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Cellulose hydrolysis by immobilized T reesei cellulase

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CELLULOSE HYDROLYSIS BY IMMOBILIZED *T. REESEI* CELLULASE

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ABSTRACT

CELLULOSE HYDROLYSIS BY IMMOBILIZED T. REESEI CELLULASE

by

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In the production of cellulosic ethanol, the pretreatment and enzymatic hydrolysis steps are the most influential in determining final ethanol yield and both steps require lower costs to be economically viable. In this work, the use of an ionic liquid (IL), 1-ethyl-3-methylimidazolium diethyl phosphate (EMIM-DEP), as a pretreatment for cellulose as well as four types of immobilized *T. Reesei* cellulase are investigated. EMIM-DEP is a low viscosity ionic liquid capable of cellulose dissolution. The addition of 2% (v/v) EMIM-DEP during hydrolysis resulted in an initial reaction rate that was 2.7 times higher than hydrolysis rates using no ionic liquid. The carrier-free immobilized cellulase (CFIC) achieved an initial yield of 0.7 g glucose/g cellulose and was able to be effectively used five times. Other carrier bound immobilization techniques used included magnetic beads, Dilbeads™ and alumina beads, which resulted in a yield of 0.2, 0.12 and 0.02 g glucose/g cellulose, respectively.

CHAPTER 1

INTRODUCTION

Ethanol (CH_3CH_2OH, ethyl alcohol) is a two carbon straight chain alcohol that can be used as a renewable fuel [1]. As a fuel, ethanol can be used in a 10% blend with gasoline(E10) or as an 85% blend (E85). E10 can be used by all internal combustion engines, while E85 can be utilized by Flex Fuel Vehicles.

Cellulosic ethanol is ethanol produced from cellulosic biomass such as sugarcane, miscanthus, switchgrass or corn stover. In the United States, corn is the preferred feedstock for ethanol production. Economically, corn based ethanol becomes competitive with gasoline at an oil price of $60 per barrel [1]. The feedstock used in Brazil is sugarcane; sugarcane derived ethanol becomes competitive with gasoline at an oil price of $40 per barrel. In addition to corn ethanol's lower cost effectiveness than cellulosic ethanol, the use of corn as a fuel substrate drives up the price of corn and corn by-products.

Cellulosic ethanol can potentially reduce greenhouse gas emissions by 86% [2] compared to gasoline. Another environmental benefit of cellulosic ethanol is in its role as a gasoline oxygenate. It replaced methyl tert-butyl ether (MTBE), which is carcinogenic in high concentrations and was associated with groundwater contamination. Ethanol is equally effective as MTBE without the potential risk of water contamination. Since ethanol uses only 10% of the fossil

energy to produce one gallon of fuel compared to gasoline, it has the potential to decrease dependence on foreign energy sources. Implementation of cellulosic ethanol also creates jobs. The ethanol industry was responsible for creating 238,000 jobs in 2007 [3].

Cellulosic ethanol can be made via three pathways: enzymatic, microbial and thermochemical conversion. Enzymatic hydrolysis involves using enzymes to break down crystalline cellulose to sugars which are then fermented to ethanol. Enzymatic hydrolysis is currently used by Iogen Corporation in their demonstration plant to produce 5000 – 6000 liters of cellulosic ethanol per day. Use of microbes combines the hydrolysis and fermentation step as they are able to do both simultaneously. Microbial technology is currently being utilized by Qteros in partnership with Valero as well as Mascoma Corporation. Thermochemical conversion involves the heating and partial oxidation of biomass to produce syngas which can then be converted to ethanol. Coskata Inc., in partnership with General Motors, uses thermochemical conversion to produce ethanol. The highest ethanol yields are produced with enzymatic hydrolysis [4].

Use of enzymatic hydrolysis has so far focused on using soluble enzymes. One way of decreasing the cost of enzymatic hydrolysis is to use immobilized enzymes. Immobilizing enzymes imparts stability to the enzyme and allows them to be easily removed and reused. Before enzymatic hydrolysis can be performed, a pretreatment step is necessary. The pretreatment step overcomes the recalcitrance of the cellulosic biomass, thereby increasing the yields obtained in

the enzymatic hydrolysis step. Pretreatment can be achieved by physical, chemical or hydrothermal processes.

This work focuses on four different immobilization techniques; carrier free immobilized enzymes of a fixed size range, magnetic beads, alumina beads and Dilbeads™. These carriers have been previously successful in immobilizing various enzymes by many researchers. They have the advantage of good mechanical stability, ease of removal and retention of activity. Chemical pretreatment using ionic liquids has been investigated by Dadi et al. [5] by dissolving the cellulose in the ionic liquid and then regenerating the cellulose before hydrolysis. In addition to increasing hydrolysis rates with the regenerated cellulose, this method allows the ionic liquid to be recovered and reused. However, recovery of the ionic liquid is cumbersome and incomplete. Use of ionic liquid in high concentrations (20 %(v/v)) by Kamiya et al. [6] in the presence of soluble enzyme has also resulted in increased hydrolysis rates. In this work, a pretreatment step with the ionic liquid 1-ethyl-3-methylimidazolium diethylphosphate (EMIM-DEP) was used in the presence of carrier-free immobilized enzyme. Since only 2% (v/v) EMIM-DEP was used during hydrolysis, recovery of the IL was not necessary.

The objective of this work is to compare the activity of cellulase bound to these carriers and the effectiveness of EMIM-DEP as a pretreatment step. The effect of precipitant, concentration of cross-linker, molarity of binding buffer and enzyme loading were considered. Kinetic parameters were determined for the

most successful immobilized enzyme. The enzyme used for immobilization was cellulase from *T. Reesei* as it is the most suited to cellulose hydrolysis.

This thesis is separated into five chapters. Chapter 1 provides an introduction to the project. Chapter 2 details a literature review. Materials and methods used and described in Chapter 3 and the results and discussion is in Chapter 4. Conclusions and recommendations are detailed in Chapter 5. Raw data can be found in the Appendix.

CHAPTER 2

LITERATURE REVIEW

2.1 General Survey

Cellulosic ethanol is ethanol produced from cellulosic biomass. Cellulosic biomass can come from a variety of cellulose rich sources including wheat straw, cornstover, miscanthus and switchgrass. Cellulosic ethanol is a 'green' alternative to fossil-based fuels since it can be produced economically, has a high octane [7] and greatly reduces green house gas emissions. The Energy Independence and Security Act of 2007 required an increase in cellulosic ethanol production to 16 billion gallons annually by 2022. Currently, four billion gallons of ethanol in the United States is produced annually, mainly from corn. Corn based ethanol is ethanol derived from corn through wet or dry milling. One drawback of corn based ethanol is that it is made from a food source. This has generated a great 'Food v. Fuel' debate as the demand for corn has pushed the selling price to an all time high. This also affects the price of other products like livestock and poultry since corn is a main ingredient in their feed. The vast amount of arable land required to make corn-based ethanol more widespread in the United States is another concern. Not only is a lot of land required, but over time, soil depletion is inevitable. To try to ward off soil depletion, nitrogen based fertilizers, which are fossil fuel based, are used when growing corn. Fertilizers contribute to pollution

of groundwater supplies. The amount of water required also increases the amount of energy needed to produce corn-based ethanol, since the water must be treated before being used for irrigation.

Researchers are split on whether corn ethanol is energy efficient. Pimental and Patzek state that more fossil energy is required to produce ethanol from corn than the amount of ethanol produced [8]. However, other groups have found that corn ethanol requires 0.74 BTU of fossil fuel to produce 1 BTU of ethanol [2, 9]. Hammerschlag reviewed ten different studies on corn and cellulosic ethanol. He normalized the data and came up with a parameter r_E, the energy return on investement. "An $r_E > 1$ indicates that the ethanol product has nominally captured at least some renewable energy, and $r_E > 0.76$ indicates that it consumes less nonrenewable energy in its manufacture than gasoline" [10]. The r_E values for the corn ethanol studies ranged from $0.84 \leq r_E \leq 1.65$ and the cellulosic ethanol studies ranged from $4.40 \leq r_E \leq 6.61$. The biomass used for cellulosic ethanol is not a food crop and is already widely available. Therefore, cellulosic ethanol is a better form of ethanol than corn-based ethanol. The major steps in producing cellulosic ethanol are pretreatment, enzymatic hydrolysis and fermentation.

2.2 Cellulose structure

Cellulose is a linear polymer of D-anhydroglucopyranose joined by β-1,4-glycosidic linkages as shown in Figure 2.1.

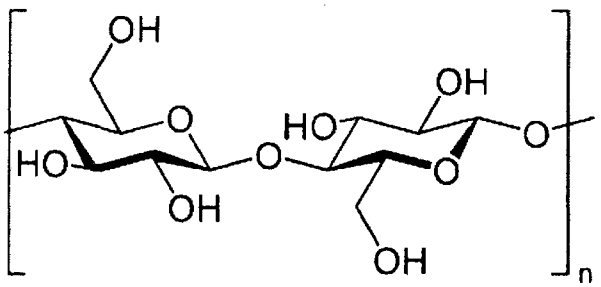

Figure 2.1 Structure of cellulose

Cellulose has a crystalline structure due to hydrogen bonds and van der Waal's forces between adjacent molecules [11]. In plant biomass, cellulose is surrounded by a matrix of lignin(phenolic propane units) and hemicellulose(a polymer of hexoses and pentoses) to form microfibrils as shown in Figure 2.2.

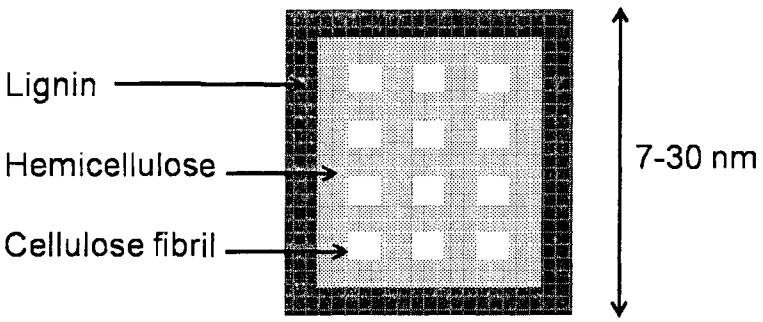

Figure 2.2 Microfibril structure [11]

Because of its stable, crystalline structure, cellulose is sparingly soluble in water. Since most enzymes require an aqueous environment, it is difficult for the enzyme to hydrolyze cellulose chains [12]. Table 2.1 shows the compositions of some types of cellulosic biomass.

Table 2.1 Composition (wt%) of cellulosic biomass [13]

Material	Cellulose	Hemicellulose	Lignin
Hardwoods stems	40-55	24-40	18-25
Softwoods stems	45-50	25-35	25-35
Grasses	25-40	25-50	10-30
Leaves	15-20	80-85	~0
Cotton seed hairs	80-95	5-20	~0
Newspaper	40-55	25-40	18-30
Waste from chemical pulp	60-70	10-20	5-10

For the production of ethanol, the feedstock used must be low cost, readily available, have high potential ethanol yield and high efficiency of conversion. Table 2.2 details the possible usage of some types of biomass. Iogen Corporation, an ethanol producing company uses wheat straw as its feedstock.

Table 2.2. Potential feedstocks [14]

Material	Potential use
Native forest – softwood	Difficult to process
Tree farms	Expensive – (Used in other markets)
Forest waste	Cellulose content too low
Mill waste	Expensive – (Used in other markets)
Straw	Leading candidate
Cane Bagasse	Localized feedstock
Corn stover	Leading candidate
Municipal waste	Not uniform enough to process

2.3 Cellulase structure

Cellulases are a group of enzymes that are able to break down cellulose to glucose. These enzymes are cellobiohydolase (CBH)- which removes cellobiose

from free end chains, endoglucanase (EG)- which attack low crystallinity areas and beta-glucosidase (BG)- which hydrolyzes cellobiose to glucose [15]. There are many microbes and fungi that produce cellulases that are being studied including *Z. mobilis* [4], *E. coli* [4], *K. oxytoca* [4], *T. reesei* [16], *Aspergillus niger* [16]. The most commonly used cellulases for cellulosic ethanol production are derived from *Trichoderma*, as they are most powerful, its composition is described in Table 2.3. Milder cellulases from other sources are more often utilized in beverage, textile and pulp and paper industries [17].

Table 2.3. Compositon of cellulases from Trichoderma

Enzyme	Concentration (%)
CBH	65-78
EG	22-35

The enzymatic hydrolysis of cellulose consists of two reactions:

$$(C_6H_{10}O_5)_n + H_2O \rightarrow (C_6H_{10}O_5)_{n-2} + C_{12}H_{22}O_{11} \quad (1)$$

$$C_{12}H_{22}O_{11} + H_2O \rightarrow 2\ C_6H_{12}O_6 \quad (2)$$

In Reaction 1, cellulose is hydrolyzed to cellobiose. This reaction is catalyzed by CBH and EG enzymes and in Reaction 2 cellobiose is hydrolyzed to glucose. This reaction is catalyzed by BG. Reaction 2 is described by Michaelis-Menten kinetics. The reactions are displayed in Figure 2.2.

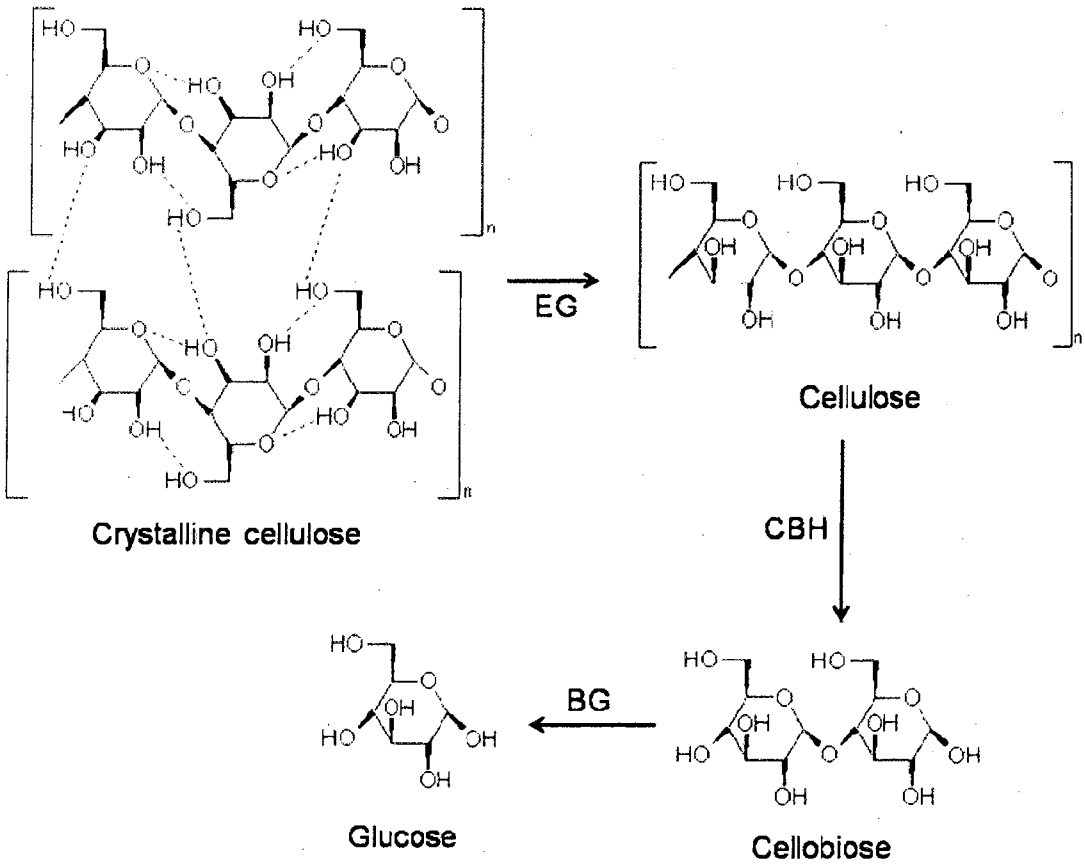

Figure 2.3 Action of cellulase on cellulose

If cellulase from Trichoderma is used, there is an absence of BG as can be seen in Table 2.3, therefore cellobiose accumulates. Cellobiose is an inhibitor to CBH and EG. Therefore, it is important to include BG in the reactor so that the hydrolysis yield can be increased. This can come from another source or from engineering Trichoderma strains to produce higher BG compositions. The hydrolysis yield is also affected by the degree of crystallinity of the cellulose, the efficiency of the pretreatment step, surface area and lignin content. The lower the lignin content in the feedstock, the better the efficiency of the enzymes. For this reason, an extra step may be added to the process where lignin is removed

before hydrolysis. The removed lignin can be used to produce energy by combustion of the lignin. This makes the process more energy efficient.

2.4 Immobilization

Immobilization enables the reuse of enzymes, thereby reducing the cost. It can also offer enhanced stability [18]. Immobilization techniques can be separated into three main areas: carrier bound, carrier-free and entrapment.

Carrier bound methods take advantage of physical, covalent or ionic bonding to fix the enzyme to an inorganic support. Physical adsorption is easy to achieve, but is non-specific and the enzyme can be desorbed with minimal changes in pH or temperature because of the weakness of the bond. Physical adsorption, therefore, cannot be used under industrial conditions. Ionic and covalent bonding result in stronger binding of the enzyme to the support, however, this is usually at the expense of enzyme activity. Examples of supports include alumina, silica, and polyacrylate beads.

Carrier-free immobilization methods involve crosslinking of flocs [19] or crystals [20] of enzyme. The most commonly used crosslinking reagent is glutaraldehyde, however dextran polyaldehyde may be used when the deactivation due to glutaraldehyde use is too large [21]. Diamines, dithiols and succinic anhydride are other important crosslinkers [22].The crosslinking with glutaraldehyde is achieved by the aldehyde groups forming bonds with some of the many free amino groups of the enzyme, thus forming an enzyme network [23].

Entrapment is the incorporation of the enzyme into a gel matrix or semi-permeable membrane. Sol-gel entrapment usually results in particle sizes that are too small to be efficiently reused [24]. To mitigate this effect, sol-gel entrapment can be used in conjunction with porous silica [25]. Other disadvantages of gel entrapment include significant enzyme leakage and diffusion limitations.

2.4.1 Carrier-free immobilized enzyme

Use of carrier-free immobilized enzymes is a simple, effective method of producing immobilized enzymes. Since it is a carrier-free method the dilution of catalytic activity is lessened [26]. The use of an organic solvent precipitates the enzyme out of solution and a crosslinking agent is used to form an enzyme network. The technique of crosslinking enzymes was originally developed by Doscher and Richards [19]. The type of organic solvent used depends on the enzyme being immobilized. Various solvents have been studied including methanol, ethanol, propanol, acetonitrile, acetone, DME, DMF and PEG [27]. The choice of precipitant affects the activity, Schoevaart et al. found that laccase precipitated with 2-propanol retained 99% of its activity, while laccase precipitated with acetonitrile retained only 27% of its activity [27]. This procedure has been successfully used in immobilizing many enzymes including penicillin G amidase [28], lipase [29], cellulase [30] and nitrilase [31]. Carrier-free immobilized enzymes are able to exhibit their activity in ionic liquids [32] and other organic media.

Filtration can be used to separate the enzymes upon completion of the reaction. In this work, the carrier-free immobilized enzymes were separated by size before reacting with the substrate. Only carrier-free immobilized enzymes smaller than twenty microns were used in this work. In this way, the separation after hydrolysis reaction with the substrate is easily achieved. Optimization studies with carrier-free immobilized enzyme produced using four different precipitants (propanol, acetone, methanol and DME) showed that acetone is the most favorable precipitant and all cross linked enzyme aggregates were then made with acetone.

2.4.2 Magnetic beads

Magnetic beads are commonly used in cell separation but were first utilized in immobilizing enzymes by Robinson et al [33]. Magnetic beads are available commercially or they can be made by suspension polymerization, emulsion polymerization or dispersion polymerization [34]. Suspension polymerization is the simplest technique, however it results in large (several hundred microns) beads with uneven distribution of functional groups on the surface of the bead [35]. Lipase has been successfully immobilized on magnetic beads with amino groups [36] as well as β-galactosidase on magnetic beads with aldehyde groups.

The magnetic beads used in this study are activated with a carboxyl group for easy binding to cellulase. The beads are only 1-4 microns in diameter and can be easily separated after the hydrolysis reaction with cellulose by use of a magnet.

2.4.3 Dilbeads™

Dilbeads™ are oxirane activated spherical polymer beads. They are larger than magnetic beads, 150-300 micron in size. Covalent bonds form between the epoxy group and the amino group of the enzyme. Because of its relatively large size, filtration can be used to separate the Dilbeads™ after the reaction with cellulose. Dilbeads™ are similar to other commercially available epoxy-activated beads like Eupergit®C and Sepabeads.

Manipulation of the pH during bonding allows a range of enzymes to be added depending upon the functional group desired. Neutral pH results in binding with thiol groups, slightly alkaline pH results in binding with amino groups and alkaline pH results in binding with phenolic groups. A major disadvantage of Dilbeads™ is diffusion limitation.

2.4.4 Alumina beads

Alumina beads are a porous carrier long used for immobilization [37]. They are inexpensive and commercially available and are stable. Many enzymes have successfully been immobilized to alumina beads including glucose oxidase [38], cellulase [39] and lipase [40].

2.5 Ionic liquid pretreatment

A pretreatment is a necessary step in the production of cellulosic ethanol since it makes the recalcitrant cellulosic biomass more accessible to enzymatic hydrolysis. There are various methods of pretreatment including chemical,

hydrothermal and physical processes, each with its own set of advantages and disadvantages.

One emerging chemical pretreatment is the use of ionic liquids. Ionic liquids(ILs) are organic salts that are liquid at room temperatures. The anions in ILs bond with cellulose at high temperatures, dissolving the cellulose [41]. This results in better enzymatic hydrolysis. However, the presence of high concentrations of some ILs results in the inactivity of the enzyme [42, 43]. Regenerating the cellulose and recovering the IL has been used to alleviate this problem [5]. Using ILs that do not denature cellulase would eliminate the need to regenerate the cellulose. It has been found that a 1:4 (v/v) 1-ethyl-3-methylimidazolium diethylphosphate to water ratio has been effective [6] in increasing hydrolysis yields.

CHAPTER 3

EXPERIMENTAL MATERIALS AND METHODS

3.1 General

Cellulase from *Trichoderma reesei* and microcrystalline cellulose were purchased from Sigma-Aldrich (St. Louis, USA). 1-Ethyl-3-methylimidazolium diethyl phosphate was purchased from Alfa Aesar. Nitex nylon mesh was purchased from Small Parts, Inc. Magnetic beads (MagnaBind^{TM} #21353) were obtained from Thermo Fisher Scientific. Dilbeads TA^{TM} were a gift from Fermenta Biotech. Roche Accu-chek Active glucose meter (Model #3184501) and test strips (Model #3146332) were used for measuring glucose concentration.

3.2 Preparation of Carrier-free immobilized cellulase (CFIC)

9 mL of chilled acetone (10°C) were added to a 25 mL glass vial with a magnetic stirrer. 150 U of cellulase enzyme were dissolved into 1 mL of 0.1 M citric acid/phosphate buffer at the isoelectric point (5.3) and the solution added to the chilled acetone. Glutaraldehyde was then added dropwise to achieve a final concentration of 5 mM. The mixture was kept at 10°C for 2.5 h with gentle stirring. 5 mL of buffer was then added and the mixture centrifuged at a relative centrifugal force of 582 for 10 min. The supernatant was decanted and the pellet washed three times with buffer. The final washed carrier-free immobilized

enzyme was kept overnight in 1mL of pH 5.0 citric acid/phosphate buffer at 4°C. To evaluate the reusability of carrier-free immobilized enzyme, the size was limited to less than 20 microns. Separation was achieved via filtration with Nitex 20 micron nylon mesh.

3.2.1 Determination of activity

The activity of the CFIC was found by reacting the CFIC with 400 mg of cellulose in 10 mL of buffer at pH 5.0 and 37°C. 50 μL samples were withdrawn after one h and the glucose concentration measured using a glucose meter. The activity was calculated as: 1U of enzyme will liberate 1 micromole of glucose from cellulose in one hour at pH 5.0 and 37°C.

3.2.2 Reusability of CFIC

50 mg of cellulose, 120 U immobilized enzyme and 10 mL of buffer were added to the reactor. 50 μL samples were withdrawn periodically and the glucose concentration measured using a glucose meter. The immobilized enzyme was recovered after 24 hours and reused. The yield of glucose was calculated as the concentration of glucose divided by the initial concentration of cellulose. Each experiment was performed twice at pH 5.0 and 45°C for 24 hours and the yield of glucose presented is shown as an average.

3.2.3 Determination of kinetic parameters of CFIC

Four cellulose concentrations (5, 10, 15, 20 mg/mL) in 10 mL of citric acid/phosphate buffer were added to the reactor and reacted with 120 U immobilized enzyme. The glucose concentration was measured after 30 minutes

and yield of glucose calculated as the concentration of glucose divided by the initial concentration of cellulose. Each experiment was performed at pH 5.0 and 45°C. A Langmuir plot was constructed from the data to determine V_{max} and K_m.

3.2.4 Use of carrier-free immobilized enzyme with IL pretreatment

When using 1-ethyl-3-methylimidazolium diethyl phosphate as a pretreatment step, 0.2 mL of 1-ethyl-3-methylimidazolium diethyl phosphate was added and the mixture heated for 10 minutes at 105 °C. 10 mL of 0.1M phosphate buffer was then added and vigorously stirred at 700 rpm for thirty minutes. The immobilized enzyme (120 U) was then added and the stirring speed was reduced to 325 rpm. 50 μL samples were withdrawn periodically and the glucose concentration measured using a glucose meter. The yield of glucose was calculated as the concentration of glucose divided by the initial concentration of cellulose. Each experiment was performed twice at pH 5.0 and 45 °C for 24 hours and the yield of glucose presented is shown as an average.

3.3 Preparation of magnetic beads

0.5 mL of magnetic beads was washed three times with 0.1 M pH 7.2 phosphate buffer. 120U of enzyme dissolved in 1 mL pH 4.7 MES buffer was added to the 0.5 mL beads, followed by 0.1 mL of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) solution (10 mg of EDC in 1mL of pH 4.7 MES buffer). The beads were then incubated at 10°C for 24 hours with gentle stirring. After incubation, the beads were then washed three times with 0.1M pH 7.2 phosphate buffer. The beads were stored in pH 5.0 citric acid/phosphate buffer at 4°C.

3.3.1 Hydrolysis with magnetic beads

50 mg of cellulose, 0.5 mL of magnetic beads and 9.5 mL of buffer were added to the reactor. 50 μL samples were withdrawn periodically and the glucose concentration measured using a glucose meter. The yield of glucose was calculated as the concentration of glucose divided by the initial concentration of cellulose. Each experiment was performed twice at pH 5.0 and 45°C for 24 hours and the yield of glucose presented is shown as an average.

3.4 Preparation of Dilbeads

100 mg of Dilbeads™ were incubated with 150U of enzyme in 3 mL of 1M pH 8.0 potassium phosphate buffer at 10°C for 24 hours with gentle shaking. The beads were removed and washed three times with pH 5.0 citric acid/phosphate buffer. The beads were stored in pH 5.0 citric acid/phosphate buffer at 4°C.

3.4.1 Hydrolysis with Dilbeads

50 mg of cellulose, 74 mg of crushed Dilbeads (see Results and Discussion section) and 10 mL of buffer were added to the reactor. 50 μL samples were withdrawn periodically and the glucose concentration measured using a glucose meter. The yield of glucose was calculated as the concentration of glucose divided by the initial concentration of cellulose. Each experiment was performed twice at pH 5.0 and 45°C for 24 hours and the yield of glucose presented is shown as an average.

3.5 Preparation of alumina beads

10 g of 2 mm alumina beads were immersed in 40 mL of 2% of 3-aminopropyl triethoxysilane (3-APTES) in acetone at 45°C for twenty-four hours. After 24 hours, the pellets were washed with DI water and immersed in 2%(v/v) glutaraldehyde for 2 hours. The beads were washed again with DI water, and then immersed in 50U/mL of enzyme solution. The beads were stored in pH 5.0 citric acid/phosphate buffer at 4°C.

3.5.1 Hydrolysis with alumina beads

300 mg of cellulose, 0.66 g of alumina beads and 10 mL of buffer were added to the reactor. 50 μL samples were withdrawn periodically and the glucose concentration measured using a glucose meter. The yield of glucose was calculated as the concentration of glucose divided by the initial concentration of cellulose. Each experiment was performed twice at pH 5.0 and 45°C for 24 hours and the yield of glucose presented are shown as an average.

3.6 Determination of kinetic parameters for soluble enzyme

Four cellulose concentrations (5, 10, 15, 20 mg/mL) in 10 mL of citric acid/phosphate buffer were added to the reactor and reacted with 150 U soluble enzyme. The glucose concentration was measured after 30 minutes and yield of glucose calculated as the concentration of glucose divided by the initial concentration of cellulose. Each experiment was performed at pH 5.0 and 45°C. A Langmuir plot was constructed from the data to determine V_{max} and K_m.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Optimal conditions for soluble enzyme and carrier-free immobilized enzyme

150 U of free enzyme was reacted with 3% (w/v) cellulose at pH 4.0, 4.5 and 5.0 at 45°C. At pH 4.0, a low yield of 0.07 g glucose/g cellulose was obtained. At this pH the enzyme was determined to be denatured. The enzyme was then reacted at a higher temperature, without repeating pH 4.0. As shown in Figure 4.1, the optimum conditions were found to be at pH 4.5 and 45°C, which gave a yield of 0.7 g glucose/g cellulose.

Upon immobilization, we observed a shift in the optimum conditions as seen in Figure 4.2. With carrier-free immobilized enzyme, higher yields were obtained at pH 5.0 and at 45°C than at pH 4.5 and 45°C.

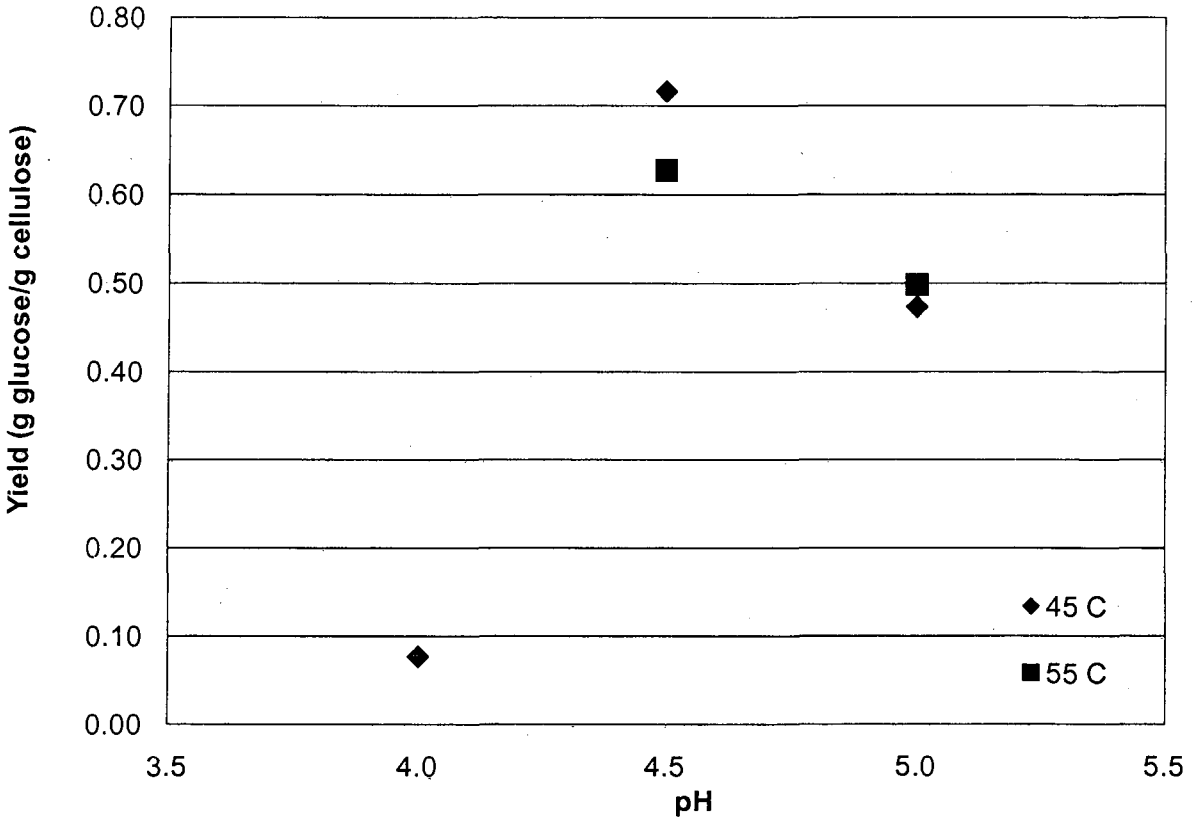

Figure 4.1 Average yield of glucose produced from 150U of enzyme and 3% (w/v) cellulose after 96 hours

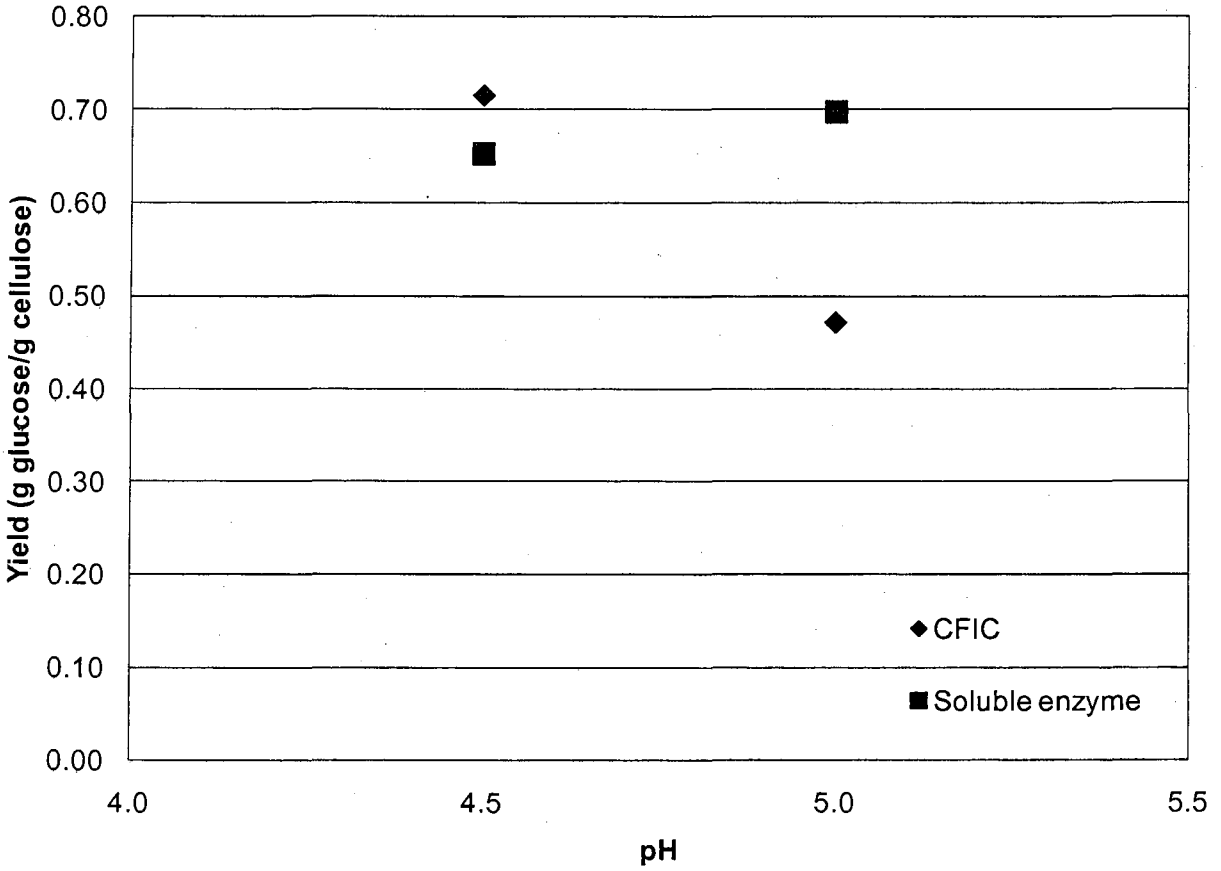

Figure 4.2 Average yield of glucose produced from 150U of enzyme and 3% (w/v) cellulose after 96 hours at pH 4.5 and pH 5.0 at 45 °C (◆), yield of glucose after 24 hours from 120 U of carrier-free immobilized enzyme with 0.5% (w/v) cellulose at pH 4.5 and pH 5 at 45 °C (■)

4.2 Carrier-free immobilized enzyme

The preparation of the immobilized enzyme was optimized by investigating the concentration of cross-linker used as well as the precipitant used. Four precipitants were investigated: methanol, n-propanol, acetone and dimethyl ether. The immobilized enzyme was able to be reused five times. Figure 4.3 shows a micrograph of carrier-free immobilized enzyme taken with Nikon Eclipse TE 200 microscope.

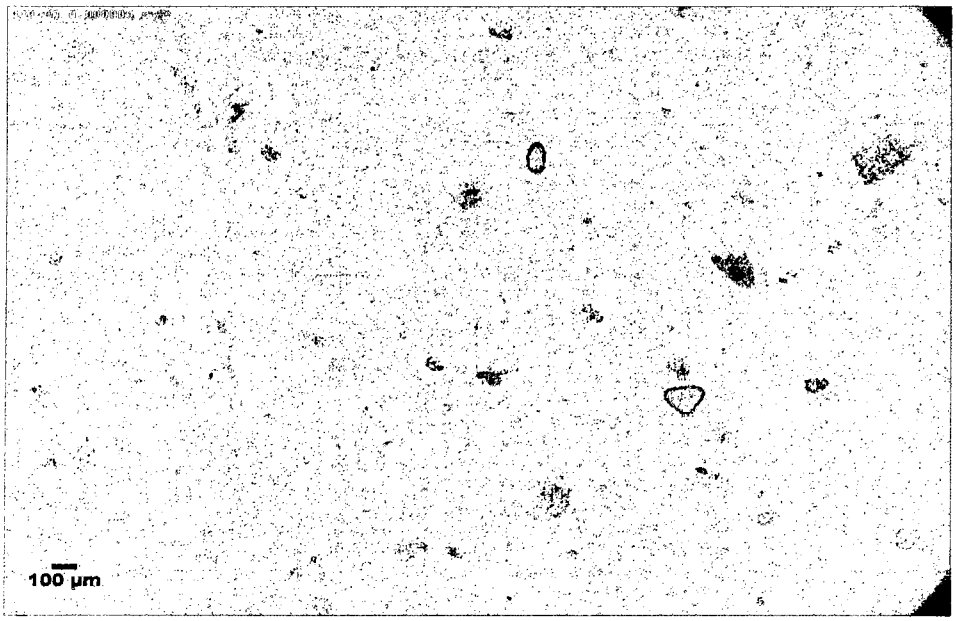

Figure 4.3 Carrier-free immobilized enzyme, magnification 4X

4.2.1 Effect of precipitant

Precipitation is the first step in manufacturing the carrier-free immobilized enzyme. Four precipitants were investigated: methanol, n-propanol, acetone and dimethyl ether. As shown in Figure 4.4, the immobilized enzyme prepared with

acetone retained the highest activity with a yield of 0.15 g glucose/g cellulose after five hours, while those prepared with methanol retained the lowest activity with a yield of 0.04 g glucose/g cellulose after five hours. Diisopropyl ether and acetonitrile were also used as precipitants; however, they did not produce viable immobilized enzyme. Dalal et al. [30] found that n-propanol was the most efficient precipitant for immobilizing Pectinex™ Ultra SP-L (a commercial enzyme preparation with pectinase, xylanase and cellulase activities). However, precipitation with n-propanol resulted in a yield of 0.07 g glucose/g cellulose after five hours, about one half of the yield achieved when precipitating with acetone. Organic solvents precipitate enzymes out of solution by changing the solubility. However, the factors affecting the solubility are quite complex. The precipitation was performed at low temperatures to prevent denaturation.

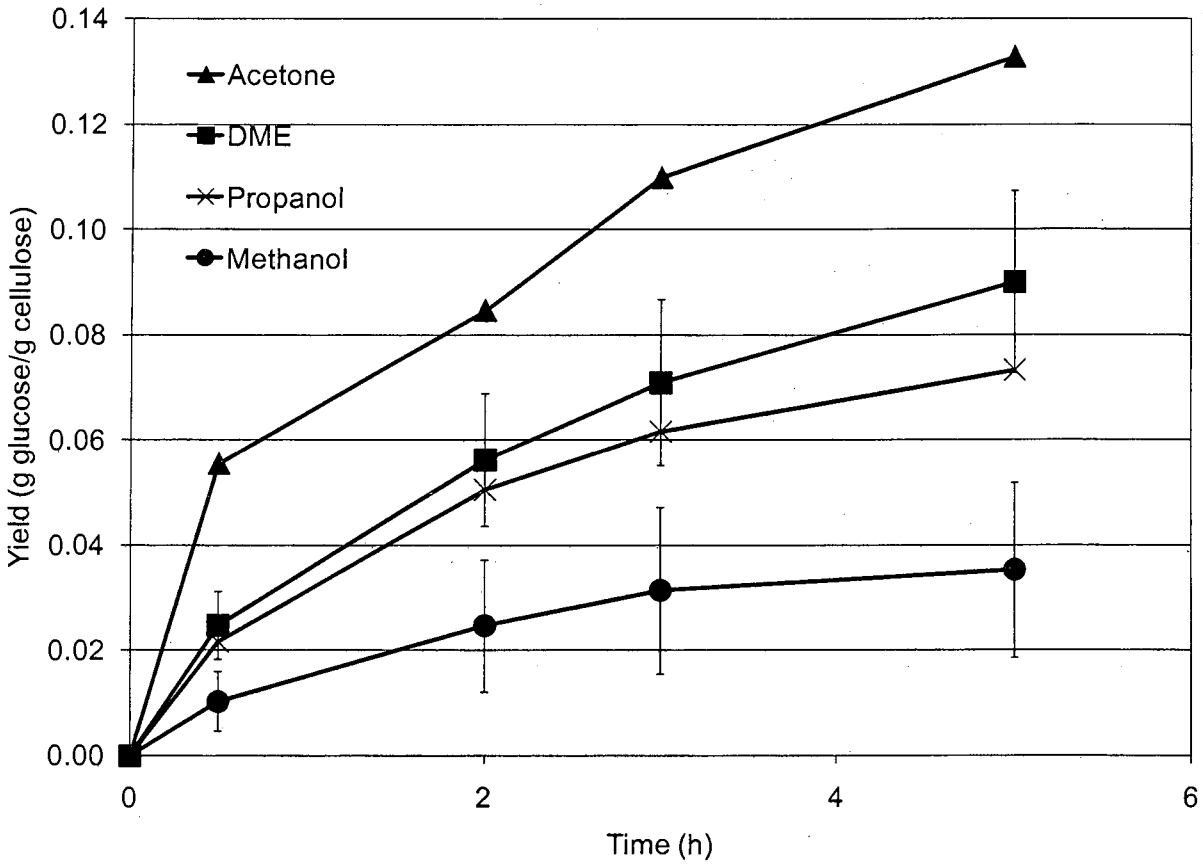

Figure 4.4 Effect of precipitant on yield. Concentration of cellulose 3% (w/v); glutaraldehyde 10 mM.

4.2.2 Effect of glutaraldehyde concentration

Addition of glutaraldehyde is the second step in preparing the carrier-free immobilized enzyme. Glutaraldehyde bonds to the amino groups of the precipitated enzymes, fixing them into position. The amount of glutaraldehyde used in this step was varied (5, 7.5 and 10mM) and it was found that the best cross-linking was achieved with 5 mM concentration of glutaraldehyde. Figure 4.5 shows that increasing glutaraldehyde concentration results in lower yields; this is expected since glutaraldehyde inactivates cellulase. Higher concentration

of glutaraldehyde may also result in larger immobilized enzyme. These larger enzymes may be mass transfer limited. However, its inexpensive cost and retention of activity with lower concentration make glutaraldehyde a suitable choice for a cross-linker in this technique.

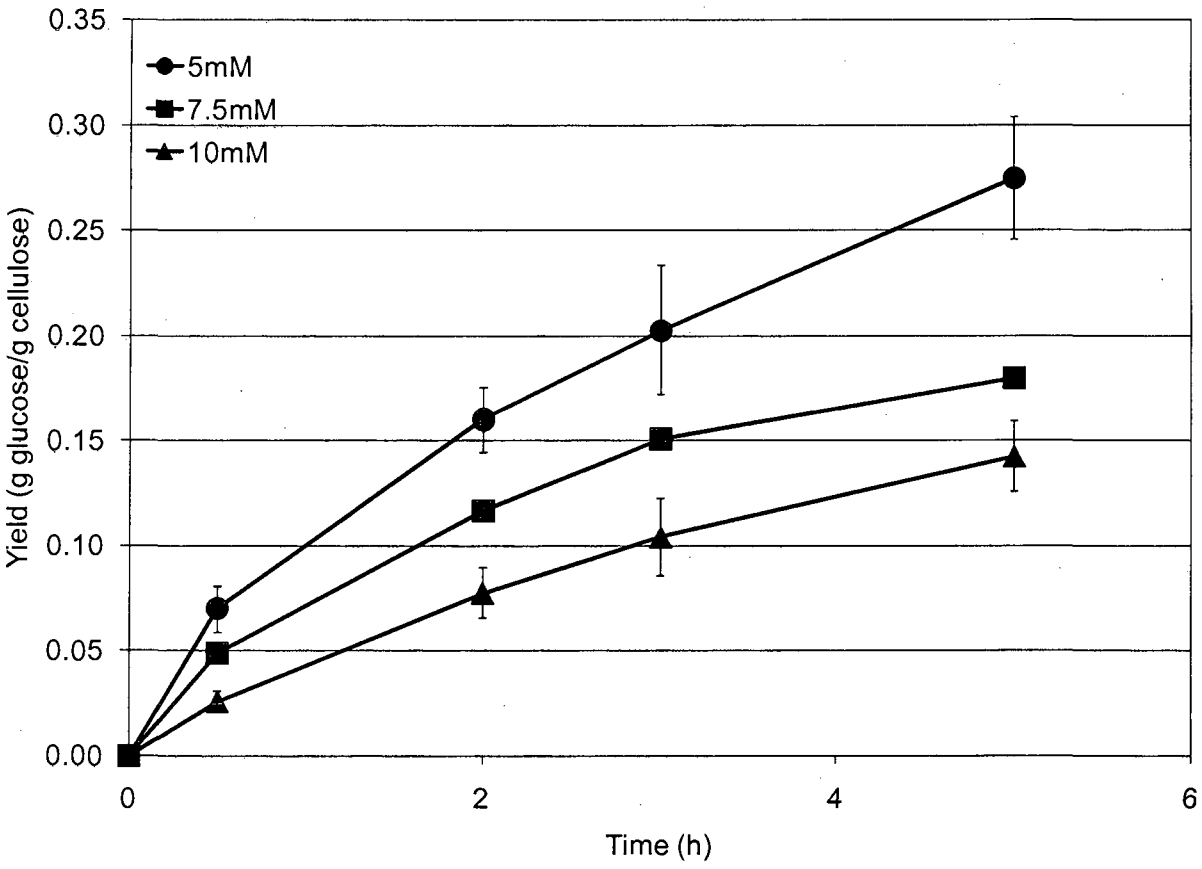

Figure 4.5 Effect of glutaraldehyde concentration on yield. Cellulose concentration 3% (w/v).

4.2.3 Kinetic parameters

The kinetic parameters were determined by plotting initial substrate concentration divided by initial reaction rate (C_s/r) versus initial substrate concentration (C_s) in a Langmuir plot as shown in Figure 4.6 and Figure 4.7

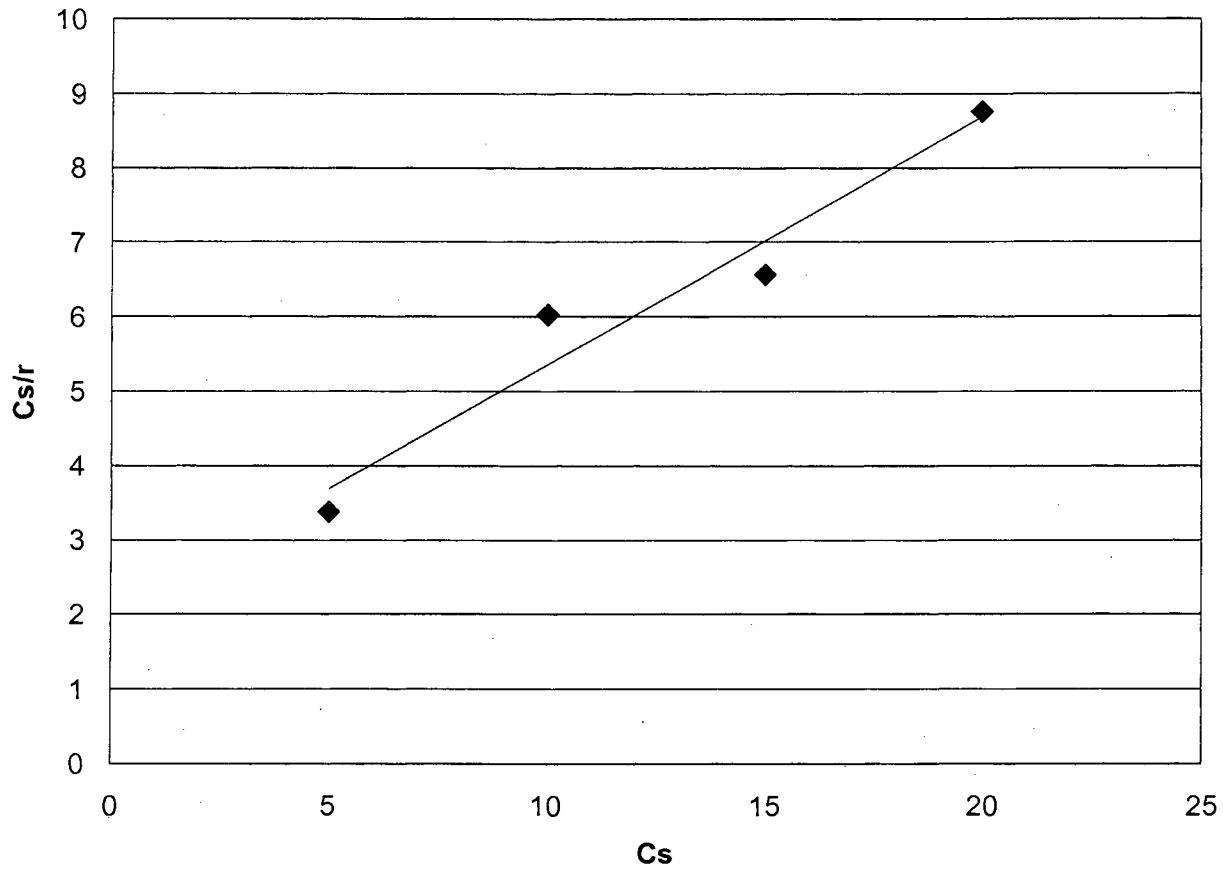

Figure 4.6 Langmuir Plot for carrier-free immobilized enzyme. Cellulose concentrations of 5, 10, 15, 20 mg/mL at pH 5.0 and 45°C

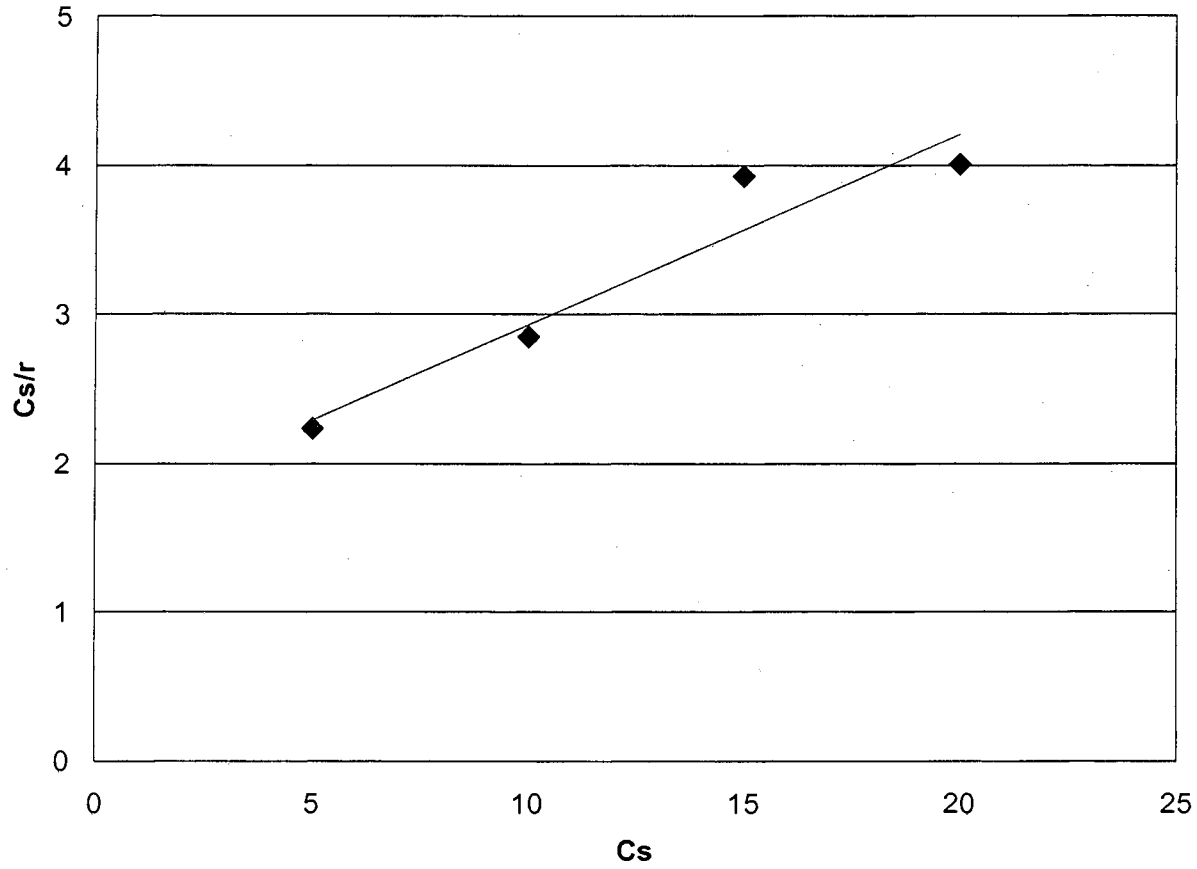

Figure 4.7 Langmuir Plot for soluble enzyme. Cellulose concentrations of 5, 10, 15, 20 mg/mL at pH 5.0 and 45°C

When compared to free enzyme, the carrier-free immobilized enzyme showed a decrease in maximum reaction rate (V_{max}) and Michaelis constant (K_m). The values of the kinetic parameters are shown in Table 4.1.

Table 4.1. Kinetic parameters for free enzyme and carrier-free immobilized enzyme

Parameter	Free enzyme	Carrier-free immobilized enzyme
V_{max} (mol/ L.s)	7.83	2.99
K_m (M)	12.96	5.99

4.2.4 Reusability

The immobilized enzyme was used five times. Figure 4.8 shows the yields obtained with each reuse. After the initial yield of 0.7 g glucose/g cellulose, the yield was level at 0.4 g glucose/g cellulose for the next three consecutive runs before decreasing to 0.3 g glucose/g cellulose in the fifth.

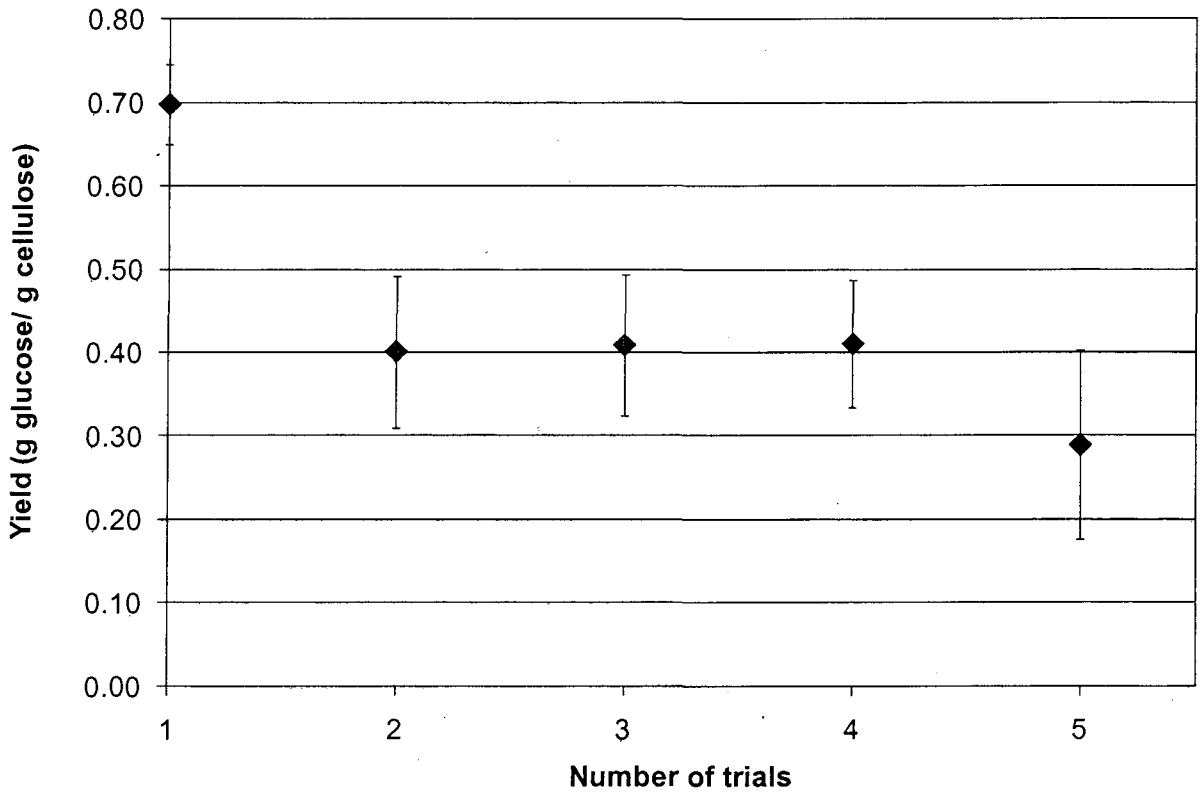

Figure 4.8 Average yield of glucose produced from 0.5% (w/v) cellulose in 10 mL of buffer and immobilized enzyme at pH 5.0 and 45°C for 24 hours. CFIC was prepared with 5mM glutaraldehyde and acetone as a precipitant.

4.3 Pretreatment with Ionic liquids

EMIM-DEP is a low viscosity ionic liquid that has been successful in cellulose dissolution [44]. The initial reaction rate was calculated over the first two hours of the reaction. We used 1, 2 and 4% (v/v) EMIM-DEP and found that using 2% (v/v) EMIM-DEP gave the greatest improvement in initial reaction rates over hydrolysis of crystalline cellulose. The use of 2% (v/v) EMIM-DEP during hydrolysis resulted in an initial reaction rate that was 2.7 times higher than with no IL, as shown in Table 4.2.

Table 4.2 Effect of IL concentration on initial reaction rate of immobilized enzymatic hydrolysis of cellulose. Glucose concentration was measured after two hours at pH 5.0 and 45°C with initial cellulose concentration of 0.5% (w/v). Each data point is the average of two experiments.

Percentage of EMIM-DEP used	Initial Rate of Reaction (mg/ml.min)	Enhancement
0	0.011	-
1	0.026	2.4
2	0.029	2.7
4	0.022	2.1

Dadi et al. [5] reported an initial rate enhancement of 2.8 times, based on the glucose liberated within the first three hours of the reaction when using *regenerated* cellulose. When using 20% (v/v) IL, Kamiya et al. reported a two-fold increase in glucose formation [6]. In our work, we did not observe an increase in initial reaction rate above 2 % (v/v) EMIM-DEP; on the contrary, the reaction rate decreased. However, after 8 h, the yields achieved with 2% and 4% (v/v) EMIM-

DEP were fairly close, namely, 1.05 g glucose/g cellulose and 0.95 g glucose/g cellulose, respectively as shown in Figure 4.9.

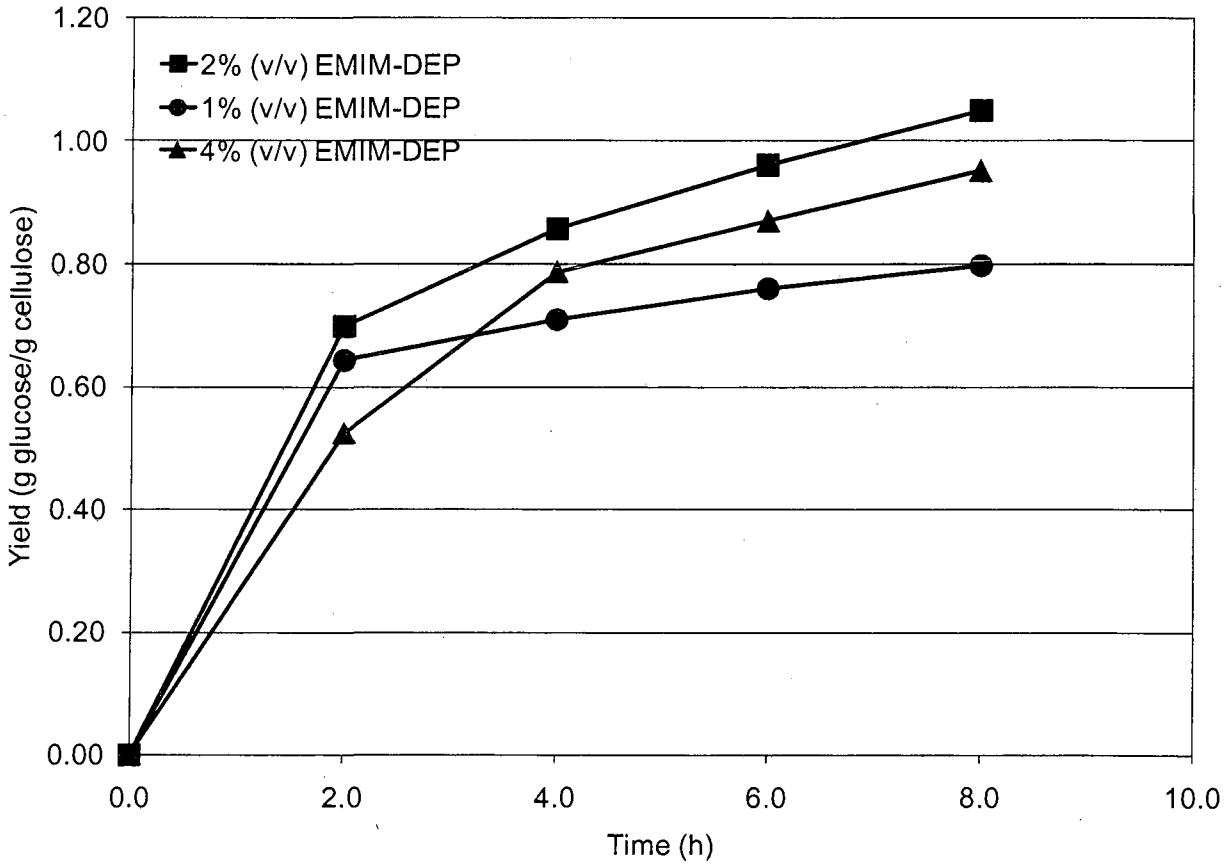

Figure 4.9 Effect of ionic liquid concentration on yield of glucose. Cellulose concentration 0.5% (w/v). CFIC was prepared with 5 mM glutaraldehyde and acetone as a precipitant.

The hydrolysis of cellulose to glucose can be described by the following reaction

$$(C_6H_{10}O_5)_n + nH_2O \rightarrow n\ C_6H_{12}O_6 \qquad (3)$$

Complete conversion of cellulose to glucose would result in a theoretical yield of 1.11 g glucose/g cellulose.

4.4 Magnetic beads

Magnetic beads have long been used as a method of immobilizing enzymes [33]. They have the advantage of being easily separated from the reaction mixture for easy reuse.

The maximum amount of enzyme loaded on the beads was determined by immersing 0.25 mL of beads into a solution containing 20, 40, 60 and 80U of cellulase as described under Experimental. As shown in Figure 4.10, the maximum loading obtained was 25 U. An increase in the concentration of the immobilizing solution above 60 U did not impact the loading of the enzyme. The activity of the enzyme is defined as: 1U of enzyme will liberate 1 micromole of glucose from cellulose in one hour at pH 5.0 and 37°C.

After determining the maximum loading possible on the magnetic beads, the enzyme was immobilized on magnetic beads as described under Experimental. Figure 4.11 compares the yields obtained with magnetic beads and CFIC. The yield obtained with the magnetic beads is 0.2 g glucose/g cellulose, while the yield obtained with CFIC is 0.7 g glucose/g cellulose as shown in Figure 4.11. This comparison is valid only to the extent that the starting concentration of the immobilizing solution was almost identical in both cases. However, the activity of the beads in the trials with CFIC and magnetic beads were 120 U and 50 U, respectively. Thus, if the amount of magnetic beads is doubled (double the enzyme activity), the yield will be much higher. However, the size of the reactor used will also be larger. Clearly, the advantage of magnetic beads is the ease of separation of the enzyme from the reaction medium.

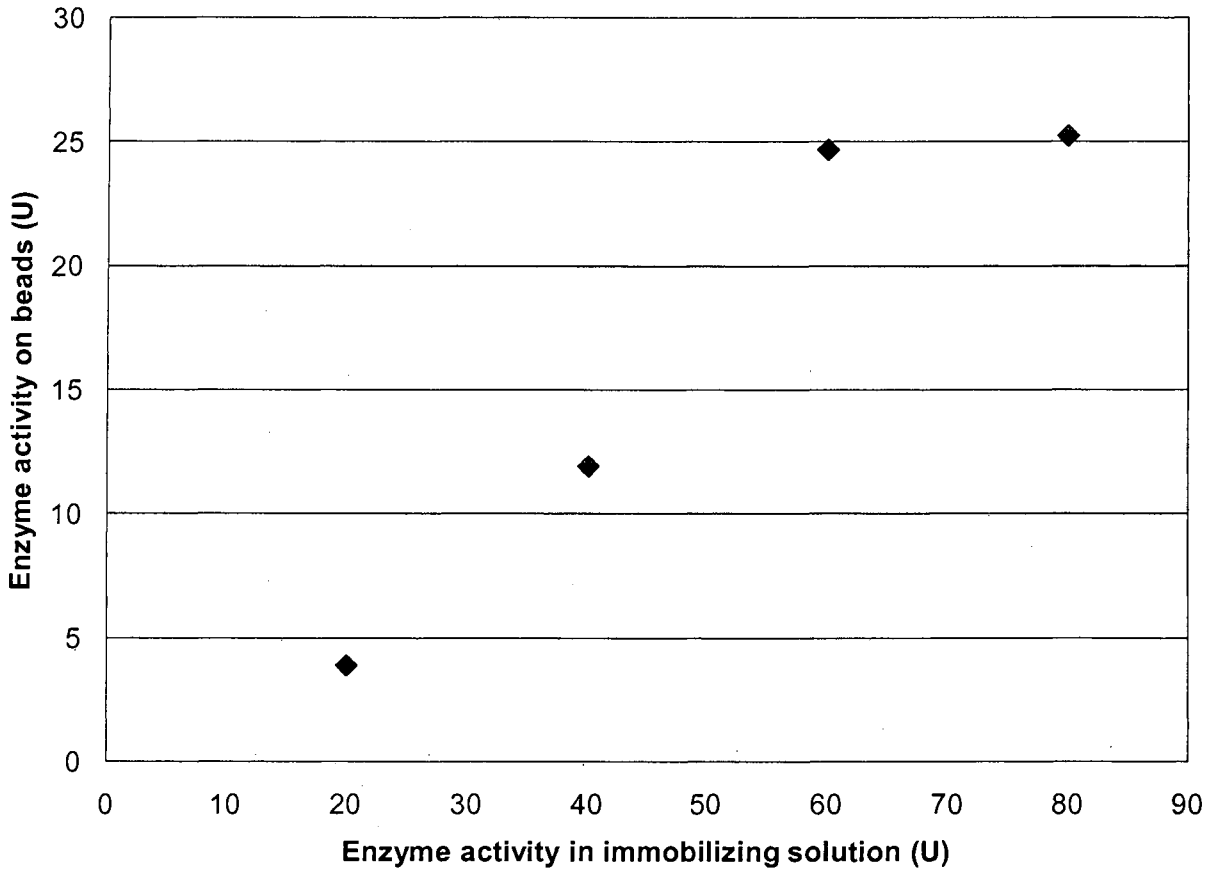

Figure 4.10 Activity observed from 0.25 mL of magnetic beads incubated with 20, 40, 60 and 80U of cellulase under identical conditions, and reacted with 4% (w/v) cellulose from magnetic beads at pH 5.0 and 37°C.

The beads used in this study were expensive commercially prepared beads. Several groups have attempted creating inexpensive magnetic beads [36, 45, 46]. However, they suffer from large size, uneven size distribution and lack of available functional groups.

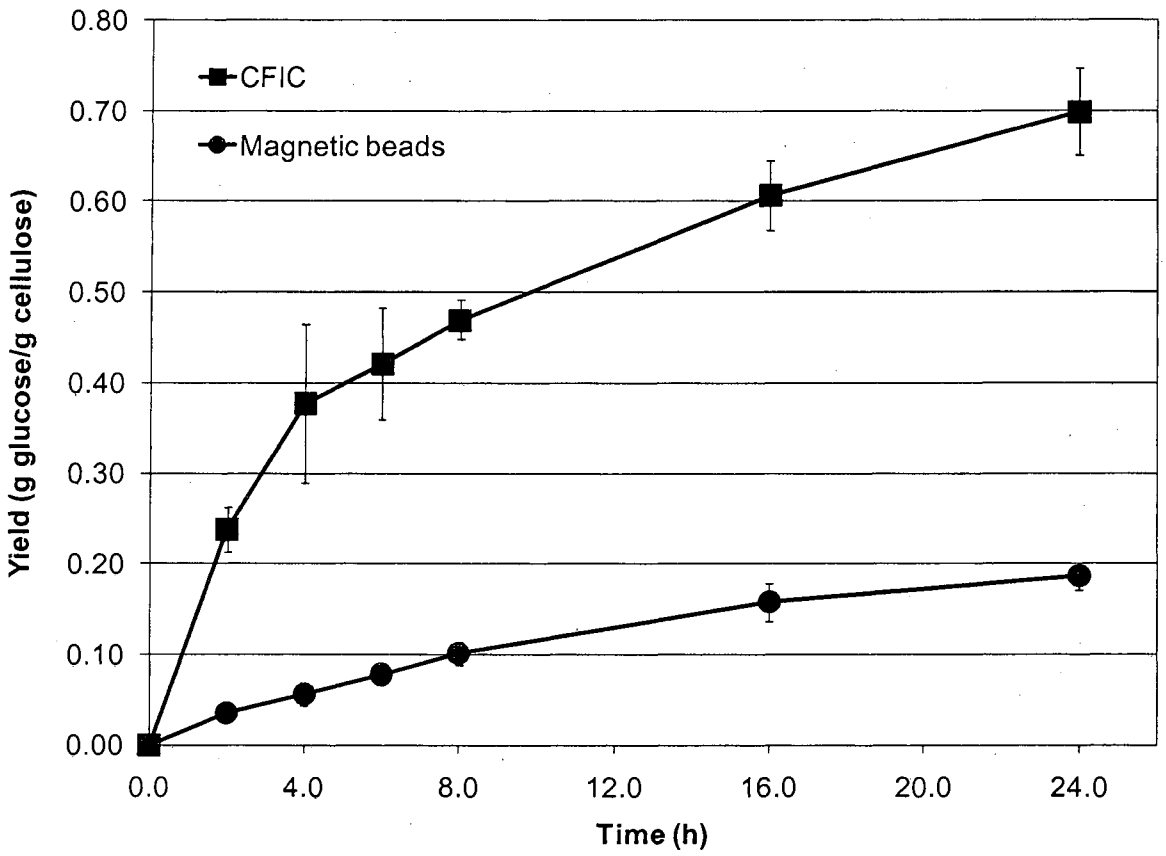

Figure 4.11 Yield of glucose produced from 0.5% (w/v) cellulose in buffer with 50 U magnetic beads (•), and 120 U of carrier free immobilized enzyme (■) at pH 5.0 and 45°C

4.5 Dilbeads

Epoxy-activated supports like Eupergit C and Sepabeads have the advantage of easy bonding of the enzyme to the support. Dilbeads™ are epoxy activated spherical polyacrylate beads 150-300 micron in size.

The molarity of the binding buffer had a significant effect on the enzyme properly binding to the bead as shown in Figure 4.12. Torres et al. [47] had success immobilizing sterol esterase on Dilbeads using a 0.3 M buffer. In this work, 0.3 M and 1 M buffer for the same quantity of beads gave a yield of 0.03

and 0.12 g glucose/g cellulose, respectively. Buffer concentration has been shown to affect immobilization efficiency [48].

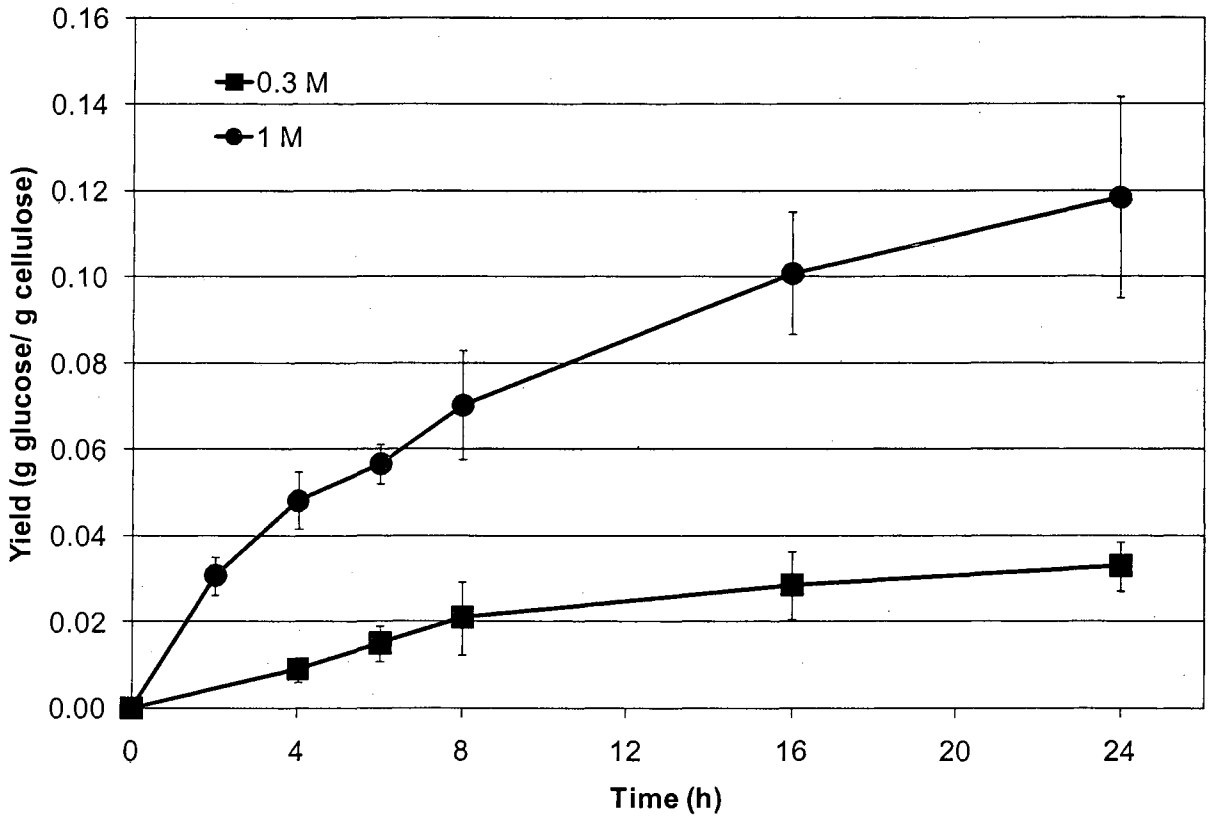

Figure 4.12 Yield of glucose produced from 0.5% (w/v) cellulose in buffer with 100 mg of Dilbeads (•) incubated with 1M buffer, 100 mg of Dilbeads (•) incubated with 0.3 M buffer (■) at pH 5.0 and 45°C

The beads did not display good mechanical stability, as they were pulverized during the reaction time by the action of the magnetic stirrer. To compensate for this, the beads were ground before immobilization, Figure 4.13 shows a micrograph of crushed Dilbeads taken with Nikon Eclipse TE 200 microscope. 100 mg of crushed beads were incubated with 150 U of cellulase. 74

mg of 5 micron size beads were separated out with 5 micron Nitex mesh and reacted with cellulose. In comparison to the carrier-free immobilized enzyme, the Dilbeads only produced 0.09 g glucose/g cellulose. As seen in Figure 4.14, this represents 13.9% of the yield achieved with the carrier-free immobilized enzyme. Even though the loading of cellulase on the Dilbeads was much lower than the loading of cellulase in CFIC, this comparison highlights the fact that the starting concentration of cellulase in the immobilizing solution was the same in both cases. However, very little cellulase is loaded onto Dilbeads.

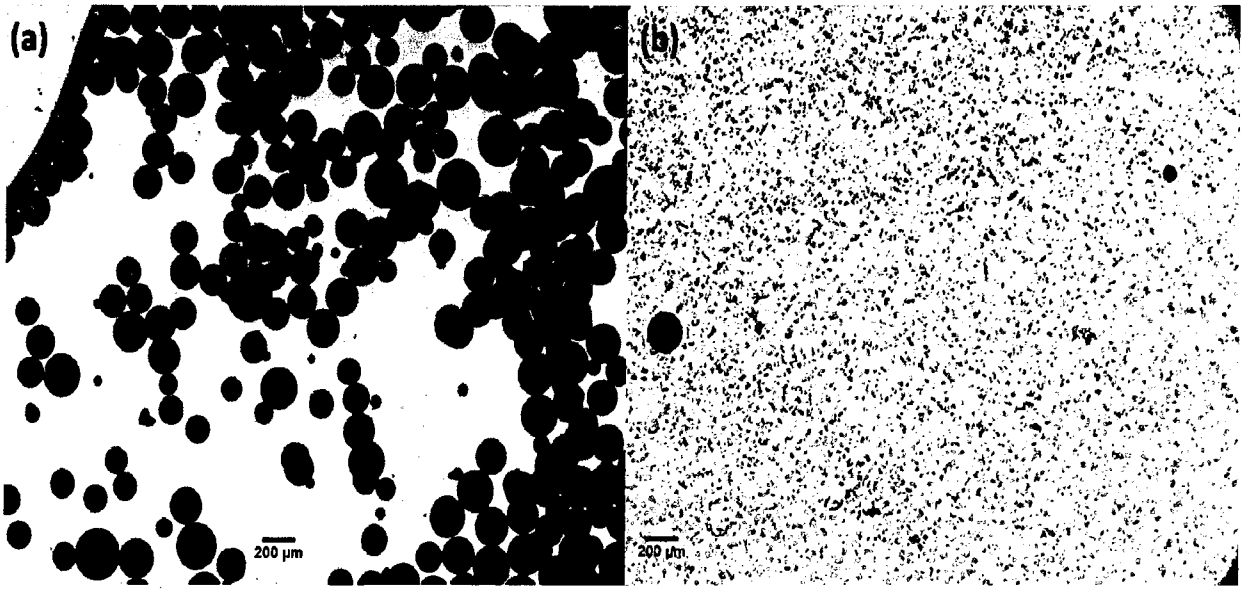

Figure 4.13 (a) Dilbeads before stirring and (b) Dilbeads after being crushed by stirring, magnification 4X

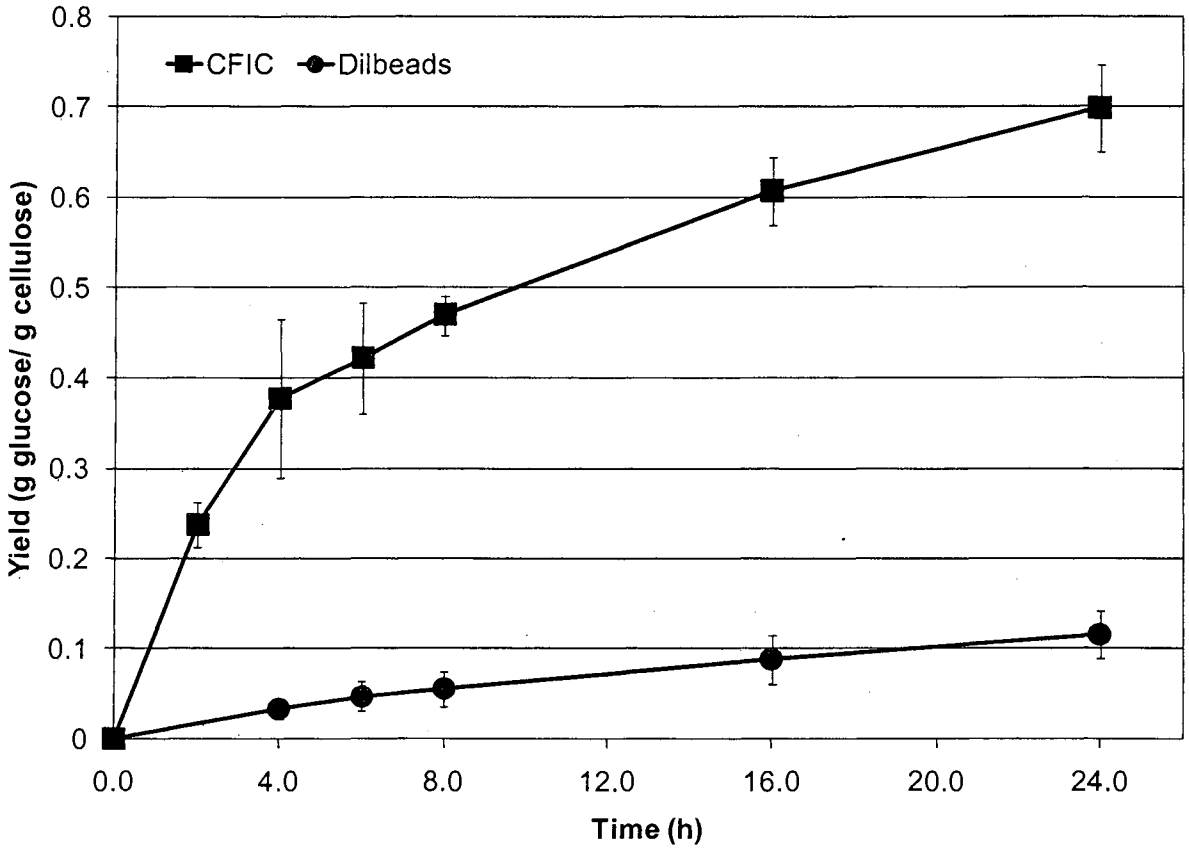

Figure 4.14 Yield of glucose produced from 0.5% (w/v) cellulose in buffer with 74mg of crushed Dilbeads (•), carrier free immobilized enzyme (■) at pH 5.0 and 45°C

4.6 Alumina beads

Kumakura [39] successfully immobilized cellulase onto alumina beads by radiation polymerization. This study used covalent bonding achieved by silanization followed by linking with glutaraldehyde. The yield achieved after 24 hours using 20 beads was 0.02 g glucose/g cellulose.

In comparison, the free enzyme produced 0.3 g glucose/g cellulose in the same period under the same conditions as seen in Figure 4.15. The alumina beads were only able to achieve 6% of the yield of the free enzyme. Therefore, the amount of enzyme required to achieve the same yield with alumina beads would be much higher, making it not cost effective.

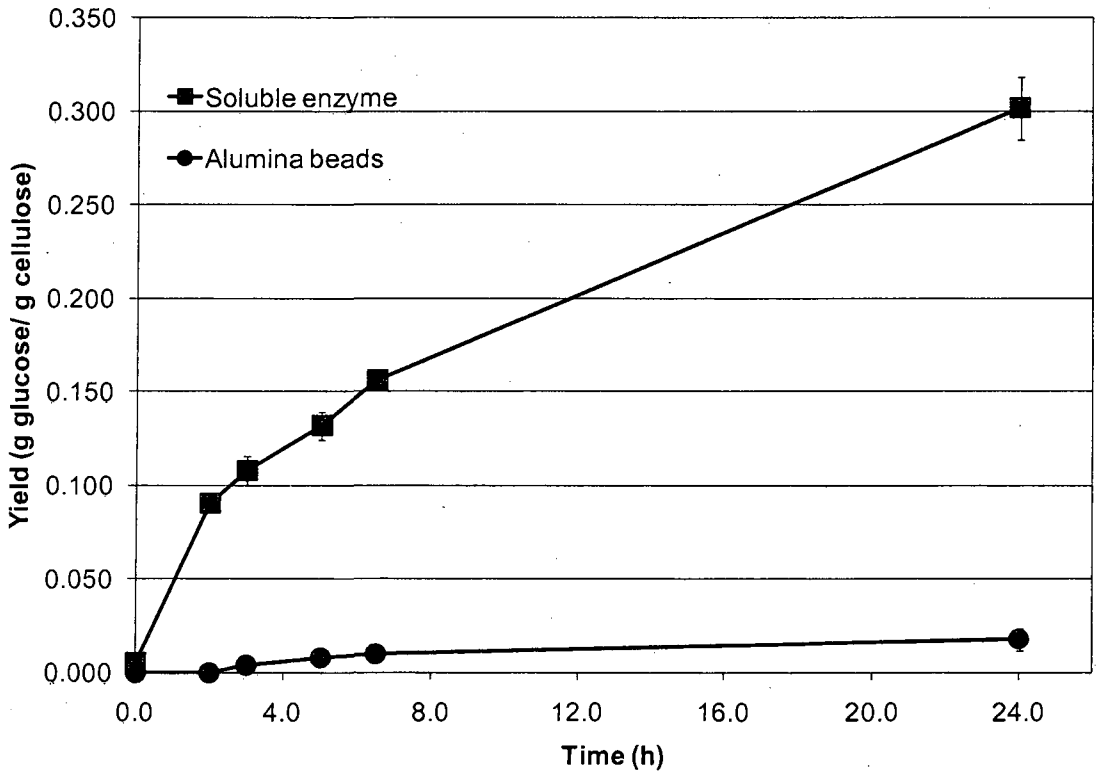

Figure 4.15 Yield of glucose produced from 3% (w/v) cellulose in buffer with 20 alumina beads (•),150U of enzyme (■) at pH 5.0 and 45°C

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The objective of this work is to examine the effectiveness of EMIM-DEP as a pretreatment step and compare the effectiveness of four methods for immobilizing cellulase.

1-Ethyl-3-methylimidazolium diethylphosphate at a concentration of 2% (v/v) increased the initial reaction rate by 2.7 times in the hydrolysis of cellulose with carrier-free immobilized enzyme. The enhancement observed here is comparable to other methods where cellulose must first be regenerated with an ionic liquid, and the IL recovered before hydrolysis.

CFIC was the most effective method of immobilization, retaining 80% of the enzyme activity. CFIC was also able to be used five times.

Magnetic beads showed exceptional ease of removal. However the yield, 0.2 g glucose/g cellulose, was only 30% of the yield achieved with carrier-free immobilized enzyme. This can be attributed to the lower loading of cellulase on the beads. Dilbeads showed poor mechanical stability and low yields of 0.12 g glucose/g cellulose. And alumina beads had the poorest yield, 0.02 g glucose/g cellulose, of all carrier bound methods investigated. It must be stated that the loading of cellulase on these supports was quite low.

For the three carrier bound immobilized enzymes: magnetic beads, Dilbeads and alumina beads, the yields achieved were far below what was achieved using the carrier-free method. Though the starting concentration of enzyme in the immobilizing solution is nearly the same as in CFIC, much less of the enzyme is immobilized resulting in lower loadings of the enzyme on the magnetic beads, Dilbeads or alumina support. In the case of magnetic beads, the yield of glucose can be increased by increasing the amount of enzyme, but this will result in a bigger reactor.

5.2 Recommendations

To further reduce the cost of enzymatic hydrolysis, enzymes can be manufactured by engineering more efficient strains.

A kinetic study of the hydrolysis process should be undertaken by monitoring cellobiose and glucose production.

An enzyme cocktail can also be used to maximize glucose yield.

REFERENCES

[1] P.T. Vasudevan, M. Gagnon, and M. Briggs, "Environmentally Sustainable Biofuels – The Case for Biodiesel, Biobutanol and Cellulosic Ethanol," *Sustainable Biotechnology Sources of Renewable Energy*, Springer, 2010.

[2] A.E. Farrell, R.J. Plevin, B.T. Turner, A.D. Jones, M. O'Hare, and D.M. Kammen, "Ethanol Can Contribute to Energy and Environmental Goals," *Science*, vol. 311, Jan. 2006, pp. 506-508.

[3] J. Urbanchuk, *Contribution of the Ethanol Industry to the Economy of the United States*, 2008.

[4] Dien, Cotta, and Jeffries, "Bacteria engineered for fuel ethanol production: current status," *Applied Microbiology and Biotechnology*, vol. 63, Dec. 2003, pp. 258-266.

[5] A.P. Dadi, S. Varanasi, and C.A. Schall, "Enhancement of cellulose saccharification kinetics using an ionic liquid pretreatment step," *Biotechnology and Bioengineering*, vol. 95, 2006, pp. 904-910.

[6] N. Kamiya, Y. Matsushita, M. Hanaki, K. Nakashima, M. Narita, M. Goto, and H. Takahashi, "Enzymatic in situ saccharification of cellulose in aqueous-ionic liquid media," *Biotechnology Letters*, vol. 30, Jun. 2008, pp. 1037-1040.

[7] Lee R. Lynd, J.H. Cushman, R.J. Nichols, and C.E. Wyman, "Fuel Ethanol from Cellulosic Biomass," *Science*, vol. 251, Mar. 1991, pp. 1318-1323.

[8] D. Pimentel and T.W. Patzek, "Ethanol Production Using Corn, Switchgrass, and Wood; Biodiesel Production Using Soybean and Sunflower," *Natural Resources Research*, vol. 14, Mar. 2005, pp. 65-76.

[9] M. Wang, N. Brinkman, T. Weber, and T. Darlington, *Well-to-Wheels Analysis of Advanced Fuel/Vehicle Systems— A North American Study of Energy Use, Greenhouse Gas Emissions, and Criteria Pollutant Emissions*, 2005.

[10] R. Hammerschlag, "Ethanol's Energy Return on Investment: A Survey of the Literature 1990−Present," *Environmental Science & Technology*, vol. 40, Mar. 2006, pp. 1744-1750.

[11] Y.P. Zhang and L.R. Lynd, "Toward an aggregated understanding of enzymatic hydrolysis of cellulose: Noncomplexed cellulase systems," *Biotechnology and Bioengineering*, vol. 88, 2004, pp. 797-824.

[12] Rose and Bennett, "Cooperative disassembly of the cellulose-xyloglucan network of plant cell walls: parallels between cell expansion and fruit ripening," *Trends in Plant Science*, vol. 4, May. 1999, pp. 176-183.

[13] J.O.D.F. Bailey, *Biochemical Engineering Fundamentals*, McGraw-Hill Education, 1977.

[14] B. Kamm, P.R. Gruber, and M. Kamm, *Biorefineries - Industrial Processes and Products*, 2006.

[15] S.I. Mussatto, M. Fernandes, A.M. Milagres, and I.C. Roberto, "Effect of hemicellulose and lignin on enzymatic hydrolysis of cellulose from brewer's spent grain," *Enzyme and Microbial Technology*, vol. 43, Aug. 2008, pp. 124-129.

[16] M. Chen, J. Zhao, and L. Xia, "Enzymatic hydrolysis of maize straw polysaccharides for the production of reducing sugars," *Carbohydrate Polymers*, vol. 71, Feb. 2008, pp. 411-415.

[17] T. Godfrey, J. Reichelt, and S. West, *Industrial Enzymology*, Nature Publishing Group, 1996.

[18] M.N. Gupta, *Thermostability of Enzymes*, Springer, 1993.

[19] M. Doscher and F. Richards, "The Activity of an Enzyme in the Crystalline State: Ribonuclease S," *J. Biol. Chem.*, vol. 238, 1963, pp. 2399-2406.

[20] F.A. Quiocho and F.M. Richards, "Intermolecular Cross Linking of a Protein in the Crystalline State: Carboxypeptidase-A," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 52, 1964, pp. 833-839.

[21] C. Mateo, Jose M. Palomo, Luuk M. van Langen, Fred van Rantwijk, and Roger A. Sheldon, "A new, mild cross-linking methodology to prepare cross-linked enzyme aggregates," *Biotechnology and Bioengineering*, vol. 86, 2004, pp. 273-276.

[22] B.H. Lee, *Fundamentals of Food Biotechnology*, Wiley-IEEE, 1996.

[23] T. Palmer, *Understanding Enzymes*, Prentice Hall, 1995.

[24] L. Cao, *Carrier-bound immobilized enzymes*, Wiley-VCH, 2005.

[25] M. Glad, O. Norrlow, B. Sellergren, N. Siegbahn, and K. Mosbach, "Use of silane monomers for molecular imprinting and enzyme entrapment in polysiloxane-coated porous silica," *Journal of Chromatography A*, vol. 347, 1985, pp. 11-23.

[26] R.A. Sheldon, "Cross-linked enzyme aggregates (CLEAs): stable and recyclable biocatalysts," *Biochemical Society Transactions*, vol. 35, Dec. 2007, pp. 1583-7.

[27] R. Schoevaart, M. Wolbers, M. Golubovic, M. Ottens, A. Kieboom, F.V. Rantwijk, L.V.D. Wielen, and R. Sheldon, "Preparation, optimization, and structures of cross-linked enzyme aggregates (CLEAs)," *Biotechnology and Bioengineering*, vol. 87, 2004, pp. 754-762.

[28] L. Cao, F. van Rantwijk, and R.A. Sheldon, "Cross-Linked Enzyme Aggregates: A Simple and Effective Method for the Immobilization of Penicillin Acylase," *Organic Letters*, vol. 2, May. 2000, pp. 1361-1364.

[29] H. Yu, H. Chen, X. Wang, Y. Yang, and C. Ching, "Cross-linked enzyme aggregates (CLEAs) with controlled particles: Application to Candida rugosa lipase," *Journal of Molecular Catalysis B: Enzymatic*, vol. 43, Dec. 2006, pp. 124-127.

[30] S. Dalal, A. Sharma, and M.N. Gupta, "A multipurpose immobilized biocatalyst with pectinase, xylanase and cellulase activities," *Chemistry Central Journal*, vol. 1, 2007, p. 16.

[31] R. Sheldon, "Enzyme Immobilization: The Quest for Optimum Performance," *Advanced Synthesis & Catalysis*, vol. 349, 2007, pp. 1289-1307.

[32] F.V. Rantwijk, F. Secundo, and R.A. Sheldon, "Structure and activity of Candida antarctica lipase B in ionic liquids," *Green Chemistry*, vol. 8, 2006, pp. 282-286.

[33] P.J. Robinson, P. Dunnill, and M.D. Lilly, "The properties of magnetic supports in relation to immobilized enzyme reactors," *Biotechnology and Bioengineering*, vol. 15, 1973, pp. 603-606.

[34] J. Ugelstad, P. Stenstad, L. Kilaas, W. Prestvik, R. Herje, A. Berge, and E. Hornes, "Monodisperse Magnetic Polymer Particles," *Blood Purification*, vol. 11, 1993, pp. 349-369.

[35] T.M. Cocker, Conan J. Fee, and Rachel A. Evans, "Preparation of magnetically susceptible polyacrylamide/magnetite beads for use in magnetically stabilized fluidized bed chromatography," *Biotechnology and Bioengineering*, vol. 53, 1997, pp. 79-87.

[36] X. Liu, Y. Guan, R. Shen, and H. Liu, "Immobilization of lipase onto micron-size magnetic beads," *Journal of Chromatography B*, vol. 822, Aug. 2005, pp. 91-97.

[37] W. Hartmeier, *Immobilized biocatalysts : an introduction*, Berlin ;;New York: Springer-Verlag, 1988.

[38] T. Tzanov, S.A. Costa, G.M. Gobitz, and A. Cavaco-Paulo, "Hydrogen peroxide generation with immobilized glucose oxidase for textile bleaching," *Journal of Biotechnology*, vol. 93, Jan. 2002, pp. 87-94.

[39] M. Kumakura, "Preparation of immobilized cellulase beads and their application to hydrolysis of cellulosic materials," *Process Biochemistry*, vol. 32, Sep. 1997, pp. 555-559.

[40] P. Padmini, S.K. Rakshit, and A. Baradarajan, "Studies on immobilization of lipase on alumina for hydrolysis of ricebran oil," *Bioprocess and Biosystems Engineering*, vol. 9, Mar. 1993, pp. 43-46.

[41] T.G.A. Youngs, C. Hardacre, and J.D. Holbrey, "Glucose Solvation by the Ionic Liquid 1,3-Dimethylimidazolium Chloride: A Simulation Study," *The Journal of Physical Chemistry B*, vol. 111, Dec. 2007, pp. 13765-13774.

[42] H. Zhao, C.L. Jones, G.A. Baker, S. Xia, O. Olubajo, and V.N. Person, "Regenerating cellulose from ionic liquids for an accelerated enzymatic hydrolysis," *Journal of Biotechnology*, vol. 139, Jan. 2009, pp. 47-54.

[43] M.B. Turner, S.K. Spear, J.G. Huddleston, J.D. Holbrey, and R.D. Rogers, "Ionic liquid salt-induced inactivation and unfolding of cellulase from Trichoderma reesei," *Green Chemistry*, vol. 5, 2003, pp. 443-447.

[44] Q. Li, Y. He, M. Xian, G. Jun, X. Xu, J. Yang, and L. Li, "Improving enzymatic hydrolysis of wheat straw using ionic liquid 1-ethyl-3-methyl imidazolium diethyl phosphate pretreatment," *Bioresource Technology*, vol. 100, Jul. 2009, pp. 3570-3575.

[45] Z. Guo, S. Bai, and Y. Sun, "Preparation and characterization of immobilized lipase on magnetic hydrophobic microspheres," *Enzyme and Microbial Technology*, vol. 32, Jun. 2003, pp. 776-782.

[46] S. Zhang, S. Gao, and G. Gao, "Immobilization of β-Galactosidase onto Magnetic Beads," *Applied Biochemistry and Biotechnology.*

[47] P. Torres, A. Datla, V. Rajasekar, S. Zambre, T. Ashar, M. Yates, M. Rojas-Cervantes, O. Calero-Rueda, V. Barba, M. Martínez, A. Ballesteros, and F. Plou, "Characterization and application of a sterol esterase immobilized on polyacrylate epoxy-activated carriers (Dilbeads(TM))," *Catalysis Communications*, vol. 9, Mar. 2008, pp. 539-545.

[48] A. Tanriseven and Y. Aslan, "Immobilization of Pectinex Ultra SP-L to produce fructooligosaccharides," *Enzyme and Microbial Technology*, vol. 36, Mar. 2005, pp. 550-554.

APPENDIX

EXPERIMENTAL RAW DATA

Table A. 1 Yield of glucose from 150 U of free enzyme at pH 4.0, T 45 °C, and 3% (w/v) cellulose

Time (h)	Yield (g glucose/g cellulose)	Yield (g glucose/g cellulose)
0	0	0
2	0.001	0.002
3	0.004	0.005
5	0.008	0.019
7	0.012	0.027
10	0.015	0.033
24	0.027	0.062
48	0.038	0.071
72	0.051	0.082
96	0.062	0.091

Table A. 2 Yield of glucose from 150 U of free enzyme at pH 4.5, T 45 °C, and 3% (w/v) cellulose

Time (h)	Yield (g glucose/g cellulose)	Yield (g glucose/g cellulose)
0	0.00	0.00
2	0.09	0.08
3	0.11	0.10
5	0.13	0.12
7	0.15	0.15
10	0.17	0.17
24	0.31	0.29
48	0.45	0.40
72	0.64	0.56
96	0.76	0.67

Table A. 3 Yield of glucose from 150 U of free enzyme at pH 5.0, T 45 °C, and 3% (w/v) cellulose

Time (h)	Yield (g glucose/g cellulose)	Yield (g glucose/g cellulose)
0	0.00	0.00
2	0.05	0.11
3	0.06	0.13
5	0.09	0.14
7	0.11	0.19
10	0.12	0.22
24	0.20	0.36
48	0.28	0.49
72	0.35	0.59
96	0.41	0.54

Table A. 4 Yield of glucose from 150 U of free enzyme at pH 4.5, T 55 °C, and 3% (w/v) cellulose

Time (h)	Yield (g glucose/g cellulose)	Yield (g glucose/g cellulose)
0	0.00	0.00
2	0.10	0.10
3	0.13	0.12
5	0.17	0.16
7	0.21	0.18
10	0.22	0.19
24	0.40	0.39
48	0.42	0.46
72	0.49	0.45
96	0.59	0.66

Table A. 5 Yield of glucose from 150 U of free enzyme at pH 5.0, T 55 °C, and 3% (w/v) cellulose

Time (h)	Yield (g glucose/g cellulose)	Yield (g glucose/g cellulose)
0	0.00	0.00
2	0.09	0.08
3	0.10	0.10
5	0.12	0.12
7	0.13	0.15
10	0.11	0.18
24	0.21	0.26
48	0.33	0.42
72	0.31	0.51
96	0.41	0.59

Table A. 6 Run 1-5 Yield of glucose produced from 0.5% (w/v) cellulose in 10mL of buffer and immobilized enzyme at pH 5.0 and 45°C

	Time (h)	Yield (g glucose/g cellulose)	Yield (g glucose/g cellulose)
1	0.0	0.00	0.00
	2.00	0.22	0.26
	4.00	0.32	0.44
	6.00	0.38	0.47
	8.00	0.45	0.49
	16.00	0.58	0.63
	24.00	0.66	0.73

	Time (h)	Yield (g glucose/g cellulose)	Yield (g glucose/g cellulose)
2	0.0	0.00	0.00
	2.00	0.01	0.11
	4.00	0.06	0.23
	6.00	0.15	0.30
	8.00	0.19	0.32
	16.00	0.26	0.39
	24.00	0.34	0.47

3

Time (h)	Yield (g glucose/g cellulose)	Yield (g glucose/g cellulose)
0.0	0.00	0.00
2.00	0.07	0.10
4.00	0.11	0.18
6.00	0.15	0.29
8.00	0.19	0.32
16.00	0.24	0.39
24.00	0.35	0.47

4

Time (h)	Yield (g glucose/g cellulose)	Yield (g glucose/g cellulose)
0.0	0.00	0.00
2.00	0.14	0.11
4.00	0.16	0.15
6.00	0.19	0.23
8.00	0.21	0.32
16.00	0.27	0.41
24.00	0.36	0.46

5

Time (h)	Yield (g glucose/g cellulose)	Yield (g glucose/g cellulose)
0.0	0.00	0.00
2.00	0.15	0.04
4.00	0.12	0.06
6.00	0.12	0.07
8.00	0.12	0.18
16.00	0.15	0.18
24.00	0.37	0.21

Table A. 7 Run 1-3 ield of glucose produced from 0.5% (w/v) cellulose in 10mL of buffer and immobilized enzyme at pH 4.5 and 45°C

	Time (h)	Yield (g glucose/g cellulose)	Yield (g glucose/g cellulose)
1	0.0	0.000	0.000
	2.00	0.265	0.252
	4.00	0.397	0.378
	6.00	0.462	0.455
	8.00	0.536	0.488
	16.00	0.617	0.597
	24.00	0.672	0.635

	Time (h)	Yield (g glucose/g cellulose)	Yield (g glucose/g cellulose)
2	0.0	0.000	0.000
	2.00	0.053	0.078
	4.00	0.066	0.128
	6.00	0.075	0.146
	8.00	0.090	0.153
	16.00	0.099	0.227
	24.00	0.135	0.330

	Time (h)	Yield (g glucose/g cellulose)	Yield (g glucose/g cellulose)
3	0.0	0.000	0.000
	2.00	0.018	0.020
	4.00	0.025	0.039
	6.00	0.032	0.058
	8.00	0.035	0.073
	16.00	0.042	0.112
	24.00	0.066	0.176

Table A. 8 Yield of glucose produced from 0.5% (w/v) cellulose, 120 U of carrier-free immobilized enzyme in buffer with 1%(v/v) EMIM-DEP

Time (h)	Yield (g glucose/g cellulose)	Yield (g glucose/g cellulose)
0.0	0	0
2.00	0.70	0.56
4.00	0.77	0.63
6.00	0.82	0.68
8.00	0.87	0.72
16.00	0.99	0.78
24.00	1.06	0.90

Table A. 9 Yield of glucose produced from 0.5% (w/v) cellulose, 120 U of carrier-free immobilized enzyme in buffer with 2%(v/v) EMIM-DEP

Time (h)	Yield (g glucose/g cellulose)	Yield (g glucose/g cellulose)
0.0	0	0
2.00	0.73	0.64
4.00	0.94	0.75
6.00	1.03	0.87
8.00	1.13	0.96
16.00	1.20	1.07
24.00	1.27	1.13

Table A. 10 Yield of glucose produced from 0.5% (w/v) cellulose, 120 U of carrier-free immobilized enzyme in buffer with 4%(v/v) EMIM-DEP

Time (h)	Yield (g glucose/g cellulose)	Yield (g glucose/g cellulose)
0.0	0	0
2.00	0.47	0.55
4.00	0.71	0.83
6.00	0.79	0.92
8.00	0.87	1.01
16.00	0.93	1.12
24.00	1.07	1.23

Table A. 11 Yield of glucose produced from Dilbeads prepared with 1M binding buffer, 0.5% (w/v) cellulose at pH 5.0 and 45°C

Time (h)	Yield (g glucose/g cellulose)	Yield (g glucose/g cellulose)
0.0	0.000	0.000
2.00	0.028	0.034
4.00	0.044	0.053
6.00	0.054	0.060
8.00	0.062	0.079
16.00	0.091	0.111
24.00	0.102	0.135

Table A. 12 Yield of glucose produced from Dilbeads prepared with 0.3 M binding buffer, 0.5% (w/v) cellulose at pH 5.0 and 45°C

Time (h)	Yield (g glucose/g cellulose)	Yield (g glucose/g cellulose)
0.0	0	0
2.00	0	0
4.00	0.011	0.007
6.00	0.018	0.012
8.00	0.027	0.015
16.00	0.034	0.023
24.00	0.037	0.029

Table A. 13 Yield of glucose produced from Alumina beads and 3% (w/v) cellulose at pH 5.0 and 45°C

Time (h)	Yield (g glucose/g cellulose)	Yield (g glucose/g cellulose)
0.0	0.00	0.00
2.00	0.00	0.00
3.00	0.01	0.00
5.00	0.01	0.01
6.50	0.01	0.01
24.00	0.02	0.01

Table A. 14 Yield of glucose produced from magnetic beads, 0.5% (w/v) cellulose at pH 5.0 and 45°C

Time (h)	Yield (g glucose/g cellulose)	Yield (g glucose/g cellulose)
0.0	0.00	0.00
2.00	0.04	0.03
4.00	0.07	0.05
6.00	0.09	0.07
8.00	0.11	0.09
16.00	0.17	0.14
24.00	0.20	0.18