Design of smart hydrogels for use as support matrices for immobilisation of cellulases in saccharification of lignocellulose

by

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RESEARCH DISSERTATION

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DECLARATION

I declare that the dissertation hereby submitted to the University of Limpopo, for the degree of Master of Science in Microbiology has not previously been submitted by me for a degree at this or any other university; that this is my work in design and in execution, and that all material contained herein has been duly acknowledged.

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DEDICATION

I dedicate this dissertation to my lovely family for earnest support. To my mother Nkhensani, my brother Ndzivalelo, my son Ntamu and my fiancée Louisa, I deeply appreciate your support and patience throughout the program and in loving memory of my grandmothers and my father. I give all the glory to the highest God for the innermost strength that pushed me throughout the program.

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ABSTRACT

Smart hydrogels could facilitate immobilisation of cellulases to allow recovery and decrease enzyme cost in the biofuel industry, as they have a soluble-gel transition. The aim of the study was to design and evaluate the use of smart hydrogels for immobilisation of cellulase system that can be recovered after hydrolysis of cellulosic biomass. Cellulases from *Aspergillus niger* FGSC A733 produced under solid state fermentation and commercial cellulases were used in immobilisation. Various support matrices prepared were *poly*-N-isopropylacrylamide (*p*-NIPAAm), *poly*-N-isopropylacrylamide-co-Methacrylic acid (*p*-NIPAAm-co-MAA) and supermacroporous *poly*-crosslinked-Acrylamide-co-N,N'-Methylenebisacrylamide (*p*-crosslinked-AA-co-MBA). Cellulases were coupled onto the support matrices by covalent attachment method through reactive groups of N-acryloxysuccinimide (NAS) or Methacrylic acid N-hydroxysuccinimide (NMS). The low critical solution temperature (LCST) of formed *p*-NIPAAm-co-MAA copolymer was determined by the inflection point method. The shrinking and swelling kinetics and pH sensitivity of *p*-NIPAAm-co-MAA copolymer and conjugates were characterised using a cloud point method. Hydrolysis of CMC using cellulase-microbeads-*p*-NIPAAm and cellulase-crosslinked-*p*-NIPAAm with different percentage gel showed activity trend of 0.05>1>10>5>0.1% and 5>2>10% respectively. HPLC analysis showed that supplementation of β-glucosidase in cellulase-crosslinked-*p*-NIPAAm conjugates increased glucose by 12 and 14-fold at 30 and 50 °C respectively in the avicel hydrolysate in comparison with no β-glucosidase supplementation. In the hydrolysis of avicel using cellulase-crosslinked-*p*-NIPAAm-co-MAA conjugate a total of 13.6 g/L of reducing sugar was liberated after three cycles. In comparison a total of 21.4 g/L of reducing sugars were released from avicel hydrolysis using cellulase-crosslinked-*p*-AA-co-MBA conjugate after 3 cycles. In contrast, reducing sugars released in thatch grass hydrolysis using free enzyme were 8 times greater than in cellulase-crosslinked-*p*-AA-co-MBA conjugate. Cellulase-crosslinked-*p*-NIPAAm-co-MAA conjugates were more stable than free enzyme at 50 and 60 °C after 24 hour and 120 minutes of incubation respectively, but lost activities at 65 °C after 120 minute. Therefore the activity loss in the immobilised enzymes was

more due to thermal inactivation during precipitation and recovery than incomplete recovery during precipitation cycles. The results show that cellulases immobilised on smart polymers with sol-gel transition could be used in hydrolysis of cellulose due to ease of recovery. Hydrolysis kinetics was efficient for both immobilised enzyme system (cellulase-crosslinked-*p*-AA-co-MBA and cellulase-crosslinked-*p*-NIPAAm-co-MAA conjugate) since were re-used in hydrolysis of avicel. Therefore the use of these smart polymers for cellulase immobilisation can contribute in cost reduction of the enzymatic hydrolysis process in the biofuel industry.

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CHAPTER 1

1. INTRODUCTION

1.1. Background information

The industrial processes for production of chemicals and fuel alcohol is increasingly implementing the use of biomass as a substrate (Hasunuma and Kondo, 2012). Biomass is mainly derived from plants and consists of association of cellulose, hemicellulose and lignin collectively called lignocelluloses (Desvaux, 2005; Matthews *et al*., 2010). Cellulose is the most abundant biopolymer that is mainly produced by plants. Natural cellulose degradation is mainly microbiological, involving production of cellulase by bacteria, fungi and protozoa (Desvaux, 2005). Biomass is cheap, abundant in nature and its potential conversion to bioethanol could lead to a reduction of greenhouse gas emission into the atmosphere (Jones and Vasudevan, 2010).

Microbial cellulolytic systems consist mainly of three enzymes namely; endoglucanase (EG) (Bedon and Legay, 2011), cellobiohydrolases I and II (CBH I & CBH II) and β-glucosidase (BGL) or cellobiase. These enzymes act synergistically on crystalline cellulose releasing mainly glucose which can be fermented to ethanol by bacteria or yeast (Hasunuma and Kondo, 2012). *Aspergillus niger* (*A. niger*) and *Trichoderma reesei* (*T. reseei*) are the most studied cellulase producing microorganisms whose cellulases have found application in brewery, wine making, animal feed, textile, laundry, pulp and paper, agriculture as well as in research (Reczey *et al*., 1996; Bhat, 2000; Howard *et al*., 2003; Kuhad *et al*., 2011).

The enzymatic hydrolysis of cellulose to fermentable sugars in biofuel industry offers the advantage over chemical processes due to lower energy costs and milder operating conditions (Yang *et al*., 2011). However, the major limitation of industrial application of cellulase in biofuel production is due to costs of enzyme production and lack of method for recycling enzymes in processes utilising these enzymes (Walsh, 2002). Other challenges in using cellulase include cellulose recalcitrance, end product inhibition and nonspecific binding to lignin (Andersson *et al*., 1998; Copeland, 2000).

Industrial enzymes are increasingly utilised in an immobilised form which is achieved using insoluble support materials. Therefore, enzyme immobilisation makes possible

the separation of enzymes from products, recovery and reuse of the expensive biocatalyst, improvement of thermal and pH stability and improvement of kinetic parameters (Walsh, 2002; Jones and Vasudevan, 2010). However, cellulosic substrates are of particulate nature and this introduces mass transfer limitations where cellulases are immobilised onto an insoluble support (Singh *et al*., 2013; Tsai and Meyer, 2014).

SMART hydrogels have gained attention as support matrices for immobilisation of enzymes due to their soluble-insoluble (sol-gel) transition in response to external stimuli such as temperature, pH, electric field *etc.* (O'Shea, 2011). Poly-N-isopropyl-acrylamide (*p*-NIPAAm) is a well-studied thermal-responsive polymer, while poly-N,N-Dimethylaminoethylmethacrylate (*p*-DMAEMA) has pH responsive character due to presence of a tertiary amine group. Studies on thermo- and pH-sensitive interpenetrating polymer network (IPN) showed that the swelling/de-swelling kinetics was largely enhanced by enlargement of pores (Liu *et al*., 2012).

Cheng *et al*. (2003) conducted a study for synthesis of macroporous *p*-NIPAAm in aqueous sodium chloride (NaCl). The improved swelling and shrinking properties were due to the presence of NaCl which initiated a phase separation and formation of a heterogeneous porous structure during the polymerizations (Cheng *et al*., 2003). Therefore, this demonstrates that synthesis of porous matrices could perhaps facilitate immobilisation of cellulase for degrading cellulose.

Enzyme immobilisation is a powerful technology that can impart superior properties to the enzyme for maximum operation during bioprocessing. Various methods are available for immobilisation and the choice of a method is dictated by purpose of immobilisation and these include covalent binding, entrapment, encapsulation, adsorption and cross-linking (Verardi *et al*., 2012). Currently cellulase immobilisation is gaining attention due to the demand of cost effective process for saccharification of cellulose and fermentation to bioethanol (Jones and Vasudevan, 2010; Alftren and Hobley, 2014; Mubarak *et al*., 2014). A study by Khoshnevisan *et al*. (2011) on immobilisation of cellulase on superparamagnetic nanoparticles showed that immobilised cellulase retained high activity than free cellulase at high temperature (80 °C) and pH.

1.2. Research problem

Cellulosic materials can be converted to fermentable sugars enzymatically using cellulases. However, application of cellulases in production of fuel ethanol is limited due the cost of enzyme, nonspecific binding to lignin and thermal deactivation of enzyme. Often enzyme immobilisation technique is used to reduce costs of enzymatic hydrolysis by facilitating the recycling of a biocatalyst and improvement of thermal stability. However, cellulosic substrates are particulate rendering them unsuitable substrates for conventional insoluble immobilised enzyme systems. Immobilisation of cellulases onto conventional insoluble supports impedes saccharification processes as a result of mass transfer limitations. Support material with sol-gel transition may be suitable for immobilisation of cellulase in saccharification of cellulosic material. The use of *p*-NIPAAm for immobilisation of cellulases is fairly established therefore this research work will offer more knowledge.

1.3. Purpose of the study

1.3.1. Aim

The aim of the current study was to design and evaluate the use of smart hydrogels as support matrices for co-immobilisation of BGL, CBHI, CBHII and EG.

1.3.2. Objectives

The objectives of the research were to:

i. Produce cellulolytic enzymes from the fungus *Aspergillus niger* using *Jatropha* seed cake as substrate
ii. To optimise efficient cellulase mixture consisting of commercial enzymes and *A. niger* FGSC A733 cellulase
iii. Synthesise thermo-sensitive cellulase-*p*-NIPAAm conjugates, pH sensitive cellulase-*p*-(NIPAAm-co-MAA) conjugates and cellulase-*p*-(Acrylamide-co-MBA) (supermacroporous monolithic gel).
iv. Determine cellulolytic activity of the immobilised enzymes
v. Apply the synthesised cellulase-*p*-(NIPAAm-co-MAA) and cellulase-*p*-(Acrylamide-co-MBA) conjugates to laboratory scale conversion of biomass such as thatched grass.

CHAPTER 2

2. LITERATURE REVIEW

2.1. Microbial cellulases

Microbial cellulase systems consist of three major groups of enzymes namely endo-(1,4)-*β*-D-glucanase (EC 3.2.1.4), exo-(1,4)-*β*-D-glucanase (EC 3.2.1.91) [cellobiohydrolase I (CBH I) and cellobiohydrolase II (CBH II)] and *β*-glucosidases (EC 3.2.1.21) commonly known as cellobiase (Reczey *et al*., 1996; Hasunuma and Kondo, 2012; Kuhad *et al*., 2012). Mechanistically these enzymes act in synergy in degrading cellulosic material with endoglucanase acting randomly on amorphous regions of cellulose chains producing oligosaccharides and new polysaccharide chain ends (Sukumaran *et al*., 2005). CBH I and CBH II act on reducing and non-reducing ends of glucan chains respectively producing glucose, cellobiose and oligosaccharides. The last step is the hydrolysis of tetrasaccharides and *β*-cellobiose (disaccharides) to glucose by the action of cellobiase (Kuhad *et al*., 2011). Figure 1 shows hydrolysis of cellulose by synergistic action of cellulase with subsequent ethanol fermentation by yeast or bacteria (Lynd *et al*., 2002).

2.1.1. Classification, structure and activity of cellulases

Cellulases are classified into Glycosyl hydrolases (GH) family according to amino acid sequence similarities (Delabona *et al*., 2012). Families which share common positions of their catalytic residues, folding and same stereochemistry (invention or retention) of catalytic site (Miettinen-Oinonen, 2004) are grouped into same category (Desvaux, 2005).

Cellulases are described to be modular consisting of two or more structural and functional domains (Ohmiyan *et al*., 1997; Miettinen-Oinonen, 2004). There are several types of domains, but only the catalytic and cellulose binding domains (CBD) are considered to be crucial for cellulose hydrolysis. The joining of catalytic domain to cellulose binding domain (CBD) through a short polylinker region is characteristic to cellulases as single enzymes (non-complexed) (Desvaux, 2005). These cellulases are synthesized and released into the extracellular medium by aerobic organisms. In addition to CBD a dockerin domain joins a catalytic domain and these characterise complex systems of cellulosomes solely from anaerobic microorganisms (Ohmiyan *et al*., 1997).

Cellulases facilitate hydrolysis of glycosidic bond via acid catalysis which involves two catalytic residues: a proton donor (HA) and nucleophile/base (B^-). Hydrolysis involves breaking of bond by adding water. Aspartic or glutamic acid residues in cellulase confer the catalytic activity (Andersen, 2007).

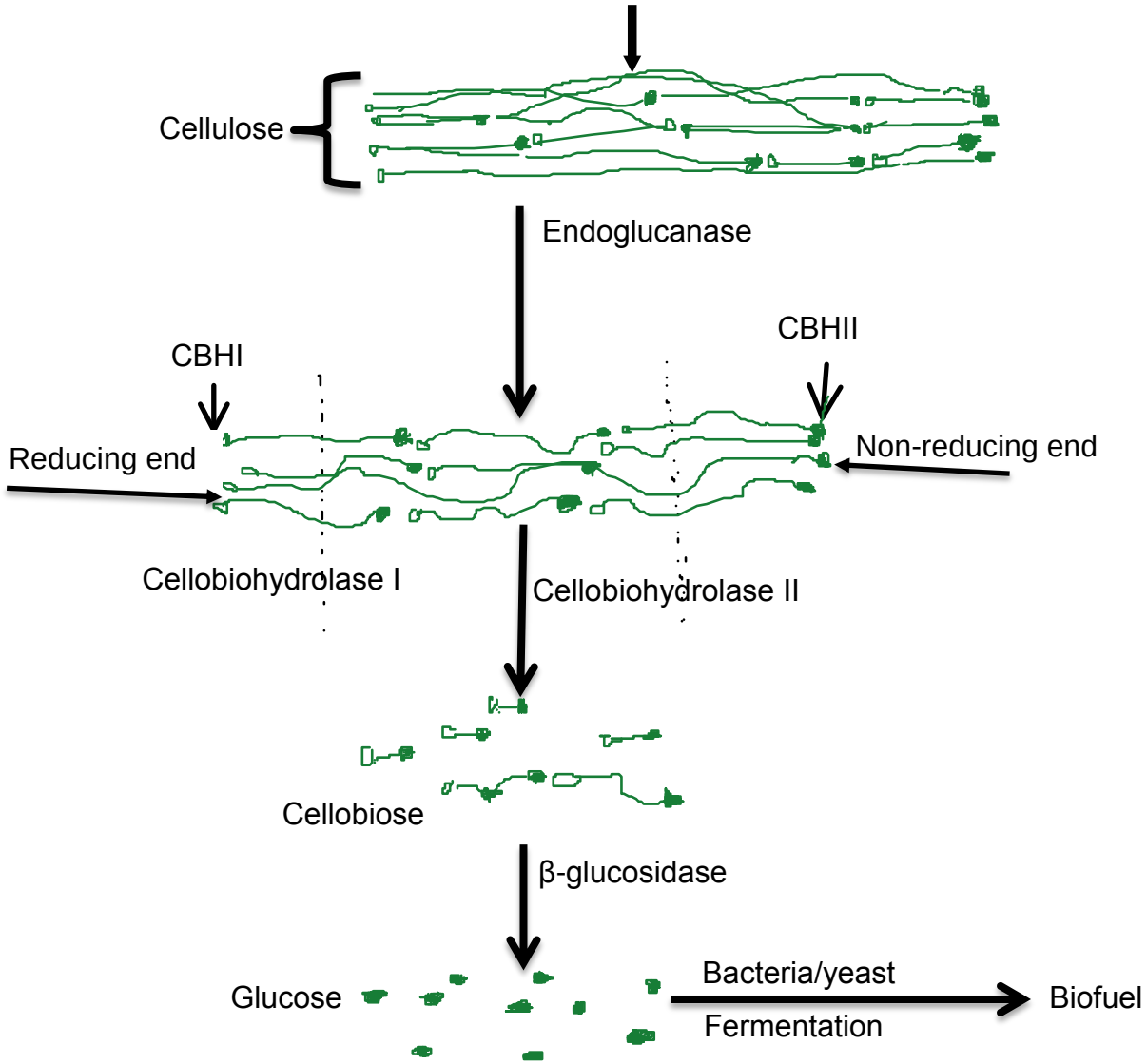


Figure 1. Hydrolysis of cellulose by synergistic action of cellulase system and subsequent ethanol fermentation. Cellulose polymer chains are not to scale; shaded and clear circles represent reducing and non-reducing ends of cellulose polymer chains respectively. Modified from Lynd *et al*. (2002).

2.1.2. Production of cellulases by fungi

Cellulases are inducible enzymes produced and secreted by number of microorganisms when growing on a cellulosic substrate (Lynd *et al*., 2002; Verma *et al*., 2012). These microorganisms can be aerobic or anaerobic bacteria, soft rot fungi, and white rot fungi. The most studied cellulase producing system is that of soft rot fungus of genera *Trichoderma* (Goldbeck *et al*., 2012), and *Aspergillus* a filamentous ascomycetes fungus (Baker, 2006). Industrial production of cellulase from *Tricoderma reesei* (*T. reesei*) is easy and inexpensive, and the strain has GRAS (generally regarded as safe) status, because it is non-pathogenic to healthy humans. The production of cellulase in *T. reesei* is controlled at transcription level, and its cellulase genes (*cbh1, cbh2, egl1, egl2, egl4 and egl5*) have been identified to be expressed co-ordinately while cbhl1 expressed in high amount (Miettinen-Oinonen, 2004).

2.1.3. Different forms of cellulase preparation

The search for new cellulase producing microorganism strains is required in order to develop biocatalyst for industrial use and screening enzymes with high hydrolytic activity (Goldbeck *et al*., 2012). Enzymatic hydrolysis of pre-treated lignocellulose contributes a large portion of total cost due to requirement of high enzyme loading (Drissen *et al*., 2007; Weiss *et al*., 2013). Different forms of cellulase preparations are available commercially; among cellulase produced by NOVOZYME cellulclast 1.5 litre or celluclast 500g, Novozyme 188 which is β-glucosidase are the most studied. Cellubrix is formed from celluclast by increasing β-glucosidase content (Drissen *et al*., 2007).

It may not be possible to hydrolyse cellulosic feed stocks with single enzyme preparation. Therefore leading commercial cellulase producing companies Genencor and Novozyme have produced different blends of cellulase which are made of mixtures of cellulase from different organisms (cocktail) (Verardi *et al*., 2012). Cellulases from *T. reesei* have low cellobiase activity and in hydrolysis high cellobiose accumulation might inhibit enzyme activity. Accelerase®1500, Accelerase®XR, Accelerase®XC and Accelerase®BG are blends from Genencor while Cellic CTec and Cellic HTec belong to Novozyme and capable of hydrolysing wide range of pre-treated feedstocks (Verardi *et al*., 2012). The use of high-purity cellulose for cellulase production inputs more cost, however methods of using lignocellulosic substrate,

such as bagasse, waste paper sludge, waste newsprint and wood has been tested to be effective in cost reduction (Reczey *et al*., 1996).

2.2. Lignocellulosic biomass

Lignocellulosic biomass includes organic matter derived from trees, grass, garbage and animal wastes (Houghton *et al*., 2006). Plant biomass is a common renewable energy source (Verma *et al*., 2012). Plant cell wall which is mainly composed of polysaccharides is the main component of biomass. Plant polysaccharide mainly include: cellulose, hemicellulose, pectin, glycoproteins, and lignin collectively called lignocellulose with cellulose the most abundant biopolymer (Desvaux, 2005; Anderson, 2007). Lignocellulosic biomass is woody, fibrous, inedible part of trees, and made of energy-rich sugars that can be converted to bioethanol (Houghton *et al*., 2006).

In industry lignocellulosic substrates, are well suited as feedstock for production of biofuels because they are abundant in nature, inexpensive and environmentally friendly (Lynd *et al*., 2002; Houghton *et al*., 2006; Maki *et al*., 2009; Vieira *et al*., 2011). Lignocellulosic biomass that can be processed into biofuels, electricity and heat is diverse and includes wheat straw, bagasse, waste paper sludge, waste newsprint and wood (Reczey *et al*., 1996). Using lignocellulosic biomass as alternative to fossil fuel (coal, petroleum, or natural gas) for energy generation reduces emission of greenhouse gases into the atmosphere and build-up of woody biomass (Hall *et al*., 1991). Implementation of biomass energy in the Republic of South Africa (RSA) can benefit rural dwellers because biomass plantation can generate income and jobs (Haw and Hughes, 2007).

2.2.1. Cellulose

Structurally cellulose is a linear polymer of D-glucose repeating unit linked together by β-1,4-linkage (Mackulak *et al*., 2012). The polymer chains are held together by hydrogen bond and van der Waals forces forming microfibrils which consist of high ordered and less ordered region termed crystalline and amorphous (formless) respectively (Andersen, 2007). Cellulose is mainly produced by plants; however cellulose of animal, algal, and microbial origin is available and its enzymatic hydrolysis is largely hampered by its crystallinity and insolubility (Desvaux, 2005).

2.2.2. Hemicellulose

Hemicelluloses are the second abundant biopolymer on earth after cellulose. Hemicellulose crosslink cellulose microfibril, and connect cellulose and lignin (Andersen, 2007), thereby providing cell wall strength. Structurally it is a branched heterogeneous polymer that is soluble in alkaline solution; chemically it is composed of different monomers, namely: non-cellulose β-D-glucans, pectic substances (polygalacturons), and heteropolysaccharides consisting of galactose and xylose (Howard *et al*., 2003). Xylan and galactoglucomannans are hemicelluloses of hard and soft wood respectively, their major difference occurs in their structures and amount (Horward *et al*., 2003; Andersen, 2007).

2.2.3. Lignin

Lignin is a complex polymer which contain high amount of aromatic compounds in plants, it is abundant in wood (15-36%) than in grass (< 20%) (Bedon and Legay, 2011). The monomers (monolignol) of lignin are coumaryl, coniferyl and sinapyl alcohols which undergo oxidative polymerization forming guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H) unit respectively (Bedon and Legay, 2011). Lignin serves to support the plant cell wall, protects plant polysaccharide from microbial attack, and assist with water transportation due to its hydrophobicity (Hossain, 2014). Unlike hemicelluloses, composition of lignin differs from species to species, cell type, and stage of tissue development (Davison *et al*., 2013).

2.3. Saccharification of lignocellulose and fermentation process

Processing of lignocellulose to bioethanol has three basic steps (i) pre-treatment of lignocellulose (ii) hydrolysis by acid or cellulases (iii) fermentation mediated by bacteria or yeast. Cellulose is packed into microfimbrils by hydrogen and van der Waals forces and is associated with the other biopolymers lignin and hemicellulose contributing to its resistance to microbial attack or enzymatic hydrolysis thus limiting its utilisation as feedstock in fermentation. Cost of biorefenery process is another limitation, therefore combining hydrolysis and fermentation in one step can reduce costs (Houghton *et al*., 2006).

2.3.1. Pre-treatment step

The main purpose of this step is to overcome structural barrier by reducing cellulose crystallinity, removing lignin and increasing surface area and porosity (Kumar *et al*., 2009). Conversion of material into fine particles increases surface area thus making cellulose and hemicellulose easily accessible to hydrolysis. Pre-treatment can produce inhibitory compounds that decrease enzyme activity. Several hydrolysis methods exist namely: physical, chemical, physico-chemical and biological (Kumar *et al*., 2009).

2.3.1.1. *Physical method*

Digestibility of lignocellulose is enhanced by reduction of particle size, degree of polymerisation, cellulose crystallinity through chipping, grinding, and milling or by combination of both. Shear forces generated during milling makes it more effective in reducing particle size and cellulose crystallinity than chipping. Vibratory milling is more effective than ordinary ball milling, however milling alone requires high energy therefore not yet feasible for industry. Combination of milling with chemical pre-treatment show to be effective in cost reduction. Other physical pre-treatment methods include the use of gamma rays which break β-1,4 glycosidic bonds (Gelbe and Zacchi, 2002).

2.3.1.2. *Chemical methods*

Biomass is delignated using ozone treatment (ozonolysis), concentrate acid such as H_2SO_4 and HCl which results in improvement of enzymatic hydrolysis of polysaccharides in biomass (Kumar *et al*., 2009). Alkali treatment uses sodium, potassium, calcium, and ammonium hydroxide of which sodium hydroxide is the most studied. As a result of less sugar degradation alkali treatment is advantageous compared to acid treatment (Kumar *et al*., 2009). The use of alkali and acid generates acidic or acaustic hydrolysate and inhibitors of enzymatic hydrolysis thus requires neutralisation which input additional costs and results in loss of sugars (Joshi *et al*., 2011).

2.3.1.3. *Biological methods*

This method is environmentally friendly; however its drawback for application in industrial scale is requirement of large space to perform biological pre-treatment and long duration (10 to 14 days) of pre-treatment (Agbor *et al*., 2011). The biological pre-treatment of biomass is mainly performed by various microorganisms such as brown, white and soft-rot fungi. Brown rot attack cellulose, while white and soft rot fungi attach both cellulose and lignin (Godliving and Mtui, 2009; Agbor *et al*., 2011). White rot fungus such as *Ceriporiopsis subvermispora* (*C. subvermispora*) degrade lignin by producing laccase and manganese peroxidase (MnP) isoenzymes (Kumar *et al*., 2009). White-rot fungi are the most effective for biological pretreatment of lignocellulosic materials due to ability to attack both lignin and cellulose (Godliving and Mtui, 2009)

2.3.1.4. *Physicochemical method*

Include uncatalysed steam explosion (SEP) where biomass is exposed to high-pressure saturated steam then to low pressure resulting in explosive decomposition of the material (Kumar *et al*., 2009; Zheng *et al*., 2009). SEP method depends on combined effect of temperature and pressure in order to disintegrate biomass material by promoting hemicellulose hydrolysis and lignin transformation as the process terminate with an explosive decompression. Therefore cellulose microfimbrils become exposed and easily hydrolysed. Improvement of catalyzed steam explosion can be achieved by addition of H_2SO_4, SO_4 or CO_2 at 0.3-3% (w/w) which reduces production of inhibitory compounds and complete removal of hemicellulose (Kumar *et al*., 2009). Ammonia fibre explosion (AFEX) is similar to SEX except liquid ammonia is added to biomass materials for period of time. Carbon dioxide (CO_2) explosion (CDEX) depends on the notion that CO_2 dissolved in water forms carbonic acid and thus the acid increases hydrolysis rate. CO_2 is able to penetrate small pores accessible to H_2O and ammonia molecules as result helpful in hydrolysing hemicellulose and cellulose (Kumar *et al*., 2009).

2.3.2. Enzymatic and acid hydrolysis step

In order to obtain fermentable sugars polymers in biomass should be easily hydrolysed by enzymes or acid. Enzymatic hydrolysis produces high sugar yield, lower inhibitory products than acid hydrolysis and reduces operation cost of the entire

process. Besides operational cost due to mild condition employed, hydrolytic enzymes themselves - cellulase and xylanase are costly and these limit their application in commercial scale. Product of pre-treatment step and enzyme dose has significant effect on rate of hydrolysis. Hydrolysis of great pre-treated material can be done in short period of time at low enzyme dose while poorly pre-treated material will require short period of time at higher enzyme dose to achieve the same effect and these input more cost to the process (Olofsson *et al*., 2008).

It has been described herein that structural features (lignin, acetyl, hemicellulose, surface area, crystallinity) affect enzyme hydrolysis; in addition reaction conditions (temperature, pH, substrate and enzyme loading) also affect enzymatic hydrolysis (Carvalho, 2009). Increasing temperature affect the rate of reaction by increasing the frequency of collision between substrate and enzyme, but very high temperature denatures enzyme resulting in activity loss. At optimal temperature enzyme reaction rates are fast and retain activity for a long period of time. pH affects activity by changing shape of active sites. Most cellulases and xylanasses have optimal condition at 50-60 °C and pH 4-5 but these conditions depend on source of enzymes (Gelbe and Zacchi, 2002).

Higher substrate loadings can result in higher glucose concentration in the liquor; however there is a threshold in which higher concentration increase contact between substrate and enzyme, but beyond the point end-product inhibition dominates. Cellobiose is known to inhibit exoglucanase, and these can be overcome by increasing β-glucosidase content although it is also inhibited by its product glucose (Lynd *et al*., 2002; Hsieh *et al*., 2014). Further hydrolysis improvement is done by simultaneous saccharification and fermentation (SSF) process when dealing with ethanol or by using membrane reactors. High substrate loading increase viscosity which makes mixing difficult and increase power consumption in stirred tank reactors. Cellulose dosage is usually expressed in terms of Filter Paper Units (FPU), defined as the micromole of reducing sugar as glucose produced by 1 mL of enzyme per minute with 10 FPU/g of substrate used in laboratory scale (Carvalho, 2009).

Acid hydrolysis of biomass is done using various types of acids, concentrated (10-30%) or diluted (2-5%) such as sulphurous (H_2SO_3), sulphuric (H_2SO_4), hydrochloric

(HCl), hydrofluoric (HF), phosphoric (H_3PO_4), nitric (HNO_3) and formic (methanoic, HCO_2H) acid with HCl and H_2SO_4 most commonly used catalyst (Verardi *et al*., 2012). Concentrated acid hydrolysis require low temperature but causes corrosion to equipment while dilute acid hydrolysis operate at high temperature which input more power consumption (Ncube, 2013). High temperatures and acid concentrations or prolonged pre-treatment time causes conversion of sugars to weak acids, furan derivatives and phenolic chemicals (Corredor, 2008). Glucose specifically convert to 5-hydromethylfurfural (5-HMF) while xylose is converted to furfural, these compounds are considered inhibitors of fermentation step (Wu *et al*., 2014). Acid hydrolyse cellulose and hemicellulose by penetrating lignin without pre-treatment required (Verardi *et al*., 2012).

2.3.3. Processing of lignocellulose to biofuel

The product of lignocellulose hydrolysis consists of pentose sugars which can be fermented to ethanol by few microorganisms while hexose sugars are readily fermented by several strains. *Saccharomyces cerevisiae* (*S. cerevisiae*) is the most studied organism capable of fermenting hexoses while some engineered strains can also ferment pentose. *Candida shehatae* (*C. shehatae*) and *Pichia stipitis* (*P. stipitis*) have genes for fermentation of both pentode and hexose sugars (Joshi *et al*., 2011; Khan and Dwivedi, 2013). Fermentation can be carried out either by simultaneous saccharification and co-fermentation (SSCF), simultaneous saccharification and fermentation (SSF) or separate hydrolysis and fermentation (SHF) (Corredor, 2008).

SSCF involves presence of co-culture which ferment mixed sugars to ethanol. *S. cerevisiae and P. stipitis* co-cultures are the most used hexose- and pentose-fermenting yeasts and have been used also in immobilised form on calcium-alginate beads (Corredor, 2008). SSF technology is advantageous because the ethanol produced can inhibit unwanted growth of microorganism, glucose is used up by yeast as it is formed in same vessel, and therefore end-product inhibition is eliminated and reduces number of steps. The drawbacks are elevated temperature for enzymatic hydrolysis than of fermentation and requirement of high solid loading which increase viscosity and hampers enzyme distribution (Verardi *et al*., 2012). In SHF, cellulose and/or hemicellulose are enzymatically converted to glucose and/or pentose first followed by fermentation into ethanol. Its main advantage is the ability to carry each

process in its optimal temperature and its drawback is cellulase and β-glucosidase inactivation (Corredor, 2008).

2.4. Application of cellulases

Biofuels consists of two main types of fuels namely bioethanol and biodiesel (Kuhad *et al*., 2011). There are several types of biofuels: first generation biofuels produced from edible plants, second generation biofuels made from conversion and fermentation of cellulose (non-edible) using cellulase and third generation biofuels produced using hybrid-processing methodologies that directly convert biomass into biofuels (Arumugam *et al*., 2007).

Cellulases are major industrial enzymes which are available in the market. Research on cellulases for conversion of lignocellulosic biomass has been conducted in the past decades and gave way for application of the enzyme to various industries (Saranraj *et al*., 2012), that include food and brewery production, animal feed processing, detergent production and laundry, textile processing and paper pulp manufacture (Juturu and Wu, 2014). Due to greater demand of biofuels in the world, cellulase application in cellulose hydrolysis for production of fermentable sugars is growing rapidly (Zhang and Zhang, 2013; Juturu and Wu, 2014).

2.4.1. Wine, Juice and Beer industry

During wine making, grape's own enzymes pectinesterase and polygalacturonase are capable of hydrolysing pectin substances but not efficiently (Ratledge and Kristiansen, 2006). Therefore, enzyme preparations such as pectinase, glucanase, hemicellulase play an important role by improving color extraction, wine quality and stability. β-Glucosidases can modify glycosylated precursors thus improving the aroma of wine (Kuhad *et al*., 2011). Beer brewing relies on activation of enzymes during malting and fermentation. Endoglucanse II and exoglucanase II preparations of *Trichoderma* cellulase system can reduce degree of polymerization and wort viscosity (Kuhad *et al*., 2011).

2.4.2. Animal feed industry

Application of cellulases, hemicellulases and xylanases can improve nutritional value and performance of animals. Feed grain contains some anti-nutritional factors such as non-starchy polysaccharides, cellulose, lignin, oligosaccharides, β-glucan,

dextrins, and pectins which can be eliminated by action of enzymes to produce soluble and edible sugars and oligormers. Pre-treatment of grass and grain feed by cellulases can improve its digestibility and nutritional value for cattle feeding (Kuhad *et al*., 2011).

2.4.3. Textile industry

In textile industry cellulases are used to process cellulose-containing materials thus creating new types of fabric (Sukumaran *et al*., 2005; Sokołowska *et al*., 2014). Cellulases are used in a process called biostoning which create fashionable stonewashed appearance of denim cotton fabric replacing the use of pumice-stone traditionally employed in industry (Kuhad *et al*., 2011). Denim fabric is woven with dye indigo wrapped within the cotton fibres, cellulase allow partial hydrolysis of cotton releasing the dye thus giving the cotton an aged appearance (Arja, 2007). Protruding microfibril on a cotton fabric can be digested using cellulase resulting in soft fabric (Sukumaran *et al*., 2005).

2.5. Enzyme immobilisation techniques

Enzyme immobilisation means enzymes are restricted to an insoluble material with restricted mobility and have become insoluble (Tischer and Wedekind, 1999). Immobilised enzymes can be reused continuously with retention of their operation stability and activity (Ratledge and Kristiansen, 2006). Immobilisation techniques have gained interest in biotechnology and application of this technology dates back to 1950's (Walsh, 2002). The most interesting idea about immobilisation is that it allows recovery and re-use of the biocatalyst, easy separation from products and convenient handling of enzyme preparations (Verardi *et al*., 2012).

2.5.1. Methods of immobilisation

Enzyme immobilisation methods are classified according to different chemical and physical principles of the reactions that took place during immobilisation (Tisher and Wedekind, 1999). These reactions are further categorized as irreversible and reversible (Guisan, 2006). Methods based on chemical mechanisms include covalent binding, adsorption and cross-linking while methods based on physical mechanism include entrapment and encapsulation as shown in Figure 2 (Brickerstaff, 1997; Girelli and Mattei, 2005; Taher *et al*., 2011). Effective method of immobilisation for a particular enzyme is one which yields high retention of activity, and achieve

operational stability and durability (Sheldona, 2007; Khan and Alzohairy, 2010). During immobilisation multipoint attachment of enzymes to the support can hamper the catalytic activity whereas non-specific interactions of enzyme and support may denature the enzyme and result in activity of enzymes significantly lower than 100% (Fang *et al*., 2011).

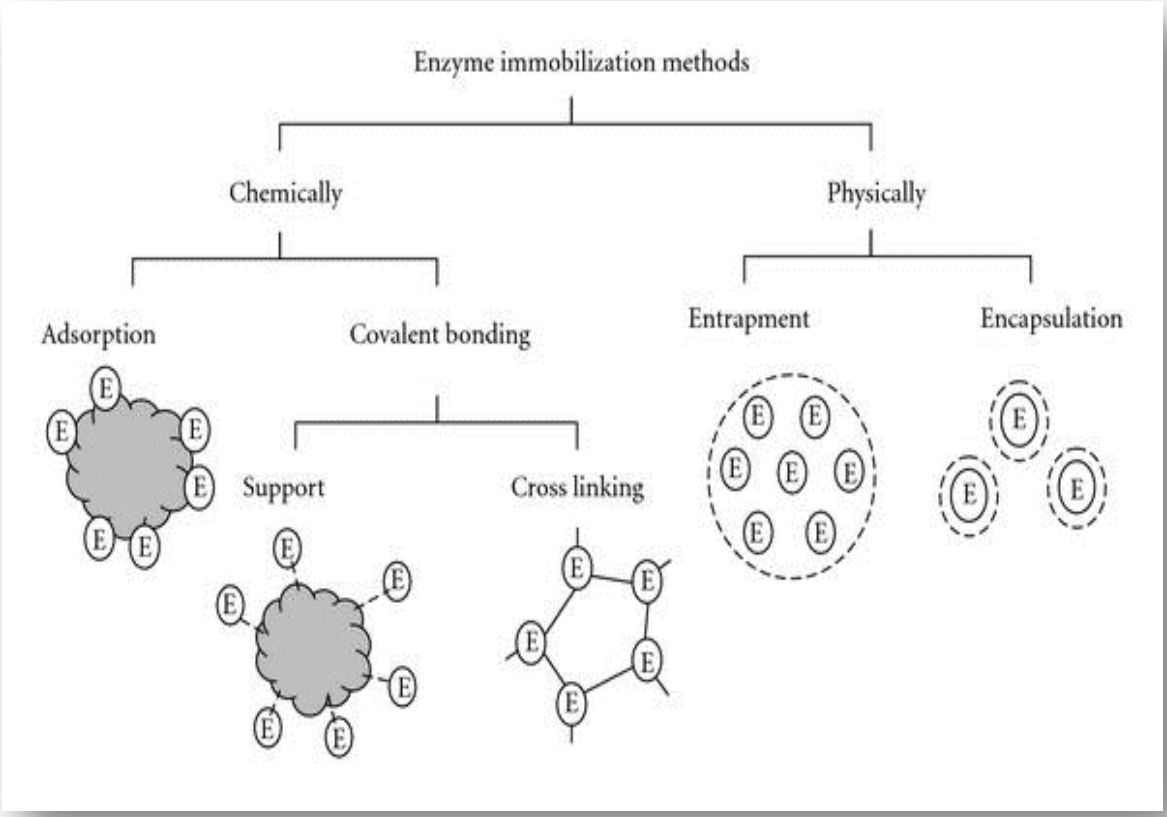


Figure 2. Various methods of enzyme immobilisation. Source: Taher *et al*. (2011).

2.5.1.1. *Covalent attachment*

This procedure involves formation of covalent bond between enzyme and support by means of spacer arm or activation molecule, therefore the result is a strong interaction between the biocatalyst and the support (Girelli and Mattei, 2005). This mode of binding offers great advantages of minimising enzyme leakage from the support thus reduces the cost of removing the enzyme from the product during purification stages. Furthermore, the covalent interaction of the enzyme with the support increases the rigidity of the protein structure and limits thermal motion of the protein at high temperatures (Mubarak *et al*., 2014). Covalent immobilisation steps

includes (1) activation of either enzyme or support material with an activation agent such as acrylic acid N-hydroxysuccinimide (NAS) ester, gluteraldehyde, cyanogen dromide, *etc* (Hermanson, 2013) and (2) immobilisation of activated enzymes on the support (Kourkoutas *et al.*, 2004). Chemical modification of enzymes occurs at different reactive groups located on the side chains of amino acids; nine side chains that are chemically active are derived from arginine, glutamic and aspartic acid, histidine, lysine, methionine, tryptophan and tyrosine (Tischer and Wedekind, 1999).

2.5.1.2. *Encapsulation*

Enzymes can be entrapped within a spherical semi-permeable membrane which is formed by spinning fibres such as cellulose acetate (Walsh, 2005). Pores of the membrane must be small enough to allow only the passage of substrates and products while other macromolecules and enzymes are retained (Walsh, 2005; Taher *et al*., 2011). The major drawback of encapsulation method is the limited diffusion of particulate substrates through the membrane (Verardi *et al*., 2012).

2.5.1.3. *Cross-linking*

Free enzymes can be bound to each other by a bifunctional cross-linker such as glutaraldehyde. In this technique there is no support material therefore it eliminate the costs of various monomers to serve as a support backbone (Górecka and Jastrzębska, 2011). The popular method is the use of gluteraldehyde which reacts with surface amino groups mostly lysine residues. Two main techniques have been developed over the last decades (1) cross-linking enzyme aggregates (CLEA) (Hanefeld *et al*., 2009) and (2) cross-linking enzyme crystals (CLEC) (Górecka and Jastrzębska, 2011). The main advantage of CLEA is ease of use, cheap and wider application while CLEC can resist proteases, thermal and organic solvent denaturation and is easy to recycle (Górecka and Jastrzębska, 2011).

2.5.1.4. *Adsorption*

Enzyme immobilisation by adsorption method is mainly based on interactions of charged surfaces on enzymes or cells with support materials (Brickerstaff, 1997). Adsorption is mediated by weak forces such as van der Waals forces, ionic, hydrophobic interactions, affinity binding and hydrogen bonding (Ratlege and Kristiansen, 2006). In spite of weak interaction between the biocatalyst and the support, the forces are large in number to support immobilisation. While the method is

cheap, easy and offers reloading of the support, its application is limited due to weak interaction between support and enzyme (Górecka and Jastrzębska, 2011).

2.5.1.5. *Entrapment*

Enzyme is entrapped in a polymer network where the polymer is synthesized in the presence of the enzyme. Only small amounts of enzyme can be entrapped and is prevented for direct contact with the environment for protection against shearing (Verardi *et al*., 2012). Other major drawbacks for this method are costs of immobilisation, diffusion limitation and deactivation of enzyme during immobilisation. Common supports that are used include carrageenan, gelatin, and polyvinyl alcohol with styrylpyridium group (Górecka and Jastrzebska, 2011).

2.6. Characteristics and types of support matrices or materials

Characteristics of a support matrix are crucial for immobilisation, this include: mean particle diameter, swelling behaviour, mechanical strength, compression behaviour, hydrophilicity, inertness toward enzymes, ease of derivatisation, biocompatibility, resistance to microbial attack, reusability and availability at low cost (Veliky and Mclean, 1994). Support matrices can be classified as organic (natural and synthetic) or inorganic polymers (natural minerals and processed materials) (Kourkoutas *et al*., 2004; Brena *et al*., 2013). Hydrophilicity is an important quality of a support matrix while pores increase the surface area and enzyme loading capacity and also prevent deactivation by proteases and other competitive inhibitors because enzyme inside a pore cannot be easily accessed (e.g. agarose) (Brena *et al*., 2013).

Inorganic support matrices have greater stability than organic support matrices; however in application organic support matrices are preferred because of their greater compatibility with enzyme (Miletic *et al*., 2012). Porous particles, gel matrices and polymeric membranes give high enzyme binding capacity, but porous particle can limit accessibility of the enzyme by particulate substrate. Non-porous carrier limits diffusion of substrate and products, therefore not suitable for immobilisation of enzymes which catalyse hydrolysis of solid substrates (Shakya *et al*., 2010).

To achieve proper enzyme attachment on the support matrix, the reactive groups on the surface of support matrix and enzyme must be able to react (geometrically

congruent) (Mateo *et al*., 2006). The reactive groups present on the protein or support should present minimal steric hindrance in the reaction.

Different support matrices have been used for immobilisation of cellulases. A study was conducted where cellulases were immobilised on polyethylene glycol (PEG)-chitosan using covalent bonding with glutaraldehyde. The best result was obtained where the immobilised enzymes were re-used in 11 cycles with retention of 80.27 % of initial activity (Walsh, 2005; Su *et al.*, 2012).

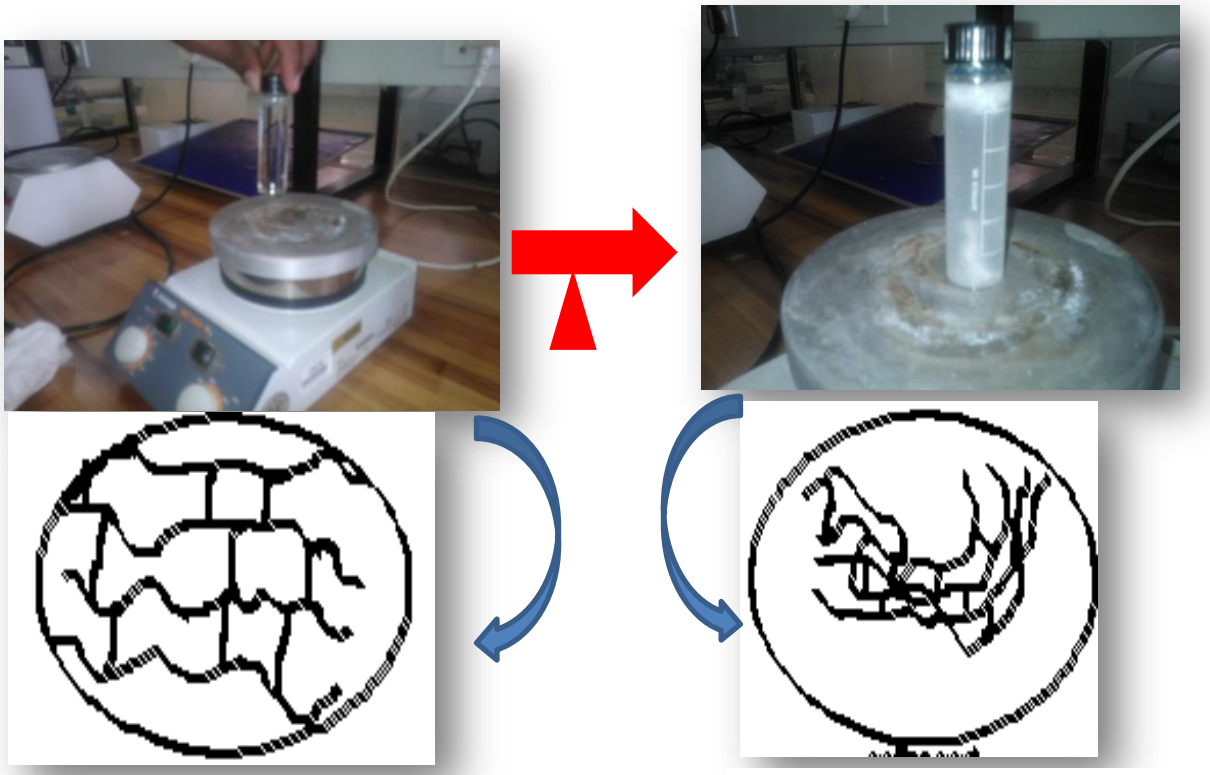
2.6.1. Smart Polymer as support matrices

Smart hydrogels are synthetic polymers that respond to environmental changes either chemical (pH, ionic factors and chemical agent in the solution) or physical (temperature, electric or magnetic fields, and mechanical stress) (Shakya *et al*., 2010; Garcia-Uriostegui *et al*., 2012; Liu *et al*., 2012). Other names coined include stimuli-sensitive, intelligent, or environmentally sensitive polymers (Gil and Hudson, 2004; Gupta *et al*., 2008). Smart polymers can be exploited in various forms such as cross-linked (permanently) hydrogels or microgel beads *etc.* (Lin *et al*., 1999; Gil and Hudson, 2004; Liu *et al*., 2012). Smart polymers are categorised according to the nature of stimuli such as temperature (thermo-) and pH responsive polymer (Gil and Hudson, 2004; Shakya *et al*., 2010).

Poly-*N*-isopropylacrylamide (*p*-NIPAAm) is the popular thermo-responsive smart-polymer with a lower critical solution temperature (LCST) of 32-33 °C in aqueous medium where it swells but does not dissolve due to hydrophilic interaction (Sheldona, 2007; O'shea *et al*., 2011). A demonstration of thermal response is shown on Figure 3 where heating above the LCST caused the polymer to precipitate (collapse, shrink or de-swell) due to hydrophobic interaction (Soppimath *et al*., 2002; Gupta *et al*., 2008; Garcia-Uriostegui *et al*., 2012). *p*-NIPAAm is chemically inert and does not contain functional groups with the ability to chemically react with bio-macromolecules, therefore, efforts have been made to introduce reactive groups into the polymer for covalent coupling of proteins onto the polymer (Rzaev *et al*., 2007).

The pH sensitive polymers consist of ionisable groups and are categorised based on the acidity (polyacids) or alkalinity (polybases) of the polymer which undergo an ionization/deionization transition from pH 4 to 8 (Gil and Hudson, 2004). The protonation and deprotonation of polymer backbone creates variation in electrostatic

interaction along the polymer that leads to alteration in hydrodynamic volume (Shakya *et al*., 2010). Most studied pH responsive hydrogel is *poly*-N,N-dimethylaminoethyl methacrylate (*p*-DMAEMA) with pKa of about 7.5. When the polymer is subjected to pH bellow 7.5 it becomes soluble due to protonation of the tertiary amine groups and above pH of 7.5 precipitation start to occur because of de-protonation (Orakdogen, 2012). The study of You and Auguste (2008) on the synthesis of *poly*-N,N-dimethylaminoethyl methacrylate/Methyl methacrylate (*p*-DMAEMA/MM) copolymer showed that the copolymer exhibited a high volume swelling ratio at low pH, low cross-linking density, and high content of *p*-DMAEMA.



a) Cross-linked polymer at low temperature (<32 °C)

b) Cross-linked polymers at high temperature (32-33 °C)

Figure 3. Thermal response of *p*-NIPAAm upon heating above its LCST. a) At low temperature *p*-NIPAAm swell due to water absorption, while b) At temperature more than its LCST (32-33 °C) the polymer collapse/shrink due to water release.

2.7. Immobilisation of cellulase onto different support materials

Khoshnevisan *et al*. (2011) demonstrated the immobilisation of cellulases onto magnetic nanoparticles (MNPs) and achieve 95% of cellulase bound to the support. With respect to temperature stability, free cellulase had high activity than immobilised

enzyme at 37, 50, 60 and 70 °C but activity of immobilised enzyme was greater than of free cellulase at 80 °C (Khoshnevisan *et al*., 2011). Jones and Vasudevan (2008) studied immobilisation of *Trichoderma reesei* cellulase using carrier free method where gluteraldehyde was used for self-crosslinking of enzymes. Minimum Gluteraldehyde and 1-ethyl-3-methylimidazolium diethyl phosphate (EMIM-DEP) concentration which gave high yield were 2% and 5 mM respectively.

2.8. Macroporous monolithic materials

Solid phase catalysts have been developed over the past years and these include polymers produced in the form of particles used in filling columns in processes that are carried out under continuous flow in different chromatography processes (Arrua *et al*., 2009). These particles are usually homogenous polymer network with low cross-linking; to be effectively used first the particles must be allowed to swell to become porous when immersed in good solvent. In contrary macroporous heteropolymer consist of macropores which are maintained in dry state or in any solvent (Arrua *et al*., 2009; Manfe *et al*., 2011). The internal structure of the macroporous polymer consists of interconnected polymers forming pores, their rigidity results from high cross-linking. Safrany (2005) has demonstrated the synthesis of heterogenous macroporous gels based on *p*-NIPAAm using e-beam method, gels were synthesized by polymerization and crosslinking in a heterogeneous media and gained faster response as compared to homogeneous macroporous gels.

2.9. Conclusion

The major limitation for making lignocellulosic based biofuel was highlighted in this review (pre-treatment, hydrolysis, microbial fermentation and biofuel separation) (Walsh, 2002; Drissen *et al*., 2007; Kumar *et al*., 2009; Goldbeck *et al*., 2012; Weiss *et al*., 2013; Balan, 2014). Commercialisation of immobilised cellulases for the purpose of reducing cost of hydrolysis is still at lower place due to crystalinity of lignocelluose which can interfere with enzyme activity (Tsai and Meyer, 2014). Therefore research needs to focus on the current limitation in order to overcome it (Datta *et al*., 2013). The use of smart polymers (*p*-NIPAAm) for immobilization of cellulases is fairly well established process (Balan, 2014; Ghizal *et al*., 2014). However through research on this subject, this technique will certainly promise the future for immobilisation of cellulases in saccharification of lignocellulosic substrates.

CHAPTER 3

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Reagents

N-isopropylacrylamide (NIPAAM) (Sigma-Aldrich) was purified by recrystallization from n-hexane. N,N'-tetramethyl-ethylenediame (TEMED) and N,N'-methylenebisacrylamide (MBA) were purchased from Sigma-Aldrich while ammonium persulfate (APS) was from Saarchem (Pty) Ltd. 2,2'-Azobis(2-methylpropionitrile) (AIBN) solution was purchased from Sigma-Aldrich and used as received. Tetrahydrofuran (THF) and Paraffin oil, the monomers 2-(dimethylamino)ethyl methacrylate (DMAEM), methacrylic acid (MAA) and methyl methacrylate (MM) were purchased from Sigma-Aldrich and used as received or purified by vacuum distillation to remove the inhibitor. Acrylic acid N-hydroxysuccinimide (NAS) ester or Methacrylic acid N-hydroxysuccinimide (NMS) ester for activation and Bicinchoninic acid (BCA) solution were purchased from Sigma-Aldrich. Water used was de-ionised unless stated otherwise. Jatropha seed cake was obtained from Polytechnic College, Harare, Zimbabwe. Sephadex® G-75 was purchased from Sigma-Aldrich.

3.1.2. Enzyme preparations

Model biocatalysts used for immobilisation were cellulase from *Aspergillus niger* FGSC A733, commercial *Aspergilus niger* β-glucosidase (commercial enzyme 1, comm1) (Sigma). Commercial cellulases: commercial enzyme 2 (comm2 and commercial enzyme 3 (comm3) were donated by particular commercial company and both names of enzymes and company were kept private for confidential purpose.

3.1.3. Equipment

Shimadzu high performance liquid chromatography (HPLC), G:Box Syngene gel imaging, Veriti 96 well Thermal cycler (Applied Biosystems), Multimode Detector DTX 880 (Beckman Coulter), BioLogic LP (BIO-RAD), Beckman Coulter Allegra X-22 Centrifuge.

3.2. Method

3.2.1. Production of cellulase from *Aspergillus niger* FGSC A733 spores

Solid-state fermentation (SSF) technique was used for production of cellulase. Cellulases were produced by inoculating duplicated 15 g of Jatropha seed cake at pH 5 with 1 mL of *Aspergilus niger (A. niger)* spore suspension (1.12 X 10^5 spores/mL), and then incubating at 40 °C for 96 hours. To extract crude enzymes, the reaction mixture was re-suspended in 50 mM acetate buffer pH 5 at 5 mL of buffer/g of fermented substrate. The content of the flask was continuously shaken at 50 rpm and 4 °C for 2 hours. Soluble enzymes were obtained by centrifugation at 3398 x *g* for 10 minutes at 4 °C. The clear enzyme solution was stored at -20 °C until used.

3.2.2. Preparation and activation of cellulase mixture

Activation of cellulases was done as described in Hao *et al*. (2001) with some modification. The activated NAS-cellulase or NMS-cellulase mixture was prepared by mixing 15 mL of 40 mg/mL of comm2 prepared in 50 mM acetate buffer pH 5 and 5 mL (1.4 FPU/mL) of *A. niger* cellulase (volume ratio 3:1). In order to obtain a clear solution, the enzyme mixture was centrifuged at 1494 x *g* for 10 minutes at 4 °C. Subsequently, 2.5 mL of 0.14 M of NAS or 0.13 M of NMS ester was added into 17.5 mL of enzyme mixture (volume ratio of 7:1) followed by incubation at 37 °C for 60 minutes. Free NAS or NMS was removed by passing the mixture through a Sephadex G25 column pre-equilibrated with 50 mM acetate buffer pH 5 or 50 Mm phosphate buffer pH 7.6. Alternatively sample was dialysed against 50 mM acetate buffer pH 5 at 4 °C for 2 hours. The activated enzyme conjugates were used immediately and stored at -20 °C when not in use.

3.2.3. Preparation of temperature sensitive cellulase-*p*-NIPAAm conjugates

To prepare a 20% (w/v) stock solution 1.2 g of NIPAAm monomer, 0.056 g of MBA cross-linker, and 0.24 g of ammonium persulfate (APS) were dissolved in 6 mL of water. Different concentrations of the gel: 10, 5, 2, 1, 0.5, 0.1, and 0.05% (w/v) of NIPAAm were prepared in 6 mL total reaction volume. Typical cellulase-2%-crosslinked-*poly*-N-isopropylacrylamide (cellulase-2%-crosslinked-*p*-NIPAAm) conjugate was prepared by mixing 0.6 mL of 20% (w/v) stock solution, 2.4 mL of water, and 3 mL (138 µmol/minute) of activated enzyme sample. To initiate free-radical

polymerisation 100 µL of TEMED was added into the reaction mixture followed by incubation for 2 hours at room temperature (Hao *et al*., 2001). After polymerisation the formed cellulase-crosslinked-*p*-NIPAAm conjugates were recovered by incubating at 45 °C for 10 minutes in the presence of 0.2 M potassium chloride (KCl). The precipitated conjugate was recovered by centrifugation at 1494 x *g* for 20 minutes at 40 °C. Recovered precipitates were washed in 20 mL of water twice using two cycles of solubilisation/precipitation in order to remove excess monomer and unbound enzymes.

The cellulase-microbeads-*poly*-NIPAAm were prepared by transferring the activated enzyme-monomer mixture into 150 mL of paraffin oil containing 1% Pluronic® L-81 detergent previously degassed (Lin *et al*., 1999). This solution was vigorously mixed in order to form aqueous droplets in the oil phase. To initiate polymerisation 100 µL of TEMED was added into the oil/enzyme/monomer mixture. The mixture was then shaken at 10 to 15 minutes intervals to maintain an emulsion over a 2 hour period at room temperature. The formed cellulase-microbeads-*p*-NIPAAm conjugates were separated out by excess deionised water and recovered in similar way as crosslinked polymers. Recovered precipitates were washed in 20 mL of water twice using two cycles of solubilisation/precipitation as. The enzyme conjugates were stored at 4 °C until use. The enzyme activity retention (% efficiency of immobilised enzyme) on the support was determined indirectly as difference of enzyme activity (µmol/minute) before immobilisation and sum of enzyme activity recovered in supernatants and washings (equation I). The % efficiency was calculated with respect to the activity of free cellulases of 264 µmol/min before immobilisation.

$$\% \text{ efficiency} = \frac{\text{Activity before immobilsation - Activity in washing}}{\text{Activity before immobilisation}} \times 100\% \qquad \text{(I)}$$

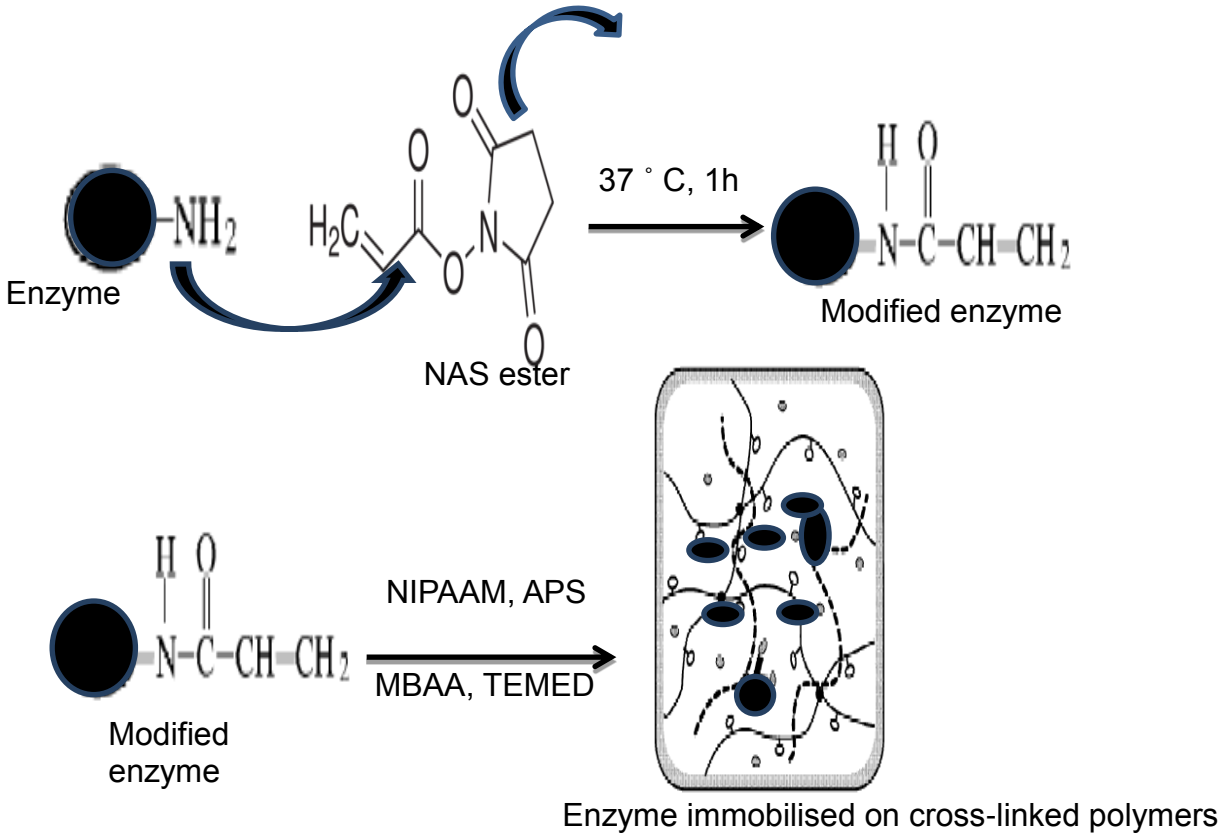


Figure 3. The reaction mechanism for the formation of amide bond between enzyme amino group and NAS ester and coupling to *p*-NIPAAm. Enzyme and polymer chains are not up to scale.

3.2.4. Preparation of pH and temperature sensitive cellulase-*p*-(NIPAAm-co-MAA) conjugates

3.2.4.1. *Synthesis of p-NIPAAm-co-MAA copolymer*

The poly-N-ispropylacrylamide-co-Methacrylic acid (*p*-NIPAAm-co-MAA) copolymers were prepared by mixing NIPAAm/MAA monomers at different molar ratios (1:0, 9:1, 8.5:1.5, 7:3, 3:2, 1:1, 2:3, 3:7, 1.5:8.5, 1:9, 0:1) with NAS fixed at 0.032 g (NIPAAm:NAS molar ratio 20:1). Typical NIPAAm/MAA copolymer was synthesized by mixing NIPAAm and MAA followed by dissolution in 20 mL of Tetrahydrofuran-Toluene mixture (1:1) (Lee *et al*., 2008). This solution was degassed under reduced pressure for 10 to 20 minutes. An amount of 4.2 mg (0.128 mL, 0.026 mM) of AIBN was added into the reaction mixture in order to initiate free-radical polymerisation which continued for 18 hours at 60 °C on a water bath. The white product formed was allowed to settle under gravity and then excess solvent decanted followed by precipitation and washing in 200 mL of diethyl ether in order to remove the homopolymer. The precipitated *p*-NIPAAm-co-MAA copolymer was recovered by

centrifugation at 1494 x *g* for 20 minutes and then air-dried and stored at 4 °C until used. The effect of cross-linking was determined by varying MBA concentration from 0 to 3 mole % with respect to the monomer.

$$\% \text{ MAA} = \frac{\text{mol of MAA}}{\text{mol of NIPAAm}} \times 100\% \qquad \text{(II)}$$

3.2.4.2. *Determination of the LCST of p-NIPAAm-co-MAA copolymer*

The lower critical solution temperature (LCST) of the copolymer was determined at the inflection point on the curve of UV absorption at 280 nm and temperature according to Lee *et al*. (2008). Polymer solutions of 60 mg/mL were prepared in 50 mM acetate buffer pH 5. The sample was filtered through 0.2 µm syringe driven filter and then 2 mL of polymer solution was added to 1 cm path length cuvette and placed in water bath at initial temperature of 25 °C The absorbance at 280 nm was measured using a UV/VIS Spectrophotometer (DU® 720, Beckman Coulter) at temperature increments of 5 °C between 25 and 65 °C and 5 minutes residence in each temperature. pH sensitivity was measured for copolymer with LCST of more than 50 °C by adjusting with 0.2 M NaOH or 0.2 M HCl.

3.2.4.3. Determination of the effect of solubilisation and shrinking kinetics of the p-NIPAAm-co-MAA copolymer

To determine the effect of solubilisation and shrinking kinetics on the recovery of the *p*-NIPAAm-co-MAA copolymer, four cycles of solubilisation and shrinking were performed (Peppas and Leobandung, 2004). The cycles were initiated by measuring the clarity of the copolymer solution followed by precipitation at 65 °C for a period of 5 to 10 minutes in the presence of 0.4 M KCl and then measured the cloudiness of the precipitated solution. The precipitated solution was allowed to re-solubilise by incubating for a period of 5 to 10 minutes at room temperature and then measure its clarity. All measurements were done at 600 nm. The clear solution used in the first solubilisation and shrinking cycle was used in the second solubilisation and shrinking cycle in the similar way as the first cycle.

3.2.4.4. *Coupling of enzyme onto p-NIPAAm-co-MAA copolymers*

For conjugation of enzyme onto *p*-NIPAAm-co-MAA, a 0.7% (w/v) solution was prepared by dissolving 0.1 g of *p*-NIPAAm-co-MAA copolymer in 10 mL of 50 mM acetate buffer pH 5. Solubilisation was assisted by adjusting pH to 6.5 then maintaining the pH at 5. Enzyme immobilisation was achieved by adding 5 mL of comm3 (24 FPU/mL; endoglucanase activity of 98 µmol reducing sugar /minute). To investigate the effect of enzyme loading dose on activity and solubility of the biocatalyst, the amount of aqueous enzyme was varied from 1960 to 19600 µmol/minute. The coupling of enzyme molecules onto the copolymer pre-activated with NAS via the amino group residues was achieved by incubating the reaction mixture at room temperature for 2 hours (Hao *et al*., 2001). Cellulase-*p*-NIPAAm-co-MAA conjugates were recovered from the solution by either adjusting the pH to 4.5 using 0.2 M HCl or by precipitation at 65 °C in the presence of 0.4 M KCl or by combination of both. This was followed by centrifugation at 193 x *g* using a bench top centrifuge for 20 minutes maintained at 65 °C. The cellulase-copolymer precipitate was washed using four cycles of precipitation and re-solubilisation in 50 mM acetate buffer pH 5 in order to remove unbound enzyme. Cellulase-copolymer, supernatant, and washings were kept at 4 °C until use.

3.2.4.5. *Determination of the effect of pH and temperature on solubility of p-NIPAAm-co-MAA copolymer and cellulase-p-NIPAAm-co-MAA conjugate*

A 0.6% (w/v) *p*-NIPAAm-co-MAA copolymer or cellulase-*p*-NIPAAm-co-MAA conjugate solution was prepared in 50 mM phosphate buffer (pH 5.5 to 6.5) and 50 mM acetate buffer pH 5. The cloudiness of the solutions was determined by incubating 2 mL of 0.6% solution in cuvette at 50, 55, 60 and 65 °C. This was followed by measuring absorbance at 600 nm in similar way as above (section 3.2.4.2).

3.2.4.6. *Determination of thermal stability of cellulase-p-NIPAAm54.5-co-MAA45.5*

To determine the residual activity of cellulase-poly-N-ispropylacrylamide54.5%-co-Methacrylic acid45.5% (cellulase-*p*-NIPAAm54.5-co-MAA45.5). A 25 mg of cellulase-*p*-NIPAAm54.5-co-MAA45.5 (adjusted to pH 5) with 0.02 µmol/min of endoglucanase activity was loaded into 2 mL Eppendorf tube. The gels were hydrated by adding 25 µL of 50 mM acetate buffer at pH 5 followed by incubating duplicate tubes at 45, 50, 55, 60 and 65 °C for 24 hours. Thereafter samples were cooled on ice for 10 minutes.

The residual activity was determined under assay conditions using CMC as substrate (section 3.2.7.3).

The thermal stability of comm3-conjugate under precipitation conditions was investigated by incubating 25 mg (0.05 µmol/min) of cellulase-conjugate adjusted to pH 5 at 60 and 65 °C in 2 mL Eppendorf tubes for a period of 2 hours. For comparison with free enzyme, 2 mL of soluble enzyme (comm3) diluted to have 155 µmol/min of endoglucanase activity was incubated under same conditions. Samples were withdrawn at 15 minute intervals, the enzyme activity before incubation was taken as 100% and residual endoglucanase activity was determined after incubation under assay conditions.

The thermo stability under operational conditions of immobilised at pH 5 and free enzyme with 0.1 U and 85 U of endoglucanase activity respectively was determined by carrying out reactions at 30 and 50 °C for 24 hours. Samples were withdrawn at 6 hours intervals and enzyme activity was determined after incubation under assay conditions (section 3.2.7.3).

3.2.5. Preparation of supermacroporous monolithic gels based on polyacrylamide

Polyacrylamide gels of concentrations ranging from 7.5 to 12% (v/v) were prepared by free radical copolymerisation of acrylamide (AA) and MBA in syringes. A stock solution (30% w/v) was prepared by dissolving 29.2 g of acrylamide monomer and 0.8 g of MBA to a total of 100 mL in distilled water. A typical 12% (w/v) gel was formed by mixing 12 mL of stock solution, 2.5 mL of 50 mM phosphate buffer pH 7.2, and 10.05 mL of comm3-NAS conjugate. To initiate copolymerisation 1 mL of 10% (w/v) APS was added followed by adding 100 µL of 0.7 M TEMED. The reaction mixture was rapidly transferred using a 20 mL syringe into a 60 mL syringe loaded with polystyrene beads which acted as porogene. The polystyrene beads contained diameter ranging from 2 to 5 mm achieved using a ruler millimeter scale. Polymerisation was allowed to occur for 6 hours at room temperature. The polystyrene beads were removed by repeatedly passing 10 mL of Toluene through the formed gel until all polystyrene beads were dissolved. The formed cellulase-AA-co-MBA supermacroporous conjugate was air dried in order to remove excess solvent. This was followed by washing with excess 50 mM acetate buffer pH 5 until insignificant amount of reducing sugar from enzyme solution remained in the gel. The

activity of cellulases and reducing sugar retained in the gel after washing were quantified using equation I (section 3.2.10.3).

The effect of crosslinking on the *p*-AA-co-MBA copolymer hardness was determined by increasing MBA to 1 g. Effect of incorporation of DMAEM into the *p*-AA-co-MBA copolymer was determined as follows: a 12% gel was formed by mixing 4 mL of 30% (w/v) of AA, 0.23 mL of DMAEM, 2.5 mL of 50 mM acetate buffer pH 5 and 3.1 mL of cellulase-NAS conjugate. The effect of increasing AA content to 15% (w/v) and enzyme concentration was determined by mixing 15 mL of 30% (w/v) of AA and 14.55 mL of cellulase-NAS.

3.2.6. Quantification of protein by Bicinchoninic acid (BCA) Assay

The BCA reagent was prepared by mixing 50 parts of BCA solution and 1 part of cupper (II) sulphate solution according to method of Pierce BCA Protein Assay Kit (2011). Triplicate reactions were performed by mixing 10 µL of protein sample with 200 µL of BCA reagent and incubating at 37 °C for 30 minutes. For estimation of quantity of protein, known concentrations of bovine serum albumin (BSA) solutions were used to create a calibration curve that was used to estimate the protein content in the samples.

3.2.7. Assay for cellulase activity

3.2.7.1. *β-glucosidase assay*

Activity of enzyme solutions on the β-glucosidase specific substrate *p*-nitrophenyl-β-D-glucopyranoside (pNPG) was performed by measuring the amount of *p*-Nitrophenol (*p*NP) released by the β-glucosidase from *p*NPG (Kaur *et al*., 2007). A volume of 25 µL of enzyme solution was mixed with 50 µL of 50 mM acetate buffer pH 5 in a flat bottomed microtitre plate and then pre-incubated at 45 °C for 5 minutes. The reaction was initiated by adding 25 µL of 10 mM pNPG in 50 mM acetate buffer pH 5. The reaction was followed over time by monitoring absorbance at 405 nm using a Beckman coulter plate reader (DTX880 multimode detector). Quantification of *p*NP released was determined from the *p*NP calibration curve. Reaction for cellulase-*p*-NIPAAm-co-MAA conjugate was done using 6 mg of copolymer loaded into 150 µL reaction volume consisting of 50 µL of *p*NPG and 100 µL of 50 mM acetate buffer pH 5. The reaction was stopped by adding 100 µL of NaOH-Glycine buffer pH 10.8,

followed by separation of the copolymer and supernatant as described in section 3.2.4.1.

3.2.7.2. *Filter paper assay for total cellulase activity*

Filter paper assay for total cellulase activity was determined according to Ghose (1987). An amount of 0.5 mL of appropriately diluted sample was added to 1 mL of 50 mM acetate buffer pH 5 and one Whatman No1 filter paper strip (1.0 x 6.0 cm) was suspended in each reaction mixture. This was followed by incubation for 60 minutes at 30 and 50 °C. To quantify the total reducing sugars liberated from the filter paper, 3 mL 3,5-Dinitrosalicylic acid (DNS) reagent was added in order to develop a dark brown colour with characteristic absorbance at 540 nm. Glucose standard solutions were used to create calibration curve. The estimation of the amount of reducing sugar liberated from the reaction was done using a linear equation of absorbance of glucose standard solutions versus concentration (mg). The activity unit (FPU) was expressed as FPU/mL (mg released*0.185).

3.2.7.3. *Endoglucanase activity on carboxymethylcellulose*

Endoglucanase activity assay was carried out by adding 100 µL of free enzyme solution into 900 µL of 1% carboxymethylcellulose (CMC) prepared in 50 mM acetate buffer pH 5 (Khoshnevisan *et al*., 2011). For the cellulase-conjugate, 50 mg of enzyme conjugate was mixed with 950 µL of 1% CMC. The reaction was incubated at 50 °C for 5 or 30 minutes in shaker incubator for immobilised enzyme then stopped by adding 1.5 mL of DNS reagent followed by boiling for 15 minutes. The absorbance (Abs) of the cooled samples was measured using UV-visible spectrophotometer at 540 nm. Reducing sugars produced were determined as previously described (section 3.2.7.2.). The enzyme unit was defined as the enzyme amount which is capable of releasing 1 µmole glucose per minute.

Alternatively, the endoglucanase activity assay was performed by adding 25 µL of free enzyme solution into 25 µL of 1% CMC in 96 well v-shaped bottomed microtitre plate according to Goldbeck *et al*. (2012) with modifications. The reaction mixture was incubated at 50 °C on a thermal cycler for 5 minutes. Thereafter the reaction was stopped by adding 75 µL of DNS reagent followed by boiling on a thermal cycler for 15 minutes. A volume of 100 µL of the solution was transferred into a 96 well flat bottomed microtitre plate and absorbance was read at 540 nm using a Beckman

coulter plate reader. For the immobilised enzyme 25 mg of cellulase-conjugate was weighed into 2 mL Eppendorf tube and then 475 µL of 1% CMC was added onto the tube then incubated at 50 °C for 30 minutes with shaking. A volume of 50 µL of the reaction mixture was transferred into a microtitre plate and then treated in similar as the reaction for the free enzyme solution. The enzyme unit was defined as the enzyme amount which is capable of releasing 1 µmole glucose per minute.

3.2.8. Hydrolysis of carboxymethylcellulose using cellulase-*p*-NIPAAm conjugates

Hydrolysis of CMC substrate using comm2-crosslinked-*p*-NIPAAm and comm2-microbeads-*p*-NIPAAm conjugate (10 to 0.05% v/v) was performed in duplicate (Jones and Vasudevan, 2010). A 1mL reaction contained 20 mg (~ 50 µL in swollen state) copolymer and 0.95 mL of 1% CMC in 2 mL microfuge. The reaction mixture was incubated at 30 and 50 °C in a shaker incubator over 6 hours. The CMC hydrolysate was withdrawn after at 0.5, 1, 2, 4, and 6 hours intervals. Cellulase-*p*-NIPAAm conjugates were separated from the products by centrifugation at 7378 x *g* for 20 minutes at 45 °C in the presence of 0.2 M KCl and stored at 4 °C until used. Quantification of reducing sugars from CMC hydrolysate samples was done by mixing 1 mL of diluted sample with 1.5 mL of DNS reagent as above (section 3.2.7.3.).

3.2.8.1. *Effect of different factors on enzyme activity of* cellulases-*p*-NIPAAm conjugates

The effect of incubation time on the activity of immobilised enzyme was determined over 48 hours using 1% CMC as substrate. In order to evaluate the effect of the copolymer quantity on the hydrolysis of CMC, the quantity of cellulases-*p*-NIPAAm conjugates was varied from 20 mg (2 wt/v) to 200 mg (21 wt/v). The concentration of comm 1 and comm 2 was varied to make 2 to 5% (v/v) and 400 mg/mL respectively in the enzyme mixture used for immobilisation in order to improve the hydrolysis yield of glucose and cellobiose.

3.2.9. Hydrolysis of microcrystalline cellulose using cellulase-*p*-NIPAAm conjugates

Hydrolysis of microcrystalline cellulose (avicel) using comm2-crosslinked-*p*-NIPAAm conjugate was performed in 450 µL reaction volume. The duplicate reactions contained 20 mg (~ 50 µL) of immobilised enzyme, 200 µL of 23% (w/v) avicel and

200 µL of 50 mM acetate pH 5 in 2 mL microfuge (Alftren and Hobley, 2014). The reaction mixture was incubated at 30 and 50 °C in a shaker incubator over 336 hours. The avicel hydrolysate (450 µL) was withdrawn initially after 1, 6, 12 and 24 hours followed by withdrawals every 24 hours. The volume of sample was adjusted to 1 mL with 50 mM acetate buffer pH 5. The biocatalyst was recovered by centrifugation as already presented (section 3.2.5.2.). The resultant supernatants were filtered through a 0.2 µm filter and kept at -20 °C for 24 hours for high performance liquid chromatography (HPLC) analysis of glucose and cellobiose formed.

3.2.9.1. HPLC analysis of glucose and cellobiose

Filtered samples were analysed for glucose and cellobiose content using Shimadzu HPLC (Tyoko, Japan) equiped with a Rezex RCM-Monosaccharide Ca+2 (8%) column (300 x 7.80 mm, Phenomenex, USA) being maintained at 85 °C (Alftren and Hobley, 2014). Separation mode was isocratic with deionised water used as mobile phase, and 20 µL of sample was injected. The flow rate was 0.6 mL/minute. Peaks were detected using a refractive index detector (RID 10A) and analysed using LC solutions computer software. The concentration of glucose and cellobiose formed was determined by extrapolation from the glucose and cellobiose standard solutions peak height.

3.2.10. Recycling of immobilised enzymes in hydrolysis of microcrystalline cellulose

3.2.10.1. *Hydrolysis using cellulase-p-NIPAAm-co-MAA conjugate*

Recycling of comm2-*p*-NIPAAm-co-MAA conjugate was done in duplicated reaction mixture. The reaction mixture contained 50 mg of immobilised enzyme, 25 mg of avicel and 100 µL of 50 mM acetate buffer pH 5 (Ikeda *et al*., 2015). The reaction mixture was incubated at 50 °C followed by withdrawal of samples after 0, 3, 6, 12 and 24 hours. The reaction volume was adjusted to 1 mL with acetate buffer in order to allow the comm2-*p*-NIPAAm-co-MAA conjugate to swell for complete recovery. The residual substrate from the first hydrolysis cycle (24 hours) was allowed to sediment while vertically placing tubes in ice for 5 to 10 minutes. The clear solution containing the conjugate was transferred using a pipette into fresh tube and then recovered the conjugate at precipitation conditions (section 3.2.4.1.). The recovered biocatalyst was washed three times with 50 mM acetate buffer pH 5 containing 0.02% (w/v) of sodium

azide. The same conditions described for the first cycle were subsequently used in the second hydrolysis cycle using the recovered biocatalyst. The amount of reducing sugars in the avicel hydrolysate was determined according to the DNS method in a microtitre plate (section 3.2.10.3.).

3.2.10.2. *Hydrolysis using* cellulase-*p*-AA15%-co-MBA (*supermacroporous monolithic gel)*

Hydrolysis of avicel in supermacroporous monolithic gel was done in the presence of 0.02% (w/v) sodium azide. The supermacroporous gel was loaded with 20 mL of 5% (w/v) of avicel prepared in 50 mM acetate buffer pH 5 and then incubated at 50 °C in shaker incubator. A 0.5 mL of avicel hydrolysate was withdrawn periodically at 0, 1, 3, 6 and then continued with 6 hours interval until a plateau was reached. At the end of the first hydrolysis cycle the gel was washed until no sugar was detected and then 20 mL of 50 mM acetate buffer pH 5 was added. The second hydrolysis cycle was performed under the same incubation conditions described from the first cycle. The control reaction was prepared using conditions described for the hydrolysis except that the gel did not contain the immobilised enzyme. The amount of reducing sugars in the avicel hydrolysate was determined according to the DNS method in microtitre plate in triplicate (section 3.2.10.3.).

3.2.10.3. DNS assay in microtitre plate for determination of reducing sugars

The analysis of reducing sugars from the avicel hydrolysate was done according to the DNS assay performed in a flat bottomed microtitre plate according to Goldbeck *et al*. (2012) with modifications. The avicel hydrolysate or glucose standard solution (60 µL) was mixed with 75 µL of DNS reagent in duplicate reaction. The mixture was heated on a Veriti 96 well thermal cycler (Applied Biosystem) at 99 °C or boiled in water bath for 15 minutes. The absorbance of the boiled mixture was read at 540 nm using a Beckman DTX880 multimode detector. The concentration of the reducing sugars formed from the avicel hydrolysate was determined from the glucose calibration curve (0 to 10 g/L).

3.2.11. Recycling of cellulase-*p*-AA15%-co-MBA in hydrolysis of thatch grass

3.2.11.1. *Pre-treatment of thatch grass*

Pre-treatment of common thatched grass (CTG) (*Hyperrhaenia sp*.) was performed using acid method according to Ncube *et al*. (2013). Thatch grass was first chopped into pieces then oven dried at 50 °C overnight to remove the moisture. The grass was ground using a grinder (MF 10 basic, IKA wereke) with 2 µm cut-off size. The ground grass (7.83 g) was suspended in 100 mL of 0.5% H_2SO_4 followed by hydrolysis in an autoclave at 121 °C for 1 hour in order to remove soluble sugars and inhibitors. The resultant slurry was filtered through a Buchner funnel fitted with a Whatman filter paper. The second washing was done using excess 50 mM acetate buffer pH 5. The slurry was stored at -20 °C and while in the moist state used.

3.2.11.2. *Hydrolysis of thatch grass and recycling of cellulase-p-AA15%-co-MBA*

Acid pre-treated thatch grass was subjected to hydrolysis by cellulase immobilised on supermacroporous gel designated cellulase-*p*-AA15%-co-MBA. The gel with 94.8% activity retention was loaded with 20 mL of 5% (w/v) of pre-treated CTG (1 g of CTG in 50 mM acetate buffer pH 5 with 0.02% sodium azide). The slurry was added in small portion to avoid clogging and uniformly spread by gentle mixing. The reaction mixture was incubated at 50 °C with 1 mL CTG hydrolysate withdrawn after 0, 1, 3, 6 and continued at 6 hour intervals. The reaction was continued until a plateau was reached. At the end of the first hydrolysis cycle the gel was washed with 50 mM acetate buffer pH 5 until no sugar was detected in the washings and then replenished with 20 mL of same buffer used in washing. The second hydrolysis cycle was repeated as described for the first cycle. The control reaction was prepared using conditions described for the hydrolysis except the gel did not contain the immobilised enzyme. The amount of reducing sugars in the CTG hydrolysate was determined according to the DNS method in microtitre plate in triplicate (section 3.2.10.3.).

3.2.12. Hydrolysis of thatch grass using free enzyme

Hydrolysis reaction was done in the same conditions as in (3.2.11.2.) except 20 mL of aqueous comm3 [0.3 mg/mL of protein, 0.63 FPU/mL (18.9 FPU/g of substrate)] at pH 5 was used. The acid pre-treated thatch grass was added into this enzyme solution to final concentration of 5% (w/v). The reaction mixture was then mixed and

incubated for 24 hours with sampling and reducing sugar determination as above (3.2.11.2).

3.2.13. Statistical analysis

Significant differences in results for different variables (effect of enzyme loading, pH, temperature and hydrolysis kinetics) were determined by one-way analysis of variance (Statistix 10, 1985-2013). Pearson r coefficient was determined using International Business Machines (IBM) Statistical Package for the Social Science (SPSS) Statistics software (Version 23).

CHAPTER 4

4. RESULTS

4.1. Effect of polymer density on enzyme binding capacity

Immobilisation results show that cellulase-10%-microbeads-*p*-NIPAAm conjugate was capable of binding to more enzymes as reflected by higher percent efficiency of 84% (Table 1). When the *p*-NIPAAm density was decreased from 5 to 1% (w/v) there was an observed decrease in the activity retention of cellulases from 95 to 76% respectively. The cellulase-microbeads-*p*-NIPAAm conjugate with *p*-NIPAAm density from 0.5 to 0.05% (w/v) had high solubility in the reaction solvent. As a result it was not possible to be recovered from the solvent after polymerisation; therefore the percentage efficiencies were not determined.

Table 1. Activity profile of cellulase during immobilisation on different microbeads-*p*-NIPAAm density

% cellulase-*p*-NIPAAm gel (w/v)	**Supernatants (µmol/minute)**	**Activity (% efficiency)**
10	42.6 ± 0.24	84 ± 0.09
5	15.7 ± 0.83	95 ± 0.31
2	63.4 ± 2.59	76 ± 0.98
1	77.9 ± 6.69	76 ± 2.54
0.5	ND	ND
0.1	ND	ND
0.05	ND	ND
NAS-cellulase before immobilisation (µmol/minute)	264	

ND: not determined

4.2. Enzyme kinetics using cellulase-*p*-NIPAAm conjugates in hydrolysis of CMC

The hydrolysis of 1% (w/v) CMC using comm2-microbeads-*p*-NIPAAm conjugates with different *p*-NIPAAm density (%) ranging from 0.05 to 10% (w/v) was performed (Figure 4). The amount of reducing sugar released by the immobilised enzyme systems within 1 hour of incubation ranged from 0.5 to 2 g/L. A sudden increase of reducing sugar was noted for comm2-0.05%-microbeads-*p*-NIPAAm conjugate after 1 hour of incubation. It was observed visually that the solubility of comm2-microbeads-*p*-NIPAAm conjugates in the reaction mixture varied with the polymer density. This is to say, low percentage gel 0.05%-microbeads-*p*-NIPAAm was more soluble than higher percentage gel 10%-microbeads-*p*-NIPAAm. It was expected that the increase of enzyme activity and maximum amount of reducing sugar released will depend on the activity retained after immobilisation. However, there was a deviation from the expected outcome: after 6 hours, the trend of increase in maximum reducing sugar was 0.05>1>10>5>0.1% with about 16, 11, 5, 4, and 2 g/L of reducing sugar released respectively.

The results for the hydrolysis of 1% CMC using comm2-crosslinked-*p*-NIPAAm conjugates shows that after 6 hours, the amount of reducing sugar released ranged between 0 to 2.5 g/L (Figure 5). The activity of comm2-10%-crosslinked-p-NIPAAm conjugate was not detected during the first 6 hours, while comm2-2%-crosslinked-p-NIPAAm and comm2-5%-crosslinked-p-NIPAAm conjugates released 2.4 and 2.5 g/L of reducing sugars respectively (Figure 5). The maximum reducing sugar released after 12 hours were 1.6, 4.2 and 3.8 g/L for 10, 5 and 2% conjugate respectively (Figure 5).

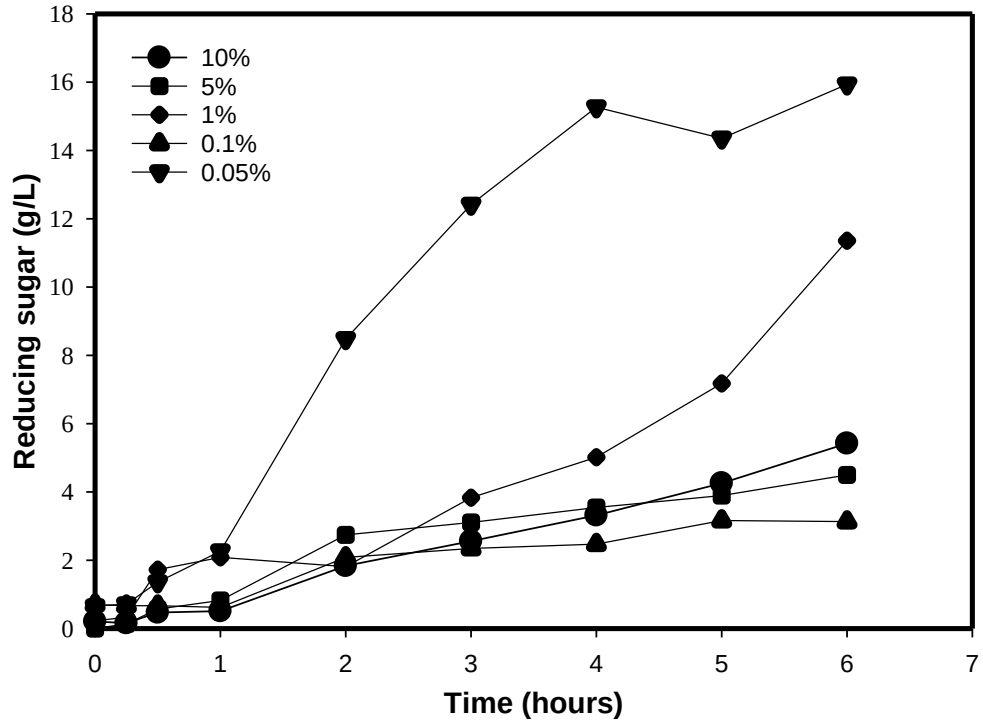


Figure 4. Effect of polymer density on the endoglucanase activity of comm2-microbeads-*p*-NIPAAm conjugate with CMC being the substrate.

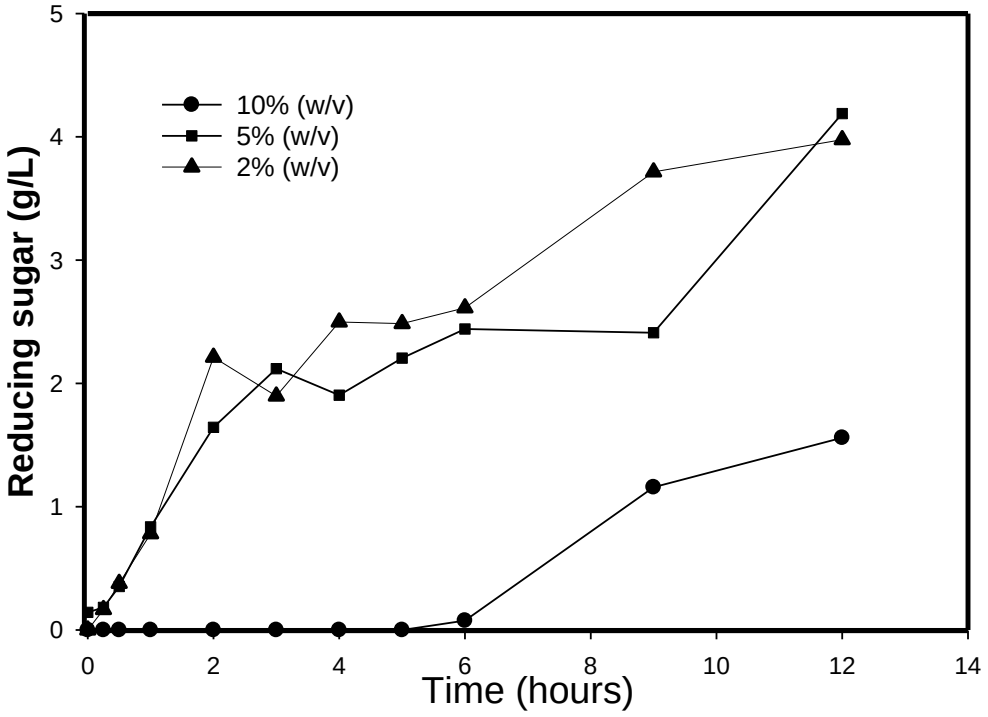


Figure 5. Reducing sugars released by comm2-crosslinked-*p*-NIPAAm conjugates from 1% CMC.

4.3. Effect of the quantity of cellulase-crosslinked-*p*-NIPAAm conjugate in hydrolysis of CMC

The effect of the quantity of comm2-2%-crosslinked-*p*-NIPAAm conjugate in hydrolysis of 1% CMC was determined in order to optimise hydrolysis conditions (Figure 6). The quantity of comm2-2%-crosslinked-*p*-NIPAAm conjugate in reaction mixture was varied between 2 and 20% (w/v). The results show that a maximum of 2.1, 4.2, 8.5 and 5.2 g/L of reducing sugar was released with loading dose of 2, 5, 11 and 20% respectively after 6 hours (Figure 6). Pearson's correlation coefficient was computed using SPSS in order to assess the relationship between quantities of immobilised enzyme and reducing sugar released. The results show that there was a weak positive linear correlation between the two variables ($r=0.36$, $n=4$, $p>0.05$). Increase of quantity of immobilised enzyme caused an increase of concentration of reducing sugar in dose dependant manner except at higher loading of 20%. However, loading dose of 5% was used in subsequent experiments for the economic reason.

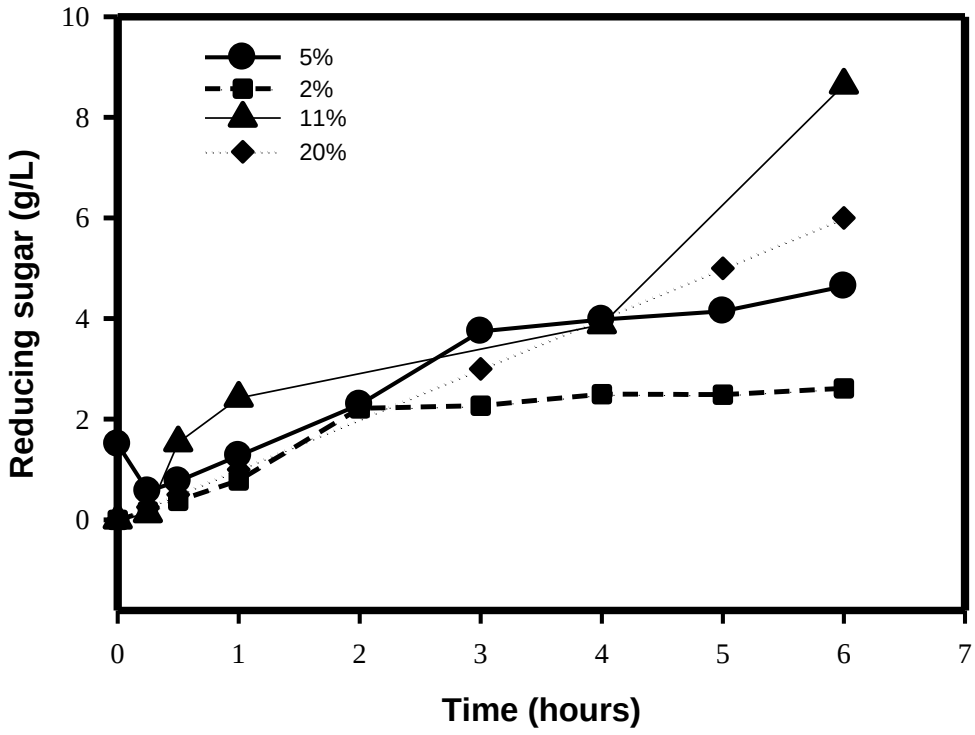


Figure 6. Effect of quantity of comm2-2%-crosslinked-*p*-NIPAAm conjugate in hydrolysis of 1% CMC.

4.4. Effect of increasing enzyme concentration during immobilisation

The effect of increasing the concentration of celluclast in the comm2 mixture from 40 (1x) to 400 (10x) mg/mL was determined (Figure 7). Hydrolysis of 1% CMC using comm2-2%-crosslinked-*p*-NIPAAm conjugates at 5.3% immobilised enzyme loading dose. The maximum reducing sugar released from the 10X immobilised enzyme increased 2-fold over the reducing sugar released from the 1X immobilised enzyme.

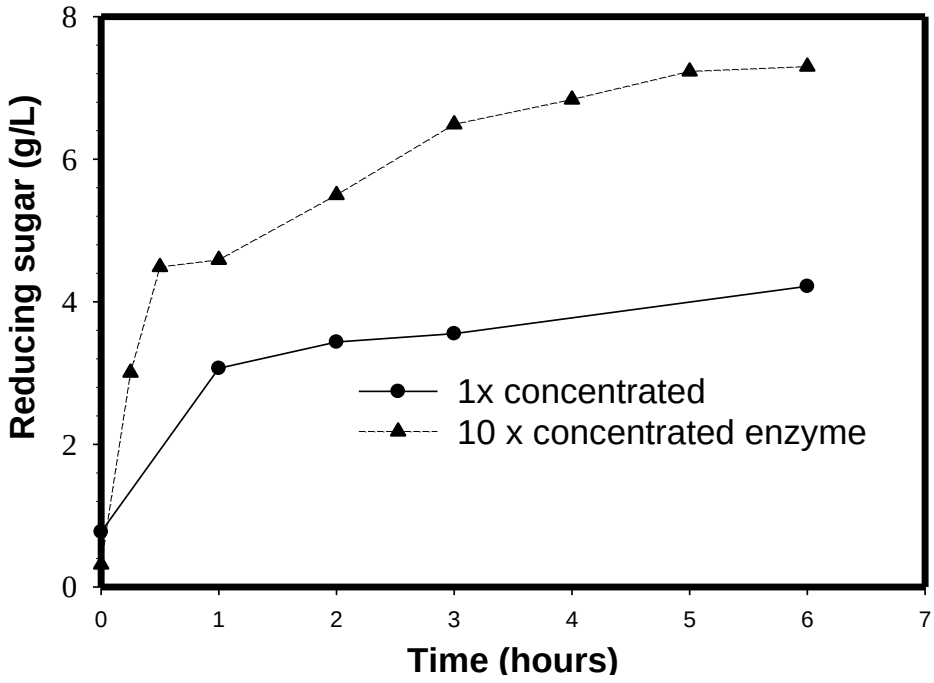


Figure 7. Kinetics study for hydrolysis of 1% CMC using comm2-2%-crosslinked-*p*-NIPAAm conjugates loaded with 1x and 10x times concentrated cellulase mixture during immobilisation.

4.5. Hydrolysis of microcrystalline cellulose using cellulase-crosslinked-*p*-NIPAAm conjugates at different temperatures

Microcrystalline cellulose (Avicel) was hydrolysed using comm2-2%-crosslinked-*p*-NIPAAm conjugates (Figure 8). Different temperatures 30 °C below the LCST of *p*-NIPAAm and 50 °C higher than the LCST were used for the reactions. HPLC analysis was done to quantify the amounts of glucose and cellobiose released over a 48 hour incubation period. The actual amount of cellobiose released was greater than the amount of glucose at both temperatures (Figure 8). Maximum release of reducing sugars was archived after 36 hours at both temperatures: cellobiose amounted to 0.3 and 0.65 g/L at 30 and 50 °C respectively while glucose amounted to 0.18 and 0.2 g/L at 30 and 50°C respectively. After 48 hours the amount of cellobiose dropped by 83 and 54% at 30 and 50 °C respectively, while glucose decreased by 50 and 30% at 30 and 50 °C respectively.

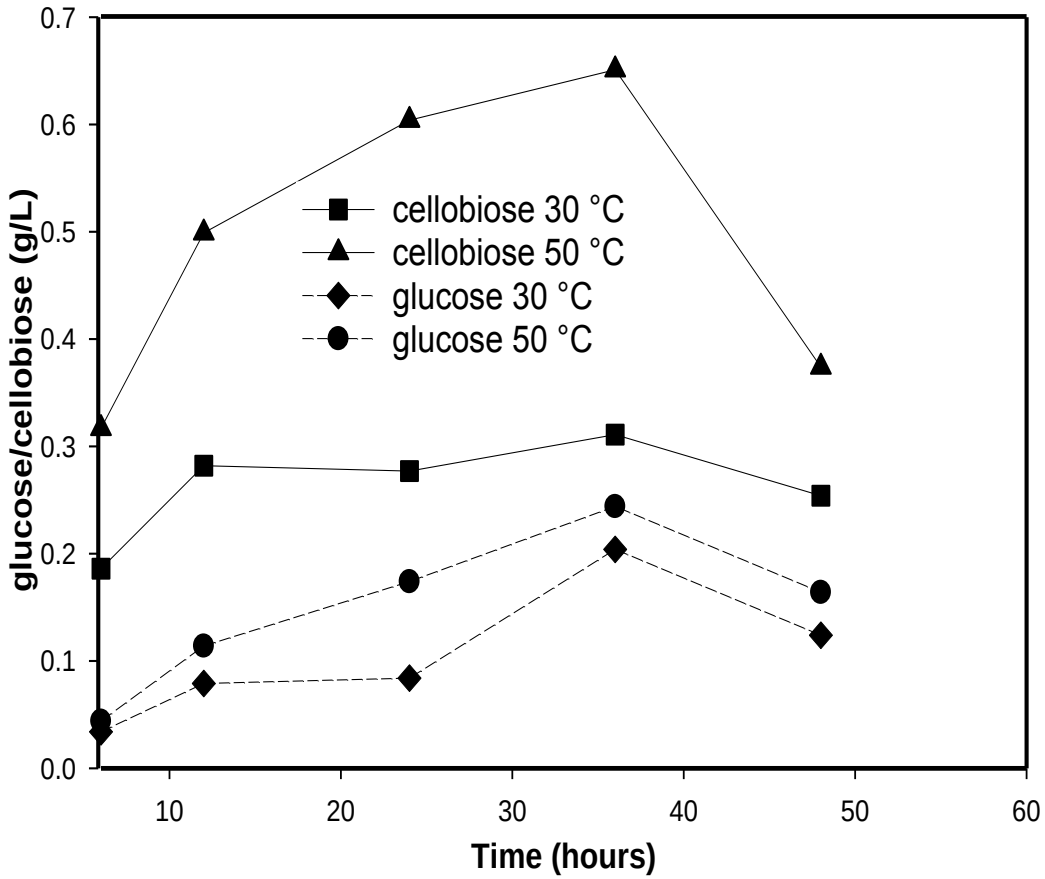


Figure 8. Glucose and cellobiose released from Avicel by comm2- 2%-crosslinked-*p*-NIPAAm conjugate at 30 and 50 °C.

4.6. Effect of β-glucosidase loading on activity of cellulase-crosslinked-*p*-NIPAAm conjugates

Commercial *A. niger* β-glucosidase with 134 U/mL (Sigma) was varied from 2 to 5% (v/v) in order to investigate the effect of β-glucosidase activity in cellulase mixture and cellulase-2%-crosslinked-*p*-NIPAAm conjugate. The resultant filter paper unit of the cellulase cocktail supplemented with 5% β-glucosidase was 10.3 FPU/mL. The maximum amount of glucose and cellobiose released from hydrolysis of Avicel after 12 hours of incubation at 30 °C were 2.3 and 0.5 g/L respectively (Figure 9). In comparison to glucose concentration at both 30 and 50 °C, the cellobiose concentration was lower than 0.6 g/L through the entire reaction at both temperatures. The hydrolysis yield of reducing sugars at 50 °C reached maximum after 84 hours with 2.5 g/L of glucose and 0.6 g/L of cellobiose.

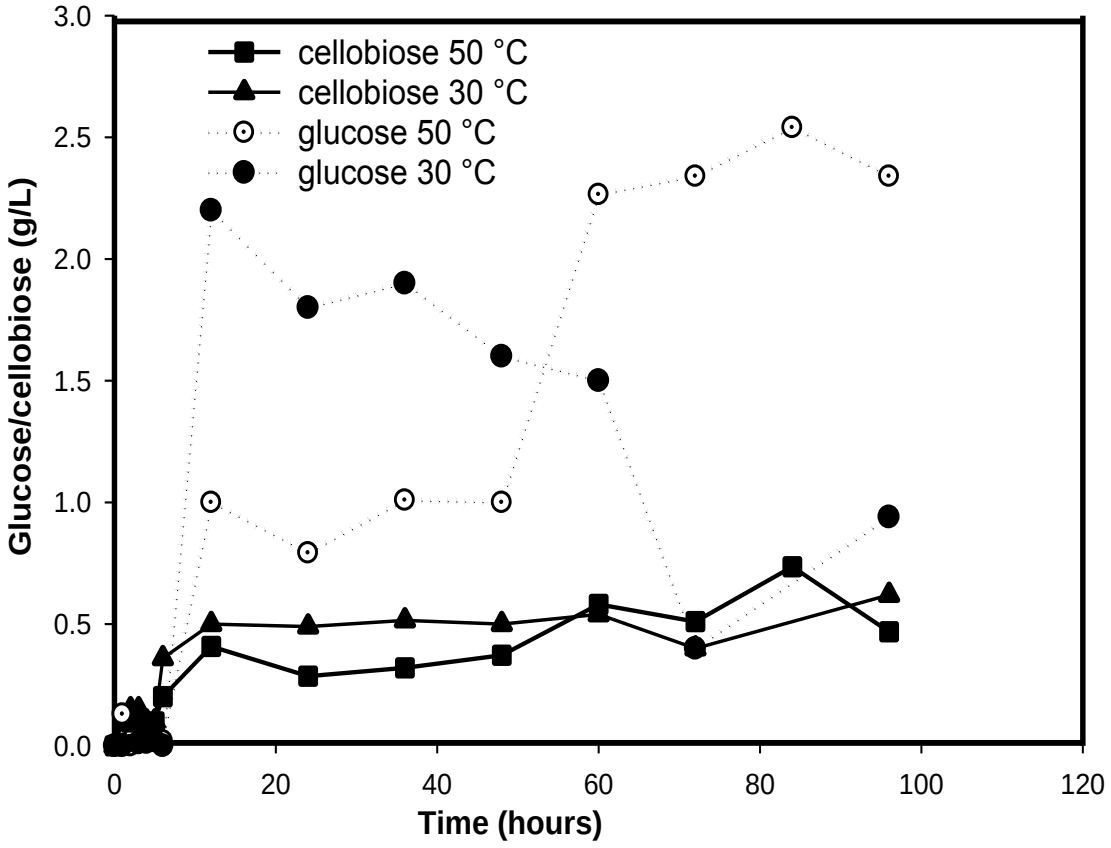


Figure 9. Reducing sugars produced during saccharification of crystalline cellulose (Avicel). Comparison of glucose and cellobiose released using cellulase-2%-crosslinked-*p*-NIPAAm conjugate modified to include 5% v/v of β-glucosidase (134 U/mL).

4.7. Effect of increasing cellulose hydrolysis time and treatment of samples with HCl

The hydrolysis of 23% (w/v) of Avicel over 312 hours was done in the presence of 0.02% of sodium azide using cellulase-2%-crosslinked-*p*-NIPAAm conjugate supplemented with 5% β-glucosidase (Figure 10). Sodium azide was used as inhibitor of microbial growth. After 216 hours about 2.5 and 5 g/L of glucose was released at 30 and 50 °C respectively. The concentration of cellobiose was constant at both 30 and 50 °C over the entire hydrolysis time (312 hours).

Acetate buffer salts interfered with glucose and cellobiose intensities during HPLC analysis as they appeared at the same retention time as glucose compound. To overcome this, glucose and cellobiose samples were treated with 0.1 M HCl and then evaporated using a rotary evaporator. The resulting solid samples were reconstituted in equal volume of water. With reference to 216 hours hydrolysis samples glucose concentration after acid treatment was lowered to 0.8 and 3.8 g/L for 30 and 50 °C sample respectively (Figure 11). Consequently, the glucose concentration in the water sample after acid treatment decreased 3 and 1-fold in the 30 and 50 °C hydrolysis samples respectively. However, the glucose compound chromatography peaks were resolved while the acetate buffer peaks eliminated from the chromatogram.

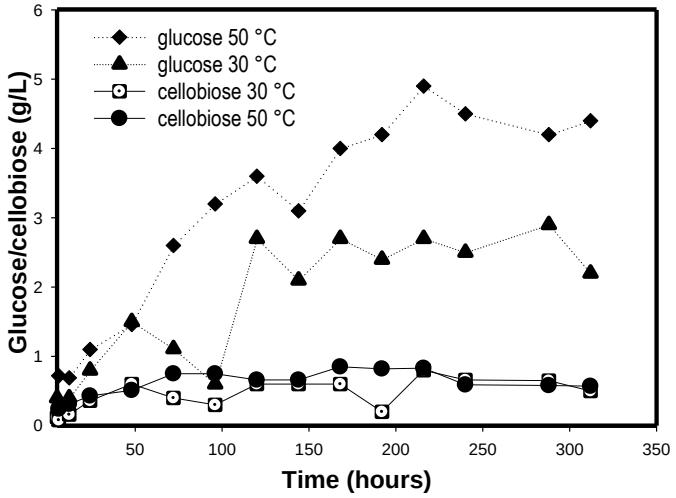


Figure 10. HPLC analysis of glucose and cellobiose produced during saccharification of crystalline cellulose (Avicel) using cellulase-2%-crosslinked-*p*-NIPAAm conjugate supplemented with 5% β-glucosidase.

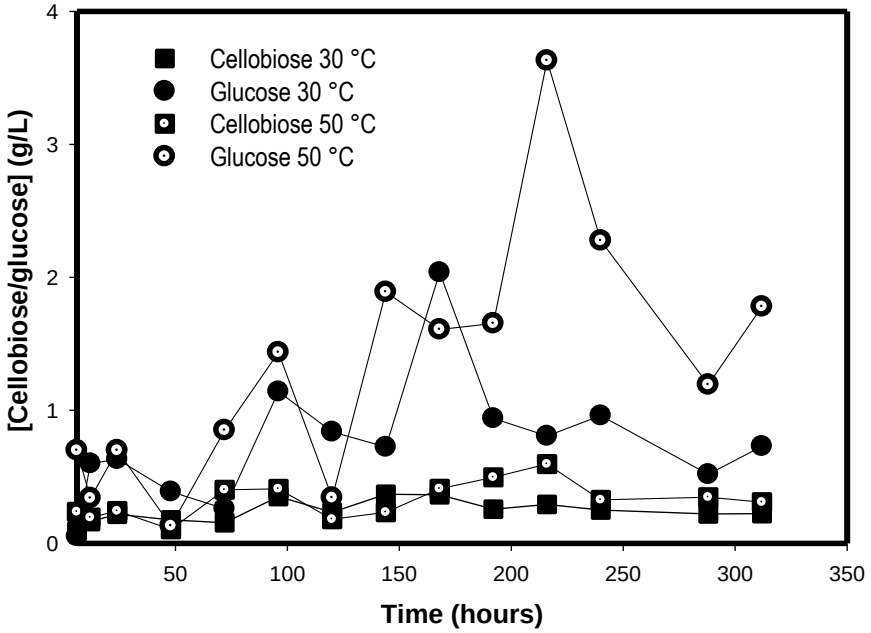


Figure 11. Treatment of avicel hydrolysate with 1 M HCl and analysis of glucose and cellobiose using HPLC after reconstitution in distilled water.

4.8. Effect of MAA content and cross-linking density on the appearance and LCST of *p*-(NIPAAm-co-MAA) copolymers after synthesis

The copolymerisation of NIPAAm and MAA monomers resulted in the formation of white product of the *p*-(NIPAAm-co-MAA) copolymers (Table 2). The native *p*-NIPPAm has its LCST at approximately 33 °C while the LCST of the formed copolymer was higher than 33 °C. When the MAA content was increased from 0 to 40 mole %, the LCST of the formed copolymer also increased from 33 to 40 °C respectively. Further increase of MAA content to 60 mole % resulted in the increase of LCST of the copolymers to more than 65 °C. Copolymers with LCST of more than 65 °C were suitable for use as support matrices for immobilisation of cellulase because the enzymes have optimal activity within 45 to 60 °C.

The LCST results of the *p*-NIPAAm85%-co-MAA15% copolymer show that when the cross-linking density of the copolymer was increased from 0 to 3 mole%, its LCST also increased from 34 to 38 (Figure 12). Even slight increase of cross-linking from 0 to 0.15 mol % had significant effect on the LCST of the copolymer. With reference to *p*-NIPAAm70%-co-MAA30% copolymer, the corresponding LCST increased from 39

to 42 °C. The *p*-NIPAAm60%-co-MAA40% copolymer achieved LCST increase from 40 to 44 °C (Table 2).

The solubility (swelling/sol state) and precipitation (shrinking/gel state) range of the copolymers below and above their LCST respectively was determined from the concavity (Figure 12). A typical solubility and precipitation range of the *p*-NIPAAm70%-co-MAA30% copolymer was from 25 to 33 °C and 34 to 60 °C respectively. Data from the inflection point method reveals that the sol state of the *p*-NIPAAm70%-co-MAA30% copolymer was reflected by the concave upwards while the gel state was reflected by the concave downwards (Figure 12). It was observed visually that a *p*-0%MBA-MAA45.5%-co-NIPAAm54.5% copolymer solution with 0.4 M KCl quickly undergoes precipitation within 5 minutes at 65 °C.

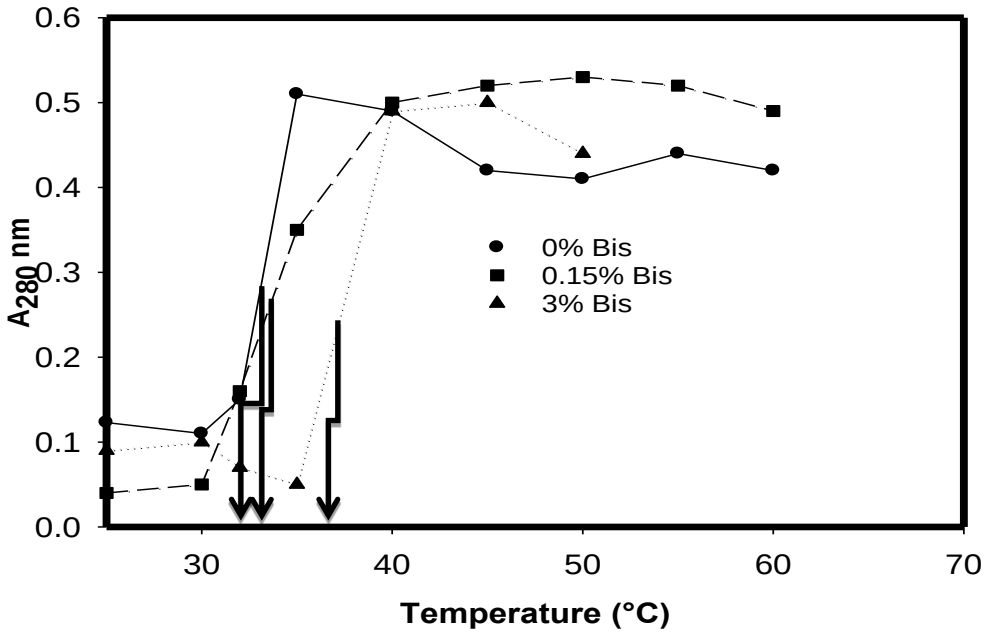


Figure 12. Low critical solution temperature (LCST) as determined by the inflection point method for MAA (15%): NIPAAm (85%) molar ratio copolymer.

Table 2. Visual appearance of *p*-(NIPAAm-co-MAA) copolymers after synthesis

MAA/NIPAAm molar ratio (%)	Visual appearance of *p*-(NIPAAm-co-MAA)	LCST (°C)* MBA molar (%)		
		0	0.15	3
0/100	white powder	33		
15/85	White powder	34	38	38
30/70	white powder	39	42	49
40/60	white powder	40	44	52
43/57	white powder	60	-	-
44/56	white powder	60	-	-
45/55	white powder	65	-	-
45.5/54.5	white powder	65	-	-
50/50	white powder	>65	-	-
60/40	white powder	>65	-	-

LCST: Determined from inflection point of Absorbance against temperature curve

4.9. Swelling/Shrinking kinetics of *p*-NIPAAm-co-MAA copolymer

The cross-linked *p*-NIPAAm45.5-co-MAA54.5 copolymer hydrogels appear to swell faster than the hydrogel without cross-linking (Figure 13). A comparison of the swelling and shrinking kinetics of the hydrogels shows a reduction of shrinking rate in sebsequent cycle. After 30 minutes of swelling and shrinking kinetics, the cloudiness of the hydrogel solutions were reduced with respect to the first cylcle at 10 minutes. When comparing the cross-linking densities, the sequence of reduction of clouding point was: 0.05%> 0.0005%> 0.05%> 0% with 2-fold, 1.5-fold, 1.3 fold and 1-fold decrease respectively. The reduced optical densities implies that the shrinking rates of the hydrogel decreased over time as seen from the graph.

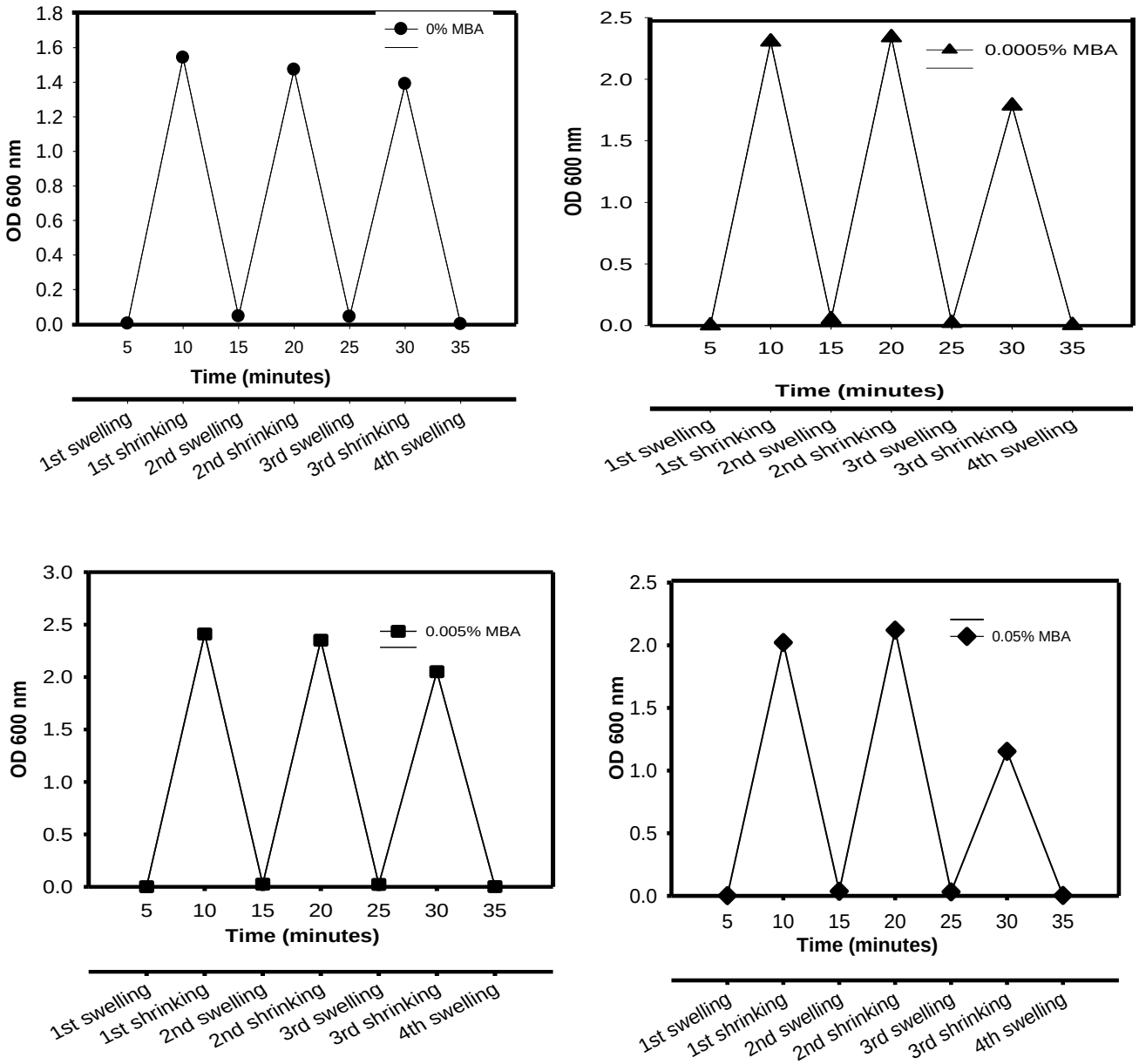


Figure 13. Swelling and shrinking kinetics over 35 minutes using *p*-NIPAAm45.5-co-MAA54.5 (a) 0 mol% MBA (b) 0.0005 mol% MBA and (c) 0.005 mol% MBA and (d) 0.05 mol% MBA. Reaction: 0.6% (w/v), 50 mM acetate buffer pH 5, swelling for 5 minutes on ice and shrinking at 65 °C for 5 minutes.

4.10. Effect of enzyme loading prior immobilisation on the activity of cellulase-*p*-(NIPAAm54.5-co-MAA45.5) conjugate

The effect of aqueous free enzyme (comm3) loading with activity of 98 µmol/minute on the activity of immobilised cellulase was determined. The result from SPSS software shows that the relationship between the activity of immobilised enzyme and enzyme loading was linear. The Pearson r coefficient (r=0.944) supports a very strong (8</r/<1) positive linear relationship between the two variables (Figure 14). However, the linear relationship between aqueous free enzyme loading and activity of the Cellulase-*p*-(NIPAAm54.4-co-MAA45.5) conjugate was not statistically significant (p>0.05). Finally the enzyme loading with 19600 µmol/minute gave the highest enzyme activity on the immobilised enzyme (0.49 µmol/minute) and was used in subsequent experiments.

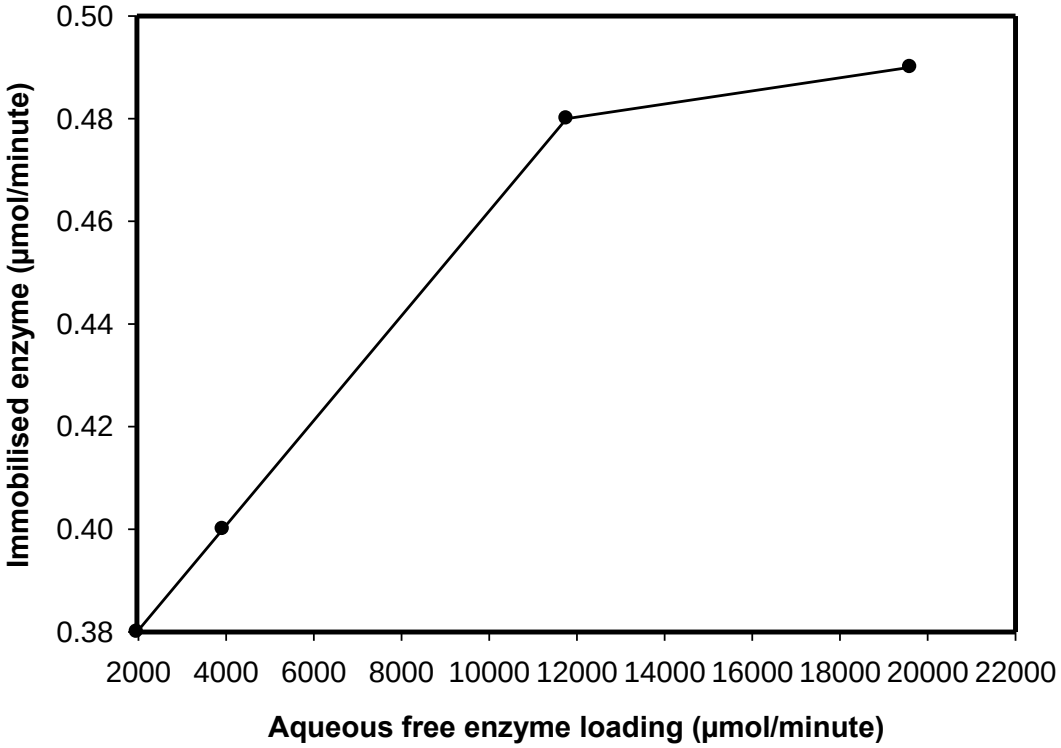


Figure 14. Effect of enzyme loading on the activity of cellulase-*p*-(NIPAAm54.4-co-MAA45.5) conjugate.

4.11. Effect of pH and temperature on the solubility of cellulase-*p*-(NIPAAm54.5-co-MAA45.5) conjugate

The immobilised enzyme prepared from various protein loading 0 to 19600 µmol/minute was analysed for solubility at various pH values and temperatures. The *p*-(NIPAAm54.5-co-MAA45.5) copolymer without enzyme was used as a control. The data for control shows that the relationship of temperature and cloudiness was linear at pH 5 as temperature increased. This was indicated by very strong Pearson coefficient r (r=0.81) (Table 3). However, as pH increased from 5 to 6.5 the r value decreased from 0.81 to 0.04 respectively.

In contrast, the immobilisation of enzymes onto the copolymer seemed to alter the solubility of the copolymer. When both pH and temperature were increased there was no significant change on the cloudiness. Therefore, the direction of the r-value seemed to shift between negative and positive depending on increase of both pH and temperature.

Table 3. Measure of cloudiness at various free enzyme loading, pH and temperature fusing cellulase-*p*-(NIPAAm55.5-co-MAA45.5) and p-(NIPAAm55.5-co-MAA45.5) copolymer

19600 µmol/minute

	Temperature (°C)					
pH	**25**	**50**	**55**	**60**	**65**	**Correlation coefficient (r)**
5	0.5	0.44	0.44	0.3	0.4	-0.73
5.5	0.54	0.6	0.55	0.52	0.47	-0.40
6	0.49	0.5	0.48	0.44	0.42	-0.69
6.5	0.48	0.47	0.41	0.5	0.42	-0.38
Correlation coefficient (r)	-0.54	-0.02	-0.34	0.68	0.04	

11760 umol/minute

	Temperature (°C)					
pH	**25**	**50**	**55**	**60**	**65**	**Correlation coefficient (r)**
5	0.22	0.23	0.21	0.21	0.24	0.17
5.5	0.26	0.35	0.28	0.28	0.27	0.14
6	0.24	0.3	0.23	0.29	0.31	0.59
6.5	0.27	0.3	0.27	0.3	0.26	0.09
Correlation coefficient (r)	0.76	0.42	0.51	0.89	0.44	

3920 umol/minute

	Temperature (°C)					
pH	**25**	**50**	**55**	**60**	**65**	**Correlation coefficient (r)**
5	0.16	0.2	0.18	0.06	0.11	-0.44
5.5	0.17	0.24	0.24	0.15	0.15	-0.09
6	0.19	0.25	0.23	0.15	0.15	-0.29
6.5	0.21	0.22	0.18	0.11	0.12	-0.73
Correlation coefficient (r)	0.99	0.41	-0.04	0.45	0.19	

1960 µmol/minute

	Temperature (°C)					
pH	**25**	**50**	**55**	**60**	**65**	**Correlation coefficient (r)**
5	0.09	0.11	0.07	0.01	0.10	-0.27
5.5	0.10	0.06	0.05	0.10	0.09	-0.21
6	0.08	0.08	0.06	0.09	0.08	0.01
6.5	0.07	0.06	0.05	0.12	0.10	0.44
Correlation coefficient (r)	-0.80	-0.71	-0.67	0.86	-0.13	

392 µmol/minute

	Temperature (°C)					
pH	**25**	**50**	**55**	**60**	**65**	**Correlation coefficient (r)**
5	0.06	0.06	0.06	0.07	0.07	0.67
5.5	0.02	0.02	0.02	0.03	0.03	0.67
6	0.03	0.03	0.03	0.03	0.03	0.00
6.5	0.03	0.02	0.03	0.03	0.03	0.04
Correlation coefficient (r)	-0.60	-0.75	-0.60	-0.77	-0.77	

0 µmol/minute: control

	Temperature (°C)					
pH	**25**	**50**	**55**	**60**	**65**	**Correlation coefficient (r)**
5	0.03	0.2	0.77	1.8	2.3	0.81
5.5	0.05	0.15	0.19	0.62	1.6	0.68
6	0.03	0.03	0.024	0.08	0.51	0.54
6.5	0.02	0.01	0.02	0.02	0.02	0.04
Correlation coefficient (r)	-0.51	-0.97	-0.88	-0.92	-0.99	

4.12. Recycling of comm3-*p*-NIPAAm-co-MAA conjugate in hydrolysis of microcrystalline cellulose

The immobilised enzyme was employed in enzyme recycling and achieved a minimum of three cycles (Figure 15). The initial enzyme loading in cycle 1 was 500 g of catalyst/L for each conjugate 1 (comm3-*p*-NIPAAm57-co-MAA43 conjugate) and 2

(comm3-*p*-NIPAAm56-co-MAA44 conjugate) both with activity of 1.4 µmol/minute. While the enzyme binding efficiencies were 74.5 and 81.1 % for conjugate 1 and 2 respectively (Table 4). This enzyme dose was reacted with avicel as substrate maintained at 250 g/L in each cycle.

The maximum reducing sugar produced in cycle 1 using conjugate 1 and 2 were 3.3 and 3.1 g/L respectively after 24 hours. Both conjugate 1 and 2 lost activity after 18 hours in cycle 2 with complete loss of activity in cycle 3. A decrease in amount of reducing sugar released using conjugate 1 and 2 was observed in subsequent cycles. The percentage loss of reducing sugar for conjugate 1 was 58% for both cycle 2 and 3 with respect to cycle 1. In contrast percentage loss of reducing sugar for conjugate 2 was 26% and 69% in cycle 2 and 3 respectively. At the end of 24 hours period the immobilised enzyme was recovered at 60 °C and pH 4.5 in the presence of 0.4 M KCl, washed and re-used up to 72 hours. Total reducing sugar released for each conjugate 1 and 2 over three cycles was 7.1 and 6.4 g/L respectively.

Table 4. Activity profile of the immobilised enzymes after washing off the unbound enzymes from the comm3-*p*-NIPAAm-co-MAA conjugate

Factor measured	**Conjugate 1**	**Conjugate 2**
	Enzyme activity (µmol/minute)	**Enzyme activity (µmol/minute)**
Comm3 free enzyme	336	
1st supernatant	76.2	57.92
1st wash	8	5.49
2nd wash	0.42	0.21
3rd wash	0.13	0.05
4th wash	0.03	0.03
% Efficiency	74.8	81.1
Comm3-copolymer conjugate	1.4	1.4

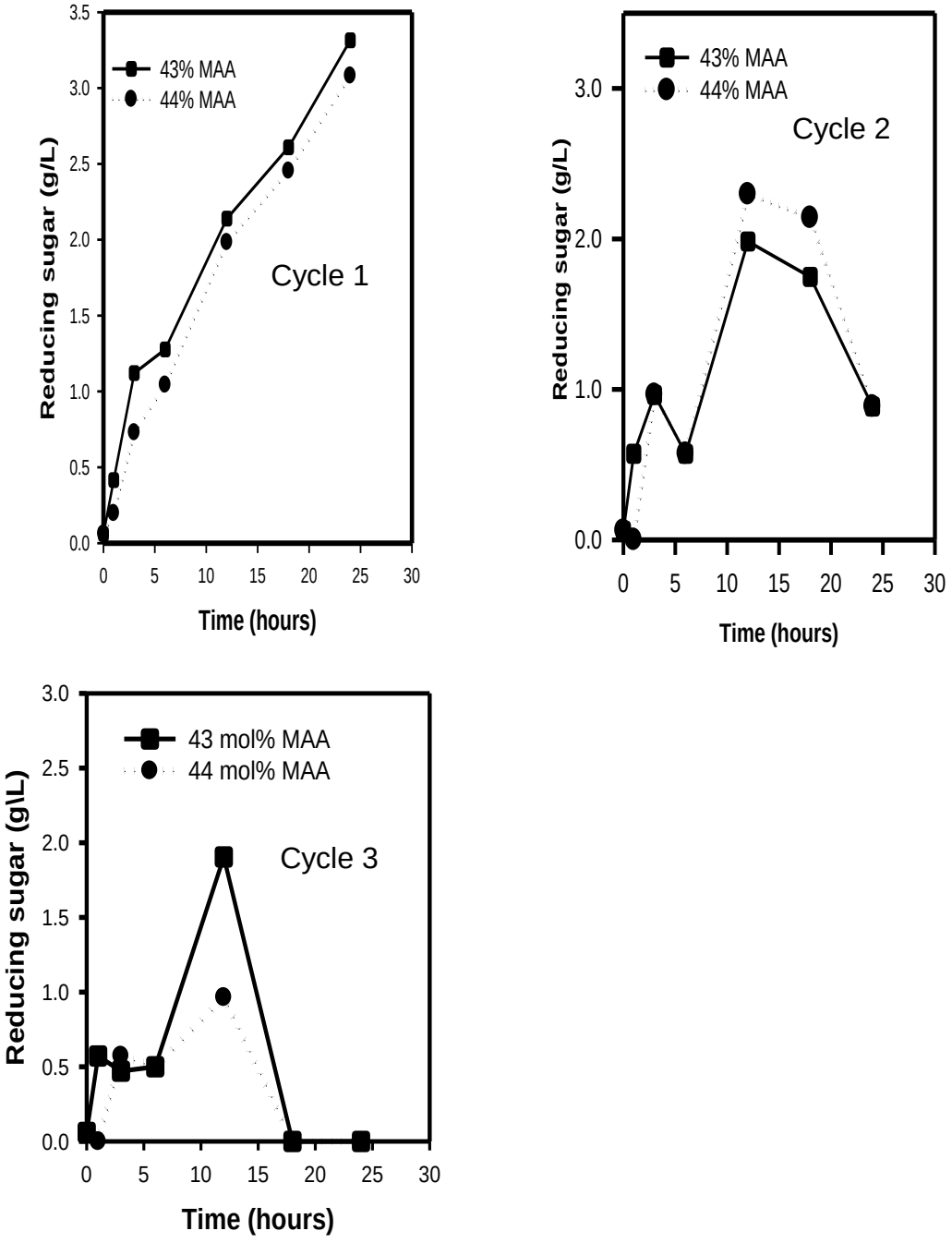


Figure 15. Recycling of comm3-*p*-NIPAAm-co-MAA conjugate in hydrolysis of microcrystalline cellulose. Reactions were performed separately at different time intervals in 2 mL microfuge tube containing 25 mg cellulose and 100 µL in the presence of 0.02 % (w/v) sodium azide.

4.13. Thermal stability of immobilised and free enzyme

The thermal stability of comm3-*p*-NIPAAm54.5-co-MAA45.5 and free enzyme was determined under optimal temperature (50 °C) of cellulases and at ambient temperature (30 °C) (Figure 17). Residual activity (%) was measured over 24 hours for both immobilised and free enzyme. There were 49 and 56% activity retention for immobilised enzyme at 30 and 50 °C respectively after 6 hours. In contrast, the free enzyme retained higher activity of 73 and 72% at 30 and 50 °C respectively after 6 hours incubation. The residual activity of the immobilised enzyme was maintained from 12 to 24 hours with maximum retained activity of 59 and 61% at 30 and 50 °C, respectively. At these incubation periods the stability of free enzyme declined to 52 and 60% at 30 and 50 °C respectively after 24 hours.

The determination of thermal stability of both immobilised and free enzyme was determined at precipitation temperatures of 60 and 65 °C (Figure 17). The thermal stability assays reveal that the activity in immobilised enzyme decreased steadily within 60 minutes. After a period of 120 minutes the minimum activity retentions for immobilised enzyme were 63 and 47% at 60 and 65 °C respectively. The stability assay for free enzyme shows that the enzyme activity declined suddenly from 100 to 80% at 60 °C and to 68% at 65 °C after 44 minutes. After 120 minutes the residual activities were 67 and 60% at 60 and 65 °C respectively.

Thermal stability of the immobilised and free enzyme at different temperatures (45 to 65 °C) over 24 hours was determined (Figure 18). This represents storage stability over 24 hour period below, near, and above the LCST of the conjugate. The immobilised and free enzyme retained 90 and 78% activity at 55 °C respectively. Free enzyme exhibited a characteristic bell shaped and was more thermal stable than immobilised enzyme between 45 to 50 °C and between 60 to 65 °C.

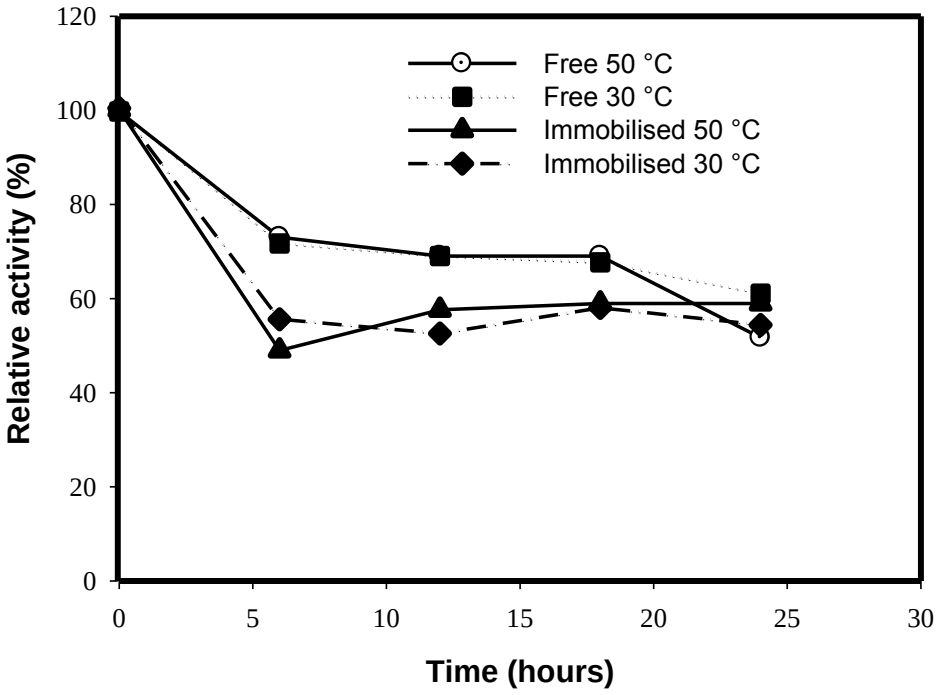


Figure 16. Effect of temperature on the residual activity of immobilised (comm3-*p*-NIPAAm54.5-co-MAA45.5) and free enzyme (comm3) at cellulase optimal temperature (50 °C) and ambient temperature (30 °C).

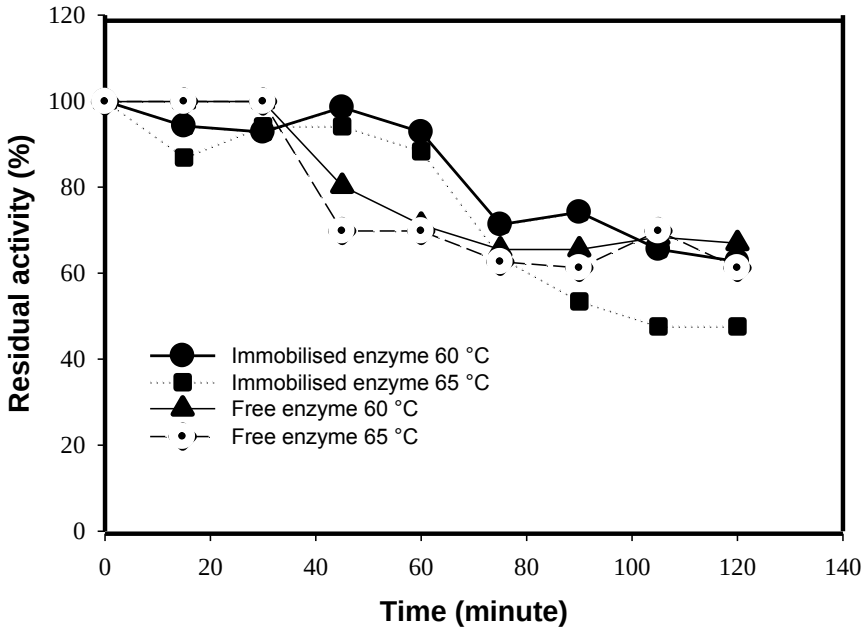


Figure 17. Stability of comm3-*p*-NIPAAm54.5-co-MAA45.5 and free comm3 in precipitation temperatures of 60 and 65 °C. Activity of samples prior incubation was taken as 100%.

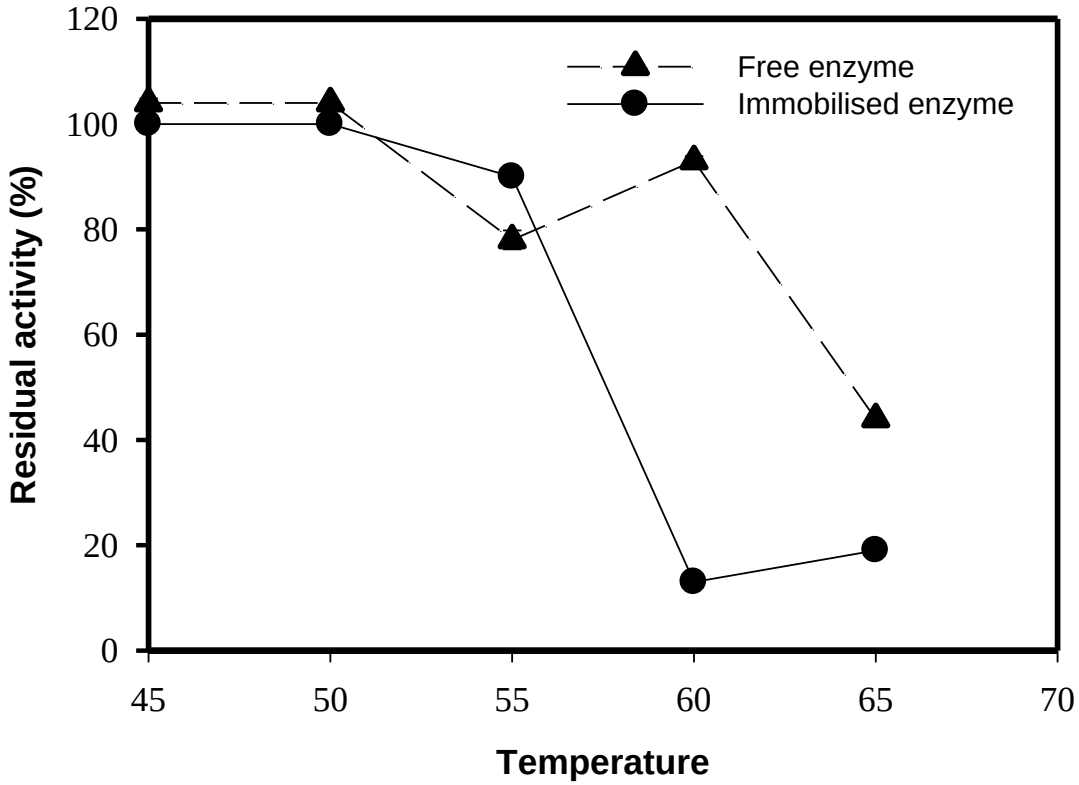


Figure 18. Residual activity of comm3-*p*-NIPAAm54.5-co-MAA45.5 and free enzyme after incubation at various temperatures over a period of 24 hour period below, near, and above LCST of conjugate. Error bar represent standard deviation of triplicate measurement.

4.14. Design of supermacroporous monolithic gels

Supermacroporous gels were prepared from polymerisation of acrylamide monomer with MBA as cross-linker in the presence of cellulase activated with NAS. During free radical polymerisation crosslinked-*poly*-Acrylamide-co-Dimethylaminoethyl methacrylate (crosslinked-*p*-AA-co-DMAEM) was formed inside a syringe only living the space occupied by the porogen, polystyrene beads (Figure 19a). The resultant pores were interconnected and continuously extended along the gel matrix referred to as monolithic (Figure 19 b and c).

Various percentage gels ranging from 7.5 to 12% were prepared, low percentage gels were found to collapse after the porogen was removed. Incorporation of DMAEM into *p*-AA confers rigidity to the gel thus lowering collapsing of the gel which disrupts the pore structure. A 15% crosslinked-*p*-AA-co-MBA gel with high crosslinking was also

observed to be rigid. Pore diameters measured using a ruler ranged from 0.2 to 0.5 cm, large pores was a result of combination of pores (Figure 19d).

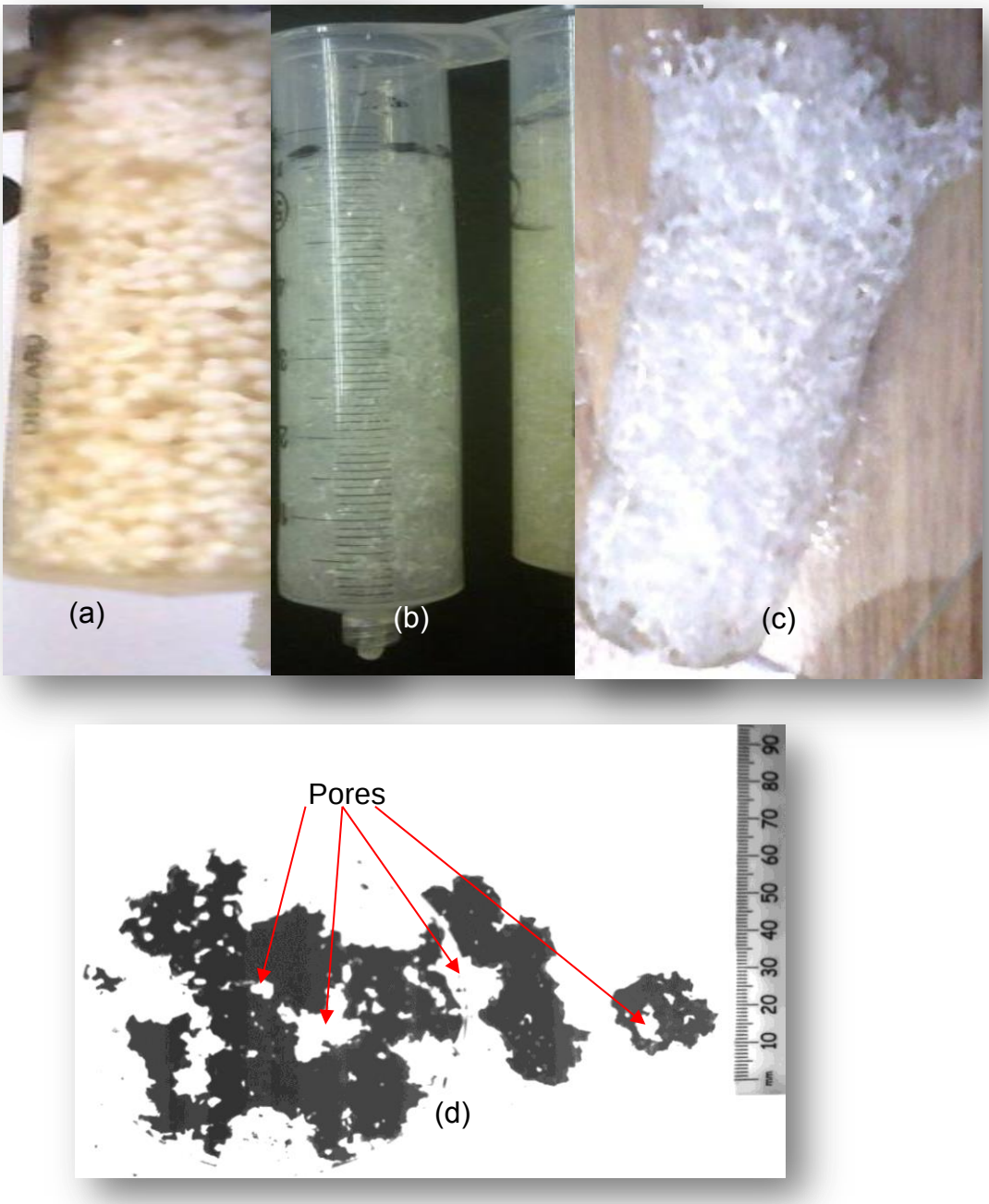


Figure 19. Supermacroporous gels with polystyrene beads serving as porogen (a) acrylamide/MBA solution polymerising in the presence of porogen (b) crosslinked-*p*-AA-co-MBA gel with porogen removed leaving macropores structure intact (c) the gel outside the syringe exhibiting monolithic properties (d) cross sectional area of the gel showing marcopores structure after staining using coomassie blue stain.

4.14.1. Recycling of cellulase-*p*-Acrylamide15%-co-NAS supermacroporous gels in hydrolysis of microcrystalline cellulose

The activity profile of unbound enzyme and elution of background sugars during washing of the supermacroporous gel after synthesis was determined (Figure 20). The background sugar decreased from 2.3 to 0.3 g/L while activity of unbound enzyme decreases from 0.06 to 0.02 µmol/minute. Therefore the sum of activity in the washing was 0.14 µmol/minute and this resulted in activity retention determined to be 91% after washing.

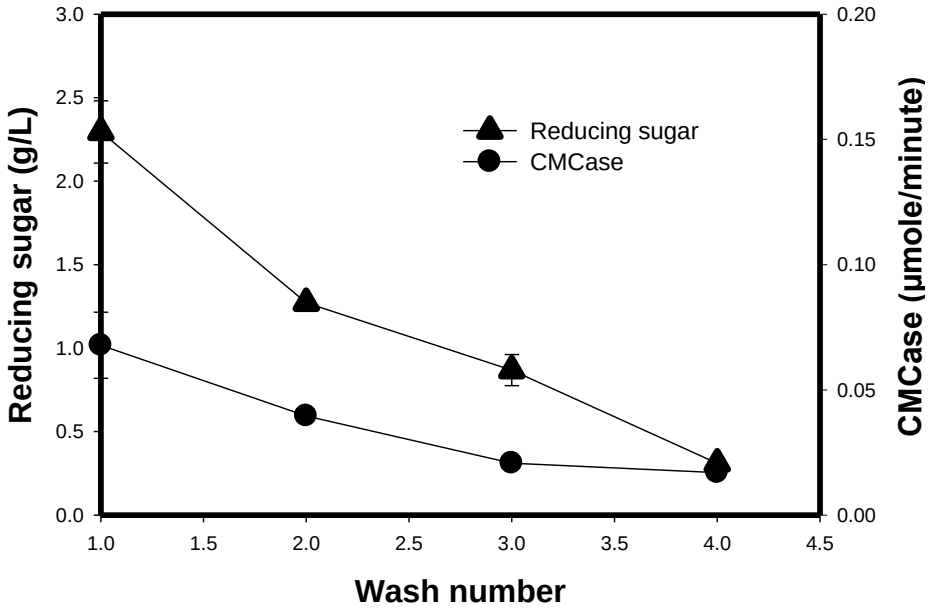


Figure 20. Measurement of background reducing sugar and activity in unbound enzyme during washing of supermacroporous gels after synthesis. Error bar represent standard deviation of triplicate measurement.

During hydrolysis of microcrystalline using cellulose comm3-*p*-Acrylamide15%-co-MBA, a maximum of three cycles were obtained with 87.9% activity retention after cycle 3 over 12 days (Figure 21). The hydrolysis reaction was discontinued as a result of lowered activity with respect to the first cycle. Under the same incubation time and cycles, the total amount of reducing sugar liberated amounted to 21.4 g/L. In cycle 1, a maximum of 15.9 g/L of reducing sugar was achieved while maximum activity of unbound enzyme in the avicel hydrolysate was 0.46 µmol/minute after 96 hours. In cycle 2 the maximum reducing sugar liberated by the immobilised enzyme was 3.6 g/L, while the free enzyme activity in samples was 0.14 µmol/min after 72 hours. The

reducing sugar was reduced by 88% in cycle 3 relative to reducing sugar in cycle 1. The corresponding enzyme activity in the avicel hydrolysate was not detected after 12 hours of cycle 3.

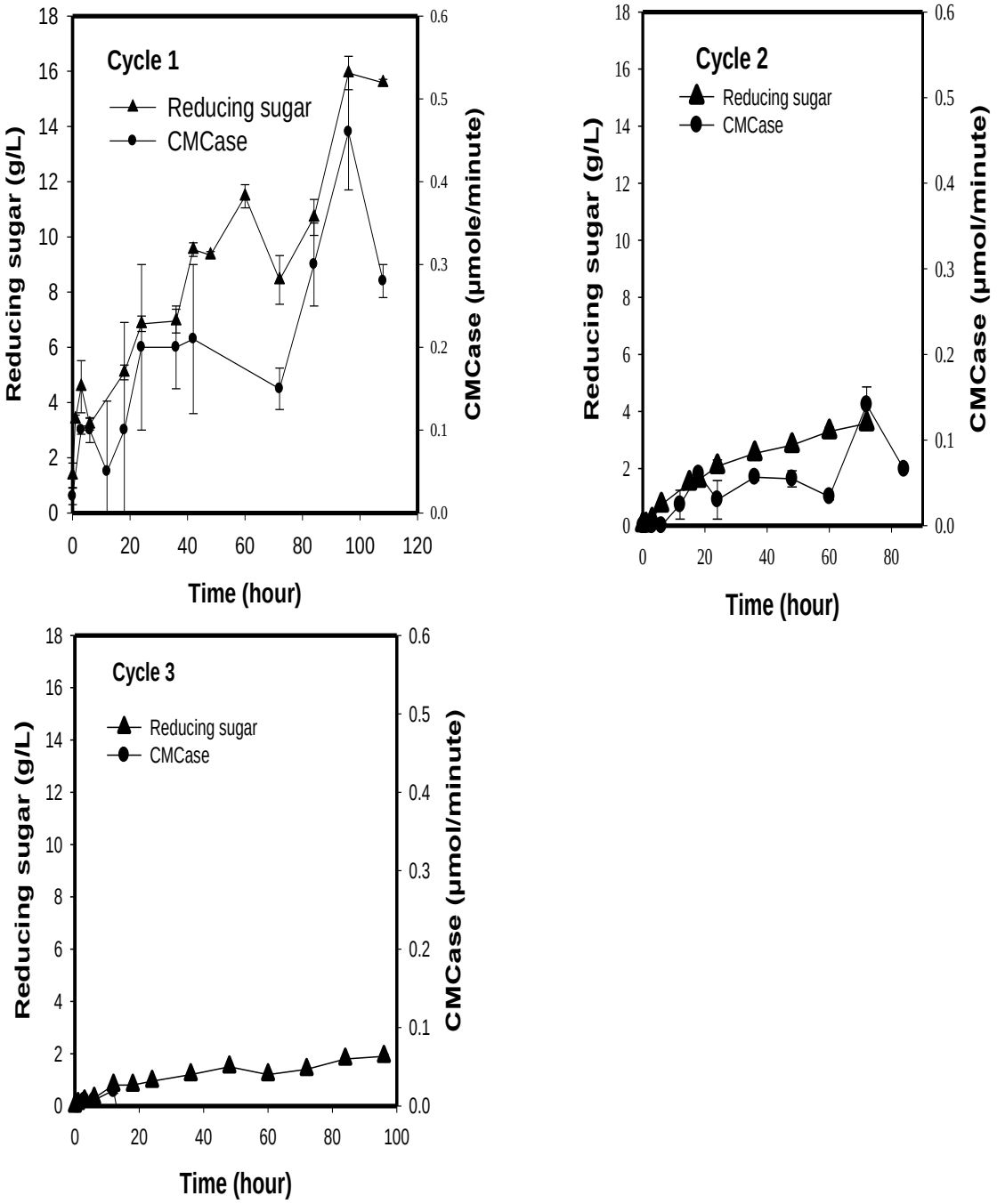


Figure 21. Recycling of comm3 immobilised on supermacroporous monolithic gels in hydrolysis of microcrystalline cellulose. Reducing sugar analysed by DNS method. Error bar represent standard deviation of triplicate measurement.

4.14.2. Saccharification of thatch grass using cellulase-*p*-Acrylamide15%-co-MBA supermacroporous conjugate and aqueous free enzyme

About 94.8% activity was retained after washing comm3-AA15%-co-MBA supermacroporous conjugate (Table 6). Acid pre-treated thatch grass with cut-off size of 0.5 µm was loaded into the gel to serve as substrate (Figure 22 a and b). After 6 hours of grass hydrolysis using comm3-AA15%-co-MBA in cycle 1 a maximum of 0.63 g/L of reducing sugar was released (Figure 23). In cycle 2 there was a maximum of 0.25 g/L of reducing sugar liberated by the immobilised enzymes which was 60% less than in cycle 1. A maximum of two cycles were achieved with total of 0.88 g/L of reducing sugar over 108 hours.

The hydrolysis of grass using free enzyme was performed as a single cycle in order to compare the total reducing sugars that can be liberated with hydrolysis using immobilised system (Figure 24). Consequently, the results shows significant (p<0.05) increase of reducing sugars in hydrolysis of thatch grass using free enzyme comm3 (0.3 mg/mL, 0.63 FPU/mL or 19 FPU/g of cellulose). A maximum amount of reducing sugar was liberated after 12 hours of incubation with 7 g/L released. The significant increase of reducing sugar was also reflected by strong Pearson coefficient of r^2= 0.686. The enzyme dilution was made such that activity and protein content in free enzyme was equivalent to activity and protein content in immobilised enzyme.

Table 5. Activity retention in supermacroporous gel

Wash number	CMCase (µmol/minute)
0	193.6
1	3.1
2	2.9
3	1.3
4	1.5
5	0.9
6	0.3
%Efficiency	94.8

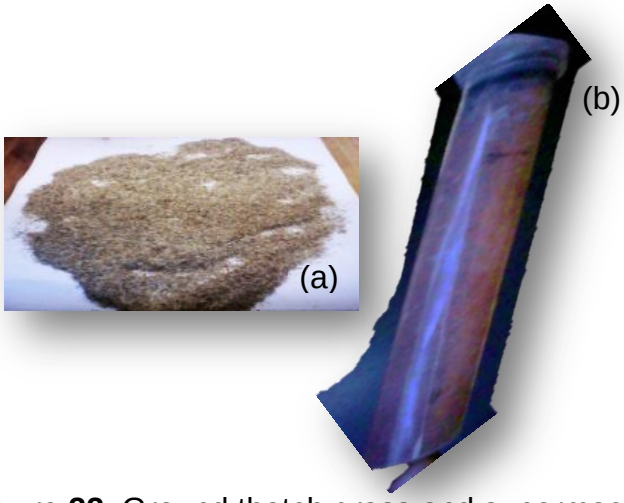


Figure 22. Ground thatch grass and supermacroporous gel containing immobilised enzyme filled with grass.

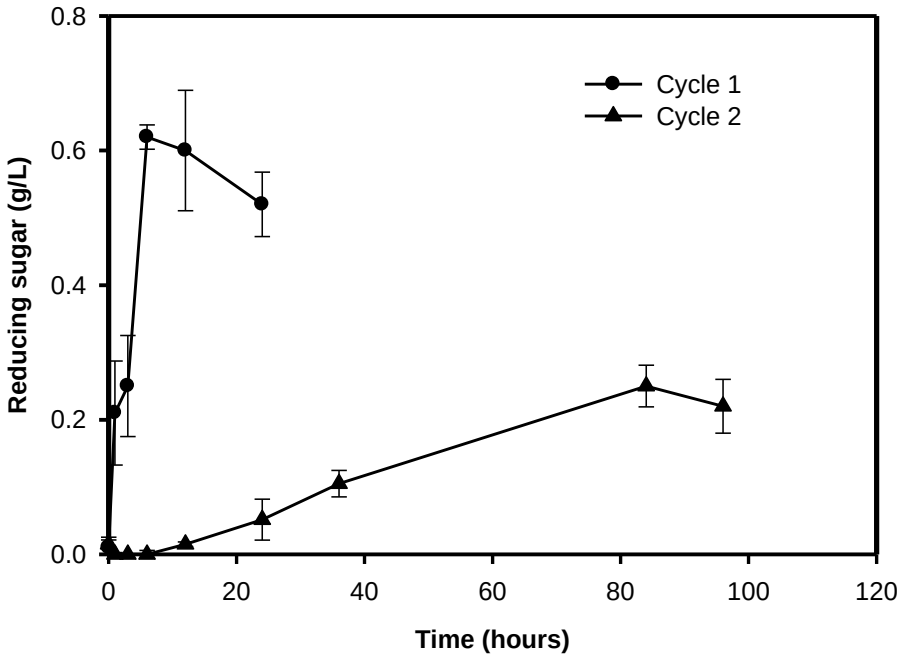


Figure 23. Saccharification of thatch grass in supermacroporous gel designated comm3-AA15%-co-MBA. Error bar represent standard deviation of triplicate measurement.

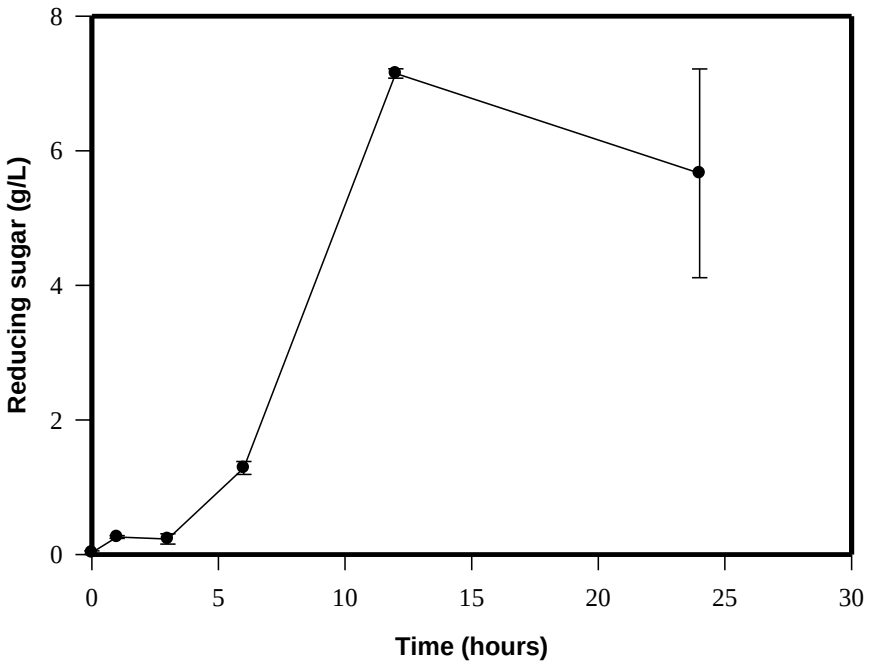


Figure 24. Analysis of reducing sugar released in saccharification of thatch grass using free comm3 by the DNS method. Error bar represent standard deviation of triplicate measurement.

CHAPTER 5

5. DISCUSSION

5.1. Synthesis of cellulase-*p*-NIPAAm cross-linked polymers and microbeads

In this study, the thermo and pH sensitivity of smart polymers have been exploited for immobilisation of cellulases. Various support materials have been designed such as the microgel beads and cross-linked polymers using inverse suspension (Lin *et al*., 1999) and free radical polymerization respectively (Hao *et al*., 2001). Selection of these supports was based on the advantage of porosity and sol-gel transition that improves contact of cellulase with particulate substrate (Tsai and Meyer, 2014).

It was observed that cellulase-10%-microbeads-*p*-NIPAAm conjugate released higher amount of reducing sugar after 6 hours while cellulase-10%-crosslinked-*p*-NIPAAm conjugate had a maximum release after 12 hours. The reason could be that microgel beads are small spherical particles and give higher surface area for enzyme loading (Brena *et al*., 2013). In addition, hydrolysis yield was also enhanced by the increased collision of enzyme and substrates than immobilisation on cross-linked polymers. This study demonstrates that porosity of the matrices plays a crucial role for enhancing the hydrolysis yield of cellulose. In the immobilisation of commercial cellulase and xylanase, the most effective support was chitosan-chitin, which has advantages of porosity and minimal structural steric hindrance over alginate-chitin (Romo-Sánchez *et al*., 2014).

Enzyme kinetics for 10, 5 and 2% cellulase-crosslinked-*p*-NIPAAm conjugates was followed over 12 hours during hydrolysis of soluble cellulose (1% CMC). The 2% and 5% conjugates had higher initial hydrolysis rate within the first 6 hours with 2% conjugate more active. After 12 hours, the reducing sugars released by 5% conjugate started to increase. Higher hydrolysis rate observed on 2 and 5% conjugate could be attributed to low mass transfer of substrate and high solubility of this biocatalyst. Gupta *et al*. (2014) studied immobilisation of β-glucosidase in various concentrations of alginate gel beads (1 to 5%). The same research team found that maximum immobilization efficiency (60% β-glucosidase activity) was obtained with 3% sodium

alginate. Zhou (2010) studied the effect of increasing the amount of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) on the activity of immobilised cellulase *N-succinyl*-chitosan-cellulase (NSCC). The research team found that optimal activity of immobilised enzyme was achieved using 10 mg of EDC; this showed that EDC introduces more carboxyl group available for coupling enzyme molecules. In contrary excessive amounts of EDC introduced cross-linking of enzymes molecules which caused activity loss due to restricted mobility of substrates and products.

The results for cellulase-10%-crosslinked-*p*-NIPAAm conjugate were characterised by a lag phase from 0 to 6 hours (Figure 5). This observation was attributed to the lower mass transfer of substrate due higher polymer (10% *p*-NIPAAm) and cross-linking density (0.5% w/v). This means substrate diffusion across the polymer chains to reach the enzyme was hindered with cellulase immobilised on higher cross-linking and polymer density. The lowered enzyme activity could be perhaps due to alteration of the enzyme active site caused by higher cross-liked polymer chains (Gupta *et al*., 2014). Chang and Juang (2007) studied the immobilisation of β-glucosidase on cross-linked chitosan-clay composite beads at different glutaradehyde concentration. The same research laboratory found that the beads that were cross-linked with a small amount of glutaradehyde presented poor mechanical strength while larger amount of glutaradehyde caused enzyme activity reduction.

Measurements of activity in the supernatants show that higher percentage gel (5 and 10%) retained more activity than low percentage gel (2 to 0.05%). These could be perhaps higher percentage gel (10 and 5%) contain higher density of reactive groups that are available to covalently react with NAS ester coupled to enzyme functional groups via the side chains of lysine, cysteine and aspartic and glutamic acids (Mohamad *et al*., 2015). In contrary the enzyme kinetics for cellulase-10%-NIPAAm conjugates revealed that its activity was lower than in low percentage gel as a result of lowered mass transfer of substrate.

5.2. Determination of minimum immobilised comm2 loading dose for hydrolysis of soluble cellulose

Increasing the quantity of cellulase-2%-crosslinked-*p*-NIPAAm conjugate from 2.1 to 10.3% (w/v) in hydrolysis of soluble cellulose resulted in an increase of yield of reducing sugar in a dose dependent manner (Figure 6). The increased quantity of immobilised enzyme allows more enzyme molecules to be in contact with the substrate which results in increased activity. However, at higher loading of 21.1% the maximum yield of reducing sugar dropped. The observed decrease of reducing sugar could be attributed to lower mass transfer of substrate. The increased quantity of polymer in the reaction mixture was reflected by increased viscosity of the solution. Therefore a minimum dose of immobilised enzyme that gives high yield of soluble sugars and that is inexpensive to prepare can be suitable for industrial scale.

The effect of enzyme loading on the activity of cellulase-2%-crosslinked-*p*-NIPAAm conjugate during immobilisation was determined. Enzyme concentration was increased from 40 to 400 mg/mL and the resulted maximum reducing sugar yield was 4.3 and 7.3 g/L respectively (Figure 7). The enzyme kinetic studies revealed that the amount of reducing sugar released, increased with enzyme concentration. Perhaps this could be due to higher activated protein concentration promoted increased covalent attachment of enzyme molecules. It was observed that the increased enzyme concentration on the polymer was directly proportional to the increased viscosity of the immobilised enzyme due to hydrophilic groups on the protein molecules. Hao *et al.* (2001) observed similar results where increased enzyme concentration (10 to 300 g/g of polymer) resulted in increased viscosity of trypsin-*p*-NIPAAm conjugate.

5.3. Hydrolysis of microcrystalline cellulose (Avicel)

The amount of cellobiose and glucose released from Avicel were determined by HPLC which had characteristic retention time (Rt) at 9.5 and 11.3 minutes respectively. Avicel hydrolysis using cellulase-2%-crosslinked-*p*-NIPAAm conjugates was performed at 30 and 50 °C below and above the LCST of *p*-NIPAAm respectively. High amounts of glucose (0.2 g/L) and cellobiose (0.65) were released at 50 °C which was the optimal temperature for cellulases. Comm2 is known to have

low amount of cellobiase the enzyme which hydrolyse cellobiose to glucose (Verardi *et al*., 2012). In this study low cellobiase activity was revealed by higher cellobiose content than glucose at both temperatures (Figure 8). Higher levels of cellobiose and glucose are known to inhibit exoglucanase and cellobiase respectively (Vanderghem *et al*., 2009).

Accumulation of cellobiose could perhaps be caused by low activity of cellobiase in the comm2-2%-crosslinked-*p*-NIPAAm conjugate system. As a result, feedback-inhibition of exoglucanase by its product cellobiose dominates (Vanderghem *et al*., 2009).

The increase of cellobiase content from 2 to 5% (v/v) in cellulase mixture during immobilisation resulted in the improvement of hydrolysis yield of glucose (Figure 9). Kovacs *et al*. (2009) conducted a study for supplementation of celluclast 1.5L with Nov 188 (cellobiase). Their results showed that glucose concentration was significantly increased from 3.5 to 8.3 g/L, while cellobiose accumulation diminished from 3.7 to 0.2 g/L. However, high amount of glucose can inhibit the activity of cellobiase and this can result in lowering of hydrolysis yield of glucose (Kovacs *et al*., 2009). In this study, the results obtained showed that, the amount of glucose was increased by more than tenfold at both temperatures. On the other hand, there was no significant change on the amount of cellobiose which was constant at 0.5 g/L over the entire reaction. This suggests that as exoglucanase catalyse the release of cellobiose from avicel chain ends, the next enzyme cellobiase rapidly hydrolyse cellobiose into two glucose units. Therefore activity of cellobiase permits accumulation of glucose while lowering the amount of cellobiose. This immobilisation system could be a suitable biocatalyst for application in biofuel industry by permitting simultaneous saccharification and fermentation when co-immobilised with yeast or bacteria.

Observing the HPLC analysis peaks (Figure 10a), at the glucose Rt there was interference by background peaks of buffer salts with Rt at about 12 minutes. Samples were treated with acid in order to protonate acetate ion and then evaporate excess buffer to remain with a pellet of sugars. The amount of glucose seemed to have been lowered after acid treatment as seen on the resultant chromatogram perhaps due to decomposition by acid treatment (Figure 10b) (Kumar *et al*., 2009).

5.4. Design of pH sensitive hydrogels using NIPAAm and MAA as co-monomers

Synthesis of *p*-NIPAAm-co-MAA copolymer was done by precipitation polymerisation using 2,2'-Azobis(2-methylpropionitrile) (AIBN) as catalyst. When the MAA/NIPAAm ratio was varied from 0:100 to 60:40 the LCST of the *p*-NIPAAm-co-MAA copolymer formed also increased from 33 t0 65 °C. The increased LCST could be due to increased carboxyl group (COO-) brought by MAA group which cause hydrophilicity of the copolymer to increase requiring more hydrophobic force to cause precipitation (Tian *et al*., 2008). Different combination of monomers was used in order to regulate the properties of the formed copolymer to meet desired thermo- and pH-sensitivity. The formed copolymer was a white powder with solubility dependent on pH due to introduced hydrophilic groups (COO-) of MAA group. A significant increase of LCST was noted at 30% MAA content which shows that the *p*-NIPAAm-co-MAA copolymer retained the thermo-sensitivity of the *p*-NIPAAm group (Tian *et al*., 2008).

5.5. Determination of LCST of *p*-NIPAAm-co-MAA copolymer

The transition temperature was defined as the LCST at 50% increase of absorption from the base line. Increasing the content of MAA in the copolymer resulted in the increase of its LCST. The increased LCST was due to increased interaction of hydrophilic groups which requires higher hydrophobic groups (alkyl group) to shrink the copolymer. Various cross-linking density [0.15 to 3% (mol/mol)] were used and this resulted in the increase of LCST from 44 to 52 °C with reference to *p*-NIPAAm60-co-MAA40 copolymer (Table 2). As a result more electrostatic repulsion was required to cause shrinkage and release of water from the copolymer chains. Liu *et al*. (2012) conducted similar studies for synthesis of interpenetrating polymer network (IPN) hydrogels. The results of Liu and co-workers showed that the LCST, hydrophilicity and enlargement of pores of the IPN hydrogel was enhanced due to increased hydrophilicity (polyaspartic acid moieties).

The LCST of polymers with 45 to 45.5% (mol/mol) MAA was within the cellulase optimal temperature, while more than 45.5% of MAA was discarded since LCST was very high. The LCST of desired copolymers varied from 54 to 65 °C above these range the copolymer is in insoluble form and below these temperatures the copolymer

is in soluble form. The optimal temperature for most cellulase activity occurs at 50 °C (Gelbe and Zacchi, 2002). Therefore this renders the copolymers with LCST of more than 50 °C suitable for use in immobilisation of cellulase because the biocatalyst will be in soluble state at this temperature. Furthermore soluble-gel transition offer advantage by allowing separation and recovery of the copolymer from the solution (Orakdogen, 2012).

It was suggested that properties of model biocatalyst and support material for immobilisation should be considered in order to achieve effective immobilisation (Walsh, 2002). Enzyme immobilisation might promote denaturation or impart thermal or pH stability compared to the free enzyme (Khan and Alzohairy, 2010; Fang et al., 2011). Hence optimal temperature for solubilising and precipitation of polymer was determined.

5.6. Swelling and shrinking kinetics

The swelling and shrinking kinetics at room temperature (25 °C) and 65 °C respectively was investigated for *p*-(NIPAAm54.5-co-MAA45.5) copolymer as shown in Figure 14. The swelling and shrinking kinetics was measured based on the clearness and cloudiness respectively of the copolymer solution at 600 nm. *p*-(NIPAAm54.5-co-MAA45.5) copolymer without cross-linking shows consistent swelling and shrinking kinetics from the first to the third cycle. The results also show that copolymers with increased cross-linking resulted in reduced cloudiness in subsequent cycles. The reason for reduced cloudiness could be perhaps due to slow shrinking rate of the copolymer compared to their swelling rate. Chen *et al.* (2010) conducted a study whereby the oscillatory deswelling–swelling kinetics of the poly (diallyldimethylammonium chloride)/poly(*N*,*N*-diethylacrylamide) (PDADMAC/PDEA) semi-IPN hydrogels was characterised. The findings of the same research laboratory showed that the magnitude of the swelling ratio (SR) of the hydrogels in consecutive cycles was reduced. It was concluded that the reduced SR magnitude of the hydrogels was due to their slow swelling rate when compared with their shrinking rate determined by gravimetric method before and after shrinking at 40 °C for 5 minutes (Chen *et al*., 2010).

The data of the current study shows that *p*-NIPAAm-co-MAA copolymers are reversible thermo-sensitive hydrogels which can be applicable in biotechnology.

Therefore separation of immobilised enzymes can be easily achieved at the shrunken state of the copolymer by precipitation at 65 °C in the presence of 0.4 M KCl and then separate by centrifugation at the mentioned temperature.

5.7. Effect of pH and temperature on the solubility of cellulase-*p*-NIPAAm54.5-co-MAA45.5

The enzyme-copolymer solution (0.62%) at all enzyme loadings (5 to 0.1 mL) did not show notable change of cloudiness as temperature was varied. The reason for weakened shrinking intensity could be perhaps the hydrophobic groups on the copolymer are occupied with hydrophilic enzyme molecules thus requiring higher driving force to shrink.

When pH was more than 5 and less than 6.5 at both temperatures, there was no variation of cloudiness of the cellulase-copolymer solutions at each enzyme loading. Only copolymer without enzyme showed significant changes, these could be perhaps the ionisable and deionisable groups were available. There was a sharp transition at pH 5.5 displayed by *p*-NIPAAm-co-MAA copolymer, this could be associated with the pKa 5.5 for *p*-MAA. Similar results were achieved by Liang and Cao. (2012) on the synthesis of the copolymer P_{MDB} containing methacrylic acid (MAA), 2-(dimethylamino) ethyl methacrylate (DMAEMA) and butyl methacrylate (BMA) as monomers. The same research laboratory studied the effects of pH on solubility of copolymer P_{MDB} and P_{MDB} with immobilized cellulase. The results showed that the was a shift in the isoelectric point (pI) for P_{MDB} and P_{MDB} with immobilized cellulase (pI 3.1 → 3.5) due to reduction in carboxyl groups of P_{MDB} after coupling of cellulases (Liang and Cao, 2012).

The data from the current study contributes in establishing reaction conditions in terms of amount of immobilised enzyme, substrate and total volume of reaction in hydrolysis of particulate substrate. These optimised factors will promote proper reaction of enzyme and substrate thus minimising mass transfer limitations of substrate.

5.8. Hydrolysis of avicel by recycling of enzyme immobilised onto *p*-NIPAAm-co-MAA hydrogels

The reuse of immobilised enzyme in number of cycles is advantageous in biofuel industry by reason of reducing cost of biofuel production process. The comm3-*p*-NIPAAm54.5-co-MAA45.5 was reused three times over 72 hours. At the end of 72 hours the total activity of immobilised enzyme was lost perhaps due to product inhibition or thermal inactivation. Thermal inactivation of immobilised enzymes can be associated with solubilising and shrinking during recovery and washing of polymer after cycle 1 and 2.

This data shows the possibility of using immobilised cellulase in industry for saccharification of cellulose. It is therefore suggested to immobilise thermo-stable cellulase preparation in order to minimise temperature inactivation. It is known that the major benefits of enzyme immobilisation technique include recycling of biocatalyst for economical purpose in industrial scale (Khan and Alzohairy, 2010). Unlike the aqueous free enzymes which are used once and difficult to recover and separate from products, immobilised enzymes can be reused and easily recovered from products (Wang *et al*., 2015).

5.9. Thermo stability test of free and immobilised cellulase

The residual activity of immobilised enzyme was less than that of free enzyme at both 30 and 50 °C after 6 hours. Further incubation shows that the immobilised enzyme regained activity and achieved 59 and 61% activity at 30 and 50 °C respectively which was more than for free enzyme. The regained activity for immobilised enzyme could perhaps be due to the NIPAAm-co-MAA copolymer which protects the cellulase against thermal inactivation. The study by Alftren and Hobley (2014); Wang *et al*. (2015) show that immobilised enzymes can exhibit improved thermal or pH stability than its aqueous free form. At higher temperatures of 60 and 65 °C the immobilised enzyme retained 63 and 47% activity respectively after 120 minutes while free enzyme retained 67 and 60% activity at same conditions. The rapid loss of activity of immobilised than free enzyme at higher temperatures could be a result of conformational change of the copolymer closer to its LCST. Viet *et al*. (2013) performed immobilisation of celluclast 1.5L from *Trichoderma reesei* on calcium

alginate beads and found immobilised celluclast 1.5L more stable at higher temperature than its free form. The same research laboratory also found that at pH 3-7 the correlation activity (%) of the immobilised and free celluclast 1.5L ranged from 69.75 to 70.38 and 62.48 to 15.06 respectively (Viet *et al*., 2013)

Thermal stability at various temperatures (45 to 65 °C) reveals a unique regain of stability of immobilised enzyme than its free form at 55 °C. The resultant residual activities for immobilised and free enzyme were 90 and 78% respectively after 24 hours incubation. Thermal conformation of the copolymer dominates at higher temperature as described earlier and resulted in more than 80% activity loss for immobilised enzyme while free enzyme regained activity at 60 °C. Similar loss of activity was observed in the study of immobilisation of Nuclease p1 whereby elevated temperature (80 °C) caused both free and immobilised enzyme molecules to outspread and ultimately loss activity (Shi *et al*., 2010). Free enzyme possess a bell shaped characteristic while in immobilised enzyme was altered due to thermal inactivation of enzyme and thermal conformation of the copolymer.

5.10. Hydrolysis of avicel by recycling of enzyme immobilised on supermacroporous gels

In hydrolysis of avicel by recycling of immobilised cellulase designated comm3-*p*-AA15%-co-MBA conjugate a total of 21.4 g/L of reducing sugar was released over 228 hours. Chen *et al*. (2007) showed that *S. cerevisiae* 316 strain was capable to ferment 95.3 g/L of glucose to 45.7 g/L of ethanol equivalent to 94% of the theoretical yield (based on the theoretical yield of 0.51 g ethanol/g glucose) after 18 hours using. Therefore, based on these findings, the amount of 21.4 g/L released in this study provided only glucose was the reducing sugar can be completely fermented to 10.8 g ethanol. This study also shows that immobilisation of cellulase on supermacroporous gels can be useful in simultaneous saccharification and fermentation.

5.11. Saccharification of common thatch grass using immobilised and free enzyme

Results for hydrolysis of acid pre-treated thatch grass by immobilised and free comm3 indicated that higher reducing sugar yield was achieved with free enzyme. The quantity of reducing sugar released by immobilised enzyme over 108 hours in

two cycles was 87% less than in free enzyme achieved over 12 hours in one cycle. The low hydrolysis yield of reducing sugar using immobilised enzyme system can be associated with hydrolysis barrier of lignocellulose crystallinity due to hydrogen bonding between cellulose fibers. During hydrolysis, cellulases break down cellulose chains to glucose while the chains become shortened and these render the substrate more crystalline. Therefore further hydrolysis using immobilised comm3 was limited as seen in subsequent cycles with low hydrolysis yield (Figure 23). Both the immobilised enzyme system and substrate were insoluble; therefore this could be another barrier of hydrolysis. This study paves the way for biofuel industry to use immobilised cellulase in supermacroporous gels due to larger pores which permit hydrolysis of particulate substrates.

5.12. Conclusion

The outcome of enzyme immobilisation technique of cellulases onto crosslinked-*p*-NIPAAm and microbeads-*p*-NIPAAm hydrogels reveals a promising application of the technique in the biofuel industry. The trend of increase of activity retention after immobilisation of comm2 onto microbeads-*p*-NIPAAm was from 1 to 10% conjugate. Enzyme kinetics for hydrolysis of soluble cellulose using *p*-NIPAAm conjugates shows that activity of 10% conjugate was lower than 5 and 2% conjugates. Optimum factors which were investigated include quantity of immobilised enzyme (10.3% w/v) during hydrolysis of soluble cellulose, concentration of enzyme (400 mg/mL) for immobilisation, addition of β-glucosidase (5%) and increase of hydrolysis time (336 hours) greatly enhanced the hydrolysis of cellulose.

Incorporation of MAA hydrophilic groups (COO-) and cross-linking significantly increase the LCST of the copolymer. The COO- groups imparted pH sensitivity to the copolymer. The pH and thermal sensitivity of *p*-NIPAAm-co-MAA allows separation of the copolymer from the solution. Comm3-*p*-(NIPAAm-co-MAA) conjugate recycling achieved minimum of 3 cycles with total of 7.1 g/L (conjugate 1) and 6.4 g/L (conjugate 2) of reducing sugar. Thermo-stability test shows that immobilised enzyme was more stable than free enzyme at 55 °C. At higher temperature closer to the LCST of the copolymer the immobilised enzyme lose activity than free enzyme.

Hydrolysis of acid pre-treated thatch grass by immobilised (comm3-*p*-AA15%-co-MBA conjugate) and free enzyme was achieved with the later releasing 87% more reducing sugar. Therefore these immobilisation system needs to be further optimised in order to achieve higher hydrolysis yield of reducing sugar using comm3-*p*-AA15%-co-MBA conjugate. This study has demonstrated possibility of application of immobilised cellulase in industrial scale for glucose production and subsequent fermentation by yeast or bacteria. *p*-NIPAAm and supermacroporous gels are well suited for use as support matrices for immobilisation of cellulases due to possibility of recycling the immobilised enzyme.

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