CAPILLARY MEMBRANE-IMMOBILISED POLYPHENOL OXIDASE AND THE BIOREMEDIATION OF INDUSTRIAL PHENOLIC EFFLUENT

THESIS

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ABSTRACT

Waste-generating industrialisation is intrinsically associated with population and economic proliferation. This places considerable emphasis on South Africa's water shortage due to the integral relationship between population growth rate and infrastructure development. Of the various types of industry-generated effluents, those containing organic pollutants such as phenols are generally difficult to remediate. Much work has been reported in the literature on the use of enzymes for the removal of phenols from these waste-streams but little application of this bioremediation approach has reached practical fruition. This study focuses on integrating and synergistically combining the advantages of enzyme-mediated dephenolisation of synthetic and industrial effluent with that of membrane technology. The ability of the enzyme polyphenol oxidase to convert phenol and a number of its derivatives to chemically reactive o-quinones has been reported extensively in the literature. These o-quinones can then physically be removed from solution using various precipitation or adsorption techniques. The enzyme is, however, plagued by a product-induced phenomenon known as suicide inactivation, which renders it inactive and thus limits its application as a bioremediation tool. Integrating membrane technology with the enzyme's catalytic ability by immobilising polyphenol oxidase onto polysulphone and poly(ether sulphone) capillary membranes enabled the physical removal of these inhibitory products from the micro-environment of the immobilised enzyme which therefore increased the phenol conversion capability of the immobilised biocatalyst. Under non-immobilised conditions it was found that when exposed to a mixture of various phenols the substrate preference of the enzyme is a function of the R-group. Under immobilised conditions, however, the substrate preference of the enzyme becomes a function of certain transport constraints imposed by the capillary membrane itself.

Furthermore, by integrating a quinone-removal process in the enzyme-immobilised bioreactor configuration, a 21-fold increase in the amount of substrate converted per Unit enzyme was observed when compared to the conversion capacity of the immobilised enzyme without the product removal step. Comparisons were also made using different membrane bioreactor configurations (orientating the capillaries transverse as opposed to parallel to the module axis) and different immobilisation matrices (poly(ether sulphone) and polysulphone capillary membranes). Conversion efficiencies as high as 77% were maintained for several hours using the combination of transverse-flow modules and novel polysulphone capillary membranes. It was therefore concluded that immobilisation of polyphenol oxidase on capillary membranes does indeed show considerable potential for future development

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ABBREVIATIONS

ANOVA	=	analysis of variance
BSA	=	bovine serum albumin
COD	=	chemical oxygen demand
HPLC	=	high performance liquid chromatography
IPS	=	Institute for Polymer Science
K_m	=	Michaelis constant
L-DOPA	=	L-β-3,4-dihydroxyphenylalanine
MC	=	methyl cellusolve
MWCO	=	molecular weight cut-off
NMP	=	N-methyl-2-pyrrolidinone
PEG	=	polyethylene glycol
RTD	=	residence time distribution
SEM	=	scanning electron microscopy
SCR	=	single-capillary reactor
V_{max}	=	maximum reaction velocity
WRC	=	Water Research Commission

PUBLICATIONS

The following is the list of publications that have arisen from the work presented in this thesis.

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Edwards, W., Bownes, R., Leukes, W.D., Jacobs, E.P., Sanderson, R., Rose, P.D., Burton, S.G. 1999. A capillary membrane bioreactor using immobilised polyphenol oxidase for the removal of phenols from industrial effluents. *Enzyme Microb.Tech.* 24(3-4): 209-217.

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CHAPTER 1

INTRODUCTION

MEMBRANE TECHNOLOGY AND EFFLUENT TREATMENT

1.1. INTRODUCTION

In 1995, the estimated population of South Africa was 43.5 million and increasing at an annual rate of 2.3%. The projected full water demand of the one million people introduced into the population in 1995, calculated as a weighted average, will be 638 ML.d^{-1} by the year 2015. This estimate is based on the assumption that the Reconstruction and Development Programme will be, to a large extent, successful in attaining its objectives. These include improving the living conditions of the poorer section of society to a level whereby acceptable water and sanitation services will be made available to all. An indication, therefore, of the tremendous pressure on this country's water resources, is that the projected full water demand of those one million people in 2015 is equivalent to a quarter of the average supply of Rand Water during the period 1994/1995 (Schutte&Pretorius,1997). As a result of the exigent circumstances that this country's water supply and demand faces, much research has been directed towards water reuse. This has been paralleled by an increase in awareness concerning the potential environmental hazards associated with chemical waste (Kempster *et al.*,1997). In conjunction with this, the need for new reactor and treatment technologies has also arisen.

According to Edgington (1994), "most toxic waste treatments rely on the traditional civil-engineering strategies of physical removal or containment". Conventional wastewater remediation methods employing physical and chemical treatment processes have, however, been associated with a number of intrinsic disadvantages. These include the production of more toxic intermediates, high operating costs, low levels of efficiency (Choi *et al.*,1992), and applicability to limited concentration ranges (Alberti&Klibanov,1981 Atlow *et al.*,1984). These methods also become exceedingly expensive when low effluent concentrations need to be achieved (Sun *et al.*,1992). A more efficient approach would be biological wastewater treatment due to it being a relatively clean technology (Dall-Bauman *et al.*,1990).

1.1.1. Phenolic pollutants

Of the various types of organic pollutants which can be classified as hazardous, phenol and its derivatives are among the more toxic. Phenols fall within the US Environmental Protection Agency's

list of "priority pollutants" (Lanouette,1977; Keith&Telliard,1979; Galli,1990). This includes pesticides, halogenated aliphatics and aromatics, nitroaromatics, polychlorinated biphenyls and polycyclic aromatic hydrocarbons. The release into the environment of large quantities of phenolic wastewater has created considerable ecotoxicological problems with severe consequences for human health and generally for all living organisms (Alberti&Klibanov,1981; Jarvis *et al.*,1985). Recently, increasingly stringent effluent standards have mandated the removal of phenol and derivatives from increasingly dilute wastewaters (Sun *et al.*,1992).

1.1.2. Distribution of phenolic pollutants

Phenols are present in the aqueous streams of many industrial processes including those from petroleum refining, petrochemical manufacturing, coking and coal conversion industries (Klein&Lee,1978), chemical plants, resin manufacturing, foundries, paint-stripping plants, and in condensate waters formed during coal gasification and liquefaction (Lanouette,1977; Alberti&Klibanov,1981; Greminger *et al.*,1982). The fundamental problem is the recalcitrance of these phenolic compounds (Galli,1990). Among these environmentally important xenobiotics, many are halogenated and halogenation is often implicated as the reason for this persistence (Chaudhry&Chapalamadugu,1991).

1.1.3. Physical and chemical treatment methods

A number of physical and chemical methods exist for the treatment of phenolic wastewater. The method of choice is influenced by a number of aspects, but the major consideration is usually the economic viability of the process (Lanouette,1977). Incineration is usually the treatment of choice for concentrated phenol waste where the complete destruction of the pollutant is required. In cases where lower concentration wastewater requires remediation, a number of other chemical treatment methods are available including activated carbon adsorption (Klein&Lee,1978) and chemical oxidation (Lanouette,1977). Due to increasingly stringent effluent standards, use of activated carbon has shown a dramatic increase (Irving-Monshaw,1990). When phenol concentrations are sufficiently high to warrant recovery, the method of choice is usually liquid solvent extraction (Klein&Lee,1978; Greminger *et al.*,1982).

1.1.4. Biological treatment methods

Increased development in the area of biological wastewater treatment has also occurred. Although the use of micro-organisms for bioremediation has a number of advantages, there are also associated disadvantages.

1.1.4.1. Advantages and disadvantages of biological treatment

The importance of the role of micro-organisms in the catabolism of both naturally-occurring and man-made organic molecules has been stressed repeatedly (Bayly&Barbour,1984). Generally, multi-step chemical transformations would require a number of sequentially acting enzymes, as well as co-factor regeneration, and the process, therefore, requires whole cells to perform these multi-tasking requirements (Klibanov,1983). Although the complete degradation/removal of the aromatic pollutant is possible using biological treatment, the problem often arises that toxic chemical compounds pass through biological effluent treatment plants unaltered (Choi *et al.*,1992). Furthermore, maintenance of steady-state conditions in environments where waste is generated discontinuously, and effluent composition varies with time, effectively limits use of microbial processes for industrial effluent treatment (Jones *et al.*,1973; Sun *et al.*,1992 and references therein).

As a result, there appear, from the literature, to be few applications of specially acclimatised microbial cultures to industrial systems. Although a culture might be capable of efficiently degrading a toxic pollutant under laboratory conditions, this will not always be the case when transferred to the harsher, less controlled aqueous environment of a wastewater treatment facility (Livingston,1993b). The inorganic composition of industrial effluent has also been shown to be deleterious to microbial growth (Livingston,1993a,b).

When single or two-step transformations are involved, the use of isolated enzymes is advantageous over the use of whole cells to avoid competing side-reactions, shear-stress due to agitation, and sterility problems (Klibanov,1983). An enzymatic approach to phenolic waste bioremediation may, therefore, be preferable.

1.2. AN ENZYMATIC APPROACH TO BIOREMEDIATION

Although much work is presented in the literature on the use of various enzymes for phenolic effluent bioremediation (refer to table 3.1), relatively little research has been done using immobilised enzymes. Most of the work done using non-immobilised enzymes for phenol removal has been carried out in batch reactors. Batch reactors, however, are prone to a number of well-documented limitations including batch-to-batch oscillations, high labour costs, frequent start-up and shut-down procedures, and the need for biocatalyst recovery after each batch (Prazares&Cabral,1994). Initial work on the use of non-immobilised enzymes for phenol removal has focused primarily on the peroxidases, specifically horseradish peroxidase (Alberti&Klibanov,1981; Davis&Burns,1990; Buchanan&Nicell,1997; Setti *et al.*,1998).

The use of laccase has also drawn considerable attention due to its phenol-removal capacity which employs the same free-radical generating mechanism of dephenolisation as the peroxidases (Shuttleworth&Bollag,1986; Bollag *et al.*,1988; Davis&Burns,1990; Crecchio *et al.*,1995). Haemoglobin, which exhibits a peroxidase activity, has also been shown to remove certain phenols (Chapsel *et al.*,1986). Recently, there has been heightened interest in the phenol removal capacity of polyphenol oxidase (Atlow *et al.*,1984), as well as the removal of the enzyme-generated *o*-quinones using various chemisorbents (Payne *et al.*,1992; Payne&Sun,1994). Lee *et al.* (1996) have also shown that a cloned thermostable β-tyrosinase from *Symbiobacterium* to be effective in removing phenol from a phenolic resin-manufacturing wastewater.

1.2.1. Immobilised enzymes

In 1967, Chibata and co-workers at the Tanake Seiyaku Company in Japan reported the earliest use of immobilised enzymes in an industrial application (Katchalski-Katzir,1993). This application involved immobilised *Aspergillus oryzae* aminoacylase for the resolution of a synthetic racemic mixture of DL-amino acids into the corresponding, optically-active enantiomers. Since then, a number of different enzymes have been investigated in the immobilised state for fundamental or application studies (Prazares&Cabral,1994). Recent examples of enzyme immobilisation studies include applications of α-amylase (Siso *et al.*,1997), β-galactosidase (Vishwanath *et al.*,1995), invertase and amyloglucosidase (Gille&Staude,1994).

Recently, immobilised enzyme systems have become prominent in bioremediation applications. The use of lignin peroxidase immobilised on controlled-pore glass beads has shown considerable potential for anthracene degradation (Presnell *et al.*,1994). Polyphenol oxidase has been immobilised on cation exchange resins (Wada *et al.*,1993), magnetite (Wada *et al.*,1992; Wada *et al.*,1995), gelatine gels (Crecchio *et al.*,1995), and chitosan gels (Sun&Payne,1996) for the removal of phenols from water.

1.2.2. Advantages of enzyme immobilisation

Although non-immobilised enzymatic dephenolisation has been relatively successful, a number of drawbacks have been associated with this approach. Specifically, in the case of polyphenol oxidase, these include the inactivation of the enzyme by the enzyme-generated quinones in solution (Wada *et al.*,1993). The use of free or non-immobilised enzymes for other applications has also been associated with numerous disadvantages. These include thermal instability, susceptibility to protease attack, activity inhibition, long-term instability, and difficulties encountered in separating and reusing the biocatalyst at

the end of the reaction (O'Fágáin *et al.*,1988; Crecchio *et al.*,1995). The immobilisation of enzymes on solid supports serves to overcome some of these limitations in that immobilisation offers the potential for readily separating the enzyme from the process stream (Sun&Payne,1996).

The economic feasibility of such industrial applications hinges almost exclusively on the catalytic lifetime of the proposed biocatalyst (Nicell *et al.*,1993). Thus, the feasibility of any immobilised enzyme system must be evaluated in terms of loss of enzyme activity over time (Busto *et al.*,1997). The effect of the physical and chemical environment is of paramount importance in maintaining an enzyme's geometry and chemical structure in relation to catalytic ability and lifetime. Perturbations in structure and geometry are a function of the physical and chemical environment in which the enzyme operates, and are usually associated with extremes in pH and elevated temperatures (Nicell *et al.*,1993).

In immobilised enzyme systems three additional types of enzyme activity loss can occur. Enzyme loss can occur due to desorption, severing of any chemical bonds involved in the immobilisation mechanism, or support material erosion. Secondly, the enzyme may become inactive due to thermal denaturation, inhibition, or destruction by proteolytic enzymes. Thirdly, the enzyme itself may become coated or blocked from contact with the substrate (Busto *et al.*,1997). These factors can be critical in determining the system's performance and the associated economics.

1.3. POLYPHENOL OXIDASE

The enzyme polyphenol oxidase is a copper-containing mono-oxygenase that catalyses the oxidation of phenol and related compounds in the presence of molecular oxygen (Vanni *et al.*,1990; Burton,1994). For a molecule to act as a substrate for the enzyme polyphenol oxidase, the presence of electron donor groups is required. A 'substrate' for polyphenol oxidase, is a phenolic compound that gives rise to a quinone intermediate (Passi&Nazzaro-Porro,1981). The electron donor (R) group of the substrate is characteristically in the *para*-position on the molecule with respect to the hydroxyl group with the electron donating nature of the R-group being a function of both the inductive (+I) and mesomeric (-M) effect of the R-group (March,1985). Phenols possessing powerful electron acceptor R-groups would be likely competitive inhibitors of polyphenol oxidase (Passi&Nazzaro-Porro,1981).

Polyphenol oxidase is able to catalyse two different reactions. The first, the *ortho*-hydroxylation of monophenols to form *o*-dihydroxybenzenes or substituted catechols, is referred to as the monophenolase or cresolase activity (EC 1.10.3.1) (refer to figure 1.1).
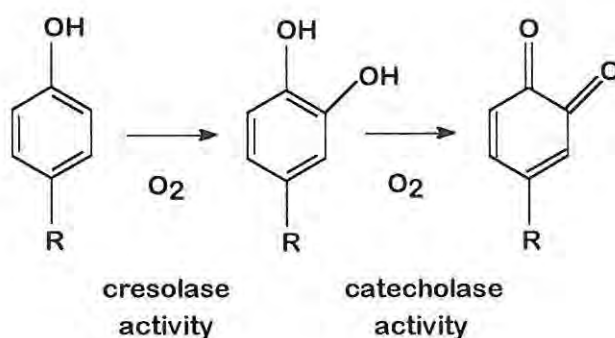
Figure 1.1 Hydroxylation and oxidation mechanism of polyphenol oxidase.

Secondly (EC 1.14.18.1), the subsequent oxidation or dehydrogenation of *o*-dihydroxybenzenes to *o*-quinones (figure 1.1) is referred to as the diphenolase or catecholase activity (Mason,1966; Sjoblad&Bollag,1981; Robb,1984; Vanni *et al.*,1990; Ros-Martínez *et al.*,1994). Non-enzymatic polymerisation of the quinones by oxidative coupling then occurs forming melanin or melanin-like compounds (Kazandjian&Klibanov,1985). This occurs *via* branched pathways consisting of cyclisation or hydroxylation reactions (Rodriguez-Lopez *et al.*,1991).

Polyphenol oxidase is ubiquitous amongst micro-organisms, animals, and plants. Some similarities in structure and functionality appear to be characteristic among the different polyphenol oxidases obtained from a variety of sources. Although polyphenol oxidase has been purified from a number of different sources, the exact molecular structure is unknown due to the occurrence of multiple isoforms of the enzyme (Gerritsen *et al.*,1994) and because of frequent pigment contamination (Lerch,1988). The molecular weight of purified polyphenol oxidase from the common mushroom *Agaricus bisporus*, which occurs in a tetrameric form, has been shown to be approximately 120kDa (Sjoblad&Bollag,1981).

The active site of polyphenol oxidase consists of two copper atoms existing in three states: 'met', 'deoxy', and 'oxy' (Ros-Martínez *et al.*,1993). Polyphenol oxidase found in *Agaricus bisporus* contains four copper atoms in a four-sub-unit protein molecule. These occur as pairs of anti-ferromagnetically coupled cupric/cuprous ions (Vanni *et al.*,1990). The accepted mechanism is that the oxidation of catechol [$C_6H_4(OH)_2$] by polyphenol oxidase [$E(Cu^{2+})_2$] involves the copper shuttling between valence states with oxygen binding to the copper(I) state (figure 1.2).

6

A suitable representation is:

$$C_6H_4(OH)_2 + E(Cu^{2+})_2 \rightarrow C_6H_4O_2 + 2H^+ + E(Cu^+)_2$$

$$E(Cu^+)_2 + O_2 + C_6H_4(OH)_2 \rightarrow C_6H_4O_2 + 2\ OH^- + E(Cu^{2+})_2$$

(Robb,1984)
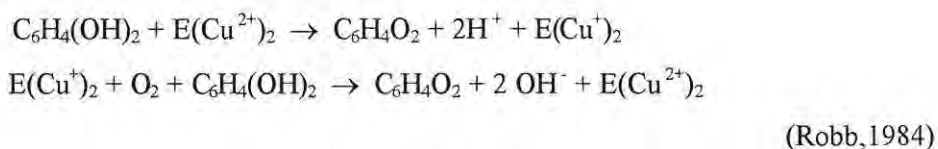
It is well established that the monophenolase or cresolase activity of polyphenol oxidase is characterised by a lag or induction period (Vanni *et al.*,1990; Ros-Martínez *et al.*,1993). This can be defined as the amount of time required by the enzyme to produce a certain concentration of diphenol (Ros-Martínez *et al.*,1993). The induction or lag period associated with the hydroxylation step has been found to be overcome by the addition of a reductant, which suggests that the enzyme reverts to its reduced bicuprous or 'deoxy' form which is able to bind oxygen (Vanni *et al.*,1990). In the absence of an exogenous reductant the lag period is overcome when the concentration of *o*-dihydroxyphenol formed reaches a level at which it is no longer rate-limiting. It has been shown that when certain catechols are used as activators the apparent K_m is two orders of magnitude smaller than the K_m evaluated when the same catechol is used as the substrate. This suggests that a specific binding site on the enzyme is actually saturated by the catechol (Vanni *et al.*,1990). Also characteristic of this lag period is that it increases when the substrate concentration increases but decreases when the enzyme concentration increases (Ros-Martínez *et al.*,1993).

Due to polyphenol oxidase's selective recognition and oxidation of phenolic compounds (Sun&Payne,1996) and the wide range of phenolic substrates with which polyphenol oxidase is capable of reacting (Passi&Nazzaro-Porro,1981), the enzyme is an ideal candidate for implementation in a bioremediation process. It has also previously been reported that a significant advantage of using polyphenol oxidase is that it is less sensitive to changes in waste stream composition and strength than other enzymes (Atlow *et al.*,1984). Unlike enzymes such as chloroperoxidase and horseradish peroxidase, the products formed during polyphenol oxidase-catalysed reactions are significantly less toxic than the parent compounds (Heck *et al.*,1992). Furthermore, the relative temperature-independence in terms of enzyme activity within the range 4°C-37°C, and the use of O_2 as the oxidant as opposed to H_2O_2, are also advantageous in a bioremediation process (Atlow *et al.*,1984). For these reasons polyphenol oxidase was chosen for determining the feasibility of implementing an immobilised-enzyme bioremediation process.
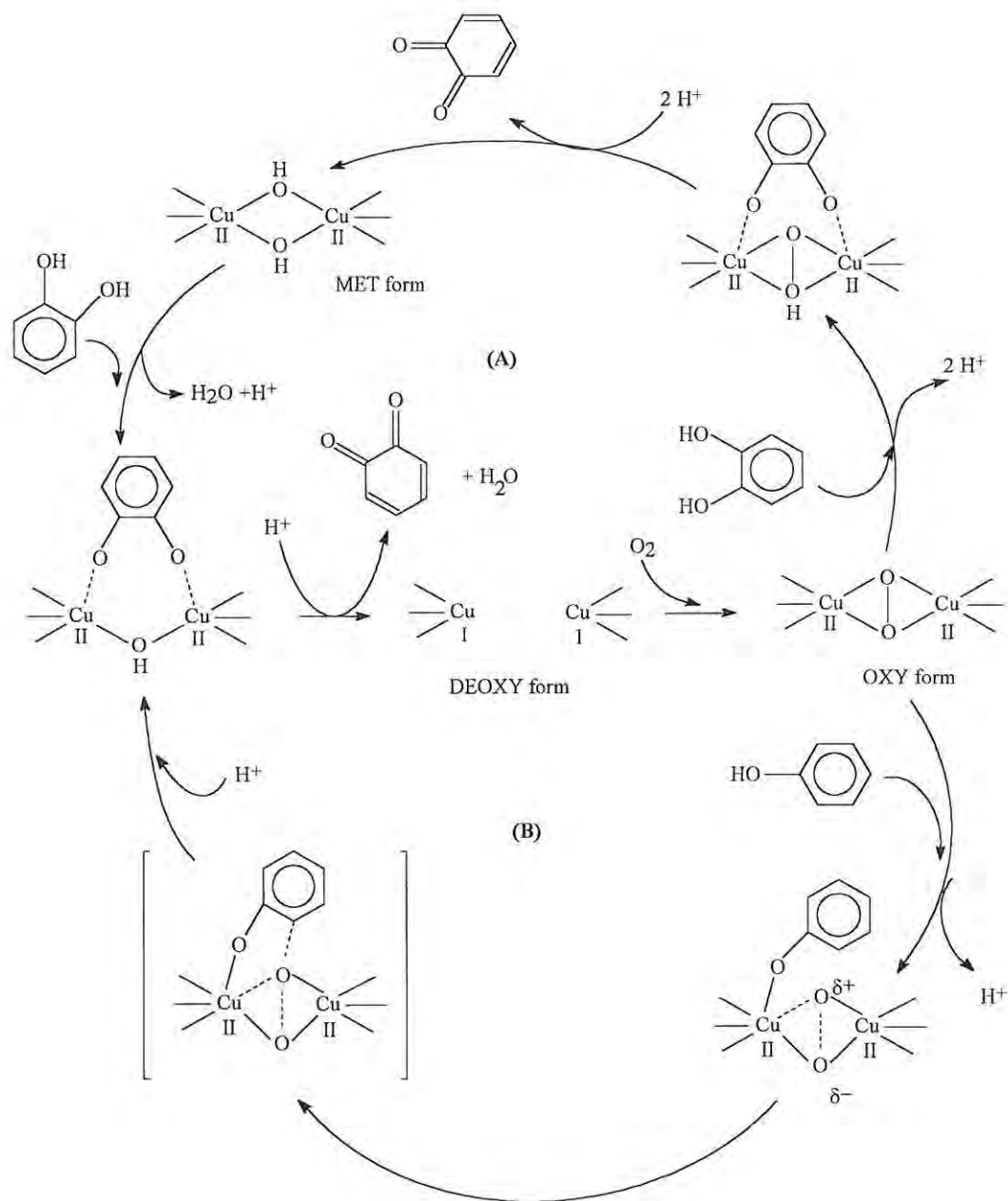
Figure 1.2 Reaction mechanism for polyphenol oxidase showing (A) the catecholase and (B) the cresolase activity. Adapted from Lerch (1983), Lerch (1988), and Martinez&Whitaker (1995).

1.3.1. Reaction inactivation of polyphenol oxidase

Polyphenol oxidase was one of the first enzymes to be shown to catalyse the incorporation of molecular oxygen into an organic molecule and it provides an early example of what is now known as suicide

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inactivation (Robb,1984; García-Cánovas *et al.*,1987; Funaki *et al.*,1991). This term is related to the rapid inactivation of the enzyme which occurs during the oxidation of catechol (Robb,1984). It is the reduction of the *o*-quinones back to the corresponding catechol which unfortunately leads to the inhibition by the reductant of the *Agaricus bisporus* polyphenol oxidase (Doddema,1988). Associated with this inactivation is the release of enzyme-bound copper (Golan-Goldhirsh&Whitaker,1985).

It was proposed that the implementation of immobilised polyphenol oxidase within a capillary-membrane bioreactor would have the effect of decreasing the effect of reaction inactivation. This would be accomplished by the effect of flux across the membrane providing an *in situ* quinone removal function, extending from the membrane itself thereby effectively including the micro-environment surrounding the enzyme. Although polyphenol oxidase has been immobilised onto nylon flat-sheet membranes (Pialis *et al.*,1996; Pialis& Saville,1998), the enzyme has not yet been immobilised onto capillary membranes.

1.4. THE APPLICATION OF PRESSURE-DRIVEN MEMBRANE PROCESSES TO EFFLUENT TREATMENT

Ultrafiltration is a pressure-driven membrane filtration process, the primary use being membrane mediated fractionation (Tutunjian,1985; Cheryan,1986; Jacobs *et al.*,1997). This can be defined as the separation of a stream into two fractions on the basis of size exclusion. It involves the separation of macrosolutes or colloidal particulate matter in a solvent, usually water, from smaller solutes also present in the stream (Fane,1986), with the result that the majority of ultrafiltration applications are directed towards aqueous separations (Eykamp,1995). In membrane processes (reverse osmosis, ultrafiltration, and microfiltration), ultrafiltration has the largest variety of applications. These include the treatment of process streams from the chemical, food, and pharmaceutical industries for the concentration, purification, and separation of macromolecules; the sterilisation, clarification and purification of beverages and biological solutions; and the production of potable as well as ultra-pure water (Cheryan,1986; Ting-Hui,1987; Jacobs *et al.*,1997). One of the more important recent developments in which membrane technology, especially ultrafiltration systems, have been applied, is environmental protection. Here commercial ultrafiltration systems have found wide application in the improvement of industrial effluent treatment processes (Ting-Hui,1987). Membrane processes also offer a number of advantages compared with conventional wastewater treatment methods. These include higher standards, reduction in the environmental impact of effluents, the potential for mobile or very compact treatment units and, therefore, reduced land requirements (Van Dijk&Roncken,1997).

Of the different membrane geometries, the capillary type is characterised by having relatively high surface area-to-volume packing densities and being of a self-supporting nature. It also offers the additional hydrodynamic advantage of open-flow passages, as in tubular-type membranes. This, however, applies only if the capillaries are pressurised from the lumen side (Jacobs *et al.*,1997). The underlying principles governing membrane operation, flux and rejection characteristics, and the numerous applications of membrane processes have recently been comprehensively reviewed (Cheryan,1986; Fane,1986; Jönsson&Trägårdh,1990; Nilsson,1990; Noble&Stern,1995; Mulder,1996).

Integrating the properties of synthetic membranes with those of biocatalysts such as cells and enzymes, in order to carry out biocatalysed reactions, is rapidly becoming one of the more advanced applications of membrane processes in biotechnology (Gekas,1986; Belfort,1989; Heath&Belfort,1992). The combination of membrane technology with biological catalysts for the treatment of industrial wastewater has led to the development of a new technology, *viz.* membrane bioreactors (Belfort,1989; Drioli *et al.*,1990; Brindle&Stephenson,1996). The first reported use of capillary membranes coupled with immobilised enzymes was by Rony (1971). Membranes may be used as either a barrier for the retention of the soluble biocatalyst, or as a high surface area per unit volume matrix on which immobilisation of the enzyme occurs (Heath&Belfort,1992). By immobilising or encapsulating enzymes on or within capillary membranes, the membrane is effectively "activated", giving it a catalytic function, while still retaining the membrane's permselective characteristics (Kitano&Ise,1984; Belfort,1989). Only the combination of both functions should, therefore, be referred to as an enzyme membrane reactor (Gille&Staude,1994). The basic concept of membrane reactors is based on the separation of enzyme and products (or substrates) by a semi-permeable membrane that effectively creates a selective barrier (Prazares&Cabral,1994). Although membranes are more expensive than porous particles or beads as enzyme supports, their advantages may in many applications outweigh this limitation. These advantages include high volumetric productivity, the possibility of carrying out a reaction simultaneously with a separation function, lower susceptibility to process upsets, and, depending on the application, decreased sterilisation requirements (Roy *et al.*,1983; Gekas,1986). The capabilities of membranes as enzyme supports compared with other immobilisation matrices have been concisely summarised by Belfort (1989). Refer to table 1.1.

Two types of immobilised biocatalyst membrane systems can be distinguished: those with the biocatalyst in the soluble form and those with the biocatalyst insolubilised on the surface or within the pores of the matrix of the membrane. The second category can be divided according to the method of insolubilisation

used, i.e. adsorption, entrapment, and chemical coupling (Gekas,1986). Adsorption traditionally refers to the binding of a biocatalyst, by weak attractive forces, to an inert carrier which has not been functionalised for covalent binding.

Table 1.1 Capabilities of membranes as enzyme supports (Belfort,1989).

Function	Membranes	Porous particles/ beads
Immobilisation	yes	yes
Transport by convection	yes	no
Compartmentalisation	yes	no
Multi-organisation	yes	no
Separation	yes	no
Concentration	yes	no

In this study, poly(ether sulphone) and polysulphone membranes were used as enzyme immobilisation matrices. Because poly(ether sulphone) (figure 1.3) and polysulphone membranes (figure 1.4), as well as polyethylene, polypropylene, and polyamide (Cheryan,1986) membranes, lack reactive groups (Seymor&Carraher,1981; Gekas,1986; Schmidt-Steffen&Staude,1992), the method of choice for immobilisation was adsorption.
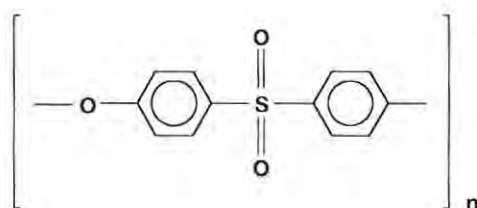
Figure 1.3 Chemical structure of poly(ether sulphone) (Mulder,1996).
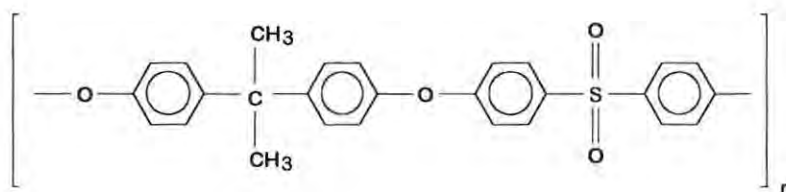
Figure 1.4 Chemical structure of polysulphone (Mulder,1996).

Two types of capillary membranes have been used in previous studies, *viz.* isotropic and anisotropic. Anisotropic membranes consist of a thin ultrafiltration membrane layer *ca.* 0.5µm thick, surrounded by a spongy support layer several hundred microns thick. It is the macroporous region of asymmetric (anisotropic) membranes that provides the means for the physical immobilisation/entrapment of enzymes or whole cells (Gekas,1986). Isotropic membranes are typically 10-30µm thick and have a uniform wall structure. Most commercial isotropic capillary membranes have diameters in the range of 0.1mm to 6mm (Roy *et al.*,1983). One of the advantages of using enzyme biocatalysts as opposed to whole cells is that if anisotropic membranes are being used, microbes would tend to grow in the spongy layer which could result in the rupture of the membrane during extended use. Isotropic membranes are not prone to this type of membrane rupture but do generally present a larger diffusion barrier (Roy *et al.*,1983). The anisotropic polysulphone capillary membranes used in this study were specially designed and manufactured, in close collaboration with Rhodes University, at the Institute for Polymer Science at Stellenbosch University, South Africa (Jacobs&Leukes,1996).

1.5. THE INTEGRATION OF CAPILLARY MEMBRANE TECHNOLOGY AND IMMOBILISED POLYPHENOL OXIDASE FOR EFFLUENT BIOREMEDIATION

1.5.1. Research objectives and rationale

The broad objective of this research programme was to develop a phenolic effluent bioremediation process based on capillary membrane-immobilised polyphenol oxidase. The main emphasis was whether immobilised polyphenol oxidase could be applied to industrial effluent treatment, thereby overcoming the aforementioned disadvantages associated with non-immobilised enzymatic dephenolisation.

The research directive could therefore be characterised as an initial feasibility study into the technical application of membrane-immobilised polyphenol oxidase for industrial effluent treatment. The technical feasibility studies then provided a basis, in terms of developmental criteria, for the subsequent scale-up of the process for the treatment of larger volumes of industrial effluent.

1.5.2. Research hypothesis

The underlying basis of this research project constituted a central hypothesis around which the research objectives were directed:

The feasibility of membrane technology integrated with enzyme immobilisation for phenolic effluent bioremediation is as yet an unutilised process technology. The developmental criteria necessary for scale-up to a suitable process for dealing with greater volumes of effluent can be obtained from the feasibility studies of membrane and enzyme immobilisation integration at the laboratory-scale level.

1.5.3. Experimental directives

In order for a research-orientated hypothesis to be validated, a step-by-step accrual of answers to the following initial questions is required:

1. What is the bioremediation potential of the enzyme membrane reactor in synthetic and real effluent situations?
2. What is the efficiency of the capillary membrane in terms of enzyme loading capacity and activity retention?
3. What are the possibilities for integrating product removal steps within the overall design of such a reactor system and the implications thereof?
4. If the combination of the above factors is effective and can be validated, what opportunities are available in terms of scaling-up such a system?

CHAPTER 2

MEMBRANE-MEDIATED EFFECTS INFLUENCING ENZYME-SUBSTRATE CONTACT AND CONVERSION

2.1. INTRODUCTION

Increasingly, synthetic membrane processes are being integrated into existing reaction, isolation, and recovery schemes for the generation of high value biological molecules in chemical and biochemical processes (Heath&Belfort,1992). One of the reasons for this is that membrane bioreactors are potentially competitive, in many aspects, with conventional bioreactors (Kleinstreuer&Agarwal,1986; Cheryan,1986), and in many cases are even replacing traditional unit processes (Heath&Belfort,1992). The advantages associated with enzyme-immobilised capillary membrane bioreactors include high volumetric productivities, negligible biocatalyst wash-out (Kleinstreuer&Agarwal,1986), permselectivity, and the potential for controlling the levels of contact or mixing between separate phases (Heath&Belfort,1992). Furthermore, from the chemical engineering perspective, the combination of immobilised enzymes and capillary membranes is advantageous due to the large membrane surface area per unit reactor volume for the permeation of reactants and products. Expensive downstream separation procedures can also be avoided due to the *in situ* separation of catalyst and product. The relatively small radial diffusion path-lengths of capillary membranes allow rapid reactions to be achieved, and inhibitory products can be removed as they are produced. The combination of these advantages means that steady-state operation can readily be achieved (Rony,1972).

Capillary membranes are physically composed of three distinct regions (figure 2.1): *viz.* the lumen (region 1), the ultrafiltration skin (region 2), and the outer annular macrovoid region (region 3) (Kleinstreuer&Agarwal,1986; Reiken&Briedis,1990). In conventional capillary membrane reactor operation, a laminar feed stream in the lumen provides a continuous source of substrate molecules that perfuse through the ultrafiltration skin to the annular macrovoid region. Transport occurs *via* diffusion or, under certain operating conditions, *via* convection. It is in the annular macrovoid region that enzymatic conversion occurs (Reiken *et al.*,1990). Ultrafiltration membranes can be distinguished from other membrane types as being depth- and not surface-filters. Therefore, the characteristics of the ultrafiltration skin-layer of the membrane define the retention properties of this particular membrane type (Jacobs *et al.*,1997). Ultrafiltration membranes possessing an anisotropic substructure are generally produced using the phase-inversion process (Aptel *et al.*,1985; Cheryan,1986; Jacobs&Leukes,1996).
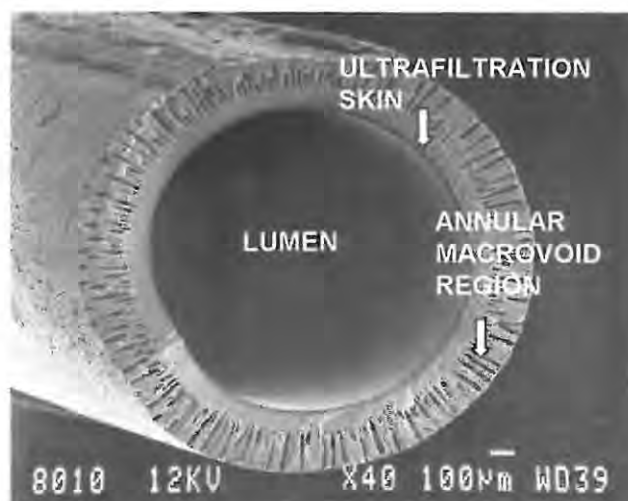
Figure 2.1 Scanning electron micrograph displaying the three distinct regions of an IPS 748 capillary membrane.

In the phase-inversion process membranes can be fabricated from any polymer which forms a homogeneous solution under specific composition and temperature conditions. Under different temperature and compositional conditions the polymer solution separates into two phases. In the wet-phase inversion process this change in phase occurs when water is used as the non-solvent medium. In terms of structural composition, these integrally skinned anisotropic ultrafiltration membranes are characterised by a dense skin-layer with the remaining substructure gradually becoming more porous with increasing distance from the ultrafiltration skin-layer (Jacobs&Leukes,1996; Jacobs *et al.*,1997).

Although numerous mathematical models exist which predict substrate conversions in enzymatic capillary membrane bioreactors (Webster&Shuler,1978; Reiken *et al.*,1990), the most complete models consider mass transfer in all three membrane regions (Opong&Zydney,1991). This includes axial flow and radial diffusion in the lumen, radial diffusion across the ultra-thin membrane, and radial diffusion and subsequent reaction in the annular spongy layer.

2.1.1. Mass-transfer limitations

Molecular diffusion can be defined as the movement of component molecules in a mixture under the influence of concentration differences present in a system (Kargi&Moo-Young,1985; Doran,1995). Although mass transfer in quiescent fluids occurs as a result of molecular diffusion, the majority of mass transfer systems consist of fluids in motion. In fluids where turbulent flow is dominant, mass transfer by molecular motion is supplemented by convective transfer (Doran,1995). To determine the

kinetic relationships for any reactor design, the possibility of mass-transfer limitations must be considered. In any bioreactor design, the significant categories of mass-transfer are intraphase diffusion in the medium concerned, interphase mass transfer at gas-liquid interfaces, mass transfer through membranes, mass transfer through porous structures, and interphase transfer at liquid-liquid interfaces (McDuffie,1991). In enzyme-immobilised capillary membrane bioreactors, mass transfer through the membrane is of greatest concern. This is because, although capillary membrane modules have been shown to facilitate rapid mass transfer, the rate of substrate conversion during enzyme-immobilised capillary membrane bioreactor operation is usually limited by constraints of substrate diffusion across the capillary wall to the enzyme (Swaisgood,1985; Reiken *et al.*,1990; Wickramasinghe *et al.*,1992). Such diffusion limitations can be decreased by forcing the substrate to flow convectively across the membrane wall (Reiken *et al.*,1990; Reiken&Briedis,1990).

Due to difficulties in determining mass transfer constants for the more complex models that best depict actual systems, it is common practice to make certain simplifying assumptions concerning the mass transfer properties of a particular system (McDuffie,1991). In a conventional stirred flask reactor containing non-immobilised enzyme and a defined mixed substrate solution with negligible molecular concentration gradients, substrate transport from the bulk solution to the surface of the biocatalyst occurs by external diffusion. This is usually a function of the stirring or linear flow rate within the reactor (Swaisgood,1985). Since external diffusion limitations are relatively easy to minimise with stirred reactors, substrate diffusion to the biocatalyst can, for all practical purposes, be assumed to be equal for all substrates present. In terms of phenol removal using polyphenol oxidase, the removal efficiency of the enzyme would, therefore, be limited by the substrate affinity (K_m) of the enzyme for the substrates in question (Clark&Switzer,1977; Bohinski,1987). In a membrane reactor, however, it is necessary to take into account the permeability or diffusion of the substrates across the membrane. The overall contact of the mixed substrate solution with the biocatalyst is, therefore, intrinsically affected by diffusion limitations imposed by the membrane.

2.2. RESEARCH OBJECTIVES

The design of a capillary membrane reactor was imperative for the initial feasibility studies on the potential use of immobilised polyphenol oxidase. A comparison between non-immobilised and immobilised polyphenol oxidase could then be determined on the basis of substrate conversion as a function of enzyme-substrate contact. For this comparison, it was necessary to evaluate any operating constraints associated with the capillary membrane bioreactor. This was important in view of the fact

that the literature reviewed stressed the association of mass transfer constraints and diffusion limitations with the operation of capillary membrane reactors. Clearly a comparative evaluation was required to determine the extent of these constraints and the role of diffusion in substrate-catalyst contact.

The following experimental objectives were identified:

1. To determine the extent of concentration-dependence on the substrate conversion efficiency for non-immobilised polyphenol oxidase.

2. To design and evaluate a capillary membrane reactor in terms of operating characteristics and catalytic potential.

3. To confirm any role that substrate permeability may play on the substrate conversion capacity of the capillary membrane-immobilised polyphenol oxidase.

2.3. MATERIALS AND METHODS

Defined synthetic substrates consisting of a mixture of phenol, *p*-cresol, 4-methoxyphenol, and 4-chlorophenol were made up using Milli-Q® water to concentrations of 1mM, 2.5mM, and 5mM (table 2.1). Phenol and *p*-cresol were purchased from BDH Laboratory Supplies (Poole, U.K.). 4-Methoxyphenol and 4-chlorophenol were obtained from Aldrich Chemical Company (Milwaukee, U.S.A). The pH of the defined mixed substrate solutions was corrected to 6.80 with 0.1M NaOH. Bovine serum albumin (BSA, Fraction V) was obtained from Boehringer-Mannheim (Mannheim, Germany). All other reagents were of analytical grade and obtained from local suppliers.

2.3.1. Enzyme activity assay using L-β-3,4-dihydroxyphenylalanine (L-DOPA) as substrate

Mushroom polyphenol oxidase (E.C. 1.14.18.1) was obtained from Sigma Chemicals (St. Louis, MO, U.S.A.) with a specific activity of 2400 Units.mg^{-1} (activity determined by supplier using tyrosine as the substrate). Enzyme activity was calibrated against that reported by the manufacturer (i.e. normalised with respect to the manufacturer's units). One of these units is equivalent to 0.325 Units when using L-β-3,4-dihydroxyphenylalanine (L-DOPA) as the substrate. The assay (Burton *et al.*,1993), is a modification of a previously described method by Gardner and Cadman (1990) and involves the addition of 100μL enzyme solution to 10mM L-DOPA (3mL) in phosphate buffer (50mM; pH 6.0) and monitoring the production of dopachrome. One unit of enzyme activity can therefore be defined as the amount of enzyme catalysing the formation of dopachrome from L-DOPA at a rate of 1μmole.min^{-1} where the extinction coefficient ($\in$) is 3600 M^{-1}cm^{-1} under these conditions.

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Table 2.1 Substrate composition for immobilised and non-immobilised experiments.

Compound	Concentration	Molecular Weight	g.L^{-1}
phenol		94.11	0.094
p-cresol	1mM	108.14	0.108
4-methoxyphenol		124.14	0.124
4-chlorophenol		128.56	0.128
phenol		94.11	0.235
p-cresol	2.5mM	108.14	0.270
4-methoxyphenol		124.14	0.310
4-chlorophenol		128.56	0.321
phenol		94.11	0.470
p-cresol	5mM	108.14	0.540
4-methoxyphenol		124.14	0.620
4-chlorophenol		128.56	0.642

2.3.2. Poly(ether sulphone) ultrafiltration membranes

Anisotropic internally-skinned poly(ether sulphone) capillary membranes (figure 2.1) were obtained from the Institute for Polymer Science (Stellenbosch University, South Africa). Membranes were manufactured using the wet-phase inversion process (Fane,1986; Cheryan, 1986; Jacobs&Leukes,1996) according to the fabrication protocol of Jacobs *et al.* (1997). The membrane formulation and coagulation conditions that were specific for this fabrication protocol (table 2.2) produced membranes characterised by a thick, dense ultrafiltration skin, irregular non-continuous macrovoids in the outer annular macrovoid region (figure 2.2), and an external surface skin surrounding the annular macrovoid region (figure 2.3). The molecular weight cut-off (MWCO) of the membranes was *ca.* 22 kDa (Jacobs, pers. comm.). Although slight perturbations during the manufacture of the membranes may have been evident, the membranes were generally consistent in terms of operating variables. These membranes were assigned a specific code (IPS 748) to designate the fabrication protocol used in the membrane manufacturing process.

Table 2.2 Membrane formulation and coagulation conditions for poly(ether sulphone) IPS 748 membrane manufacture (Jacobs *et al.*,1997; WRC,1997).

Component	
Poly(ether sulphone)	26 %
Solvent	51 %
Non-solvent additive	2 %
Low-molecular mass polymer additive	11 %
High-molecular mass polymer additive	10 %
Aqueous external coagulant	80 % solvent
Pre-rinsing condition	short air-gap
Final rinse medium	water
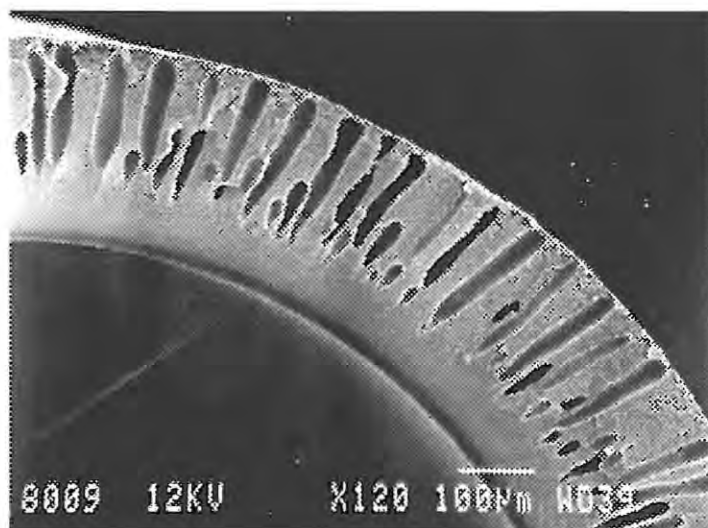
Figure 2.2 Scanning electron micrograph of a cross-section through an IPS 748 capillary membrane (magnification 120x) showing the thick, dense ultrafiltration skin layer and non-continuous macrovoids in the annular spongy layer.
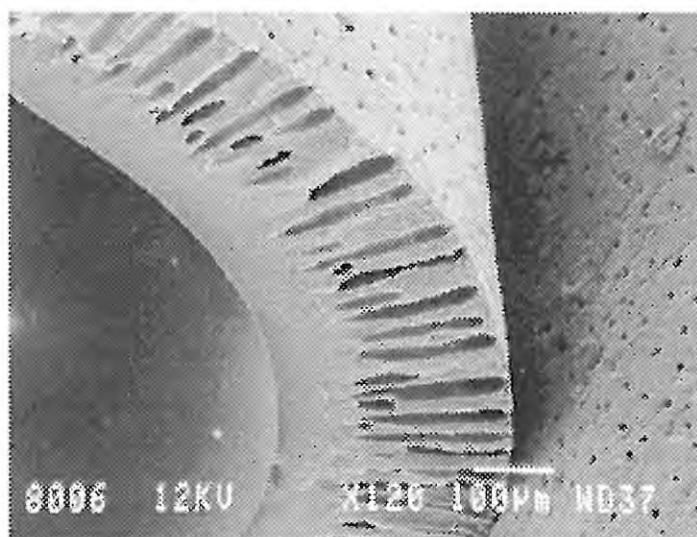
Figure 2.3 Scanning electron micrograph of a cross-section through an IPS 748 capillary membrane (magnification 120x) showing the external surface skin surrounding the annular macrovoid region.

2.3.3. Scanning electron microscopy of IPS 748 capillary membranes

Visualisation of the IPS 748 capillary membrane morphological structure was achieved using a JEOL JSM-840 scanning electron microscope (JEOL Ltd, Tokyo, Japan). Scanning electron microscopy (SEM) proved necessary to confirm that the capillary membranes displayed the morphological characteristics associated with the fabrication protocol of Jacobs *et al.* (1997). Furthermore, specific batches of membranes that were received from the Institute for Polymer Science could be periodically checked for defects or other fabrication artifacts which could result in any compromise in membrane integrity that could detract from proper functioning.

2.3.4. Membrane preparation for SEM

Membrane deformation during the preparation of the capillary membranes for SEM was prevented by obtaining membrane sections using a procedure involving a modified cryo-fracture technique. This involved immersing the capillary membranes in liquid nitrogen followed by pressure-induced fracture of the frozen samples. This prevented morphological deformation of the membrane sections previously shown to occur when preparing the sections by cutting with a surgical blade. After cryo-fracture, the membrane sections were prepared further for SEM using the sputter-coating technique as outlined in Appendix I.

2.3.5. Non-immobilised enzymatic removal of phenols from a defined mixed substrate solution

All experiments with non-immobilised polyphenol oxidase were conducted in 250mL Erlenmeyer flasks under stirred conditions at 25°C. 50mL of substrate (table 2.1) was aerated for 1 hour prior to inoculation with 171 Units of polyphenol oxidase (section 2.3.1). Samples were removed at specified time intervals, placed in Eppendorf® tubes and immediately immersed in a boiling water bath for 10 minutes to inactivate the enzyme and prevent further reaction. These were then stored at -20°C until they were analysed.

2.3.6. Substrate utilisation determination

In both immobilised and non-immobilised experiments using polyphenol oxidase, the substrate removal capacity of the enzyme is represented as the amount of substrate converted as opposed to the amount of product formed. The reason for this representation is that the o-quinone product of the reaction is unstable in aqueous solution and reacts to form oligomeric and polymeric phenols upon extended incubation. For short-term incubations (15 minutes and less) the primary product is likely to be o-quinone and, therefore, the short-term product concentration or quinone equivalent can be estimated using spectrophotometry at 390nm. Using this method, Sun and Payne (1996), using phenol as the substrate, calculated a molar extinction coefficient for the quinone equivalent of 1320 $M^{-1}cm^{-1}$ at 390nm. To determine product concentration under conditions of longer term incubation, reaction times of 24-36 hours are necessary for accurate product level estimations, because of the oligomerisation and polymerisation reactions that o-quinones undergo during longer term incubations. Under these conditions, an extinction coefficient of 2150 $M^{-1}cm^{-1}$ for phenol has been reported (Sun&Payne,1996). Two limitations, therefore, exist for representing phenol removal capacity as a function of product formation. Firstly, levels of quinone intermediate cannot be accurately determined at the intermediate stages between short- and long-term incubations. Secondly, quinone intermediates from phenol and associated derivatives cannot easily be distinguished from each other in a substrate mixture. Due to these constraints in determining levels of polyphenol oxidase-generated products, substrate depletion was determined using High Performance Liquid Chromatography (HPLC).

2.3.7. HPLC of samples

All analyses were conducted in triplicate. Reported results are expressed as mean values. Samples were analysed by HPLC (Beckman Instruments, San Ramon, U.S.A.) using a reverse-phase Nucleosil 5 μm C$_{18}$ (250 x 4.6mm i.d.) column (Machery-Nagel, Düren, Germany). The mobile-phase consisted of

water/acetonitrile (6:4; v/v) with a flow rate of 1.0 mL.min^{-1}. Peaks were detected using a Beckman 168 diode array detector at 270nm and analysed using Beckman System Gold® software version 6.0.

2.4. CAPILLARY MEMBRANE-IMMOBILISED POLYPHENOL OXIDASE

2.4.1. Capillary membrane bioreactor construction

Single-capillary reactors (SCR) were used for the initial feasibility studies with immobilised polyphenol oxidase because of convenience in terms of disposability, simplicity, and the small amounts of biochemicals required for the bioconversion tests (Reiken&Breidis,1990; Reiken *et al.*,1990). The SCR consists of a single capillary membrane in a shell-and-tube configuration (figure 2.4). The single capillary membrane was encased in a borosilicate glass shell 110mm long with an outer diameter of 7mm and inlet and outlet tubes of 3mm outer diameter. The total effective membrane area for the SCR was 4.838 cm^2. The SCR was assembled by threading the capillary membrane into the glass shell and applying a sealant consisting of a mixture of an epoxy resin (Henkel Chemical Company, S.A.) to either side of the glass shell. The epoxy resin was cured for 48 hours prior to using the SCRs. The SCR used was assumed to be representative of the characteristics of other poly(ether sulphone) membranes used in subsequent studies, although the characteristics of individual poly(ether sulphone) membranes may have varied slightly due to perturbations in the manufacturing process.
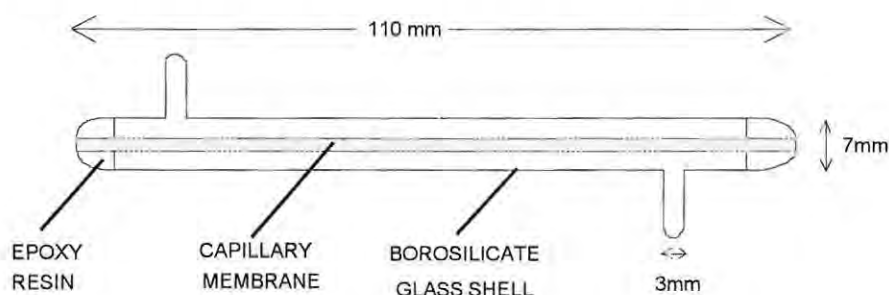
Figure 2.4 Single-capillary reactor (SCR) showing the dimensions of the shell-in-tube configuration.

2.4.2. Reactor operation

The SCR was installed in a laboratory reactor system (figure 2.5) with all hydraulic delivery elements of the reactor system consisting of Norprene® tubing (Cole-Parmer, Instr. Co., Chicago, IL, U.S.A.) to prevent substrate absorption evident in tubing such as silicone. Fluid-flow was facilitated using a Watson-Marlow peristaltic pump (Aeromix, S.A.) and pressure monitored using a standard 0-250 kPa pressure gauge (Wika Instruments, S.A.). Both the substrate reservoir and the reactor were held at

constant temperature (25°C) in a temperature-regulated room. The capillary membrane bioreactor was operated in a semi-continuous mode similar to that of Prenosil and Hediger (1988) and only the substrate solution was circulated, i.e. the permeate was not recycled back into the feed solution. In terms of mass transfer, the SCR was operated using a convective mode of transport and the "blank" solution on the shell-side was static as opposed to being circulated.
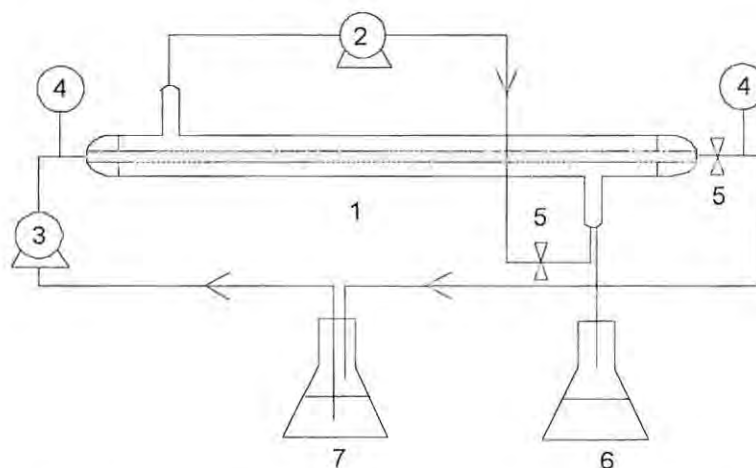
Figure 2.5 Capillary reactor system configuration: 1) capillary module; 2) inoculation peristaltic pump; 3) substrate feed peristaltic pump; 4) pressure gauge; 5) regulator clamps; 6) permeate solution; 7) feed solution.

The operating conditions of the capillary membrane bioreactor are summarised in Table 2.3.

Table 2.3 Capillary reactor system operational characteristics.

Membrane area (cm^2)	Operating pressure (kPa)	Flux (L.m^{-2}.h^{-1})	Flow volume (L.h^{-1})	Flow rate (m.s^{-1})
4.838	50	6.366	20.4	36.807

2.4.3. Determination of substrate conversion during reactor operation

To measure the "effective" concentrations of the substrates in the shell-side of the reactor, control reactions were carried out without any immobilised enzyme present. Prior to operation, the shell-side of the SCR was filled with 0.1M K$_2$HPO$_4$/KH$_2$PO$_4$ buffer (pH 6.80). The respective substrate solution was then supplied, according to operating conditions (section 2.4.2), to the lumen. As a consequence, the permeate from the perfusing substrate solution effectively displaced the phosphate buffer solution

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from the shell-side of the SCR. This permeate was then collected at regular time intervals for analysis by HPLC; the composition of the permeate was thus a mixture of the perfusing substrate solution and the buffer it was displacing. The effective substrate concentration, therefore, increased until a steady-state concentration was reached. This occurred once mixing was constant and all the residual buffer had been displaced.

To measure the enzyme-catalysed substrate conversion, the same procedure as outlined above was followed using SCRs in which the enzyme had been immobilised as described below (section 2.4.4). The substrate concentration in the permeate was determined using HPLC. The difference in permeate substrate concentration between the control experiments and the immobilised enzyme experiments, measured at equivalent time intervals, was quantitatively indicative of the amount of substrate that had been enzymatically converted.

2.4.4. Immobilisation of polyphenol oxidase onto capillary membranes

The capillary membrane in the SCR was subjected to an initial pre-treatment prior to immobilisation according to the following procedure: the membranes were initially flushed prior to use with Milli-Q® water to remove any residual storage solutions. In the case of these membranes, the capillary membranes were stored according to the fabrication protocol of Jacobs *et al.* (1997) in 50% glycerol:water solution. The membranes were then pre-cleaned using 0.1M NaOH for *ca.* 1 hour. A second wash with Milli-Q® water was followed by a conditioning rinse with 0.1M phosphate buffer (pH 6.80) for 1 hour to equilibrate the membrane to the appropriate buffer conditions. The pre-treated membranes were then used as the support for the enzyme immobilisation procedure. Commercial polyphenol oxidase (section 2.3.1) was re-suspended in 0.1M phosphate buffer (pH 6.80) and 1.0mL aliquots of the enzyme suspension, equivalent to 171 Units, were stored at -20°C until required. A solution containing 171 Units (section 2.3.1) of the enzyme was immobilised by cross-flow circulation (figure 2.5) in 0.1M phosphate buffer (pH 6.80) on the shell-side of the membranes for 1 hour at 25°C using a small peristaltic pump (Tokyo Rikakikai Co. Ltd., Japan).

2.4.5. Determination of membrane protein-loading capacity using Bradford's reagent

The extent of enzyme immobilisation was determined during inoculation to quantify the protein loading capacity of the membrane. Samples were removed at specified time intervals during the immobilisation procedure (section 2.4.4) and assayed for protein content, as outlined in Appendix II, according to the

method of Bradford (1976). After the immobilisation was complete, the reactor was run with $0.1M$ phosphate buffer through the lumen under standard operating conditions (section 2.4.2). Permeate samples were taken at specific intervals and also assayed for protein. This was done to determine any enzyme wash-out from the annular sponge layer after inoculation and during reactor operation. The method of Ko *et al.* (1994), using total hydrolysis for the determination of total adsorbed protein, was considered as an alternative method for determining the amount of immobilised polyphenol oxidase. However, hydrolysis using HCl would result in destruction of the capillary membranes, leading to the presence of artifacts which were shown to influence the ninhydrin step of the method, thus decreasing the sensitivity of the assay method.

2.4.6. Substrate permeability and diffusivity determination

According to Reiken *et al.* (1990), permeability and diffusivity determination for certain substrate species in a feed solution can be calculated by following the concentration changes between the lumen and the shell-side of a capillary membrane, operated in dialysis mode, with buffer solution being circulated on the shell-side to collect the diffusing species. Using a modification of this method, the permeability of phenol, *p*-cresol, 4-methoxyphenol, and 4-chlorophenol in a defined mixed substrate feed solution was determined by driving the diffusion of the substrate species convectively across the capillary-wall and determining the species concentration in a static solution on the reactor shell-side. The advantage of this modification for membrane permeability determination is that after a 4-hour period the overall shell-side substrate concentration for all diffusing species reaches a steady-state. Solute concentration on the shell-side was therefore unaffected by any influence that a dilution effect might cause if the buffer solution on the shell-side had been circulated. The overall membrane permeability (K_0) of the transported species could then be calculated from the material balances between the tube- and shell-side of the capillary membrane reactor according to equation 1.

$$- \ln \left(\frac{m - (V_t + V_s) S_s}{V_t (S_{t0} - S_{s0})} \right) = \frac{K_0 A}{V_t V_s} (V_t + V_s) t \tag{1}$$

V_t and V_s are the solute solution volumes in the tube- and shell-sides respectively; S_t and S_s are the concentrations of solute in the tube- and shell-sides; S_{t0} and S_{s0} are the initial concentrations of solute in the tube- and shell-sides; $m = S_t V_t + S_s V_s$; A is the mass transfer area of the membrane and t is the time of sample. These are all known or measured quantities and therefore the term $\{-\ln[(m-(V_t+V_s)S_s \,/\, V_t (S_{t0}-S_{s0}))]\}$ may be plotted versus time. The slope of the plot is equal to $K_0 A (V_t + V_s) \,/\, V_t V_s$ as shown in

equation (1). The overall membrane permeabilities of the transported species may then be calculated directly.

According to Reiken *et al.* (1990), the effective diffusivities (D_{eff}) of the individual species can be calculated from the overall membrane permeability using the wet membrane thickness as the diffusion path length (refer to equation 2).

$$D_{eff} = K_0 \text{ x wet membrane thickness} \qquad (2)$$

A method for accurate determination of the wet membrane thickness for calculating the overall diffusivities of the transported species was unavailable. However, membrane thickness can be accurately determined with the aid of scanning electron micrographs of the capillaries (figure 2.2). Due to the method of preparation of membrane samples for SEM, the determined membrane thickness is only representative of the dry or non-wetted membrane and does not take into account the marginal swelling characteristically seen when a capillary membrane is subjected to any wetting procedure. For the purpose of calculating solute diffusivity it is, therefore, necessary to assume that the marginal increase in membrane thickness is negligible. The calculated capillary membrane thickness was measured as 58.3μm.

2.5. RESULTS

2.5.1. Substrate conversion using non-immobilised polyphenol oxidase

Initial experiments using polyphenol oxidase were conducted under non-immobilised stirred-flask batch conditions (section 2.3.5). Figures 2.6, 2.7, and 2.8 depict non-immobilised enzyme substrate conversion at initial substrate concentrations of 1, 2.5, and 5mM respectively. It was found in these preliminary experiments that a trend in interaction effects developed between the biocatalyst and the individual substrates. At equal concentrations and under well-mixed, stirred conditions, individual species are assumed to experience negligible concentration gradients (section 2.1.1). Mass transfer constraints between catalyst and each individual species should, therefore, be negligible. According to Passi and Nazzaro-Porro (1981), the greater the electron donor nature of the R-group, the more effective certain *p*-monophenols act as substrates for polyphenol oxidase. Therefore, arranged in decreasing order according to the electron donor nature of the R-group (although phenol has been included, the absence of a R-group should be noted):

4-methoxyphenol > *p*-cresol > phenol

Generally, the presence of an electron-acceptor R-group denotes a substrate as a competitive inhibitor of polyphenol oxidase; however, 4-chorophenol, of which the halogen is of modest electron acceptor nature, is still oxidised by the enzyme. The observed effect (figures 2.6, 2.7, and 2.8), in terms of enzymatic conversion, supports this trend with 4-chlorophenol falling between *p*-cresol and phenol. Over the concentration range tested (1-5mM), the order of conversion of substrate species to the corresponding *o*-quinone appears to be a function of the electron donor nature of the R-group. Although each individual substrate in the defined mixture is at either 1, 2.5, or 5mM, the *total* concentration of phenols is 4, 10, and 20mM respectively, due to the presence of four substrates, in equal concentrations, per mixture.
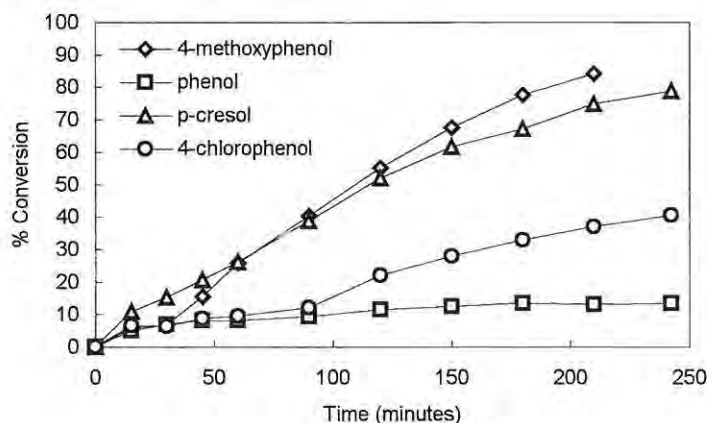
Figure 2.6 Substrate conversion using non-immobilised polyphenol oxidase under stirred conditions at an initial individual substrate concentration of 1mM.
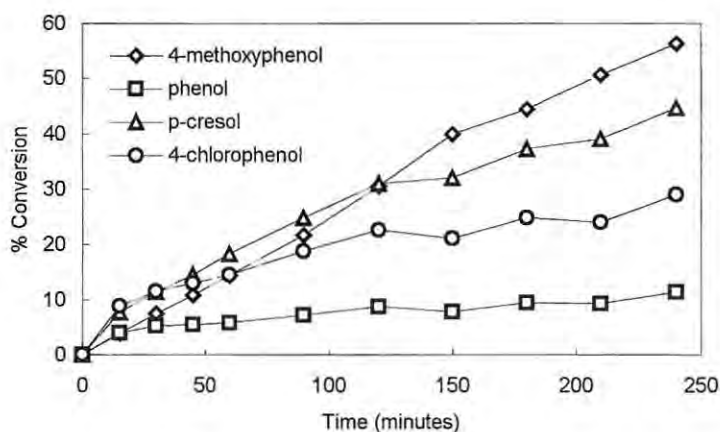
Figure 2.7 Substrate conversion using non-immobilised polyphenol oxidase under stirred conditions at an initial individual substrate concentration of 2.5m*M*.
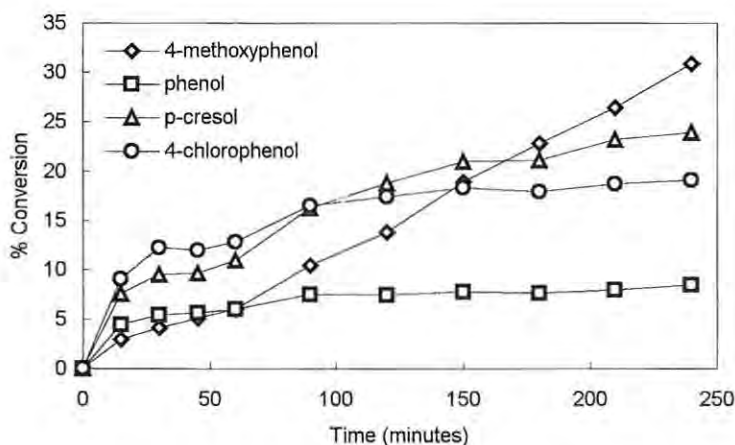
Figure 2.8 Substrate conversion using non-immobilised polyphenol oxidase under stirred conditions at an initial individual substrate concentration of 5m*M*.

Table 2.4 summarises the amount of substrate converted after four hours using non-immobilised polyphenol oxidase. Although the reaction volumes involved were 50mL batches, values are represented as the amount of substrate converted per litre. Additionally, results also indicate the amount of substrate converted per 50mL batch volume. Since 171 Units (section 2.3.5) of polyphenol oxidase was used for each batch experiment, an effective comparison can be made by considering the amount of substrate converted per Unit of enzyme.

Maintaining a constant amount of enzyme for each individual substrate concentration (i.e. 1, 2.5, and 5mM) allowed comparisons to be made for the determination of whether enzyme activity displayed any trends indicating a concentration-dependence. From the calculated values obtained for the amount of substrate converted as a function of volume (mg.L^{-1}), and those obtained as a function of the amount of enzyme (μg.Unit^{-1}), the total amount of converted substrate for the different initial concentrations was plotted to ascertain whether activity varied linearly with respect to concentration. Figure 2.9 indicates that a concentration-dependent effect is evident. As initial substrate concentration was increased, an inverse relationship developed where no further increase in enzyme activity was observed. Thus, a linear trend was not evident, indicating that substrate saturation was occurring.

Table 2.4 Substrate conversion by non-immobilised polyphenol oxidase in 50mL reaction volumes under stirred conditions (section 2.3.5).

	Initial substrate concentration (mM)	4-methoxyphenol	phenol	p-cresol	4-chlorophenol
Substrate converted (mg.L^{-1})	1	133.0	13.0	91.8	64.0
	2.5	183.4	27.3	120.7	108.85
	5	192.9	39.2	127.4	138.7
Substrate converted (mg)	1	6.65	0.65	4.59	3.20
	2.5	9.17	1.37	6.04	5.44
	5	9.65	1.96	6.37	6.93
Substrate converted per unit enzyme (μg.Unit^{-1})	1	38.75	3.78	26.74	18.65
	2.5	53.43	7.95	35.17	31.72
	5	56.20	11.42	37.12	40.41
Substrate converted (mM)	1	1.07	0.14	0.85	0.50
	2.5	1.48	0.29	1.12	0.85
	5	1.55	0.42	1.19	1.08
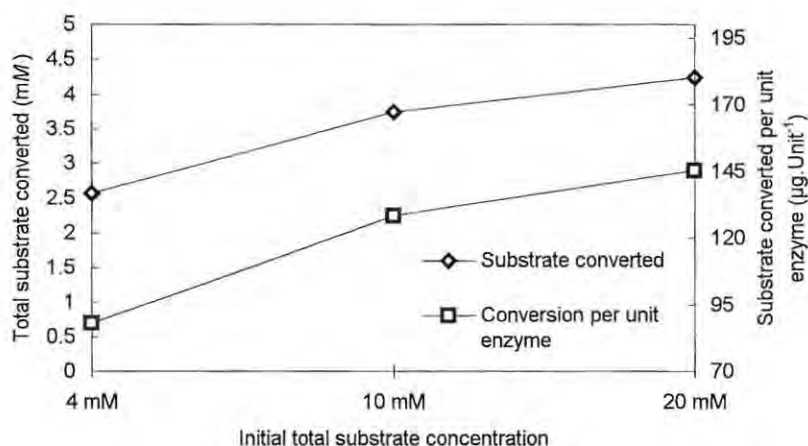
Figure 2.9 Trend indicating the decrease in the amount of substrate conversion as a function of increasing initial substrate concentration for non-immobilised polyphenol oxidase.

The total amount of defined mixed substrate converted per Unit of enzyme at an initial substrate concentration of 4mM after 4 hours was 87.92 µg.Unit^{-1} and at 10mM, 128.27 µg.Unit^{-1}. If the activity of non-immobilised polyphenol oxidase was not affected by the substrate concentration, i.e. not concentration-dependent, a linear increase in projected substrate conversion to *ca.*170 µg.Unit^{-1} would be expected when the initial substrate concentration was 20mM. However, the results obtained confirmed a substrate conversion of only 145.15 µg.Unit^{-1} after a 4-hour period. Clearly, under non-immobilised conditions, as the initial substrate concentration is increased, so the conversion efficiency of polyphenol oxidase becomes limited due to this apparent substrate saturation. In addition, when non-immobilised polyphenol oxidase is subjected to high initial substrate concentrations, it can be presumed that the resultant inhibitory enzyme-generated product concentration also increases. This effectively translates to a decrease in the conversion efficiency of the enzyme with time.

To decrease the product inhibition effect, product removal, or at least product separation, would be necessary. It was envisaged that this could be achieved by immobilising the enzyme onto capillary membranes. However, in order to determine whether any concentration-dependent phenomena occurred with polyphenol oxidase in an immobilised state, it was first necessary to determine the existence of any diffusional constraints imposed by the capillary membrane immobilisation matrix on enzyme-substrate contact.

2.5.2. Permeability and diffusivity determination of substrate species

Single-capillary reactors were constructed and operated as previously described (section 2.4.1 and 2.4.2). All substrate permeability experiments were conducted without the presence of immobilised polyphenol oxidase. To determine the overall membrane permeabilities and diffusivities of the transported species according to the equations described in section 2.4.6, it was necessary to determine quantitatively the amount of perfusing substrate in the shell-side of the SCR using HPLC. Figures 2.10, 2.11, and 2.12 show the increase in substrate concentration on the shell-side of the membranes as a function of time for initial individual substrate concentrations of 1, 2.5, and 5mM respectively. As described in section 2.5.1, the initial substrate concentrations relate to each individual substrate species in a mixture. Effectively, then, initial total substrate concentrations were in the order of 4, 10, and 20mM.

As described in section 2.4.3, during reactor operation the perfusing substrate effectively displaces the phosphate buffer solution from the shell-side of the SCR. As a result, the substrate concentration within the shell-side increases relative to the displaced buffer until a steady-state concentration is reached.
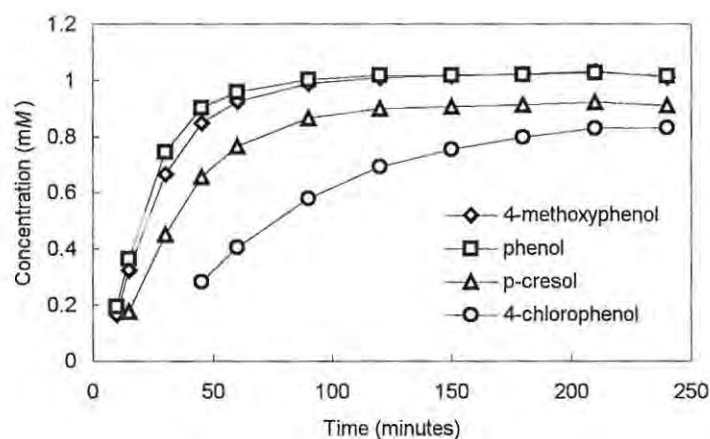
Figure 2.10 Perfusing substrate species determination for 4-methoxyphenol, phenol, *p*-cresol, and 4-chlorophenol across an IPS 748 poly(ether sulphone) capillary membrane. The individual substrate feed concentration was 1mM.

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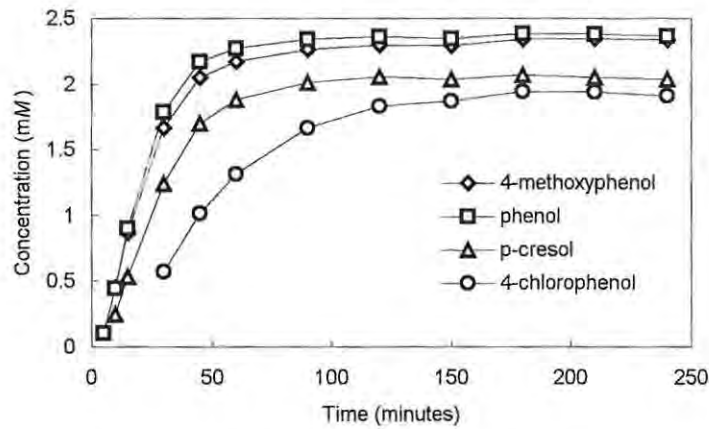
Figure 2.11 Perfusing substrate species determination for 4-methoxyphenol, phenol, *p*-cresol, and 4-chlorophenol across an IPS 748 poly(ether sulphone) capillary membrane. The individual substrate feed concentration was 2.5mM.
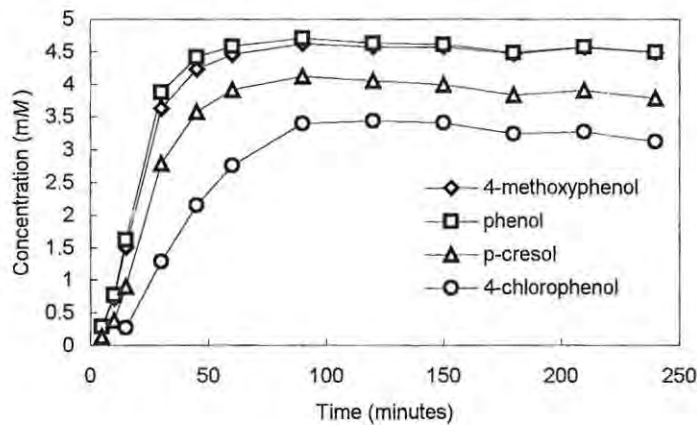
Figure 2.12 Perfusing substrate species determination for 4-methoxyphenol, phenol, *p*-cresol, and 4-chlorophenol across an IPS 748 poly(ether sulphone) capillary membrane. The individual substrate feed concentration was 5mM.

This results in the hyperbolic appearance of the curves as indicated in Figures 2.10, 2.11, and 2.12. From the individual substrate concentrations, S_s could be determined (equation 1, section 2.4.6). From the curves generated, it was observed that not all substrate species attain the same shell-side steady-state concentration although the initial feed concentrations were equal. The total species concentration in the permeate, once steady-state was achieved, should theoretically be 4, 10, and 20mM, depending on

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initial feed concentrations. From the results obtained it is evident that this does not occur and that the observed concentration is less than it theoretically should be. This was attributed to the fact that although the individual solute species in the circulating feed are not retained by the membrane (because the solute molecular mass is less than the molecular weight cut-off of the membrane), the solute tends to accumulate at the membrane surface during the filtration process. Thus, the concentration of solute at the wall is considerably higher than the bulk concentration. This increase in solute concentration at the membrane wall during the ultrafiltration process is termed concentration polarisation (Nilsson,1990; Jönsson&Trägårdh,1990; Mulder,1991). The dynamic nature of this layer, however, means that a concentration gradient exists across the membrane wall resulting in the permeate having a lower solute concentration than is theoretically possible.

Once S_s was known, it was possible to determine the overall membrane permeability as outlined in section 2.4.6. To calculate the overall membrane permeability (K_0), it was necessary to determine the slope of the plot of $\{-\ln[(m-V_t+V_s)S_s)/(V_t(S_{t0}-S_{s0}))]\}$ versus time. Figures 2.13, 2.14, and 2.15 are indicative of the plots obtained at initial individual substrate concentrations of 1, 2.5, and 5mM respectively. Due to the mode of operation of the SCR configuration (section 2.4.6), i.e. diffusing species were not collected using circulating buffer, the generated curves were hyperbolic in nature. As a result the slopes for each of the perfusing substrate species were determined from the initial linear sections of each curve. Slopes are reported individually for each species in the figure legends.

According to equation 1 (section 2.4.6), the slopes of the curves are equal to $K_0A(V_t+V_s)/V_tV_s)$, thus, K_0 could be determined. Since the effective diffusivity (D_{eff}) is a function of the overall membrane permeability, the wet membrane thickness, D_{eff} could be calculated directly (equation 2, section 2.4.6). The results for K_0 and D_{eff}, for each of the individual species, are shown in Table 2.5. From the results obtained it was observed that the order of permeability for the individual species at 1mM, 2.5mM, and 5mM was:

phenol > 4-methoxyphenol > p-cresol > 4-chlorophenol
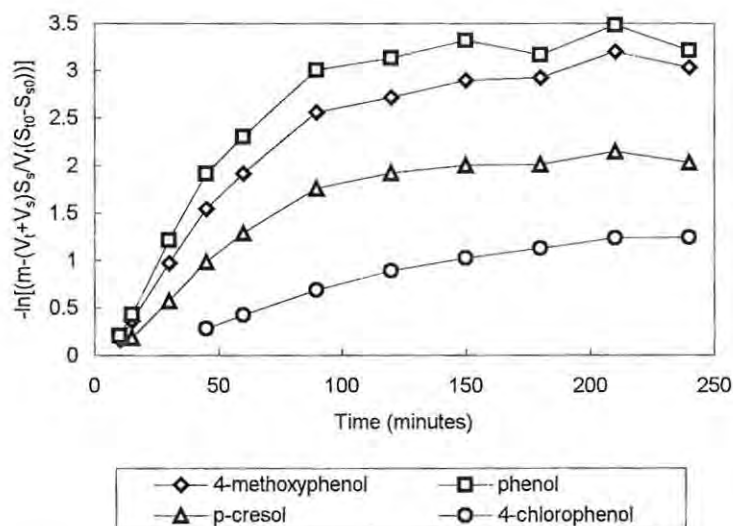
Figure 2.13 Plot of $\{-\ln[(m-(V_t+V_s)S_s / V_t (S_{t0}-S_{s0}))]\}$ versus time at a substrate feed concentration of $1mM$ (refer to section 2.4.9). Initial slope values calculated from the generated curves for the individual substrates are: 4-methoxyphenol $0.039\pm2\times10^{-4}$; phenol $0.048\pm6\times10^{-4}$; p-cresol $0.027\pm1\times10^{-4}$; 4-chlorophenol $0.009\pm2\times10^{-4}$.
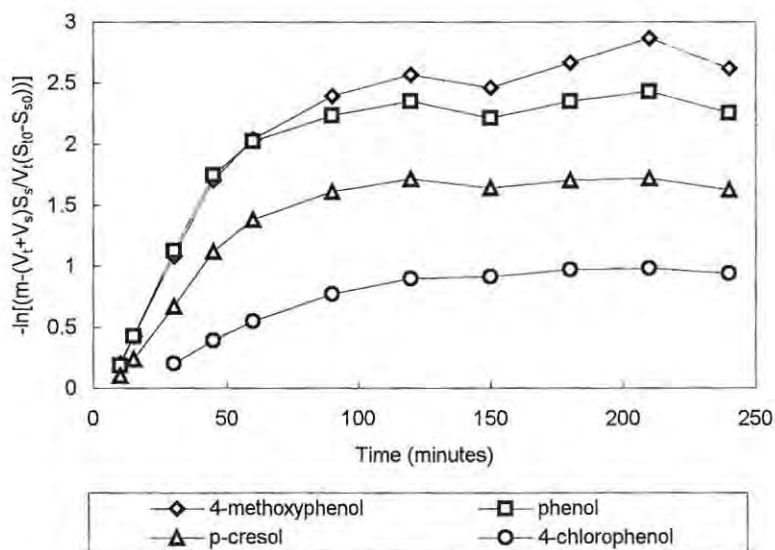
Figure 2.14 Plot of $\{-\ln[(m-(V_t+V_s)S_s / V_t (S_{t0}-S_{s0}))]\}$ versus time at a substrate feed concentration of $2.5mM$ (refer to section 2.4.9). Initial slope values calculated from the generated curves for the individual substrates are: 4-methoxyphenol $0.043\pm8\times10^{-4}$; phenol $0.045\pm8\times10^{-4}$; p-cresol $0.029\pm5\times10^{-4}$; 4-chlorophenol $0.011\pm1\times10^{-4}$.
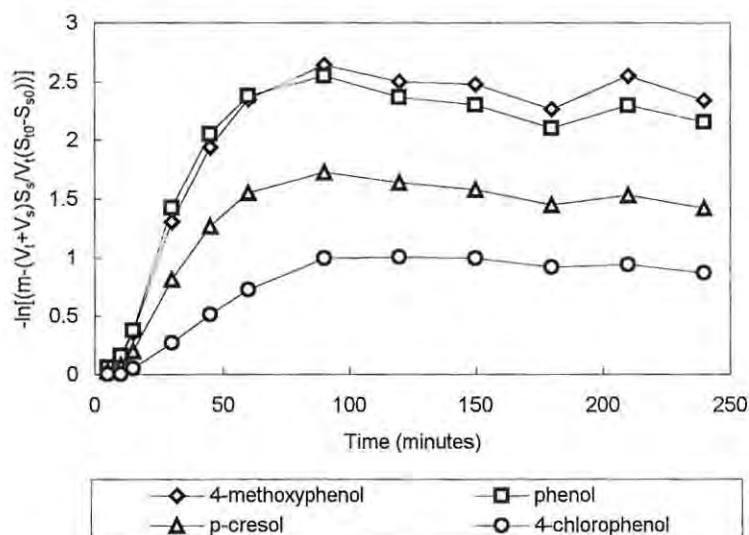
Figure 2.15 Plot of $\{-\ln[(m-(V_t+V_s)S_s / V_t (S_{t0}-S_{s0}))]\}$ versus time at a substrate feed concentration of 5mM (refer to section 2.4.9). Initial slope values calculated from the generated curves for the individual substrates are: 4-methoxyphenol $0.063\pm7\times10^{-4}$; phenol $0.070\pm4\times10^{-4}$; *p*-cresol $0.038\pm1\times10^{-4}$; 4-chlorophenol $0.015\pm1\times10^{-4}$.

From Table 2.5, it is also evident that the overall permeability and diffusivity for the individual species increased as the initial feed concentration was increased. However, as the initial feed concentration increased to 5mM, K_0 and D_{eff} were significantly lower than the K_0 and D_{eff} at initial substrate concentrations of 2.5mM.

Table 2.5 Overall membrane permeability (K_0) and diffusivity (D_{eff}) values for individual substrates at 1mM, 2.5mM, and 5mM feed concentrations. The mean and standard deviation values are indicated.

		4-methoxyphenol	phenol	*p*-cresol	4-chlorophenol
1 mM	K_0(cm.min^{-1})	1.05 ± 0.007	1.31 ± 0.016	0.71 ± 0.003	0.24 ± 0.005
	D_{eff} (cm^2.s^{-1})	$6.13 \times 10^{-3} \pm 0.007$	$7.61 \times 10^{-3} \pm 0.016$	$4.13 \times 10^{-3} \pm 0.003$	$1.37 \times 10^{-3} \pm 0.005$
2.5mM	K_0(cm.min^{-1})	1.70 ± 0.019	1.87 ± 0.011	1.01 ± 0.004	0.40 ± 0.005
	D_{eff} (cm^2.s^{-1})	$9.91 \times 10^{-3} \pm 0.019$	$1.09 \times 10^{-2} \pm 0.011$	$5.90 \times 10^{-3} \pm 0.004$	$2.33 \times 10^{-3} \pm 0.005$
5mM	K_0 (cm.min^{-1})	1.16 ± 0.023	1.21 ± 0.023	0.78 ± 0.014	0.31 ± 0.003
	D_{eff} (cm^2.s^{-1})	$6.77 \times 10^{-3} \pm 0.023$	$7.05 \times 10^{-3} \pm 0.023$	$4.57 \times 10^{-3} \pm 0.014$	$1.78 \times 10^{-3} \pm 0.003$

Table 2.6 Species diffusion and permeability comparison using a one-way ANOVA and employing the Bonferroni and Tukey-Kramer multiple comparisons tests. P values of < 0.05 are deemed statistically significant.

Substrate species comparison	Concentration		
	1mM	**2.5mM**	**5mM**
4-methoxyphenol *vs.* phenol	P < 0.001	P < 0.001	P < 0.05
4-methoxyphenol *vs. p*-cresol	P < 0.001	P < 0.001	P < 0.001
4-methoxyphenol *vs.* 4-chlorophenol	P < 0.001	P < 0.001	P < 0.001
phenol *vs. p*-cresol	P < 0.001	P < 0.001	P < 0.001
phenol *vs.* 4-chlorophenol	P < 0.001	P < 0.001	P < 0.001

In situations where a solute is comparable in size to the pores in a membrane, the effective diffusion coefficient of the solute is usually lower than its bulk solution value. This is known as 'hindered' or 'restricted' diffusion and occurs as a result of the hydrodynamic restrictions on the solute, due to the presence of the pore wall (Davidson&Deen,1988). For the diffusion of phenols, the solute is considerably smaller than the pore diameters. Under conditions of purely convective transport then, taking into consideration that the phenols in question are of similar molecular weight and dimension, transport through the pores should be relatively similar, if not equal, for all species. From the results obtained (table 2.5), it is apparent that all solute species are not transported across the membrane at the same rate. This was statistically validated (table 2.6) and implies that the rate at which the immobilised enzyme will effectively contact the different substrate species is largely defined by the capillary membrane and the diffusion limitations imposed by it. To explain this, it is necessary to consider the processes occurring at the membrane wall. However, a detailed description of the transport mechanisms and diffusional phenomena occurring within the membrane does not fall within the scope of this study. Briefly, the individual solute species in the circulating feed are not retained by the membrane (because the solute molecular mass is less than the molecular weight cut-off of the membrane). Under conventional filtration conditions, however, due to the feed velocity tending to zero at the membrane wall due to frictional resistance (V_{wall}), the solute will tend to accumulate at the membrane surface during the filtration process prior to crossing the capillary membrane wall (refer to figure 2.16). Thus, the concentration of solute at the wall (C_{wall}) is considerably higher than the bulk concentration (C_{bulk}).

This increase in solute concentration at the membrane wall during the ultrafiltration process is termed concentration polarisation (Nilsson,1990; Jönsson&Trägårdh,1990; Mulder,1991).
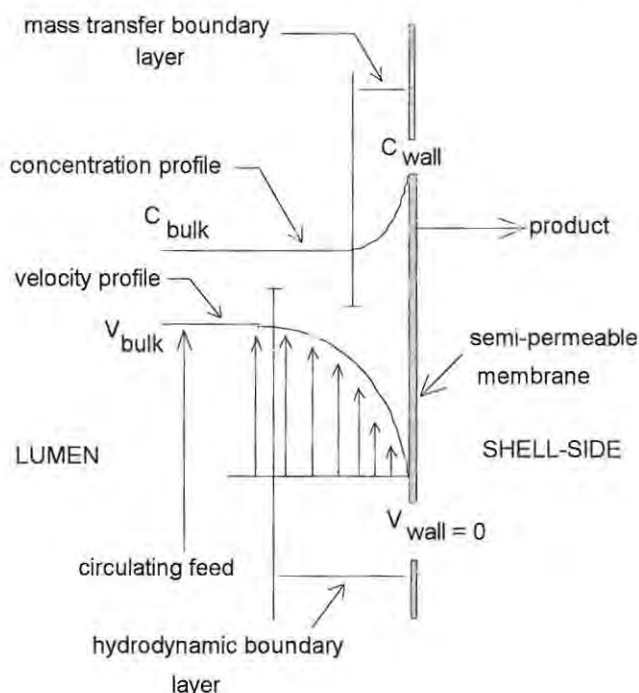
Figure 2.16 Schematic indicating the boundary layer conditions existing during laminar flow. At the mass transfer boundary layer the concentration at the membrane wall (C_{wall}) is considerably higher than the bulk fluid concentration (C_{bulk}) in the lumen. Adapted from Eykamp (1995).

During ultrafiltration, high solute concentration at the wall is compensated by the dispersion of solute molecules back into the bulk feed solution due to the flow velocity of the feed stream parallel to the membrane. The rate at which this accumulated material is dispersed back into the bulk fluid has been shown to be a function, amongst other factors, of the concentration difference between the membrane surface and the bulk feed solution (Eykamp,1995). However, these processes have been shown not to apply for laminar flow conditions (Reynolds Number < 1800) in thin capillary membranes due to virtually negligible concentration variations between the wall and bulk fluid.

Therefore, two transport processes become evident for the diffusion of phenols across a capillary membrane. Firstly, surface diffusion involves the molecules adsorbed on the pore wall diffusing on the surface due to concentration gradients in the adsorbed phase. Transport through the membrane pores is, therefore, a combination of convective flow resulting in an accumulation of substrate species at the

membrane wall, and diffusion into the pores where the solute concentration is lower (hence an apparent C_{wall} concentration that is greater than the solute concentration within the pores). However, if this were the dominant mechanism involved in the transport of phenols across the membrane, transport would be equal for all species. Secondly, at apparently higher C_{wall} concentrations, however, diffusion into the pores may become limiting due to the development of a bottleneck effect resulting in lower diffusion rates (table 2.5).
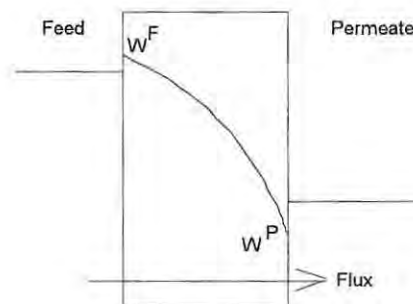
Figure 2.17 Mass transport through a membrane according to the solution-diffusion model. W^F and W^P are the weight fractions of the solute components in the membrane at the feed side and the permeate side respectively (Cen&Lichtenthaler,1995).

As a consequence, frequent contact with the wall, and thus the polymeric material, results in the solute molecules being subject to a partitioning effect where solute separation is a function of differences in the solubility of the solutes within the membrane polymer matrix (Cen&Lichtenthaler,1995), and is referred to as the solution-diffusion model (figure 2.17). Trans-membrane solute transport *via* this solution-diffusion mechanism occurs in three steps. Sorption of the solute first occurs at the membrane surface. Diffusion of the absorbed solute then occurs through the membrane matrix with subsequent desorption of the solute from the membrane matrix occurring at the permeate side. Subsequent movement of solute molecules through the polymer matrix, therefore, occurs as a cascade with the driving force being a function of high solute concentration within the polymer matrix at the lumen side (w^F) and lower concentration at the shell-side of the membrane (w^P). Variation in permeability rate is therefore a function of the solubilities of the various solute species within the membrane matrix.

Thus, the demonstration that substrate species permeate the membrane at different rates was necessary in order to make any predictions as to the enzymatic conversion of individual substrates in the permeating feed. This is discussed with relevance to substrate conversion in section 2.5.4.2.

2.5.3. The protein-loading capacity of IPS 748 capillary membranes

To determine the conversion efficiency of immobilised polyphenol oxidase it was necessary to determine the amount of enzyme immobilised on the membrane. The protein-loading capacity of the capillary membranes was determined using the method of Bradford (1976) as outlined in section 2.4.5.

2.5.3.1. Protein-loading determination using the method of Bradford (1976)

Using the method of Bradford (1976), as outlined in section 2.4.5, it was determined that 32.51% of the protein was immobilised. This was equivalent to 55.8 Units of polyphenol oxidase.

2.5.3.2. Determination of kinetic parameters for immobilised polyphenol oxidase

Although apparent Michaelis-Menten constants (K_m) and maximal reaction velocities (V_{max}) can be determined for immobilised enzymes, these apparent kinetic constants are highly dependent on the extent of external and internal mass transfer influences. For practical considerations, this information would have little meaning other than to provide an indication of the existence of transport impedance (Venkatsubramanian, 1980). As shown in section 2.5.2, transport impedance in the form of diffusional constraints imposed by the capillary membrane have a considerable effect on substrate conversion by immobilised polyphenol oxidase. Because of these support matrix effects, determining if any variation in K_m was due to the actual physical immobilisation or due to permeability and diffusion effects would be inconclusive.

2.5.4. Substrate conversion using immobilised polyphenol oxidase

Initial experiments using poly(ether sulphone) membrane-immobilised polyphenol oxidase were done using a single substrate (*p*-cresol) at different initial feed concentrations. Subsequent experiments were carried out using defined mixed synthetic substrates containing phenol, *p*-cresol, 4-methoxyphenol, and 4-chlorophenol at different initial feed concentrations.

2.5.4.1. Conversion of *p*-cresol by immobilised polyphenol oxidase

As described in section 2.4.3, determination of the 'effective' substrate conversion levels using SCRs required comparison between control reactions, with no immobilised enzyme, and the experimental

reactions with immobilised enzyme present. Figure 2.18 shows the variation in substrate concentration between control and experimental (exp.) conditions. Theoretically, if no substrate conversion were to occur, the control and experimental curves would be identical. As indicated in Figure 2.18, at an initial substrate concentration of 1mM, conversion levels of 100% were maintained for 3.5 hours, dropping to 63.10% at 4 hours. No substrate conversion is indicated within the first 60 minutes because the shell-side substrate concentration was too low for detection in the control experiments. The percentage substrate conversion has been superimposed to facilitate comparison between the control and experimental conditions.
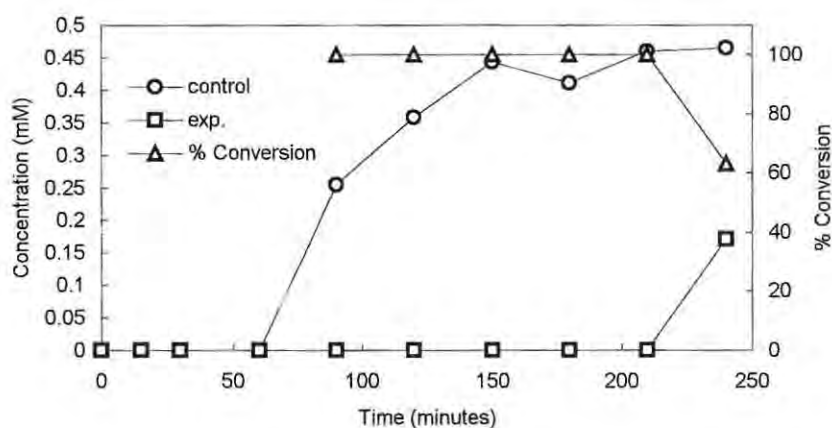
Figure 2.18 Conversion of *p*-cresol (1mM) using IPS 748 membrane-immobilised polyphenol oxidase.

Further experiments were conducted with *p*-cresol as the sole substrate at initial substrate concentrations of 2.5 and 5mM. These were conducted to ascertain whether the levels of substrate conversion were in any way affected by an increase in initial substrate concentration when polyphenol oxidase was immobilised. From Figure 2.19, it is apparent that the initial substrate concentration has less influence when the enzyme is immobilised than what was theoretically expected. The initial expectation was that as initial substrate concentration increased, this would be paralleled by a decrease in conversion efficiency due to the concomitant increase in the concentration of enzyme-generated product. As can be seen from Figure 2.19, this is not evident as 100% conversion of *p*-cresol, initially at a concentration of 2.5mM, occurs over the 4-hour reaction period. In comparison, at an initial substrate concentration of 1mM, substrate conversion drops from 100% to 63.10% between 3.5 and 4 hours after reaction initiation (figure 2.18). A decrease in enzymatic conversion was observed with an initial *p*-cresol concentration of 5mM.
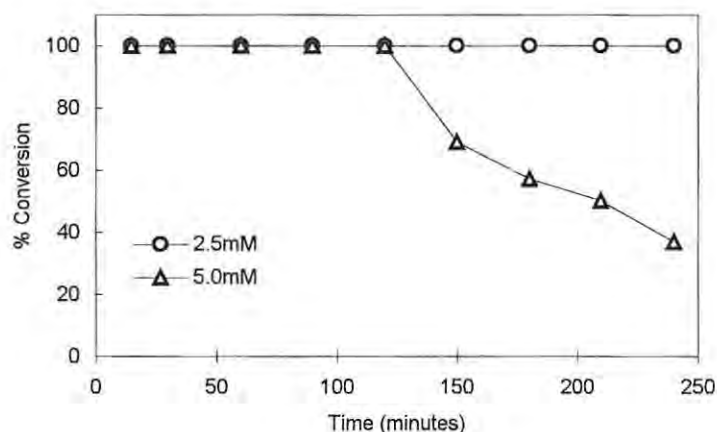
Figure 2.19 Conversion of *p*-cresol using IPS 748 membrane-immobilised polyphenol oxidase. Initial substrate concentrations are 2.5, and 5m*M*.

It is evident, therefore, that a certain correlation exists between these results and the permeabilities and diffusivities observed for the mixed feed solutions. As described in section 2.5.2, generally as the initial substrate feed concentration increases from 4 to 10m*M*, so the rate of perfusion increases up to a point. Since a feed solution containing one solute, i.e. *p*-cresol, was found not to be subject to interactions at the wall from other solute species, higher initial feed concentrations would result in greater perfusion rates. Referring back to Table 2.5, it is apparent that as the total substrate concentration is increased to 10m*M*, so the rate of diffusion increases. At higher concentrations (20m*M*) a bottleneck effect results in a decrease in the rate of diffusion. For *p*-cresol, however, initial concentrations were below 10m*M*, and, therefore, diffusivity and permeability would increase as the initial substrate concentration was increased within the concentration range tested. This appears to result in increased substrate conversion due to the more rapid enzyme-substrate contact occurring at higher concentrations. Thus, apparent substrate conversion for *p*-cresol is greater at high feed concentrations than at lower concentrations. Table 2.7 summarises the substrate conversion of *p*-cresol at various concentrations by immobilised polyphenol oxidase.

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Table 2.7 Amount of *p*-cresol converted using IPS 748 membrane-immobilised polyphenol oxidase over a 4-hour period. Flux = 3.08 mL.h^{-1}; total reaction volume = 12.32mL.

Initial feed concentration (m*M*)	Total *p*-cresol converted (mg)	*p*-cresol converted (mg.L^{-1})	*p*-cresol converted (μg.Unit^{-1})	*p*-cresol converted (m*M*)
1	0.25	20.18	4.52	0.19
2.5	0.94	76.59	17.16	0.71
5	1.57	127.54	28.56	1.18

Referring back to Figure 2.19, it appears that enzymatic substrate conversion decreases as the initial substrate concentration increases. However, quantitatively the relative *total* amounts of substrate converted are higher as the feed substrate concentration is increased (table 2.7).

2.5.4.2. Defined mixed substrate conversion by immobilised polyphenol oxidase

As discussed in section 2.5.2, the inter-relationship between enzyme-substrate contact and the enzyme-catalysed conversion of the contacted substrate is complex. Diffusion constraints affecting the convective transport of substrate species would impose considerable limitation on the order in which enzyme-substrate contact occurs. The implication of this is that the order of substrate conversion would be affected by the order in which enzyme-substrate contact occurred. Figures 2.20, 2.21, and 2.22 show the substrate conversions of the defined mixture of phenols by immobilised polyphenol oxidase, at initial substrate species concentrations of 1, 2.5, and 5m*M* each respectively. The amount of substrate converted is represented in Table 2.8.

From these results it is observed that the percentage overall conversion of *p*-cresol, over the concentration range tested, is greater than the other substrates in the defined feed mixtures. The order of substrate conversion, for initial *total* feed concentrations of 4 and 10m*M*, is:

p-cresol > 4-chlorophenol > phenol > 4-methoxyphenol
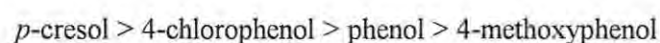
As the total feed concentration is increased to 20mM, the order of substrate conversion is:

p-cresol > phenol > 4-methoxyphenol > 4-chlorophenol
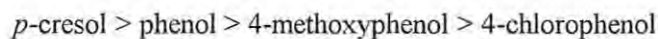
It might be expected that the order, and subsequently, the rate at which substrate molecules are converted to their respective quinone products should reflect the order in which the substrate molecules make contact with the enzyme. From the preliminary studies to determine the permeability and diffusivity of individual substrate species, this would be firstly phenol, followed by 4-methoxyphenol, p-cresol, and finally 4-chlorophenol.
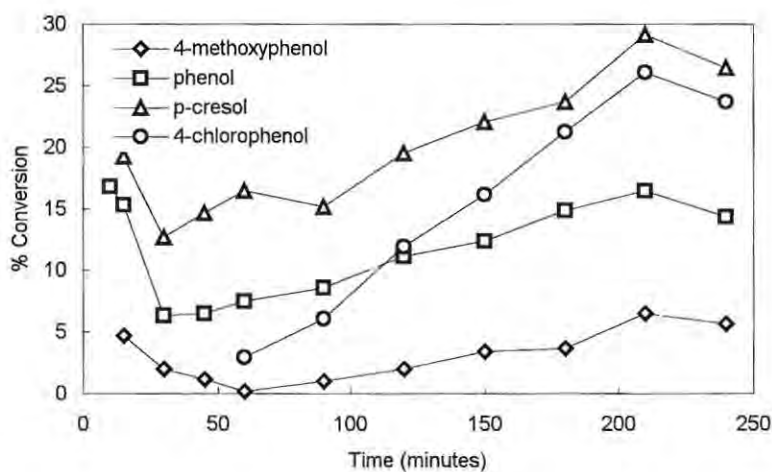
Figure 2.20 Substrate conversion using capillary membrane-immobilised polyphenol oxidase with each individual species at an initial substrate feed concentration of 1mM. Therefore, total initial substrate concentration is 4mM.
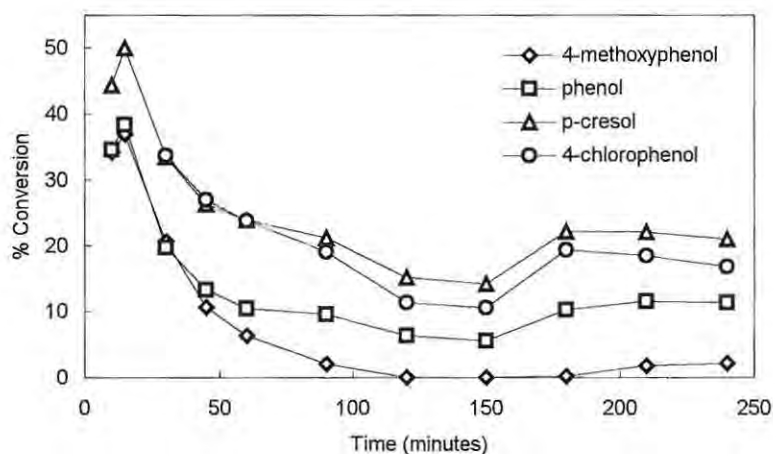
Figure 2.21 Substrate conversion using capillary membrane-immobilised polyphenol oxidase with each individual species at an initial substrate feed concentration of 2.5mM. Therefore, total initial substrate concentration is 10mM.
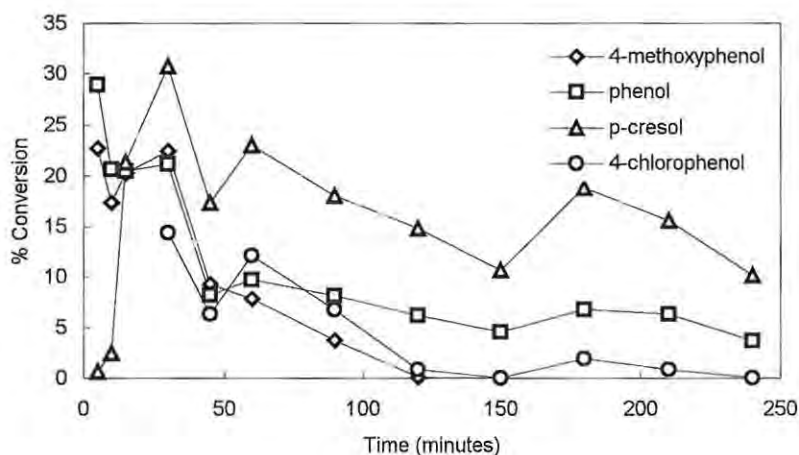
Figure 2.22 Substrate conversion using capillary membrane-immobilised polyphenol oxidase with each individual species at an initial substrate feed concentration of 5mM. Therefore, total initial substrate concentration is 20mM.

As described above, this is not the case and furthermore, the order in which the substrate species are converted appears to be variable with respect to initial substrate concentration. From these results it is apparent that the variation in the order in which substrate conversion occurs correlates with a decrease in the rate of permeability of individual species as the initial substrate concentration is increased. The shapes of the curves in Figures 2.20 and 2.21 differ markedly from each other. However, they appear

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consistent for the individual species at the respective concentrations. The generated curves for the individual species at 20mM (figure 2.22) correlate well with the observable trend indicated in Figure 2.21, i.e. substrate conversion decreases with time.

Table 2.8 is a quantitative summary of the substrate conversion of defined mixed substrates by immobilised polyphenol oxidase.

Table 2.8 Substrate conversion by IPS 748 membrane-immobilised polyphenol oxidase.

	Initial substrate concentration (mM)	4-methoxyphenol	phenol	*p*-cresol	4-chlorophenol
Substrate	1	2.5227×10^{-2}	8.7158×10^{-2}	0.1546	0.1104
converted	2.5	1.6925×10^{-2}	0.1769	0.3457	0.2879
(mg)	5	0.1331	0.2511	0.5284	8.9013×10^{-2}
Total substrate	4	0.3774			
converted	10	0.8274			
(mg)	20	1.0016			
Substrate converted	1	0.46	1.58	2.81	2.01
per unit enzyme	2.5	0.31	3.22	6.29	5.23
(μg.Unit^{-1})	5	2.42	4.56	9.61	1.62
Total substrate	4	6.86			
converted per unit	10	15.04			
enzyme (μg.Unit^{-1})	20	18.21			

Results are expressed for the individual species concentrations and as a function of the *total* substrate concentration in the feed stream.

Comparison of the data expressed in Table 2.7 and 2.8 is shown in Figure 2.23, where the amount of substrate converted is expressed as a function of the total initial substrate concentration.
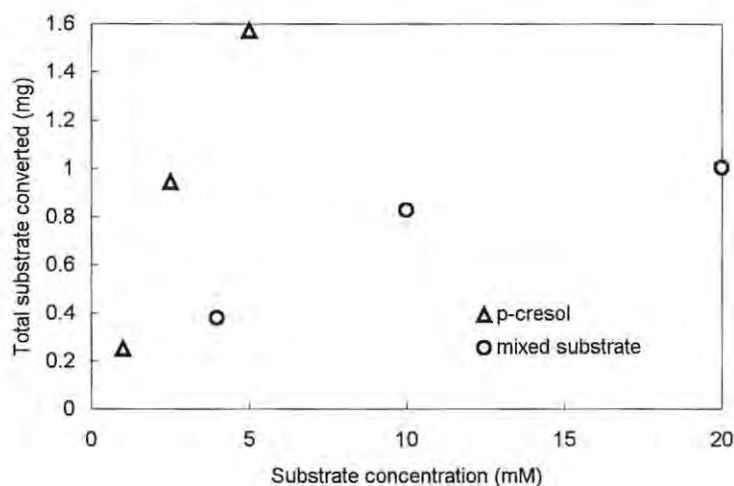
Figure 2.23 A comparative indication of the total amount of substrate converted by immobilised polyphenol oxidase as a function of the total initial substrate concentration.

As indicated, the total amount of substrate converted was determined with *p*-cresol as the sole substrate, and for the defined mixed synthetic substrates at 1, 2.5, and 5m*M* initial respective substrate concentrations (i.e. total concentrations of 4, 10, and 20m*M* respectively). Evident from the plot of total substrate conversion as a function of initial substrate concentration is that at comparable concentrations, e.g. 4m*M* mixed substrate and 5m*M* *p*-cresol, significantly less total substrate is converted when more than one substrate is present in the feed mixture. This could be attributed to a number of factors including competitive inhibition of the substrate, lower conversion of some substrates due to differing affinities (K_m), or the enzyme-generated products of some of these substrates exhibiting a greater inhibitory effect than certain other substrates within the mixture. As indicated in section 2.5.1, a concentration-dependent effect is evident with non-immobilised polyphenol oxidase when the substrate consists of an equal concentration mixture of phenol, *p*-cresol, 4-methoxyphenol, and 4-chlorophenol. From Figure 2.23, it is apparent that a significant decline in substrate conversion occurs as the substrate concentration is increased from 10m*M* to 20m*M* for the mixed substrate feed. This concentration-dependent effect is thus also evident for the immobilised enzyme when subjected to the same substrate and initial concentration conditions as the non-immobilised enzyme, i.e. as initial substrate concentration is increased, so a decline in immobilised-enzyme substrate conversion is observed. It appears, therefore, that under immobilised and non-immobilised conditions, the substrate conversion efficiency of polyphenol oxidase is limited by substrate saturation.

2.5.5. Comparison between non-immobilised and immobilised polyphenol oxidase

Table 2.9 shows a quantitative comparison of the substrate conversion capacity for non-immobilised polyphenol and immobilised polyphenol oxidase. The immobilised enzyme data reflects variation in conversion capacity between *p*-cresol as a single substrate and the defined mixed feed substrate.

Table 2.9 Quantitative comparison of the substrate conversion capacity between non-immobilised polyphenol oxidase and immobilised polyphenol oxidase.

	Volume (mL)	Concentration (m*M*)	Total substrate available (mg)	Total substrate converted to quinone (mg)	% conversion of total substrate	Total substrate converted per unit enzyme (μg.Unit^{-1})
Non-immobilised	50	4	22.74	15.09	66.35	87.92
		10	56.86	22.02	38.72	128.27
		20	113.73	17.98	15.80	145.15
Immobilised; *p*-cresol	12.32	1	1.33	0.2486	18.69	4.52
		2.5	3.33	0.9436	28.34	17.16
		5	6.66	1.5713	23.59	28.56
Immobilised; mixed feed	12.32	4	5.60	0.3774	6.74	6.86
		10	14.01	0.8274	5.91	15.04
		20	28.02	1.0016	3.57	18.21

From the above quantitative analysis it appears that considerably greater substrate conversion capacity is achieved when polyphenol oxidase is in a non-immobilised state as opposed to the membrane-immobilised situation. This is clearly evident when comparing the percentage conversion of the total substrate available to the enzyme. However, as previously described, *ca*. 171 Units of polyphenol oxidase were used under non-immobilised conditions whereas only 55 Units were immobilised on the capillary membranes. Thus, the amount of immobilised enzyme was *ca*. 3.1 times less than for the non-immobilised conditions. Increasing the amount of immobilised enzyme to levels comparable to non-immobilised conditions would achieve percentage conversions of *ca*. 57, 87, and 73% for *p*-cresol conversion at 1, 2.5, and 5m*M* initial substrate concentrations respectively. Under conditions where the feed stream is a substrate mixture, the percentage conversion is less significant but high nonetheless,

with conversions approaching 21, 18, and 11% at 4, 10, and 20mM initial feed concentrations respectively.

2.6. DISCUSSION

Although the results documented above indicate non-immobilised polyphenol oxidase to be more efficient at conversion of phenols to the associated quinone products, in the long term, the aforementioned disadvantages (section 1.2.2) of using non-immobilised enzymes are still an important consideration. The first consideration to be taken into account in the assessment of the above described enzyme-immobilised system, is the protein-loading capacity of the capillary membranes. As discussed in section 2.5.5, comparable enzymatic conversion may be feasible for immobilised polyphenol oxidase if the amount of enzyme per unit membrane area can be increased to levels comparable with the non-immobilised enzyme. Secondly, the practicality of any immobilised enzyme system, in situations where product retention in the micro-environment of the biocatalyst becomes inhibitory, lies in the removal of these products. In the case of polyphenol oxidase these are the enzyme-generated o-quinones. In a capillary membrane-immobilised situation the rate of product removal is dependent on the flux conditions of the system.

Conventional descriptions of membrane processes generally relate the productivity of a specific reactor unit as a function of the membrane area and the time for the conversion to occur. Productivity is, therefore, related to standardised units of measurement for membrane area (m^2) and time (h). Since the reported substrate conversion values (table 2.8) are effectively a function of the membrane area for the SCR (refer to table 2.3), correlating the potential conversion efficiency of sufficient SCRs to constitute an equivalent membrane area of 1m^2 would be representative of the bioremediation potential of this system. This is best illustrated by calculating the amount of substrate converted per m^2 per hour. At an initial substrate concentration of 1mM, potential substrate conversion levels of 0.195 g.m^{-2}.h^{-1} can be obtained. At initial substrate concentrations of 2.5 and 5mM, conversion levels are higher at 0.427 g.m^{-2}.h^{-1} and 0.517 g.m^{-2}.h^{-1} respectively. As discussed above, increasing the enzyme loading to comparable levels with the non-immobilised system has the potential to achieve productivities as high as 1.6 g.m^{-2}.h^{-1}.

Although low substrate conversion by IPS 748 membrane-immobilised polyphenol oxidase is evident (table 2.9), immobilised polyphenol oxidase would be more susceptible to the effects of substrate saturation due to the low levels of enzyme immobilised (refer to section 2.5.3.1). Therefore, increasing

the amount of polyphenol oxidase that is immobilised would lessen this influence thus increasing the substrate conversion capability of immobilised polyphenol oxidase. From the results shown it is obvious that although polyphenol oxidase is efficient at converting phenolic species when immobilised, there appears to be considerable potential for productivity improvement. Furthermore, it is clearly evident that the role of the immobilisation matrix on influencing enzyme-substrate contact is marked. As such, this is therefore an important implication to consider if a future scale-up of this process is implemented.

2.7. CONCLUDING REMARKS

From these results, it is apparent that under non-immobilised conditions, as initial substrate concentration is increased, the amount of substrate conversion does not increase to the same extent. This absence of a linear trend indicates the occurrence of substrate saturation. Substrate saturation also appeared to be the limiting factor influencing the substrate conversion efficiency of immobilised polyphenol oxidase. However, preliminary investigations using a different immobilisation matrix demonstrated that this is not the case. From these preliminary investigations it was evident that the crucial requirement in extending the half-life of polyphenol oxidase is dependent on increasing the rate at which inhibitory products are removed. Optimisation of the described system can, therefore, be achieved by focusing on two crucial aspects. Firstly, optimisation of the reactor configuration, focusing primarily on the immobilisation matrix, and secondly, physical removal of the inhibitory products. Investigation of these issues is described in the following chapters.

CHAPTER 3

THE APPLICATION OF IMMOBILISED POLYPHENOL OXIDASE IN THE TREATMENT OF INDUSTRIAL EFFLUENT

3.1. INTRODUCTION

Although much fundamental work has been done on the use of enzymes for the removal of phenols from wastewater, the majority of applications have focused primarily on synthetic effluents. The pollutant range that has been subject to enzymatic treatment studies is extensive (table 3.1), but knowledge of the potential for the industrial use of such systems is limited, because of the lack of application to industrial effluents.

Table 3.1 Reported enzymatic removal of selected substituted phenols.

Compound	Reference	Compound	Reference	Compound	Reference
phenol	a,c,d,f,i,j	2,4-dichlorophenol	d,e,h	3,3'-dichlorobenzidine	g
o-chlorophenol	a,d,h	2,6-dichlorophenol	d	3,3'-dimethylbenzidine	g
m-chlorophenol	a,d	2,6-dimethylphenol	d,h	p-phenylazoaniline	g
p-chlorophenol	a,d,f,i	2,6-dimethoxyphenol	d,e	2-naphthylamine	g
o-methoxyphenol	a,d	α-naphthol	d,e	5-nitro-1-naphthylamine	g
m-methoxyphenol	a	4-chloro-2-methylphenol	d,h	4-aminobiphenyl	g
p-methoxyphenol	a,d,i	vannilic acid	e	3,4-dichloroaniline	a
o-cresol	a,d,h	syringic acid	e	1-naphtholamine	g
m-cresol	a,d	catechol	e	p-chloroaniline	a,e
p-cresol	a,b,d,h,i	caffeic acid	e	benzidine	g
catechol	a,i	3,3'-diaminobenzidine	g	aniline	a
hydroquinone	a	3,3'-dimethoxybenzidine	g	p-hydroxyphenoxyacetate	k

[a]Wada *et al.*,1995: polyphenol oxidase immobilised on magnetite; [b]Payne *et al.*,1992: non-immobilised polyphenol oxidase; [c]Sun&Payne,1996: polyphenol oxidase immobilised on chitosan gels; [d]Shuttleworth &Bollag, 1986: non-immobilised laccase and laccase immobilised on celite; [e]Crecchio *et al.*, 1995: laccase and polyphenol oxidase immobilised in gelatine gels; [f]Nicell *et al.*,1993: non-immobilised horseradish peroxidase; [g]Klibanov&Morris,1981: non-immobilised horseradish peroxidase; [h]Bollag *et al.*, 1988: non-immobilised laccase; [i]Wada *et al.*,1993: polyphenol oxidase immobilised on cation exchange resins; [j]Buchanan&Nicell,1997: non-immobilised horseradish peroxidase; [k]Payne&Sun,1994: non-immobilised polyphenol oxidase.

The application of polyphenol oxidase for the treatment of industrial effluent has been limited to the removal of *p*-hydroxyphenoxyacetate, a contaminant in the manufacturing process of penicillin V production (Payne&Sun,1994). Laccase and horseradish peroxidase have been utilised in a non-immobilised state, and entrapped within alginate beads for the treatment of pulp mill, cotton mill hydroxide, and cotton mill sulphide effluents. In all cases, the laccase and horseradish peroxidase underwent rapid and irreversible enzyme inactivation (Davis&Burns,1990). Horseradish peroxidase has also been used in the treatment of an effluent generated during the caustic extraction stage of a bleach plant process (Paice&Jurasek,1984).

3.1.1. Application of novel capillary membranes for polyphenol oxidase immobilisation

3.1.1.1. Rationale behind the formation of externally unskinned capillary membranes

Collaborative work between the Department of Biochemistry and Microbiology, Rhodes University, and the Institute for Polymer Science, Stellenbosch University, was initiated for the development of externally unskinned capillary membranes to serve as unique immobilisation matrices facilitating fungal biofilm development for secondary metabolite production (Leukes *et al.*,1996; Jacobs&Leukes,1996). Problems encountered with IPS 748 membranes (section 2.3.2) were manifested as the inability of the fungal hyphae to penetrate the macroporous substructure of the capillary membrane. The reasons for this appeared to be firstly, the presence of an outer support skin and secondly, the non-continuous nature of the cavities making up the macroporous region of the membrane. The development of the new membrane was initiated in an attempt to circumvent these shortcomings associated with the IPS 748 membranes. The membranes that were produced displayed continuous, narrow-bore, finger-like cavities as well as the absence of the external supporting skin. The internal skin of the membrane was also thinner than previous membranes, contributing to decreased membrane resistance, and resulting in an increased flux.

It was envisioned that similar advantages might be derived for enzyme immobilisation applications. These included the open, continuous macrovoid region possessing greater surface area than IPS 748 membranes, and the increased flux due to decreased membrane resistance facilitating more rapid enzyme-catalysed product removal. The new membranes, designated as IPS 763, were, therefore, tested to determine their potential as an enzyme-immobilisation matrix.

3.1.1.2. Membrane formation and manufacture

The spinning of capillary membranes is often the preparation technique used for the formation of ultrafiltration membranes. In the spinning process itself, the shape and morphology of the capillary membranes is influenced by both phase separation and rheological phenomena (Ventoza&Loyd,1985; Wienk *et al.*,1995). This is of great significance because the hydraulic pattern of both feed and permeate solutions are affected by the fibre geometry. It is this hydraulic pattern which in turn has an influence on the mass transfer coefficient and pressure drop within capillary membrane structures (Mok *et al.*,1995). In membrane formation, phase-inversion membranes are made from any polymer or mixture thereof that, under certain compositional or temperature conditions, a homogeneous solution is formed. At different composition and temperature conditions, however, the homogeneous solution separates into two phases (Jacobs *et al.*,1997). A number of factors can induce this phase separation, including quenching of the solution to a lower temperature, solution immersion into a non-solvent bath, evaporation of the solvent, and contacting the polymer solution with a vapour of the non-solvent. Although immersion precipitation is the most important process for membrane formation, phase separation induced by evaporation or non-solvent vapour contact are closely related because they are also diffusion induced (Wienk *et al.*,1995). In the immersion precipitation process, also known as wet-dry phase inversion, water is the most common non-solvent medium used to bring about this phase separation (Jacobs&Leukes,1996; Jacobs *et al.*,1997).

In the wet-dry phase inversion process, the membrane spinning solution consisting of the viscous, degassed and filtered polymer or polymer mixture solution, is forced into a coaxial tube spinneret which can be schematically represented as a tube-in-orifice configuration (figure 3.1). The bore or lumen side of the emerging nascent membrane thread is kept open and stabilised by the co-extrusion of an internal coagulating fluid. For internally skinned membranes a strong non-solvent such as water is used as the internal coagulating fluid (Jacobs *et al.*,1997). The formation of the anisotropic morphology is facilitated by contact of the external membrane surface with an external coagulant bath high in solvent composition (Aptel *et al.*,1985; Jacobs&Leukes,1996).
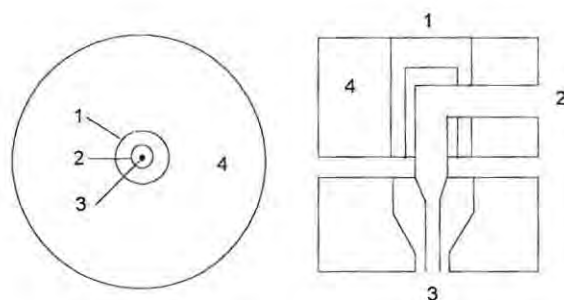
Figure 3.1 Schematic representation of the tube-in-orifice extrusion die. (1) Polymer solution entry port; (2) Internal coagulant port; (3) Internal coagulant extrusion orifice; (4) Spinneret body.

3.2. RESEARCH OBJECTIVES

In this study, the effect of using a different immobilisation matrix (polysulphone IPS 763 membranes), for the application of immobilised polyphenol oxidase, was compared to the use of poly(ether sulphone) IPS 748 membranes, for the treatment of two industrial effluents. By using a different immobilisation matrix, it was envisaged that the problems associated with the IPS 748 membranes (section 2.7) could be circumvented. Initial comparisons between the two membrane types were made using synthetic effluent to determine variations in efficiency under more controlled conditions, prior to application using the industrial effluent. Comparisons were also made between the commercially available purified polyphenol oxidase and a partially-purified crude extract of the enzyme from the common mushroom *Agaricus bisporus*.

3.3. MATERIALS AND METHODS

3.3.1. Substrate preparation and characterisation

Defined synthetic substrates consisting of a mixture of phenol, *p*-cresol, 4-methoxyphenol and 4-chlorophenol was made up using Milli-Q® water as previously described (section 2.3). A phenolic industrial effluent were obtained from a South African coal-gas plant. A detailed chemical analysis was provided with the received sample (table 3.2). The industrial effluent was not subject to any pre-treatment other than dilution (1/10) with Milli-Q® water and, in some cases, pH adjustment using 0.1M NaOH.

Table 3.2 Detailed chemical analysis of phenolic industrial effluent.

Compound	Concentration	Concentration Units
Phenol	0.197	mM
COD	1673	mg.L^{-1}
Acetonitrile	0.00418	mass %
Methanol	0.00542	mass %
Ethanol	0.01236	mass %
1-Propanol	0.00186	mass %
i-Propanol	0.00024	mass %
n-Butanol	0.00062	mass %
Acetone	0.00047	mass %
2-Propanone	0.00236	mass %
Suspended Solids	218.4	mg.L^{-1}
Total Dissolved Solids	866	mg.L^{-1}

3.3.2. Crude polyphenol oxidase extraction from *Agaricus bisporus*

An extract of polyphenol oxidase was also prepared from the common mushroom *Agaricus bisporus* according to the method of Burton *et al.* (1993). Fresh mushrooms (1kg) were frozen at -20°C and then homogenised in cold acetone (2.5L; 4°C) using a Waring blender. The resultant slurry was rapidly filtered through a Buchner funnel and the residual pulp was air dried briefly prior to freezing with liquid N_2 and stirring into H_2O (500mL). The mixture was stored overnight at 4°C, filtered through cheesecloth and residual acetone was removed by gently bubbling N_2 through the solution. The protein was precipitated with ammonium sulphate to 40% saturation and centrifuged for 10 minutes (10 000g). The supernatant was then brought to 52% saturation and re-centrifuged. The resultant pellet was resuspended in 50mL of 0.05M phosphate buffer (pH 7.0) prior to dialysis in 0.01M phosphate buffer (pH 7.0) for 12 hours at 4°C. This was further dialysed against cold Milli-Q$^{®}$ water for 4 hours. The aqueous extract was then lyophilised (Edwards Modulyo freeze drier, BOC Ltd., U.K.) yielding a pale brown powder. The freeze-dried extract was resuspended in cold phosphate buffer (pH 6.80) and aliquoted into 1mL samples which were stored at -20°C until required. The activity of the partially-purified crude extract was determined using the previously described method (section 2.3.1).

3.3.3. HPLC of samples

Samples were analysed as described in section 2.3.7. An HPLC chromatogram of the phenolic effluent is shown in Figure 3.2.
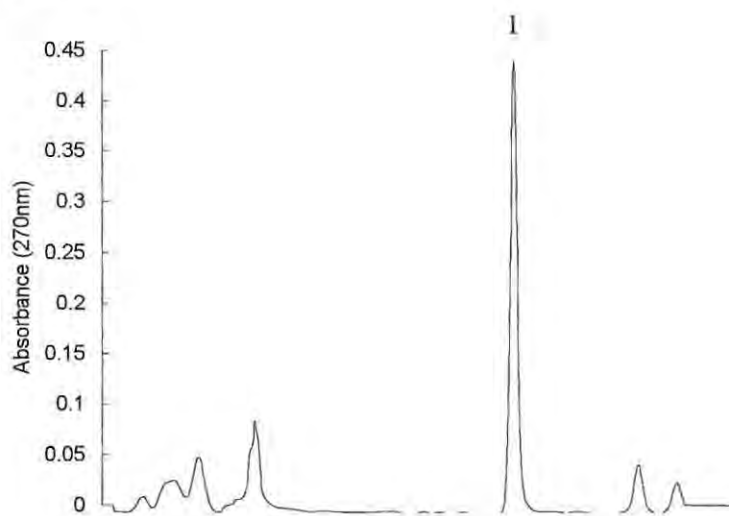
Figure 3.2 HPLC chromatogram of phenolic industrial effluent. 1) phenol, retention time 5.45 minutes.

3.3.4. Chemical oxygen demand (COD) determination

Due to the high organic load of the industrial effluent (table 3.2) it was necessary to determine if the ultrafiltration characteristics of the IPS 763 membranes facilitated any reduction in the organic load of the effluents. The organic load reduction potential of the membranes was quantitatively determined by measuring the COD of the feed and permeate streams. COD was determined by adding 3mL sample solution to 0.3mL COD solution A and 2.3 mL COD solution B (Merck, Darmstadt, Germany). This was heated for 2 hours (148°C) on a TR205 heating block (Merck, Darmstadt, Germany) and COD levels determined using a Merck Spectroquant® SQ118 spectrophotometer.

3.3.5. Membrane preparation and manufacture

3.3.5.1. Casting solution composition

Polysulphone (Grade 3010 Ultrason S) was used as the membrane material. The solvent, *N*-methyl-2-pyrrolidinone (NMP), and non-solvent methyl cellusolve (MC) were stored over a 0.3 nm molecular sieve after vacuum distillation. The low molecular mass polymer (600 Da) additive was polyethylene glycol (PEG600). The composition of the casting solution is given in Table 3.3.

Table 3.3 Casting solution formulation designed to produce membranes characterised with increased porosity, and finger-like macrovoids within the substructure.

Component	Mass percent
Ultrason S (polysulphone)	22
High-boiling point solvent (NMP)	36
Low-boiling point non-solvent additive (MC)	10
Low molecular mass additive (PEG 600)	32

3.3.5.2. Preparation of membrane casting solution

Solution preparation was done in 2L resin kettles which were placed in an oil bath. The kettles were equipped with high-speed overhead stirrers and the solvation temperature was maintained at 60°C. By passing the shaft of the stirrer through a Liebig condenser, loss of low-boiling point solvent was prevented. Homogeneity was achieved after a minimum period of 48-hours after which the solution was decanted into Schott bottles and subjected to rotation at ambient temperatures. After 48-hours, the polymer solution was filtered through a 5μm stainless-steel filter and degassed in a desiccator for 24-hours prior to use.

3.3.5.3. Membrane manufacture

Capillary membrane formation is facilitated by means of extruding the casting solution through a tube-in-orifice spinneret (figure 3.1). The polymer extrusion rate is controlled by a precision-gear metering pump. A schematic of the membrane spinning apparatus is shown in figure 3.3. Unlike previously reported membrane formation processes where the spinneret is positioned above the coagulation bath separated by an air gap (Liu *et al.*,1991; Boom *et al.*,1992; Wienk *et al.*,1995; Mok *et al.*,1995), the spinning die was positioned at the bottom of the coagulation tank (Jacobs&Leukes,1996). The extruded polymer was therefore drawn vertically from the extrusion die at a linear production rate of 4m.min^{-1}. The dense inner skin layer was formed by metering pure water through the lumen side as the internal coagulant. Due to the high solvent content of the coagulation bath, the outside or shell-side of the membrane was still gel-like and highly swollen as the nascent membrane was withdrawn from the external coagulation fluid. The membrane structure was subsequently fixed following withdrawal from the coagulation bath by contact with humidified air which served as the non-solvent vapour atmosphere. The formed membrane could then be guided, *via* a system of rollers, to a final aqueous rinse tank

without any damage to the membrane itself. The MWCO of the membranes was *ca.* 65 kDa (Jacobs, pers. comm.).
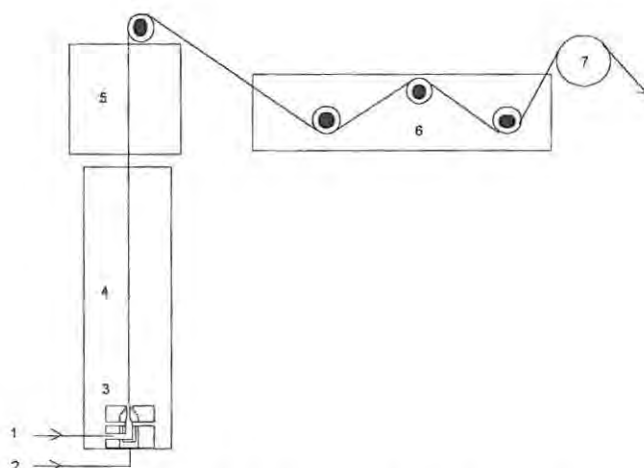
Figure 3.3 Schematic representation of the spinning set-up showing transport of the membrane to the rinsing bath. 1) spinning solution; 2) internal water coagulant; 3) spinneret; 4) aqueous non-solvent coagulation tank; 5) non-solvent vapour chamber; 6) final rinsing bath; 7) membrane take-up.

3.3.5.4. Post-treatment of fabricated membranes

Membranes were subjected to a final 24-hour rinse in pure water in the final rinse tanks prior to a conditioning period in a 1:1 aqueous glycerol solution. They were then dried in a high humidity chamber at ambient temperature for a period of seven days prior to use. The membranes were not subjected to any other form of post-manufacturing treatment.

3.3.5.5. SEM of IPS 763 capillary membranes

Specimen membranes were visualised using scanning electron microscopy (figure 3.4) as previously described (section 2.3.3 and 2.3.4).
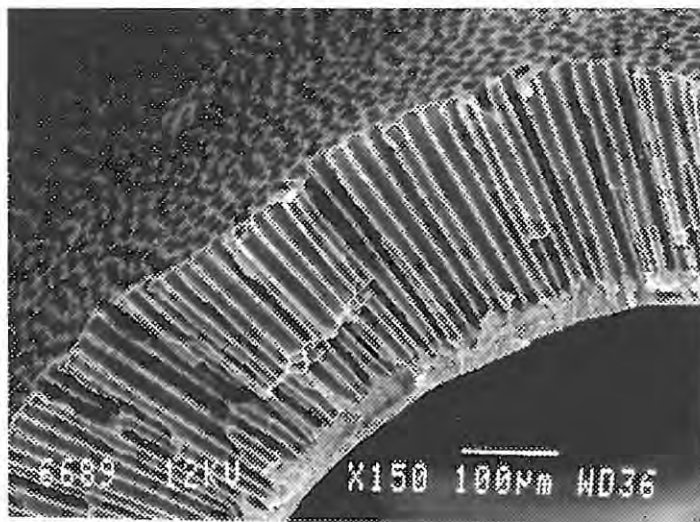
Figure 3.4 Scanning electron micrograph of a cross-section through an IPS 763 capillary membrane (magnification 150X) showing the absence of an external surface skin surrounding the annular macrovoid region and the presence of continuous finger-like macrovoids.

3.3.5.6. Capillary membrane bioreactor construction and operation

Single-capillary reactors were constructed as in section 2.4.1 and operated under the same conditions as described in section 2.4.2.

3.3.5.7. Membrane protein-loading capacity determination

Subsequent to inoculation of the capillary membranes with polyphenol oxidase (section 2.4.4), the protein-loading capacity of the membranes was determined according to the method of Bradford (1976) as outlined in section 2.4.5.

3.3.5.8. Operating characteristics of IPS 763 capillary membrane bioreactor

The operating conditions of the capillary membrane bioreactor with IPS 763 membranes are summarised in table 3.4.

Table 3.4 Capillary reactor operational characteristics.

Membrane area (cm^2)	Operating pressure (kPa)	Flux (L.m^{-2}.h^{-1})	Flow volume (L.h^{-1})	Flow rate (m.s^{-1})
4.838	50	197.189	19.6	35.36

3.4. RESULTS

3.4.1. COD analysis of industrial effluent

IPS 763 membranes were evaluated for their organic load (COD) reduction potential for the phenolic effluent, according to the procedure outlined in section 3.3.4. The effluent was analysed prior to passing through the membrane, and subsequently the permeate was analysed and compared to determine if the capillaries facilitated any reduction in COD due to the ultrafiltration characteristics of the membrane (MWCO *ca.* 65kDa).

Table 3.5 A comparison of the COD reduction capacity of IPS 763 membranes for the phenolic effluent.

	Phenolic effluent (mg.L^{-1})
Pre-membrane treatment	1673 ± 70.24
Post-membrane treatment	1393 ± 94.52

From the results obtained (table 3.5), it was determined that IPS 763 ultrafiltration membranes facilitated a 16.7% reduction in the COD of the phenolic effluent.

3.4.2. Substrate conversion of a defined synthetic substrate using IPS 763 capillary membranes

Results of the IPS 748 and IPS 763 membrane-immobilised polyphenol oxidase conversion of phenol-containing effluent could be compared in considering the potential of membrane-immobilised polyphenol oxidase as an effective bioremediation process. Initial experimentation with the new IPS 763 membranes was conducted with a defined mixed synthetic substrate (section 2.3) to facilitate comparisons with the IPS 748 capillary membranes (see Chapter 2). As discussed in section 2.5.1, preliminary experiments with the capillary membranes were carried out with each individual substrate species in the feed mixture at a concentration of 1.0mM, thereby effectively constituting a *total* concentration of 4mM. The substrate conversion attained using polyphenol oxidase immobilised on IPS 763 membranes (figure 3.5) showed a considerable increase in comparison to polyphenol oxidase immobilised on IPS 748 membranes (835µg.Unit^{-1} vs. 6.86µg.Unit^{-1}).
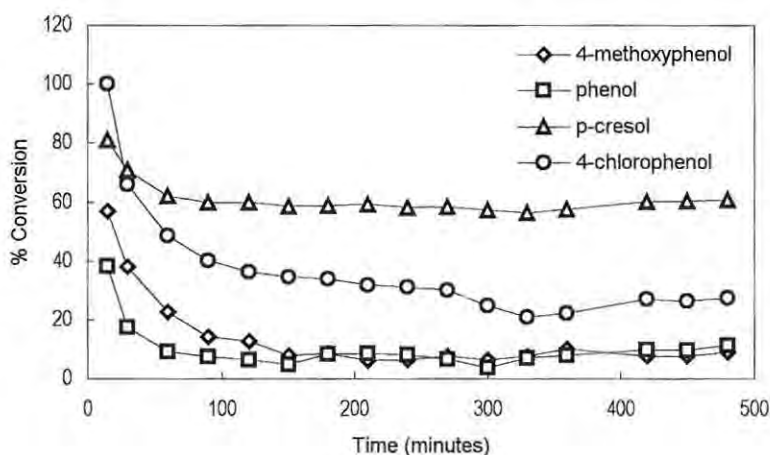
Figure 3.5 Simultaneous conversion of phenols in a defined synthetic effluent (1mM)
using commercial polyphenol oxidase extract immobilised on IPS 763 membranes.

Enzyme activity was also extended, such that substrate conversion was evident for *ca.* 8 hours
compared with only 4 hours for polyphenol oxidase immobilised on IPS 748 membranes. As can be
seen from Table 3.4, the associated flux for IPS 763 membranes is *ca.* 30 times greater than the
operating flux for IPS 748 membranes (table 2.3). Although this effectively means that the
immobilised enzyme is exposed to increased levels of substrate, the rate at which inhibitory *o*-quinones
are removed from the micro-environment of the biocatalyst is significantly higher. This translates to a
considerable decrease in the associated product inhibition. Furthermore, of the total amount of enzyme
immobilised, only 32.5% (55 Units) was immobilised onto IPS 748 membranes (section 2.5.3.1),
whereas *ca.* 82% (140 Units) of the initial amount of enzyme was immobilised onto IPS 763
membranes, as determined according to the method of Bradford (1976) as outlined in section 3.3.5.7.

The considerable increase in the amount of substrate conversion observed with polyphenol oxidase
immobilised on IPS 763 membranes is summarised in Table 3.6. From these results it is apparent that
over an 8-hour period a considerable amount of substrate comes into contact with the enzyme due to
the high flux associated with the IPS 763 membranes.

Table 3.6 Substrate conversion by IPS 763 membrane-immobilised polyphenol oxidase.

	4-methoxyphenol	phenol	*p*-cresol	4-chlorophenol
Substrate converted (mg)	10.43	5.34	70.93	30.25
Conversion per unit enzyme (μg.Unit^{-1})	74.50	38.14	506.64	216.07
Total available substrate (mg)	330.87			
Total substrate converted (mg)	116.95			
Total substrate converted per unit enzyme (μg.Unit^{-1})	835.35			

Of this substrate, *ca*. 35% of the total substrate available to the IPS 763 immobilised polyphenol oxidase was converted to the corresponding *o*-quinone. In comparison, only 6.74% of the total available substrate was converted with IPS 748 membranes as the immobilisation matrix (section 2.5.5). Although substrate availability was increased from 5.60mg to 330mg, a substrate conversion increase of 28.3% was observed with the IPS 763 membranes. This appears to indicate that substrate saturation was not affecting the conversion efficiency of the immobilised enzyme. This will be discussed further in section 3.5.

3.4.3. Substrate conversion of industrial effluent using IPS 748 capillary membranes

Preliminary evaluation of the substrate conversion potential of immobilised polyphenol oxidase exposed to industrial effluents (table 3.2) was conducted using IPS 748 membranes as the immobilisation matrix. Initial experiments using the IPS 748 membranes were carried out at the original pH of both industrial effluents (pH 5.0) to determine whether this pH would allow substrate conversion by the immobilised enzyme. According to Flurkey (1991), the pH at which mushroom polyphenol oxidase exhibits optimal activity is *ca*. 6.8-7.0, and therefore, subsequent experiments were carried out at pH 6.80 (effluents adjusted using 0.1M NaOH).

From the results obtained, it was apparent that pH plays a considerable role in the conversion efficiency of polyphenol oxidase. Figure 3.6 shows the results of phenolic effluent treatment with a crude extract of the enzyme, immobilised on IPS 748 membranes, with the feed at pH 5.0 and pH 6.80. Further evidence that substrate saturation has relatively little effect on the substrate conversion capacity of immobilised polyphenol oxidase is provided by the observation that increasing the pH of the effluent has the effect that greater substrate availability is observed at higher pH values. A

quantitative analysis of substrate availability to the enzyme and substrate conversion of the perfusing substrate is shown in Table 3.7. A possible explanation for this is that the pH increase might induce disassociation or association of certain effluent components resulting in decreased resistance to trans-membrane permeation.
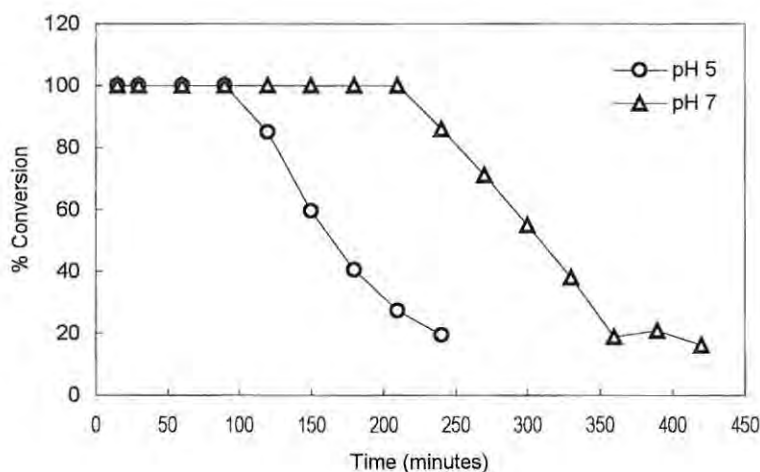
Figure 3.6 Conversion of phenolic effluent at pH 5 and pH 6.8 using crude polyphenol oxidase immobilised on IPS 748 membranes.

From these results (table 3.7), it is observed that as the feed stream pH is increased from 5.0 to 6.8, a 34% increase (24.83mg to 38.09mg) in substrate availability to the immobilised enzyme occurs. Furthermore, at pH 5.0, 59.75% of the available substrate was converted to *o*-quinones. At pH 6.8, however, substrate conversion increased to 66.34% (25mg converted). The correlation, therefore, is that a 34% increase in the amount of substrate contacting the immobilised enzyme corresponds to a 6.5% *increase* in the percentage substrate conversion to the corresponding *o*-quinone products. Although polyphenol oxidase is more active at pH 6.8 (Flurkey,1991), the increase in substrate conversion coupled with greater substrate availability indicates that product inhibition, and *not* substrate saturation, is responsible for the decrease in substrate conversion over time culminating in total activity loss.

3.4.4. Substrate conversion of industrial effluent using IPS 763 capillary membranes

The results obtained with IPS 748 membrane-immobilised enzymatic conversion of the industrial effluents indicated that greater substrate conversion to the *o*-quinone products occurred at pH 6.8 as opposed to pH 5. Subsequent experiments with IPS 763 membranes were, therefore, conducted with

the effluent pH adjusted to 6.8. Figure 3.7 represents the substrate conversion of the phenolic effluent with 140 Units of IPS 763 immobilised commercial polyphenol oxidase. Of the 62.70mg available to the enzyme, only 4.11mg was converted to the *o*-quinone product. Conversion efficiency of the enzyme was extremely low, at only 6.56% conversion.
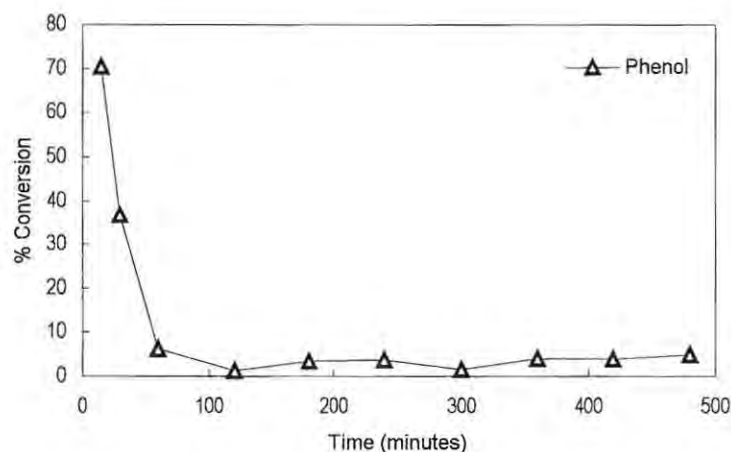
Figure 3.7 Conversion of phenolic effluent at pH 6.8 using commercial polyphenol oxidase immobilised on IPS 763 membranes.

Table 3.7 Comparison of the substrate conversion efficiency of IPS 748 and IPS 763 membrane-immobilised polyphenol oxidase exposed to the phenolic industrial effluent.

Membrane type	pH	Total substrate available (mg)	Substrate converted to *o*-quinone (mg)	% conversion of total substrate	Total substrate converted per unit enzyme (μg.Unit^{-1})
IPS 748	5.0	24.83	14.83	59.75	105.93
IPS 748	6.8	38.09	25.27	66.34	180.50
IPS 763	6.8	62.70	4.11	6.56	29.35

3.5. DISCUSSION

Differences in terms of morphological characteristics associated with IPS 748 and IPS 763 membranes are fundamentally significant, including the different MWCO's of *ca.* 22kDa and 65kDa respectively. As shown above, the high flux and greater protein-loading capacity associated with the IPS 763 membranes effectively results in greater substrate conversion when the feed stream is composed of a defined mixed synthetic feed. The high operating flux associated with IPS 763 membranes should

theoretically be associated with a greater potential for substrate saturation. However, it is evident from the results obtained for the synthetic substrate feed that substrate saturation appears less deleterious to the maintenance of enzyme activity than the effect of product inhibition.

Under conditions where the feed stream was composed of a defined mixed synthetic substrate, associated substrate conversion for IPS 763 membranes was considerably greater than for IPS 748 membranes. Initial indications from the results obtained suggest that substrate saturation of the immobilised polyphenol oxidase is, therefore, not the limiting factor affecting the substrate conversion efficiency of the enzyme. A rigorous investigation requiring further work would, however, be necessary in order to conclusively determine this.

Furthermore, the associated lower substrate conversions for the industrial effluent may be attributed to the higher MWCO of the IPS 763 membranes facilitating transport of high molecular weight components present in the phenolic effluent across the membrane. Possible interference with transport of substrate molecules to the enzyme active site may subsequently occur, resulting in lower substrate conversion.

3.6 CONCLUDING REMARKS

In Chapter 2, two key areas were identified where systems using membrane-immobilised polyphenol oxidase for bioremediation purposes could potentially be optimised. From the results reported here, it is apparent that substrate saturation is not the primary factor influencing the decrease in polyphenol oxidase activity over time, but rather product inhibition. Moreover, because substrate saturation did not appear to be the dominant limitation, the primary objective for subsequent experimentation was focused on decreasing the effect of product inhibition. Chapters 4 and 5 focus on two possible methods whereby the effects of product inhibition can be minimised.

CHAPTER 4

REMOVAL OF POLYPHENOL OXIDASE-GENERATED PRODUCTS

4.1. INTRODUCTION

Although polyphenol oxidase is effective in converting phenol and a number of associated derivatives to their corresponding o-quinones, these resultant enzyme-generated o-quinones and low-molecular weight polymers still remain in the treated effluent. This presents a two-fold problem. Firstly, it was shown in chapter 4 that the o-quinones remaining in solution deleteriously affect the activity of polyphenol oxidase due to product inhibition. Secondly, although the goal of reducing the toxicity of the phenol-containing effluent is achieved, the resultant treated stream is of a highly coloured nature which is unacceptable for discharge purposes. Therefore, to complete the wastewater treatment process, it is necessary to remove the polyphenol oxidase-generated products from the capillary membrane bioreactor permeate stream. At low concentrations the remaining coloured products do not achieve high enough degrees of polymerisation to precipitate out of solution. This has been observed previously in a number of independent studies (Sun *et al.*,1992; Payne *et al.*,1992; Wada *et al.*,1993; Wada *et al.*,1995). Since conventional treatment methods including aerated lagoons and activated sludge plants have been shown to be ineffectual in removing this colour, a number of physical and chemical treatment methods have been considered (Paice&Jurasek,1984). These have included ultrafiltration, ion exchange, and lime precipitation (Davis&Burns,1990). Due to these chemical and physical treatment methods being considerably expensive, biotreatment processes appear to be far more attractive alternatives. For o-quinone removal, biosorption using activated carbon and chitosan have been shown to be very effective (Sun *et al.*,1992; Wada *et al.*,1993; Juang *et al.*,1996).

4.1.1. The use of chitosan as a biosorbent

Chitin (an N-acetylglucosamine polymer) is obtained from abundant natural resources of crustaceans such as crabs, shrimps, lobsters and krill, where the shells are the major component of seafood processing waste (Siso *et al.*,1997). Chitosan, $(1\rightarrow4)$-2-amino-2-deoxy-β-D-glucan (figure 4.1), is a polycationic polymer obtained commercially by the alkaline deacetylation of chitin (Hall&Yalpani,1980; Krajewska *et al.*,1996; Siso *et al.*,1997). Prior to deacetylation, the chitin is first subjected to deproteination, conventionally accomplished by alkali extraction, and then demineralisation by extraction using dilute acid (Bough *et al.*,1978).
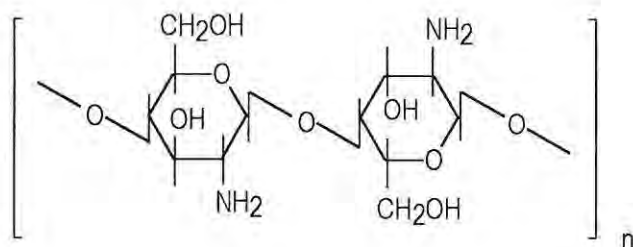
Figure 4.1 The chemical structure of chitosan (Hsien&Rorrer,1995).

Since chitosan is a shellfish-industry waste-product, it is inexpensive as well as being inert, hydrophilic, and biocompatible. Due to the presence of a reactive amine functional group on each of the glucosamine units within the chitosan biopolymer chain, chitosan has been shown to be of potential use in a number of different fields. In wastewater treatment, chitosan has shown considerable potential as a flocculent and coagulating agent for organic matter (Chavasit&Torres,1990; Bough,1976). It has also been shown to act as an ion-chelating agent for heavy metal removal (Hsien&Rorrer,1995), and as an adsorption medium for certain acid pesticides and polychlorinated biphenyls (PCB's) present in many polluted water sources (Van Daele&Thomé,1986). In these wastewater treatment applications, chitosan has been shown to be more effective than other polymers and resins including activated charcoal, cellulosic derivatives, and synthetic polyelectrolytes.

Selective chemisorption of contaminating *o*-quinones is possible using chitosan. Although the chemistry behind the reaction has not yet been fully elucidated, it appears that the amine group in the chitosan structure reacts with carbonyl groups to form covalent bonds (Hall&Yalpani,1980; Muzzarelli *et al.*,1982; Sun *et al.*,1992). Two possible mechanisms have been suggested for the reaction paths leading to bond formation. The first is imine formation, which occurs rapidly as a reversible reaction, and the second mechanism involves an irreversible oxidative reaction path leading to the formation of aminoquinones (Parris,1980). In studies by Sun *et al.* (1992) to characterise quinone-chitosan interactions using calorimetry, the adsorption enthalpy for quinone adsorption onto chitosan was found to be in the order of -24.7 kcal.mol^{-1}. Since adsorption enthalpies of this magnitude generally result from strong, presumably, covalent bond formation, such adsorption can be classified as chemisorption. In comparison, the observed enthalpy for quinone-activated carbon interactions was only found to be -7 kcal.mol^{-1}. This suggests the involvement of low-energy physical forces such as hydrophobic interactions. Due to the effectiveness of chitosan as a quinone biosorbent, chitosan has been used in a number of studies involving the removal of polyphenol oxidase-generated *o*-quinones originating from a variety of

different substrates. These include phenol, *p*-chlorophenol, *p*-methoxyphenol, *p*-cresol, and catechol (Sun *et al.*,1992; Payne *et al.*,1992; Wada *et al.*,1993), as well as *p*-hydroxyphenoxyacetate, a fermentation contaminant formed during the production of penicillin V (Payne&Sun,1994).

4.2. RESEARCH OBJECTIVES

Initial objectives were to test observations by Payne *et al.* (1992) that no adsorption of phenols to chitosan occurred, and to confirm the selective adsorption of *o*-quinones to chitosan. Although these observations have already been reported by Payne and Sun (1994), the use of an uncharacterised industrial effluent required that the biosorbent selective nature of chitosan be demonstrated using this example. Initial experiments involved the contact of defined phenolic substrates and the industrial phenol-containing effluent with chitosan. Confirmation of the selective adsorption of the enzyme-generated quinones to chitosan was achieved using HPLC. Furthermore, the integration of the chitosan-facilitated quinone removal step in the overall SCR configuration was investigated.

4.3. MATERIALS AND METHODS

Chitosan from crab shells was purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). The chitosan was not subject to further treatment prior to use.

4.3.1. Quinone removal using chitosan

The quinone removal potential of a chitosan-packed column, integrated into the immobilised enzyme bioreactor configuration (IPS 763 membranes), was determined. Integration of a chitosan-containing unit facilitated contact between the chitosan and reactor permeate immediately upon exit from the immobilised polyphenol oxidase membrane reactor. The immobilised enzyme reactor was constructed and operated as previously described (section 2.4.1 and 2.4.2) but with the inclusion of the chitosan-containing column (figure 4.2). The chitosan-packed column consisted of a borosilicate glass shell 110mm long with an outer diameter of 7mm and inlet and outlet tubes of 3mm outer diameter. For all subsequent experiments the average packing mass of the chitosan per column was 0.494 g. Product removal within the unit was highly effective at a flow rate of *ca*. 3mL.h^{-1}.
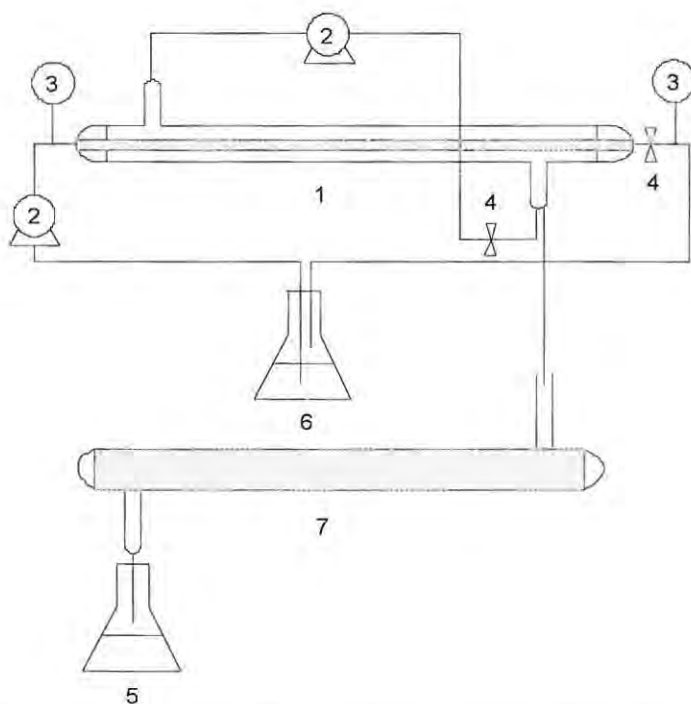
Figure 4.2 Reactor configuration showing the integration of the chitosan-packed column within the system facilitating contact of the quinone-containing permeate with the chitosan.

KEY: 1. Single-fibre reactor   2. Peristaltic pump

   3. Pressure gauge   4. Flow regulatory clamps

   5. Post-chitosan permeate   6. Circulated substrate feed

   7. Chitosan-packed column

4.3.2. HPLC comparison to determine *o*-quinone removal

To supplement the spectrophotometric analyses, the chitosan-treated permeate was also analysed using HPLC. HPLC conditions were as previously described (section 2.3.7).

4.3.3. Spectrophotometric method for determining colour removal

In this study it was necessary to determine the colour of samples due to the presence of *o*-quinones and associated derivatives, and hence a spectrophotometric method was used. In principle, the colour of a sample is expressed in terms describing the sensation realised when viewing the sample. In this context the term 'colour' refers to true colour. This is distinguished from 'apparent' colour by the absence of suspended solids giving rise to turbidity. Due to the difficulties encountered quantifying sensation, colour can be expressed using certain specific characteristics. These are the specific hue of the sample (green, yellow, red, etc.), the "dominant wavelength" associated with the sample, and the degree of brightness or "luminance" of the sample. These are best determined from the light transmission characteristics of the

sample using spectrophotometry (Clesceri&Greenberg,1989). The transmittance values (in percent) were determined for a set of specific visible wavelengths (Clesceri&Greenberg,1989) using a Shimadzu UV-160A spectrophotometer (Shimadzu Corporation, Kyoto, Japan) with a spectral bandwidth of 2nm. The transmittance values were then totalled and multiplied by the appropriate factors to obtain the tristimulus values X, Y, and Z, with Y being the percentage luminance. These values were determined using 10 selected ordinates. Trichromatic coefficients x and y could then be calculated from equations (1) and (2).

$$x = X/(X+Y+Z) \qquad (1)$$
$$y = Y/(X+Y+Z) \qquad (2)$$

Using the trichromatic coefficients x and y as co-ordinates, the dominant wavelength and purity could be determined from the chromaticity diagram (Clesceri&Greenberg,1989). The "hue" of the sample could be determined using the dominant wavelength (table 4.1).

Table 4.1 Colour hues as determined from the dominant wavelength ranges (Clesceri&Greenberg,1989).

Wavelength range (nm)	Hue
400-465	violet
465-482	blue
482-497	blue-green
497-530	green
530-575	greenish yellow
575-580	yellow
580-587	yellowish orange
587-598	orange
598-620	orange-red
620-700	red

The samples were also compared based on absorbance, as opposed to transmittance, over a 200nm wavelength range by doing wide-range wavelength scans between 340 and 540nm.

4.4. RESULTS

4.4.1. HPLC analysis

Due to the selective chemisorption capabilities of chitosan (section 4.1.1), phenol and derivatives do not interact with chitosan. This has previously been reported by Payne *et al.* (1992). To confirm these observations it was necessary to distinguish the respective constituents present in the permeate stream that was subjected to treatment with chitosan. Since the technique of spectrophotometry is unable to distinguish between individual species in a mixture of phenols, an effective alternative such as HPLC was necessary. Subsequent analyses using HPLC enabled the separation of the respective phenol peaks from the enzymatically-generated quinones. Figure 4.3 shows a representative HPLC chromatogram of a reaction mixture from a defined mixed synthetic substrate. Peak retention times are indicated in the figure legend with all peaks characterised by retention times of less than 4 minutes being indicative of quinones. Identification of substrate peaks, i.e. retention times greater than 4 minutes, was confirmed by spiking the individual peaks with higher concentrations of each individual species within the mixture.
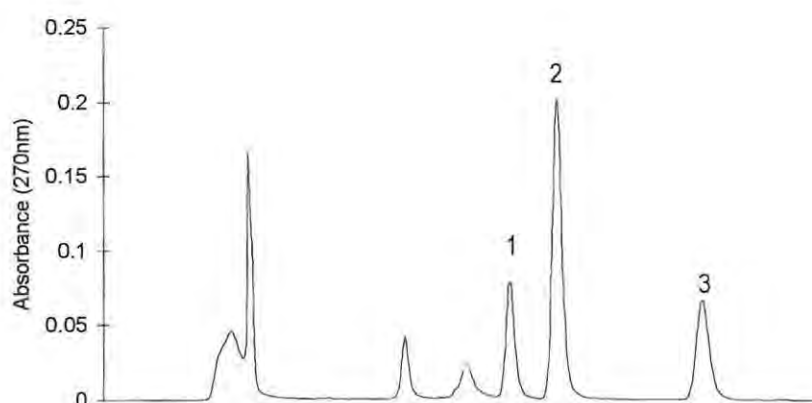
Figure 4.3 Representative HPLC chromatogram of defined synthetic substrate indicating the presence of quinone product peaks when the permeate is not treated with chitosan. Peaks are indicated as: 1) 4-methoxyphenol, retention time 4.16 minutes; 2) phenol, retention time 4.56 minutes; 3) *p*-cresol, retention time 5.91 minutes. Peaks with retention times less than 4 minutes are indicative of *o*-quinones.
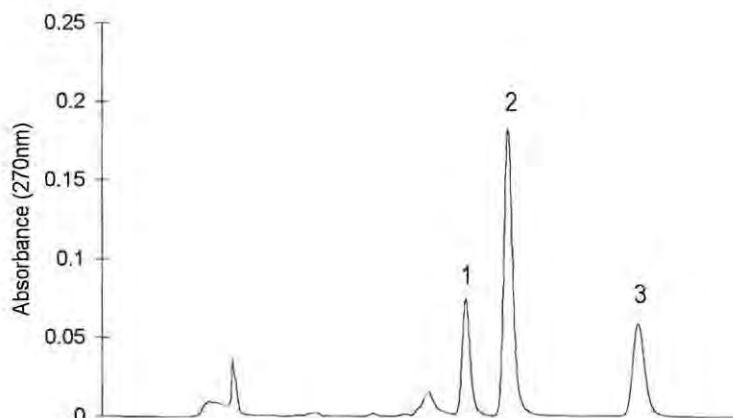
Figure 4.4 HPLC chromatogram indicating the disappearance of quinone product peaks when the permeate is subjected to treatment with chitosan. Peaks are indicated as: 1) 4-methoxyphenol, retention time 4.16 minutes; 2) phenol, retention time 4.56 minutes; 3) *p*-cresol, retention time 5.91minutes. Peaks with retention times less than 4 minutes are indicative of *o*-quinones.

Figure 4.4 shows an HPLC chromatogram of the same sample (figure 4.3), subsequent to chitosan-treatment. From the chromatogram it is evident that a considerable amount of quinone product was removed from the sample thus lending confirmation to the results obtained in sections 4.4.2 and 4.4.3. Comparing Figure 4.3 and 4.4 also show that the substrate peaks were unaffected by chitosan treatment. The HPLC analysis of chitosan-treated and untreated quinone-containing permeate, confirmed the observation of Payne *et al*. (1992) as to the selective chemisorptive nature of chitosan. The results shown were found to be representative over the concentration range of defined mixed synthetic substrate analysed, as well as for the treated industrial effluents (chromatograms not shown).

4.4.2. Spectrophotometric analysis of defined mixed synthetic substrates

The effectiveness of chitosan in the removal of colour-generating *o*-quinones was investigated by comparing spectrophotometric scans of the reactor permeate before and after passing it through the chitosan-containing column. Initial comparisons were done with the defined synthetic effluents at the specific substrate concentrations used under standard operating conditions (section 2.4.2). Figures 4.5, 4.6, and 4.7 show the spectrophotometric scans of the defined synthetic substrates within the wavelength range of 340 to 540 nm. Specific absorbance values are indicated on the individual curves representing the absorbance at 400nm, because the λ_{max} values associated with quinones is *ca*. 400nm (Duckworth&Coleman,1970).
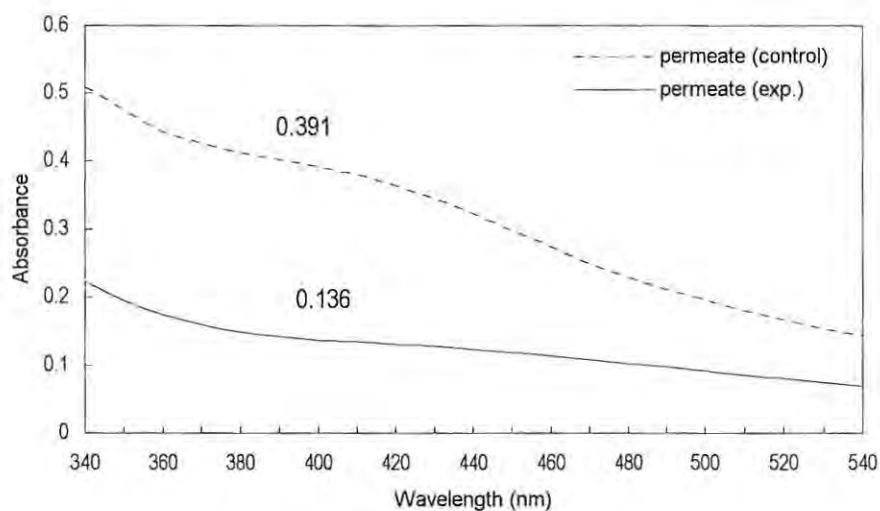
Figure 4.5 Spectrophotometric scan of defined mixed substrate (4mM). The absorbance values indicated on the curves were determined at 400nm. The permeate (control) curve represents the untreated quinone-containing SCR permeate. The permeate (exp.) curve represents the chitosan-treated quinone-containing SCR permeate.

Due to the random nature of the oligomerisation and polymerisation reactions that o-quinones undergo following formation (Kazandjian&Klibanov,1985), the rates of formation and resultant amounts of melanin-like polymeric products that form vary considerably. As a result, the rates and amounts cannot be correlated with concentration. This is confirmed by comparing Figures 4.5, 4.6, and 4.7, with attention to the absorbance values indicated on each curve. A trend that is evident, however, is that the permeate (control) curves all display characteristic shoulders between the wavelength range 390-410nm. This is indicative of the presence of o-quinones and melanin-like polymers. The curves generated for the permeate (exp.) show a general absence at all concentrations of these shoulders characteristic of the presence of o-quinones.
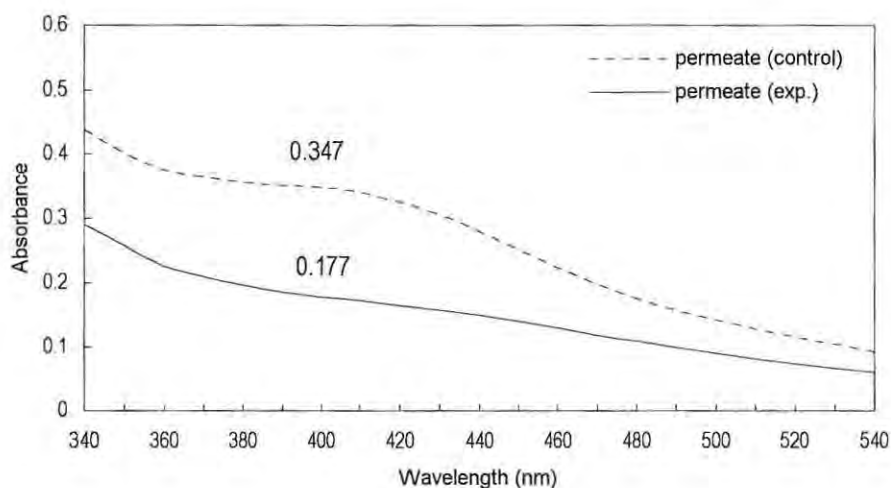
Figure 4.6 Spectrophotometric scan of artificial mixed substrate (10m*M*). The absorbance values indicated on the curves were determined at 400nm. The permeate (control) curve represents the untreated quinone-containing SCR permeate. The permeate (exp.) curve represents the chitosan-treated quinone-containing SCR permeate.
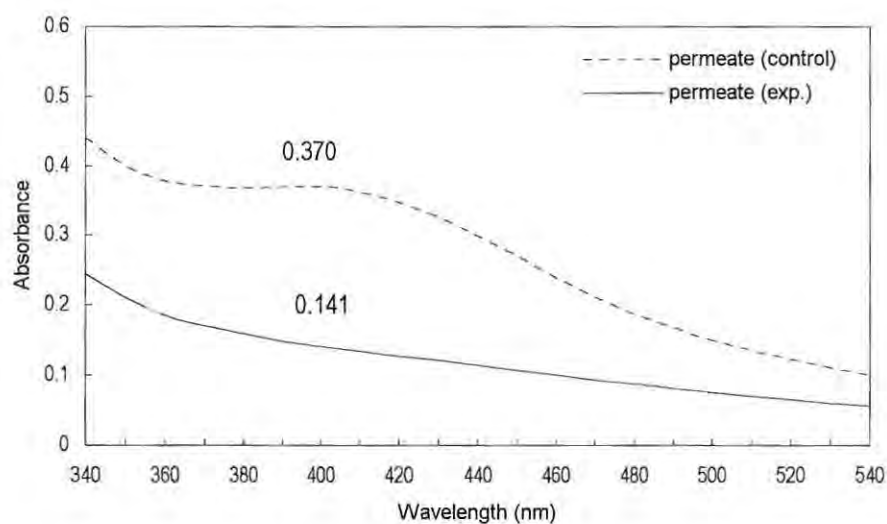
Figure 4.7 Spectrophotometric scan of artificial mixed substrate (20m*M*). The absorbance values indicated on the curves were determined at 400nm. The permeate (control) curve represents the untreated quinone-containing SCR permeate. The permeate (exp.) curve represents the chitosan-treated quinone-containing SCR permeate.

These results show conclusively the colour-removal effectiveness of the integrated chitosan column in the overall SCR configuration. Similar results were envisaged for the treatment of the industrial effluent. This was applied and confirmed in section 4.4.3.

4.4.3. Spectrophotometric analysis of industrial effluent

In view of the effectiveness of chitosan in *o*-quinone removal (demonstrated using defined synthetic effluents), the phenolic industrial effluent previously analysed were also subjected to spectrophotometric analysis to determine the effect of chitosan on *o*-quinone removal. The effluent, prior to any treatment, exhibited a characteristic colour (table 4.3) possibly related to a combination of the organic and inorganic composition (table 3.2). Initial observations from the spectrophotometric analysis was that a significant amount of the nascent effluent colour was removed due to the ultrafiltration characteristics of the capillary membranes. This colour removal capacity of ultrafiltration membranes has previously been reported for a number of different applications (Rose,1992; Jacobs *et al.*,1997). Prior to treatment by polyphenol oxidase, each effluent was subjected to membrane filtration and the subsequent permeate analysed spectrophotometrically. Figure 4.8 shows the spectrophotometric scan of the phenolic effluent (table 4.2) prior to either membrane or enzymatic treatment (substrate-control). In comparison, a significant amount of colour removal, facilitated by the capillary membrane, and manifested as the decrease in absorbance over the wavelength range scanned, is evident after subjecting the effluent to membrane filtration (permeate-control).

As outlined in section 2.4.2, the substrate is fed through the lumen, with enzyme-substrate contact being facilitated by perfusion of the substrate across the membrane. Thus, a considerable amount of colour removal occurs because of the substrate perfusing across the membrane (permeate-control), and the subsequent increase in absorbance of the permeate following enzymatic conversion is due to the enzyme-generated *o*-quinones. The curve labelled permeate (exp.) shows this increase in absorbance over the entire wavelength range scanned (figure 4.8).
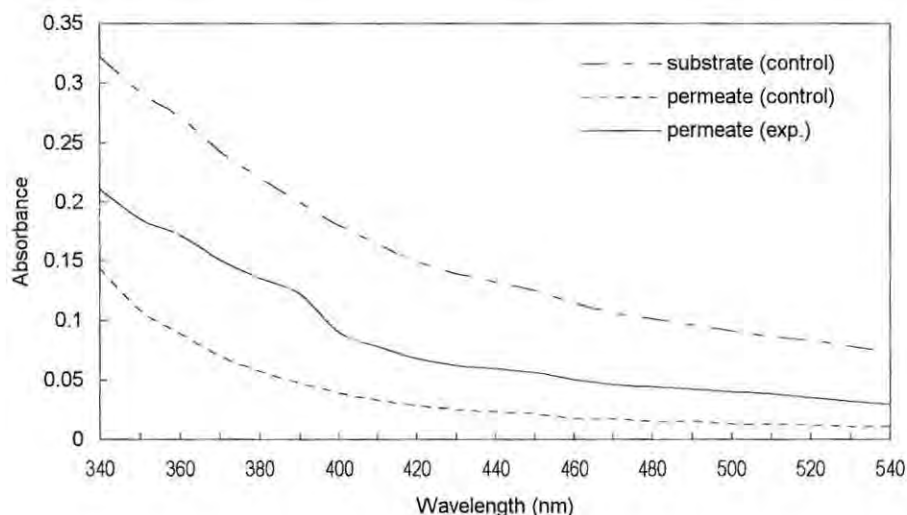
Figure 4.8 Spectrophotometric scans of the phenolic effluent during various stages of treatment. The substrate (control) curve represents the phenolic effluent. The permeate (control) curve represents the substrate after passing through the capillary membrane. The permeate (exp.) curve represents quinone-containing permeate after enzymatic conversion.

Chitosan appeared relatively ineffectual when applied to the phenolic effluent quinone-containing permeate (figure 4.9). No observable variation in the spectrophotometric analysis confirmed the chemisorptive selectivity of chitosan. Comparison of the scan of the permeate (exp.+chitosan), consisting of the quinone-containing permeate treated with chitosan, with that of the untreated quinone-containing permeate shows only extremely slight removal at *ca*. 380nm. One plausible explanation for this may be related to the nature of the effluent as shown in Table 3.2, with certain components present in the phenolic effluent permeate blocking many of the binding sites of the glucosamine units comprising the chitosan matrix. Although this conclusion is speculative, it appears to provide the most likely explanation, in view of the considerable effectiveness of chitosan in quinone removal, as already shown in section 4.4.2. Effectively the possibility of freeing up more binding sites within the chitosan matrix would circumvent this problem. A method of accomplishing this is described in Chapter 5.
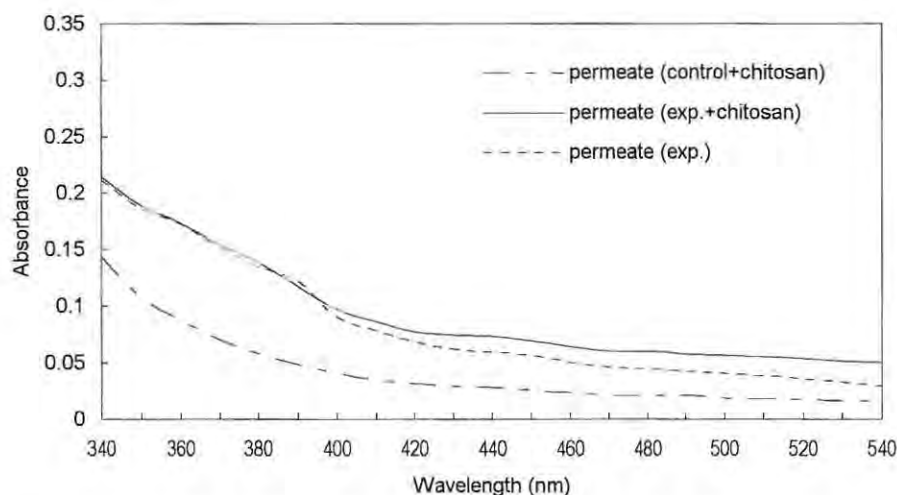
Figure 4.9 Spectrophotometric scans of phenolic effluent during various stages of treatment in the presence of chitosan. The permeate (control+chitosan) curve represents the contacted phenolic substrate, after passing through the membrane, with chitosan. The permeate (exp.+chitosan) curve represents quinone-containing permeate after contact with chitosan. The permeate (exp.) curve is representative of the quinone-containing permeate as shown in Figure 4.8. The spectrophotometric scan of the phenolic effluent (substrate-control) prior to membrane treatment is indicated in Figure 4.8.

4.4.4. Quantitative spectrophotometric analysis of defined mixed synthetic substrates

While 'colour' is representative of a subjective sensation experienced at different levels by different individuals, a quantitative determination of 'colour' can be determined as a function of certain measurable characteristics (section 4.3.3). The colour (quinone) removal capacity of chitosan can be tabulated for comparative purposes. Results obtained by the chitosan treatment of permeates produced using the defined mixed synthetic substrates are shown in Table 4.2.

From Table 4.2 it is evident that the dominant wavelengths, designated as λ, for both the untreated (control), and the chitosan-treated (experiment) permeates fall within the range 576-581nm. These results indicate that little of the dominant colour-generating species within the permeates were removed. Quinone formation as a result of the conversion by polyphenol oxidase of individual substrates, however, yields λ_{max} values of *ca.* 400nm (Duckworth&Coleman,1970) and 390nm (Sun&Payne,1996).

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Table 4.2 Results of the spectrophotometric method to determine the extent of colour removal by chitosan of the permeate from the SCRs in which mixed synthetic effluent was converted by polyphenol oxidase. Data expressed as the control (no chitosan present) and as the experiment (quinone removal due to chitosan).

Concentration	Sample	λ (nm)	Hue	Luminance (%)
1.0 mM	control	577	yellow	74.30
	experiment	576	yellow	85.96
2.5 mM	control	576	yellow	82.48
	experiment	581	yellow/orange	86.70
5.0 mM	control	579	yellow	80.95
	experiment	579	yellow	88.67

Although the defined mixtures of individual substrate species, after enzymatic treatment, are associated with λ_{max} values of much longer wavelengths, it was shown previously that significant amounts of the quinones in the permeates were removed (refer to figures 4.5, 4.6, and 4.7). As the sample hue is determined from the dominant wavelength (refer to table 4.1), from the above results it is apparent that hue and dominant wavelength are not indicative of the quinone-removal capacity of chitosan. Contrary to this, the luminance of the sample is indicative of its degree of brightness. It is evident, then, that the removal of o-quinones from the permeate contributes considerably to the brightness of the permeate.

4.4.5. Quantitative spectrophotometric analysis of industrial effluent

Low to medium molecular mass cut-off ultrafiltration membranes, due to the pores in the membrane skin-layer falling within the 10-50nm size range, are highly effective in removing components present in a feed stream that contribute to colour and turbidity (Cheryan,1986; Jacobs *et al.*,1997). Table 4.3 shows the results obtained from the quantitative spectrophotometric analysis of the phenolic effluent during various untreated and treated stages as described in section 4.4.3. Evident from Table 4.3 is the effectiveness of the ultrafiltration membrane in increasing the brightness or luminance of the filtered effluent by *ca.* 13%. These results indicate that a substantial amount of colour removal is facilitated by the membrane itself. The effects of chitosan treatment on the phenolic effluent permeate support the findings outlined in section 4.4.3, with chitosan being relatively ineffectual in decreasing the colour of the quinone-containing permeate. From Table 4.3 it can be seen that there is in fact a decrease in the luminance or brightness of

the permeate.

Table 4.3 Spectrophotometric determination of colour removal for the phenolic industrial effluent. The substrate (control) represents the phenolic effluent. The permeate (control) represents the substrate after passing through the capillary membrane. The permeate (experiment) represents quinone-containing permeate after enzymatic conversion. The permeate (control+chitosan) represents the contacted phenolic substrate, after passing through the membrane, with chitosan. The permeate (experiment+chitosan) represents quinone-containing permeate after contact with chitosan.

	Substrate (control)	Permeate (control)	Permeate (experiment)	Permeate (control+chitosan)	Permeate (experiment+chitosan)
λ (nm)	575	570	570	570	570
Hue	yellow	greenish/yellow	greenish/yellow	greenish/yellow	greenish/yellow
Luminance (%)	85.71	98.4	94.63	97.13	90.21

4.5. DISCUSSION

The question of whether chitosan is effective in the removal of enzyme-generated o-quinones from SCR permeate was resolved by the reported combination of HPLC, spectrophotometry, and quantitative spectrophotometric analysis. Due to the dominant wavelengths, as determined using the trichromatic method, falling in the range 576-581nm, direct quantitative comparison of o-quinone removal cannot be made due to the λ_{max} for o-quinones being *ca*. 400nm. A quantitative comparison would be possible using HPLC to show the effectiveness of o-quinone removal using chitosan, however, due to the unstable nature of o-quinones in aqueous solution (Sun&Payne,1996), attempts at determining o-quinone concentrations using HPLC at 400nm proved non-reproducible (chromatograms not shown). The combination of HPLC analysis and the results obtained from the trichromatic method were therefore deemed significant enough to provide a useful means by which the effectiveness of chitosan on colour removal could be determined. Although chitosan was relatively ineffectual in the treatment of the quinone-containing phenolic effluent permeate, the reason for this is possibly that chitosan is affected by the major limitation inherent when using any adsorption material, namely a small surface area.

4.6. CONCLUDING REMARKS

The effectiveness of chitosan for the removal of enzyme-generated o-quinones is evident from the data

presented here. However, further use of chitosan could warrant careful consideration in two main areas:

- Firstly, the use of chitosan in scale-up operations would necessitate a requirement for large volumes of chitosan. As chitosan is a waste-product of the shell-fish industry, the economic implications for obtaining large volumes of biosorbent are not necessarily limiting.

- *In situ* quinone removal using biosorption with activated carbon and chitosan has, in the past, been limited mainly by low adsorptive surface area (Sun *et al.*,1992; Wada *et al.*, 1993; Juang *et al.*,1996).Therefore, the second consideration, requiring considerable attention, is the possibility of increasing the reactive surface area of the biosorbent thus facilitating greater *o*-quinone removal efficiencies. In conjunction with an increased surface area, it would be necessary to engineer greater and more efficient contact of the chitosan with the *o*-quinones at a micro-environment level as close to the site of *o*-quinone generation as possible.

It is the second consideration that is dealt with in detail in the following chapter.

CHAPTER 5

CHITOSAN-COATED CAPILLARY MEMBRANES FOR INDUSTRIAL EFFLUENT TREATMENT

5.1. INTRODUCTION

The effectiveness of chitosan-flakes in a packed column for the removal of colour-generating o-quinones from immobilised polyphenol oxidase-process permeate has been demonstrated (see Chapter 4). Two major material limitations exist, however, when using chitosan for this application. Firstly, chitosan is non-porous and therefore to provide a sufficient surface-area-to-mass ratio for high capacity adsorption, the chitosan must be ground to a powder form. Secondly, chitosan powder is unsuitable for packed-bed adsorption columns due to associated high-pressure drop and the potential for bed fouling at low void volumes (Hsien&Rorrer,1995).

Although the chitosan biosorbent step was integrated within the capillary reactor configuration (section 4.3.2), greater colour-removal efficiency would be achieved if quinone-chitosan contact occurred at the site of o-quinone generation, as opposed to downstream contact within the reactor configuration. Due to its solubility in dilute organic acids, chitosan can be processed into different geometrical configurations including membranes, fibres, hollow-fibres, capsules, and beads (Krajewska et al.,1996). Acetic acid is the most widely used solvent for this biopolymer, while oxalic and dichloroacetic acid also show significant interactions with chitosan (Muzzarelli et al.,1982; Payne&Sun,1996).

Of the various configurations, chitosan beads have found application as an immobilisation matrix for α-amylase and invertase for starch and sucrose hydrolysis respectively (Siso et al.,1997), as well as for cadmium ion adsorption (Hsien&Rorrer,1995). Chitosan membranes have also found application in the separation of water-ethanol mixtures by pervaporation (Uragami&Takigawa,1990; Lee&Shin,1991).

5.2. RESEARCH OBJECTIVES

Of importance to the current investigation is the amalgamation of the directives identified in the preceding chapter. These can be summarised as the potential for increasing the surface area for the quinone-chitosan interaction in conjunction with increasing the contact of quinone with chitosan at a

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micro-environment level i.e. the membrane surface. The following research objectives were therefore identified for further investigation:

- The feasibility of contacting capillary membranes with a viscous chitosan solution thereby facilitating the formation of a double composite membrane i.e. polysulphone and chitosan

- Whether the reactive amine group of the individual glucosamine units would facilitate increased enzyme immobilisation resulting in high enzyme-per-unit-surface-area loading capacity.

- Whether greater reaction-product adsorption would be achieved due to the high surface-area-to-mass ratio as a result of the chitosan coating.

- If the combination of the two previously mentioned objectives would result in greater enzyme-catalysed phenol conversion efficiency.

5.3. MATERIALS AND METHODS

Mushroom polyphenol oxidase (E.C. 1.14.18.1) was obtained from Sigma Chemicals (St. Louis, MO, U.S.A.) and assayed as described in section 2.3.1. Chitosan was obtained from Sigma Chemicals (St. Louis, MO). A phenolic industrial effluent was obtained from a South African coal-gas plant (table 4.3).

5.3.1. Preparation of chitosan-coated membranes

Chitosan-coated membranes were prepared according to the following procedure: 1% chitosan (w/v) was dissolved in 0.8% acetic acid (v/v) and stirred overnight. Undissolved chitosan and debris was removed by centrifugation at 5000 rpm for 20 minutes. The viscous chitosan solution (pH 4.13) was recirculated across the capillary membrane at a flow rate of $0.150L.h^{-1}$ for 8 hours. Following this coating procedure the chitosan coating was then neutralised with 8% NaOH (pH 13.40) for 30 minutes. The coated membranes were then rinsed with 0.1M phosphate buffer until a pH of 6.80 was reached.

5.3.2. Immobilisation of polyphenol oxidase on chitosan-coated membranes

SCRs were constructed and operated as previously described (section 2.3.1 and 2.3.2).

5.3.3. Determination of the enzyme loading capacity of chitosan-coated membranes

Variations in the protein-loading capacity of the membranes was investigated. The extent of enzyme immobilisation for chitosan-coated and non-coated membranes was compared by sampling during the immobilisation procedure. These samples were subjected to protein determination according to the method of Bradford (1976) as outlined in section 2.4.5. After immobilisation the membranes were washed with phosphate buffer (pH 6.71) to remove residual protein not immobilised by adsorption.

5.4 RESULTS

5.4.1. Phenol removal capacity of polyphenol oxidase on chitosan-coated membranes

Experiments using the composite membrane for phenol removal were done only to demonstrate the effectiveness of the chitosan coating. An in-depth study was not deemed necessary because the chitosan coating could not be removed from the membrane once saturation of the chitosan was complete (section 5.6). Significant differences in the capacity to bind polyphenol oxidase and to remove phenol were observed between bioreactors where polyphenol oxidase was immobilised on chitosan-coated and non-coated membranes (table 5.1).

Table 5.1 Substrate conversion (phenolic industrial effluent) by chitosan-coated, and non-coated, membrane-immobilised polyphenol oxidase.

	Immobilised enzyme	Total substrate converted	Substrate converted per Unit enzyme
Chitosan-coated membranes	143 Units	1224.4 mg	8.6 mg.Unit^{-1}
Non-coated membranes	55 Units	20.3 mg	0.4 mg.Unit^{-1}

5.4.2. Membrane- and chitosan-mediated colour removal

The industrial effluent used in this study had considerable colour initially, and the ultrafiltration capability of the membrane was found to remove a significant amount of suspended solids which contributed to this initial brown colour. Figure 5.1 shows the colour removal of the effluent facilitated by the membrane itself. The membrane-mediated removal of a significant amount of suspended solids as well as part of the soluble colour-generating fraction of the industrial effluent is indicated in Figure 5.2 as permeate 1. Comparisons were then made of colour in the permeates from bioreactors in which polyphenol oxidase was immobilised in the presence and absence of the chitosan coating, and with chitosan which had not been subjected to acetic acid and NaOH treatment. The colour generated by the

o-quinones was almost completely removed (figure 5.2 shows this as permeate 2) by the viscous chitosan coating, as opposed to a significantly lower colour removal capacity when the permeate was treated with non-acid/NaOH treated chitosan and stirred overnight (permeate 3). No colour was removed in the absence of chitosan.
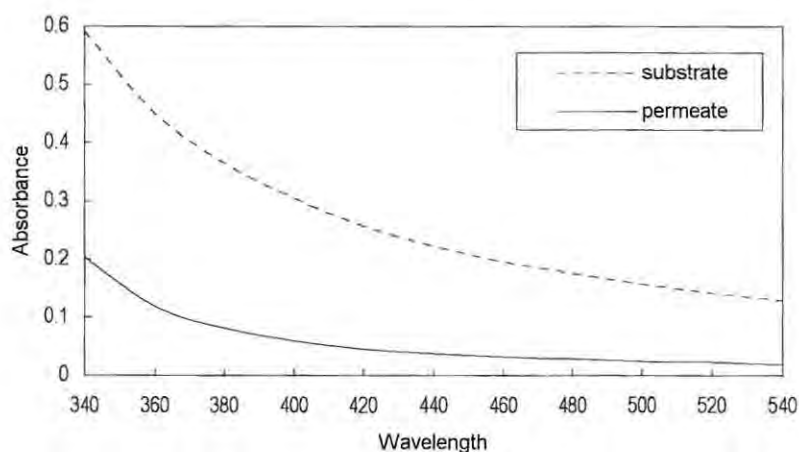
Figure 5.1 Spectrophotometric analysis showing membrane-facilitated colour-removal of the phenolic effluent. The substrate curve represents the phenolic effluent. The permeate curve represents the substrate after passing through the membrane.
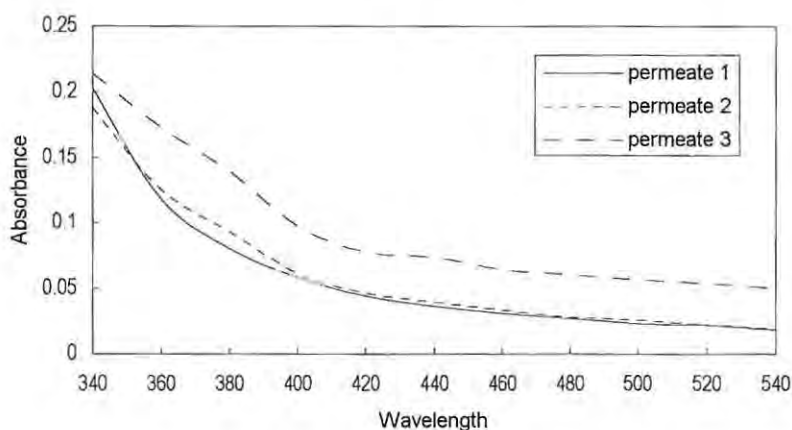
Figure 5.2 Spectrophotometric analysis showing the decrease in permeate colour as a result of quinone removal facilitated by different chitosan treatment methods. The permeate 1 curve represents the substrate after passing through the membrane. The permeate 2 curve represents the colour removal facilitated by the chitosan coating. Permeate 3 represents colour removal by chitosan flakes.

5.5. DISCUSSION

Over an 8-hour period, a maximum total phenol conversion of 1224.4 mg phenol was achieved using 143 Units polyphenol oxidase on chitosan-coated membranes as compared with 20.3 mg removed using 55 Units of polyphenol oxidase with non-coated capillary membranes (table 5.1). While loss in activity was observed for polyphenol oxidase on both coated and non-coated membranes, the activity retention was far greater for polyphenol oxidase immobilised on the coated membranes. The level of phenol conversion in this case decreased to 53.9 mg removed over a final two-hour period. In comparison, for non-coated membranes, phenol conversion decreased to 4.6 mg in the final two hours. The greater retention of productivity can be attributed, firstly, to the greater amount of immobilised enzyme on the coated membranes, and secondly, to the removal of the resultant *o*-quinones from the immediate environment of the enzyme due to adsorption by the chitosan, which would decrease the *o*-quinone-related inactivation of polyphenol oxidase.

Although a 62% increase (table 5.1) in enzyme immobilisation was achieved using coated-membranes compared with non-coated membranes, the difference in substrate conversion over an equivalent time period was not only attributed to a greater enzyme-loading capacity. From Table 5.1, it is evident that immobilised polyphenol oxidase in the presence of the chitosan-coating exhibited a 21.5-fold increase in the amount of substrate converted per Unit enzyme ($8.6mg.Unit^{-1}$) in comparison to immobilised polyphenol oxidase on non-coated membranes ($0.4mg.Unit^{-1}$).

5.6. CONCLUDING REMARKS

The initial objective of forming a composite chitosan-polysulphone membrane was effectively achieved using a 1% (w/v) concentration of chitosan as outlined in section 5.3.1. Secondly, the major advantage of forming a gel-like layer of chitosan on the capillary membranes is that greater protein loading capacities can be achieved, as compared with non-coated membranes. This, combined with the effect of chitosan providing an *in situ* product removal function at a micro-environment level, in combination with a greater enzyme loading capacity, allows for high levels of phenol removal to be achieved. Two functions are therefore served using chitosan-coated capillary membranes and immobilised polyphenol oxidase: an initial highly efficient dephenolisation process and effective *in situ* colour removal from the resultant permeate. In addition, the presence of chitosan contributes considerably to decreasing the product inhibition which is characteristic of polyphenol oxidase.

However, a significant limitation of using chitosan to coat the membranes is that the gel-like coating has a finite loading-capacity for either polyphenol oxidase or o-quinones. Attempts at removing the coating using alkaline, acidic conditions, and using organic solvents proved ineffectual. This culminated as a severe disadvantage due to polysulphone being prone to degradation under acidic conditions and under conditions of contact with most organic solvents. Thus, reuse of the membranes after removing the coating was not feasible. As a result of the severe limitations associated with reusing the polysulphone membranes, an alternative reactor design was envisaged as being more practical for maintaining high productivities, as demonstrated using chitosan-coated membranes, but at the same time obviating the need to carry on the application of the chitosan-polysulphone composite membrane for achieving these high productivities. Hence, the use of transverse-flow membrane modules with membranes arranged in a plane perpendicular to the longitudinal axis of the module as opposed to the axial membrane orientation within the single-capillary reactor.

CHAPTER 6

DESIGN AND CONSTRUCTION OF A TEST RIG FOR THE OPERATION OF TRANSVERSE-FLOW MEMBRANE MODULES

6.1. INTRODUCTION

It was envisaged that the polyphenol oxidase/membrane bioremediation system could be more accurately assessed in the context of remediation efficiency by treating a larger volume of industrial effluent. It was therefore proposed that a modular scale-up approach from the single-fibre reactors to a membrane module configuration with greater industrial application was necessary to determine the efficiency of the enzyme-immobilised membrane approach to bioremediation.

The scale-up procedure can be considered to involve the process or group of activities by which one moves from the calculations, studies and demonstrations to a successful commercial operating facility (Bisio&Kabel,1985). When the stage of designing a reactor is reached, the project, of which the reactor is integral, will as a whole have already acquired a fairly definite form (Richardson&Peacock,1994). The primary objective of rational design can therefore be to produce the plans necessary to build economically viable systems that satisfy well-considered performance criteria. According to Lyderson *et al.* (1994), scale-up criteria usually fall into the following categories:

- Primarily theoretical, i.e. based on assumptions and simplifications that are not valid for most reactions.
- Single-parameter objectives (e.g. maintenance of oxygen transfer level).
- Experiments involving scale-up from very small to small.
- Experiments done under very idealised conditions which are impractical for commercial applications.

These scale-up criteria require careful overall consideration prior to the outset of reactor design because scale-up based solely on any of these defined criteria will result in a reactor prone to severe short-comings. These may manifest as singular inefficiencies or as combinations thereof.

6.2 RESEARCH OBJECTIVES

The basic objectives in the design of a reactor can be determined by considering the overall purpose of the reactor. According to Richardson and Peacock (1994), the generalised aim in any reactor design is "to produce a *specified product* at a *given rate* from *known reactants*". Within the confines of these broadly defined constraints, the objective of designing a bioreactor for bioremediation purposes would be the maximisation of reactor output defined as the most efficient minimisation of influent pollutant concentration.

At the outset of design, certain fundamental considerations need to be considered:

    1. The method of reactor operation and more specifically, the type of reactor to be used.

    2. Mode of operation, i.e. batch, continuous, or a combination.

    3. If the reactor is to be operated isothermally, adiabatically, or under intermediate conditions.

    4. The physical conditions of the influent reactants and effluent products.

Due to the composition of the reactants (phenolic effluent) and the initial feasibility studies already described, the majority of these considerations have already been dealt with. Within the context of this project, the fundamental objective to be reached was to develop a modular test unit capable of operating under different, but controlled, conditions based on a reactor type with greater productivity than single-fibre capillary membrane reactors.

6.3. REACTOR AND OPERATIONAL CONSIDERATIONS

6.3.1. Reactor type selection

Although the main focus of a scale-up procedure is the operating characteristics in terms of the specific goal of the scaled-up reactor, reactor selection and reactor design optimisation are iterative procedures. In the context of the design of this reactor, important considerations to be taken into account were flexibility, cost minimisation, and product selectivity or reactor productivity. In practice, minimisation of reactor cost is considerably less significant than the reactor's ability to maximise product selectivity or productivity (Kabel,1985).

When choosing a membrane reactor, an important consideration to be taken into account is that the membrane module geometry generates a number of influencing factors apart from the positioning of the capillary membranes. It also has to provide a well-defined hydrodynamic feed and permeate flow across

the membrane surface. This in turn leads to control of concentration polarisation and optimisation of the trans-membrane driving force.

6.3.2. Transverse-flow membrane modules

Although conventional capillary membrane modules have a high membrane area per unit volume and the capillaries are self-supported against pressures applied from both the inside and outside, in practice less than optimal performance occurs as a result of flow maldistribution and poor control of concentration polarisation (Yang&Cussler,1986; Futselaar,1993). This is attributed to:

- The shape of the capillaries preventing the application of turbulence promoters near the membrane surface.

- Regular arrangement of capillaries within modules, necessary to provide well-defined flow distribution in the module shell-side, is difficult because of the long length and small diameters of the capillaries.

- The internal diameters of the capillaries should be large enough to allow for acceptable pressure drop within the capillary module and small enough to ensure good packing densities within the module.

- In economically large scale modules, relatively large back-pressures occur within the lumen due to the length of the capillaries.

These limitations can be overcome by orientating the capillaries transverse instead of parallel to the module axis. The regular arrangement of capillaries within the transverse-flow membrane module prevents flow maldistribution over the cross-section of the module. As a result, optimal use of the installed membrane area in the absence of channelling is possible. By orientating the capillaries perpendicular to the direction of the feed-flow, turbulence is generated by the fibres themselves, thus obviating the need for higher feed flow velocities or auxiliary turbulence promoters (Futselaar *et al.*, 1993; Futselaar,1993). The transverse-flow membrane module (figure 6.1) is an attempt to circumvent the limitations associated with conventional capillary membrane modules. Transverse-flow modules were obtained from the Institute for Polymer Science, Stellenbosch University.

A transverse-flow module (shown schematically in figure 6.2) is composed of an inner body comprised of a number of grids stacked together. Each grid comprises a membrane support and a series of

capillary membranes regularly arranged in a plane which is perpendicular to the longitudinal axis of the module, i.e. transverse to the direction of flow (Futselaar,1993). The membranes are arranged in a regular pattern to achieve optimum geometry. Optimum membrane spacing has been shown to be critical in achieving maximum cell density in applications of immobilised mammalian cell culture (Chresand *et al.*,1988). Although this application involves immobilised enzymes as opposed to immobilised cells, membrane spacing is critical because of its influence on oxygen mass transfer to the immobilised polyphenol oxidase.

In the present application, the stream through the lumen is the feed stream and the shell-side is the permeate stream. The inner body of a transverse-flow module comprises the assemblage of one or more grids as a fluid-tight connection. The outer body or module housing is composed of the inner body and the channels or connections for feed stream supply. The importance of the membrane arrangement being regular and transverse to the direction of flow is that high-pressure drop over the grids can be avoided. Secondly, flow maldistribution over the cross-section of the module can effectively be eliminated (Futselaar,1993).
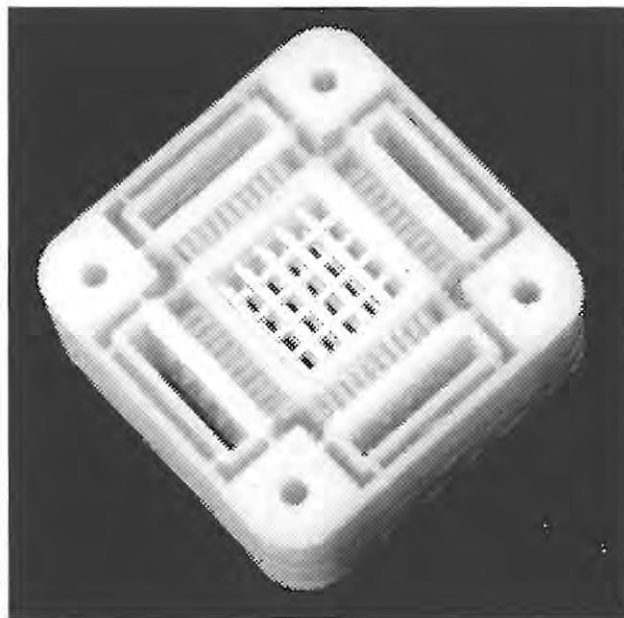
Figure 6.1 The transverse-flow capillary membrane module.

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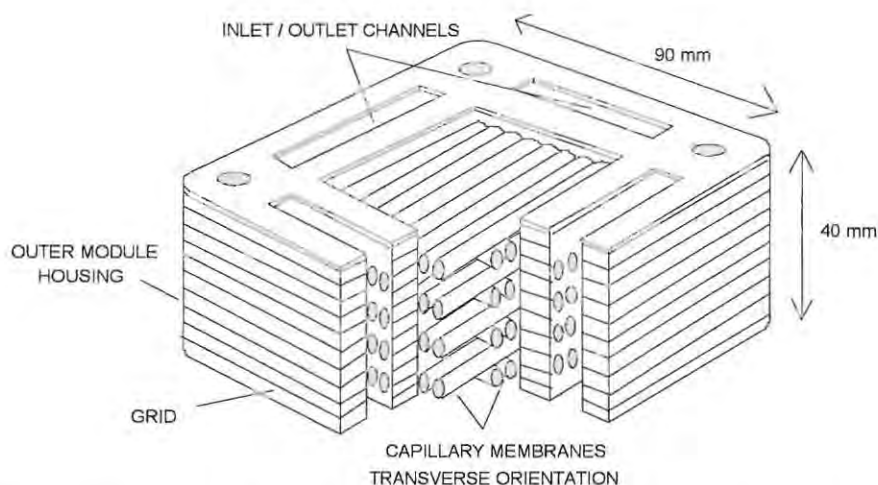
Figure 6.2 Cut-away schematic of the transverse-flow capillary membrane module.

6.3.3. Manifold design and development

The development of a novel manifold (figure 6.3) was found to be essential for maintaining high levels of efficiency during the operation of the transverse-flow modules. It was found through previous experience that conventional flat polypropylene manifolds were unfavourable in terms of promoting hydraulic hold-up within the shell-side of the modules. As a result, permeate exit from the module was erratic and extreme variations in exiting permeate volume were observed. In view of the inactivation effect of polyphenol oxidase-generated products, the retention of the *o*-quinones within the module itself was highly undesirable. Thus, a cone-shaped permeate dissipater was included in the manifold design, and this proved efficient in decreasing the hydraulic hold-up that occurred with the previous manifold design. A number of other advantages were also found to be associated with the new manifold. These included:

1) a decreased "bowing" effect of the manifold. This was attributed to the thickness (30mm) of the manifold. The previous manifold (10mm) was found to bend under stress during the clamping procedure when the transverse-flow modules were sealed.

2) The thickness of the polypropylene manifold also facilitated the inclusion of flow channels. The transverse lumen inlet and outlet configuration could therefore be fed using one inlet channel (figure 6.3).
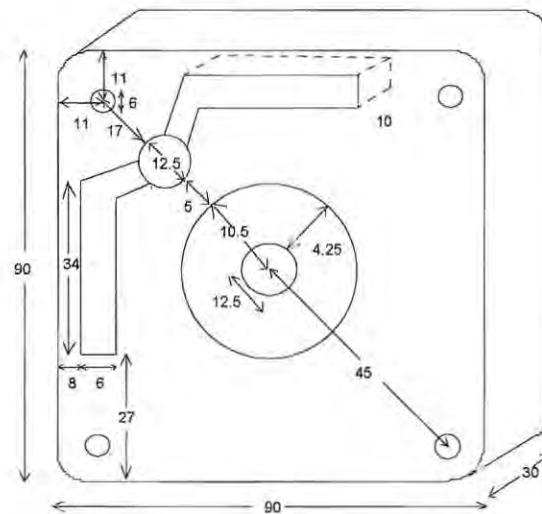
Figure 6.3 Schematic of the manifold design showing the single inlet channel and permeate dissipater. Dimensions are given in millimetres.

6.3.4. Transverse-flow membrane module characteristics

IPS 763 unskinned polysulphone membranes were used in the transverse-flow modules giving a total internal surface area of $0.0113m^2$. The total extra-capillary reactor volume of the transverse-flow module was $36cm^3$ and $45cm^3$ with the extra volume contributed by the cone-shaped dissipaters integrated within the novel manifold design.

6.4. DESIGN OF THE TRANSVERSE-FLOW MEMBRANE MODULE TEST-RIG

For the initial design of the modular enzyme-immobilised capillary membrane reactor test-rig, a rudimentary knowledge of the general configuration of the reactor, and necessary integral features within the reactor itself, was fundamental in initiating preliminary construction. Because transport of reactants to, and products from, the vicinity of the biocatalyst can have serious implications in the performance of biological systems, serious consideration of the operational characteristics of the reactor were elementary to the design of the test-rig (Kargi&Moo-Young,1985).

The basic requirements and components necessary for the development of an enzyme-immobilised capillary membrane reactor capable of treating industrial effluent are indicated in Figure 6.4. Thus, in the preliminary stages of the reactor design, the principal features of the bioreactor outlining the main and associated components in its construction, were decided upon.

Because of the scale and application of the envisioned reactor, six essential parameters needed to be assessed concerning the overall design. These are summarised as:

1. The arrangement of the reactor, i.e. horizontal or vertical.

2. Whether the reactor would be fixed or portable.

3. The pressure range of the reactor, i.e. vacuum, atmospheric, or pressurised.

4. The material of construction, i.e. whether the majority of the reactor was to be plastic, metal or some other non-metal.

5. If the reactor was required to handle liquids, gases or solids, or a combination of these.

6. In terms of size, could construction be implemented within a workshop (less than $10m^3$) or would construction necessarily be on-site.
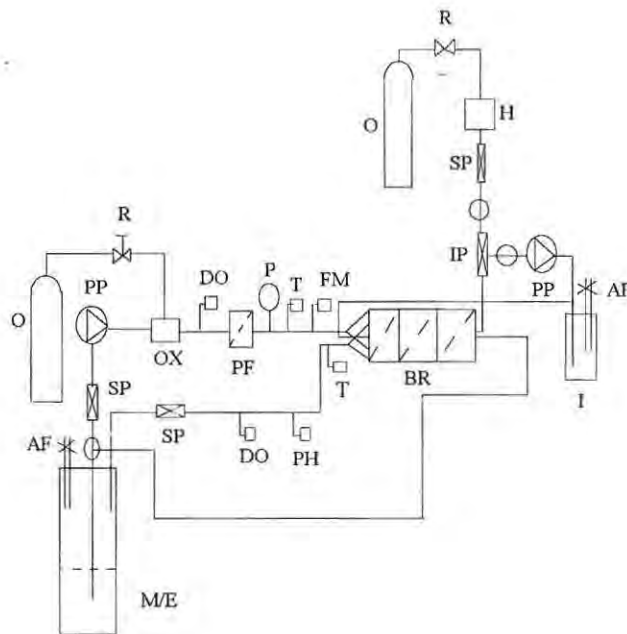
Figure 6.4 Initial bioreactor design indicating the proposed components thought necessary to be integrated within the overall reactor configuration.

Key:	O: oxygen	PP: peristaltic pump	PH: pH probe	H: humidifier
	T: temperature probe	R: regulator	FM: flow meter	IP: inoculation port
	OX: oxygenator	SP: sample port	PF: pre-filter	AF: air filter
	M/E: medium/effluent	DO: dissolved O_2 probe	BR: bioreactor	P: pressure gauge
	I: inoculum			
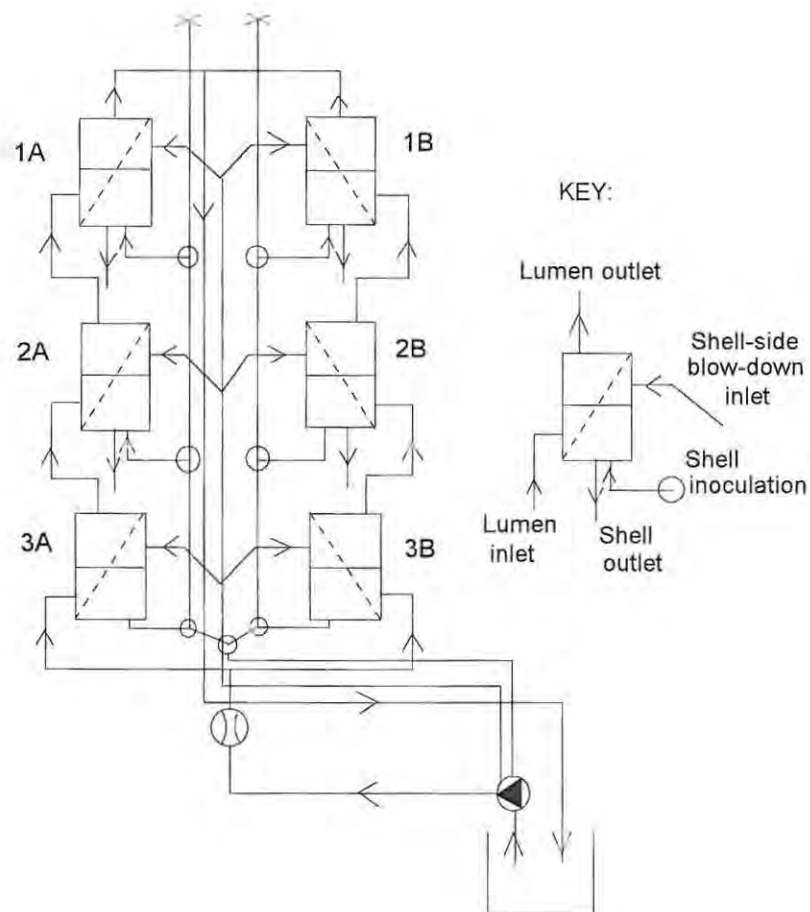
Figure 6.5 Schematic of the final reactor design indicating series and parallel arrangement of the transverse-flow capillary membrane modules.

In accordance with these parameters, and based on the type of effluent to be treated, a number of criteria concerning the design could be identified. Firstly, the reactor was designed as a vertical arrangement within a frame enabling portability of the entire assembly. Assembly was therefore possible within a workshop environment. Stainless steel was used for the reactor construction because of the necessity to pressurise the reactor feed. The use of stainless steel was supported by the fact that the industrial effluent to be tested in the bioreactor was highly corrosive in nature due to chemical composition and pH. The final reactor design is shown schematically in Figure 6.5. Figures 6.6 and 6.7 show the completed reactor as seen from the front and behind respectively.
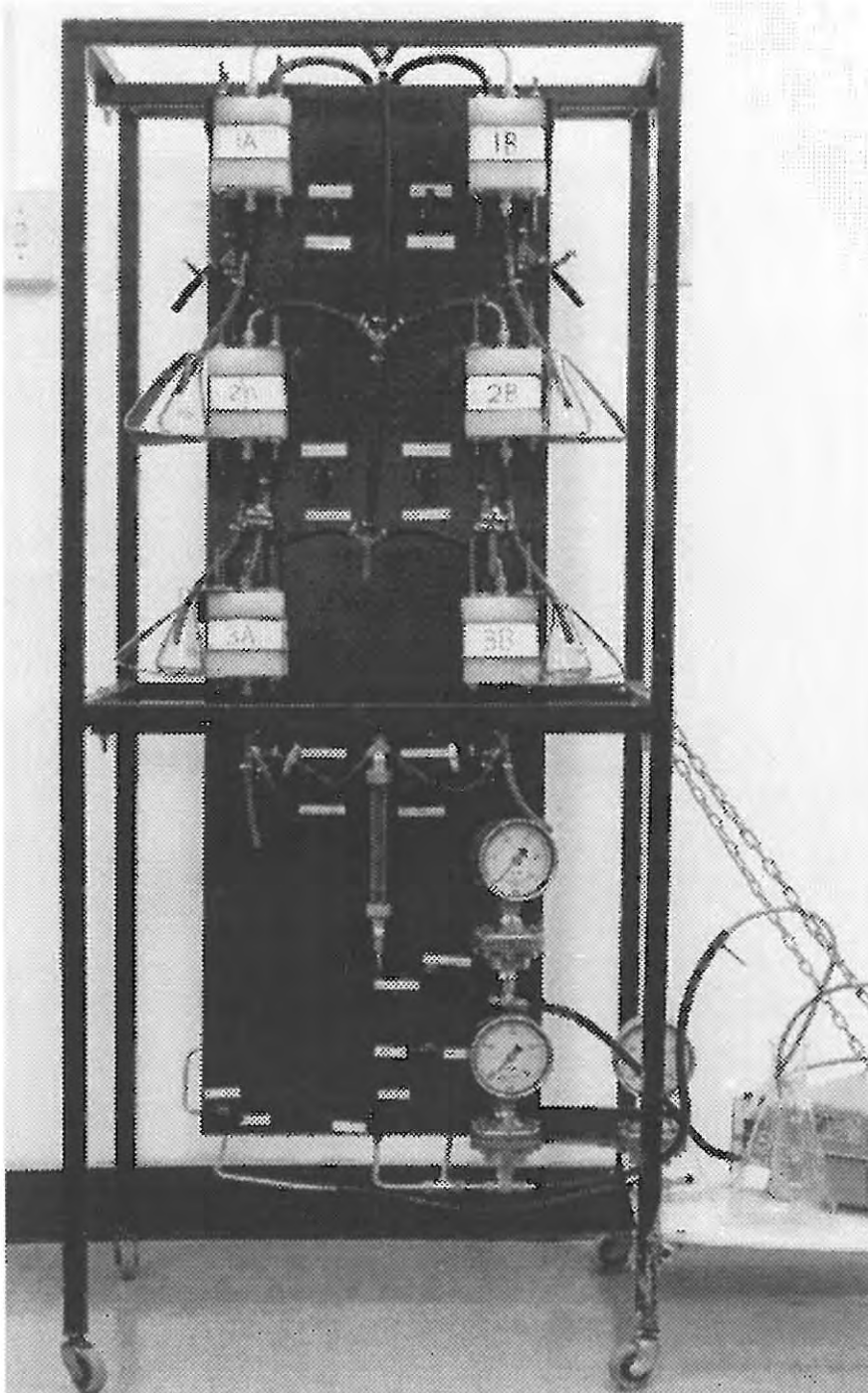
Figure 6.6 Complete modular reactor as seen from the front.
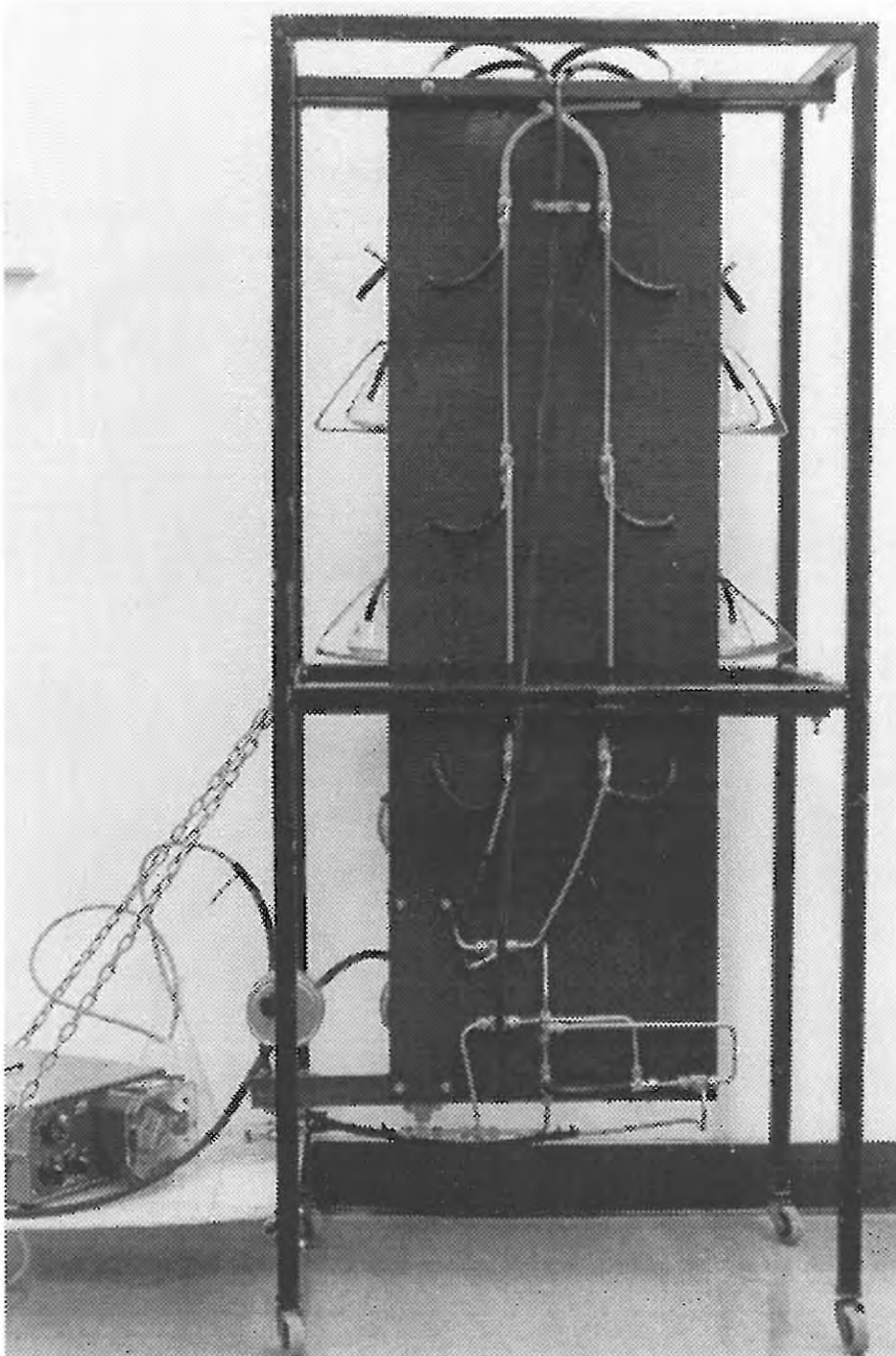
Figure 6.7 Complete modular reactor as seen from behind.

6.4.1. Cost analysis of demonstration unit construction

The total cost breakdown for the demonstration unit is shown in Appendix III. However, the two most fundamental considerations for an accurate economic assessment of the reactor test-rig described have been omitted. The major economic drawback in the scale-up of membrane-based reactors is the cost of

the membranes and the modules in which they are housed due primarily to the cost of the injection mold. As part of the collaborative research with the Institute for Polymer Science, transverse-flow membrane modules were provided by the institute at no charge. Secondly, the reactor was constructed in-house, therefore labour costs were not accounted for.

6.5. CONCLUDING REMARKS

As was previously reported in Chapter 2, the influence of enzyme-substrate contact was shown to play a considerable role in the substrate conversion potential of the immobilised polyphenol oxidase. The rationale behind determining what influence a fundamentally different reactor configuration would have on the potential productivity of the immobilised polyphenol oxidase system would therefore be based on determining the effect on productivity of varying rates of enzyme-substrate contact within the transverse-flow membrane bioreactor. To test this hypothesis, it was necessary to construct a test-rig capable of operating a number of transverse-flow membrane modules under conditions where enzyme immobilisation, feed make-up, and oxygen mass transfer could be controlled. The operating condition that had to be variable was the rate at which enzyme-substrate contact occurred. Thus, the fundamental requirement for the test-rig was to be able to operate each transverse-flow module under different conditions of trans-membrane flux. It was thus decided that certain considerations needed to be taken into account for the design of the test-rig.

Substantial evidence is required to confirm the practicality of the reactor design outlined in this chapter. This was obtained by evaluating the hydrodynamic characteristics of the modular scaled-up bioreactor prior to an evaluation of the efficiency of the reactor as defined at the outset of the initial reactor design. An accrual of empirical observations concerning the hydrodynamic flow characteristics of the reactor will therefore have direct implications for theoretical considerations of the behaviour of effluent feed streams within the reactor. These considerations are dealt with in detail in the following chapter. The acquired knowledge of the flow characteristics within the reactor was then applied to determining the efficiency of this reactor in the treatment of industrial phenol-containing effluent.

CHAPTER 7

THE USE OF TRANSVERSE-FLOW MEMBRANE MODULES FOR THE TREATMENT OF INDUSTRIAL EFFLUENT

7.1. INTRODUCTION

Theoretical considerations associated with novel processes in biotechnology are closely linked to an inherent requirement for practical demonstration. Thus, progress and potential need to be demonstrated at both the theoretical and applied levels. The accrual of fundamental, theoretical, and speculative data and results acquired earlier in this study require amalgamation in the form of a technical demonstration of the system proficiency.

7.2. RESEARCH OBJECTIVES

The broad objective of the final part of this investigation could be divided into two sections. These are defined as follows:

- To demonstrate the hydrodynamic characteristics of the transverse-flow module test rig.
- To initiate and monitor the successful operation of the reactor in the applied treatment of industrial effluent.

The fundamental goal of these objectives is the overall determination of the reactor efficiency as a unit in the minimisation of industrial effluent pollutant concentration. The fundamental application is therefore directed at industrial effluent treatment as opposed to defined mixtures of synthetic substrates.

7.3. MATERIALS AND METHODS

7.3.1. HPLC of samples

Analysis of samples was performed as in section 2.3.7.

7.3.2. Residence time determination (RTD)

7.3.2.1. Determination of reactor fluid dynamics

In tubular-flow reactors operation is ideal when back-mixing of reactants and products is absent. Thus in the *ideal* tubular-flow reactor the feed or reaction mixture is transported in a state of *plug flow*.

Under plug flow conditions it also assumed that not only is the local mass flow-rate uniform across any section normal to the flow-rate, but the fluid properties such as temperature, pressure, and composition are also uniform. Under ideal conditions, all elements of the fluid within the reactor pass through the reactor at the same time and are subject to the effect of temperature, pressure, and compositional changes in the same sequence and at the same intensity. In reality, however, fluid flow within reactors is far from ideal. Because of this, any departure from ideal flow needs to be taken into account (Levenspiel,1972; Richardson&Peacock,1994).Various types of non-ideal flow patterns in reactors can be identified. Of these, the most relevant appear to be channelling, internal recirculation, and the presence of stagnant regions. The identification of variations in reactor flow-rate characteristics is fundamental in any scale-up procedure because one of the major problems is that flow-patterns often change with a change in scale (Richardson&Peacock,1994). By having a velocity distribution map for fluid within the reactor it is possible to predict to some extent the behaviour of a vessel as the reactor. Although this applies in principle, it is invariably an impractical approach to use due to many attendant complexities (Levenspiel,1972).

7.3.2.2. Tracer methods for flow characterisation

Whether the fluid dynamics or flow characteristics of a reactor are ideal or non-ideal can be determined by the introduction of a non-reactive tracer into the reactor. Analysis of the exit stream following the injection of a tracer can provide valuable information regarding the nature of the flow. In the present study, the fluid characteristics of the test-rig were determined by applying an instantaneous tracer pulse to the stream entering the vessel (stimulus-response experiment). The exit stream of this pulse input experiment was then sampled and the data analysed to determine flow conditions operating within the test-rig. This provided information showing that certain elements of fluid taking different routes within the transverse-flow membrane module test-rig require different lengths of time to pass through the modules as well as the hydraulic delivery elements of the test-rig. It is the distribution of these times for the reactor effluent stream leaving the test-rig that is referred to as the residence time distribution (or exit age distribution).

7.3.2.3. Determining RTD using tracer methods

The RTD was determined by spiking the reactor inlet with a concentrated NaCl solution (40mL; 3M) and monitoring the NaCl concentration exiting in the permeate of each transverse-flow module in the reactor using a salinity refractometer (Atago, Japan). NaCl concentrations were also determined in the

outlet feed to monitor the exit of the tracer solution. The feed solution (Milli-RO® H_2O) was not recirculated. Permeate from each transverse-flow module was not recirculated back into the feed solution and was collected separately. It was envisaged that analysis of the tracer residence time within the reactor would indicate variations in the flow characteristics of the reactor and any variation to which each transverse-flow module might be subject. Due to the influence of permeation rate on the exit of the tracer solution across the membranes, the trans-membrane flux of each transverse-flow module was accurately measured. Table 7.1 indicates the trans-membrane flux for the individual membrane modules. From the flux values obtained, it can be seen that variation in trans-membrane flux is evident, with the reactor at the bottom of the reactor (3A) having the highest flux with those at the top (1B) exhibiting the lowest flux (refer to figures 6.5 and 6.6 for the vertical arrangement of the reactor). Reactor 1A, however, did exhibit slightly higher flux than reactor 2B. This may be due to slight perturbations in the membranes themselves. Due to this vertical stratified arrangement of the individual transverse-flow modules, hydraulic pressure is greater within the lower regions of the reactor than at the top. As the mode of mass-transfer within the modules is convective, a higher pressure within the capillaries results in the lower modules exhibiting higher flux. During operation of the test-rig membrane rupture occurred in transverse-flow membrane module 3B, thus preventing further experimentation using this module, hence reported results are only for five transverse-flow membrane modules.

Table 7.1 Flux values for each module in the reactor. These values were obtained at a flow rate of 2.870L.h^{-1} and an operating pressure of 15 kPa.

Module code	Average flux (mL.h^{-1})	Average flux (L.m^{-2}.h^{-1})
1A	19.10	1.69
1B	15.33	1.36
2A	20.66	1.83
2B	17.33	1.53
3A	49.33	4.37

7.3.3. Operation of the transverse-flow module test rig

7.3.3.1. Enzyme preparation for the transverse-flow module test rig

Due to the large amount of immobilised polyphenol oxidase necessary for the test-rig, a crude extract of polyphenol oxidase was used as opposed to the commercially available enzyme. A crude extract of polyphenol oxidase was prepared as described in section 3.3.2 and the activity of the crude extract was determined using the previously described method (section 2.3.1).

7.3.3.2. Immobilisation of polyphenol oxidase within the transverse-flow membrane modules

Due to the arrangement of the individual modules, attempts at immobilising polyphenol oxidase within all the modules as a single unit would necessitate considerable residence time within the hydraulic delivery tubing network within the reactor at medium-to-high flow rates. As a result, the circulating biocatalyst would be susceptible to considerable attrition due to the resultant shear stress (Swaisgood,1985). Each individual membrane module was therefore inoculated separately as indicated in the schematic (figure 7.1). Using the inoculation of reactor 2B as an example, the inoculation procedure consisted of circulating the enzyme extract as indicated in Figure 7.1 for 15 minutes on the shell-side of the capillary membranes. Once circulation was complete, the crude enzyme extract was forced into the annular macrovoid region of each capillary by closing off the module circulation outlet thus creating a situation of dead-end filtration. To compensate for the pressure increase (monitored using the pressure gauge (P) as indicated), the inoculation pump was operated so that a constant pressure of *ca.* 50-100kPa was maintained within the module.
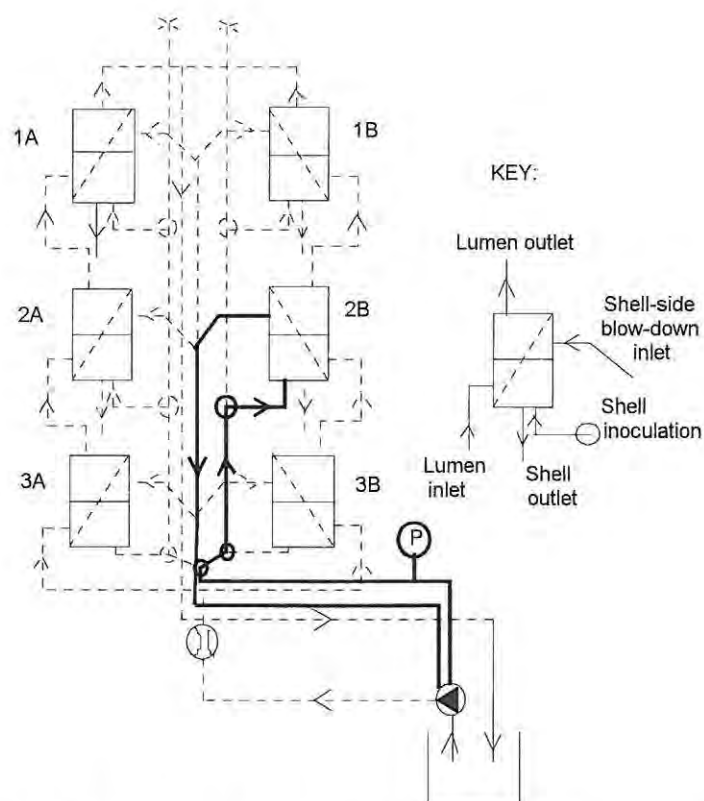
Figure 7.1 Schematic of the demonstration unit with the highlighted flow pattern indicating the inoculation flow path for module 2B.

Similar enzyme loading capacities for the transverse-flow modules as for the SCRs (section 3.4.2) were observed. Thus, from a total of 510 Units per reactor, approximately 418 Units were effectively immobilised.

7.3.3.3. Reactor operation

The transverse-flow membrane module test-rig was operated with the substrate solution (phenolic industrial effluent) circulated around the reactor. In the schematic shown in Figure 6.5, the flow path of the circulating substrate is indicated with the substrate entering the module at the lumen inlet (refer to key) and exiting via the lumen outlet back to the substrate reservoir.

7.4. RESULTS

7.4.1. Reactor fluid dynamics

The response curves obtained from the tracer study of the various modules operated using the test-rig are shown in Figures 7.2, 7.3, 7.4, and 7.5. The tracer study was performed with the tracer impulse

introduced to each module in a cross-flow mode of operation as opposed to dead-end. As a result, the tracer pulse was monitored at the outlet (figure 7.2). Since the tracer solution was not circulated back to the source of the tracer input, the concentration of NaCl decreased to zero over a period of 70 minutes.
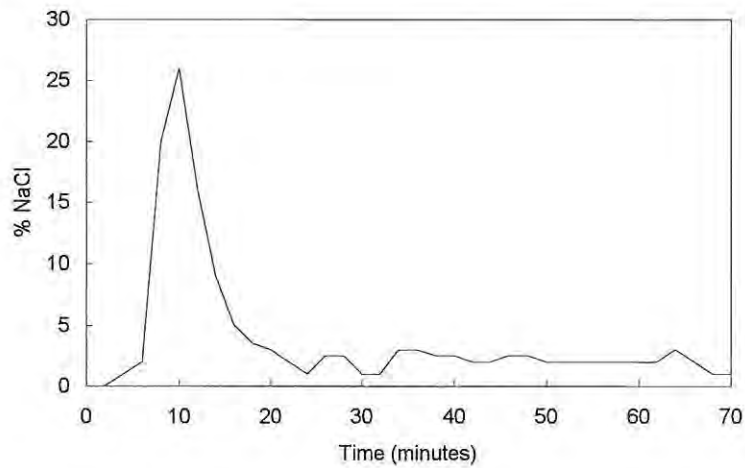
Figure 7.2 Residence time distribution of the tracer input as measured at the feed outlet.
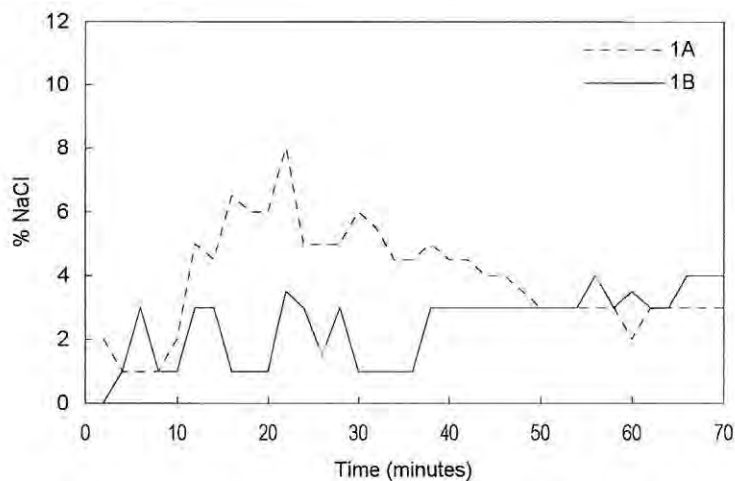
Figure 7.3 Residence time distribution of the tracer input as measured in the permeate of reactors 1A (1.69L.m^{-2}.h^{-1}) and 1B (1.36 L.m^{-2}.h^{-1}). Refer to Figure 6.5 for the positioning of 1A and 1B within the test-rig configuration.
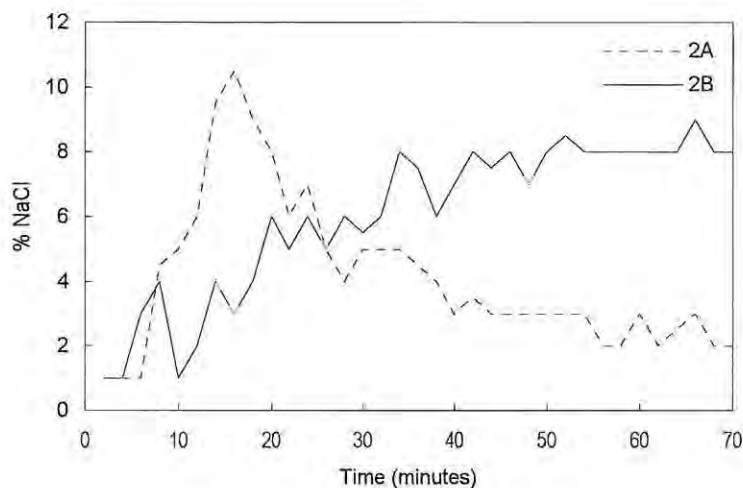
Figure 7.4 Residence time distribution of the tracer input as measured in the permeate of reactors 2A (1.83 L.m^{-2}.h^{-1}) and 2B (1.53 L.m^{-2}.h^{-1}). Refer to Figure 6.5 for the positioning of 2A and 2B within the test-rig configuration.
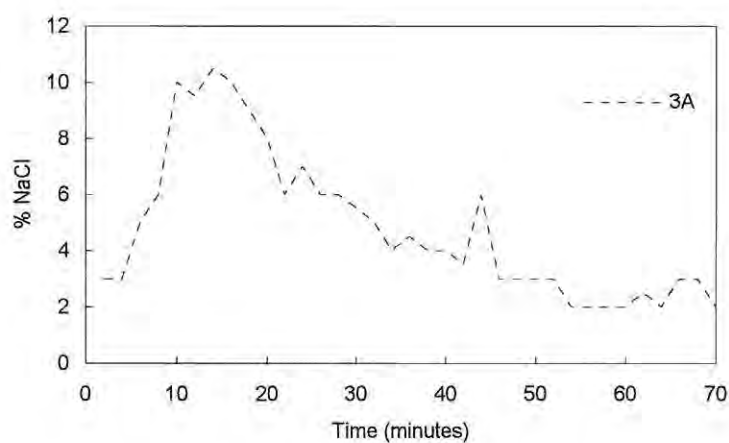
Figure 7.5 Residence time distribution of the tracer input as measured in the permeate of reactors 3A (4.37 L.m^{-2}.h^{-1}). Refer to Figure 6.5 for the positioning of 3A within the test-rig configuration.

Although the tracer response curves show variations within the hydrodynamic flow characteristics of each of the modules there is little indication of the nature of these flow characteristics. However, what is evident from observation of the graphical trend of the tracer responses is that flow is non-ideal due to none of the curves displaying well known trends that would be indicative of plug flow or completely

mixed flow (Levenspiel,1972). It is therefore apparent that the hydrodynamic flow characteristics of the test-rig and associated transverse-flow modules are a combination of mixed and plug flow.

From comparisons between the flux (refer to figure legends) and the tracer response curves, a direct correlation is evident. This enabled tentative prediction as to what substrate feed rate the individual transverse-flow modules would be subjected to. These predictions could be semi-quantitatively confirmed by determining the age distribution (E) of the fluid leaving the reactor using the following mathematical relationship (Levenspiel,1972).

$$Q = \sum C \, \Delta t$$

Where Q is the area under the concentration time curve which is an indication of the total amount of tracer introduced, t represents the sampling time, and C is the various tracer concentrations measured at specific time intervals. Values of Q for the various transverse-flow membrane modules are shown in Table 7.2.

Table 7.2 Q values for the transverse-flow membrane modules.

	1A	1B	2A	2B	3A
Q (g.min.L^{-1})	27.2	17.1	27.8	42.2	33.63

Once the Q values have been determined, the age distribution of the fluids can be calculated according to the equation:

$$E = \frac{C}{Q}$$

The age distribution (E) of the NaCl tracer for the various transverse-flow modules is shown in Figures 7.6, 7.7, 7.8, 7.9, and 7.10.
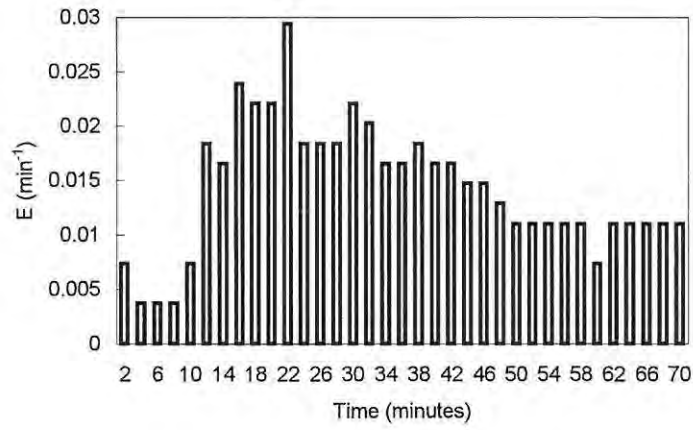
Figure 7.6 Plot of the exit age distribution for transverse-flow module 1A.
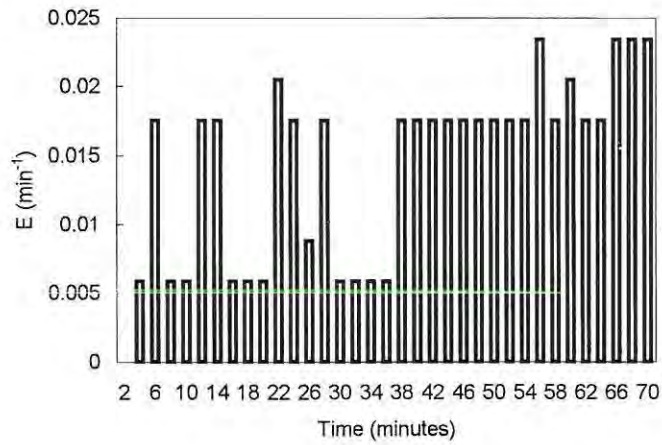
Figure 7.7 Plot of the exit age distribution for transverse-flow module 1B.
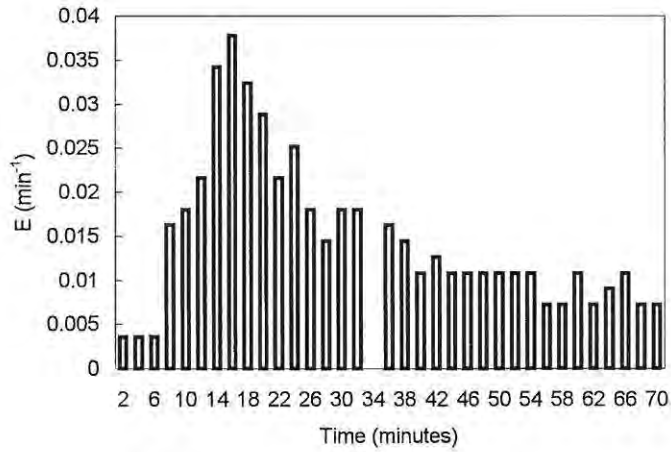
Figure 7.8 Plot of the exit age distribution for transverse-flow module 2A.
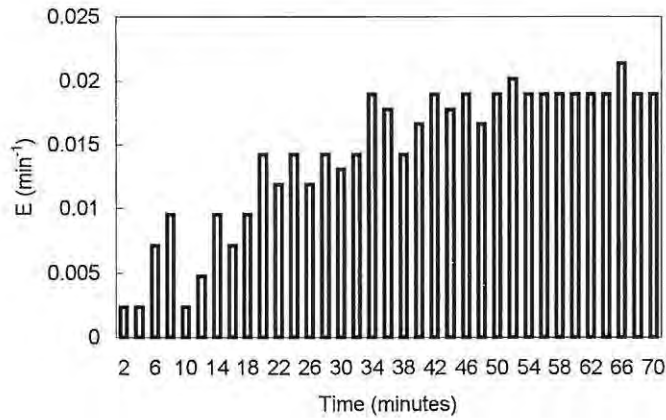
Figure 7.9 Plot of the exit age distribution for transverse-flow module 2B.
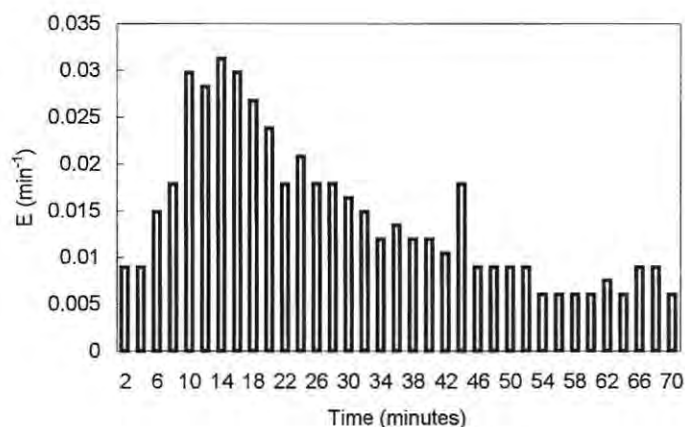
Figure 7.10 Plot of the exit age distribution for transverse-flow module 3A.

What is evident from the analysis of the exit age distribution is that the residence time of the tracer within the test-rig varies considerably for all the transverse-flow modules. The implication of this lends further evidence to the fact that the flow conditions within the test-rig are a combination of mixed and plug flow conditions. These mixed flow conditions and RTD variance within each module are a direct result of the differences in hydrostatic pressure as a result of the vertical arrangement of the test-rig configuration. The objective of determining if these variations in RTD were present within the test-rig configuration was to provide quantitative evidence that substrate availability to the immobilised enzyme upon reactor scale-up is also a function of the hydrodynamic environment as well as due to substrate and diffusivity constraints imposed by the individual membranes themselves (Chapter 2). A rigorous analysis of the combination of these effects, however, does not fall within the scope of this study.

7.4.2. Substrate conversion by polyphenol oxidase immobilised within transverse-flow capillary-membrane modules

By operating each individual transverse flow module under different flux conditions, it was envisaged that a relationship between flux and substrate conversion would emerge. Table 7.3 and Figure 7.11 show the substrate conversion capacities of the individual transverse-flow membrane modules operated under different flux conditions.

As indicated in section 7.3.2.3, transverse-flow modules at the bottom of the test-rig (module 3A) exhibited greater flux than those at the top (1A and 1B), although 1A did, however, exhibit greater flux

than 2B. From Table 7.3, it is evident that over a flux range of 1.35 $L.m^{-2}.h^{-1}$ to 1.83 $L.m^{-2}.h^{-1}$, the percentage substrate conversion ranged between 37% and 50%. As the flux increased to 4.37 $L.m^{-2}.h^{-1}$, substrate conversion increased dramatically to *ca*. 77%.

Table 7.3 Phenol industrial effluent conversion by immobilised polyphenol oxidase using transverse-flow membrane modules operated under different flux conditions.

Reactor	Flux (ml.h^{-1})	Flux (L.m^{-2}.h^{-1})	Control (mM)	Experiment (mM)	Substrate converted (mM)	% Substrate conversion
1A	19.10	1.69	4.291	2.287	2.0039	46.70
1B	15.33	1.35	3.712	2.100	1.6117	43.42
2A	20.66	1.83	3.232	1.605	1.6266	50.32
2B	17.33	1.53	2.351	1.474	0.8776	37.33
3A	49.33	4.37	3.617	0.830	2.7862	77.03
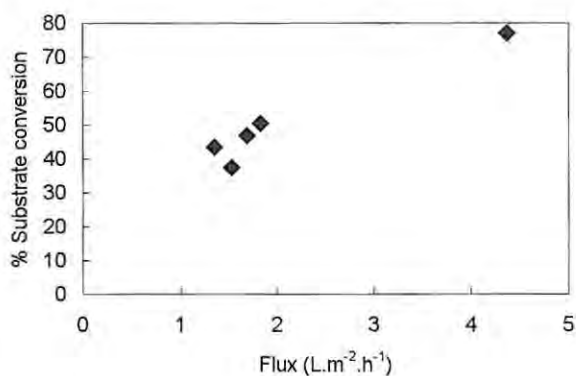
Figure 7.11 Phenol conversion using transverse-flow membrane module-immobilised polyphenol oxidase showing the non-linear trend between the operating flux and associated substrate conversion.

From these results, the general trend appears to be that as the operating flux increases, so the percentage substrate conversion increases. However, a linear relationship is not evident.

7.5. DISCUSSION

Due to the design of the transverse-flow module test-rig, and more importantly, the design and manifold-sealing system of each individual transverse-flow module, it was not possible to measure individual pressure differences within each module. As a result, it was not possible to relate any pressure differences within each individual module as a function of the hydrostatic pressure imposed by the physical design of the test rig. From the results obtained, a correlation between flux and substrate conversion capacity is evident. Within a flux range of 0.4 $L.m^{-2}.h^{-1}$, a 13% variation in conversion efficiency is evident. Although non-linear in nature the general trend appears to be that the higher the flux, the greater the percentage substrate conversion. This is particularly evident in module 3A where a flux of 4.37 $L.m^{-2}.h^{-1}$ is associated with 77% substrate conversion. Assuming that there was an absence of any significant back-pressure hindering air-flow across the individual membrane stacks within each module, it is probable that each individual membrane stack experienced equal air flow, and this therefore tends to the premise that an equal oxygen mass transfer coefficient applied to each individual membrane. It must be noted that these are assumptions as the oxygen concentrations within each module were not able to be measured due to the unavailability of oxygen microelectrodes.

In view of the assumed equal oxygen mass transfer, the fundamental influencing factor affecting the substrate conversion capacity of each individual module may then be attributed to the differences observed concerning the rate at which the immobilised enzyme was contacted by the perfusing substrate. As indicated in section 7.4.2., as operating flux is increased, so the amount of substrate conversion also increased (although non-linearly). Although the oxygen mass transfer was assumed to be equal for all transverse-flow membrane modules operated within the configuration of the test-rig, slight deviations in mass transfer, which were not able to be measured, may have influenced the substrate conversion significantly enough to contribute to the non-linear nature of substrate conversion vs. trans-membrane operating flux. Secondly, although modules operated in parallel on the test-rig (i.e. 1A and 1B; 2A and 2B) were assumed to experience the same hydrostatic pressure (due to the test-rig configuration), variation in operating flux between transverse-flow membrane modules 1A and 1B, and modules 2A and 2B, indicated that these modules were either not subject to equivalent hydrostatic pressure, manifesting as flux variation, or slight perturbations in the manufacturing of the individual modules or the capillaries therein resulted in these observed flux deviations.

7.6. CONCLUDING REMARKS

In comparison with the previously described SCR experiments, the higher percentage substrate conversion of the transverse-flow membrane modules may be attributed to a number of factors. These can be summarised as:

- improved oxygen mass transfer to the immobilised polyphenol oxidase.
- more rapid product removal.
- the operating configuration of the transverse-flow modules being vastly different from that of the SCRs.

From these observations, the SCRs and transverse-flow modules could be compared and the disadvantages and advantages associated with each configuration identified.

Firstly, improved oxygen mass transfer is possible using transverse-flow membrane modules as opposed to the SCRs. The has been reported previously by Ahmed *et al.* (1996) and Ahmed and Semmens (1996), where it was shown that oxygen mass transfer is considerably improved when air flow is transverse, as opposed to parallel, to the capillary membranes. By improving the oxygen mass transfer to the immobilised enzyme, greater substrate conversion proved possible. This would be expected due to the nature of the reaction mechanism of polyphenol oxidase and the requirement for oxygen (see figure 1.1). In the operating configuration of the SCRs, because the substrate permeation effectively displaced the static buffer solution in the shell-side of the SCR, an airflow could not be established across the shell-side of the capillary membranes. Thus, the only oxygen available to the enzyme was the oxygen in solution from the feed. Although air was bubbled constantly through the feed solutions during all experiments, mass transfer would still be limited due to the relatively low solubility of oxygen in water (7.93mg.L^{-1} at 25°C). From the stoichiometry of the reaction, a maximum of 3.96mg.L^{-1} phenol could then be converted to the corresponding *o*-quinone. In comparison, by having a constant airflow across the capillaries, as in the operational configuration of the transverse-flow membrane modules, mass transfer of O$_2$ at the membrane-enzyme interface would be considerably greater thus increasing the amount of substrate conversion.

Secondly, due to the shell-side of the capillaries in the SCR configuration being filled with a buffer solution that was displaced during substrate permeation (section 2.4.2 and 2.4.3), effectively the product in the shell-side can increase to a point where inhibition plays a role in affecting the ability of

the immobilised polyphenol oxidase in converting the permeating substrate. Although means of removing these inhibitory products have been investigated and found to be effective (Chapter 4 and Chapter 5), the accumulation of inhibitory products due to the operating configuration of the SCRs is a major disadvantage. In the transverse-flow membrane configuration, the necessity for airflow across the shell-side of the lumen means that the permeating solution after enzymatic conversion is efficiently removed by the permeate dissipating cone within the novel manifold designed for the transverse-flow modules (section 6.3.3). Hence, polyphenol oxidase-generated *o*-quinones tended not to accumulate near the membrane-enzyme interface thus decreasing the effect of product accumulation and the associated decrease in substrate conversion.

As was suggested in Chapter 3, the limiting factor in the substrate conversion by immobilised polyphenol oxidase is not substrate saturation, as was initially indicated in Chapter 2, but rather product inhibition. The greater the removal efficiency of the *o*-quinone products from the vicinity of the enzyme-membrane interface, the greater the substrate conversion capacity of the immobilised enzyme. In comparison to the amount of substrate converted using IPS 763 membranes in the SCR configuration (section 4.4.4), only $29.35\mu g.Unit^{-1}$ total substrate was converted, as opposed to an average of $67\mu g.Unit^{-1}$ using the same membranes in the transverse-flow module configuration. For module 3B, substrate conversion as high as $185\mu g.Unit^{-1}$ was attained. These results attest to the advantages of using the transverse-flow membrane module configuration as opposed to the SCR configuration.

Although the maximum substrate conversion capacity for polyphenol oxidase immobilised within the transverse-flow module was considerably less than that of the composite chitosan-polysulphone membranes (section 5.4.1), the aforementioned disadvantage (section 5.6) of using chitosan-coated membranes significantly impacts on any long-term usage of the composite membrane.

CHAPTER 8

CONCLUSIONS

The application and integration of membrane technology with biocatalyst immobilisation is not a technology that is still in its infancy. Rather, the advent and integration of membrane technology with established biotechnological processes has made non-membrane-integrated technology seem almost antediluvian.

Experimentally, this project focused on synergistically combining the advantages of enzyme-mediated dephenolisation of synthetic and industrial effluent with that of membrane technology. Within the context of membrane biotechnology, the use of an integrated system aimed at the use of membrane-immobilised enzymatic bioremediation of phenol-containing effluents is as yet a novel and unestablished technology. The project reported here was therefore undertaken as an initial feasibility study, with the specific aim being flexibility, allowing modification of this novel technology to achieve high levels of optimisation.

It was demonstrated that the use of capillary membrane-immobilised polyphenol oxidase as a technological alternative to several conventional bioremediation methods showed considerable potential. Initial experimentation was to determine any comparisons between non-immobilised and immobilised polyphenol oxidase based on substrate conversion as a function of enzyme-substrate contact. It was therefore imperative that a capillary membrane reactor was designed and constructed for these initial feasibility studies on the potential use of immobilised polyphenol oxidase. For this comparison, it was necessary to evaluate any operating constraints associated with the capillary membrane bioreactor and to determine any role that substrate permeability and diffusivity may play on the substrate conversion capacity of the capillary membrane-immobilised polyphenol oxidase. From the initial experimental findings (Chapter 2), it was evident that substrate permeability and diffusivity do affect enzyme-substrate contact. These initial findings also indicated that substrate saturation appeared to be the limiting factor influencing the substrate conversion efficiency of immobilised polyphenol oxidase. However, later investigation using a different immobilisation matrix showed that this was not the case (Chapter 3). It was therefore apparent that the crucial requirement in extending the half-life of polyphenol oxidase was dependent on increasing the rate at which inhibitory products are removed.

Thus, several incumbent restrictions on the half-life of the enzyme were shown to be decreased depending on which configuration modifications were applied to the system.

The first configuration modification was to determine the effect of using a different immobilisation matrix (polysulphone IPS 763 membranes) for immobilising polyphenol oxidase. Using polysulphone IPS 763 membranes it became apparent that substrate saturation was not the primary factor influencing the decrease in polyphenol oxidase activity over time, but rather product inhibition. Moreover, elimination of substrate saturation as the dominant limitation enabled the primary focus and objective for subsequent experimentation to be on decreasing the effect of product inhibition. The integration of the chitosan-facilitated quinone removal step in the overall capillary membrane-immobilised polyphenol oxidase configuration was, therefore, investigated. From these investigations (Chapter 4), the limiting factor that emerged was the low surface area of the chitosan flakes. The possibility of increasing the reactive surface area of the biosorbent, thus facilitating greater *o*-quinone removal efficiencies, was investigated. In conjunction with increasing the surface area of the chitosan, it would be necessary to engineer greater and more efficient contact of the chitosan with the *o*-quinones at a micro-environment level as close to the site of *o*-quinone generation as possible. This was achieved by contacting capillary membranes with a viscous chitosan solution thereby facilitating the formation of a double composite membrane i.e. polysulphone and chitosan (Chapter 5).

However, a significant limitation of using chitosan to coat the membranes is that the gel-like coating has a finite loading-capacity for either polyphenol oxidase or *o*-quinones. Furthermore, reuse of the membranes after removing the coating was not feasible. It was therefore envisaged that an alternative reactor design would be more practical for maintaining high productivities, as demonstrated using chitosan-coated membranes, but at the same time obviating the need to carry on the application of the chitosan-polysulphone composite membrane for achieving greater enzymatic conversion of the phenols. Transverse-flow membrane modules with membranes arranged in a plane perpendicular to the longitudinal axis of the module as opposed to the axial membrane orientation within the single-capillary reactor were therefore investigated (Chapter 6). Due to the radical differences in configuration between the single-capillary reactors used for the initial feasibility studies and the transverse-flow membrane modules, preliminary investigations involved demonstrating the hydrodynamic characteristics of the transverse-flow modules. Once this was determined, the overall determination of the reactor efficiency as a unit in the minimisation of industrial effluent pollutant concentration was investigated. The fundamental application was therefore directed at industrial effluent treatment as opposed to defined

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mixtures of synthetic substrates. In comparison with the initial feasibility studies using single-capillary reactors, higher productivity using transverse-flow membrane modules with immobilised polyphenol oxidase was observed (Chapter 7). This was attributed to a number of factors, namely improved oxygen mass transfer to the immobilised polyphenol oxidase, and more rapid product removal facilitated by the operating configuration.

Future considerations for improving and optimising the process should be focused on site-directed immobilisation using functionalised membranes. This would decrease any masking of the enzyme active-site as a result of random immobilisation by adsorption. By controlling the orientation of the active-site, increased substrate conversion may be attained. Furthermore, improved control of product removal warrants further investigation. Although sufficient product removal was obtained using the transverse-flow membrane modules, a certain amount of hydraulic hold-up due to module and manifold configuration will have manifested as a decreased substrate conversion efficiency over time. Use of the composite chitosan membrane met with limited success (even though substrate conversion was high) due to the chitosan coating being permanent and therefore subject eventually to saturation. An efficient technique for removing and replacing the chitosan coating will increase the viability of this product-removal approach immensely. When these considerations are investigated further, optimisation of the system would facilitate the use of membrane-immobilised polyphenol oxidase operated as a continuous process as opposed to a semi-continuous or batch mode.

To summarise:

- Polyphenol oxidase can be immobilised onto capillary membranes with a significant retention of enzyme activity.
- The immobilised enzyme was efficient in the substrate conversion of both a synthetic effluent as well as an industrial effluent.
- The substrate conversion capacity of the enzyme is influenced by a number of factors such as substrate availability, membrane-imposed diffusion limitations, and rate and efficiency of product removal.
- The substrate conversion efficiency of the immobilised enzyme is also influenced by the reactor configuration.

In conclusion, the initial research directive for this programme was characterised as an initial feasibility study into the use of membrane-immobilised polyphenol oxidase for the bioremediation of phenol-

containing industrial effluent. The research objectives and rationale underlying this technical feasibility study then provided a basis whereby certain developmental criteria could be identified. These provided the foundation for the initial fundamental experiments to evolve to a point where different reactor configurations and matrix supports were evaluated in terms of their dephenolisation potential. From the results presented here, it seems that there is considerable potential for using capillary membrane-immobilised polyphenol oxidase for removing phenols in industrial waste streams. However, due to the well documented fouling potential of ultrafiltration membranes, the use of membrane-immobilised polyphenol oxidase may be limited to low-concentration phenol-containing wastewater as an effective polishing step prior to discharge.

APPENDIX I

Sample preparation for Scanning Electron Microscopy

Sputter-Coating Technique

After cryo-fracture of the sample membrane sections (section 2.3.3), the samples were mounted on the SEM stub, clamped into position in the sputter-coat chamber, and the chamber closed. The chamber was then evacuated until a vacuum of $ca.$ 10^{-1} Torr was achieved.

Residual air was then flushed out using argon for a period of 5-10 minutes depending on the size and number of the specimens. The argon leak valve was then closed and the chamber allowed to evacuate further. When a vacuum of better than 5×10^{-2} Torr had been achieved, the high tension set switch was turned on and the field potential adjusted to 2.5kV. The sputtering current was allowed to rise to 20mA by admitting argon into the chamber up to a maximum pressure of 1×10^{-1} Torr. The timer was then set to 120 seconds and the sputtering allowed to proceed.

After the coating was complete the high tension set switch and vacuum pump were turned off and air admitted to the chamber. Specimens were then removed and examined in the scanning electron microscope.

APPENDIX II

Protein quantification using the method of Bradford (1976)

The method of Bradford (1976) depends on quantitating the binding of Coomassie Brilliant Blue (G-250), to an unknown protein and comparing this binding to that of a standard protein (bovine serum albumin).

Materials:

$0.5mg.mL^{-1}$ bovine serum albumin

0.15M NaCl

Coomassie Brilliant Blue (G-250) solution: 100mg Coomassie Brilliant Blue (G-250) dissolved in 50mL 95% (v/v) ethanol added to 100mL 85% (v/v) phosphoric acid and made up to 1L with Milli-Q® water.

Method:

1. Into 30 microfuge tubes triplicate amounts of 0.5 $mg.mL^{-1}$ bovine serum albumin (5, 10, 15, 20, and 25μL) are made up to 100μL with 0.15M NaCl. Blank tubes are made up with 100μL 0.15M NaCl.

2. 1mL Coomassie Brilliant Blue solution is added to each microfuge tube and vortexed. This is then allowed to stand for 2 minutes at room temperature.

3. Determine the absorbance of the unknown solutions (100μL samples + 1mL Coomassie Brilliant Blue solution) at 595nm using a 1-cm path length microcuvette (1mL) and determine the protein concentration from the standard curve.

APPENDIX III

Cost analysis:

Watson-Marlow peristaltic pump.	R9 471.12
Steel mounting/backing plate	R108.30

Stainless steel Swagelock fittings:

¼ s/s female fittings x 3	R 264.00
8mm 3-way valves x 11	R 4398.90
¼ 8mm 2-way valves x 1	R 375.09
6m 8mm s/s tubing x 1	R 119.70
¼ x ¼ male connectors x 40	R 1000.00
¼ x ½ male tees x 3	R 458.85
Total (incl. VAT)	R 7542.86

Rotameter (2-25 L/h)	R 537.60
Polypropylene rod Sufficient for 30 manifolds. Reactor requires 12.	R 889.33

Pressure gauges
2 x 0-400 KPa
1 x 0-250 KPa
3 x chemical seal gauges

Total (incl. VAT)	R 2935.73
Cost per Transverse-flow membrane module	R80.00
Cost of poly(ether sulphone) and polysulphone membranes	R1.00/m

The Transverse-flow membrane modules and membranes are used for experimental purposes only and are not for commercial sale. As such, the cost of the injection mold (R25 000) should be taken into account in terms of an overall costing analysis. However, on a commercial scale, because a large number of module grids are made and function as a saleable product, the costs of the injection mold are drastically reduced due to the commercial nature of the grid-production process.

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