NANOFIBER IMMOBILIZED CELLULASES AND HEMICELLULASES FOR FRUIT WASTE BENEFICIATION

A thesis submitted in fulfilment of the requirements for the degree of

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By

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ABSTRACT

Large quantities of waste are generated from fresh fruit processing for wine, juice and soft drink production. This fruit waste consists mainly of lignocellulose, a combination of highly recalcitrant plant polysaccharides such as cellulose, lignin, pectin and hemicellulose. Conversion of the lignocellulosic waste is therefore of particular interest and provides a potential solution to large scale pollution, allowing greater economic benefit and a cleaner environment. In addition to its role in bioremediation, lignocellulosic biomass conversion also offers a potential step towards the production of other value added products including biofuels and other chemical compounds. However, the use of free enzymes for this process on an industrial scale is not economically viable. Viscozyme L® and Celluclast 1.5L® are commercial enzyme cocktails that can be used for the degradation of lignocellulose; however, little is known about the immobilization of commercial enzyme mixtures containing cellulases or hemicellulases, particularly when using complex substrates, such as apple pomace (AP).

Immobilized enzymes offer potential advantages over free enzymes including increased stability, reusability, and ease of recovery, although some activity is often lost. This is particularly problematic when insoluble substrates are used, due to decreased mobility of the immobilized enzyme and mass transfer limitations.

Electrospinning is a relatively simple and useful technique used for the synthesis of nanofibers. Electrospun nanofibers exhibit the following qualities: increased surface area to volume ratios, potential for surface modification, pore sizes tailored to protein dimensions, increased porosity, interconnectivity and low mass-transfer limitations. These features make them excellent candidates for enzyme immobilization.

The aim of this study therefore, was to synthesize and utilize electrospun nylon 6 nanofibers for the immobilization of hemicellulases and cellulases in Viscozyme L® and Celluclast 1.5L® solutions, using apple pomace as a hydrolysis substrate.

The nanofibers were synthesized by electrospinning 22% (w/v) nylon 6 dissolved in a 1:1 (v/v) ratio of formic and acetic acid, at 25 kV, with a needle tip-collector distance of 10 cm

and a flow rate of 0.8 ml/h. The nanofiber morphology was verified using Scanning Electron Microscopy (SEM).

The enzymes in Viscozyme L® and Celluclast 1.5L® were immobilized onto the nanofibers by crosslinking with glutaraldehyde, and showed 17.5 and 9.9 units of activity per g of fibrous nylon 6 membrane, respectively. The optimal conditions for immobilizing the enzymes in Viscozyme L® and Celluclast 1.5L® were investigated, and 4% and 2% glutaraldehyde (GA) was used, with 4 mg/ml and 2 mg/ml initial protein concentrations, respectively. An immobilization time of 5 h was used for both enzyme solutions. Fibres immobilized with the enzymes in Celluclast 1.5L® were pre-activated with 1.91 M HCl. Fourier transform infrared spectroscopy (FT-IR) and energy dispersive spectroscopy (EDS) were used to confirm enzyme binding of the enzymes to the nanofibrous support.

The immobilized enzymes were used for the hydrolysis of apple pomace and the reducing sugars produced were quantified using the 3,5-dinitrosalicylic acid (DNS) method. The viability of the immobilization was determined by characterization and comparison of the free and immobilized enzymes. The free and immobilized enzymes showed pH optima between pH 2.5 and 5 (for Viscozyme L®, and pH 3 and 5 (for Celluclast 1.5L®). However, the temperature optima of the free and immobilized enzymes shifted from 50˚C to 60˚C for Viscozyme L® and from 60˚C to 65˚C for Celluclast 1.5L, respectively. The K_m and V_{max} values for the immobilized enzymes in Viscozyme L® were 5.361 mg/ml and 0.039 μmol/cm^2/min, as compared to the free enzyme K_m and V_{max} of 7.876 mg/ml and 0.480 μmol/ml/min, respectively. For Celluclast 1.5L, the K_m and V_{max} values for the free enzymes were 4.345 mg/ml and 0.068 μmol/ml/min, respectively. The K_m and V_{max} values for the immobilized enzymes were 4.835 mg/ml and 0.035 μmol/cm^2/min, respectively. For the reusability studies, the immobilized enzymes retained approximately 40% activity after 6 cycles for Viscozyme L®, and 60% after 5 cycles for Celluclast 1.5L® at 37˚C. Although the free enzymes appeared to be quite stable, ANOVA single factor analysis did reveal occurrences of slight improved stability of the immobilized enzymes, relative to the free enzymes. Even though this increased stabilization was minimal, the combined collection of all the above mentioned benefits suggests that the proposed system for immobilization of these enzymes provides a reasonable basis and contribution towards the eventual successful

implementation of the immobilized system. This was a pilot study and still requires further investigation and it is anticipated that the eventual success of this system should be pursued through the future recommendations outlined in Chapter 6, as well as by using slightly cheaper resources and materials as hosts for enzyme immobilization.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

AA	**Acetic acid**
AFM	**Atomic force microscopy**
ANOVA	**Analysis of variance**
AP	**Apple pomace**
BSA	**Bovine serum albumin**
BWX	**Beechwood xylan**
C	**Carbon**
CBM	**Carbohydrate binding modules**
Cl	**Chlorine**
CMC	**Carboxymethyl cellulose**
DMF	**Dimethylfuran**
DNS	**3,5-Dinitrosalicylic acid**
EDS	**Energy dispersive spectroscopy**
FA	**Formic acid**
GA	**Glutaraldehyde**
HCl	**Hydrochloric acid**
LBG	**Locust bean gum**
LB	**Lineweaver-Burk**
MM	**Michaelis-Menten**
N	**Nitrogen**
Na	**Sodium**
O	**Oxygen**
P	**Phosphorous**
PAN	**Polyacrylonitrile**
PEC	**Pectin (apple)**
PEI	**Polyethylenimine**
PGA	**Polygalacturonic acid**
PS	**Polystyrene**
PVA	**Polyvinyl alcohol**
RT	**Room temperature**
S	**Sulphur**
SD	**Standard deviation**
SEM	**Scanning Electron Microscopy**
SS	**Simultaneous saccharification**
THF	**Tetrahydrofuran**
V	**Velocity**
WRC	**Water Research Commission**

Chapter 1

Literature Review and Introduction

1.1 INTRODUCTION AND BACKGROUND

There is a significant amount of lignocellulosic waste that is being incinerated or wasted (Sun and Cheng, 2002). Lignocellulose consists mainly of cellulose, hemicellulose and lignin and is the most common plant cell wall component of the biosphere and the most abundant waste produced by society (Figure 1) (Bayer *et al*., 2007). Pomace constitutes a large proportion of this waste and is generated from fresh fruit processing for wine, juice and soft drink production (Nawirska and Kwasniewska, 2005). For example, during apple juice production, approximately only 75% of the fruit is used and the remaining pomace is discarded (Shalini and Gupta, 2010). In some processing industries partially bruised or spoiled fruit is also discarded which contributes to the total fruit waste (Rachana and Gupta, 2010). The high transportation costs to dispose of these wastes results in the accumulation of waste heaps outside the manufacturing plants, which violates pollution control regulations, causes industrial safety issues and poses a serious health hazard (Rani and Nand, 2004). These waste piles are detrimental to the aesthetic quality of the natural environment and may result in putrid smells due to the fast degradation by natural microorganisms (Bhushan *et al*., 2008).

If this waste reaches water-bodies, it may lead to possible production of fruit waste water. Wastewaters that result from processing exhibit high chemical oxygen demands (CODs) of 4 400 and 15 000 mg/ ml for the canning and juicing processes, respectively (Pletschke *et al*., 2014). For example, a fruit processing plant in Stellenbosch produces 20 million litres of wastewater annually and current management consists of storing the waste water in concrete ponds or irrigating it onto agricultural land. This wastewater often exceeds the South African legal pH range of 6 to 9 and the COD of 400 mg/L (Pletschke *et al*., 2014).

In addition to its role in bioremediation, biomass hydrolysis and utilization of waste as a resource allows the production of fermentable sugars which can be used for manufacturing value added products. For example, discarded apple pomace (AP) resulting from fruit juice

production, contains approximately 79% sugar that could potentially be used for production of value added products such as bioethanol (Shalini and Gupta, 2010). Lignocellulosic biomass conversion is therefore of particular interest and provides a potential solution to large scale pollution allowing greater economic benefit and a cleaner environment.

1.2 LIGNOCELLULOSE

Recognition has been given to lignocellulose as a key source for production of biofuel and other value added products. The main components of lignocellulose (Figure 1) are cellulose, hemicellulose, lignin and sometimes pectin (Sticklen, 2008).

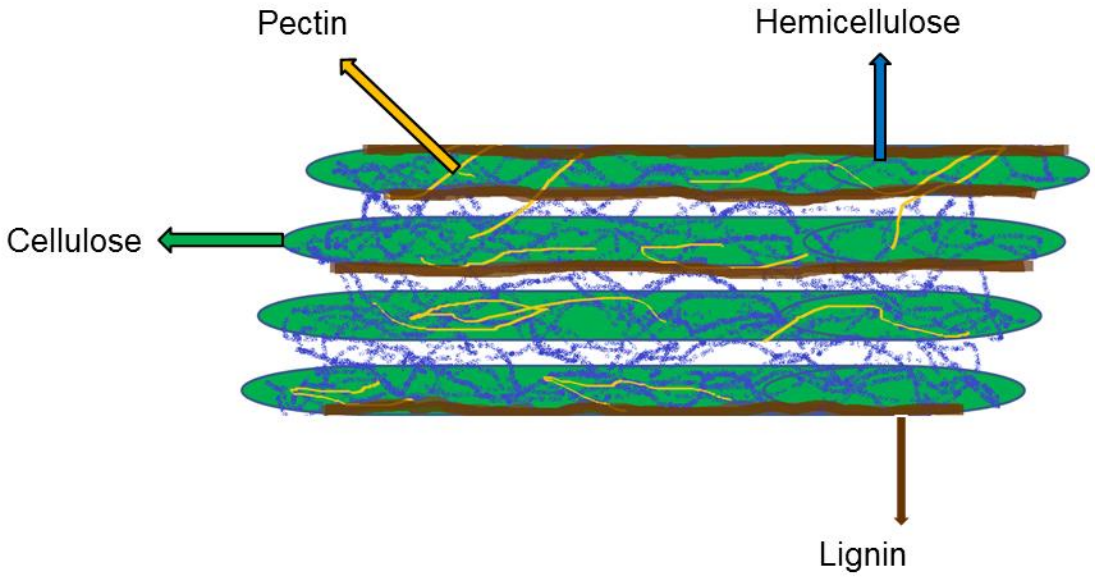


Figure 1: Schematic diagram of the components forming lignocellulose (Image adapted from Sticklen, 2008).

Cellulose is the main component, forming between 40 and 50% of lignocellulosic biomass (Beukes *et al.*, 2008). Cellulose consists of cellobiose units which are essentially two glucose units, linked by β1-4 glycosidic bonds (Figure 2). Cellulose is arranged in long chains called microfibrils that lie parallel to each other and these rigid, insoluble structures are stabilised by intermolecular hydrogen bonds and hydrophobic interactions (Bayer *et al.*, 2007).

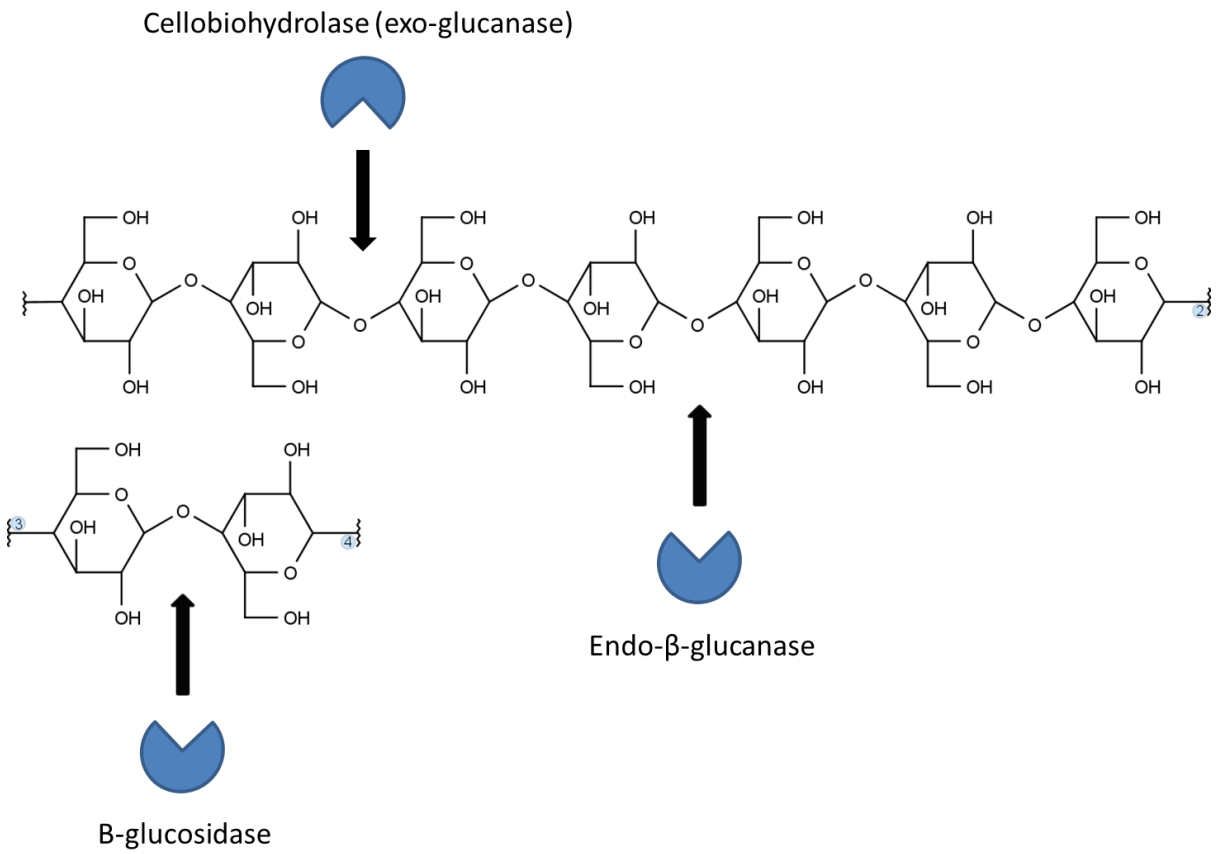


Figure 2: Molecular structure of cellulose and site of action of endoglucanase, cellobiohydrolase and β-glucosidase (Image adapted from Kumar *et al*., 2008).

According to Saha (2003), hemicellulose forms between 20-30% of lignocellulose and is the second most abundant polysaccharide in nature and consists of sugar acids, pentoses such as xylose and arabinose (Figure 3), and hexoses such as mannose and glucose. Xylans are predominant in the hemicellulose of hardwood, while hemicellulose in softwood is composed mostly of glucomannans (Saha, 2003). According to Shallom and Shoham (2003), xylose is the most predominant type of hemicellulose and constitutes as much as 40% of the hemicellulose content in plants. Yang *et al*. (2011) claim that removal of hemicellulose significantly increases enzymatic hydrolysis of cellulose, indicating that hemicellulose plays a key role in physically obstructing the action of cellulases.

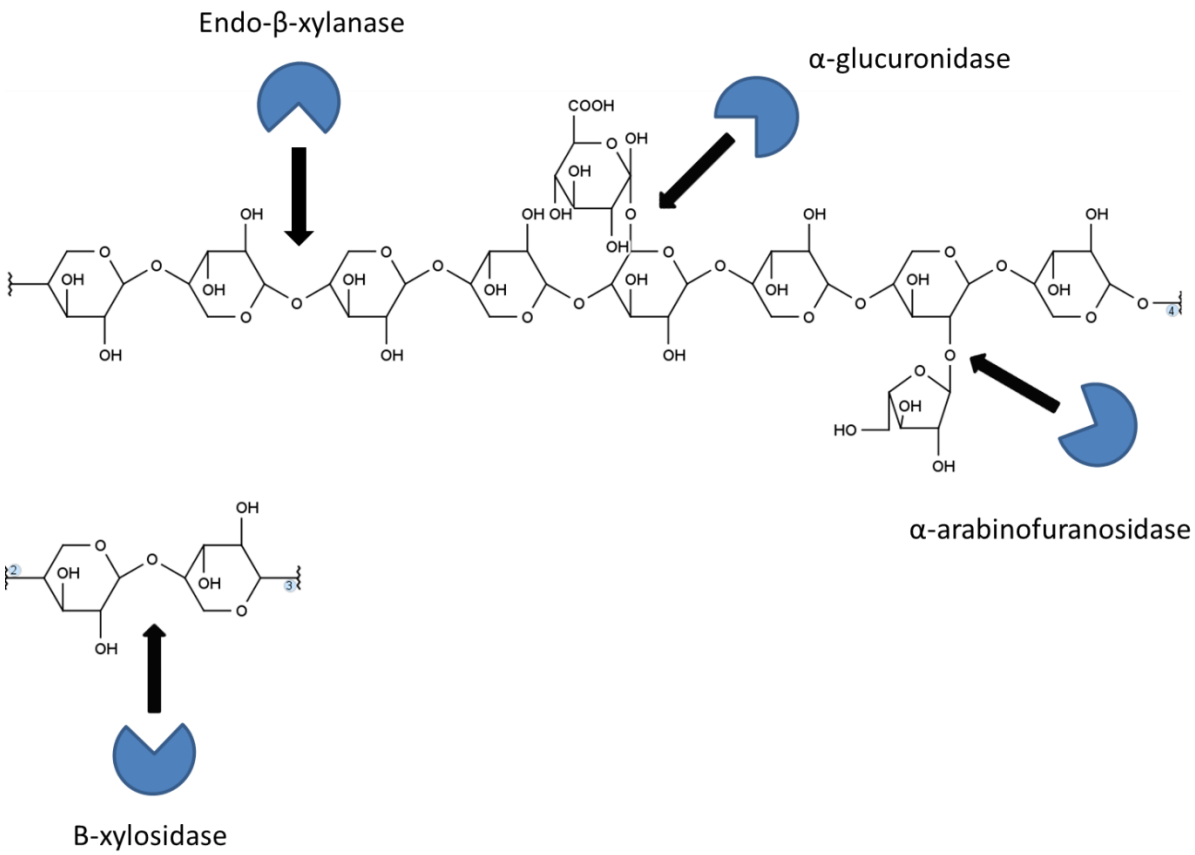


Figure 3: Molecular structure of arabinoxylan and targets of hydrolytic enzymes involved in hemicellulosic polymer degradation (Image adapted from Kumar *et al.*, 2008).

Kumar *et al*. (2008) indicated that lignin accounts for 10-25% of lignocellulosic biomass. Lignin is a heterogeneous polyphenylpropane polymer which is responsible for the recalcitrant nature of lignocellulose due to the high amounts of cross linking which add rigidity and strength to the structure (Howard *et al.*, 2003). According to Krongtaew *et al*. (2010) the recalcitrance of lignin is a major limiting factor in microbial and enzymatic digestion of biomass for the utilization of value added products. Removal of lignin can enhance enzymatic hydrolysis by reducing non-productive binding of enzymes and increasing cellulase accessibility (Yang *et al*., 2011).

The third most abundant structural polysaccharide in plant cell walls, especially in fruits such as citrus and apple, is pectin (Voragen *et al*., 2009) (Figure 4). According to Voragen *et al.* (2009), pectin is possibly the most complex macromolecule in nature, since it may consist of as much as 17 different monosaccharides containing more than 20 different linkages. Kumar

et al. (2008) describe pectin as being composed of a homo-galacturonic acid backbone linked by α-(1,4) glycosidic linkages with neutral sugar side chains including L-rhamnose, arabinose, galactose and xylose. Voragen *et al*. (2009) described these portions with neutral sugars as "hairy" regions, and the homogalacturonic regions as "smooth".

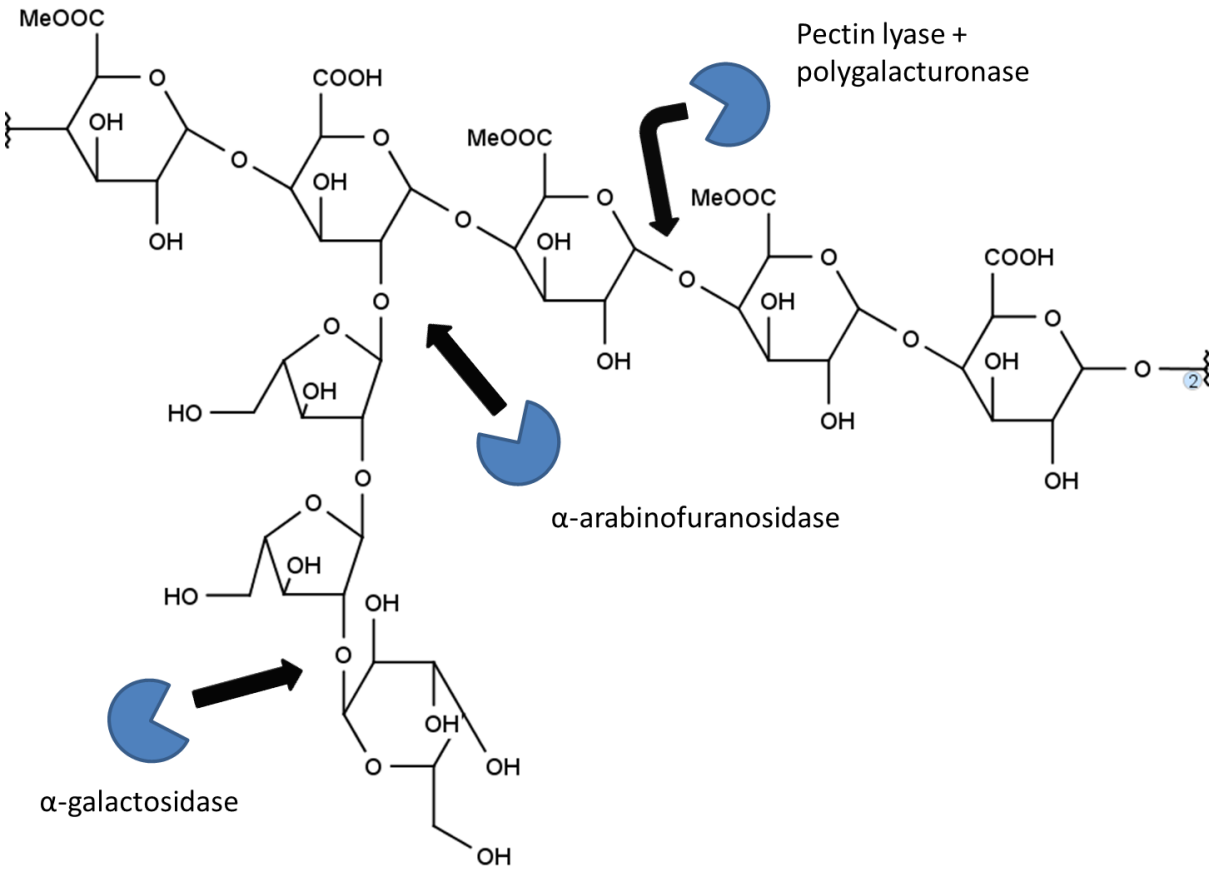


Figure 4: Molecular structure of pectin and enzymatic sites for pectin lyase, endo-β-polygalacturonase, α-arabinofuranosidase and α-galactosidase (Image adapted from Kumar *et al*., 2008).

1.3 FRUIT WASTE COMPOSITION

Plant cell walls are composed primarily of polysaccharides with cellulose micro-fibrils fixed in a network of hemicellulose and sometimes pectin and lignin. Different fruit wastes consist of varying amounts of lignocellulosic components, as seen in Table 1. Various factors may affect the composition of different fruit wastes depending on, for example, the ripeness of the fruit when it was picked, the processing methods used and the type or variety of the fruit (Pletschke *et al*., 2014).

Table 1: Composition of different fruit wastes on a percentage of dry weight basis.

Waste	Lignin (%)	Hemicellulose (%)	Cellulose (%)	Pectin (%)	Other (such as protein and ash) (%)	Reference
Apple pomace	20.4	24.4	43.6	11.6	-	Narwirska and Kwaniewska (2005)
Citrus waste	2.19	11.1	22.0	25.0	39.71	Pourbafrani *et al.* (2010)
Pineapple peel	11.0	6.5	12.0	7.1	63.4	Rani and Nand (2004)

The most abundant fruit crop in the world is citrus and the primary reason for its processing is for juice production. Over 88 million tons of citrus fruits are produced worldwide per year and nearly half is processed for juice (Pourbafrani *et al*., 2010). Some of the waste from citrus processing includes peels, seeds and citrus pulp which are high in lignocellulose (Pletschke *et al*., 2014). One of the challenges with utilizing citrus waste as a biomass conversion resource is that it contains limonene which can be toxic to microorganisms involved in its processing and thus needs to be removed prior to fermentation (Pourbafrani *et al.*, 2010).

In China up to 1 million tons of apple pomace is produced as a result of juice processing (Wang *et al.*, 2010). Apple pomace consists of the core, seeds, peel, stem and fleshy part of the fruit. According to Pletschke *et al*. (2014), apple pomace is comprised of varying amounts of cellulose, hemicellulose, lignin and pectin (Figure 5). Apple processing wastewater and apple pomace contain high amounts of pectin (Nawirska and Kwasniewska, 2005). Pectin is therefore one of the potential value added products that can be extracted from apple pomace and its exploitation is currently being investigated (Rachana, and Gupta 2010).

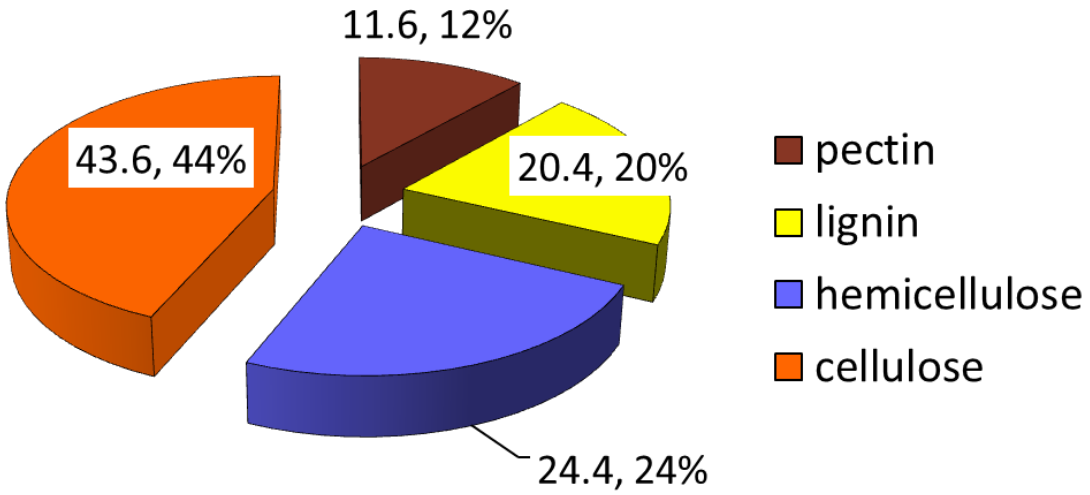


Figure 5: Composition of apple pomace as a percentage of dry mass (Narwirska and Kwaniewska (2005).

The residual component after the processing of grapes for the fermentation of wine is known as grape pomace and consists of skins, pulp, seeds and stems (Pletschke *et al*., 2014). The growth and cultivation of grapes is found predominantly in temperate regions around the world, where 80% of these crops are harvested and utilized in the wine industry (Schieber *et al*., 2001). Grape pomace is also known to contain high amounts of phenolic compounds, lignocellulose and grape seed oil (Schieber *et al*., 2001).

In the Ivory Coast, 40-80% of the 100 000 tons of pineapple produced annually is discarded as waste, consisting mostly of peel and cores (Ban-Koffi and Han, 1990). Pineapple waste has previously been reported for the production of bromelain, vinegar, animal feed and organic acids (Ban-Koffi and Han, 1990). Different parts of pineapple peel are composed of varying amounts of lignin, cellulose and hemicellulose as seen in Table 1. The crown contains a greater proportion of cellulose (29.6%) and therefore can be considered a model target for ethanol production as glucose is the main hydrolysis product which is the preferred sugar utilized by *S. cerevisiae*.

1.4 ENZYMES CAPABLE OF DEGRADING LIGNOCELLULOSE (LIGNOCELLULOLYTIC ENZYMES)

Capek *et al*. (1995) argue that although chemical hydrolysis of lignocellulosic biomass is faster and more complete, it may be corrosive, hazardous and produce toxic by-products. Consequently, interest has been diverted to enzymatic degradation. Pletschke *et al*. (2014) also point out that enzymatic degradation is more efficient, uses more moderate conditions, and is more specific and less hazardous.

The efficiency of hydrolysis by cellulase is limited by the presence of lignin and hemicellulose, by providing inadequate access of the cellulases to cellulose, and by irreversibly binding hydrolytic enzymes (Sun and Cheng, 2002). This also depends on the structural features of the substrate such as cellulose crystallinity, polymerisation, surface area and lignin content. Sun and Cheng (2002) suggested that the irreversible binding of cellulases on cellulose may be reduced by altering the surface property of cellulose via the addition of surfactants during hydrolysis. Eriksson *et al*. (2002) found that non-ionic surfactants were the most effective, particularly Tween and Triton surfactants. However, Triton surfactants may contribute to negative environmental effects due to the existence of the aromatic ring in the surfactant and is therefore not considered suitable. Enzyme hydrolysis may also be improved by adopting pre-treatment techniques that aid in the removal of lignin and hemicellulose, reduce cellulose crystallinity and increase porosity (Ishizawa *et al.*, 2009). This may involve treating pomace with strong and concentrated acids that hydrolyse the lignin, thus making the desired substrates accessible to enzyme degradation. Other pre-treatment techniques include alkali and mechanical methods, as well as steam explosion (De Vries *et al.*, 2000). Krongtaew *et al*. (2010) recommend pre-treatments at atmospheric pressure due to its low cost and simplicity. Krongtaew *et al*. (2010) indicated that mild pre-treatment techniques are preferred due to the decreased production of fermentation inhibitors such as furfural and hyroxymethylfurfural. Various pre-treatments have been investigated to remove lignin and hemicellulose, however limited focus has been placed on understanding how compounds released during pre-treatment and hydrolysis may affect enzymatic hydrolysis of lignocellulose (Yang *et al*., 2011).

1.4.1 Cellulases

The enzymatic hydrolysis of cellulose by cellulases is highly specific and the hydrolysis products include reducing sugars such as glucose and oligosaccharides of glucose (Sun and Cheng, 2002). There are three main enzymatic groups associated with the complex and synergistic degradation of cellulose (Figure 2) (Bayer *et al*., 2007). Endoglucanases (EC 3.2.1.4) are one of the groups of enzymes present in a cellulase enzyme mixture and are responsible for generating free chain ends by targeting internal, amorphous regions at random points along the glucose chain (Sun and Cheng, 2002). Endoglucanases can hydrolyse non-crystalline cellulose, but are unsuccessful at hydrolysing crystalline cellulose substrates (Samayam and Schall, 2010). Another enzyme group found in a cellulase mixture is exoglucanases or cellobiohydrolases (EC 3.2.1.91), which cleave cellobiose units (glucose dimers) from the free chain ends, thus degrading the molecule further. Type I cellobiohydrolases attack from the reducing end while Type II attack from the non-reducing end (Teeri, 1997). The final enzymatic group found in a cellulase mixture is β-glucosidase (EC 3.2.1.21) which is responsible for hydrolysing glucan oligomers and cellobiose to produce glucose monomers. Most cellulases contain carbohydrate binding modules (CBM) which may enhance enzyme activity by allowing the enzymes to bind to their substrate during hydrolysis (Yang *et al*., 2011). Pletschke *et al*. (2014) explain that the CBM holds the enzyme's active site in contact with the substrate thus increasing the concentration of the enzyme at the surface of the substrate. Recent studies have also reported a new class of enzymes; bacterial Auxiliary Activity family 10 (previously classified as family 33 Carbohydrate Binding Domain) and fungal Auxiliary Activity family 9 (previously classified as family 61 Glycoside Hydrolase). These enzymes catalyse the oxidative cleavage of the polysaccharide chains, exposing the cellulose crystalline structures for further degradation (Horn *et al*., 2012).

Cellulase activity is inhibited by cellobiose and to a lesser degree by glucose, but procedures are available to limit inhibition, including the removal of sugars during hydrolysis by ultrafiltration or fermentation, and the use of high concentrations of enzymes, as suggested by Sun and Cheng (2002). However, this is a critical issue because the commercial application of cellulosic ethanol production is hindered by the high cost of enzymes. One possible solution to limiting inhibition at reduced costs involves the immobilization of enzymes such as β-glucosidase. Lee *et al*. (2010) were able to successfully adopt this principle in

combination with simultaneous saccharification and fermentation to demonstrate the relief of glucose inhibitory effects, which maintains a low level of glucose due to continuous consumption of glucose by the fermentative process.

1.4.2 Hemicellulases

Enzymes that are involved in the biodegradation of hemicellulose are known as hemicellulases and have drawn attention from the paper and pulp industry due to their bleach-boosting properties which decrease non-environmentally friendly chlorine consumption. Additional applications of hemicellulases include high fibre baked goods and coffee processing as well as fruit and vegetable degradation (Nawirska and Kwasniewska, 2005).

Saha (2003) states that, although xylan (main component of hemicellulose) structure is more complex, it is not tightly wound in a crystalline structure (unlike cellulose), and is therefore more accessible to enzymatic hydrolysis. This group of enzymes hydrolyse the hemicellulose component of the lignocellulose and consists of different subcategories of enzymes that target either the backbone xylan chain or its substituents (Samayam and Schall, 2010) (Figure 3). Many enzymes (hemicellulases) are required for the biodegradation of xylan including endo-β-1,4-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37) and several accessory enzymes such as α-L-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.31), acetylxylan esterase (EC 3.2.1.72) and ferulic acid esterase (EC 3.2.1.73), which are required for hydrolysing different substituted xylans (De Vries *et al*., 2000). Arabinofuranosidase is an important enzyme and can synergistically degrade arabinose containing polysaccharides with other carbohydrate hydrolysing enzymes (Beukes and Pletschke, 2010).

1.4.3 Pectinases

Pectinases is the general term used to describe enzymes that are able to break down pectin to its monomers and several enzymes are involved in the degradation of pectin (Figure 4) (Jayani *et al*., 2005). The mechanism of pectinase activity is via hydrolysis or by transelimination (lyases). Pectinases can hydrolyse the polygalacturonic acid chain of pectin by the addition of a water molecule (Kumar *et al.*, 2008). Pectin methyl esterases (EC 3.1.1.11) remove the methyl groups in pectin which allow access to the backbone for action by depolymerising enzymes such as polygalacturonase (EC 3.2.1.15), pectin lyase (EC

4.2.2.10) and pectate lyase (EC 4.2.2.2) (Jayani *et al.*, 2005). According to Voragen *et al.* (2009), the complex nature of pectin means that enzyme degradation requires the addition of several enzymes simultaneously or in a specific sequence and it has been established that several pectin degrading enzymes have shown synergistic associations.

1.5 ENZYME IMMOBILIZATION

The term 'immobilized enzymes' was first used at an Enzyme Engineering Conference in 1971, and was described as enzymes that could be reused since they were confined or localized to a particular region or area (Costa *et al*., 2004). The potential of free and immobilized enzymes in commercial applications has since been recognised, and further development of cheaper methods is an on-going area of research. Immobilization of enzymes has attracted much interest and plays a significant role in optimizing the performance of an enzyme-catalysed process in various industrial applications including the food, textile and chemical industries as well as in biotechnological and pharmaceutical uses (Costa *et al*., 2004; Wu *et al*., 2005). Many different enzymes have been immobilized in different ways for various industrial bioprocesses. For example, glucose isomerase has been used for the production of fructose syrup from glucose (Cao, 2006); β-galactosidase has been used for the manufacturing of lactose-free dairy products and processing of commercial wastewater; and glycosidases have been used for improvement and clarification of juices and wines (Volkov *et al*., 2003).

The main reasons why enzyme immobilization shows so much potential in commercial processes is that it may improve long-term operational stability, recovery and reuse of the enzyme, as well as allow convenient handling (Sheldon, 2007). Lupoi and Smith (2011) claim that enzyme immobilization may also provide protection from protein degradation and aggregation. Cellulases in particular are commonly used in different industries such as the food, juice, wine, bioenergy, paper and pulp, animal feed and textile industry. Some of the applications of these enzymes require them to function with high stability at extreme temperatures and pH (Dincer and Telefoncu, 2007). Therefore, immobilized cellulases and hemicellulases may be a potential solution to reduce production costs for efficient biotechnological processes such as the degradation of lignocellulose and therefore the production of value added products such as bioethanol.

1.5.1 Immobilization support

The support used to immobilize the enzyme, as well as the method used, can affect the activity or function of the enzyme (Lupoi and Smith, 2011). Properties that need to be considered when selecting an immobilization support include hydrophilicity, inertness, biocompatibility, microbial resistance, mechanical strength, availability and cost (Bickerstaff, 1995). Types of immobilization supports can be categorised according to their chemical composition as inorganic or organic, where the latter can be subcategorised into synthetic or natural supports. Both organic and inorganic support materials have been used for the immobilisation of cellulases and hemicellulases. However, the latter is not as well established, although some inorganic support polymers have been used such as acrylamide (Roy *et al.,* 2008), glass (Mandali and Dalaly, 2010) and benzoquinone silochrome (Tavobilov *et al*., 1985). Polysulfone (Wei *et al*., 2009), silica (Afsahi *et al*., 2007), polyurethane (Chakrabarti and Storey, 1988), commercial agarose gels (Chim-anage, 1986), chitin, chitosan, nylon and polyvinyl alcohol (PVA) have been used as support carriers for the immobilization of cellulases (Hung *et al*., 2011).

PVA is a non-toxic, water soluble synthetic polymer with good chemical and thermal stability. PVA is an appropriate matrix for immobilized enzymes due to its biocompatibility (Dincer and Telefoncu, 2007). However, the applications of PVA are limited due to its high water dissolution and poor mechanical strength (Wu *et al*., 2005).

A possible, alternative synthetic polymer that may be used is polyacrylonitrile (PAN) due to its waterproof property and increased mechanical strength (Hung *et al*., 2011). Li *et al*. (2007) report that PAN nanofibers have been used as an alternative support for immobilization of lipase. However, research on their applications to cellulase and hemicellulase immobilization (Hung *et al.,* 2011) has been limited.

Nylon 6 consists of amide groups separated by linear chains of methylene units (Figure 6A) (Zhang *et al.,* 2009). Nylon is also an ideal synthetic matrix that can be used for enzyme immobilization due to its chemical and physical properties, non-toxicity, inert nature, availability in different forms (beads, nets, tubes, film, sheets) as well as its low cost (Isgrove *et al*., 2001). For these reasons nylon was selected as the polymer for immobilization in this study. Nylon has previously been electrospun into nanofibers (Chigome *et al*., 2011;

Supaphol *et al.*, 2005; Xu *et al*., 2010) and different forms used as an immobilization support for different types of enzymes (Andrews and Mbafor; 1991; Goldstein *et al*., 1974; Lozano *et al*., 1988; Zaidi *et al*., 1995). However, the use of electrospun nylon 6 nanofibers for the immobilization of enzyme cocktails containing cellulases and hemicellulases, to the best of our knowledge, has not been investigated.

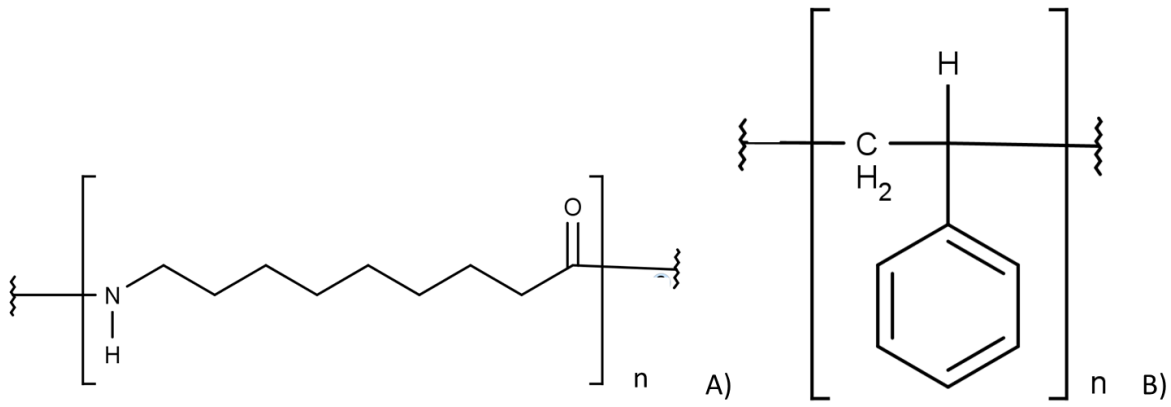


Figure 6: Chemical structures of nylon 6 A) and polystyrene B).

Partial acid hydrolysis of the nylon is often required to generate free amino and carboxyl groups on the surface of the nylon, which can be coupled to proteins with cross linkers such as glutaraldehyde (GA) or carbodiimide (Isgrove *et al*., 2001; Mandali and Dalaly, 2010). Jain and Wilkins (1986) immobilized cellulase on nylon and nylon incorporated with glass through coupling with GA. Apart from acid hydrolysis, alkylation of the nylon can also be used as a surface modification technique to functionalize the nylon through the use of agents such as dimethyl sulphate. Despite the toxic and carcinogenic properties of alkylating agents, they have been used widely as functionalizing agents for enzyme immobilization (Ngo and Yam, 1978; Sundaram and Hornby, 1970).

Polystyrene (PS) (Figure 6B) is a commonly used, hydrophobic, thermoplastic polymer with a low surface energy because of the presence of CH groups (Li *et al*., 2009). PS is also a synthetic polymer that offers high chemical and mechanical stability and is readily available at low costs.

1.5.2 Immobilization methods

In addition to various supports that can be used, there are also many different methods that can be carried out for enzyme immobilization. According to Cao (2006), there have been more than 5 000 peer reviewed articles published on enzyme immobilization techniques. Sheldon (2007) describes three categories of enzyme immobilization, including binding to a carrier support either by adsorption, electrostatic interactions or covalent attachment, via a linker or spacer arm, entrapment and cross-linking (Figure 7). Due to the limitations of each of these methods, many variations of combinations of these basic techniques have been developed (Cao, 2006).

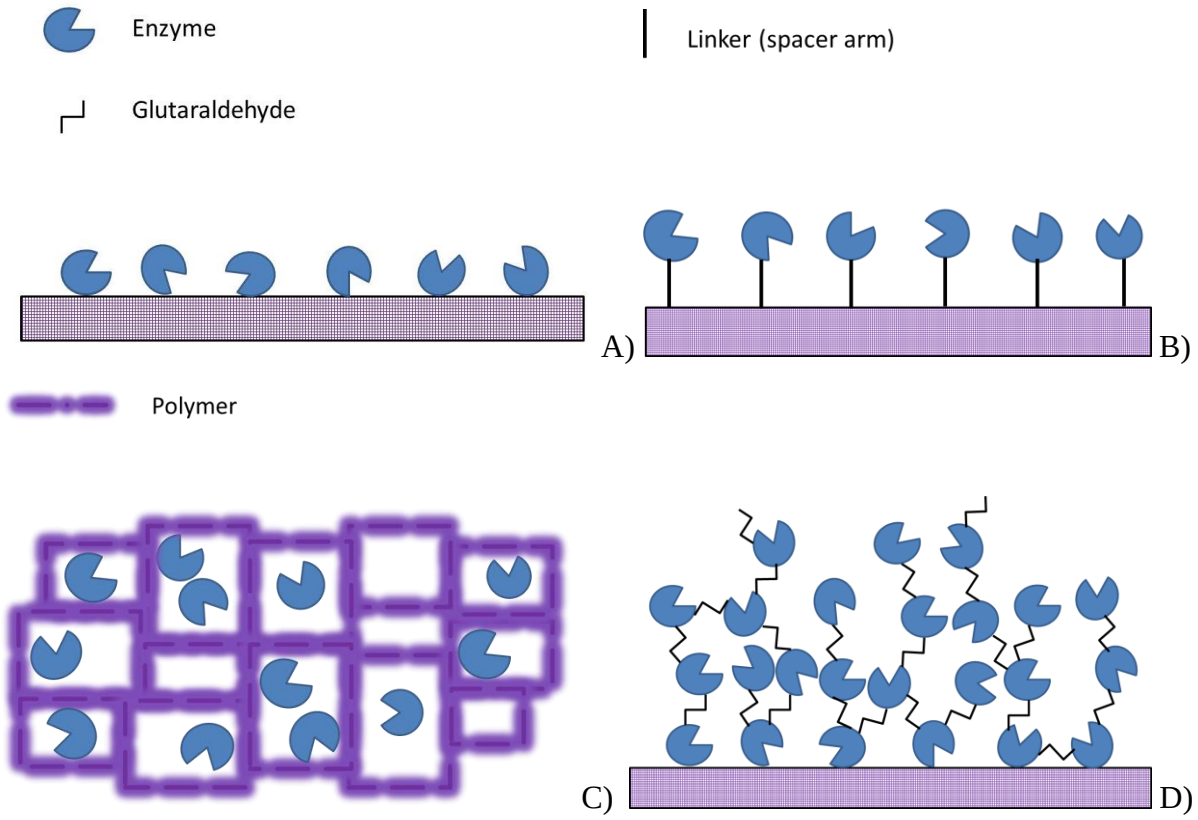


Figure 7: Different methods of enzyme immobilization including binding to a resin (A), via a linker (B), encapsulation (C) or crosslinking (with GA) (D). (Images adapted from Costa *et al.*, 2004, Sienko *et al.*, 2005 and Wang *et al.*, 2009).

Encapsulation (Figure 7C) involves trapping the enzymes within a porous gel, fiber, film or microencapsulation. The main advantages are that the enzymes may retain their native form and the large surface area between substrate and enzyme within a relatively small volume.

Adsorption (Figure 7A) is a simple method of enzyme binding to a support and normally involves non-covalent linkages and a pre-activation step is usually not required. The enzymes typically bind to the support via weak interactions such as hydrophobic interactions, hydrogen bonding, van der Waals forces and the slightly stronger ionic interactions (Cao, 2006; Costa *et al*., 2004). Apart from simplicity, the main advantage of this method of immobilization is that the enzymes' conformation is often unaltered due to the nature of the binding mechanism (Sheldon, 2007).

The covalent binding of an enzyme to a support usually involves covalent bonds with the amino group (NH_2) of lysine or arginine, the carboxyl group (COOH) of aspartic or glutamic acid, the hydroxyl group (OH) of threonine or serine or the sulphydryl group (SH) of cysteine (Costa *et al*., 2004; Hanefeld *et al*., 2009). There are various possible mechanisms involved including Schiff's base formation, amidination, thiol-disulphide, peptide bond, diazotation, amino bond or alkylation reactions (Costa *et al*., 2004). The binding between an enzyme and the support may be direct or via a linker or spacer arm (Figure 7B). This provides a link between the enzyme and support, thus allowing increased mobility. The principal advantages of covalently attached enzymes are that they often lead to increased enzyme stability and decreased enzyme leaching (Costa *et al*., 2004; Sheldon, 2007).

Crosslinking (Figure 7D) is a method that uses covalent attachment of enzymes via bi- and multi-functional reagents. Crosslinking agents that have been used include glyoxal (Costa *et al*., 2004), epichlorohydrin (Costa *et al*., 2004; Dincer and Telefoncu, 2007), carbodiimide (Jordan *et al.*, 2011; Mandali and Dalaly, 2010; Zhang *et al*., 2010), diisocyanates (Costa *et al*., 2004) and GA (Afsashi *et al*., 2007; Isgrove *et al*., 2001; Kulys and Vidziunaite, 2003; Mandali and Dalaly, 2010). According to Chae (1998), GA is also the most commonly used crosslinking agent as it offers simplicity and the gentlest coupling method. GA is readily available, relatively inexpensive, easy to handle and shows high reactivity with amino groups of proteins around neutral pH (Isgrove *et al*., 2001; Poddar and Jana, 2011).

GA exists in many different forms depending on the solution conditions such as pH, concentration and temperature. Because of this, its reaction mechanism during the immobilization of enzymes is highly debated in literature (Alexa *et al*., 1971; Habeeb and Hiramoto, 1968; Isabelle *et al*., 1964; Migneault *et al*., 2004; Peters and Richards, 1977; Walt

and Agayn, 1994), and some of the more common forms and explanations relevant to this study are summarized here (Figure 8). GA exists as a monomer under acidic conditions either as a free aldehyde (structure I), monohydrate (structure II), dihydrate (structure III) or cyclic hemiacetal (structure IV) (Figure 8). GA tends to undergo polymerization at higher concentrations to form oligomeric hemiacetals (Figure 8, structure V) (Walt and Agayn, 1994). According to Hardy *et al*. (1969), GA exists as a mixture of these forms, in varying proportions, in equilibria as a function of temperature. As the pH increases towards more basic conditions, GA tends to undergo intermolecular aldol condensations to form α, β-unsaturated multimeric aldehydes (Figure 8, structure VI). Once diluted, polymerized GA tends to revert back to its monomeric form, as would be expected from the reversibility of the hydration reaction (Walt and Agayn, 1994). Each form of GA is capable of reacting with proteins in different ways, and since polymeric GA has been reported to show improved immobilization capabilities, its reaction with enzyme is shown in Figure 8, through either a stabilized Schiff base formation (structure VII) or Michael-type addition (structure VIII) (Migneault *et al.*, 2004). Hardy *et al.* (1969) argued that the reaction mechanism involved dimerization in the presence of the amino group, resulting in the formation of quaternary pyridinium compounds (structure IX). In the present study, GA was diluted and therefore the more likely reaction with enzyme may have proceeded via Schiff base formation from the monomeric forms of GA (structures X and XI) (Walt and Agayn, 1994).

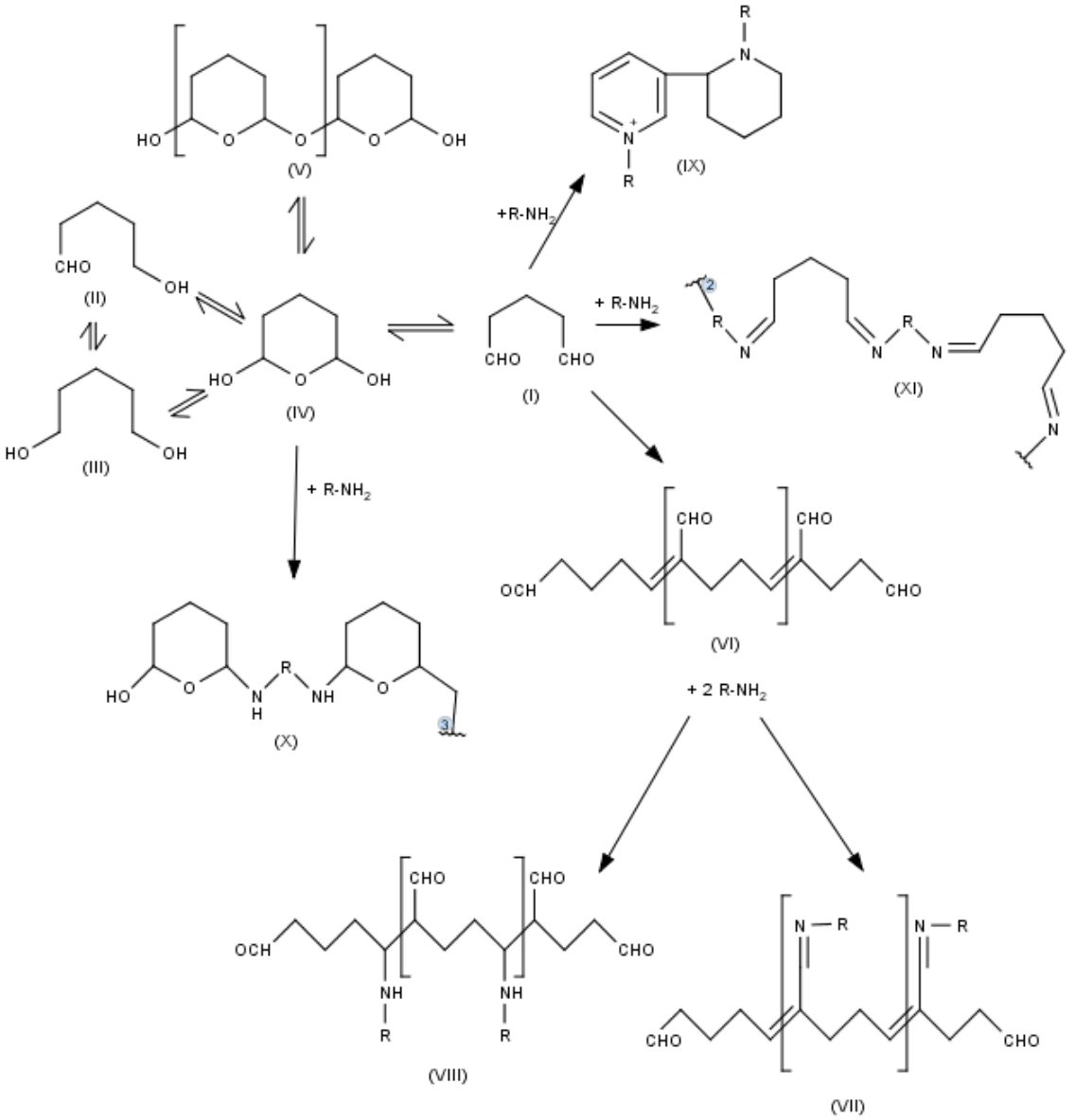


Figure 8: Schematic summary of the possible forms of GA in aqueous solution and the reaction mechanisms with enzyme (Image adapted from Walt and Agayn, 1994; Migneault, 2004).

As seen from Figure 8, the structure of GA and the forms in which it exists is a complex system and the solution conditions govern which coupling reaction mechanism is favoured. Some of the reactive groups that have been reported to be involved in crosslinking of GA and proteins are the ε-amine of lysine, thiol of cysteine, phenol hydroxyl of tyrosine and imidazole of histidine; because the most reactive amino acid side chains are nucleophiles (Chae, 1998; Costa *et al.*, 2004; Habeeb and Hiramoto, 1968; Walt and Agayn, 1994). Tryptophan, phenylalanine (Hopwood *et al*., 1968), proline, serine, glycine and arginine

(Alexa *et al*., 1971) have also been reported to be involved in the reaction with GA. The ε-amino group of lysine residues is commonly reported to be involved in binding of proteins to other proteins as well as to a support, such as nylon (Andrews and Mbafor, 1991). According to Migneault *et al*. (2004), the unprotonated amino groups of lysine residues are very reactive as nucleophilic agents. Due to the polarity of the amino group on lysine residues, they are usually located on the protein surface and not in the enzyme active site. This is one of the advantages of crosslinking with GA since it allows preservation of enzyme conformation because the active site is not involved in immobilization and therefore catalytic activity is better retained. However the disadvantage involving crosslinking is that other residues that may be involved in the immobilization reaction may alter enzyme conformation, resulting in decreased flexibility and mobility and therefore activity (Walt and Agayn, 1994).

There are more forms of GA that have been reported in literature under different conditions, however, only the more common ones are mentioned here. Therefore the simplistic structure of GA is not indicative of the complexity of its behaviour and reactivity in aqueous solution due its chemical nature and existence of monomeric and polymeric forms in equilibrium. For this reason, an empirical approach towards its use for enzyme immobilization is required to optimize and balance the interaction of the various factors that influence its reactivity, including enzyme concentration, GA concentration, pH, temperature and reaction time, which were investigated in this study.

Each immobilization technique therefore has its limitations, for example, entrapment or encapsulation methods often result in enzyme leakage, diffusion constraints, lower stability compared to other immobilization techniques as well as high enzyme concentration requirements (Cao, 2006). The main disadvantage is that this technique is prone to mass transfer limitations which is particularly problematic for hydrolysis of complex/insoluble substrates (Grauz and Waldmann, 2002). Adsorption often also leads to enzyme leakage due to weak binding interactions and is easily affected by changes in the environment such as pH, temperature, organic solvents and ionic strength (Costa *et al*., 2004). Covalent attachment may lead to enzyme inactivation and loss in catalytic activity, particularly if the enzyme active site is involved in the immobilization. The disadvantage of immobilizing an enzyme on a carrier is that more than 50% of the enzyme activity may be lost. In addition, if the enzyme,

covalently linked to a support, is irreversibly deactivated, then both the enzyme and (often expensive) support will be unusable (Sheldon, 2007).

There are ways around this, such as the immobilization of enzymes producing cross-linked enzyme aggregates (CLEAs). These are simply enzymes precipitated out of solution through extensive crosslinking and there have been reports of this technique in literature (Dalal *et al*., 2007; Sheldon, 2007). The cost effective advantage is that a support carrier is not required. However, the principal disadvantage of this approach is that the CLEAs are easily broken down, particularly when stirring, which is essential with insoluble/complex substrates such as apple pomace (AP). Therefore this approach was not used in this study.

Crosslinking with GA may be used to bind additional enzyme molecules to a support matrix and therefore potentially increase enzyme loading. The multi-attachment of enzymes has also been reported to increase enzyme stability (Walt and Agayn, 1994). The binding reaction of GA seldom involves the active site residues of the enzyme and therefore loss of catalytic activity is less compared to other methods (Poddar and Jana, 2011). Although a loss in enzyme activity is still possible, this approach was chosen for immobilization in this study.

Loss in enzyme activity during immobilization is often unavoidable; however, the reasons for differences between the free and immobilized enzymatic activities can be explained. For example, the optimum conditions for the immobilized enzyme may not coincide with those for the free enzyme. Although identical protein or enzyme masses may be used for the immobilized and free enzyme reactions, not all of the immobilized enzymes may be able to take part in the reaction. The enzymes are immobilized in a random fashion and therefore, depending on their orientation, the active sites may be inaccessible to the substrate (Lupoi and Smith, 2011). Attempts have been made to tackle this problem by site-directed enzyme immobilization using different genetically engineered tags, which helps orientate the enzyme on the carrier surface, thereby improving retention of activity (Cao, 2006).

Despite the apparent loss in activity, the associated benefits of enzyme immobilization allow it to be a feasible concept worth investigating. One such benefit includes improved stability, particularly at non-optimal conditions (Lupoi and Smith, 2011). Multiple-point attachment of the enzyme to a support allows for this by restricting the undesirable conformational change

of the enzyme in harsh environments. For example, a cellulase has been shown to have increased pH and temperature stability after being immobilized on polysulfone (Wei *et al.*, 2009) and non-porous ultrafine silica particles (Afsahi, *et al*., 2007), compared to the free enzyme.

Another benefit associated with enzyme immobilization is recovery and reuse of the enzyme. This may reduce costs, which is particularly important in biotechnological applications involving immobilized cellulases. There have been reports of cellulases and hemicellulases immobilized on various supports that were able to successfully hydrolyse their substrates. Operational studies carried out by Roy *et al*. (1984) suggested that cellulolytic and hemicellulolytic enzymes immobilized in acrylamide polymer retained the original activities up to 25 times in the reuse cycle. Mishra *et al*. (1983) showed that immobilized cellulase could be reused three times, producing the same sugar yields each time. Recycling studies, carried out by Mandali and Dalaly (2010), indicated that higher residual cellulase and hemicellulase activities were maintained with enzymes that were covalently bound to the support than non-covalently bound enzymes.

1.5.3 Immobilized enzyme hydrolysis of insoluble/complex substrates

A decrease in activity of immobilized cellulases and hemicellulases becomes more apparent when insoluble substrates are used. This is one of the major obstacles associated with immobilization of this type of enzyme. This is because movement is restricted, and the mechanism of cellulases requires adsorption onto its substrate and consequent desorption (Jain and Wilkins, 1986). If the enzymes are in the immobilized form, then adsorption onto the surface of the substrate would be impaired, implying that the notion of immobilized cellulases is not feasible. However, there have been reports in literature that state otherwise. For example, immobilization of β-glucosidase is still practical because its substrate, cellobiose, is soluble and it does not require adsorption onto the surface of the substrate in order for hydrolysis to take place (Woodward, 1989). Isgrove *et al*. (2001) were able to successfully immobilize β-glucosidase on nylon film via a relatively inexpensive method and the use of non-toxic reagents. Supplementation with immobilized β-glucosidase is of particular interest because it can reduce product inhibition of cellobiose in the cellulosic ethanol production by hydrolysing cellobiose into glucose (Lee *et al*., 2010).

Entrapment does not appear to be a viable method for immobilization of cellulases where insoluble substrates are involved, because effective interaction between the enzyme and its insoluble substrate would seem unlikely (Woodward, 1989). Nevertheless, Roy *et al.* (1984) entrapped cellulase in an acrylamide polymer and were able to hydrolyse filter paper and cotton up to 41% and 6.8%, respectively. However, no evidence was given to suggest this activity was not due to free enzyme as a result of leakage. This is the main disadvantage of using entrapment methods of immobilization. Woodward (1989) suggested that enzyme leakage can be limited by including additives, such as silica gel or PEG 6000, within the gel.

Woodward (1989) also suggested that individual cellulase components may interact with an insoluble substrate by immobilizing the enzymes onto a water-soluble polymer. For example, Mishra *et al*. (1983) immobilized crude cellulase onto PVA and demonstrated that there was 30% and 59% substrate conversion by free and immobilized enzyme, respectively. The major disadvantage with this technique is recovery of the enzyme after hydrolysis (Zhang *et al.*, 2010).

A similar, yet relatively novel method that deals with the problem of enzyme recovery is to immobilize the enzymes on stimulus responsive or smart polymers. These matrices undergo conformational changes, which can be manipulated by changes in environmental conditions such as temperature, pH and ionic strength (Sheldon, 2007). For example, Zhang *et al*. (2010) immobilized cellulase on the smart polymer Eudragit L-100 by altering the pH, which affected the solubility of the matrix, thus allowing recovery of the enzyme. According to Sheldon (2007), temperature-controlled smart polymers have the advantage that runaway conditions can be avoided, because when the reaction exceeds the threshold temperature, precipitation of the immobilized enzyme occurs, forcing the reaction to stop.

Another solution to hydrolysing insoluble substrates is to temporarily immobilize enzymes onto a support matrix, where the cellulases desorb from the support to hydrolyse the substrate, and then re-adsorb back onto the support after hydrolysis is complete. Woodward *et al*. (1984) used the basis of this principle to immobilize cellulase onto concanavalin A which was covalently linked to Macrosorb granules. In this instance, the Macrosorb granules had a higher density than the substrate which allowed separation of the support and the substrate. This type of immobilization occurs via the sugar (glucose or mannose) residues on

the enzyme, which is a glycoprotein (Woodward, 1989). Immobilization through these carbohydrate side chains allows for an increased retention of enzyme activity, which is likely due to the following reasons: i) the carbohydrate moiety is not crucial for enzyme activity and ii) the protein moiety is not restricted by immobilization and still largely accessible to the cellulose surface (Woodward and Zachry, 1981).

An alternative approach to maximising the interaction between immobilized enzymes and the insoluble substrate is via the use of spacer molecules. Placing the enzyme some distance from the carrier support may allow the interaction between the enzyme and insoluble substrate to be more effective (Woodward, 1989). Polyethylenimine (PEI), polylysine (Andrews and Mbafor, 1991), and chitosan (Isgrove *et al*., 2001) have previously been used as spacer molecules to help reduce steric hindrance caused by insoluble substrates.

An additional approach that can be used to overcome the problem with immobilized cellulases accessing the insoluble substrate is to immobilize whole microorganisms capable of producing the necessary enzymes. According to Woodward (1989), the same methods used for enzyme immobilization can be used to immobilize fungal mycelia for the continuous production of cellulase. For example, Linko *et al*. (1983) immobilized *Trichoderma reesei* by entrapment in polyurethane and showed that immobilization was not detrimental to cellulase production.

A different approach could also be used to immobilize the cellulases on nano-structured supports. Non-porous nanoparticles provide a large enough surface area to allow sufficient enzyme binding without compromising the ability of the immobilized enzymes to reach the insoluble substrate. This was demonstrated by Lupoi and Smith (2011), who successfully immobilized cellulase on non-porous silica nanoparticles. Although the small size of the nanoparticles allowed sufficient interaction between the enzyme and substrate, recovery of the enzyme may become an issue if the insoluble substrate is not completely hydrolysed. This problem may be overcome with magnetic capturing. Lee *et al*. (2010) were able to demonstrate this by incorporating magnetic nanoparticles within the carrier matrix, which allowed for easy recovery and reuse of the enzyme.

Recently, the novel research area of nanobiocatalysis has shown its potential in effective enzyme stabilization, improved enzyme activity and loading by utilizing nanostructured materials such as nanoparticles, nanotubes and nanofibers (Lee *et al.*, 2010). The large surface area to volume ratio of nanomaterials makes them excellent candidates for enzyme immobilization by promoting enzyme loading and activity per unit mass of support (Lee *et al*., 2010). For these reasons, this was the approach that was used in this study.

1.5.4 Electrospinning

One of the methods used for production of nanoscale supports that has been successfully developed is electrospinning (Figure 9). With electrospinning, nanofibers are formed from a viscoelastic polymer solution that has been charged to high voltages (Chigome *et al.*, 2011; Chigome and Torto, 2011; Doshi and Reneker, 1995; Taylor *et al*., 1969; Teo and Ramakrishna, 2006). An electric field is applied to the metal capillary of a syringe containing the polymer solution. The spherical droplet is deformed into a Taylor cone from which a cone-jet of the charged solution emanates when a threshold voltage is reached. The jet is projected towards a stable metal collector and the solvent evaporates resulting in the production of fibrous membranes (Wu *et al*., 2005). The physical properties of the electrospun nanofibrous membranes are determined by factors such as solvent systems, applied voltage, flow rate, the distance between the needle tip and the collector, as well as the support material (Huang *et al*., 2003), all of which were investigated in this study.

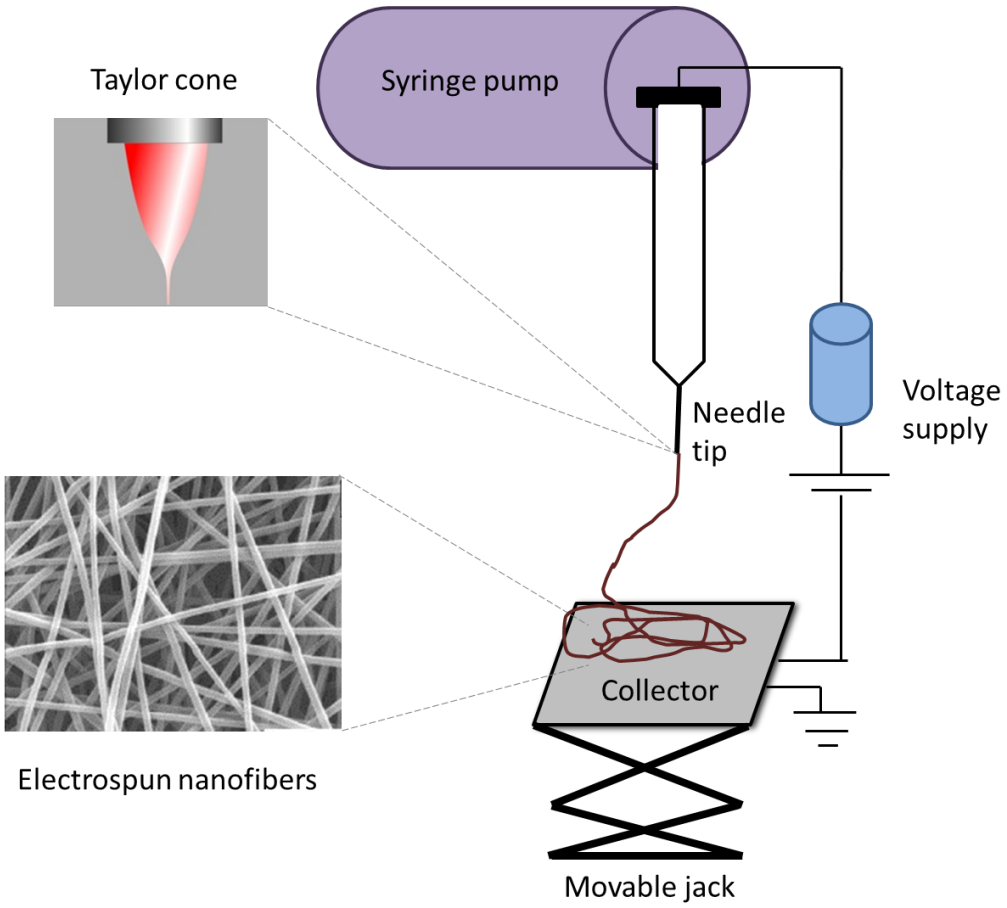


Figure 9: Schematic representation of the electrospinning apparatus used. (Image adapted from Lee *et al.*, 2010, Chigome *et al.*, 2011 and Kim *et al.*, 2005).

Electrospinning offers a simple and versatile approach to synthesising nanofibers using a variety of different polymers. There are other methods that have been reported for the synthesis of nanofibers, including self-assembly (Hartgerink *et al.*, 2001), drawing (Ondarcuhu and Joachim, 1998), phase separation (Ma and Zhang, 1999) and template synthesis (Feng *et al.*, 2002). Electrospinning is considered superior over the other methods due to its versatility, simplicity and its ability to control the nanofiber orientation (Chigome and Torto, 2011). Electrospun fibers have been used as membrane filters, reinforcements of dental materials, supports of chemical catalysts, sorbent material for solid phase extraction, tissue engineering scaffolds, electrochemical and optical detection systems and wound dressings due to their high specific surface area and porous structure (Chigome *et al.*, 2011; Chigome and Torto, 2011; Huang *et al.*, 2003). These properties of electrospun fibrous membranes also make them excellent candidates for immobilization of enzymes (Wu *et al.*, 2005). Electrospinning has also recently drawn much attention and interest due to its potential

to produce ultrafine fibers with diameters in the nanometer range with the potential of surface modification to promote enzyme binding and activity (Wu et al., 2005). The advantage of nanofibrous supports over other nanostructured supports is the increased porosity and interconnectivity, and is therefore less affected by mass transfer limitations (Wang *et al*., 2009). In addition, recovery and reuse of nanofibrous enzyme support matrices are more feasible than nanoparticles since they can be further processed into different structures such as non-woven mats, films and membranes (Kim *et al*., 2005).

Although enzymes are more often successfully immobilized onto the surface of nanofibers using different methods as previously described (Figure 7), it is also possible to encapsulate the enzymes by co-electrospinning the polymer with the enzymes. The advantage of this is that the surface tension of the electrospinning solution may be reduced, thus promoting the production of bead-free nanofibers (Wang *et al*., 2009). There are however, more disadvantages associated with this method of immobilization, including inaccessibility of the substrate due to confinement of the enzyme active site within the nonporous fiber. A water soluble polymer is usually required in order to form a homogeneous solution with the enzyme. This method of immobilization is also prone to enzyme leaching. Crosslinking may be used to reinforce enzyme binding to the support, however, it also reduces enzyme activity and crosslinking of the fibers can reduce porosity and therefore limit accessibility of the substrate to the enzyme active site. For these reasons, in this study the enzymes in Viscozyme L® and Celluclast 1.5L® were immobilized on the surface of the nanofibers (rather than through encapsulation).

The first report of utilizing electrospun nanofibers as an enzyme immobilization support was described by Jia *et al*. (2002). In their study, α-chymotrypsin was covalently attached to polystyrene modified with a hydroxyl initiator. There have since been reports on the use of nanofibers for the immobilization of cellulases on PAN (Hung *et al*., 2011) and PVA (Wu *et al*., 2004). The use of electrospun nanofibrous supports for immobilization of hemicellulases is less established.

Research on immobilization of commercial enzyme mixtures containing cellulases or hemicellulases is also limited. However, there have been reports on the immobilization of Celluclast 200L® on modified nylon (Jain and Wilkins, 1986) and an enzyme complex

(Novozymes; cellulases and hemicellulases) on porous glass beads (Mandali and Dalaly, 2010). Commercial enzyme cocktails often contain a consortium of enzymes, which is advantageous when complex/insoluble substrates are concerned. There is therefore potential in developing this concept for improving the commercial viability of lignocellulose-derived value added products such as biofuels.

1.6 VALUE ADDED PRODUCTS

In addition to its role in bioremediation, lignocellulosic biomass conversion offers a potential step towards the production of value added products, including biofuels such as bio-ethanol, bio-hydrogen and bio-gas (Figure 10). Over the past few decades, the increased demand for energy and depletion of petroleum reserves provides an interesting incentive to develop and investigate alternative energy sources (Samayam and Schall, 2010). Furthermore, the continuous dependence and use of petroleum-based fuels has caused serious environmental problems such as air pollution, acid rain and emission of greenhouse gases (Ni *et al*., 2007).

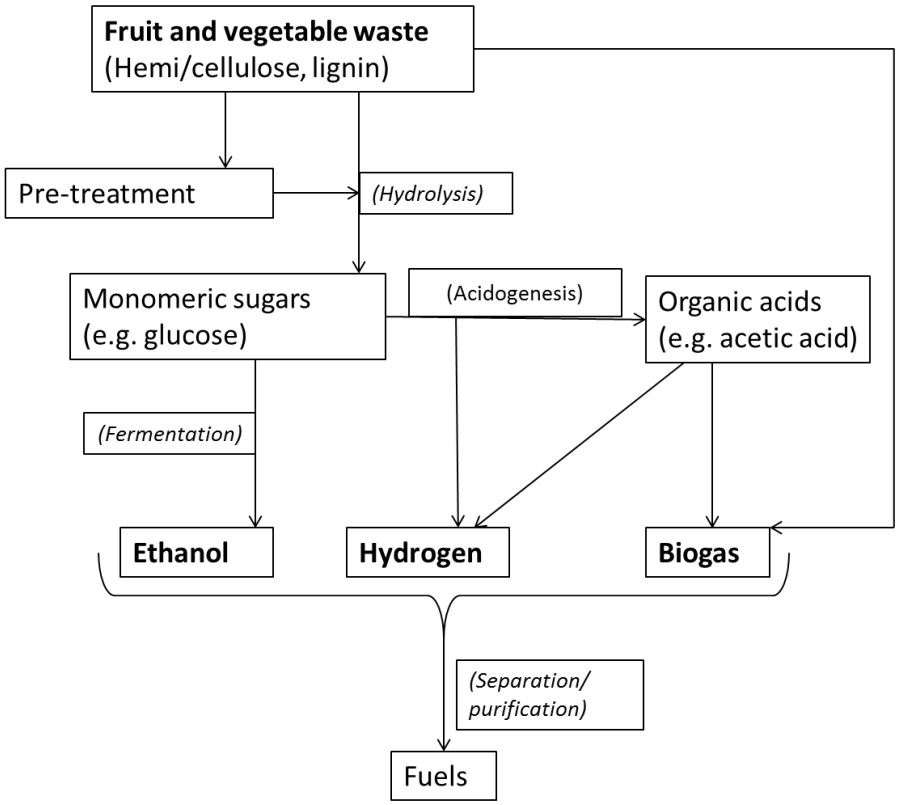


Figure 10: Schematic overview of lignocellulosic waste conversion to biofuel.

1.6.1 Bio-ethanol

Biomass feedstock, such as fruit waste, is desirable for bio-ethanol production as it is a source that does not compete with food crops. Focus has therefore been shifted to ethanol derived from fermentation of cellulosic substrates. The principle is based on the hydrolysis of lignocellulose to produce monomeric sugars (Anderson *et al*., 2008; Bayer *et al*., 2007; Beukes *et al*., 2008; Capek *et al.*, 1995; De Vries *et al*., 2000; Hoshino *et al*., 1997; Nidetzky *et al.*, 1994; Roy and Gupta, 2003; Spangnuolo *et al.*, 1997; Yang *et al.*, 2006) which are then fermented by yeast to produce bio-ethanol (Ban-Koff and Han, 1990; Fujita *et al*., 2004; Kaparaju *et al*., 2009; Kumar *et al.*, 2008; Lee *et al*., 2010; Ni *et al.*, 2007; Philippidis *et al*., 1993; Sticklen, 2008; Sun and Cheng., 2002).

Ethanol is a liquid, transport fuel that can also be combined with or used as a partial replacement of gasoline (Sun and Cheng, 2002). In countries where the vast majority of fuel sources are imported, bio-ethanol can be produced locally and therefore contribute towards domestic economies (Pletschke *et al*., 2014).

The biomass conversion of lignocellulose to produce fermentable sugars is a complex process as many enzymes are required which results in the production of many different hexose and pentose sugars. *Saccharomyces cerevisiae* is a fungus that is commonly used to ferment these sugars into ethanol. However, the disadvantage of using *S. cerevisiae* is that it preferentially utilizes glucose (Van Dyk *et al*., 2013). Therefore, it could be beneficial to find an alternative way to utilize the other sugars that are produced. For example, xylose can be used for the production of furfural and xylitol. Furfural is used in the manufacture of varnishes and pesticides (Howard *et al*., 2003). Xylitol is a five carbon sugar alcohol which is used in tooth paste, in artificial sweeteners and as a sugar substitute for diabetics (Howard *et al*., 2003). Saha (2003) stated that xylitol can be produced by chemical reduction in alkaline conditions of the xylose derived from hemicellulose hydrolyzate. However, some of the disadvantages associated with this process include; high temperature and pressure requirements, expensive catalyst and removal of by-products via extensive separation and purification steps (Saha, 2003). Pourbafrani *et al*. (2010) suggest another alternative use of non-fermentable sugars is that they can be dried with the solid polymer residue and used as cattle feed.

1.6.2 Bio-hydrogen

Hydrogen is also an ideal alternative to fossil fuels as it is cleaner and has high energy content per unit weight (Wang *et al.,* 2010). Hydrogen is commonly used in fuel cells to generate electricity (Ni *et al*., 2007). The combustion of hydrogen does not generate any toxic by-products as only water is produced (Wang *et al.,* 2010). Traditional methods of hydrogen production such as water electrolysis (Su *et al*., 2010), auto-thermal processes (Kothari *et al*., 2010), steam reforming and partial oxidation of fossil fuels have been known to be quite costly and require large amounts of energy (Wang *et al*., 2010). A more environmentally friendly method of producing hydrogen is through biological processes involving algae, photosynthetic bacteria, cyanobacteria or anaerobic fermentation bacteria (Yang *et al*., 2006) which can utilize industrial and agricultural wastes as substrates (Wang *et al*., 2010).

Various species of bacteria can be used in the production of bio-hydrogen, some of which include *Bacillus coagulans, Enterobacter aerogenes* and *Clostridium butyricum* (Pletschke *et al*., 2014). Mixed cultures of bacteria can be obtained from sources such as municipal waste, cow dung, river sludge, compost heaps and soil. The disadvantage with using mixed cultures of bacteria is that the source may also contain hydrogen consuming bacteria that affect the production of bio-hydrogen, such as sulphate reducing bacteria or methane producing bacteria (Pletschke *et al*., 2014).

Feng *et al*. (2009) investigated the production of bio-hydrogen from apple pomace combined with river sludge in the presence of anaerobic bacteria. They were able to produce sustainable quantities of bio-hydrogen, as well as other useful by-products such as acetic acid, ethanol and butyric acid (Feng *et al*., 2009). Anaerobic digestion is a complex process and may involve many different bacteria and enzymatic processes (Van Dyk *et al*., 2013). Initially lipids, proteins and carbohydrates are hydrolysed to their monomers which are then metabolised and fermented to produce organic acids, hydrogen and carbon dioxide by hydrogenases and nitrogenases (Kothari *et al*., 2010) in a process called acidogenesis. Acetic acid may then be used to produce methane by the action of methanogenic bacteria (Pletschke *et al*., 2014).

Utilization of organic wastes to produce bio-hydrogen offers a solution to economical energy production with simultaneous waste treatment. Bio-hydrogen can therefore be considered a

sustainable energy supply and shows great potential for future research. It is an environmentally friendly fuel and its ability to burn cleanly and minimize environmental problems such as acid rain and the greenhouse effect, make it the ultimate candidate for a future alternative energy supply.

1.6.3 Biogas

Biogas is another possible example of a value added product that can be obtained from lignocellulosic waste. Coalla *et al*. (2009) investigated the production of biogas from apple pulp and slaughter house waste. Under anaerobic conditions, biogas and organic fertilizer can be produced from organic wastes by the action of various microorganisms (Kothari *et al*., 2010). Biogas consists of mostly methane (50-70%) and is used to supply energy for lights, cooking, water pumps and electric generators (Kothari *et al*., 2010). Some advantages of producing biogas through anaerobic digestion, compared to other waste treatment strategies, include increased effectiveness in pathogen removal, reduced production of biomass sludge compared to aerobic treatment methods and decreased odour emissions (Kothari *et al.,* 2010).

1.6.4 Other value added products

Other chemical compounds can also be produced from fruit waste such as organic acids, amino acids and vitamins. Pectin can also be extracted from fruit and it is an industrially important product as it is used as a gelling agent, texturizer, thickener, emulsifier and stabilizing agent in food processing, cosmetics and pharmaceutical industry (Bhushan *et al*., 2008). Fruit pectin also has the added application as use for a drug carrier due to its non-toxicity and biocompatibility (Bhushan *et al*., 2008).

Some value added products that can be derived from lignin, include vanillin and gallic acid (Howard *et al*., 2003). Vanillin has previously been used in industry for the production of herbicides, anti-foaming agents and drugs such as papaverine and L-dopa (Walton *et al*., 2003). Walton *et al*. (2003) also reported the use of vanillin in domestic products such as floor polish and air-freshener.

There has been an increased interest in lignocellulosic biomass degradation of agro-industrial wastes to produce multiple products since it maximises the economic value of biomass. In

order for a bio-process to be economically feasible and sustainable, it is necessary to maximise the amount of value added products by utilizing as much of the lignocellulosic waste as possible at minimal costs. Kaparaju *et al*. (2009) investigated and developed a bio-process in which wheat straw was hydrolysed and fermented to produce bio-ethanol, bio-hydrogen and bio-gas. Pourbafrani and co-workers were able to produce 39.64 L of ethanol, 45 m^3 methane, 8.9 L limonene and 38.8 kg pectin from one ton of citrus waste (Pourbafrani *et al*., 2010). In order to compete with other fuel conversion processes, it is therefore ideal to develop bio-processes that fully utilize biomass waste, at low cost.

1.7 TILE BIOREACTOR

Pletschke *et al*. (2014) proposed the use of a system of selected enzymes acting synergistically in a bioreactor to successfully degrade lignocellulosic material in fruit waste water. This system was referred to as the Tuneable Immobilized Lignocellulosic Enzyme (TILE) system (Figure 11). The combination of enzymes was variable (tuneable) so that it may be applied to a range of fruit waste substrates. Pilot studies utilized AP as a model substrate to facilitate comparative results. The advantages of utilizing enzymes over microorganisms include increased specificity, easier handling and storage, and that enzyme concentration is independent of microbial growth (Pletschke *et al.*, 2014). In this study, the enzymes were proposed to be immobilized to improve stability in terms of storage, pH and temperature as well as recovery and reusability.

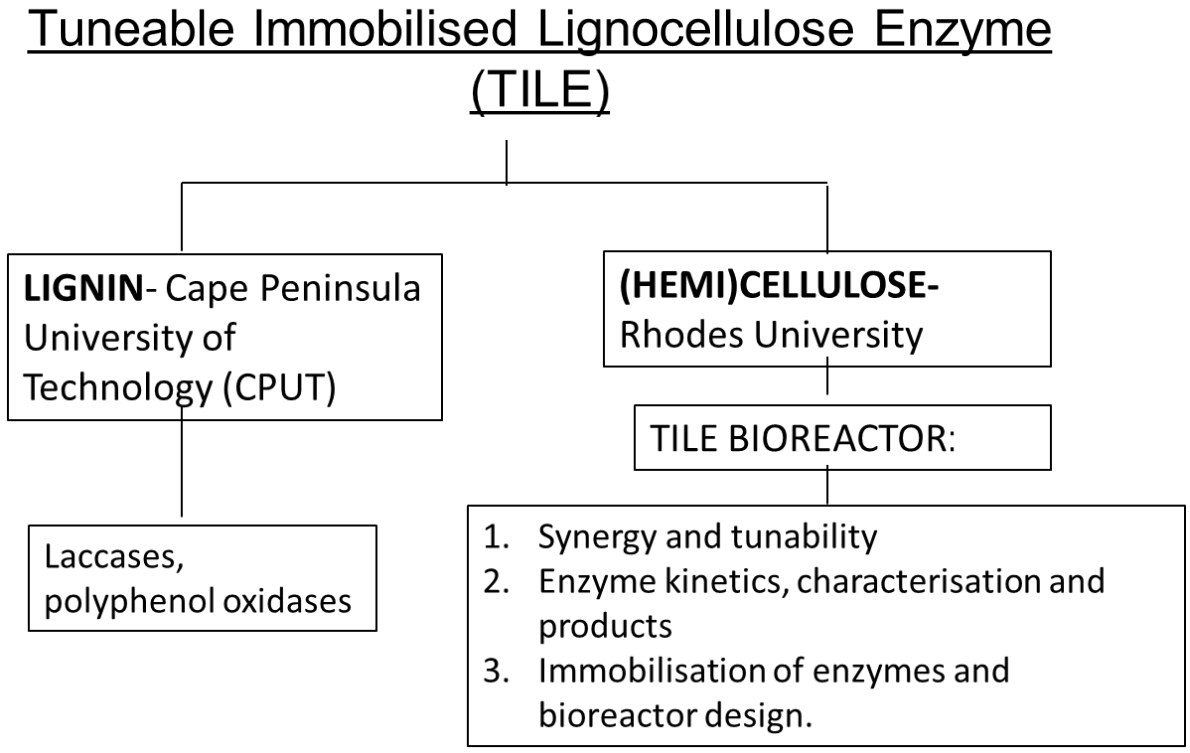


Figure 11: Different areas of investigation for the TILE project (Pletschke *et al*., 2014).

Colleagues in our research group were investigating different aspects of the TILE project (Figure 11). However, the focus of my project was on immobilization of the commercial enzyme mixtures, Celluclast 1.5L® and Viscozyme L®, on electrospun nanofibers for the hydrolysis of apple waste to demonstrate its potential to produce value added products such as bio-ethanol.

1.8 VISCOZYME L®

Viscozyme L® (catalogue No. V2010, Novozyme) is a plant cell wall degrading enzyme complex from *Aspergillus sp*. (Sigma- Aldrich, 2011). It is described as a multi-enzyme complex containing a wide range of carbohydrases, including arabanase, cellulase, β-glucanase, hemicellulase, pectinase, polygalacturonase and xylanase (Zheng *et al*., 2010). Viscozyme L®, in previous experiments in our laboratory, has been reported to have varying amounts of the following enzyme activities: β-glucosidase, α-arabinofuranosidase, endoxylanase, pectinase, endomannanase, polygalacturonase and endoglucanase (Gama, 2011). Viscozyme L® has also been reported to be active in the pH and temperature range of 3-6 and 35-60˚C, respectively (Adamsen *et al*., 2002; Anthon and Barrett, 2008; Combo *et*

al., 2012). Viscozyme L® has been reported to have been used in various processes, including the retting of flax, antioxidant scavenging and extraction of valuable compounds such as starch and oils (Adamsen *et al*., 2002; Guan and Yao, 2008; Lim *et al*., 2008). However, to the best of our knowledge, the immobilization of this enzyme cocktail has not been reported.

1.9 CELLUCLAST 1.5L®

Celluclast 1.5L® (catalogue No. C2730, Novozyme) is a commercial enzyme mixture containing cellulases from *Trichoderma reesei*. Gama (2011) has reported Celluclast 1.5L® to contain the following enzyme activities: endoglucanase, endoxylanase, β-xylosidase, β-glucosidase and exoglucanase. Celluclast 200L® Type N, a similar commercial cocktail made by submerged fermentation of a selected strain of *Trichoderma reesei*, has been immobilized on modified nylon incorporated with glass beads (Jain and Wilkins, 1986).

Research is currently being done with the intention of developing a method for reducing the overall costs relating to the enzymes (particularly cellulases) involved in ethanol production. Enzyme immobilization offers a positive solution to this challenge, as it offers increased stability, recovery and reuse of enzymes and therefore potentially lowers costs. In this study, the hemicellulases and cellulases in Viscozyme L® and Celluclast 1.5L® were immobilized onto nylon 6 nanofibers, using apple pomace as a substrate. The nanofibers were synthesized by electrospinning as they offer an increased surface area for more enzymes to bind, as well as reduced mass transfer limitations associated with bulk supports, all of which are important considerations for degradation of insoluble/complex substrates (such as AP). The electrospinning process was optimized to produce smooth nylon 6 nanofibers and their morphology analysed by SEM. The immobilization process was also optimized and the feasibility of the immobilized enzymes was determined by biochemical characterization studies and comparison with the free enzymes.

Chapter 2

Research Motivation and Hypothesis

2.1 PROBLEM STATEMENT

Large quantities of waste are generated from fresh fruit processing for wine, juice and soft drink production. The high transportation costs to dispose of these wastes results in the accumulation of waste heaps outside the manufacturing plants, which violates pollution control regulations and causes industrial safety issues. These landfill waste heaps are also detrimental to the aesthetic quality of the natural environment and may result in putrid smells due to the fast degradation by natural microorganisms. This fruit waste consists mainly of lignocellulose, a combination of highly recalcitrant plant polysaccharides such as cellulose, lignin, pectin and hemicellulose. Conversion of the lignocellulosic waste is therefore of particular interest and provides a potential solution to large scale pollution, allowing greater economic benefit and a cleaner environment. In addition to its role in bioremediation, lignocellulosic biomass conversion offers a potential step towards the production of other value added products including biofuels and other chemical compounds. However the use of free enzymes for this process on an industrial scale is not economically viable. Viscozyme L® (catalogue No. V2010, Novozyme) and Celluclast 1.5L® (catalogue No. C2730, Novozyme) are commercial enzyme cocktails that can be used for the degradation of lignocellulose; however, little is known about the immobilization of commercial enzyme mixtures containing cellulases or hemicellulases, particularly when using complex substrates, such as apple pomace.

2.2 HYPOTHESIS

Cellulases and hemicellulases in Celluclast 1.5® and Viscozyme L® can be immobilized on electrospun nanofibers for the beneficiation of fruit waste, such as apple pomace.

2.3 AIMS AND OBJECTIVES

1. To synthesize electrospun nylon 6 nanofibers and optimize with regards to polymer concentration, solvent system, flow rate, applied voltage and needle tip-collector distance.

2. To immobilize the enzymes in Celluclast1.5L® and Viscozyme L® on electrospun nanofibers and optimize with reference to method of immobilization, enzyme and crosslinker (GA) concentration, HCl activation concentration and immobilization times.

3. To verify nanofiber morphology and confirm enzyme binding using SEM, FT-IR and EDS.

4. To determine the optimum operation conditions, substrate specificity, pH, thermal and storage stability, as well as compare the kinetic characteristics between the free and immobilized enzymes, mainly using AP as a substrate.

5. To determine the reusability of the immobilized enzymes for repeated cycles of conversion.

Hydrolysis of lignocellulosic substrates, such as fruit waste, via enzymatic methods offers greater benefits over chemical methods such as increased yields and selectivity, lower energy costs and milder operating conditions. However, the enzymatic hydrolysis mechanism is complex and the relationship between the substrate structure and function of the enzymes involved is not well understood. There is therefore potential for further investigation in developing and improving lignocellulosic biomass conversion processes with maximum yields and minimal costs. This may be achieved through optimizing the interaction between enzymes for a particular substrate and through the use of immobilized enzymes. A biomass-ethanol production system in South Africa may introduce economic and social benefits for the rural community such as new employment opportunities and a novel source of income. Utilization of fruit waste as a fermentation feedstock can also serve bioremedial purposes and does not directly compete with food resources. Therefore, the saccharification of fruit waste biomass via immobilization of Celluclast 1.5® and Viscozyme L® on electrospun nanofibers for fermentation and production of bio-ethanol provides a positive outlook on the benefits

associated with this dual process. In this study, the synthesis of the electrospun fibers was optimized as well as the enzyme immobilization procedure. The feasibility of the immobilization was determined by comparing and characterizing the immobilized and free enzymes.

Chapter 3

Electrospinning of Nanofibers for Enzyme Immobilization

3.1 INTRODUCTION

Nanotechnology has advanced towards the immobilization of enzymes for the development of biosensors, however nanotechnological applications for immobilization of enzymes for the purpose of bioethanol production are not well established (Lu *et al*., 2007). Nanofibrous supports offer advantages over other supports for enzyme immobilization, such as increased porosity and interconnectivity, and are therefore less affected by mass transfer limitations (Wang *et al*., 2009). In addition, recovery and reuse of nanofibrous enzyme support matrices are more feasible than nanoparticles since they can be further processed into different structures such as non-woven mats, films and membranes (Kim *et al*., 2005). One of the methods used for production of nanoscale supports, that has been successfully developed, is electrospinning (Figure 12).

According to Wu *et al*. (2004), the first report of electrospinning was in 1934 and since then this technique has received much attention due to its ability to produce fibers in the nanometer range. Electrospinning allows the synthesis of nanofibrous supports with increased porosity, interconnectivity, surface attachment, diversity in composition and pore sizes tailored to protein molecule dimensions (Wang *et al.,* 2009). These qualities make them excellent candidates for enzyme immobilization.

The main components of the electrospinning apparatus are: the voltage supply, the collector and the spinning system (syringe, needle and pump) (Figure 12) (Chigome *et al.,* 2011). For the experiments in this study, a polymer solution was fed into the spinning system using a peristaltic pump. The needle was connected to a high voltage supply which injects a charge of a certain polarity into the polymer solution. When the electrostatic force is high enough to overcome the polymer surface tension, the droplet at the tip of the needle is deformed into a Taylor cone (Figure 13) and a liquid jet is emitted from the needle tip. As the solvent evaporates, tensile forces from surface charge repulsion cause whipping, bending and

stretching of the fiber jet before being deposited on the collector, covered in aluminium foil (Chigome *et al*., 2011; Dai *et al.*, 2011).

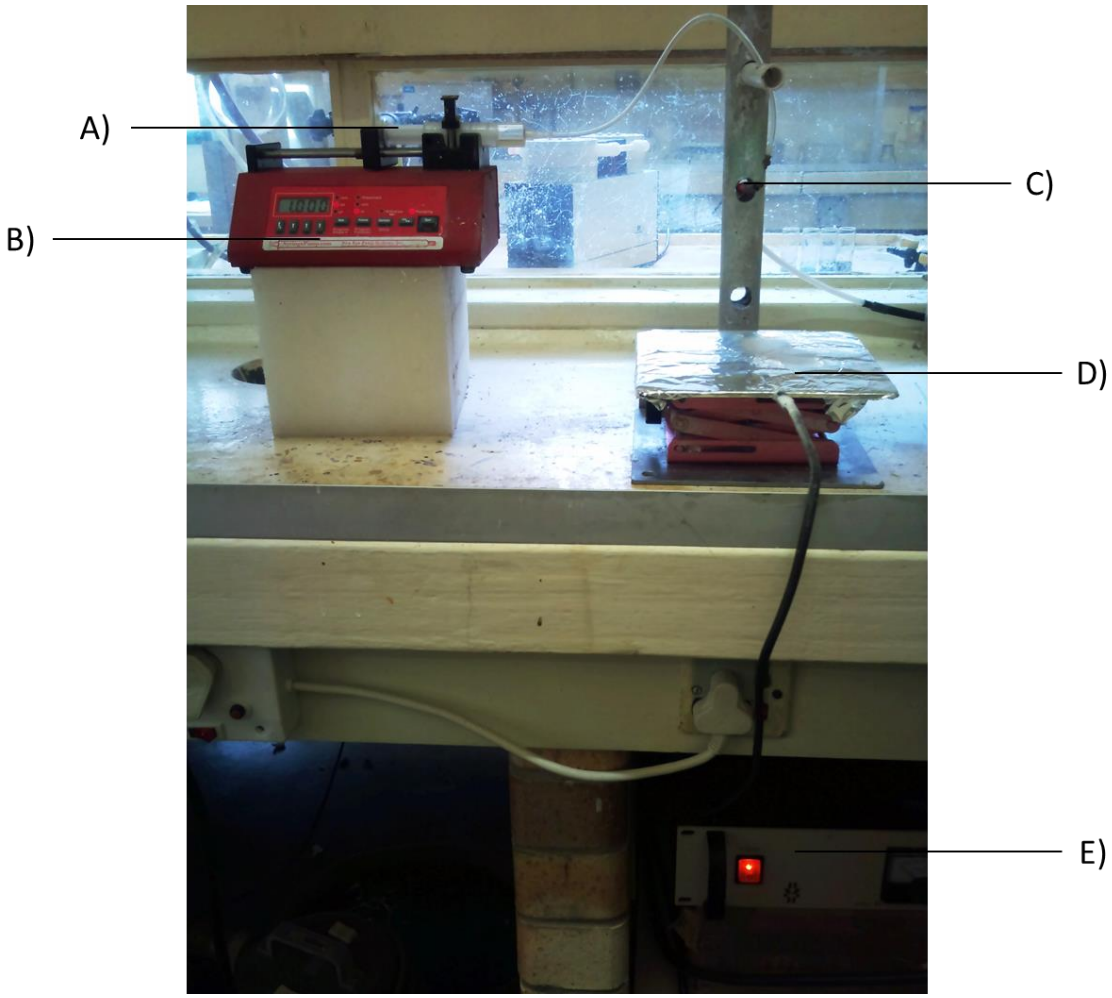


Figure 12: Electrospinning apparatus with each of its components including the spinning system; A) syringe containing the polymer solution, B) pump, C) needle tip, D) the collector covered with aluminium foil and E) the voltage supply.

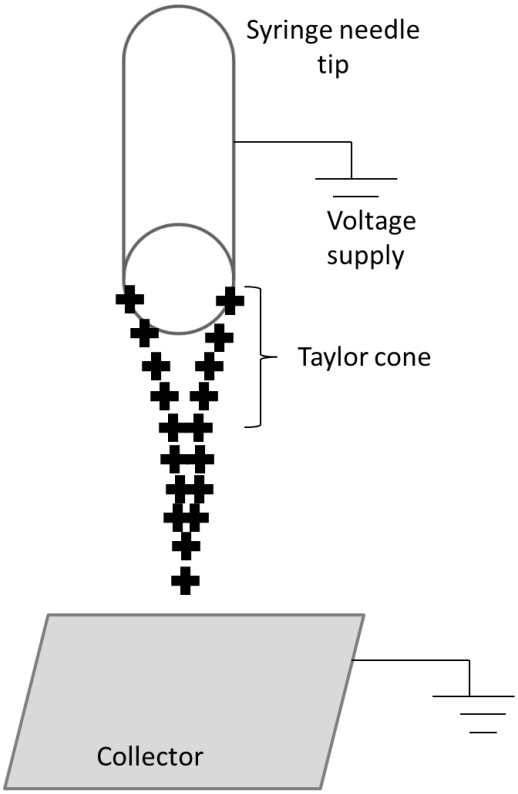


Figure 13: Schematic representation of Taylor cone formation. (Image adapted from Chigome *et al.*, 2011).

Nylon was selected as the main polymer used for electrospinning as it is non-toxic, possesses ideal chemical and physical properties, and is readily available at low cost (Isgrove *et al.*, 2001). However, due to the inert nature of nylon, its reactivity and biocompatibility was increased by co-electrospinning the nylon with polystyrene to provide more reactive groups for enzymes to bind, and by pre-treating the nylon with HCl.

An important consideration for steady state electrospinning is the solvent system that is used and some studies have used more than one solvent to dissolve the polymer (Wannatong *et al*., 2004). For this to be possible, the solvents need to be miscible with each other. (Formic acid (FA) (Figure 14A) and acetic acid (AA) (Figure 14B) resemble each other structurally, as do the heterocyclic compounds tetrahydrofuran (THF) (Figure 14C) and dimethylfuran (DMF) (Figure 14D), which explains their miscibility. Therefore, in this study, various concentrations of these solvents were used to dissolve nylon and polystyrene (PS).

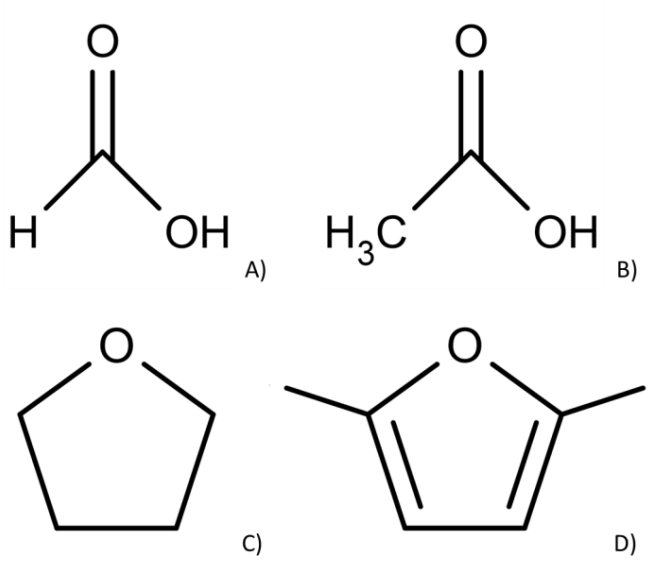


Figure 14: Chemical structures of formic acid A), acetic acid B), THF C) and DMF D).

The spinnability and stability of the polymer is governed by several factors that affect the electric current and charge density in the polymer. These process parameters are inter-dependent and crucial for optimisation and strongly influence the nanofiber properties and morphology (Bhardwaj and Kundu, 2010). Such parameters include polymer concentration, solvent system, applied voltage, needle tip-collector distance and the flow rate. Therefore, in this study, all of these conditions were investigated and optimized.

3.2 MATERIALS AND METHODS

3.2.1 Synthesis and optimisation of nylon-6 electrospun nanofibers

Electrospun nanofibers were synthesized using the polymer nylon 6 (22% w/v), which was dissolved in a 1:1 ratio of FA and AA (v/v) under magnetic stirring for 2 hours. The formation of nanofibers was optimized with regards to polymer concentration, flow rate, applied voltage and distance between the needle tip and collector. The electrospinning was carried out using a self-made electrospinning apparatus (Figure 12) at room temperature (24 ± 2˚C). In a typical procedure the polymer solution was first loaded into a 25 ml syringe with a 0.5 mm (inner diameter) needle attached. A syringe pump was set to inject the polymer solution at a flow rate of 0.8 ml. h^{-1}. Electrospinning was conducted at a voltage of 25 kV, and the distance between the needle tip and earthed collector was 10 cm. The fibers were collected on a level plate covered with aluminium foil after 1 h.

3.2.2 Synthesis and optimisation of polystyrene electrospun nanofibers

Polystyrene nanofibers were synthesized using a similar method previously described for nylon 6 and optimized with different conditions. Polystyrene (20%) was dissolved in a 4:1 ratio of DMF and THF (v/v). PS was electrospun at a flow rate of 0.5 ml/h at 20 kV and with distance of 13 cm from needle tip to collector.

3.2.3 Co-electrospinning

Both PS and nylon 6 were electrospun simultaneously and sequentially, each under its own optimum conditions as previously described. However, for simultaneous electrospinning, polymers were in separate syringes, a single voltage supply was used at 20 kV and fibers formed on a single collector 11.5 cm from the needle tip.

3.2.4 Characterisation

Specimens of the electrospun polymer nanofibers were analysed with scanning electron microscopy (SEM) (Zeiss Evo MA 15) after sputtering with gold using a JEOL JFC-1200 fine coater. Vega TC Software (Tescan Digital Microscopy Imaging) was used to visualize the nanofibers. The nanofiber morphology and diameters were analysed using the image visualization software analySIS.

3.3 RESULTS

3.3.1 Optimization of electrospinning conditions

Solvent conditions and process parameters for steady state electrospinning of nylon 6 and PS were investigated and the optimum conditions are shown in Table 2.

Table 2: Electrospinning parameters for nylon 6 and polystyrene nanofibers after optimization.

Parameter	Nylon 6	Polystyrene
Solvent system	1:1 (v/v) AA: FA	1:4 (v/v) THF: DMF
Polymer %	22%	20%
Applied voltage	25 kV	20 kV
Needle tip- collector distance	10 cm	13 cm
Flow rate	0.8 ml/h	0.5 ml/h

Different process parameters were investigated for both nylon 6 and PS and the optimum conditions resulted in smooth, uniform fibers. The nanofiber morphology was analysed using SEM with a thin layer of gold coating to prevent charging for both nylon 6 (Figure 15) and polystyrene (Figure 16), each before and after optimization.

3.3.2 Characterization of nanofibers using SEM

The surface morphology of the fibers was determined using SEM, before and after optimization of the electrospinning process parameters for both nylon 6 (Figure 15) and PS (Figure 16). Typical magnifications ranged between 100X and 8 000X.

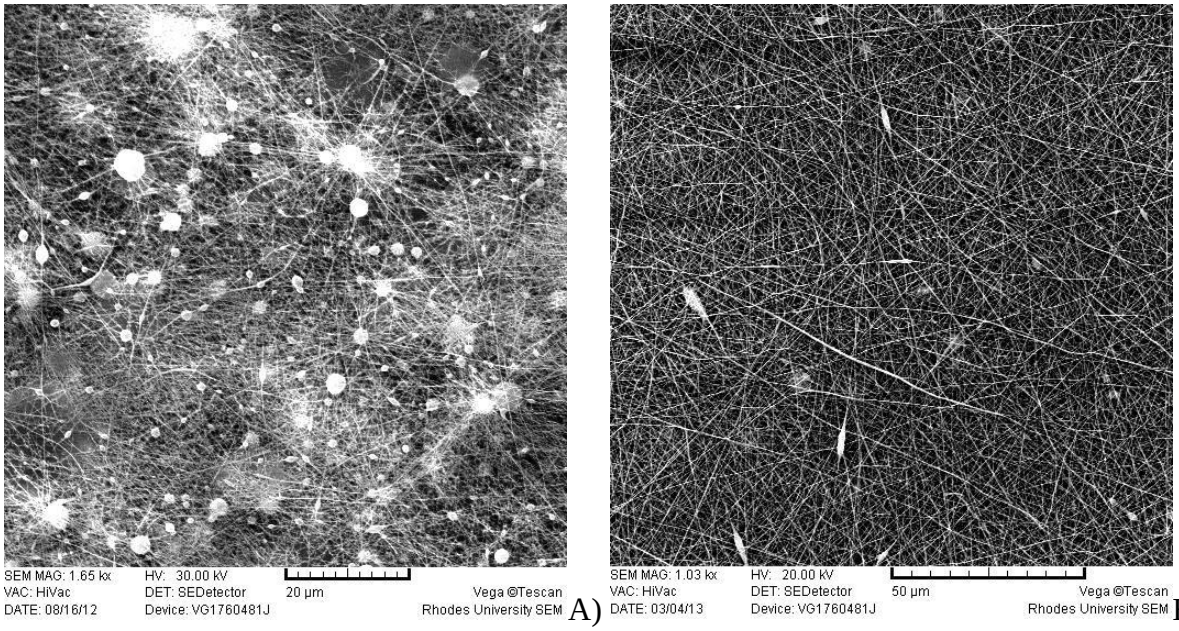


Figure 15: SEM images of electrospun nylon 6 nanofibers before A) and after B) optimization.

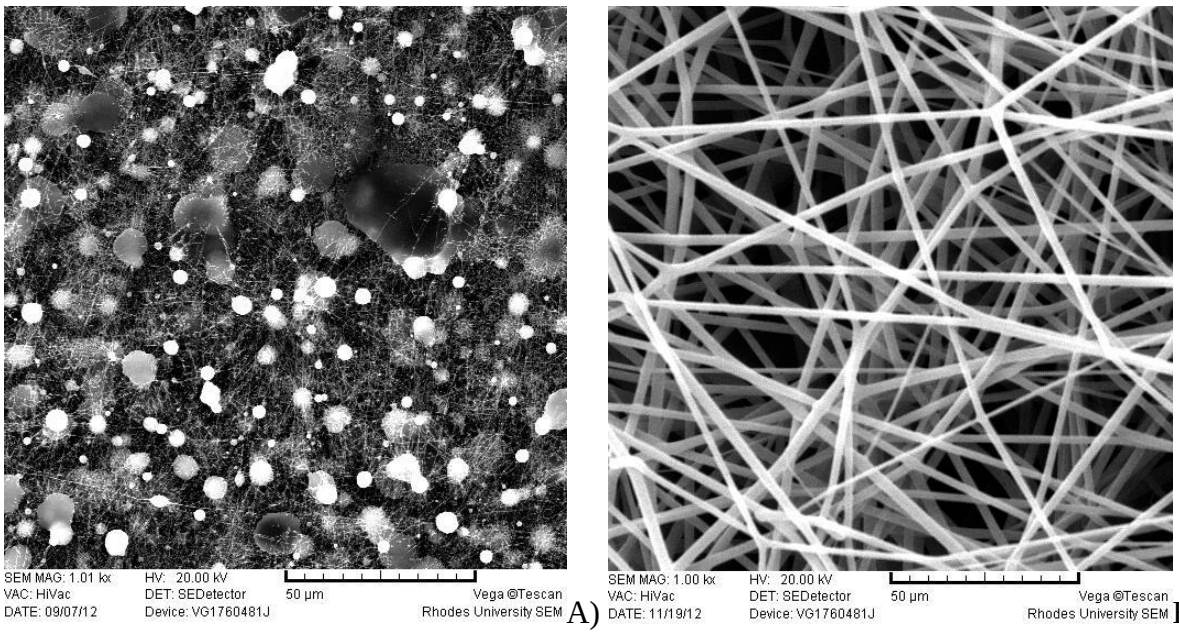


Figure 16: SEM images of electrospun polystyrene nanofibers before A) and after B) optimization.

3.3.3 Co-electrospinning

After optimization of the electrospinning parameters for nylon 6 and PS, the polymers were co-electrospun simultaneously on the same collector and using the same voltage supply. Each polymer was electrospun under its own optimum conditions but with a voltage of 20 kV and a

needle tip to collector distance of 11.5 cm. SEM images of the co-electrospun nylon 6 and PS nanofibers are shown in Figure 17.

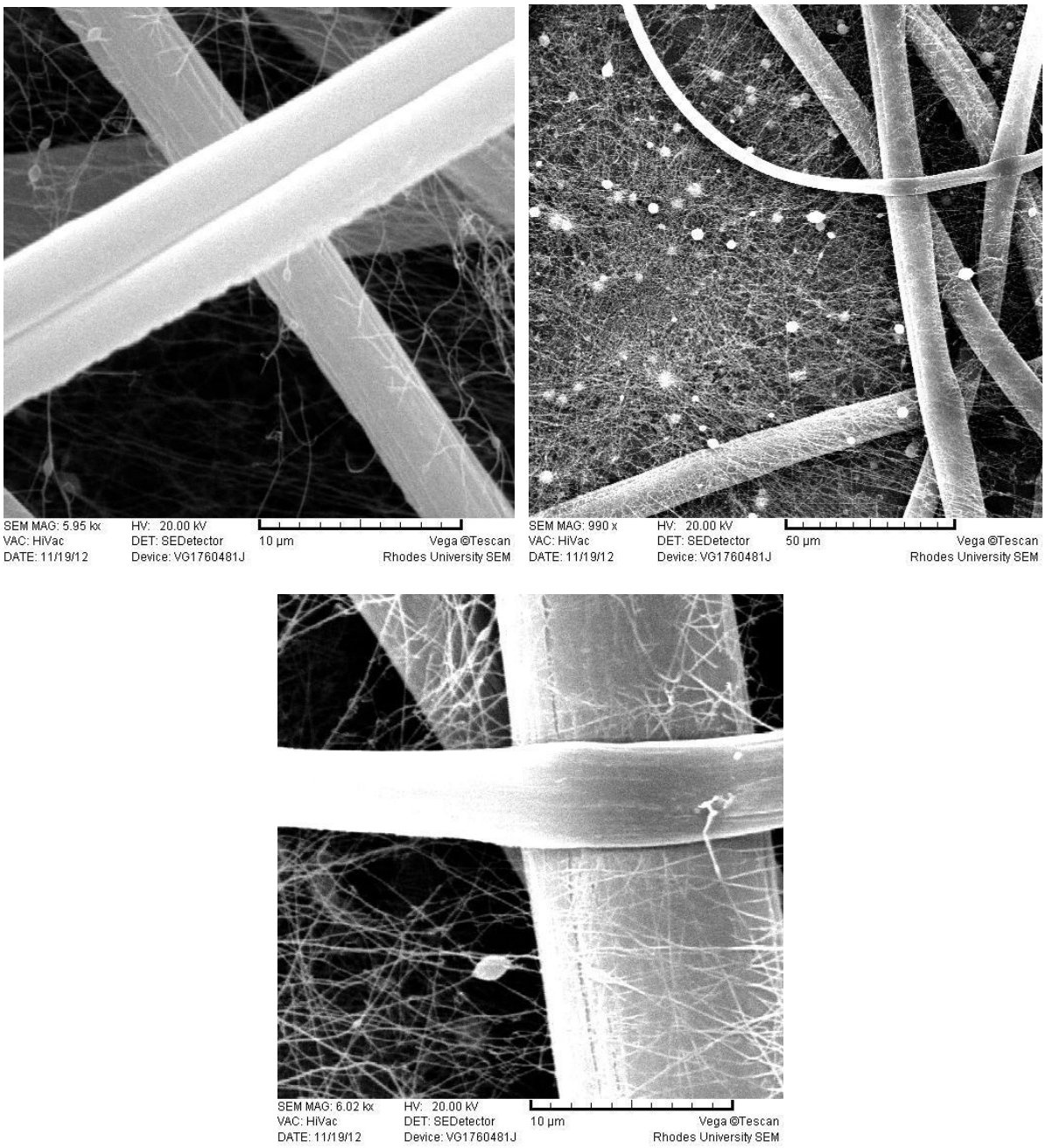


Figure 17: SEM images of simultaneously co-electrospun polystyrene (20%) and nylon 6 (22%) nanofibers. Polystyrene and nylon 6 were electrospun with a flow rate of 0.5 ml/h and 0.8 ml/h respectively, and both with a voltage of 20 kV and a needle tip to collector distance of 11.5 cm.

The PS nanofibers are distinguished from the nylon 6 nanofibers because of their increased fiber diameter which is visible in the SEM images in Figure 17. There is evidence of beading and breaking of the nylon 6 nanofibers, as well as inconsistent distribution of the two different polymer fiber types.

An attempt was made to co-electrospin PS and nylon 6 sequentially to even out the fiber distribution. The SEM images of this are shown below, where nylon 6 was electrospun first and PS second (Figure 18A), and vice versa (Figure 18B).

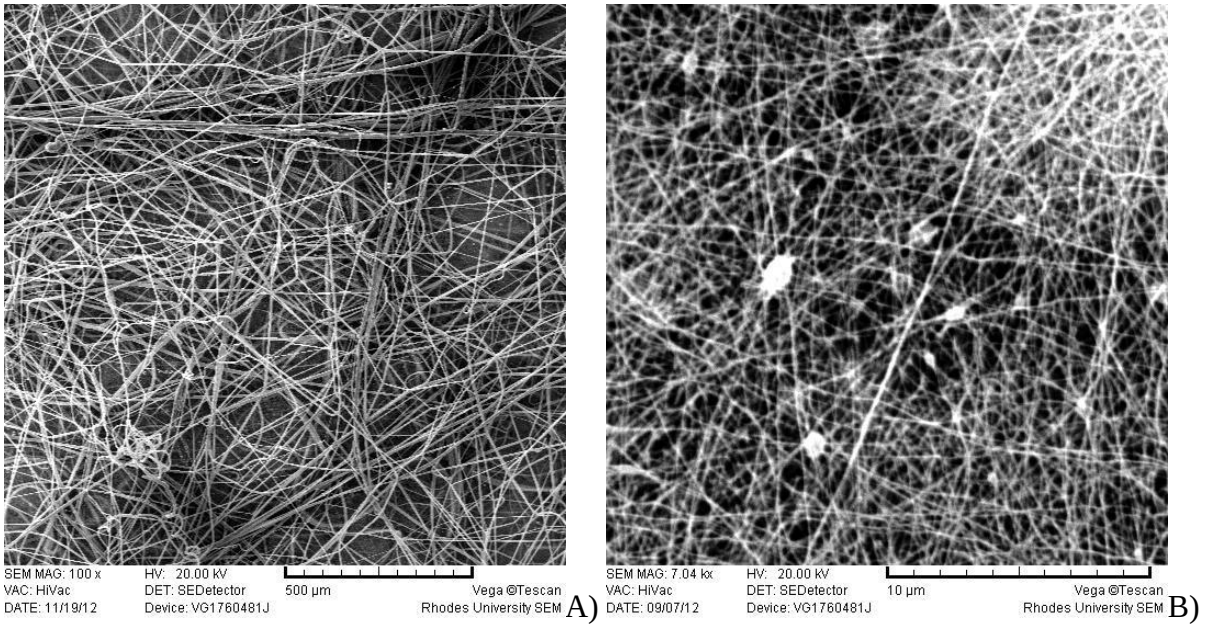


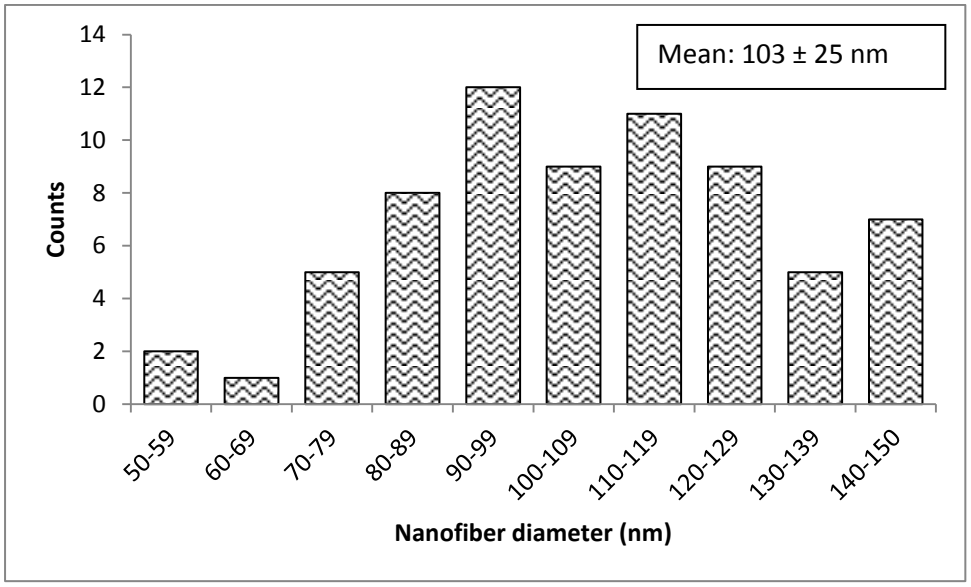
Figure 18: SEM images of sequentially co-electrospun polystyrene (20%) and nylon 6 (22%) nanofibers. Each polymer was electrospun under optimum conditions. Nylon 6 was electrospun first, polystyrene second A) and polystyrene first, nylon 6 second B).

Due to the dense layering of the fibers, the polymer that was electrospun first (at the bottom) could not be visualized (Figure 18), and therefore even distribution of the two different fiber types could not be determined by SEM. Due to the limited success of co-electrospinning the two polymers, nylon 6 was selected as the principle polymer for further experiments.

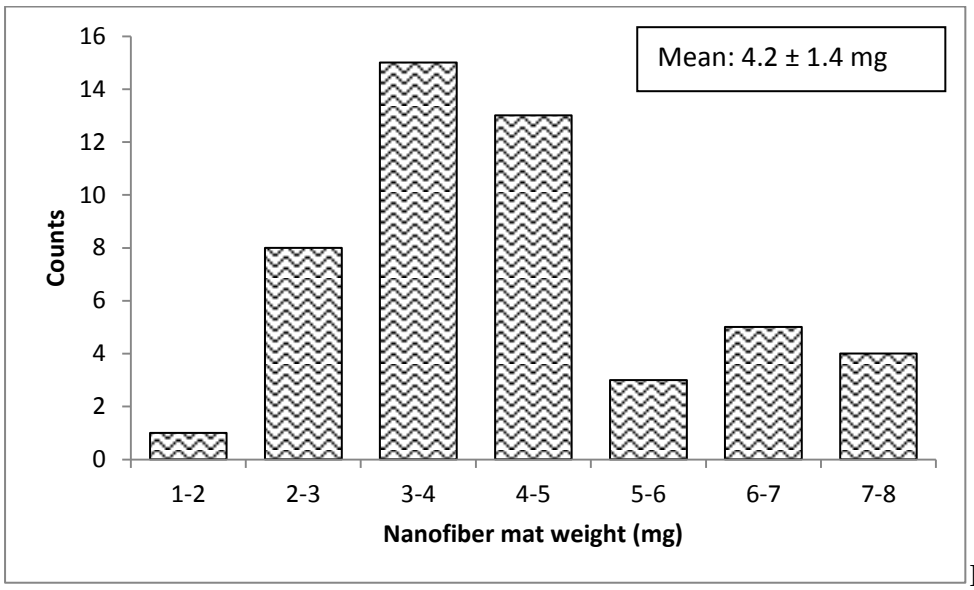
3.3.4 Size and weight distribution

Imaging and visualization software analySIS, was used to measure the nanofiber diameters of the nylon 6 nanofibers and their distribution is shown in Figure 19A. The fiber mats were then cut into 1 cm x 1 cm squares to be used later for the immobilization experiments.

Although each of the fiber mats were electrospun for the same period of time to ensure consistent thickness, each of the squares were also weighed and their distribution is shown in Figure 19B.



A)



B)

Figure 19: Distribution graphs of nylon 6 nanofiber diameter A), and the cut nanofiber square mat weight (mg) B).

The majority of the nylon 6 nanofibers had a diameter in the range of 90-99 nm with an average (mean) of 103 nm and a standard deviation of 25 nm (Figure 19A). The majority of the cut nanofiber squares had a net mat weight in the range of 3 and 5 mg, with an average (mean) of 4.2 mg and a standard deviation of 1.4 mg (Figure 19B).

3.4 DISCUSSION

3.4.1 Solvent system

Initially, an attempt was made to electrospin a polymer blend to improve biocompatibility and to introduce additional reactive groups for enzymes to bind during the immobilization process. However, access to polymers with mutual or compatible solvents was limited. According to Supaphol (2005), this is one of the major challenges associated with electrospinning polymer blends. Chitosan and nylon 6 were considered and have been reported to be soluble in formic acid (FA) and acetic acid (AA) and both solvents are miscible with each other as they resemble each other chemically (Figure 14A and B, respectively) (De Vrieze *et al*., 2009; Supaphol *et al*., 2005; Zhang *et al*., 2009). However, it was established that some of the enzymes in Viscozyme L® and Celluclast 1.5L®, such as pectinases, would be able to degrade chitosan (Roy and Gupta, 2003); therefore this polymer could not be used. Therefore, attempts were made to co-electrospin two different polymers (PS and nylon 6) with different solvent systems, using separate pumps and syringes, but on the same collector and using the same voltage supply.

3.4.2 Optimization of electrospinning conditions

The solvent and electrospinning conditions were optimized for each of the polymers and the optimal ratios are shown in Table 2. Nylon 6 was dissolved in a 1:1 (v/v) ratio of AA and FA, and PS was dissolved in a 1:4 (v/v) ratio of THF: DMF. These solvents have previously been used for the dissolution of nylon in ratios between 1:1 and 2:1 (Daels *et al*., 2010; De Vrieze *et al*., 2009; Supaphol, 2005;) and PS in a ratio of 1:3 (Li *et al*., 2009).

Before optimisation of the electrospinning of nylon 6 (Figure 15A) and PS (Figure 16A), significant bead formation was observed. These structures are generally considered as unwanted defects and can be influenced by process parameters such as solution concentration and applied voltage (Fong *et al.*, 1999). It may also be partially caused by instability of Taylor cone formation during the electrospinning process (Kim *et al*., 2005). Similar observations were made by Deitzel *et al*. (2001), and Liu and Hsieh (2002) who demonstrated that electrospinning of low molecular weight nylon solutions at higher voltages resulted in unwanted bead formation due to jet instability. Therefore, the electrospinning conditions were adjusted and optimized (Table 2) to minimize bead formation, for nylon 6 (Figure 15B)

and PS (Figure 16B), and relatively smooth uniform fibers were produced. In this study, the electrospinning of nylon 6 solutions above 20% resulted in little or no bead formation. This trend can be explained by increased chain entanglement when the polymer solution concentration was increased. The rise in molecular weight caused an increase in viscosity leading to increased visco-elastic forces resulting in elongation of fibers (Ryu *et al*., 2003).

3.4.3 Co-electrospinning

Figure 17 illustrates the morphology of simultaneously co-electrospun nanofiber mats. PS and nylon 6 in the blended fibrous mats were easily distinguished due to the observable difference in fiber diameters. Obtaining homogenously blended mats proved to be challenging and was evident in Figure 17. This may have been due to like charges of each polymer jet repelling each other and therefore producing two separate fiber mats (PS and nylon 6) on the same collector with limited overlap. Li *et al*. (2009) co-electrospun polystyrene and polyamide 6 (PA6) via a four-jet electrospinning process and were able to obtain evenly distributed fibrous mats. However, they had access to a rotating drum collector which allowed even dispersion of both polymer fiber mats with sufficient overlap. Furthermore, the four-jet electrospinning apparatus that was used allowed them to control the ratios of PS and PA6.

An attempt was therefore made to sequentially electrospin nylon 6 and PS, i.e. to electrospin nylon 6 first and PS on top (Figure 18A), and PS first and nylon 6 on top (Figure 18B). This also proved to be challenging as whichever polymer was electrospun second (on top) was the only one that could be visualized on the SEM image. The reproducibility of this method was limited and therefore it was decided to proceed with experiments utilizing primarily nylon 6. Nylon 6 was selected over PS because PS also forms a partially three-dimensional mesh, rather than a smooth mat which makes it difficult to work with.

3.4.4 Nanofiber diameter and weight distribution

In Figure 15B, it was also observed that the nylon 6 fibers showed a smooth fiber surface with a wide fiber diameter distribution and an average nanofiber diameter of 103 ± 25 nm (Figure 19A). This was close to the values reported by Li *et al*. (2009) who obtained an average nanofiber diameter of 132 ± 28 nm for PA6. The small fiber diameters can be

explained by the ionization of the amino groups under acidic conditions; this causes an increase in charge density on the surface of the polymer jets formed during electrospinning. The increased charge density leads to higher elongation forces on the polymer jets which results in the eventual formation of fibers with small diameters (Zong *et al*., 2002).

There was also no evidence of nanowebs which have previously been reported in literature (Li *et al*., 2009). Nanowebs are interlinked one-dimensional nanowires, one tenth the size of normal nanofibers, which form as a result of electrically forced fast phase separation of the charged droplets (Lie *et al*., 2009). This uniformity in nanofiber size is ideal for obtaining reproducible results.

The samples used for enzyme immobilization were largely bead free. The nanofibrous membranes were cut into 1 cm x 1 cm square mats. These square mats were weighed and displayed a wide fiber mat weight distribution and the majority of the cut nanofiber squares had a net mat weight in the range of 3 and 5 mg, with an average (mean) of 4.2 ±1.4 mg (Figure 19B).

3.4.5 Concluding statements

Bead free, electrospun nylon 6 nanofibers were successfully synthesized with an average fiber diameter of 103 nm ± 25 nm. The electrospinning process was optimized with regards to polymer concentration, flow rate, needle tip collector distance and applied voltage, and the nanofiber morphology was verified by SEM.

Polymer nanofibers, providing a large surface area for the attachment of enzymes, show potential as a support for enzyme immobilization. This is particularly beneficial when insoluble/complex substrates are being used, such as apple pomace, which was used in this study. A nanofibrous support also offers reduced mass transfer limitations, associated with bulk immobilization supports. Therefore, in this study, electrospun nylon 6 nanofibers were used as hosts for the immobilization of the hemicellulases and cellulases in Viscozyme L® and Celluclast 1.5L®, as discussed in the following chapter.

Chapter 4

Immobilization of Enzymes in Viscozyme L® and Celluclast 1.5L® on Prepared Nanofibers

4.1 INTRODUCTION

In the bioconversion of lignocellulosic substrates, enzyme cost is still a major factor that affects the economic sustainability of the process. One possible way to overcome this obstacle is through enzyme coupling with a suitable support matrix, such as electrospun nylon 6 nanofibers. This would allow for recovery and recycling of enzymes through multiple rounds of hydrolysis. However, a common problem with immobilization is a loss of enzyme activity that occurs upon immobilization.

This can be partially attributed to the immobilization reaction and enzyme-support interactions. This is particularly problematic when insoluble substrates are used, such as AP, which was used in this study. This is because movement is restricted, and because the mechanism of the enzymes involved, particularly the cellulases, requires adsorption onto its substrate and consequent desorption (Jain and Wilkins, 1986). If the cellulases are in an immobilized form, then adsorption onto the surface of the substrate will be impaired. However, there have been reports in literature that offer solutions to this problem. Some of these that were discussed in Chapter 1 include: immobilization onto smart polymers, water soluble polymers, or via linkers (spacer arms), immobilization of whole microorganisms instead of the enzymes, or immobilization of the enzymes onto nanostructured supports. A similar approach was used for this study where the enzymes in Viscozyme L® and Celluclast 1.5L® were immobilized onto electrospun nylon 6 nanofibers as they offer an increased surface area for more enzymes to bind and potentially reduced mass transfer limitations.

Nylon was chosen as the support polymer due to its chemical and physical properties, non-toxicity, inert nature, availability in different forms, as well as its low cost (Isgrove *et al.*, 2001). However, due to the mildly inert nature of nylon, attempts were made to increase the reactive surface of the fibers. This was done by pre-activating the fibers with HCl to produce

more reactive groups for surface attachment of the enzymes, as well as crosslinking with GA to act as a spacer arm and to facilitate binding of the enzymes to the support matrix.

The precise mechanism of enzyme coupling with GA is still under investigation but many suggestions have been put forward, as discussed in Chapter 1. GA has been reported to exist as a monomer in dilute solutions up to 10% (w/v) (Kawashara *et al*., 1997) and as an α,β-unsaturated polymer under basic conditions. These were the conditions (pH > 7; GA% < 10%) used in the present study for immobilization and the possible mechanisms of binding with protein for both forms of GA were described in Chapter 1. Although the most likely or favoured reaction under these conditions involves Schiff base formation, the complex nature of GA suggests that there may be several possible reaction mechanisms occurring simultaneously.

The conditions for efficient immobilization with GA requires an empirical approach as it is dependent on many factors, such as the nature of the enzyme and support, the pH and temperature, the concentration of the enzyme and GA, as well as the crosslinking time (Poddar and Jana, 2011). Therefore, these factors were taken into consideration for the immobilization procedure used in this study.

In order to immobilize the enzymes in Viscozyme L® and Celluclast 1.5L®, two different methods were investigated (Figure 20). The first method involved pre-activating the fibers with GA (as a spacer/linker arm), followed by a wash step and then incubation with the enzyme solution. The second method involved binding of seed enzymes onto the fibers, followed by crosslinking of additional enzymes and aggregates to the seed enzyme molecules using GA. Although this method is prone to loss in enzyme activity due to the harsh treatment of GA, the increased enzyme loading as a result of the crosslinking enzyme aggregates, compensates for this loss.

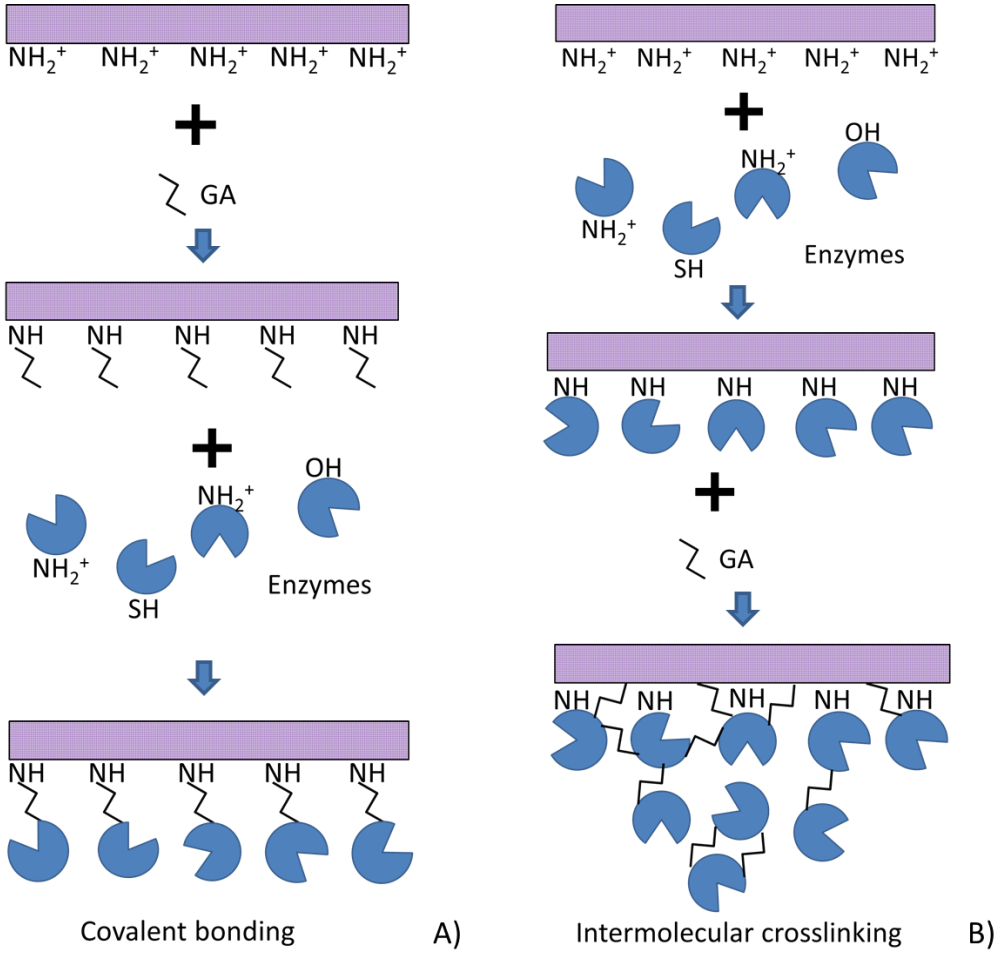


Figure 20: Schematic representation of enzyme immobilization by crosslinking with GA using method 1 A) and method 2 B). (Image adapted from Wang *et al.*, 2009).

Finally, FT-IR and EDS were used to confirm enzyme binding to the nanofibrous support. EDS analysis was performed coupled to SEM. The SEM images were produced from the backscattered electrons that result from different elements (with different atomic numbers) and their distribution. The generation of an X-ray spectrum from the entire scanned area of the SEM image is produced and EDS measures the energy of the emitted X-rays. This energy is measured in electron volts (eV) and is characteristic for the atomic structure of an element and therefore allows the identification of particular elements and their relative proportions (Ngo, 1999).

4.2 MATERIALS AND METHODS

4.2.1 Preparation of AP

Golden delicious apples (2 kg) were cut up into small pieces and placed into a juicer until a fine pulp remained. The pulp was then mixed with an equal volume of deionized water and homogenized with a blender. The pulp was filtered with cheese cloth to remove any liquid that was present. The pulp was then washed with 3 times its volume of deionized water and stirred magnetically at 200 rpm for 30 minutes before filtering again. This was repeated 3 times. The remaining pulp was placed into a plastic 500 ml beaker and frozen at -20°C overnight and freeze dried. The substrate was autoclaved at 120°C, at 24 x atmospheric pressure for 20 min and the dry substrate was then further blended to reduce particle size.

4.2.2 Activity assay

Dinitrosalicylic acid (DNS) method

Reducing sugars were quantified using the DNS assay described by Miller (1959). Enzyme was added to AP in 50 mM sodium citrate buffer (pH 5.0) to a total volume of 400 µl and assayed at 37° C for 1 h on a rotor at 8 rpm to maintain the substrate in suspension. An enzyme control was used where substrate was substituted with buffer and a substrate control was also used where enzyme was substituted with buffer. Samples were centrifuged at 10 000 rpm for 1 minute and 150 µl of each sample was added to 300 µl of DNS reagent. Samples were then heated in a dry bath at 100˚C for 5 minutes and cooled on ice for 5 minutes. The absorbance of the resultant colour development was measured at 540 nm using a Powerwave$_x$ microplate reader from Biotek instruments with KC junior software. A glucose standard curve (Appendix A) was produced in the range of 0.1-1 mg/ml. Assays were performed in triplicate and the reducing sugars released were calculated in units (U) of activity and reported as relative activity, where 1 U was defined as the quantity of enzyme required to release 1 µmol of reducing sugar per minute under the specified assay conditions.

4.2.3 Effect of % GA on protein assay

Various concentrations of GA (1-5%) were added to aliquots of Viscozyme L® and Celluclast 1.5L® (2.5 mg/ml). The buffer volume was adjusted accordingly to allow for a total reaction volume of 1.5 ml for the required GA concentrations. Enzyme-GA mixtures (5

µl) were each added to 245 µl of Bradford's reagent and left to stand at room temperature for 5 minutes. The absorbance of the resultant colour development was measured at 595 nm using a Powerwave$_x$ microplate reader from Biotek instruments with KC junior software. Controls were included where enzyme (protein) was substituted with buffer (blank) and where GA was substituted with buffer (0% GA). Assays were performed in triplicate and total protein was calculated using a bovine serum albumin (BSA) standard curve (Appendix B). Relative total protein was reported, where 100% referred to protein without GA (0% GA).

4.2.4 Effect of % GA on soluble/free enzyme activity

Various concentrations of GA (1-5%) were added to Viscozyme L® and Celluclast 1.5L® (2.5 mg/ml). The buffer volume was adjusted accordingly to allow for a total reaction volume of 1.5 ml for the required GA concentrations. Enzyme-GA mixtures (50 µl) were each added to substrate (1% AP in 50 mM sodium citrate buffer, pH 5) to a total volume of 400 µl and assayed for 1 hour using the DNS method. An enzyme control was used where substrate was substituted with buffer and a substrate control was also used where enzyme was substituted with buffer. Assays were performed in triplicate and activity was reported as relative activity, where 100% activity referred to enzyme without GA (0% GA).

4.2.5 Enzyme immobilization

Method 1

Electrospun nylon 6 nanofibers were cut into 1 cm x 1 cm squares. The fibers were then placed in varying concentrations of GA (0-5%) for 4 hours and washed thoroughly in 50 mM sodium phosphate buffer (pH 7.7). The fibers were incubated in enzyme solution, 2 mg/ml (for Viscozyme L®) and 1 mg/ml (for Celluclast 1.5L®), in 50 mM sodium phosphate buffer (pH 7.7) for 24 hours at 4˚C. The fibers were assayed for activity using the DNS method. The protein (x) was determined indirectly by Bradford's method and by measuring the absorbance at 280 nm of the solutions before (a) and after (b) immobilization, as well as the wash (c) solutions, and calculated using the equation below:

$x= a- (b+c)$

Method 2

Electrospun nylon 6 nanofibers were cut into 1 cm x 1 cm squares and immersed in Viscozyme L® (4 mg/ml); and HCl pre-treated (1.91 M for 2 h) fibers were immersed in Celluclast 1.5L® (2 mg/ml) in 50 mM sodium phosphate buffer (pH 7.7) and allowed to equilibrate for 1 hr at 4˚ C on a rotor at 8 rpm. GA was added to Viscozyme L® and Celluclast 1.5L®-fiber mixtures to a final concentration of 4% and 2%, respectively, and left on a rotor at room temperature for half an hour to promote initial polymerization/crosslinking. The samples were then left for a further 3.5 hours (5 hours total immobilization time) at 4˚C. Non-selectively bound protein was removed by washing with 50 mM phosphate buffer (pH 7.7) and then the fibers were incubated in 50 mM Tris-HCl buffer (pH 7.7) for capping of the aldehyde groups. Immobilization yield was determined as a % of immobilized and free enzyme activity. Immobilization efficiency was calculated by determining the ratio of the enzyme activity (U) on the nanofiber membrane to the whole membrane mass (g).

4.2.6 Effect of %GA on enzyme immobilization

Immobilization method 2 was used whereby electrospun nylon 6 nanofibers were immersed in 50 mM sodium phosphate buffer (pH 7.7) containing the relevant enzymes and allowed to equilibrate for 1 hr at 4˚C on a rotor at 8 rpm. GA was added to the enzyme solutions to final concentrations of 1-5% (GA) at 4˚C. The buffer volume was adjusted accordingly to allow for a total reaction volume of 1.5 ml for the required GA concentrations. The immobilization was carried out and the immobilized enzymes were then assayed for activity using the DNS method as described previously. Assays were performed in triplicate and appropriate enzyme and substrate controls were used. Activity was reported as relative activity, where 100% activity was defined as the immobilized enzyme activity on the fibers that were not treated with GA (0% GA) (control).

4.2.7 Effect of pretreatment with HCl

Electrospun nylon 6 nanofibers were treated with various concentrations (0 - 3.18 M) of HCl for 2 h at room temperature (± 24˚C) to pre-activate the fibers. The enzymes were immobilized on the fibers using method 2 and assayed for activity using the DNS method, as described previously. Assays were performed in triplicate and appropriate enzyme and

substrate controls were included. The activity was reported as relative activity, where 100% activity was defined as immobilized enzyme activity on the fibers that were not treated with HCl. An additional control was included where the fibers were not immobilized with GA (or HCl).

4.2.8 Effect of initial protein concentration

Electrospun nylon 6 nanofibers were immersed in various concentrations (1-5 mg/ml) of Viscozyme L® and Celluclast 1.5L®. The immobilization was carried out using method 2 and the immobilized enzymes assayed for activity using the DNS method as previously described. Assays were carried out in triplicate and appropriate enzyme and substrate controls were used. The activity was reported as % relative activity where 2.5 mg/ml was stated as 100% activity, since this was the condition used for experiments up to this point of optimization.

4.2.9 Immobilization (and crosslinking) time

The immobilization was carried out using method 2, as described previously. The immobilized enzymes were assayed for activity at various time intervals (1, 5, 16, 24, 32 and 48 h). Assays were carried out in triplicate and appropriate enzyme and substrate controls were used. The relative activity at 1 h was defined as 100%.

4.2.10 SEM

SEM images of the electrospun nylon 6 nanofibers were taken, as described previously (Chapter 3), prior to and post GA and HCl pre-treatment, and post Viscozyme L® and Celluclast 1.5L® immobilization, using method 1 and 2.

4.2.11 FT-IR analysis

Analysis of the nylon 6 nanofibers, prior and post HCl pre-treatment, and post Viscozyme L® and Celluclast 1.5L® immobilization, was carried out using Fourier Transform Infrared Spectroscopy (FT-IR) with a Nicolet 17DSX FT-IR Spectrometer in the wavelength range 600-4000 cm^{-1} and Spectrum Software.

4.2.12 EDS analysis

EDS analysis was performed using an Oxford instruments DAAD spectrophotometer coupled to the SEM and INCAPentaFETx3 software. The presence of carbon, oxygen, nitrogen and particularly sulphur were monitored to qualitatively confirm the presence of enzyme bound to the nanofibers.

4.2.13 Statistical analysis

All data points were plotted as mean ± standard deviation (SD). ANOVA single factor analysis (Microsoft Excel) was used to determine significant differences, where $P \leq 0.05$ indicates 95% confidence, and $P \leq 0.01$ indicates 99% confidence.

4.3 RESULTS

The electrospun nylon 6 nanofiber mats that were synthesized in Chapter 3 were cut into 1 cm x 1 cm squares and used as matrices for the immobilization of the hemicellulases and cellulases in Viscozyme L® and Celluclast 1.5L® via crosslinking with GA.

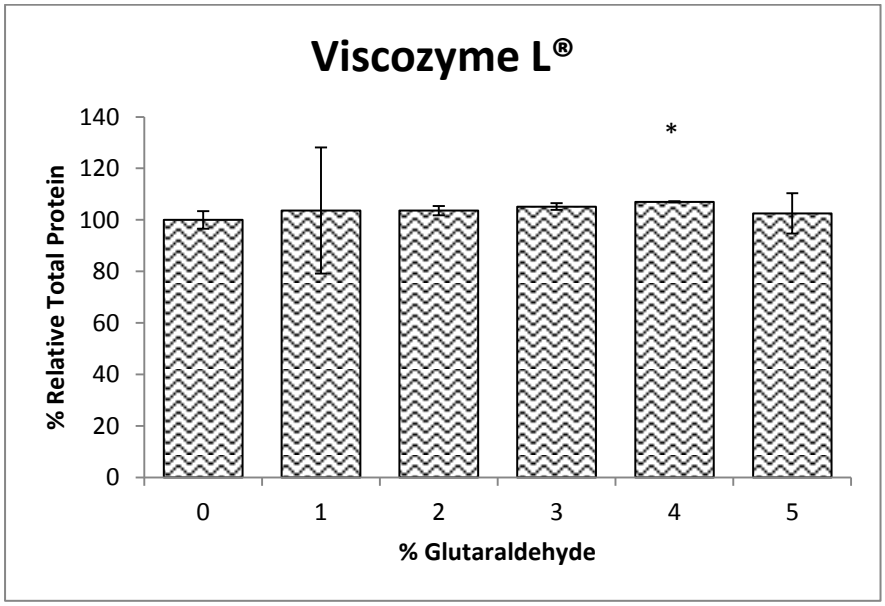
Initially, method 1 was used whereby the fibers were pre-activated with GA, washed and then immobilized with the enzymes. However, due to limitations of this approach, method 2 was employed whereby the fibers were equilibrated with enzyme first and then crosslinked with GA.

The immobilization was monitored initially via both protein assays (Bradford method and A_{280}) and enzyme activity assays (reducing sugar quantification by DNS method). However, due to limitations of the protein assay using this immobilization system, the results of the activity assays were used as the guiding factor during optimization. The effects of GA on free/soluble enzyme protein (Figure 21) and activity assays (Figure 22) were assessed to further explain these limitations.

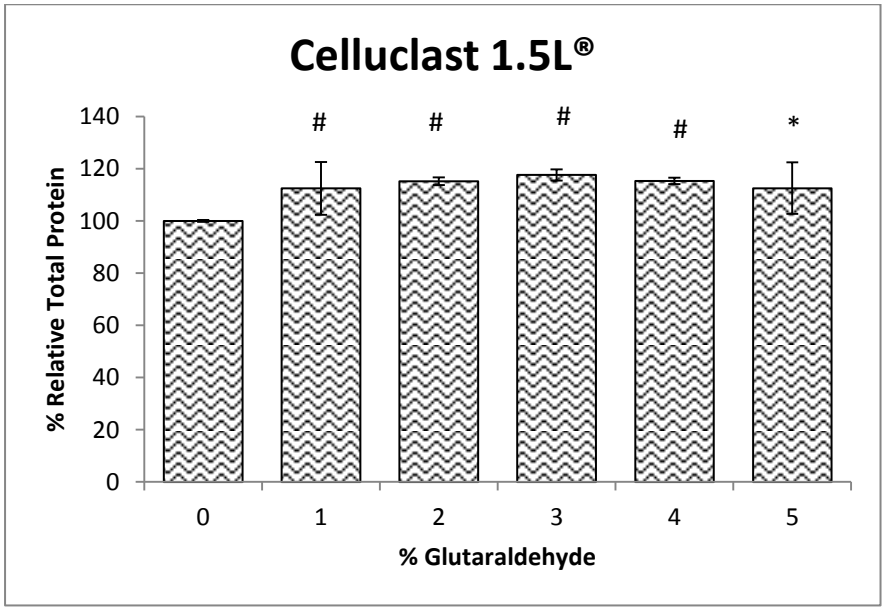
The immobilization was optimized with regards to GA concentration (Figures 23 and 24), HCl pre-activation of the nanofibers (Figure 25), initial enzyme/protein concentration (Figure 26) and immobilization time (Figure 27).

4.3.1 Effect of %GA on protein assay

GA (1-5% final concentration) was added to equal enzyme/protein concentrations of Viscozyme L® and Celluclast 1.5L® solutions. After 4 h of crosslinking time, the protein concentration was determined using Bradford's method. A control was also used where enzyme/protein was substituted with buffer. The relative total protein was plotted where 100% was taken as the enzyme solution that was not exposed to GA (Figure 21).



A)



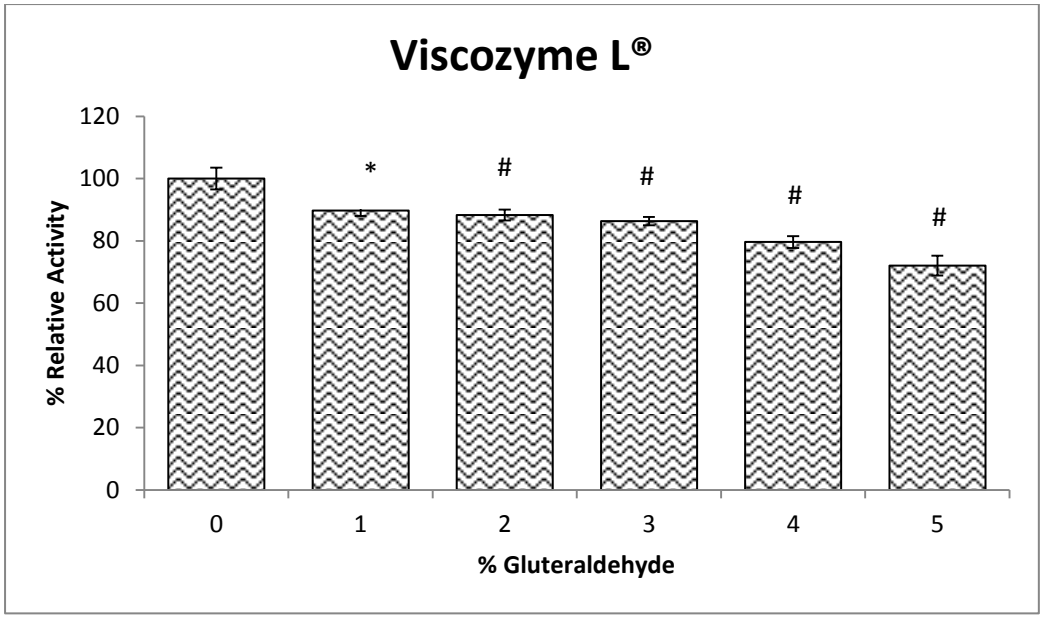
B)

Figure 21: Effect of crosslinker concentration (GA) on free Viscozyme L® A) and Celluclast® B) using Bradford's method. Data points represent the means ± SD (n=3). The untreated control (0% GA) was taken as 100%. ANOVA single factor analysis was used to determine significant differences from the untreated control *P ≤ 0.05, #P ≤ 0.01.

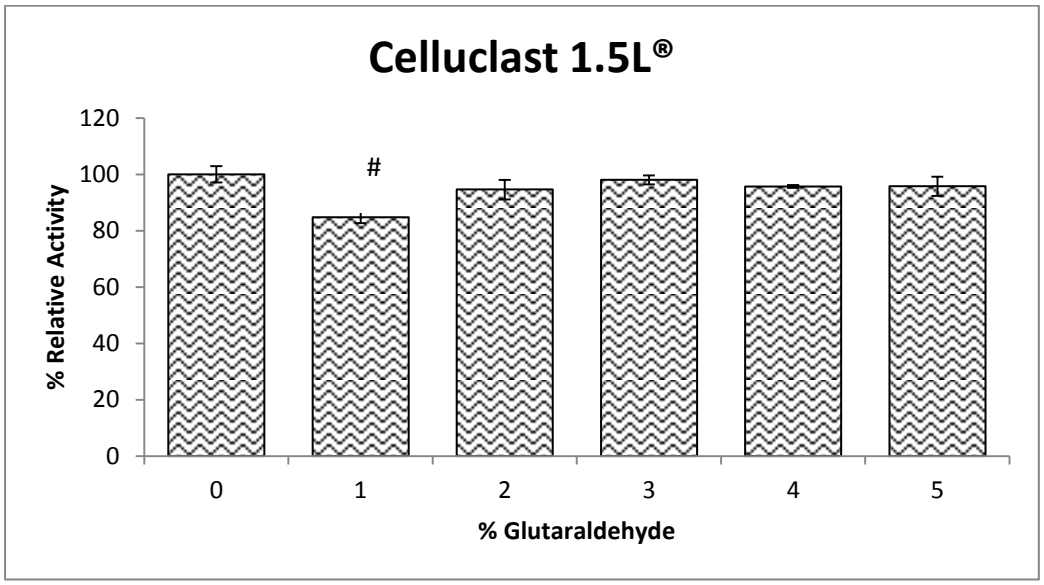
With increasing concentrations of GA there were statistically significant increases in the absorbance and therefore the calculated total protein for both Viscozyme L® and Celluclast 1.5L® (Figure 21), although no additional protein was added and appropriate controls were used. Random instances of high standard deviations also occurred.

4.3.2 Effect of %GA on soluble/free enzyme activity assay

GA (1-5% final concentration) was added to equal enzyme/protein concentrations of Viscozyme L® and Celluclast 1.5L® solutions. After 4 h of crosslinking time, the enzyme-GA mixtures were assayed with AP using the DNS method. Appropriate enzyme and substrate controls were used as well as controls where GA was substituted for buffer. The residual activity was plotted relative to the enzyme solution that was not exposed to GA (Figure 22).



A)



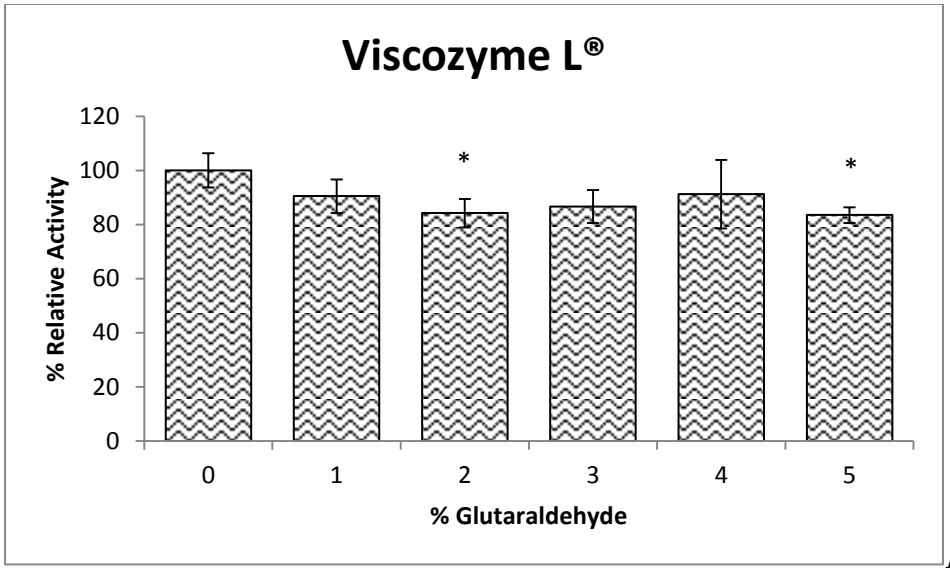
B)

Figure 22: Effect of crosslinker concentration (GA) on free Viscozyme L® A) and Celluclast 1.5L® B) activity. The data points represent the means ± SD, (n=3). The untreated control (0% GA) was taken as 100%. ANOVA single factor analysis was used to determine significant differences from the untreated control *P ≤ 0.05, #P ≤ 0.01.

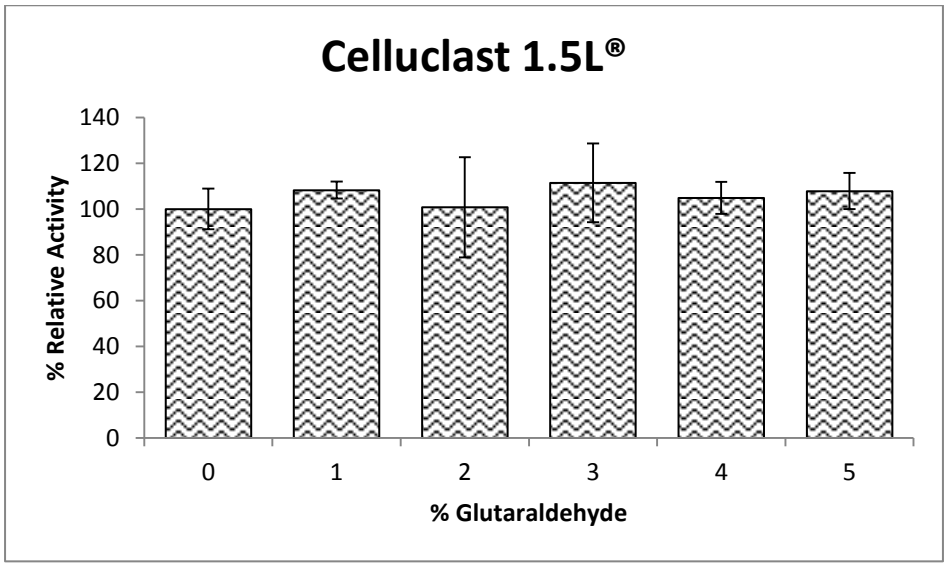
For Viscozyme L®, increasing concentrations of GA resulted in a statistically significant decrease of enzyme activity in its soluble form with up to 30% loss in activity using 5% GA ($P \leq 0.01$) (Figure 22A). In contrast, for Celluclast 1.5L®, the effect of GA on enzyme activity was minimal (Figure 22B).

4.3.3 Effect of % GA on enzyme immobilization

Immobilization method 1 was used and the fibers were pre-treated with various concentrations of GA (0-5%). The immobilization was carried out and the immobilized enzymes were then assayed for activity using the DNS method. Appropriate enzyme and substrate controls were used as well as controls where GA was substituted with buffer. The activity of the immobilized enzymes was plotted relative to the immobilized enzymes that had not been exposed to GA (Figure 23).



A)

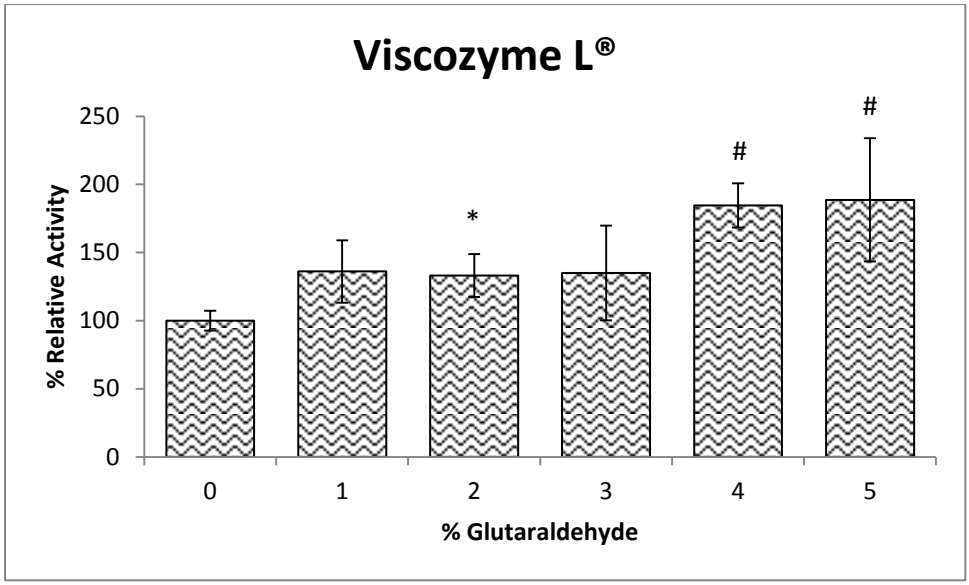


B)

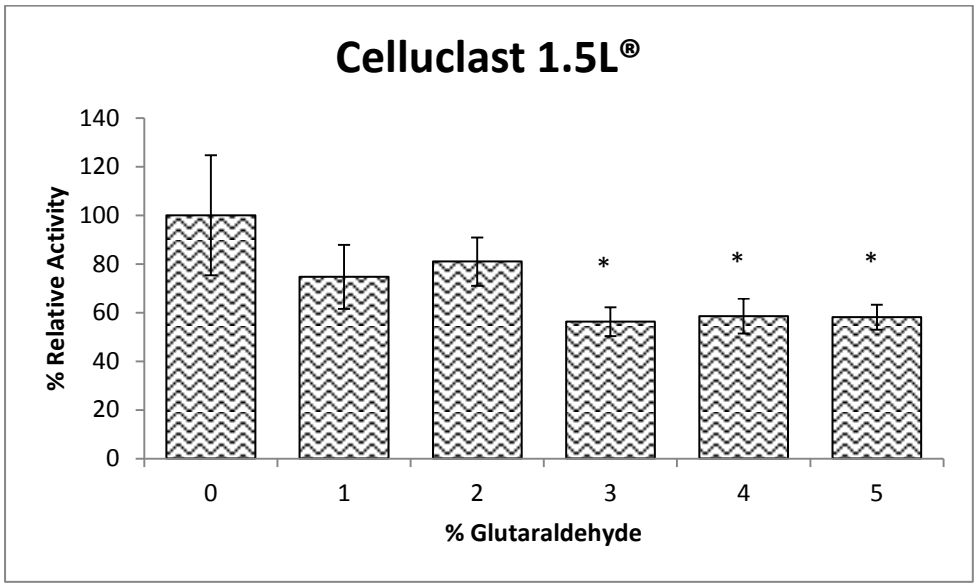
Figure 23: Effect of crosslinker concentration (GA) on the immobilized Viscozyme L® A) and Celluclast 1.5L® B) activity. The data points represent the means ± SD (n=3) using method 1. Untreated (0% GA) fibers were taken as 100%. ANOVA single factor analysis was used to determine significant differences from the untreated control *P ≤ 0.05, #P ≤ 0.01.

Pre-treatment of the nanofibers with GA showed a negative effect on the immobilized enzyme activity as up to 15% activity was lost for Viscozyme L® when pre-treated with 2% GA ($P \leq 0.05$) (Figure 23A). For Celluclast 1.5L®, although the general trend indicated a slight improvement in activity when pre-treating with GA, none of the sample data were statistically significant ($P > 0.05$) and high standard deviations were observed (Figure 23B).

Immobilization method 2 was used and various concentrations of GA (0-5%) were added to the enzyme solutions after 1 h of equilibration. The immobilization was carried out and the immobilized enzymes were then assayed for activity using the DNS method. Appropriate enzyme and substrate controls were used, as well as controls where GA was substituted with buffer. The activity of the immobilized enzymes was plotted relative to the immobilized enzymes that were not exposed to GA (Figure 24).



A)



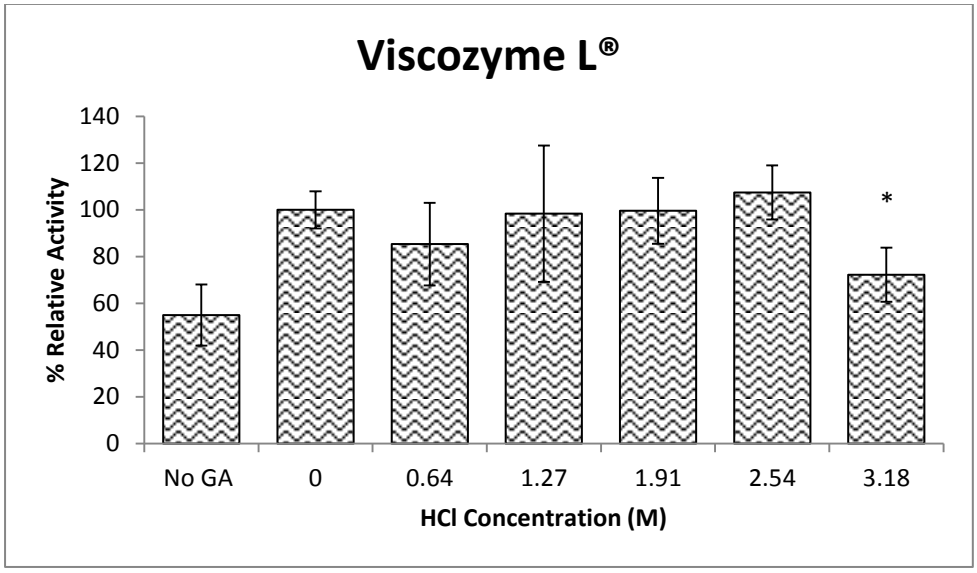
B)

Figure 24: Effect of crosslinker concentration (GA) on the immobilized Viscozyme L® A) and Celluclast® B) activity. Data points represent the means ± SD (n=3) using method 2. The untreated control (0% GA) was taken as 100%. ANOVA single factor analysis was used to determine significant differences from the untreated control *P ≤ 0.05, #P ≤ 0.01.

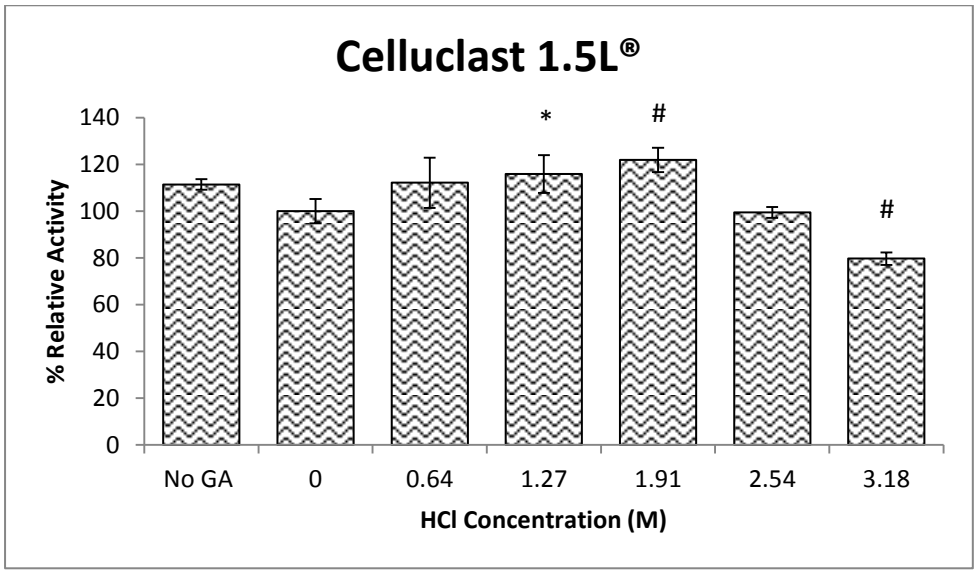
For Viscozyme L®, there was a significant increase in activity of up to 75% when treated with 4% GA ($P \leq 0.01$), compared to the untreated immobilized enzymes (Figure 24A). Alternatively, for Celluclast 1.5L®, the activity of the immobilized enzymes decreased when treated with GA. When treated with 3% GA or higher, there was ~40% loss in activity ($P \leq 0.05$) (Figure 24B).

4.3.4 Effect of pre-treatment with HCl

The fibers were pre-treated with various concentrations of HCl (0-3.18 M) to pre-activate the fibers and create more reactive groups available for immobilization. The enzymes were immobilized on the fibers using method 2 and assayed for activity using the DNS method. The activity of the immobilized enzymes on the HCl pre-treated fibers was plotted relative to the immobilized enzymes on fibers that were not pre-treated with HCl (Figure 25). A control was also included, where fibers were not immobilized with GA (or HCl).



A)



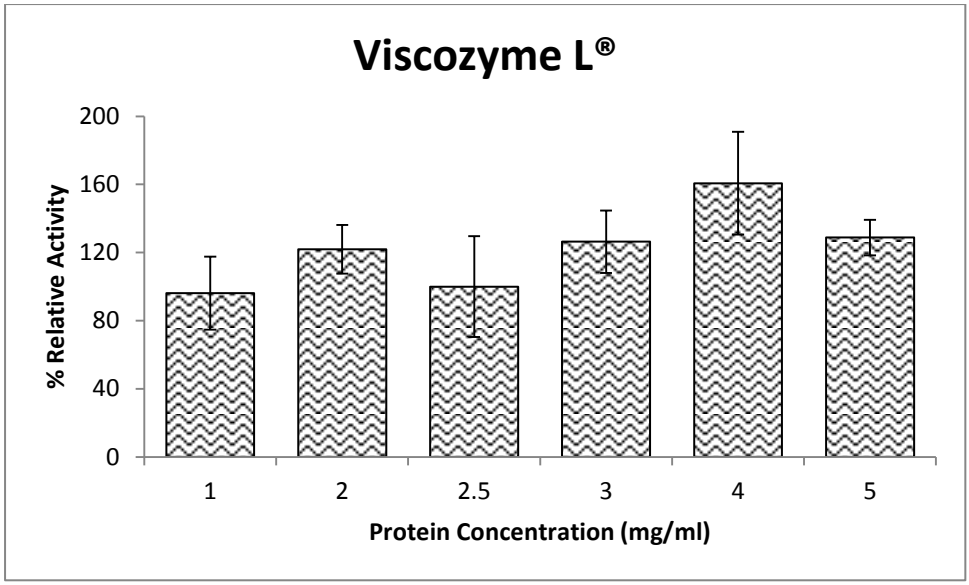
B)

Figure 25: Effect of HCl activation on immobilized Viscozyme L® A) and Celluclast 1.5L® B) activity. Data points represent the means ± SD (n=3). The untreated control (0 M HCl) was taken as 100%. ANOVA single factor analysis was used to determine significant differences from the untreated control *P ≤ 0.05, #P ≤ 0.01.

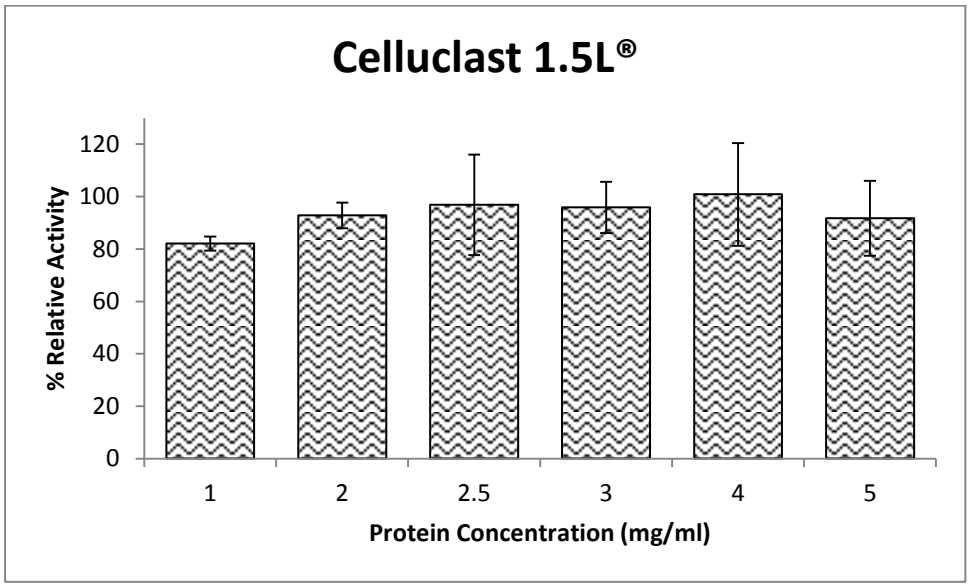
For Viscozyme L®, HCl pre-treatment did not have a significant improvement on enzyme immobilization (Figure 25A). However, for Celluclast 1.5L® there was a statistically significant increase in relative activity (~20%) when the fibers were pre-treated with 1.91 M HCl ($P \leq 0.01$), compared to fibers that were not pre-treated with HCl. With higher concentrations of HCl up to 3.18 M, the relative activity decreased to ~80% relative activity (Figure 25B) ($P \leq 0.01$).

4.3.5 Effect of initial protein concentration

The fibers were immersed in various concentrations (1-5 mg/ml) of Viscozyme L® and Celluclast 1.5L®. The immobilization was carried out using method 2 and the immobilized enzymes were assayed for activity using the DNS method. The relative activity was plotted where 100% was taken as the activity with 2.5 mg/ml as the initial protein concentration (Figure 26), since this was the enzyme concentration that had been used up to this optimization step.



A)



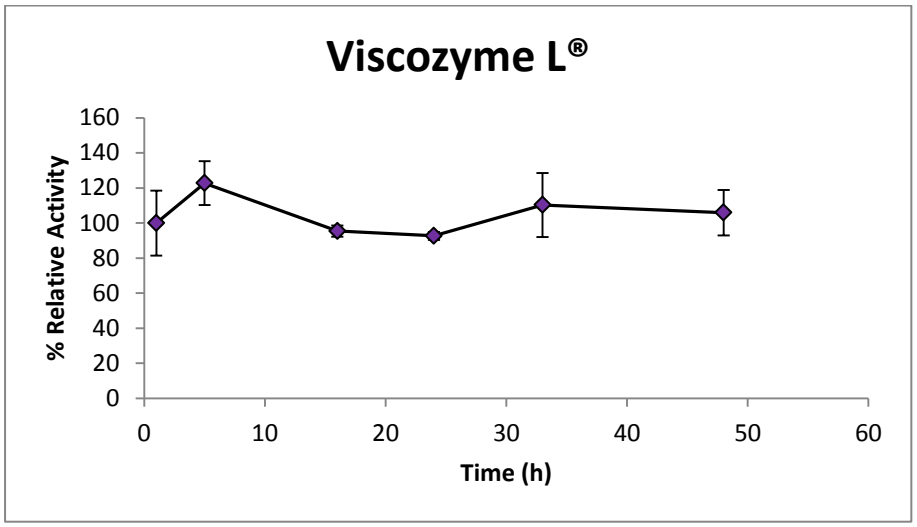
B)

Figure 26: Effect of initial enzyme concentration on the amount of immobilized Viscozyme L® A) and Celluclast 1.5L® B) activity. Data points represent the means ± SD (n=3). Protein concentration of 2.5 mg/ml was taken as 100%. ANOVA single factor analysis revealed that there were no statistically significant increases from the 100% value P > 0.05.

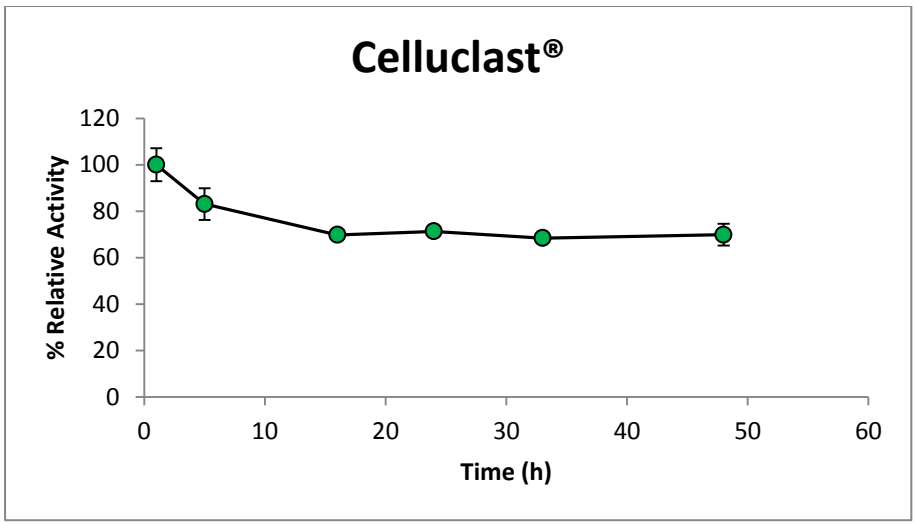
For Viscozyme L®, the highest activity was observed when an initial concentration of 4 mg/ml was used; therefore this concentration was used for further experiments (Figure 26A). For Celluclast 1.5L®, there was no significant increase in activity with initial protein concentrations higher than 2 mg/ml, therefore this initial protein concentration was used (Figure 26B).

4.3.6 Immobilization (and crosslinking) time

The immobilization was carried out using method 2, and at various time intervals (1, 5, 16, 24, 32 and 48 h) the immobilized enzymes were assayed for activity. The relative activity at 1 h was defined as 100% activity (Figure 27). This is because the enzymes were allowed to equilibrate with the fibers for 1 h and only then was GA added.



A)



B)

Figure 27: Effect of immobilization time on the amount of immobilized Viscozyme L® A) and Celluclast 1.5L® B) activity. Data points represent the means ± SD (n=3). The relative activity at the immobilization time of 1 h was defined as 100%.

For Viscozyme L®, the highest activity occurred after 4 hours of crosslinking (5 hours total immobilization time). Relative activity decreased thereafter but remained relatively stable (Figure 27A). For Celluclast 1.5®, enzyme activity immediately decreased after the addition of GA but remained stable at ~70% relative activity (Figure 27B).

4.3.7 SEM

SEM images of the fibers were taken prior and post GA and HCl pre-treatment, and post-Viscozyme L® and Celluclast 1.5L® immobilization using method 1 (Figure 28) and 2 (Figure 29).

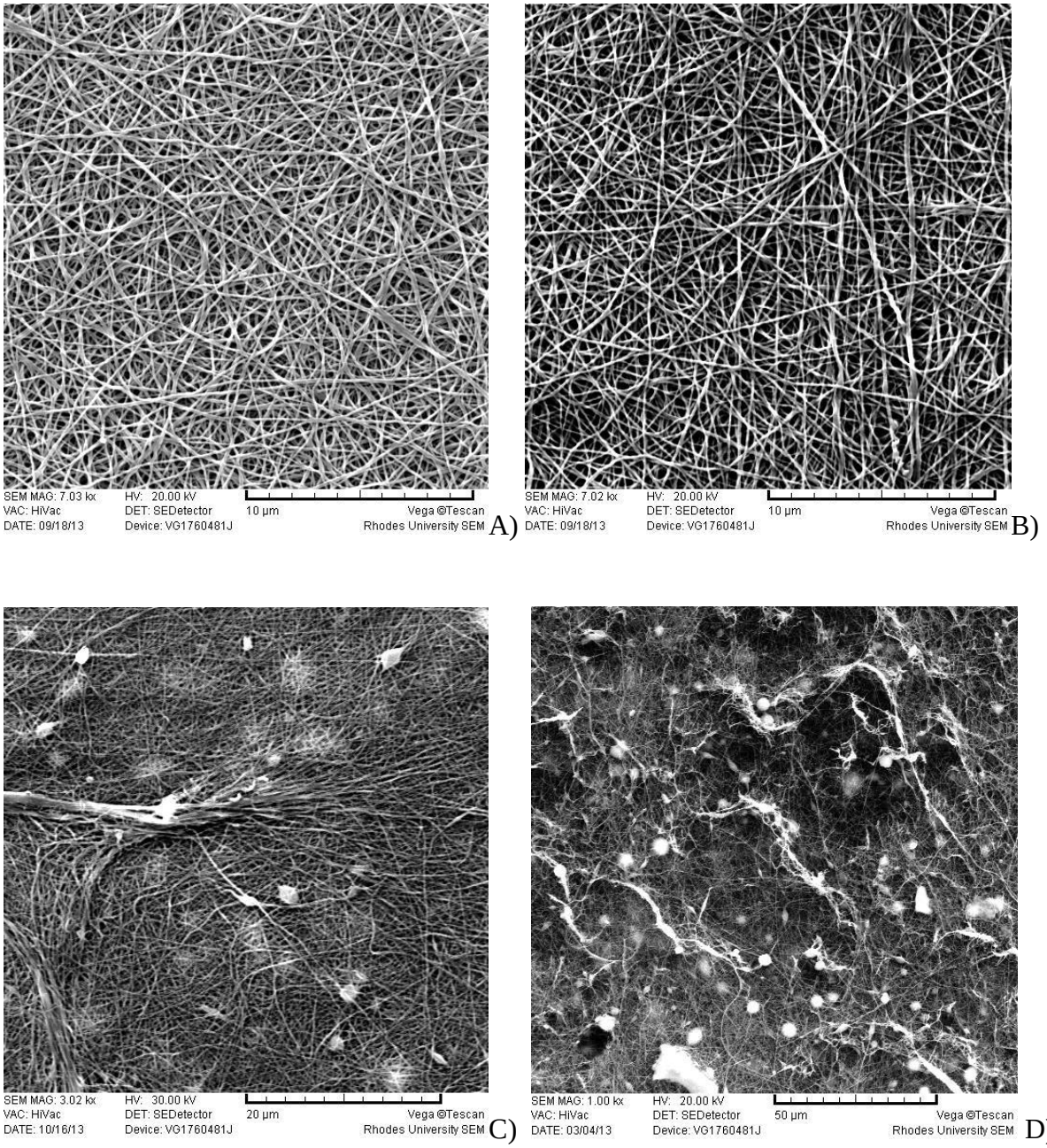


Figure 28: SEM images of electrospun nylon 6 nanofibers prior A) and post HCl B) and GA C) pre-treatment, and post enzyme immobilization D), using method 1.

From Figure 28 it can be seen that there was no change in morphology of the nanofibers before A) and after B) HCl pre-treatment. However, after pre-treatment with GA C) and

immobilization D) there was a significant change in morphology of the fibers as they appear to have clumped together.

SEM images were also taken under similar conditions for fibers that were immobilized using method 2 (Figure 29).

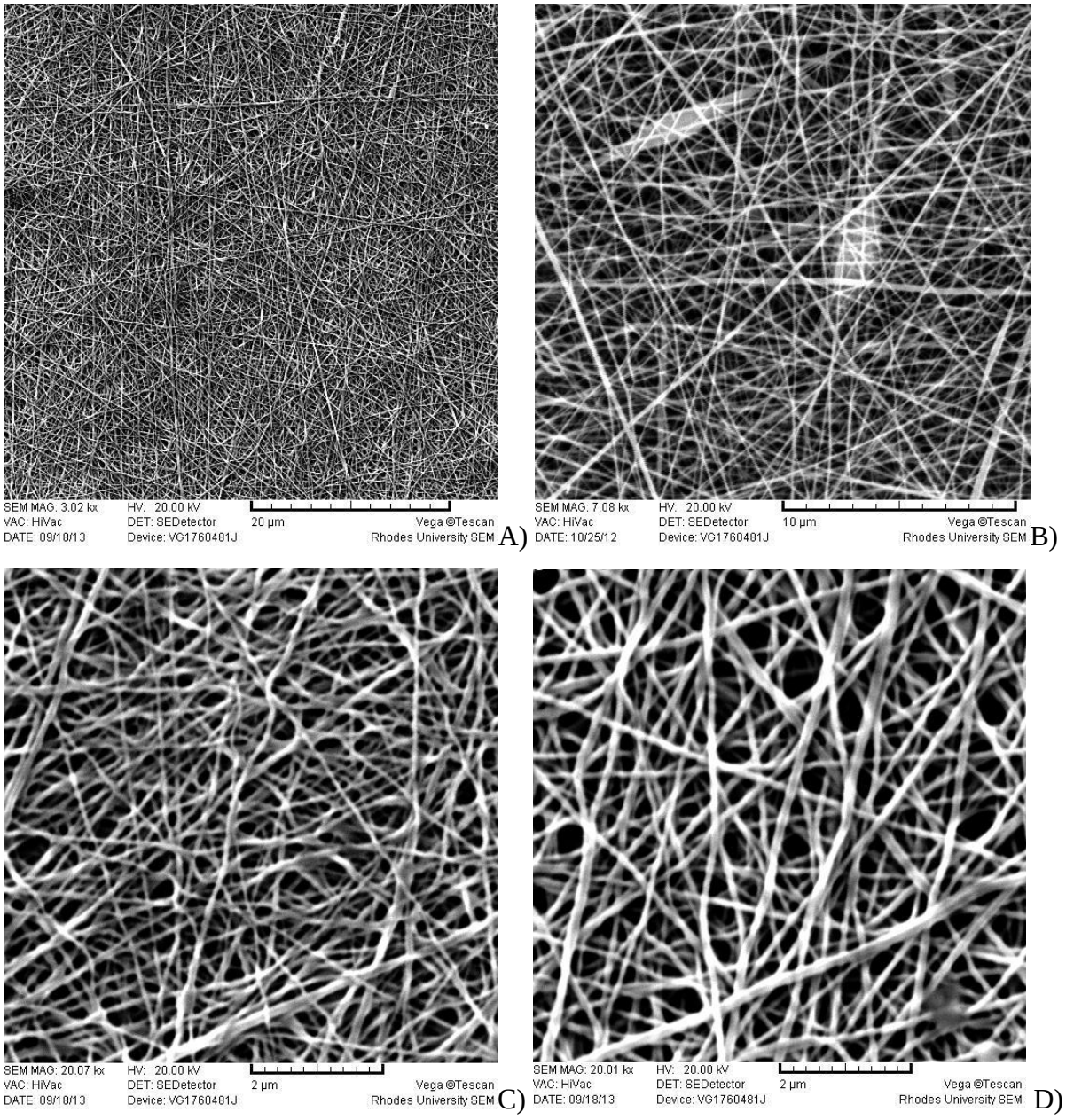
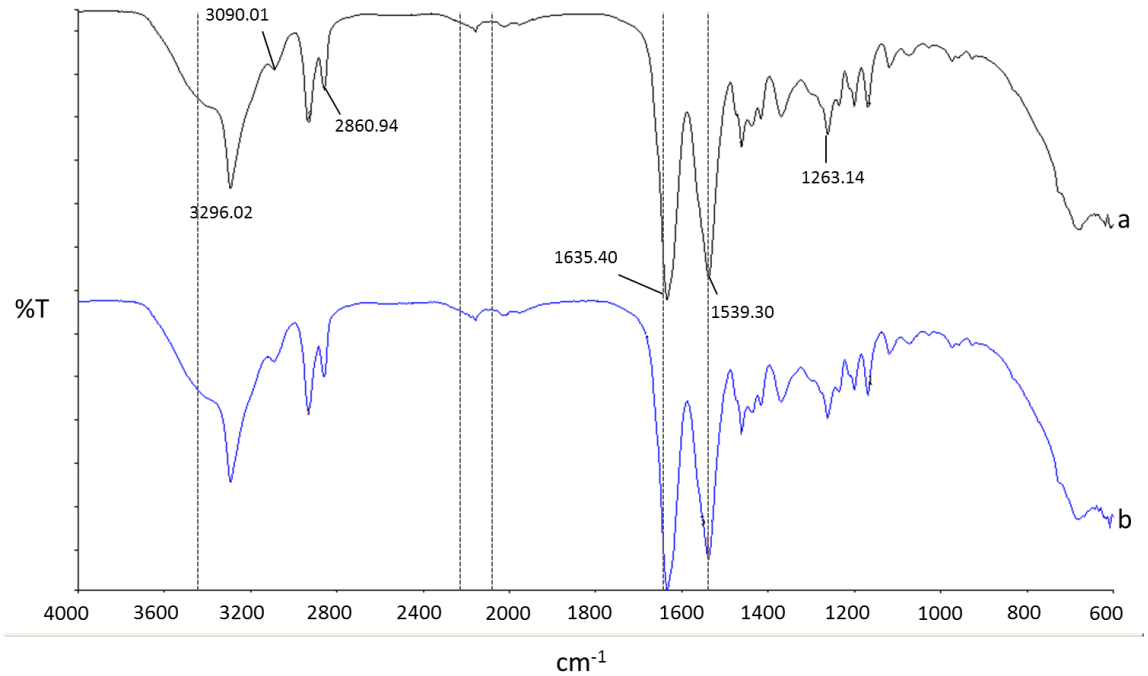


Figure 29: SEM images of electrospun nylon 6 nanofibers prior A) and post HCl B) and prior addition of GA C), and post enzyme immobilization (post addition of GA) D), using method 2

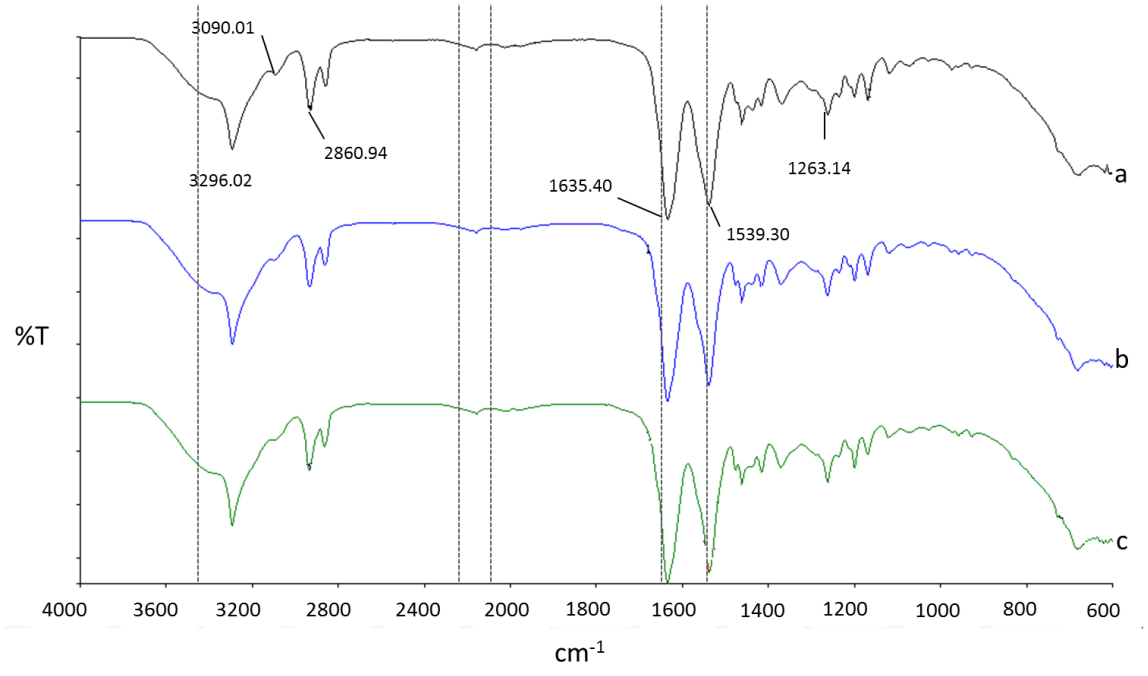
From Figure 29, it can be seen there were no visible changes in morphology before A) and after HCl treatment B) or before C) and after the addition of GA (post immobilization) D).

4.3.8 FT-IR analysis

In order to confirm enzyme binding to the nanofibers, FT-IR spectra of the nanofibers, before and after HCl pre-treatment (Celluclast 1.5L® only) and after immobilization with Viscozyme L® (Figure 30A) and Celluclast 1.5L® (Figure 30B), were measured.



A)



B)

Figure 30: FT-IR spectra of electrospun nylon 6 nanofibers, before a), and after b) immobilization with Viscozyme L® A) as well as for Celluclast 1.5L® B), before a) and after HCl pre-treatment b) and after immobilization c).

Characteristic peaks in the spectra shown in Figure 30, for un-immobilized nylon 6 were identified (3296, 3090, 2860, 1635 and 1539 cm^{-1}). Similar spectra for nanofibers before and

after HCl pre-treatment and immobilization with Viscozyme L® and Celluclast 1.5L® were obtained.

4.3.9 Energy Dispersive Spectroscopy (EDS)

Due to the limited success of FT-IR to confirm enzyme binding, EDS was used for elemental analysis, and particularly the presence of a peak at approximately 2.3 keV was monitored to determine the presence of sulphur (S) and therefore confirm the presence of enzymes in Viscozyme L® (Figure 31) and Celluclast 1.5L® (Figure 32).

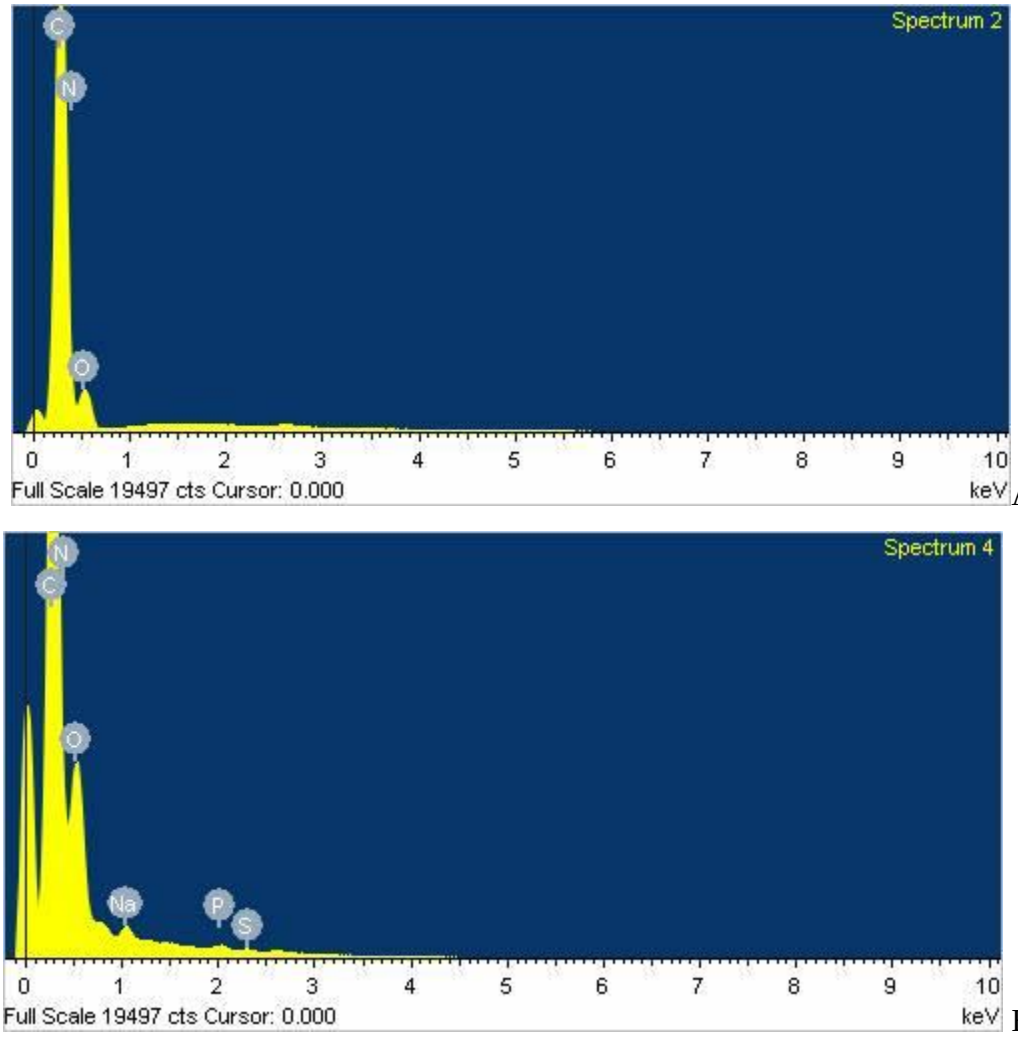
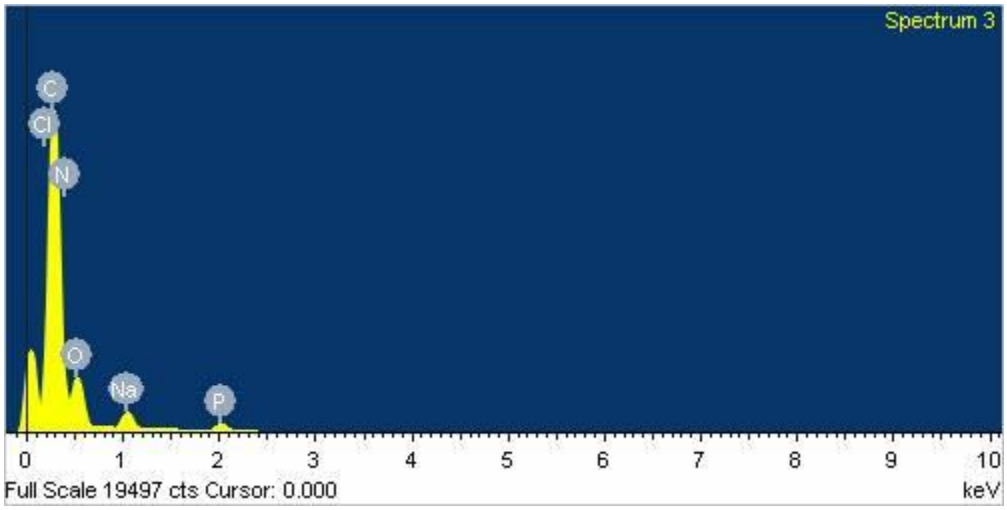
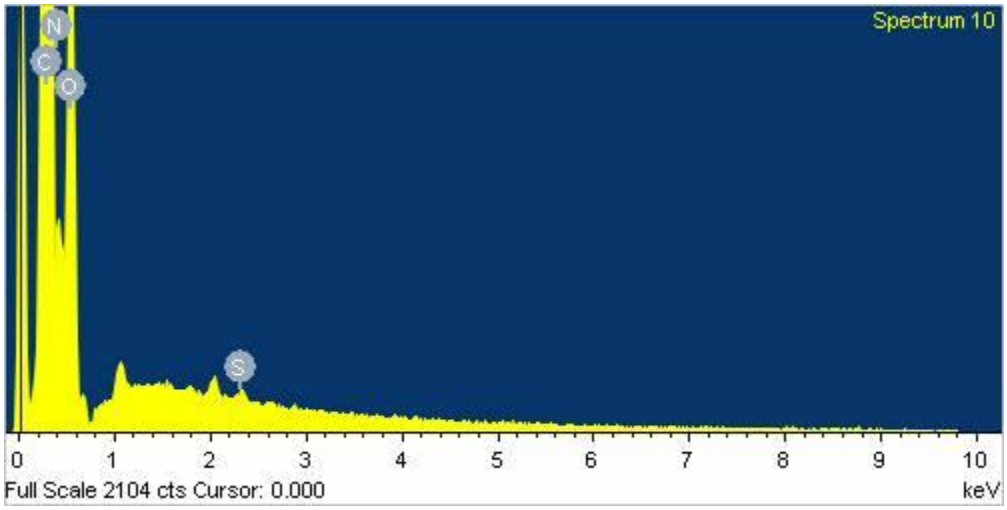


Figure 31: EDS spectra of electrospun nylon 6 nanofibers before A) and after B) immobilization of Viscozyme L®. Elemental labels are as follows; N= nitrogen, C= carbon, O= oxygen, Na= sodium, P= phosphorous, S= sulphur.

EDS analysis confirmed the presence of a very small peak at 2.3075 keV and strong peaks were observed for C, N and O before (Figure 31A) and after (Figure 31B) immobilization with Viscozyme L®, as well as smaller peaks for Na and P after immobilization (Figure 31B).



A)



B)

Figure 32: EDS spectra of electrospun nylon 6 nanofibers after pre-treatment with HCl (before immobilization) A) and after immobilization B) of Celluclast 1.5L®. Elemental labels are as follows; N= nitrogen, C= carbon, O= oxygen, Na= sodium, P= phosphorous, S= sulphur, Cl= chlorine.

For Celluclast 1.5L®, there was a small peak present at 2.3075 keV, after immobilization (Figure 32B) that was not present in the spectrum before immobilization (Figure 32A). Strong peaks were also observed for C, N and O before and after immobilization as well as smaller peaks for Na and P. There was also evidence of a peak forming for Cl after HCl pre-treatment (before immobilization) (Figure 32A).

4.4 DISCUSSION

The electrospun nylon 6 nanofiber mats that were synthesized in chapter 3 were cut into 1 cm x 1 cm squares and used as matrices for the immobilization of the hemicellulases and cellulases in Viscozyme L® and Celluclast® via crosslinking with GA.

The immobilization was monitored initially via both protein assays (Bradford method and A_{280}) and enzyme activity assays (reducing sugar quantification by DNS method). However, due to limitations of the protein assay using this immobilization system, the results of the activity assays were used as the guiding factor during optimization. The effect of GA on free/soluble enzyme activity (Figure 22) and protein assays (Figure 21) were determined to further explain these limitations.

Bradford's method was initially used as the principal protein assay in this study due to its high sensitivity, rapid response, simplicity and its lack of susceptibility to carbohydrate interferences (Bradford, 1976). Different protein assays such as the Folin-Lowry method (Lowry *et al*., 1951) have also been used to measure immobilized protein (Afsahi *et al*., 2007); this assay was also considered. However, this method has been reported as 4 times less sensitive than the Bradford method and is subject to more interferences, including carbohydrates and Tris buffers (Bradford, 1976), which are important reagents used in these assays. The Biuret reaction (Gornall *et al*., 1949) is also less sensitive and subject to interference from Tris buffers (Bradford, 1976), and therefore could not be used with this immobilization system.

The absorbance at 280 nm (A_{280}) was also used in this study to verify protein quantification. However, there were occasional, slight deviations between the results using this method compared to the Bradford's method. This may have been due to differences in the detection mechanisms since both assays are dependent upon the amino acid composition of the protein; Bradford's relies on arginine and lysine residues, and to a lesser extent, the aromatic amino acid residues (phenylalanine, tryptophan and tyrosine) (Kruger, 2009). Conversely, A_{280} relies predominantly on the presence of aromatic amino acid residues (tyrosine, tryptophan and phenylalanine) (Whitaker and Granum, 1980). However, the A_{280} assay has also been reported to show interferences with phosphate and Tris buffers, as well as sucrose (Stoscheck, 1990), which were relevant to the immobilization system used in this study. For

the above mentioned reasons, as well as the fact that Bradford's method is more commonly used in literature, Bradford's method was initially used as the principal assay in this study.

4.4.1 Effect of GA on the free enzyme

The effect of GA on Bradford's assay, using Viscozyme L® and Celluclast 1.5L®, was investigated in this study. It was found that with increasing concentrations of GA there were statistically significant increases in the absorbance and therefore the calculated total protein for both Viscozyme L® and Celluclast 1.5L® (Figure 21A and B, respectively), despite the fact that all assays contained identical amounts of protein and appropriate controls had been used. Random instances of high standard deviations were also observed. This may be explained by the crosslinking reaction with GA, which typically involved the ε-amine of lysine residues (Costa *et al*., 2004), while Bradford's detection mechanism also relies on basic amino acids such as lysine (and arginine) (Kruger, 2009). Although this is the most likely explanation for the protein interference that was observed, another contributing factor is the possible presence of additional interfering compounds in the commercial enzyme cocktails, since their exact composition is unknown. These include proteins (other than the active enzymes) as well as non-proteinaceous compounds such as detergents, stabilizers and preservatives. These components may have caused interferences with the protein (and activity) assays. The Bradford's assay has been reported to show interferences with detergents as well as acetic acid (Bradford, 1976), which was the solvent used to dissolve the nylon prior to electrospinning of the nanofibers. For the immobilization studies, using method 1, high standard deviations and inconsistent trends were also observed using both Bradford's method (Appendix D.3 and D.4). For these reasons, accurate quantification of protein during immobilization could not be determined and therefore activity assays were used as a reliable guide for further investigations.

It has been well documented in literature that the use of crosslinkers, such as GA, may affect the active site of enzymes and therefore its activity (Wu *et al.,* 2004). The effect of GA on free and immobilized enzyme activity in Viscozyme L® and Celluclast 1.5L® was therefore investigated in this study. As shown in Figure 22A, with increasing GA concentration, the catalytic efficiency of the free enzymes in Viscozyme L® was gradually reduced with a statistically significant decrease of enzyme activity up to 30% with 5% GA ($P \leq 0.01$). This

was likely due to the above mentioned reasons. This finding is in accordance with Chui and Wan (1997), who claimed that enzyme activity is inversely proportional to the concentration of GA used due to distortion of the enzyme structure as a result of crosslinking; the distortion of enzyme structure can lead to reduced accessibility of the substrate and ultimately results in reduced catalytic activity. Although the DNS assays relies on the detection of reducing sugars (and therefore involves reaction with aldehyde groups), it is not likely that the trends observed were due to a reaction of GA (instead of reducing sugars) with DNS. This is because the data were normalized to controls (tubes with buffer and the appropriate concentration of GA). Conversely, for Celluclast 1.5L®, the effect of GA on enzyme activity was minimal and not statistically significant (Figure 22B).

4.4.2 Effect of GA on immobilized enzyme

Low concentrations of GA may lead to insufficient crosslinkages and increased chances of intramolecular crosslinking (Zaborsky, 1973). Higher concentrations may lead to extensive crosslinking, insolubilization of the enzyme and distortion of its structure, such that the enzyme active site is not accessible to the substrate, thus reducing the catalytic activity (Poddar and Jana, 2011). Therefore, the effect of crosslinking by GA in varying concentrations on the activity of the immobilized enzymes was investigated in this study (Figure 24). Interference of GA with the DNS reagent was eliminated through the use of appropriate controls as mentioned previously, as well as thorough washing and incubation of the immobilized enzyme fibers in Tris-HCl buffer (pH 7.7) to cap the unreacted aldehyde groups. A similar capping step was also used by Lee *et al*. (2010).

The pre-activation step with GA in method 1 was carried out at pH 6.8, as GA is more stable at lower pH. The reaction with protein was carried out at pH 7.7, because a higher pH is ideal for efficient nucleophilic attack by the protein's lysine residues (Walt and Agayn, 1994; Weetal, 1974). The immobilization reaction used in method 2 in this study was also carried out at pH 7.7 because of the high reactivity of GA with proteins at around neutral pH (Poddar and Jana, 2011). The acid dissociation constant (pK_a) of lysine ε-amino groups is 10.5 which is > pH 7.7; the resulting protonated amino groups are very reactive towards nucleophilic agents (Guisan, 1988). Although GA has been reported to react with proteins over a wide pH range, the reaction is irreversible between pH 7 and 9 (Okuda *et al*., 1991). Because of this it

was initially decided that a reduction step, for example treatment with $NaBH_4$, was not necessary. Reduction of the Schiff base to a stable secondary amine is often reported in literature and may cause a slight increase in the enzymatic activity (Isgrove *et al*., 2001; Migneault *et al.*, 2004; Quiocho and Richards, 1964; Walt and Agayn, 1994). However, the use of reducing agents may also interfere with possible disulphide linkages responsible for maintaining protein structure (Walt and Agayn, 1994).

Two different methods of enzyme immobilization using GA were investigated in this study (Figure 20); the first method involved pre-activation with GA, and the second method involved initial adsorption of the enzymes followed by crosslinking with GA. Both these methods have been previously reported in literature for immobilization of various enzymes on different supports (Goldstein *et al.*, 1974; Sousa *et al.*, 2001; Stoilova *et al*., 2010; Wang *et al*., 2009).

Method 1- Pre-treatment of the nanofibers with GA showed a negative effect on the immobilized enzyme activity as up to 15% activity was lost for Viscozyme L® when pre-treated with 2% GA ($P \leq 0.05$) (Figure 23A). For Celluclast 1.5L®, although the general trend indicated a slight improvement in activity when treating with GA, none were statistically significant ($P > 0.05$) and high standard deviations were observed (Figure 23B). High standard deviations and inconsistent trends were also observed when attempting further optimization such as the effect of HCl pre-treatment (Appendix D.1 and D.2).

As previously mentioned, due to the nature of enzyme cocktails, such as Viscozyme L® and Celluclast 1.5L®, the exact contents are unknown. Therefore, saturation of binding sites may have been contributed to by non-specific binding of additional ligands present in the commercial cocktails, such as proteinaceous material that does not display any enzyme activity. The amino and carboxyl groups on the nylon can form electrostatic bonds to oppositely charged groups on protein molecules and give rise to non-specific binding of ligands, as well as hydrophobic interactions. Similarly, unreacted or residual aldehyde groups after GA activation that had not reacted with enzyme, can also bind non-specifically to any unreacted NH_2-group containing material present in the commercial cocktails. In addition to this, unreacted GA is capable of reacting with some carbohydrate functional groups (Russell and Hopwood, 1976). This may also explain the inconsistent results that were observed using

this method. The potential presence of unknown, non-proteinaceous material such as preservatives, as previously mentioned, may also have been a contributing factor to the high standard deviations and inconsistent trends that were observed. Due to the limitations obtained using this method, modifications to the approach were made and the effect of GA using method 2 was investigated.

Method 2- For Viscozyme L®, there was a significant increase in activity of up to 75% when treated with 4% GA ($P \leq 0.01$), compared to the untreated (0% GA) immobilized enzymes (Figure 24A). These results suggest a cascade reaction of extensive enzyme cross linking, orientating the enzymes in such a way that favours their activity, thus promoting catalytic efficiency. These findings are in agreement with El-Zahab *et al*. (2004) and Lupoi and Smith (2011), who also stated that the immobilization of multiple enzymes on the same support can promote overall catalytic efficiency through close proximity, ideal positioning and orientation of the immobilized enzymes on the support matrix. This method of immobilization also gives rise to potential inter-molecular cross linking (Figure 20B), in which additional enzymes bind to the originally adsorbed seed enzymes.

Conversely, for Celluclast 1.5L®, it appeared that GA did not improve immobilization in terms of the enzyme activity, as there was a reduction in immobilized enzyme activity with increased concentrations of GA. When treated with 3% GA or higher, there was ~40% loss in activity ($P \leq 0.05$) (Figure 24B). This may also have been due to extensive inter-molecular cross linking of enzymes, orientating the enzymes in such a way that their active sites were blocked or distorted or were too closely positioned in such a way that led to steric hindrances for facile substrate accessibility. Another possible contributing factor is the presence of cellulases (particularly exo-glucanases and β-glucosidases) hydrolysing the AP to produce glucose and cellobiose which are known product inhibitors for this type of reaction (Lee *et al*., 2010). However, 2% GA was still selected for further experiments because GA is able to form intermolecular crosslinks with enzymes and larger enzyme aggregates and thus reduce the likelihood of enzyme leeching (Dai *et al*., 2011; Hanefeld *et al*, 2009). Recycling studies have also shown that covalently bound enzymes displayed greater retention of residual enzyme activity than non-covalently bound enzymes (Mandali and Dalaly, 2010). Afsashi *et al*. (2007) immobilized cellulase on non-porous silica particles using a similar method, and

also found that the addition of GA did not improve the immobilization in terms of enzyme activity.

Although GA did not improve immobilization with regards to Celluclast 1.5L®, it did improve immobilization of the enzymes in Viscozyme L®. The standard deviations were lower compared to method 1 and the trends were more consistent and reproducible. Therefore, method 2 was selected for further immobilization studies.

4.4.3 HCl pre-treatment

The composition, morphology and surface characteristics of the support polymer contribute to the binding capacity and regulation of catalytic activity (Kishore *et al.*, 2012). In this study the peptide bonds of nylon were cleaved hydrolytically by HCl, thus improving the availability of more reactive groups (amino and carboxyl groups) for enzymes to bind. For Viscozyme L®, however, HCl pre-treatment did not have a significant improvement on enzyme immobilization (Figure 25A). Therefore, for further experiments regarding Viscozyme L® immobilization, the nanofibers were not pre-treated with HCl. On the other hand, for Celluclast 1.5L®, there was a statistically significant increase in relative activity (~20%) when the fibers were pre-treated with 1.91 M HCl ($P \leq 0.01$), compared to fibers that were not pre-treated with HCl (Figure 25B). Therefore, in further experiments involving Celluclast 1.5L®, the nanofibers were pre-treated with 1.91 M HCl. Jain and Wilkins (1986) also pre-treated nylon blocks with 3.65 M HCl before immobilization with Celluclast 200L® and reported 0.39 U per 650 mg of support (0.6 $U.g^{-1}$ of nylon). This is in comparison to Celluclast 1.5L® where 9.9 $U.g^{-1}$ of nylon was obtained. Although Celluclast 200L® is also reported to contain a mixture of enzymes from *T. reesei* (Jain and Wilkins, 1986), its composition and initial soluble enzyme activity may differ slightly from the Celluclast 1.5L® used in this study. Another possible reason for the increased activity retained for Celluclast 1.5L® (compared to Celluclast 200L®) may simply be the increased surface area of the nanofibers, thus exposing more reactive groups for enzymes to bind, as well as possible reduced mass transfer limitations associated with using nano-scaled supports.

For HCl molarities above 1.91 M (2.54 and 3.18 M), the retained immobilized enzyme activity decreased for both Viscozyme L® and Celluclast 1.5L® (Figure 25). Particularly for

Celluclast 1.5L®, with HCl concentrations up to 3.18 M, the relative activity decreased to ~80% (Figure 25B) ($P \leq 0.01$). This was likely due to partial disintegration of the fibrous mats. Similar findings were observed by Isgrove *et al*. (2001) who reported a critical level of 3.0 - 3.1 M HCl, before disintegration of the nylon film.

4.4.4 Effect of protein concentration

One of the major limitations of the viability of an applied immobilization system is the cost. Enzymes are expensive and therefore the effect of initial enzyme concentration on immobilization was assessed. Enzyme concentrations between 1 and 5 mg/ml were tested and the relative activity was reported (Figure 26).

For Viscozyme L® (Figure 26A), the relative activity increased with increasing concentration of enzyme up to 4 mg/ml. This may be explained by the increase in contact frequency between the enzymes and fibrous membrane active sites as the enzyme concentration increased. The highest activity was observed when an initial concentration of 4 mg/ml was used; therefore this concentration was used for further experiments.

It is also possible that increasing the initial protein concentration may have caused increased stacking of the enzymes due to the crosslinking reaction (inter-molecular cross linking) (Figure 20B). Therefore the increased enzyme loading would be responsible for the increased activity, rather than availability of binding sites on the fibrous membrane. Specific activity would therefore be useful to verify this, but unfortunately since the amount of protein immobilized could not be quantified for reasons discussed earlier, this could not be confirmed.

For Celluclast 1.5L® (Figure 26B), there was no significant increase in activity with initial protein concentrations higher than 2 mg/ml; therefore this protein concentration was used for further experiments with Celluclast 1.5L®. It is possible that saturation of the binding sites on the fibrous membrane had already been reached at this point. According to Wanjari *et al*. (2011), the decrease in enzyme activity with protein loadings above the saturation point may be explained by internal diffusional restrictions resulting from the increased protein concentrations at the external surface. This could have caused underestimation of the

immobilized enzyme activity. Similar findings were reported by Isgrove *et al*. (2001) and Mansour and Dawoud (2003), who found very little increase in immobilization with initial protein concentrations above 1 mg/ml.

In this case it is also possible that increasing the initial protein concentration may have caused stacking of the enzymes due to the crosslinking reaction (inter-molecular cross-linking) (Figure 20B). The crosslinking reaction may have taken place in such a way that the active sites of the original seed enzyme molecules were blocked (by further stacking of additional enzymes) and were therefore not accessible to the substrate. Again, the amount of immobilized protein could not be determined for reasons discussed earlier. However, if the amount of protein loading had increased and the activity had remained constant, as it did in this case, then this hypothesis could have been confirmed.

The possible presence of additional components in the commercial enzyme cocktails, as previously discussed, may also have contributed to the occupation of membrane binding sites and therefore the saturation point may have been reached sooner.

4.4.5 Immobilization time

As previously discussed, the amount of immobilized enzyme is influenced by the number of available active sites on the nylon 6 nanofibrous membranes. Since a crosslinking method was used, the enzymes may stack on top of the seed enzymes (Figure 20B). Therefore, the enzyme loading may also be influenced by time. Hence the effect of immobilization time was investigated to determine the optimal time for the amidination reaction. The immobilization was carried out over 48 hours (1 h equilibration followed by crosslinking), and at various time intervals the immobilized enzymes were assayed for activity.

For Viscozyme L®, the relative activity increased initially up to 5 h (4 h crosslinking time), then decreased and plateaued (Figure 27A). The increase may have been due to increased enzyme loading (stacking of enzymes on top of seed enzymes). Occupation of remaining nanofibrous membrane binding sites may also have been taking place. The decrease and eventual plateau in activity may have been due to extensive crosslinking of the enzymes. The enzyme active sites may have been blocked or crosslinking of enzymes may have occurred

between enzymes in the solution, rather than to enzymes that were already bound to the support. Since 5 h resulted in the highest activity, this immobilization time was used for further studies.

For Celluclast 1.5L®, the enzyme activity immediately decreased after the addition of GA and plateaued around 70% relative activity after 16 hours (Figure 27B). This is in accordance with Figure 24B, which also demonstrated that the addition of GA negatively affected the immobilization of the enzymes in Celluclast 1.5L®. Again, this may simply be due to obstruction and inaccessibility of the enzyme active sites to the substrate, resulting from the crosslinking reaction.

The decrease observed after 5 h for Viscozyme L® and after 1 h for Celluclast 1.5L® may also have been due to crosslinking of the enzymes in solution with other free enzymes, rather than with the immobilized seed enzymes. Another possible reason for the decrease that was observed may be enzyme leaching and denaturation as a result of stirring (rotation) (Wanjari *et al*., 2011).

4.4.6 SEM

Method 1- Evidence of the internal crosslinking of fibers, using method 1, was shown in Figure 28C and D as the fibers appeared to have clumped together. This caused areas of the fibrous mats to become densely packed, reducing the surface area for enzymes to bind, and therefore potentially decreasing the catalytic activity, which may also explain the high standard deviations and inconsistent trends observed in Figure 23 (and Appendix D). Wu *et al*. (2004) also experienced internal crosslinking of the fibers (PVA) through the use of GA, as was illustrated in the SEM images.

Method 2- There were no observable differences in the morphology of the fibers before and after HCl pre-treatment and immobilization (Figure 29). This showed that the structure of the fibrous membranes was not definitively affected by any of the pre-treatments and particularly by the amidination reaction. Similar findings were reported by Hung *et al.* (2011) who immobilized cellulase on electrospun PVA nanofibers. FT-IR and EDS were therefore used to confirm enzyme binding to the nanofibrous membranes.

4.4.7 FT-IR

FT-IR was used to confirm binding of the enzymes to the nylon 6 nanofibers, and the resulting spectra is shown in Figure 30A and B for Viscozyme L® and Celluclast 1.5L®, respectively. The region between 600 and 1450 cm^{-1} of IR spectra has been referred to as the fingerprint region (Kishore *et al.*, 2012) due to its complexity and distinctive patterns that have been reported in that area. However, this also causes difficulty in assignment of all absorption bands. Conversely, the spectra in the 1450 to 4000 cm^{-1} region have been referred to as the group frequency region and typically results from stretching vibrations of diatomic units (Kishore *et al*., 2012).

The un-immobilized nylon 6 nanofibrous mats showed several FT-IR absorption features in the range 600- 4000 cm^{-1} (Figure 30A a). The characteristic peaks of nylon 6 that were identified were the hydrogen bonded N-H stretch vibration (3296 cm^{-1}), N-H in plane bending vibration (3090 cm^{-1}), symmetric -CH_2- stretching vibration (2860 cm^{-1}), amide I, C-O stretch (1635 cm^{-1}), amide II, C-N stretch and CO-N-H bend (1539 cm^{-1}) and the band at 1263 cm^{-1} was as a result of the C–N–H group of nylon 6 (Andrews and Mbafor, 1991; Griffiths and Hasseth, 2007; Li *et al*., 2009).

From Figure 30A and B, it can also be seen that there was very little difference between spectra of the nanofibers before and after HCl pre-treatment and immobilization for both commercial enzyme cocktails. Although some very small differences could be observed, they may not be significant enough to suggest that the enzymes were covalently bound to the support matrix. From literature, changes that have been reported between spectra before and after enzyme immobilization include the presence of, or band broadening at approximately 3400 cm^{-1} due to the combination of the stretching vibration of O-H and N-H (Hung *et al*., 2011; Liao *et al.*, 2010; Wu *et al*., 2005). Another difference that has been reported in literature, that particularly involves attachment of GA to –NH_2 groups of an immobilization support, is the presence of a band at around 2100 cm^{-1} (Hung *et al*., 2011) and 2250 cm^{-1} (-N=C=O stretch) (Kishore *et al.*, 2012). Since a prominent band was not observed in this region, it is possible that the enzymes were not bound covalently. It was also expected that the IR spectra after immobilization would reveal a peak or shift at approximately 1650 cm^{-1} (amide I) and 1540 cm^{-1} (amide II), as these were characteristic peaks for a cellulase complex that was detected in its crude form and when immobilized, as well as a shift in frequency

from 1542 cm^{-1} to 1522 cm^{-1}, suggesting amide bond formation (C-O to C-N conversion) (Jordan *et al*., 2011). The expected results mentioned here are based on literature; a more accurate prediction could have been made with IR spectra of Viscozyme L® and Celluclast 1.5L® in their crude/free form for comparison. However, the available instrumental facilities did not allow for analysis of liquid samples.

Wu *et al.* (2005) also showed similar IR spectra for their immobilization supports before and after immobilization. A possible reason for the limited differentiation observed, in this study with Viscozyme L® and Celluclast 1.5L®, is that the characteristic, functional groups present in nylon (carbonyl and amine) are also present in proteins, resulting in overlapping peaks. This masking effect may also have been contributed to by the presence of additional unknown compounds in the commercial enzyme cocktails. It is also possible, however, that these results can be explained by low enzyme loading. Unfortunately, as previously mentioned, the amount of enzyme (protein) bound to the immobilization matrix could not be determined.

UV-Vis was also considered, as absorbance at 280 nm could potentially confirm the presence of enzyme on the nanofibers, however the available instrumental facilities did not allow for solid sample analysis.

4.4.8 EDS

Due to limitations experienced using FT-IR, qualitative EDS analysis was used to examine the surface structure of the fibers since elemental components and functional groups can be detected by EDS spectra. The presence of nitrogen (N), oxygen (O) and particularly sulphur (S) was monitored. Wu *et al*. (2004) and Jordan *et al.* (2011) used X-ray photoelectron spectroscopy (XPS), a similar technique, to verify that cellulase was bound to the immobilization supports by analysing the presence of N, O and S.

Figure 31 and 32 display EDS spectra of electrospun nylon 6 nanofibers before A) and after B) immobilization of Viscozyme L® and Celluclast 1.5L®, respectively. Characteristic peaks present before and after immobilization at 0.2774, 0.3924 and 0.5249 keV corresponded to the presence of C, N and O, respectively. These elements also displayed a slight overlap in

characteristic peaks/ principal lines (Kα). These three peaks may be attributed to the amine and carboxyl groups on the nylon 6 nanofibers. These are, however, also the characteristic groups present in enzymes. Therefore the presence of S at 2.3075 keV, corresponding to the sulfhydryl group on cysteine (and methionine) amino acid residues, was monitored to indicate the presence of enzymes.

For Viscozyme L® (Figure 31B) and Celluclast 1.5L® (Figure 32B), small peaks at approximately 2.31 keV were observed, confirming the presence of enzyme for both commercial enzyme cocktails.

The peaks shown at approximately 1.04 and 2.01 keV (Figures 31A, 32A and B) corresponded to the elements Na and P, most likely from the sodium phosphate buffer that was used to wash the fibers.

Since qualitative EDS was used, the amount of each element could not be quantified specifically and was relative to the other elements that were present. The small size of the peaks observed corresponding to S may have been due to masking of cysteine residues resulting from the crosslinking reaction (Costa *et al*., 2004). In addition to this, another possible explanation could be a low enzyme loading, however, as previously mentioned, the amount of enzyme immobilized could not be determined.

Activity assays, together with EDS, provide evidence for the presence of enzymes bound to the electrospun nylon 6 nanofibers.

4.4.9 Yield

Immobilization yield is usually reported in literature using the equation shown below (Afsahi *et al.*, 2007; Dincer and Telefoncu, 2007; Hung *et al*., 2011; Jia *et al*., 2011; Jordan *et al*., 2011; Kishore *et al*., 2012).

Specific activity of immobilized enzymes / specific activity of free enzymes x100

There have been reports in literature of low immobilization yields, between 7% and 40% when immobilizing cellulases on various solid supports by crosslinking with GA (Jain and Wilkins, 1986; Mandali and Dalaly, 2010; Mao *et al*., 2006; Vaillant *et al*., 2000). These low yields may be attributed to enzyme aggregation or occupation of active sites involved in the immobilization. In this study, the immobilization yield of Viscozyme L® and Celluclast 1.5L® was 15.1% and 42.7%, respectively (see Appendix E). However, due to the limitations of the immobilization system used in this study with Viscozyme L® and Celluclast 1.5L®, the amount of protein on the nylon 6 membranes could not be determined, therefore the yield was calculated using activity (not specific activity). This may explain the low yields that were obtained. For example, a study that was carried out by Dincer and Telefoncu (2007), reported the activity, protein and specific activity values of the free and immobilized enzymes. This allowed the yield to be calculated as 1.34% using activity, and 87% using specific activity. This study therefore showed that, depending on whether activity or specific activity was used to calculate the yield, it can have a significant effect on the final yield value. Therefore the yield values for Viscozyme L® and Celluclast 1.5L® may not have been as low if specific activity could have been used for the calculation.

4.4.10 Efficiency

In literature, protein loading efficiency is usually calculated by determining ratio of the amount (mg) of enzyme on the membrane to the whole membrane mass (mg) (Hung *et al*., 2011; Lozano *et al*., 1988; Wu *et al*., 2004; Zaidi *et al*., 1995). However, due to the limitations previously mentioned in this study, the amount of protein on the nylon 6 membranes could not be determined. For this reason, efficiency was reported by determining the ratio of enzyme activity (U) on the membrane to the whole membrane mass (g) (see Appendix E). The enzymes in Viscozyme L® and Celluclast 1.5L® were immobilized onto the nanofibers by crosslinking with glutaraldehyde, and showed 17.5 and 9.9 units of activity per g of fibrous nylon 6 membrane, respectively. This method of reporting efficiency has also been described by Lee *et al*. (2010) and Lu *et al*., (2013).

4.4.11 Concluding statements

The enzymes in Viscozyme L® and Celluclast 1.5L® were successfully immobilized onto electrospun nylon 6 nanofibers under optimum conditions, with 17.5 and 9.9 units of activity per g of fibrous nylon 6 membrane, respectively.

GA appeared to interfere with Bradford's protein assay and had a negative effect on the free enzymes in Viscozyme L® and the immobilized enzymes in Celluclast 1.5L® (using method 2). Although GA did not improve immobilization with regards to Celluclast 1.5L®, it did improve immobilization of the enzymes in Viscozyme L® using method 2, which was used for further immobilization studies.

The optimal conditions for immobilizing the enzymes in Viscozyme L® and Celluclast 1.5L® were investigated, and 4% and 2% GA concentrations were used, with 4 mg/ml and 2 mg/ml initial protein concentrations, respectively, and an immobilization time of 5 h was used for both enzyme solutions. Fibres immobilized with the enzymes in Celluclast 1.5L® were pre-activated with 1.91 M HCl.

In this chapter, the hemicellulases and cellulases in Viscozyme L® and Celluclast 1.5L® were successfully immobilized onto nylon 6 nanofibers. In industry, enzyme immobilization offers several benefits relating to the reduction in costs; such as increased stability, recovery and reuse of enzymes for the hydrolysis of lignocellulosic waste. Therefore, these factors were investigated in Chapter 5, as well as the optimum operating conditions.

Chapter 5

Comparison and Characterization of Free and Immobilized Enzymes

5.1 INTRODUCTION

Changes in chemical and physical characteristics have been known to occur in enzymes when they are in the immobilized form and this may result in the immobilized enzymes behaving differently to the free form of enzymes. For this reason, comparative studies were performed involving the free and immobilized enzymes of Viscozyme L® and Celluclast 1.5L®, under various conditions.

Factors that may affect the immobilization efficiency include method of immobilization, the nature of the immobilization matrix and the orientation of the enzymes on the support matrix. Although similar protein or enzyme concentrations may be used for the immobilized and free enzyme reactions, not all of the immobilized enzymes may be able to take part in the reaction. The enzymes are immobilized in a random fashion and therefore, depending on their orientation, the active sites may be inaccessible to the substrate (Lupoi and Smith, 2011) and therefore activity may be lost.

Despite this possible loss in activity, immobilized enzymes do offer potential benefits. These include increased stability, reusability, simplified prevention of microbial growth and ease of recovery (Jordan *et al.,* 2011). The operational and non-operational (storage) stability and reusability are therefore important factors when considering the economic viability of a bioprocess involving immobilized enzymes (Dincer and Telefoncu, 2007). These factors were therefore investigated in this study for the immobilization of the enzymes in Viscozyme L® and Celluclast 1.5L®.

5.2 MATERIALS AND METHODS

5.2.1 Substrate specificity studies

DNS assay

The DNS assay was used to determine the different enzyme activities in Viscozyme L® and Celluclast 1.5® in both the free and immobilized forms. Enzyme (1 square or 50 µl) was added to 350 µl of substrate (2%) (w/v) in 50 mM sodium citrate buffer (pH 5.0). The substrates used included AP, apple pectin (PEC) for pectinase activity, carboxymethylcellulose (CMC) for endoglucanase activity, beechwood xylan (BWX) for endoxylanase activity, locust bean gum (LBG) for endomannanase activity and polygalacturonic acid (PGA) for polygalacturonase activity. Assays were performed in triplicate and appropriate enzyme and substrate controls were set up. The activity was calculated as glucose equivalents in µmol/cm^2/min for the immobilized enzyme and in µmol/ml/min for the free enzyme using a glucose standard curve (Appendix A).

4-nitrophenol assay method

Additional enzyme activities were identified for the free and immobilized forms of the enzymes, using the 4-nitrophenol assay method described by Berghem and Pettersson (1974). Enzyme (1 square or 50 ul) was added to 450 µl of various substrates (0.5 mM) and assayed for 1 h at 37˚C. The substrates used included 4-nitrophenyl-β-D-glucopyranoside for β-D-glucosidase activity, 4-nitrophenyl-β-D-xylopyranoside for β-D-xylosidase activity, 4-nitrophenyl-β-D-mannopyranoside for β-D-mannosidase activity, 4-nitrophenyl-β-D-galactopyranoside for β-D-galactosidase activity and 4-nitrophenyl-α-L-arabinofuranoside for α-L-arabinofuranosidase activity. The enzyme reaction was terminated by the addition of 500 µl Na_2CO_3 (2 M). The absorbance of the resultant colour development was measured at 405 nm using a Powerwave$_x$ microplate reader from Biotek instruments with KC junior software. Assays were performed in triplicate and appropriate enzyme and substrate controls were set up. The activity was calculated as Units per square of nanofiber (µmol/cm^2/min) for the immobilized enzyme and as Units per ml (µmol/ml/min) for the free enzyme using a 4-nitrophenol standard curve (Appendix C) which was prepared in the range of 0.001 – 0.08 µmol/ml.

5.2.2 Temperature and pH optima

The pH and temperature optima were determined for the enzymes in Viscozyme L® and Celluclast 1.5L®. AP (1%) (w/v) was used as the substrate for the pH studies, and CMC (1%) (w/v) was used as the substrate for the temperature studies. For pH optimum determination, enzyme assays were carried out in triplicate at pH values ranging from pH 2.4-9.8 using the universal buffer system described by Britton and Robinson (1931). For the temperature optimum determination, enzyme assays were carried out in 50 mM sodium citrate buffer (pH 5.0) between RT (24°C) and 80°C, in triplicate. Reducing sugars liberated were quantified using the DNS method as previously described (see section 4.2.2). The pH and temperature optima of the free and immobilized enzymes were reported as a percentage of maximal activity.

5.2.3 Temperature and pH stability

The temperature stability of each enzyme cocktail was determined by pre-incubating the enzymes (free and immobilized) at various temperatures (RT, 37˚C and 50˚C) over 24 hours. At various time intervals (0, 1, 3, 6, 12 and 24 hours), samples were assayed using the DNS method. The pH stability of each enzyme was determined at pH 3.0 and 5.0, using the universal buffer system described in section 5.2.2. The enzymes (free and immobilized) were pre-incubated in the corresponding buffers and the residual activities were determined at various time intervals (0, 1, 3, 6, 12 and 24 hours) using the DNS method. The non-pre-incubated control (0 hours) was defined as 100%.

5.2.4 Storage stability of the immobilized enzyme

The storage stability of the free and immobilized enzymes was monitored over 15 days at 4˚C and at RT (24 ± 2˚C). At various time intervals (0, 1, 3, 6, 10 and 15 days), the residual activity was determined using the DNS method. The non-pre-incubated control (0 hours) was defined as 100%.

5.2.5 Kinetic studies

The K_m and V_{max} values for the free and immobilized enzymes in Viscozyme L® and Celluclast 1.5L® were determined in sodium citrate buffer (pH 5.0) at 37˚C. Different

concentrations of AP (2.5, 5.0, 10, 20, 30 and 40 mg/ml) were used and the activity calculated using the DNS method. Lineweaver-Burk plots (Lineweaver and Burk, 1934) were derived from the Michaelis-Menten plots in order to determine the kinetic parameters.

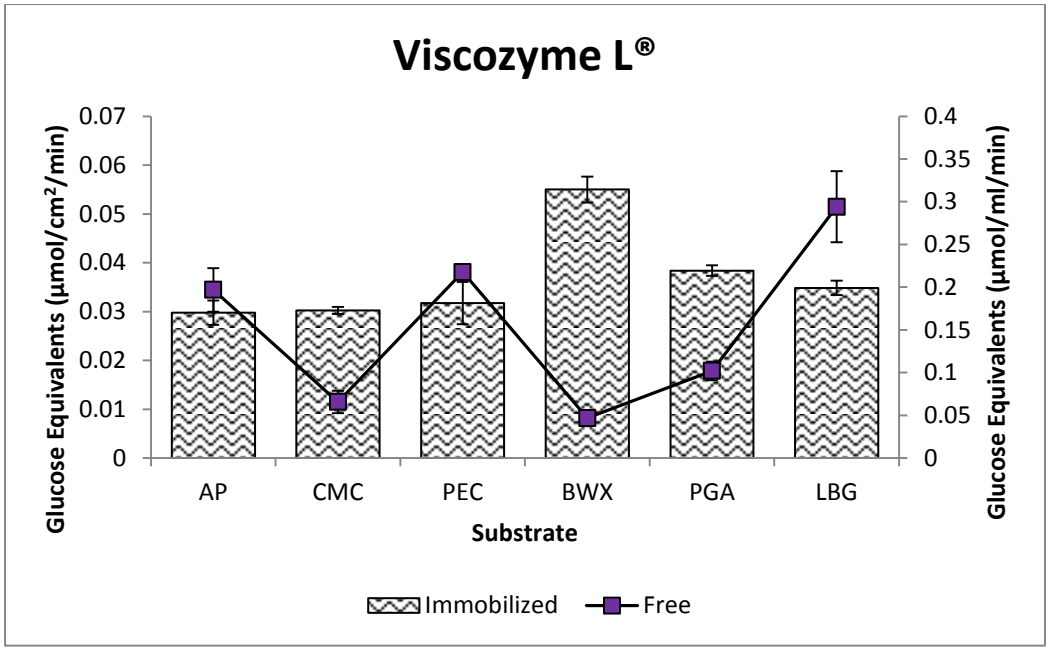
5.2.6 Reusability of the immobilized enzyme

The reusability of the immobilized enzymes was determined using the DNS method where the same functionalized nanofiber squares were assayed at 2 h intervals. After each reaction, the nanofiber squares were washed 3x with distilled water to remove any residual substrate and reintroduced into a new reaction tube with fresh substrate. Assays were performed in 50 mM sodium citrate buffer (pH 5.0) at 37˚C and at room temperature (RT) (24 ± 2˚C). The activity was reported as % activity remaining relative to the first cycle of use.

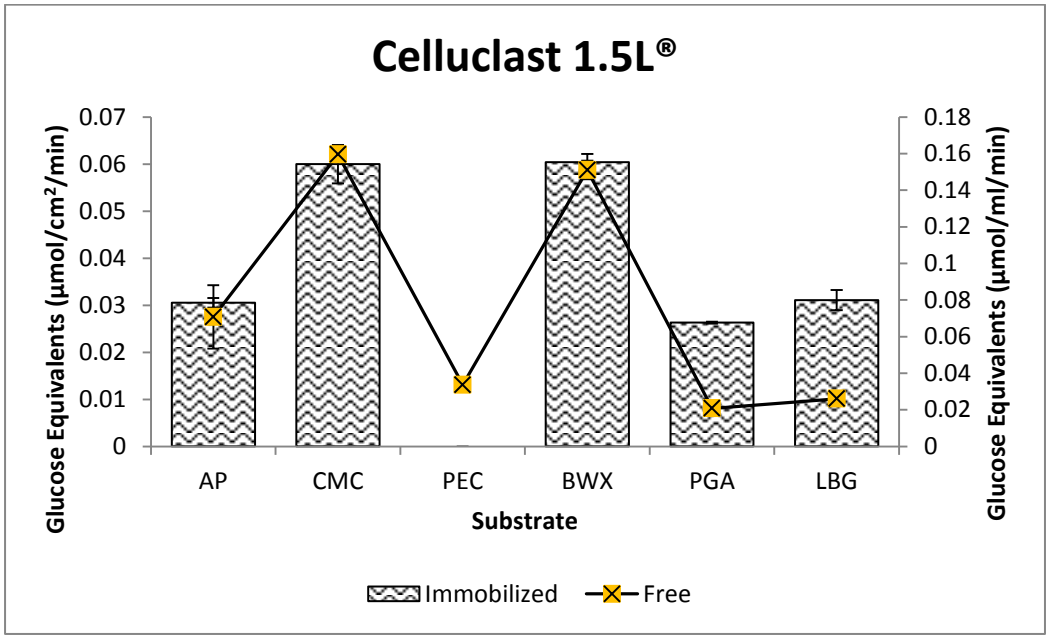
5.3 RESULTS

5.3.1 Substrate specificity

The DNS assay was used to determine the different enzyme activities in Viscozyme L® and Celluclast 1.5L® for both the free and immobilized forms. The activity was plotted as glucose equivalents in µmol/cm^2/min for the immobilized enzyme and in µmol/ml/min for the free enzyme (Figure 33).



A)



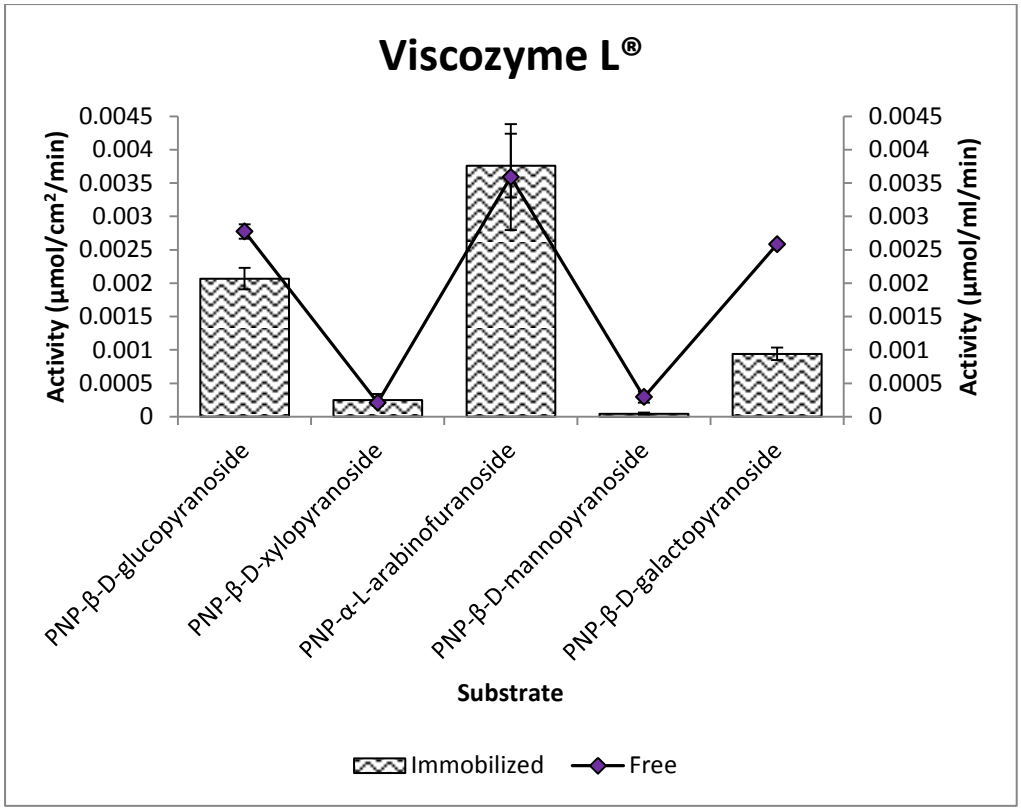
B)

Figure 33: Substrate specificity comparison of free and immobilized enzymes in Viscozyme L® A) and Celluclast 1.5L® B). Data points represent the means ± SD (n=3). The reactions were carried out at 37˚C in 50 mM sodium citrate buffer (pH 5.0) and the activities determined using the DNS method.

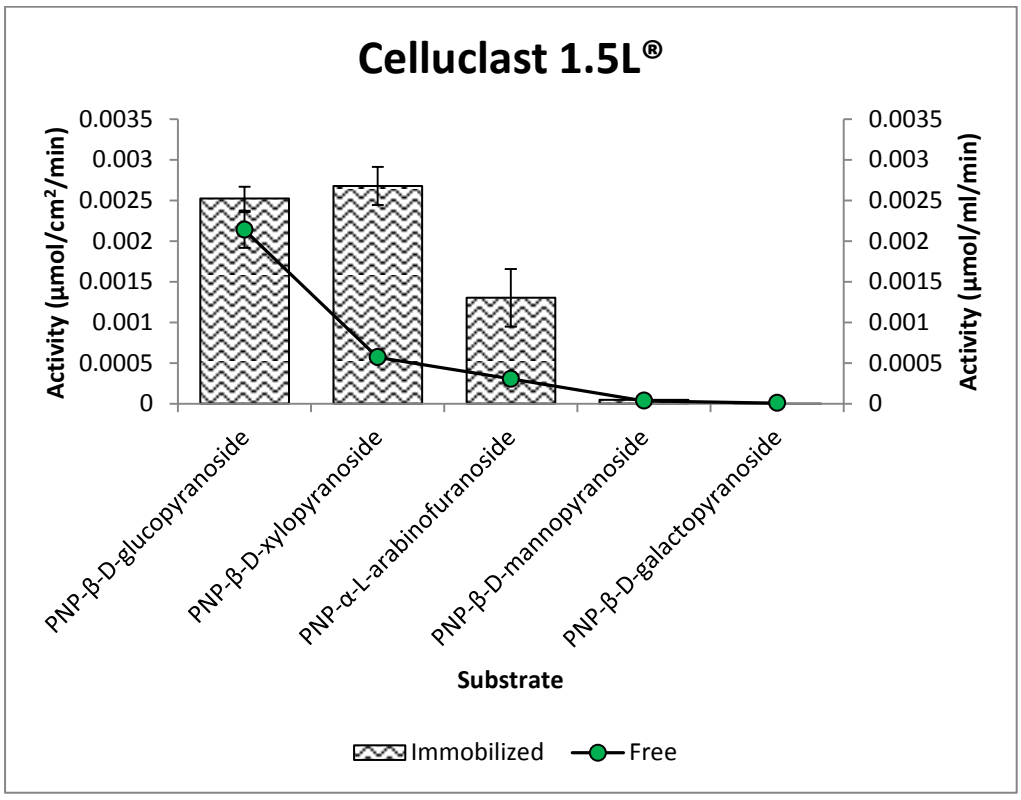
For Viscozyme L® (Figure 33A), similar activity profiles were observed for the free and immobilized enzymes, except for CMC (endoglucanase), BWX (endoxylanase) and PGA (polygalacturonase) which displayed increased retention of enzyme activity in the immobilized form relative to the other enzyme activities and compared to the free enzymes.

For Celluclast 1.5L® (Figure 33B), similar activity profiles were also observed for the free and immobilized enzymes, except for PEC (pectinase) and to a lesser extent for PGA (polygalacturonase) and LBG (mannanase). Pectinase activity was not retained in the immobilized form at all, and there was an increase in retained polygalacturonase and mannanase activity in the immobilized form relative to the other enzyme activities and compared to the free enzymes.

Additional enzyme activities were identified using the 4-nitrophenol assay method (Figure 34). The activity was calculated as Units per square of nanofiber (μmol/cm^2/min) for the immobilized enzyme and as Units per ml (μmol/ml/min) for the free enzyme.



A)



B)

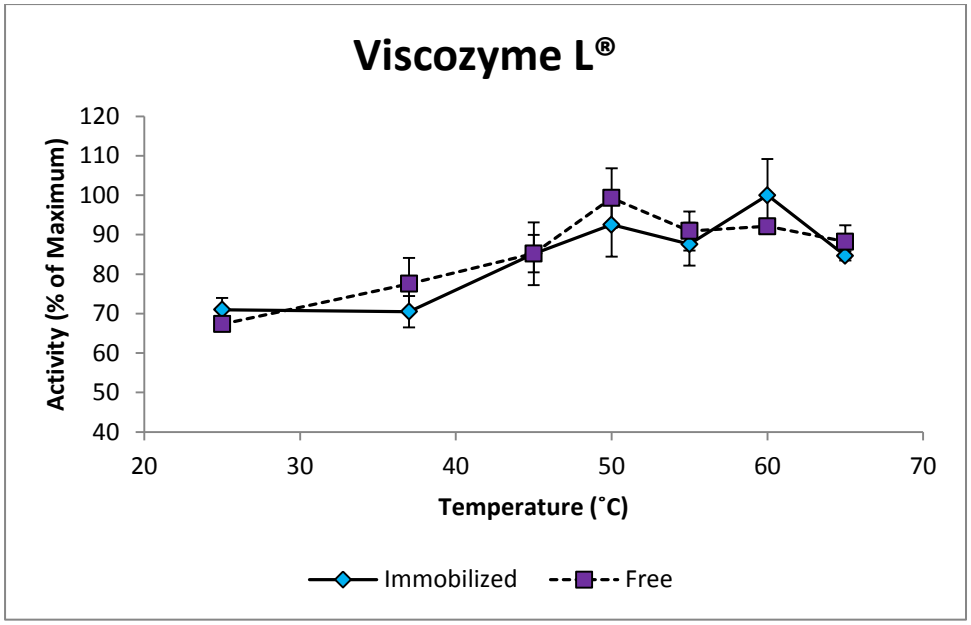
Figure 34: Substrate specificity comparison of free and immobilized enzymes in Viscozyme L® A) and Celluclast 1.5L® B). Data points represent the means ± SD (n=3). The reactions were carried out at 37˚C in 50 mM sodium citrate buffer (pH 5.0) and the activities determined using the 4-nitrophenol assay method.

For Viscozyme L® (Figure 34A), similar activity profiles were observed for the free and immobilized form of the enzymes, with a slight loss in retained β-D-galactosidase activity in the immobilized form compared to the free enzyme, relative to the other enzyme activities.

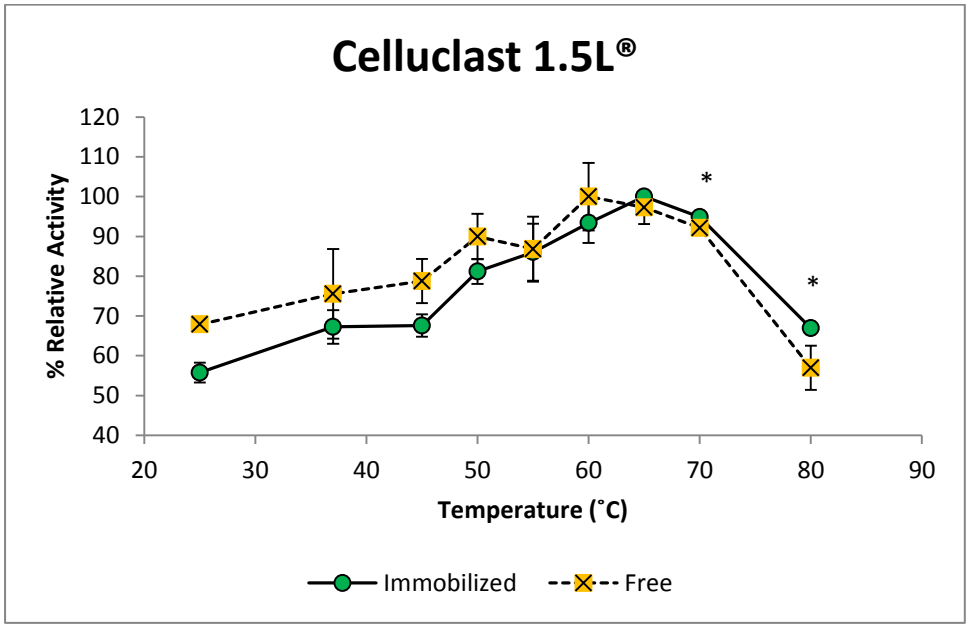
For Celluclast 1.5L® (Figure 34B), similar activity profiles were also observed for the free and immobilized enzymes, except for an increase in retained β-D-xylosidase and α-L-arabinofuranosidase activity in the immobilized form, relative to the other enzyme activities.

5.3.2 pH and temperature optimum

The pH and temperature optima were determined for the enzymes in Viscozyme L® and Celluclast 1.5L® using the DNS assay. CMC was used as the substrate for the temperature studies due to limitations in maintaining the substrates in suspension at certain temperatures. Therefore, a soluble substrate was used which did not require constant rotation/mixing. The effect of temperature on the activities of the free and immobilized enzymes was plotted as a percentage of maximal activity (Figure 35).



A)



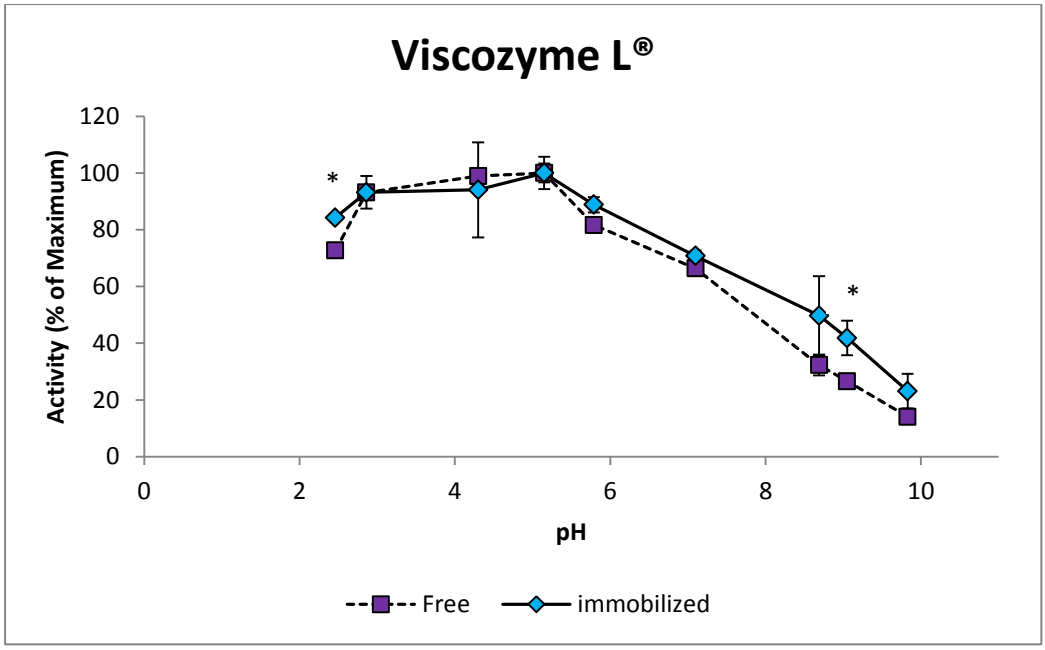
B)

Figure 35: The effect of temperature on free and immobilized Viscozyme L® A) and Celluclast 1.5L® B) enzyme activity. Data points represent the means ± SD (n=3). The reactions were carried out in 50 mM sodium citrate buffer (pH 5.0). The maximum activity was taken as 100%. ANOVA single factor analysis was used to determine significant differences between the free and immobilized enzymes *P ≤ 0.05, #P ≤ 0.01

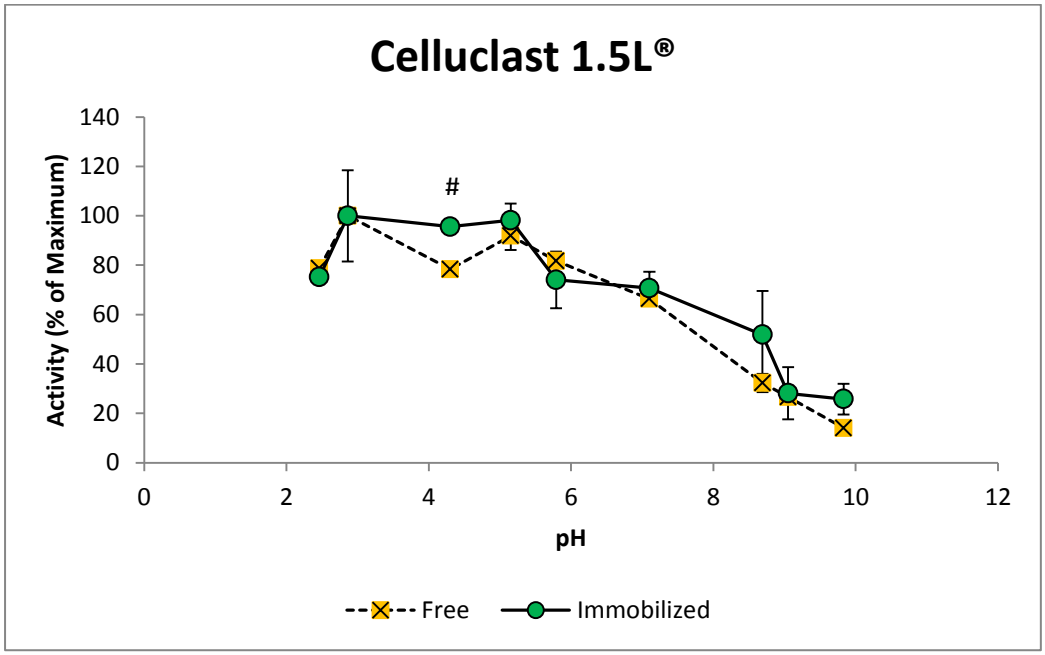
For Viscozyme L® (Figure 35A), both the free and immobilized enzymes showed a relatively high activity over a broad range of temperature (24˚C-65˚C), with 65% relative activity as the lowest at 24˚C. The highest activity for the free enzyme was at 50˚C and at 60˚C for the immobilized enzyme, although ANOVA single factor analysis could not confirm a statistically significant difference between the free and immobilized enzymes.

For Celluclast 1.5L® (Figure 35B), both the free and immobilized enzymes showed activity over a broad range of temperatures (24˚C-80˚C) as their relative activity never dropped below 50%. The highest activity observed for the free and immobilized enzymes occurred at 60˚C and 65˚C, respectively. The difference in activity between the free and immobilized enzymes was statistically significant ($P \leq 0.05$) at 70˚C and 80˚C, indicating an increased thermal stability for the immobilized enzymes.

The effect of pH on the free and immobilized enzymes was also studied and AP was used as the substrate. The pH optima of the free and immobilized enzymes in Viscozyme L® (Figure 36 A) and Celluclast 1.5L® (Figure 36B) were plotted as a percentage of maximal activity.



A)



B)

Figure 36: The effect of pH on free and immobilized Viscozyme L® A) and Celluclast 1.5L® B) enzyme activity. Data points represent the means ± SD (n=3). The reactions were carried out at 37˚C in universal buffer. The maximum activity was taken as 100%. ANOVA single factor analysis was used to determine significant differences between the free and immobilized enzymes *P ≤ 0.05, #P ≤ 0.01.

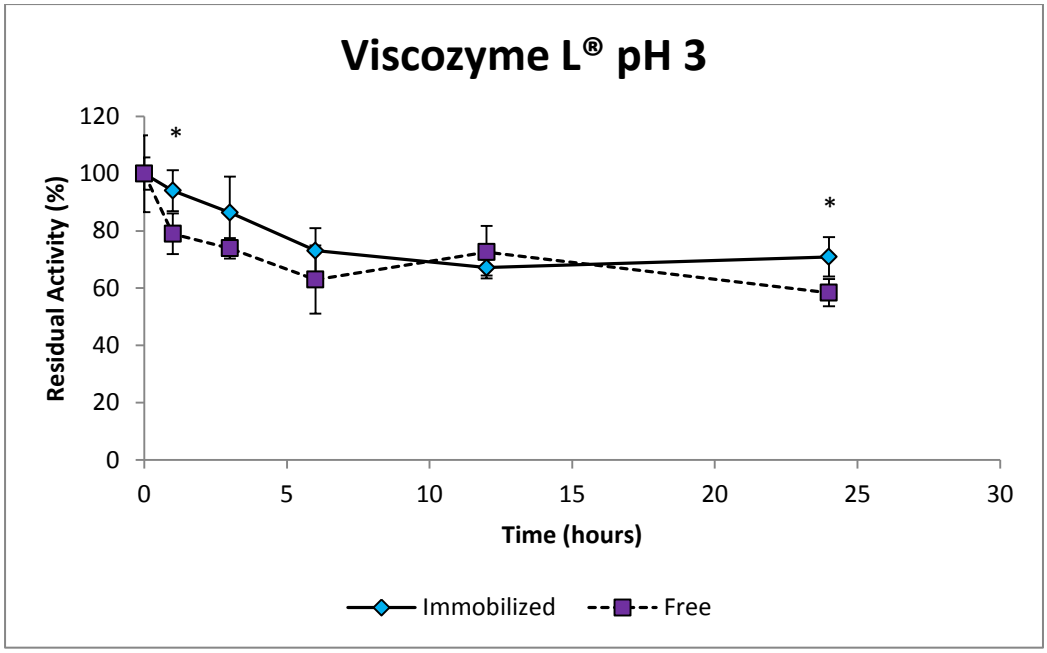
The free and immobilized enzymes in Viscozyme L® showed pH optima between pH 2.5 and 5.0 (Figure 36A). The differences were not statistically significant and the maximum activity was observed at pH 5.0 for both the free and immobilized enzymes. The immobilized

enzymes also demonstrated an increased tolerance to pH, as supported by the statistically significant increase at pH 2.5 and pH 9.0.

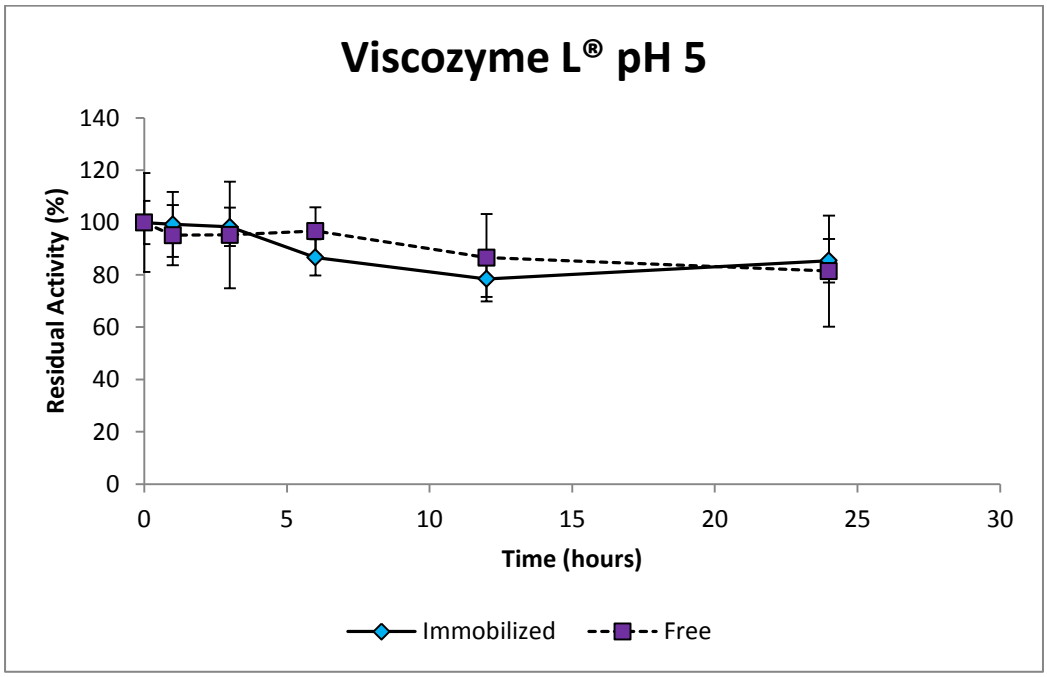
The free and immobilized enzymes in Celluclast 1.5L® showed pH optima between 3.0 and 5.0 (Figure 36B). Although the differences were also not statistically significant the maximum activity was observed at pH 3.0 for both the free and immobilized enzymes. There was however, a statistically significant difference at pH 4.0

5.3.3 pH and temperature stability

The pH stability was further investigated at pH 3.0 and pH 5.0 for the free and immobilized enzymes in Viscozyme L® (Figure 37) and Celluclast 1.5L® (Figure 38) by pre-incubating the enzymes in the respective buffers at 37˚C over 24 hours. At regular time intervals the residual activities of the enzymes were determined.



A)

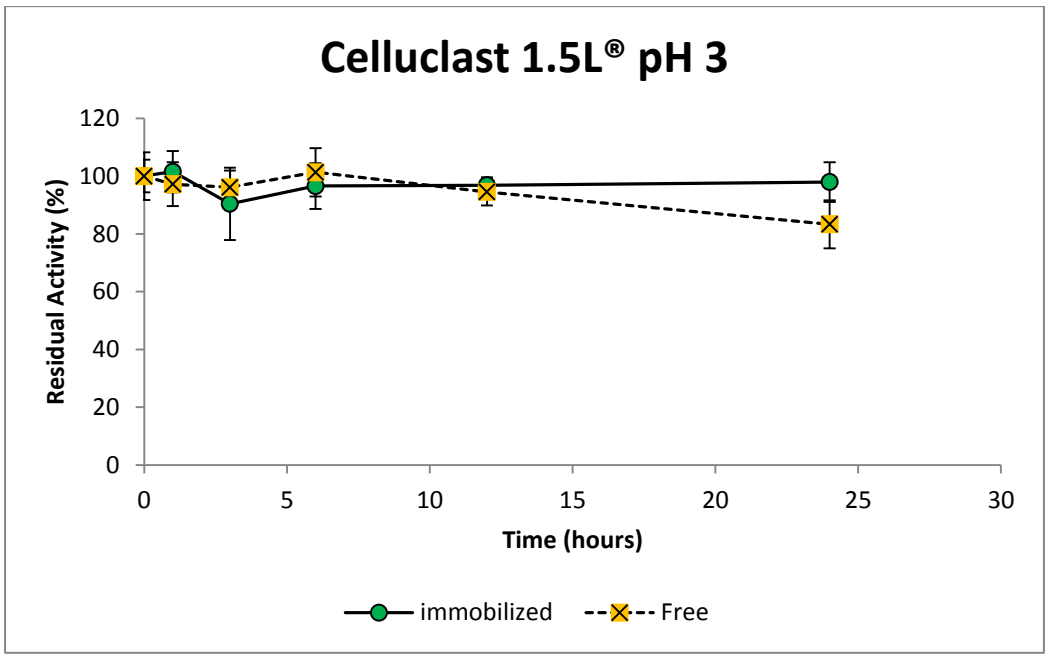


B)

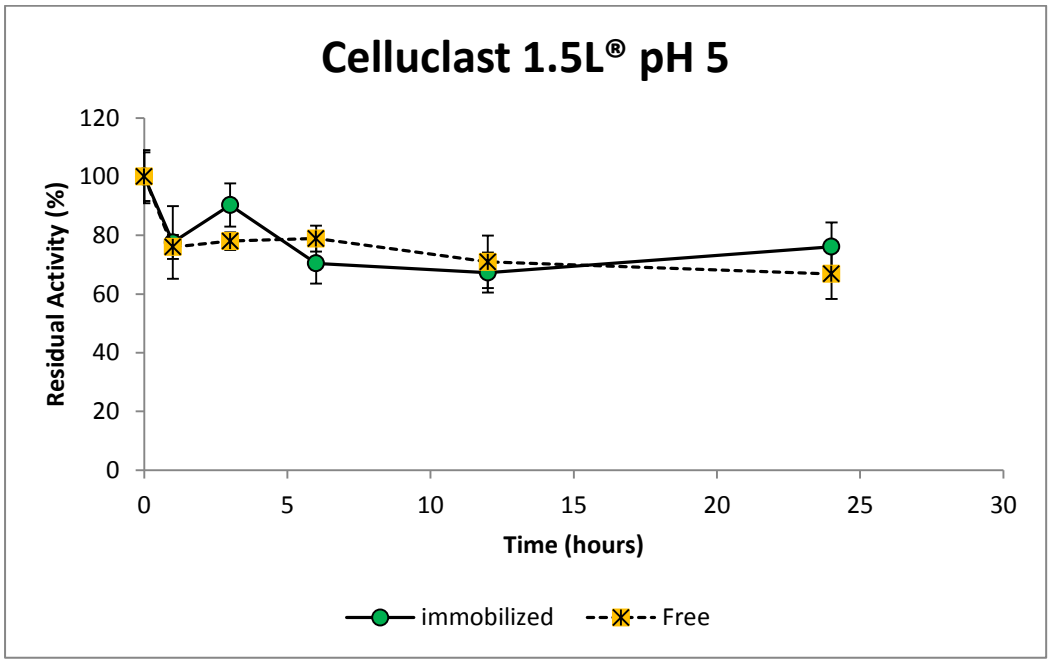
Figure 37: pH stabilities of free and immobilized enzymes in Viscozyme L® after pre-incubation in universal buffer (pH 3.0) A) and pH 5.0 B) over time. Data points represent the means ± SD (n=3). The reactions were carried out at 37˚C. The non-pre-incubated control (0 h) was defined as 100%. ANOVA single factor analysis was used to determine significant differences between the free and immobilized enzymes *P ≤ 0.05, #P ≤ 0.01.

Both the free and immobilized enzymes in Viscozyme L® were relatively stable over the 24 hour period. The residual activity never dropped below ~60% at pH 3.0 (Figure 37A) and ~80% at pH 5.0 (Figure 37B) for either the free or immobilized enzymes. At pH 3, although

the immobilized enzymes generally appeared more stable than the free enzymes as validated by ANOVA single factor analysis ($P \leq 0.05$) where statistically significant differences were observed after 1 h and 24 h, the enzymes (both free and immobilized) showed higher residual activity at pH 5 (~80% after 24 h).



A)

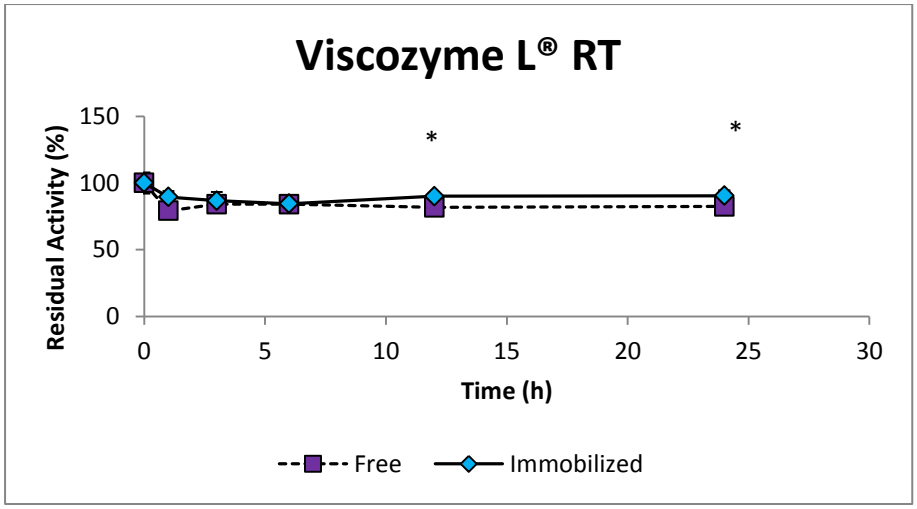


B)

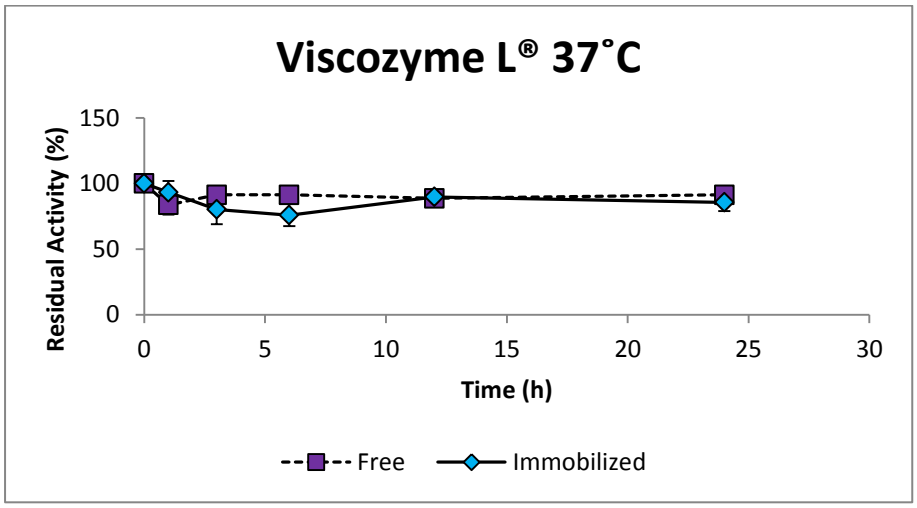
Figure 38: pH stabilities of free and immobilized enzymes in Celluclast 1.5L® after pre-incubation in universal buffer (pH 3) A) and pH 5 B) over time. Data points represent the means ± SD (n=3). The reactions were carried out at 37˚C. The non-pre-incubated control (0 h) was defined as 100%. ANOVA single factor analysis was used to determine significant differences between the free and immobilized enzymes *P ≤ 0.05, #P ≤ 0.01.

Both the free and immobilized enzymes in Celluclast 1.5L® were relatively stable over the 24 h period and there were no statistically significant differences between the free and immobilized enzymes. The residual activity never dropped below ~80% (free) or ~90% (immobilized) at pH 3.0 (Figure 38A) and ~70% at pH 5.0 (Figure 38B) for either the free or immobilized enzymes. There was a statistically insignificant increase in stability of the immobilized enzymes at pH 3.0 and pH 5.0 after 24 h ($P > 0.05$).

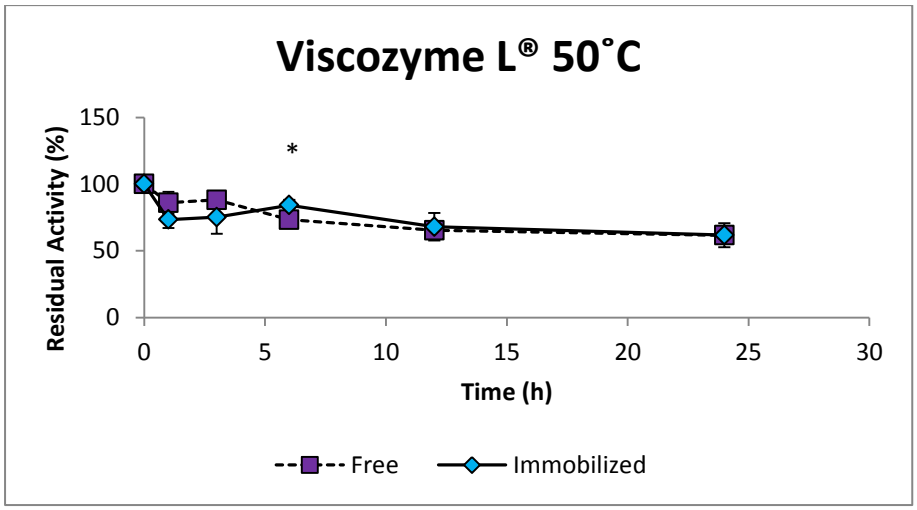
The thermal stabilities of the free and immobilized enzymes in Viscozyme L® (Figure 39) and Celluclast 1.5L (Figure 40) were studied at RT A), 37˚C B) and at 50˚C C). The enzymes were pre-incubated at the respective temperatures in 50 mM sodium citrate buffer (pH 5.0) over 24 hours. At regular time intervals the residual activitis of the enzymes were determined.



A)



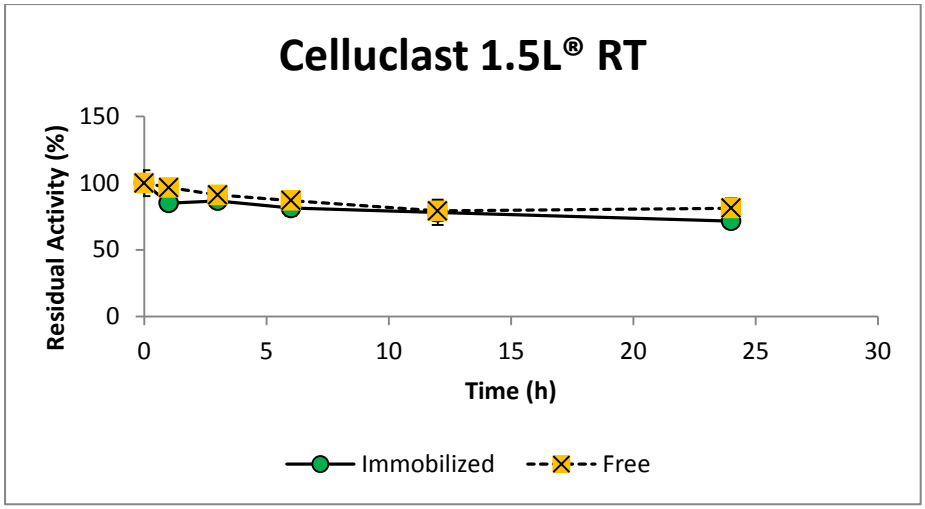
B)



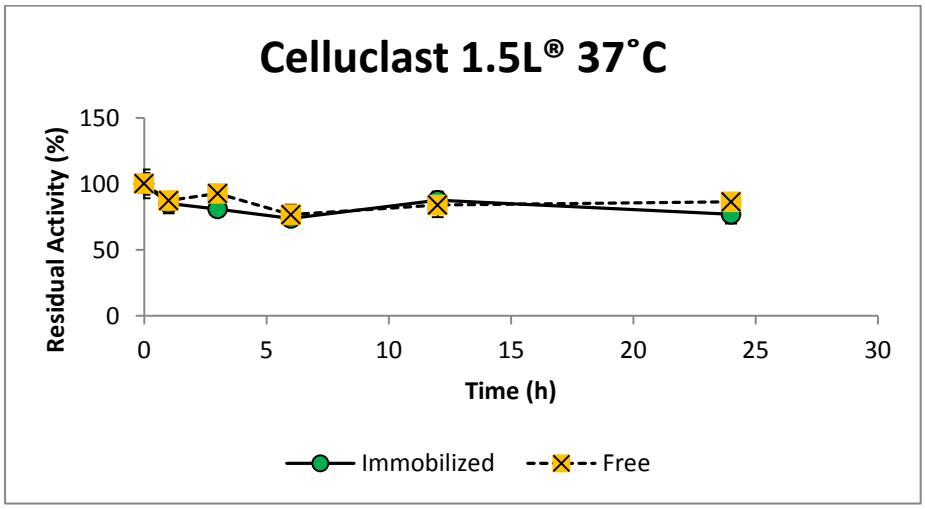
C)

Figure 39: Thermal stabilities of free and immobilized enzymes in Viscozyme L® after pre-incubation in sodium citrate buffer (pH 5) at RT A), 37˚C B) and at 50˚C C) over time. Data points represent the means ± SD (n=3). The reactions were carried out at 37˚C. The non-pre-incubated control (0 h) was defined as 100%. ANOVA single factor analysis was used to determine significant differences between the free and immobilized enzymes *P ≤ 0.05, #P ≤ 0.01.

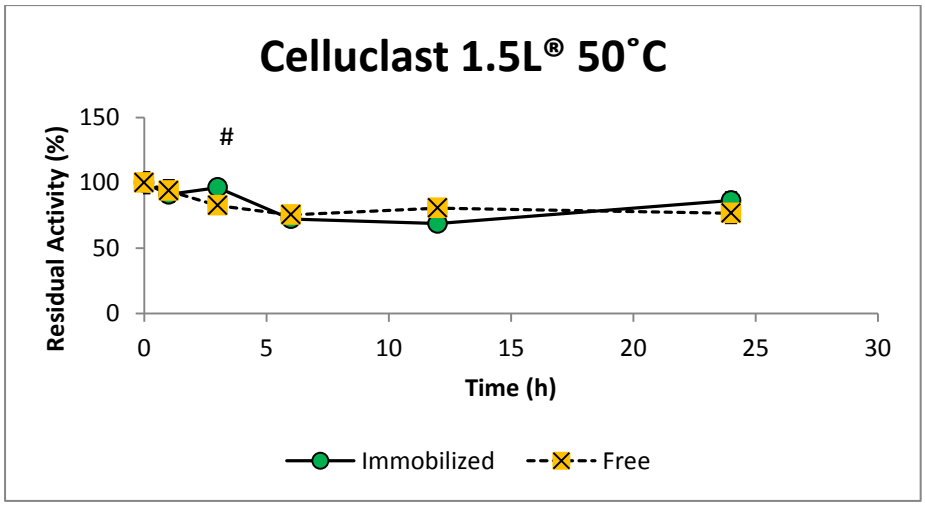
Both the free and immobilized enzymes in Viscozyme L®, at all three temperatures (RT, 37˚C and 50˚C) were relatively thermally stable (Figure 39). After incubation for 24 h, both the free and immobilized enzymes retained ~85% activity at 37˚C and ~65% activity at 50˚C. However, at RT, there was a statistically significant increase ($P \leq 0.5$) in thermal stability of the immobilized enzymes after 12 h and 24 h.



A)



B)



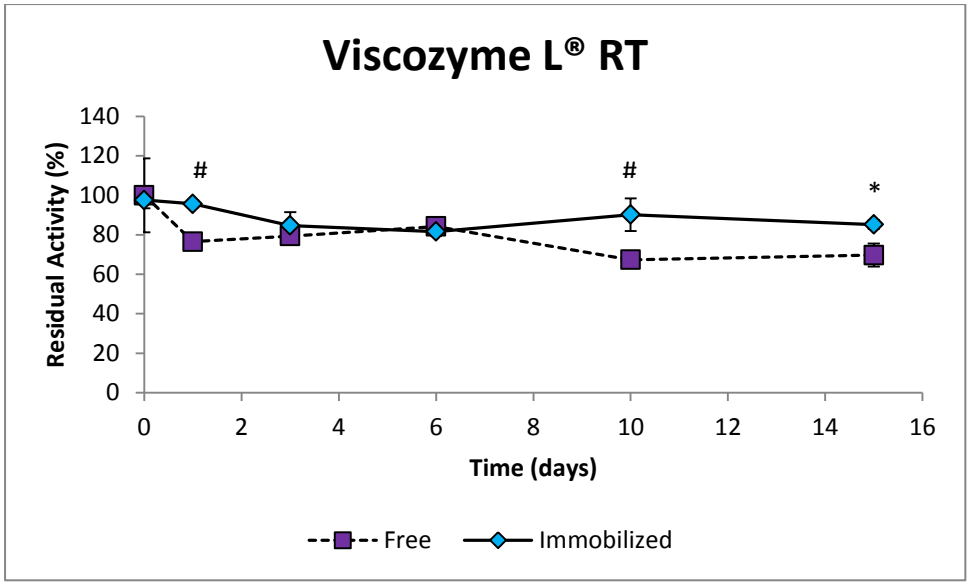
C)

Figure 40: Thermal stabilities of free and immobilized enzymes in Celluclast 1.5L® after pre-incubation in sodium citrate buffer (pH 5) at RT A), 37˚C B) and at 50˚C C) over time. Data points represent the means ± SD (n=3). The reactions were carried out at 37˚C. The non-pre-incubated control (0 h) was taken as 100%. ANOVA single factor analysis was used to determine significant differences between the free and immobilized enzymes *P ≤ 0.05, #P ≤ 0.01.

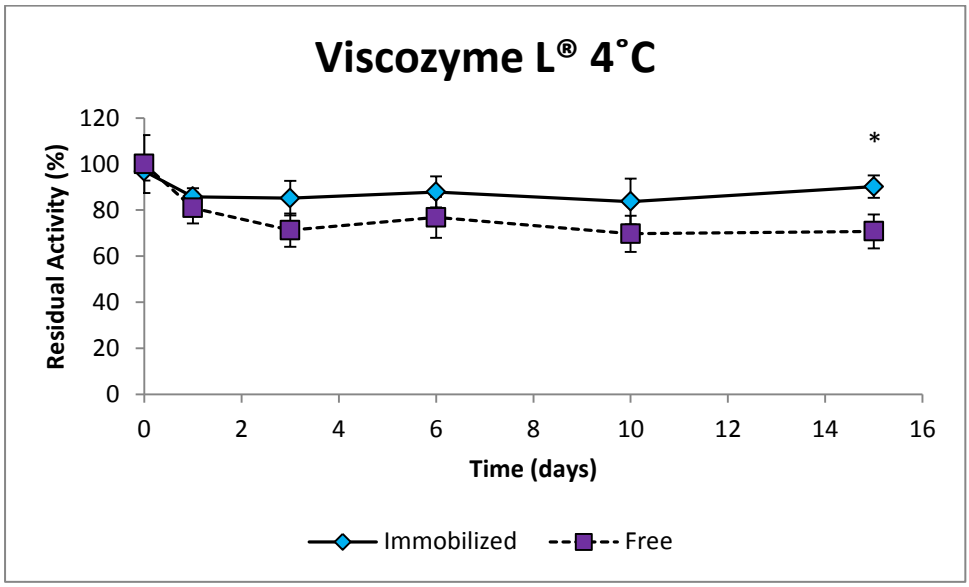
Both the free and immobilized enzymes in Celluclast 1.5 L® were relatively thermally stable at all three temperatures (RT, 37˚C and 50˚C) (Figure 40). The free and immobilized enzymes both retained approximately 80% activity at RT (Figure 40A) and at 37˚C (Figure 40B). At 50˚C, there was a statistically significant increase ($P \leq 0.1$) in thermal stability of the immobilized enzymes after 3 h and an increase after 24 h that was not statistically significant (Figure 40C).

5.3.4 Storage stability of the free and immobilized enzyme

The storage stability of the free and immobilized enzymes in Viscozyme L® (Figure 41) and Celluclast 1.5L® (Figure 42) was determined over 15 days. The enzymes were pre-incubated in 50 mM sodium phosphate buffer (pH 7.7) at RT and at 4˚C. At regular time intervals the residual activity of the enzymes was determined.



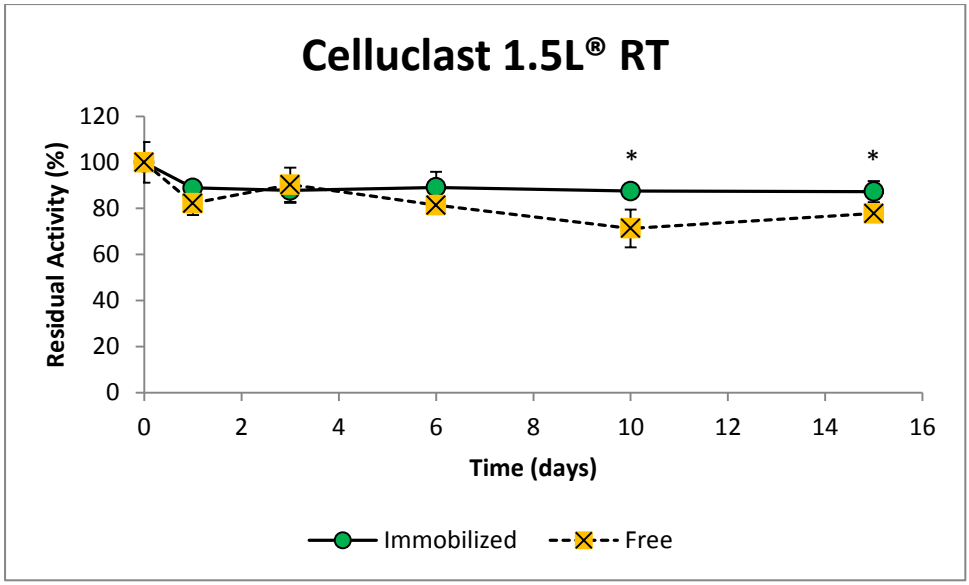
A)



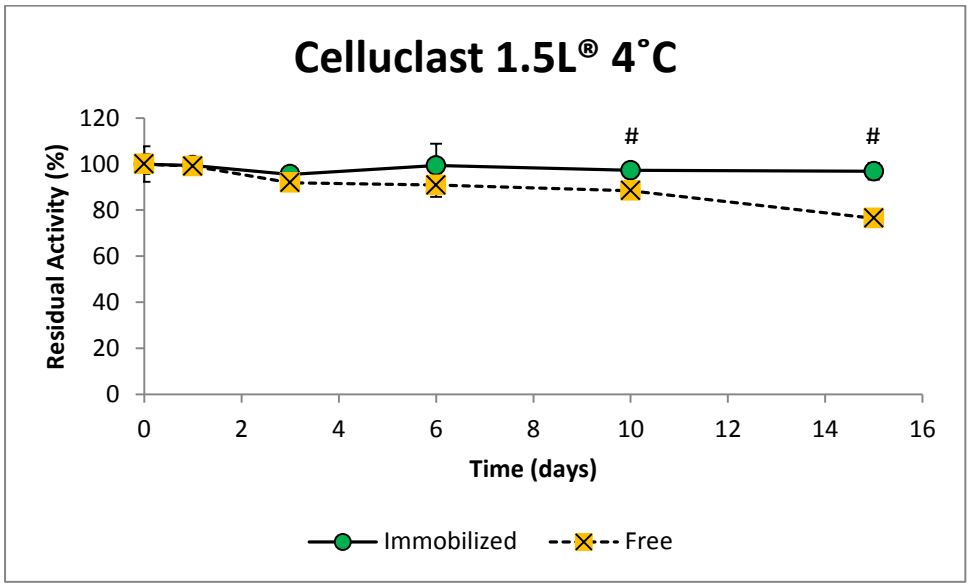
B)

Figure 41: Storage stability of free and immobilized enzymes in Viscozyme L® at RT A) and at 4˚C B) in sodium phosphate buffer (pH 7.7) over time. Data points represent the means ± SD (n=3). The reactions were carried out at 37˚C in sodium citrate buffer (pH 5). The non-pre-incubated control (0 h) was taken as 100%. ANOVA single factor analysis was used to determine significant differences between the free and immobilized enzymes *P ≤ 0.05, #P ≤ 0.01.

The general trend showed an increase in storage stability of the immobilized enzymes in Viscozyme L® at RT (Figure 41A) and at 4˚C (Figure 41B), compared to the free enzymes, particularly over longer periods. This was validated by an ANOVA single factor analysis which revealed a statistically significant increase in stability of the immobilized enzymes after 1, 10 ($P \leq 0.01$) and 15 days ($P \leq 0.05$) at RT, and after 15 days ($P \leq 0.05$) at 4˚C, compared to the free enzymes.



A)



B)

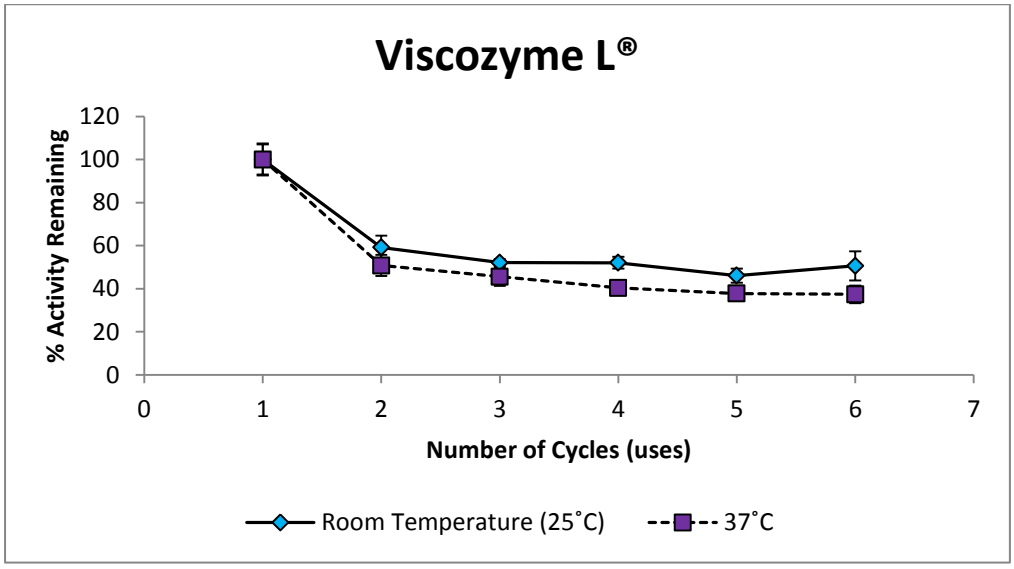
Figure 42: Storage stability of free and immobilized enzymes in Celluclast 1.5L® at RT A) and at 4˚C B) in sodium phosphate buffer (pH 7.7) over time. Data points represent the means ± SD (n=3). The reactions were carried out at 37˚C in sodium citrate buffer (pH 5). The non-pre-incubated control (0 h) was defined as 100%. ANOVA single factor analysis was used to determine significant differences between the free and immobilized enzymes *P ≤ 0.05, #P ≤ 0.01.

The general trend also showed an increase in storage stability of the immobilized enzymes in Celluclast 1.5L® at RT (Figure 42A) and at 4˚C (Figure 42B), compared to the free enzymes, particularly over longer periods. This was validated by an ANOVA single factor analysis which revealed a statistically significant increase in stability of the immobilized enzymes

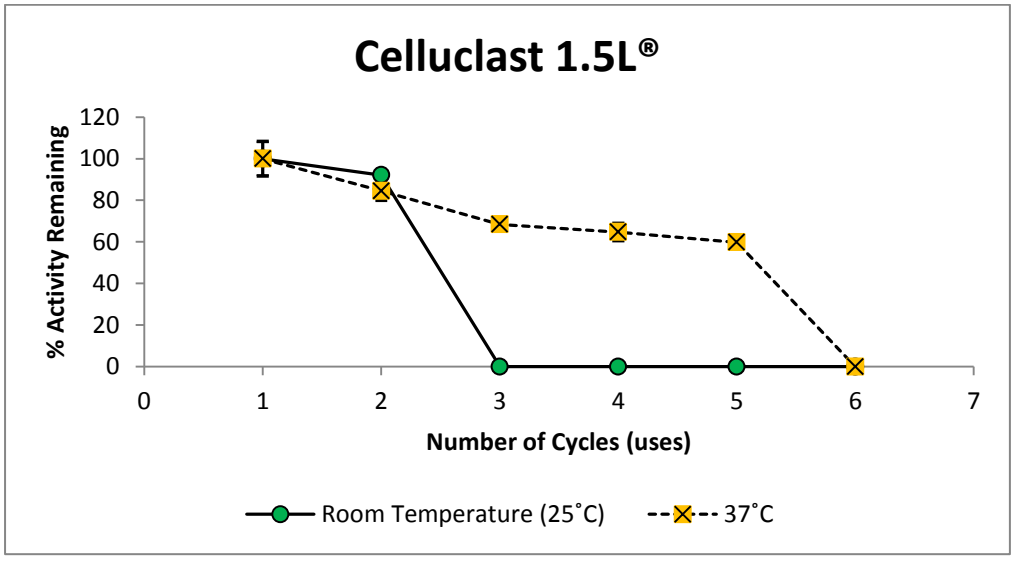
after 10 and 15 days ($P \leq 0.05$) at RT, and after 10 and 15 days ($P \leq 0.01$) at 4˚C, compared to the free enzymes.

5.3.5 Reusability of the immobilized enzyme

The reusability of the immobilized enzymes in Viscozyme L® (Figure 43A) and Celluclast 1.5L® (Figure 43B) was determined using the DNS method where the same functionalised nanofiber squares were assayed at 2 h intervals. The activity was plotted as % activity remaining relative to the first cycle of use.



A)



B)

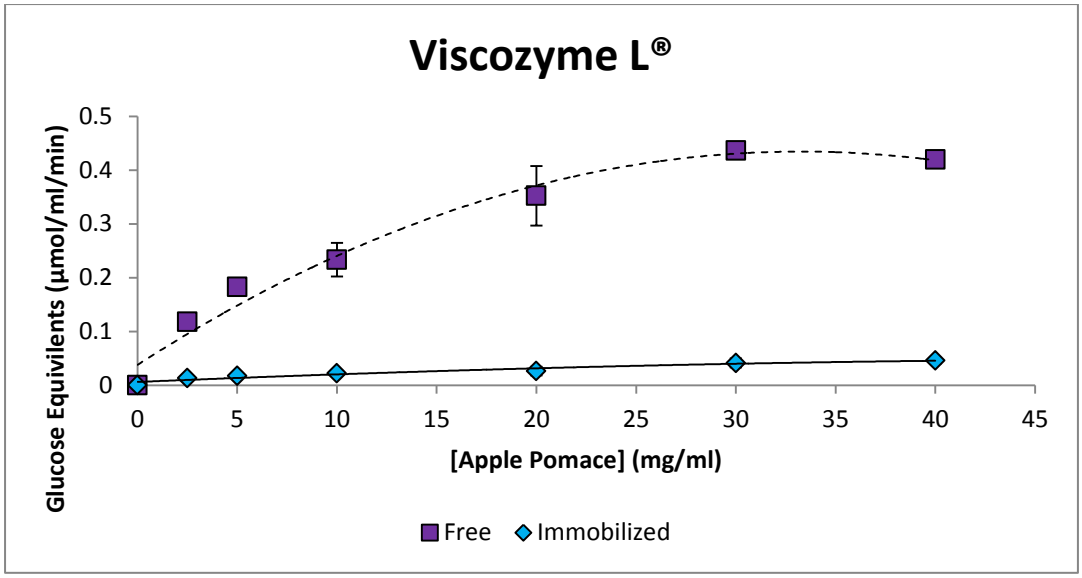
Figure 43: Reusability of the immobilized enzymes in Viscozyme L® A) and Celluclast 1.5L® B) at RT and at 37˚C. Data points represent the means ± SD (n=3). Cycle number 1 was defined as 100%.

For Viscozyme L® (Figure 43A), there was up to 40 % loss in activity after the first cycle at RT, however the enzyme activity remained stable at ~50%, even after 6 cycles. At 37˚C, there was a 50% loss in activity and after 6 cycles there was still ~40% remaining activity.

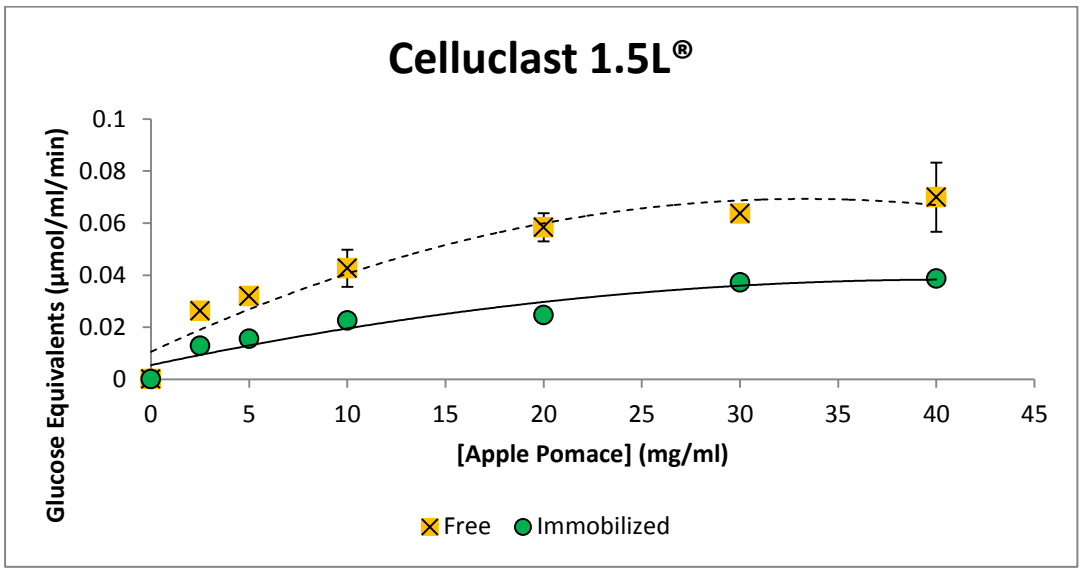
For Celluclast 1.5L® (Figure 43B), there was a gradual loss in activity at 37˚C after each cycle, with ~60% activity remaining after the 5th cycle. However, at RT by the 3rd cycle, all activity was lost.

5.3.6 Michaelis-Menten (MM) and Lineweaver-Burk (LB) plots

The Michaelis constant (K_m) and maximal velocity (V_{max}) values of free and immobilized enzymes in Viscozyme L® and Celluclast 1.5L® were determined from Lineweaver-Burk plots (Figures 45A and B, respectively). These were constructed by measurement of enzyme activity using varying concentrations of AP shown in the Michaelis-Menten plots in Figure 44.



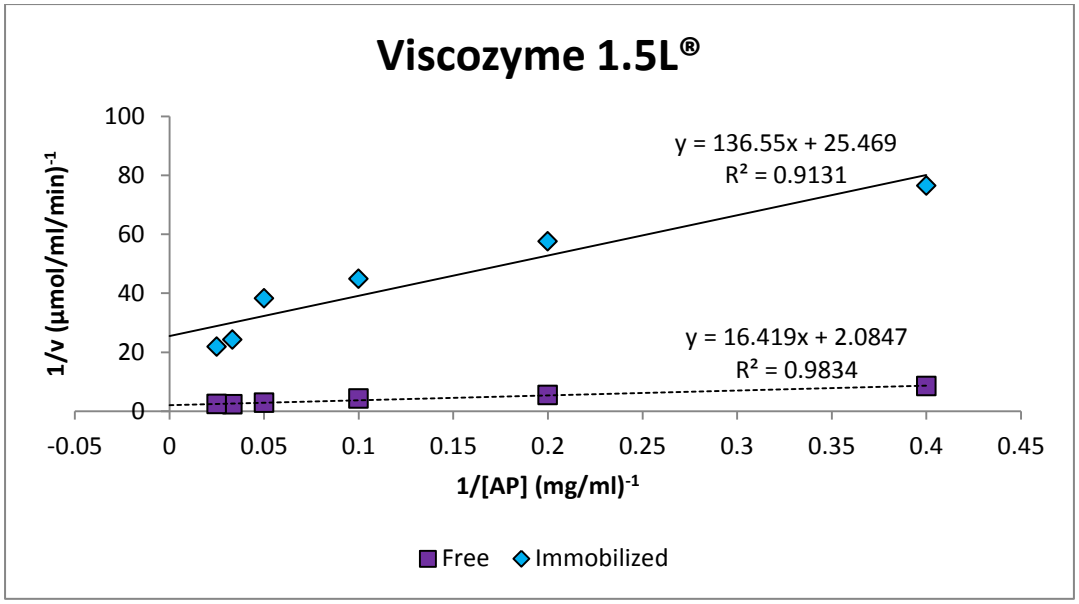
A)



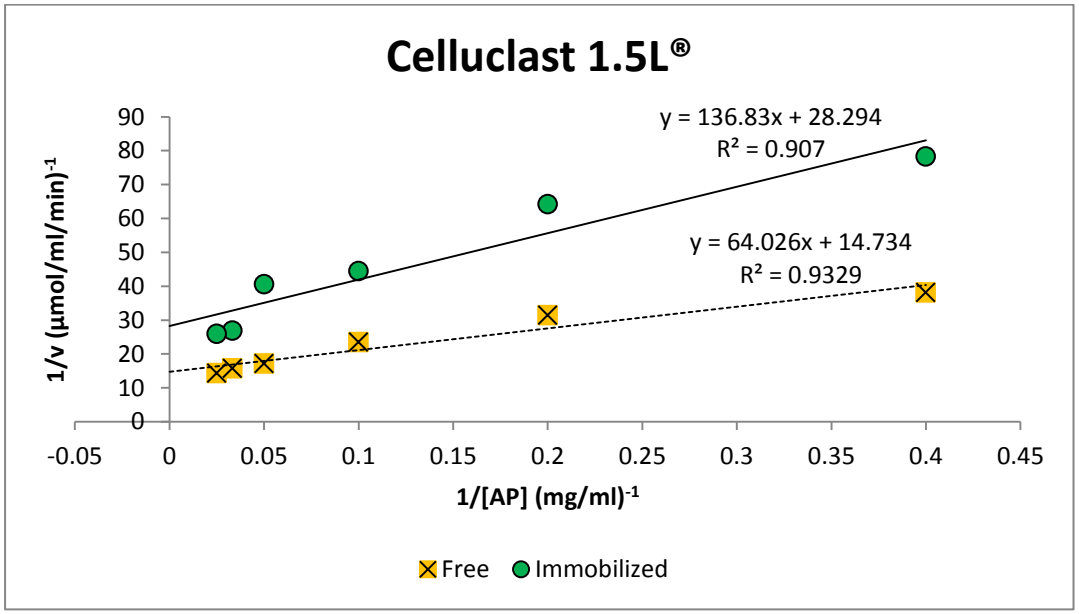
B)

Figure 44: Michaelis Menten plots for free and immobilized Viscozyme L® A) and Celluclast 1.5L® B). Data points represent the means ± SD (n=3). The reactions were carried out at 37˚C in sodium citrate buffer (pH 5).

The Michaelis-Menten plots show the velocity (*v*) of the reaction vs substrate concentration ([AP]), resulting in the hyperbolic graphs shown in Figure 44. The reciprocal of *v* and [AP] were used to construct the Lineweaver-Burk plots shown in Figure 45.



A)



B)

Figure 45: Lineweaver-Burk plots for estimation of K_m and V_{max} for the free and immobilized enzymes in A) Viscozyme L® and B) Celluclast 1.5L®.

The kinetic constants K_m and V_{max} were determined from the Lineweaver-Burk plots shown in Figure 45A and B for Viscozyme L® and Celluclast 1.5L®, respectively. The K_m and V_{max} values of the free and immobilized enzymes were calculated from the slope and y intercept of the Lineweaver-Burk plots, respectively. These values are shown in Table 3.

Table 3: Kinetic parameters of the free and immobilized enzymes in Viscozyme L® and Celluclast 1.5L®

	Viscozyme L®		**Celluclast 1.5L®**	
	Free	Immobilized	Free	Immobilized
K_m	7.876	5.361	4.345	4.835
V_{max}	0.480	0.039	0.068	0.035

From Table 3, it can be seen that the K_m (5.361 mg/ml) and V_{max} (0.039 μmol/cm^2/min) values for the immobilized enzymes in Viscozyme L® were lower than the K_m (7.876 mg/ml) and V_{max} (0.480 μmol/ml/min) values for the free enzymes. In contrast, for Celluclast 1.5L®, the K_m for the immobilized enzymes (4.835 mg/ml) was larger than that of the free enzymes (4.345 mg/ml). The V_{max} (0.035 μmol/cm^2/min) value of the immobilized enzymes, however, was also smaller than that of the free enzymes (0.068 μmol/ml/min).

5.4 DISCUSSION

The general and expected loss in activity observed between free and immobilized enzymes, in this study, may be explained by three main reasons. The first is that not all of the immobilized enzymes may have been able to partake in the reaction. The enzymes are immobilized in a random fashion and therefore, depending on their orientation, the active sites may have been inaccessible to the substrate (Lupoi and Smith, 2011) and therefore activity may have been lost. This is of particular concern when crosslinking of enzymes is involved as the active site of the enzyme may have been involved in the amidination reaction. Secondly, steric hindrances between the immobilized enzyme and insoluble/complex substrate may have partially prevented the usual binding of the enzyme to the substrate (Woodward and Zachry, 1981). Nevertheless the enzyme was still able to access the surface of the complex substrate, as evidenced by the production of reducing sugars. The third and final obvious reason is that the amount of free and immobilized enzymes (protein) assayed were different, since protein in the immobilized enzymes could not be determined as previously discussed in Chapter 4.

5.4.1 Substrate specificity

The range of different enzyme activities present in the free and immobilized enzymes in Viscozyme L® and Celluclast 1.5L® was investigated using the DNS method (Figure 33) and the 4-nitrophenol assay method (Figure 34). Since the amount of enzymes (protein) used for the free and immobilized enzyme assays were different, only relative (qualitative) observations and conclusions could be made. Although the immobilized enzymes retained similar proportions of enzymatic activities, and therefore similar trends, relative to the free enzymes, there were a few exceptions. The immobilized enzymes in Celluclast 1.5L® showed slightly higher levels of activity for arabinofuranosidase, xylosidase (Figure 34B), endomannanase and polygalacturonase activity (Figure 33B), relative to the other enzyme activities and compared to the free enzyme. Tavobilov *et al*. (1985) immobilized a complex of glycosyl hydrolases and also reported an increase (in the specific activity) of arabinan and galactomannan (LBG) hydrolysing enzymes. Pectinase activity was not retained in the immobilized form at all for Celluclast 1.5L® (Figure 33B). The differences in retained enzyme activities may be explained by the nature of the support and enzymes as well as the crosslinking effects. These reasons may also be used to explain the increased retention of endoglucanase, endoxylanase, polygalacturonase and reduced retention of endomannanase activities in Viscozyme L® (Figure 33A), relative to the free enzymes. The immobilization and crosslinking procedure may have bound the enzymes in such a way that their orientation or conformation favoured and exposed the enzyme catalytic site for substrate hydrolysis. Alternatively, for the reduced retention of immobilized enzyme activity, these enzymes may have been bound in such a way that the active site was either blocked or distorted, or the substrates were too ‘bulky’ which hindered the binding of substrates and release of products.

5.4.2 pH and temperature optima

The temperature (Figure 35) and pH (Figure 36) optima were determined for the enzymes in Viscozyme L® and Celluclast 1.5L® using the DNS assay. According to Lupoi and Smith (2011), modification of a support matrix may influence the pH of an immobilized enzyme due to surface ionic charges on the support matrix. Jordan *et al.* (2011) stated that the nature of the enzyme functional groups also play a role on pH dependency. This is because of the charges on the enzyme active site, which will determine if the enzymes’ ideal operating conditions result from an acidic or basic microenvironment. The immobilization of enzymes

in this study involved positively charged amino groups; therefore a shift in pH optimum was expected. However, in this study it was found that there was very little difference between the free and immobilized enzymes and the optimum pH remained the same.

The free and immobilized enzymes in Viscozyme L® showed a pH optima between 2.5 and 5 (Figure 36A). Although the differences were not statistically significant the maximum average was observed at pH 5 for both the free and immobilized enzymes. The immobilized enzymes also demonstrated an increased tolerance, as supported by the statistically significant increase at pH 2.5 and pH 9. Similar observations have been made by Cheng *et al.* (2010), Dalal *et al*. (2007), Hung *et al.* (2011), Roy and Gupta (2003) and Zhou (2010). Enzyme stability at a higher or lower pH range in an industrial process may be considered advantageous as additional chemicals would not be required to raise or lower the pH.

The free and immobilized enzymes in Celluclast 1.5L® showed a pH optima between 3 and 5 (Figure 36B). Although the differences were also not statistically significant the maximum activity was observed at pH 3 for both the free and immobilized enzymes. Similar pH optima were found by Lu *et al.* (2013) who immobilized β-glucosidase on carbon nanotubes. Jain and Wilkins (1986) reported a shift in the pH optimum of the immobilized enzymes in Celluclast 200L® towards a lower value (optimum pH 4.8), using CMC and untreated saw dust as substrates. Dincer and Telefoncu (2007) immobilized cellulase on modified polyvinyl alcohol coated chitosan beads and reported a shift in the optimum pH in the opposite direction (towards a higher value) from free (pH 4) to immobilized (pH 7) enzymes. It was suggested that this was due to the polyanionic surroundings. Jordan *et al.* (2011) also reported a shift in the optimum pH of cellulase bound magnetite nanoparticles from pH 4 to 5.

The unexpected similarity in pH optima between the free and immobilized enzymes in Viscozyme L® and Celluclast 1.5L® may be due to the high stability of the enzymes present in the commercial enzyme cocktails. As previously mentioned, these enzyme mixtures may contain unknown preservatives, stabilizers and detergents that may mask the ionization effects responsible for pH optimum shifts that are commonly reported in literature. Lupoi and Smith (2011) also reported identical pH optima for free and nanoparticle-immobilized cellulase. They suggested that there was little or no change in the protein's pI at the interface,

and that reasonable pH modifications at the confined micro-environment of the charged support matrix surface did not affect ionisation states in structural or active site amino acids.

The retained activities, over a wide pH range, that were observed for both Viscozyme L® and Celluclast 1.5L® may be due to high stability as well as the presence of many different enzymes in the commercial enzyme cocktails that may each have different pH optima. This may also explain the statistical significance that was observed at pH 4 in Figure 36B. The reduction in enzyme activity under non-optimal pH conditions may be attributed to variations of the ionization state of the amino acid residues that are involved in either the active site or maintaining the enzymes' structure (Lupoi and Smith, 2011).

Broader temperature optimas and shifts were expected in this study, since the thermal characteristics of an enzyme can also be affected by immobilization and often lead to increased thermal stability. In this study, CMC was used as the substrate instead of AP due to limitations in maintaining the complex/insoluble substrate in suspension at certain temperatures. Therefore, a soluble substrate was used which did not require constant rotation/mixing and could be assayed at a wide range of temperatures in a dry bath. CMC was selected over the other soluble substrates that were tested as it showed sufficient activity in both Viscozyme L® and Celluclast 1.5L, in the free and immobilized forms (Figure 33). Although using CMC as the substrate only measured endoglucanse activity, this activity was present in both commercial enzyme cocktails and this limitation was identified and considered during the analysis of the temperature optima results. CMC and citrate have been reported to have an effect on the colour development of the DNS assay (Miller, 1959), but appropriate substrate and buffer controls were used to assess the effects of these interferences.

Denaturation of free enzymes at higher temperatures happens as a result of protein unfolding from intra-molecular disruption of tertiary structure. In the immobilized form, the increased thermal stability may be explained by stabilization of the weak ionic forces and hydrogen bonding (Jordan *et al*., 2001). Jain and Wilkins (1986) immobilized the enzymes in Celluclast 200L® and reported its optimum temperature as 48˚C using CMC as a substrate and 58˚C using untreated sawdust as a substrate. An increase in temperature optima was also observed in this study, as the highest activity observed for the free and immobilized enzymes in

Celluclast 1.5L® occurred at 60˚C and 65˚C, respectively (Figure 35B). The difference in activity between the free and immobilized enzymes was statistically significant ($P \leq 0.05$) at 70˚C and 80˚C, indicating an increased thermal tolerance of the immobilized enzymes and resistance to denaturation caused by the rise in temperature. This may be attributed to the increased rigidity of the enzyme molecules that resulted from the crosslinking immobilization process. Similar findings were reported for Afsahi *et al*. (2007), Hung *et al*. (2011), Jordan *et al*. (2011), and Roy and Gupta (2003).

Both the free and immobilized enzymes in Celluclast 1.5L® (Figure 35B) showed high activity over a broad range of temperatures (24˚C-80˚C) as their relative activity never dropped below 50%. The free and immobilized enzymes in Viscozyme L® (Figure 35A), also showed a relatively high activity over a broad range of temperatures (24˚C-65˚C), with 65% relative activity as the lowest at 24˚C. Similar findings were reported by Afsahi *et al*. (2007). The highest activity for the free enzymes in Viscozyme L® was at 50˚C and at 60˚C for the immobilized enzymes, although ANOVA single factor analysis could not confirm a statistical significance between the free and immobilized enzymes.

Improved thermal tolerance can have significant industrial benefits as the enzymes are able to survive higher temperatures without undergoing denaturation and therefore compromising catalytic activity. An added value to this benefit would be if the enzymes were sufficiently thermally stable.

5.4.3 Stability

Although an increase in temperature may improve enzyme activity to a certain point, due to increased kinetic energy and therefore increased frequency of enzyme substrate collisions, the higher temperature also requires energy and therefore greater costs. This is not ideal for industrial applications involving bioreactors. For this reason, and because maintaining suspension of the substrate at elevated temperatures was problematic, the temperature stability studies of the immobilized enzymes was carried out by pre-incubating the enzymes in Viscozyme L® (Figure 39) and Celluclast 1.5L® (Figure 40) at 50˚C, 37˚C and at RT (24 ± 2˚C) over 24 hours, using AP as a substrate.

Both the free and immobilized enzymes in Viscozyme L®, at all three temperatures (RT, 37˚C and 50˚C) were relatively thermally stable (Figure 39). After incubation for 24 h, both the free and immobilized enzymes retained ~85% activity at 37˚C and ~65% activity at 50˚C. However, at RT, there was a statistically significant increase ($P \leq 0.5$) in thermal stability of the immobilized enzymes after 12 h and 24 h. Both the free and immobilized enzymes in Celluclast 1.5 L® were also relatively, thermally stable at all three temperatures (RT, 37˚C and 50˚C) (Figure 40). The free and immobilized enzymes both retained approximately 80% activity at RT (Figure 40A) and at 37˚C (Figure 40B). At 50˚C, there was a statistically significant increase ($P \leq 0.1$) in thermal stability of the immobilized enzymes after 3 h and a statistically insignificant increase after 24 h (Figure 40C). Although there were instances of statistically significant increased thermal stability, for both Viscozyme L® and Celluclast 1.5L®, in the application of these immobilized enzymes the increase may not be significant enough to compensate for the loss in activity. Therefore the feasibility of this immobilization process, in terms of thermal stability, could be questioned.

The free and immobilized enzymes in Viscozyme L® (Figure 37) and Celluclast 1.5L® (Figure 38) were also relatively stable in terms of pH. Their pH stability was investigated at the optimum pH range of 3 and 5, by pre-incubating the enzymes for various periods of time up to 24 h. For Viscozyme L®, the residual activity never dropped below ~60% at pH 3 (Figure 37A) and ~80% at pH 5 (Figure 37B) for either the free or immobilized enzymes. At pH 3, although the immobilized enzymes generally appeared more stable than the free enzymes as validated by ANOVA single factor analysis ($P \leq 0.05$) where statistically significant differences were observed after 1 h and 24 h (Figure 37A), the enzymes (both free and immobilized) showed higher residual activity at pH 5 (~80% after 24 h) (Figure 37B).

For Celluclast 1.5L® the residual activity never dropped below ~80% (free) or ~90% (immobilized) at pH 3 (Figure 38A) and ~70% at pH 5 (Figure 38B) for either the free or immobilized enzymes. There was a statistically insignificant increase in stability of the immobilized enzymes at pH 3 and pH 5 after 24 h.

The improved thermal and pH stability may be attributed to multi-point attachment of the enzymes, effectively contributing towards prevention of denaturation. Enhanced stability is a

desirable characteristic of enzymes in industrial applications. With regards to bioreactor design, improved enzyme stability may contribute to reduced costs of the necessary processes. For example, the enzymes used in this study were relatively stable between RT and 37˚C, which is the optimal conditions (28-37˚C) for SSF where the fermentative microorganisms ideally convert the resulting sugars to ethanol. This concept has been widely reported in literature (Lee *et al*., 2010; Lupoi and Smith, 2011; Philippidis *et al*., 1993; Xin *et al*., 1993).

5.4.2 Storage

The non-operational (storage) stability is also an important factor that needs to be considered in order to define the economic viability of a bioprocess involving immobilized enzymes (Dincer and Telefoncu, 2007). The free and immobilized enzymes were stored in sodium phosphate buffer (pH 7.7) for 15 days at 4˚C and at RT (±24˚C). The neutral/ mildly basic pH was selected because under acidic conditions the reaction with GA is reversible (Walt and Agayn, 1994), which may lead to enzyme leaching. At various time intervals the enzymes in Viscozyme L® and Celluclast 1.5L® were assayed and the residual activity was plotted as shown in Figures 41 and 42, respectively.

The general trend showed an increase in storage stability of the immobilized enzymes in Viscozyme L® at RT (Figure 41A) and at 4˚C (Figure 41B), compared to the free enzymes, particularly over longer periods. This was validated by an ANOVA single factor analysis which revealed a statistically significant increase in stability of the immobilized enzymes after 1, 10 ($P \leq 0.01$) and 15 days ($P \leq 0.05$) at RT, and after 15 days ($P \leq 0.05$) at 4˚C, compared to the free enzymes. The general trend also showed an increase in storage stability of the immobilized enzymes in Celluclast 1.5L® at RT (Figure 42A) and at 4˚C (Figure 42B), compared to the free enzymes and also over longer periods of time. An ANOVA single factor analysis revealed a statistically significant improvement in stability of the immobilized enzymes after 10 and 15 days ($P \leq 0.05$) at RT, and after 10 and 15 days ($P \leq 0.01$) at 4˚C, compared to the free enzymes. It was previously mentioned that the immobilized enzymes in both commercial mixtures were relatively stable in the free form in terms of pH and temperature. Similar observations have been made been made in this study, in terms of storage stability. However, this study revealed that the enzymes were more stable over longer

periods of storage (10-15 days). Although the study was conducted over 15 days, a longer time study may have revealed more significant differences between the free and immobilized enzymes. This should therefore be considered as a future study to facilitate the determination of the feasibility of this immobilization system on an industrial scale, as increased storage stability capabilities may be highly advantageous.

Concluding the stability studies; although the free enzymes remained stable, with reasonably high activity for both commercial enzyme cocktails, there was evidence of enhanced storage stability of the immobilized enzymes particularly over longer periods of time. In addition, although the immobilized enzyme activity was lower than the free enzyme, the slight enhancement of thermal (and minor pH) stability, as well as storage stability, highlights the value of the immobilized enzymes in these commercial enzyme cocktails. A stable immobilization system and long storage life offer additional advantages over soluble enzyme systems with biotechnological relevance, as they may contribute towards reduced costs associated with hydrolytic bioprocesses.

5.4.3 Reusability

Enzyme reusability is another important consideration for economic viability of a bioprocess. The recycling stability of the immobilized enzymes in Viscozyme L® and Celluclast 1.5L® was investigated by measuring the enzyme activity repeatedly. From Figure 43 A, it can be seen that the potential for reusability of Viscozyme L® was promising. There was up to 40 % loss in activity after the first cycle at RT, however the enzyme activity remained stable ~50%, even after 6 cycles. At 37˚C, after the first cycle there was a 50% loss in activity and after 6 cycles there was still ~40% remaining activity. Such reusability is vastly beneficial for recycling and repeated use of enzymes in industrial applications. These results are consistent with previous recycling studies that indicate a significant loss in enzyme activity after the first cycle, followed by a tapering off trend in relative activity after subsequent cycles (Lupoi and Smith, 2011 and Jordan *et al*., 2011).

For Celluclast 1.5L®, there was a gradual loss in activity at 37˚C after each cycle, with ~60% activity remaining after the 5th cycle (Figure 43B). Jain and Wilkins (1986) also reported a gradual loss in activity of the enzymes in Celluclast 200L® at 50˚C, with ~25% remaining

activity after the 5th cycle. The higher retained activity for Celluclast 1.5L® in the present study could be attributed to the different temperatures of the assays or possibly the crosslinking method. Jain and Wilkins (1986) conducted the immobilization using a method similar to method 1 described earlier in Chapter 4 where the support was pre-activated with GA before being immobilized. Method 2 (chapter 4), as was used for Celluclast 1.5L® in the present study, combined the GA and enzyme incubation steps where the GA may have played a role in stabilizing the binding or retention of the enzyme to the nylon nanofiber support.

At RT, the enzymes in Celluclast 1.5L® showed no activity by the 3rd cycle (Figure 43 B). This may simply have been due to the lower temperature at which the assay was conducted, with the enzymes being less active. Although 100% activity was shown as the same point for 37˚C and RT, they were both calculated independently, relative to their own activity value after 1 cycle. The actual activity value after the first cycle at RT (0.01 μmol/cm^{-1}.min) was lower than that at 37˚C (0.03 μmol/cm^{-1}.min). Since this activity is quite low, particularly at RT, for the activity to decrease to 0 by the 3rd cycle is, in fact, not a large decrease or difference in activity.

The loss in activity after each cycle for both Viscozyme L® and Celluclast 1.5L® may also be explained by possible enzyme leaching from the nylon nanofiber support, particularly the physically adsorbed enzymes (non-covalently reinforced by GA). Although the remaining reaction solutions were tested for the presence of protein (enzyme) (data not shown) after each cycle, none was present, or the levels were too low to be detected. However, it is also possible that the enzymes detached from the nylon nanofiber squares and bound to the substrate which was pelleted during centrifugation, since glycosyl hydrolases, particularly cellulases, tend to bind to their substrate during hydrolysis. The supernatant was used to perform protein assays (not the pellet), and this could explain why no protein was detected in the remaining solutions. Although the use of GA may play a role in stabilizing the binding of enzymes to the immobilization matrix, under certain conditions the condensation reaction between amino groups and aldehydes is reversible. It has been reported in literature that the use of reducing agents such as $NaBH_4$ stabilizes the Schiff's base intermediate and therefore facilitates further stabilization and retention of the enzyme bound to the support (Isgrove *et al.*, 2001). However, the effect of this reducing agent was not investigated in this study for the following reasons: firstly, it often causes inactivation of bound enzyme (Isgrove *et al*., 2001)

and may interfere with the disulphide bridges responsible for maintaining the protein's tertiary structure (Walt and Agayn, 1994). Secondly, the reaction is only reversible under acidic conditions (Migneault *et al*., 2004), and the immobilization was carried out at pH 7.7 in this study.

Another reason that could explain the loss in activity after each cycle for both Viscozyme L® and Celluclast 1.5L® is denaturation of the enzymes due to shear stress under the rotating and shaking (mixing) conditions. Similar observations were made by Lee *et al.* (2010).

5.4.4 Kinetic parameters

The kinetic behaviour of immobilized enzymes often differs from that of the free enzyme due to variations in the microenvironment caused by substrate hydrolysis. Therefore, in this study the kinetic properties were determined for Viscozyme L® and Celluclast 1.5L® and expressed in terms of Michaelis-Menten parameters (Figure 44). The kinetic constants K_m and V_{max} were determined from the Lineweaver-Burk plots shown in Figures 45 A and B for Viscozyme L® and Celluclast 1.5L®, respectively. These values are summarized in Table 3.

There have been reports in literature indicating that the optimum activity of immobilized enzymes occurs at higher substrate concentrations, compared to the free enzyme (Saiyavit *et al*., 2002; Poddar and Jana, 2011). Therefore, in an immobilized system, it was expected that the K_m value would increase (reduced enzyme substrate affinity), due to diffusional restriction of large, complex substrate molecules from the bulk solution to the microenvironment of an immobilized enzyme. However, this was not the case for the enzymes in Viscozyme L®. The K_m (5.361 mg/ml) and V_{max} (0.039 μmol/cm^2/min) of the immobilized enzymes were lower than the K_m (7.876 mg/ml) and V_{max} (0.480 μmol/ml/min) of the free enzymes. Similar observations were found by Kishore *et al*. (2012) who immobilized β-galactosidase on graphene nano-sheets. These findings may have been due to reduced mass transfer limitations associated with the use of nano-structured supports.

In contrast, for Celluclast 1.5L®, the K_m of the immobilized enzymes (4.835 mg/ml) was only slightly higher than that of the free enzymes (4.345 mg/ml). An increased K_m (and therefore reduced enzyme substrate affinity) for various immobilized glycosyl hydrolases has

been commonly reported in literature (Dalal *et al*., 2007; Migneault *et al.,* 2004; Poddar and Jana, 2011; Saiyavit *et al*., 2002; Vu and Le, 2008; Wanjari *et al.,* 2011; Wei *et al*., 2009; Woodward, 1989) and was usually attributed to mass transfer limitations that exist for the immobilized enzymes. The V_{max} (0.035 μmol/cm^2/min) of the immobilized enzymes, however, was also smaller than that of the free enzymes (0.068 μmol/ml/min). Lower V_{max} values for immobilized enzymes, as was the case with Celluclast 1.5L® (and Visczoyme L®), were also reported by Wanjari *et al*. (2011) and Vu and Le (2008). The increased K_m and decreased V_{max}, for Celluclast 1.5L®, indicates a reduced enzyme-substrate affinity and a decreased velocity in enzyme reaction. As previously mentioned, this was likely due to lower substrate accessibility to the immobilized enzyme active site and decreased transporting of substrate and products to and from the surface of the nylon support matrix. However, the comparison between the V_{max} values for the free and immobilized enzymes was made with discretion, since the units are slightly different. The free enzyme activity values were calculated per ml and the immobilized enzymes were calculated per cm (of nylon membrane). This makes comparison of the kinetics between the free and immobilized enzymes challenging, and may be one of the reasons why the free enzymes showed higher activity than the immobilized enzymes (Figure 44). In literature, there are very few studies that characterize nanofiber immobilized enzymes in terms of their kinetic parameters. Some of the papers that have reported and compared free and immobilized cellulase activities (on nanofibers) have used different dimensions. For example, Hung *et al*. (2011) cut the nanofibers into 2 cm x 2 cm squares, unlike the 1 cm x 1 cm squares used in this study. Kim *et al*. (2005) and Lee *et al*. (2009) used weight (± 1 mg) instead of size to standardize their fibers, and Wu *et al*. (2005) did not state how they standardized their nanofiber dimensions at all. In addition to this, all of these studies either reported the enzyme activities as specific activity or in percentages (rather than the actual values with units).

The R^2 values, for the linear regression analysis of the Lineweaver-Burk plots, suggest a reliable line of best fit ($R^2 > 0.9$). The slight deviations may be as a result of product inhibition, thermal inactivation (Woodward and Zachry, 1981) and release of different sugars due to the complexity of the substrate.

5.4.5 Concluding statements

To reduce production costs, the application of immobilized enzymes, which allows for enzyme recycling, has been considered as a possible solution. In this study, the immobilized enzymes in Viscozyme L® and Celluclast 1.5L® have shown advantages over the free enzymes including: 1) Reusability of immobilized enzymes (up to 6 cycles for Viscozyme L® and 5 cycles for Celluclast 1.5L at 37˚C), with adequate retention of activity (17.5 and 9.9 units of activity per g of fibrous nylon 6 membrane, respectively), 2) slightly improved pH (Viscozyme L® and Celluclast 1.5L®) and thermal (Celluclast 1.5L® at higher temperatures) tolerance, 3) increased stability profiles in terms of temperature (Viscozyme L® at RT), pH (Viscozyme L® at pH 3) and storage (Viscozyme L® and Celluclast 1.5L® at RT and 4˚C), 4) Recovery of enzyme (relatively easy separation) and easy handling, and 5) Potential for use in a continuous system for production of fermentable reducing sugars.

Although the free enzymes appeared to be quite stable, ANOVA single factor analysis did reveal instances of slightly improved stability of the immobilized enzymes, relative to the free enzymes. Even though this increased stabilization was minimal, the combined collection of all the above mentioned benefits coupled with further optimization in immobilization efficiency, suggests that the proposed system for immobilization of these enzymes offers great potential for use in industrial applications involving lignocellulose hydrolysis, bioremediation and biofuel production.

Chapter 6

General Discussion and Future Recommendations

Conversion of lignocellulosic waste is of particular interest and provides a potential solution to large scale pollution, allowing greater economic benefit and a cleaner environment. In addition to its role in bioremediation, lignocellulosic biomass conversion also offers a potential step towards the production of other value-added products, including biofuels and other chemical compounds. Immobilization of the enzymes required for these processes offers certain economic advantages, such as recovery and reusability, as well as increased stability, compared to free enzymes. Viscozyme L® and Celluclast 1.5L® are commercial enzyme cocktails that can be used for the degradation of lignocellulose; however, little is known about the immobilization of commercial enzyme mixtures containing cellulases or hemicellulases, particularly when using complex substrates, such as AP. Therefore, in this study, the hemicellulases and cellulases in Viscozyme L® and Celluclast 1.5L® were immobilized onto nylon 6 nanofibers, using AP as a substrate. The nanofibers were synthesized by electrospinning as they offer an increased surface area for more enzymes to bind, as well as reduced mass transfer limitations associated with bulk supports, all of which are important considerations for the degradation of insoluble/complex substrates (such as AP). The electrospinning process was optimized to produce smooth nylon 6 nanofibers and their morphology was analysed by SEM. The immobilization process was also optimized and the feasibility of using the immobilized enzymes was determined by characterization studies and comparison with the free enzymes.

6.1 Electrospinning of nanofibers for enzyme immobilization

Electrospun nanofibers have demonstrated excellent qualities that make them ideal candidates for enzyme immobilization. As mentioned previously, such qualities include increased surface area to volume ratios, potential for surface modification, pore sizes tailored to protein dimensions, increased porosity, interconnectivity and low mass-transfer limitations.

However, the use of nanofibers in large scale applications requires further investigation, although there have been reports in literature on the use of multiple spinnerets (Dai *et al*., 2011; Wang *et al*., 2009). Access to this type of equipment would also allow simultaneous co-electrospinning of polymers without compatible solvent systems, as was the difficulty experienced in this study.

In addition to solvent compatibility, the spinnability and stability of the polymer is also governed by several other factors that affect the electric current and charge density in the polymer. These parameters, including polymer concentration, applied voltage, needle-tip collector distance and flow rate, were therefore optimized to produce bead-free nylon 6 and polystyrene nanofibers. Two polymers were initially selected to improve biocompatibility of the immobilization support, and since the process parameters for each polymer were different and did not have compatible solvent systems, simultaneous and sequential co-electrospinning was investigated. Unfortunately, due to limitations associated with this process, nylon 6 was selected as the sole polymer for further experiments. It is an ideal polymer as it is readily available at low cost, is non-toxic and easier to handle in its nanofibrous form, compared to polystyrene.

Polymer nanofibers, providing a large surface area for the attachment of enzymes, show potential as a support for enzyme immobilization. As mentioned previously, this is particularly beneficial when insoluble/ complex substrates are being used, such as AP, which was used in this study. A nanofibrous support also offers reduced mass transfer limitations, associated with bulk immobilization supports. Therefore, in this study, electrospun nylon 6 nanofibers were used as hosts for the immobilization of the hemicellulases and cellulases in Viscozyme L® and Celluclast 1.5L®.

6.2 Optimization of Enzyme Immobilization

There are many different methods of enzyme immobilization which were outlined in Chapter 1. This study involved an enzyme crosslinking method with GA. The precise mechanism of enzyme coupling with GA is still under investigation, but many suggestions have been put forward, as discussed in Chapter 1. GA has been reported to exist as a monomer in dilute solutions up to 10% (w/v) (Kawashara *et al*., 1997) and as an α,β-unsaturated polymer under

basic conditions. These were the conditions used in this study for immobilization and the possible mechanisms of binding with protein for both forms of GA were described in Chapter 1. Although the most likely or favoured reaction under these conditions involves Schiff base formation, the complex nature of GA suggests that there may be several possible reaction mechanisms occurring simultaneously.

The conditions for efficient immobilization with GA requires an empirical approach as it is dependent on many factors, such as the nature of the enzyme and support, the pH and temperature, the concentration of the enzyme and GA, as well as the crosslinking time (Poddar and Jana, 2011). Therefore, these factors were taken into consideration for the immobilization procedure used in this study.

In order to immobilize the enzymes in Viscozyme L® and Celluclast 1.5L®, two different methods were investigated (Figure 20). The first method involved pre-activating the fibers with GA (as a spacer/ linker arm), followed by a wash step and then incubation with the enzyme solution. The second method involved binding of seed enzymes onto the fibers, followed by crosslinking of additional enzymes and aggregates to the seed enzyme molecules using GA. Although this method is prone to loss in enzyme activity due to the harsh treatment of GA, in theory the increased enzyme loading, as a result of the crosslinking enzyme aggregates, makes up for it. Unfortunately, this could not be confirmed for the current study due to protein determination limitations discussed in Chapter 4. However, in terms of activity it was found that method 2 showed more consistent trends, reproducibility and smaller standard deviations; therefore this method was chosen for further studies.

To further improve immobilization, rather than using either method 1 or method 2 as previously described, it is recommended that using both methods should be attempted together, i.e. pre-activating the fibers with GA, washing and incubating with the enzyme solution and adding GA again (Isgrove *et al*., 2001).

In this study, the effect of GA on both free and immobilized enzymes was investigated and it was shown that GA had a negative effect on the free enzymes in Viscozyme L® and the immobilized enzymes in Celluclast 1.5L®. It has been suggested that this was due to damage at the active site and distortion of the enzymes' native structure caused by the crosslinking

reaction. It would be interesting to investigate the effect of GA vapour (rather than in solution), on enzyme immobilization. Wu *et al*. (2004) used this method and reported an improved activity due to the reduced toxicity of GA vapour as a crosslinking agent. Another possible way to reduce the enzyme active site deformation during immobilization could be to carry out the immobilization in the presence of its substrate (ligand), which would shield the enzyme active site.

As discussed previously, it was suggested that crosslinking with GA is affected by pH (Migneault *et al*., 2004; Walt and Agayn, 1994). However, this is in disagreement with Isgrove *et al*. (2001), who claimed that the coupling reaction is not pH dependent. It would therefore be interesting to study the effect of pH on immobilization of the enzymes in Viscozyme L® and Celluclast 1.5L® to determine if the immobilization can be improved further. In this study, the immobilization was carried out at pH 7.7 for reasons discussed in Chapter 4.

Loss in activity of immobilized enzymes compared to the free enzymes, which is often unavoidable, was observed in this study and can be explained by the following reasons: Firstly, mobility of the enzyme is restricted due to multipoint attachment of the enzyme to the support and may result in an undesirable enzyme conformation for catalysis. Changes in the microenvironment and non-biospecific interactions between the enzyme and support may also contribute to this. Finally, electron transfer involved in the catalytic reaction may be affected by the support matrix (Wang *et al*., 2009). All these aspects are interrelated and affected by the surface chemistry of the support. In this study, the inert and hydrophobic nature of nylon 6 was addressed and attempts were made to improve enzyme binding to the nanofiber surface. This was done by hydrolytically cleaving nylon 6 with HCl and it was shown to assist with immobilization of Celluclast 1.5L®, but not Viscozyme L®, measured in terms of activity. Another possible approach that could be used to activate a support surface is through non-hydrolytic cleavage, for example, with amines. Jain and Wilkins (1986) cleaved nylon bonds with HCl and N, N-dimethylaminopropylamine (amino) and reported almost a doubling of the retained activity per 650 mg of support.

There are additional approaches that involve the surface modification of supports for improved enzyme immobilization that could be considered for future studies. One such

approach could be through the use of biomimetics layers. This works by mimicking the environment or conditions in which the enzymes would occur naturally and thus promoting their stability and therefore retention of activity (Wang *et al*., 2009). The biomimetics layer also aids in the reduction of nonspecific interactions. This was demonstrated by Huang *et al*. (2006) who utilized phospholipid moieties attached to acrylonitrile/2-methacryloyloxyethyl phosphorylchlorine (MPC) nanofibers as a biomimetic layer to promote immobilized lipase activity. It is therefore worth considering the anchoring of phospholipid moieties to nylon 6, for the immobilization of the enzymes in Viscozyme L® and Celluclast 1.5L®. This biomimetics layer may compensate for the highly hydrophobic nylon surface by providing a more stable, hydrophilic and biocompatible environment for enzyme immobilization.

The use of spacer molecules or linkers, as mentioned in Chapter 1, is another possible surface modification to improve enzyme binding. These are also beneficial when hydrolysing insoluble/complex substrates because the use of spacer molecules, for example polyethylene glycol, helps reduce the steric hindrance by the substrate. Other examples of spacer molecules that have been used in literature include polyethyleneimine (PEI), chitosan, polylysine (Isgrove *et al.,* 2001) and GA (Wang *et al*., 2009). These may also provide more binding sites for each amino group that originally existed on the nylon surface. The use of GA as a spacer molecule was investigated in this study (described previously using immobilization method 1). However, there were difficulties experienced using method 1 as inconsistent trends, non-reproducible results and high standard deviations were observed.

When optimization studies on initial protein concentrations were carried out, the highest activity for Viscozyme L® was observed when an initial concentration of 4 mg/ml was used; therefore this concentration was used for further experiments. For Celluclast 1.5L®, there was no significant increase in activity with initial protein concentrations higher than 2 mg/ml, therefore this protein concentration was used for further experiments with Celluclast 1.5L®. In Chapter 4 the possibility that saturation of the binding sites on the fibrous membrane had been reached by this point was discussed. If this was true, a possible way to improve the immobilization and reduce the costs involved would be to immobilize the enzymes using lower initial protein concentrations and longer immobilization times. This study could offer substantial scope for reducing the amount of enzymes required and improving the immobilization efficiency, provided long immobilization times can be tolerated.

When optimizing immobilization time, GA was only added after the first hour to allow for equilibration and initial binding of seed enzymes. Since 5 h (4 h crosslinking time) resulted in the highest activity for Viscozyme L®, this immobilization time was used for further studies. The same immobilization time was used for further studies with Celluclast 1.5L®, even though activity decreased (possibly due to effects of GA as previously discussed in detail in Chapter 4), as it was thought that GA would assist with maintaining enzyme stability. However, subsequent stability studies revealed very limited improvements in enzyme stability. This suggests that the use of GA for immobilization of Celluclast 1.5L may not be necessary.

In order to confirm enzyme binding to the fibers, FTIR analysis was used. However, limitations were also experienced here and discussed in more detail in Chapter 4. Therefore, qualitative elemental analysis using EDS was used where the presence of S was monitored to confirm the presence of enzyme. This, together with the enzyme assays, confirmed the presence of enzymes on the nanofibers (for both enzyme cocktails).

6.3 Characterization and comparison of the free and immobilized enzymes

Changes in chemical and physical characteristics have been known to occur in enzymes when they are in the immobilized form and this may result in the immobilized enzymes behaving differently to the free forms of enzymes. For this reason, comparative studies were performed involving the free and immobilized enzymes in Viscozyme L® and Celluclast 1.5L®, under various conditions. Also, in industry, enzyme immobilization offers several benefits relating to reduction of costs, such as increased stability, recovery and reuse of enzymes for the hydrolysis of lignocellulosic waste. Therefore, these factors were investigated and discussed in Chapter 5, as well as the optimum operating conditions.

The general and expected loss in activity observed between free and immobilized enzymes, in this study, may be explained by three main reasons. The first is that not all of the immobilized enzymes may have been able to take part in the reaction. The enzymes are immobilized in a random fashion and therefore, depending on their orientation, the active sites may have been inaccessible to the substrate (Lupoi and Smith, 2011) and therefore activity may be lost. This is of particular concern when crosslinking of enzymes is involved

as the active sites of the enzymes may have been involved in the amidination reaction. Secondly, steric hindrances between the immobilized enzyme and insoluble/complex substrate may have partially prevented the usual binding of the enzyme to the substrate (Woodward and Zachry, 1981). Nevertheless, the enzymes were still able to access the surface of the complex substrate, as evidenced by the production of reducing sugars. The third and final obvious reason is that the amount of free and immobilized enzymes (protein) assayed were different, since protein in the immobilized enzymes could not be determined as previously discussed in Chapter 4.

The pH and temperature optima were determined for the enzymes in Viscozyme L® and Celluclast 1.5L® and it was found that there was very little difference between the free and immobilized enzymes in terms of optimum pH. This unexpected similarity in pH optima between the free and immobilized enzymes may be due to the high stability of the enzymes present in the commercial enzyme cocktails. As previously mentioned, these enzyme mixtures may contain unknown preservatives, stabilizers and detergents that may mask the ionization effects responsible for pH optimum shifts that are commonly reported in literature. Lupoi and Smith (2011) also reported identical pH optima for free and nanoparticle-immobilized cellulases. It was suggested that there was little or no change in the protein's pI at the interface, and that reasonable pH modifications at the confined micro-environment of the charged support matrix surface did not affect ionisation states in structural or active site amino acids.

In terms of temperature optima, the highest activity observed for the free and immobilized enzymes in Celluclast 1.5L® occurred at 60˚C and 65˚C, respectively. The highest activity for the free enzymes in Viscozyme L® was at 50˚C and at 60˚C for the immobilized enzymes. This increased thermal tolerance was attributed to stabilization of weak ionic forces and hydrogen bonding (Jordan *et al*., 2001).

Broader temperature optima and shifts were expected in this study, since the thermal characteristics of an enzyme can also be affected by immobilization and often lead to increased thermal stability.

Although the stability studies indicated that the free enzymes were quite stable, ANOVA single factor analysis did reveal instances of slightly improved pH, thermal and storage stability of the immobilized enzymes, relative to the free enzymes. However, these statistically significant increases do not necessarily indicate systematically significant increases. Therefore, stability alone is not beneficial enough in this immobilization system to establish it as a feasible process. For this reason, recovery and reusability are important factors in determining the viability of this immobilization system.

One of the characteristics of the immobilized enzymes in Viscozyme L® and Celluclast 1.5L®, that also shows potential for application in industry, is their reusability. This is particularly true at 37˚C, where the enzymes retained 60% activity after 5 cycles for Celluclast 1.5L® and 40% activity after 6 cycles for Viscozyme L®. Possible reasons for loss of enzyme activity that were discussed include enzyme leaching, deactivation or denaturation and adsorption between enzyme and separated substrate. Due to the nature of AP (being an insoluble substrate), the type of bioreactor suited for its saccharification could be a batch or fed-batch bioreactor. This is where ease of recovery of the immobilized enzymes may become advantageous. A different approach that has been reported in literature is through magnetic capturing (Kim *et al*., 2005; Lee *et al*., 2010; Liao *et al*., 2010). Therefore, embedding magnetic nanoparticles into the support matrix may be recommended for future work regarding the immobilization of the enzymes in Viscozyme L® and Celluclast 1.5L®, as it would provide additional value to these immobilized enzymes.

Generally, in an immobilized system, it is expected that the K_m and V_{max} values would increase (reduced enzyme substrate affinity), due to mass transfer limitations. However, this was not the case for the enzymes in Viscozyme L®. The K_m (5.361 mg/ml) value of the immobilized enzymes was lower than the K_m (7.876 mg/ml) of the free enzymes. Similar observations were found by Kishore *et al*. (2012) who immobilized β-galactosidase on graphene nano-sheets. These findings may have been due to reduced mass transfer limitations associated with the use of nano-structured supports, which supports the reasoning for choosing nanofibers as an immobilization support in this study. However, it may also be inferred that product inhibition (affecting the free enzymes more than the immobilized enzymes) contributed to these findings. Inhibition studies would need to be carried out to

confirm this, since glucose and cellobiose have been reported in literature as end product inhibitors for these types of reactions (Xiao *et al*., 2004).

The slightly increased K_m value (4.835 mg/ml) for the immobilized enzymes in Celluclast 1.5L®, compared to the free enzyme (4.345 mg/ml) was suggested to have been due to a potential change in enzyme conformation leading to reduced substrate binding and accessibility of the substrate to the enzyme catalytic site.

The R^2 values for the linear regression analysis of the Lineweaver-Burk plots suggest a reliable line of best fit ($R^2 > 0.9$). The slight deviations may be as a result of product inhibition, thermal inactivation (Woodward and Zachry, 1981) and release of different sugars, due to the complexity of the substrate.

6.4 Additional considerations and future recommendations

As previously suggested, future work may also include the investigation of inhibitory affects, particularly that of ethanol, glucose and cellobiose. These two sugars have been known to cause feedback inhibition during hydrolysis of lignocellulose (Lee *et al*., 2010; Xiao *et al*., 2004). One possible solution to this type of inhibition that has previously been used is through the addition of β-glucosidase in its free form (Duff *et al.,* 1985; Flachner *et al.,* 1999; Xiao *et al*., 2004; Xin *et al*., 1993) and in the immobilized form (Lee *et al*., 2010, Lupoi and Smith, 2011), to hydrolyse cellobiose to its glucose monomers. Another strategy is simultaneous saccharification (SS) and ethanol production (Lee *et al*., 2010; Lupoi and Smith, 2011; Philippidis *et al*., 1993; Xin *et al*., 1993,). Since the presence of ethanol and the optimal temperature (28-38˚C) operating conditions of the fermentative yeast are not the same for the hydrolytic enzyme activity, the increased stability of the immobilized form of the enzyme may retain and promote enzyme activity under non-optimal conditions.

The proposed immobilization system in this study could also be further improved by investigating the maintenance of enzyme activity under vigorous shaking conditions (Kim *et al*., 2005). This is particularly important for the effective application of immobilized enzymes in heterogeneous systems.

In addition, in this study, optimization of the immobilization and operating conditions was investigated one factor at a time. The disadvantage with this approach is that the combined interaction of each factor is not taken into consideration. A multivariate approach involves experimental designs for which the levels of all the variables are altered simultaneously. Optimization of these parameters may further be improved through the use of more accurate modelling techniques such as response surface methodology (RSM) (Hung *et al*., 2011; Kishore *et al*., 2012) or an artificial neural network (ANN) (Zhang *et al*., 2010).

Also, in this study, the enzyme cocktails were immobilized and used to hydrolyse AP and the extent of hydrolysis (and therefore enzyme activity) was measured using the DNS method. The mechanism of this assay involves the reaction of the aromatic compound, 3,5-dinitrosalicylic acid, with reducing sugars to form 3-amino-5-nitrosalicylic acid for which the absorbance can be measured at 540 nm (Miller, 1959). A reducing sugar here is defined as a sugar that has an open chain form with an aldehyde group. Therefore sugars such as sucrose and trehalose, which have monomers that are linked together via the anomeric carbons, cannot form open ring structures in which the aldehyde groups are exposed (Rivers *et al*., 1983). Therefore this assay only measures the extent of hydrolysis that results in products with an available aldehyde group. Another limitation of this assay is its susceptibility to colour interferences from complex lignocellulosic hydrolysates such as inks and tannins (Rivers *et al*., 1983). For these reasons, it is recommended that future studies involving a more specific approach investigate the hydrolysis products, using methods such as HPLC or sugar quantifying kits.

An approach that has been used to combat the recalcitrant nature of lignocellulose is through synergistic degradation. Synergy is defined in this context, as two or more enzymes working together being more efficient than the sum of the activities of the enzymes working individually (Anderson *et al*., 2008). Therefore, a combination of enzymes can be used where the addition of one enzyme acting on its substrate reduces the degree of complexity and inter-linking, thus giving access to another enzyme to act on that particular substrate. This concept can be applied to the use of Viscozyme L® and Celluclast 1.5L® since collectively they contain a diverse range of glycosyl hydrolases that can be used to synergistically degrade complex substrates such as AP. Synergistic combinations of various glycosyl hydrolases in their native or free form has been studied (Beukes *et al*., 2008; Beukes and Pletschke, 2010;

Fujita *et al.*, 2004; Hoshino *et al.*, 1997; Murashima *et al.*, 2003; Nidetzky *et al.*, 1994; Spangnuolo *et al.*, 1997), but is not fully established. However, the immobilization of these enzymes in their synergistic combinations is not well-known and this can therefore be recommended for future studies. The optimal synergistic ratio of the free enzymes in Viscozyme L® and Celluclast 1.5L® for hydrolysis of AP has been determined as 50:50 (v/v) (Gama, 2011). For future studies it may be interesting to immobilize these enzymes simultaneously on the same support, in their optimal synergistic combination. This was not done in the present study as each enzyme cocktail was optimized under different conditions. Monitoring and managing the amount of enzyme immobilized using the method described in this study would also have proved to be challenging, due to protein determination limitations. Therefore, a different immobilization approach, such as entrapment or the use of CLEAs (Costa *et al*., 2004; Wilson *et al*, 2004) may be considered to immobilize multiple enzymes in their synergistic ratios.

6.4 Overall concluding statements

It has been demonstrated that Viscozyme L® and Celluclast 1.5L® can be used to degrade fruit wastes such as AP. To facilitate the application of these enzymes in an industrial setting, these enzymes were immobilized to improve enzyme stability, reusability and ease of recovery. To improve the feasibility of enzyme immobilization when insoluble substrates (such as apple pomace) are used, electrospun nylon 6 nanofibers were synthesized as the immobilization support to provide an increased surface area for more enzymes to attach and to reduce mass transfer limitations associated with bulk supports. The potential for these enzymes in a scaled-up, bioreactor setting for the production of value added products such as ethanol still needs to be investigated. Although this proposed immobilization system is not ready for scaling up, it was a pilot study that provided a good basis for further investigation and contribution towards the eventual successful implementation of an immobilized system. The additional cost of this approach for the recycled use of enzymes and production of bio-ethanol is being considered. It is anticipated that the eventual success of this system should be pursued through the future recommendations suggested above, as well as by using slightly cheaper resources and materials as hosts for enzyme immobilization.

Chapter 7

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APPENDICES

APPENDIX A: Glucose standard curve

DNS reagent was prepared by adding the following:

100 ml 2% (w/v) NaOH

2 g Dinitrosalicylic acid (DNS)

40 g Sodium potassium tartrate (Roschelle salts)

0.1g Phenol

0.1 g Sodium Metasulfite

100 ml Distilled MilliQ water

A sugar standard curve was generated using the reducing sugar assay described by Miller (1959). Glucose standards were prepared in various concentrations between 0 and 1 mg/ml, of which, 150 µl of each was added to 300 µl DNS reagent. The sugar-DNS reagent mixture was incubated at 100°C for 5 minutes, followed by 5 minutes incubation on ice. The absorbance of the resultant colour development was measured at 540 nm using a Powerwave$_x$ microplate reader from Biotek instruments with KC junior software.

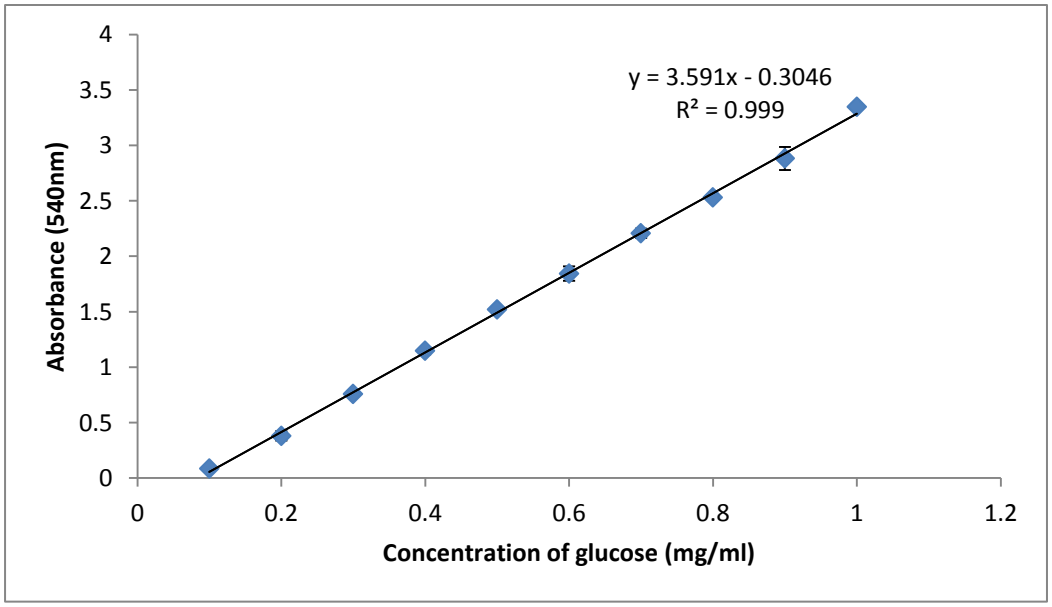

Figure A: Glucose standard curve. Data points represent the means ± SD (n=3).

APPENDIX B: Protein standard curves

The protein standard curve was generated using a modified Bradford protein assay (Bradford, 1976) and bovine serum albumin (BSA) as the protein standard. Various concentrations of

BSA were made ranging from 0 and 1.4 mg/ml. The standard curves were produced by adding 5 µl of BSA to 245 µl of the Bradford's reagent (Figure A.1) or 25 ul of BSA to 225 ul of the Bradford's reagent (Figure A.2) and allowed to stand at room temperature for 5 minutes. The samples were gently shaken for 10 seconds, and the absorbance of the resultant colour development was measured at 595 nm using a Powerwave$_x$ microplate reader from Biotek instruments with KC junior software.

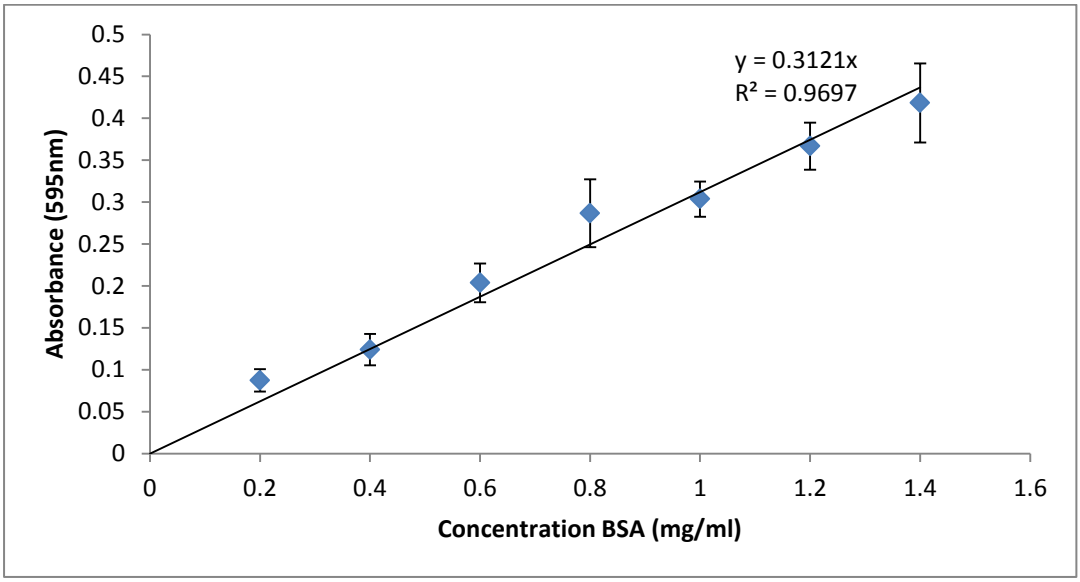

Figure B.1: Protein standard curve using 5µl of sample and 245µl of Bradford's Reagent and BSA as the standard. Data points represent the means ± SD (n=3).

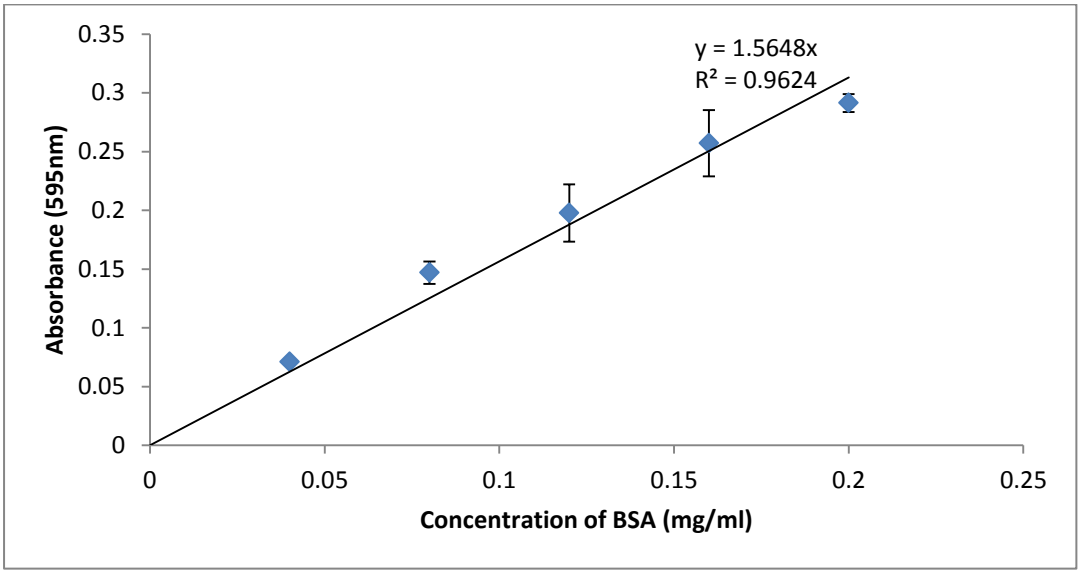

Figure B.2: Protein standard curve using 25µl of sample and 225µl of Bradford's Reagent and BSA as the standard. Data points represent the means ± SD (n=3).

APPENDIX C: 4-nitrophenol standard curve

A 4-nitrophenol standard curve was prepared using 4-nitrophenol in the range of 0.001 – 0.08 µmol/ml. Phenol standards were prepared in 50 mM citrate buffer (pH 5), with a total volume of 500 ul. After the addition of 500 ul of 2 M Na_2CO_3, the absorbance of the resultant colour development was measured at 405 nm using a Powerwave$_x$ microplate reader (from Biotek instruments with KC junior software).

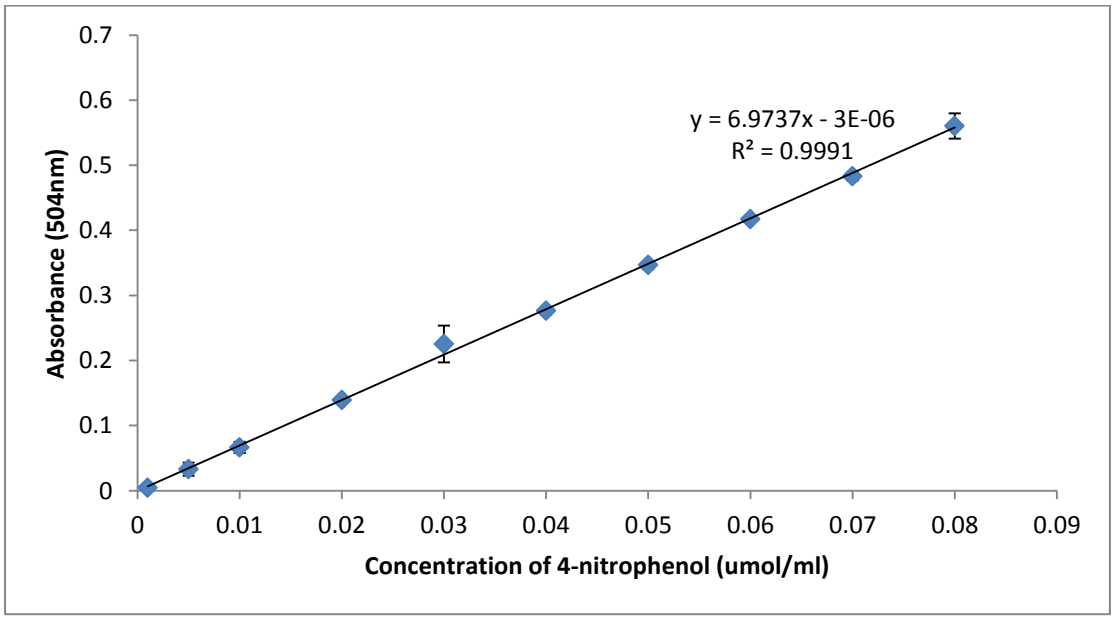

Figure C: 4-nitrophenol standard curve. Data points represent the means ± SD (n=3).

APPENDIX D: Effect of HCl activation on immobilized Viscozyme L® and Celluclast 1.5L® activity using method 1

Electrospun nylon 6 nanofibers were treated with various concentrations (0-3.18 M) of HCl for 2 h at room temperature (± 24˚C) to pre-activate the fibers. The enzymes were immobilized on the fibers using method 1 and the protein quantified using Bradford's method and A_{280}. The immobilized enzymes were also assayed for activity using the DNS method, as previously described. Assays were performed in triplicate and appropriate enzyme and substrate controls were used. The activity was reported as relative activity where 100% activity was taken as immobilized enzyme activity on the fibers that were not treated with HCl. An additional control was used where the fibers were not immobilized with GA (or HCl).

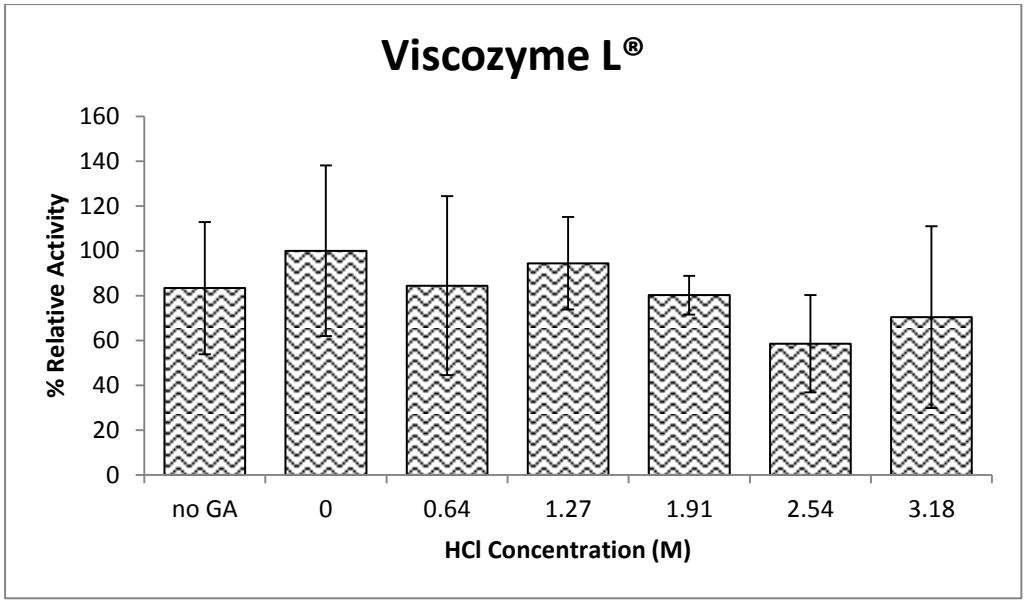

Figure D.1: Effect of HCl activation on immobilized Viscozyme L® activity, using method 1. Data points represent the means ± SD (n=3). The untreated control (0M HCl) was defined as 100%.

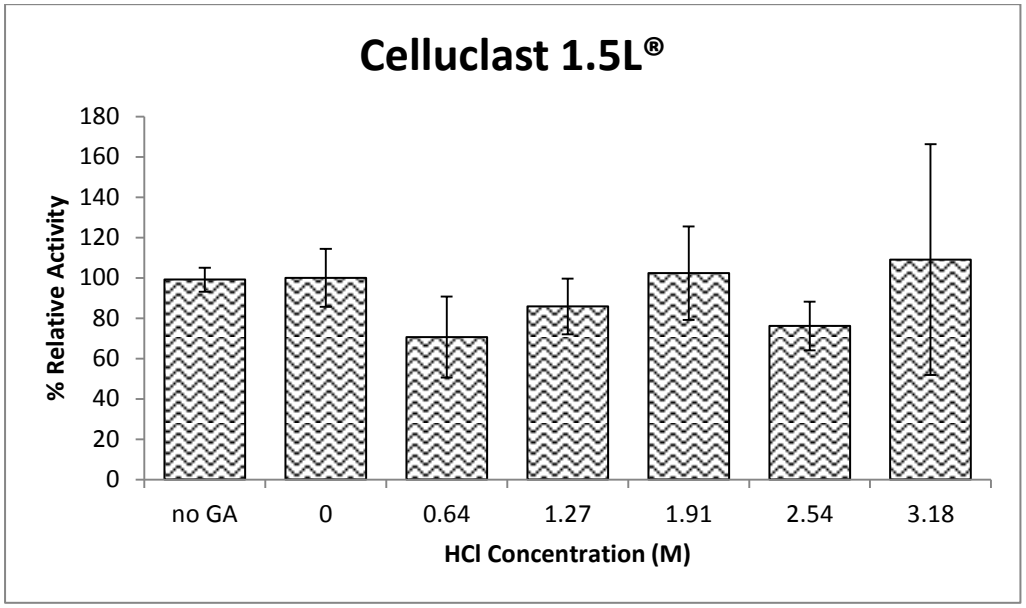

Figure D.2: Effect of HCl activation on immobilized Celluclast 1.5L® activity, using method 1. Data points represent the means ± SD (n=3). The untreated control (0M HCl) was defined as 100%.

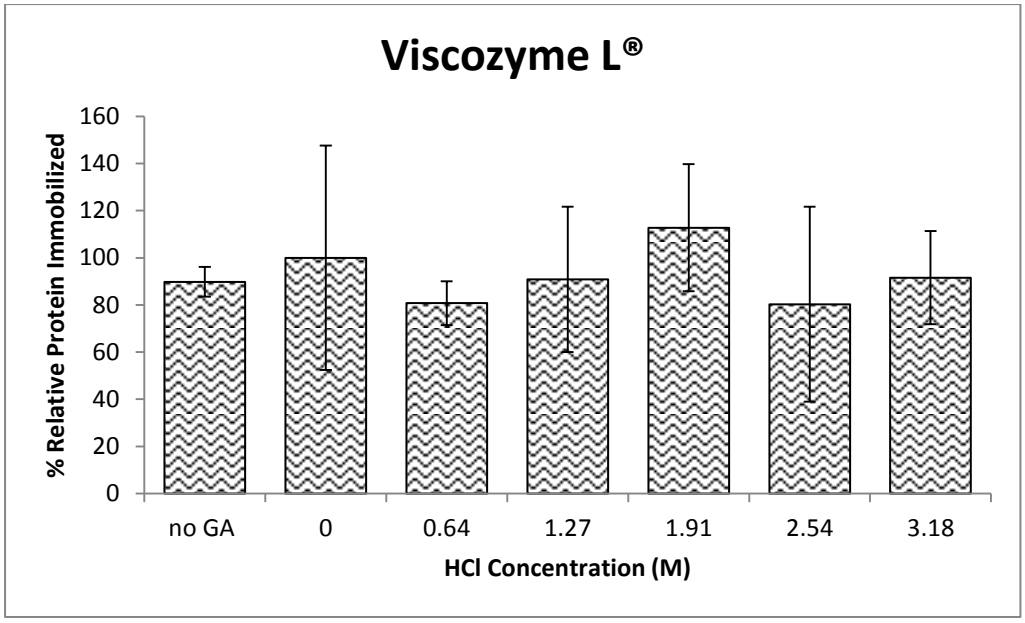

Figure D.3: Effect of HCl activation on immobilized Viscozyme L® with Bradford's assay, using immobilization method 1. Data points represent the means ± SD (n=3). The untreated control (0M HCl) was defined as 100%.

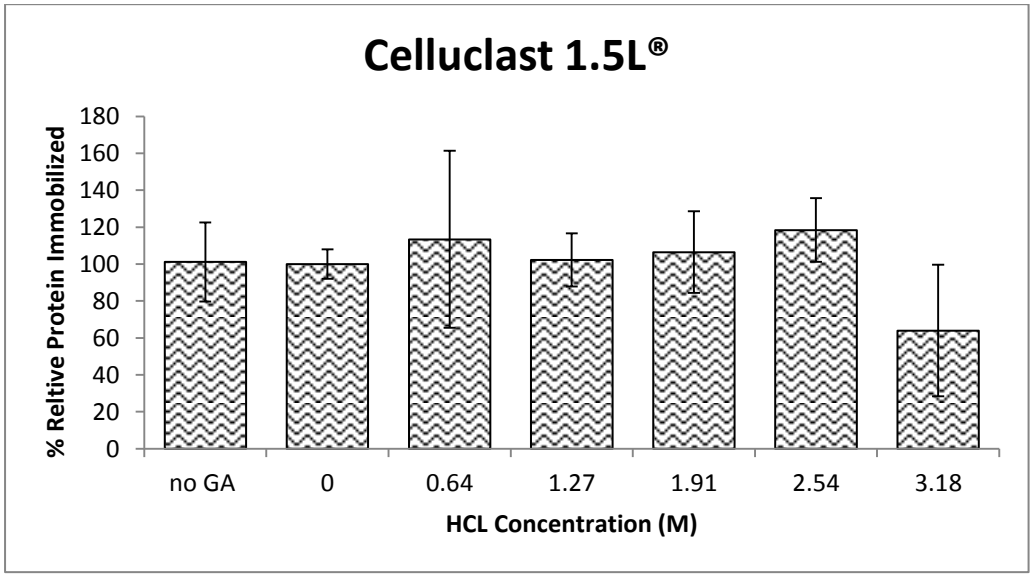

Figure D.4: Effect of HCl activation on immobilized Celluclast 1.5L® with Bradford's assay, using immobilization method 1. Data points represent the means ± SD, where n=3). The untreated control (no HCl) was defined as 100%.

APPENDIX E: Immobilization yield and efficiency calculations for Viscozyme L® and Celluclast 1.5L®

Immobilization Yield: activity of immobilized enzymes/ activity of free enzymes x 100

Viscozyme L®: 0.031 U/ 0.205 U x 100

= 15.1%

Celluclast 1.5L®: 0.032 U/ 0.075 U x 100

= 42.7%

Immobilization Efficency: Units of activity per g nylon membrane

Viscozyme L®: 0.0725 U is the average activity of enzymes on 1 square of nylon membrane with an average weight of 0.0042g

Therefore, 1 g of nylon membrane has 0.0753 U/ 0.0042 g

= 17.5 U / g nylon membrane

Celluclast 1.5L®: 0.0416 U is the average activity of enzymes on 1 square of nylon membrane with an average weight of 0.0042g

Therefore, 1 g of nylon membrane has 0.0416 U/ 0.0042 g

= 9.9 U / g nylon membrane

LIST OF REAGENTS

3,5-Dinitrosalicylic acid	Sigma (Cat. No. D0550)
Acetic acid	Merck (Cat. No. 1.00063)
Bovine Serum Albumin (BSA)	Sigma Aldrich (Cat. No.A7906)
Bradford reagent	Sigma (Cat. No. B6916)
Carboxymethyl cellulose	Sigma (Cat. No. C5678)
Celluclast 1.5L®	Sigma (Cat. No. ATCC 26921)
Citric acid	Sigma-Aldrich (Cat. No. C0759)
Formic acid	Sigma-Aldrich (Cat. No. F0507)
Glutaraldehyde (25%)	Sigma-Aldrich (Cat No. G6257)
Hydrochloric acid (32%)	Fluka (Cat No. 38281)
Locust bean gum	Fluka (Cat. No. 62631)
Nylon 6	Aldrich (Cat. No. 181110)
Pectin (Apple)	Sigma (Cat. No. P8471)
Phenol	Merck (Cat. No. 8.22296)
Polygalacturonic acid	Sigma (Cat. No. P3850)
Polystyrene	Fluka (Cat. No. 81401)
Sodium azide	Merck (Cat. No. 8.22335)
Sodium citrate	Aldrich (Cat. No. W302600)
Sodium hydroxide	Saarchem (Cat. No. 5823200)
Sodium potassium tartrate	Merck (Cat. No. 1.08087)
Sodium phosphate (di)	Sigma (Cat. No. S5136)
Sodium phosphate (mono)	Sigma (Cat. No. S3522)
Viscozyme L®	Sigma-Aldrich (Cat. No. V2010)
Xylan (beechwood)	Fluka (Cat. No. 95588)