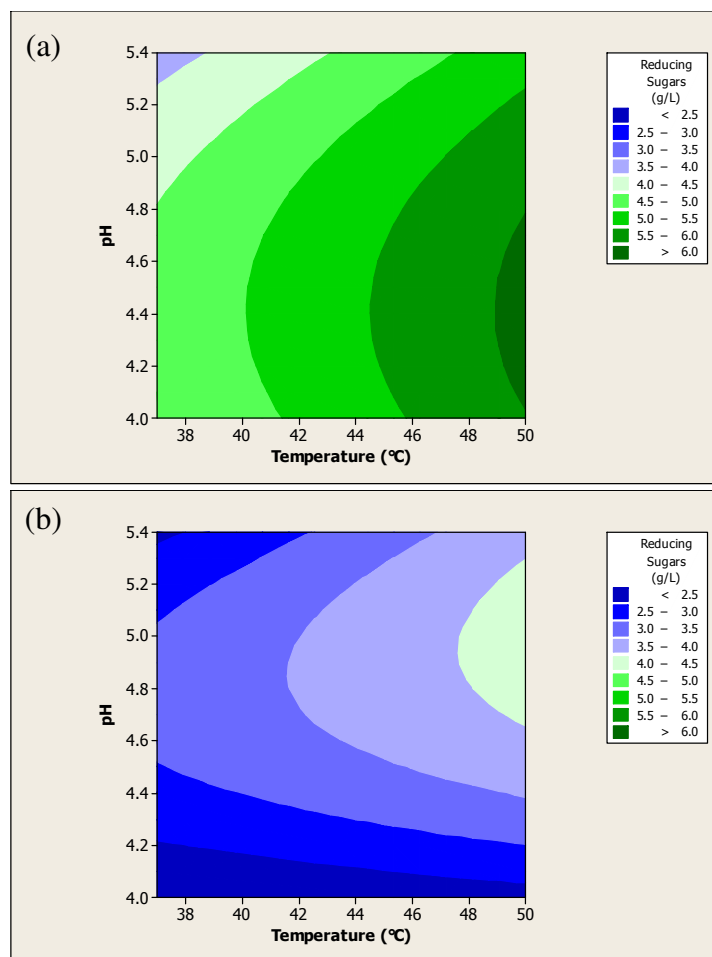
Figure 5.3: Reducing sugars produced by (a) free and (b) attached cellulase, modeled by response surface methodology for pH and temperature.

The optimal pH for producing the most reducing sugars from hydrolysis with free cellulase was found to be 4.4 (figure 5.3a). However, the most common hydrolysis pH for cellulases ranges from 4.8 to 5 (Jeya et al., 2009; Tu et al., 2007a; Tu et al., 2009). Furthermore, a study by Balsan et al. (2012) indicates that the maximum activity of NS50013 for agro-industrial residues was between pH 5.2 to 5.5 for the temperatures of 40°-50°C. However, another study showed that optimal pH for NS50013 will differ based on substrates used (Ferreira et al., 2009). Therefore, the optimum pH found here may not be indicative of that for substrates other than Solka-Floc.

Modeling was also carried out for total soluble sugars (the sum of glucose and the glucose equivalents of cellobiose) in addition to glucose and total reducing sugars. Though the desired end product is glucose, the presence of cellobiose and its derivatives represents the efficacy of exoglucanases. Furthermore, the cellulase mixture used in this study does not contain sufficient β-glucosidase; total activity of endoglucanases and exoglucanases will not be evident if only glucose was considered to test cellulase efficacy. In this study, the models and the contour plots for total soluble sugars for attached and free cellulase were very similar to that of reducing sugars. This indicates that endoglucanases and exoglucanases performed well and had cleaved longer cellobiose chains, without limiting the formation of soluble sugars. The results and the models for total soluble sugars are not included in this paper due to similarity to that of total reducing sugars.

5.4.2. Recovery of attached cellulase

Protein recovery after 120 h of hydrolysis was also modeled for attached cellulase. Higher than 80% of loaded protein was recovered for all pH and temperature conditions tested. However, approximately 30% of substrate remained as unhydrolyzed residue even at the most favorable pH and temperature. Protein recovery was determined by testing protein concentrations of supernatant after centrifugation. While enzymes attached to the brushes get pelleted during centrifugation, enzymes released from the carrier during hydrolysis may also be pelleted because of attachment to unhydrolyzed residue. Separation of free enzymes with unhydrolyzed residue has been documented in literature (Gregg and Saddler, 1996). Such recovery of free enzymes with unhydrolyzed residue could cause higher enzyme recovery than would be facilitated by attachment to polymer brush particles. Therefore, the enzyme recovery models are not presented in this paper.

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5.4.3. Efficacy of attached β-glucosidase

Table 5.4 shows the experimental data and the model predicted values for glucose

concentrations and protein recovery from hydrolyzates using free and attached β-glucosidase.

Glucose concentrations obtained with free β-glucosidase ($G_{F\beta}$) was modeled.

$$G_{F\beta} = -1.84 - 0.211T + 7.57P - 1.22P^2 + 0.0615TP \qquad (5.6)$$

Table 5.4: Treatment conditions, data, and predicted values for glucose concentrations and protein recovery after hydrolysis of cellobiose with free and attached β-glucosidase.

Run	Uncoded Levels		Glucose (g/L)				Protein Recovery (%)	
			Free		Attached		Attached	
	T[a]	P[b]	Exp[c]	Pre[d]	Exp[c]	Pre[d]	Exp[c]	Pre[d]
1	37	4	10.08	10.15	2.94	2.84	87.8	86.8
2	37	4.7	9.61	9.58	3.31	3.35	73.7	75.5
3	37	5.4	7.82	7.82	2.24	2.12	64.4	64.2
4	43.5	4	10.49	10.38	4.09	4.14	86.6	86.8
5	43.5	4.7	10.15	10.09	4.35	4.42	76.9	75.5
6	43.5	4.7	10.15	10.09	4.36	4.42	76.1	75.5
7	43.5	4.7	10.13	10.09	4.39	4.42	75.7	75.5
8	43.5	4.7	10.14	10.09	4.36	4.42	77.3	75.5
9	43.5	4.7	10.12	10.09	4.36	4.42	76.2	75.5
10	43.5	5.4	8.61	8.61	2.87	2.97	62.5	64.2
11	50	4	10.61	10.61	5.37	5.43	85.8	86.8
12	50	4.7	10.49	10.60	5.76	5.49	70.6	75.5
13	50	5.4	9.47	9.40	3.78	3.81	64.8	64.2

[a] T = temperature (°C)
[b] P = pH
[c] Exp = experimental results
[d] Pre = predicted values by model

The quadratic term for temperature was removed since it was statistically not significant

(p>0.05) as shown in table 5.5.The experimental hydrolysis glucose concentrations with free β-

glucosidase were within 1% of the model predicted values. The R^2 for these results is 0.995 and

the adjusted R^2 is 0.992.

Table 5.5: ANOVA for glucose concentrations and protein recovery after hydrolysis of cellobiose with free and attached β-glucosidase.

	Degrees of freedom	Sequential sum of squares	Adjusted sum of squares	F-value	p-value
Glucose by free β-glucosidase					
Regression	4	7.682	7.682	372.22	<0.001
T	1	1.561	0.164	31.81	<0.001
P	1	4.646	0.418	80.95	<0.001
P^2	1	1.161	1.161	225.07	<0.001
T*P	1	0.314	0.314	60.78	<0.001
Residual	8	0.041	0.041		
Lack of fit	4	0.041	0.041	59.70	0.001
Pure error	4	0.001	0.001		
Total	12	7.723			
Glucose by attached β-glucosidase					
Regression	4	11.536	11.536	176.58	<0.001
T	1	6.869	0.575	35.19	<0.001
P	1	2.053	2.331	142.72	<0.001
P^2	1	2.415	2.415	147.86	<0.001
T*P	1	0.198	0.198	12.13	0.008
Residual	8	0.131	0.131		
Lack of fit	4	0.130	0.130	141.01	<0.001
Pure error	4	0.001	0.001		
Total	12	11.666			
Protein recovery by attached β-glucosidase					
Regression	1	763.882	763.882	402.02	<0.001
P	1	763.882	763.882	402.02	<0.001
Residual	11	20.901	20.901		
Lack of fit	1	0.060	0.060	0.03	0.869
Pure error	10	20.842	20.842		
Total	12	786.783			

The quadratic term for temperature was also not significant (p>0.05) (table 5.5) for glucose concentrations with attached β-glucosidase, and was therefore, removed from the model.

$$G_{A\beta} = -47.8 + 0.395T + 17.9P - 1.76P^2 - 0.0489TP \tag{5.7}$$

The experimental glucose concentrations for attached β-glucosidase are within 4% of the model predicted values. The R^2 for glucose production is 0.989, while the adjusted R^2 is

0.975.The contour plots for glucose concentrations with free and attached β-glucosidase are shown in figure 5.4.
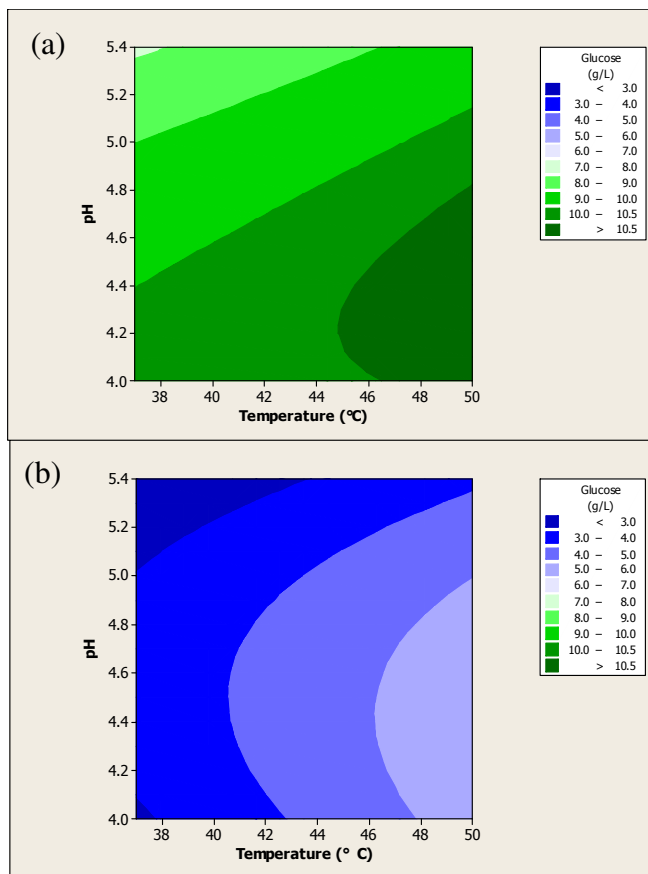
Figure 5.4: Glucose concentrations with (a) free and (b) attached β-glucosidase, modeled by response surface methodology for pH and temperature.

According to figure 5.4, the highest hydrolysis glucose concentration by attached β-glucosidase is 53% of that by free β-glucosidase, similar to the case of attached and free cellulase.

Figure 5.4 also shows that free β-glucosidase was more stable over the temperature range tested compared to attached β-glucosidase in terms of glucose con β-glucosidase concentrations. The fraction of glucose concentration at lowest temperatures (37℃) by that at highest temperature (50℃) at optimum pH for each case was higher for free β-glucosidase than attached β-glucosidase. Substrates for endoglucanases and exoglucanases are insoluble while substrates

for β-glucosidase are soluble. Endoglucanases and exoglucanases attached on a brush particle could simultaneously cleave one cellulose chain while cellobiose substrates will be in random motion throughout the hydrolysis solution for attached β-glucosidase. Therefore, trends for immobilized cellulase and β-glucosidase could differ. When applying cellulases immobilized on polymer brushes for manufacturing industrial sugars, it may be more beneficial to use attached cellulase while using free β-glucosidase. However, high glucose concentrations (near complete hydrolysis) were achieved at 6 h for free β-glucosidase. This could make free enzymes appear more stable over the temperature range since the majority of substrate has been converted to glucose.

5.4.4. Recovery of attached β-glucosidase

For enzyme recovery results with attached β-glucosidase, unhydrolyzed residue was not a concern since the substrate, cellobiose, is soluble. Protein recovery after hydrolysis for attached β-glucosidase ($P_{A\beta}$) was modeled by the equation 5.8. The linear term for temperature, the quadratic terms for temperature and pH, and the temperature and pH interaction term were statistically not significant ($p > 0.05$), and therefore, removed from the model.

$$P_{A\beta} = -151.3 - 16.12P \tag{5.8}$$

The experimental results for percentage protein after hydrolysis with attached β-glucosidase were within 2% of the model predicted values. The R^2 for protein recovery results was 0.973 and the adjusted R^2 was 0.971. Temperature did not have an impact on recovery of attached β-glucosidase. The plot for protein recovery after hydrolysis with attached β-glucosidase is shown in figure 5.5.
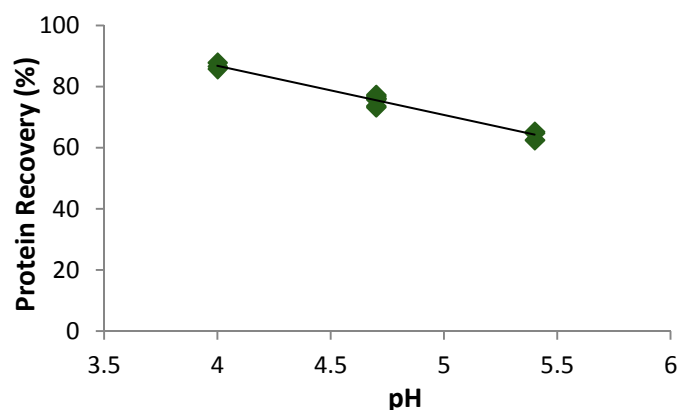
Figure 5.5: Protein recovery after hydrolysis for attached β-glucosidase, with respect to pH.

The relationship between pH and enzyme recovery was linear. Recovery of attached β-glucosidase decreased with increasing pH. The decreasing affinity between PAA brushes and the enzymes with increasing pH is consistent with the low recovery at high pH. Therefore, a low pH is desirable for recovering attached β-glucosidase.

5.5. Conclusions

The ideal temperature for hydrolysis with both the attached and free cellulase and β-glucosidase was 50°C. Hydrolysis sugar concentrations were higher with free enzymes than attached both cellulase and β-glucosidase. The highest reducing sugar concentration for attached cellulase and highest glucose concentration for attached β-glucosidase were 71% and 54% of that with free enzymes, respectively. A higher attached enzyme loading must be used for hydrolysis since the sugar rates are lower than that of free enzymes. Still this could be beneficial since the majority of attached enzymes may be recovered for reuse.

The models indicate that attached cellulase have a higher optimal pH (5) than free enzymes (4.4) with respect to both the responses glucose and reducing sugar concentrations.

The optimal pH for hydrolysis of Solka-Floc with free cellulase at 50°C was found to be 4.4, contrary to the most widely used pH of 4.8. However, the optimal pH will differ for each substrate.

The ideal pH for attached and free β-glucosidase in terms of glucose concentrations from hydrolysis were similar with pH of 4.39 and 4.36, respectively. Free β-glucosidase is more stable over the range of temperatures tested. Therefore, when producing industrial sugars from cellulosic biomass, there is potential for using attached cellulase for recovery and reuse while using β-glucosidase as a free enzyme.

True enzyme recovery aided by immobilizing cellulase on polymer brush particles was not observed for attached cellulases due to the presence of unhydrolyzed residue. Temperature did not have an impact on attached β-glucosidase recovery. Protein recovery had a linear relationship with pH; recovery decreased with increasing pH. The ideal pH for attached β-glucosidase was 4. However, with the higher pH needed for attached cellulase to perform well, a compromising pH may be established to get the desired sugar yields and enzyme recovery.

6. CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

The objectives of this project were (1) to determine the impact of polymer brush enzyme density on efficacy and recovery of immobilized cellulase (NS50013) and β-glucosidase (Novozyme188), and (2) to model the effect of temperature and pH on sugar concentrations and enzyme recovery for enzymatic hydrolysis using immobilized cellulase and β-glucosidase. These objectives were successfully accomplished.

6.1.1. Efficacy of attached enzymes

Hypothesis 1: Attachment to polymer brushes will cause only minor reduction in the activity of β-glucosidase and cellulase such that the glucose yields will be within 90% or higher when compared to free β-glucosidase and cellulase.

Total soluble sugars from hydrolysis with attached cellulase as percentage of sugars with free were averages of 45% and 53% for filter paper and Solka-Floc, respectively. The highest glucose concentration from hydrolysis of attached β-glucosidase, obtained for the highest enzyme density on the brushes, as a percentage of glucose from free β-glucosidase was 38%. When the effect of pH and temperature were modeled, the highest reducing sugar concentration for attached cellulase and highest glucose concentration for attached β-glucosidase were 71% and 54% of that with free enzymes, respectively, at the most favorable pH for each case. The difference in sugar concentrations with free and attached enzymes could be due to (1) reduced activity of attached enzymes or (2) physical limitations of substrates reaching the enzymes attached on the brushes. A higher attached enzyme loading must be used for hydrolysis since the sugar rates are lower than that of free enzymes. Still this could be beneficial since the majority of attached enzymes may be recovered for reuse.

6.1.2. Enzyme attachment to PAA brushes

Hypothesis 2: PAA brush particles have a saturation limit for attachment to cellulase and β-glucosidase.

Enzyme saturation limits for the brush particles containing PAA brushes were determined for both cellulase and β-glucosidase. This saturation occurred at approximately 103 µg protein attached/ mg of brush for cellulase and 111 µg protein attached/mg of brush for β-glucosidase. Maximum enzyme density will minimize brush loading and will reduce costs related with the brush particles.

6.1.3. Effect of brush particle enzyme density on efficacy

Hypothesis 3: Varying enzyme densities on polymer brush particles will result in different sugar yields for cellulase and β-glucosidase due to inaccessibility of enzymes to substrates as the enzyme density increase.

Increasing cellulase densities on the brush particles did not affect hydrolysis rates. Increasing cellulase densities on the brushes hydrolyzed statistically similar sugar concentrations for both filter paper and Solka-Floc. Therefore, a smaller amount of brush particles with a higher enzyme density can be used. However, the proportion of cellobiose in hydrolyzates was higher for attached enzymes than for free enzymes. This could be due less affinity of β-glucosidase to polymer brushes compared to endoglucanases and exoglucanases, reduced efficacy of attached β-glucosidase, or reduced accessibility of substrates to attached β-glucosidase.

Increasing β-glucosidase densities on the brushes led to increasing glucose concentrations from hydrolysis. This was contrary to results with attached cellulase and expectations based on mass transfer of substrates. Increasing enzyme densities on the brushes implies decreasing amounts of brush particles per unit volume since attached enzymes were loaded with the same

total protein concentrations. This could be due to the swelling of the PAA brushes that occur when cellulases are attached. This swelling could increase the efficacy of attached β-glucosidase since soluble cellobiose substrates could reach more attached β-glucosidase while it may not alter the efficacy of attached cellulase due to the insoluble nature of substrates.

6.1.4. Effect of brush particle enzyme density on recovery

Hypothesis 4: Varying enzyme densities on polymer brush particles will result in varying recovery results due to decreasing strength of attachment as the enzyme density increases on the brushes.

The high recovery of attached cellulases seen was affected by the presence of unhydrolyzed residue. Varying enzyme density on the brushes did not impact the recovery of attached β-glucosidase after hydrolysis. Approximately 66% of attached β-glucosidase was recovered.

6.1.5. Ideal pH and temperature for attached enzymes

Hypothesis 5: The ideal operating conditions (temperature and pH) for sugar yields and recovery of attached enzymes (β-glucosidase and cellulase) are different than for the free enzymes.

The models obtained via response surface methodology indicate that the ideal temperature for hydrolysis with both the attached and free cellulase and β-glucosidase was 50ºC. The attached cellulases have a higher optimal pH (5) than free enzymes (4.4) when analyzed in terms of both glucose and reducing sugar concentrations. The optimal pH for hydrolysis of Solka-Floc with free cellulase at 50°C was found to be 4.4, contrary to the most widely used pH of 4.8. However, the optimal pH will differ for each substrate.

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The ideal pH for attached and free β-glucosidase in terms of glucose concentrations from hydrolysis were similar with pH of 4.39 and 4.36, respectively. Free β-glucosidase is more stable over the range of temperatures tested. Therefore, when producing industrial sugars from cellulosic biomass, there is potential for using attached cellulase for recovery and reuse while using β-glucosidase as a free enzyme.

True enzyme recovery aided by immobilizing cellulase on polymer brush particles was not observed for attached cellulases due to the presence of unhydrolyzed residue. Temperature did not have an impact on attached β-glucosidase recovery. Protein recovery had a linear relationship with pH; recovery decreased with increasing pH. The ideal pH for attached β-glucosidase was 4. However, with the higher pH needed for attached cellulase to perform well, a compromising pH may be established to get the desired sugar yields and enzyme recovery.

6.2. Recommendations

For future studies, effects of pH and temperature for hydrolysis of lignocellulosic biomass such as switchgrass or corn stover with attached enzymes can be modeled since the optimal pH and temperature differs for each substrate.

Models of the effect of pH and temperature on sugar concentrations for attached cellulase and β-glucosidase suggest hydrolysis may be carried out for higher temperatures above the range tested, as sugar concentrations kept increasing towards the highest temperature of 50°C. Therefore, temperatures beyond what was tested in this study can be modeled for cellulases immobilized on PAA brush particles.

The brush particles used in this study consist of PAA brushes grafted on silica nanoparticles. These brush particles with silica cores were used due to particle availability. While the brushes and their interaction with enzymes are comparable, the ultimate goal is to use

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brush particles with magnetic cores. Therefore further hydrolysis and recovery experiments at various operating conditions and biomass sources can be carried out since the recovery technique of magnetic separation is different from centrifugation. The impact of unhydrolyzed residue of real biomass on recovery of attached cellulase could be overcome with magnetic separation since only the magnetic brushes will be recovered via magnetic separation.

Furthermore, particle diameters have an effect on magnetic separation. Therefore, when using magnetic brush particles with various particle diameters, the surface area available for brushes will also change. This will present another opportunity to study efficacy and recovery of enzymes immobilized on particles of varying core diameters.

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