SphereZyme™ Technology for enhanced enzyme immobilisation: Application in biosensors

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Abstract

Self-immobilisation enzyme technologies, such as SphereZyme™, suffer from the lack of applicability to hydrolyse large substrates. Solid support immobilisation is usually a method of choice, to produce a stable biocatalyst for large substrates hydrolysis in the industry. In order to investigate this limitation, a commercial protease called Alcalase® was chosen as a model enzyme due to its natural activity (hydrolysis of large substrates-proteins).

Prior to immobilising through the SphereZyme™ technology, Alcalase® was partially purified through dialysis followed by CM Sepharose™ *FF* cation exchanger. Sample contaminants, such as salts and stabilisers can inhibit protein crosslinking by reacting with glutaraldehyde. Alcalase® was successfully separated into 3 proteases with the major peak correlating to a positive control run on native PAGE, indicating that it was likely subtilisin Carlsberg.

A 16% alkaline protease activity for azo-casein hydrolysis was retained when 5% v/v PEI: 25% v/v glutaraldehyde solution was used as a crosslinking agent in Alcalase® SphereZyme™ production. An increase in activity was also observed for monomeric substrates (PNPA) where the highest was 55%. The highest % activities maintained when 0.33 M EDA: 25% v/v glutaraldehyde solution was initially used as crosslinking agent were 4.5% and 1.6% for monomeric and polymeric substrates, respectively. PEI is a hydrophilic branched polymer with an abundance of amine groups compared to EDA.

A comparison study of immobilisation efficiencies of SphereZyme™, Eupergit® and Dendrispheres was also performed for large substrate biocatalysis. The two latter technologies are solid-support immobilisation methods. Dendrispheres reached its maximum loading capacity in the first 5 minute of the one hour binding time. Twenty minutes was chosen as a maximum binding time since there was constant protein maintained on the solid support and no enzyme loss

was observed during the 1 hour binding time. PEI at pH 11.5, its native pH, gave the highest immobilisation yield and specific activity over the PEI pH range of 11.5 to 7. SphereZyme™ had the highest ratio for azocasein hydrolysis followed by Dendrispheres and Eupergit®.

The SphereZyme™ was also shown to be applicable to biosensors for phenol detection. Different modifications of glassy carbon electrode (GCE) were evaluated as a benchmark for the fabrication of SphereZyme™ modified phenol biosensor. GCE modified with laccase SphereZyme™ entrapped in cellulose membrane was the best modification due to the broad catechol range (<0.950 mM), high correlation coefficient (R^2, 0.995) and relative high sensitivity factor (0.305 $\mu A.mM^{-1}$). This type of biosensor was also shown to be electroactive at pH 7.0 for which its control, free laccase, lacked electroactivity. From the catalytic constants calculated, GCE modified with laccase SphereZyme™ entrapped in cellulose membrane also gave the highest effectiveness factor (I_{max}/K_m^{app}) of 1.84 $\mu A.mM^{-1}$. The modified GCE with Alcalase® SphereZyme™ was relatively more sensitive than GCE modified with free Alcalase®.

Output

Poster presentation

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List of Abbreviations

A	Amperes
BAPNA	N-benzoyl-L-arginine-*p*-nitroanilide
(BSA)	Bovine Serum Albumin
CA	Chronoamperometry
CE	capillary electrophoresis
CLEA	Crosslinked Enzyme Aggregates
CLEC	Crosslinked Enzyme Crystals
CM	Carboxymethyl weak cation
CNT	Carbon-nanotubes
CV	Cyclic voltammetry
D-A	Dextran Aldehyde
DEAE	Diethylaminoethyl weak anion
D-E	Dextran Epoxy
DET	Direct electron transfer
DMAEMA	Dimethylaminoethyl methacrylate
DMSO	Dimethylsulfoxide
DP-A	DMSO PEG Aldehyde
EDA	Ethylenediamine
E	Extinction Coefficient
FF	Fast Flow
GA	Glutaraldehyde
GCE	Glassy Carbon Electrode
GC-MS	Gas chromatography, mass spectrometry
HEMA	2-Hydroxyethyl methacrylate
I	Current response
I_{max}	Limiting current response
ISFET	Ion-selective field effect transistors
K_m^{app}	Michaelis-Menten constant

LiTCNE	Lithium tetracyanoethylenide
mGCE	Modified Glassy Carbon Electrode
MPc	Metallophthalocyanine complexes
NP	Nanoparticles
OD	Optical density
P-A	PEG-Aldehyde
PCMCs	Protein-coated microcrystals
P-E	PEG Epoxy
PEG	Polyethylene glycol
PEI	Polyethyleneimine
Ph	Phenol
pI	Isoelectric Point
PLL	Poly-L-lysine
PNP	*p*-Nitrophenyl
PNPA	*p*-Nitrophenol Acetate
Poly/NIPAM	poly-N-isopropylacrylamide
PSCC	Palm seed coat carbon
PVA	Polyvinyl alcohol
RT	Rotavirus
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis
SEN	Single enzyme nanoparticles
S	Substrate concentration
SP	Sulfopropyl strong cation
TEMED	N,N,N, N"tetramethylethylenediamine
TNT	Titanate nanotubes
Tris	2-Amino-2-hydroxymethyl)-1,3-propanediol
V	Volts

1 Literature Review

1.1 General Introduction

Enzymes are important bio-macromolecules and considered nature‟s catalysts (Berger *et al*., 1992; Panke *et al*., 2004). Their catalytic function and superior features such as regioselectivity and chiral specificity and reduced by-products has led to their application in replacing chemical catalysts as more and more industries are resorting to environmentally safer processes (Kilara and Shahani, 1979). The application of enzymes for performing chemical reactions is known as biocatalysis (Kilara and Shahani, 1979). Furthermore, enzymes are becoming popular for applications such as research tools, in manufacturing, and in the food and medical industries (Berger *et al*., 1992; Christen and Lopez-Munguia, 1994; Chung and Baek, 1999).

Enzymes are however, unstable in conditions such as high temperature, pH, organic solvent and mechanical stresses such as shear which are often required for their use as biocatalysts (Cao, 2005; Alcalde *et al*., 2006). They may also be prone to autolysis and proteolysis due to their biological nature (Reezt and Jaeger, 1998; Cao *et al*., 2003; Christensen *et al*., 2003). To limit these drawbacks, numerous efforts have been devoted to the development of stabilised, immobilised enzymes (Kilara and Shahani, 1979; Bornscheuer, 2003; Cao, 2005). The general definition of enzyme immobilisation is the inter-crosslinking (using a suitable crosslinking agent) of biocatalysts and/or their attachment or adsorption to a functionalised support (Michaelis and Menten, 1964). In addition to stability, enzyme immobilisation offers several advantages for their application to biocatalysis including reduced product contamination (simple separation from reaction) and simplified recovery for recycling or re-use which can reduce process costs (Tischer and Kasche, 1999; Christensen *et al*., 2003; Bornscheuer, 2003).

Immobilisation methods can generally be divided into 2 groups, namely support-based and self-immobilisation (Cao *et al*., 2003). Carrier-bound or solid support immobilisation involves linking enzyme molecules covalently or non-covalently to a solid support, which renders them insoluble, and further imparts chemical, mechanical and thermal stability to the enzyme (Reetz and Jaeger, 1998). Non-covalent linkages such as adsorption or entrapment may result in enzyme leaching out into the reaction medium which may lead to substrate and product contamination (Boller *et al*., 2002; Cao, 2005; Sheldon, 2007). This is less of a concern with covalent immobilisation due to improved bond strength between the solid support and the enzyme (Sheldon, 2007).

A second immobilisation method, known as self-immobilisation involves the covalent linking of enzymes to each other (rather than a solid support) using crosslinking chemicals or agents such as glutaraldehyde (Cao *et al*., 2001; Roy and Abraham, 2004). The major advantage of this technique is considered to be high activity to weight ratio improving the volumetric productivity of the catalyst, as well as the potential for reduced catalyst cost, as there is no requirement for a non-catalytic support (Cao, 2005; Sheldon, 2007). The other advantages of self-immobilisation include the potential use of impure enzymes for immobilisation (e.g. CLEA"s) and also the ability to achieve improved chemical and mechanical stability as compared to solid support immobilisation (Tischer and Kasche, 1999; Cao *et al*., 2003).

The immobilisation of biocatalysts does however suffer from several drawbacks, including the lack of applicability for the hydrolysis of large polymeric substrates (Nakagomi and Ajisaka, 1990; Galaev and Mattiasson 1999; Hamerska-Dudra *et al.,* 2007; Mateo, 2007a). For instance, in self-immobilisation techniques, enzymes are tightly bound together within the crosslinking network. This can result in substrate diffusional limitations of the immobilised enzyme preparation, especially for the hydrolysis of high molecular weight substrates (e.g. starch). The current work describes ascertaining the limitation of large substrate diffusion

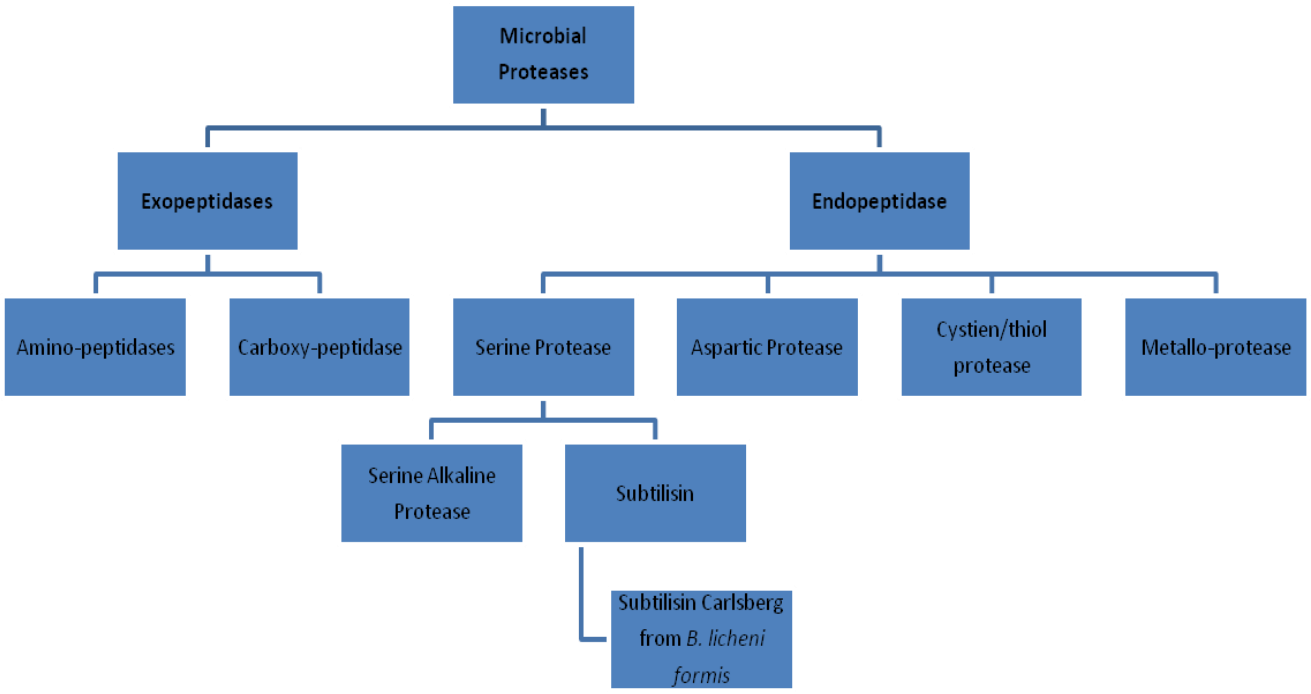
on a proprietary self-immobilisation technology, SphereZyme™, and further investigates methods of reducing this limitation.

In order to investigate the aforementioned limitation, proteases were chosen as the model enzymes. They are known for their biocatalysis of monomeric and, to a larger extent, polymeric substrates such as proteins in a variety of commercial industries (Rao *et al*., 1998; Chung and Baek, 1999; Alcalde *et al*., 2006). However, their immobilisation severely reduces their activity towards polymeric substrates. Microbial proteases are industrially relevant and are considered important enzymes, second only to lipases (Rao *et al*., 1998). Proteases are further important in the development of biosensors. We intend to evaluate high activity SphereZyme™ preparations for this application.

1.2 Protease

Proteases are important industrial enzymes that are primarily used for the biocatalysis of large substrates (Rao *et al*., 1998). This makes protease the ideal enzyme for the evaluation of the limitation to large substrate biocatalysis and the adaptation of the SphereZyme™ technology to address this limitation.

Proteases are in general small sized, compact, and spherical proteins (Kalisz, 1988). Their activity in biological systems is primarily for the hydrolysis of large proteins for efficient nutrient transport and metabolism (Kalisz, 1988). Proteases also play a role in gene expression and enzyme modification (Robert *et al*., 1977; Hershko *et al*., 1984). Proteases are categorised according to the amino acids in their active site, mechanism of action and evolutionary relationship of their 3-dimensional structures (scheme 1.1; Rao *et al.* 1998). For instance, aspartic acid proteases are a subclass of endopeptidases that contain aspartic acid at their active site which is vital for their catalytic function (Rao *et al*., 1998).



Scheme 1.1: Schematic classification of microbial proteases (Rao *et al.* 1998)

Serine proteases are defined as having a serine amino acid in their active site, together with histidine and aspartate residues, forming the so-called catalytic triad (Perona and Craik, 1997). They are ubiquitous in nature and consist of omega peptidases, exopeptidases, endopeptidases and oligopeptidases (McDonald, 1985; Barett, 1995). Alkaline serine proteases, from *Bacillus* spp. hydrolyse peptide bonds at leucine, tyrosine and phenylalanine (McDonald, 1985). These are further classified by the fact that they are not inhibited by amino acid derivatives such as tosyl-L-lysine chloromethyl ketone and tosyl-L-phenylalanine chloromethyl/ketone (Rao *et al*., 1998). However, they are inhibited by diisopropylfluorophosphate, similar to other serine proteases (Rao et al., 1998). Optimal enzyme activity of alkaline serine proteases occurs at pH 10, they have pI‟s in the region of pH 9 and have a molecular mass of between 16 and 30 kDa (Rao *et al*., 1998). Other commonly known proteases from *Bacillus* spp. are the subtilisins which include subtilisin Carlsberg (Boguslawski *et al*.,

1983). It is commercially important due to its broad substrate specificity, a valuable feature in for instance the detergent industry (Chen *et al*., 1993).

1.2.1 Protease substrates

Proteases hydrolyze amide and some ester bonds of proteins and peptide thio-esters. For visualisation and kinetic monitoring of activity, peptides and amino acids have been linked to chromogenic/fluorogenic substrates (Juliano *et al*., 1998; Müller and Bordusa, 2000; Bhaskar *et al*., 2002). Proteases have broad substrate specificity hydrolyzing either from the C- or N-terminus of proteins (exopeptidase) or internally (endopeptidase). Most proteolytic assays employ large protein substrates such as collagen, casein, gelatin and haemoglobin (Rao *et al*., 1998). Even though these substrates have been widely used and have several advantages, including low cost and well defined products, they do suffer from drawbacks (Dunn and Hung, 2000). These drawbacks include possible product inhibition, side reactions, inability to quantitate the cleavage kinetics of individual bonds and sequence variation (Bhaskar *et al*., 2002). Since most assays are based on the measurement of tyrosine release from the substrate used, they are insensitive to endopeptidases or exopeptidases which do not cleave the carboxyl terminus of the substrate (Dunn and Hung, 2000; Bhaskar *et al*., 2002). Proteolytic assays using these substrates are time consuming as they often require centrifugation. Derivatisation of the product using dyes such as diazonium ions or phenol reagent may be required in order to quantify activity spectrophotometrically (Müller and Bordusa 2000; Bhaskar *et al*., 2002). These drawbacks led to the development of more convenient, cost-effective and non-laborious methods e.g. the use of dye impregnated substrates such as azocasein (Šafařík, 1988). Azocasein eliminates the requirement for a derivatisation of the reaction products, since the azo-dye released from substrate hydrolysis is indicative of proteolysis.

Small synthetic substrates such as peptide 4-nitroanilides, peptides thioesters, glycosylated and acetylglycosylated peptides and peptidic derivatives of 7-amino-4-methylcoumarin may also be used (Juliano *et al.*, 1998; Müller and Bordusa, 2000; Bhaskar *et al.*, 2002). These substrates allow for more accurate kinetic characterisation since they tend to be more specific for the enzyme and allow real-time reaction monitoring (Müller and Bordusa, 2000). These substrates further minimize the unwanted side reactions due to a limited number of bonds susceptible to enzyme cleavage, wide range of detection methods and allow for fast, flexible, sensitive and accurate protease activity measurements (Müller and Bordusa, 2000).

The following section discusses the preferences and specificities of proteases to particular peptide sequences.

Aspartic acid proteases which in general have wide substrate specificity are further grouped according to their specificity (Rao *et al*., 1998; Harris *et al*., 2000). Pepsin, an aspartic acid protease, will preferentially cleave at carboxylic groups of aromatic amino acids such as phenylalanine and tyrosine (table 1.1; Keil, 1992; Rao *et al*., 1998), while no cleavage at peptide bonds containing valine, alanine or glycine have previously been reported. Chymotrypsin, found in rennet, is particularly active with glutamic acid (Litvinova and Balandina, 1999).

Serine proteases and metallo-endopeptidases are particularly active towards peptide thio-esters and display high activity for these substrates. The thiol-leaving group can be detected at low concentrations (James *et al.,* 1992). Juliano *et al*., (1998) reported that the chromogenic substrates with the presence of a sugar moiety not only improved the solubility of the substrate but increased its susceptibility for enzymatic cleavage of some enzymes (e.g trypsin, tissue kallikrein and rat tonin; Juliano *et al.,* 1998). Juliano *et al*., (1998) also reported that the acetylation of hydroxyl groups of a sugar residue improved hydrolysis of the peptide (except for tonin).

Table 1.1: Protease specificity

Enzyme	Cleavage Site
Chymotrypsin or subtilisin	-Trp(or Tyr, Phe, Lue) ↓---
Papain	-Phe(or Val, Leu)-XX↓---
Pepsin	-Phe(or Tyr, Leu)↓Trp(or Phe, Tyr)
Staphylococcus V8 protease	-Asp(or Glu) ↓---
Thermolysin	---↓Leu(or Phe)---
Trypsin	-Lys(or Arg) ↓---

(↓) The arrow indicate the cleavage site of proteases and XX, is any amino acid residue (Rao *et al*., 1998)

1.2.2 Alcalase®

Alcalase®, also known as Alkaline protease A, is a proteolytic enzyme preparation from *Bacillus Licheniformis*, containing the major enzyme component subtilisin Carlsberg (Chen *et. al.,* 1993). The serine proteases are widely used as a detergent additive, as well as for proteolysis of proteins due to their non-specificity and high tolerance to alkaline pH‟s (Chen *et., al.,* 1993). Their optimal conditions for activity are temperatures of around 60 °C and pH 10. Molecular weights and pI are around 27.5 kDa and 9, respectively (Rao, *et al.,* 1998). Subtilisin Carlsberg, in comparison to other similar enzymes, has broader substrate specificity and does not depend on Ca^{2+} for stability, further contributing to its broad range of applications (Rao, *et al.,* 1998). Alcalase® peptide cleavage sites include peptide bonds at a protein‟s carboxylic terminus and hydrophobic amino acids such as tyrosine, leucine and valine (table 1.1; Kukman *et al*., 1995).

1.2.3 Applications of Protease

Proteases, primarily alkaline proteases, are largely used in detergent industries to remove protein based stains (Gupta *et al*., 2002). For an enzyme to be used as a detergent additive, it should work optimally at alkaline pH and must be detergent compatible (Anwar and Saleemuddin, 1998). They are also involved in the production of enantiopure *L*-amino acids (Christen and Lopez-Munguia, 1994). Proteases have applications in the food industry for production of sweeteners, extracts for seasoning and amino acid rich protein hydrolysates (Christen and Lopez-Munguia, 1994). They are also used for improving the physical properties of food, e.g. thermitase improved foaming and emulsifying properties, solubility, water and fat-binding capacity for yeast protein lysate (van Boven *et al*., 1988; Matsui *et al*., 1993; Gonzalez-Tello *et al*., 1994). There is also an interest in proteases for peptide synthesis where the advantages over chemical catalysis of peptides include the freedom from racemisation, regio- and stereoselectivity and a reduced requirement for protection chemistry (Miyazawa *et al*., 2002).

Alcalase® is used in the proteolysis of milk protein for the production of infants‟ milk formula, while Savinase is used in the same industry to prevent the growth of *Staphylococcus aureas* (Smith *et al*., 2003). An advantage of proteases used as a disinfectant over alternative chemical disinfectants, is that proteases can remove protective layers of pathogens such as that of rotavirus (RT), rendering RT sensitive to environmental parameters such as pH and toxic natural compounds contained in faecal matter (Walker and Toth, 2000).

Proteases are used in the treatment of raw silk fibre for removal of a stiff and dull gum layer known as sericine, improving its lustre and softness (Najafi *et al*., 2005). They can provide unique finishes to wool and are further used in the hide-dehairing process (Najafi *et al*., 2005). Alkaline proteases were reported to have potential application in the degradation of gelatinous coatings of X-ray films for

the recovery of silver (Sakiyama *et al*., 1998). These enzymes are also useful in biopharmaceutical products such as enzymatic contact-lens cleaners (Nakagawa, 1994; Anwar and Saleemuddin, 2000). Proteolytic enzymes are also involved in the efficient removal of necrotic material to support the natural healing process in the treatment of skin ulcers (Kudrya and Simonenko, 1994; Sjobahl *et al*., 2002).

Proteases have further found application in the development of biosensors. Sarkar, (2000) reported using protease together with amino acid oxidase to detect the presence of proteins in a solution. Since the limitations of applying proteases to biosensors, relatively low activity and reduced signal due to protein coating (particles rather than coating) can potentially be addressed using a high activity immobilised enzyme, we intend to evaluate proteases for this application.

1.3 Biosensors

Biosensors are analytical devices that convert any signal (e.g. electrochemical, optical or thermal) in a solution or environment to measurable data via a transducer and electronic amplifier coupled with an immobilised biological recognition element (Tuner *et al*., 1987; Thévenot *et al*., 2001). They may be applied for the routine analysis of blood or urine samples in clinical diagnosis (Koncki, 2007) or for analysis of water and air samples in the environment (Leonard *et al*., 2003; Stergiou, 2006; Wutor *et al*., 2007). Biosensors can also find application in drug screening by analysing a wide spectrum of active compounds for drug discovery application (Keusgen, 2002).

Biosensors are grouped according to their transduction element (e.g. electrochemical, optical, piezoelectrical or thermal) or biorecognition principle (e.g. enzymatic, DNA or whole cell sensor; Keusgen 2002; Dzyadevych *et al*., 2008). Of these biosensors, amperometric biosensors have received the most

research interest and have resulted in successful commercial sensing devices (Keusgen, 2002; Hanrahan *et al*., 2004).

Electrochemical biosensors such as amperometric and voltammetric sensors are typically of low cost, have a high degree of reproducibility and their electrodes are replaceable (Koncki 2007; Dzyadevych *et al*., 2008). Instrumentation is easy to obtain, inexpensive and compact, thereby providing the possibility of remote measurement (Park *et al*., 2002). However, electro-active compounds present in the sample may interfere with analyte detection (Fu *et al*., 1996; Dzyadevych *et al*., 2008). The insulating property of the protein coat may dampen the response. This can result in limited interaction of the enzyme and the electrode surface (Dzyadevych *et al*., 2008; Farré *et al*., 2009). Much research focus has thus been concentrated on addressing these concerns through measures including the nature of the protein immobilisation onto the electrode surface (Anderson *et al*., 2000; Keusgen, 2002).

Potentiometric biosensors are pH-sensitive devices that transform analytical/electro-active signals into a measurable potential at a working electrode (Tuner *et al*., 1987). The devices commonly used for this sensor are ion-selective field effect transistors (ISFET), pH gas or pH glass electrodes and the electrodes are selected based on the analyte to be detected (Thévenot *et al*.,2001; Keusgen 2002).

Several alternate detection methods have been developed including conductivity-based sensing devises and optical-based biosensor (Thévenot *et al*., 2001). For instance optical transducers involve the interaction between analyte and optical fibre receptor (Farré *et al*., 2009). The measurable optical phenomenon includes linear optical measurements (e.g. adsorption or fluorescence) and non-linear optics (second harmonic generation). Benefits of optical transducers include reproducibility and rapid response time. However, the instrumentation is currently of high cost (Farré *et al*., 2009).

Biosensors based on instrumentation such as gas or liquid chromatography have limited applications (Jáuregui *et al*., 1997). Despite being sensitive and reliable they are time consuming, costly and require trained personnel (Banik *et al*., 2008). Samples containing low concentrations of target molecules need to be concentrated down prior to analysis (Rodriguez *et al*., 1997). Detection of some compounds with high polarity, e.g. nitrophenol, gives poor chromatographic results (Rodriguez *et al*., 1997). The result of these limitations is reduced application to on-site or on-line measurement, in for instance the analysis of drinking water for bacterial contamination. This sensor is based on counts of colony forming units and data capturing and analysis may take days due to long incubation periods for colony formation (Nistor *et al*., 2001; Ercole *et al*., 2002).

Biological entities commonly used as biosensing agents are oxidoreductases, for their oxidation and reduction properties and thereby ability to elicit an electroactive response (Schuhmann, 2002). Enzyme based sensors are one of the common sensing devices used especially in food and environmental applications (Baeumner, 2003). They may further be used for diagnostic applications such as sensing of glucose for blood glucose monitoring in diabetic patients. The method of detection usually monitors the current associated with oxidation of hydrogen peroxide (H_2O_2), which is a byproduct in glucose hydrolysis by glucose oxidase (GOX; Wang, 2001).

1.3.1 Recent Advances in Biosensors

Fabrications of biosensors with modified sensing agents (synthetic and biological) have been shown to enhance the operation and stability of sensors (Schuhmann, 2002; Bakker and Pretsch, 2005). The introduction of nanomaterials, such as carbon-nanotubes (CNT), titanate nanotubes (TNT) and metal nanoparticles (NP), in the fabrication of amperometric biosensors has improved electrical responses by improving electron transport from the active site

of a redox enzyme to the electrode surface (Gooding *et al*., 2003; Pandey *et al*., 2007), effectively acting as direct-electron transporters. The use of nanomaterials is thus set to improve the performance of biosensors for bio-analytical assay (Dhawan *et al*., 2009).

Modification of the electrode with already pre-immobilised analyte has previously been shown to simplify the fabrication of a biosensor while providing increased sensitivity (Gomes and Rebelo, 2003; Bakker and Pretsch 2005). For instance, the dynamic range of a disposable biosensor for phenol detection was increased by co-immobilisation of laccase and tyrosinase on a sol-gel matrix composed of diglycerysilane (Brook *et al*., 2004; Montereali *et al*., 2009).

1.3.2 Protein Biosensors

Exposure to proteases and proteins in work environments for prolonged periods, e.g. in the detergent industry, can sensitise individuals with consequent detrimental effects on their health (Behizad *et al*., 1989; Koochaki *et al*., 1995). Reliable and sensitive methods of detection for these biological compounds are also of importance for analysis of air samples (Saum *et al*., 1998). Potentiometric biosensors are the most promising device for detection of environmental proteins and their by-products (Koncki, 2007). However, the lack of electrochemically active species in proteins, poses a problem for their electrochemical detection (Sarkar, 2000). A possible way to propagate electro-active species is to hydrolyse the protein to amino acids using proteases. The released amino acids can then be oxidised by amino acid oxidase to release hydrogen peroxide (H_2O_2) which is electro-active (Sarkar, 2000; Sarkar *et al*., 2005).

A variety of electrodes such as graphite, platinum and glassy carbon have been modified with amino acid oxidase (Rosini *et al*., 2008). Few articles report on the detection of proteins (Sarkar, 2000). Protein-based sensors for the detection of

proteases are further in demand due to the need to detect medically relevant proteases (Cooper *et al*., 2005; Grant *et al*., 2007; Weilbaecher *et al*., 2007).

1.3.3 Laccase-based Biosensors

There are numerous reports on the use of laccase biosensors for detection of phenolic substrates (Xu *et al*., 2000; Freire *et al*., 2001). Laccase catalyses the oxidation of a number of organic compounds followed by reduction of molecular oxygen to water (Ghindilis *et al*., 1988).

Advantages of using laccase biosensors for detection of phenols over other oxidases are that, H_2O_2, a potentially interfering and toxic electroactive species, is not required for the oxidation of phenols (Rosatto *et al*., 1999). Other oxidases such as tyrosinase tend to be less stable and are further prone to by-product inhibition (Adeyoju *et al*., 1996; Daigle and Leech, 1997).

Fabrication of laccase biosensors may involve direct immobilisation by the use of glutaraldehyde or polyethylenemine at the surface of electrodes including carbonaceous, platinum, gold and glass (Quan *et al*., 2002). Several findings are yet to be made in the application of laccase based biosensors for phenols through tailoring of immobilisation strategies.

1.4 Enzyme Immobilisation

Enzyme immobilisation offers several benefits such as improved storage, thermal and pH stability, and improved stability against denaturation caused by organic solvent and autolysis (Fortier and Bélanger, 1990; Mateo, *et al*., 2007a).

Since immobilised enzymes are insoluble, this assists in their recovery and simplifies downstream processing (Cao, 2005). They can subsequently be recycled thus reducing the cost of the enzyme for biocatalytic processes.

Immobilisation can provide improved specificity and even higher activity when compared to their free form (Gianfreda and Scarfi, 1991; Reetz *et al*., 2003; Sharma, *et al*., 2007; López-Gallego *et al*., 2007). The improvement in activity, in the case of lipases, has been attributed to the fixation of the active site lid in an open conformation by a crosslinking agent resulting in super activation of lipase after immobilisation (Brady *et al*., 2008). Further benefits include enabling the use of multi-enzyme and chemo-enzymatic cascade processes (Monti *et al*., 2009). For instance, Dalal *et al.* (2007) showed that CLEAs (Crosslinked Enzyme Aggregates) can be made from a heterogeneous population of enzymes (termed as Combi-CLEA). The Combi-CLEA showed enzyme activity retention of up to 100% for almost all immobilised enzymes (Dalal *et al*., 2007).

Although immobilisation increases the cost of the biocatalyst (cost of preparation or support), this can be offset by the recovery and reuse of the biocatalyst. Examples of this cost reduction include the conversion of 600 kg of 6-aminopenicillanic acid (6-APA) produced per kg immobilised penicillin G amidase (Tischer and Kasche, 1999) and the production of fructose from glucose isomerase, 11 000 kg per kg immobilised enzyme (Bhosale *et al*., 1996). These examples obey the rule of thumb for biocatalytic processes, the enzyme cost must not amount to more than a few percent of the production cost of the product of interest (Kilara and Shahani 1979; Christensen *et al*., 2003; Cao *et al*., 2003).

Enzyme immobilisation, specifically solid support immobilisation, involves the use of often expensive matrices and is usually associated with reduction in the specific and volumetric activity (Matsuno, *et al*., 2007). This drawback results from the limited loading capacity of the supports (Cao, 2005), incorrect orientation of the biocatalyst after binding results in an inactive conformation (Mateo *et al*., 2007b) and potentially protein denaturation (Matsuno *et al*., 2007). The molecular complexity of the biocatalyst can further limit the potential for immobilisation to a solid support (Mateo *et al*., 2007b). For instance, if the enzyme has more than one subunit, it is likely that the enzyme may not be

optimally immobilised on a support, i.e. all components are not attached, leading to dissociation of the subunits (Pessela *et al*., 2007).

Major losses of catalytic activity have been reported with physical adsorption to a solid support, which is attributable to enzyme leaching (Li *et al*., 2009). Covalent grafting of the enzyme to the support tends to reduce this limitation. However, irreversible covalent binding of the biocatalyst to the support limits their applications to signal transduction and further prevents re-use of the support (Minett *et al*., 2002; Pierre *et al*., 2006).

Self-immobilisation technologies were reported to remedy some of these limitations; however, they too suffer from several drawbacks. Examples of these limitations have been illustrated in CLEA and CLEC technology which can result in substrate and product diffusional limitation (Sheldon, 2007). A contributing factor to this limitation is particle size. This, to an extent, can be overcome by controlling the surface to volume ratio, i.e. smaller particles result in improved diffusion within the particle and improved interaction between enzyme and substrate (Rusling and Foster, 2003; Brady and Jordaan, 2009). Combining nanotechnology with enzyme immobilisation for the production of nanoparticles can improve these properties. However, recovery of the nanoparticles through centrifugation or precipitation is a challenge (Betancor *et al.,* 2005; Brady and Jordaan, 2009).

Substrate diffusional limitation is therefore a major feature of self-immobilisation techniques (Manrich *et al*., 2008). Although good activity maintenance has previously been reported for proteases e.g. an activity yield of 100% after immobilisation was achieved for trypsin immobilised on aldehyde activated chitosan and agarose gel. These activities are often monitored using small synthetic substrates such as N-benzoyl-L-arginine-*p*-nitroanilide (BAPNA) and not towards large substrates such as proteins (Pierre *et al*., 2006; Manrich *et al*., 2008).

The hydrophobicity of a solid support plays a role in the decreased activity of an immobilised biocatalyst towards a hydrophilic substrate (Reetz and Jaeger, 1998; Mateo *et al*., 2007a; Knežević-Jugovic *et al*., 2008). A polymeric substrate may further be inaccessible to the enzyme‟s active site due to steric hindrance of the solid support, or the degree of crosslinking in the self-immobilised network (Ferreira *et al*., 2003). Orientation of the enzyme active site maybe a factor as some of the active site may face toward or interact with the solid support or may be hindered by other enzymes in the case of self-immobilisation (Ferreira *et al*., 2003; Betancor *et al*., 2005). This is therefore a limitation for both self- and support-based immobilisation techniques (Blanco *et al*., 1991; Rao *et al*., 2006; Hamerska-Dudra *et al*., 2007).

1.4.1 Considerations for Enzyme Immobilisation Method

From the abovementioned information, it is evident that several factors influence the selection of a suitable protein immobilisation technique (Cao *et al*., 2003; Sheldon, 2007). It is vital to select a suitable immobilisation method that can not only meet the catalytic requirements (e.g. productivity, specificity or space time yield), but the non-catalytic needs of a given application such as downstream processing requirements which entails recovery and purification of both the enzyme and product at reduced cost (Roy and Gupta, 2002; Cao *et al*., 2003).

1.4.1.1 Enzyme active site modification

It is necessary when choosing an immobilisation method to prevent loss of enzyme activity without changing the chemical nature of the enzyme or reactive groups in the enzyme active site (Wei *et al*., 2000). Knowledge of the active site of the enzyme can further improve the chance of successful immobilisation since some enzyme active sites may contain functionalised groups (such as lysine) that may take part in their coupling to the support or react with the crosslinking agent

(Palomo *et al*., 2003; Berglund and Park, 2005; Wang, *et al*., 2008). An active site can be protected during immobilisation as long as the protective groups can be removed later without loss of enzyme activity (Chae *et al*., 2000; Wang, *et al*., 2008). In some cases, a substrate or a competitive inhibitor of the enzyme can fulfil this protective role (Chae *et al*., 2000).

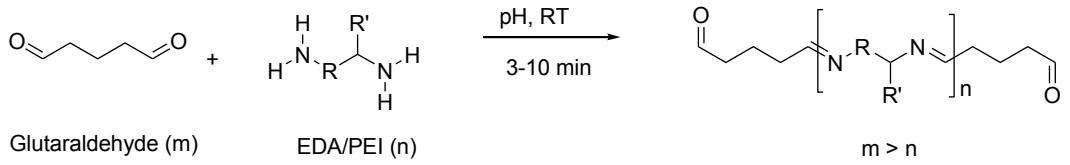
1.4.1.2 Surface chemistry

A bi-functional crosslinking reagent, glutaraldehyde is often used for immobilisation techniques as either a crosslinking or an activating agent (Payne 1973; Molin *et al*., 1978). For instance, it has been used to crosslink protein molecules together via the amino groups of the surface-based lysine residues of the proteins. This step polymerises, insolubilises and stabilises the protein as part of immobilisation (Cao *et al*., 2000; Cabirol *et al*., 2006). Thermal and operational stability was reported to be directly proportional to the number of linkages of the enzyme to the support (Pedroche *et al*., 2007). Covalent binding and larger pore size of the support increases the specific activity, pH stability, temperature stability and reusability of the enzyme (Kannan and Jasra, 2009).

The surface, on which the enzyme is immobilised, is responsible for retaining the structure in the enzyme (Mateo *et al*., 2007a). These links will prevent vibration of the enzyme and thus increase thermal stability (Grazú *et al*., 2005). The charged micro environment, i.e. the surface of the support and enzyme has been shown to lead to a possible shift in the optimum pH of the enzyme of up to 2 pH units (DeSantis and Jones, 1998). This may be accompanied by a general broadening of the pH region in which the enzyme can work effectively, allowing enzymes that normally do not have similar pH regions to work together in an application such as dual enzyme biosensor systems (Freire *et al*., 2003; Wang and Caruso, 2005). Immobilised enzymes have a limited life span, so proper disposal mechanisms are needed, especially for large-scale application (Katchalski-Katzir and Kraemer, 2000).

The nature of the enzyme can dictate the method of stabilisation required (e.g. nitralases) as they may be inactivated by the use of glutaraldehyde which can react with amino groups of enzyme active sites (Chae *et al*., 1998; Chae *et al*., 2000). Inactivation can occur through the small size and reactivity of glutaraldehyde, which can easily penetrate the protein (Chae *et al*., 1998). Furthermore, certain enzymes contain a low abundance of lysine residues which are often used for coupling, rendering routine immobilisation chemistry unsuitable for their immobilisation (Chae *et al*., 1998; Křenková and Foret, 2004). In the instance where small chemical coupling agents are unsuitable, large agents such as dextran polyaldehyde can be used, resulting in improved enzyme activity maintenance. This technique has previously resulted in retention of more than 50% enzyme activity (Mateo *et al*., 2004). An amine rich co-precipitant such as polyethyleneimine can be used to improve crosslinking of lysine deficient enzymes (López-Gallego *et al*., 2005b) and especially when immobilisation of a low concentration of enzyme is required (Cabana *et al*., 2007).

Lysine rich protein such as bovine serum albumin (BSA) has been referred to as a „proteic feeder", and used to increase the lysine content of the enzyme preparation to be immobilised thereby improving the efficiency of enzymes with low lysine content (Shah *et al*., 2006). Lysine is an amino acid group that is easily accessible on the enzyme surface and has a high affinity for reaction with aldehyde via its α-amino group (Křenková and Foret, 2004). Combining glutaraldehyde with PEI (scheme 1.2) was reported to increase the stability as well as the activity recovered by the immobilised enzyme (L pez-Gallego *et al*., 2005b).



Scheme 1.2: Bifunctional crosslinking agent (glutaraldehyde) which contains two terminal aldehyde groups, reacts with primary amines (L pez-Gallego *et al*., 2005b).

1.4.2 Methods of Enzyme Immobilisation

Immobilised enzymes may either be linked to a support, entrapped or crosslinked in a process known as self-immobilisation (Cao *et al*., 2003). In the following section these groups are discussed separately.

1.4.2.1 Support-based immobilisation

Immobilisation of biocatalysts on fabricated matrices imparts rigidity and stability to the biocatalyst. Usually the catalytic masses of immobilised enzymes make up less than 10% of the total mass. A solid support may account for up to 20% of the reactor volume (Cao, 2005). Studies have further shown that enzyme loading and activity is related to the accessible surface and pore size in the matrix (Blanco *et al*., 1991). Support-based enzyme immobilisation can further be subdivided into entrapment, adsorption, covalent coupling and affinity interactions (Spahn and Minteer, 2008).

Adsorption is the term used to define when the enzyme is attached to the surface of the support through relatively weak physical interactions such as hydrophobic and or ionic linkages (Sheldon, 2007; Spahn and Minteer, 2008). Application of enzymes immobilised by adsorption can result in desorption from the matrix through high shear forces, ionic strength of the reaction medium, as well as denaturation by the reactant and/or product (Sheldon, 2007). Desorption or

leaching can result in contamination of the product, in turn leading to difficulties in down-stream processing and overall increased cost of the process (Kumakura and Kaetsu, 2003).

Enzymes may alternately be covalently linked to the carrier via the enzyme‟s native functional groups (Bornscheuer, 2003). Covalent binding is irreversible and stronger than ionic and physical binding (Pierre *et al*., 2006). It reduces or eliminates enzyme leakage and often provides improved stability of the immobilised enzyme (Mateo *et al*., 2000; Suh *et al*., 2005; Pierre *et al*., 2006). Optimal conditions for binding can vary with respect to the pH and temperature employed and may further vary according to the chemistry and number of functional groups employed (Dong *et al*., 1996; Pedroche *et al*., 2007; Mateo *et al*., 2007b).

I) Types of solid supports

Supports, also known as carriers or matrices, are widely available and include a variety of inorganic and organic materials. These supports are discussed in the following section.

a) Inorganic supports

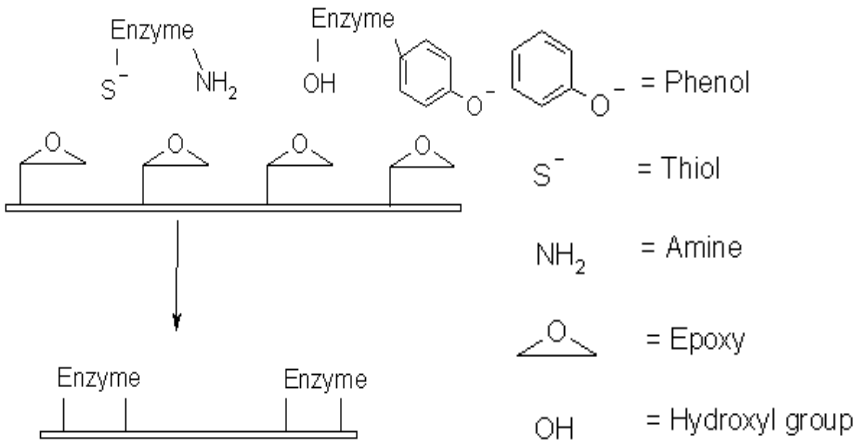
A variety of inorganic supports such as alumina, silica, zeolites and mesoporous silica (Diaz and Balkus 1996; Takahashi *et al.,* 2001; Wang *et al.,* 2001; Yan *et al.,* 2002; Borole *et al.,* 2004; Moelans *et al.,* 2005; Petri *et al.,* 2005) have been used for enzyme immobilisation. The most commonly used support is silica due to its simplicity and low cost (Kirk and Christensen, 2002). Silica has a high surface area and uniform small sized pore diameters which can accommodate small sized enzymes; they are further inert and stable at elevated temperatures (Yiu and Wright, 2005). A variety of enzymes have been successfully immobilised

on this support including subtilisin Carlsberg, lipases and oxidoreductases (Ferreira *et al*., 2003; Lei *et al*., 2004; Yadav and Jadhav 2005).

b) Synthetic Organic supports

The most prominent mode of interaction between an organic carrier and enzyme is through covalent binding (Křenková and Foret, 2004; Sheldon, 2007). These kinds of solid support normally have a high content of functionalised groups (usually covalent) thus resulting in multipoint attachment of the enzyme leading to improved stability (Mateo *et al*., 2007b).

Epoxy groups are very stable and are able to chemically react with all nucleophilic groups on the protein surface such as lysine, histidine, cysteine and tyrosine (scheme 1.3). The epoxy activated supports are thus suitable to stabilise enzymes by multipoint covalent attachment, both for industrial and laboratory use (Sheldon, 2007). The other advantage is the controlled and partial modification of epoxy groups with sodium sulphide which has permitted the preparation of thiol-epoxy supports. This resulted in the specific immobilisation of enzymes through their thiol groups via thiol-disulphide bridges (Grázu *et al*., 2005).



Scheme 1.3: Representation of enzyme coupling on epoxide functionalised solid supports (adapted from Boller *et al*., 2002).

An example of a commercially available epoxide support is Eupergit® epoxy-activated acrylic beads (scheme 1.3; Boller *et al*., 2002). Two commercial products are available, namely Eupergit® C and Eupergit® C 250 L, the latter named due to the relatively larger bead diameter in the range of 100-250 μm. Eupergit C has an average pore size with radius of 10 nm and an oxirane density of 600 μmol.g^{-1} dry beads, Eupergit® C 250 L has larger pores (r =100 nm) and a lower functional group density of 300 μmol.g^{-1} dry beads. These supports are hydrophilic and stable, both chemically and mechanically, over a pH range of 0-14 (Boller *et al*., 2002).

Immobilisation on Eupergit results in multipoint attachment with associated improvements in operational stability (Boller *et al*., 2002; Sheldon 2007) and increased thermo- and organic solvent stability (Kennedy and Cabral, 1995). Furthermore, immobilisation on this support has further resulted in increased enantioselectivity of the enzyme (Koszelewski *et al.,* 2007).

Another popular organic carrier is Sepabeads (Mitsubishi chemical company) which consists of poly-methacrylate-based polymer beads. The polymer is functionalised with oxirane groups, thus immobilising enzymes through the same chemistry as Eupergit C (Mateo *et al*., 2002). Sepabead supports are also stable in a range of solvents, have a low swelling tendency and can be used in stirred tank or packed bed reactors due to its excellent mechanical properties (Guisan *et al*., 2001; Knežević-Jugović *et al*., 2008). Enzymes immobilised on this support have demonstrated improved stability especially in organic solvents due to their high water content and multipoint protein immobilisation (Guisan *et al*., 2001; Mateo *et al*., 2002; Knežević-Jugović *et al*., 2008). Sepabeads are available with various functionalities, including epoxide (Sepabeads EC-EP), primary amine (EC-EA) and butyl residues (EC-BU; Torres *et al*., 2003; Segura *et al*., 2004; Torres *et al*., 2004; López-Gallego *et al*., 2005c; Alonso *et al*., 2005). Sepabeads EP was reported to be a robust enzyme carrier and has been applied in industrial processes (López-Gallego *et al*., 2005c). Mateo *et al*., (2002) ascribed the

robustness of the matrix to the internal geometry of the support which consists of cylindrical pores and high functional group density (~100 μmol.ml^{-1}).

Sepabeads EA is a primary amine support that is used for cationic adsorption of protein. The presence of primary amino groups from the Sepabeads EA turns them into a suitable support for enzyme immobilisation through glutaraldehyde activation (López-Gallego *et al*., 2005c; López-Gallego *et al*., 2005b).

c) Biopolymers and hydrogels

These are biologically produced polymers such as polysaccharides (cellulose, starch, chitosan and agarose) and proteins (gelatin and albumin). The use of biopolymers as a support is a more recent advancement for enzyme immobilisation (Sheldon, 2007). The most studied biopolymer is poly-N-isopropylacrylamide (poly/NIPAM; Bergbreiter *et al*., 1998; Sheldon, 2007). The effectiveness of this polymer was shown by Ivanov *et al*., (2003), where penicillin G amidase was immobilised by condensation with polyNIPAM (functionalised with ester groups). The immobilised enzyme exhibited hydrolytic activity close to that of the free enzyme (Ivanov *et al*., 2003). Another more recent example is the use of a thermo-responsive polymer which consists of 2(2-methoxyethoxy)ethylmethacrylate and oligo(ethylene glycol) methacrylate. The major advantage is its lack of toxicity and immunogenicity in addition to its thermo-responsive properties (Clutz *et al*., 2006). The first industrial application of biopolymers was the Tanabe process, where the enzyme was immobilised by ionic adsorption on DEAE-Sephadex, a diethylaminoethyl functional support (Chibata, 1982).

Hydrogels are hydrophilic polymeric materials which swell in water while maintaining a three dimensional structure (Wichterle and Lím, 1960). They are classified according to their source (natural and synthetic gels), type of network and interaction, pore size and bio-degradability (Kopeĉek and Yang, 2007). Due

to their high water content and low toxicity, they may also be applied as biomaterials (Kopeĉek and Yang, 2007). The hydrophilic nature and large pore size are advantageous for its application in organic media where the hydrophobic solvent prevents leaching from the support (Sheldon, 2007). Materials used in hydrogels include natural products such as gelatin, starch, pectin and synthetics such as polyvinyl alcohol (PVA) cryogels, 2-hydroxyethyl methacrylate (HEMA), dimethylaminoethyl methacrylate (DMAEMA) and composites such as poly-N-isopropylacrylamide-gelatin (poly/NIPAM-gelatin; Wichterle and Lím, 1960; Lozinsky *et al.,* 2003; Ohya *et al*., 2004).

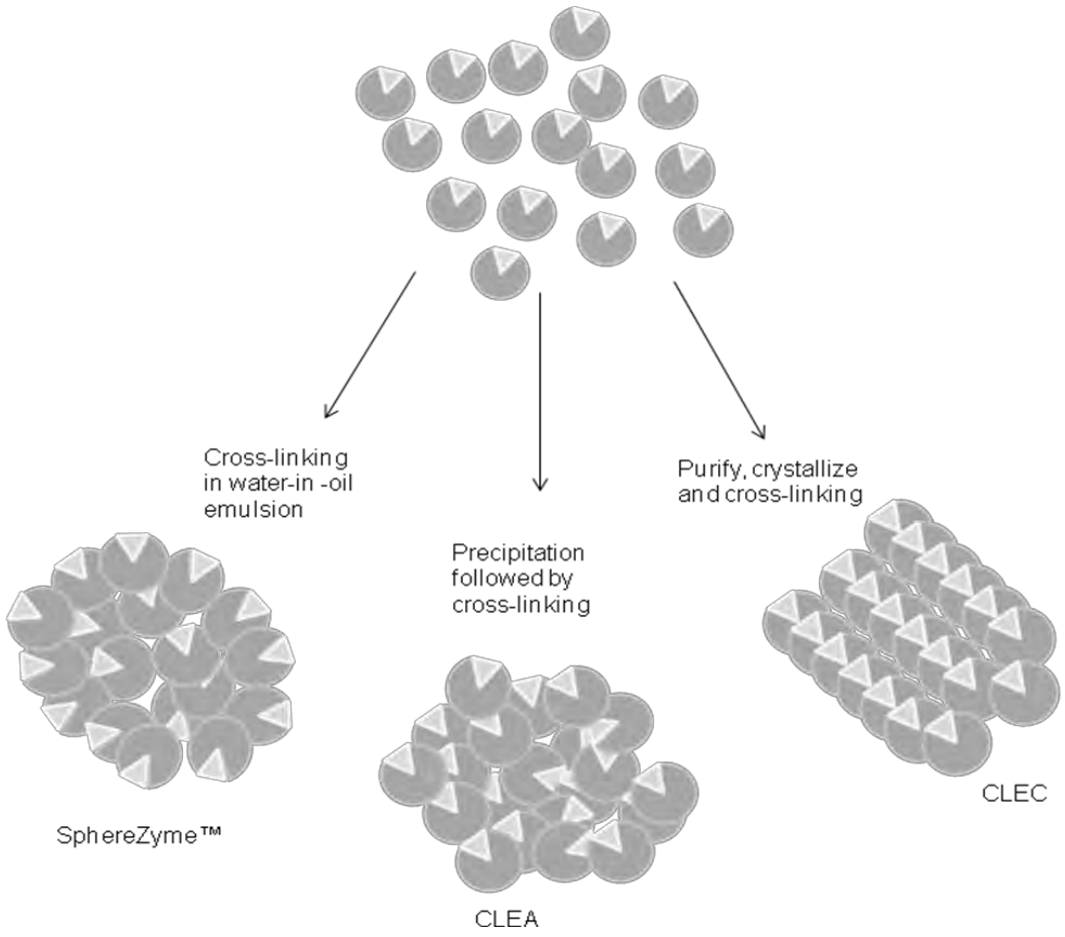
Biopolymers and hydrogels exhibit controlled release properties, making them suitable for drug and protein delivery. It can be used in conjunction with the immobilised or crosslinked enzyme to limit leaching out of the enzyme into the environment (Hennink *et al*., 1997; Kim *et al*., 2006). For instance crosslinked enzyme crystals (CLEC) of subtilisin Carlsberg were encapsulated in a hydrogel which was subsequently tested for oral delivery of proteins (Simi and Abraham, 2007).

Dendrispheres are a relatively new PEI-based hydrophilic solid-support (scheme 1.4 and 1.5). In addition to its relatively high functional group density, its loose and interpenetrating network allows for high enzyme loading as compared to other supports (Brady and Jordaan, 2009). The preparation of the support involves the use of a water-in-oil emulsion where aqueous phase PEI is crosslinked with an excess glutaraldehyde resulting in spherical polymeric particles. The particles are easily recovered from the emulsion after which they are used for protein immobilisation (Jordaan *et al*., 2009b). Exposed aldehyde groups on the particles are subsequently used to bind proteins through covalent interaction with the primary amine groups on the enzymes surface (Harris *et al.,* 1984; Roberts *et al.,* 2002). The use of an emulsion technology allows particle uniformity and monodispersity. This technology may overcome the limitation of solid support technology (i.e. the low catalytic load). However, it may suffer from

the major disadvantage of enzyme self-immobilisation techniques, namely substrate diffusional constraints (Brady and Jordaan, 2009).

1.4.2.2 Self-immobilisation (crosslinking)

Crosslinking is often achieved through the reaction of primary amine groups from lysine available on the protein surface (e.g. lysine) and an aldehyde functional crosslinker such as glutaraldehyde (Haring and Schreier, 1998; Roy and Abraham, 2004). The mechanism of crosslinking is illustrated in scheme 1.4. These techniques have not realised wide industrial adoption due to several drawbacks such as poor reproducibility, low activity and poor mechanical stability (Sheldon, 2007).



Scheme 1.4: Comparison of the self-immobilisation techniques such as crosslinked enzyme aggregates (CLEA), crosslinked enzyme crystals (CLEC) and SphereZyme™ technologies (adapted from Brady and Jordaan, 2009).

a) Crosslinked Enzyme Crystals (CLEC)

In 1966, Quiocho and Richards, re-established enzyme self-immobilisation by demonstrating that crystallized enzymes can be crosslinked using glutaraldehyde. This was further improved as an industrial biocatalyst in the early 1990‟s (St. Clair and Navia, 1992; Sheldon, 2007). The first enzyme immobilised using CLEC technology was thermolysin which was used in the manufacture of aspartame (St. Clair and Navia, 1992). The thermolysin CLECs were shown to be applicable for the production of a variety of peptides (Persichetti *et al*., 1995). The technology was later shown to be applicable to a range of enzymes, including

lipases, oxidoreductases (Lalonde *et al*., 1997; Margolin and Navia, 2001; Roy and Abraham 2004; Roy and Abraham, 2006). CLEC"s robustness, operational stability, ease of recycling, high catalyst load and volumetric productivities, made the technology ideally suited for industrial biotransformation and oral drug and protein delivery (Govardhan, 1999; Simi and Abraham, 2007). However, the need to crystallize the protein, often a labour intensive task requiring high purity protein, has detracted from its use for industrial biocatalysis due to its cost implications (scheme 1.4; Cao *et al*., 2000).

b) Crosslinked Enzyme Aggregates (CLEAs)

CLEA technology involves the formation of aggregates by precipitation, using precipitating agents such as ammonium sulphate or polyethylene glycol, followed by crosslinking with crosslinking agents (scheme 1.4; Cao *et al*., 2003). This technique not only immobilises the enzyme, but the precipitation step also results in partial protein purification (advantage over CLEC). This makes the technology applicable to lower purity enzyme, thereby reducing the cost for industrial bio-catalytic applications (Cao *et al*., 2003; Cao, 2005; Sheldon, 2007). The formation of insoluble aggregates is an essential step for the retention of enzyme activity during crosslinking. The close proximity of the enzyme molecules to each other after precipitation increases the crosslinking efficiency (Cao *et al.,* 2003). However, precipitation can result in a loss of enzyme activity, as well as the blocking of active sites due to the close proximity of enzyme particles (López-Serrano *et al*., 2002). The technology does not allow for particle size control which further limits its biocatalytic application.

The first industrially important enzyme used to show the applicability of CLEA"s for biocatalysis was penicillin acylase (Cao *et al*., 2000). The immobilised enzyme showed improved thermal stability and improved tolerance to organic solvents. Other enzymes that have been immobilised include lipase, the most prominent biocatalytic enzyme (López-Serrano *et al*., 2002). Several additives

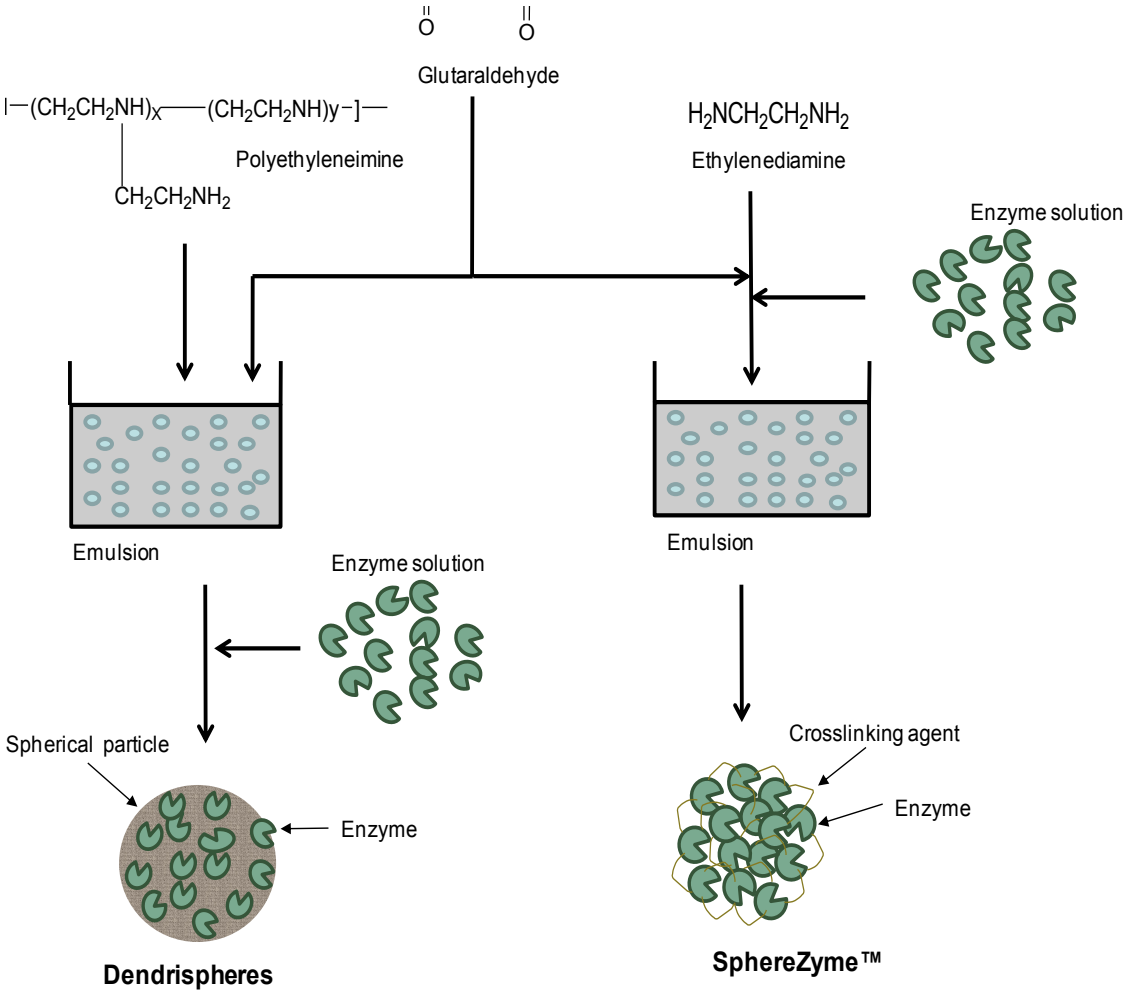
were evaluated during particle manufacture in an attempt to super activate lipase through active site modification (Theil, 2000). Thereafter the active conformation can be preserved by crosslinking using glutaraldehyde. Since the additive is not covalently linked to the enzyme it can be easily washed away (Sheldon, 2007).

Particle size distribution of the CLEA is an important factor in large-scale application as it can negatively affect mass transfer limitations and may not be suitable for recovery by filtration (Yu *et al*., 2006, Brady and Jordaan, 2009). Factors that are directly linked to particle size distribution are the nature of the enzyme and crosslinking agent (Lee *et al*., 2005; Yu *et al*., 2006).

c) SphereZyme™ Technology

The SphereZyme™ technology involves the emulsification of a hydrophilic phase, containing the enzyme, with a hydrophobic phase (scheme 1.4 and 1.5; Brady *et al*., 2008). The proteins are subsequently crosslinked within individual emulsion droplets with bi- or multifunctional chemical reagents to form stabilized enzyme particles. The structural properties of the enzyme may affect enzyme orientation, i.e. it may orientate at the interfacial boundaries of the water and oil droplets (Moolman *et al*., 2005; Brady and Jordaan, 2009).

Immobilisation of enzymes, especially lipases, using the SphereZyme™ technology does offer several advantages. For instance, it uses an agent that results in the protection of the active site and fixation of the active site lid (in the case of lipases) after crosslinking. SphereZyme™ preparation is relatively simple, inexpensive and has the advantage of particle size control by controlling the emulsion parameters (Moolman *et al*., 2005). However, partial purification of the enzyme sample is required for immobilisation (Brady *et al*., 2008). Being a self-immobilisation technology, there may be an inherent disadvantage for the large substrate biocatalysis.



Scheme 1.5: A brief representation of the preparation of SphereZyme™ and Dendrispheres propriety technologies (Brady *et al*., 2008; Brady and Jordaan, 2009).

1.4.2.3 Entrapment

Entrapment refers to a process where the immobilisation is achieved by capturing the enzyme within a matrix rather than through the attachment to the surface of a matrix (Sheldon, 2007). Entrapped enzymes may be further sub-categorized according to their carrier system- (1) encapsulation involving a single micro space (microcapsules) and (2) multiple linked micro spaces such as a matrix or membrane (Yiu and Wright, 2005). Matrices used for encapsulation or

entrapment are often natural polymers such as agar, agarose and gelatin (Sheldon, 2007). Immobilisation occurs through thermally-reversible polymerization, sol-gel, nanoscale polyelectrolyte and other inorganic materials (Buisson *et al*., 2001; Jin and Brennan, 2002; Trau and Renneberg, 2003; Brook *et al*., 2004; Mateo *et al*., 2006). Poly (3,4-ethylenedioxy thiophene) is a further example of an entrapment matrix in which biomolecules are incorporated through a shrinkage phenomenon during a washing step (Fabiano *et al*., 2002; Chen, 2006).

Entrapment techniques are rarely used for enzymes and are preferably used for cell immobilisation. This is due to the high porosity of these matrices resulting in slow enzyme leakage during continuous operation (Li *et al*., 2009). The other major disadvantage of entrapment is their relatively large particle size resulting in substrate diffusional limitations especially for macromolecular substrates such as starch and proteins (Diaz and Balkus, 1996; Yiu and Wright, 2005). These disadvantages have in part been resolved. For example, the pore size of mesoporous silica after enzyme encapsulation was reduced through silanition with 3-amino-propyltriethoxysilane to reduce leaching (Yiu and Wright, 2005). The encapsulation of enzymes pre-adsorbed onto mesoporous materials was also reported to decrease enzyme leakage (Ribeiro *et al*., 2004; Yadav and Jadhav, 2005).

1.5 Protease Immobilisation

Due to the extensive industrial applications of proteases, it is important to develop stabilized enzyme preparations which have high operational, mechanical and chemical stability (Gianfreda and Scarfi, 1991; Berger *et al*., 1992). Protease immobilisation can also prevent autolysis, a common problem in the application of proteases (Gianfreda and Scarfi, 1991). This can allow for the continuous use of the enzyme and ease of downstream processing due to reduced product contamination (Anwar and Saleemuddin, 1998). Proteases have previously been

stabilized via immobilisation on a variety of supports including glyoxyl agarose, silica derivatives, crosslinked thermo-sensitive carriers (TH8-NH_2), Lewatit R258-K, synthetic organic carrier-Eupergit® C, tri(4-formyl phenoxy) cyanurate, chelating sepharose and celite (Kukman *et al*., 1995; Miyazama *et al*., 2002; Ramos *et al*., 2003; Rao *et al*., 2006). Proteases such as Alcalase® have been immobilised using the self-immobilisation CLEA technique. A protease CLEA formulation of Alcalase® is commercially available (Sigma-Aldrich Cat# 30079). They include Alcalase® CLEA-ST, Alcalase® CLEA-OM and Alcalase® CLEA-UF. The latter is a CLEA preparation with ultra fine particle size to provide improved surface area to volume ratio for increased substrate diffusion (Internet Reference 1).

1.6 Current Trends in Enzyme Immobilisation

The importance of protein immobilisation in realising the variety of applications of enzymes makes this an ever-expanding area of research. New developments in the field include the use of polymer crosslinking agents, e.g. PEG-aldehyde (Sheldon, 2007; Caramori and Fernandes, 2008). The use of polymers has been reported to increase the activity recovery for bulky macromolecular substrates, and increased operational and thermal stability in cases where the proteins had been pre-treated with these agents (Chae *et al*., 2000; Betancor *et al*., 2005; Ramos *et al*., 2006).

There are some new emerging techniques for protein immobilisation, an example of which is the preparation of single enzyme nanoparticles (SEN) which are subsequently immobilised in nano-porous silica (Kim *et al*., 2006). Their solubility in water and their nano-sized characteristics means they have to be used in conjunction with a solid support. The current cost implications limit the large scale applications of this technique for biocatalysis but they could find application in the development of biosensors and other devices where enzyme cost is less important (Sheldon, 2007).

It was recently shown that different immobilisation technologies can be combined in order to produce a more robust immobilised biocatalyst (Hilal *et al*., 2004). For instance, encapsulation via layer-by-layer assembly of multilayered nanocomposite thin shells of immobilised enzymes in mesoporous silica spheres has recently been demonstrated. This resulted in improved enzyme stability and activity retention compared to the use of mesoporous silica alone (Wang and Caruso 2005).

Another recently demonstrated method for enzyme immobilisation involved the preparation of protein-coated microcrystals (PCMCs; Kreiner *et al*., 2001). The major benefit is that the enzyme molecules are dehydrated using a method that leaves the majority of the enzymes in an active conformation resulting in high enzyme activity maintenance for the immobilisation procedure (Kreiner *et al*., 2001).

More recently developed carrier-free immobilisation techniques, such as CLEA and SphereZyme™ are showing potential for industrial biotransformations (Sheldon, 2007; Brady *et al*., 2008). This is particularly relevant to lipases where both of these techniques have reported more than 100% activity recovery (López-Serrano *et al*., 2002; Brady *et al*., 2008).

1.7 Research Project

A good immobilisation technique should maintain high catalytic activity after immobilisation. The limitations to enzyme self-immobilisation techniques can be summarised as follows:

a) Low permeability from high degree of crosslinking (Brady *et al*., 2008; Cao, 2005).

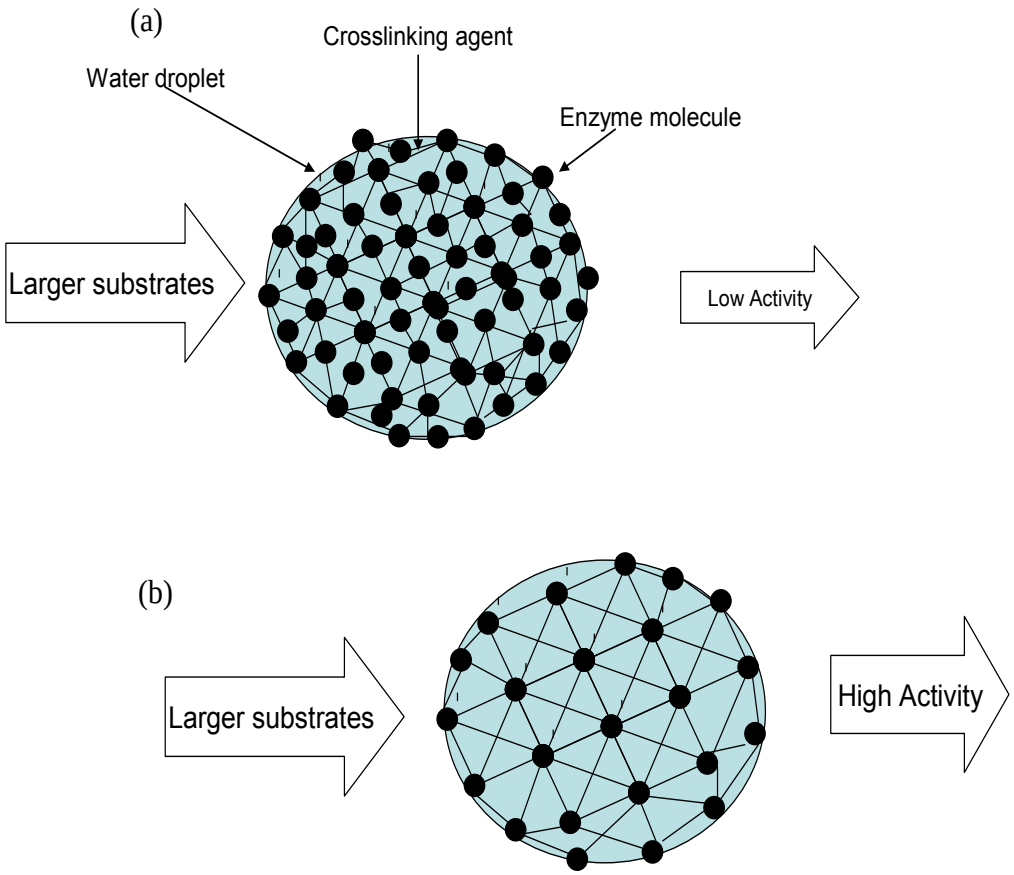
b) Reduced immobilisation of enzymes with low content of functional reactive groups for crosslinking (López-Gallego *et al*., 2005b)

c) Large particle size distribution, crucial for self-immobilisation technologies as it affects substrate and product diffusion and biocatalyst recovery.

Although the SphereZyme™ technology has to a large extent alleviated b and c, the limitation to large substrate biocatalysis has not yet been addressed.

A major drawback then associated with self-immobilised enzyme technologies is the potential for low enzyme activity yield for the biocatalysis of large substrates. The aim of this thesis is to elucidate the extent of this limitation to the recently developed SphereZyme™ technology and evaluate methods of alleviating this drawback (scheme 1.6). This method of enzyme immobilisation will be benchmarked against solid-support immobilisation technologies. Alcalase® was chosen as a model enzyme due to the activity for the hydrolysis of proteins, a large substrate and the industrial relevance of this enzyme. We aim to examine methods to improve enzyme activity towards these large substrates by investigating the use of longer crosslinking agents to develop looser matrices and thereby improve substrate and product diffusion. We believe there is potential benefit for the use of self-immobilised enzymes in the development of biosensors and therefore intend to evaluate it against free enzyme for this application.

The project aims to determine the extent of the foreseen limitation and address it using scheme 1.6 below.



Scheme 1.6: Proposed mechanism for enhancing SphereZyme™ activity toward polymeric substrates.

(a) Self-immmobilised enzymes constitute a tightly packed network, thereby decreasing efficiency for large substrate catalysis (b) Proposed method of alleviating limitation, using larger crosslinking agent to alleviate the limitation by providing improved substrate permeation.

1.8 Hypothesis:

The use of elongated crosslinking agents can assist in overcoming the limitation of the self-immobilisation enzyme technology, SphereZyme™, for large substrate biocatalysis.

1.8.1 Research Objectives

Objectives of the study are:

- Achieve partial purification of subtilis Carlsberg from crude Alacalase® (chapter 2)
- To determine the extent of the limitation of SphereZyme™ for large substrate biocatalysis (chapter 3)
- Optimise the immobilisation of Alcalase® using elongated crosslinking agents allowing for improved conformational flexibility and substrate permeability (chapter 3)
- Compare the SphereZyme™ immobilisation of Alcalase® to solid support immobilisation technologies (chapter 4)
- Evaluate the optimised biocatalysts for the development of novel a) laccase based biosensors for phenol detection and b) Alcalase® based biosensor for PNP (chapter 5)

2 Alcalase® Purification

2.1 Introduction

Alcalase® is a commercial protease preparation from *Bacillus Licheniformis* with a major enzyme component being subtilisin Carlsberg (Chen *et al.,* 1993). This enzyme was found to be very stable in organic solvents, which has in part led to its numerous biocatalytic applications (Miyazawa *et al*., 2002). The immobilised product of subtilisin Carlsberg was reported to be more efficient than the lyophilized powder for transesterification of *N*-acetyl-L-phenylalanine ethyl ether (Kreiner *et al*., 2001; Shah *et al*., 2008). Subtilisin Carlsberg was selected as the target protein due to its enzymatic activity towards large proteinaceous substrates and its value as an industrially important enzyme (Chen *et al*., 1991; Miyazawa *et al*., 2002).

Alcalase® commercial preparation was shown to be of low purity (Ferreira *et al*., 2003). In addition to the major enzyme, subtilisin Carlsberg (27.3 kDa) which constitute <20% of the overall protein content, it contains several other proteins (Tardioli *et al*., 2003). These proteins are for instance enzyme hydrosylate products and make up about 30-40% of the total protein content (Tardioli *et al*., 2003). There are few references reporting on the purification of subtilisin Carlsberg from the crude commercial Alcalase® preparation.

Before immobilising enzymes using the SphereZyme™ technology and solid supports such as Eupergit® or Dendrispheres, protein samples require partial purification since contaminants can affect immobilisation by reacting with the active chemical species required for crosslinking and thereby reducing immobilisation efficiency (Ferreira *et al*., 2003). Sample contaminants, such as salts and stabilisers can further serve as enzyme inhibitors. These inhibitors could potentially interfere in data interpretation (Tardioli *et al*., 2003).

Alcalase® is known to contain stabilizers including glycerol and monopropylene glycol (manufacturer"s specifications). Washing through dialysis or filtration is therefore required before application (Ferreira *et al*., 2003; Tardioli *et al*., 2003). We intend to investigate cation exchange as a suitable method for subtilisin purification using HiTrap™ test columns (SP XL, SP FF & CM FF). Cation exchange is the method of choice since the pI of the enzyme is 8.89 (Rao *et al*., 1998; Tardioli *et al*., 2003). Due to its trypsin-like nature, affinity chromatrography using Benzamidine Sepharose™ 6B was further investigated (Barata *et al*., 2002; De-Simone *et al*., 2005).

2.2 Aims

- Optimise the purification of subtilisin Carlsberg from crude Alcalase®
- Obtain enzyme of sufficient purity for immobilisation

2.3 Material and Methods

2.3.1 Theory of the Techniques

2.3.1.1 Desalting –Diafiltration, PD-10 desalting columns and Dialysis

These methods are employed for the removal of contaminating substances from protein solutions. Diafiltration and dialysis use a similar mechanism to remove small contaminants such as salts and rely on the retention of a protein using a membrane. Dialysis is a passive technique where diafiltration requires the application of pressure in the form of inert gas. Diafiltration further allows for the concentration of the protein of interest.

Diafiltration can be performed in a gas pressured unit (e.g. stirred cell ultrafiltration unit) to force a sample liquid across a semi-permeable membrane. It is a non-destructive method for desalting. The sample is initially concentrated by

ultrafiltration followed by washing with water or buffer to reach the required ionic strength and pH. Higher concentrations of protein may decrease the flux rate and hence increase the processing time.

Desalting using PD-10 desalting columns relies on the principles of size exclusion chromatography. The resin within these columns is sephadex G25. Proteins of molecular weight above the exclusion limit 5 kDa do not interact with the resin and will therefore be eluted first, while smaller molecules such as salts will interact with the matrix (pores) and elute at higher volumes. The column may be pre-equilibrated with a buffer of choice to achieve buffer exchange (Amersham Biosciences, PD-10 desalting column).

Dialysis: the protein sample is placed inside a semi-permeable dialysis bag with defined pore size. The bag containing a protein solution will be placed in distilled water or buffer. Exchange of liquid and salts molecules will occur, using osmotic principles. Small molecules can pass freely across the membrane whilst larger molecules, larger than the exclusion limit of the bag, will be retained. The semi-permeable dialysis bag is usually made up of cellulose acetate with pores of between 1-20 nm in diameter.

2.3.1.2 Ion exchange and size exclusion chromatography

The principle of protein purification by ion exchange is based on the relative differences in charge of proteins below or above their respective isoelectric point (pI). At a pH above a protein‟s pI the protein will have a net negative charge and vice versa. The relative charge is related to the ionisable amino acid derivatives (side chains) of the protein. The initial step of binding the protein to the chromatographic column relies on ionic attraction between opposite charges on the particles and the protein. There are two classifications of ion-exchange resins, namely cation and anion exchangers. Anion exchangers are positively charged for binding negatively charged proteins, while cation exchangers are

negatively charged and are used to bind positively charged proteins. Selection of ion exchange principle for protein purification relies on the nature of the protein, including pI and pH stability.

Functional ionic residues in ion-exchange resins include sulphonate (SO_3^-) for cation exchange and qauternary ammonium ($-N^+R_3$) for anion exchange, both of which are considered strong exchangers since they are totally ionized at working pH while carboxylate ($-COO^-$) and diethylammonium ($-HN(CH_2CH3)_2$) are considered weak because they are ionized within a narrow pH range (Wilson, 1994). The selection of a weak or a strong exchanger depends on the nature of the impurities and the relative strength of the protein binding to the support. Weak exchangers have a number of advantages such as inability to bind weakly charged impurities, enhanced elution properties and most importantly reduced tendency to cause sample denaturation (Wilson, 1994).

Size exclusion chromatography relies on separation of protein samples based on their relative size. Larger proteins having higher molecular weights will elute first (Wilson, 1994).

2.3.1.3 Protein Electrophoresis

a) SDS-PAGE

SDS-PAGE is the most frequently used method for monitoring protein purification and for estimation of a protein"s molecular mass (separates protein based on size; size standards are included for molecular weight estimation). Samples to be separated are first heated for 5 min at around 95ºC in a sample buffer which contains β-mercaptoethanol and sodium dodecyl sulphate (SDS). β-mercaptoethanol reduces protein disulfide bridges while SDS (anionic detergent) binds amino acid residues to retain the protein in a denatured state. On average one SDS molecule binds for every 2 amino acid residues. Ionisable tracking dye

usually bromophenol blue (a constituent of the sample buffer) allows for the monitoring of the progress of the electrophoretic run while glycerol gives the sample solution increased density, allowing it to settle easily during loading. The sample first passes through a stacking gel which serves to concentrate the sample to a sharp band under the effect of an electric field before it enters the separating component of the gel (Walker, 1994).

b) Zymogram

This section describes gel electrophoresis used in the study of proteins in terms of their biological activity. It is similar to SDS-PAGE however, denaturing procedures and additives are not included before the electrophoretic run. Proteins separate according to the sieving properties of the acrylamide gel under electric current (Walker, 1994). They separate according to their electrophoretic mobility at the pH of the gel (e.g. pH 8.7). Since the proteins remain functional, they may subsequently be detected using a suitable assay. The overlay method involves placing an electrophoresis gel on top of activity a gel (usually agarose) with incubation for chromophore formation or substrate clearance (protease; Lee *et al*., 1997). The coloured product or clear zone corresponds to the protein of interest. An alternate method employs the copolymerisation of the substrate in the electrophoresis gel and colour will develop after incubation at the desired pH or the addition of a secondary substrate. Another method of zymography incorporates the incubation of the electrophoretic gel in an appropriate substrate with subsequent colour formation identifying the band of interest (Walker, 1994).

2.3.2 Chemicals and Reagents

Alcalase® was a gift from Novo Industri A/S. This sample had a specific activity of 2.4 AU/G and was stored at 4 °C. Omega™ 10 kDa, low protein binding, polyethersulphone ultrafiltration membranes were purchased from Pall Life Sciences. Benzamidine Sepharose™ 6B, CM Sepharose FF and HiTrap™ cation

exchange protein chromatography cartridges (SP FF, SP XL and CM FF) were purchased from GE Healthcare. SnakeSkin® Dialysis Tubing (10kDa cut-off) was purchased from Pierce. A Mini-PROTEAN 3 Electrophoresis Cell and Protein Assay Dye Reagent Concentrate were from Bio-Rad. Unstained Protein Molecular Weight Markers were purchased from Fermentas. N,N,N‟,N‟-tetramethylethylenediamine (TEMED), Ammonium persulphate, Acrylamide/bis-Acrylamide solution (30%); 2-Amino-2-(hydroxymethyl)-1,3-propanediol (TRIS), Bromophenol Blue, Azocasein, 4-Aminobenzamidine dihydrochloride, Bovine Serum Albumin (BSA), Sodium dodecyl sulphate (SDS) and Subtilisin Carlsberg were obtained from Sigma-Aldrich Pty (Ltd).

2.3.3 Purification of Alcalase®

Two methods of protein chromatography were evaluated for Alcalase® purification, namely affinity chromatography (Benzamidine Sepharose™ 6B) and cation exchange chromatography. Three alternate cation exchange columns were evaluated for the purification of the Alcalase®, namely HiTrap™ SP XL, SP FF & CM FF). Protein chromatography was performed using an ÄKTAprime™ Plus System (GE Healthcare). Protein concentration and salt gradient were monitored using the instruments conductivity monitor (mS/cm) and absorbance measurement capabilities in OD (optical density) 280 nm. Fractions were collected (5 ml) and assayed in triplicate for alkaline protease activity and protein content as specified in section 2.3.6 and 2.3.7.

2.3.3.1 Affinity Chromatography: Benzamidine Sepharose™ 6B

A 10 ml sample of crude Alcalase® solution was dialysed using Snakeskin™ dialysis tubing (Pierce) with 10 kDa molecular weight cutoff for 3 washes in 1 litre of MilliQ water. This was subsequently lyophilized and stored at 4 °C until required. A 5 ml suspension of 80 $mg.ml^{-1}$ protein was prepared in binding buffer (50 mM Tris buffer, 500 mM NaCl, pH8) and subsequently loaded onto

Benzamidine Sepharose™ 6B column with specified binding capacity of 13 mg trypsin.ml^{-1}. The column was pre-equilibrated with binding buffer (according to manufacturer"s protocol). Bound protein was eluted with elution buffer (components as per binding buffer containing 20 mM ρ-amino-benzamidine) at a flow rate of 0.8 mg.ml^{-1} collecting 2 ml fractions (Barata *et al*., 2002; De-Simone *et al*., 2005). Eluted fractions were assayed for alkaline protease activity.

2.3.3.2 Cation exchange Chromatography

a) HiTrap™ Cation Exchange Screening

Three HiTrap™ screening columns namely CM FF, SP FF and SP XL, were used to identify the ideal cation exchange matrix for Alcalase® purification. A 1 ml Alcalase® sample (10 mg.ml^{-1}) was diafiltrated twice with 10 ml MilliQ water in an Amicon (2800) ultrafiltration stirred cell reactor with a 10 kDa cut-off polyethersulphone membrane to remove preservatives and salts. The washed preparation was resuspended to a volume of 2 ml in column loading buffer (20 mM MES, pH 6.0). Samples of 500 µl were loaded onto the three different HiTrap™ cation exchange columns pre-equilibrated in loading buffer. Bound protein was eluted with a 20 ml linear salt gradient from 0 to 500 mM NaCl in 20 mM MES pH 6. Fractions of 1 ml were collected and assayed. The chromatography resin of choice was Sepharose FF which was subsequently used for larger batch purification. Fractions containing activity were pooled and concentrated using an ultra-filtration stirred cell (Amicon 2800). The active enzyme fractions were desalted (PD-10 desalting column) according to the manufacturers protocol (Amersham Biosciences). The desalted protein was lyophilized and stored at 4 °C for further analysis.

b) CM Sepharose™ FF purification

Crude Alcalase® (50 ml) was dialysed using 10 kDa molecular weight cutoff Snakeskin™ dialysis tubing (Pierce) for 3 washes of 1 L MilliQ water. Thereafter the sample was made up to a final volume of 250 ml with MilliQ water. A 10X buffer concentrate (20 ml of 200 mM MES, pH 6.0) was added to a sample of 150 ml and made up to 200 ml (final buffer concentration of 20 mM MES, pH 6.0). This was subsequently applied to a CM Sepharose™ FF column (200 ml, XK 16/20; Amersham) column pre-equilibrated with start buffer (20 mM MES, pH 6.0). Bound protein was eluted with an 800 ml linear salt gradient from 0 mM to 500 mM NaCl in 20 mM MES buffer pH 6.0 at flow rate of 5 $ml.min^{-1}$. Five ml fractions samples were collected and assayed for alkaline protease activity. Active fractions were pooled washed and concentrated by ultrafiltration. This sample was lyophilized for storage until required for immobilisation studies.

2.3.4 Gel electrophoresis

Gel electrophoresis was performed according to a protocol by Bio-Rad adapted from Laemmli (1970). The proteins were stained using Coomassie protein stain consisting of 40% methanol, 0.7% acetic acid, 0.075% Coomasie dye, and destained using a solution containing 40% methanol, 0.7% acetic acid and MilliQ water. Sample loading buffer contained 0.0625 M Tris-HCl pH 6.8, 10 % glycerol, 2% SDS, 0.05% bromophenol blue and 5% β-mercaptoethanol. The approximate subunit molecular mass of electrophoresed proteins was determined by calibration against Protein Molecular Weight Marker (Fermentas) containing markers of 14.4 kDa, 18.4 kDa, 25.0 kDa, 35.0 kDa, 45.0 kDa, 66.2 kDa and 116.0 kDa.

2.3.5 Zymography

A protease zymogram (overlay method) was adapted from Lee *et al*., (1997) to identify protease activity after non-denaturing SDS-PAGE. Native PAGE was prepared as in section 2.3.4 but omitted the protein heat treatment step before resolution. After electrophoresis the gels were incubated in renaturing buffer (25% v/v Triton X-100) for 30 minutes at room temperature with gently agitation. The gel was overlaid with a pre-cast 1% agarose slab containing 0.6% casein in 50 mM Tris-HCl buffer at pH 7.5 for 30 minutes at 37 ºC. Protease activity was detected by clear zones.

2.3.6 Enzyme Assays

The azocasein reagent was prepared by dissolving azocasein (2.5% w/v) in warm (35 ºC) 50 mM Borax-NaOH buffer pH 9.5. Protease samples of 12.5 µl were added to a reagent volume of 250 µl and incubated for 20 minutes at 30 °C. Water was used in the reagent blank. The reaction was terminated with 1 ml of 4 °C TCA (5%) with incubation for 30 minutes at 4 °C. The sample was centrifuged for 5 minutes at 17000 x *g* in Thermo Scientific, Heraeus Pico 17 microcentrifuge. A 200 µl volume of the assay supernatant (containing released azo dye) was sampled into a flat-bottomed microtiter plate and the absorbance was read at 340 nm (Iversen and Jørgensen, 1995). One unit of total proteolytic activity (U) was defined as the amount of enzyme required to produce a change of 1 OD per minute at 340 nm (D'Ambrosio *et al*., 2003).

2.3.7 Protein Quantification

Protein assays were performed using the Bio-Rad-Coomassie® Protein Dye Reagent according to the manufacturer‟s protocol. This assay is based on the method of Bradford (1976) for protein quantification. Dye reagent was prepared by diluting 1 part dye reagent with 4 parts MilliQ water. The reagent was filtered

through Whatman #1 filter paper to remove dye particulates as per the manufacturers‟ specification.

Dilutions of BSA protein standards were prepared and assayed to generate a standard curve and compared to the manufacturer‟s protocol. The linear range of the assay was found to be from 0.05 $mg.ml^{-1}$ to 0.5 $mg.ml^{-1}$. Samples of 10 µl protein standards or protein sample were mixed with 200 µl diluted dye reagent in a flat-bottomed microtiter plate. The plate was incubated at room temperature for at least 5 min and the absorbance of samples was measured using spectrophotometric analysis at 595 nm in a PowerWave™ HT microtitre plate reader (BioTek® Instruments). Protein concentration was determined by linear regression analysis of the standard curve of OD at 595 nm (fig. 2.1 and Eq. 2.1).

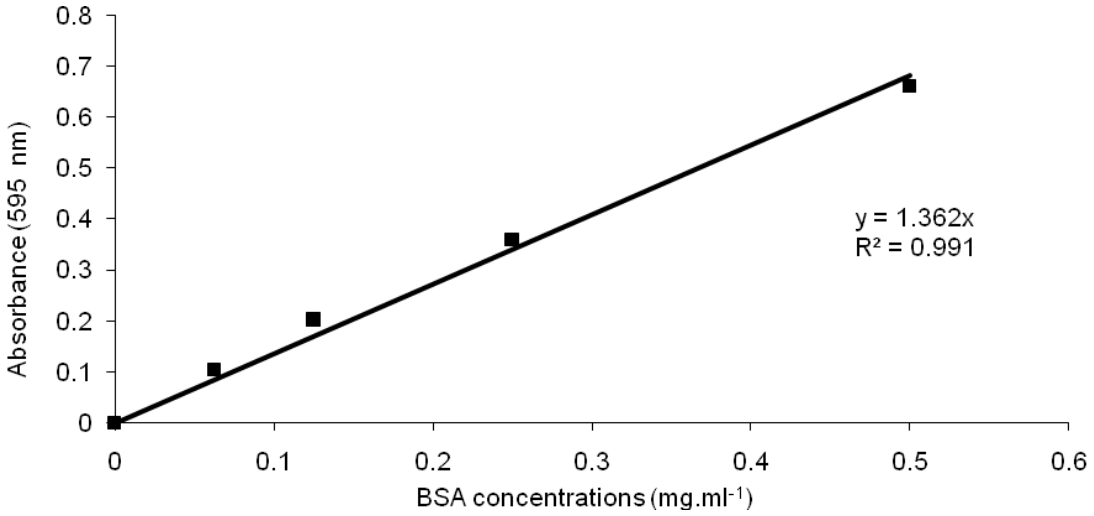


Figure 2.1: Standard Curve of standard protein solution (BSA) in Milli-Q water

All assays were performed in triplicate and data was represented as mean ± standard deviation

Equation 2.1: Protein concentration ($mg.ml^{-1}$) = (A/1.3622)*DF

Where:

A = Absorbance at 595 nm

DF = Dilution factor

2.4 Results and Discussion

2.4.1 Alcalase® Purification

Chromatographic resins were assessed for the purification of Alcalase®. These included the affinity matrix Benzamidine Sepharose® 6B and three HiTrap™ cation exchange test columns, namely SP FF, SP XL and CM FF. These resins were assessed with respect to the relative ability to purify protease and protein capacity.

Benzamidine Sepharose 6B is an affinity resin for trypsin like proteases. Although Alcalase® is considered to be a trypsin-like protease, it appeared to have no affinity for this matrix as demonstrated by the elution profile.

Cation exchange was chosen as a suitable purification method due to the relatively high pI of Alcalase®. Three HiTrap™ screening columns (SP XL, SP FF and CM FF) were evaluated for the purification of Alcalase®.

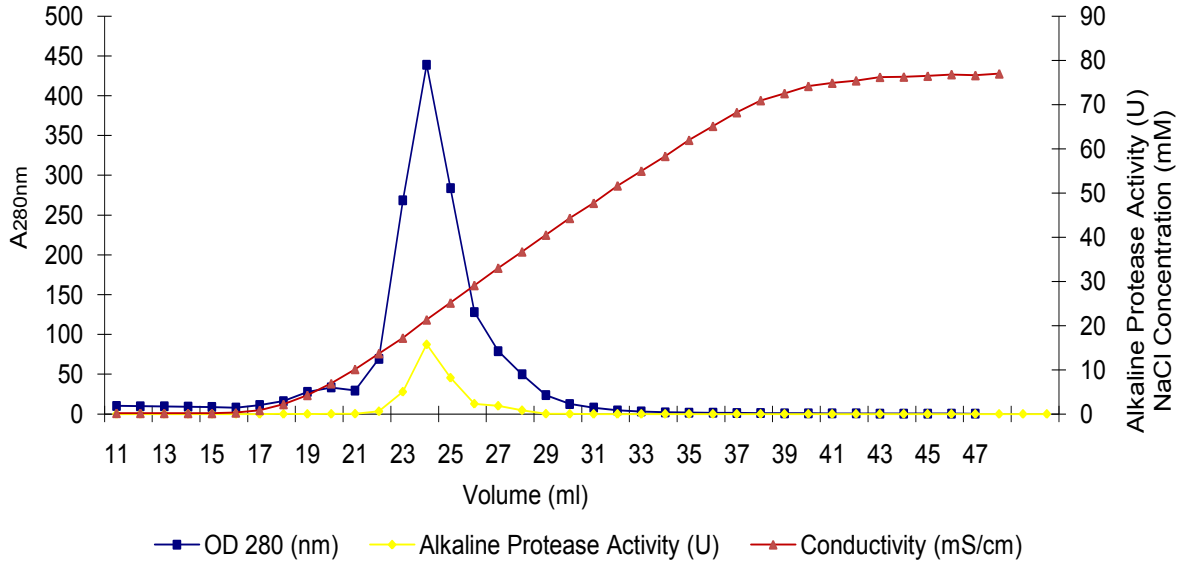


Figure 2.2: Elution profile during purification of Alcalase® sample through HiTrap™ CM FF column.

The protein eluate from CM FF showed the highest enzyme purity, measured by specific activity, as well as the highest protein binding capacity (fig. 2.2) and was thus selected as the matrix of choice for protease purification (section 2.4.2).

2.4.2 Alcalase® purification through CM Sepharose® FF

The high alkaline protease activity was found to be between the elution volumes of 80 ml and 140 ml (designated F1; fig. 2.3). Other protease containing peaks corresponding to 150-220 ml and 275-380 ml were designated F2 and F3 respectively. However, these samples were not analysed further due to their comparatively low enzyme activity as seen in the purification table (table 2.1). Of interest was the purification of the major protease. The presence of alternate proteases could potentially complicate immobilisation studies.

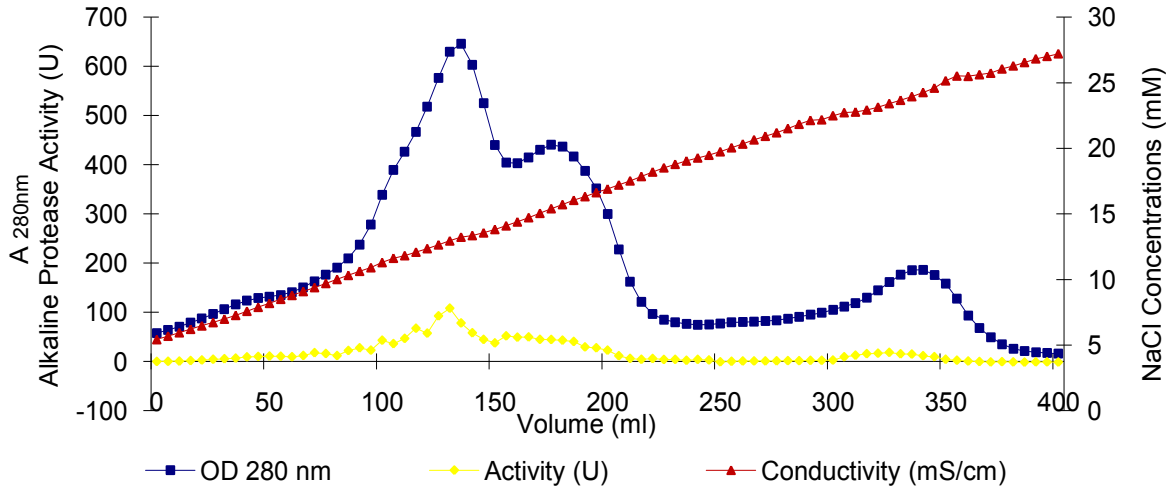


Figure 2.3: Elution profile of Alcalase® through 200 ml, CM Sepharose® FF (strong cation exchanger) in XK 16/20 Amersham column at a flow rate of 5 ml.min^{-1}.

Table 2.1: Alcalase® purification table

Sample Description	Protein (mg)	Alkaline Activity (U)	Specific Activity (U.mg^{-1})	Yield (%)	Fold Purification
Alcalase®*	403.4	2280	5.65	100	1.00
Dialysis*	290.6	2217	7.63	97	1.40
Anion* Exchange	207.8	1419	6.83	62	1.21
F1#	107.7	830	7.71	36	1.40
F2#	63.8	446	7.00	20	1.20
F3#	36.3	142	3.93	6.0	0.70

* Samples adjusted for column loading (60% of starting sample)

Values are totals of the different fractions purified

Different proteins from the Alcalase® sample were separated as depicted by the three peaks (denoted as F1, F2, and F3) from the elution profile (fig. 2.3) as determined by the protease activity assay (table 2.1) and protease zymogram (fig. 2.4). Thus, protease corresponding to the peak F1 (later referred to as partially purified Alcalase®) was deemed sufficiently pure for SphereZyme™ immobilisation.

A collective yield of 62% of the protein was recovered after cation exchange purification (table 2.1). The yield was further subdivided into 3 protease containing fractions, the largest of which had a fold purity of 1.4 and yield of 36% of the starting enzyme activity. Dialysis resulted in high enzyme activity yield of 97% but approximately 25% loss in the overall protein content. This was likely due to the loss of stabilisers and peptides. Since each protease would behave differently during immobilisation, the separation of each protease was important for subsequent immobilisation and characterisation.

Native and SDS-PAGE (fig. 2.4a and fig. 2.5, respectively) were performed to determine the extend of purity on the main fraction (F1, refered to as partially pure Alcalase®) compared to Alcalase® crude sample. The crude sample (fig. 2.4a, lane 1; fig. 2.5, lane 1-2) was partially purified as depicted in fig. 2.4a (lane 2) and fig 2.5 (lane 5-6). The partially purified sample had a similar band to that of the positive control (commercially available subtilisin Carlsberg; fig. 2.4a, lane 3). The samples tested were also shown to possess alkaline protease activity. For instance the activity profile of the samples shown in zymogram gel (fig. 2.4b) corresponded to that of native-PAGE gel (fig. 2.4a).

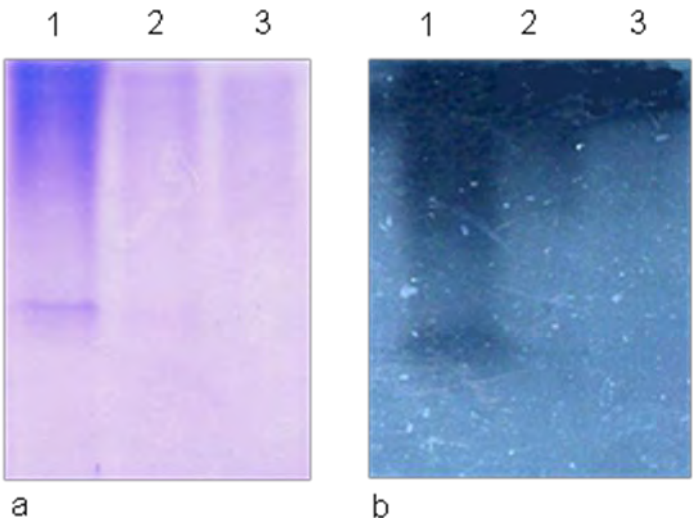


Figure 2.4: Electrophoretic comparison of the Alcalase® crude sample (lane 1) and its partially purified form (lane 2) to the positive control (commercially available subtilisin Carlsberg; lane 3) using Native PAGE (15% polyacrylamide gel; a) and Protease zymogram (b).

All three samples were shown to contain alkaline protease activity while the native PAGE showed similar enzyme profiles for the partially purified and purified commercial preparation.

Figure 2.5 shows an SDS-PAGE profile of Alcalase® crude sample, compared to its dialysed and partially purified sample. It appeared that the lower molecular weight compounds in the crude preparation (lane 1-2) and the dialysed fractions (lane 3-4) were removed using cation exchange chromatography (CM Sepharose, lane 5-6). Although there was a 25% reduction in total protein content after dialysis as measured by Bradford, there appeared to be no reduction in the protein content (as per SDS PAGE, lane 3-4 are dialysed vs lane 1-2 are crude Alcalase®). This may have been due to the removal of potentially interfering stabilisers. Similar results of SDS-PAGE were reported by Tardioli *et al*., (2007) where a comparison of the crude and dialysed sample was made. Ferreira *et al*., (2003) also reported on the composition and characterisation of crude commercial Alcalase® sample.

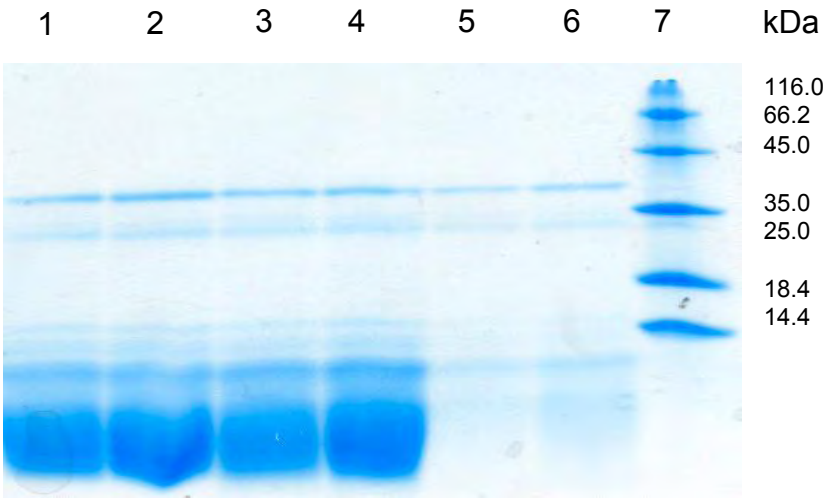


Figure 2.5: SDS-PAGE (15% polyacrylamide) of crude (lane 1-2), dialysed (lane 3-4) and partially pure Alcalase® (27.8 kDa; lane 5-6) at 2 concentrations.
Lane 7 is the molecular weight standard consisting of proteins of 14.4, 18.4, 25.0, 35.0, 45.0, 66.2 and 116.0 kDa.

2.5 Conclusions

The commercial alkaline protease preparation, Alcalase® was successfully purified into 3 protease containing fractions using cation exchange chromatography. The fraction with the highest protease activity correlated well with subtilisin Carlsberg from a purified commercial preparation of the enzyme. There are few reported instances of the purification of subtilisin Carlsberg from Alcalase®, but the method of purification described in this chapter compares favourably to previously reported values.

The major protease containing fraction was used for SphereZyme™ immobilisation to ascertain the limitation of this self-immobilisation technology for the catalysis of large substrates and the subsequent modification of the immobilisation method to overcome this limitation.

3 Immobilisation of Alcalase® using SphereZyme™ Technology

3.1 Introduction

The selection of a technique for immobilisation is based on the intended application of the immobilised product, taking into consideration the environment and/or enzyme (Cao, 2005). An ideal enzyme immobilisation technique is chosen to address and circumvent the drawbacks faced by the biocatalyst for the application as stipulated in section 1.4.5.

The applicability of immobilised enzymes is still hindered by several factors, including the hydrolysis of relatively large polymeric substrates. Their immobilisation limits the interaction between the enzyme and the substrate resulting in low activity maintenance for the immobilisation technique (Nakagomi and Ajisaka 1990; Galaev and Mattiasson 1999; Hamerska-Dudra *et al.,* 2007). This limitation is more pronounced in self-immobilisation techniques due to high substrate diffusional limitations as compared to solid support immobilisation methods (Brady *et al*., 2008; Sangeetha and Abraham, 2008). This limitation is thought to be as a result of many factors including a relatively tight crosslinking network between enzyme molecules and their incorrect orientation in the network (Cao, 2005; Brady, *et al*., 2008). Other factors include reduced immobilisation efficiencies due to the low content of lysine residues on the enzyme‟s surface (López-Gallego, *et al*., 2005b).

The crosslinking capability of glutaraldehyde is well documented (Payne 1973; Cao *et al*., 2000). However, due to its small size and reactivity, it can easily penetrate the protein and lead to deactivation (Chae *et al*., 1998). Over-cross-linking of the protein can also lead to distortion and deactivation of the protein. Ethylenediamine (EDA) possesses two terminal amine groups which can react with the terminal aldehyde groups of glutaraldehyde, thus increasing the chain

length of the crosslinking agent (Brady *et al*., 2008; scheme 3.2). This elongated crosslinker was reported to have positive impact on the activity of the immobilised enzyme (Moolman *et al*., 2005; Kaul *et al*., 2007). Elongating glutaraldehyde with PEI or replacing glutaraldehyde with dextran polyaldehyde was also reported to increase the stability as well as the activity retention of the immobilised enzyme (Mateo *et al*., 2004; L pez-Gallego *et al*., 2007). High molecular weight (i.e. >100 kDa) poly-functional polymers are reported to enhance the activity of the immobilised enzyme as compared to glutaraldehyde coupled proteins (Fernández-Lafuente *et al*., 1999; Manta *et al*., 2003). The enhancement in activity was proposed to be due to protection of the active site and looser crosslinking network (Mateo *et al*., 2004). The protective role of polyfunctional crosslinkers results from their inability to penetrate the enzyme‟s active site to react with amino acids that are essential for catalysis. The loose crosslinking network allows for increased substrate and product diffusion enabling improved substrate catalysis (Fernández-Lafuente *et al*., 1999; Mateo *et al*., 2004).

Epoxide functional groups are able to react with a variety of amino acids groups on the enzyme surface (e.g. lysine, histidine, cysteine and tyrosine) to form secondary amino, ether or thio-ether bonds (Mateo *et al*., 2002). Their applications as functional groups and as crosslinking agents are of further interest.

The limitations of self-immobilisation techniques for the biocatalysis of polymeric substrates have resulted in most of the research to date, being based on improving solid support immobilisation (Tardioli *et al*., 2003; Kannan and Jasra, 2009). The proposed research aims at evaluating the novel immobilisation technique, SphereZyme™ for its limitation for polymeric substrate (protein) hydrolysis. In order to investigate the potential drawback for large substrate biocatalysis, Alcalase® was chosen as the model enzyme. We further evaluate mechanisms of overcoming any observed limitations through the use of large polyfunctional crosslinking agents.

3.2 Aims

- Examine the limitation of SphereZyme™ for large substrate biocatalysis
- Investigate the use of alternate crosslinking agents to alleviate this limitation

3.3 Materials and Methods

3.3.1 Chemicals and Reagents

Mineral oil and Nonoxynol were purchased from Castrol and BASF respectively. Trizma Base, casein, azocasein, ethylenediamine, 50% v/v polyethyleneimine, *p*-Nitrophenol Acetate (PNPA), 6.0 kDa polyethylene glycol, 15-30 kDa Dextran, sodium periodate, sodium borohydride, sodium hydroxide, acetic anhydride, dryethyl ether, digylcidyl ether and dimethylsulfoxide (DMSO) were purchased from Sigma Aldrich Pty.(Ltd). Trichloroacetic acid was purchased from Merck. Alcalase® was a gift from Novozymes®.

3.3.2 Esterase and Protease Zymogram

To determine whether an esterase assay could indeed be used to monitor protease activity (Polgár, 1990), an esterase/lipase and protease zymogram of the partially purified Alcalase® (section 2.4.3) was deemed necessary such that the esterase activity towards the substrate correlated with protease and not residual esterase or lipase activity. The presence of lipase or esterase in the sample would interfere in the assay and subsequent characterisation of the immobilised enzyme. Corresponding clearing zones in the protease zymogram and development of dark purple colour in the esterase zymogram would indicate the ability to use esterase substrates for protease activity determination.

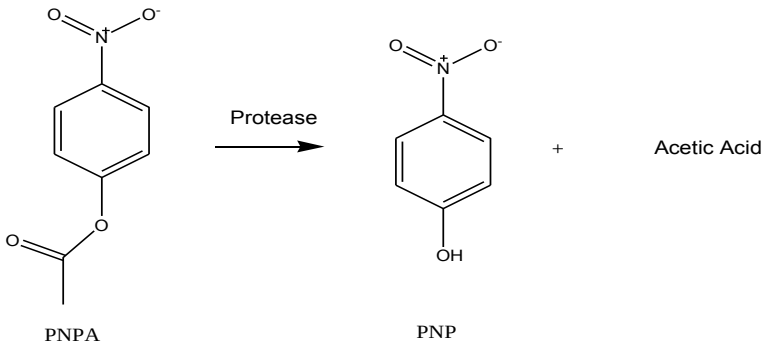
A zymogram protocol was adapted from Gudelj *et al*., (1998) with the following modifications: Native PAGE was run according to section 2.3.4 but omitted the protein heat treatment step. After electrophoresis, the gel was overlaid in a solution comprising: 10 ml of 0.1 M sodium phosphate pH 7, 1 ml substrate stock (1% α-naphthyl acetate w/v in acetone) and 0.25 ml of fast blue B stock solution (2% w/v in water). Ester hydrolysis by protease was detected by the development of a purple band on the gel within 5 minutes.

3.3.3 Protease Assays

The molecular mass difference of azocasein (large) and PNPA (small) used for activity assays enables the analysis of the mass transfer properties of the immobilised enzyme particles based on substrate size (Tardioli *et al*., 2003; Yu *et al*., 2006; Brady *et al*., 2008).

3.3.3.1 *p*-Nitrophenyl Acetate (PNPA)

An assay using PNPA as a substrate was adapted from Dillion and Lane (1993) and modified according to Brady *et al*., (2008). This assay is based on the cleavage of an ester bond (scheme 3.1). This reaction liberates a yellow product *p*-nitrophenol which is followed spectrophotometrically at 410 nm.



Scheme 3.1: The release of PNP and acetic acid from PNPA hydrolysis by enzymatic cleavage using esterase/protease.

PNPA was dissolved in isopropanol at 30ºC. This constituted the substrate stock solution which was mixed with 0.05 M Tris Buffer pH 7.5 to attain a final concentration of 1 mM PNPA in the assay reagent. Thereafter, 10 µl of enzyme solution was mixed with 240 µl of assay reagent and the kinetic activity was followed at 410 nm for 5 minutes in a microtiter-plate well (light path of 0.67 cm). The control contained 10 µl of water. Units of enzyme activity were calculated from the experimentally determined extinction coefficient.

3.3.3.2 Azocasein

The large substrate assay was performed as described previously, refer to 2.3.6. All assays were performed in triplicate and data was represented as mean ± standard deviation.

3.3.4 Protein Crosslinking

Solutions of both 100 and 50 $mg.ml^{-1}$ purified Alcalase® sample (section 2.3.3.2) were prepared in 50 mM Tris-HCl buffer pH 8. Although Tris has a primary amine, we have previously demonstrated that this does not quench aldehyde groups at pH 8 (Jordaan *et al*., 2009a). Enzyme samples of 200 µl were evaluated for their ability to crosslink using agents described in section 3.3.6.1. The crosslinking agents were added in each well as depicted in table 3.1. Experiments were performed in a microtitre plate at room temperature and were allowed to crosslink for 24 hours.

To another microtitre plate, albumin was used as a model protein for evaluation of crosslinking efficiency of polyfunctional polymers described in section 3.3.6.2 (b)-(d). Two concentrations (100 and 50 $mg.ml^{-1}$) of albumin were prepared in either MilliQ water or 20 mM universal buffer pH 7 and pH 10. A 50 µl protein sample was subsequently added to micro-titer plate wells. Thereafter crosslinking agents were added in each well in the volumetric ratios as depicted in table 3.2.

The plate was left at room temperature for 24 hours. Optimum crosslinking efficiency was determined qualitatively using the solidification or gelation of the protein solution (Jordaan *et al*., 2009a).

3.3.5 SphereZyme™ Preparation

Protease SphereZyme™ particles were prepared according to Brady *et al*., (2008) with some modification as reported in Jordaan *et al*., (2009a). A solution of 100 $mg.ml^{-1}$ purified protease (section 2.3.3.2) with and without 10% albumin was prepared in 50 mM Tris-HCl buffer pH 8.0. Protein solution volumes of 0.2 ml were mixed with 0.05 ml active site protectant (2.5% w/v casein) for 5 minutes prior to emulsification. After 5 minutes the crosslinking agents (section 3.3.6.1 and 3.3.6.2) were added to the protein-protectant solutions. Immediately after mixing, the protein solution was emulsified in 5 ml of 4 ºC mineral oil containing 0.05 ml nonoxynol with stirring at 700 rpm for 10 minutes. The emulsified enzyme preparation was allowed to crosslink for 12 hours at 4 ºC (to prevent enzyme denaturation) with stirring at 300 rpm.

The crosslinked particles were recovered using centrifugation at 3901 x *g* for 5 minutes in Beckman Coulter™, Allegra™ X-22R benchtop centrifuge, fitted with swinging bucket rotor. Thereafter, the SphereZyme™ particles were washed 6 times in 50 ml of 50 mM Tris-HCl buffer pH 8 containing 1 mM ethanolamine to quench all the aldehyde groups that may react with the substrate. After washing, the particles were resuspended in their respective buffer and assayed according to the methodology described in section 2.3.6 (for the azocasein assays) and in section 3.3.3.1 as per the PNPA assay to determine enzyme activity maintenance.

3.3.5.1 Evaluation of variable EDA concentrations

Glutaraldehyde: EDA crosslinking agents at different concentrations of EDA as mentioned in section 3.3.6.1 (b) were evaluated for improving the % activity maintenance of Alcalase® SphereZyme™. Reaction time for each crosslinking agents was changed to 10 minutes to ensure efficient reaction of the glutaraldehyde with EDA, prior to mixing with the protein to initiate crosslinking (section 3.3.5).

3.3.5.2 Effect of enlarged crosslinking agents

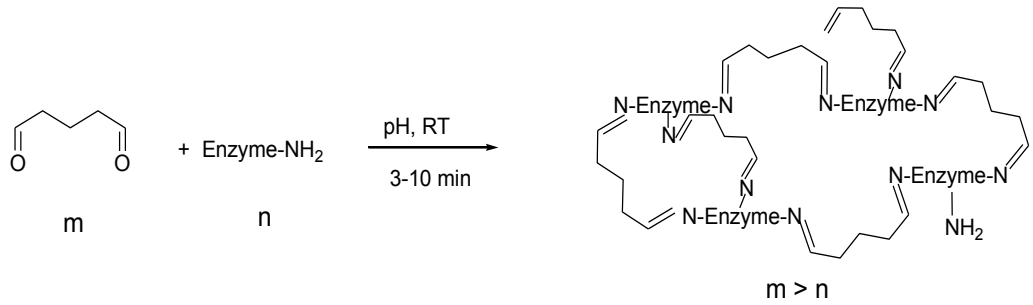
Protease SphereZyme™ particles were prepared as in section 3.3.5. However, the glutaraldehyde and glutaraldehyde: EDA were replaced with agents indicated in 3.3.6.2 such as dextran and PEG epoxy, dextran and PEG aldehyde and glutaraldehyde: PEI solutions. All the experiments were conducted in duplicates.

3.3.6 Crosslinking Agents

3.3.6.1 Standard SphereZyme™ immobilisation protocols

a) Glutaraldehyde

A 25% v/v Glutaraldehyde Grade II solution from Sigma Aldrich Pty.(Ltd) was used, as its crosslinking capabilities are well documented (Payne 1973, Cao *et al*., 2000). Crosslinking occurs through a Schiff base reaction between the aldehyde groups of the crosslinking agent and primary amines of lysine on the enzyme surface (scheme 3.2).



Scheme 3.2: Proposed method for enzyme crosslinking using a bifunctional crosslinking agent.

b) Glutaraldehyde: Ethylenediamine

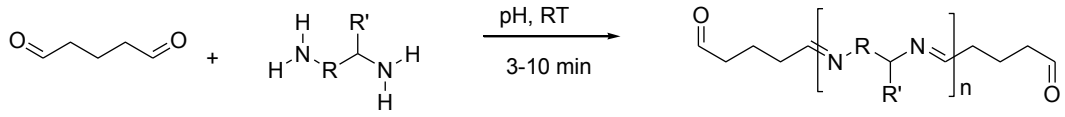
Ethylenediamine (EDA) can be used to increase the length of glutaraldehyde and has been shown to improve the crosslinking efficiency of proteins (Cao and Elzinga, 2003). EDA contains two terminal primary amines which will react with terminal aldehyde groups of glutaraldehyde. Molar excesses of glutaraldehyde are required so that the reaction contains terminal aldehyde groups. The elongation of the glutaraldehyde using EDA could potentially provide improved substrate diffusion and thereby improved reactivity between the crosslinked enzyme and the substrate (Cao and Elzinga, 2003).

Different molar ratio"s (0.33, 0.66, 0.99 M) of aqueous (EDA) were prepared and mixed with glutaraldehyde (25%) at 1:1 volumetric ratio for 5 minutes prior to addition to the protein as a crosslinking agent. The principle behind the reaction is indicated in scheme 3.2.

3.3.6.2 Polymeric crosslinking agents

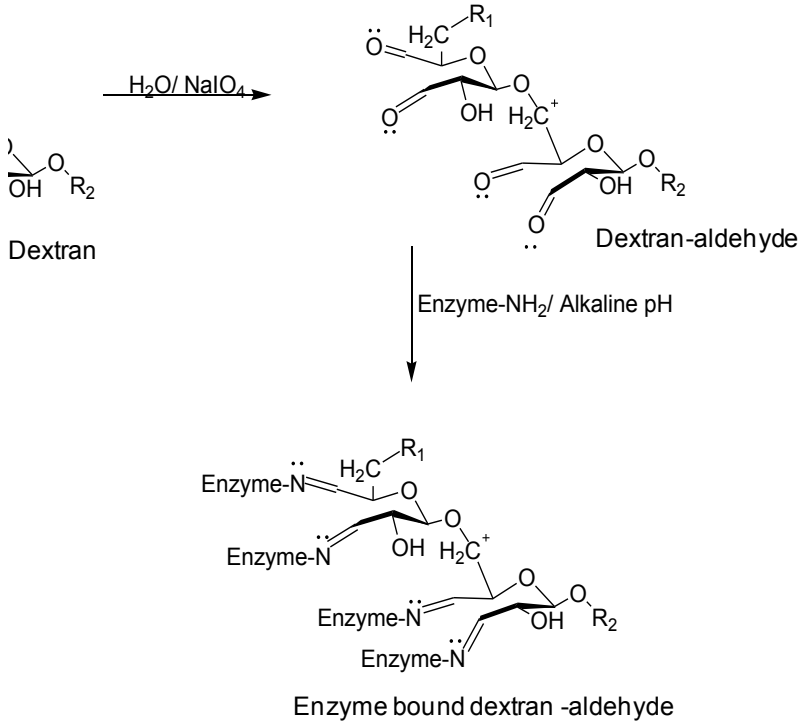
a) Glutaraldehyde: Polyethyleneimine

A longer crosslinking agent was prepared by reacting glutaraldehyde (section 3.3.2.1) with aqueous alkaline Polyethyleneimine (PEI) solution (3% and 5%) with a 1:1 volumetric ratio (López-Gallego *et al*., 2007). The two solutions were reacted for 2 minutes, longer reaction periods resulted in gelation.



Scheme 3.3: Elongation chain reaction of glutaraldehyde with primary amine compound (e.g. EDA or PEI).

b) Dextran and PEG Aldehyde

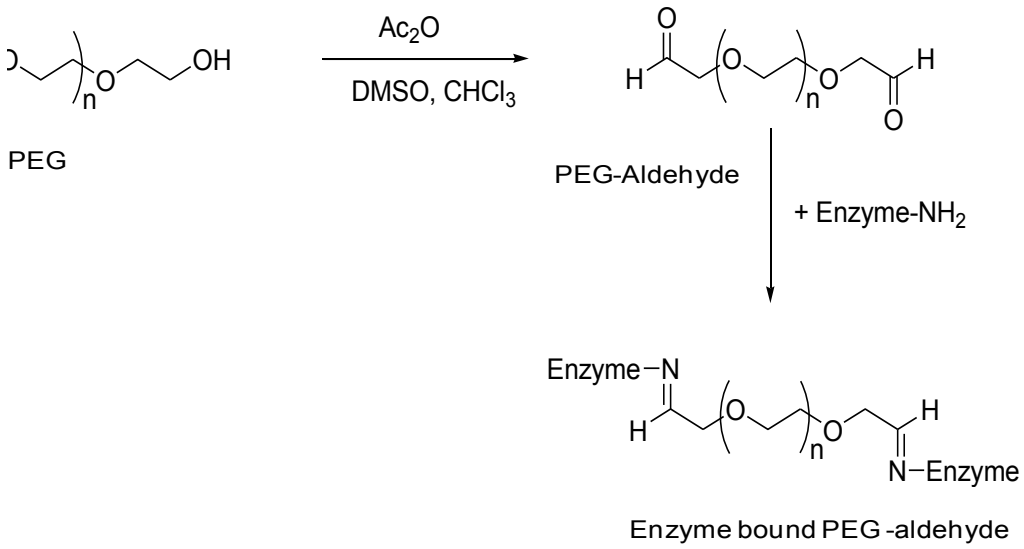


Scheme 3.4: Dextran oxidation with sodium periodate to dextran aldehyde and demonstration of application to enzyme coupling.

Preparation of these crosslinking agents involved oxidation of either 20 kDa dextran or PEG (3.33 g in 100 ml MilliQ water) with 8 g of sodium periodate. The reaction was incubated for 3 hours at 25 ºC (scheme 3.4). The oxidised dextran solution was dialysed against 50 volumes of MilliQ water to remove un-reacted periodate (Guisan *et al*., 1997; Betancor *et al*., 2005). The dialysed solution was concentrated to 10 ml using an Amicon (2800) ultrafiltration stirred cell reactor with a 10 kDa cut-off polyethersulphone membrane.

Preparation of PEG-aldehyde was further performed using a method adapted from Harris *et al*., (1984) and Ramos *et al*., (2006). To 17 ml dimethylsulfoxide (DMSO), a 4.6 ml acetic anhydride solution containing 5 g of PEG (20 kDa), was added. After 30 hours of stirring at room temperature the solution was added dropwise to 100 ml of dry ethyl ether. The precipitant was re-dissolved into

chloroform and the PEG-aldehyde re-precipitated with dry ethyl ether. This was repeated three times for removal of the chloroform (scheme 3.5).

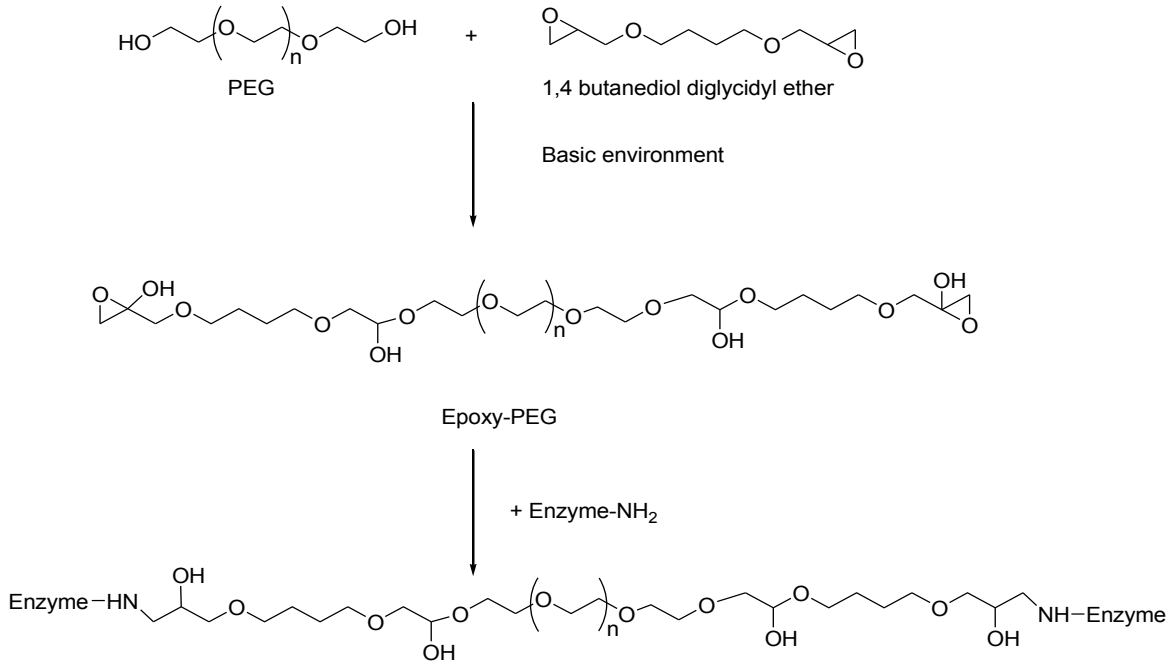


Scheme 3.5: Oxidation reaction of the terminal hydroxyl groups of PEG with Ac_2O in DMSO.

Mechanism of enzyme coupling using product is also shown.

c) Epoxy PEG or Dextran

PEG or Dextran was activated with epoxy groups using a method adapted from Head *et al*., (1989). Solutions, 10 ml of 8 % w/v PEG (20 kDa) or dextran (20 kDa) were prepared. To these, 1 ml of diglycidyl ether and 3 ml of 2 M sodium hydroxide containing 20 mg sodium borohydride were added. The solutions (scheme 3.6) were agitated for 15 hours at room temperature. A washing step was performed using an Amicon (2800) ultrafiltration stirred cell reactor with a 10 kDa cut-off polyethersulphone membrane using Milli-Q water.



Scheme 3.6: Preparation of PEG-Epoxy (P-E) using a bis-epoxide in a basic environment followed by reduction with $NaBH_4$.

The scheme further shows that this agent can be used to couple to the primary amine groups of enzymes.

3.4 Results and Discussion

3.4.1 Calculation of Extinction Coefficient

From the linear regression analysis of the standard curve in fig. 3.1, the extinction coefficient was calculated to be 7 500 M^{-1} for a 0.67 cm path length. The extinction coefficient is indicated by the slope of the straight line. For a 1 cm path length, the extinction coefficient was calculated to be 11194.03 $M^{-1}.cm^{-1}$ according to Eq. 3.1.

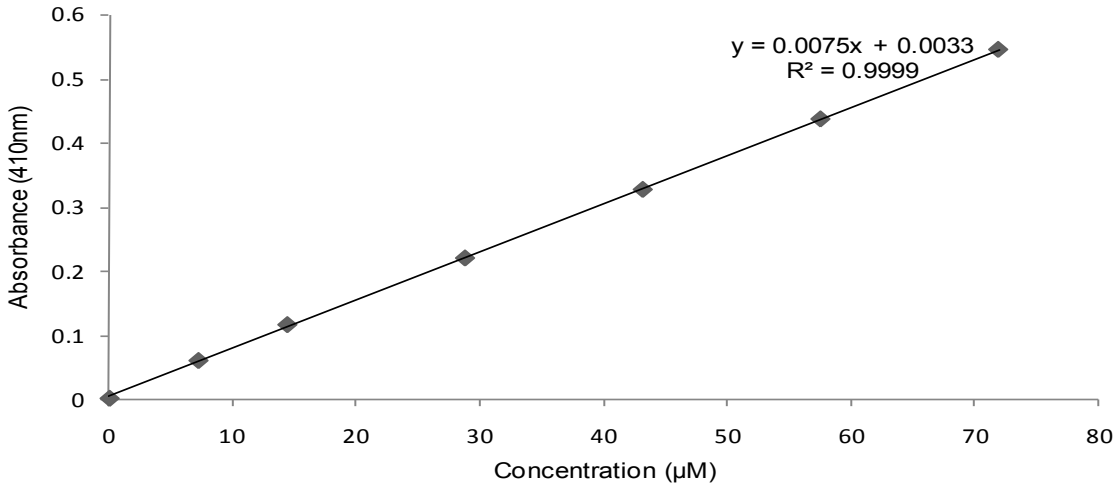


Figure 3.1: Standard curve of *p*-Nitrophenol (PNP) for the determination of the extinction coefficient at pH 7.5.

Equation 3.1: E= A/cl (Beer-Lambert Law)

Where:

E= extinction coefficient

A= absorbance at 410 nm

c= concentration (µM)

l= path length (cm)

3.4.2 Esterase and Protease zymogram

The zymograms compared favourably in that the crude Alcalase® (lane 1) and partially purified Alcalase® (lane 2) exhibited similar profiles for protease (fig. 3.2b) and esterase activity (fig 3.2a). The purified enzyme compared well to commercial enzyme (fig. 3.2, lane 3; Tardioli *et al*., 2003). This verified that Alcalase® proteases could hydrolyse ester bonds as previously reported (Müller and Bordusa 2000; Bhaskar *et al*., 2002). The results indicated further that ester cleavage could indeed be used to monitor protease activity since no ester activities without corresponding protease activity could be detected.

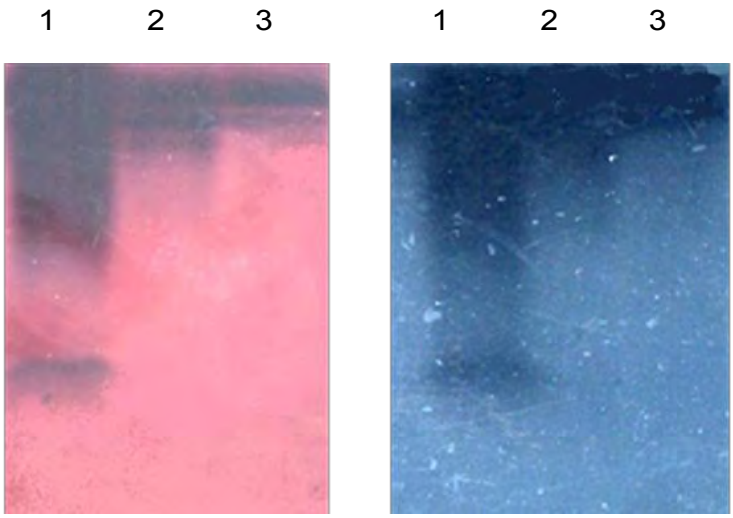


Figure 3.2: Esterase (a) and Protease (b) zymogram gels for verification of both alkaline protease and esterase activity

Lane 1 depicts Alcalase® crude sample, lane 2 represent a lane for partially purified Alcalase® sample (this thesis) while lane 3 contains the positive control (commercially available subtilisin Carlsberg).

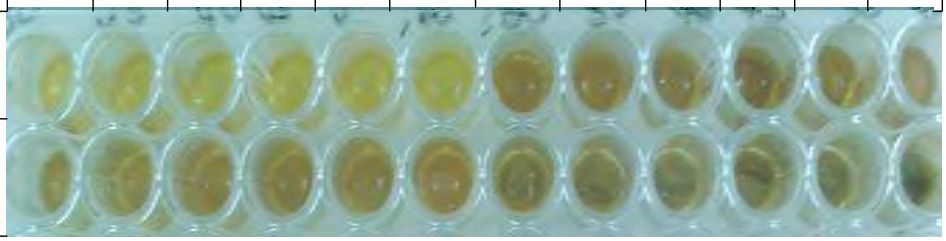
The use of esterase substrates such as PNPA is beneficial as the assay is more sensitive for enzyme activity maintenance determination and subsequent

experimentation, especially for enzyme activity maintenance experimentation where low levels of activity were expected.

3.4.3 Protein Crosslinking

Prior to Alcalase® SphereZyme™ manufacture, the crosslinking agents were tested for their efficiency to couple proteins. These results would further indicate the concentration required to achieve protein crosslinking.

Table 3.1: Crosslinking efficiency of glutaraldehyde and glutaraldehyde: EDA reactions for purified Alcalase®.

Crosslinking agents		Glutaraldehyde						Glutaraldehyde: EDA					
Volume (µl)		30	35	40	45	50	60	30	35	40	45	50	60
Enzyme sample	Well #	1	2	3	4	5	6	7	8	9	10	11	12
50 mg.ml^{-1}	A												
100 mg.ml^{-1}	B												

The formation of an orange-brown precipitate in the well indicated the suitability for crosslinking of protein; wells B10 and B11.

Crosslinking was visualised as the formation of solid mass or precipitate and an orange-brown colour formation after 24 hours as reported in Jordaan *et al*., (2009a). Cross-linked protein-to-protein network was observed in well # B10 and B11 (glutaraldehyde: EDA solution at 45 and 50 µl respectively, mixed with 100 mg.ml^{-1} enzyme solution; table 3.1). These conditions were used for the preparation of SphereZyme™ immobilised enzymes. The lack of optimum crosslinking in glutaraldehyde solution at both enzyme concentrations might be due to the low abundance of lysine groups (<9 residues) on the surface of subtilisin Carlsberg (Pal *et al*., 2002).

Dextran-aldehyde appeared to be the only polymeric crosslinking agent capable of crosslinking with albumin as the model protein. Crosslinking was seen as a brownish jelly-like material after 24 hours (Jordaan *et al*., 2009a). The colour and „jelly-like" material formed with dextran-aldehyde intensified with an increase in alkalinity of the albumin solution. This trend was expected due to the more nucleophilic nature of primary amines at alkaline pH, sensitising it to reaction with electrophiles (e.g. carbonyls; López-Gallego *et al*., 2005b). The best conditions for crosslinking using this agent were with 50 µl of 100 $mg.ml^{-1}$ protein and 150 µl of 33.3% w/v dextran aldehyde solution at pH 10 (well # F7, table 3.2).

Table 3.2: Crosslinking efficiency of large crosslinkers PEG Aldehyde (P-A), Dextran Aldehyde (D-A), PEG Epoxy (P-E), Dextran Epoxy (D-E) and DMSO PEG Aldehyde (DP-A) with Albumin.

Cross-linking agents		P-A	D-A	P-E	D-E	DP-A	P-A	D-A	P-A	D-E	DP-A
Protein Concentration	Well #	1	2	3	4	5	6	7	8	9	10
50 $mg.ml^{-1}$	A										
100 $mg.ml^{-1}$	B										
50 $mg.ml^{-1}$	C										
100 $mg.ml^{-1}$	D										
50 $mg.ml^{-1}$	E										
100 $mg.ml^{-1}$	F										

Milli-Q water — pH 7.5 — pH 10 (1:2 Enzyme: CA)

Milli-Q water — pH 7.5 — pH 10 (1:3 Enzyme: CA)

The effectiveness of DA over PEG aldehyde preparations is likely due to the fact that dextran has a higher functional group density i.e. more hydroxyl side chains that can be oxidised than PEG (terminal hydroxyls), providing more functional groups for effective crosslinking (Veronese, 2001; Roberts *et al*., 2002).

The albumin solution appeared turbid immediately after adding PEG-aldehyde; however, the solution did not solidify during the 24 hour incubation (well # A1-D1, A6-D6, E1-H1, A6-D6 and E7-H7, table 3.2). The turbidity of the protein solution could potentially be due to the precipitating action of PEG at high concentrations.

Epoxy-oxirane activated PEG and dextran did not appear to induce protein crosslinking under the conditions evaluated. Epoxides are more stable functional groups than aldehydes and require longer periods to react with primary amine groups (Mateo *et al*., 2007b). The standard crosslinking procedure for epoxide functionality is recommended as 24 hours (Mateo *et al*., 2007b), while protocols for immobilisation of enzymes to Eupergit® recommend up to 48 hours.

3.4.4 SphereZyme™ Preparation

The use of a protectant, defined as a compound that protects the enzyme active site during crosslinking, is an important component of SphereZyme™ preparation as it has been shown to increase the activity maintenance for the immobilised enzyme (Brady *et al*., 2008). This was indeed the case for protease where the % activity maintenance doubled for large substrate catalysis and increasing over 10 fold for small substrate activity. For azocasein the enzyme activity maintenance increased from 2.26% to 4.03%, while PNPA increased from 0.021% to 2.19% (table 3.3).

3.4.4.1 Addition of EDA

Table 3.3: The influence of variable EDA concentrations.

*Sample	Activity (U)		Activity Maintenance (%)	
	Azocasein	PNPA	Azocasein	PNPA
Free enzyme	15.28 ± 0.02	3.51 ± 0.029	100 ± 0.14	100 ± 0.027
#0.33 M EDA	0.35 ± 0.02	0.0012 ± 0.004	2.26 ± 0.13	0.021 ± 0.01
0.33 M EDA	0.62 ± 0.12	0.077 ± 0.040	4.03 ± 0.79	2.19 ± 0.14
0.66 M EDA	0.20 ± 0.044	0.026 ± 0.0010	1.33 ± 0.29	1.09 ± 0.5
0.99 M EDA	0.19 ± 0.065	0.073 ± 0.012	1.27 ± 0.43	2.08 ± 0.037
1.32 M EDA	0.037 ± 0.0014	0.073 ± 0.010	0.24 ± 0.009	2.07 ± 0.072

*EDA and glutaraldehyde solution was reacted for 5 minutes

#No protectant was added in this sample

Equation 3.2: $U = [((\Delta OD.min^{-1}_{340nm} * 1ml)/ V_S)/20 \text{ minutes}] * DF * V_T)$

Where:

V_T = particle re-suspension volume (ml) or total volume used of 100 $mg.ml^{-1}$ enzyme solution for SphereZyme™ preparation

V_S = sample volume = 0.0125 ml for azocasein (340nm)

DF = Dilution factor

Equation 3.3: $U = [(V_T/ (V_S * \varepsilon_{410\ nm} * l)) * (OD.min^{-1} * DF)] * V_R$

Where:

V_T= Total assay volume

V_S = sample volume = 0.01ml PNPA (410 nm)

DF = Dilution factor

V_R = particle re-suspension volume (ml)

$\varepsilon_{410\ nm}$ at pH 7.5 = 7.50 $mM^{-1}.cm^{-1}$ (for a path-length of 0.67 cm)

Equation 3.4: % Activity maintenance = (activity (U) of SphereZyme particles / activities (U) for start material) * 100

The highest activity maintenance after immobilisation towards both the small and large substrates was achieved with the addition of protectant and EDA at a concentration of 0.33 M (table 3.3). Lower activity for the large substrate was achieved at increased EDA concentrations while the small substrate activity remained fairly constant (table 3.3). In the case of low EDA concentrations, the improved activity maintenance towards azocasein indicates improved accessibility for large substrates into the cross-linked enzyme matrix (Ferreira *et al*., 2003). The decrease in activity maintenance at high EDA concentrations for this substrate is therefore not as a result of enzyme deactivation (small substrate activity). It rather appears that the degree of crosslinking is potentially higher, and thereby the network at high EDA concentrations has caused the decrease in activity maintenance towards azocasein. Another potential explanation is possibly that the higher EDA concentration has led to the incomplete incorporation of the enzyme into the immobilized enzyme particles. This coupled with an increase in the enzyme activity maintenance (reduced aldehyde reactive groups) could have resulted in the data obtained. Increasing the time of pre-reaction between the EDA and glutaraldehyde also appeared to have a positive impact on the activity maintenance of the immobilised preparations (table 3.4).

Table 3.4: Effect of Glutaraldehyde: EDA reaction time on enzyme activity maintenance

Sample	Activity (U)		Activity Maintenance (%)	
	Azocasein	PNPA	Azocasein	PNPA
Free enzyme	60.4 ± 1.05	2.70 ± 0.19	100 ± 0.69	100 ± 0.069
*0.33 M EDA	0.55 ± 0.092	0.032 ± 0.0078	0.90 ± 0.12	1.20 ± 0.50
#0.33 M EDA	0.98 ± 0.099	0.12 ± 0.012	1.63 ± 0.18	4.46 ± 0.51
#0.66 M EDA	0.28 ± 0.073	0.032 ± 0.0085	0.47 ± 0.046	1.20 ± 0.55

*Reaction time between Glutaraldehyde: EDA was 5 minutes

Reaction time between Glutaraldehyde: EDA was 10 minutes

Increasing the time of reaction between glutaraldehyde and EDA can potentially increase the length of the crosslinking agent, and thereby result in the formation of a looser protein network (Cao and Elzinga, 2003). This could have resulted in the improved enzyme activity toward azocasein in the case of 0.33 M EDA reacted with 25% v/v glutaraldehyde for 10 minutes (0.98 U) than that reacted for 5 minutes (0.55 U; table 3.4). However, increasing the reaction time seemed to have little effect at higher EDA concentrations. At higher EDA concentrations the available aldehydes for crosslinking may not be sufficient for complete incorporation of the enzyme as described earlier. The low % enzyme activity maintenance for PNPA supports this explanation, as it decreases with an increase in concentration of EDA (Yu *et al*., 2006).

3.4.4.2 Addition of PEI

PEI is a hydrophilic polymer and acts as a poly-cation or poly-ion at alkaline or acidic pH, respectively (López-Gallego *et al*., 2007). However, PEI is more nucleophilic at an alkaline pH and thereby more reactive toward electrophiles such as the carbonyls of aldehyde groups (Boussif *et al*., 1995).

Incorporating PEI as an additive seemed to vastly improve the activity maintenance (table 3.5) for protease SphereZyme™ preparations with a maximum yield of 17% enzyme activity maintenance using azocasein as the substrate. There was an activity increase of about 17% towards azocasein at both PEI concentrations used in the experiment. However, about 54% of activity was maintained for PNPA at 5% PEI and only 26% at 3% PEI. The incorporation of PEI (5%) may have resulted in improved enzyme coupling through improved protein polymer interaction or reduction of the potential destructive effects of glutaraldehyde. The improved enzyme activity recovery towards azocasein indicates improved large substrate permeability due to a loose network created by PEI incorporation.

Table 3.5: The influence of larger crosslinking agents on SphereZyme™ manufacture.

Sample	Activity (U)		Activity Maintenance (%)	
	Azocasein	PNPA	Azocasein	PNPA
Free enzyme	60.4 ± 1.05	2.70 ± 0.19	100 ± 0.69	100 ± 0.069
[#]0.33 M EDA	0.55 ± 0.092	0.032 ± 0.0078	0.90 ± 0.12	1.20 ± 0.50
5% PEI	10.01 ± 0.35	1.45 ± 0.00033	16.57 ± 0.37	53.68 ± 0.48
3% PEI	10.3 ± 0.42	0.70 ± 0.0081	17.05 ± 2.01	25.84 ± 0.50

[#]reproduced from table 3.4

No particles were formed when dextran aldehyde was used as a crosslinking agent for partially purified Alcalase® SphereZyme™ production. Although the dextran aldehyde appeared to function as a crosslinking agent (section 3.4.3 and table 3.2) it was ineffective in crosslinking of the Alcalase® even when the protein was supplemented with albumin as a proteic feeder.

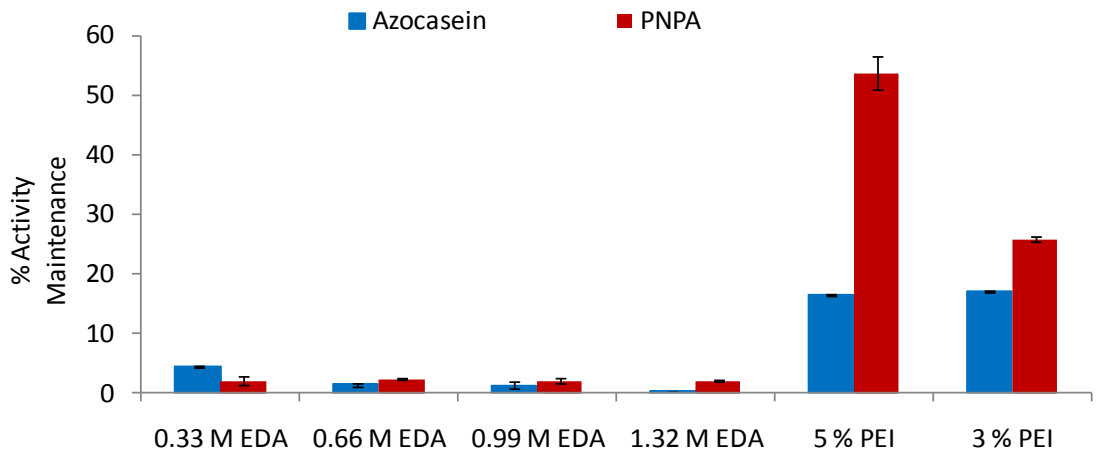


Figure 3.3: Percentage activity maintenance of SphereZyme™ with primary amine additives.

Figure 3.3 summarises the percentage activity maintenance of SphereZyme™ for azocasein and PNPA substrates utilising the different amine additives. PEI served the role of a high molecular weight polymeric crosslinking agent and as expected the activity for both (azocasein and PNPA) substrates increased when it was employed. This increase in activity was attributed to the development of a „looser" network (Mateo *et al*., 2004) which reduced the substrate diffusional limitation. The increase in associated azocasein activity was linked to a marked increase of up to about 50% for enzyme activity towards PNPA. This potentially indicates a protective effect of PEI for the preservation of enzyme activity. This correlates well with previous reports by Kawaguti *et al*., (2006) who noted improvement in the enzyme activity when glutaraldehyde activated PEI was used as a crosslinking agent.

Table 3.6: SphereZyme™ Activity profile

#Sample	Activity (U)	Mass Recovery (mg)	Specific Activity ($U.g^{-1}$)
Azocasein	250.25 ± 8.8	50.1	5000
PNPA	13.59 ± 0.03	50.1	270

#(5% PEI: 25% Glutaraldehyde in 1:1 ratio, used as a crosslinking agent)

In comparison to previous reports on the immobilisation of Alcalase® by Simi and Abraham (2007) who reported a specific activity of 0.034 $U.mg^{-1}$ for a CLEC preparation of Alcalase® with casein as a substrate, we reported a final activity of 5 $U.mg^{-1}$ (table 3.6) which resulted in about 150 fold improvement for the Alcalase® SphereZyme™ preparation. Sangeetha, and Abraham (2008) also reported a specific activity of 0.065 $U.mg^{-1}$ for a CLEA preparation of subtilisin. The small substrate (PNPA) specific activity of 0.27 $U.mg^{-1}$ (table 3.6) was however, lower than a commercially available CLEA preparation at 0.4-0.6 $U.mg^{-1}$ (Internet Reference 1).

Table 3.7: Comparison of the initial SphereZyme™ immobilised Alcalase® using standard methodology with SphereZyme™ preparation using enlarged crosslinking agent.

#Sample	Azocasein Activity (U)	PNPA Activity (U)	Activity Ratio (Azocasein/PNPA)
Glutaraldehyde SphereZyme™	0.55 ± 0.092	0.032 ± 0.0078	17
PEI-Glutaraldehyde SphereZyme™	10 ± 0.35	1.5 ± 0.00033	6.9

Table 3.7 shows the activity of initial preparation (0.33 M EDA: 25% v/v Glutaraldehyde as a crosslinking agent) and optimised preparation (5% PEI: 25% v/v Glutaraldehyde) of immobilised Alcalase® preparations. The initial preparation had very low azocasein and PNPA activity as compared to the

preparation using PEI as the crosslinking agent. The ratio of azocasein to PNPA activity decreased, showing that the enzyme activity maintenance was not only potentially due to improved substrate permeability but also to improved amount of bound enzyme particles.

3.5 Conclusions

The study showed the limitation of SphereZyme™ technology for large substrate biocatalysis. The use of an enlarged crosslinking agent in the form of PEI modified glutaraldehyde improved the activity maintenance from 1% to 17% (table 3.5 and fig. 3.3) with respect to the standard preparation technique described by Brady *et al*., 2008. This was attributed to the increased availability of the enzyme for large substrate biocatalysis resulting from increased substrate permeability and overall improved enzyme activity maintenance.

The activity maintenance achieved, compares favourably with the current commercial self-immobilised CLEA preparation. Alcalase® SphereZyme™ may therefore prove to be a commercially viable technology. The specific activity of Alcalase® SphereZyme™ was approximately 150 and 100 times higher than the CLEC and CLEA techniques respectively using casein as a substrate (Simi and Abraham, 2007; Sangeetha and Abraham, 2008). Dextran aldehyde was also previously reported to improve the activity of enzymes towards large substrates. However, the use of this crosslinking agent was unsuccessful using the SphereZyme™ technique. This may have been due to the incomplete oxidation of the hydroxyls to aldehydes, or the requirement for longer crosslinking periods.

4 Comparison of SphereZyme™ self-immobilised Alcalase® preparations to Solid Support Technologies

4.1 Introduction

Immobilisation of enzymes via solid support has several benefits over self-immobilisation (Cao, 2005). For instance, this method is preferred for large substrate biocatalysis and provides a more rigid product which can assist in recovery of the enzyme and application to continuous bioreactor systems (Cao, 2005; Mateo *et al*., 2007a). However, the attachment to a support can result in protein leaching, leading to major enzyme loss and thereby increase the process and consequently the product cost (Lasch and Janowski 1988). Furthermore, solid-support technologies may not be ideal for biocatalytic applications due to the high non-catalytic requirement, increase in cost and potential instability of the support in organic solvent (Balcão *et al*., 1996; Cao *et al*., 2003).

Commercially available epoxy supports include Sepabeads and Eupergit® C. Eupergit® C is a synthetic organic solid support which uses stable epoxy groups for protein immobilisation (Boller *et al*., 2002). Sepabeads contain the same functional group, epoxide, but provide a potentially larger surface area for protein attachment.

Dendrispheres is a recently developed proprietary solid-support immobilisation technology. The support is prepared through an emulsion based process in which the branched polymer polyethyleneimine is crosslinked with a bi-functional crosslinking agent (usually glutaraldehyde). This results in the formation of a loosely linked hydrophilic polymeric support with aldehyde functionality. These free aldehyde groups can subsequently be used for protein immobilisation through the primary amines of lysine groups of proteins (Harris *et al.,* 1984; Roberts *et al.,* 2002; Jordaan *et al*., 2009b). The main advantage of this technology over alternative supports is that the controllable polymerisation results

in a loosely-linked polymer network capable of higher enzyme loading capacities of >300 $mg.g^{-1}$ (Jordaan *et al*., 2009b). This is in comparison to Sepabeads, which when pre-treated with 25 kDa PEI for increased surface area, and functionalised with glutaraldehyde to achieve covalent attachment, resulting in a loading capacity of only 15 $mg.g^{-1}$ support (López-Gallego *et al*., 2005c). Other benefits of the Dendrispheres technology include controllable particle size and reduced substrate diffusional limitation (Brady and Jordaan, 2009). The limitation of this technology for the biocatalysis of high molecular weight substrates is as yet unknown. This support was chosen for comparison since in many respects it alleviates the current limitations of solid-support technologies, but could potentially suffer from similar disadvantages to self-immobilisation techniques.

4.2 Aim

This chapter aims at benchmarking the improved large substrate biocatalytic properties of SphereZyme™ technology with solid support technologies including Eupergit® and Dendrispheres.

4.3 Materials and Methods

4.3.1 Chemicals and Reagent

Mineral oil and Nonoxynol were purchased from Castrol and BASF respectively. Trizma Base, azocasein, 50% w/v polyethyleneimine, and *p*-Nitrophenol Acetate (PNPA) were purchased from Sigma Aldrich Pty.(Ltd). Trichloroacetic acid was purchased from Merck. Alcalase® was a gift from Novozymes®. Eupergit® C 250L was purchased from Rohm GmbH.

4.3.2 Dendrispheres Preparation

The particles are prepared using a bi-emulsion process. To 2 different solutions both comprising 5 ml mineral oil and 50 µl nonoxynol-4 (NP4), 250 µl of either 20% v/v glutaraldehyde or 10% v/v PEI were added. The solutions were emulsified separately by magnetic stirring at 500 rpm for 30 minutes. The emulsions were subsequently mixed to initiate crosslinking with magnetic stirring at 700 rpm for 90 minutes.

The solid support was recovered by centrifugation at 3901 x *g* for 5 minutes in Beckman Coulter™, Allegra™ X-22R benchtop centrifuge, fitted with swinging bucket rotor. The particles were washed six times with 50 ml of Milli-Q water. Recovery between each washing step was achieved using centrifugation as mentioned above. The particles were suspended to a final volume of 1 ml in water.

Since the pH of the PEI affects the nature of the primary amine substituents, i.e. it is nucleophilic at alkaline pH and hence more reactive with aldehydes; particles were prepared at various pH"s (Boussif *et al*., 1995). A more crosslinked and dense support is expected for preparation using alkaline pH. This is demonstrated by the formation of a more (intense orange-brown colour, indicative of Schiff base formation). The effect of the degree of crosslinking was evaluated with respect to protein binding capacity and enzyme activity maintenance.

PEI (10% v/v) solutions were prepared at pH"s of 7 to 10 at 1 pH unit intervals. These PEI solutions were used in the manufacture of solid-support mentioned above. The resulting supports were re-suspended to a 1 ml aqueous suspension.

4.3.3 Protein Binding Capacity

To 1 ml of Dendrispheres suspension (section 4.3.2), 3 ml of purified 5 $mg.ml^{-1}$ Alcalase® solution in MilliQ water (section 2.3.3.2) was added. The solution was allowed to react for 1 hour and centrifuged at 17000 x *g* for 1 minute in Thermo Scientific Heraeus Pico 17 microcentrifuge, for particles recovery. The recovered pellets were washed six times with 50 mM Tris-HCl buffer pH 8.0 containing 1mM ethanolamine to quench unreacted aldehyde groups. The particles and Alcalase® supernatant were subsequently assayed to determine particle binding capacity and enzyme activity retention using the large and small substrates (section 2.3.6 and 3.3.3.1). The supernatant was assayed for total protein according to 2.3.7 to quantify unbound protein. The quantity of bound protein was determined by the difference in concentration between the starting protein suspension and residual protein in solution after particle recovery.

The optimal protein immobilisation time was determined using a time course experiment where 300 µl samples were recovered every 5 minutes and assayed for residual enzyme in the supernatant. All assays were performed in triplicate and data was represented as mean ± standard deviation.

4.3.4 Immobilisation of Alcalase® on Eupergit® C 250 L

The theoretical loading capacity of Eupergit® C 250 L according to Boller *et al.,* (2002) was reported to be approximately 0.1% (w/w). Thus, a solution of 1.5 ml 50 mM Tris-HCl buffer pH 8.0 containing 0.5 $mg.ml^{-1}$ enzyme solution was added to 75 mg of the commercial solid support (excess protein based on suppliers recommended capacity to ensure saturation). The enzyme was immobilised over a period of 24 hours with gentle stirring at 25 °C. All binding experiments were performed in duplicate.

4.4 Results and Discussion

4.4.1 Immobilisation Time Course

Figure 4.1 shows a time course binding capacity for the partially purified Alcalase® to the Dendrispheres solid support. It appeared that the solid support could immobilise protein within the first five minutes of incubation as there was no further protein binding after this period. Increasing the time for immobilisation did not appear to result in the loss of enzyme activity and therefore 20 minutes was selected for subsequent experimentation.

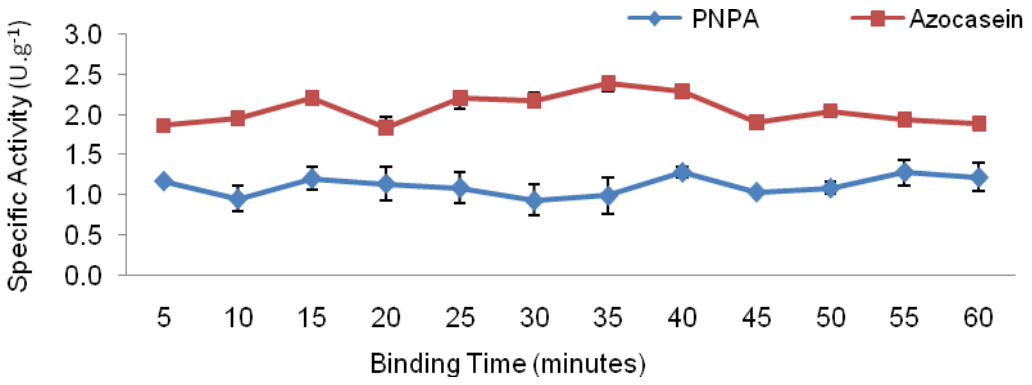


Figure 4.1: Binding profile of 5 mg.ml^{-1} Alcalase® on Dendrispheres support over 60 minutes.

Table 4.1: Enzyme activity retention for Alcalase® immobilised on Dendrispheres after 20 minute incubation.

Sample	Immobilisation Yield (%)	Specific Activity (U. total weight, g^{-1})	Binding capacity (mg.g^{-1} support)
Azocasein	2.49 ± 0.14	1.84 ± 0.045	243 ± 0.40
PNPA	25.5 ± 0.68	1.14 ± 0.027	243 ± 0.40

Enzyme activity maintenance was calculated according to the equations below.

Equation 4.1: Immobilisation yield = Activity (U) of enzyme bound / Activity yield {Activity yield = activity (U) in start material - activity (U) left in supernatant after binding}

Equation 4.2: Specific activity = Activity bound (U)/ total weight (g)

Equation 4.3: Binding Capacity = Protein bound (mg)/ mass of the support (g)

In general, Alcalase® has a higher activity for azocasein rather than for PNPA since this is a natural substrate for Alcalase® (Kannan and Jasra, 2009). The enzyme immobilisation yield for the smaller substrate was markedly higher at 25.5% over 2.5% for the large substrate. This indicates that this solid support suffers from similar drawbacks to self-immobilisation techniques, i.e. substrate diffusional limitations.

4.4.2 The effect of PEI pH on Dendrispheres Properties

The immobilised enzyme activity of Alcalase® immobilised on various preparations of Dendrispheres was evaluated. Figure 4.2 shows the effect of pH of the PEI on the immobilisation yield (%) of immobilised Alcalase®. The control, using PEI at its native pH of 11.5, had the highest immobilisation yield (%) of 12.9% for PNPA while pH 7 exhibited the lowest at around 3.61%. The general trend was that the more alkaline the pH of the preparation the greater the immobilisation yield (%). This was attributed to the speed of reaction and hence the degree of crosslinking of the support. The subsequent higher crosslinking prevented penetration of the large substrate (azocasein) into the particle even though the particles had a higher protein binding capacity. For this substrate, the use of PEI at pH 10 resulted in the highest immobilisation yield (%) of 5.40% (fig 4.2). These results correlate well with the improved reactivity of the primary amine groups at alkaline pH (López-Gallego, 2005b).

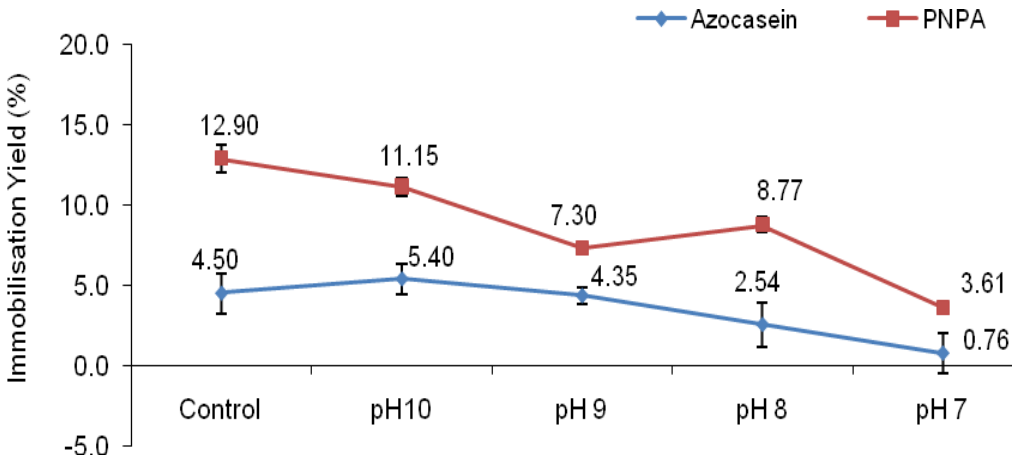


Figure 4.2: Immobilisation yields (%) on the various Dendrispheres preparations using small and large substrates.

The specific activity toward PNPA (small substrate) followed a similar trend to that of Azocasein, displaying higher enzyme activity with increasing pH (fig. 4.3)

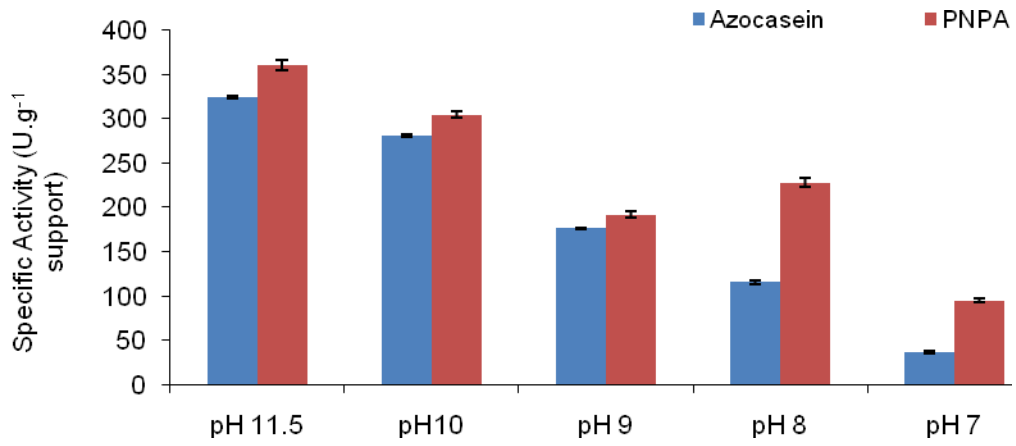


Figure 4.3: Specific Activities ($U.g^{-1}$) on the various Dendrispheres preparations using small and large substrates.

PEI has previously been used in the reversible binding of enzymes on polymer coated glyoxyl support (Pessela *et al.*, 2005; López-Gallego, 2005c). For comparison to other immobilisation techniques (SphereZyme™ and Eupergit® C

250L), the sample having the highest immobilisation yield (%) was used (PEI ≈ pH 11.5).

Table 4.2: Binding profile of purified Alcalase® on Eupergit® C 250L support

Sample	Immobilisation Yield (%)	Specific Activity (U. g^{-1}support)	Binding Capacity ($mg.g^{-1}$ support)
Azocasein	9.73 ± 0.66	0.59 ± 0.50	0.71 ± 0.19
PNPA	63.4 ± 3.5	2.62 ± 2.1	0.71 ± 0.19

The enzyme immobilisation yield of Eupergit® C 250L toward azocasein for purified Alcalase® was 9.73% (table 4.2). The binding capacity of Dendrispheres was calculated to be about 243 $mg.g^{-1}$ support and that of Eupergit® C 250L was 0.71 $mg.g^{-1}$ support (Boller *et al*., 2002).

4.4.3 Comparison of the immobilisation techniques for large substrate catalysis

Figure 4.4 shows the comparative ratios (azocasein/PNPA activity (U)) of the evaluated immobilised techniques. The ratios provided information about surface to volumetric activity of immobilised enzyme (Brady, *et al*., 2008). SphereZyme™ yielded the highest efficiency (6.9) for azocasein hydrolysis compared to Dendrispheres and Eupergit® C 250L at 1.7 and 0.23, respectively (fig. 4.4).

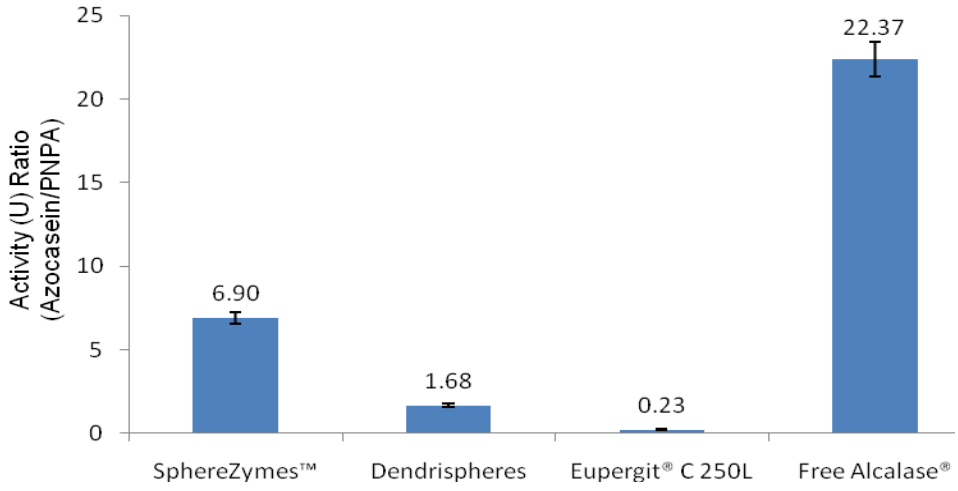


Figure 4.4: The ratio of azocasein to PNPA activity of different immobilisation techniques compared to the free form of the Alcalase®.

Due to its low molecular weight, PNPA was chosen as a control substrate to determine enzyme activity maintenance and gives a measure of the enzyme activity lost due to enzyme denaturation. Azocasein, a protein, was chosen as a model of large substrate. This substrate thus gives a relative indication of enzyme accessibility for large substrate catalysis. From these results it can be drawn that the SphereZyme™ immobilised preparation is more efficient for azocasein biocatalysis than the two solid supports. However, a threefold difference in ratios of free Alcalase® (22) and SphereZyme™ (6.9) shows that most of the enzyme was not accessible for large substrate catalysis after immobilisation (Hamerska-Dudra *et al*., 2007; Caramori and Fernandes, 2008).

4.5 Conclusions

Numerous studies for the immobilisation of subtilisin from Bacillus have been conducted to date, most of which involve immobilisation on to a solid support. For instance Ferreira *et al*., (2003) reported a specific activity of 0.063-0.1124 $U.mg^{-1}$ for bound subtilisin on modified Silica. The binding efficiency/capacity and enzyme immobilisation yield were 0.0028-0.0035 mg protein. mg^{-1} support and

14.1-31.6% respectively, an improvement of the unmodified support with specific activity of 0.0053 $U.mg^{-1}$, binding capacity of 0.0024 $mg.mg^{-1}$ silica and 1.7% enzyme immobilisation yield respectively. The activity yield and binding efficiency of Eupergit® and Dendrispheres preparations were lower as compared to the SphereZyme™ technology.

The results for the immobilised enzyme reported in this chapter represent a substantial improvement over the current state of the art. The superior activity maintenance for large substrate biocatalysis makes the immobilisation product potentially suitable for replacing current immobilisation techniques.

5 Evaluation of immobilised enzyme preparations for application to biosensors: Modification of Glassy Carbon Electrode with Laccase and Protease SphereZyme™ Particles for phenol detection

5.1 Introduction

Phenols are aromatic hydrocarbons that contain a hydroxyl group. About 95% of phenol worldwide is produced from the Raschig-Hook process which involves a three step cumene synthesis and oxidation step (Schmidt, 2005). Other processes for phenol production involve amongst others oxidation of toluene via benzoic acid or through coal coking (Internet Reference 2; Gelbein and Nislick, 1978). Phenols are also generated via emissions through combustion of fossil fuel and through decomposition of organic materials (Busca *et al*., 2008). It is also produced naturally in biosynthetic pathways in plants (Busca *et al*., 2008). Phenols span a broad range of molecules incorporating beneficial and toxic properties to living organisms.

Phenols such as flavonoids have a broad range of potential health benefits. Phenols are known for their germicidal and local anesthetic properties and can be used as disinfectants. They have also been used in veterinary medicine as antiseptics and gastric anaesthetics (Busca *et al*., 2008). In addition to sensory characteristics, flavonoid-plant pigments can also serve as powerful anti-oxidants (Deiana *et al*., 1999). The anti-oxidant role was reported to be through the shielding of biomolecules such as DNA from oxidative damage by free radicals (Deiana *et al*., 1999). Beneficial phenols have thus found application in chemotherapy and chemoprevention through the activation of the carcinogen detoxifying enzyme system (Johnson *et al*., 1994; Deiana *et al*., 1999; Kerry and Rice-Evans, 1999). It is thought that these phenols increase the activity of enzyme systems involved in the detoxification of carcinogens (Kerry and Rice-

Evans, 1999). There have also been claims that these molecules can prevent heart diseases and cancer (Steenken and Neta, 1982; Filipiak, 2001). Flavonoids are also found in fruits, vegetables, beverages (wine and tea), nuts, and are incorporated into herbal medicine and dietary supplements (Riemersma *et al*., 2001; Galati and O'Brien, 2004).

Toxic phenols such as nitrophenols, chlorophenols and aminophenols are ubiquitous in industrial wastewater. Industries generating these toxic by-products include petrochemicals, paper and pulp refineries and pharmaceutical industries (Banik *et al*., 2008). Phenols may be present in agricultural waste (Kavitha and Palanivelu 2005), in the atmosphere, soil treated with pesticides, rivers, and in ground water (Hallas and Alexander, 1983; Spain and Gibson, 1991; Hanne *et al*., 1993; Ko and Chen, 2008). *p*-Nitrophenol (PNP) is one of the more important phenols due to its role in the manufacture of pesticides, dyes, plasticizers, explosives, industrial solvents and pharmaceuticals (Hallas and Alexander, 1983; Spadaro and Renganathan, 1994; Lante *et al*., 2000; Yi *et al*., 2006). This compound has been reported to be mutagenic and carcinogenic in microoganisms and mammals (Megharaj *et al*., 1990; Benigni *et al*., 2000; Franke *et al*., 2001) and is responsible for the off-flavour in drinking water. *p*-Nitrophenol and other phenols are thus under strict regulation by US Environmental Protection Agency, with the set limit in drinking water being 0.1 ppb (Nistor *et al*., 2001).

Given the toxicity of phenols, several processes have been utilised in the removal or remediation of toxic phenols in wastewater. Phenol adsorption on ion exchange resins (Oprea and Sandulescu, 2006), low cost clay (Nayak and Singh 2007) and on activated palm seed coat carbon (PSCC; Rengaraj *et al*., 2002) have shown promise. Other processes include separation by steam distillation, membrane-based solvent extraction or membrane pervaporation and activated sludge (Kujawski *et al*., 2004; Busca *et al*., 2008; Smith *et al*., 2009). Chemical modification of the adsorbents or polymers, for instance Amberlite® IRA 900

treatment with metallophthalocyanine complexes (MPc) increased the polymer adsorption rate for phenols (Marais and Nyokong, 2008).

The uses of biological catalysts such as phenoloxidases (e.g. laccase or tyrosinase) or peroxidases (e.g. horseradish peroxidase) were also reported to increase the effectiveness of the adsorbent by improving its capacity for removal of phenols (Peralta-Zamora *et al*., 1998; *ElKaoutit et al*., 2007; Sulak *et al*., 2010). The main benefit of using biocatalysts instead of conventional methods is its application to recalcitrant materials.

The detection and monitoring of phenols is thus of importance in remediation of industrial wastewater, and in air, soil and in food analysis (Stanca and Popescu 2004, Sulak *et al*., 2010). Analysis is usually carried in the laboratory through the use of liquid and gas chromatography, mass spectrometry (GC-MS; Aramendia *et al*., 1996; Puig and Barcelo, 1995), capillary electrophoresis (CE; Kaniansky *et al*., 1997) and immuno assays (Oubiña *et al*., 1999). Applications of these techniques are however, limited due their high cost, time consumption, the need for skilled technicians and unsuitability for on-site or field application (Andreson *et al*., 2000). Thus, there are numerous reports on the use of enzyme-based sensors for detection and monitoring of phenols (Marko-Varga *et al*., 1995; Roy *et al.,* 2005). Such enzymes include laccase, horseradish peroxidase, tyrosinase and catechol dioxygenases amongst others (Durán and Esposito, 2000).

Laccases (1.10.3.2) are known for their simultaneous oxidation of phenols and reduction of O_2 (Robles *et al*., 2000). The copper containing di-phenolic oxido-reductases (Mason *et al*., 1961) were also reported to catalyse decolourisation of recalcitrant dyes due to it oxidative nature. This step follows through non-specific oxidation of hydroxyl groups resulting in an electrochemically detectable compound without direct azo bond cleavage (Zille *et al*., 2005). Reduced laccase can oxidise phenols (e.g. catechol, pyrogallol, guaiacol, ferulic acid and catechin), chlorophenols, lignin-related diphenylmethanes, organophosphates,

benzopyrenes and nonphenolic compounds, amongst others (Marko-Varga *et al*., 1995).

There are numerous benefits for the application of biocatalysts to biosensors including increased pH, temperature and salinity range, easy control processes and operation in a wide dynamic range of pollutant concentrations. There are numerous reports on the use of enzyme-based sensors for detection and monitoring of phenols (Marko-Varga *et al*., 1995; Roy *et al.,* 2005). Such enzymes include laccase, horseradish peroxidase, tyrosinase and catechol dioxygenases amongst others (Durán and Esposito, 2000). The oxidative nature of laccases has enabled their role in electrochemistry for the detection of phenols and to a smaller degree, proteins (Durán *et al*., 2002). However, due to their reliance on mediators for improved sensitivity and broadening of substrate range (Mayer and Staples, 2002; Couto and Sanromán, 2007), their stability can be affected. Most mediators and/or their by-products are harmful and inhibitory to the enzyme and the environment (Durán and Esposito, 2000; Durán *et al*., 2002; Majeau *et al*., 2010). Some mediators are expensive, increasing the cost of biosensor (Li *et al*., 1999; Bourbonnais *et al*., 1998). Thus, there is a continuing need for non-toxic organic mediators or for finding alternative methods of increasing the substrate range without the use of mediators.

As previously mentioned in section 1.4, enzyme immobilisation can impart some of the benefits the mediator has on the biosensor in addition to improved enzyme stability (Zille *et al*., 2003; Kunamneni *et al*., 2008). SphereZyme™ laccase was reported to be stable toward the oxidative effects of mediators such as 2-hydroxyphthalimide and N-hydroxybenzotriazole free radicals. It was also stable in acidic pH and high temperatures (Jordaan *et al*., 2009a).

The use of biocatalysts in biosensor has lead to numerous studies and most of these studies report on modified electrodes (Anderson *et al*., 2000). Modification can be through adsorption, covalent binding, ionic interaction and attachment of

the biocatalysts (Schuhmann, 2002). Coating of working electrodes with PEI films or glutaraldehyde for covalent enzyme coupling provided some advantages. For instance, short response times improved shelf life and mechanical strength. Modifications of the electrode with biocatalysts also form a direct electronic communication between the electrode and the biocatalyst (Quan *et al*., 2002). These enhancements were observed by employing self-immobilised biocatalyst (laccase CLEC) instead of its free form (un-immobilised), in the modification of electrodes (Bakker and Pretsch, 2005).

However, research has tended to focus on solid-support immobilisation. Zille *et al*., (2005) used graphite carbon electrode for the adsorption of the enzyme which required long process of pre-treatment processes. Another author reported on a time- consuming gold electrode modification with thiol monolayer and enzyme. This was followed by treatment with glutaraldehyde (preventing enzyme leaching) to covalently couple the enzyme to the electrode. However, at higher concentrations of glutaraldehyde, toxicity of this compound and over-crosslinking can lead to enzyme denaturation (Migneault *et al*., 2004). Prior to modification, the electrode was also pre-treated by polishing, sonicating and electrochemical washes (Gupta *et al*., 2002). The use of solid supports for enzyme immobilisation can thus contribute to the complexity of biosensors.

Electrochemical detection of para-nitrophenol (PNP) was reported to follow a reduction peak catalysed at modified GCE during a cyclic voltammetry (CV) cathodic sweep. The GCE was modified with lithium tetracyanoethylenide (LiTCNE) post-treated with poly-L-lysine (PLL; Luz *et al*., 2004). Modified carbon paste with hydroxyapatite was also used in the detection PNP by CV via electro-reduction (Mhammedi *et al*., 2009). The potential scans for both reports were in the negative range. However, there are reports of electrochemical oxidation of PNP to less toxic quinones at an anodic current (Lei *et al*., 2005; Quiroz *et al*., 2005).

Owing to the complexicity of improving the operation and stability of biosensors mainly through the use of solid support concerns associated with mediator (such as toxicity; Anderson *et al*., 2000), there is a need to investigate alternative methods of immobilisation. SphereZyme™ technology could potentially provide the benefit of improved biosensor sensitivity through its high activity to mass ratio (Brady *et al*., 2008; Brady and Jordaan, 2009).

SphereZyme™, a self-immobilisation technology, could potentially provide unique advantages in the development of biosensors by overcoming the limitations of low specific activity of the immobilised recognition element and improved exposed surface area for electro-active species measuring. In this study Denilite® laccase and Alcalase® SphereZyme™ will be evaluated as the recognition element for phenol biosensors in comparison to the free form of the enzymes to ascertain potential improvements in the use of the SphereZyme™ immobilisation technique.

5.2 Aims

- The study is aimed at investigating the application of self-immobilised enzymes (SphereZyme™) in electrochemical biosensor assemblies, specifically employing laccase and Alcalase® immobilised via the SphereZyme™ technique for detection of catechol (as a model phenolic substrate), and PNPA, respectively.
- Utilising catechol as a model substrate, several electrode modifications were examined to determine the application of SphereZyme™ for contacting laccase with the electrode in a biosensor. Where possible these were compared with “free laccase” routinely used in biosensor assemblies for phenols.
- The following modifications for catechol detection were examined:
    - Solution phase catalysis (in which the biocatalyst is free in solution)

- Cross-linking (in which biocatalyst were co-immobilised with glutaraldehyde
- Membrane entrapment (in which enzymes are trapped by a permeable membrane)
- Polymeric entrapment (in which enzymes are co-immobilised within a polymeric support)

5.3 Materials and Methods

5.3.1 Chemicals and reagents

Catechol, *p*-Nitrophenol Acetate (PNPA), *p*-Nitrophenyl (PNP), D9402 Dialysis tubing cellulose membrane (12 kDa,), Poly-L-lysine, sodium hydroxide (NaOH), Trizma Base, succinic acid and hydrochloric acid (HCL) were purchased from Sigma Aldrich. Commercial Denilite® II Base (Laccase), Denilite® Assist and Alcalase® were obtained from Novozymes. Laccase and Alcalase® SphereZyme™ particles were prepared by Enzyme Technologies Group (CSIR). Commercial Denilite® II Base was the source of laccase used for the preparation of laccase self-immobilised in SphereZyme™ particles and referred to as SphereZyme™ laccase in these studies). Laccase (E.C. 1.10.3.2) from *Trametes versicolor* commonly used for biosensor assembly studies was sourced from Sigma Aldrich. This enzyme referred to as “free laccase” was used in these studies as a comparison to studies utilising SphereZyme™ laccase. The same terminology was used for studies incorporating Alcalase®.

5.3.2 Enzyme purification

The method for purification of crude Commercial Denilite® II Base sample was adapted from Jordaan *et al*., (2009a). This sample (10 g) was dissolved in 200 ml of 20 mM Tris–HCl buffer (pH 8.0). The suspension was magnetically stirred for 2 hours at 200 rpm (4 °C) and was clarified by centrifugation for 15 minutes at

15,330 x g. The solution was concentrated to 50 ml using an Amicon (2800) ultrafiltration stirred cell reactor with a 10 kDa cut-off polyethersulfone membrane (PALL filtration). This concentrate was dialysed against Milli-Q water (changing water at 3 hour intervals) at 4 °C in 10 kDa cutt-off Snakeskin® dialysis tubing (Pierce). The dialysed solution was lyophilised and stored at 4 °C until required for SphereZyme™ preparation.

Purification of Alcalase® was performed according to section 2.3.3.2

5.3.3 Laccase Assay

The laccase activity assay for both free and SphereZyme™ samples, adapted from Bergmeyer (1974), was analysed spectrophotometrically by monitoring the oxidation of guaiacol in 100 mM succinate buffer pH 4.5 at 450 nm (ε = 0.0266 $mM^{-1}.cm^{-1}$). The final concentration of guaiacol in the buffer was 1 mM and guaiacol oxidation was initiated by mixing 180 µl of the substrate with 20 µl laccase at 25 °C.

The conversion was monitored using a PowerWave HT (Biotek Instruments) with medium shaking between reads (intensity 3, for 1 s).

Equation 5.1: $U.ml^{-1} = (\Delta A_{450}.min^{-1} \times TV \times Dil) / (\varepsilon \times SV)$
Where:

TV = total reaction volume (ml) = 0.200

SV = sample volume (ml) = 0.02

ε = millimolar extinction coefficient of tetraguaiacol ($mM^{-1}.cm^{-1}$) = 0.0266

5.3.4 Enzyme immobilisation through SphereZyme™ preparation

Two sets of SphereZyme™ particles were prepared using the standard preparation protocol as stipulated in section 3.3.5. The first set contained Denilite® II Base laccase, which was purified via dialysis against Milli-Q water (the method is thoroughly described in section 5.3.2). The second set was made of partially purified Alcalase® (section 2.3.3.2). The preparations contained 2.5% w/v PEI: 25% v/v glutaraldehyde solution which was used as a crosslinking agent at 1:1 volumetric ratio. Active site protectants incorporated in the preparation were 2.5% casein and 10 % Denilite® II Assist (laccase substrate). The particles were recovered as previously reported (section 3.3.5). After which, they were suspended to 5 ml in MillQ water and stored at 4 °C until required for immobilisation to biosensor.

The immobilised and free enzyme samples were immobilised on the surface of a glassy carbon electrode (GCE). The samples were loaded accordingly to compensate for differences in enzymatic activity. Activity determination assays were according to section 3.3.3.1 and 5.3.3. All assays were performed in triplicate and data was represented as mean ± standard deviation.

5.3.5 Electrochemical Analysis

5.3.5.1 Principles of Cyclic voltammetry

Cyclic voltammetry (CV) is mainly used in the study of redox species. It is reported to be the most versatile electroanalytical technique and is often the first experiment performed in an electrochemical study (Shippy and Lu, 2007). In order to detect electroactive (redox) species through CV, a potential window is scanned over a set range. Once detected an anodic or cathodic current at the working electrode is registered resulting in an oxidation or reduction peak (Shippy and Lu, 2007). CV is able to recycle the electrons by rapidly generating new

redox species during the forward and reverse scan (Shippy and Lu, 2007). Cyclic voltammetry scans were performed for the examination of the performance of an Alcalase® biosensor for detection of PNPA.

5.3.5.2 Principles of Chronoamperometry

Chronoamperometry is an electrochemical technique in which the potential of the working electrode is stepped and the resulting current from faradaic processes occurring at the electrode is monitored as a function of time at a set potential. The faradaic process is the electron transfer between the electrode and solution species. Current correlates to substrate concentration, thus current increases until substrate is depleted (Bard and Faulkner, 2001). Unlike CV, CA has diverse applications such as measurement of concentration by measuring current (I) versus concentration at fixed potential (Anderson *et al*., 2000). It can also be used to analyse the shape of the current-time curve in order to study coupled chemical reactions (Portaccio *et al*., 2006). Due to broad applications of CA, it is an important method on which other techniques (for instance, enzyme kinetics and sensitivity factor of the biosensor) are based (Anderson *et al*., 2000; Roy *et al*., 2005). CA was used to examine laccase based biosensor performance.

5.3.5.3 Apparatus and measurement procedure

A modified or unmodified 1.5 mm radius GCE together with a platinum wire auxiliary electrode and Ag/AgCl (saturated with 3M KCl) reference electrode were fitted to Metrohm 663 VA Stand in a glass cell. This system was controlled by Autolab (Netherlands) potentiostat (PGSTAT 30) with IME663 interfaced to a computer with Autolab software where both chronoamperometry (CA) and cyclic voltammetry was performed. The conditioning potential was -0.3 V for 10 seconds and equilibration time was 5 seconds. CA was utilised for the detection of catechol as a model substrate for examining the performance of SphereZyme™ laccase biosensors for detection of phenols. For CA

measurements the working electrode (modified with laccase enzymes as detailed below was polarized at -100 mV for quinone reduction in 0.1 M succinate buffer at pH 4.5 or 0.1 M phosphate buffer pH 7.0 as reported in literature (Freire *et al*., 2001; Portaccio *et al*., 2006). Current was measured following the incremental introduction of catechol to the voltammetric cell in different buffer media as outlined further.

CV measurements of PNP and PNPA were conducted separately by scanning a potential window of 0-1.1 V at both unmodified and Alcalase® modified working electrodes. Five consecutive CV scans were run in 0.05 M Tris-HCl buffer pH 7.5 in which instance the 5^{TH} scan was used to represent the data unless otherwise stipulated.

5.3.5.4 Optimisation and fabrication of detection system

Variable modifications were examined in the fabrication of a biosensor for detection of model substrate (catechol) or PNPA to PNP. The enzymes for the biosensor were either free/unimmobilised in solution during solution phase electrocatalysis, or immobilized onto the electrode in different immobilisation strategies to generate a modified GCE (mGCE). Enzyme loadings of variable volumes were added in order to account for differences in activity (U) of samples as detailed below for i) catechol and ii) PNP.

Catechol detection

a) Solution-phase biosensor

Twenty five microliters of 1 $mg.ml^{-1}$ free laccase (1.018 U) and 100 µl SphereZyme™ laccase (0.255 U) were immersed in either 0.1 M succinate buffer pH 4.5 or 0.1 M phosphate buffer pH 7.0 to a final volume of 5 ml. The reaction

was started with increment introduction of 0.1 M catechol (in MilliQ water) to the buffer solution.

b) Glutaraldehyde (GA) cross-linking

Five micro-litres of 1 mg.ml^{-1} free laccase (1.018 U) and 20 µl of SphereZyme™ laccase (0.255 U) were separately dropped on the surface of the glassy carbon electrode (GCE) followed by 5 µl of 10% v/v GA solution treatment for coupling to the electrode (Freire *et al*., 2003). The electrode was air dried for 30 minutes before analysis. CA studies were performed in 0.1 M succinate buffer pH 4.5.

c) Polymer entrapment

A solution of 2 µl of 2 mg.ml^{-1} poly-L-lysine was place on the electrode followed by 20 µl SphereZyme™ laccase (0.255 U) and 5 µl 10% v/v GA. The solutions were mixed thoroughly. CA studies were conducted at this modified electrode in 0.1 M succinate buffer pH 4.5.

d) Membrane entrapment

Five micro-litres of 1 mg.ml^{-1} free laccase (1.018 U) and 20 µl of SphereZyme™ laccase (0.255 U) were separately dropped on the (GCE) and dried in an oven at a temperature of 32 °C for 30 minutes. After drying, the enzyme modified electrodes was entrapped in cellulose membrane (12 kDa) and secured with 3 *o*-rings. Prior to use, cellulose membrane was washed in 250 ml warm (30 °C) MillQ water with stirring at 250 rpm. MillQ water was changed once after 30 minutes. The electrode was connected to the potentiostat and the redox reaction was monitored amperometrically in 0.1 M succinate buffer pH 4.5 or 0.1 M phosphate buffer pH 7.0 buffers.

Optimisation of the electrode surface for amperometric analysis of PNPA and PNP

a) Glutaraldehyde crosslinking

To 3 ml 50 mM Tris-HCl pH 7.5, 100 µl of 1 mM PNP or PNPA was mixed for a baseline CV at an unmodified GCE. For PNPA and PNP detection, 5% v/v glutaraldehyde was used for cross-linking 10 µl of 4.4 $mg.ml^{-1}$ free Alcalase® (2.7 U) to the electrode surface immersed in 3 ml 50 mM Tris-HCl pH 7.5. The GCE was also modified with 100 µl of 1 $mg.ml^{-1}$ Alcalase® SphereZyme™ (0.54 U) solution was air dried, without glutaraldehyde cross-linking. CV was performed in the presence and absence of 100 µl PNPA or PNP in 0.05 M Tris-HCl pH 7.5.

5.3.5.5 Measurement of linearity and kinetics

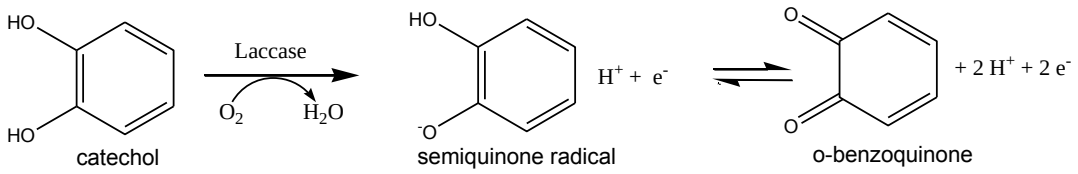
Measurement of linearity (sensitivity factor) was only calculated for CA plot in order to evaluate the effectiveness of SphereZyme™ for application to biosensors. The concentration of substrate used was from 0.0 mM until 2.191 mM in either 0.1 M succinate buffer pH 4.5 or 0.1 M phosphate buffer pH 7.0. The increase in current was monitor at a fixed potential of -0.1 V over 20 minutes. The kinetics of the biosensor was estimated using Hanes Woolf methodology.

5.4 Results and Discussion

5.4.1 Catechol as a model substrate

The electro-activity and sensitivity of the modified GCE (mGCE) was tested using catechol as a substrate. Catechol is a well studied model for phenol biosensors and is highly soluble in aqueous solutions at pH 7 (Partaccio *et al*., 2006). The potential for one electron transfer is at 100 mV (Eberson, 1985) and for the

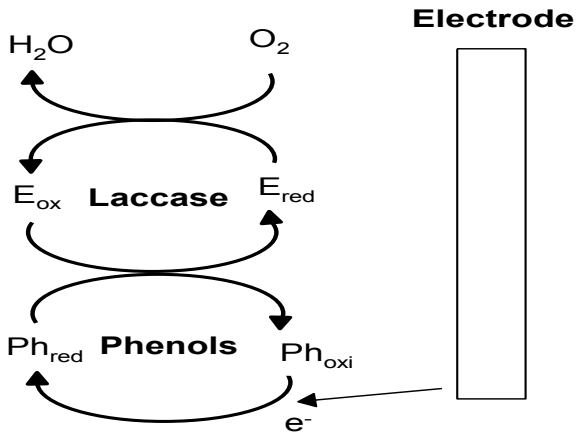
second electron transfer, a potential of 530 mV was reported (Steenken and Neta, 1979) both at pH 7.



Scheme 5.1: Redox cycling of catechol ($C_6H_6O_2$) to its two redox states (Schweigert *et al*., 2001).

Scheme 5.1 shows catalytic oxidation of catechol ($C_6H_6O_2$) into two redox species. During the first step, production of semi-quinone radical ($-C_6H_5O_2^-$), only one electron is transferred to oxygen to produce a superoxide (O_2^-). The reaction can further be catalysed by heavy metal or oxygen to produce *o*-benzoquinone ($C_6H_4O_2^{2-}$) and compounds such as hydrogen peroxide (H_2O_2) and hydroxyl ion (-OH, Mason *et al*., 1961; Schweigert *et al*., 2001).

Scheme 5.2 shows the direct electron transfer (DET) from the electrode caused by the oxidation of catechol to its quinone at the electrode surface (Zille *et al*., 2005).



Scheme 5.2: Electron movement of the oxidation of phenols at a laccase modified GCE (Dzyadevych *et al*., 2008).

Ph $_{red}$ = reduced phenol, Ph $_{oxi}$ = oxidised phenol. E $_{red}$ = reduced enzyme, E $_{ox}$ = oxidised phenol.

Laccase contains three types of copper atoms. Type I initiates four mono-electronic oxidations of the substrate. Electrons generated are relayed to tri-nuclear cluster (type II and type III) which will in turn reduce dioxygen (O_2) to water (Claus, 2004). In the presence of O_2, the released electrons are transferred to O_2 (oxidising agent) producing water. In absence of O_2, a lower positive redox potential of 0.34 V shows that laccase has low affinity for reducing substrates compared to oxidising agents (Gomes and Rebelo, 2003; Morozova *et al*., 2007).

Figure 5.1 shows a representative chronoamperogram, obtained after several additions of catechol at 100 µl of 1mg.ml^{-1} SphereZyme™ laccase immobilised via cellulose membrane entrapment.

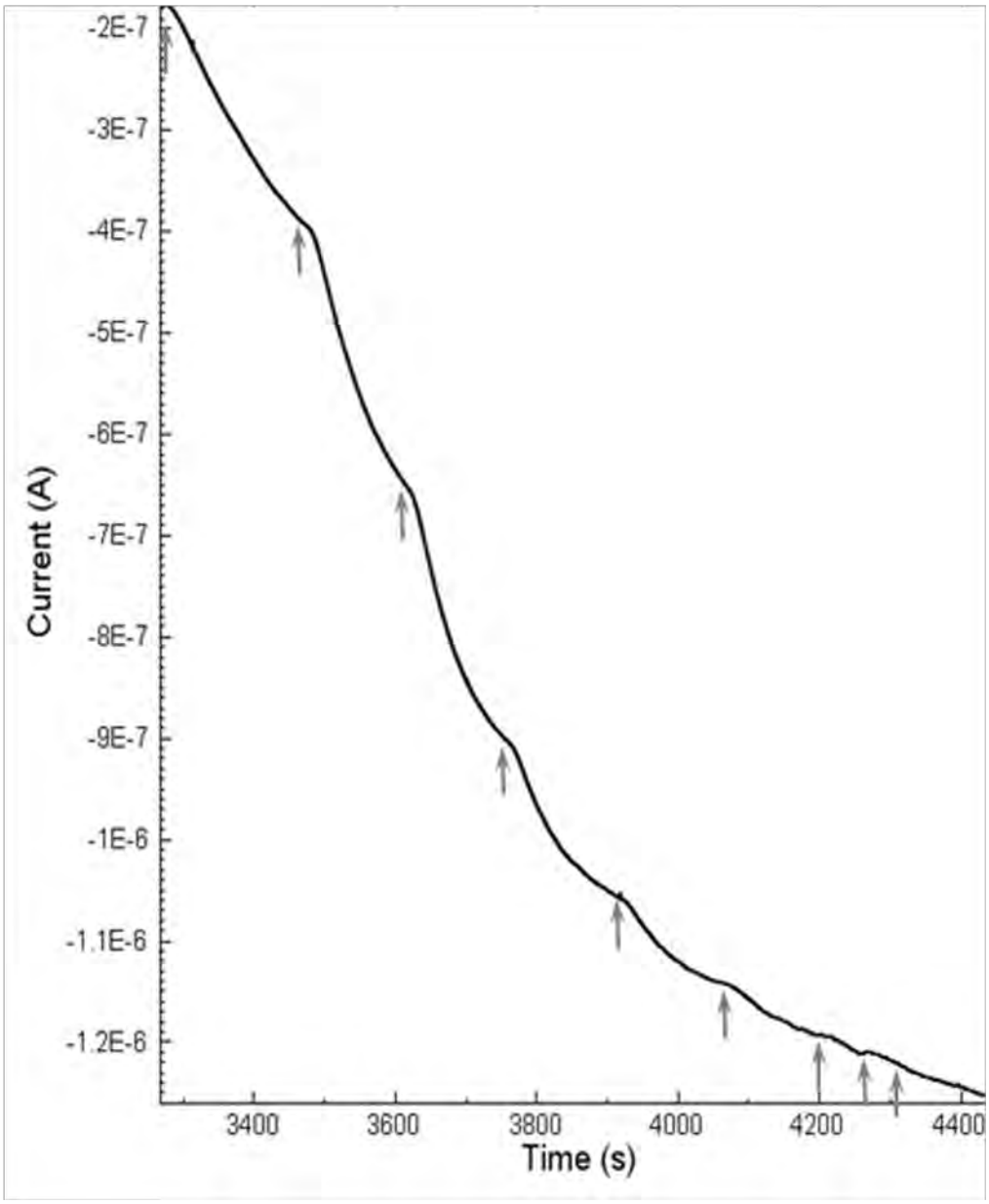


Figure 5.1: CA plot showing current response behaviour of successive additions of catechol (0-1400 µM) at a GCE modified with 100 µl of 1 mg.ml^{-1} SphereZyme™ laccase immobilised via cellulose membrane entrapment. Buffer: 0.1 M succinate buffer pH 4.5.

The current measured during CA was used to create plots of current response versus catechol concentration, generated for all modified surfaces examined.

5.4.2 Solution-phase biosensor

In fig. 5.2 plots of current vs. catechol concentration of SphereZyme™ laccase in 0.1 M succinate buffer pH 4.5 and 0.1 M phosphate buffer pH 7.0 are shown. The maximum current, which was extrapolated from the plot, of both the biosensors was below 0.015 µA. The sensitivity factor, which is the slope of the linear regression line of the plot, was 0.094 µA.mM^{-1}at pH 7.0 and 0.100 µA.mM^{-1} at pH 4.5.

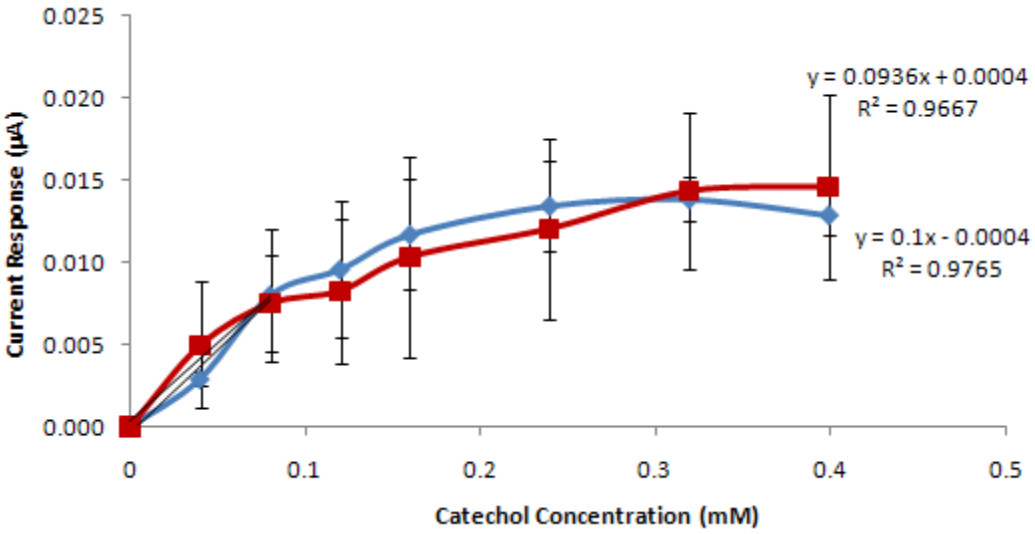


Figure 5.2: Current response of 100 µl 1 mg.ml^{-1} SphereZyme™ laccase in 0.1 M succinate buffer pH 4.5 (♦) and 0.1 M phosphate buffer pH 7.0 (■) at increasing concentration of catechol.

Applied potential -0.1 V vs. mGCE (Solution phase catalysis).

The sensitivity factor of the free laccase in solution phase in succinate buffer at pH 4.5 (1.05 µA.mM^{-1}) as shown in fig. 5.3 was higher than SphereZyme™ laccase at both pH 4.5 and 7.0. The maximum current of this biosensor was also higher at +/-0.400 µA.

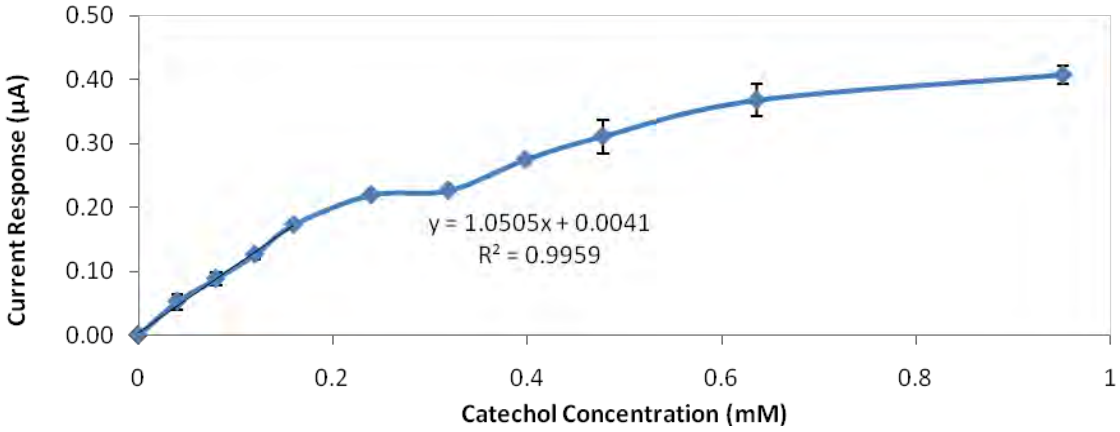


Figure 5.3: Current response of 25 µl of 1 mg.ml^{-1} free laccase in 0.1 M succinate buffer pH 4.5 at increasing concentrations of catechol.

Applied potential -0.1 V vs. Ag/AgCl.

The low sensitivity reported for both free laccase and SphereZyme™ laccase in solution phase catalysis was expected, due to the lack of direct electron communication between the biocatalyst and the electrode (Bard and Faulkner, 2001). However, the solution phase biosensor served to indicate the utility of the SphereZyme™ laccase for catechol biosensor applications. It provides a benchmark against which surface modifications can be compared given that the unimmobilised enzymes are theoretically free in solution and hence non-denatured.

5.4.3 Glutaraldehyde crosslinking

Figure 5.4 shows the current versus concentration plots obtained for catechol detection at GCEs modified with both free laccase and SphereZyme™ laccase cross-linked at the electrode surface with glutaraldehyde at pH 4.5. The peak current was reported as a function of catechol concentration. In CA, the current response correlates to substrate concentration up to a certain point. The increase in current response results in substrate reduction to product, hence increases in current reponse were observed until substrate depletion at the GCE (Bard and

Faulkner, 2001). Both of these plots describe a Michaelis-Menten like-curve was observed as in Eq. 5.2:

Equation 5.2: $I = I_{max}[S]/([S]+K_m^{app})$

Where I is the current response, I_{max} is the limiting current response, K_m^{app}, which is the Michaelis-Menten constant and [S] is the substrate concentration (Bard and Faulkner, 2001).

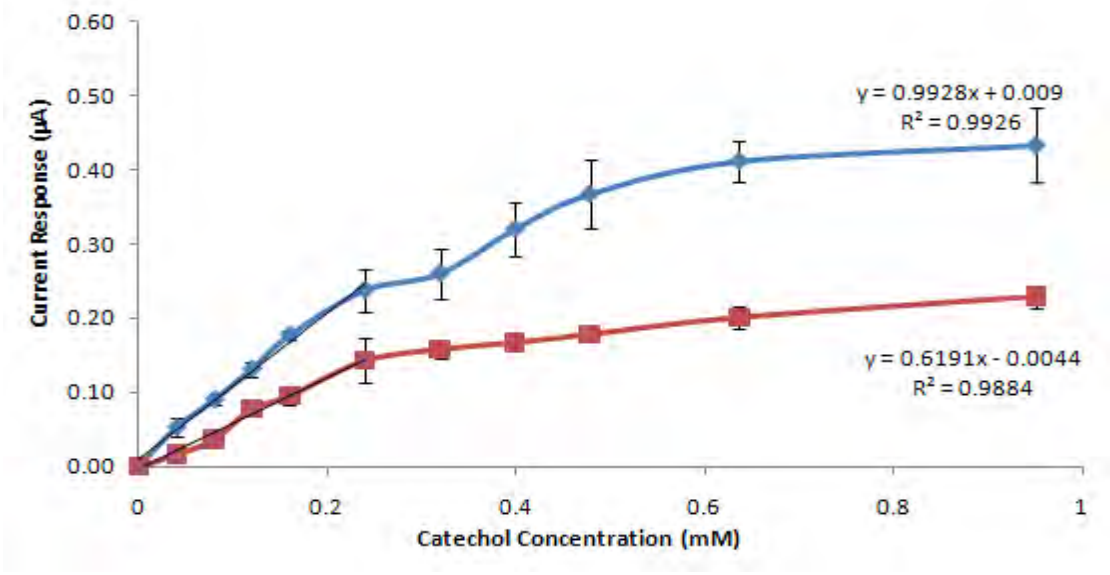


Figure 5.4: Current response of GCE modified with 5 µl of 1 $mg.m^{-1}$ free laccase (♦) and 20 µl SphereZyme™ laccase (■) cross-linked with 5 µl of 10% v/v GA in 0.1 M Succinate buffer pH 4.5 at increasing concentration of catechol.
Applied potential -0.1 V vs. mGCE.

Sensitivity factor recorded for mGCE with free laccase cross-linked with glutaraldehyde (0.993 $\mu A.mM^{-1}$; fig. 5.4) was similar to that monitored using the solution phase catalysis (1.05 $\mu A.mM^{-1}$; fig. 5.5) at pH 4.5. However, modifying the GCE with SphereZyme™ laccase resulted in improved sensitivity (0.619 $\mu A.mM^{-1}$; fig. 5.4) over the biosensor of the same biocatalyst in solution phase (0.100 $\mu A.mM^{-1}$; fig. 5.2).

The close proximity and a more direct contact of the biocatalyst with the electrode should in theory serve to increase the sensitivity over solution phase catalysis, as observed for modified GCE with SphereZyme™. The similarity between the sensitivity factor for glutaraldehyde cross-linked and solution phase free laccase biosensors may indicate denaturation of the free laccase in precence of glutaraldehyde. While glutaraldehyde is commonly used for enzyme immobilisations, it is known for its protein denaturation properties (Migneault *et al*., 2004). The relative increase in sensitivity of the glutaraldehyde cross-linked SphereZyme™ laccase suggests that the SphereZyme™ may afford protection against the denaturation by glutaraldehyde.

5.4.4 Cellulose Membrane Entrapment

The current responses versus catechol concentration for enzymes entrapped via cellulose membrane are shown in fig. 5.5-5.7. The effect of pH on cellulose membrane entrapped SphereZyme™ laccase is also shown in fig 5.5 and 5.6.

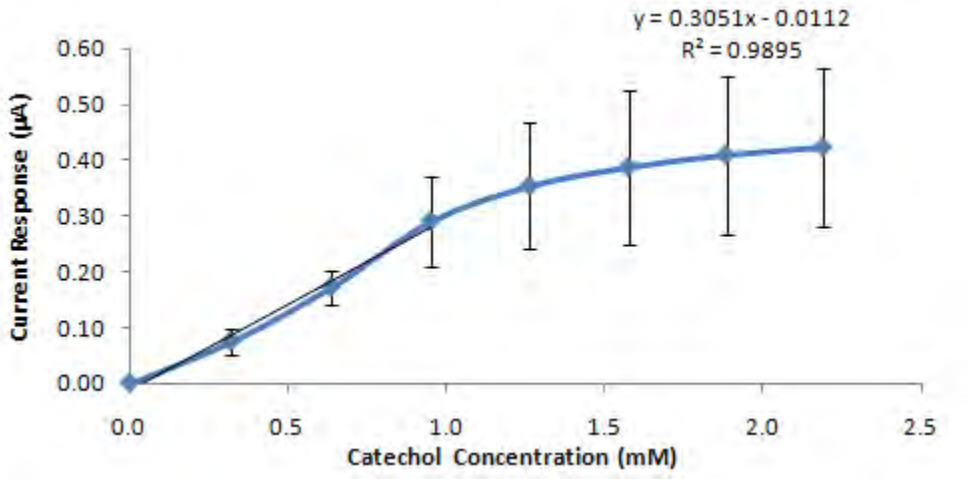


Figure 5.5: Current response and CA plots of GCE modified with 20 µl of 1 mg.ml^{-1} SphereZyme™ laccase via cellulose membrane entrapment in 0.1 M succinate buffer pH 4.5 at increasing concentration of catechol.

Applied potential -0.1 V vs. Ag/AgCl.

The cellulose membrane entrapped SphereZyme™ laccase biosensor showed a reasonably good linear response in a catechol range of less than 0.95 mM in succinate buffer, pH 4.5 with R^2 of 0.990 (fig. 5.5). At higher catechol concentration, the standard deviations of the three replicates were high indicating a lack of reproducibility for the detection of phenol at high catechol concentrations (>0.950 mM). At pH 4.5 a decrease in sensitivity of cellulose membrane entrapped SphereZyme™ laccase (0.305 µA.mM^{-1}; fig 5.5) relative to glutaraldehyde crosslinked SphereZyme™ laccase (0.619 µA.mM^{-1}; fig 5.4) may indicate mass tranfer limitation for catechol through the SphereZyme™ membrane.

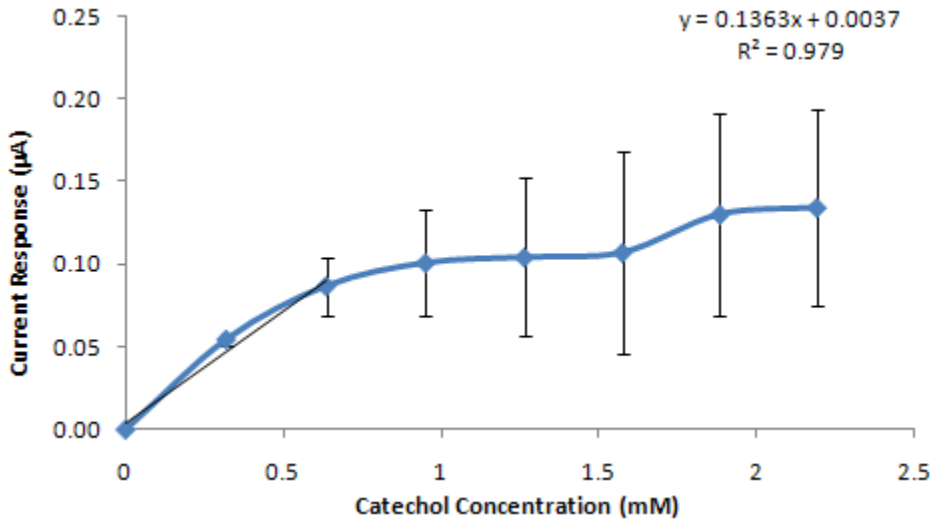


Figure 5.6: Current response of GCE modified with 20 µl of 1 $mg.ml^{-1}$ SphereZyme™ laccase via cellulose membrane entrapment in 0.1 M phosphate buffer pH 7.0 at increasing concentration of catechol.

Applied potential -0.1 V vs. mGCE.

At pH 7.0 (fig. 5.6), the cellulose membrane entrapped SphereZyme™ laccase biosensor was less efficient relative to the same surface at pH 4.5. Firstly, the maximum current response (0.136 µA) recorded at pH 7.0 was less than at pH 4.5 (0.423 µA). The catechol range with linear response was less than 0.686 mM with an R^2 of 0.979 (fig. 5.6).

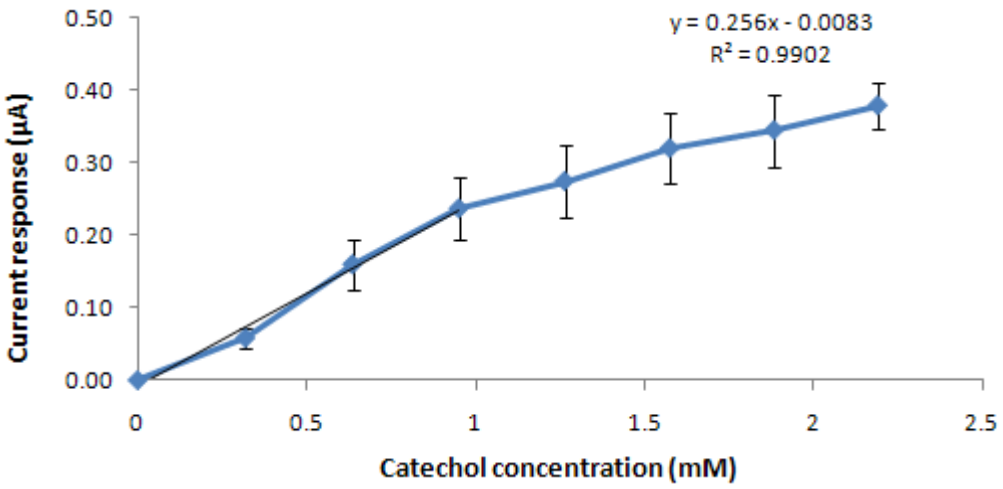


Figure 5.7: Current response of GCE modified with 5 µl 1 mg.ml^{-1} free laccase via cellulose membrane entrapment in 0.1 M succinate buffer pH 4.5 at increasing concentration of catechol.

Applied Potential: -0.1 V vs. mGCE.

As shown in fig 5.7, free laccase biosensor had low sensitivity relative to the sensitivity factor recorded for SphereZyme™ laccase (0.305 µA.mM^{-1}; fig 5.5), both entrapped with cellulose membrane at pH 4.5. While a similar linear range was observed for both modified enzyme surfaces at pH 4.5 (<0.950 mM of catechol), SphereZyme™ laccase had the highest maximum current response (0.420 µA). No recordable current response was observed for free laccase biosensors for catechol at pH 7.0.

The GCE modified with SphereZyme™ laccase imparted certain advantages over GCE modified with free laccase alone (fig. 5.7). Even though the laccase biosensor was capable of measurements at concentrations >0.950 mM, the linear range of operation of the two biosensors (SphereZyme™ laccase and its free form) were similar (<0.950 mM).

The pH of the solution also places a major role on the electro-activity and hence the sensitivity of the biosensor. Laccase has acidic optimal pHs and was shown

that at neutral to alkaline pHs, enzyme was inactive (Madzak *et al*., 2006). However, through immobilisation using SphereZyme™ technique, the shift in the pH profile towards the alkaline range was reported (Jordaan *et al*., 2009a). Hence the recordable sensitivity of SphereZyme™ laccase biosensor at pH 7.0 (0.136 $\mu A.mM^{-1}$, fig 5.6) compared to the lack of a current response for the free laccase biosensor at that pH, shows a particular advantage for the use of SphereZyme™ technology for laccase immobilisation.

Fig. 5.8 shows that the GCE modified with SphereZyme™ laccase and poly-L-lysine resulted in no current response at pH 4.5. Poly-L-lysine blocked redox-active centres of the SphereZyme™ laccase which were already restricted (Anderson *et al*., 2000).

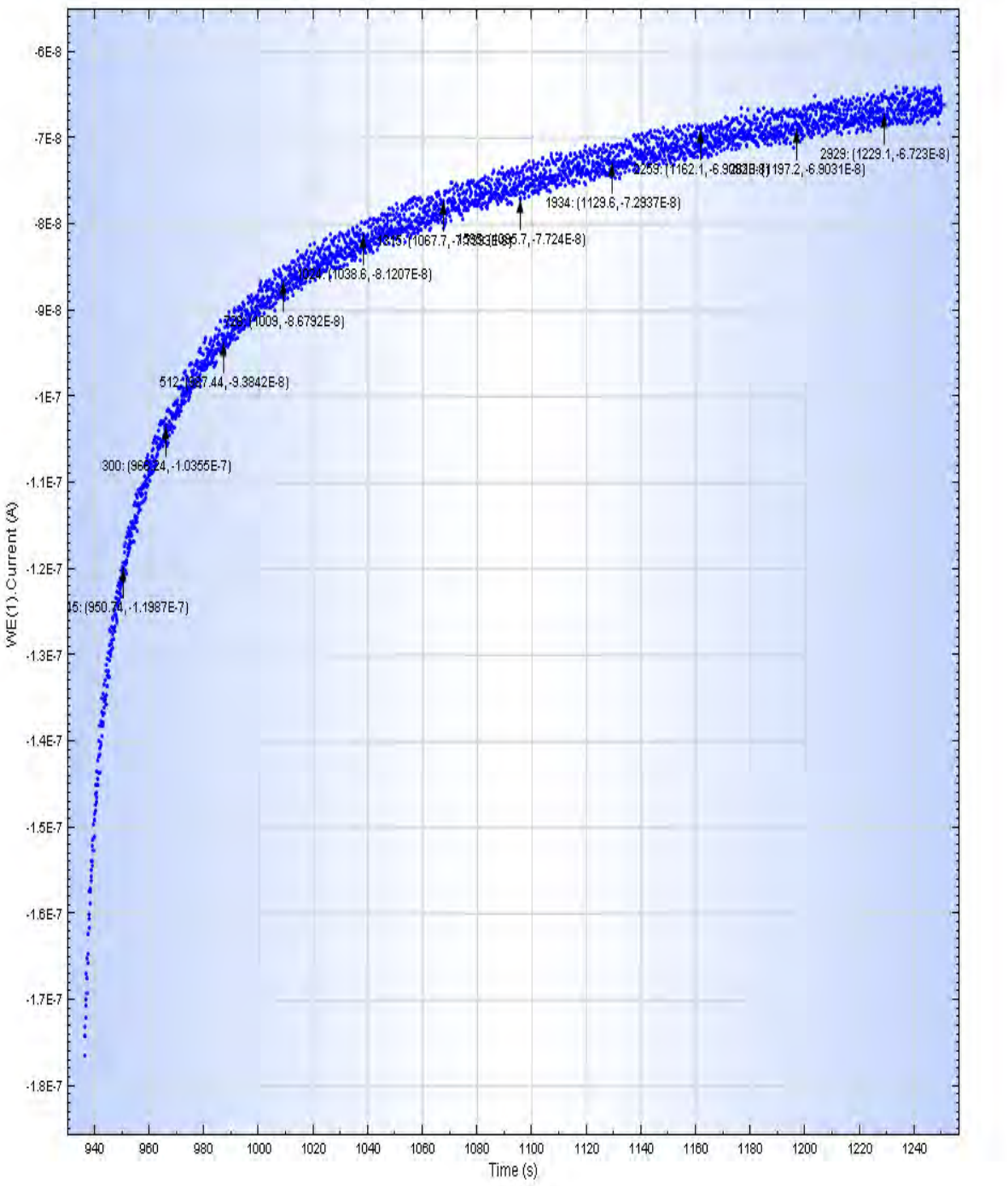


Figure 5.8: CA of GCE modified with 1 mg.ml^{-1} SphereZyme™ laccase and 2 mg.ml^{-1} poly-L-lysine in 0.1 M succinate buffer pH 4.5 at increasing concentration of catechol.

Applied Potential: -0.1 V vs. mGCE.

Tabulated comparisons of evaluated biosensors at pH 4.5 and pH 7.0 are given in table 5.1. Modification of GCE with SphereZyme™ laccase cross-linked with glutaraldehyde (GA) resulted in the highest sensitivity factor (0.619 $\mu A.mM^{-1}$) of the SphereZyme™ laccase biosensors (table 5.1). However, the substrate linearity range was lower (<0.300 mM) compared to the cellulose membrane entrapped SphereZyme™ laccase biosensor (<950 mM) which had a sensitivity factor of 0.305 $\mu A.mM^{-1}$. Free laccase biosensors yielded greater sensitivities in general than the SphereZyme™ laccase biosensor (+/- 1.05 $\mu A.mM^{-1}$) for solution phase biosensor except for the membrane entrapped biosensor. The lack of sensitivity of SphereZyme™ biosensor might be linked to substrate diffusional limitation which is the major drawback of the SphereZyme™ technique (Brady *et al*., 2008).

Table 5.1: Comparison of free laccase biosensor and SphereZyme™ laccase biosensor at pH 4.5 and pH 7.0.

Biosensors	#Sensitivity Factor ($\mu A.mM^{-1}$)	Catechol Range (mM)	Correlation Coefficient (R^2)	Maximum Current (μA)
Solution phase: Free laccase pH 4.5	1. 05 ± 0.007	<0.200	0.996	0.400
Solution phase: SphereZyme™ laccase pH 4.5	0.100 ± 0.094	<0.100	0.977	0.0140
Solution phase: Free laccase pH 7	0.094 ± 0.050	<0.100	0.967	0.0149
GA cross-linking: Free laccase pH 4.5	0.993 ± 0.263	<0.300	0.993	0.420
GA cross-linking: SphereZyme™ laccase pH 4.5	0.619 ± 0.073	<0.300	0.988	0.195
Membrane entrapment: Free laccase pH 4.5	0.256 ± 0.052	<0.950	0.990	0.360
Membrane entrapment: SphereZyme™ laccase pH 4.5	0.305 ± 0.087	<0.950	0.990	0.420
Membrane entrapment: SphereZyme™ laccase pH 7.0	0.136 ± 0.014	<0.686	0.979	0.120

#The sensitivity factor is the slope of the linear regression line in fig 5.2-5.7.

Modification of the GCE with SphereZyme™ laccase was more sensitive than the same biocatalyst in solution phase biosensors. This result follows the logic that the shorter the distance is between the biocatalytic membrane (SphereZyme™ laccase) and the electrode, the better the electronic communication between the two. As mentioned above, the same did not hold true for the free laccase preparations in solution phase. Laccase molecules are in a homogeneous state in solution phase and there is even distribution of electrons from the enzyme active site to the electrode (Shleev *et al*., 2005). Another reason might be potential denaturation caused by glutaraldehyde on the enzyme coupled to the electrode.

However, the SphereZyme™ biosensor when compared to other similar reported biosensors was not as sensitive. Freire *et al*., (2001) for example reported K_m^{app} values of 61 μM and 390 μM for immobilised and unimmobilised laccase, respectively. Roy *et al*., (2005) reported a more sensitive biosensor (827.0 $\mu A.mM^{-1}$) which entailed the use of CLEC laccase in the modification of the electrode. Freire *et al*., (2001) however, reported a value of 16.0 $\mu A.mM^{-1}$ for immobilised laccase via carbodiimide and 10% v/v glutaraldehyde on the electrode surface. The high sensitivity of CLEC biosensor was due to high activity to mass ratio of self-immobilisation techniques compared to solid support (Freire *et al*., 2001).

Since entrapment of SphereZyme™ laccase membrane on the GCE surface with cellulose membrane resulted in increased substrate linearity range and high correlation coefficient (R^2) at both pH 4.5 and 7 (relative to free laccase at pH 4.5), its kinetic parameters were calculated for demonstration purposes. These parameters shown in table 5.2 for comparison of free laccase and SphereZyme™ laccase were calculated through the linearization of Michaelis-Menten using Hanes Woolf Eq. 5.3:

Equation 5.3: $[S]/I_o = K_m^{app}/I_{max} + [S]/I_{max}$

Where:

X-intercept $= K_m^{app}$

Y-intercept $= K_m^{app}/I_{max}$

Slope $= 1/I_{max}$

The data sets utilised were cellulase membrane entrapped free laccase and SphereZyme™ laccase both at pH 4.5 (and pH 7.0 in the case of SphereZyme™ laccase biosensors for catechol).

Table 5.2: Kinetic constants of cellulose membrane entrapped free laccase and SphereZyme™ laccase biosensors

Samples	I_{max} (µA)	K_m^{app} (mM)	I_{max}/K_m^{app} (µA.mM^{-1})
Free laccase pH 4.5	0.593 ± 0.119	1.578 ± 0.095	0.379 ± 0.09
SphereZyme™ laccase pH 4.5	0.538 ± 0.205	0.297 ± 0.114	1.840 ± 0.352
#SphereZyme™ laccase pH 7.0	0.091± 0.004	0.179 ± 0.087	0.566 ± 0.230

#Number of replicates (n=2)

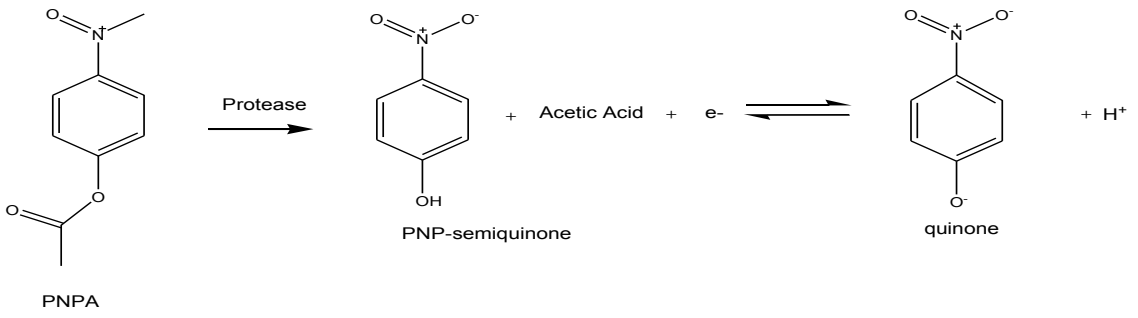
The SphereZyme™ immobilised enzyme promoted a significant increase (5 times higher) in the selectivity of the biosensor for detection of catechol as seen with the low K_m^{app} value of 0.297 mM compared to free laccase alone (1.578 mM).

However, a slight decrease in maximum current response (0.538 µA) compared to free laccase (0.593 µA) showed that SphereZyme™ may interfere with conductivity of GCE for electron passage (Zille *et al*., 2003). The recorded K_m^{app} and I_{max} at pH 7.0 was the lowest of the three modification. This showed that immobilisation of laccase through the SphereZyme™ shifted the pH of the enzyme (Roy *et al*., 2005; Jordaan *et al*., 2009a). It showed very good selectivity

for catechol at pH 7.0. Even though the SphereZyme™ laccase biosensor was electro-active at pH 7, the maximum current and K_m^{app} were lower (0.091 µA and 0.179 mM, respectively) than at pH 4.5. The I_{max}/K_m^{app} ratio estimates the effectiveness of the biosensor for catechol detection (Zille *et al*., 2005). As illustrated in table 5.2 it is seen that the SphereZyme™ laccase cellulose membrane entrapped biosensor at pH 4.5 recorded the highest effectiveness (1.84 $\mu A.mM^{-1}$) for detection of catechol at the electrode surface of the cellulose membrane entrapped enzyme biosensors. SphereZyme™ laccase at pH 7.0 recorded second highest at 0.566 $\mu A.mM^{-1}$ followed by free laccase which recorded 0.379 $\mu A.mM^{-1}$ at its optimal pH (pH 4.5). This represents a distinct advantage conferred to SphereZyme™ technology for entrapped enzymes.

5.4.5 Electrochemical behaviour of PNP at the modified GCE with SphereZyme™ particles

Conversion of PNPA to PNP and acetic acid entails the cleavage of an ester bond in PNPA by a protease (Alcalase®). The released PNP can then be electro-oxidised to its quinone compound (scheme 5.3, Mason *et al*., 1961).



Scheme 5.3: Proposed hydrolysis of PNPA ($C_6H_8O_3$) by a protease to an alcohol (PNP-$C_6H_7O_3$).

The alcohol will then be electrochemically oxidised to its quinone ($C_6H_5O_2^-$) under aerobic conditions.

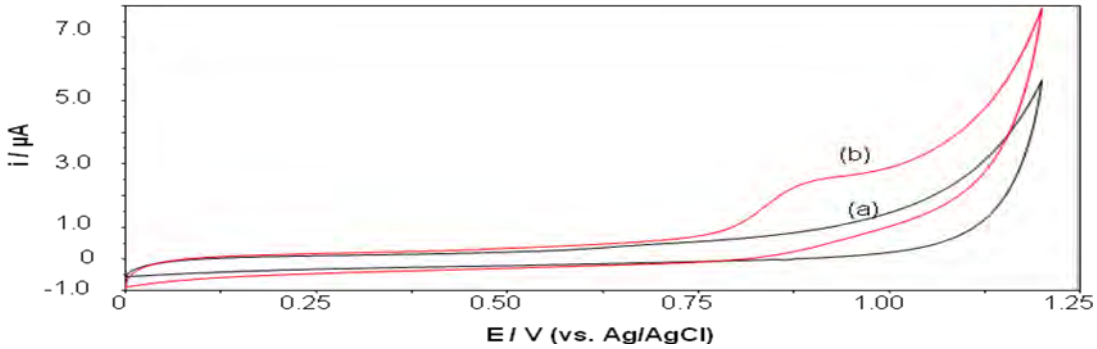


Figure 5.9 Cyclic voltammogram of a) the buffer solution (blank) and b) PNP (1 mM final concentration) at bare GCE. Electrolyte: 0.05 M Tris-HCL buffer pH 8.0 and 1 mM PNP. Scan rate: 0.1 V/s.

The CV results show the irreversible electrochemical oxidation of PNP (peak at 0.88 V) as seen in fig. 5.9b) compared to the control in the absence of PNP, fig 5.9a).

No peak related to PNPA was observed in the absence of Alcalase® since PNPA is not electroactive, fig. 5.10.

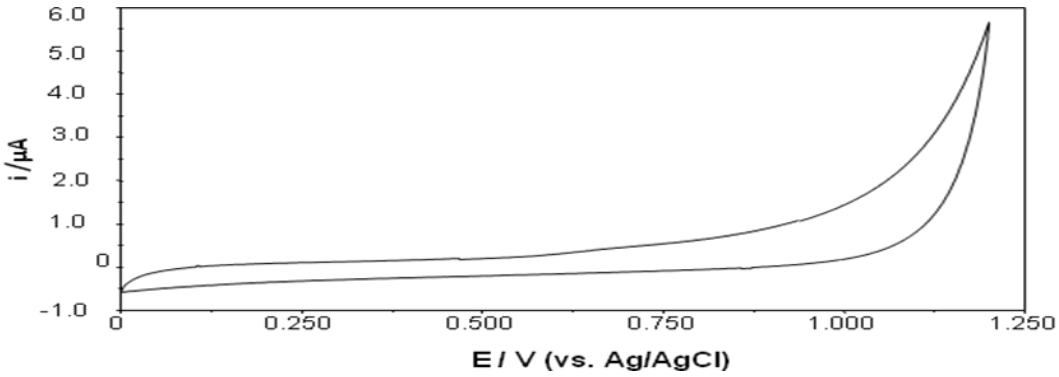


Figure 5.10: Cyclic voltammogram of PNPA (1 mM final concentration) at bare GCE.

Electrolyte: 0.05 M Tris-HCL buffer pH 8.0 and 1 mM PNPA at final concentration. Scan rate: 0.1 V/s

Figure 5.11 examines the biocatalytic conversion of PNPA to PNP at differently modified electrodes. At all GCE modified with Alcalase® SphereZyme™ a current response of 0.991 µA at a potential of 0.952 V was observed which was the same potential region as that observed for electro-oxidation of PNP (fig. 5.10; Liu *et al*., 2009). This suggests that Alcalase® in the biosensor converts PNPA to PNP followed by electro-oxidation of PNP to its quinone resulting in a detectable waveform. A lower current response of 0.136 µA at 0.958 V potential was observed at the free Alcalase® modified GCE compared to the Alcalase® SphereZyme™ GCE, suggesting enhanced production of PNP at Alcalase® SphereZyme electrode.

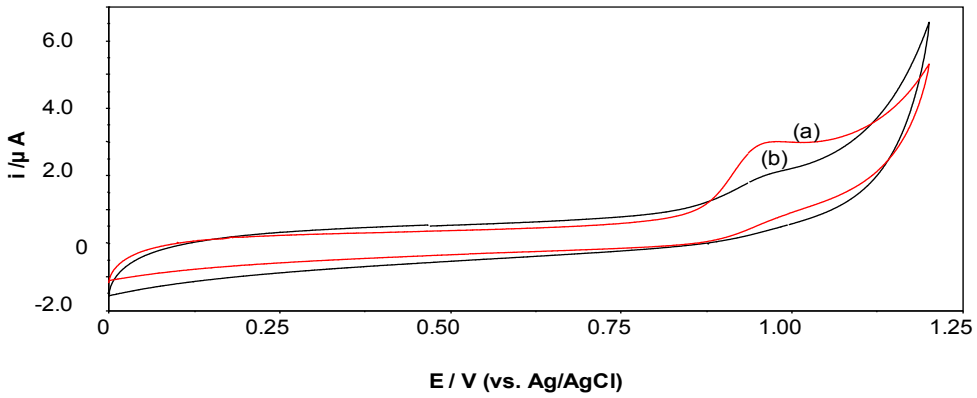


Figure 5.11: Cyclic voltammogram of biocatalysis of 1 mM PNPA to PNP followed by PNP oxidation at modified-GCE with Alcalase® SphereZyme™ (a) and Free Alcalase® cross-linked with glutaraldehyde (b).

Electrolyte: 0.05 M Tris-HCL buffer pH 8.0 and 1 mM PNPA at final concentration. Scan rate: 0.1 V/s.

Figure 5.12 shows the increase in production of PNP quinone at GCE modified with Alcalase® SphereZyme™ (fig 5.13a) or with free Alcalase® (fig 5.13b). There is a higher current response at GCE modified with Alcalase® SphereZyme™ (0.540 U) compared to free Alcalase® (2.70 U). These results suggest that immobilisation of Alcalase® within the SphereZyme™ enhanced the sensitivity of the sensor for detection of phenols.

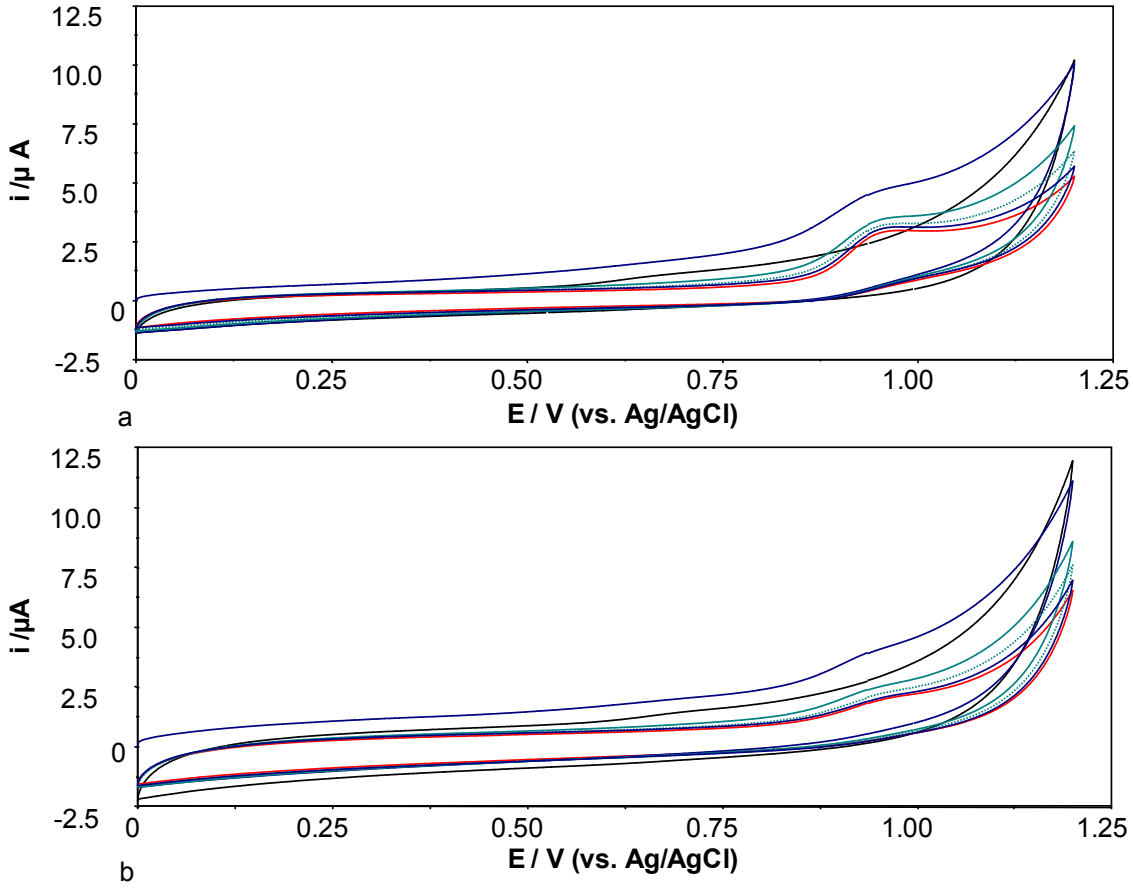


Figure 5.12: CV of modified GCE showing production of PNP from PNPA hydrolysis.

Increase in PNP production from PNPA due to catalysis at modified GCE over 5 successive scans. GCE was modified with Alcalase® SphereZyme™ (a) and free Alcalase® (b). Electrolyte: 0.05 M Tris-HCl buffer pH 8.0 and 1 mM PNPA final concentration. Scan rate: 0.1 V/s

Figure 5.13 is the graphical presentations of CV scans (fig. 5.12) of two differently modified GCE namely, free Alcalase® and Alcalase® SphereZyme™ monitoring PNP production. The increase in PNP detection possibly showed the increase in PNP production by the biocatalyst on the electrode surface through the cleavage reaction of the ester bond in PNPA (Klis *et al*., 2006).

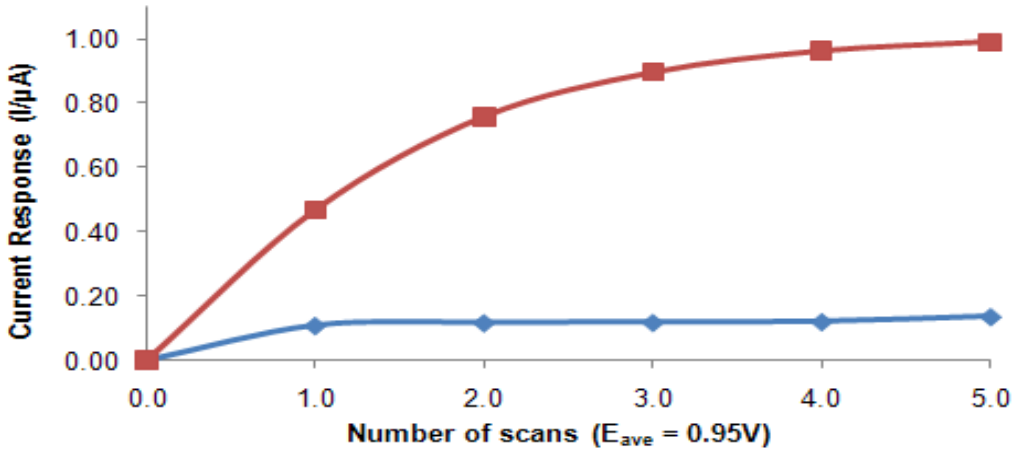


Figure 5.13: Graphical presentation of PNP production from PNPA at modified GCE with free Alcalase® (♦) and Alcalase® SphereZyme™ (■) through 5 successive CV scans.

Electrolyte: 0.05 M Tris-HCl buffer pH 8.0 at 1 mM PNPA final concentration. Scan rate: 0.1 V/s.

Free Alcalase® (cross-linked with glutaraldehyde) at the GCE, showed a lower production rate of PNP from PNPA (fig. 5.13 and table 5.3). The low production rate might be due to denaturation of Alcalase® by glutaraldehyde or leaching (Freire *et al*., 2001). This current response was even lower than PNP detection at bare electrode (table 5.3).

Table 5.3: Comparison of different GCE modifications for PNP detection after production from PNPA hydrolysis (results reproduced from fig. 5.11 and 5.12)

GCE modifications	I (µA)	E/V
*Alcalase® SphereZyme™	0.99	0.95
*Alcalase®	0.14	0.96
#Bare electrode	0.52	0.89

*The electrolyte solution comprised 1 mM PNPA in 0.05 M Tris-HCl pH 7.5

#The electrolyte solution comprised 1 mM PNP in 0.05 M Tris-HCl pH 7.5

The comparison of results of different modifications made on GCE in Table 5.3 showed that the highest current response was at the Alcalase® SphereZyme™ GCE. The results prove the advantage of using SphereZyme™ for GCE modification in biosensors over free enzyme. PNP was electro-oxidised from PNPA (not detectable) hydrolysis by Alcalase®. This shows the possible increase in the substrate range for phenol biosensors which can only detect PNP (Liu *et al*., 2009).

5.5 Conclusions

This work provided the first evaluation of the application of SphereZyme™ technology as a means of self-immobilisation of enzymes for biosensor applications. The responses were variable dependent on the method of immobilisation relative to free laccase preparations. The sensitivity factor of the free laccase in solution phase in succinate buffer was greater than that at SphereZyme™ laccase. The increase in sensitivity for detection of catechol when SphereZyme™ laccase was immobilised with glutaraldehyde suggests substrate diffusion as a limitation.

The main short fall of applying the SphereZyme™ in phenol biosensors is that it interferes with the conductivity of the electrodes for electron flow. Other disadvantages include its lack of sensitivity and reliability at substrate concentration higher than 0.95 mM. Reducing the size of the SphereZyme™ particles could solve the mass transfer problems.

The kinetics data however, showed that modifying the GCE with cellulose membrane entrapped SphereZyme™ particle increased not only the specificity but also increased the affinity for catechol detection relative to free laccase. This type of modified biosensor with SphereZyme™ laccase was also able to detect catechol with higher affinity at pH 7.0 while the free laccase biosensor was less efficient at that pH. This is a significant achievement in terms of further exploring

the role and application of SphereZyme™ self-immobilisation technologies for biosensor applications under differing pH conditions. Production of PNP from PNPA and detection of its quinone was improved in Alcalase® SphereZyme™ biosensor compared to Alcalase® biosensor due to enhanced PNP production.

6 Final Conclusions

6.1 General Discussion

In the past decade focus has been placed on establishing new enzyme immobilisation techniques or improving the existing ones to alleviate limitations preventing the realisation of applications (Sheldon, 2007 and Brady and Jordaan, 2009). The focus of this study was to optimise the novel SphereZyme™ self-immobilisation technique for the hydrolysis of polymeric substrates. This self-immobilisation technique was compared to a commercial enzyme solid support (Eupergit® C 250 *L*) and a proprietary microsphere technology, Dendrispheres. The immobilised enzyme preparation was subsequently evaluated for application to biosensors to assess the potential for improving the development of this technology.

However, a partially pure enzyme is one of the requirements of the SphereZyme™ technology since contaminants can react with the crosslinking agents and thereby reducing the immobilisation efficiency. Thus the first part of the thesis was the purification of Alcalase®, crude commercial enzyme preparation. The crude enzyme preparation was firstly dialysed to remove stabilisers and other non-protein additives. The dialysed sample was passed through a CM Sepharose Fast Flow column to separate the major subtilisin Carlsberg protease component from potentially interfering protease activities. The crude preparation was successfully purified into three fractions for which the fraction with the most activity, F1, correlated well with major protease component (subtilisin Carlsberg) in the preparation. There are few reported instances of the purification of subtilisin Carlsberg from Alcalase®, of which only dialysis was used as a method of purification. The different enzyme components of this enzyme preparation work in synergy to hydrolyse proteins hence the lack of need for purification.

After purification the enzyme sample was thus ready for immobilisation and the first method used was the SphereZyme™. Self-immobilisation techniques also suffer from a lack of applicability to the hydrolysis of large substrates due to substrate diffusional limitations. Protease, known for their hydrolysis of proteins, was chosen as a suitable demonstration enzyme. This limits the use of immobilised proteases for industrial application and further limits the development of biosensors using the same biocatalyst.

The SphereZyme™ preparation process was successfully optimised to address the aforementioned limitation. The modified process involved the addition of PEI as an enlarged crosslinking agent. The activity demonstrated in this study, 17 %, was the highest activity recorded to date. This modified method of preparation could prove useful for enabling new applications of the SphereZyme™ technology.

The third part of the thesis was immobilisation of purified Alcalase® on solid supports for comparative assessment of the self-immobilised enzyme for large substrate biocatalysis. The SphereZyme™ technique was the most efficient immobilisation method for the biocatalysis of proteins having superior activity to a commonly used support, Eupergit® and a newly developed solid support technology, Dendrispheres.

The optimised SphereZyme™ method was further used in the modification of GCE for detection of phenols. Modified GCE with SphereZyme™ laccase particles demonstrated suitable activity. Particularly notable was the detection of catechol at neutral pH, as the free enzyme was not capable of detecting the metabolite at pH 7.0. This may be suitable for the development of new applications for biosensors, such as real time monitoring of pollutant concentrations. The use of SphereZyme™ also simplified the fabrication of biosensors by eliminating the step of electrode modification with glutaraldehyde.

Alcalase® was shown to cleave ester bonds of non electroactive PNP derivatives, releasing PNP which produced measurable responses at the electrode surface. Greater current response was achieved at SphereZyme™ Alcalase® modified surfaces than at Alcalase® modified with glutaraldehyde. This can potentially allow improved monitoring of PNP for applications such as pollutant monitoring. The SphereZyme™ however, interferes with the conductivity of the electrodes for electrons flow. Other disadvantages include its lack of sensitivity and reliability at high substrate concentrations.

6.2 Future Work

We intend to evaluate the subtilisin Carlsberg SphereZyme™ immobilised enzyme for real world biocatalytic examples to demonstrate industrial applicability. We further intend to alleviate the limitations observed for the application of biosensors. The monitoring of phenols at elevated pH"s could potentially find application in the analysis of alkaline waste streams such as the pulp and paper industry. The high volumetric activity can potentially result in the development of a novel range of biosensors using SphereZyme™ immobilised enzyme preparations.

7 References

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