

PROBING CELLULASE STABILITY IN LOW COST MINERAL ACID IONIC LIQUIDS

LATIFFAH KARIM

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IMPERIAL COLLEGE LONDON**

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DECLARATION

I hereby declare that the work described in this thesis is the result of my own independent investigation, unless otherwise stated.

This work has not been previously submitted for any degree and is not being concurrently submitted in candidature for any other degree.

Latiffah Karim

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ABSTRACT

Ionic liquid (IL) pretreatment has gained significant attention for biomass deconstruction. However, residual IL left in biomass can cause enzyme deactivation, leading to problems with further processing of biomass to sugars. Cellulases are enzymes commonly used in the saccharification process and the activity of this enzyme is severely affected by ILs. This thesis focuses on understanding how ILs affect the activity of cellulases in the enzymatic saccharification process. Sigmacell cellulose was used in the enzymatic saccharification process instead of biomass plant. Seven different ILs were added in the enzymatic saccharification mixtures and the activity of a mixture of commercially available cellulases was measured using high-performance liquid chromatography (HPLC) to measure glucose release. Sulphate based ILs were more harmful for cellulase action than [EMIM][OAc], [BMIM]Cl and phosphate based ILs. [HBIM][HSO₄] inactivated commercial cellulases (Celluclast®) and cellobiase (Novozyme188) in 4 h in the enzymatic saccharification process. In this thesis, it was observed that the main factor that affects the activity of cellulase is pH. To overcome this, acidophilic cellulases were suggested to improve the activity of the enzymes in ILs. Endoglucanase from *Cryptococcus sp.*S-2 and β -glucosidase from *Thermofilum pendens* were studied and tested with ILs. The result of this study has shown endoglucanase from *Cryptococcus sp.* as a promising enzyme in IL biomass deconstruction. There was a slight improvement in the activity of β -glucosidase from *T.pendens* with cellobiose in 1% [TEA][HSO₄] but when both of these enzymes were combined and acting against carboxymethylcellulose (CMC) with 10% (v/v) of [TEA][HSO₄], the enzymes were still active. Based from these findings, pretreatment process using low cost IL is favourable as it is cost effective and more efficient.

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LIST OF EQUATIONS

$$U/mL = (Absorbance/min \times V)/(e \times d \times v) \dots\dots\dots 2.1$$

$$\text{Specific activity [U/mg protein]} = \text{enzymatic activity volume} \dots\dots\dots 2.2$$

$$\text{Absorbance} = E \cdot b \cdot c \dots\dots\dots 2.3$$

$$\text{Glucose yield} = (\text{Grams cellulose digested/grams cellulose added}) \times 100 \dots\dots\dots 3.1$$

$$I = \frac{1}{2} \cdot \sum \cdot \frac{m_i}{m^0} \cdot z_i^2 \dots\dots\dots 3.2$$

ABBREVIATIONS

-	micro-
Abs	absorbance
[BMIM]Cl	1-butyl-3-methylimidazolium chloride
[BMIM][HSO ₄]	1-butyl-3-methylimidazolium hydrogen sulfate
[BMIM][H ₂ PO ₄]	1-butyl-3-methyl-imidazolium dihydrogen phosphate
BSA	bovine serum albumin
CMC	Carboxymethyl cellulose
DNA	deoxyribonucleic acid
DTT	dithiothreitol
[EMIM][OAc]	1-ethyl-3-methylimidazolium acetate
EDTA	ethylenediaminetetraacetic acid
GH	Glycoside hydrolase
[HBIM][HSO ₄]	1-butylimidazolium hydrogen sulfate
[HBIM][H ₂ PO ₄]	1-butylimidazolium dihydrogen phosphate
HPLC	High-performance liquid chromatography
HCl	Hydrochloric acid
IPTG	Isopropyl-β-D-thio-galactopyranoside
IUB	International Union of Biochemistry
O.D.	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
<i>p</i> NPG	<i>p</i> -Nitrophenyl-β-D-glucopyranoside
<i>p</i> NP	<i>p</i> -Nitrophenol
rpm	rotations per minute
SDS	sodium dodecyl sulfate
[TEA][HSO ₄]	Triethylammonium hydrogen sulfate
TEMED	tetramethylethylenediamine
U	unit
UV	ultra violet

CHAPTER 1: LITERATURE REVIEW

The non-renewable fossil fuels that provide energy for heat, electricity, and transportation are anticipated to run out during the latter half of the 21st century. There is therefore a growing interest in the production of transport fuels such as ethanol from renewable resources. Mabee (2011) stated that currently, sugarcane or sugarbeet, corn and wheat are popular feedstocks for the bioethanol industry. However, those biomass feedstocks are also the food supply for livestock and humans. Thus, there is competition between food and fuel. An alternative feedstock to develop a bio-based ethanol industry is to explore lignocellulosic biomass from agricultural and forest activities. Lignocellulosic biomass is generally composed of cellulose (40-50%), hemicellulose (20-30%), and lignin (20-30%). Cellulose comprises glucose moieties and represents an abundant renewable biomaterial (Ding and Himmel, 2006; Doblin et al, 2002; Saxena and Brown, 2005; Scheible and Pauly, 2004). In biofuel production, the first process is a pretreatment step. This step is important to separate the components of the biomass into cellulose, hemicellulose and lignin. The second step is enzymatic saccharification which uses enzymes to convert cellulose to sugars. The final step is fermentation, which converts the sugars into biofuel (figure 1.1).

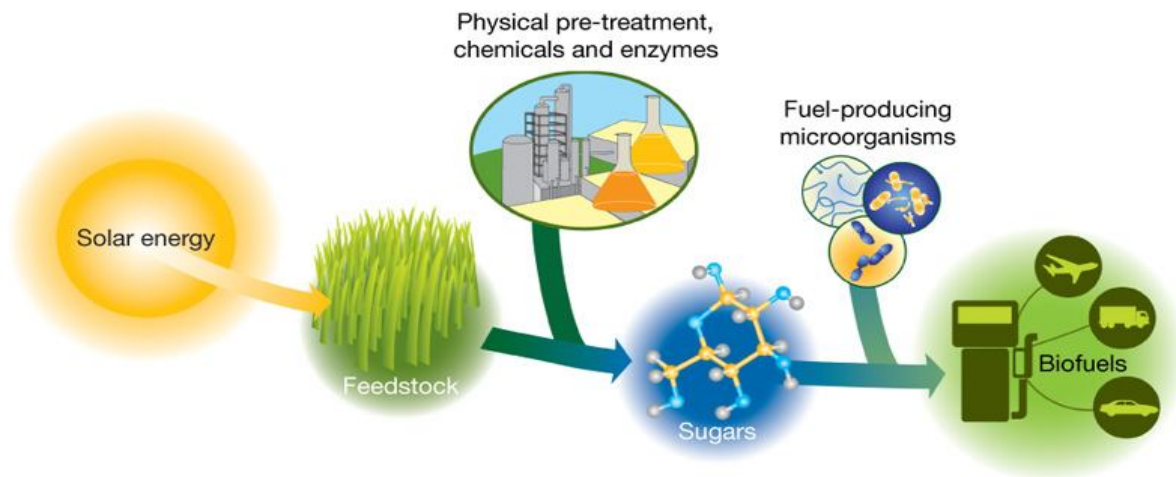


Figure 1.1: Flowchart for biofuel production from lignocellulosic biomass (Gilbert, 2008).

1.1 Biorefinery and lignocellulosic biomass

The biorefinery concept is analogous to an oil refinery. Refineries take petroleum or crude oil and fractionate all of the potential feedstocks for further processing and blending to produce the fossil-fuel based products that we use today, for example plastics, detergents, solvents and fibres such as nylon and polyesters. In a biorefinery, oil is replaced with a renewable biological feedstock such as biomass. Biomass consists of organic and inorganic compounds. Cereal grains, nuts and oil seeds are direct food sources for humans and animals, and they are perfect raw materials for chemicals, energy and materials. Woody biomass, on the other hand, is a structural material for plants. There are four main categories of substances in woody biomass: cellulose, hemicellulose, lignin, and extractives. Fractionation and conversion of the lignocellulosic biomass can produce a vast array of chemicals, energy and materials (Liu et al., 2012). Figure 1.2 shows a schematic diagram of biorefinery.

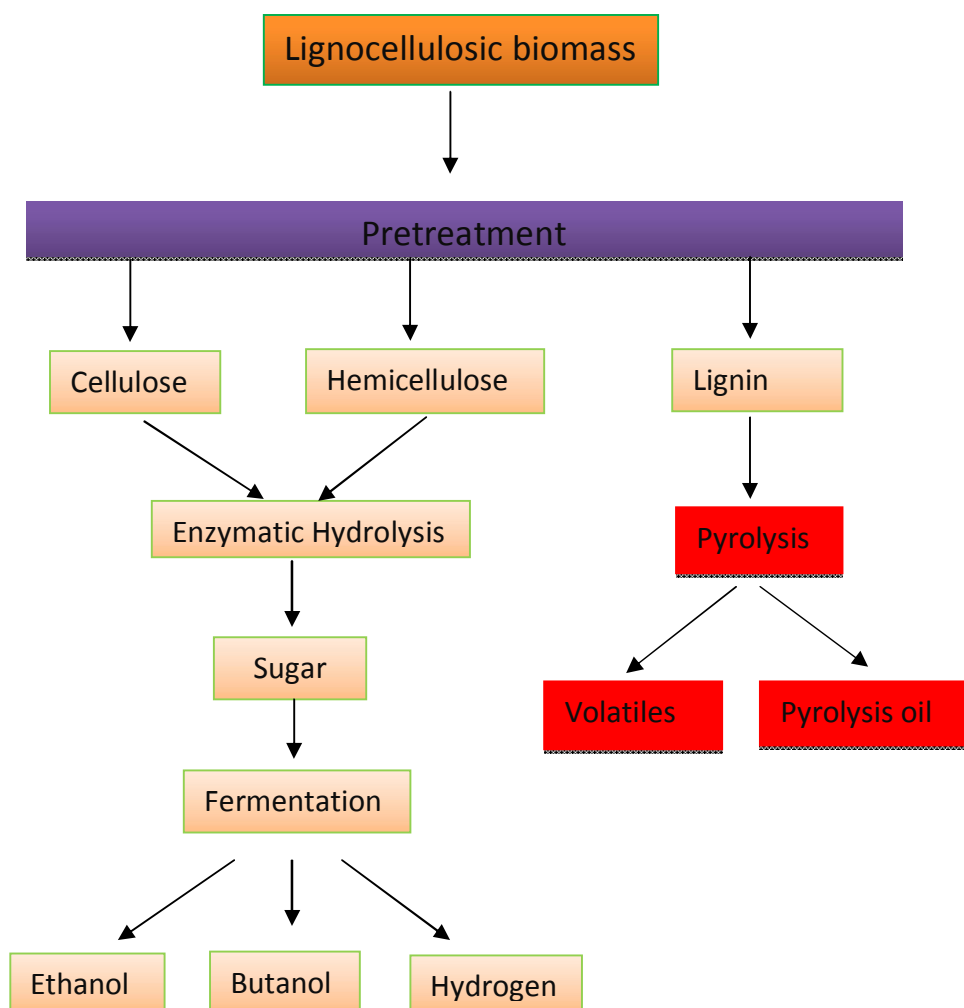


Figure 1.2: Schematic diagram of biorefinery

Lignocellulose is comprised of three polymers interlinked in a dense matrix (figure 1.3). It consists of cellulose, hemicellulose and lignin. It can be seen that cellulose is enclosed by a lignin-hemicellulose complex lignin-carbohydrate polymeric structure (Padmanabhan et al., 2011).

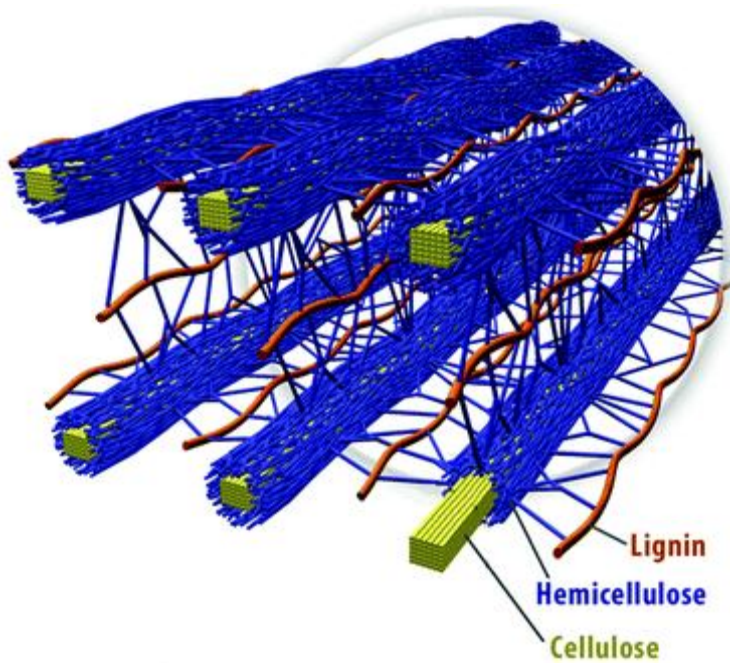


Figure 1.3: Lignocellulose structure (Padmanabhan et al., 2011).

Cellulose has a complex and heterogeneous structure and the chemical composition of cellulose consists of D-glucose residues linked by β -1,4-glucosidic bonds (Figure 1.4). Cellulose is insoluble in water because it has over 10,000 glucose residues. Crystalline microfibrils are formed from the polymeric chains which attach to each other in a parallel fashion. Normally, cellulose contains highly crystalline and amorphous regions along with microfibrils. Highly crystalline cellulose can only be found in algae or bacteria and these organisms have been used as a model substrate in order to understand how different types of cellulases can attack and degrade crystalline cellulose (Martin, 2000).

In addition, hemicellulose consists of five sugars which are glucose, galactose, mannose, xylose, and arabinose and lignin consists of phenolic compounds. An example of lignocellulosic biomass normally found in North America and Europe are cereal straws which contain 35 to 40% cellulose, 26 to 27% hemicellulose and 15 to 20% lignin (Mabee et.al, 2011).

Furthermore, lignin is the most complex polymer in lignocellulose. It is made up of a few units of phenylpropene derivatives, namely p-coumaryl, coniferyl and sinapyl alcohols. Lignin is a high polydispersity polymer with different branching and bonding; this makes the polymer insoluble in water. It is highly resistant to impact, compression and binding; this enhances the durability of plant cells (Harmsen et al., 2010).

Moreover, the production of bioethanol from biomass relies on the chemistry of the feedstock. Table 1.1 shows percentages of cell wall composition in biomass. Herbaceous lignocellulosics (wheat straw) are relatively simple to convert when compared to other feedstocks. In addition, biomass from hardwood (oak) consists of more cellulose and less hemicellulose which indicates that more glucose can be obtained. The hemicellulose in hardwoods has more xylose, a C5 sugar which can be converted into ethanol (Biely and Kremnicky, 1998). However, softwood species (loblolly pine) are more difficult for bioconversion due to the presence of more lignin, which is more difficult to extract from softwoods than from hardwoods. The cell wall of softwoods is thicker and more rigid. Delignification of softwood is very challenging because lignin is very stable (Shimada et al., 1997).

Table 1.1: Cell wall composition (%) in biomass

Biomass	Cellulose	Hemicellulose	Lignin
<i>Miscanthus</i> spp. (Vrije et al., 2002)	40	24.3	25
Wheat straw (Bowyer et al., 2003; Kristensen et al., 2008)	47	32	10
Rice straw (Jin and Chen, 2007)	42	32	14
Willow (Ray et al. 2012, Serapiglia et al. 2013)	30	40	25
Loblolly pine (Shaobo Pan et al., 2013)	23	14	44
Oak (Le Floch et al., 2015)	38	29	25

1.1.1 Cost

ILs are said to be an expensive solvent in biomass dissolution. This is because large volumes are required for pretreatment. Brandt considers the ideal cost of IL to be used as USD 2.50 / kg or less (Brandt et al., 2013). It has been reported that the cost to produce acetone is less than USD 1.50 / kg (Chen et al., 2014). However, the cost of [EMIM] [OAc] is USD 20/kg (Simmons 2013) which is more expensive than the acetone. To solve this issue, cheaper ILs are required to reduce the cost of IL biomass processing. Therefore, Chen (Chen et al., 2014) conducted a study on triethylammonium hydrogen sulfate [TEA] [HSO₄] and 1-methylimidazolium hydrogen sulfate which cost USD 1.24/kg and USD 2.96-5.88/kg, respectively. In order to prove the efficiency of using this cheaper IL, a comparative study on switchgrass using [TEA] [HSO₄] and [EMIM] [OAc] had been performed and this study found that more than 75% glucose yield was obtained when [TEA] [HSO₄] was used (George et al., 2015). These findings suggest that IL cost can be reduced in order to lower the cost of the biomass processing.

1.2 Cellulases

Enzymatic cellulose hydrolysis involves endoglucanase (EC 3.2.1.4), exoglucanase or cellobiohydrolase (EC 3.2.1.91) and β -glucosidase (EC 3.2.1.21) (Henrissat, 1994; Knowles et al., 1987; Lynd et al., 2002; Teeri, 1997; Wood and Garica- Campayo, 1990; Zhang and Lynd, 2004).

Endoglucanases hydrolyse intramolecular β -1,4-glucosidic bonds of cellulose chains randomly to produce new chain ends. Exoglucanases are important in releasing soluble cellobiose or glucose by cleaving cellulose chains at the ends. In order to eliminate cellobiose

inhibition, β -glucosidases are used to hydrolyse cellobiose to glucose. Figure 1.4 shows three types of reaction catalysed by cellulases.

It has been reported that cellulases have two domain structures which are catalytic domain (CD) and a cellulose binding domain (CBD). CBD is also known as carbohydrate binding module (CBM). CD has catalytic site but CBDs assist in binding of enzyme to cellulose. Moreover, cellulases are inducible enzymes and the regulation of the cellulase is controlled by activation and repression mechanism. The production of cellulolytic enzyme is induced in the presence of the substrate, and is repressed by glucose. Cellobiose, δ -cellobiose and other oxidized products of cellulose hydrolysis can act as inducers of cellulase. Moreover, promoter is involved in cellulase production. CBH I promoter of *T. reesei* is a highly efficient promoter with a high rate of expression and this promoter has been used to drive expression of β -glucosidases and endoglucanases thus improving cellulase profile of host strain (Meenu et al., 2014).

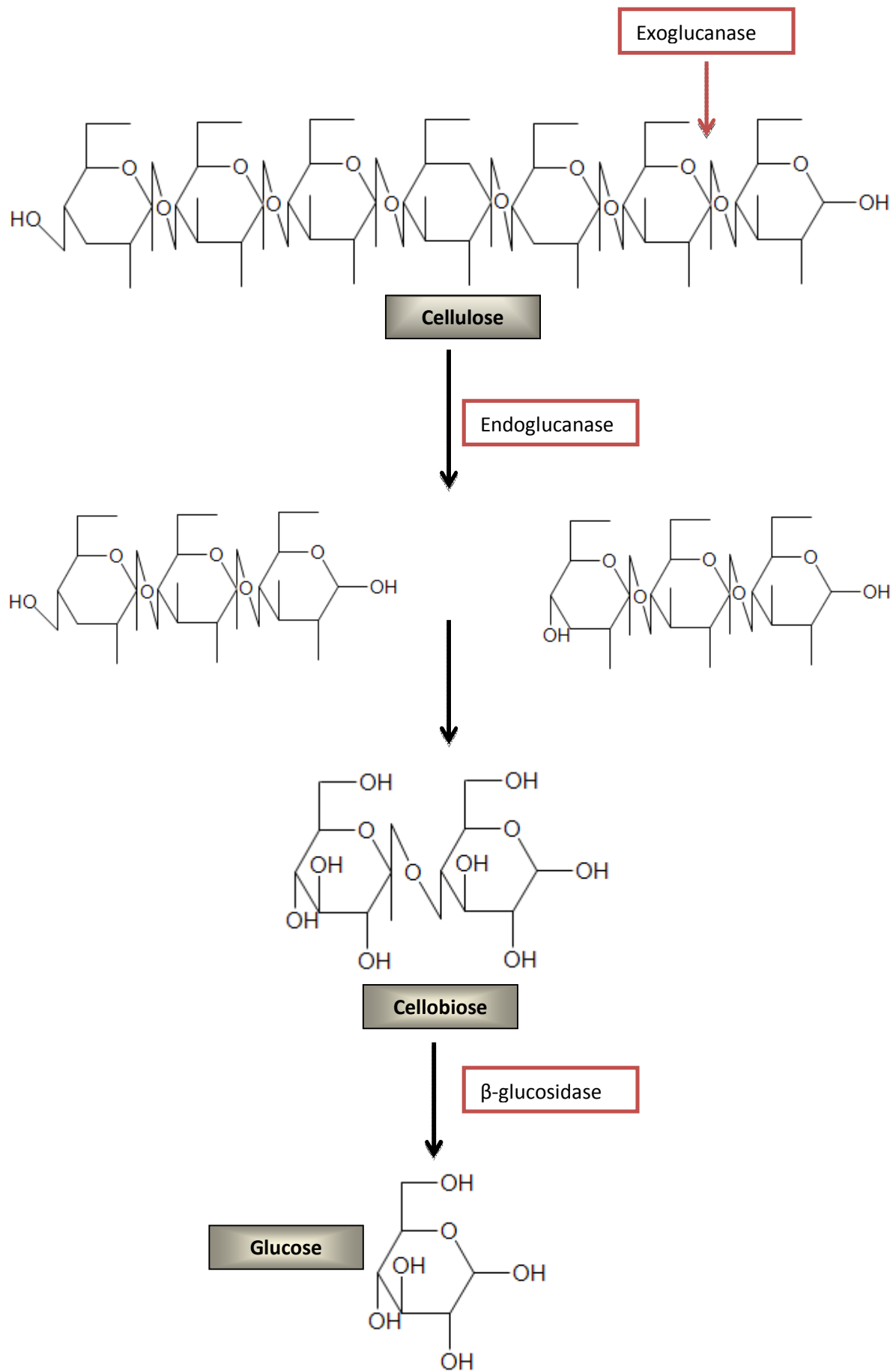


Figure 1.4: Reactions catalysed by cellulases

1.2.1 Occurrence of cellulases

Cellulases are produced by a wide variety of organisms which cover a number of ecological niches. This includes bacteria and fungi, living both in the presence and absence of oxygen. These can be mesophiles or thermophiles (Bhat and Bhat, 1997; Coutinho and Henrissat, 1999; Maijala, 2000; Lynd et al., 2002). Within bacteria, industrially relevant organisms such as the Clostridia are commonly used to manufacture cellulases or used in consolidated bioprocessing to convert cellulose to small molecules by fermentation (Higashide et.al. 2011). Within fungi, the most widely studied organisms for cellulase production are the *Trichoderma* and *Aspergillus* species.

1.2.2 Cellulase expression system

Cellulases produced naturally from fungi and bacteria sometimes do not fulfill the demand of industry due to some properties such as temperature and pH optima. Biomass pretreatment often conducted in a high temperature and acidic condition which can deactivate the activity of cellulases. Therefore, recombinant cellulases have been investigated extensively by researchers to meet this demand. Bacterial, fungal and plant are three common expression system for recombinant cellulase production.

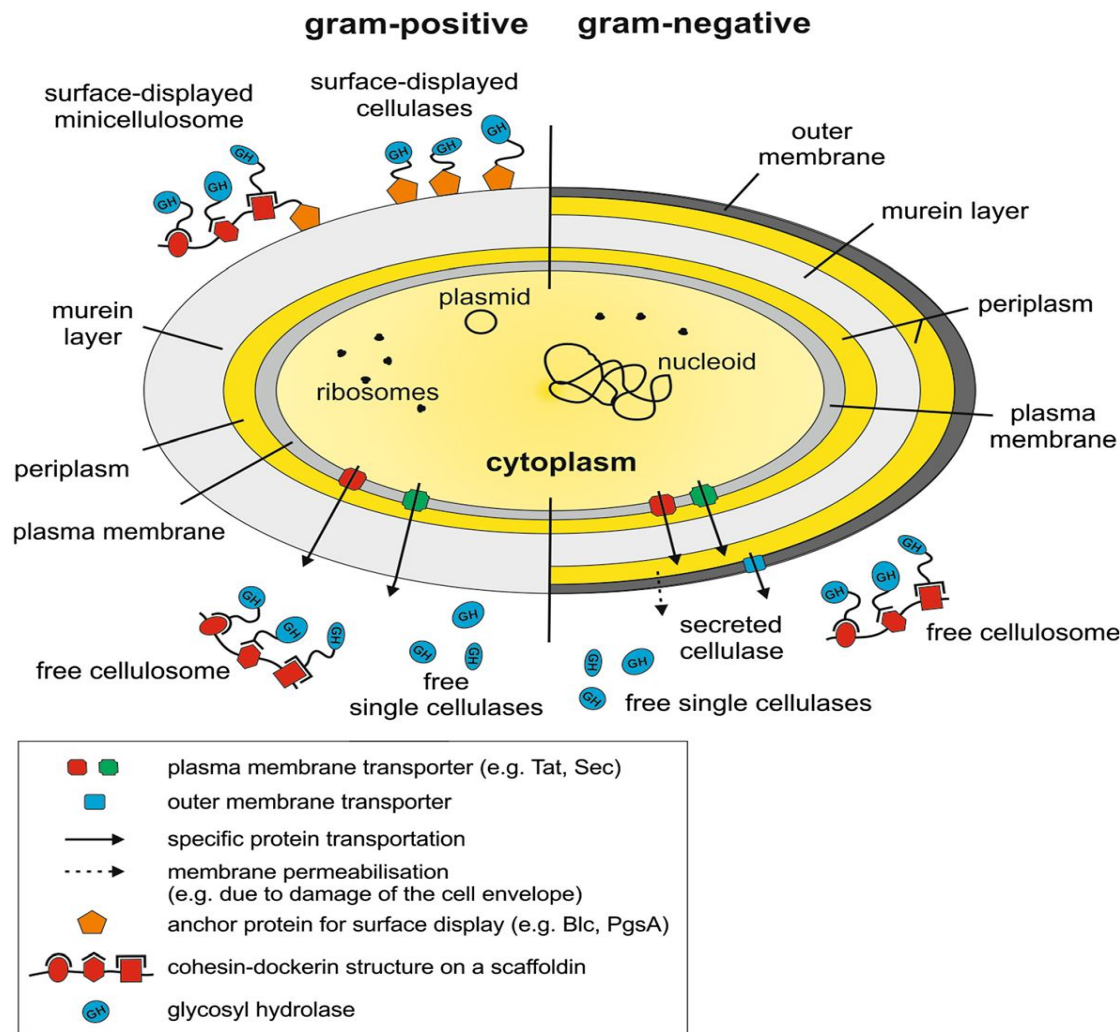


Figure 1.5: Diagram of a bacterial cell displayed the localization of recombinant cellulases and cellulosomes. GH: glycosyl hydrolase, Sec: secretion protein, Tat: twin-arginine-transporter (Lambertz et al., 2014). Reproduced with permission.

In the bacterial expression systems, recombinant cellulases are expressed and directed to the periplasm by specific protein transporters for example Tat- and Sec-pathways or stored in the cytoplasm. It has been speculated that Gram-positive bacteria (left) are able to secrete cellulase more effectively because there is no outer membrane. Cellulases can be secreted as single cellulases, cellulosomes or as mini-cellulosomes which are presented on the cell surface using anchor protein such as Blc or PgsA (Figure 1.5).

Cellulase secretion in Gram-negative bacteria (right) requires a specific protein transporter or membrane permeabilisation because Gram-negative bacteria have a thick outer membrane which can limit extracellular protein transport. Cellulases can be secreted as single cellulases or cellulosomes (Figure 1.5). Bacterial expression system such as *E.coli* is easy to manipulate and cost effective and it takes a few days to get the cellulase. However, the yield of the enzyme is not much compared to fungal and plant system (Brondyk, 2009).

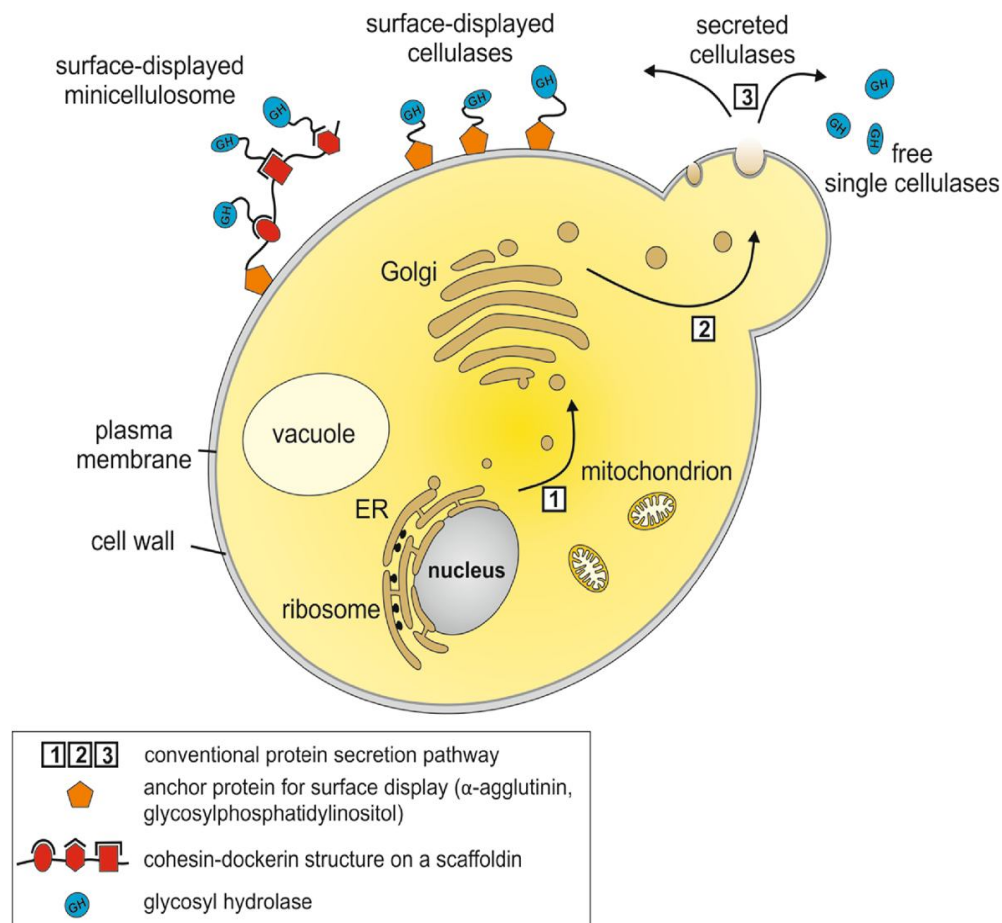


Figure 1.6: Diagram of a single yeast cell presenting the main cell compartments involved in recombinant cellulase expression. GH: glycosyl hydrolase, ER: endoplasmic reticulum (Lambertz et al., 2014). Reproduced with permission.

In yeast expression systems, first cellulases are expressed in the ER and then transported to the Golgi apparatus and then cellulases are secreted from the medium as a single cellulase or cellulosomes. Cellulase can also be secreted by α -agglutinin which is an anchor protein and forms surface-displayed mini-cellulosomes or a single cellulose (Figure 1.6).

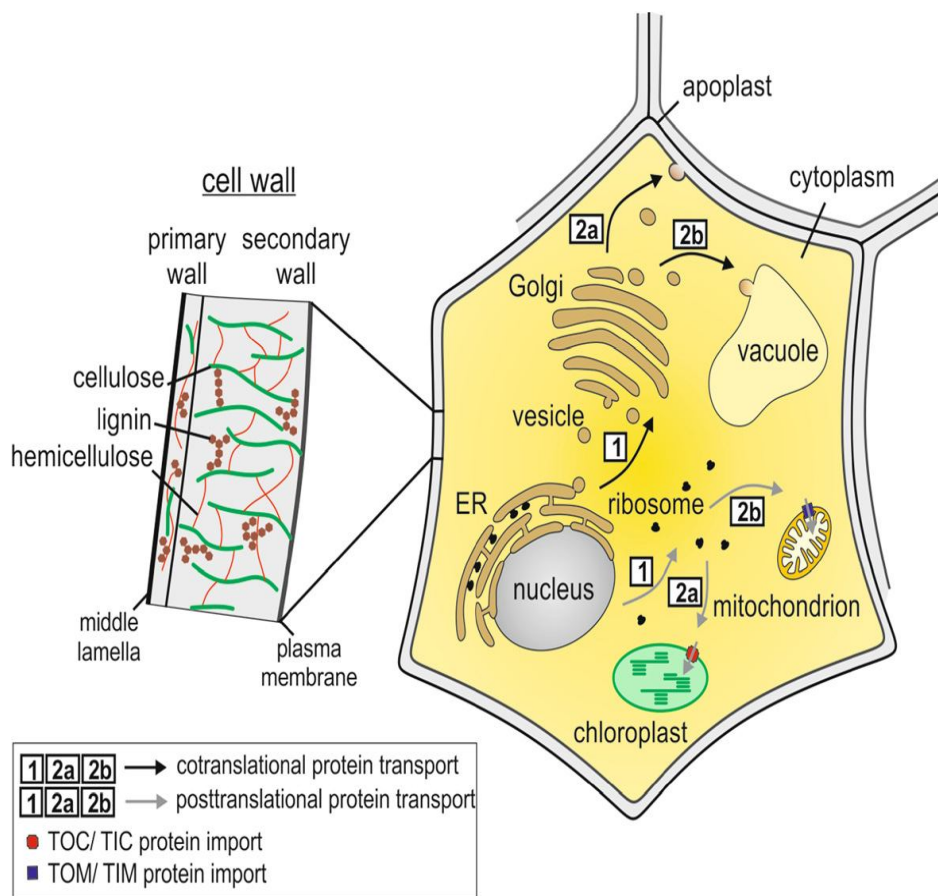


Figure 1.7: Diagram of a plant cell with the main cell compartments for recombinant cellulases expression. TOC: Translocon at the outer envelope of chloroplasts, TIC: Translocon at the inner envelope of chloroplasts, TOM: Translocase of the outer mitochondrial membrane, TIM: Translocase of the inner membrane (Lambertz et al., 2014). Reproduced with permission.

In plant expression system, there are two different routes for cellulase expression which can be directed to different subcellular compartments. One route is where cellulases are transported into vesicles from the Golgi apparatus to the apoplast or vacuole and secreted into the medium. In this route, translation takes place directly into the ER lumen by

ribosomes. In the other pathway, cellulases are transported to the chloroplast or mitochondrion by peptides and protein import mechanisms (Figure 1.7).

1.2.3 Origins of common industrial cellulases

Cellulases are enzymes that have commonly been used in industry for textile processing as well as biorefining. Industrially used enzymes are mainly produced by the genera of bacteria such as *Clostridium* (*Clostridium thermocellum*), *Cellulomonas*, *Thermobifida* (*Thermobifida fusca*), and fungi for example *Trichoderma* (*Trichoderma reesei*) and *Aspergillus* (*Aspergillus niger*). *T.reesei* and *A.niger* can secrete cellulase and cellobiase with a high activity and stability and they can hydrolyse insoluble cellulose substrates such as Sigmacell.

1.2.4 Commercial enzymes used in this study

The most commonly used industrial cellulase is from *Trichoderma* strains (Hawksworth et al., 1983; Gupta et al., 2013). According to Harman et al (2004), 100 types of *Trichoderma* species have been recognized. The strains can be found in different types of soil for example in desert, agricultural, forest, and prairie soils (Domsch et al., 1980; Gupta et al., 2010). *Trichoderma reesei* is the most well-known commercial cellulase used in research and industry. *T.reesei* is a filamentous soft-rot fungus which was found in the Solomon Islands during World War II. The US Army faced a problem at that time in that their cotton materials (such as tents and parachutes) were deteriorating and they found that *T.reesei* was the causes of the problem (Reese et al., 1950; Reese, 1976).

Celluclast® (Sigma Aldrich) comprises endoglucanase, exoglucanase and β -glucosidase. However, β -glucosidase from *T.reesei* is only 0.5% of the total cellulases

produced and is thus inadequate to complete the hydrolysis of cellobiose to glucose. Because of this, β -glucosidase from *Aspergillus niger* is required in the enzymatic saccharification procedure used in chapter 4 (see section 3.1).

1.2.5 β -glucosidases

β -glucosidases are categorised as glycoside hydrolases (GHs) in the (International Union of Biochemistry) IUB Enzyme Nomenclature (1984) taking into account the kind of substrate they catalyse. β -glucosidases have been designated the number EC 3.2.1.21 which characterizes hydrolysis of terminal, non-reducing beta-D-glucosyl with release of glucose.

The CAZY database (<http://www.cazy.org/>) is a database dedicated to providing data on Carbohydrate-Active enzymes, containing genomic, structural and biochemical data. Information in the database includes glycoside hydrolases (GHs), glycosyl transferases (GTs), polysaccharide lyases (PLs), and starch esterases (CEs). A classification system for GHs based on sequence similarity has led to the definition of 85 different families. In the CAZY database, β -glucosidases are classified into families 1, 3, 9, 30 and 116. GH family 1 comprises enzymes with a number of known activities such as beta-glucosidase, beta-galactosidase, 6-phospho-beta-galactosidase, 6-phospho-beta-glucosidase, lactase-phlorizin hydrolase, beta-mannosidase and myrosinase. GH family 3 comprises enzymes with a number of known activities such as beta-xylosidase, N-acetyl beta-glucosaminidase, glucan beta-1,3-glucosidase, cellodextrinase, exo-1,3-1,4-glucanase. GH family 9 consists of enzymes such as endoglucanase and cellobiohydrolase. GH family 30 consists of only one enzyme which is mammalian glucosylceramidase. Human acid beta-glucosidase (D-glucosyl-N-acylsphingosine glucosylhydrolase) cleaves the glucosidic bonds of glucosylceramide and synthetic beta-

glucosides. GH family 116 consists of enzymes such as beta-xylosidase, acid beta-glucosidase/beta-glucosylceramidase and beta-N-acetylglucosaminidase.

Bacterial, archaeal, plant, animal, and some fungal β -glucosidases have been categorised in family 1 (Cantarel et al., 2009).

1.2.6 β -glucosidases from Novozymes

Commercial β -glucosidase has been used widely in the hydrolysis of lignocellulose biomass. According to Chauve et al (2010), the optimum temperature for Novozyme 188 is 65°C. In terms of pH activity, β -glucosidase from Novozyme has an optimum pH of 4.3 to 4.5 (Bravo et al., 2001; Calsavara et al., 2001; Chauve et al., 2010). Apart from Novozyme 188, Cellic CTec2 is another enzyme product from Novozyme which contains a mixture of cellulases, including more β -glucosidase than Celluclast[®] and it can be operated at temperatures of 45 to 50°C and pH 5.0 -5.5.

1.2.7 Cellulases from extremophiles

One option for isolating cellulases that can operate at extreme conditions of temperature, pH, or even salt content is to find cellulases from organisms that live in these types of environments (also called extremophiles). Because cellulases are enzymes that are secreted into the environment, they must have adequate stability to facilitate the growth of the organism in the environment they live in.

There are many examples of cellulases from extremophiles in the literature and often they are more stable than those isolated from other sources. In particular, to identify enzymes that are stable in ILs, one might look for enzymes from halophilic or halotolerant organisms. Recombinant enzymes from *Haloferax volcanii* showed high

stability to heat, alkali and ILs in the presence of salts and because of this the researchers suggested that the enzyme was stable in the presence of salt and consider it as halophilic enzyme (Zhang et al., 2011). A study conducted by Raddadi et al (2013) stated that over 90% and 76% of carboxymethyl cellulase activity from *Paenibacillus tarimensis* can be detected in the presence of 20% (v/v) [EMIM][OAc] and [BMIM]Cl at 50 and 80 °C. When the reactions were carried out in a high salt contents (5M NaCl), at 50 or 80°C, more than 80% and 76% activity is maintained in the presence of 20% [EMIM][OAc] and [BMIM]Cl. These results indicated that cellulases were active in the presence of ILs and a high tolerance was observed in 20% (v/v) IL. The presence of acidic amino acids resulting in a protein with an overall negative surface allows interaction with a hydrated salt cations forming a hydration shell which allows the protein to be resistant to heat, alkali and ILs as occurred in a salt-dependent enzyme known as Hu-CBH1 identified from *Halorhabdus* (Datta et al., 2010). So far, only a few halophilic cellulases have been identified. A study conducted by Trivedi et al (2013) stated that cellulase from *Pseudoalteromonas* sp. had 88.45% remaining activity in the presence of 20% NaCl. Raddadi (Raddadi et al., 2013) studied the enzyme from *P. tarimensis* and showed that at pH 7, CMCase activity can be detected in solutions containing up to 2M KCl, 1.7M NaCl and 5% (v/v) of organic solvents such as ethanol, methanol, isopropanol, acetone, hexane or DMSO and 10 mM EDTA, CoCl₂, MgCl₂, CaCl₂, MnCl₂, BaCl₂, NiCl₂ or FeCl₂. On the other hand, the presences of 10 mM CuSO₄, AgNO₃ or ZnSO₄ were inhibitors of CMCase activity.

1.2.8 Measurement of cellulose hydrolysis activity

The physical diversity of cellulosic substrates and the complexity of multiprotein cellulase systems produced by different microorganisms have led to the development of assay procedures for the measurement of cellulase activities. In 1984, the International Union of

Pure and applied Chemistry (IUPAC) Commission on Biotechnology published standard assay procedures for measuring cellulase activities (Sharrock, 1988; Bhat and Bhat, 1997). Some of these recommendations have been accepted by biotechnologists, but enzymologists think that these procedures are restrictive and not suitable for understanding the detailed mechanism of action and substrate specificities of cellulases. Consequently, Wood and Bhat (Wood and Bhat, 1988) reviewed the cellulase assays used by laboratories working on *Trichoderma* cellulases and emphasised the advantages and disadvantages of the procedures.

The main technical use of cellulases is the conversion of cellulosic fractions into glucose. Filter paper, microcrystalline cellulose (for example Sigmacell), cotton fibers and soluble carboxymethyl cellulose are the most widely used substrates for total activity.

The filter paper hydrolysis method determines the total cellulase activity in units of micromoles of reducing sugar (measured as glucose) liberated per one minute under standard assay conditions. The units of measurement this activity are filter paper units (FPU), or international Units (IU). While quantifying the cellulase hydrolysis from different organisms, important properties such as pH and heat stability, specific molecular inhibitors and activators, adsorption of enzyme onto substrate, and transglycosylation reactions must be considered. Transglycosylation reactions are usually the side reactions leading to the formation of unwanted reaction by-products. The adsorption capacity of cellulases is extremely important factor to consider when examining potential biotechnological applications of the enzymes to the hydrolysis of cellulose. The ability of cellulase to hydrolyse cellulose is directly related to its adsorption capacity. The tighter the adsorption of cellulase to crystalline cellulose, the higher the rate of reaction and the yield of glucose (Klyosov, 1990; Kawaguchi et al., 1996; Janne Lehtio, 2003). If a cellulase binds weakly to crystalline cellulose, the rate of hydrolysis decreases, irrespective of the amount of enzyme used and the time given for reaction (Fan et al., 1980; Goto et al., 1992; Janne Lehtio, 2003).

Enzymatic saccharification of crystalline cellulose needs a carbohydrate binding module (CBM) that attaches the catalytic segments on the surface of the substrate. In fungal cellulases, there are two domain structures which are the catalytic domain and cellulose binding domain. However, not all cellulases have a cellulose binding domain (Morana et al., 2011)

1.2.9 Methods for detecting cellulase activity

One of the methods used to measure cellulase activity is cellulose-azure. Cellulose-azure contains insoluble cellodextrins coupled with water-soluble reactive dyestuffs. The activity of cellulose azure was measured in terms of dye released per unit time or by using a standard curve of cellulose azure degrading activity versus activity in international filter paper units (IFPU). The hydrolytic activity in a sample is proportional to the amount of dye released. However, the size of the cellulose-azure particles is uneven which can increase the release of dye due to the particle attrition when applied to a consistent concentration of cellulase. The use of cellulose-azure to measure the activity of cellulase is easy and fast but the cost of cellulose-azure is expensive, which not a good option if a large amount of samples are required to examine the cellulase activity (Lai et al., 2006).

The DNS Assay is another technique used to determine the activity of cellulase by using 3,5-dinitrosalicylic acid reagent. CMC, filter paper and Sigmacell are used as the substrate and the analysis of reducing sugar (RS) release is determined using a spectrophotometer. The reducing groups react with 3,5-dinitrosalicylic acid and then from this reaction 3-amino-5-nitrosalicylic acid, a coloured product that absorbs light at 540 nm is formed. The units of enzyme activity are expressed as micromoles of reducing sugar released per minute per milligram of protein (Ilmberger and Streit, 2012). The DNS Assay

is a very fast method to measure the cellulase activity but this method is not able to detect glucose directly.

Another popular method to detect glucose is by using high-performance liquid chromatography (HPLC). A standard curve of carbohydrate monomers can be prepared to determine the amount of glucose present from the reaction between cellulase and the substrates. The advantage of using this method is that glucose can be detected directly, but the time required to quantify the glucose is longer than the above methods.

1.3 Pretreatment process

Bioconversion of lignocellulosic biomass requires a pretreatment step in order to separate carbohydrates and lignin. There are many choices for pretreatment processes namely dilute acid, concentrated acid, ammonia fibre expansion (AFEX), lime, organosolv and ionic liquids (Brandt et al., 2011). Pretreatment is very important in biofuel production as the type of pretreatment has significant impacts on the subsequent enzymatic hydrolysis. In addition, an efficient method of pretreatment can help reduce the cost of biofuel production (Mabee et al., 2011; Brandt et al., 2011).

1.3.1 Steam explosion pretreatment

In steam explosion pretreatment, high pressure steam is used and then this steam will absorb in the lignocellulose matrix which then separates the components of a biomass (Kumar & Murthy, 2011). The advantages of using steam explosion pretreatment is an increase of the surface area of cellulose, the removal of hemicellulose and the transformation of lignin which assist in enhancing the hydrolysis process of biomass with the enzymes (Singh et al., 2015).

Although the steam pretreatment method has its advantages, there are also disadvantages such as that the low yield of hemicellulose sugar and lignin cannot be separated perfectly and produced degradation of products such as furfural, HMF and soluble phenolic compounds that can prevent the growth of microbes (Chiaramonti et al., 2012; Martin-Sampedro et al., 2011; Jorgensen et al., 2007).

1.3.2 Using Ionic Liquids for biomass pretreatment

Ionic liquids (ILs) have been studied for pretreatment of lignocellulosic biomass. ILs are a class of salts that are liquid at temperatures below 100 °C. The use of ILs in the process of pretreatment is to remove the physical and chemical barriers which prevent enzymatic hydrolysis of the cellulose to sugar.

The solubility of lignocellulose in ILs has been described in various hydrogen-bond basic ILs, proposing that ILs which are cellulose solvents can be used for lignocellulose processing (Fort et al., 2007; Lee et al., 2009). IL pretreatment can be divided into two types. The first one is to obtain regenerated cellulose in which the whole of the biomass was dissolved and then cellulose selectively precipitated with water. These regenerated celluloses from ILs are less crystalline and much more accessible by cellulases than untreated substrates, which results in improved cellulose hydrolysis rates (Zhao, Baker, & Cowins, 2009). The second option is a pretreatment that alters or removes lignin from the lignocellulosic materials. After recovery the separate lignin fraction can be converted to aromatic, value-added chemicals (Brandt et al., 2011) and the cellulose fraction turned into sugars by cellulase treatment.

The objective of the pretreatment process is to make the surface of the cellulose after the pretreatment amorphous. Thus, enzymatic hydrolysis of this material becomes more effective and it helps to increase the amount of sugars compared to the untreated material (Dadi et al., 2007). However, enzymes can be deactivated by residual IL left behind in the

recovered cellulose (Turner et al., 2003) probably due to protein unfolding or inhibition (Bose et al., 2010). ILs frequently induce enzyme conformational changes which then cause inactivation and in some cases, the enzymes will not dissolve in the ILs, leading to a bi- (or tri-) phasic system (Lau et al., 2004; Sheldon et al., 2002).

1.3.2.1 Types of ionic liquids used for biomass dissolution and cellulose regeneration

[EMIM][OAc] and [BMIM]Cl are ILs used in the pretreatment process of biomass. These ILs had been studied extensively by researchers to dissolve biomass and then regenerate the cellulose by precipitation (Da Costa Lopes et al., 2013). Table 1.2 describes the use of these ILs in the pretreatment process.

Table 1.2: The conditions required in biomass pretreatment using [EMIM][OAc] and [BMIM][Cl]

	ILs	Explanation
1	[EMIM][OAc]	[Emim][OAc] is used in biomass pretreatment to solubilise lignocellulose. Then, cellulose was obtained by filtration after precipitation occurred. The molarity used was 0.594 mol. The temperature for IL pretreatment using [Emim][OAc] is 120 °C for 12 h (Brandt et al., 2013).
2	[BMIM][Cl]	[Bmim][Cl] is used in biomass pretreatment. The process is similar to that of [Emim][OAc] where dissolution process occurred. [Bmim][Cl] was used together with a strong acid such as H ₂ SO ₄ . This IL solubilised cellulose and hemicellulose. Then cellulose was precipitated upon dilution with water. The molarity used was 0.594. The temperature for IL pretreatment using [Bmim][Cl] was 120 °C for 12 h (Brandt et al., 2013).

1.3.2.2 Types of ionic liquids used for selective dissolution of lignin

[HBIM][HSO₄] is a type of IL which can separate the lignin from the biomass (Brandt et al., 2012). Sulphate and phosphate based ILs can separate the lignin from the lignocellulosic biomass by performing acid hydrolysis of the lignin. Table 1.3 describes the use of these ILs in the pretreatment process.

Table 1.3: The conditions required in biomass pretreatment using sulphate and phosphate based ILs

	ILs	Explanation
1	[BMIM][HSO ₄]	[Bmim][HSO ₄] is used to separate cellulose and hemicellulose from lignin. This IL can dissolve lignin and it is good for softwood as it contain higher amount of lignin. The temperature used for pretreatment was 120°C for 10 h. The molarity used was 0.682 mol. [Bmim][HSO ₄] reacts with the hydroxyl group in lignocellulose and bind with lignocellulose through covalent bonds and coulombic forces (Brandt et al., 2013).
2	[HBIM][HSO ₄]	[Hbim][HSO ₄] is used to separate cellulose and hemicellulose from lignin. It was observed that this IL is a good solvent for lignin separation and there was an improvement in term of cellulose digestibility. The molarity used was 0.682 mol. The temperature used for pretreatment was 120°C for 22 h. In an imidazolium-based IL, the cation and the anion interact via H-bonding. [HC4im] cation forms stronger H-bonding to the [HSO ₄] ⁻ anion because of the availability of the strong H-bonded site as the [HC4im] ⁺ cation has an acidic N-H proton. As a result, there is a strong H-bonding within the [HC4im][HSO ₄] which can prevent the [HSO ₄] ⁻ anion to form H-bonding with the protons of the 4-nitroaniline. Because of this, the B value will decrease which then decrease the ability of [HC4im][HSO ₄] to swell or dissolve the cellulose (Shikh Zahari SMSN (2015).
3	[TEA][HSO ₄]	Pretreatment was conducted at 150°C for 1 hour. This IL is cost effective as the price is cheaper than the imidazolium

		based ILs. The reaction of this IL with lignocellulose is similar with [HBIM][HSO ₄] (Chen et al., 2014).
4	[HBIM][H ₂ PO ₄]	Pretreatment was conducted at 160°C, 42 h. The molarity used was 1.2 mol. The reaction of this IL with lignocellulose is similar with [HBIM][HSO ₄] but a moderate anion [H ₂ PO ₄] ⁻ was chosen because there was a decrease in enzyme activity when using acidic anion [HSO ₄] ⁻ (Li, 2013).
5	[BMIM][H ₂ PO ₄]	Pretreatment was conducted at 160°C, 42 h. The molarity used was 1.2 mol. The reaction of this IL with lignocellulose is similar with [HBIM][H ₂ PO ₄] but the difference is the length of the alkyl chain of the cation (Li, 2013).

1.4 Activity of cellulases in ILs

The interest in IL pretreatment has led to a number of studies on the effects of different ILs on subsequent hydrolysis reactions. As salts, ILs could be expected to have an effect on the activity and/or stability of cellulase enzymes. Due to differences in the IL physicochemical properties and sequence and structure of the enzymes, most studies have made empirical observations of the effects of a handful of ILs on the activity of one or more enzymes under study. To date, there is no unifying theory about the effects of ILs on enzyme stability generally. Table 1.4 provides some examples of previous research on the stability of enzymes in ionic liquids. Many of the investigated cellulases lost activity drastically in the presence of ILs (Pottkämper et al. 2009, Turner et al. 2003).

Table 1.4: Cellulases activities in the presence of ILs

Cellulase	Specific activity (U/mg)	Relative activity (%) in (vol/vol%) IL	Organism	Reference
Cel5A	294	65 in 20% [BMIM]Cl	<i>Thermoanaerobacter tengcongensis</i>	(Liang et al.2011)
Celluclast (Novozyme)	0.096	40 in 10% [MMIM] [DMP]	<i>Trichoderma reesei</i>	Engel et al. 2010
T.viride	11	40 in 5% [EMIM][OAc]	<i>Trichoderma viride</i>	(Datta et al.2010)
Tma Cel5A	30	40 in 20% [EMIM][OAc]	<i>Thermotoga maritima</i>	(Datta et al.2010)
Pho EG	1.9	95 in 20% [EMIM][OAc]	<i>Pyrococcus horikoshii</i>	(Datta et al.2010)
Cellulase preparation	n/a	n/a	<i>Penicillium janthinnellum</i>	(Adsul 2009)
CelA10	2.4	74 in 30% [BMPY][OTf]	Metagenome	(Pottkamper et al.2009)
UniMAP AA-6	n/a	50 in 20% (v/v) [BMIM]Cl	<i>A. terreus</i>	(Gunny et al.2014)

Metagenomics is the study of the genomes of all microbes living in a particular habitat. Metagenomic libraries can be formed by extracting total microbial DNA of the chosen habitat and then ligated into appropriate vectors and cloned into a suitable host. A study investigating metagenomic cellulases from mesophilic habitats showed that most of the discovered enzymes lost their activity completely in the presence of 30% (vol/vol) IL, but one enzyme showed moderate activity in the presence of different ILs (Pottkämper et al. 2009). Nevertheless, the thermostable cellulase from *Thermoanaerobacter tengcongensis* MB4 still

showed 65% activity in 1.0 M (approx. 20% (vol/vol) [BMIM]Cl (Liang et al. 2011). A thermophilic enzyme from *Pyrococcus horikoshii* was very active in the presence of 1-ethyl-3-methylimidazolium acetate [EMIM][OAc], and it showed 95% relative activity in 20% (vol/vol) IL and 79% relative activity after 15 hours in 15% (vol/vol) IL (Datta et al. 2010). Moreover, a marine microorganism known as *A. terreus* showed 50% activity in 20% (v/v) [BMIM]Cl. Altogether, the industrial need for novel cellulases which tolerate specific conditions, i.e. the presence of high IL concentrations, and especially for those that are active over a long time period in the presence of ILs is rising.

1.5 Protein stability in IL

Alkaline phosphatase was the first enzyme studied by Magnuson in order to understand the stability of this enzyme in ethylammonium nitrate (Magnuson et al., 1984). According to Kragl (Kragl et al., 2002), lipases are among the enzymes which have been reported to be active in ILs. Cations and anions can be categorized according to the Hofmeister series depending on their ability to promote or destroy water structure. In general, the kosmotropic anions stabilize proteins and chaotropic cations may destabilize protein.

However, in a study conducted by Zhao, the enzymes were stabilized by kosmotropic anions and chaotropic cations but destabilized by chaotropic anions and kosmotropic cations. These experimental results are in contrast with general theory (Zhao et al., 2006). The enzyme inactivation rates and inactivation reversibility or irreversibility have been proposed to be mostly anion dependent (Kaar, et al. 2003).

The important of understanding enzyme deactivation in ILs is to find a solution that can be done to improve the activity of enzyme with the present of small amount of IL after biomass pretreatment. The aim is to find a cellulase compatible with ILs so that this cellulase enables the efficiency of biomass deconstruction using IL and more importantly it will reduce the overall cost of biomass processing.

1.6 Objectives

The objectives of the research presented in this thesis were:

1. To study the effect of different ILs on the activity of a commercial cellulase mixture (*T. reesei* cellulase mix) and Novozyme 188 (β -glucosidase) using Sigmacell as a model substrate.
2. To identify the physicochemical causes of the loss of glucose yield in the presence of ILs.
3. To clone and overexpress an endoglucanase from *Cryptococcus sp.* S-2 identified in the literature as acid-stable and study its activity in the presence of ILs.
4. To overexpress β -glucosidase from *Thermofilum pendens* identified in the literature as acidophilic and study its activity in the presence of ILs.

1.7 Outline of the thesis

A general introduction to this research study and a review of the literature is presented in Chapter 1 which starts with a background of biorefinery and lignocellulosic biomass, IL pretreatment, origin of cellulase and cellulase stability in ILs.

The experimental methods of the thesis are presented in chapter 2. Chapter 3 presents a study on the effect of ILs on the activity of commercial cellulase mixture (Celluclast®, a *T.reesei* cellulase mix) and Novozyme 188 (β -glucosidase) and this chapter suggests an explanation for the physicochemical effect of ILs on cellulase activity.

Chapter 4 discusses the results of the cloning and expression of endoglucanase from *Cryptococcus sp* S-2. In this chapter, the effect of ILs on the activity of endoglucanase is presented.

In chapter 5, activity of β -glucosidase from *Thermofilum pendens* and the effect of ILs on the activity of this β -glucosidase are discussed. A combined assay using both acidophilic enzymes cloned as part of this thesis is also presented in Chapter 5.

CHAPTER 2: MATERIALS AND METHODS

All experiments and solutions were prepared using high quality water produced by a Millipore Milli-RO.

Super Broth (1 Litre)

35 g Tryptone
20 g Yeast extract
5 g NaCl
2.5 ml 2N NaOH

Terrific Broth (1 Litre)

Tryptone, 12 g
Yeast extract, 24 g
Glycerol 4 ml
After autoclaving the media, 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4 was added in the media.

Phosphate-Buffered Saline (PBS)

137mM NaCl, 2.7mM KCl, 2mM KH_2PO_4 , 10mM Na_2HPO_4

Phosphate-Buffered Saline containing NaCl and MgCl_2 (PBS/NaCl)

As PBS, but with the addition of an additional 0.5M NaCl and 1mM MgCl

Polyacrylamide Gel electrophoresis (PAGE)

SDS-PAGE loading buffer (5x stock)

50% (v/v) glycerol, 60mM Tris-HCl pH 6.8, 10% SDS, 200mM β -mercaptoethanol, 25mg bromophenol blue.

SDS-PAGE Running Buffer

25mM Tris base, 250mM glycine, 0.1% SDS.

12 % Separating gel

3.3ml H_2O , 4ml 30% Bis/Acrylamide, 2.5ml 1.5 M Tris/HCl (pH8.8), 100 μ l 100% SDS, 100 μ l 10% Ammonium persulfate (APS), 4 μ l TEMED.

5 % Stacking gel

3.4ml H₂O, 833μl 30% Bis/Acrylamide, 630μl 0.5M Tris/HCl (pH 6.8), 50μl 10% SDS, 50μl 10% APS, 5μl TEMED.

SDS-PAGE Coomassie stain

40% methanol, 10% acetic acid, 0.25% Coomassie brilliant blue (R-250).

Western Blot Transfer Buffer

48mM Tris base, 39mM glycine, 0.04% (w/v) SDS, 20 % (v/v) methanol.

Lysis buffer

50 mM NaH₂PO₄
300 mM NaCl
10 mM imidazole

Wash buffer

300 mM NaCl
20 mM Imidazole
pH 8.0

Elution buffer

20 mM Phosphate buffer
300 mM NaCl
500 mM Imidazole
pH 8.0

2.1 Enzymatic saccharification

The enzymatic saccharification was performed according to LAP “Enzymatic saccharification of lignocellulosic biomass” (NREL/TP-510-42629), which can be accessed from <http://www.nrel.gov/biomass/pdfs/42629.pdf>. 100 mg of Sigmacell was added to a 15 ml Sterilin tube. Then, 5 ml of 0.1M citrate buffer (pH 4.8), antibiotics (30 μ l kanamycin and 40 μ l tetracycline) and distilled water were mixed to a final volume of 10 ml as described in the standard protocol. For each Sterilin tube, suitable volume of the cellulase enzyme was added equal to nearly 60 FPU/g cellulose and volume of β -glucosidase enzyme equal to 64 p NPGU/g cellulose. Commercial enzyme preparations used in this study were 60 μ l Celluclast[®] (*T. reesei* cellulase mix) and 60 μ l Novozyme 188 (β -glucosidase, both from Sigma). The reactants were incubated at 50°C for 1 hour and the pH of the samples was measured. Subsequently, the samples were placed in the incubator at 50°C for up to 96 hours. The standard protocol was modified in order to add the IL with different concentrations (1, 2, 5, 10% (v/v)). Table 2.1 and 2.2 shows different ILs used in this study.

Table 2.1: List of ionic liquids

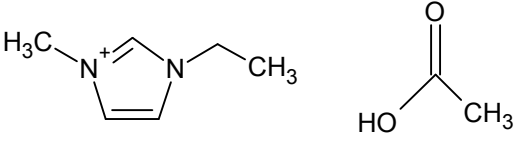
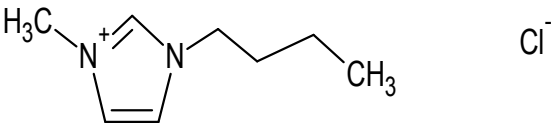
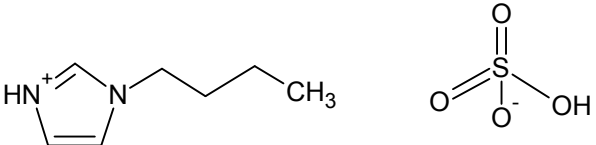
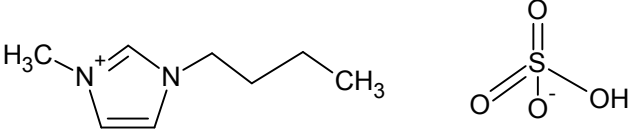
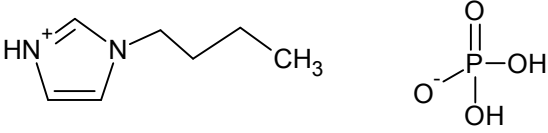
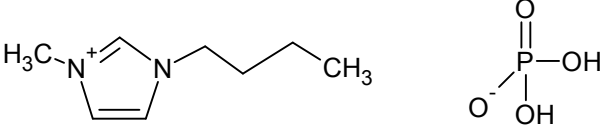
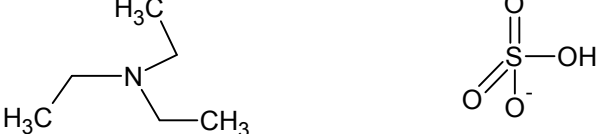
Ionic Liquid	Chemical structures
1-ethyl-3-methylimidazolium acetate[EMIM][OAc]	 The cation is 1-ethyl-3-methylimidazolium, consisting of an imidazole ring with a methyl group at position 3 and an ethyl group at position 1, with a positive charge on the nitrogen at position 1. The anion is acetate, consisting of a methyl group bonded to a carbonyl group, which is also bonded to a hydroxyl group.
1-butyl-3-methylimidazolium chloride[BMIM] Cl	 The cation is 1-butyl-3-methylimidazolium, consisting of an imidazole ring with a methyl group at position 3 and a butyl group at position 1, with a positive charge on the nitrogen at position 1. The anion is chloride, represented as Cl⁻.
1-butyliimidazolium hydrogen sulphate [HBIM][HSO₄]	 The cation is 1-butyliimidazolium, consisting of an imidazole ring with a hydrogen atom at position 3 and a butyl group at position 1, with a positive charge on the nitrogen at position 1. The anion is hydrogen sulfate, consisting of a central sulfur atom double-bonded to one oxygen and single-bonded to three others, one of which is a hydroxyl group and the other two are negatively charged.
1-butyl-3-methylimidazolium hydrogen sulphate [BMIM][HSO₄]	 The cation is 1-butyl-3-methylimidazolium, consisting of an imidazole ring with a methyl group at position 3 and a butyl group at position 1, with a positive charge on the nitrogen at position 1. The anion is hydrogen sulfate, consisting of a central sulfur atom double-bonded to one oxygen and single-bonded to three others, one of which is a hydroxyl group and the other two are negatively charged.
1-butyliimidazolium dihydrogen phosphate [HBIM][H₂PO₄]	 The cation is 1-butyliimidazolium, consisting of an imidazole ring with a hydrogen atom at position 3 and a butyl group at position 1, with a positive charge on the nitrogen at position 1. The anion is dihydrogen phosphate, consisting of a central phosphorus atom double-bonded to one oxygen and single-bonded to three others, one of which is a hydroxyl group and the other two are negatively charged.
1-butyl-3-methyl-imidazolium dihydrogen phosphate [BMIM][H₂PO₄]	 The cation is 1-butyl-3-methylimidazolium, consisting of an imidazole ring with a methyl group at position 3 and a butyl group at position 1, with a positive charge on the nitrogen at position 1. The anion is dihydrogen phosphate, consisting of a central phosphorus atom double-bonded to one oxygen and single-bonded to three others, one of which is a hydroxyl group and the other two are negatively charged.
Triethylammonium hydrogen sulfate [TEA][HSO₄]	 The cation is triethylammonium, consisting of a central nitrogen atom with a positive charge bonded to three ethyl groups. The anion is hydrogen sulfate, consisting of a central sulfur atom double-bonded to one oxygen and single-bonded to three others, one of which is a hydroxyl group and the other two are negatively charged.

Table 2.2: Source of ILS

	ILs	Source of ILs
1	[EMIM][OAc]	Commercially synthesised by BASF
2	[BMIM][Cl]	Obtained from Welton's lab
3	[BMIM][HSO ₄]	Obtained from Welton's lab
4	[HBIM][HSO ₄]	Synthesised in the lab
5	[TEA][HSO ₄]	Obtained from Hallett's lab
6	[HBIM][H ₂ PO ₄]	Obtained from Welton's lab
7	[BMIM][H ₂ PO ₄]	Obtained from Welton's lab

2.2 Enzymatic assays

2.2.1 HPLC detection of released glucose

Glucose release was analysed on a Jasco HPLC system with a refractive index detector equipped with an Aminex HPX-87H column (Biorad). The mobile phase was 10 mM sulfuric acid, the column oven temperature 35°C, the flowrate 0.6 ml/min and the acquisition time 15 minutes. Glucose yields were then calculated. The mobile phase for phosphate based ILs was 10mM phosphoric acid and was analysed on Shimadzu HPLC with the same column as mentioned above. The ratio of biomass to IL pretreatment was 1:10

2.2.1.1 Preparation of glucose standards

A glucose standard was prepared in order to get the standard curve. 4 different concentrations of glucose used were 1, 2, 5 and 10 mg/ml. Moreover, glucose standards with the addition of ILs were also prepared.

2.2.2 Enzyme Assay (3,5-Dinitrosalicylic Acid (DNSA) reagent)

The standard assay mixture contained 1 ml of 1% carboxymethylcellulose (CMC), 3 ml of 3,5-Dinitrosalicylic acid (DNS or DNSA), 1 ml 0.1 M Citrate buffer at pH 4.8, 1 ml of diluted enzyme and 1 ml of potassium sodium tartrate. A glucose standard was measured in order to get the standard curve. Different volumes of 10 mg/ml glucose solution were taken and diluted to a final volume of 1 ml as shown in Table 2.3 below:

Table 2.3: Concentrations of glucose for DNSA Assay standard curve

No	Glucose (ml)	Distilled water (ml)	Concentration of glucose (ml)
1	0.0	0.5	0.0
2	0.1	0.4	0.2
3	0.2	0.3	0.4
4	0.3	0.2	0.6
5	0.4	0.1	0.8
6	0.5	0.0	1.0

The enzymatic activity volume was calculated according to the following formula (Ilmberger and Streit, 2010):

$$U/mL = (\text{Absorbance}/\text{min} \times V) / (e \times d \times v) \dots\dots\dots 2.1$$

V = volume of the test reaction mix.

d = thickness of the cuvette [cm].

e = ascendant of straight calibration line.

v = sample volume.

$$\text{Specific activity [U/mg protein]} = \text{enzymatic activity volume[U/mL]}/\text{protein concentration [mg/mL]} \dots\dots\dots 2.2$$

2.2.3 p-nitrophenyl-beta-D-glucoside (pNPG) Assay

The standard assay mixture contained 1 ml of 50mM citrate buffer, 1 mM of pNPG and 200 μ l enzyme solution. First, the substrate, pNPG was incubated at 90°C for 5 minutes, and then 0.2 ml of the enzyme (β -glucosidase) was added and incubated for 20 minutes at 90°C. Then, 1 ml of 100mM NaOH was added to stop the reaction. The solutions were placed in a 96-well

plate and the absorbance was measured at 430 nm using the Synergy HT reader (Park et al., 2007). A solution of p-nitrophenol (PNP) was measured in order to get a standard curve. 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml of PNP were prepared. 0.2 ml for each solution was mixed with 0.6 ml of 1M Na₂CO₃. The absorbance of the sample was measured at 410nm. Then, a graph was plotted between absorbance and concentration of PNP. The slope of the curve was calculated and used for estimating the β -glucosidase activity.

2.3 Molecular Cloning Techniques

2.3.1 Bacterial strains, plasmids and primers

The *E.coli* strains, bacterial plasmids and primers used are listed in Table 2.4, 2.5 and 2.6

Table 2.4: Bacterial strains

<i>E.coli</i> strain	Use
TOP10 competent cell	Cloning
BL21 (DE3)	Protein expression
Rosetta	Protein expression
Shuffle T7	Protein expression

Table 2.5: Bacterial plasmids and manufacturer

Plasmid	Manufacturer
pRSET A	Life Technologies
pUC18	Control
pTPEN1494*	DNA 2.0

*This gene sequence was obtained from the literature (Li et al., 2013) and the backbone was from DNA 2.0

Table 2.6: Forward and reverse primer

Forward primer with restriction site BamHI	5' GATTAGGATCCATGAAAACAGCTACCTTGTTG 3'
Reverse primer with restriction site HindIII	5' CGTTGGAAGCTTCAAGGTAGATTTGGCTTTAC 3'

2.3.2 PCR reaction

Oligonucleotides were purchased from IDT and PCR reactions were carried out using Phusion® High-Fidelity DNA Polymerase (New England Biolabs). Table 2.7 shows the amounts and reagents used.

Table 2.7: Composition of a standard PCR reaction

Components	50 µl Reaction
Nuclease-free water	To 50 µl
5X Phusion HF	10 µl
10 mM dNTPs	1 µl
10 µM Forward Primer	2.5 µl
10 µM Reverse Primer	2.5 µl
Template DNA	10 ng
Phusion DNA Polymerase	0.5 µl

PCR was run according to the program below:

Initial denaturation: 98°C for 2 minutes

98°C for 30s (Denaturation)

60°C for 30s (Annealing of primers)

72°C for 2 minutes (Extension)

30 cycles

Final polishing step: 72°C for 5 minutes

Hold at 4°C

2.3.3 Plasmid DNA extraction

Plasmid DNA was purified using the Qiagen QIAprep kit according to the manufacturers protocol, based on an alkaline lysis method. Briefly, the bacterial pellet was lysed in alkaline buffer and the solution neutralised to precipitate cell debris. The DNA was bound to a silica-based resin column and washed with an ethanol-containing buffer to remove genomic DNA, RNA and other contaminants. Plasmid DNA was eluted using the buffer provided or sterile water.

2.3.4 Measuring DNA concentration and purity

The absorbance of the sample DNA was measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). This device allowed accurate determination of concentration and purity. Basically 1 µl of neat sample was pipetted onto the receiving fibre; avoiding dilutions, which can introduce error. The sample absorption at 260nm and 280nm was measured. A reading of 1.0 at 260nm corresponds to 50 µg/ml of DNA.

2.3.5 Restriction digests of plasmid DNA

All digests were carried out in the buffer according to the manufacturer's recommendations. Digests were incubated at 37°C for a minimum of 1 hour. If the digested products (vector) were to be subsequently used in ligation reaction, 1 unit of Alkaline Phosphatase was added and incubated for 10 minutes at 37°C. This was to prevent the DNA from re-circularising. The reaction was stopped by heating to 65°C. The DNA bands were excised from agarose gel with a sharp knife and purified using a gel purification kit (Qiagen) based on the

manufacturer's instructions. The resulting DNA was eluted with 50 μ l of elution buffer provided with the kit.

2.3.6 Ligation of DNA

Ligations were carried out in the buffer supplied by the manufacturer with total volume 20 μ l. T4 DNA ligase has been used for the ligation process. The molar ratio of linearised plasmid DNA: insert DNA were 3:1 (typically 50-100ng plasmid DNA). Reactions were incubated at room temperature for 1-2 hours or overnight at 4°C prior to transformation into competent *E. coli* cells. Table 2.7 shows the components and the amounts of ligation reaction (New England Biolabs):

Table 2.8: The components and the amounts of ligation reaction

Components	20 μ l Reaction
10X T4 DNA Ligase Buffer	2 μ l
Vector DNA	50 ng
Insert DNA	37.5 ng
Nuclease-free water	To 20 μ l
T4 DNA Ligase	1 μ l

2.3.7 Agarose gel electrophoresis of DNA

A 1% agarose solution (w/v) was prepared in TAE buffer, heated to melt the agarose and cooled slightly before the addition of GelRed to a concentration of 1 μ g/ml. The gel was cast in a gel system and allowed to set. The tank was filled with 1 x TAE buffer and DNA samples loaded into gel wells by the addition of DNA loading buffer. Gels were separated at approximately 100V and subsequent visualisation of the DNA was achieved by exposure to a non-UV blue light box.

2.3.8 Plasmid transformation (Chemically-competent *E.coli*)

One tube of chemically competent cells (see Table 2.4) per transformation required was allowed to thaw on ice. Between 10-100ng plasmid DNA was added to each tube, the cells mixed by gentle agitation with the pipette. The cells were then incubated on ice for 30 minutes. Cells were heat-shocked for 30 seconds in a 42°C water bath and returned to ice for 5 minutes. Then 950µl of pre-warmed SOC media was added followed by incubation for 1 hour at 37°C with shaking at 250 rpm in an orbital shaker. Typically, 10, 50,100 and 200 µl of the transformed cell culture was plated on LB Agar with an appropriate antibiotic with concentrations as shown in table below:

Table 2.9: Antibiotics used in this research

Antibiotic	Stock concentration	Working concentration
Ampicillin	50 mg/ml	50 µg/ml
Kanamycin	50 mg/ml	50 µg/ml

2.3.9 Plasmid transformation (Electrocompetent *E.coli*)

One tube of chemically competent cells (see Table 2.4) per transformation required was allowed to thaw on ice. Between 1-50ng plasmid DNA was added to each tube and placed on ice for approximately 10 minutes. Cells were transferred to a chilled electroporation cuvette (0.2cm electrode gap) and electroporated in a BioRad GenePulser Plus at the following settings:

Voltage: 2.5 kV

Resistance: 200 Ω

Immediately following electroporation, 1ml of pre-warmed LB media was added to the cuvette and the cells were transferred into a sterile tube. Cells were allowed to recover at

37°C for 1 hour with 250 rpm shaking in an orbital shaker before plating on agar plates containing the appropriate antibiotic. Plates were incubated inverted at 37°C overnight to obtain single colonies.

2.3.10 Protein expression in *E.coli*

2.3.10.1 Small-scale expression

The expression strain (see table 2.4) was transformed with the DNA construct and a single colony used to inoculate an overnight 5ml starter culture in LB medium containing appropriate antibiotic. This culture was centrifuged at 4000 rpm for 10 minutes and the bacterial pellet resuspended in fresh LB medium. The resuspended cells were then used to seed a 50ml culture at 1:100 ratio, in a sterile 250ml conical flask with LB, 2 x YT, superbroth or terrific broth medium (see Appendix I) and grown at 37°C with 250rpm shaking until an optical density (600nm) of 0.6-0.9 was reached. Following this, IPTG (Isopropyl- β -D-thio-galactopyranoside) was added to a final concentration of 0.4mM. This was to allow induction of protein expression. The cultures were grown at 30°C overnight with 250rpm shaking to allow expression of the protein of interest.

2.3.10.2 Large-scale expression

Larger scale expression was achieved by seeding a 500ml culture of LB, 2YT, superbroth and terrific broth medium in a sterile 2L conical flask and continuing the steps for small-scale inductions until the point of induction. The cells were then induced with IPTG and grown at 30°C overnight with 250rpm shaking to allow expression of the protein of interest. Then,

culture supernatant was harvested by centrifugation at 4000rpm in a Sorvall rotor for 20 minutes. Cell pellet was resuspended in PBS and sonicator was used to lyse the cell. Sonicator amplitude setting was 45% and sonication pulse rate was set up for 20 seconds ON and 20 seconds OFF. The lysate was centrifuged at 4000rpm for 10 minutes to separate the soluble and insoluble fractions.

2.3.11 Protein purification (Ni-NTA)

Protein purification was conducted using Ni-NTA agarose resin (Qiagen). The resins are supplied as 50% slurries. Three types of buffers were used lysis, wash and elution buffer. The lysis buffer comprises 10mM imidazole to reduce binding of untagged and contaminating proteins. 1 ml of the 50% Ni-NTA slurry was added to a lysate and shake at 200 rpm at 4°C for 1 hour. Then, the lysate-Ni NTA mixture was loaded into a column. Then, column flow-through was collected. After that, column was washed twice with 4 ml wash buffer. Wash fractions were collected for SDS-PAGE analysis. Then, the protein was eluted 4 times with 0.5 ml elution buffer (Qiagen).

2.3.12 Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Polyacrylamide gels were cast in the supplied gel-casting apparatus according to the manufacturer's instructions (Bio-Rad), typically consisting of a resolving gel of 12% (4ml acrylamide/bis, 2.5ml 1.5M Tris/HCl pH 8.8, 100µl 10% SDS, 3.3 ml distilled water, 100µl 10% APS, 4µl TEMED) and a 5% of stacking gel (833 µl acrylamide/bis, 630 µl 0.5M Tris/HCl pH 6.8, 50µl 10% SDS, 3.4 ml distilled water, 50µl 10% APS, 5µl TEMED). Protein samples were boiled in SDS loading buffer, loaded into SDS-PAGE gel wells and

electrophoresed at 20mA per gel in SDS running buffer to achieve separation of proteins (approximately 1 hour). Gels were either stained with Coomassie blue and visualised following destaining for several hours in SDS-PAGE destaining buffer or used for Western blotting.

2.3.13 Western blotting

Acrylamide gels were soaked in transfer buffer for at least 10 minutes along with filter paper (Bio-Rad) and a nitrocellulose PVDF membrane. Then the filter papers, nitrocellulose membrane and the gel were assembled on the bed of the transblotter (BioRad Semi-Dry Transfer Cell) with care taken to eliminate any air bubbles. Gels were electrophoresed at a constant potential of 15 volts for 45 minutes to transfer the proteins to the nitrocellulose PVDF membrane. Visualisation was conducted using the WesternBreeze anti-mouse chromogenic kit (Invitrogen) using the buffers supplied with the kit, but exchanging the chromogenic substrate for a chemiluminescent one as described below. The membrane was blocked in 10 ml blocking solution for 1 hour at 37°C or at 4°C overnight. The blocking solution was discarded and the membrane was incubated with the primary antibody (mouse anti-6x- His tag) at an appropriate dilution (1:000 dilution) for 1 hour at room temperature. After incubation with primary antibody, PVDF was washed three times with prepared antibody wash and then incubated with secondary antibody (anti-mouse) for one hour before washing with antibody wash three times for 10 minutes. Finally, blots were developed by enhanced chemiluminescence (Amersham Biosciences) according to the manufacturer's instructions. Developing reagents were mixed at a ratio of 1:1 and the solution was allowed to cover the membrane. After 5 minutes of incubation, developing reagent was discarded and the PVDF was visualised using an imaging system (non-UV blue light box).

2.3.14 Measuring protein concentration (Nanodrop)

The absorbance of the protein sample was measured using NanoDrop Spectrophotometer (Thermo Scientific). This device allowed accurate determination of concentration. Basically 1 μ l of neat sample was pipetted onto the receiving fibre and avoiding dilutions which can present an error. The protein concentration was measured at 280 nm. NanoDrop used Beer-Lambert equation ($A = E \cdot b \cdot c$) for all protein calculations to relate absorbance with concentration.

$$A = E \cdot b \cdot c \dots\dots\dots 2.3$$

A= The absorbance value (A),

E= The wavelength-dependent molar absorptivity coefficient (or extinction coefficient) with units of liter/mol-cm,

b= The path length in cm,

c=The analyte concentration in moles/liter or molarity (M)

2.3.15 Viscosity measurement

The viscosities of ILs were measured using an AR 2000 ex cone-plate viscometer under a nitrogen atmosphere at room temperature. The viscometer was calibrated with Fluka Viscosity & Density Standard Solution N35. The measurements were repeated three times.

CHAPTER 3

ENZYMATIC SACCHARIFICATION USING COMMERCIAL ENZYMES ON SIGMACELL IN THE PRESENCE AND ABSENCES OF ILs

Chapter 3 discusses the experimental data obtained from the enzymatic saccharification of Sigmacell with addition of different ILs. Studies conducted by previous researchers have shown that residual IL left on biomass results in a lower percent of glucose obtained after saccharification. Therefore, this research was conducted to understand what the factors influencing the activity of the cellulase are. How does residual IL affect the cellulase? Until now, the effects of [HBIM][HSO₄], [BMIM][HSO₄] and [TEA][HSO₄] on the activity of cellulase have not yet been studied. Therefore, in this study, three types of sulphate based ILs had been included in order to understand how these ILs affect the activity of cellulase. In addition, sulphate based ILs are good in the biomass pretreatment process for separating lignin from biomass. Based on previous studies, two common types of ILs [EMIM][OAc] and [BMIM]Cl were included as controls with well-known effects on enzymes. In addition, two IL based on phosphate anions ([HBIM][H₂PO₄], [BMIM][H₂PO₄]) were included as the phosphate anion is a common buffering agent in biochemical experiments and therefore should be relatively 'protein-friendly'.

The enzyme chosen in this study is cellulase because cellulase is very important in the process of converting cellulose to glucose. In this study, pH, viscosity and ionic strength were the primary physico-chemical effect of ILs on cellulase considered. The effect of pH on the enzyme is very important as enzyme may not work properly when the pH is not suitable. It has been reported that the optimum pH for Celluclast from *T.reesei* and cellobiase from *A.niger* was pH 4.8 (Hu & Catchmark, 2011). Also, the viscosity can affect the activity of enzyme; for example higher viscosity can reduce enzyme activity due to inhibition of enzyme diffusion as a result of a high viscosity. In a study conducted by Engel and co-workers

(2010), the ionic strength can also affect enzyme activity but this effect has a smaller impact than other physical factors such as pH and viscosity.

3.1 HPLC glucose standard

A standard curve was required to calculate glucose content when using HPLC. Figure 3.1 depicts one of the representative curves of HPLC glucose standard. The amount of glucose from the samples was calculated by converting the area of the glucose peak using the standard curve equation. Then, the glucose reading was multiplied by 0.9 to correct for the water molecule added during hydrolysis. In order to get 0.9, molar mass of water is subtracted from molar mass of glucose.

Molar mass of water = 18

Molar mass of glucose = 180

$$\frac{180 - 18}{180}$$

= 0.9

After that, the glucose reading was multiplied by 10 mL for total volume of mixtures. Then, the percentage of digestion was calculated using this formula:

(Grams cellulose digested/ Grams cellulose added) X 100.....3.1

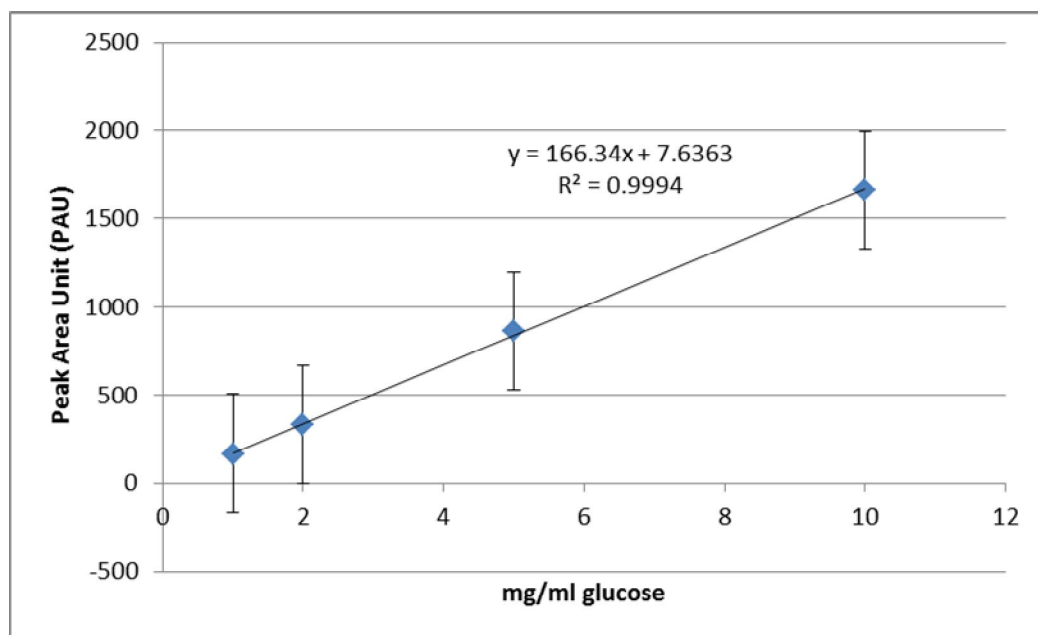


Figure 3.1: Representative standard curve for HPLC analysis of glucose release. Standards of 1, 2, 4, and 10 mg/mL glucose were run on a Jasco HPLC with a flowrate of 0.6 ml/min and a total run time of 15 minutes. 10 mM sulfuric acid was used as the mobile phase. Error bars represent standard errors between three determinants.

Table 3.1 shows the results obtained when 7 different type of ILs were added in the enzymatic saccharification mixture. The figures indicated that ILs with a hydrogen sulphate anion gave the lowest yield of glucose. Overall comparison of the results obtained from this data suggested that the anion plays a greater role in the production of glucose than the cation. Inactivation of cellulase depends on anions as mentioned by Kaar previously (Kaar et al., 2003), the enzyme inactivation have been suggested to be mostly anion dependent.

Moreover, in 10% (v/v) of [EMIM][OAc] and [BMIM]Cl, there was a decrease of 60-90% of glucose yield. According to Engel, there were 70-85% decreases in cellulase activity in the presence of ([DMIM][DMP], [BMIM]Cl, [EMIM][OAc] and [AMIM]Cl) with a commercial *T. reesei* cellulase cocktail (Engel et al., 2010), which is similar to the result obtained here.

Moreover, it seems that phosphate based ILs have better glucose yield than sulphate based ILs. It can be observed that pH value of phosphate based ILs were higher than sulphate based ILs.

Finally, acetate anion resulted in higher yield compared to chloride and hydrogen sulphate, most likely because of the basicity of the anion (Gräsvik et al., 2014). Table 3.1, 3.2, 3.3 and 3.4 presented results on glucose yield obtained in this chapter.

Table 3.1: Average glucose yield at 72 hours and pH after the addition of different types and concentrations of ILs on Sigmacell.

Ionic liquid	Concentration	Glucose yield (%)	pH
[EMIM][OAc]	0%	99.8	4.8
	1%	79.91	5.12
	2%	79.64	5.17
	5%	74.05	5.44
	10%	40.37	5.71
[BMIM]Cl	0%	99.8	4.8
	1%	78.83	4.45
	2%	50.20	5.13
	5%	21.71	6.09
	10%	4.74	6.78
[HBIM][HSO ₄]	0%	99.8	4.8
	1%	35.98	3.31
	2%	n.d	2.41
	5%	n.d	1.96
	10%	n.d	1.15
[BMIM][HSO ₄]	0%	99.8	4.8
	1%	35.03	3.43
	2%	5.07	2.36
	5%	n.d	1.77
	10%	n.d	1.59
[TEA][HSO ₄]	0%	99.8	4.8
	1%	5.48	3.24
	2%	n.d	2.31
	5%	n.d	1.55
	10%	n.d	1.27
[HBIM][H ₂ PO ₄]	0%	99.8	4.8
	1%	86.51	4.32
	2%	70.93	3.55
	5%	65.92	3.01
	10%	n.d	2.42
[BMIM][H ₂ PO ₄]	0%	99.8	4.8
	1%	78.98	4.55
	2%	70.01	4.75
	5%	34.37	5.06

	10%	4.9	5.41
[EMIM][OAc]			
(change to pH 2)	1%	25.0	2.0
(change to pH 3)	1%	27.33	3.0
[HBIM][HSO ₄]			
(change to pH 4)	1%	45.3	4.0
(change to pH 5)	1%	51.6	5.0

Table 3.2: Average glucose yield at pH 1 to 5 on Sigmacell at 72 hours.

pH	Glucose yield (%)
1	16.5
2	64.7
3	78.0
4	85.2
5	88.2

Table 3.3: Kinetic study on enzymatic saccharification of Sigmacell without IL for 6, 24, 48 and 72 hours.

Hour	Glucose yield (%)
6	60.8
24	81.8
48	94.6
72	99.9

Table 3.4: Kinetic enzyme deactivation studies for duration of 4, 24, 48 and 72 hours with the presence of 1, 2, 5 and 10% [HBIM][HSO₄].

Concentration of IL (%)	Glucose Yield (%)			
	4 hr	24 hr	48 hr	72 hr
1	16.6	19.2	26.7	36
2	19.4	n.d	n.d	n.d
5	14.6	n.d	n.d	n.d
10	10	n.d	n.d	n.d

- n.d = not detected

3.2 pH effect

The results in Table 3.1 suggest a negative correlation between glucose release and the pH of the saccharification solution. Therefore, this effect was explored in solutions containing [EMIM][OAc] and [BMIM]Cl both of which had relatively mild effects on glucose yield. The pH of the saccharification solution was reduced using sulfuric acid to pH 2 and 3. The concentration of IL used was 1%. The result on the effect of pH of [EMIM][OAc] and [BMIM]Cl is depicted in Figure 3.2. At pH in the range of 4.5 – 5.5, the yield of glucose was 78 – 80%. As the pH decreased to pH 2 and 3, the yield of glucose was lower than 30%. Also, from the graph shown below, when the pH reached up to pH 6 and 7, the percent of glucose was only 22% and 4.7%, respectively. Celluclast and Novozyme 188 from Sigma showed a good activity at pH 4.8 (Hu & Catchmark, 2011) and this was the reason why at pH lower than 4.8, the yield of glucose was very low.

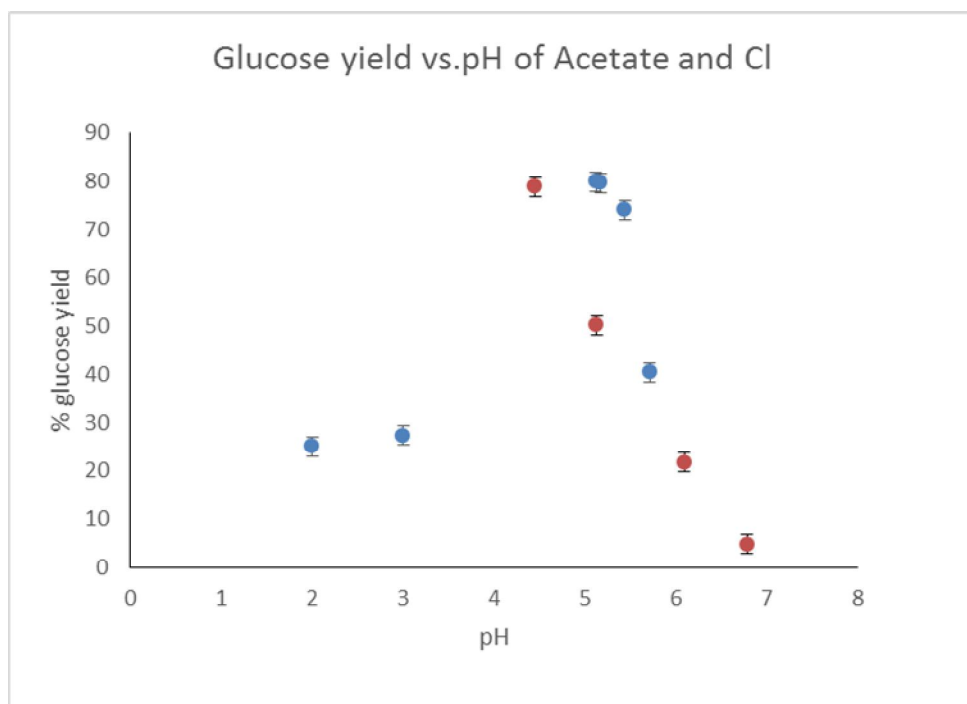


Figure 3.2: The effect of pH of [EMIM][OAc] (blue circle) and [BMIM]Cl (orange circle) on glucose yield. The pH of the Sigmacell cellulose saccharification solution was reduced using sulphuric acid to create pH 2 and 3. Error bars represent standard errors between (n=3). The concentration of IL used was 1%.

The results in Table 3.1 suggest sulphate based ILs result in a very low glucose release and also have a very low pH. Therefore, pH effect was studied in solutions containing [HBIM][HSO₄]. The pH of the saccharification solution was increased using NaOH to create pH 4 and 5. The result on the effect of pH of [HBIM][HSO₄] is depicted in Figure 3.3. At pH lower than 2.3, no glucose can be detected. The yield of glucose increased to 52% when the pH of [HBIM][HSO₄] was modified to pH 5.

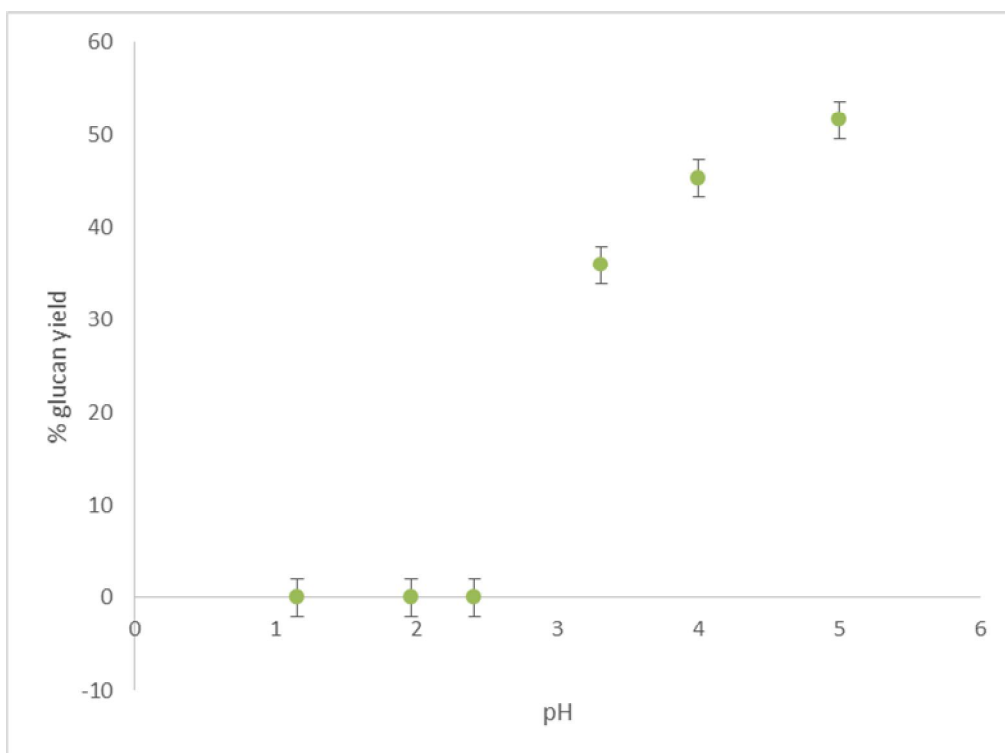


Figure 3.3: The effect of pH of [HBIM][HSO₄] on glucose yield. The pH of the saccharification solution was increased using NaOH to create pH 4 and 5. Error bars represent standard errors between n=3. The concentration of IL used was 1%.

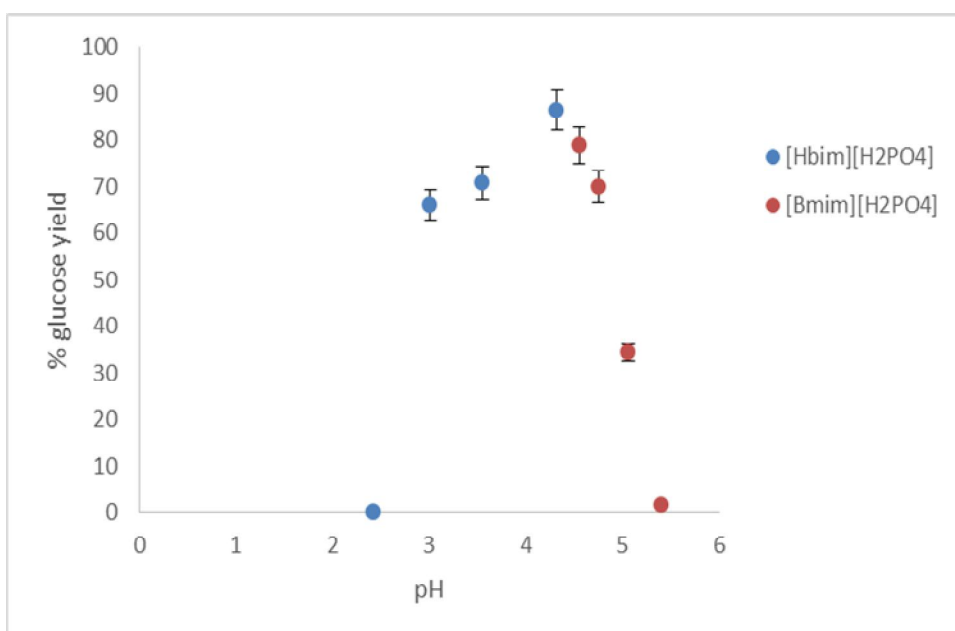


Figure 3.4: The effect of pH of [HBIM][H₂PO₄] and [Bmim][H₂PO₄] on glucose yield. Error bars represent standard errors between three determinants.

Based from the graphs shown above (figure 3.2, 3.3 and 3.4), low pH, as well as higher pH can decrease the yield of glucose content.

3.2.1 pH effect of [H₂SO₄] on percentages of glucose yield.

The results depicted in Table 3.1 and Figures 3.2-3.4 showed that low pH can affect the activity of cellulase. In this experiment, the effect of sulfuric acid on the percentages of glucose yield was studied. This experiment was conducted in citrate buffer with no IL. Sulfuric acid was used to change from pH 1 to 5 in order to understand pH effect of the enzyme without the influence of ILs. Figure 4.5 demonstrates the effect of pH on the enzymatic activity of the cellulase mixture. The result indicated that the amount of glucose yield decreased as the pH decreased. This experiment showed that cellulase mixture from *Trichoderma reesei* and cellobiase from *Aspergillus niger* were not stable in a very acidic solution.

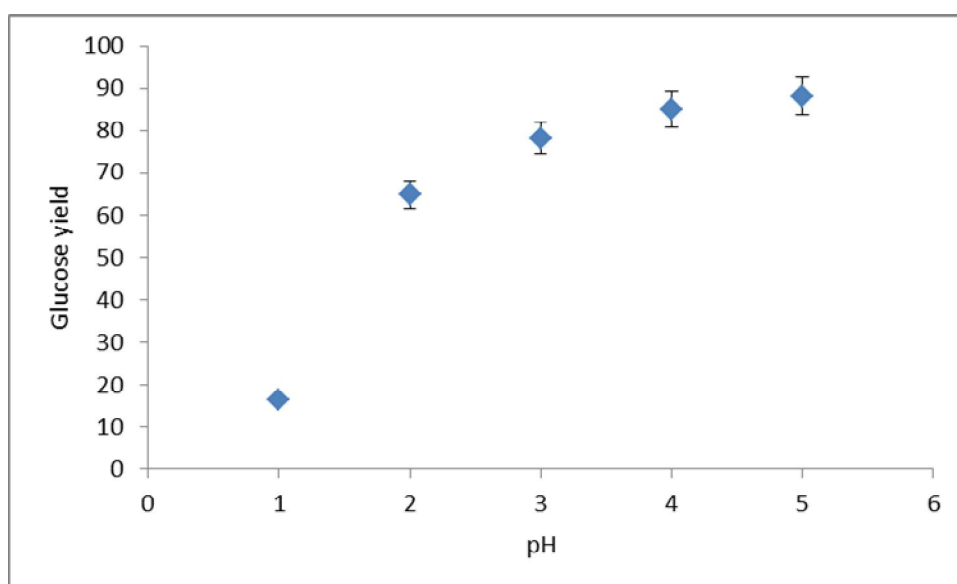


Figure 3.5: Effect of pH 1- 5 on the enzymatic activity of celluclast and cellobiase. Samples were run on a Jasco HPLC with a flowrate of 0.6 ml/min and a total run time of 15

minutes. 10 mM sulfuric acid was used as the mobile phase. Sulfuric acid was used to get pH 1 to 5. Error bars represent standard errors between three determinants.

3.2.2 Specific Ion effects

As results depicted in Table 4.1 showed that low pH can affect the activity of cellulase, specific ion effect was studied in order to understand what will happen if with the pH of the solution is changed with different acids (in this case H₂SO₄ and HCl). This should allow us to separate the effects of pH change from the effects of adding more sulfate ions. In this study, the pH of solutions containing different volume percentage of [EMIM][OAc] was adjusted to pH 2 (figure 3.6). In this reaction, when H₂SO₄ was added to [EMIM][OAc], [EMIM][HSO₄] and acetic acid were formed. $[\text{EMIM}][\text{OAc}] + \text{H}_2\text{SO}_4 \longrightarrow [\text{EMIM}][\text{HSO}_4] + \text{AcOH}$. When HCl was added to [EMIM][OAc], [EMIM]Cl and acetic acid were formed. $[\text{EMIM}][\text{OAc}] + \text{HCl} \longrightarrow [\text{EMIM}]\text{Cl} + \text{AcOH}$.

Only solutions containing 1% (v/v) of [EMIM][OAc] had glucose released after saccharification whereas no glucose was detected in 2,5 and 10% (v/v) [EMIM][OAc]. There was a slightly difference between these two results in which 27% glucose can be observed when the pH was changed with H₂SO₄ and 25% glucose when the pH was changed with HCl. There was no significant difference between these results when t-test was calculated as the p-value was 0.7 (Table 3.5).

Table 3:5: Statistical analysis of changing pH using H_2SO_4 and HCl

	<i>Variable</i>	
	<i>1</i>	<i>Variable 2</i>
Mean	27.32953	25.30609775
Variance	33.33654	18.92300907
Observations	2	2
Hypothesized Mean Difference	0	
df	2	
t Stat	0.395842	
P(T<=t) one-tail	0.365229	
t Critical one-tail	2.919986	
P(T<=t) two-tail	0.730457	
t Critical two-tail	4.302653	

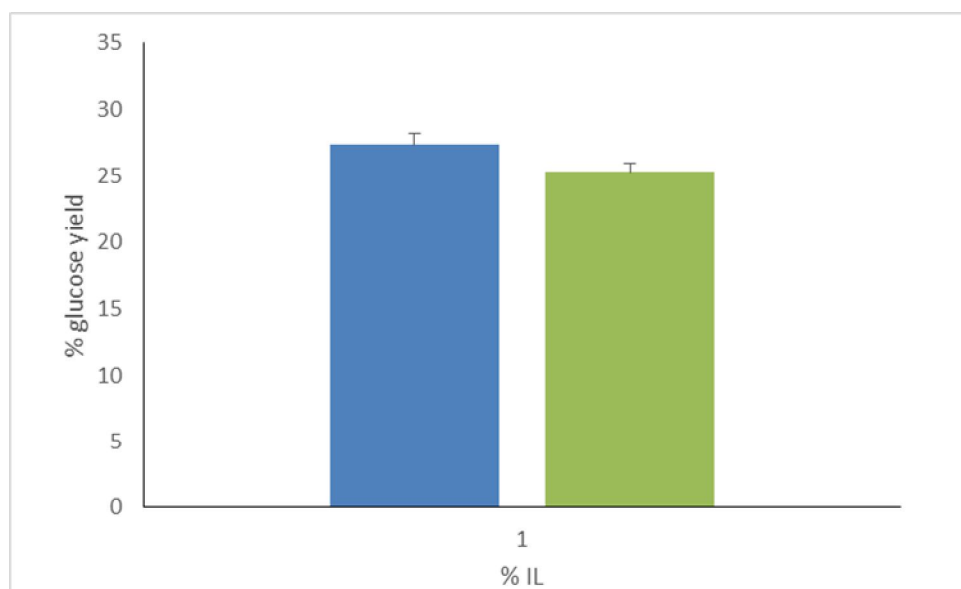


Figure 3.6: Effect of changing pH of $[\text{EMIM}][\text{OAc}]$ to pH 2 using H_2SO_4 (blue) and HCl (grey). Samples were run on a Jasco HPLC with a flowrate of 0.6 ml/min and a total run time of 15 minutes. 10 mM sulfuric acid was used as the mobile phase. Error bars represent standard errors between three determinants.

It was observed that there was a small effect if different acids were used to change the pH of [EMIM][OAc] containing solutions to pH 2. The major effect on the enzyme activity was the pH value.

3.3 Kinetic study

A kinetic study for enzymatic saccharification on Sigmacell cellulose has been conducted to determine the yield of glucose after 4, 24, 48 and 72 hours. From figure 3.7, it can be observed that the yield of glucose increased gradually from 6 hours to 72 hours. After 6 hours of the incubation, 61% of glucose yield was achieved and increased up to 82% after 24 hours of study. The yield of glucose increased to 95% after 48 hours and 99.97% after 72 hours. By looking at the data obtained, a very high increase in glucose yield was between 6 and 24 hours.

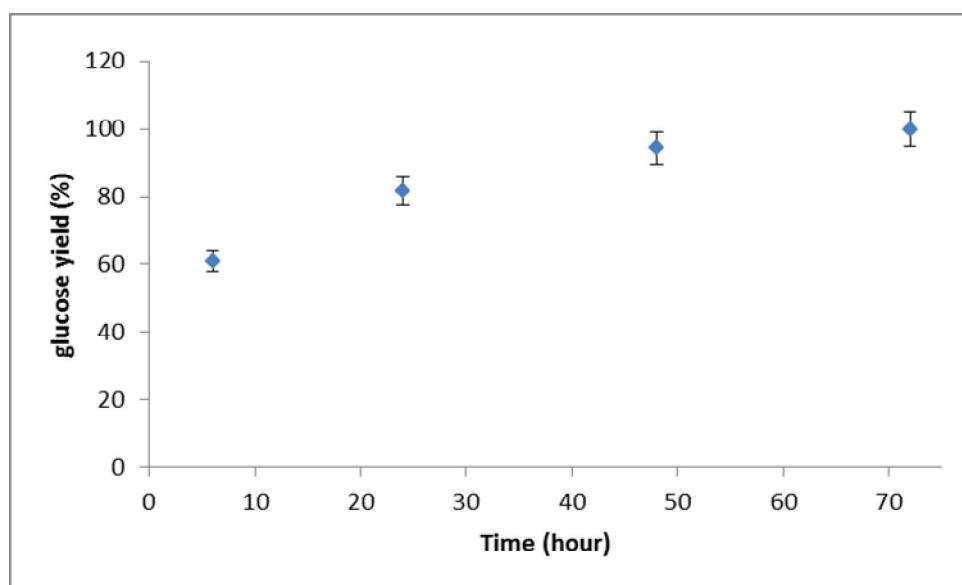


Figure 3.7: Kinetics study on enzymatic saccharification of Sigmacellulose without IL for 6, 24, 48, and 72 hours. Samples were run on a Jasco HPLC with a flowrate of 0.6 ml/min and a total run time of 15 minutes. 10 mM sulfuric acid was used as the mobile phase. Error bars represent standard errors between three determinants.

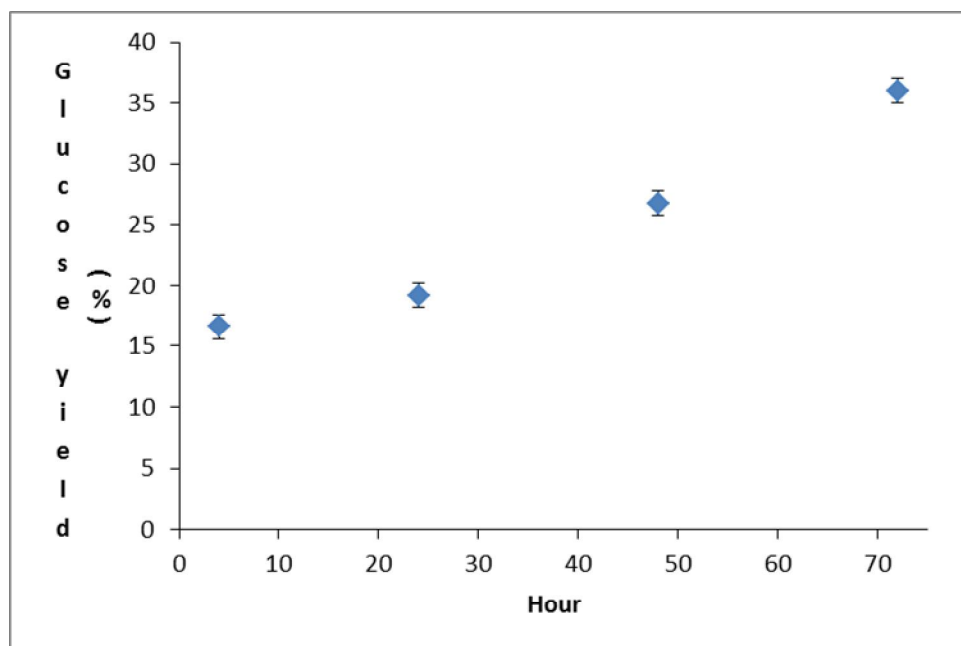


Figure 3.8: Kinetic enzyme deactivation studies for duration of 4, 24, 48 and 72 hours with the presence of 1% [HBIM][HSO₄]. Sample swere run on a Jasco HPLC with a flowrate of 0.6 ml/min and a total run time of 15 minutes. 10 mM sulfuric acid was used as the mobile phase Error bars represent standard errors between n=3.

To investigate the time required for the IL to deactivate the enzyme, a kinetic study on enzymatic saccharification with the presence of IL had been performed. For 1 % of [HBIM][HSO₄], glucose yield shows an increased from 16.6% at 4 hours to 36% at 72 hours (figure 3.8). When the concentration of [HBIM][HSO₄] increased to 2, 5, and 10%, the yield of glucose was below 20% and it was deactivated after 4 hours of saccharification process (see figure 3.9, figure 3.10 and figure 3.11). It seems that only 1% (v/v) of [HBIM][HSO₄] produced glucose at 72 hours, but all of the higher concentrations deactivated at 4 hour. This indicates that [HBIM][HSO₄] deactivates the cellulase enzymes very rapidly.

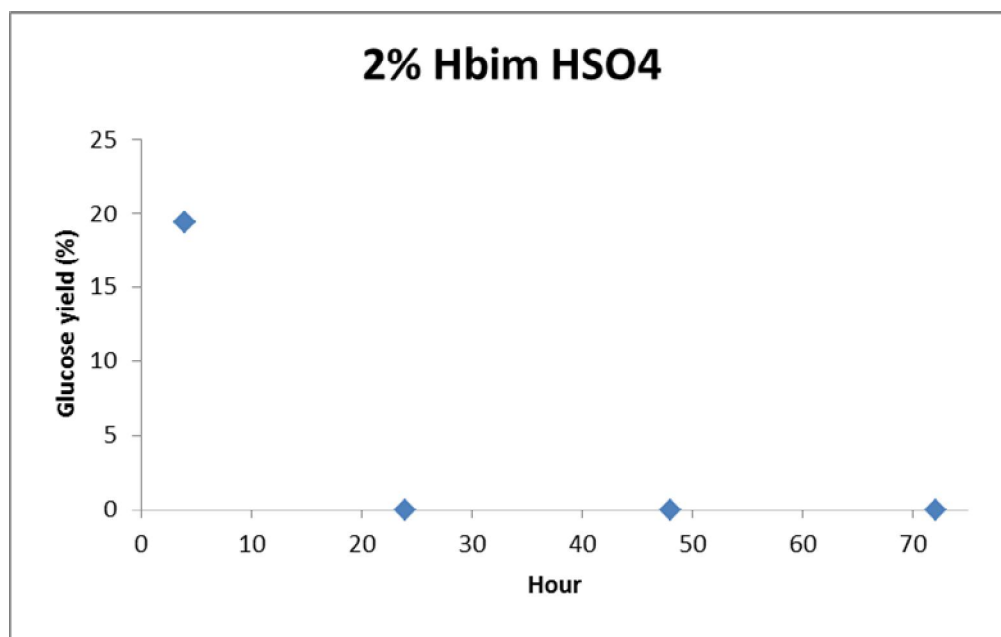


Figure 3.9: Kinetic enzyme deactivation studies for duration of 4, 24, 48 and 72 hours with the presence of 2% [HBIM][HSO₄]. Samples were run on a Jasco HPLC with a flowrate of 0.6 ml/min and a total run time of 15 minutes. 10 mM sulfuric acid was used as the mobile phase. Error bars represent standard errors between n=3.

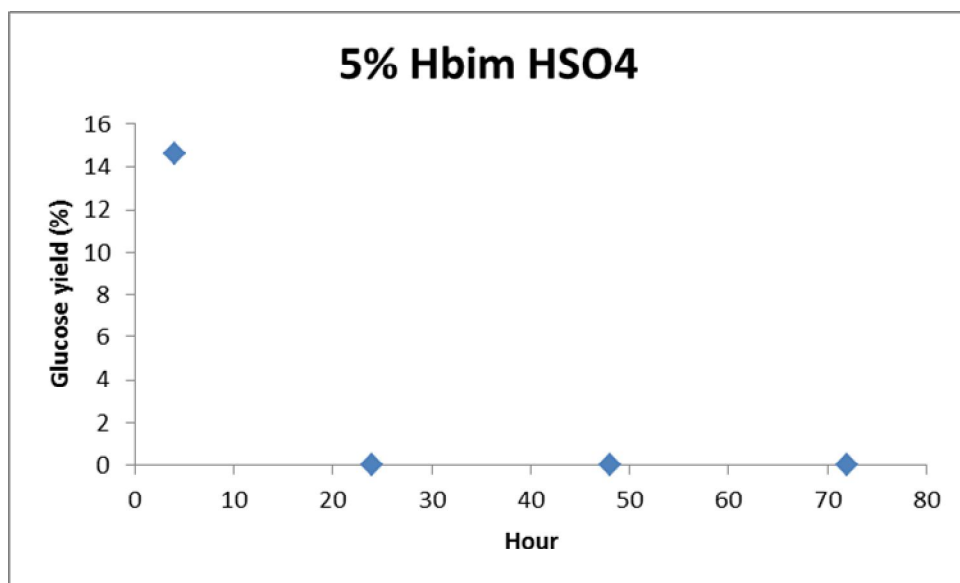


Figure 3.10: Kinetic enzyme deactivation studies for duration of 4, 24, 48 and 72 hours with the presence of 5% [HBIM][HSO₄]. Samples were run on a Jasco HPLC with a flowrate of 0.6 ml/min and a total run time of 15 minutes. 10 mM sulfuric acid was used as the mobile phase. Error bars represent standard errors between n=3.

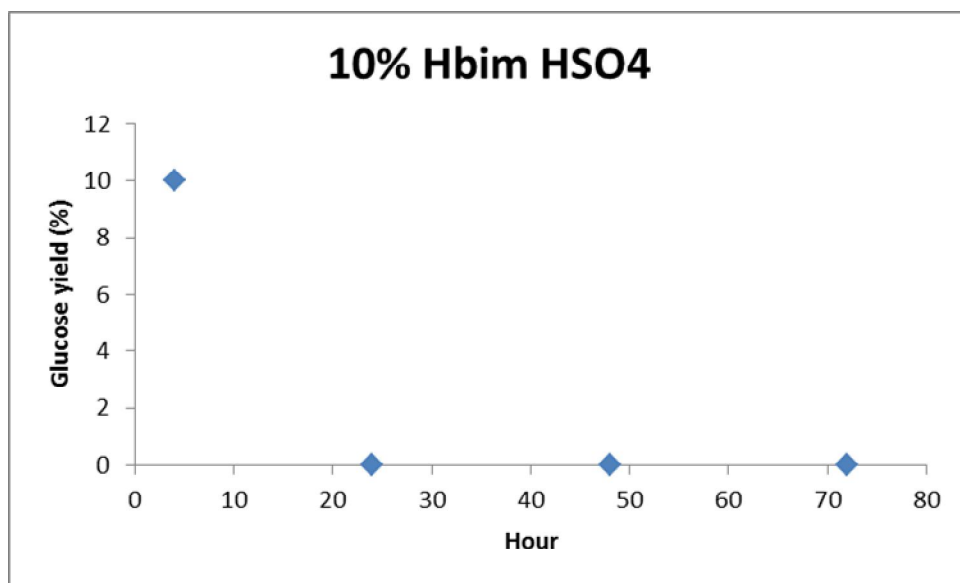
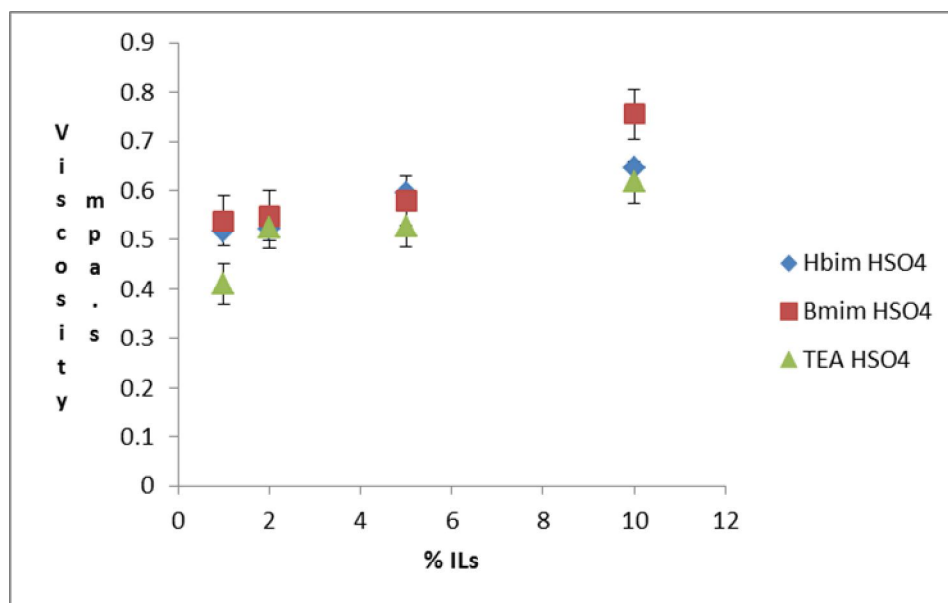


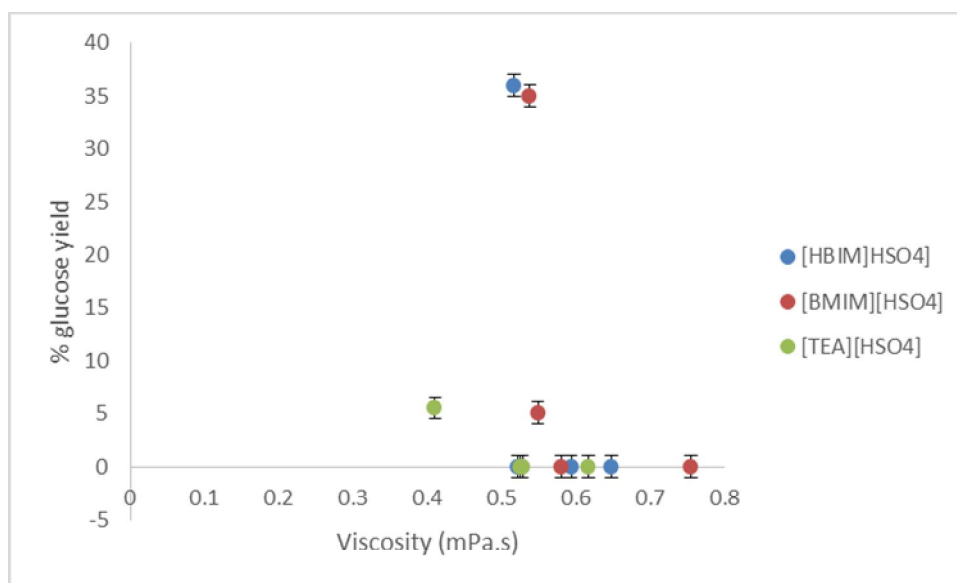
Figure 3.11: Kinetic enzyme deactivation studies for duration of 4, 24, 48 and 72 hours with the presence of 10% [HBIM][HSO₄]. Samples were run on a Jasco HPLC with a flowrate of 0.6 ml/min and a total run time of 15 minutes. 10 mM sulfuric acid was used as the mobile phase. Error bars represent standard errors between n=3.

3.4 Effect of viscosity

The effect of viscosity on the enzyme was studied as to investigate what are the other physical factors can influence the activity of the enzyme. The viscosity of 1, 2, 5 and 10% (v/v) IL were measured using viscometer at room temperature. As the concentration of ILs increased, the viscosity of the solutions containing [HBIM][HSO₄] and [BMIM][HSO₄] and [TEA][HSO₄] increased (figure 3.10a). The viscosity of 1% [HBIM][HSO₄] was 0.52 mPa.s and increased up to 0.59 mPa.s at 10%. For [BMIM][HSO₄], the viscosity was 0.54 mPa.s at 1% and rose to 0.76 mPa.s at 10%. For [TEA][HSO₄], the viscosity was 0.41 mPa.s at 1% and increased to 0.62 mPa.s at 10%. For each sulphate based ILs, as the viscosity increased, the yield of glucose decreased. It has been reported by Engel and co-workers (2010), as the concentration of [MMIM][DMP] increased, the viscosity increased and an increase of viscosity of [MMIM][DMP], had decreased the relative enzyme activity.



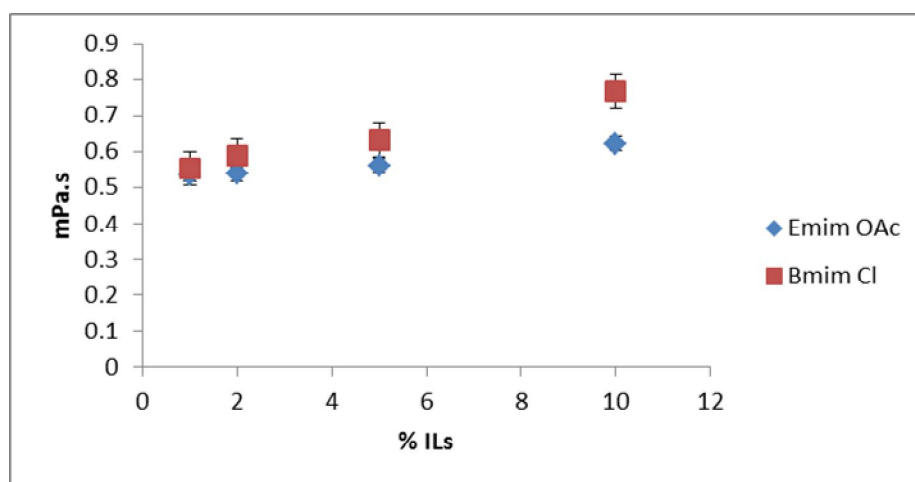
a) Viscosity vs. % IL



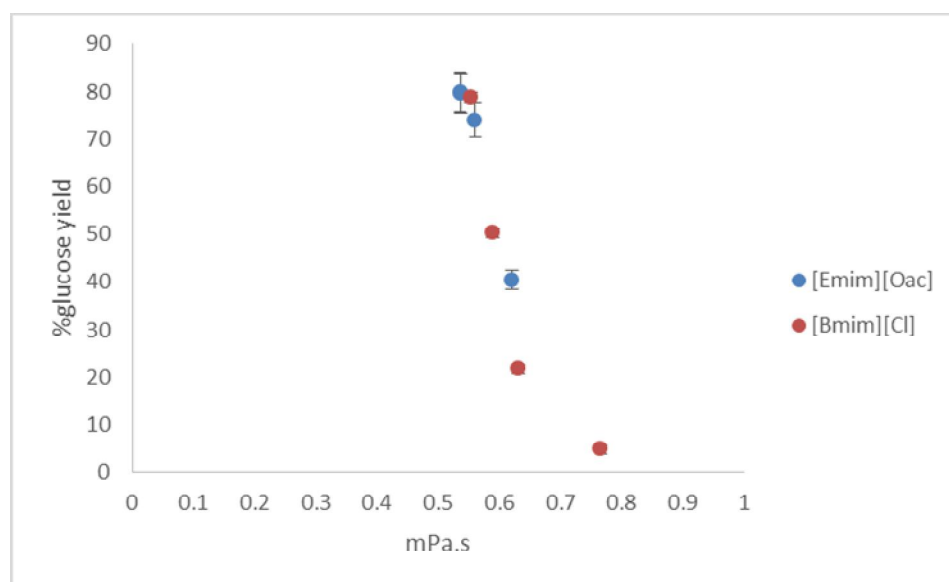
b) Glucose yield vs. viscosity

Figure 3.10: Effect of viscosity of [HBIM][HSO₄], [BMIM][HSO₄] and [TEA][HSO₄] on glucose yield. a) Viscosity vs. % IL. b) Glucose yield vs. viscosity. Error bars represent standard errors between n=3.

The correlation in figure 3.11 was not perfect, although higher viscosity results in low glucose release. Therefore, viscosity is not the only determinant of glucose yield because all sulphate IL have low yield (with them the main effect is pH). It has been reported previously that higher viscosity results in low glucose release (Engel et al., 2010) and this concept was applied to [EMIM][OAc] and [BMIM]Cl where higher viscosities resulted in lower percentage of glucose yield (figure 3.11).



a) Viscosity vs. % IL



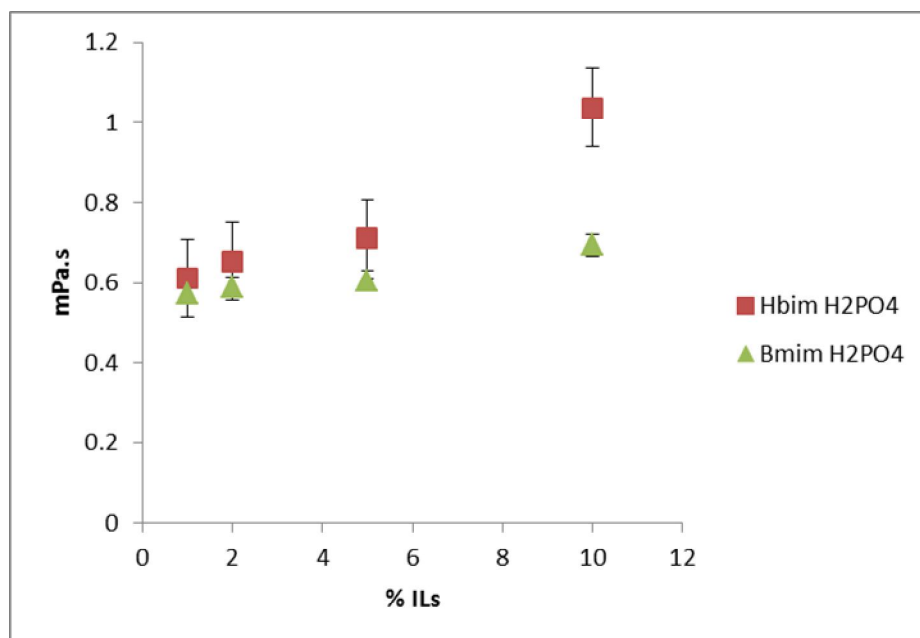
b) Glucose yield vs. viscosity

Figure 3.11: Effect of viscosity of [EMIM][OAc] and [BMIM]Cl on glucose yield. a) Viscosity vs. % IL. b) Glucose yield vs. viscosity. Error bars represent standard errors between three determinants.

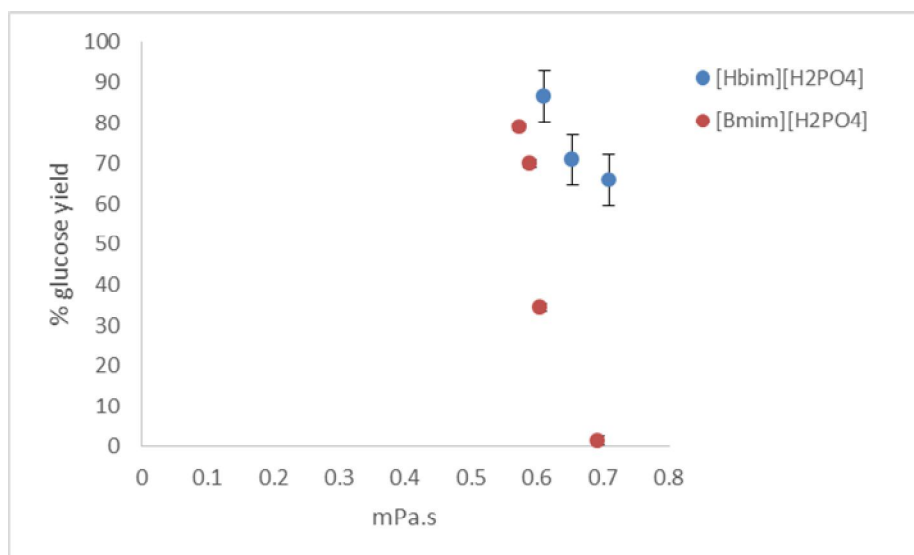
There is a strong correlation in figure 3.11 where higher viscosities of [EMIM][OAc] and [BMIM]Cl result in lower yield of glucose released. Viscosity can inhibit enzyme diffusion to the substrate and therefore it can reduce the enzyme activity.

Moreover, the effect of viscosity on phosphate based ILs were studied. For each of [HBIM][H₂PO₄] and [BMIM][H₂PO₄], as the viscosity increased, the percentage of glucose

yield decreased (figure 3.12b). However, comparing these two ILs, the viscosity of [HBIM][H₂PO₄] was higher than [BMIM][H₂PO₄], but the yield of glucose in [HBIM][H₂PO₄] was higher. Clearly, in this case, the cation plays an important role in the activity of the enzymes. Besides that, pH effect was the main factor that influences the percentage of glucose yield.



a) Viscosity vs. % IL



b) Glucose yield vs. viscosity

Figure 3.12: Effect of viscosity of [HBIM][H₂PO₄] and [BMIM][H₂PO₄] on glucose yield. a) Viscosity vs. % IL. b) Glucose yield vs. viscosity. Error bars represent standard errors between three determinants.

3.5 Effect of ionic strength

The effect of ionic strength on the activity of the enzyme was studied in order to understand other factors which can influence the activity of the enzyme. Ionic strength was calculated by using a dimensionless ionic strength equation as defined by Atkins (1986):

$$I = \frac{1}{2} \cdot \sum \frac{m_i}{m^0} \cdot z_i^2 \dots\dots\dots 3.2$$

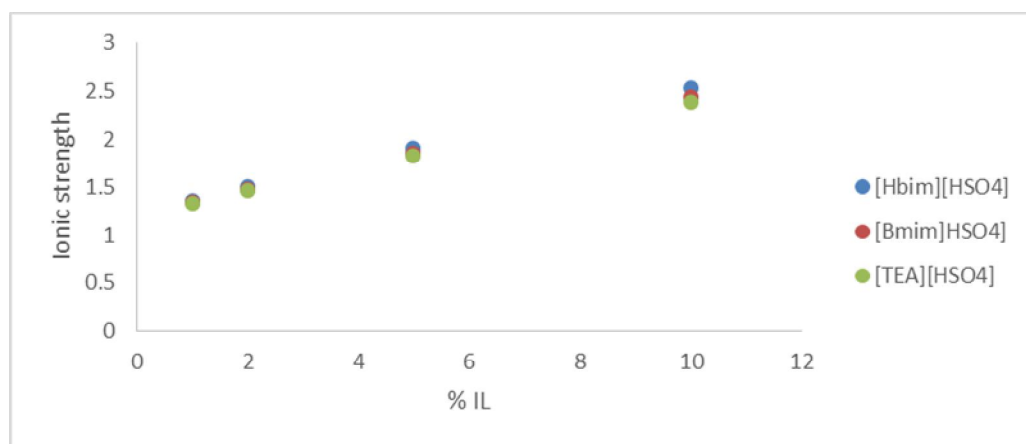
I = Ionic strength

m_i = Ionic concentration in units of molality, mol kg⁻¹

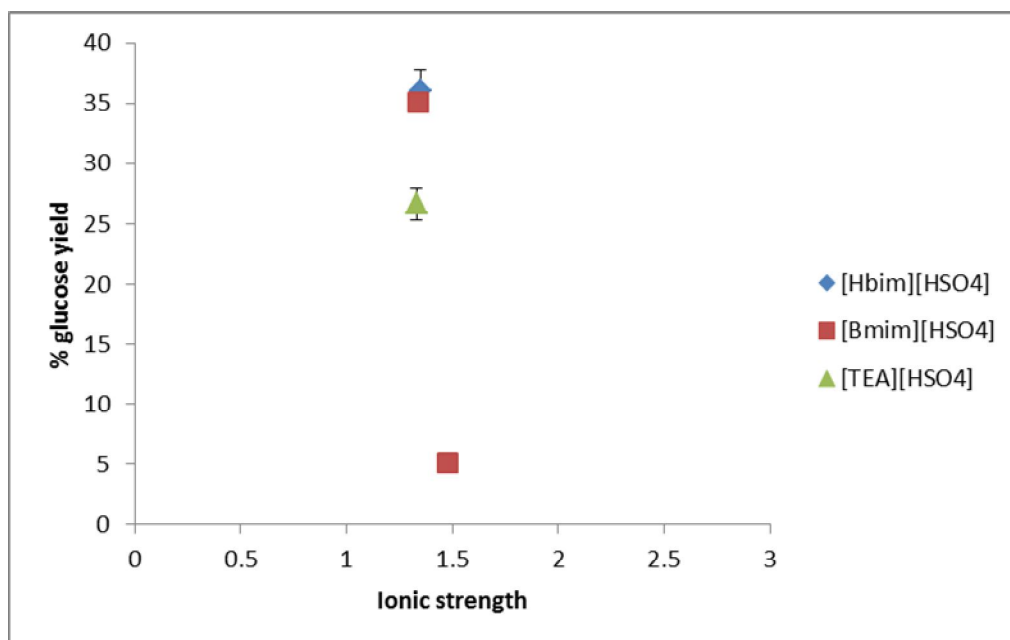
m^0 = 1 mol kg⁻¹

z_i^2 = Number of charges on the ion

Based from the data obtained (figure 3.13, 3.14, and 3.15), as ionic strength increased, the enzyme activity decreased. According to Engel and co-workers (2010), the relative enzyme activity decreased when the ionic strength of [MMIM] [DMP] increased. For each type of IL studied (figure 3.13, 3.14, and 3.15), as the concentration increased, the ionic strength increased and the yield of glucose decreased, which was similar to the result reported by Engel and co-workers (2010).

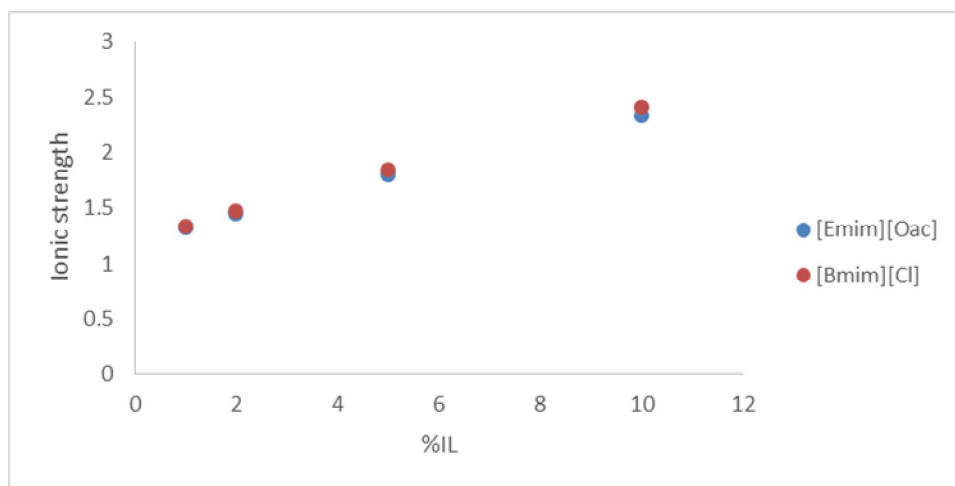


a) Ionic strength vs. % IL

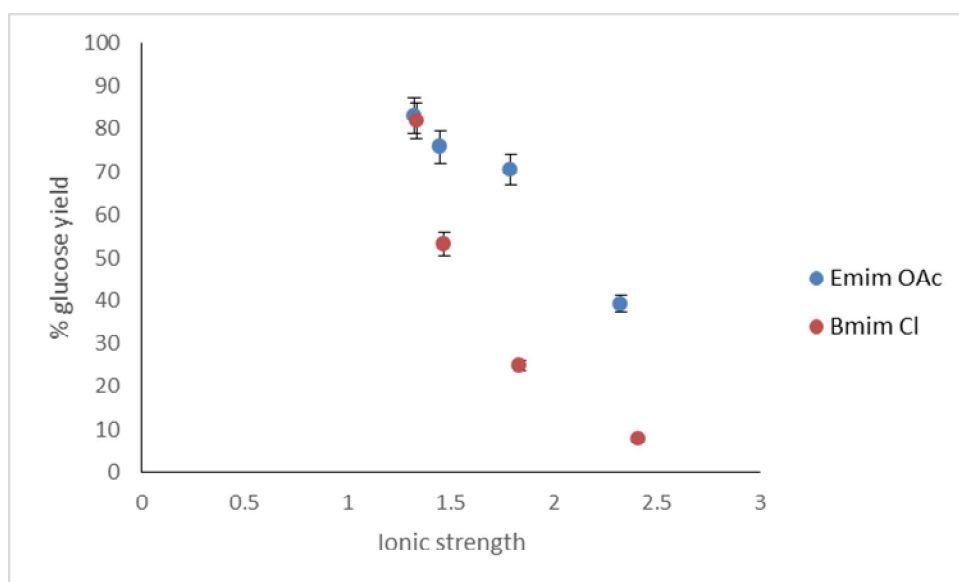


b) %glucose yield vs.Ionic strength

Figure 3.13: Effect of ionic strength of [HBIM][HSO₄], [BMIM][HSO₄] and [TEA][HSO₄] on glucose yield. Error bars represent standard errors between three determinants.

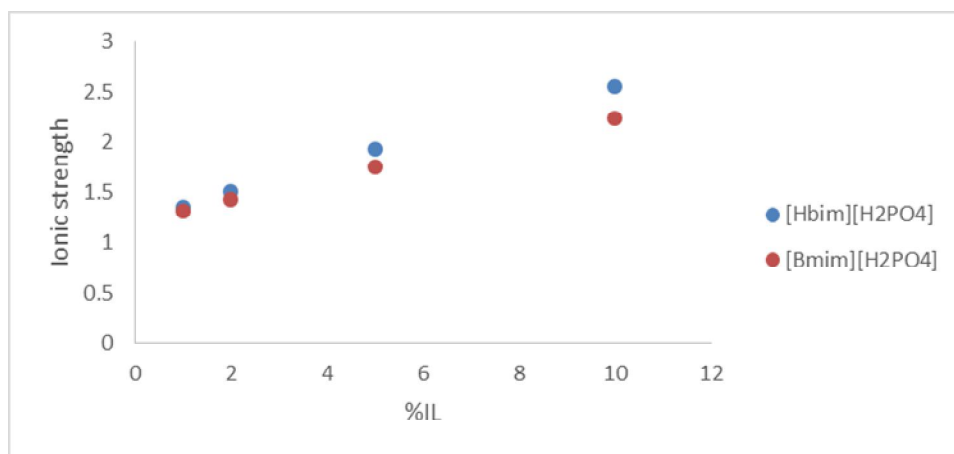


a) Ionic strength vs. % IL

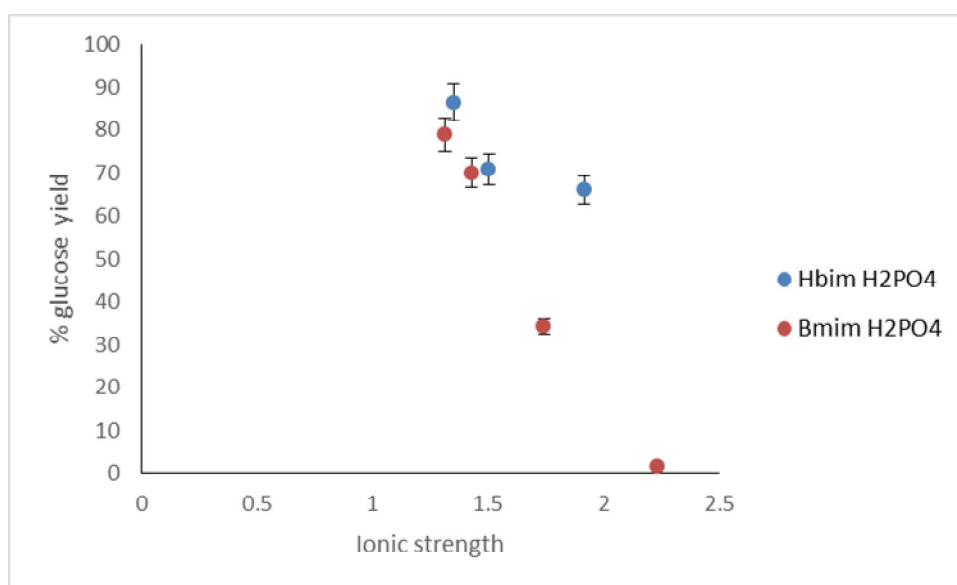


b)%glucose yield vs.Ionic strength

Figure 3.14: Effect of ionic strength of [EMIM][OAc], [BMIM]Cl on glucose yield. Error bars represent standard errors between three determinants.



a) Ionic strength vs. % IL



b) %glucose yield vs. Ionic strength

Figure 3.15: Effect of ionic strength of [HBIM][H₂PO₄], [BMIM][H₂PO₄] on glucose yield. Error bars represent standard errors between three determinants.

However, ionic strength of ILs with different cation and anion, did not agree with the statement increased ionic strength will decrease the enzyme activity. Based on this results, ionic strength of [HBIM][H₂PO₄] is higher than [BMIM][H₂PO₄], but the yield of glucose from saccharifications in the presence of [HBIM][H₂PO₄] was better than [BMIM][H₂PO₄]. It seems that ionic strength had small effect on cellulase activity than other effects such as concentrations and pH.

3.6 Statistical analyses

Statistical analyses was performed by using SPSS software version 19. Analysis of variance (ANOVA) was performed on each set of data which state the occurrence of significant difference of 4 parameters used in this study (ILs concentrations, pH, viscosity, ionic strength). ANOVA indicated significant results for ILs concentrations and pH effects as $P=0.031$ and $P=0.001$ respectively. However, there was no significant effects on viscosity and ionic strength as $P=0.995$ and $P=0.551$ (Table 3.6). A large number of ANOVA tables are presented in Appendix I.

Table 3.6: ANOVA; The effects of ILs concentrations, pH, viscosity and ionic strength on glucose yield

Parameters	F	P-Value
Ionic liquids concentrations	3.494	0.031
pH effects	16.418	0.001
Viscosity	0.239	0.995
Ionic strength	0.972	0.551

3.7 A study on the effect of sulfate based IL with commercial cellobiase from *A.niger* using cellobiose as the substrate.

The results depicted in Table 3.1 showed that low pH can affect the activity of cellulases. In this experiment, the effect of [TEA][HSO₄] on cellobiase from *A.niger* was conducted (figure 3.16). The objective of this experiment was to examine how ILs affect the cellobiase specifically as this is often the rate limiting step in total reaction yield (Rani et al., 2014)). It has been reported previously, β -glucosidase (Novozyme 188) from *A.niger* was more sensitive to deactivation than cellulase from *T.reesei* (Engel et al., 2012). The result

obtained in this thesis showed that only 3.85% glucose yield was observed in 1% (v/v) of [TEA][HSO₄] compared to 68.6% glucose released in the absence of [TEA][HSO₄].

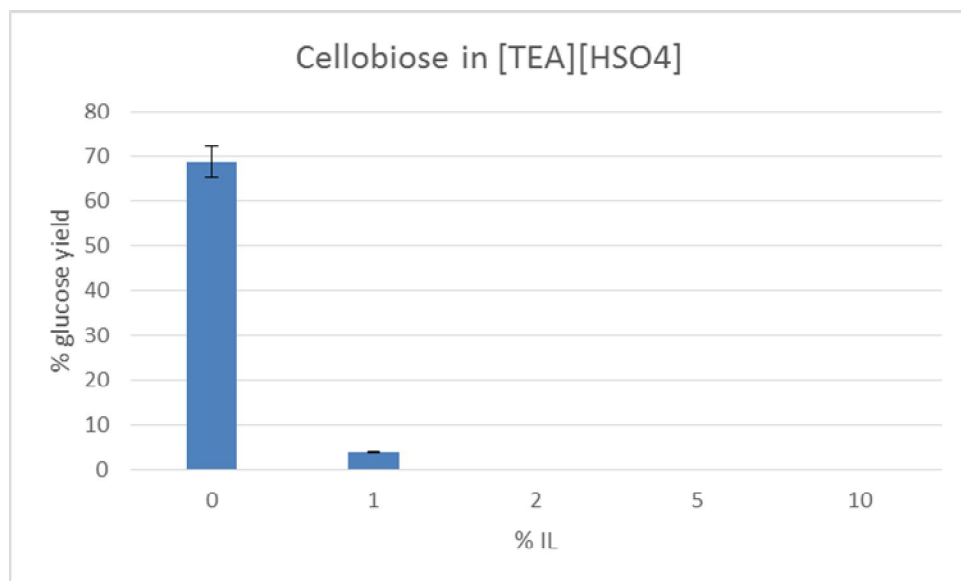


Figure 3.16: Effect of 1,2,5,10% (v/v) of [TEA][HSO₄] on cellobiase. Samples were run on a Jasco HPLC with a flowrate of 0.6 ml/min and a total run time of 15 minutes. 10 mM sulfuric acid was used as the mobile phase. Error bars represent standard errors between (n=3).

3.8 DISCUSSION

The first part of this study was an enzymatic saccharification assay with the addition of ILs. Enzymatic saccharification was performed following the NREL standard protocol LAP-009 “Enzymatic Saccharification of Lignocellulosic Biomass” (Selig et al., 2008) with some modifications. In this research, ILs containing sulphate shows the strongest inhibition with lowest glucose yields (Table 3.1). Research done by other groups has suggested that other parameters such as ionic strength, pH and viscosity might be responsible for the inactivation of cellulases. (Engel et al, 2010). In addition, the pH of ILs could also increase or decrease greatly when the concentration of ILs increase in aqueous solution (Cui et al, 2010).

The ILs used in this study are all water-miscible and therefore dissociate into the respective ions in aqueous solutions. The activity of proteins in aqueous salt solutions can be predicted along the rules of the Hofmeister series (figure 3.17) in which ions are classified according to their chaotropicity or kosmotropicity (Hofmeister 1888, Kunz et al.2004). Chaotropic ions have a low charge density and are normally large in dimension size. Thus, chaotropic ions are known as structure-breakers as they break the structure of water due to their weak interactions with water molecules. Kosmotropic ions are small and strongly charged. All multivalent ions are highly hydrated and therefore kosmotropic. As their interaction with water is strong, they are called “structure-makers” (Zhao 2006). Kosmotropic ions induce higher hydrophobic effects in the protein and therefore stabilise its structure. Nevertheless, at higher concentrations of kosmotropic salts, proteins precipitate as they are excluded from water (salting out). Many studies have demonstrated that kosmotropic anions and chaotropic cations usually stabilise proteins while kosmotropic cations and chaotropic anions tend to destabilise them (Zhao 2005).

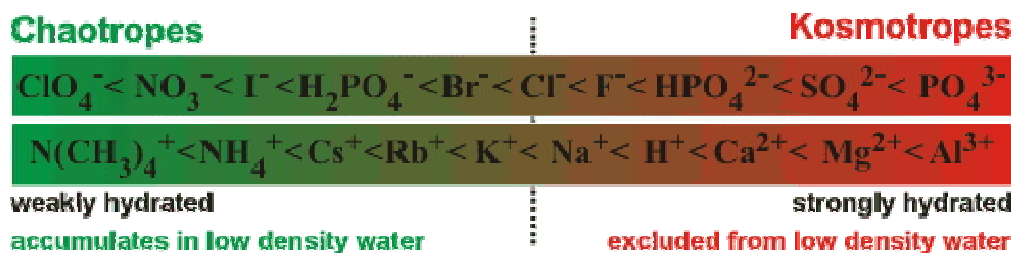


Figure 3.17: Ionic chaotropes and kosmotropes in approximate order of strength (Chaplin, 2001)

The same tendencies have also been reported in studies using aqueous IL solutions (Maruyama et al. 2002, Park and Kazlauskas 2001). Nevertheless, in this thesis the results did not always follow the Hofmeister series (figure 3.18) as the sulphate anion destabilizes the activity of cellulase but chloride anion stabilizes the enzyme. Studies conducted by Yang (Yang 2009, Yang et al. 2010) also mentioned that several enzymes have been shown to exhibit activity not in accordance with the rules of the Hofmeister series. However, phosphate anion agreed with Hofmeister series as the activity of cellulase was higher in phosphate based ILs.

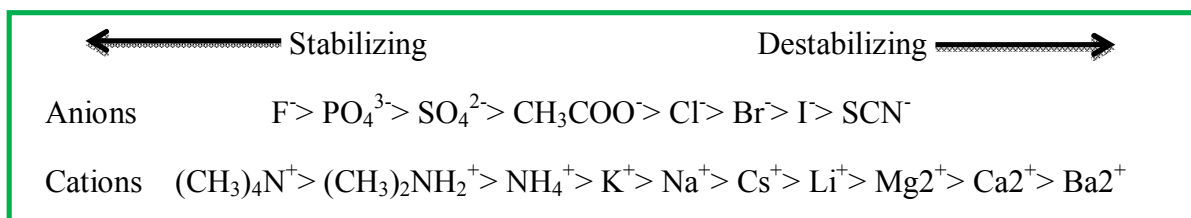


Figure 3.18: The Hofmeister series as an order of the ion effect on protein stability

The effect of viscosity and ionic strength of ILs on the enzymatic saccharification were observed but these two parameters were not the main factors that influenced the enzyme activity. The effects of viscosity and ionic strength can be observed in [EMIM][OAc] and [BMIM]Cl in which an increase of viscosities and ionic strength decrease the release of glucose. The main contribution of enzymes activity in this study was pH. The optimum pH for cellulase by *T. reesei* and for β -glucosidase was 4.5 and 5.5, respectively (Li et al., 2013). Sulphate based ILs had pH lower than 3.5 but [EMIM][OAc] and [BMIM]Cl had pH in the range of 4.5 – 5.7. Phosphate based ILs had pH in the range of 4.32 – 5.4 and the activity of

cellulase in [EMIM][OAc], [BMIM]Cl and phosphate based ILs was higher than the sulphate based ILs. It can be concluded that the activity of cellulase and β -glucosidase were significantly affected by the pH. This is problematic since the ionic liquids with sulphate anions, particularly [TEA][HSO₄], are useful for biomass processing as they effectively dissolve lignin and can be made at low cost (Chen et al., 2014). Therefore in order to adopt these IL as solvents for biomass processing new acid-stable cellulases must be sought.

CHAPTER 4: ACIDOPHILIC ENDOGLUCANASE FROM *CRYPTOCOCCUS SP.* S-2

Celluclast and β -glucosidase seem to be inactive in acidic conditions and sulphate based ILs are very acidic as discussed in chapter 3. To solve this problem, a cellulase with acid-tolerance is required to improve the enzyme performance in converting the cellulose to glucose in acidic environments. Moreover, acidophilic enzymes are important in bioethanol production from lignocellulosics because the pretreatment process is more efficient in acid in order to obtain high sugar yields (Galbe and Zacchi 2007). It has been reported that some industrial processes required extreme pH conditions (very acidic), so if acid stable cellulase is available, the industry can minimise the cost for pH adjustment. In order to find an acid-tolerant cellulase, studies conducted by previous researchers were examined and it was observed that endoglucanase from *Cryptococcus sp.* S-2 showed maximum activity at pH 3.5 (Thongekkaew et al., 2008). More importantly it still retains activity at pH 2 in the original study. This chapter presents results on the cloning, overexpression, and testing of recombinant endoglucanase from *Cryptococcus sp.* The enzyme was expressed in *E.coli* with two different expression vectors and its activity on carboxymethylcellulose (CMC) tested in the presence and absence of sulphate-based ionic liquids. Therefore this chapter aims to obtain endoglucanase which is stable in a very acidic condition.

4.1 Cloning of endoglucanase from *Cryptococcus sp.* S-2

The gene encoding *Cryptococcus* endoglucanase was synthesised commercially by Integrated DNA Technologies (see Appendix II). First the gene was isolated from the original plasmid by conducting a PCR reaction and the size of the gene was 1035bp (figure 4.1). The insert and the purified vector pRSET-A (Appendix III) were double digested with BamHI and HindIII (figure 4.2). After that, the gene was ligated into this pRSET-A, transformed into *E.*

coli TOP10 cells, and screened by plating on an LB agar plate containing 50 µg/mL ampicillin to select for cells which had taken up the plasmid. DNA from a transformant was then purified and sequenced. The *Cryptococcus* cellulase comprises 322 amino acids. Furthermore, a 100% DNA sequence match was also detected (Appendix IV). This suggests that the full-length of the cellulase gene was cloned successfully and was not modified by any mutations during the cloning process.

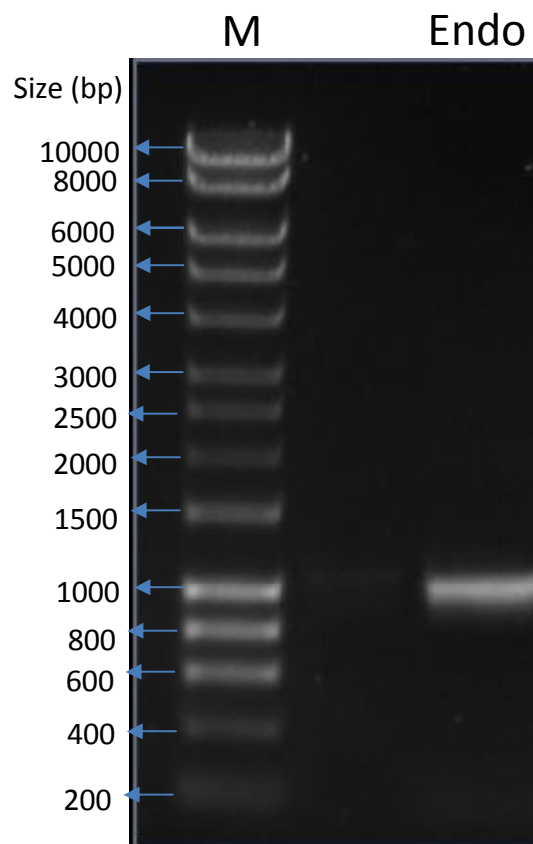


Figure 4.1: The size of the gene was 1035 bp. PCR was done using Phusion[®] High-Fidelity DNA polymerase. Primers used to amplify the gene were BamHI (forward) and HindIII (reverse). M represents DNA marker (Hyperladder I) and Endo represents endoglucanase from *Cryptococcus sp.* Primers sequences can be found in Table 2.5

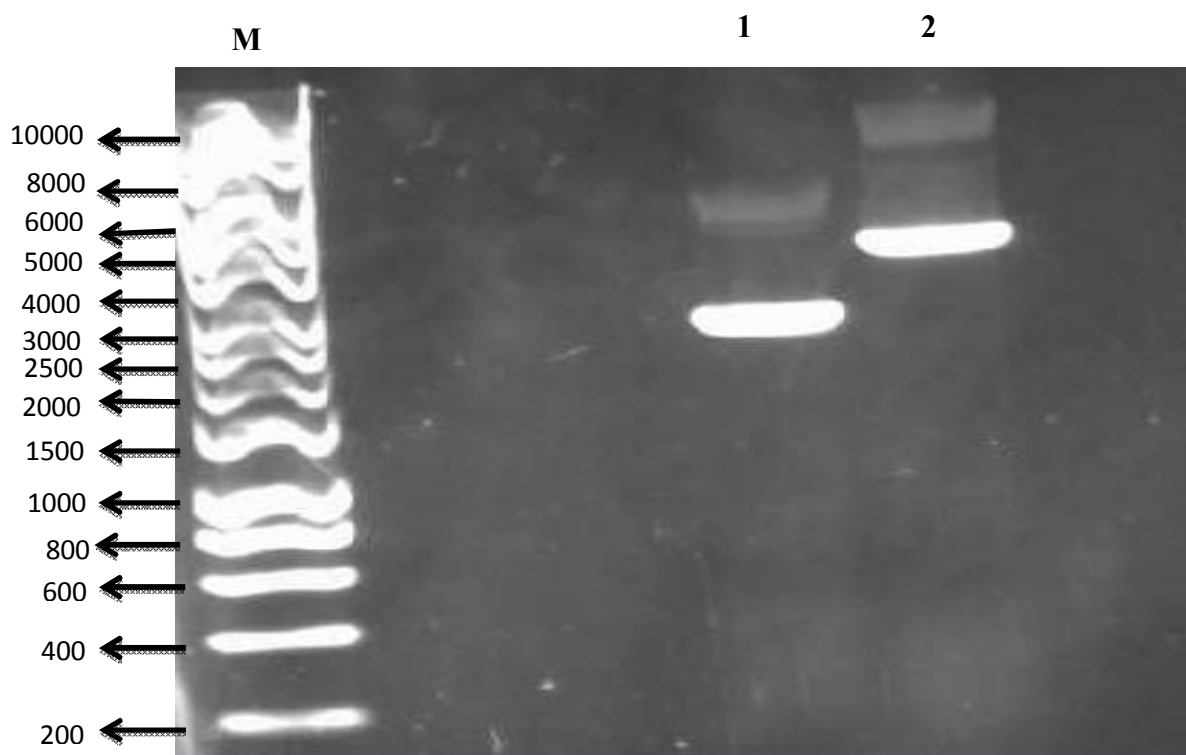


Figure 4.2: Digestion of pRSET-A vector. The pRSET-A underwent double digestion with BamHI and HindIII which resulted in two linearized DNA fragments. M represents DNA marker (Hyperladder I). Lane 1: digested pRSETA. Lane 2: Backbone of plasmid

4.2 Protein Expression

E. coli BL21 (DE3) was chosen first as it was common and widely used for higher expression levels of recombinant protein. BL21 (DE3) was grown in LB supplemented with ampicillin to an OD_{600} of 0.6 at 37°C. The expression was induced with the addition of 0.1mM IPTG and the culture was then incubated at 30°C for 4 hours. Then, SDS-PAGE was conducted and the molecular weight of the purified endoglucanase was determined (figure

4.3). Theoretically, the molecular weight of the protein is 34 kDa (Thongekkaew, J., et al. 2008).

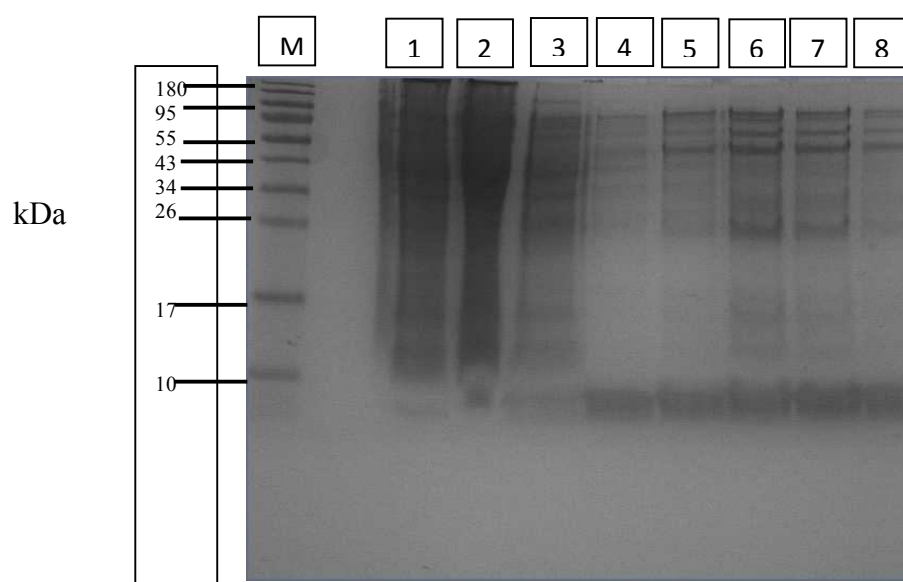


Figure 4.3: Expression using LB Media. Lane M: protein ladder (NEB #P7706), Lane 1: crude extract, Lane 2: flow-through, Lane 3 - 4: washing fraction, Lane 5 - 8: purified protein. Protein samples were extracted from cells grown at 30°C with the addition of 0.1mM IPTG to induce the expression. Protein samples were separated on 12% SDS-PAGE.

By looking at the gel, the intensity of the protein band was not very high, so an optimisation was required to improve the expression and the intensity of the band. In order to optimise the expression, different types of media were used such as 2YT, super broth and terrific broth. Crude extracts of the enzyme (the cell was boiled in loading buffer) were loaded onto an SDS gel to determine which media gave the best expression (figure 4.4). The temperatures used for the expression were 30°C and 37°C. Based on the result, 2YT medium produced a better expression than the other types of media. Therefore, in the next step of experiment, 2YT medium was used to determine the size of the purified protein.

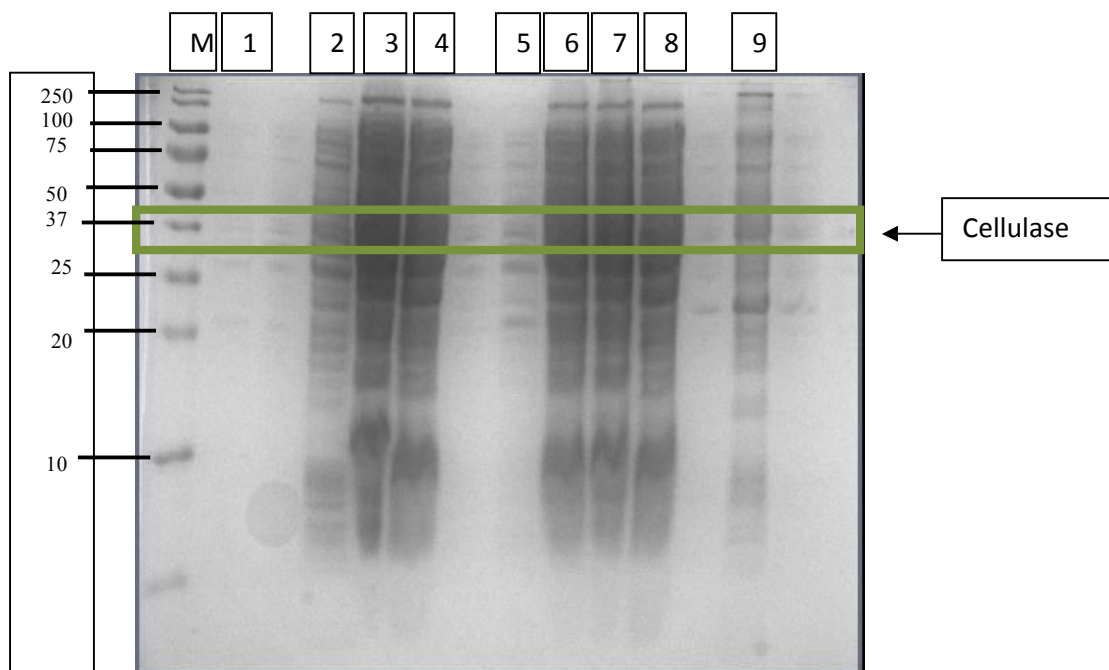


Figure 4.4: Expression using different media. Crude extracts were loaded on SDS gel. Lane 1 to 4, the temperature for the expression was 30°C and Lane 5 to 8, the temperature was 37°C. Lane M: protein ladder (NEB #P7703), Lane 1: Terrific broth, Lane 2: Super broth, Lane 3: 2YT medium, Lane 4: LB medium, Lane 5: Terrific broth, Lane 6: Super broth, Lane 7: 2YT medium, Lane 8: LB medium, Lane 9: Negative control. Protein samples were extracted from cells grown at 30°C and 37°C with the addition of 0.1 mM IPTG to induce the expression. Protein samples were separated on 12% SDS-PAGE.

Figure 4.5 shows the purified protein expressed in 2YT medium and the same expression host BL21 (DE3) and 0.1 mM IPTG was induced at 30°C for 4 hour. However, the intensity of the protein band was not really improved over the initial results in Figure 5.3. This could indicate that the expression was in inclusion bodies because the whole cell was boiled in loading buffer. Proteins produced as inclusion bodies were inactive, aggregated, and insoluble (Demain & Vaishnav, 2009).

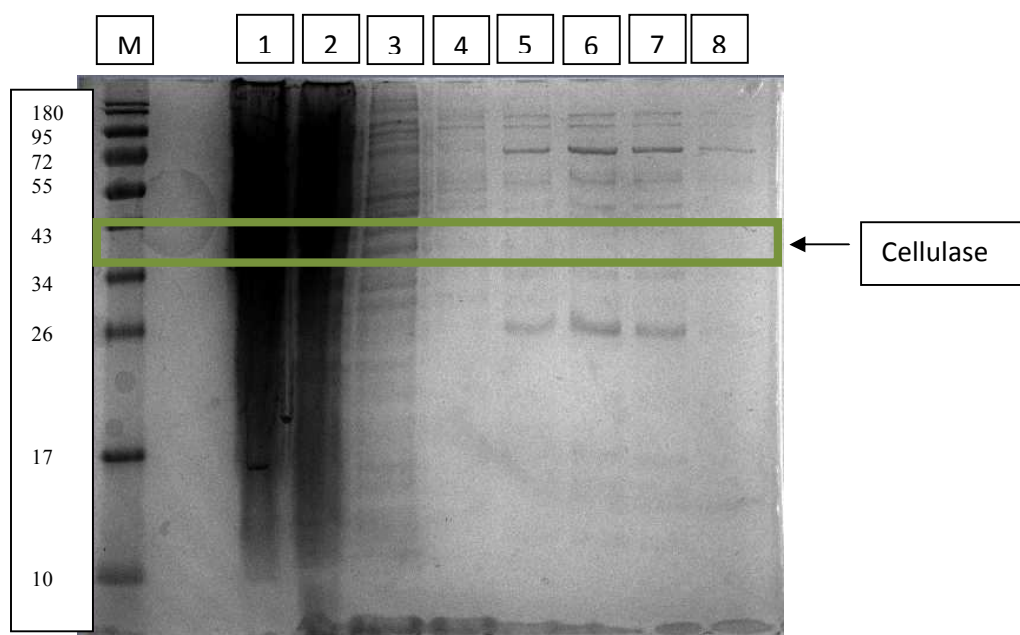


Figure 4.5: Expression using 2YT Media. Lane M: protein ladder (NEB #P7706), Lane 1: crude extract, Lane 2: flow-through, Lane 3 - 4: washing fractions, Lane 5 - 8: purified protein. Protein samples were extracted from cells grown at 30°C for 4 hour with the addition of 0.4 mM IPTG to induce the expression. Protein samples were separated on 12% SDS-PAGE.

A different expression host, Shuffle T7 Express Competent *E.coli* was used instead of BL21 (DE3). Because of the low concentration of protein obtained, SHuffle® T7 Express Competent *E.coli* was chosen to enhance the protein concentration. This type of competent cell is specifically designed to express proteins containing disulphide bonds in the cytoplasm and it is fit for T7 promoter driven protein expression.

The gene was transformed in Shuffle T7 and then grown in 2YT medium supplemented with ampicillin to an O.D₆₀₀ of 0.7 at 37°C. The expression was induced with the addition of 0.4mM IPTG and the culture was then incubated at 30°C for 4 hours (figure 4.6). However, the concentration of the protein was still not very high.

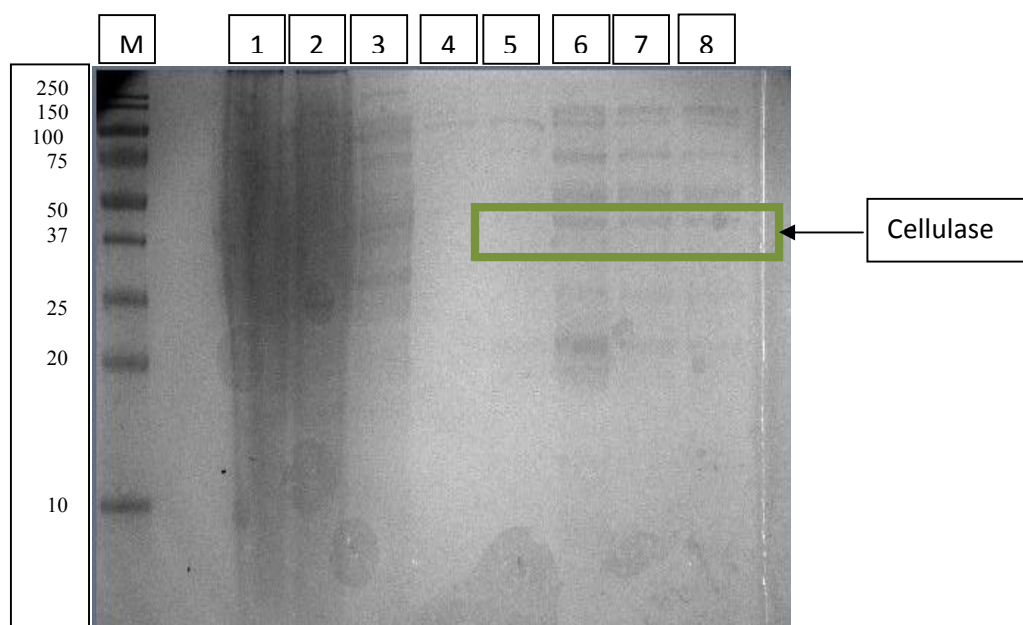


Figure 4.6: Expression using 2YT Media with SHuffle® T7 Express for 16 hours. Lane M: protein ladder (NEB #P7703), Lane 1: crude extract, Lane 2: flow-through, Lane 3 - 4: washing fractions, Lane 5 - 8: purified protein. Protein samples were extracted from cells grown at 30°C with the addition of 0.4 mM IPTG to induce the expression. Protein samples were separated on 12% SDS-PAGE.

Further optimisation was conducted by performing an expression at longer time with the same temperature (30°C). This time, the cells were grown in LB Medium. The expression was induced with 0.4 mM IPTG at 30°C for 16 hours and the intensity of the protein band was similar to the previous results (figure 4.7).

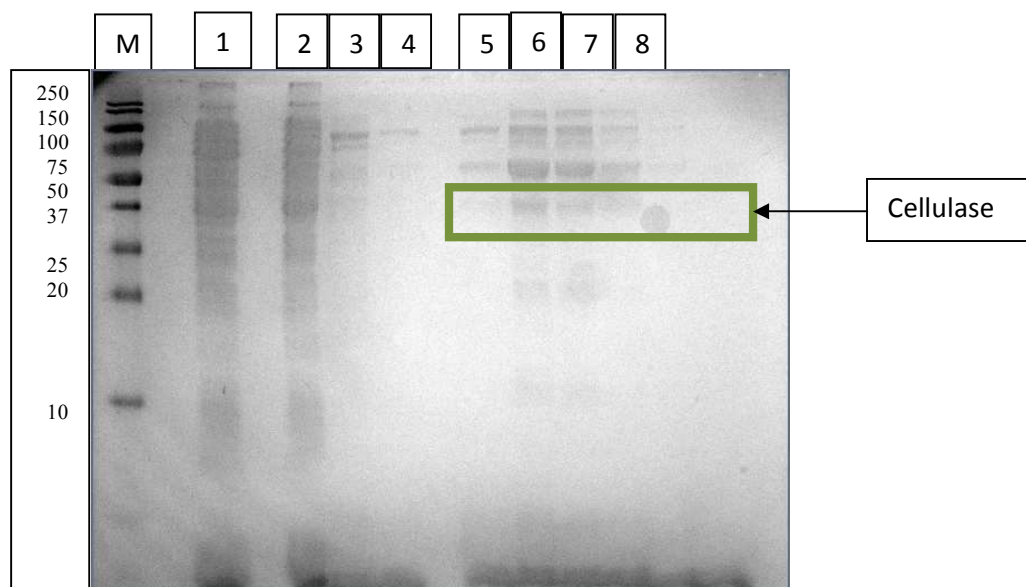


Figure 4.7: Expression in LB Media with SHuffle® T7 Express for 16 hours. Lane M: protein ladder (NEB #P7703), Lane 1: crude extract, Lane 2: flow-through, Lane 3 - 4: washing fractions, Lane 5 - 8: purified protein. Protein samples were extracted from cells grown at 30°C with the addition of 0.4 mM IPTG to induce the expression. Protein samples were separated on 12% SDS-PAGE.

Perhaps, the enzyme required lower temperature for the expression so 0.4 mM IPTG was used to induce expression at 25°C for 16 hours. The protein was purified with Ni-NTA and at this condition, the intensity of the protein band is higher as can be seen in figure 4.8.

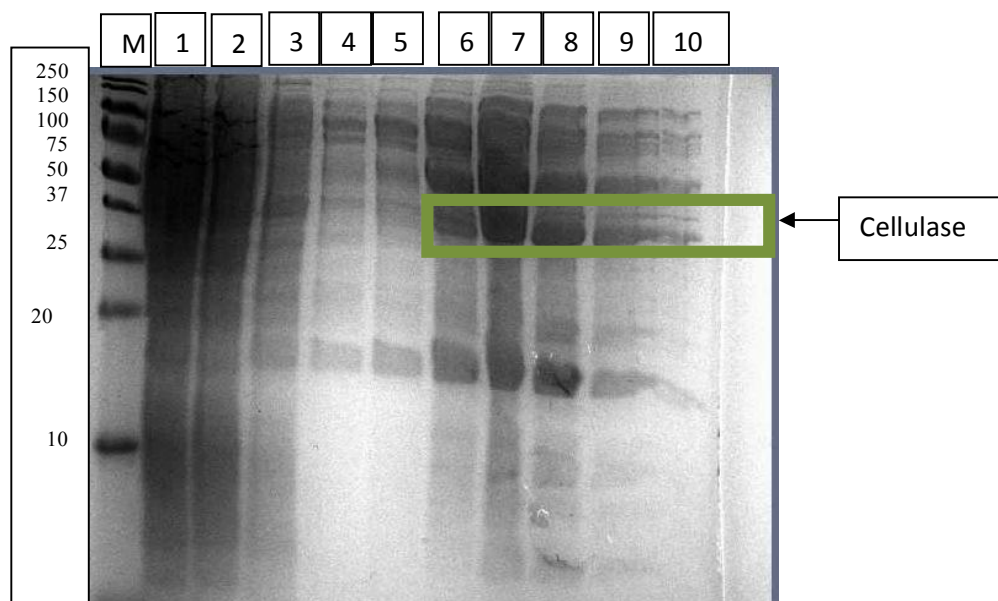


Figure 4.8: Expression in LB Media with SHuffle® T7 Express. Lane M: protein ladder (NEB #P7703), Lane 1-2: crude extract, Lane 3: flow-through, Lane 4-5: washing fractions, Lane 6 - 10: purified protein. Protein samples were extracted from cells grown at 25°C with the addition of 0.4 mM IPTG to induce the expression. Protein samples were separated on 12% SDS-PAGE.

In the figures above, the purified fractions contain many bands in addition to that of the expected size. Perhaps, the concentration used was very high. Therefore, another SDS-gel with less protein concentration was run. The concentration used to load the gel was 0.5 mg/ml. (figure 4.9).

Moreover, another experiment with Congo red was conducted. Endoglucanase activity can be detected in electrophoretic gel with Congo red. This experiment is very specific because only band with endoglucanase will appear yellow against the Congo red (figure 4.10).

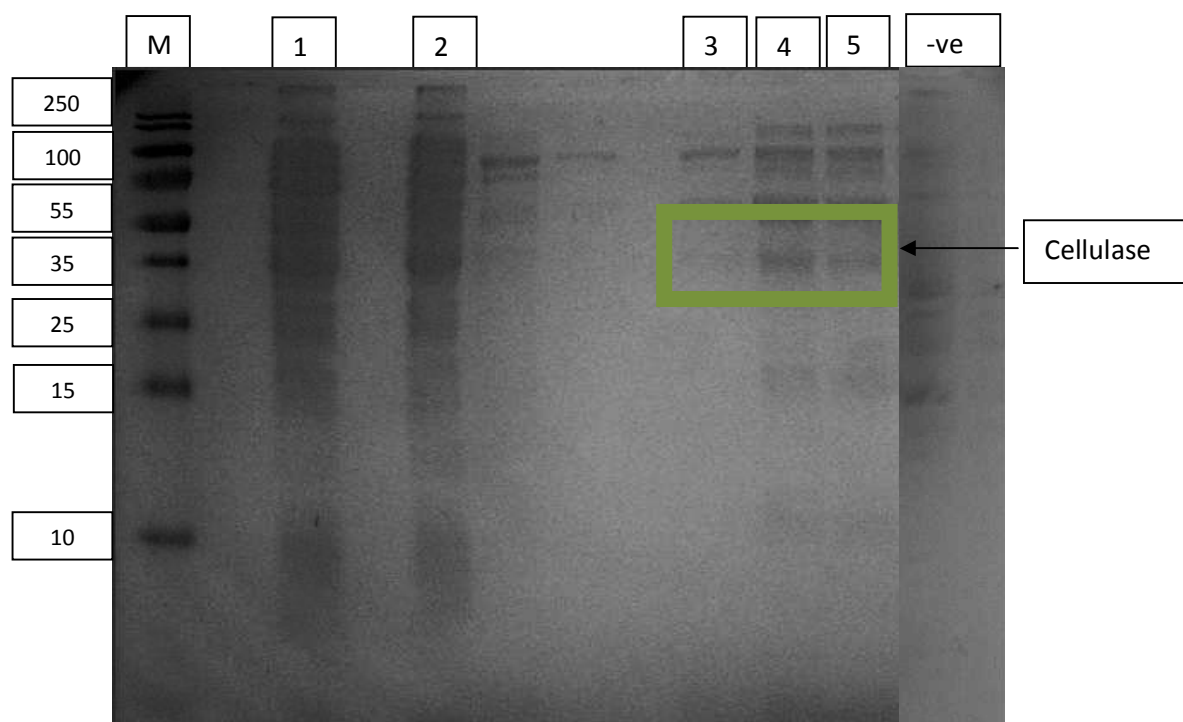


Figure 4.9: Expression in LB Media with SHuffle® T7 Express with less concentration of protein. Lane M: protein ladder (NEB #P7703), Lane 1-2: crude extract, Lane 3-5: purified protein. Lane 6: negative control. Protein samples were extracted from cells grown at 25°C with the addition of 0.4 mM IPTG to induce the expression. Protein samples were separated on 12% SDS-PAGE.

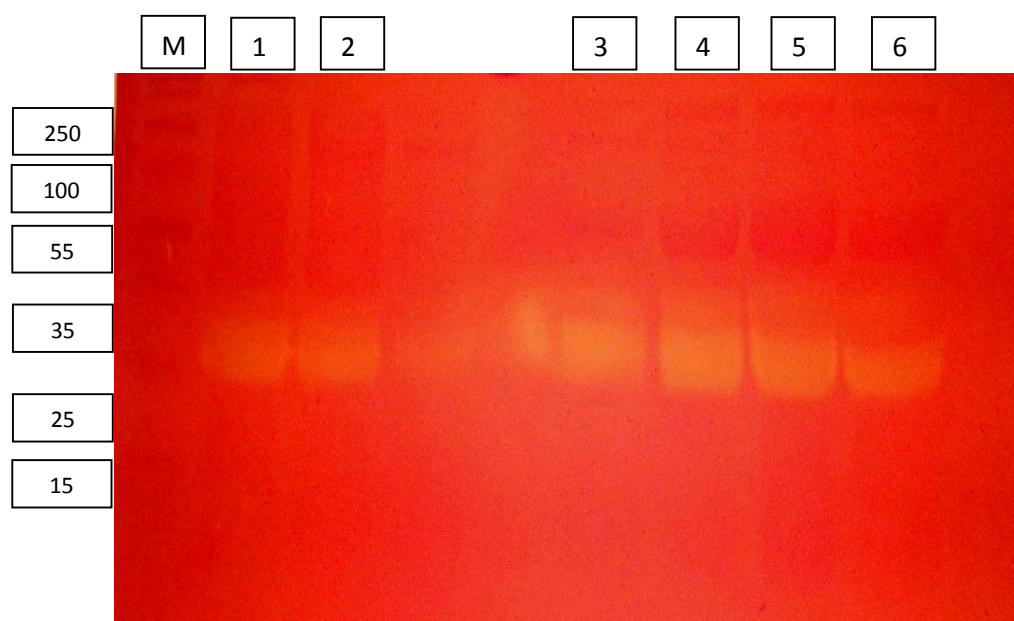


Figure 4.10: Detection of endoglucanase activity in electrophoretic gel with congo red. Lane M: protein ladder (NEB #P7703), Lane 1-2: crude extract, Lane 3-6: purified protein.

Western-blot analysis was conducted to confirm the size of the protein using a mouse anti-his antibody and a chemiluminescence detection kit for mouse antibodies. The size of the protein was 34 kDa (figure 4.11). The size of the protein was in accordance with the one reported previously (Thongekkaew, J., et al. 2008). In addition, very few of the other bands are stained in the Western blot, suggesting that they are contaminating proteins, rather than undenatured oligomers of the protein of interest.

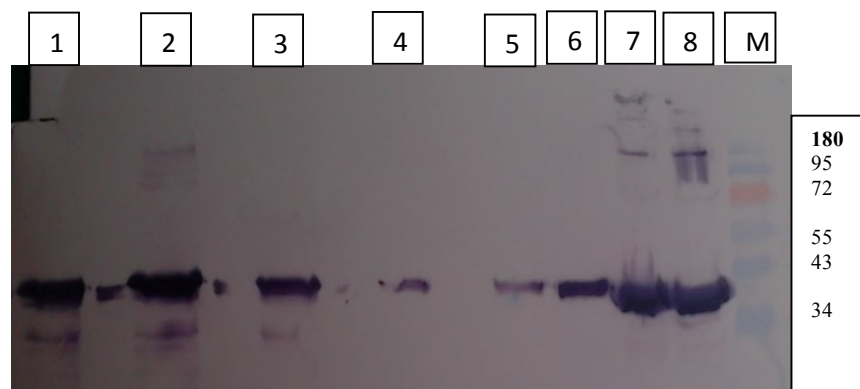


Figure 4.11: Western-blot analysis of endoglucanase expression. Protein samples were extracted from cells grown at 25°C with the addition of 0.4 mM IPTG to induce the expression. Lane 1-4: purified endoglucanase, lane 5-6: washing fractions, lane 7: flow-through, lane 8: crude extracts, lane M: marker (NEB #P7703). Protein samples were separated on 12% SDS-PAGE and transferred onto a PVDF. The endoglucanase size was 34 kDa.

4.3 Specific activity against CMC

The enzyme's specific activity was assayed according to the standard DNSA assay protocol (Ilmberger and Streit, 2010). Figure 4.12 shows a representative glucose standard curve used for calculating the specific activity of the endoglucanase from *Cryptococcus* sp. DNS assay standard plus ILs was also conducted and presented in Appendix VI.

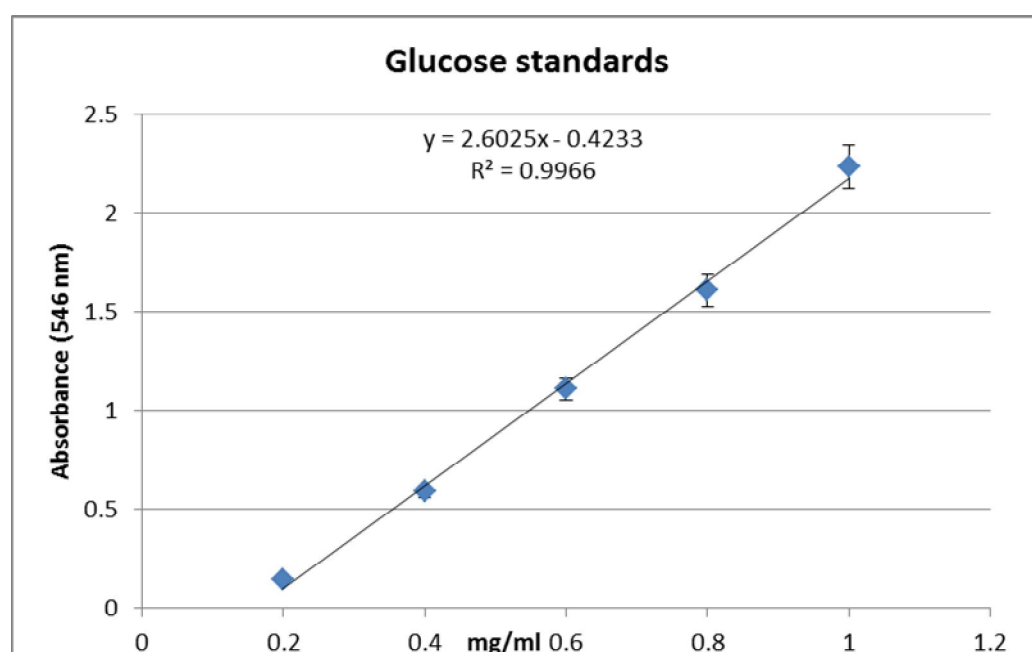


Figure 4.12: Representative standard curve for DNSA Assay for glucose release. Standards of 0.2, 0.4, 0.6, 0.8 and 1 mg/mL glucose were used and placed in a 96-well plate. Absorbance was measured at 546 nm using a synergy HT reader. Error bars represent standard errors between n=3.

The specific activity of recombinant endoglucanase was calculated to be 5.59 U/mg in the standard assay condition of 50 mM citrate buffer pH 4.8. It has been reported previously the specific activity was 4.93 U/mg (Thongekkaew et al., 2008). Specific activity in this research study was better than the previous one possibly because the host expression used was different. In a previous research reported by Thongekkaew et al (2008), they used *P.*

pastoris as host expression. *P. pastoris* is a fungal expression system which has been reported does not ferment sugar and it can avoid the toxic fermentative product ethanol. The lack of toxic products results in very high cell densities and this is why some researchers preferred fungal expression system than *E.coli* (Eugene et al., 2002). However, it was reported by Jeoh et al. (2008), the specific activity of cellobiohydrolases decreased due to the higher N-glycan content on cellobiohydrolases and this enzyme was expressed in fungal expression.

Perhaps, the endoglucanase in *P.pastoris* undergo more glycosylation which can reduce the specific enzyme activity. *E. coli* lack post-transformational modification such as glycosylation therefore endoglucanase from *Cryptococcus sp.* expressed in *E.coli* had higher activity.

Furthermore, specific activity of recombinant endoglucanase in different pH was evaluated. At pH 1, the specific activity was 12.99 U/mg and the activity was decreased to 5.63 U/mg at pH 4 (figure 4.13). The optimum pH for endoglucanase from *Cryptococcus sp.* was 3.5 but in this study the activity of cellulase was higher at pH 1 which was different than what had been reported previously (Thongekkaew et al., 2008). This may be because HCl was used to adjust the pH instead of H₂SO₄ in the previous study. Endoglucanase in this case works better in a stronger acid such as H₂SO₄.

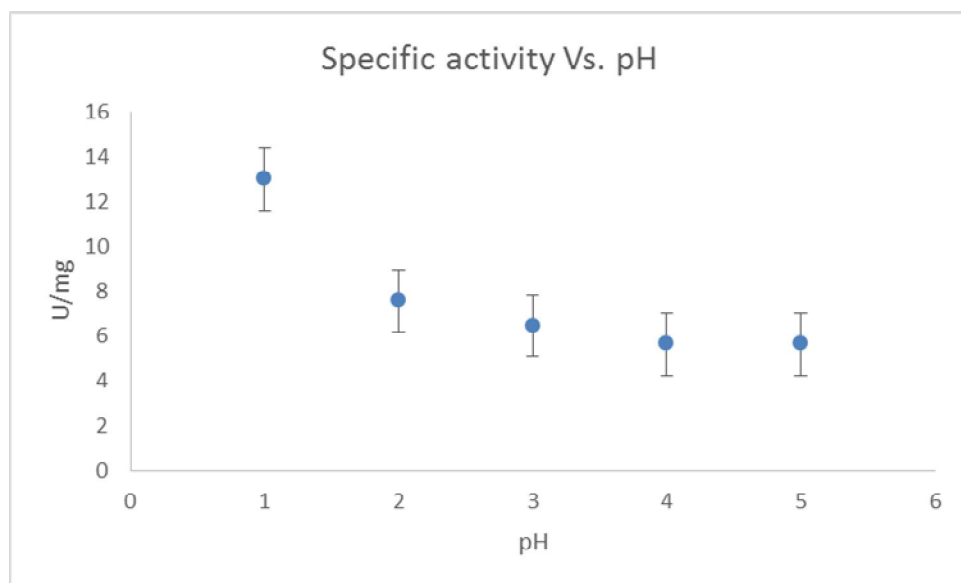


Figure 4.13: pH study of recombinant endoglucanase activity. The enzyme was incubated at pH 1-5 at 37 °C for 15 min. H_2SO_4 was added in the solution to get pH 1-5. Absorbance was measured at 546 nm using a synergy HT reader. Error bars represent standard errors between three determinants. Controls of each pH were conducted to measure the specific activity of the enzyme (Appendix VII).

Furthermore, the activity of recombinant endoglucanase was assayed in the presence of 5, 10 and 20 % (vol/vol) of [HBIM][HSO_4]. As the concentration of this IL increased, the specific activity was increased. The specific activity of this recombinant endoglucanase at 5, 10 and 20% were 5.62, 6.28 and 11.98 U/mg, respectively (figure 4.14).

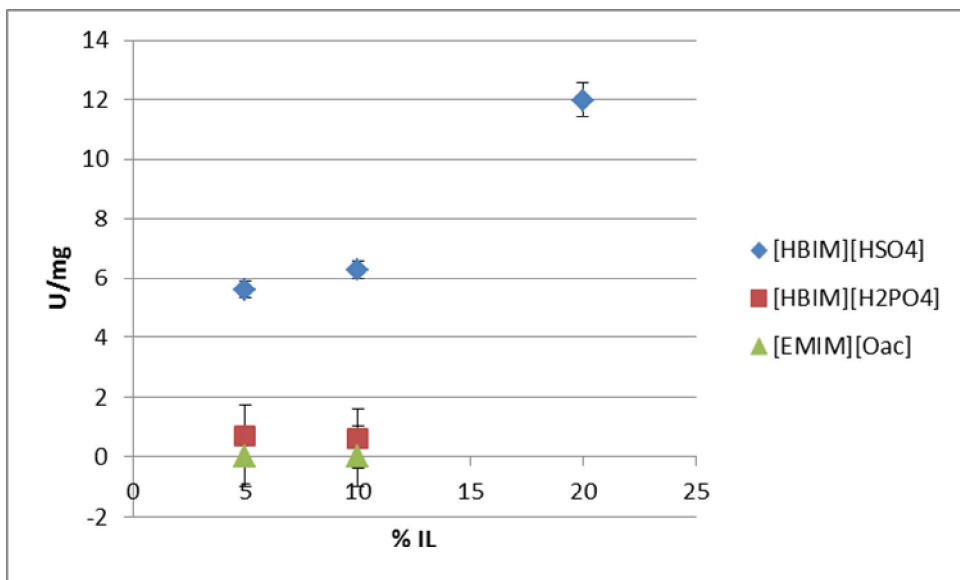


Figure 4.14: Specific Activity of recombinant endoglucanase from *Cryptococcus sp.* with ILs. The enzyme was incubated in 5,10 and 20% [HBIM][HSO₄], 5 and 10% [HBIM][H₂PO₄] and 5 and 10% [EMIM][OAc] for 20 min. Absorbance was measured at 546 nm using a synergy HT reader. Error bars represent standard errors between three determinants.(see Appendix VI for the glucose standards with each ILs).

Moreover, the enzyme had almost similar activity in 5% and 10% [EMIM][OAc], but the activity was very low. At the concentration of 5 and 10% (vol/vol), the specific activities were 0.04 U/mg and 0.03 U/mg, respectively (figure 4.14). This was most likely because the optimum pH of endoglucanase from *Cryptococcus sp.* was 1 and the pH of [EMIM][OAc] is 13. Therefore, it can be observed that endoglucanase activity from *Cryptococcus sp.* was higher in [HBIM][HSO₄] than in [EMIM][OAc].

The activity of this recombinant endoglucanase with the presence of 5 and 10% (vol/vol) [HBIM][H₂PO₄] was conducted and the specific activity was 0.72 U/mg and 0.62 U/mg, respectively. In chapter 4, phosphate based IL had more yield of glucan than in sulphate based IL but because endoglucanase from *Cryptococcus sp.* works better in a lower pH, activity of this enzyme is lower in [HBIM][H₂PO₄] than in [HBIM][HSO₄].

A temperature study was conducted in order to evaluate the optimum temperature for this enzyme at pH 1. The optimum temperature of this enzyme was 37°C as shown in figure 4.15. It has been reported by Thongekkaew (Thongekkaew et al., 2008) the optimum temperature was 40 - 50°C. One of the reasons why the result reported here might differ from the previous result was due to the different host expression used. In the previous study, they used *P. pastoris* but in this study Shuffle T7 *E.coli* had been used. Perhaps, *P. pastoris* was a better host expression for higher temperature stability compared to endoglucanase expressed in *E.coli*. One of the reasons might be that glycosylation is important post-translational modification in achieving cellulase with higher thermal stability. (Voutilainen et al. 2010), found that glycosylation improved thermal stability of cellobiohydrolase.

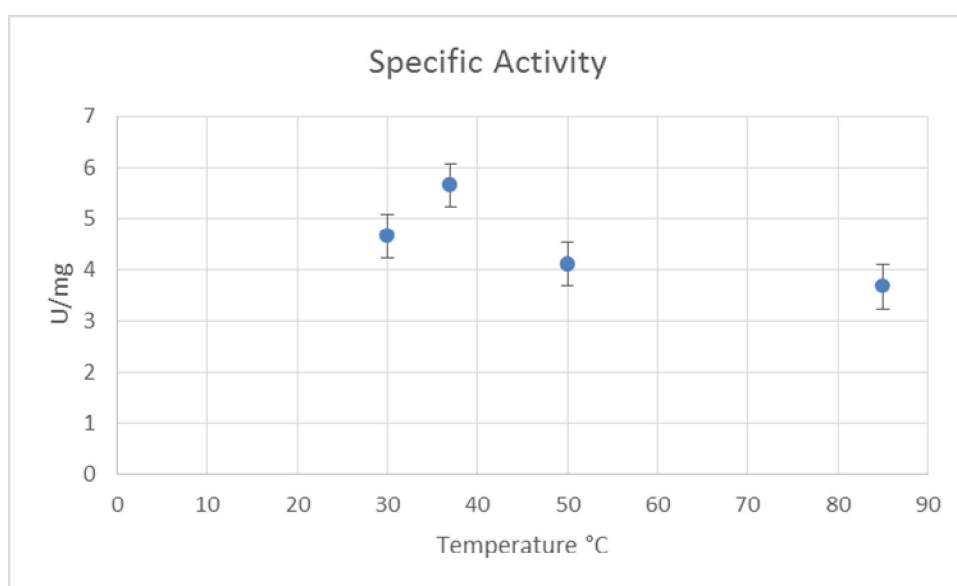


Figure 4.15: Temperature activity of the enzyme at 30°C, 37°C, 50°C and 85°C. The enzyme was incubated in 100mM citrate buffer (pH 1) for 20 min. Absorbance was measured at 546 nm using a synergy HT reader. Error bars represent standard errors between three determinants.

4.4 DISCUSSION

In previous research, *Pichia pastoris* has been used as the host to express recombinant endoglucanase from *Cryptococcus* SP-2 (Thongekkaew et al., 2013). However, in this study *E. coli* T7 shuffle was used and the advantages of using *E. coli* strains are that it is easy to get commercial vectors for use with this organism, easy to perform genetic manipulation and cost effective (Lambertz et al., 2014). However, *E. coli* also has disadvantages in terms of low production of a soluble protein and the ability to secrete proteins is low. It has been reported by Garvey et al. 2013, different host production system will result in different yields of cellulase. For example 1000 mg/l crude enzyme solution (cellulase) can be produced in *Saccharomyces cerevisiae* and only 11.2 to 90 mg/l enzyme solution was produced in *E. coli*. It appears that using yeast as a host production can produce higher amount of enzyme compared to *E. coli*. In this research study, *E. coli* was selected as the expression host, so time was invested in small scale expression for optimisation of yield. *E. coli* can grow very fast and the process of producing recombinant protein in *E. coli* takes only one week. Development in yeast can take over a month, while in mammalian cells after development of a stable cell line required a minimum of three months (Brondyk, 2009). The enzyme specific activity was higher for enzyme produced in shuffle T7 *E. coli* than in *Pichia pastoris* probably because Shuffle T7 *E. coli* produces a disulphide bond isomerase in the cytoplasm to facilitate protein folding. It was reported that disulphide bonds can enhance the stability of a protein (Khoo and Norton, 2012). Furthermore, in terms of temperature stability, endoglucanase expressed in *P. pastoris* exhibited a higher temperature than endoglucanase expressed in *E. coli*. It was known that some proteins require glycosylation which can be absent in *E. coli* but exists in *P. pastoris*. Although endoglucanase expressed in *E. coli* had lower temperature

stability, the enzyme activity in [HBIM][HSO₄] showed a promising activity. Until now, there was no study reported the activity of endoglucanase in the presence sulphate based ILs. The investigated endoglucanase from *Cryptococcus sp.* showed remarkable activity in the presence of 5, 10, and 20% [HBIM][HSO₄]. Therefore, this enzyme can be a promising enzyme in the future research on biomass processing using sulphate based ILs as the pretreatment method.

CHAPTER 5: ACIDOPHILIC β -GLUCOSIDASE FROM *THERMOPHILUM PENDENS*

This chapter describes expression and purification of β -glucosidase from *Thermophilum pendens* and the activity of this enzyme with IL. β -glucosidase is a type of enzyme that hydrolyses β -glucosidic bonds and then releases glucose (Li et al., 2013). A β -glucosidase with acid-tolerance is required to improve the enzyme performance in converting the cellobiose to glucose in acidic environment. Moreover, acidophilic enzymes are important in bioethanol production from lignocellulose because the pretreatment process is more efficient in acidic environment in order to obtain high sugar yields (Galbe and Zacchi 2007).

Based on the results of the experiments in chapter 3, commercial β -glucosidase from *Aspergillus niger*, resulted in low percent of glucan release, which was 3.9% in the presence of 1% [TEA] [HSO₄]. Therefore, β -glucosidase compatible with IL is required to improve the percentage of glucan produced with the presence of sulphate based ILs. In order to find acid-tolerant β -glucosidase, a literature review was conducted and it was found that β -glucosidase from *T.pendens* showed maximum activity at pH 3.5. *T.pendens* is an anaerobic microorganism and is sulphur-dependent (Li et al., 2013). In chapter 3, the results showed that sulphate based ILs and low pH were two main factors that affect the activity of the enzyme, so *T.pendens* was selected in this research study because it is known as sulphur-dependent and has a maximum activity at pH 3.5. This chapter presents results on recombinant β -glucosidase from *T.pendens* and its activity with the presence of [TEA][HSO₄]. In addition, the combined activity of the endoglucanase from *Cryptococcus* sp. SP-2 and the β -glucosidase from *T.pendens* on two different cellulosic substrates in the presence and absence of IL was studied.

5.1 β -glucosidase expression

E.coli Shuffle T7 transformed with a commercial plasmid containing β -glucosidase (see Appendix VIII for the gene sequences) from *T.pendens* (GeneArt) was induced with 0.4 mM IPTG for 18 hours at 250 rpm and 30°C in a flask containing 500 ml LB supplemented with kanamycin (50 μ g/ml). Then, purification of β -glucosidase was performed with Nickel Immobilised Metal Affinity Chromatography (Ni-IMAC). Soluble protein was incubated with and bound to the resin to maximise the binding and elution was carried out with the elution buffer and analysed for purity via SDS-PAGE. The size of the protein was 77 kDa (figure 5.1). The size of this enzyme reported previously (Li et al., 2013) was 77.8 kDa which is similar in this research study and it has 701 amino acids.

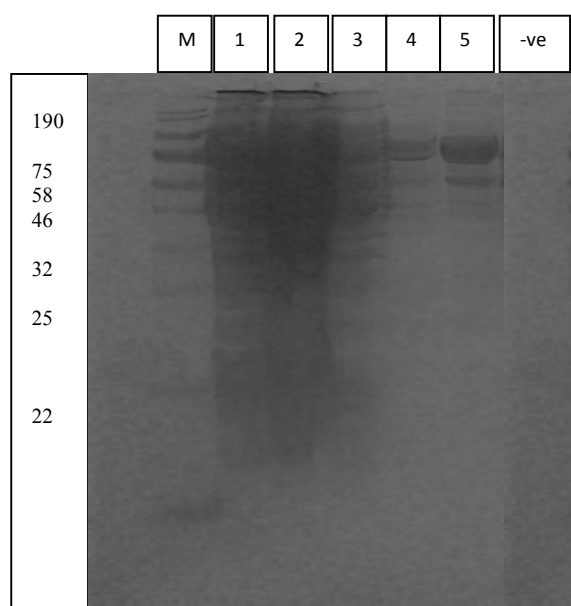


Figure 5.1: Expression and purification of β -glucosidase. Lane M: protein ladder (NEB #P7706), Lane 1: crude extract, Lane 2: flow-through, Lane 3 - 4: washing fraction, Lane 5 : purified protein. Lane 6: Negative control. Protein samples were extracted from cells grown at 30°C with the addition of 0.4 mM IPTG to induce the expression. Protein samples were separated on 12% SDS-PAGE.

5.2 β -glucosidase assay using p-Nitrophenyl- β -D-Glucoside (pNPG)

β -glucosidase was assayed according to the standard pNPG assay protocol (Zhang et al., 2009). Figure 5.2 was one of the p-nitrophenol standard curve used for calculating the enzyme activity of the β -glucosidase from *T.pendens*. The enzyme activity was calculated on the basis of the linear range between absorbance and enzyme concentrations.

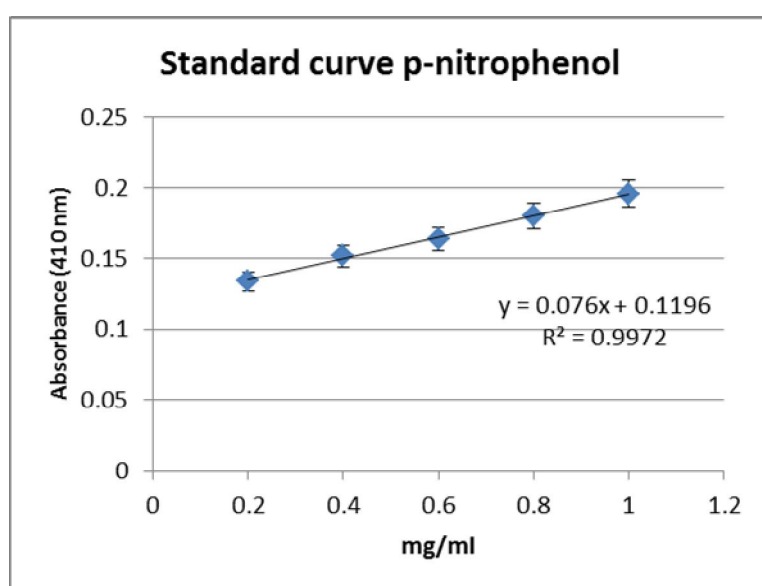


Figure 5.2: Representative of standard curve of p-nitrophenol. Standards of 0.2, 0.4, 0.6, 0.8 and 1 mg/mL p-nitrophenol were used and placed in a 96-well plate. The pH solution is 3.5. Absorbance was measured at 430 nm using a synergy HT reader.

β -glucosidase specific activity was calculated to be 27.73 U/mg. It has been reported previously, the specific activity was 7.6 U/mg (Li et al., 2013). The specific activity was higher in this study and perhaps this was because of different host expression used. In this research study, β -glucosidase gene was expressed in shuffle T7 competent *E.coli* because this competent cell has a chaperone known as DsbC which can improved protein folding even of proteins that do not have disulphide bonds (Chen et al., 1999).

Moreover, a temperature study was conducted in order to evaluate the optimum temperature for this enzyme. The optimum temperature of this enzyme was 90°C as shown in figure 5.3. This result was in accordance with the one reported previously in which the enzyme exhibited a high activity at 90°C (Li et al., 2013). Besides *T.pendens*, β -glucosidase from *T.neapolitana*, *S.solfataricus*, and *P.kodakarensis* exhibited a high thermostability but these enzymes did not show a high activity at lower pH. *T.pendens* had a high activity at lower pH and at higher temperature (Li et al., 2013).

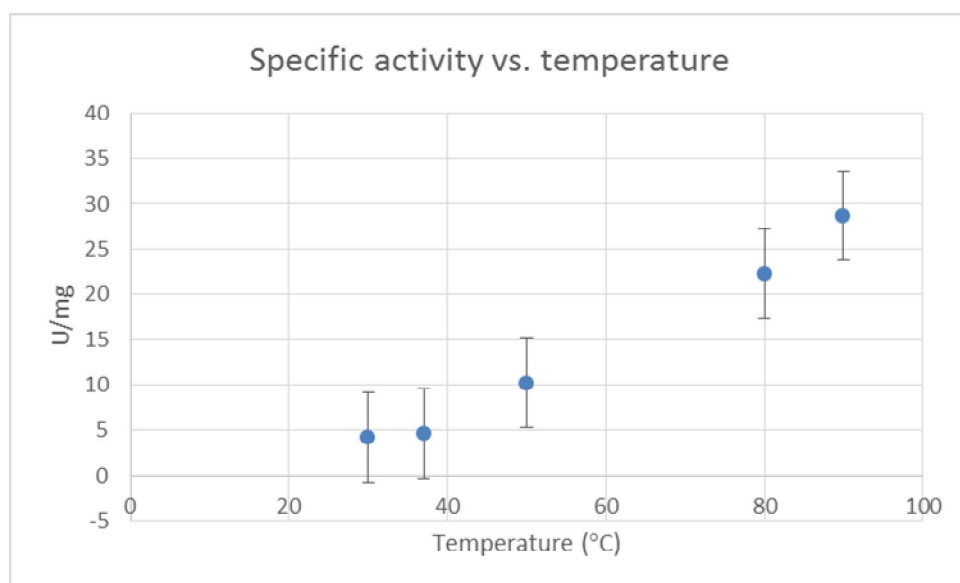


Figure 5.3: Temperature activity of the enzyme at 30°C, 37°C, 50°C, 80°C and 90°C. The enzyme was incubated in 100mM citrate buffer pH 3.5 for 30 min. Error bars represent standard errors between three determinants.

5.3 Standard curve of p-nitrophenol in IL

Moreover, the activity of β -glucosidase in the presence of IL was tested. Standard curve of p-nitrophenol in IL was constructed to ensure that IL do not interfere the absorbance of p-nitrophenol. Figure 5.4 showed that, when IL was added in p-nitrophenol, there was a problem with the absorbance as the curve did not form a straight line. Because of this, PNPG

assay cannot be used to test the activity of β -glucosidase in ILs. Because of this, activity of β -glucosidase in IL was conducted with HPLC using cellobiose as the substrate.

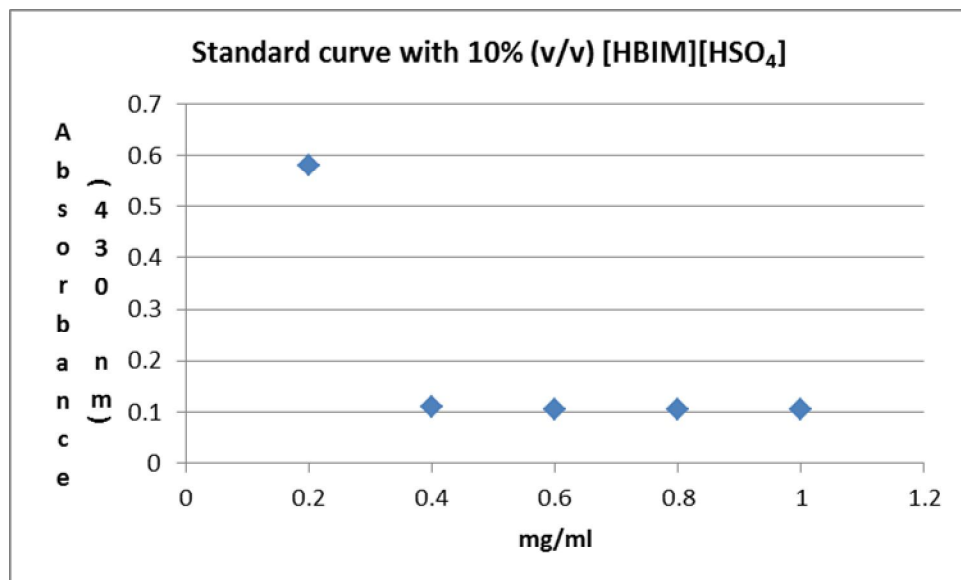


Figure 5.4: Standard curve of p-nitrophenol in 10% (v/v) [HBIM][HSO₄]. Standards of 0.2, 0.4, 0.6, 0.8 and 1 mg/mL p-nitrophenol were used and placed in a 96-well plate. Absorbance was measured at 430 nm using a synergy HT reader.

5.4 β -glucosidase activity with cellobiose

β -glucosidase activity was studied using cellobiose as the substrate. HPLC was used to analyse the conversion of cellobiose to glucose using β -glucosidase from *T.pendens*. Figure 5.5 shows the kinetic activity of this enzyme from 24 to 72 hours. It seems that the percentage of glucan yield increased from 24 to 72 hours, which results in 65% of glucan released. This experiment was conducted to determine how long it takes for the β -glucosidase from *T.pendens* to change cellobiose to glucose. It seems it required 72 hours to get 65% of glucan. In chapter 3, when 1% [TEA] [HSO₄] was present in the enzymatic saccharification mixture containing β -glucosidase from *A. niger*, only 3.9% glucan was produced (compared to more than 65% in the absence of IL) but β -glucosidase from *T.pendens* showed 7.7% of glucan in 1% [TEA] [HSO₄] which is less of a decrease.

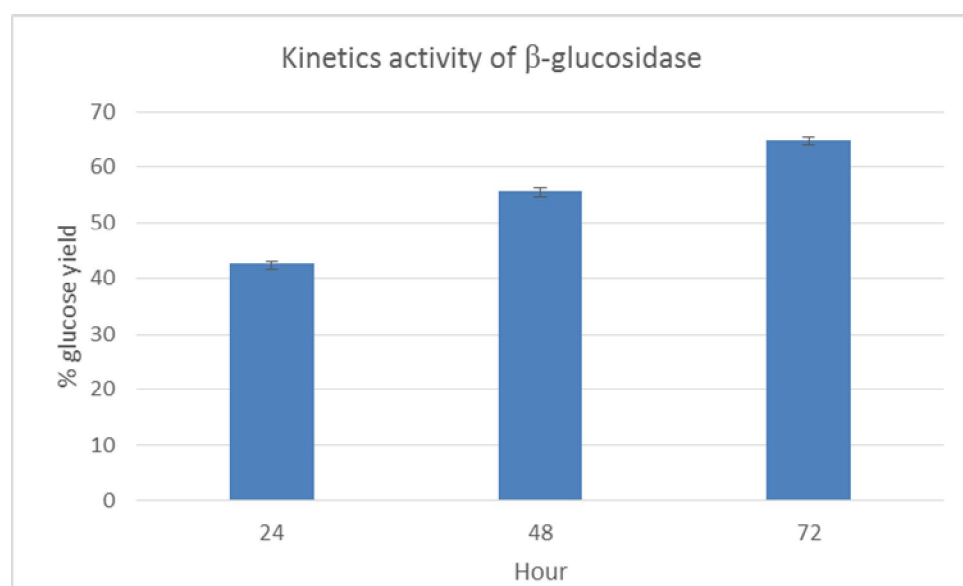


Figure 5.5: Kinetic activity of β -glucosidase (*T.pendens*) from 24 to 72 hour. No IL was added in the solution. HPLC was used to determine the percentage of glucose yield. Error bars represent standard errors between three determinants.

When compared with the result in chapter 4, there was a slight improvement between β -glucosidase from *T.pendens* compared with commercial β -glucosidase from *A.niger*. So far, no experimental studies have been conducted on the effect of β -glucosidase activity with sulfate based IL. The effects of β -glucosidase from *A.niger* with [DMIM] [DMP] had been studied previously and it was observed that β -glucosidase was more sensitive than endoglucanase and lost its activity in 15% [DMIM] [DMP] (Engel et al., 2012).

5.5 Saccharification using endoglucanase from *Cryptococcus sp.* and β -glucosidase from *T.pendens*

Enzymatic saccharification using endoglucanase from *Cryptococcus sp.* and β -glucosidase from *T.pendens* together was conducted. Similar procedure from NREL was applied except that the enzyme used was different. Figure 5.7 was one of the standard curves used for HPLC analysis of cellobiose released. Based on the result obtained, there was no cellobiose or glucose detected by the HPLC when using both of these enzymes with the presence of 1, 2, 5, and 10% of [HBIM][HSO₄]. However, the presence of cellobiose was detected when 1 and 2 % of [TEA][HSO₄] were added in the enzymatic saccharification solution with Sigmacell as the substrate (Retention Time =7.245 min) (Table 5.1).

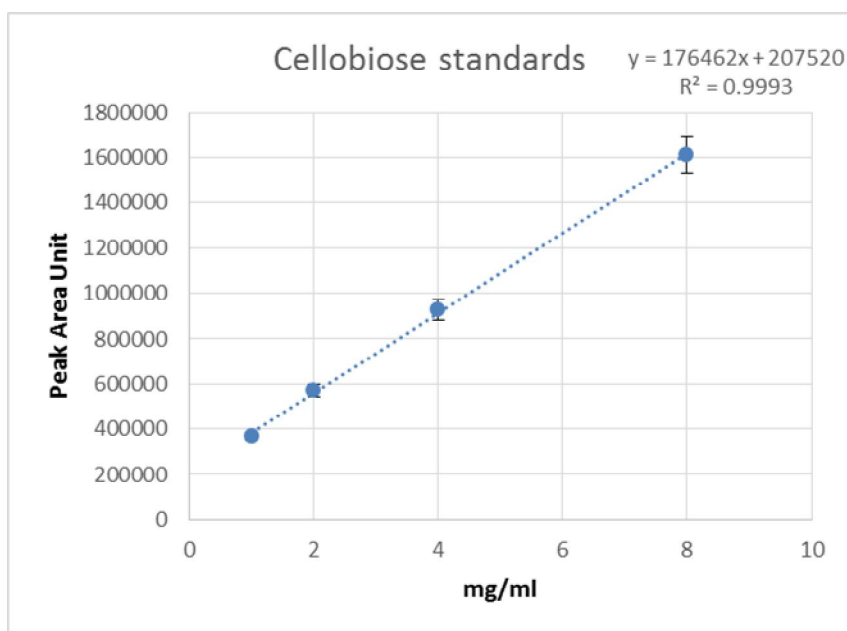


Figure 5.7: Representative standard curve for HPLC analysis of cellobiose release. Standards of 1, 2, 4, and 8 mg/mL cellobiose were run on a Shimadzu HPLC with a flowrate of 0.6 ml/min and a total run time of 10 minutes. 10 mM sulfuric acid was used as the mobile phase.

Table 5.1: Peak area unit showing the presence of cellobiose after performing enzymatic saccharification with 1 and 2% [TEA][HSO₄] with both enzymes. Samples were run on a Shimadzu HPLC with a flowrate of 0.6 ml/min and a total run time of 10 minutes. 10 mM sulphuric acid was used as the mobile phase.

No		Retention time	Peak Area Unit
1	1%[TEA]HSO ₄	6.342	708760
2	2%[TEA]HSO ₄	6.342	1353437
3	Cellobiose	7.245	4816 (1%[TEA]HSO ₄) 4107 (2%[TEA]HSO ₄)
4	Citrate buffer	8.066	1425035

Table 5.2: Peak area unit showing no presence of cellobiose after performing enzymatic saccharification with [TEA][HSO₄] only. Samples were run on a Shimadzu HPLC with a flowrate of 0.6 ml/min and a total run time of 10 minutes. 10 mM sulphuric acid was used as the mobile phase.

No		Retention time	Peak Area Unit
1	1%[TEA][HSO ₄]	6.342	573910
2	2%[TEA][HSO ₄]	6.342	1194328
3	5%[TEA][HSO ₄]	6.342	3164178
4	10%[TEA][HSO ₄]	6.342	5858894
5	Citrate buffer	8.066	1538703

In order to confirm that Sigmacell was not hydrolysed by [TEA][HSO₄], another experiment on enzymatic saccharification was conducted. This time, no enzymes were added. Based on table 5.2, there was no peak for cellobiose present. Also, a control experiment was conducted in which no enzyme or [TEA][HSO₄] was added in the saccharification solution and from the chromatogram, only a peak for citrate buffer was present. Based on the data, it seems that those enzymes had an activity on Sigmacell since the HPLC can detect the presence of cellobiose.

Furthermore, another experiment was performed in order to determine the activity of endoglucanase only on Sigmacell. It was found that no cellobiose was detected. Also, when only β -glucosidase was tested on Sigmacell, there was no cellobiose present. Cellobiose can only be detected when endoglucanase from *Cryptococcus* sp. and β -glucosidase from *T.pendens* were added in the mixture of saccharification with Sigmacell together.

According to Thongekkaew et al (2008), endoglucanase from *Cryptococcus* sp. cannot hydrolyse Sigmacell and Avicel but it can hydrolyse CMC. CMC is soluble and less crystalline than Sigmacell. This might be the reason why there was no glucose detected when enzymatic saccharification on Sigmacell was performed using endoglucanase from *Cryptococcus* sp. and β -glucosidase from *T.pendens*. One of the reasons why endoglucanase from *Cryptococcus* sp. cannot hydrolyse insoluble substrate was because this enzyme has no

cellulose binding domain (CBD) (Thongekkaew et al. 2013). *T.reesei* cellulase has CBD and this helps the cellulase from *T.reesei* to hydrolyse insoluble cellulose substrates. Further, a study conducted by Thongekkaew et al., (2013) showed that when CBD of *T.reesei* was fused with endoglucanase from *Cryptococcus sp.*, this enzyme improved cellulolytic activity against Sigmacell. Since it was known that endoglucanase from *Cryptococcus sp.* cannot hydrolyse insoluble substrate, the next experiment was to investigate the effect of the combination of β -glucosidase and endoglucanase from *Cryptococcus sp.* with CMC. Figure 5.11 was one of the standard curves used for HPLC analysis of carboxymethylglucose released.

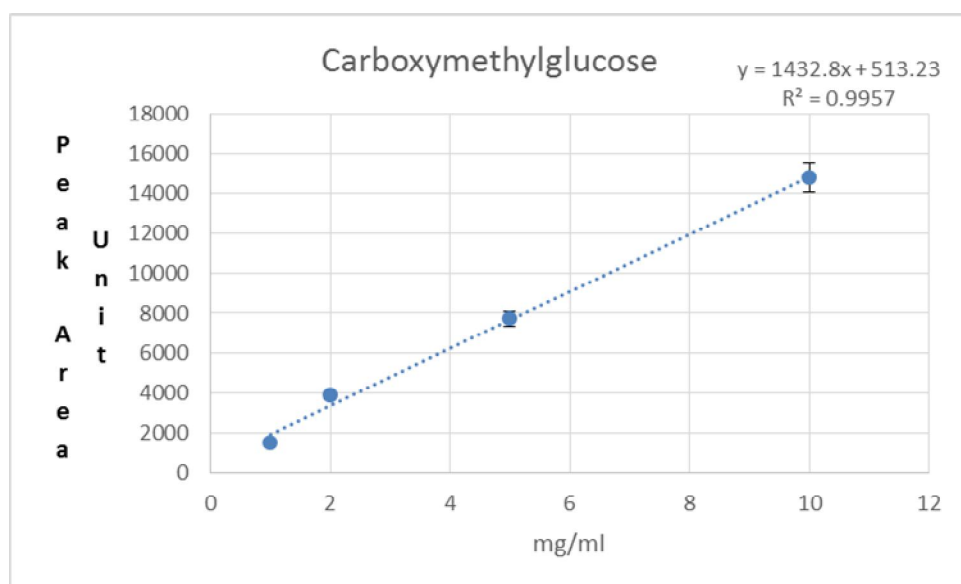


Figure 5.11: Representative standard curve for HPLC analysis of carboxymethylglucose release. Standards of 1, 2, 5, and 10 mg/mL carboxymethylglucose were run on a Shimadzu HPLC with a flowrate of 0.6 ml/min and a total run time of 10 minutes. 10 mM sulphuric acid was used as the mobile phase. Error bars represent standard errors between three determinants.

It was observed that, at the concentrations of 2 and 5% (v/v) of [TEA][HSO₄] in the saccharification mixture, there was a considerable increase in carboxymethylglucose released compared to 1% [TEA][HSO₄]. The activity slightly decreased at 10% (v/v) [TEA][HSO₄], but still the results showed that 52% carboxymethylglucose released from saccharification solutions containing 10% [TEA] [HSO₄] (figure 5.12). When compared this result in chapter

3, this result showed an improvement in the activity of enzyme with 2,5, and 10% of [TEA] [HSO₄].

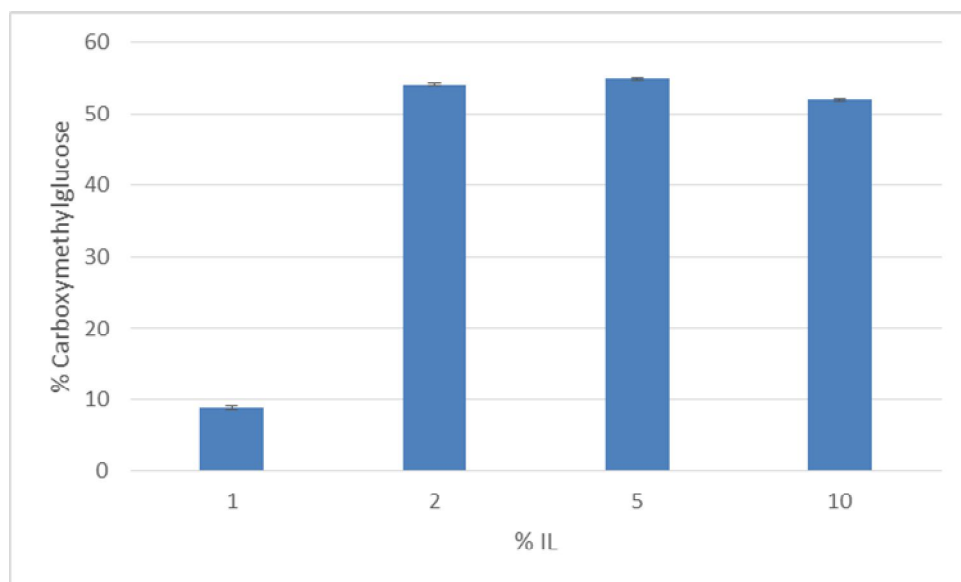


Figure 5.12: Activity of β -glucosidase (*T.pendens*) and endoglucanase from *Cryptococcus sp.* with 1,2,5 and 10% of [TEA] [HSO₄] on CMC as the substrate. Enzymatic saccharification was conducted for 24 hours and HPLC was used to analyse the glucan yield. Error bars represent standard errors between three determinants.

5.6 DISCUSSION

The results of experiments carried out in this chapter showed that there was an improvement in terms of the specific activity of β -glucosidase from *T.pendens* expressed in shuffle T7 *E.coli*. The specific activity was 27.73 U/mg which was higher than the one reported previously (Li et al., 2013). This could be due to the presence of a chaperone known as DsbC which can promote the folding of proteins which do not have disulphide bonds. In term of thermal stability, this enzyme showed optimum activity at 90°C which is considered high. Li et al., (2013) reported the same thing for thermal stability so this enzyme can be used in

industrial application where higher temperature was required. Moreover, there was a slight improvement in the activity of β -glucosidase from *T.pendens* in 1% (v/v) [TEA][HSO₄] compared to β -glucosidase from *A.niger*. According to Singhanian et al., (2013), some β -glucosidases demonstrate high activity on pNPG but not on cellobiose. Perhaps, β -glucosidase from *T.pendens* works better on pNPG than on cellobiose

In addition, it was observed that there was little cellobiose released when β -glucosidase from *T.pendens* combined with endoglucanase from *Cryptococcus sp.* was used in the saccharification of Sigmacell. This was due to the incompatibility of the substrate used in which endoglucanase from *Cryptococcus sp.* cannot hydrolyse insoluble cellulose substrates. However, when CMC was used to test the activity of the enzyme, there was 52% activity detected in the presence of 10% [TEA] [HSO₄]. As mentioned previously, both of these enzymes can react in a very acidic condition. Compared with the previous experiments as portrayed in chapter 3 where no enzyme activity can be observed when 10% [TEA][HSO₄] was included in the enzymatic saccharification mixtures the activity of these enzymes is higher. Based on the results obtained, β -glucosidase from *T.pendens* can be a useful enzyme with sulphate based ILs as this enzyme showed an activity in the presence of 10% [TEA][HSO₄] with CMC.

Based from the data obtained, the specific activity of β -glucosidase with the presence of IL using pNPG assay cannot be measured. Therefore, CMC was used to investigate the reaction of β -glucosidase and endoglucanase. Interestingly, there was a synergistic action when both of these enzymes were used. In previous chapter, DNS assay was used to measure the specific activity of endoglucanase with ILs but this assay was not specific as it can only measure the reducing sugar and not glucose. Unlike saccharification assay, the assay is more specific and we can analysed how much of the carboxymethylglucose present from the CMC. What is interesting is that β -glucosidase from *T.pendens* can produce carboxymethylglucose

when combine with endoglucanase from *Cryptococcus sp.* It seems that there is a synergistic action between endoglucanase from *Cryptococcus sp.* and β -glucosidase from *T.pendens*.

CHAPTER 6: CONCLUSIONS AND FUTURE WORK

6.1 Thesis summary

The work presented in this thesis studied the effect of ILs toward commercial cellulase and cellobiose activity. ILs have a negative impact on the enzymatic saccharification with *Trichoderma reesei* cellulases and *Aspergillus niger* cellobiase. The enzyme activity was influenced by pH, viscosity and ionic strength and it was observed that pH was the main factor causing low cellulase activity as discussed in chapter 4. Several studies conducted previously showed cellulase is sensitive to pH condition. Therefore acidic cellulase and β -glucosidase were needed to enhance the production of glucose in the presence of IL. The results of the study in chapter 5 were promising in terms of cellulase activity with sulphate based IL. This was because endoglucanase activity was higher in the presence of [HBIM][HSO₄] than in [EMIM][OAc]. Experimental results in chapter 6 showed there was a slightly improvement in terms of β -glucosidase activity (*T.pendens*) with [TEA][HSO₄] but when both of these enzymes combined and acting against carboxymethylcellulose (CMC) with 10% (v/v) of [TEA][HSO₄], the enzymes were still active. It seems that there was a synergistic reaction when both of these enzymes were used.

6.2 Overall thesis achievements

The contributions of this thesis are:

1. Enzymatic saccharification of Sigmacell with the addition of ILs, specifically low-cost mineral acid ILs

In the present study, the effect of ILs on the activity of the commercial cellulase and Novozyme 188 was tested. The experiment as presented in chapter 3 showed that sulfate based ILs deactivated the cellulase and cellobiase more than other type of ILs. Based on previous literature it was known that the effect of pH on cellulase activity was pronounced (Hailong et al., 2011). Apart from pH, other physical factors such as viscosity and ionic strength were studied to understand the effect of these factors on the activity of the cellulases. It was found that viscosity and ionic strength are not the main factors that influenced the activity of the cellulase. Moreover, there was an interest in understanding at what time the cellulase and cellobiase deactivated by the ILs. It was observed that at concentration more than 2% (v/v) of [HBIM][HSO₄], the enzyme was deactivated at 4 hour of enzymatic saccharification process. It seems that the property of these commercial cellulases are not compatible with sulphate based ILs so acidic cellulases which can enhanced the yield of glucose are needed. Many researchers previously conducted a study on [EMIM][OAc], [BMIM]Cl and phosphate based ILs but no studies were performed using [TEA][HSO₄], then this was the main reason why this thesis was focused more on [TEA][HSO₄].

2. Acidophilic endoglucanase from *Cryptococcus sp.*S-2

The identification of acidic cellulases was conducted by examining previous literature for enzymes which were reported to have optimum activity at low pH. Endoglucanase from *Cryptococcus sp.* was chosen as this enzyme had optimum activity at pH 3.5. This enzyme was expressed in *E.coli* and purified and then tested with ILs. It was observed that this enzyme had better specific activity in sulfate based IL than in phosphate based IL and [EMIM][OAc]. The results obtained in this chapter showed endoglucanase from *Cryptococcus sp.* can be a useful enzyme in biomass processing using IL for biomass pretreatment. Moreover, *E.coli* was chosen as host expression because it was more efficient in term of cost and time consumption. However, it should be noted that this enzyme lacks a cellulose binding domain so it cannot hydrolyse insoluble substrates. Therefore, further work is needed before it can be applied to biomass saccharification (see below).

3. Acidophilic β -glucosidase from *Thermofilum pendens*

As mentioned in chapter 3, endoglucanase was not the only enzyme became deactivated with ILs. Cellobiase also known as β -glucosidase can be deactivated by IL and it was claimed in a literature that β -glucosidase was more sensitive with IL than endoglucanase. Therefore, acidic β -glucosidase is required to meet the demand of industrial application especially in biomass processing using IL for pretreatment. The results presented in chapter 5 showed that there was a slightly improvement when β -glucosidase from *T.pendens* were tested with [TEA][HSO₄] compared to commercial β -glucosidase (Novozyme 188). However, when both of these enzymes combined and acting against carboxymethylcellulose (CMC) with 10% (v/v) of [TEA][HSO₄], the enzymes were still active.

4. Synergistic action of different enzymes

In chapter 6, it can be seen that there was a synergistic action between cellulase from *Cryptococcus sp.* and β -glucosidase from *T. pendens*. This is very interesting outcome as this combination can be explored in a future work.

6.3 Future work and application

There are several things can be performed in the future to improve this research study:

1. Variants of *Cryptococcus* SP-2 that can bind cellulose

The endoglucanase from *Cryptococcus* shows promise for biomass treatment due to its high activity in acidic IL. However, currently its use is limited due to its lack of activity on insoluble cellulose substrates. To solve this problem, future work could fuse the current enzyme to an acidotolerant cellulose binding domain to improve the activity on insoluble substrates, but retain the pH optimum at low pH (Thongekkaew et al., 2013).

2. Immobilised cellulase

Immobilised cellulase is another way to improve enzyme stability. It has been reported that the coating of immobilised cellulase particles with ILs has been shown as a useful means to improve enzyme stability at extreme condition. It was reported that immobilized cellulase has the capability to adsorb on solid supports such as polystyrene which is stable and the enzyme become reusable (Lozano et al., 2011).

3. Directed evolution

Another method to improve the cellulases activity with ILs is directed evolution. Molecular directed evolution can enhance the activity of cellulases by changing the properties of cellulases such as pH and thermal stability. Three main methods of directed evolution which have been reported previously are DNA shuffling, error-prone PCR, and combination of error-prone PCR and DNA shuffling (Lin et al., 2011).

4. Different type of host expression

In this thesis, bacterial expression system was chosen as cellulase host expression. In the future, yeast and plant expression system can be carried out to enhance the activity and the yield of cellulases. Yeast and plant have a better post-translation modification process which can avoid the production of insoluble protein (Lambertz et al., 2014).

5. Alkaline cellulases

This thesis mainly focuses on acidic cellulases. Perhaps, metagenomics screening on alkaline cellulases from soil can be performed and tested with ILs such as [EMIM][OAc] and [BMIM] [Cl]. Alkaline cellulases can be examined from previous literature and expressed in bacteria, yeast or plant expression system.

6. Designing IL-based consolidated bioprocessing

Consolidated bioprocessing is a process which combines enzyme production, cellulose hydrolysis, and fermentation. This technique is performed by hydrolysing cellulose to glucose after being treated with ILs and directly converted to ethanol by recombinant cellulases. This method helps to decrease costs as it can reduce unit of operations.

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APPENDICES

Appendix I: ANOVA tables

ILs concentrations Vs.Glucose yield

Descriptives

GY								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1=1%	7	57.2457	31.44780	11.88615	28.1613	86.3301	5.48	86.51
2=2%	7	39.4071	36.40119	13.75836	5.7417	73.0726	.00	79.64
5=5%	7	28.0071	31.59818	11.94299	-1.2163	57.2306	.00	74.05
10=10%	7	7.1443	14.82638	5.60384	-6.5678	20.8564	.00	40.37
Total	28	32.9511	33.58244	6.34648	19.9292	45.9730	.00	86.51

ANOVA

GY					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9256.402	3	3085.467	3.494	.031
Within Groups	21193.664	24	883.069		
Total	30450.066	27			

pH effects Vs. Glucose yield

Descriptives

pH								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0	7	1.7986	.48396	.18292	1.3510	2.2462	1.00	2.41
5.07	1	2.3600	.	.	.	.	2.36	2.36
5.48	1	3.2400	.	.	.	.	3.24	3.24
7.81	1	6.7800	.	.	.	.	6.78	6.78
24.79	1	6.0900	.	.	.	.	6.09	6.09
25	1	2.0000	.	.	.	.	2.00	2.00
27.33	1	3.0000	.	.	.	.	3.00	3.00
35.03	1	3.4300	.	.	.	.	3.43	3.43
35.98	1	3.3100	.	.	.	.	3.31	3.31
39.22	1	5.7100	.	.	.	.	5.71	5.71
45.3	1	4.0000	.	.	.	.	4.00	4.00
51.6	1	5.0000	.	.	.	.	5.00	5.00
53.28	1	5.1300	.	.	.	.	5.13	5.13
70.59	1	5.4400	.	.	.	.	5.44	5.44
75.8	1	5.1700	.	.	.	.	5.17	5.17
81.9	1	4.4500	.	.	.	.	4.45	4.45
82.98	1	5.1200	.	.	.	.	5.12	5.12

Descriptives

pH								
					95% Confidence Interval for Mean			
	N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
0	7	1.7986	.48396	.18292	1.3510	2.2462	1.00	2.41
5.07	1	2.3600	.	.	.	.	2.36	2.36
5.48	1	3.2400	.	.	.	.	3.24	3.24
7.81	1	6.7800	.	.	.	.	6.78	6.78
24.79	1	6.0900	.	.	.	.	6.09	6.09
25	1	2.0000	.	.	.	.	2.00	2.00
27.33	1	3.0000	.	.	.	.	3.00	3.00
35.03	1	3.4300	.	.	.	.	3.43	3.43
35.98	1	3.3100	.	.	.	.	3.31	3.31
39.22	1	5.7100	.	.	.	.	5.71	5.71
45.3	1	4.0000	.	.	.	.	4.00	4.00
51.6	1	5.0000	.	.	.	.	5.00	5.00
53.28	1	5.1300	.	.	.	.	5.13	5.13
70.59	1	5.4400	.	.	.	.	5.44	5.44
75.8	1	5.1700	.	.	.	.	5.17	5.17
81.9	1	4.4500	.	.	.	.	4.45	4.45
82.98	1	5.1200	.	.	.	.	5.12	5.12
Total	23	3.6009	1.69129	.35266	2.8695	4.3322	1.00	6.78

ANOVA

pH					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	61.525	16	3.845	16.418	.001
Within Groups	1.405	6	.234		
Total	62.930	22			

Viscosity Vs.Glucose yield

Descriptives

VISCOCITY

					95% Confidence Interval for Mean			
	N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
0	9	.64278	.167857	.055952	.51375	.77180	.517	1.037
4.74	1	.76600	.	.	.	.	.766	.766
4.9	1	.69300	.	.	.	.	.693	.693
5.07	1	.54900	.	.	.	.	.549	.549
5.48	1	.41100	.	.	.	.	.411	.411
21.71	1	.63100	.	.	.	.	.631	.631
34.37	1	.60500	.	.	.	.	.605	.605
35.03	1	.53800	.	.	.	.	.538	.538
35.98	1	.52200	.	.	.	.	.522	.522
40.37	1	.62100	.	.	.	.	.621	.621

50.2	1	.58900	.	.	.	.	.589	.589
65.92	1	.70900	.	.	.	.	.709	.709
70.01	1	.58900	.	.	.	.	.589	.589
70.93	1	.65400	.	.	.	.	.654	.654
74.05	1	.56100	.	.	.	.	.561	.561
78.83	1	.55400	.	.	.	.	.554	.554
78.98	1	.57300	.	.	.	.	.573	.573
79.64	1	.53700	.	.	.	.	.537	.537
79.91	1	.53600	.	.	.	.	.536	.536
86.51	1	.61100	.	.	.	.	.611	.611
Total	28	.60836	.114406	.021621	.56400	.65272	.411	1.037

ANOVA

VISCOCITY					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.128	19	.007	.239	.995
Within Groups	.225	8	.028		
Total	.353	27			

Ionic strength Vs. Glucose yield

Descriptives

IS								
					95% Confidence Interval for Mean			
	N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
0	9	2.04658122E0	.431808435	.143936145	1.71466388	2.37849857	1.461619	2.550247
4.74	1	2.40536700E0	.	.	.	.	2.405367	2.405367
4.9	1	2.23372800E0	.	.	.	.	2.233728	2.233728
5.07	1	1.47701600E0	.	.	.	.	1.477016	1.477016
5.48	1	1.32962700E0	.	.	.	.	1.329627	1.329627
21.71	1	1.83356100E0	.	.	.	.	1.833561	1.833561
34.37	1	1.74210900E0	.	.	.	.	1.742109	1.742109
35.03	1	1.33709700E0	.	.	.	.	1.337097	1.337097
35.98	1	1.34783600E0	.	.	.	.	1.347836	1.347836
40.37	1	2.32390200E0	.	.	.	.	2.323902	2.323902
50.2	1	1.46653400E0	.	.	.	.	1.466534	1.466534
65.92	1	1.91638800E0	.	.	.	.	1.916388	1.916388
70.01	1	1.42751100E0	.	.	.	.	1.427511	1.427511
70.93	1	1.50441700E0	.	.	.	.	1.504417	1.504417
74.05	1	1.78939800E0	.	.	.	.	1.789398	1.789398
78.83	1	1.33212800E0	.	.	.	.	1.332128	1.332128
78.98	1	1.31283000E0	.	.	.	.	1.312830	1.312830
79.64	1	1.44735700E0	.	.	.	.	1.447357	1.447357

79.91	1	1.32267300E0	.	.	.	.	1.322673	1.322673
86.51	1	1.35064400E0	.	.	.	.	1.350644	1.350644
Total	28	1.76140550E0	.427583922	.080805766	1.59560576	1.92720524	1.312830	2.550247

ANOVA

IS					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.445	19	.181	.972	.551
Within Groups	1.492	8	.186		
Total	4.936	27			

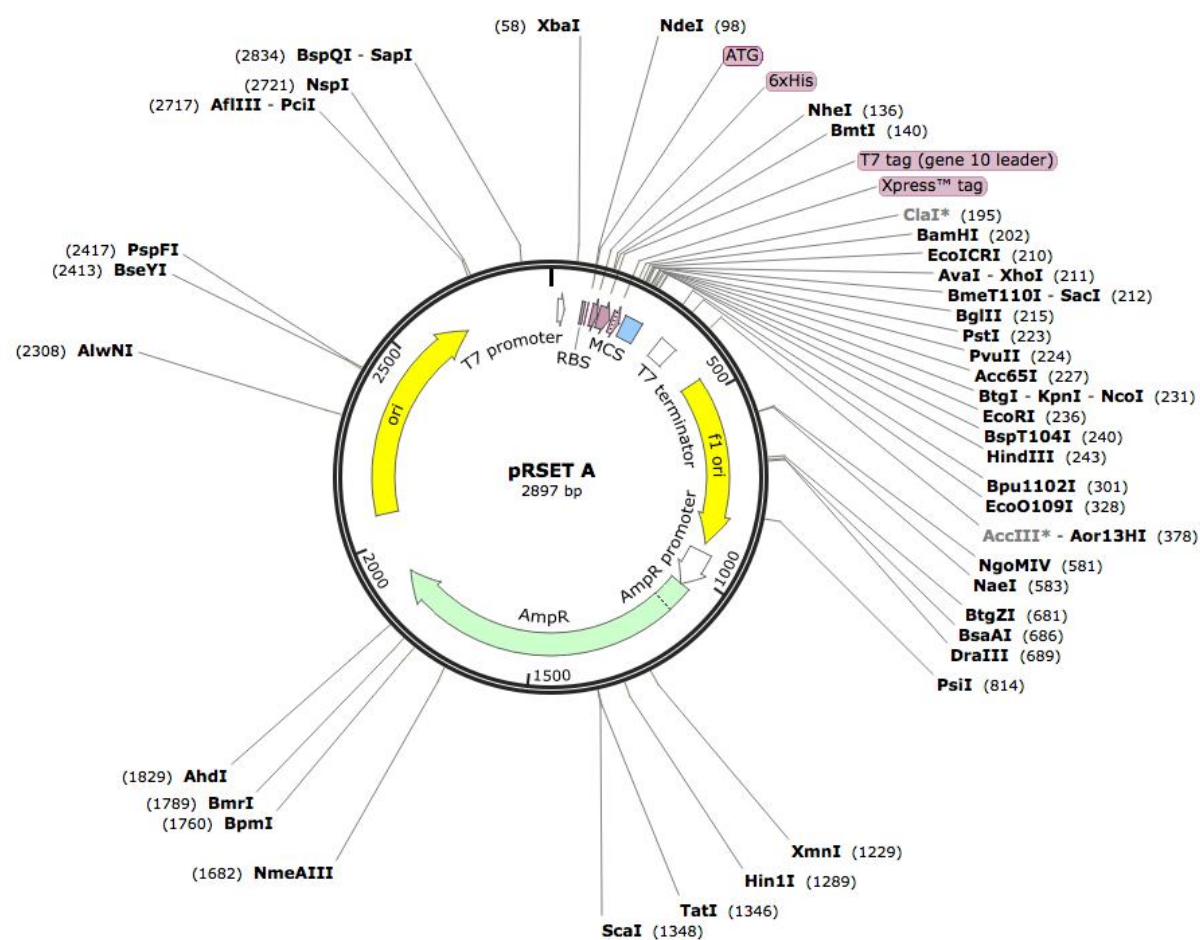
Appendix II: Gene sequence of *Cryptococcus sp.*S-2

```

CTCGAGTCAA  GGTAGATTG  GCTTTACGGC  GATATCGAAA  AGTTGGTTAT
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CCTGCTGCCC  ACACAGCAAA  ACCTAGAACG  GAAGGATAAT  CTTCTTGAT
ACCATGCAAA  AACTCTCCAA  GGTCGGTTTC  GCAAGAAGTG  GTATGACCAC
CGCCGGTTTC  GGAAACAATA  GCTTTTCTTC  CATTTCCTTG  TAGCCAGGAG
TCAAGTGAGG  AAAGAACATC  CAAACTGTTT  GTAGTACATT  CAGCATGGGT
ACCACTAAAA  TCAACATCAT  TGTAACGGTG  AACGTCTAAC  AACAATTTAT
CAGTGCCTCC  AACTGGGTCA  GTTACACCTA  GCAAAGCATT  ATCTTGACCT
TCAAACCAGG  CATTTGCATT  TGTGAAACCG  GTTCCGGGAA  CTAGAATGTA
CTGACTTGTA  GCACCAGCAG  CACGAATAGC  ATTAACAGCA  GCCTGCACAC
TTCCAGCCCA  GGTAGGGACA  TCCAAGTCAT  GAGGCTCATT  CATTAAACCA
AAGATCACGT  TAGGATCGTT  CTTGTACTTT  GTTGCCAATA  GGGTCCACAG
GTTTGCAAAG  TCTTGATTAG  ATGGCCCACC  TTGACCAATG  ATAGCGCCGT
TCCATCTAGC  GTAATTATGG  ACGTCAATGA  TAGCATAAGC  TCCTTTGGAA
ATAACAGCCT  GGACAAGAGC  ATCATACTGT  GCGAAAAAAT  CTGGAGCTAA
ACTGGTACTT  GCTTGATTGT  TACCAACTAA  ATACTGCCAA  CCGGCGGGTA
GCCTGAAAAG  GTTAGCCCCG  TCGGCAATGA  AATGCCCGAC  CTGCTCAGTC
CCGGGGCATG  CAGGGGTACC  ACTGTTACCG  TAAATATCAA  TACCAAAGTC
ACAGCCAGCC  AGATTAACAC  CTCCAATCT  TGGTAGGGTT  CGTCTATGCA
AGGCTGACTC  TCCGGCCAAA  GGTGCTGCCA  AAGCACCAGC  CAGCACAGAT
AAAGAAGCCA  ACAAGGTAGC  TGTTTTCATG  AATTC

```

Appendix III: Plasmid map of pRSET A



Appendix IV: DNA sequence alignment shown by ClustalW

CLUSTAL 2.1 multiple sequence alignment

```

original      -----
sample        ATGGTATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGGGATCTGTACGACGATGACG  60

original      -----ATGAAAACAGCTACCTTGTGGCTTCTTTATCTGTGCTGG  40
sample        ATAAGGATCGATGGGGATCCATGAAAACAGCTACCTTGTGGCTTCTTTATCTGTGCTGG  120
                *****

original      CTGGTGCTTTGGCAGCACCTTTGGCCGGAGAGTCAGCCTTGCATAGACGAACCTTACCAA  100
sample        CTGGTGCTTTGGCAGCACCTTTGGCCGGAGAGTCAGCCTTGCATAGACGAACCTTACCAA  180
                *****

original      GATTGGGAGGTGTTAATCTGGCTGGCTGTGACTTTGGTATTGATATTTACGGTAACAGTG  160
sample        GATTGGGAGGTGTTAATCTGGCTGGCTGTGACTTTGGTATTGATATTTACGGTAACAGTG  240
                *****

original      GTACCCCTGCATGCCCCGGGACTGAGCAGGTCGGGCATTTTCATTGCCGACGGGGCTAACC  220
sample        GTACCCCTGCATGCCCCGGGACTGAGCAGGTCGGGCATTTTCATTGCCGACGGGGCTAACC  300
                *****

original      TTTTCAGGCTACCCGCCGGTTGGCAGTATTTAGTTGGTAACAATCAAGCAAGTACCAGTT  280
sample        TTTTCAGGCTACCCGCCGGTTGGCAGTATTTAGTTGGTAACAATCAAGCAAGTACCAGTT  360
                *****

original      TAGCTCCAGATTTTTTCGCACAGTATGATGCTCTTGTCAGGCTGTTATTTCCAAAGGAG  340
sample        TAGCTCCAGATTTTTTCGCACAGTATGATGCTCTTGTCAGGCTGTTATTTCCAAAGGAG  420
                *****

original      CTTATGCTATCATTGACGTCCATAATTACGCTAGATGGAACGGCGCTATCATTGGTCAAG  400
sample        CTTATGCTATCATTGACGTCCATAATTACGCTAGATGGAACGGCGCTATCATTGGTCAAG  480
                *****

original      GTGGGCCATCTAATCAAGACTTTGCAAACCTGTGGACCCTATTGGCAACAAAGTACAAGA  460
sample        GTGGGCCATCTAATCAAGACTTTGCAAACCTGTGGACCCTATTGGCAACAAAGTACAAGA  540
                *****

```

original	ACGATCCTAACGTGATCTTTGGTTTAAATGAATGAGCCTCATGACTTGGATGTCCCTACCT	520
sample	ACGATCCTAACGTGATCTTTGGTTTAAATGAATGAGCCTCATGACTTGGATGTCCCTACCT	600

original	GGGCTGGAAGTGTGCAGGCTGCTGTTAATGCTATTTCGTGCTGCTGGTGTACAAGTCAGT	580
sample	GGGCTGGAAGTGTGCAGGCTGCTGTTAATGCTATTTCGTGCTGCTGGTGTACAAGTCAGT	660

original	ACATTCTAGTTCCCGGAACCGGTTTCACAAATGCAAATGCCTGGTTTGAAGGTCAAGATA	640
sample	ACATTCTAGTTCCCGGAACCGGTTTCACAAATGCAAATGCCTGGTTTGAAGGTCAAGATA	720

original	ATGCTTTGCTAGGTGTAAC TGACCCAGTTGGAGGCACTGATAAAATTGTTGTTAGACGTTT	700
sample	ATGCTTTGCTAGGTGTAAC TGACCCAGTTGGAGGCACTGATAAAATTGTTGTTAGACGTTT	780

original	ACCGTTACAATGATGTTGATTTTAGTGGTACCCATGCTGAATGTACTACAAACAGTTTGG	760
sample	ACCGTTACAATGATGTTGATTTTAGTGGTACCCATGCTGAATGTACTACAAACAGTTTGG	840

original	ATGTTCTTTCCTCACTTGACTCCTGGCTACAAGGAAATGGAAGAAAAGCTATTGTTTCCG	820
sample	ATGTTCTTTCCTCACTTGACTCCTGGNTACAAGGAAATGGAAGAAAAGCTATTGTTTCCG	900

original	AAACCGGCGGTGGTCATACCACTTCTTGCGAAACCGACCTTGGAGAGTTTTTGCATGGTA	880
sample	AAACCGGCGGTGGTCATACCACTTCTTGCGAAACCGACCTTGGAGAGTTTTTGCATGGTA	960

original	TCAAGGAAGATTATCCTTCCGTTCTAGGTTTGTGCTGTGTGGGCAGCAGGTTTCATTTCGATC	940
sample	TCAAGGAAGATTATCCTTCCGTTCTAGGTTTGTGCTGTGTGGGCAGCAGGTTTCATTTCGATC	1020

original	CCAGTTACGTCTTGTCCATAACTCCTACCAACGGAGTCGATAACCAACTTTTCGATATCG	1000
sample	CCAGTTACGTCTTGTCCATAACTCCTACCAACGGAGTCGATAACCAACTTTTCGATATCG	1080

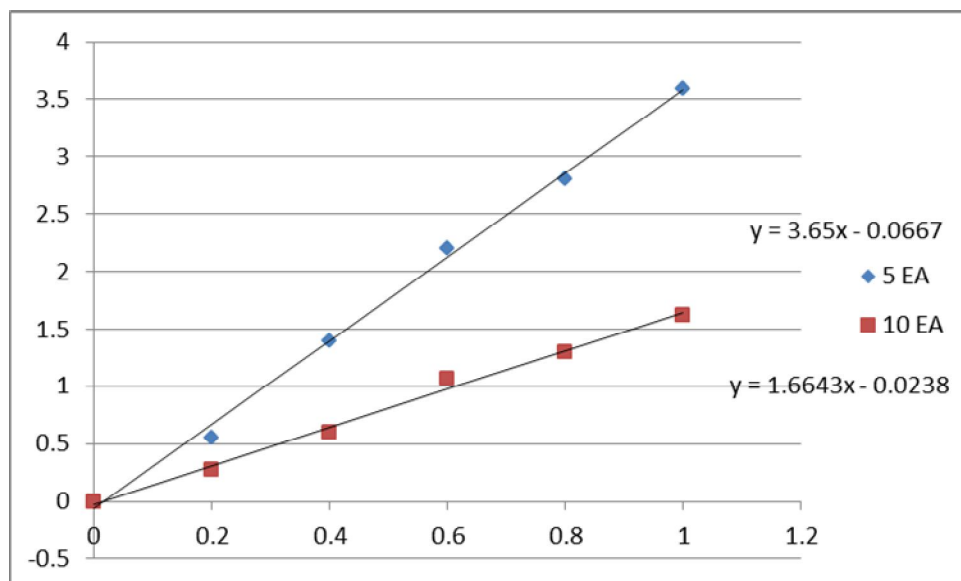
original	CCGTAAAGCCAAATCTACCTTGA-----	1023
sample	CCGTAAAGCCAAATCTACCTTGAGTATCAANGGAANATTATCCTTCCNTTCTNGNN	1136

Appendix V: Amino acid sequences of cellulase from *Cryptococcus sp.*

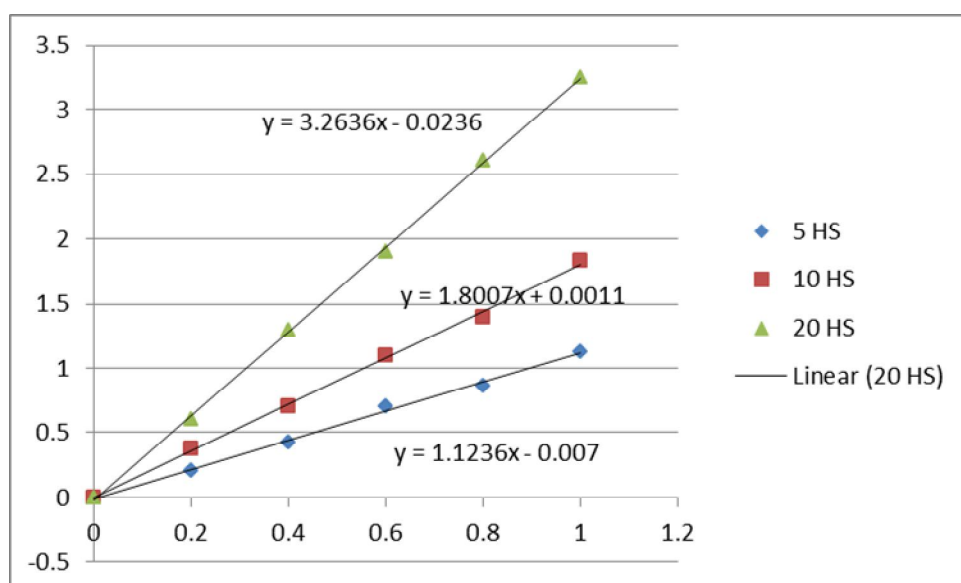
LESRIWLYG DIEKLVIDSV GRSYGQDVTG IETCCPHSK TNGRIIFLD TMQKLSKVG
 ARSGMTTAGF GNNSFSSISL PGVKGKNI QTVCSFMSG TTKINIIVTV NVQQFISAS
 NWVSYTQSI ILTFKPGICI CETGSGNNV LTCSTSSTNS INSSLHTSSP GRDIQVMRLI
 HTKDHVRIV LVLCCQGPQ VCKVLIRWPT LTNDASVPSS VIMDVNDSIS SFGNNSLDKS
 IILCEKIWS TGTCLIVTN ILPTGGPEK VSPVGNEMPD LLSPGACRGT TVTVNINTKV
 TASQINTSQS WGSSMQGL SGQRCCQSTS QHRRSQQS CFHEF

Appendix VI: DNS Assay standard plus ILs

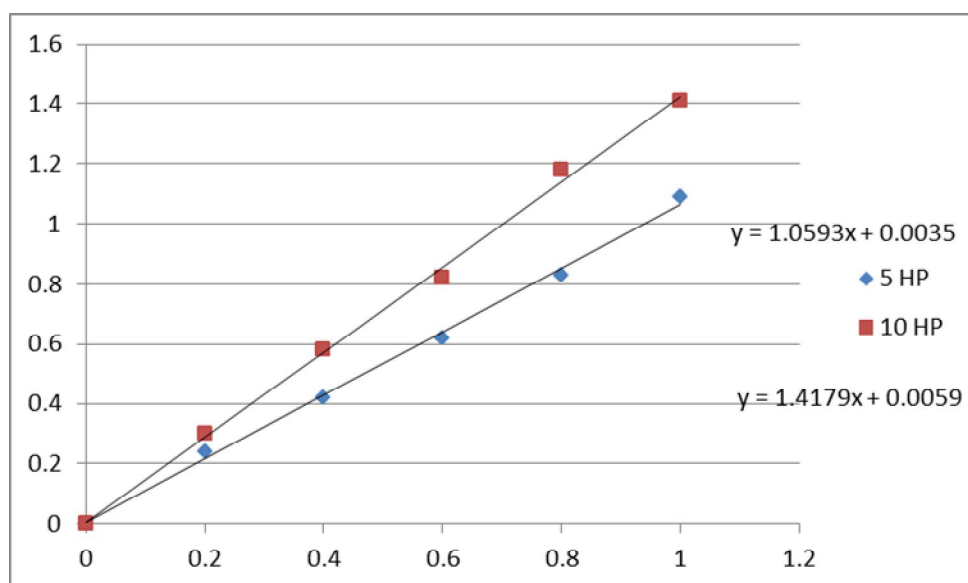
a) 5% and 10% [EMIM][OAc] as a control



b) 5% , 10% and 20% [HBIM][HSO₄] as a control

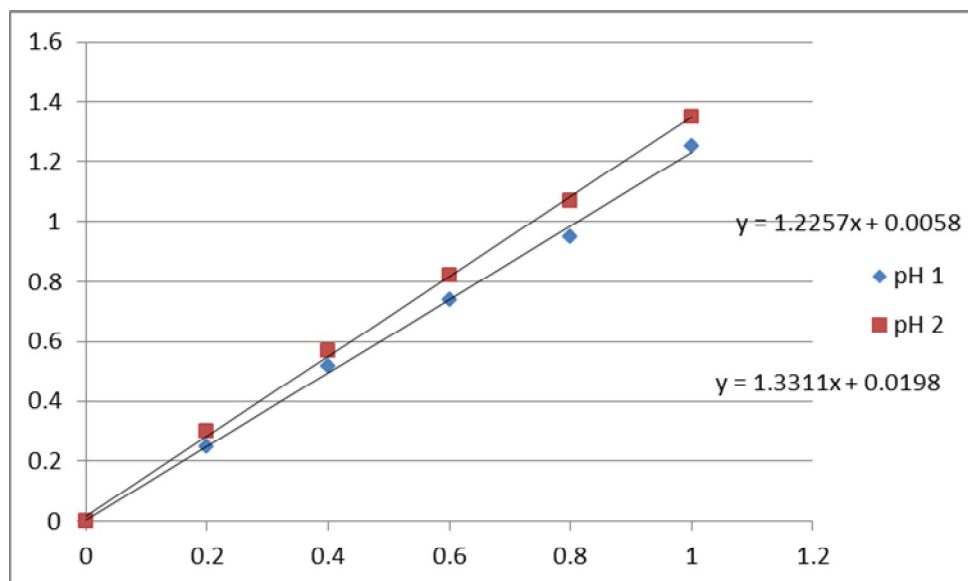


c) 5% and 10% [HBIM][H₂PO₄] as a control

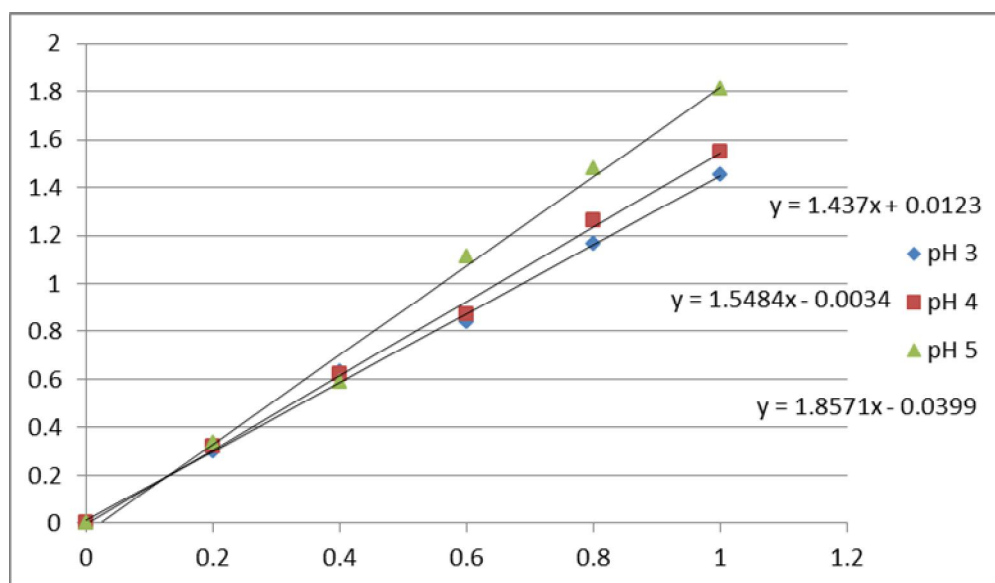


Appendix VII:DNS Assay standard plus pH

a) pH 1 and 2



b) pH 3, 4 and 5



Appendix VIII: Gene sequences of β -glucosidase from *T.pendens*

ATGCGCGGCCGCGGCCCGGGCCTGAGCCTGGAAGAAAAAGCGAGCCTGGTGGTG
GGCTGGGGCAGCAGCCGCCGCTGCCGGGCGCGGCGGGCGAAACCCGCCCGGTG
CGCGTGCCGAGCATTGTGACCGCGGATGGCCCGAGCGGCCTGCGCGTGGAACCG
AGCGGCGGCCGCGCTGGTTTGCACCGCGTTTCCGGTGCCGACCATGCTGGCGG
CGACCTGGAACCCGGAAGTGGTGGAACGCGTGGGCCGCGCGATTGGCGAAGAAT
GCCGCGCGTATGGCGTGATGTGCTGCTGGCGCCGGGCGTGAACATGCATCGCC
ATCCGCTGTGCGGCCGCAACTTTGAATATTTTAGCGAAGATCCGCTGCTGAGCGG
CGAAATGGCGGCGGCGTATGTGCGCGGCGTGACAGAGCGTGGGCGTGGGCGCGAC
CCTGAAACATTTTGCGGCGAACGATCAGGAAACCAACCGCACCGTGATTGATAC
CGTGGTGAGCGAACGCGCGCTGCGCGAAATTTATCTGAAACCGTTTGAAATTGC
GGTGAAAAAAGCGAAACCGTGGTGCCTGATGAGCAGCTATAACAACTGAACG
GCAAATATAGCAGCCAGAACGAATGGCTGCTGACCCGCGTGCTGCGCGAAGAAT
GGGGCTTTGATGGCGTGGTGTGATGACCGATTGGGGCGCGGGCGATGATAGCGTGG
AACAGGTGAACGCGGGCAACGATCTGATTATGCCGGGCAGCGATGAAGCGGTGG
AAAACTGCTGGAAGCGGCGCGCAGCGGCCCGCTGCGCCTGGAAGCGCTGGAAG
CGAGCGCGGAACGCGTGCTGCGCCTGGTGCAGAAAAGCCTGACCTATCGCGGCT
ATCGCCCGCGCGGGCGCGCCGGATCTGGATGGCCATGCGCGCGTGCGGTATGAAG
CGGCGAGCGAAGGCGTGGTGTGCTGCTGAAAAACGAAGGCGCGCTGCCGCTGGGCC
CGGGCGCGCGCGTGCGCTGTTTGGCACCGGCCAGGTGGAAACCTGAAAGGCG
GCATGGGCAGCGGCCATACCCATCCGCGCTATGTGGTGACCGTGCTGGAAGGC
CTGAAAAGCGGCGGCCTGCTGGTGGATGAAGAACTGAGCAGCATTTATGAACGC
TATGTGCGCGAAGCGCGCGGCGAAGAATTTCTGGAAAACTGTATCTGGATGAA
GTGTATGCGGATCCGCTGCCGCAGGATATTGTGAGCGAAGGCGATGCGGCGCGC
TTTGCGGAACGCAACGATGCGGCGGTGGTGGTGTGCTGTATCGCGTGAGCGGCGAA
GGCTGGGATCGCCGCCCGGTGCGCGGCGATTTTTATCTGACCGAAAGCGAAGAA
CGCCTGCTGCGCCTGGTGAAGCCGCAATTTGCGGGCCGCGGCAAAAAAGTGGTG
GTGGTGTGTAACGTGTGCGGCCCGATTGAAGTGGCGAGCTGGCGCGATCTGGTG
GATGCGATTCTGGTGGTGTGGCTGCCGGGCCAGGAAGCGGGCCGCGTGGTGGCG
GATGTGCTGGCGGGCCGCGTGAACCCGAGCGGCAAACTGCCGATGACCTGGCCG
CGCGATTGGACCGATGTGCCGGCGGCGAAAGCGCCGGAATGCTATCCGGGCCTG
CCGGTGGAAAGATCCGCGCCGCGTGGTGTATTGCGAAGGCGTGTATGTGGGCTAT
CGCTATTATGATACCTTTGGCGTGGAACCGGCGTATGAATTTGGCTATGGCCTGA
GCTATACCAAATTTGAATATCGCGGCCTGCGCGTGCGCTGAGCCGCGGCGCGCT
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TGAAAGCGTTTCGCAAAACCCGCCTGCTGGAACCGGGCGAAGCGGAACGCATTA
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GGGTGGTGGAAACCGGGCGAATATGAAGTGCCTGGGCGAGCAGCAGCCGCGAT
ATTCGCCTGACCGAACATTTTGAAGTGAAACAGGAACTGCGCTTTGCGCCG

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