THE DEVELOPMENT OF AN IMMOBILISED-ENZYME BIOPROBE FOR THE DETECTION OF PHENOLIC POLLUTANTS IN WATER

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by

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ABSTRACT

The possibility of developing an immobilised-enzyme bioprobe, based on mushroom polyphenol oxidase, for the purely biological detection and quantification of phenolic pollutants in water was investigated. Polyphenol oxidase catalyses the bioconversion of many phenolic compounds into quinone-related coloured products. Thus, in an immobilised form, the enzyme serves as a visible indicator of the presence and concentration of phenolic pollutants in water. The objective of this research was to develop a portable, disposable bioprobe incorporating polyphenol oxidase for this purpose. The intensity of the colour changes produced by the enzyme on reaction with *p*-cresol, *p*-chlorophenol and phenol was found to increase proportionally with increasing concentrations of these substrates in solution. Immobilisation of the enzyme on various supports did not appear to significantly affect the catalytic activity of the enzyme. The enzyme was immobilised by adsorption and cross-linking on polyethersulphone, nitrocellulose and nylon membranes with the production of various colour ranges on reaction with the phenolic substrates. The most successful immobilisation of the enzyme, in terms of quantity and distribution of enzyme immobilised and colour production, was obtained with the enzyme immobilised by adsorption on nylon membranes in the presence of 3-methyl-2-benzothiazolinone hydrazone (MBTH). The enzyme, immobilised using this method, produced ranges of maroon colours in phenolic solutions and orange colours in cresylic solutions. The colour intensities produced were found to increase proportionally with increasing substrate concentration after 5 minutes exposure to the substrates. The bioprobe had a broad substrate specificity and was sensitive to substrate concentrations down to 0.05 mg/L. The enzyme activity of the bioprobe was not significantly affected in a pH range from 4 to 10

and in a temperature range from 5-25^{0}C. The bioprobe activity was not affected by various concentrations of salt and metal ions and the bioprobe was able to detect and semi-quantify phenolic substrates in industrial effluent samples. These features of the bioprobe indicate that the commercialisation of such a bioprobe is feasible and this technology has been patented (Patent No. SA 97/0227).

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PUBLICATIONS

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POSTER PRESENTATIONS

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PATENTS

1. **PATENT - in the name of Water Research Commission (Patent No. SA 97/0227)**

An immobilised-enzyme bioprobe for the detection of phenolic pollutants in water

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CHAPTER 1:
Introduction

Monitoring of pollutants in the environment is becoming increasingly important as the need to preserve our resources becomes more urgent. One of the major resources requiring strict control of pollutants is water. Biological reactions are one of the tools used in this monitoring process, since they allow for the sensitive and specific detection of pollutants. Enzymes are especially useful, since each enzyme group interacts with a specific substrate or specific group of substrates. This thesis describes the use of the enzyme, polyphenol oxidase, which reacts with phenolic substrates, for the detection of these polluting chemicals in water.

1.1 ENVIRONMENTAL MONITORING OF PHENOLS

Phenol and its derivatives, including cresols, chlorophenols and naphthol etc., are a class of polluting chemicals which are harmful to animals and humans. They are easily absorbed through the skin and mucous membranes and can cause damage to a variety of organs and tissues, primarily the lungs, kidneys and genito-urinary system. In addition to being toxic, phenols, even in low concentrations, give an undesirable taste and odour to drinking water, particularly if they are combined with chlorine to form chlorophenols (Canofeni *et al.*, 1994).

A large number of industries release phenolic compounds into the environment. These include the coal conversion, petroleum refining, resins and plastics, dyes, textiles, timber, mining and paper industries (Atlow *et al.*, 1984). In most countries, strict limits on phenol concentrations in the environment are laid down by law. Acceptable levels of phenols in wastewater in South Africa are 0.01-0.1 mg/L (South African Water Act No. 54 of 1956). The maximum allowable level of phenols in potable water is 0.002 mg/L (Cox, 1969). It is therefore important to monitor and control the levels of these compounds in water. A number of sophisticated techniques have been developed for the determination of phenols at trace concentration levels. These include high performance liquid chromatography, gas chromatography, and spectrophotometry. These techniques, however, in addition to being expensive, sometimes need preconcentration or extraction steps which can lead to sample loss and are time consuming (Besombes *et al.*, 1995). The use of systems like these also does not allow for

continuous monitoring of phenols *in situ*.

1.2 ROLE OF BIOSENSORS

Analytical methods used for environmental monitoring have to be rapid, inexpensive and capable of being used *in situ*. Biosensors have been "hailed" as the solution to the analytical problems experienced in many industries using the more complicated techniques previously mentioned (Griffiths and Hall, 1993).

A biosensor is made up of an immobilised biological molecule (most often an enzyme) which interacts with an analyte of interest. The interaction produces a chemical signal which is detected by a transducer which converts the signal into a measurable response, most often an electronic signal (Scouten *et al.*, 1995). A generalised scheme of biosensor operation is shown in Figure 1.1. A bioprobe, however, comprises only a biological sensing element and does not require electronic amplification of the signal. Bioprobes could therefore be considered more appropriate for applications in areas such as field testing.

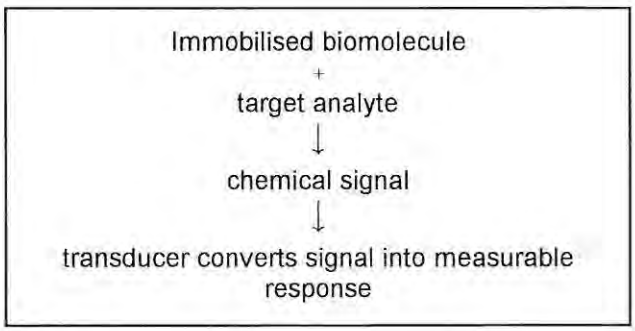


Figure 1.1. Generalised scheme of biosensor operation

Biosensors are increasingly becoming important tools in environmental monitoring as well as other fields such as medicine and food quality control. In principle, they can be manipulated to suit the analytical demands for almost any target molecule or compound which interacts specifically with a biological system (Scouten *et al.*, 1995). The immobilisation of the biomolecules allows for their reuse, minimising costs, and also allows for continuous monitoring *in situ*.

Biosensors based on polyphenol oxidase have shown great potential for the detection of phenolic compounds in wastewaters. Polyphenol oxidases are a group of oxidoreductase enzymes which catalyse the oxidation of phenolic substrates using oxygen. Oxidoreductases, in combination with amperometric electrodes have been found to be the best combination so far since they can easily and sensitively measure phenolic compounds by electrochemical means (Scouten *et al.*, 1995).

1.3 POLYPHENOL OXIDASE

1.3.1 Oxidation reactions catalysed by polyphenol oxidase

Polyphenol oxidase, also called tyrosinase, phenol oxidase or polyphenolase, is a copper containing enzyme which catalyses two types of oxidation reactions. It can act as a monooxygenase (incorporation of one oxygen atom from molecular oxygen into an organic substrate) by hydroxylating monophenols to form *ortho*-dihydroxyphenols (catechols)(Aitken *et al.*, 1994). Polyphenol oxidase is an unusual monooxygenase because it does not require a reducing cofactor such as NADH in order to carry out this reaction (Aitken *et al.*, 1994). Polyphenol oxidase also acts as an oxidase (oxidation of a substrate by molecular oxygen without incorporating oxygen into the product) by further oxidising the catechol to an *ortho*-quinone (Aitken *et al.*, 1994). These reactions are shown in Figure 1.2.

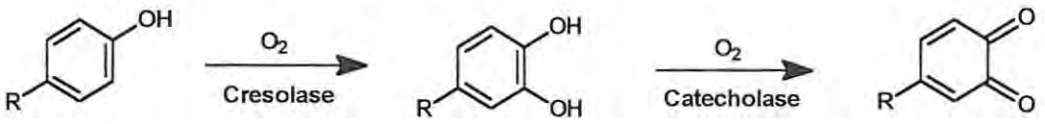


Figure 1.2. Oxidation reactions catalysed by polyphenol oxidase (Burton, 1994a)

The hydroxylation of phenols is often referred to as the "cresolase" or "phenolase" activity while the oxidation of the catechol is called the "catecholase" activity (Burton, 1994a). Most *ortho*-quinones are unstable and undergo a nonenzymatic polymerisation to form water-insoluble pigments called melanins (Atlow *et al.*, 1984). This process is responsible for the well-known phenomenon of browning of fruits and vegetables at open surfaces (Burton, 1994a). It is also responsible for the production of ink in squids and for sclerotization of

arthropod cuticula (Jacobsohn *et al.*, 1988). Eumelanin, the black skin pigment, and phaeomelanins, the pigments responsible for red and yellow colours in hair, feathers and fur are further examples of melanins formed in this way (Burton, 1994a). These coloured polymeric pigments can be monitored spectrophotometrically or visually and therefore, a measure of these coloured compounds produced in the presence of polyphenol oxidase could be used as an indication of the phenols present.

1.3.2 Mechanism of oxidation by polyphenol oxidase

The active site of polyphenol oxidase contains two closely associated anti-ferromagnetically coupled copper ions. These ions bind dioxygen to form a dioxygen-dicopper(II) complex (**A** in Figure 1.3). According to a mechanism proposed by Solomon and co-workers cited by Burton (1994a), the phenolic substrate coordinates initially from the axial position, and electron density is donated from the substrate into the lowest unoccupied molecular orbital of the oxy-dicopper complex (**B**). Oxygen transfer to the *ortho* position of the phenyl ring is then initiated, resulting in the formation of bound catechol (**C**). Electrons are then transferred from the catechol to the copper atoms generating the deoxy site and releasing the *o*-quinone.

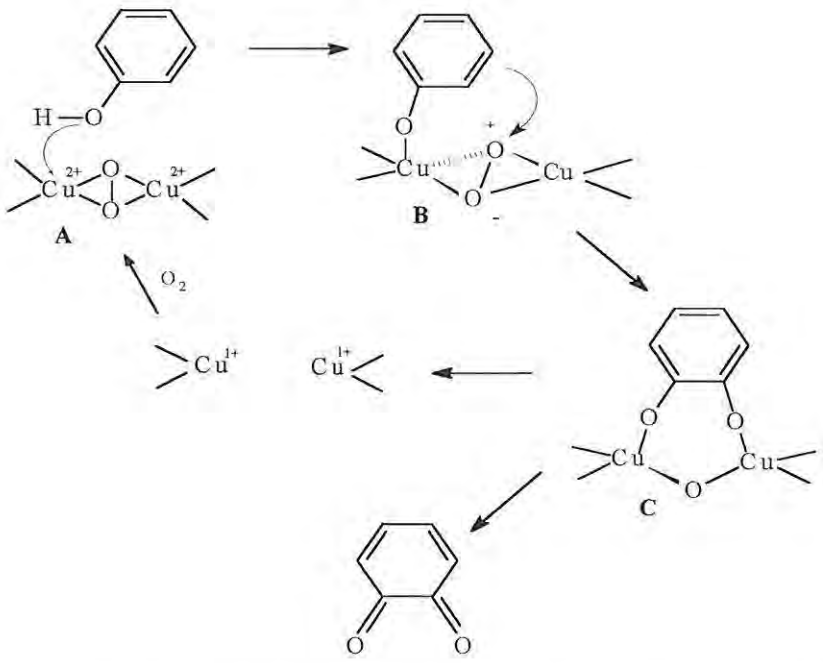


Figure 1.3. Proposed mechanism of oxidation by polyphenol oxidase (Burton, 1994a)

1.3.3 Sources of polyphenol oxidase

Polyphenol oxidases have a wide range of sources, from prokaryotes to higher plants and mammals. Their substrate specificity varies from plant sources to animal sources. Plant polyphenol oxidases oxidise a number of different phenolic and catecholic substrates while polyphenol oxidases from higher animals have a much narrower specificity. Polyphenol oxidase from mammalian cells utilises only dihydroxyphenylalanine (DOPA) or closely related substrates (Burton, 1994a). The polyphenol oxidase found in the common mushroom, *Agaricus bisporus*, has been used in most biocatalytic studies since commercial preparations are readily available (Kumar and Flurkey, 1991), although the extraction and characterisation of polyphenol oxidase from apples (Espin *et al.*, 1995), pears (Carlos-Espin *et al.*, 1996), avocados (Espin *et al.*, 1997a) and artichoke heads (Espin *et al.*, 1997b) has also been described. Mushroom polyphenol oxidase is unusual since it has two non-identical subunits in its tetrameric structure, a "heavy" subunit of 43000 Da and a "light" subunit of 13400 Da. The most common of four isozymes currently recognised is made up of two of each of these subunits. These four isozymes differ in their ratios of cresolase and catecholase activities (Burton, 1994a). Considerable quantities of an inactive or latent form of polyphenol oxidase have also been reported to be present in mushrooms. This latent form of the enzyme was activated using 0.1 % sodium lauryl sulphate and it was found to constitute almost 95 % of the total polyphenol oxidase present. The latent enzyme was present in only white, golden cream and sobexas mushroom strains (Yamaguchi *et al.*, 1970). The extraction of a latent form of polyphenol oxidase from iceberg lettuce has also been described. This latent form was activated using sodium dodecyl sulphate (Chazarra *et al.*, 1996).

1.3.4 Inhibitors of polyphenol oxidase

A number of substances have been shown to inhibit polyphenol oxidase and these have to be taken into consideration in the application of polyphenol oxidase in biosensors as well as other biotechnological processes. There are three main groups of polyphenol oxidase inhibitors (Burton, 1994a):

i) small ions which bind to the copper atoms in the active site;

ii) aromatic inhibitors which compete with phenolic substrates in binding to the active site;

iii) compounds which reduce or oxidise the copper ions.

These inhibitors include azide (Healey and Strothkamp, 1981), methimazole (Andrawis and Kahn, 1986), mimosine (Winkler *et al.*, 1981; Hider and Lerch, 1989), tropolone, fusaric acid, 3-aminotyrosine, hydroquinone (Burton, 1994a), benzoic acid, cinnamic acid and ferulic acids (Kermasha *et al.*, 1993). High concentrations of ascorbic acid have also been shown to inhibit mushroom tyrosinase (Burton, 1994a).

1.4 ENZYME IMMOBILISATION

1.4.1 Immobilisation techniques

A critical step in the development of biosensors is effective enzyme immobilisation and no other topic in applied biocatalysis has received as much attention over the past decade (Clark, 1994). An immobilised enzyme must retain its catalytic activity and must remain stable in order for a biosensor to be operational. Over a hundred immobilisation techniques have been described in the literature (Klibanov, 1983) which can be divided into five groups (shown diagrammatically in Figure 1.4).

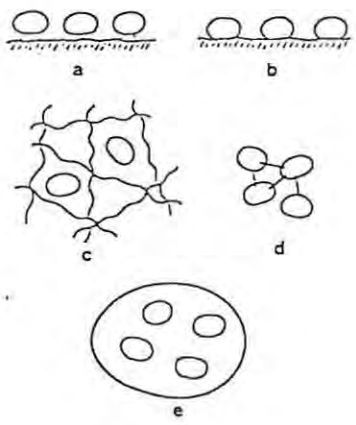


Figure 1.4. Diagrammatic representation of immobilisation techniques (Klibanov, 1983)

1.4.1.1 Covalent attachment on solid supports (Figure 1.4a)

A variety of supports have been used including porous glass and ceramics, stainless steel, sand, charcoal, cellulose, synthetic polymers and metallic oxides (Klibanov, 1983). The immobilisation procedure usually consists of two stages:

i) activation of the support;

ii) attachment of the enzyme.

The enzymes are often covalently attached to the activated supports via glutaraldehyde, OHC-$(CH_2)_3$-CHO, whose one carbonyl group forms a Schiff base with an amino group on the support while the other reacts with amino groups of the enzyme (Klibanov, 1983).

1.4.1.2 Adsorption on solid supports (Figure 1.4b)

Ion-exchangers readily adsorb most proteins and have therefore been widely used for enzyme immobilisation. Anion exchangers such as diethylaminoethyl cellulose (DEAE-cellulose) and Sephadex, as well as cationic exchangers such as carboxymethyl cellulose (CM-cellulose) have been used industrially to adsorb enzymes (Klibanov, 1983). This immobilisation technique is very simple, involving only the simple addition of an enzyme solution to the support, stirring and the removal of the remaining enzyme by washing (Klibanov, 1983).

1.4.1.3 Entrapment in polymeric gels (Figure 1.4c)

In this approach, an enzyme is added to a solution of monomers before the gel is formed. A polymerising agent is added to initiate gel formation and the enzyme becomes entrapped in the gel (Klibanov, 1983; Uhlich *et al.*, 1996).

1.4.1.4 Cross-linking with bifunctional agents (Figure 1.4d)

The most commonly used cross-linkers are glutaraldehyde, dimethyl adipimidate, dimethyl suberimidate and aliphatic diamines. The cross-linking may be both intermolecular (forming water-insoluble aggregates) and intramolecular (Klibanov, 1983).

1.4.1.5 Encapsulation of enzymes (Figure 1.4e)

In this approach, enzymes are encapsulated with various forms of membranes which are impermeable for enzymes and other macromolecules but permeable for low molecular weight substrates and products (Klibanov, 1983).

1.4.2 The effect of immobilisation on enzyme stability

Immobilisation of enzymes can result in their improved stability in a number of ways:

i) the enzyme molecules may become sterically inaccessible for the action of microorganisms, proteases or other inhibitors;

ii) spatial fixation of the enzyme may prevent intermolecular inactivation processes such as autolysis;

iii) multipoint attachment of the enzyme to the support may prevent unfolding, particularly at elevated temperatures where enzymes are susceptible to denaturation (O'Fagain *et al.*, 1988; Gupta, 1991);

iv) a support with buffering surface groups may prevent pH inactivation;

v) in a dry state, enzymes are stable (Klibanov, 1979), and therefore dehydration of the enzyme molecules and their fixation to the support may prevent their unfolding or denaturation;

vi) increased rigidity due to cross-linking may enhance an enzyme's stability by preventing critical unfolding of the molecule (Klibanov, 1979).

1.4.3 The advantages and disadvantages of immobilisation techniques

The advantage of covalent methods (Figure 1.4a and d) is that they result in strong chemical bonds between the enzyme and the support. The disadvantages are that covalent bonding is relatively laborious, expensive and often leads to significant inactivation of enzymes due to attachment through their active sites. This problem of inactivation can, however, be overcome by immobilising the enzyme in the presence of other ligands which selectively bind to the active sites and protect them from attachment. Methods of immobilisation such as adsorption and gel entrapment (Figure 1.4b and c) are very simple and efficient, but since these methods do not create strong bonds between the enzyme and the support, enzymes often leak from the supports. This problem can be overcome by treating these adsorbed or entrapped enzymes with cross-linking reagents such as glutaraldehyde (Klibanov, 1983).

Other factors which need to be considered in the development of an immobilised enzyme process (such as a bioprobe or biosensor) are the cost of the enzyme, its stability, the

parameters determining its mode of action, the price of the carrier and the need to eliminate contaminating waste products (Katchalski-Katzir, 1993).

1.5 APPROACHES TO BIOSENSOR DESIGN

Biosensor technology can be divided into two main components:

i) the biological-sensing element (to confer specificity);

ii) the transduction technique (to convert the biological interaction into a response that can be further processed and displayed) (Griffiths and Hall, 1993).

The biological components used in biosensor technology can be divided into two groups. The first is the catalytic group, where enzymes are by far the most commonly used components but microbial (*eg.* Karube and Nakanishi, 1994), plant or animal cells (*eg.* Chibata *et al.*, 1986) have been used where the use of enzymes has been impractical or impossible. The second group comprises a non-catalytic or affinity group where antibodies (*eg.* Sadana and Ram, 1996), receptors (*eg.* Wijesuriya and Rechnitz, 1993) or nucleic acids (*eg.* Maeda, 1993) are used.

A wide range of transducers can be used in biosensors and these can be divided into four broad categories: electrochemical (amperometric, potentiometric and conductimetric), optical, acoustic and calorimetric transducers.

1.5.1 Electrochemical transduction

1.5.1.1 Amperometric

Amperometry is the most commonly used biosensor-transduction method and the majority of commercial biosensor devices are amperometric enzyme electrodes (Griffiths and Hall, 1993). A constant potential is maintained with respect to a reference electrode and the current measured is due to the oxidation or reduction of an electroactive species at the surface of the working electrode. The current is therefore directly proportional to the concentration of the analyte at the working electrode surface (Newman and Turner, 1992).

1.5.1.2 Potentiometric

These transducers measure the difference in potential between the active electrode and a second reference electrode under conditions of zero current flow. The relationship between the potential generated at the electrode surface and the ion of interest is logarithmic. These sensors therefore have a very wide range of sensitivity (Griffiths and Hall, 1993).

1.5.1.3 Conductimetric

Conductance is generally measured by applying a current between two electrodes immersed in the solution. Some enzyme reactions convert neutral substrates into charged products, which cause a change in the conductance of the medium. This technique is not widely used in biosensors due to interference in "real" samples (Griffiths and Hall, 1993).

1.5.2 Optical transduction

The simplest form of optical transduction involves the detection of changes in the light absorbance characteristics of a reagent layer due to interaction with a particular analyte of interest (Newman and Turner, 1992). Optical fibres are commonly used to carry light to and from the reaction area. These fibres, carrying light by multiple internal reflections, are made of glasses and plastics (Newman and Turner, 1992). An optical biosensor for lysine has been developed using this type of transduction (Li *et al.*, 1992).

1.5.3 Acoustic/Piezoelectric transduction

Piezoelectric crystals which are able to generate and transmit acoustic waves in a frequency dependant manner are mass produced cheaply for the electronics industry. Adsorption of molecules onto the surface of these crystals, causes a change in the frequency of the waves transmitted and therefore the molecules can be detected. The major limitation of these types of transducers at present, is the insufficient knowledge of the acoustic characteristics of biological samples (Newman and Turner, 1992). Other drawbacks are non-specific binding and poor sensitivity in liquid media (Griffiths and Hall, 1993).

1.5.4 Calorimetric transduction

Calorimetric transduction is still an area of very limited research activity, although almost all biological reactions are exothermic and should therefore provide a universal transduction technique. Calorimetry also has the advantages of being relatively independent of the chemical properties of the sample (Griffith and Hall, 1993). Thermal interference, however, is a problem which needs to be overcome in using these transducers (Newman and Turner, 1992).

1.6 BIOSENSOR APPLICATIONS

Medical diagnostic requirements for the instant analysis of clinical samples (which is appealing to both patient and physician) have been the major driving force in biosensor development. Most of the early work done in this field was directed at blood glucose level determination in insulin-dependent diabetic patients (Newman and Turner, 1992), and one of the first commercially successful biosensors was a blood glucose meter, the ExacTech (Medisense, Cambridge, MA, USA). The enzyme used in this electrochemical biosensor, and which is most widely used in biosensors, is glucose oxidase. Glucose oxidase catalyses the oxidation of glucose to produce gluconic acid and hydrogen peroxide using oxygen. In the ExacTech sensor, a mediator is used to replace oxygen as an electron acceptor so that oxygen is not required and hydrogen peroxide is not produced. The mediator used is a ferrocene derivative (Newman and Turner, 1992). Attention has been directed recently to *in vivo* sensors, which present a particular challenge since these biosensors would not only need to overcome the traditionally experienced problems, but would also need to be biocompatible (Newman and Turner, 1992).

Interest in non-clinical applications of biosensors has also increased in recent years. New legislation and increasing awareness of environmental issues has led to a flood of research into biosensors for pollutants such as pesticides and herbicides (*eg.* Wang *et al.*, 1996), gases (*eg.* Saini *et al.*, 1995) and important environmental contaminants (*eg.* Luong *et al.*, 1995). Hygiene regulations and the need for stricter quality control in the food industry has led to research into biosensors for use in this field (Newman and Turner, 1992). The military have shown an interest in portable sensors for the detection and/or identification of agents of

chemical warfare such as mustard and nerve gases (Paddle, 1996). The development of these biosensors, however, will depend on political factors and on the technology itself (Griffiths and Hall, 1993). Biosensors have therefore been developed for the detection of a wide variety of diagnostically, environmentally or otherwise important compounds. These compounds include amino acids, glucose, phenols, organophosphates, ammonium, urea, cholesterol, penicillin and hydrogen peroxide (Klibanov, 1983; Bertocchi *et al.*, 1996).

The most intense interest at the moment is in the monitoring of the environment and the next "wave" of biosensor products have been predicted to address applications in this field (Griffiths and Hall, 1993). The largest single area of application for the environment lies in water analysers. These biosensors would be useful to the water industry itself, as well as to water-using and policing industries such as municipalities for monitoring of pollutants in sea water, wastewater, rivers, reservoirs and water supplies (Griffiths and Hall, 1993).

1.7 BIOSENSORS WITH POLYPHENOL OXIDASE

Polyphenol oxidase is relatively stable and versatile *in vitro*, making it ideal for use as an experimental enzyme for research (Burton, 1994a). The versatility of the enzyme has led to its incorporation into biosensors for a number of uses. The importance of monitoring phenolic compounds in water has led to the majority of biosensors utilising polyphenol oxidase being developed for this purpose. Polyphenol oxidase-modified electrodes have been used for the detection of phenols and catechols, and immobilisation procedures, electrode materials, stability and sensitivity of these electrodes have been studied (Burestedt *et al.*, 1996).

1.7.1 Biosensors incorporating polyphenol oxidase, for use in aqueous systems

The first work done in this area involved the immobilisation of polyphenol oxidase in polyacrylamide gel on carbon electrodes. Ferrocyanide was used as a chemical mediator and the system was used to measure different phenol concentrations (Schiller *et al.*, 1970, cited by Burton, 1994a). A number of carbon paste electrodes based on polyphenol oxidase have been described for the monitoring of phenolic compounds. Such an electrode has been described for the detection of micromolar quantities of phenolic compounds in untreated river and

groundwater samples (Wang and Chen, 1995). The use of carbon paste electrodes has also been described for the flow injection analysis of phenolic compounds (Lindgren *et al.*, 1996; Petit *et al.*, 1995). Such an electrode had a lower detection limit of 900 nM (0.1 mg/L) for catechol (Ortega *et al.*, 1994). The use of hydrocarbon pasting liquids for improved polyphenol oxidase-based carbon paste phenol biosensors has been described. Such a biosensor had a detection limit of 6 nM (0.7 μg/L) for catechol (Wang *et al.*, 1997).

Polyphenol oxidase immobilised on Clark-type oxygen electrodes has also been shown to detect phenols. The oxygen consumption during phenol oxidation was monitored using these electrodes, giving an indirect method of phenol detection (Burton, 1994a). Three different methods of immobilising polyphenol oxidase on a Clark-type oxygen electrode for the detection of phenols in wastewater were compared by Campanella *et al.* (1993). Of the three methods used, immobilisation involving adsorption onto a functionalised nylon membrane gave the best results with respect to precision, accuracy, sensitivity (detection limit of 1 μM (0.09 mg/L) for phenol), lifetime and minimum activity loss under working conditions (Campanella *et al.*, 1993).

Polyphenol oxidase has been immobilised on various solid graphite electrodes. Polyphenol oxidase immobilised by entrapment and covalent adsorption on graphite electrodes previously modified by tetracyanoquinodimethane showed a detection limit for phenol of 0.23 μM (0.02 mg/L) (Kulys and Schmid, 1990). The use of a polyphenol oxidase graphite electrode for the post-column detection of phenolic drugs after liquid chromatographic separation has been described by Ortega *et al.* (1992). The enzyme electrode, when compared with an enzyme reactor with respect to sensitivity, was found to give the highest sensitivity for the quantification of dopamine (Ortega *et al.*, 1992). The enzyme immobilised into a solid paraffin-graphite-particle matrix had a detection limit for dopamine of 50 nM (0.01 mg/L) (Petit *et al.*, 1995). An amperometric biosensor prepared by immobilising polyphenol oxidase onto a wax-impregnated graphite electrode had a linear detection range from 2×10^{-7} M (0.02 mg/L) to 1.25×10^{-5} M (1.2 mg/L) phenol. This biosensor lost no enzyme activity during 5 months of storage (Hu, 1995). Electrodes based on the entrapment of polyphenol oxidase in

epoxy-graphite matrices have also been described. The limits of detection for phenol and catechol were 1 μM (0.09 mg/L) and 0.04 μM (0.004 mg/L), respectively (Oennerfjord *et al.*, 1995; Lutz and Dominguez, 1996).

A number of immobilisation procedures for the development of electrochemical polyphenol oxidase-based biosensors were compared by Canofeni *et al.* (1994). Both crude and pure enzyme solutions were used for the immobilisation techniques which included covalent attachment, cross-linking with glutaraldehyde and physical entrapment. Membranes containing the biocatalysts were then assembled on the surface of Clark-type electrodes. The electrode based on pure polyphenol oxidase covalently immobilised on a nylon net showed the greatest sensitivity and the longest lifetime (30 days). This biosensor detected phenol in a 1-10 mM (94-940 mg/L) linear range (Canofeni *et al.*, 1994). An electrochemical biosensor in which polyphenol oxidase was immobilised by electropolymerisation in a cationic derivatised pyrrole polymer has been described for the amperometric detection of phenols, catechols and catecholamines. This sensor had a detection limit of 2 x 10^{-7} M (0.02 mg/L) for phenol (Burton, 1994a). This form of enzyme immobilisation has been utilised in a number of other polyphenol oxidase-based biosensors for phenol detection (Cosnier and Popescu, 1996; Besombes *et al.*, 1995). An improvement to this method of immobilisation by the incorporation of synthetic laponite-clay-nanoparticles has been described. The nanoparticles resulted in increased sensitivity of these biosensors (Besombes *et al.*, 1997). A biosensor constructed by the use of a novel immobilisation technique involving the physical freezing of polyphenol oxidase resulted in a biosensor that was able to detect 0.02 μM (0.002 mg/L) catechol and was stored for more than three months without any loss of activity (Deng *et al.*, 1996).

A number of biosensors based on the inhibition of polyphenol oxidase have been described. This type of biosensor has been used in a flow-through system for the detection of the herbicide atrazine. In this system, the degree of inhibition of polyphenol oxidase activity by atrazine was measured amperometrically and used as an indirect assay of atrazine concentration (McArdle and Persaud, 1993). A biosensor for cyanide has also been described using an

electrochemical cell to monitor the degree of inhibition of polyphenol oxidase by cyanide. The substrate of the enzyme was replaced by the electrochemical mediator, ferricyanide (Smit and Rechnitz, 1993). A similar polyphenol oxidase enzyme electrode has been described for the amperometric detection of phenol, 3-chlorophenol and 4-chlorophenol. This electrode was also used for the detection of 2-chlorophenol, several polychlorophenols and pentachlorophenol by measuring the degree of inhibition of the bioelectrode functioning due to these compounds (Besombes *et al.*, 1995). Other polyphenol oxidase biosensors based on this principle have been described for the detection of respiratory poisons (Robinson *et al.*, 1995), and pesticides and herbicides (Wang *et al.*, 1996).

A number of bienzyme biosensors have been described utilising polyphenol oxidase immobilised together with other enzymes. Polyphenol oxidase immobilised together with laccase (Yarapolov *et al.*, 1995) and horseradish peroxidase (Cosnier and Popescu, 1996) was incorporated into sensors for the flow injection analysis of phenols. Polyphenol oxidase co-immobilised with glucose dehydrogenase on a Clarke-type oxygen electrode has been used for the highly sensitive measurement of phenolic compounds. The resulting detection limits for catechol and phenol were 0.6 nM (0.006 μg/L) and 0.9 nM (0.008 μg/L), respectively (Makower *et al.*, 1996). Polyphenol oxidase immobilised together with glucose dehydrogenase has also been used for the monitoring of biologically important peptides containing tyrosine (Eremenko *et al.*, 1997).

Various plant tissues rich in polyphenol oxidase have been incorporated into biosensors as an enzyme source. Mushroom tissue has been used in a cobalt phthalocyanine dispersed amperometric biosensor for the determination of phenolic compounds. Cobalt phthalocyanine was shown to result in a shorter response time compared to conventional tissue biosensors (Ozsoz *et al.*, 1996). A biosensor constructed by the immobilisation of sweet potato crude extract with glutaraldehyde onto an oxygen membrane has been proposed for the amperometric detection of phenols (Vieira and Fatibello-Filho, 1997). Potato, banana and apple tissues have been incorporated into carbon paste electrodes for the determination of catechol related components in beers (Eggins *et al.*, 1997).

A polyphenol oxidase-based chemically amplified biosensor for the determination of *Escherichia coli* density has recently been described. This biosensor is based on the substrate recycling of polyphenolic compounds driven by polyphenol oxidase catalysed oxidation and chemical reduction of *o*-quinones by L-ascorbic acid (Hasebe *et al.*, 1997).

1.7.2 Biosensors incorporating polyphenol oxidase, for use in organic solvents

A range of biosensors based on polyphenol oxidase have been used for the detection of phenolic compounds in organic solvents. A biosensor described by Hall *et al.* (1988, cited by Burton, 1994a) was able to detect *p*-cresol and various other phenols in chloroform solutions using a graphite foil electrode in which the enzyme was present in a thin aqueous film. Polyphenol oxidase was found to be stable when stored in chloroform having a lifetime of 160 days (Burton, 1994a). Phenols were also detected in olive oils using polyphenol oxidase on a graphite electrode in a chloroform medium (Burton, 1994a). Wang and Reviejo (1993) cited by Burton (1994a) have described a polyphenol oxidase containing electrode which was used for water detection in organic phases, the activity of the enzyme being directly dependant on its degree of hydration. Another polyphenol oxidase based enzyme electrode has been described which was able to detect phenol and phenol derivatives in *n*-hexane and water saturated chloroform. The sensitivity of the sensor varied with each particular phenol (Campanella *et al.*, 1994). Polyphenol oxidase immobilised by simple adsorption onto an electrochemically pretreated carbon-fibre surface was shown to be useful for the on-line biomonitoring of organic streams. This biosensor responded very rapidly to dynamic changes in the concentration of phenolic compounds in flowing chloroform and acetonitrile solutions (Wang and Lin, 1993). The construction of a polyphenol oxidase based biosensor for the detection of phenols in pure organic phase has also been reported. This biosensor was constructed using a novel immobilisation technique which gave the enzyme a hydrated shell allowing it to maintain its activity in pure chloroform and chlorobenzene. It was also stored for 3 months without loss of any activity (Deng and Dong, 1995). Mushroom and banana tissues rich in polyphenol oxidase have been incorporated into organic-phase biosensors resulting in very fast and sensitive amperometric detection of phenol with detection limits of 3×10^{-5} M (3 mg/L) (Wang *et al.*, 1992).

1.8 OTHER BIOSENSORS FOR THE DETECTION OF PHENOLIC COMPOUNDS

1.8.1 Enzyme-based biosensors

A laccase electrode has been described for the continuous-flow concentration measurement of a wide range of phenolic substrates. The electrode response for a hydroquinone concentration of 0.25 mM (28 mg/L) was stable with repeated use for at least 800 determinations without significant loss of activity (Zouari *et al.*, 1994). Horseradish peroxidase modified solid graphite and carbon paste electrodes have been described for the determination of phenol and related compounds. The graphite electrode was more efficient and was able to detect nine different phenolic compounds. Its lowest detection limit was 0.5 μM (0.07 mg/L) for 2-amino-4-chlorophenol (Ruzgas *et al.*, 1995).

1.8.2 Microbial biosensors

A number of microbial biosensors for the detection of phenol and chlorophenols have been developed. Amperometric biosensors based on *Rhodococcus* and *Trichosporon beigelii* (*cutaneum*) have been described for phenol determination having detection limits of 4 μM (0.4 mg/L) and 2 μM (0.2 mg/L), respectively (Riedel *et al.*, 1993; 1995). A high-temperature operating biosensor based on living phenol-oxidizing *Bacillus stearothermophilus* cells has also been described. This amperometric sensor showed a very fast response time of 2 minutes, was operative over a wide temperature range (35-55^{0}C) and pH range (4.5-8.0) and gave a reproducible response for months. The sensor had a lower detection limit of 1 μM (0.1 mg/L) for *o*-, *m*- and *p*-cresol and 2.5 μM (0.2 mg/L) for phenol (Rella *et al.*, 1996).

1.9 COMMERCIALISATION OF BIOSENSORS

Biosensors have been proposed in the literature for many, diverse areas of application. The features and potential benefits that biosensors can offer include selected measurement in complex samples, targeted specificity, continuous measurement, small size and fast measurement. A biosensor can be very useful to the user even when only some of these features are incorporated into its development (Griffiths and Hall, 1993).

Despite the prolific publications of research papers and patents by the many research groups

worldwide, there are as yet, very few successful commercial biosensor products available (Griffiths and Hall, 1993). Probably the most serious limitation in commercialising biosensors is the relative lack of long-term stability of biological molecules (Scouten *et al.*, 1995). Other factors affecting the commercial realisation of these products include the technical and manufacturing feasibility and market development (Griffiths and Hall, 1993).

The most popular biosensor types to date are amperometric sensors and these have been found to be useful for many applications but they are not ideal in all cases (Griffiths and Hall, 1993). Much research has been done on biosensors based on optical transduction and some promising devices have been developed. These biosensors, however, are very expensive to manufacture and therefore it is not likely that they will become available within the next few years (Griffiths and Hall, 1993). Piezoelectric and thermal-based biosensors are limited to only very specialised applications since further development will be necessary before practical devices can be realised. The manufacturing technology of all these biosensor types will need to be improved before commercial products can be mass-produced (Griffiths and Hall, 1993). Other problems experienced in the mass production of biosensors is the lack of uniformity and reproducibility (Scouten *et al.*, 1995). Most of the signal transducers discussed use covalent immobilisation methods which do not always result in uniform and reproducible coupling (Scouten *et al.*, 1995). New methods of immobilisation able to immobilise specific sites on biomolecules to the support surface may lead to new and more effective biosensors (Scouten *et al.*, 1995).

1.10 OBJECTIVES OF THE STUDY

This study was proposed for the development of an immobilised-enzyme bioprobe which would be a purely biological sensor based on polyphenol oxidase for the detection of phenolic pollutants in water. This sensor was intended to detect phenolic substrates by a "dipstick" type colour test (a unique method for the detection of these compounds). Polyphenol oxidase-based enzyme electrodes have been shown to be useful for the selective determination of phenolic substrates in environmental samples. These electrodes, however, are not always practical for field use. This "dipstick" would be made up of polyphenol oxidase immobilised on a support.

On reaction with phenolic compounds present in a water sample, the enzyme would produce coloured products on the support according to the phenol concentration. The colour produced would be compared with a colour chart made from reacting the bioprobe with a set of standard phenol solutions of known concentrations. By finding the corresponding colour on the colour chart, the concentration of phenolic substrates in the sample would be determined. The intensity of the colour produced would therefore give an indication of the phenolic substrate levels present. The colour change would be visible and would not require any amplification by a transducer. The bioprobe could therefore be described as a simplified biosensor having only a biological sensing element and requiring no transduction technique. This portable, disposable bioprobe could be manufactured at a cost which is attractive to the market and it could be used by non-experts. It would therefore be more appropriate, than many of the detection methods previously described, for field use, particularly in rural areas. The objectives of this study were as follows:

i) To become familiar with current and recent literature on the characteristics and applications of polyphenol oxidase which are relevant to the project;

ii) To isolate and immobilise polyphenol oxidase on a support in such a way as to maintain its stability and catalytic activity;

iii) To adapt assay procedures to suit their application to the immobilised enzyme and to develop an appropriate protocol for quantifying the bioprobe response to various pollutants;

iv) To adapt laboratory-scale experimental data and information already available to enable the incorporation of the biocatalyst into a module suitable for the field testing of water;

v) To evaluate the success, accuracy and suitability of the bioprobe.

CHAPTER 2:
Extraction and analysis of polyphenol oxidase

2.1 INTRODUCTION

A number of extraction procedures and modifications to these procedures have been described in the literature for the extraction of polyphenol oxidase from mushrooms (*eg.* Frieden and Ottesen, 1959; Bouchilloux *et al.*, 1963; Nelson and Mason, 1970; Miletic *et al.*, 1990; Rescigno *et al.*, 1997). Polyphenol oxidase was relatively simply and cheaply extracted from mushrooms (*Agaricus bisporus)* throughout the course of this project as described by Burton (1994b).

In order to evaluate the success of each extraction, samples were taken after each step in the purification procedure and analysed for protein content and enzyme activity. Numerous methods have been utilised for assaying polyphenol oxidase activity (Pomerantz, 1976; Winder and Harris, 1991; Gauillard *et al.*, 1993). These methods are chiefly based on the measure of the oxygen uptake during the enzymic reaction, on the reduction of the oxidised phenolic substrate by ascorbic acid or on the spectrophotometric determination of the oxidation products (Pifferi and Baldassari, 1973). The most widely used *in vitro* assay for the DOPA oxidase activity of polyphenol oxidase is the "Dopachrome assay" (Gardner and Cadman, 1990, cited by Burton, 1994b). This assay measures the catecholase activity (see section 1.3.1) of enzyme samples using DOPA as the substrate. The conversion of DOPA to dopachrome, which has a molar extinction coefficient of 3600 $M^{-1}cm^{-1}$ at 475 nm, is measured spectrophotometrically (Burton, 1994b). The most frequently used and reliable procedures for protein determination are the Bradford (Bradford, 1976) and Lowry (Lowry *et al.*, 1951) methods. The Bradford method, which is simple and rapid to complete, was used in this work. This assay involves the binding of Coomassie Brilliant Blue G-250 to protein to form a protein-dye complex with a high extinction coefficient thereby allowing the sensitive measurement of small quantities of protein. The binding of the dye to the protein occurs very rapidly (approximately 2 minutes)

resulting in a quick and sensitive assay.

Samples of the various extracts were analysed by Polyacrylamide Gel Electrophoresis (PAGE) together with the commercial enzyme in order to compare the purity of the extracts with that of the commercially prepared enzyme. Polyphenol oxidase has been extracted and analysed by PAGE fairly extensively in the literature with some varying results (*eg.* Czapski, 1994; Zhang and Flurkey, 1997). A significant degree of variation in protein content and in protein distribution has also been found amongst commercial preparations of the enzyme analysed by PAGE (Kumar and Flurkey, 1991). Despite the variation in the literature, PAGE separations of the enzyme extracts were useful for the comparison of the extracts with each other and the commercially prepared enzyme.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Polyvinylpolypyrrolidone (PVPP), ammonium sulphate, commercially prepared polyphenol oxidase, marker proteins and L-β-3,4-dihydroxyphenylalanine (DOPA) were bought from Sigma Chemical Co. USA. Sodium chloride (NaCl) was purchased from BDH Chemicals, South Africa. Mushrooms were bought fresh from a local vegetable store.

2.2.2 Methods

2.2.2.1 Extraction of polyphenol oxidase

Mushrooms (500 g) were homogenised with approximately 1 L of ice cold acetone in a Waring blender. The resulting homogenate was then filtered on a Buchner funnel with a vacuum applied. The residue was dried between sheets of Whatman No.1 filter paper and then frozen with liquid nitrogen. Cold water (500 ml) was added to the frozen residue along with 40 g of PVPP to absorb any phenolic substrates present in the extract (Vilter, 1994). The mixture was mixed thoroughly and allowed to stand overnight at 4^0C. It was then filtered through a cloth on a funnel and the filtrate was flushed of residual acetone by bubbling nitrogen through it for 2 hours. The enzyme was further purified by performing a number of ammonium sulphate precipitations on this filtrate. A 40% saturation ammonium sulphate precipitation was done by

adding 22.6 g ammonium sulphate/100 ml of solution and leaving overnight at 4°C. The solution was then centrifuged at 10 000g for 10 minutes. A 52% saturation ammonium sulphate precipitation was performed on the resulting supernatant (40% saturation supernatant) by adding 7.35 g ammonium sulphate/100 ml of supernatant and leaving overnight at 4°C. This was then centrifuged at 10 000g for 25 minutes. The resulting pellet (52% saturation pellet) was resuspended in 30 ml water and stored at 4°C. A 60% saturation ammonium sulphate precipitation was done on the resulting supernatant by adding 4.8 g ammonium sulphate/100ml of supernatant. This was done in order to determine whether all the enzyme had precipitated out during the 52% saturation fractionation. The supernatant was stored overnight at 4°C and then centrifuged at 10 000g for 25 minutes. The resulting pellet (60% saturation pellet) was resuspended in 30 ml water and stored at 4°C. All of the described procedures were carried out on ice or in a cold room (4°C) as far as possible to avoid denaturing of the enzyme. Samples (1 ml) were taken of the crude enzyme extract, the 40% saturation supernatant, the 52% saturation pellet and the 60% saturation pellet and protein and enzyme activity assays (see section 2.2.2.2) were done on these samples. The samples containing ammonium sulphate were dialysed against water for an hour before doing these assays to remove the salt which may have interfered with the assay results. The 52% saturation pellet was dialysed against phosphate buffer (50 mM, pH 7) overnight at 4°C and then freeze-dried. This method describes the extraction procedure used for 500 g mushrooms. The volumes/masses were adjusted for extractions conducted using larger or smaller amounts of mushrooms. Protein determinations and activity assays were carried out on the freeze-dried extracts to determine the percentage protein and specific activity of each. The specific activities of the freeze-dried enzyme extracts were calculated as the polyphenol oxidase activity present per mg of each extract. All centrifuging was carried out using a Beckman model J2-21 centrifuge.

Polyphenol oxidase was extracted initially from both fresh and frozen open, brown mushrooms and from white button mushrooms in order to ascertain which mushroom type gave the enzyme extract with the highest specific activity. Polyphenol oxidase was then extracted from frozen, open, brown mushrooms for the remainder of the project.

2.2.2.2 Enzyme activity and protein assays

The enzyme activity in the samples was measured using the "Dopachrome assay". A fresh DOPA solution (10 mM DOPA in 50 mM phosphate buffer, pH 6) was made up and 0.1 ml enzyme solution (sample) was added to 3 ml of the DOPA solution in a cuvette. The change in absorbance over 3 minutes was then measured spectrophotometrically at 475 nm. The change in absorbance per minute was then used to calculate the enzyme activity present in the sample. The calculation of enzyme activity (Units/ml) and the definition of polyphenol oxidase Units are given in Appendix A.

Protein determinations were carried out on the enzyme samples using "Bradford's assay". Standard protein concentrations (0, 50, 100, 150, 200, 250 μg/ml) were made up using a 0.5 mg/ml solution of bovine serum albumin (BSA). These standards were made up to 100 μl volume using a 0.15 M NaCl solution and 1 ml Bradford's reagent was added to each standard. The absorbance at 595 nm was read for each standard after 2 minutes and a protein standard curve was generated (Appendix B). The protein content of the enzyme samples was then determined using the same procedure but with 50 μl enzyme solution in place of the BSA solution.

The enzyme activity and protein assays were conducted in duplicate and mean values were calculated. All spectrophotometric measurements were made using a Shimadzu UV-160A spectrophotometer.

2.2.2.3 Discontinuous Polyacrylamide Gel Electrophoresis (DISC-PAGE) analysis of polyphenol oxidase

PAGE separations of the enzyme extracts were carried out using Hoefer "Tall Mighty Small" electrophoresis apparatus (Model SE280). Gels were prepared with 4% stacking and 10% resolving gel concentrations. The extracts (in various quantities) were run on denaturing gels (containing Sodium Dodecyl Sulphate (SDS)) and on non-denaturing gels (without SDS) together with the commercial enzyme for approximately 3 hours at 100V. Marker proteins (listed in Table 2.5) were loaded onto the denaturing gels together with the enzyme samples.

The enzyme samples run on the denaturing gels were made up by adding 1 mg enzyme extract to 300 μl dissociation buffer. The samples were then boiled for approximately 2 minutes before loading onto the gels. After the electrophoresis, the denaturing gels were stained with Coomassie Brilliant Blue G-250 to visualise the protein bands. The enzyme samples run on the non-denaturing gels were made up by adding 1 mg enzyme extract to 300 μl 10% glycerol. After the electrophoresis, these gels were soaked in a DOPA s lution (10 mM DOPA in 50 mM phosphate buffer, pH 6) to detect enzyme activity. The molecular weights of the separated proteins/protein subunits could then be determined by plotting a graph of log molecular weight versus distance migrated for the marker proteins (Appendix C) and interpolating from the graph. This method is known to accurately determine the molecular weights of polypeptide chains for a wide variety of proteins (Weber and Osborn, 1969).

2.3 RESULTS AND DISCUSSION

2.3.1 Extraction of polyphenol oxidase

2.3.1.1 Comparison of enzyme extractions from different mushroom types

The analysis of the samples taken during the enzyme extractions from fresh brown; white button and frozen brown mushrooms are tabulated in Tables 2.1, 2.2 and 2.3, respectively. Polyphenol oxidase was extracted once from white button mushrooms and numerous times from fresh and frozen brown mushrooms. Tables 2.1 and 2.3 show the most successful extraction performed for fresh and frozen brown mushrooms, respectively.

Table 2.1. Purification table of polyphenol oxidase extracted from fresh brown mushrooms (500g)

Fraction	Crude sample	AS 40% supt.	AS 52% pellet	AS 60% pellet
Volume (ml)	300	312	30	30
Protein (mg/ml)	1.138	0.213	0.426	0.410
Activity (µmols/min/ml)	6.986	2.640	25.917	12.155
Specific activity (µmols/min/mg)	6.139	12.394	60.837	29.647
Total activity (µmols/min)	2096	824	778	365
Total protein (mg)	341.4	66.5	12.8	12.3
Yield (%)	100	39	37	17
Fold purification	1.00	2.02	9.87	4.83

AS = ammonium sulphate
supt.= supernatant
Refer to Appendix A for formulae used for the calculation of the data.

Table 2.2. Purification table of polyphenol oxidase extracted from white button mushrooms (250g)

Fraction	Crude sample	AS 40% supt.	AS 52% pellet	AS 60% pellet
Volume (ml)	176	190	30	30
Protein (mg/ml)	0.502	0.174	0.173	0.212
Activity (µmols/min/ml)	0.709	0.282	0.336	0.811
Specific activity (µmols/min/mg)	1.412	1.621	1.942	3.825
Total activity (µmols/min)	125	54	10	24
Total protein (mg)	88.4	33.1	5.2	6.4
Yield (%)	100	43	8	19
Fold purification	1.00	1.15	1.38	2.71

AS = ammonium sulphate
supt. = supernatant
Refer to Appendix A for formulae used for the calculation of the data.

Table 2.3. Purification table of polyphenol oxidase extracted from frozen brown mushrooms (750g)

Fraction	Crude sample	AS 40% supt.	AS 52% pellet	AS 60% pellet
Volume (ml)	478	511	30	30
Protein (mg/ml)	0.900	0.296	0.290	0.333
Activity (µmols/min/ml)	13.151	7.044	27.263	20.251
Specific activity (µmols/min/mg)	14.612	23.797	94.010	60.814
Total activity (µmols/min)	6286	3600	818	608
Total protein (mg)	430.2	151.3	8.7	9.9
Yield (%)	100	57	13	10
Fold purification	1.00	1.63	6.43	4.16

AS = ammonium sulphate
supt. = supernatant
Refer to Appendix A for formulae used for the calculation of the data.

The average protein contents of the three different extracts are shown in Table 2.4.

Table 2.4. Average protein contents of different mushroom extract

Mushroom type	Percentage protein
Fresh brown	24.53
White button	8.20
Frozen brown	20.17

The average specific activities of the extracts obtained from the fresh brown, frozen brown and white button mushrooms are represented graphically in Figure 2.1.

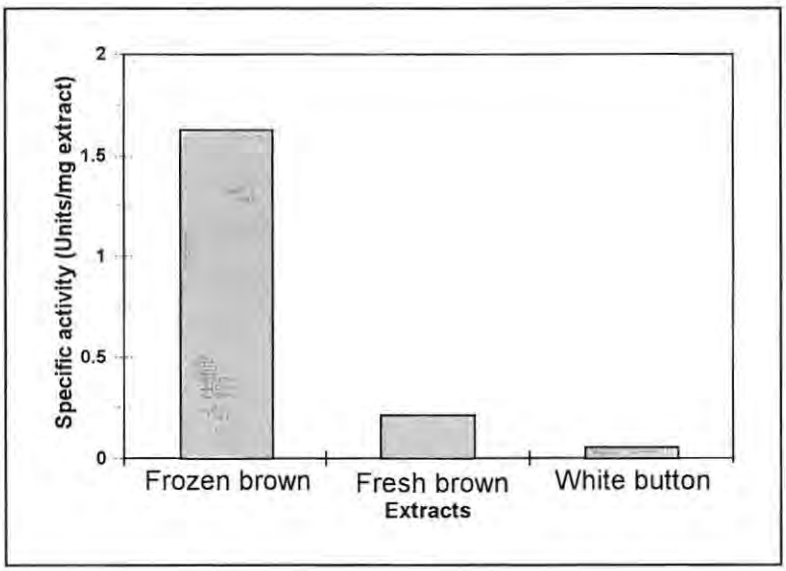


Figure 2.1. Average specific activities of polyphenol oxidase measured in different mushroom extracts

The average specific activity of the enzyme extracts from the frozen brown mushrooms was 1.629 Units/mg extract. This value was far higher than the values obtained for the fresh brown (0.213 Units/mg) or white button mushrooms (0.05421 Units/mg). These results indicate that frozen brown mushrooms produce enzyme extracts with higher specific activities than fresh brown mushrooms and white button mushrooms. Frozen brown mushrooms were therefore used for extracting polyphenol oxidase for the remainder of this study. Although polyphenol oxidase was only extracted from white button mushrooms once, the low yield of enzyme activity obtained from these mushrooms agrees with the yields obtained in the literature by other researchers. Yamaguchi *et al.* (1970) found that the white strain of *Agaricus bisporus* contained a large amount of an inactive or latent form of polyphenol oxidase which could be activated by 0.1 % sodium lauryl sulphate. The latent form constituted almost 95 % of the total polyphenol oxidase (Yamaguchi *et al.*, 1970).

2.3.1.2 Extraction of polyphenol oxidase from frozen brown mushrooms

Polyphenol oxidase was extracted from frozen brown mushrooms over the course of the study with varying degrees of success. The percentage protein present in these extracts varied from 5.9-31.2 %. The specific activities of the extracts obtained varied from 0.478-3.116 Units/mg.

The polyphenol oxidase enzyme was most successfully extracted where the specific activity increased with each step of the extraction and was the highest in the 52% pellet (Tables 2.1 and 2.3), the fraction used as the final enzyme preparation. In a number of the extractions the highest specific activity was found in the 60% pellets (Table 2.2) indicating that the enzyme had not precipitated out fully in the 52% pellet. The reason for the inconsistencies in the results obtained for the extractions is not clear but there are some possibilities:

i) There may have been differences in the mushrooms used for the extractions. Kertesz and Zito cited by Nelson and Mason (1970) have pointed out that the polyphenol oxidase content of mushrooms is low and variable. This would account for the inconsistent specific activities of the extracts;

ii) Slight variations in the incubation times of the ammonium sulphate precipitations may have resulted in inconsistent results. All the ammonium fractionations were left at least overnight at 4^0C but some were left for 48 hours which may have made a difference.

2.3.2 DISC-PAGE analysis of polyphenol oxidase

2.3.2.1 PAGE analysis of three different mushroom extracts

The protein profiles of the enzyme extracts produced from fresh brown mushrooms (**A**), white button mushrooms (**B**), frozen brown mushrooms (**C**) and the commercial enzyme (**D**) are shown in Figure 2.2.

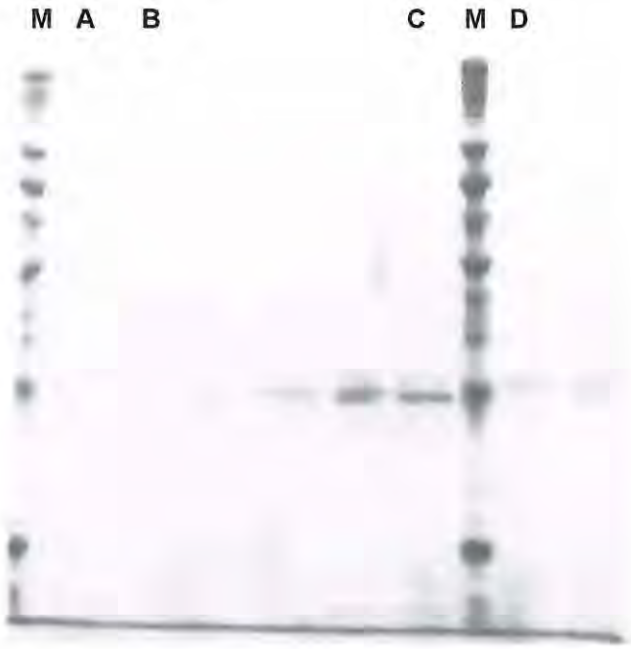


Figure 2.2. SDS-PAGE analysis of three different mushroom extracts and the commercial enzyme (A=fresh brown mushroom extract; B=white button mushroom extract; C=frozen brown mushroom extract; D=commercial enzyme extract; M=marker proteins)

The extract from fresh brown mushrooms produced three protein bands; the extract from white button mushrooms produced one protein band; the extract from frozen brown mushrooms produced five protein bands and the commercial enzyme extract produced four protein bands. These bands were visible in the gel but they are not all clearly visible in Figure 2.2. One of the five bands produced from the frozen brown mushroom extract was more intense than the other bands showing that this protein band was more concentrated than the others. Other protein bands generated from the three extracts were very faint and were therefore not visible in Figure 2.2. This may have been due to the low protein contents of the extracts or due to poor binding of the staining dye to the proteins. The distances migrated by the proteins and their molecular weights are tabulated in Table 2.5. The protein bands produced by the different extracts corresponded to bands produced by the commercial enzyme extract showing that the extracts contained protein subunits similar to the commercial enzyme extract but the protein subunits present in each varied.

Table 2.5. SDS-PAGE analysis of three different mushroom extracts and the commercial enzyme

Component	Molecular weight (Da)	Distance migrated (mm)
Myosin	205 000	10.0
β-Galactosidase	116 000	13.9
Phosphorylase b	97 400	17.8
Albumin, bovine	66 000	23.0
Albumin, egg	45 000	36.5
Carbonic anhydrase	29 000	54.5
Band 1 (**A**)	71 837	27.5
Band 2 (**A**)	55 743	33.9
Band 3 (**A**)	53 365	35.0
Band 1 (**B**)	69 871	28.2
Band 1 (**C**)	71 836	27.5
Band 2 (**C**)	52 944	35.2
Band 3 (**C**)	48 331	37.5
Band 4 (**C**)	20 861	58.7
Band 5 (**C**)	18 670	61.5
Band 1 (**D**)	51 495	35.9
Band 2 (**D**)	47 760	37.8
Band 3 (**D**)	21 027	58.5
Band 4 (**D**)	19 043	61.0

A = fresh brown mushroom extract; **B** = white button mushroom extract; **C** = frozen brown mushroom extract; **D** = commercial enzyme extract

The molecular weights of the protein subunits were interpolated from the graph of the log of the molecular weights of the protein markers versus the distances migrated (Appendix C).

The non-denaturing PAGE analysis of the three different mushroom extracts and the commercial enzyme is shown in Figure 2.3.

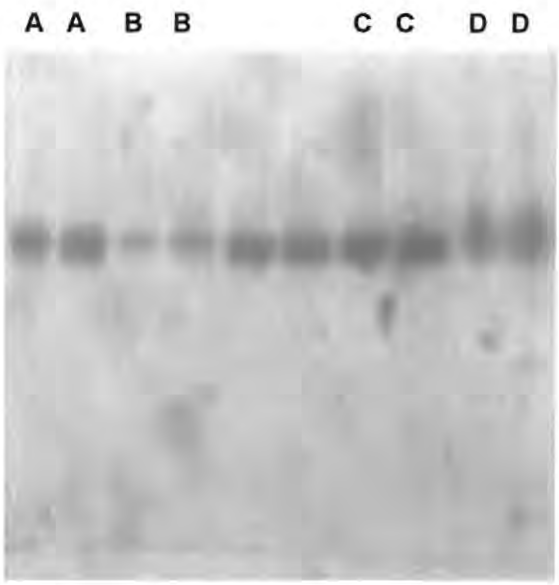


Figure 2.3. Non-denaturing PAGE analysis of three different mushroom extracts and the commercial enzyme (A=fresh brown mushroom extract; B=white button mushroom extract; C=frozen brown mushroom extract; D=commercial enzyme extract)

One active band was visible for each of the extracts after staining with DOPA. The active bands migrated to identical positions on the gel showing that their molecular weights were similar. The active band of the frozen brown mushroom extract was more intense than the other bands while the active band of the white button mushroom extract was the least intense. These results correspond favourably with the specific activities calculated for the extracts (Figure 2.1). The bands produced from the extracts compared favourably with the commercial enzyme extract showing that polyphenol oxidase had been isolated in each case.

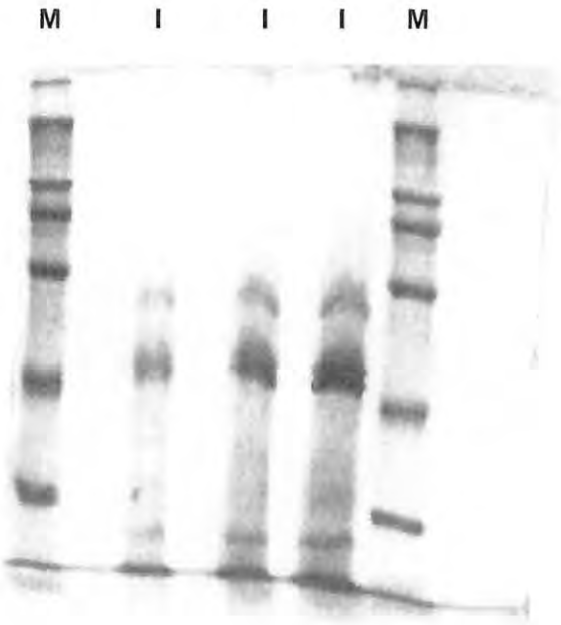
2.3.2.2 PAGE analysis of polyphenol oxidase extracts from frozen brown mushrooms

The protein bands generated by SDS-PAGE analysis of three frozen brown mushroom extracts (I, II and III) and the commercial enzyme (IV) are shown in Figure 2.4. Each of the extracts produced four visible protein bands. The molecular weights and the distances migrated for the different proteins are shown in Tables 2.6 and 2.7. The protein banding patterns shown in Figure 2.4 are similar to those obtained for the frozen brown mushroom extract and the commercial enzyme extract shown in Figure 2.2. The frozen brown mushroom extract shown

in Figure 2.2 produced five protein bands while only four bands were visible in the extracts shown in Figure 2.4. The fifth band visible in Figure 2.2 corresponded to a molecular weight of approximately 18 000 Da. Four faint protein bands are visible in the commercial extract shown in Figure 2.2 while four different bands are visible in the commercial extract in Figure 2.4 identifying a fifth protein band of 64 275 Da which was not visible in the previous gel. Altogether, the frozen brown mushroom extracts produced five different protein bands while the commercial enzyme extract produced five corresponding protein bands showing that the polyphenol oxidase extracts compared favourably with the purity of the commercially prepared enzyme. The results obtained in this study are similar to other PAGE analyses of polyphenol oxidase extracts obtained by other researchers (Kumar and Flurkey, 1991; Burton, 1994b). Variation in commercially prepared extracts as seen in Figures 2.2 and 2.4 has been reported by Kumar and Flurkey (1991) who attribute these variations to different isoenzyme distributions and to different purities of the extracts. The protein profiles obtained for the three enzyme extracts (I, II and III) are very similar to those obtained by Burton (1994b) who also obtained between four and five protein bands for polyphenol oxidase extracts obtained by the same method used in this study.

The non-denaturing DISC-PAGE analysis of extracts I, II and III and the commercial enzyme (IV) is shown in Figure 2.5. Each of the extracts produced one active band of identical molecular weight after staining with DOPA (Figure 2.5). The intensity of the bands, however, varied from one extract to another. The active band produced from extract I was similar in intensity to the band produced by the commercial enzyme extract indicating that these two extracts had similar enzyme activity. The active bands produced from extracts II and III, however, were less intense, particularly the band produced from extract III, showing that these extracts had lower enzyme activities. The bands obtained were identical to the commercial enzyme showing that polyphenol oxidase had been isolated in each case while the difference in intensity of the bands confirmed the different specific activities of the extracts.

(A)



(B)

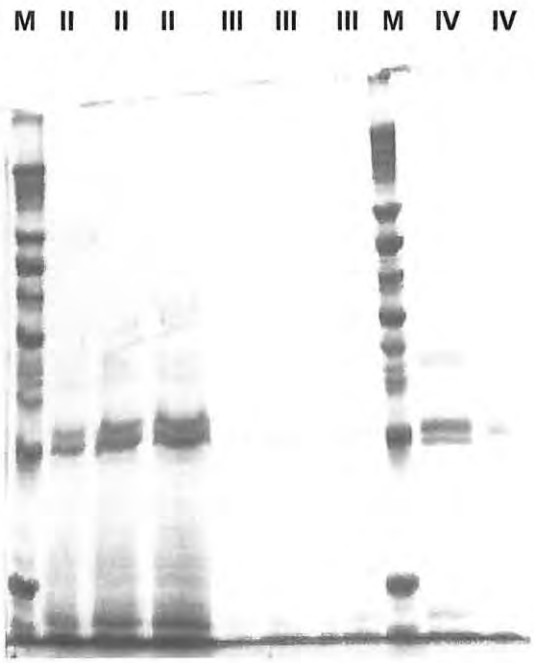


Figure 2.4. SDS-PAGE analysis of frozen brown mushroom extracts and the commercial enzyme (A) Extract I (B) Extracts II, III and the commercial enzyme (IV)

Table 2.6. SDS-PAGE analysis of frozen brown mushroom extract I

Component	Molecular weight (Da)	Distance migrated (mm)
Myosin	205 000	4.5
β-Galactosidase	116 000	10.8
Phosphorylase b	97 400	13.3
Albumin, bovine	66 000	19
Albumin, egg	45 000	30
Carbonic anhydrase	29 000	39.8
Band 1 (I)	72 796	20.5
Band 2 (I)	53 180	26.5
Band 3 (I)	49 682	27.8
Band 4 (I)	23 631	42

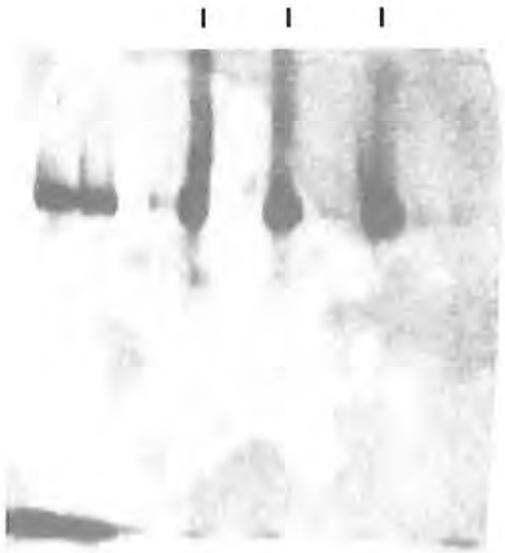
The molecular weights of the protein subunits were interpolated from the graph of the log of the molecular weights of the protein markers versus the distances migrated (Appendix C).

Table 2.7. SDS-PAGE analysis of frozen brown mushroom extracts II and III and the commercial enzyme (IV)

Component	Molecular weight (Da)	Distance migrated (mm)
Myosin	205 000	8.9
β-Galactosidase	116 000	19.5
Phosphorylase b	97 400	24
Albumin, bovine	66 000	34.2
Albumin, egg	45 000	50.3
Carbonic anhydrase	29 000	69.5
Band 1 (II)	62 510	40.9
Band 2 (II)	50 177	48
Band 3 (II)	46 441	50.5
Band 4 (II)	22 439	74
Band 1 (III)	66 296	39
Band 2 (III)	50 960	47.5
Band 3 (III)	48 049	49.4
Band 4 (III)	21 092	76
Band 1 (IV)	64 275	40
Band 2 (IV)	49 868	48.2
Band 3 (IV)	45 870	50.9
Band 4 (IV)	21 621	75.2

The molecular weights of the protein subunits were interpolated from the graph of the log of the molecular weights of the protein markers versus the distances migrated (Appendix C).

(A)



(B)

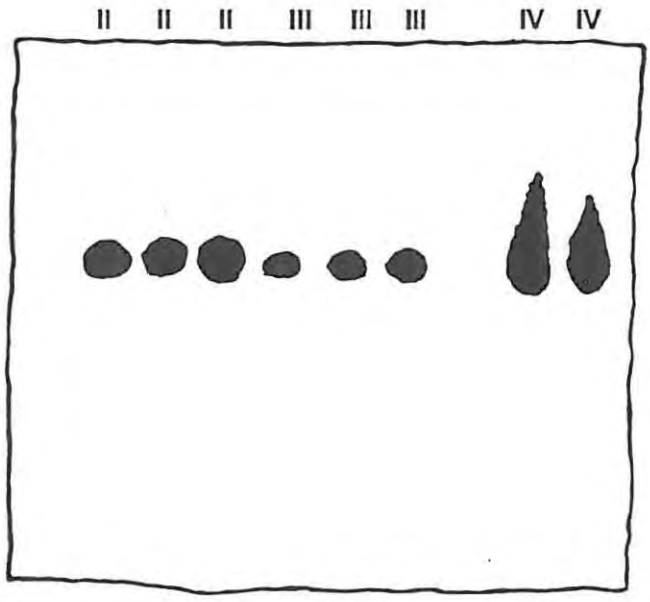


Figure 2.5. Non-denaturing PAGE analysis of frozen brown mushroom extracts and the commercial enzyme (A) Extract I (B) Diagrammatical representation of the bands generated by PAGE of extracts II, III and the commercial enzyme (IV)

2.4 CONCLUSION

Polyphenol oxidase was most successfully extracted from frozen brown mushrooms since this extract contained the highest enzyme activity per mg of protein extracted (Table 2.3). The banding patterns generated from the enzyme extracts by DISC-PAGE compared favourably with the commercial enzyme extract and with those reported by other researchers. The protein banding patterns of the extracts also compared favourably with each other showing that the extraction method was able to produce enzyme extracts of uniform composition. The varying intensity of the activity bands, however, showed that the enzyme activity present in each of the extracts did vary considerably and this was taken into account with the use of these extracts in subsequent experiments.

CHAPTER 3:
Colour production by polyphenol oxidase on reaction with phenolic substrates

3.1 INTRODUCTION

Since the development of the bioprobe was to depend on the coloured products produced by the enzyme in the presence of phenolic substrates, a number of aspects of this colour-producing reaction had to be investigated. Firstly, the quantity of enzyme required to give a visible colour change in the presence of phenolic substrates had to be determined. Secondly, and most importantly, the relationship between phenolic substrate concentration and colour intensity produced had to be established. It had to be shown that this relationship was linear since the development of the bioprobe would be based on this, i.e. the colour intensity produced would have to be proportional to the concentration of phenolic substrates present in a sample in order for an accurate quantification of concentration to be made. The third aspect to be investigated was the length of time taken for the enzyme to react with the substrates. This was investigated in order to determine the length of time a bioprobe would take to produce a result. This investigation was repeated for the immobilised enzyme in order to determine any change in the reaction time (chapter 5). Since the colours produced were quantified using spectrophotometric assays, the wavelengths of maximum absorbance for the quinones formed from the standard phenolic substrates used were found and used in these investigations.

3.2 MATERIALS AND METHODS

3.2.1 Materials

The *p*-cresol used in this and all subsequent experiments was obtained from Aldrich Chemical Company Inc. while the phenol and *p*-chlorophenol used were purchased from BDH Chemicals Ltd.

3.2.2 Methods

3.2.2.1 Determining the wavelengths of maximum absorbance for the quinones formed from some standard phenolic substrates

The wavelengths of maximum absorbance were determined for the quinones formed from phenol, *p*-cresol and *p*-chlorophenol by reading the spectrophotometric absorbance of these quinones over a wavelength range from 220-520 nm. These substrates were chosen since they are common phenolic compounds. Solutions (1 mM) of phenol, *p*-cresol and *p*-chlorophenol were made up and 0.1 ml of a 1 mg/ml polyphenol oxidase solution was added to 3 ml of each of these substrate solutions. The absorbance was then read after an obvious colour had developed in each of the 3 ml solutions, i.e. after approximately 2 hours.

3.2.2.2 Determination of the quantity of enzyme required to produce a change in absorbance of 0.5 (obvious colour) after reacting with phenolic substrates for 5-10 minutes

Various quantities of polyphenol oxidase were added to 3 ml of 1 mM, 0.1 mM and 0.01 mM solutions of *p*-cresol, *p*-chlorophenol and phenol. The absorbance of each was measured spectrophotometrically after 5 and 10 minutes. Increasing quantities of the enzyme were added until an absorbance change of 0.5 was obtained after this time interval.

3.2.2.3 Establishing the relationship between phenolic substrate concentration and colour intensity produced

A series of concentrations from 1-100 mg/L of *p*-cresol, *p*-chlorophenol and phenol were made up. This unit of concentration (mg/L) was used since this is the unit of concentration used most commonly in the literature dealing with pollutants. Equal amounts (1.032 Units) of polyphenol oxidase were added to 3 ml of each substrate concentration and the colours in the solutions were allowed to develop until a visible spectrum of colours could be seen over the range of substrate concentrations. The relationship between the (starting) phenol concentrations and the corresponding colour intensities produced was then determined by measuring the absorbance of the solutions spectrophotometrically and plotting the absorbance readings versus the (starting)

substrate concentrations. Linear regressions were then drawn through the points in order to assess the linearity of the relationship. This experiment was done in duplicate and mean values were calculated.

3.2.2.4 Determination of the length of time required by polyphenol oxidase in order to produce colours with intensities proportional to the substrate concentrations

This experiment was carried out using the same procedure as used in the previous experiment (section 3.2.2.3), except that the absorbance was read for each substrate concentration after 10 minutes, 1 hour and 3 hours. The colour intensities produced after each time interval were plotted against the (starting) substrate concentrations so that the time interval after which the relationship between the colour intensities that had developed and the (starting) substrate concentrations was linear could be determined.

3.3 RESULTS AND DISCUSSION

3.3.1 Determining the wavelengths of maximum absorbance for the quinones formed from some standard phenolic substrates

The wavelengths of maximum absorbance for the quinones formed from phenol, *p*-cresol and *p*-chlorophenol are shown in Table 3.1.

Table 3.1. Wavelengths of maximum absorbance for quinones formed from standard phenolic substrates

Substrate	Wavelength of maximum absorbance (nm)	Colour observed
Phenol	380	orange/red
p-Cresol	399	yellow
p-Chlorophenol	391	yellow

It was found that the optimum wavelengths for measuring the absorption of the quinones formed from phenol, *p*-cresol and *p*-chlorophenol were 380, 399 and 391 nm, respectively (Table 3.1).

Hence, these wavelengths were used in all subsequent assays involving these quinones.

3.3.2 Determination of the quantity of enzyme required to produce a change in absorbance of 0.5 (obvious colour) after reacting with phenolic substrates for 5-10 minutes

It was found that for each concentration, a point was reached where the addition of more enzyme no longer increased the absorbance reading obtained. It was therefore not possible to obtain an absorbance reading of 0.5 for the lower concentrations of the substrates. These results indicated that for each concentration, a point was reached where the enzyme no longer had substrate to react with and therefore the maximum absorbance for that substrate concentration had been reached. The next step in the investigation was to establish the relationship between the substrate concentration and the colour intensity produced (section 3.3.3).

3.3.3 Establishing the relationship between phenolic substrate concentration and colour intensity produced

Visible spectra of colours were seen over the ranges of substrate concentrations after 3 hours. The absorbance readings measured for the different substrate concentrations were therefore plotted against the (starting) substrate concentrations after this length of time. The relationship between the (starting) substrate concentrations and the corresponding colour intensities produced is shown graphically in Figure 3.1 for phenol, *p*-cresol and *p*-chlorophenol.

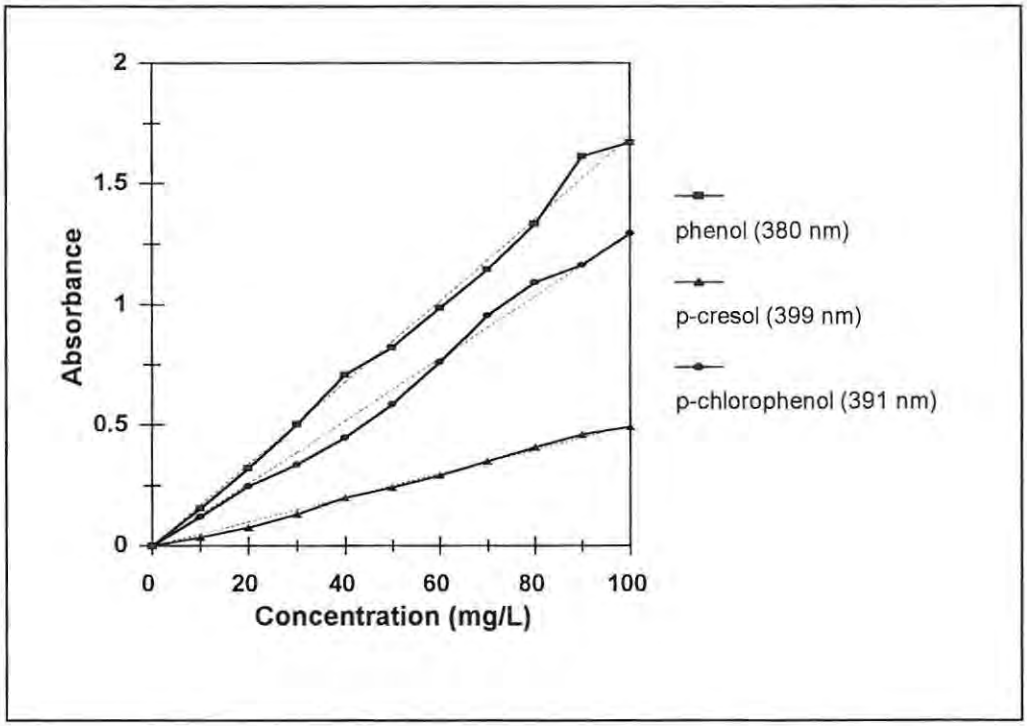


Figure 3.1. Graph of concentration versus colour intensity (expressed as absorbance) for three standard phenolic substrates (phenol: r^2=0.996; *p*-cresol: r^2=0.991; *p*-chlorophenol: r^2=0.995)

Figure 3.1 showed that the relationship between the (starting) concentration of the substrates and the intensity of the colours produced was linear. This was a crucial result since, as already stated, the development of the bioprobe would be based on this relationship, i.e. the colour intensity produced by the bioprobe would have to be proportional to the concentration of the phenolic substrates present in a sample in order for the test to be meaningful.

3.3.4 Determination of the length of time required by polyphenol oxidase in order to produce colours with intensities proportional to the substrate concentrations

The absorbance readings taken at 10 minutes, 1 hour and 3 hours for the different concentrations of *p*-cresol, *p*-chlorophenol and phenol are represented graphically in Figures 3.2, 3.3 and 3.4, respectively.

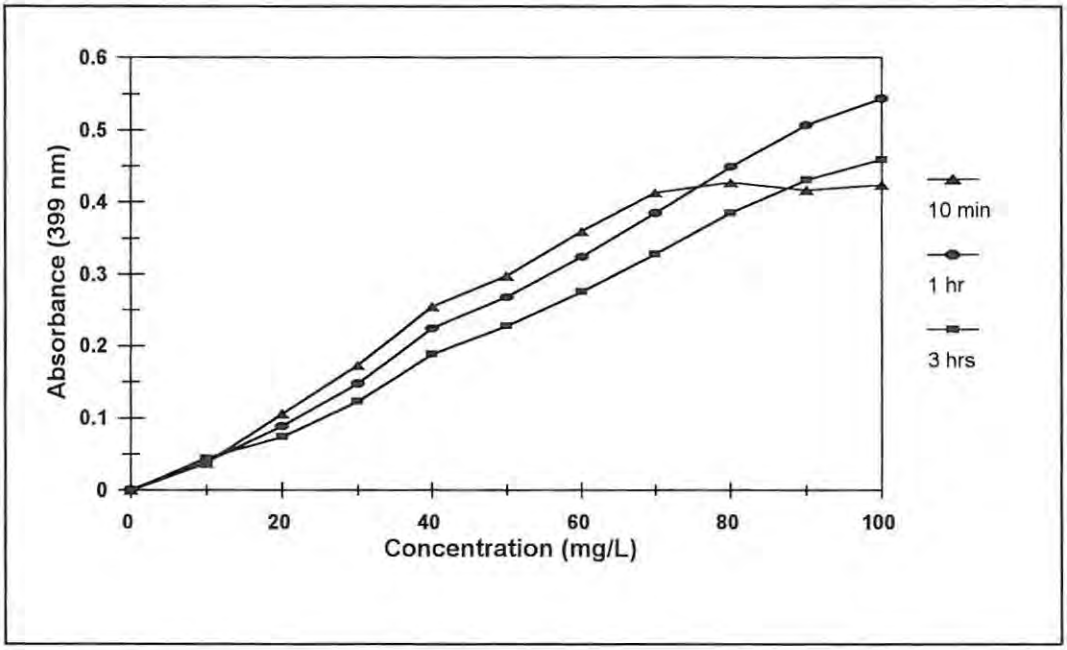


Figure 3.2. Graph of concentration versus colour developed (expressed as absorbance) with time for *p*-cresol

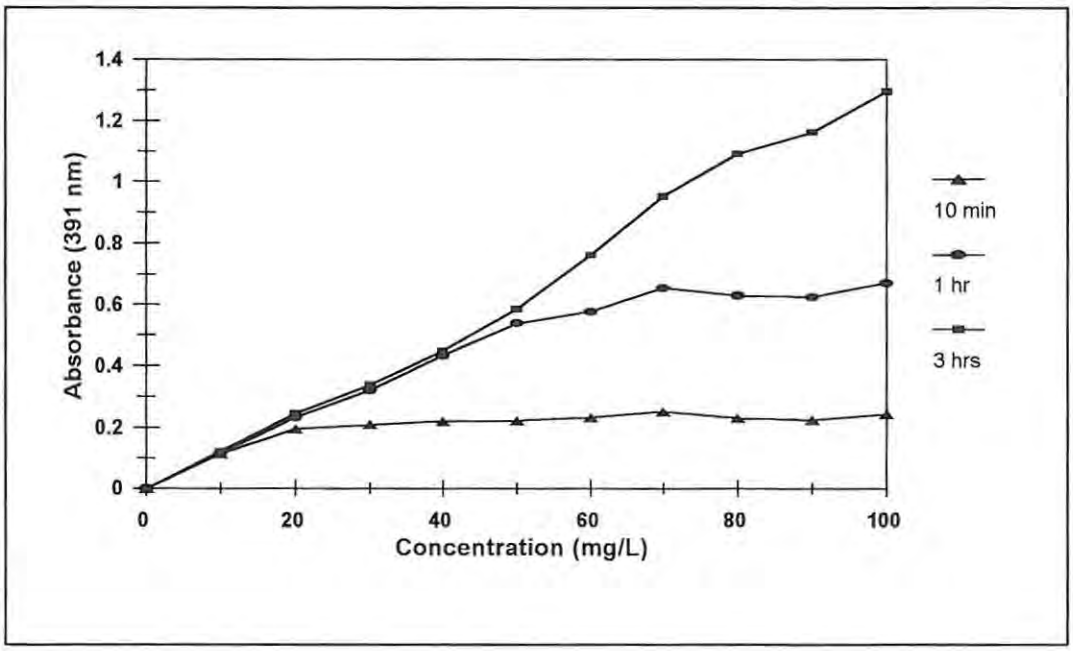


Figure 3.3. Graph of concentration versus colour developed (expressed as absorbance) with time for *p*-chlorophenol

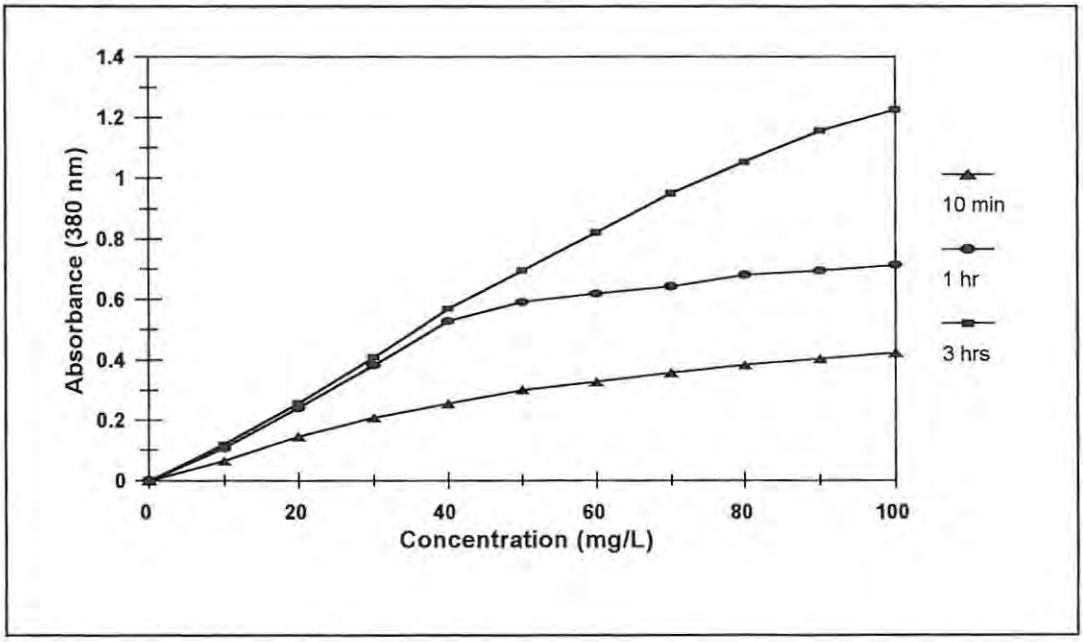


Figure 3.4. Graph of concentration versus colour developed (expressed as absorbance) with time for phenol

It was found that the time taken for the colour intensities to become proportional to the (starting) substrate concentrations varied for the three substrates. The colour intensities of the *p*-cresol solutions became proportional to the (starting) substrate concentrations after 1 hour, while the colour intensities of the *p*-chlorophenol and phenol solutions only became proportional to the (starting) substrate concentrations after 3 hours. This indicated that *p*-cresol was a more favourable substrate for polyphenol oxidase than the other two substrates and therefore reacted more quickly with the enzyme. The reaction times may have been influenced by a lag phase in the reactions due to substrate inhibition at higher substrate concentrations. These results suggested that a bioprobe test in solution would need to be 3 hours in length in order to accurately quantify phenolic substrates in a sample since the intensity of the colour produced would only be proportional to the concentration of the phenolic substrates in a sample after this length of time. The reaction time required for the bioprobe comprising the immobilised enzyme was reassessed later (section 5.3.9).

3.4 CONCLUSION

The relationship between the concentration of a phenolic substrate present in a sample and the intensity of the colour produced by polyphenol oxidase in solution on reaction with the phenolic substrate was found to be linear after three hours (for *p*-cresol, *p*-chlorophenol and phenol).

CHAPTER 4:
Immobilisation of polyphenol oxidase

4.1 INTRODUCTION

This investigation was carried out in order to establish the effect of immobilising polyphenol oxidase on the colour reaction of the enzyme. The immobilisation of polyphenol oxidase on a variety of supports has been described in the literature. These include cation exchange resins (*eg.* Wada *et al.*, 1993), glass beads, celite (*eg.* Day and Legge, 1995), various membranes (*eg.* Campanella *et al.*, 1993; Pialis and Saville, 1998) and electrodes (Burestedt *et al.*, 1996). In the present study, polyphenol oxidase was first immobilised on filter paper and on the inner surfaces of test-tubes by simple adsorption (air drying), and later it was immobilised on three different membranes. On reaction with solutions of phenolic substrates, the enzyme, immobilised on filter paper and test-tubes, leached into the substrate solutions and produced the same colour changes in the solutions of phenolic substrates as the free enzyme. The colours were therefore produced in solution rather than on the filter paper or the test-tubes. The enzyme was immobilised on membranes in an attempt to immobilise it in such a way as to produce a colour change on the support on which it was immobilised. The three membranes used were polyethersulphone, nitrocellulose and nylon. The immobilisation methods used were adsorption and cross-linking of the enzyme on the membrane surfaces. Adsorption relies on the immobilisation of the enzyme to the membrane by either an electrostatic attraction of opposite charges on the surface of the enzyme and the membrane or by hydrophobic interactions between the enzyme and the membrane. Cross-linking involves the covalent attachment of the enzyme to the membrane by the formation of covalent bonds between the membrane and the cross-linking agent and between the cross-linking agent and the enzyme. The enzyme is therefore much more strongly attached to the membrane by the second immobilisation method. The cross-linking agent used in these experiments was glutaraldehyde ($OHC\text{-}(CH_2)_3\text{-}CHO$). The enzyme was therefore covalently attached to the membranes by the formation of covalent bonds between the carbonyl groups on the glutaraldehyde and amino groups on the surfaces of the enzyme and/or the membranes.

The immobilisation of polyphenol oxidase on the various membranes, and the subsequent reaction with the phenolic substrates, resulted in ranges of beige/brown colours on the membranes. A third immobilisation procedure was carried out in which a colour reagent, 3-methyl-2-benzothiazolinone hydrazone (MBTH), was added to the immobilisation mix in an attempt to change the hue of the colours produced on the membranes. This reagent undergoes a coupling reaction with the quinone products of the oxidation of various monophenols in the presence of polyphenol oxidase (Rodriguez-Lopez *et al.*, 1994). The *o*-benzoquinone produced by this coupling reaction is red in colour (Pifferi and Baldassari, 1973). A pathway proposed by Rodriguez *et al.* (1994) for the hydroxylation of phenol catalysed by polyphenol oxidase in the absence and presence of MBTH is shown in Figure 4.1. This reaction has been used for the continuous spectrophotometric measurement of the cresolase and catecholase activities of polyphenol oxidase (Mazzocco and Pifferi, 1976; Rodriguez-Lopez *et al.*, 1994; Espin *et al.*, 1997c). The reaction has also been used in a recent study of the mechanism of *o*-semiquinone production in the polyphenol oxidase catalysed oxidation of various catechols (Ferrari *et al.*, 1997). In the present study, an attempt was made to immobilise polyphenol oxidase together with MBTH in the hope that the membranes would be coloured red on reaction with the phenolic substrates, an aesthetic improvement on the beige/brown colours produced without this reagent.

Once the enzyme had been immobilised on the various supports, a preliminary investigation was made into the effect of the immobilisation methods and the supports on the durability of the enzyme. This was done in order to further evaluate the immobilisation methods and supports so that the most suitable combination of support and immobilisation method could be chosen for the development of the bioprobe.

During the course of the immobilisation experiments, it was found that the enzyme solution coloured the membranes slightly brown. An experiment was therefore also performed in an attempt to remove this colour from the enzyme solution by further purifying the enzyme by molecular exclusion chromatography.

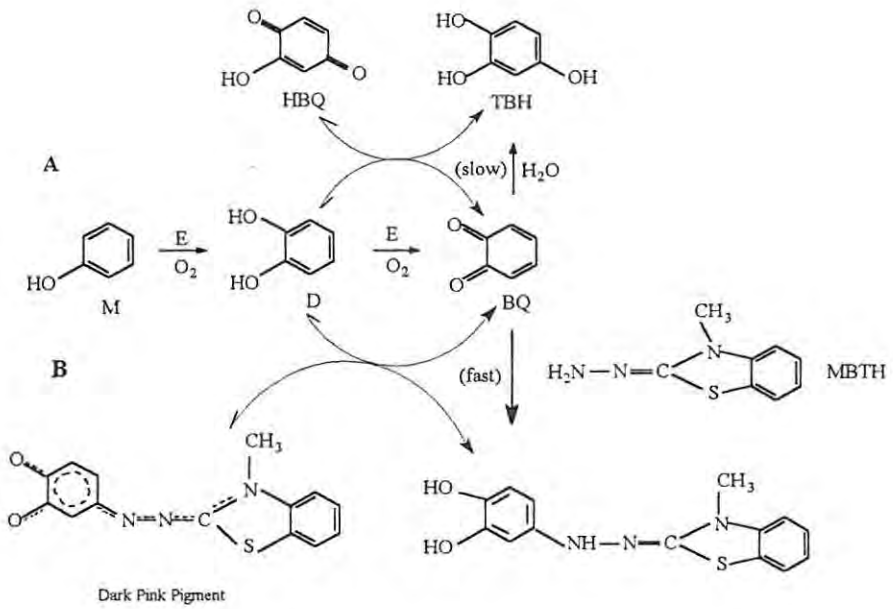


Figure 4.1. Proposed pathways for the hydroxylation of phenol catalysed by polyphenol oxidase in the absence (A) and presence (B) of MBTH (E=polyphenol oxidase; M=phenol; D=catechol; BQ=*o*-benzoquinone; THB=1,2,4-trihydroxybenzene; HBQ=hydroxybenzoquinone) (Rodriguez-Lopez *et al.*, 1994)

4.2 MATERIALS AND METHODS

4.2.1 Materials

Glutaraldehyde and 3-methyl-2-benzothiazolinone hydrazone (MBTH) were purchased from BDH Chemicals Ltd. and Sigma Chemical Company, respectively. Polyethersulphone, nitrocellulose and nylon (0.45 micron pore size) membranes were purchased from The Institute of Polymer Science (Stellenbosch), Amersham and Micron Separations Inc., respectively.

4.2.2 Methods

4.2.2.1 Polyphenol oxidase dried on filter paper

Equal amounts (1.032 Units) of polyphenol oxidase were allowed to air dry on strips of Whatman No.1 filter paper. These were then placed into 1-100 mg/L solutions (3 ml) of *p*-cresol, *p*-chlorophenol and phenol for 3 hours and the colour intensities produced were measured spectrophotometrically. The absorbance readings measured for the various substrate

concentrations after 3 hours were plotted against the substrate concentrations and linear regressions were plotted.

4.2.2.2 Polyphenol oxidase dried in test-tubes

The same amount of enzyme as used in the previous experiment (1.032 Units) was air dried on the inner surfaces of test-tubes. Equal volumes (3 ml) of the solutions of *p*-cresol, *p*-chlorophenol and phenol (1-100 mg/L) were then added to the tubes and the colour intensities produced after 3 hours were measured spectrophotometrically. The absorbance readings measured for the various substrate concentrations were plotted against the substrate concentrations and linear regressions were plotted.

4.2.2.3 Immobilisation of polyphenol oxidase on membranes

A 10 ml volume of a 10 Units/ml solution of polyphenol oxidase was made up and kept on ice to avoid denaturing of the enzyme. Since the specific activities of the enzyme extracts varied, the mass of enzyme extract dissolved was adjusted so that the number of Units of enzyme activity per ml in the solution remained constant. The enzyme solution was added to 20 ml phosphate buffer (0.1 M, pH 7) and 33 equal sized pieces of polyethersulphone, nitrocellulose or nylon membranes were added. The nylon membranes were soaked in 3 M HCl for 10 minutes in order to increase the presence of carboxyl and amino functional groups on the surfaces (Pialis *et al.*, 1996). They were then rinsed thoroughly with distilled water before they were added to the enzyme solution. The solutions containing the enzyme and membranes were incubated at 4^0C for 12 hours with gentle agitation to distribute the enzyme evenly in the solutions. The membrane pieces were removed from the enzyme solutions after the incubation and rinsed with phosphate buffer before being left to air dry. Protein determinations according to Bradford (1976) were carried out on the enzyme solutions before and after each immobilisation in order to calculate the average amount of protein immobilised on each membrane piece. Once dry, the pieces of membranes were placed into 1-100 mg/L solutions (3 ml) of phenol, *p*-cresol and *p*-chlorophenol and the colours produced on the membranes were photographed after 3 hours.

The immobilisation of the enzyme with glutaraldehyde was carried out using the same procedure as above. Glutaraldehyde was added to the enzyme solution to make a final concentration of 2.5% (w/v) before adding the membrane pieces. This concentration of glutaraldehyde was found to be sufficient to achieve acceptable immobilisation yields for polyphenol oxidase on nylon membranes by Pialis *et al.* (1996).

The immobilisation of the enzyme with MBTH was carried out only with nylon membranes. MBTH (0.022 g) was dissolved in 20 ml distilled water to make a final concentration of 5 mM and this solution was then added to the 10 ml enzyme solution (10 Units/ml). The nylon discs (after activation by soaking in 3 M HCl for 10 minutes and thorough rinsing in distilled water) were then added and the solution was incubated as above. After incubation, the discs were removed from the solution and allowed to air dry. Protein determinations and testing of the discs in the various phenol solutions were carried out as above.

Polyphenol oxidase was immobilised on:
i) polyethersulphone, by adsorption and by cross-linking with glutaraldehyde;
ii) nitrocellulose, by cross-linking with glutaraldehyde;
iii) nylon, by adsorption and cross-linking with glutaraldehyde;
iv) nylon, by adsorption and cross-linking with glutaraldehyde in the presence of MBTH.

4.2.2.4 Effects of various immobilisation methods and supports on the durability of polyphenol oxidase activity

Polyphenol oxidase immobilised on filter paper (by adsorption), test-tubes (by adsorption), nitrocellulose (by cross-linking) and nylon (in the presence of MBTH by adsorption and cross-linking) was stored in a desiccator for a month. After this storage period, the activity of the immobilised enzyme was tested by observing the colour change produced in a 100 mg/L phenol solution (3 ml) after 3 hours and comparing it with the colour change produced in a 100 mg/L phenol solution (3 ml) after 3 hours by the freshly immobilised enzyme.

4.2.2.5 Removal of the brown colour from the polyphenol oxidase solution

A 2 mg/ml solution (10 ml) of polyphenol oxidase was made up in phosphate buffer (0.1 M, pH 7). This solution was run through a Sephadex G-25-150 column using phosphate buffer (0.1 M, pH 7) and 2 ml fractions were collected. Protein determinations according to Bradford (1976) were carried out on the enzyme solution before the chromatography and on the fractions collected from the column. The fractions were also assayed for enzyme activity and those containing the highest activity were combined. The level of brown colour remaining in the combined fractions was then observed.

4.3 RESULTS AND DISCUSSION

4.3.1 Polyphenol oxidase dried on filter paper

On placing the filter paper strips with the immobilised polyphenol oxidase into the substrate solutions, the enzyme leached into the substrate solutions and the colour reactions therefore took place in the solutions. The absorbance readings measured for the ranges of substrate concentrations (1-100 mg/L) after 3 hours, plotted against the substrate concentrations for phenol, *p*-cresol and *p*-chlorophenol are shown in Figure 4.2. The colour changes produced in the phenolic substrates were very similar to the colour changes produced by the free enzyme placed directly into solution. Drying polyphenol oxidase on filter paper therefore did not adversely affect the catalytic activity of the enzyme.

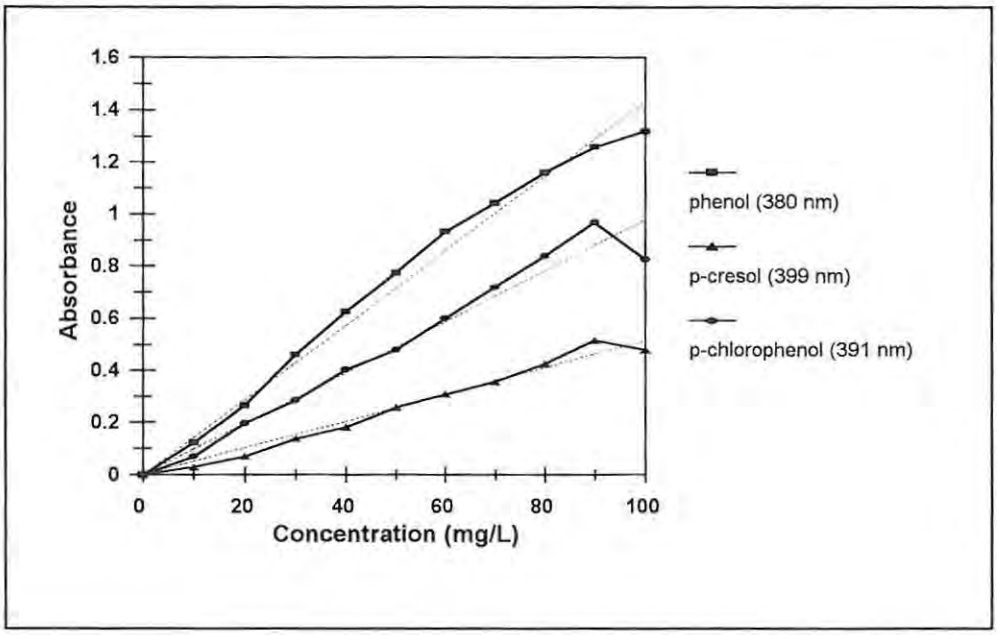


Figure 4.2. Graph of phenolic substrate concentration versus colour developed (expressed as absorbance) using polyphenol oxidase dried on filter paper (phenol: r^2=0.986; *p*-cresol: r^2=0.980; *p*-chlorophenol: r^2=0.967)

4.3.2 Polyphenol oxidase dried in test-tubes

As with the filter paper experiment, the immobilised polyphenol oxidase leached off the test-tube surfaces and went into solution. The substrate solutions therefore became coloured. The absorbance readings measured for the range of substrate concentrations (1-100 mg/L) after 3 hours, plotted against the substrate concentrations for phenol, *p*-cresol and *p*-chlorophenol are shown in Figure 4.3. The colour changes produced in the substrate solutions were very similar to the colour changes produced by the free enzyme in solution indicating that drying the enzyme on test-tubes did not have an adverse affect on the catalytic activity of the enzyme.

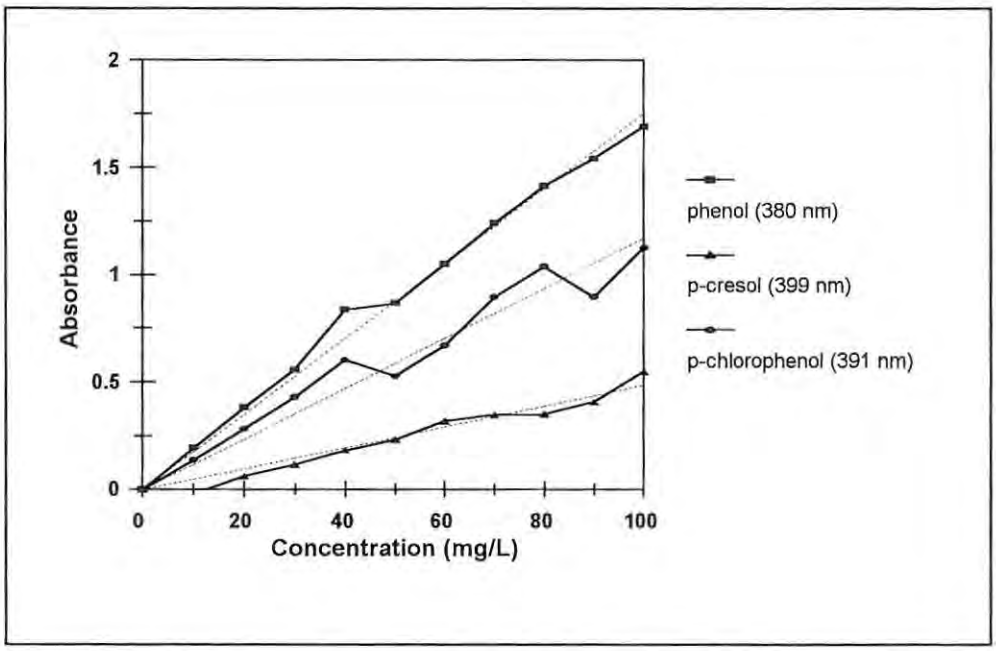


Figure 4.3. Graph of phenolic substrate concentration versus colour developed (expressed as absorbance) using polyphenol oxidase dried in test-tubes (phenol: r^2=0.992; *p*-cresol: r^2=0.958; *p*-chlorophenol: r^2=0.945)

4.3.3 Immobilisation of polyphenol oxidase on membranes

The amounts of protein immobilised per piece of membrane for the different membranes are shown in Table 4.1.

Table 4.1. Immobilisation of polyphenol oxidase on membranes

Membrane type	Protein immobilised per piece of membrane (mg)			
	with glut.	% bound	without glut.	% bound
Polyethersulphone	0.027	1.78	0.012	0.79
Nitrocellulose	0.066	4.36	-	-
Nylon	0.066	4.36	0.031	2.05
Nylon with MBTH	0.056	3.70	0.036	2.38

glut. = glutaraldehyde

Of the three membranes used, nylon showed the best uptake and the most even distribution of the enzyme. In each case, more enzyme was immobilised per piece of membrane in the presence of glutaraldehyde. The results obtained with the nylon membranes are similar to those

obtained by Thompson *et al.* (1985) who obtained 3.8% immobilisation of glucose oxidase on nylon with glutaraldehyde using a similar immobilisation procedure.

In contrast to the enzyme immobilised on filter paper and in test-tubes, the enzyme immobilised on membranes produced colours on the surfaces of the membranes. The various colours produced on the membranes are described in the following sections (section 4.3.3.1 to 4.3.3.4).

4.3.3.1 Immobilisation of polyphenol oxidase on polyethersulphone

Polyphenol oxidase did not immobilise evenly or in large quantities on the polyethersulphone membranes and therefore the quality of the colours produced on the membranes was poor. The ranges of light brown and beige colours produced on this membrane in the presence of phenol, *p*-cresol and *p*-chlorophenol are shown in Figures 4.4 and 4.5. The colours on the membranes with the enzyme immobilised with glutaraldehyde (Figure 4.5) were slightly darker than those with the enzyme immobilised without glutaraldehyde (Figure 4.4) indicating that more enzyme was immobilised in the presence of glutaraldehyde.

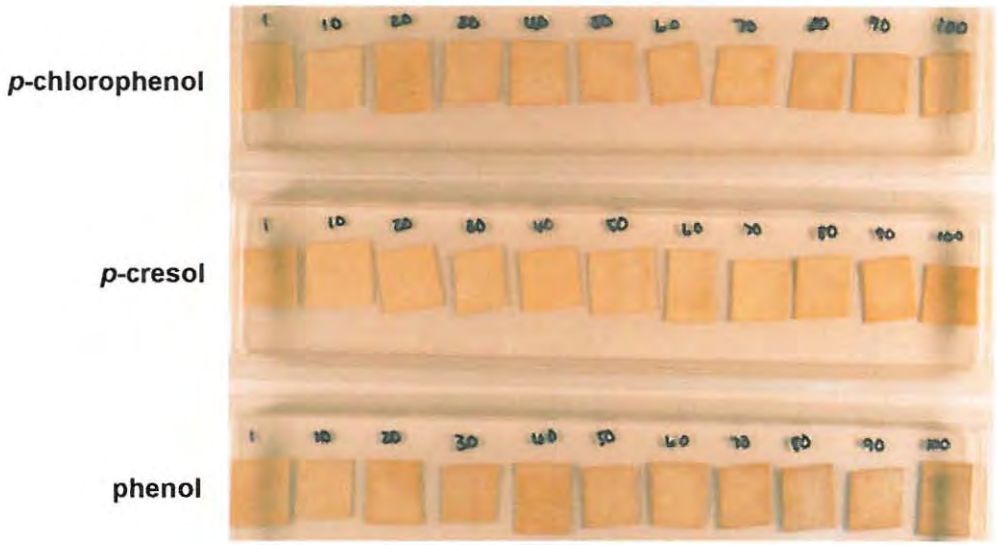


Figure 4.4. Colours produced in 1-100 mg/L solutions of phenol, *p*-cresol and *p*-chlorophenol by polyphenol oxidase immobilised on polyethersulphone (without glutaraldehyde)

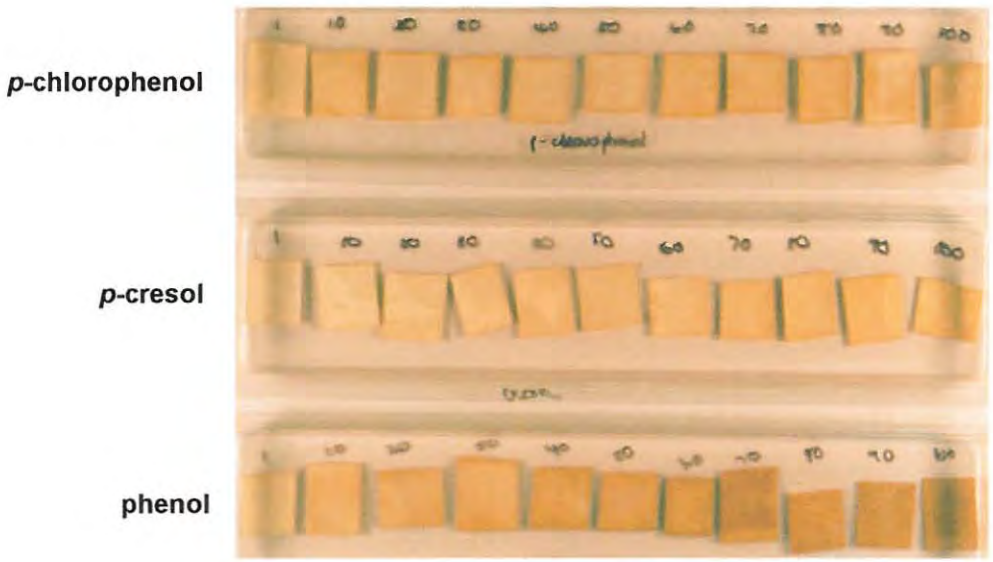


Figure 4.5. Colours produced in 1-100 mg/L solutions of phenol, *p*-cresol and *p*-chlorophenol by polyphenol oxidase immobilised on polyethersulphone (with glutaraldehyde)

4.3.3.2 Immobilisation of polyphenol oxidase on nitrocellulose

Polyphenol oxidase immobilised far more evenly and in larger quantities on the nitrocellulose than on the polyethersulphone resulting in the production of darker colours on the membranes. The ranges of cream and brown colours produced by the enzyme on this membrane in the presence of phenol, *p*-cresol and *p*-chlorophenol are shown in Figure 4.6.

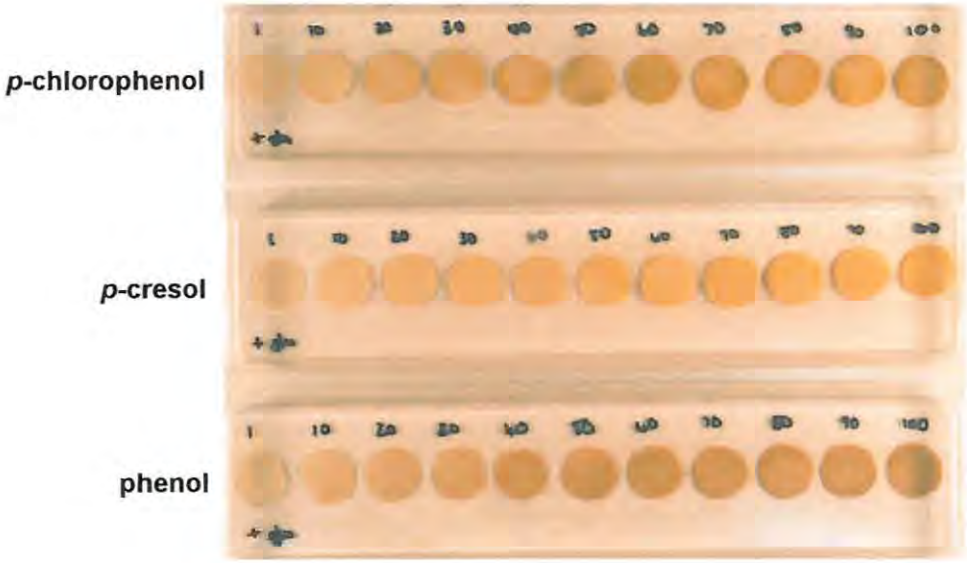


Figure 4.6. Colours produced in 1-100 mg/L solutions of phenol, *p*-cresol and *p*-chlorophenol by polyphenol oxidase immobilised on nitrocellulose (with glutaraldehyde)

4.3.3.3 Immobilisation of polyphenol oxidase on nylon

The polyphenol oxidase immobilised on nylon more evenly than on the other membranes and in similar quantities as on nitrocellulose. Furthermore, as with the polyethersulphone membranes, the enzyme appeared to immobilise in larger quantities and more evenly in the presence of glutaraldehyde than without it since the colours on the membranes with the enzyme immobilised with glutaraldehyde were darker and more even. The ranges of beige and brown colours produced by the enzyme on this membrane in the presence of phenol, *p*-cresol and *p*-chlorophenol are shown in Figures 4.7 and 4.8.

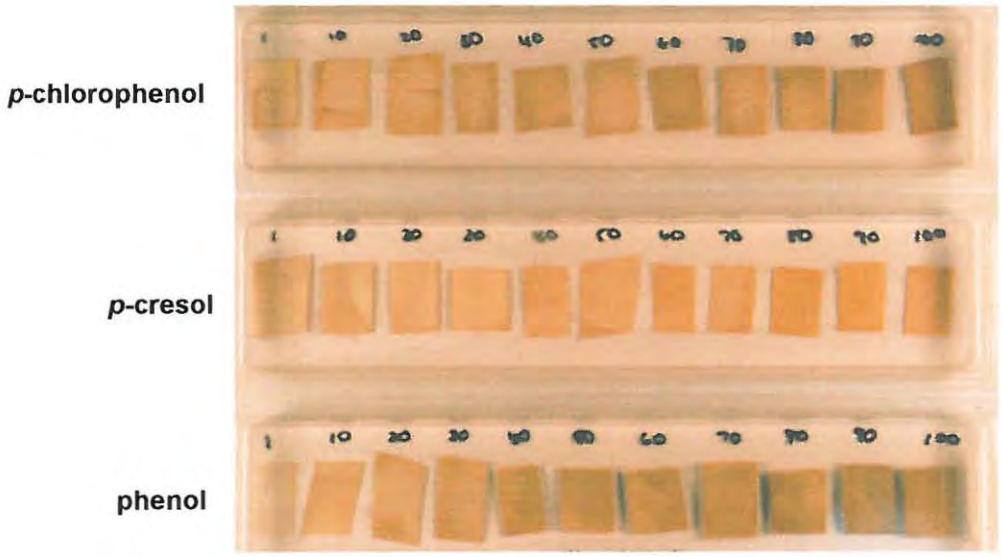


Figure 4.7. Colours produced in 1-100 mg/L solutions of phenol, *p*-cresol and *p*-chlorophenol by polyphenol oxidase immobilised on nylon (without glutaraldehyde)

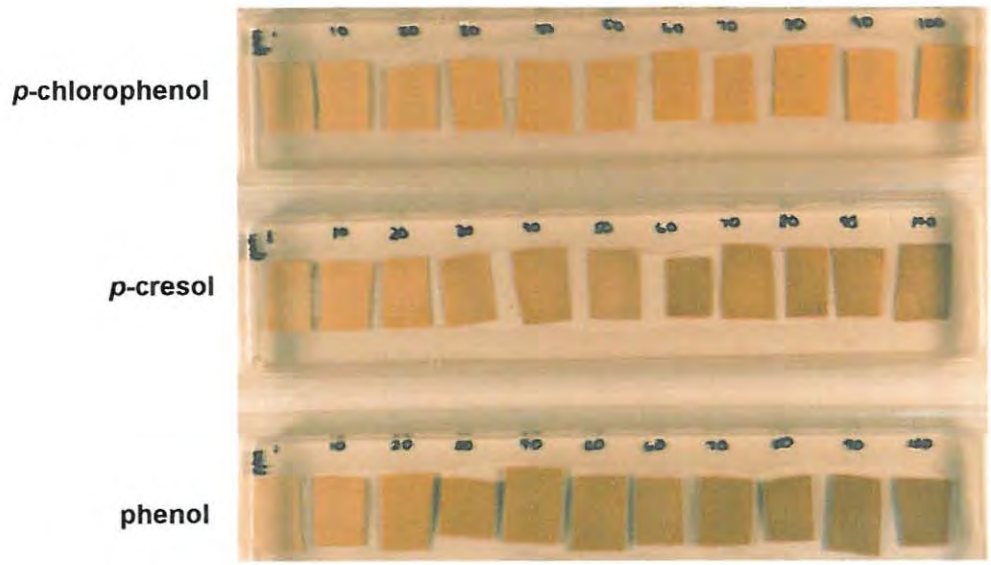


Figure 4.8. Colours produced in 1-100 mg/L solutions of phenol, *p*-cresol and *p*-chlorophenol by polyphenol oxidase immobilised on nylon (with glutaraldehyde)

4.3.3.4 Immobilisation of polyphenol oxidase on nylon in the presence of MBTH

The colours produced by polyphenol oxidase immobilised on nylon in the presence of glutaraldehyde and MBTH were very similar to the colours produced by the enzyme immobilised on nylon in the presence of glutaraldehyde without MBTH (Figure 4.8). These colours are shown in Figure 4.9. The enzyme immobilised in the presence of MBTH without glutaraldehyde, however, produced ranges of maroon colours for phenol and *p*-chlorophenol and orange colours for *p*-cresol shown in Figure 4.10.

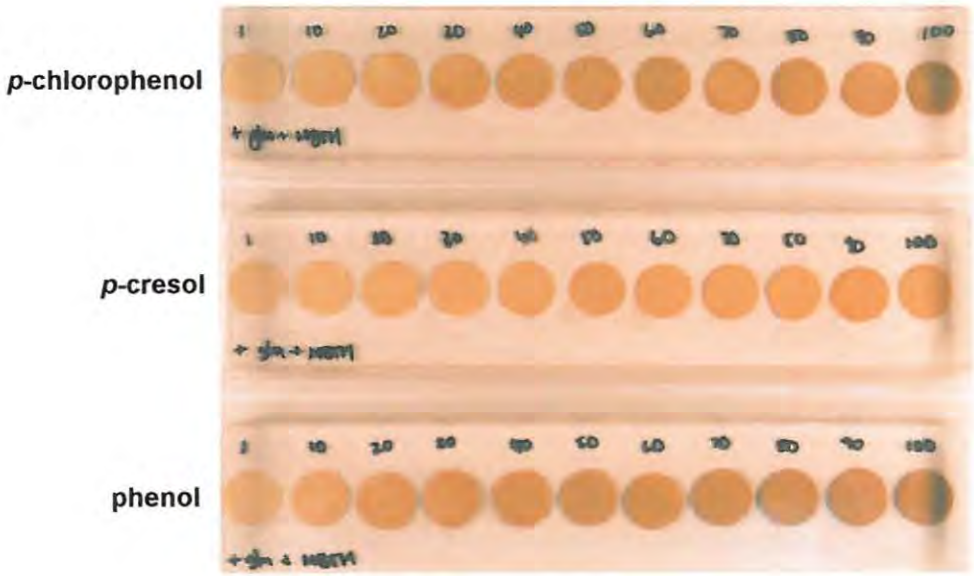


Figure 4.9. Colours produced in 1-100 mg/L solutions of phenol, *p*-cresol and *p*-chlorophenol by polyphenol oxidase immobilised on nylon in the presence of MBTH and glutaraldehyde

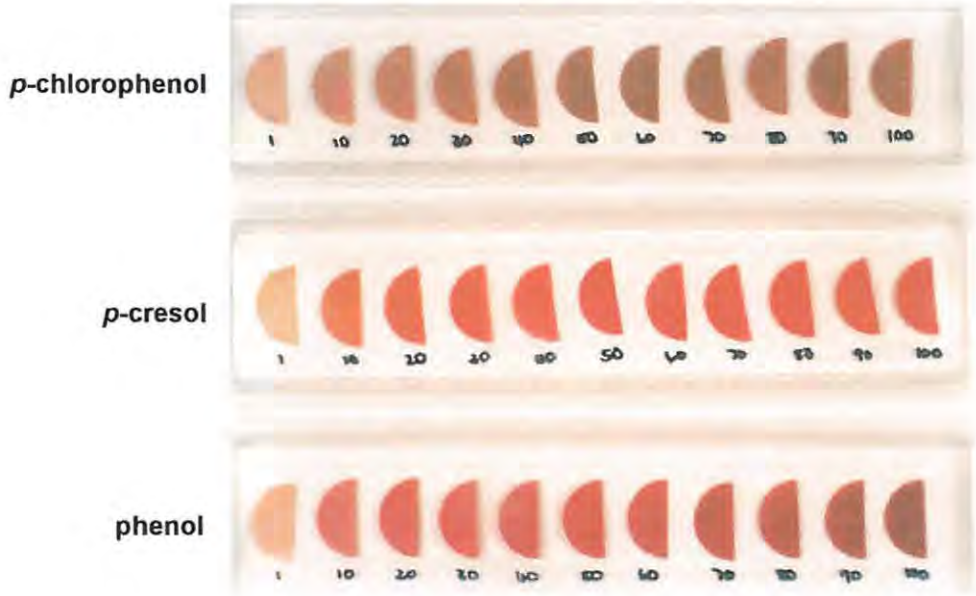


Figure 4.10. Colours produced in 1-100 mg/L solutions of phenol, *p*-cresol and *p*-chlorophenol by polyphenol oxidase immobilised on nylon in the presence of MBTH without glutaraldehyde

The results showed that the nylon membrane gave the best enzyme uptake and distribution. Nylon has, in fact, been shown to be an effective support for the immobilisation of a number of enzymes in the literature (Gekas, 1986; Michalon *et al.*, 1990; Alkorta *et al.*, 1996). For all the membranes, more enzyme was immobilised in the presence of glutaraldehyde than without it (Table 4.1) and the glutaraldehyde did not appear to have any adverse affect on the activity of the enzyme. Furthermore, the enzyme appeared to be more evenly distributed on the membrane pieces when immobilised in the presence of this cross-linker, showing that this method of immobilisation was more successful than adsorption.

The immobilisation of polyphenol oxidase on nylon in the presence of MBTH without glutaraldehyde was very successful in terms of colour production. The enzyme immobilised under these conditions produced ranges of aesthetically pleasing maroon colours when reacted with phenol and *p*-chlorophenol, and orange colours when reacted with *p*-cresol (Figure 4.10). In the presence of glutaraldehyde, the presence of MBTH made no significant difference to the colours produced on the membranes. This was probably due to a reaction between the

glutaraldehyde and the amino group of the MBTH which was therefore then not available for reaction. The enzyme immobilised on nylon membranes by adsorption in the presence of MBTH was therefore chosen as the best combination of support and immobilisation method for the development of the bioprobe.

The colour intensities produced on the membranes appeared to be proportional to the corresponding substrate concentrations for most of the experiments. In some cases though, the colour gradations did not seem to match the increasing concentrations of the substrate. One of the reasons for this may have been that the enzyme was not immobilised in exactly equivalent amounts on the membrane pieces. The colour intensities produced on the nylon membranes with polyphenol oxidase immobilised in the presence of MBTH could not be easily differentiated at the higher substrate concentrations. The reaction time allowed for the colours to develop (3 hours) may have been too long, resulting in the possible saturation of the enzyme with inhibiting quinones at the higher substrate concentrations. Rodriguez-Lopez *et al.* (1994) state that the hydroxylation of phenol catalysed by polyphenol oxidase in the presence of MBTH to produce the corresponding quinone-MBTH adduct is a much quicker reaction compared to melanin formation. These researchers showed that the addition of MBTH to the reaction medium in fact almost completely eliminated the lag period of the reaction. The response time required by the bioprobe to give colour intensities proportional to the corresponding substrate concentrations was therefore further investigated (section 5.3.9).

4.3.4 Effects of various immobilisation methods and supports on the durability of polyphenol oxidase activity

The colour changes observed in 100 mg/L phenol with polyphenol oxidase immobilised on various supports, after a one month storage period in a desiccator, are described in Table 4.2.

Table 4.2. Effect of storage (1 month) on polyphenol oxidase activity

Support/immobilisation conditions	Colour changes observed in 100mg/L phenol
Filter paper	solution turned pale pink
Test-tubes	solution turned orange/brown
Nitrocellulose with glut.	membrane turned light brown
Nylon with MBTH	membrane turned pale pink
Nylon with MBTH and glut.	membrane turned light brown

glut. = glutaraldehyde

In each case, the colours produced after storage were not as intense as the colours produced by the freshly immobilised enzyme indicating that the enzyme had lost activity over the one month storage period in a desiccator. A more meaningful assessment of the durability of the enzyme's activity would have been made if the stored enzyme had been tested for activity after a series of shorter time periods so that the actual time period for which the enzyme had retained its initial activity could have been determined. Storage in a desiccator was not a successful storage method since a significant amount of enzyme activity was lost. A number of other storage methods were evaluated later (section 5.3.7) using the potential bioprobe (comprising polyphenol oxidase immobilised on nylon in the presence of MBTH).

4.3.5 Removal of brown colour from polyphenol oxidase solution

The protein concentrations and enzyme activities measured in the fractions collected from the molecular exclusion chromatography column are represented graphically in Figure 4.11.

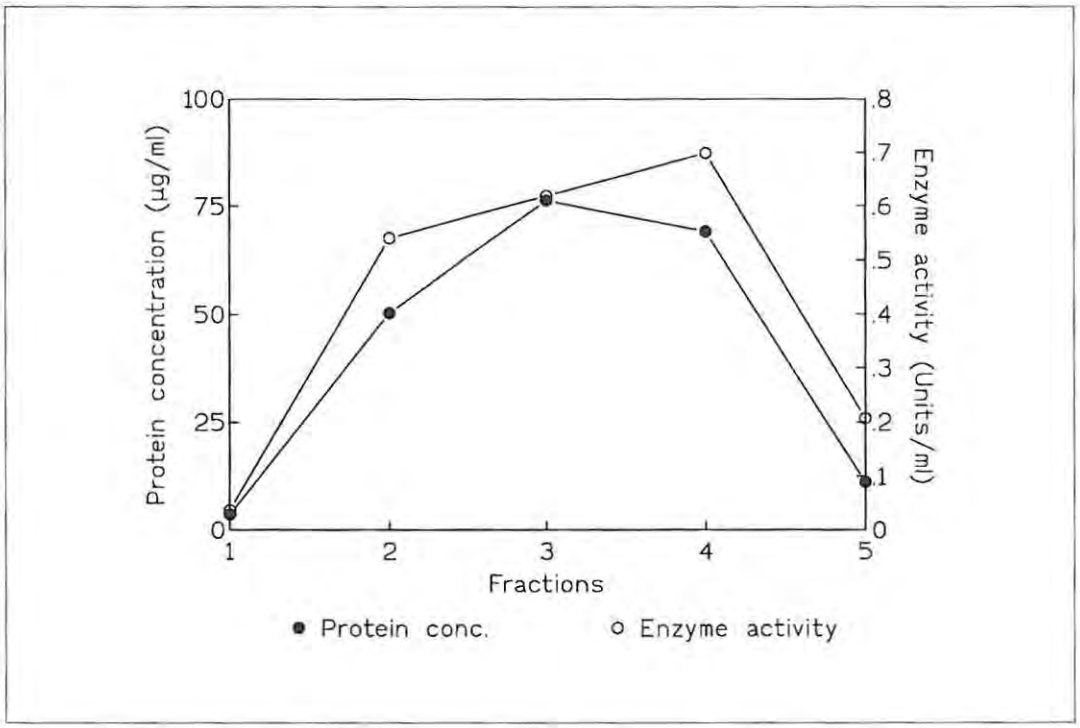


Figure 4.11. Graphical representation of protein concentrations and enzyme activities measured in the fractions collected from the molecular exclusion chromatography column (Sephadex G-25-150)

The highest protein concentrations and enzyme activities were found in fractions 2-4 (Figure 4.11) and these fractions were therefore combined. The colour of this solution was, however, still brown and therefore this particular purification procedure was not successful in removing the brown colour from the enzyme solution.

4.4 CONCLUSION

Immobilisation of polyphenol oxidase on a number of different supports did not appear to significantly affect its catalytic activity. The enzyme was most effectively immobilised on nylon membranes in the presence of MBTH, and this resulted in the production of ranges of maroon colours when the biocatalyst was reacted with phenol and *p*-chlorophenol, and orange colours with *p*-cresol. Immobilisation of the enzyme in the presence of the cross-linker, glutaraldehyde, resulted in more successful immobilisations (in terms of enzyme uptake and distribution), but interference between the glutaraldehyde and the MBTH resulted in a loss of the maroon and orange colours. The enzyme immobilised by simple adsorption on nylon membranes in the

presence of MBTH was therefore chosen as the most suitable combination for the development of the bioprobe.

CHAPTER 5:
Features of the bioprobe response

5.1 INTRODUCTION

Up to this point in the investigation, various combinations of polyphenol oxidase had been immobilised on different supports using different immobilisation methods. These combinations were evaluated in terms of enzyme uptake and distribution, and colour production. As a result, the enzyme immobilised on nylon in the presence of MBTH was chosen as the most promising combination for the development of the bioprobe. The use of the term, bioprobe, in the following chapters therefore refers to this combination of enzyme, support and immobilisation method. It was now necessary to evaluate the feasibility of such a bioprobe for use in the field. This was done by investigating the effects of a variety of different parameters on the activity and colour producing reaction of the bioprobe. The bioprobe was tested in a mixture of phenolic substrates in order to observe the effect of combined substrates on the colours produced. The colours produced by the bioprobe in phenolic substrates other than phenol, *p*-cresol and *p*-chlorophenol were also observed. The potential lower detection limit for the bioprobe was determined. The effects of pH, temperature and the presence of various ions were investigated. The bioprobe was stored under a number of different conditions in order to evaluate the durability of the bioprobe activity. Up to this point, the potential bioprobe had been tested in solutions of pure phenolic substrates only. It was therefore important to test it in "real" samples in order to further evaluate the feasibility of the bioprobe for use in the field. It was therefore finally tested in industrial effluent samples.

Polyphenol oxidase free in solution had required a response time of 3 hours in order to produce colour intensities proportional to substrate concentrations. The response time required by the bioprobe in order to produce colour intensities proportional to the corresponding substrate concentrations was investigated.

Finally, the colours produced on the membrane were measured numerically by using the software package, Adobe Photoshop (V.4.). This was done in order to make a more accurate assessment of the colours produced. Up to this point in the investigation, all assessments had been made by simply visualising the colours on the membranes and photographs. Although the human eye is an excellent discriminator of colour, this method of evaluation is not always easy to report. The photographs also did not always represent the colours accurately. An attempt at obtaining a numerical representation of the colours was therefore made in order to find a more precise method of reporting the results.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Catechol, 4-methylcatechol and gelatin were obtained from Sigma Chemical Company. Ammonium persulphate (APS) was purchased from Saarchem (Pty) Ltd. and N,N,N',N'-Tetramethyl-ethylendiamin (Temed) was obtained from Merck. Hair spray (Nuhair) containing no perfume, colouring or conditioner was obtained from a local hair salon. Cytological fixative was obtained from a local medical supplier. Effluent samples containing phenols were obtained from a local coal gasification plant.

5.2.2 Methods

The bioprobe was now formulated consistently, by immobilising polyphenol oxidase by adsorption on nylon in the presence of MBTH, according to the exact method described in section 4.2.2.3. Freshly prepared bioprobe discs were used for each experiment. All the experiments were carried out in duplicate and duplicate bioprobe discs are shown in all the photographs. Adobe readings were measured for duplicate discs and average values were plotted.

5.2.2.1 Observation of the colours produced by the bioprobe in a mixture of phenolic substrates

The bioprobe was tested in 0-100 mg/L (total concentration) solutions containing equimolar amounts of *p*-cresol, *p*-chlorophenol and phenol, and the colours produced by the bioprobe

were observed after 3 hours.

5.2.2.2 Observation of the colours produced by the bioprobe in other phenolic substrates

The bioprobe was tested in 0-100 mg/L solutions of catechol and 4-methylcatechol and the colours produced were observed after 3 hours.

5.2.2.3 Bioprobe response to low substrate concentrations (lower than 1 mg/L)

The bioprobe was tested in phenol, *p*-cresol and a mixture of phenolic substrates (containing equimolar amounts of phenol, *p*-cresol and *p*-chlorophenol) at concentrations of 0, 0.05, 0.1 and 1 mg/L. The colours were observed after 3 hours. The colours produced in 0.1, 0.5 and 1 mg/L phenol were observed over 24 hours in order to determine whether a longer response time would allow for the determination of lower concentrations. From this investigation the potential lower detection limit of the bioprobe was determined.

5.2.2.4 The effect of pH on the bioprobe response

The effect of pH on the activity of the bioprobe was investigated by observing the colour changes produced in 100 mg/L phenol solutions at various pH values. Phenol solutions (100 mg/L) were adjusted to a range of pH values from 2 to 13 using a pH meter and HCl and NaOH. The bioprobe was then tested in the various phenol solutions and the colour changes produced were observed after 3 hours.

5.2.2.5 The effect of temperature on the bioprobe response

The bioprobe was tested in 100 mg/L phenol solutions at 5, 15, 25, 35 and 40^{0}C and the effects of the various temperatures on the bioprobe activity were determined by observing the colour changes produced after 3 hours.

5.2.2.6 The effect of salt and metal ion concentration on the bioprobe response

The bioprobe was tested in 50 mg/L phenol and *p*-cresol solutions containing various concentrations of NaCl and ammonium sulphate. These salts were tested at the concentrations at which they are commonly found in effluents. The bioprobe was also tested in 50 mg/L

phenol solutions having concentrations of various metal ions above the acceptable level for drinking water (Kempster *et al.*, 1982). The effects of the various ions on the bioprobe activity were observed by observing the colour changes produced after 3 hours.

5.2.2.7 Investigation of the bioprobe activity after storage

Samples of the bioprobe were stored in N_2 and in air at room temperature (approximately 25^{0}C) and at 4^{0}C. Others were stored in a vacuum desiccator at room temperature. Samples of the bioprobe were also stored at room temperature between thin sheets of tin foil, and in clear plastic, sealed with Scotch tape. Any air trapped between the sheets was pressed out before sealing with the tape and therefore this method could effectively be described as storage in the absence of air, with and without light. The bioprobe activity in 100 mg/L phenol was tested weekly, over a month, for each storage method and the degree of activity preservation was evaluated for each method.

The effects of various covering layers on the preservation of the bioprobe activity were also investigated. Samples of the bioprobe were coated with gelatin, acrylamide, cytological fixative and hair spray. Gelatin and acrylamide were used in an attempt to produce thin, porous layers on the surfaces of the bioprobe samples which would protect the enzyme and would simultaneously be permeable to phenolic substrates. Cytological fixative and hairspray were used in an attempt to produce coatings on the surfaces of the bioprobe samples which would keep the membrane and enzyme dry during storage, and would then dissolve once the bioprobe was placed in a water sample. The effects of the coatings on the bioprobe activity in 100 mg/L phenol and *p*-cresol solutions were observed by comparing the colours produced by the coated bioprobe samples with the colours produced by an uncoated bioprobe sample (control).

A 25 % (w/v) solution (80 ml) of gelatin was made up and heated to 80^{0}C in order to dissolve the gelatin. The solution was allowed to cool and samples of the bioprobe were then dipped into the solution. The samples were then placed on wax paper until the gelatin layer had set before they were tested in 100 mg/L phenol and *p*-cresol solutions. The colours produced were

observed after 3 hours.

A 20% (w/v) acrylamide solution (5 ml) was made up. A 80 μl volume of 10% APS (freshly prepared) and 40 μl Temed were added to the acrylamide solution. Samples of the bioprobe were immediately dipped into the solution and then placed on wax paper until the acrylamide layer had set. The samples were then tested in the same manner as the gelatin coated samples.

Samples of the bioprobe were placed on wax paper and then sprayed with hairspray and cytological fixative. The samples were allowed to dry and were then tested in the same manner as the gelatin coated samples.

The bioprobe coated with gelatin was stored in a vacuum desiccator for a month and the effect of the gelatin on the preservation of the bioprobe activity was tested in the same manner as the other storage methods.

5.2.2.8 Observation of the colours produced by the bioprobe in effluents

Samples of the bioprobe were initially tested in three different phenolic industrial effluents. The colour changes produced in these undiluted effluents were observed after 3 hours and compared with the colour changes produced in pure phenol solutions. The effluent samples were:

i) Fischer Tropsch acid water (RW) before concentration

ii) Stripped gas liquor blow down (SGL-BD)

iii) SGL + RW blow down

The phenolic contents of these effluents (analysed by the supplier) are given in Appendix D.

The bioprobe was also tested in a phenolic and a cresylic industrial effluent. The organic compositions of these effluents (analysed by the supplier) are shown in Appendix E. The phenolic contents of these effluents were high and therefore dilution of the effluents was necessary. The colours produced by the bioprobe in these diluted effluent samples were observed and compared with those produced in similar concentrations of pure phenolic

substrates.

5.2.2.9 Determination of the response time for the bioprobe

The bioprobe was tested in 10 mg/L and 100 mg/L phenol, *p*-cresol and a mixture of phenols (containing equimolar amounts of *p*-cresol, *p*-chlorophenol and phenol) at 2 hour intervals over 24 hours and at 30 minute intervals over 3 hours. The colours produced were observed and the minimum length of time required by the bioprobe to produce colours with intensities that could clearly distinguish between the two substrate concentrations was determined. The colour gradations produced by the bioprobe in 0-100 mg/L phenol and *p*-cresol solutions after 30 minutes and after 5 minutes were then observed in order to ascertain whether the colour intensities produced after these time intervals were proportional to the corresponding substrate concentrations.

5.2.2.10 Photographic and numerical representations of the colours produced by the bioprobe

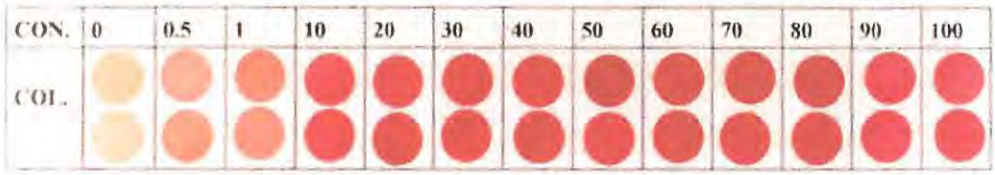
Photographs were taken of the coloured discs after each experiment using a Nikon camera with AGFA ASA 100 photographic film. A blue filter was used during photographing. Numerical measurements of the colours were obtained by scanning the coloured discs using a Hewlett Packard ScanJet II cx scanner, and measuring the levels of red colour in each disc with the Adobe Photoshop (V.4.) software package (Adobe Systems Inc.). Adobe colour units are expressed as a measure of "lightness" (as opposed to "darkness") on a scale of 0 to 255, where 0 is pure black and 255 is pure white. The readings obtained therefore decreased as the red colour on the discs became darker. By subtracting the Adobe colour units measured from the maximum possible colour unit measurement (255), a measure of the red colour on the discs was obtained. These measurements increased with a visible increase in red colour intensity.

5.3 RESULTS AND DISCUSSION

5.3.1 Observation of the colours produced by the bioprobe in a mixture of phenolic substrates

The colours produced by the bioprobe in 0-100 mg/L solutions containing equimolar amounts

of *p*-cresol, *p*-chlorophenol and phenol were visually the same as the maroon colours obtained with the phenol solutions (Figure 4.10). The colours produced by the bioprobe after 3 hours in the mixture of substrates are shown in Figure 5.1 and the numerical representation of the colours is shown in Figure 5.2.



CON. = Concentration (mg/L)
COL. = Colours produced

Figure 5.1. Photograph showing the colours produced by the bioprobe (after 3 hours) in 0-100 mg/L substrate solutions containing equimolar amounts of *p*-cresol, *p*-chlorophenol and phenol

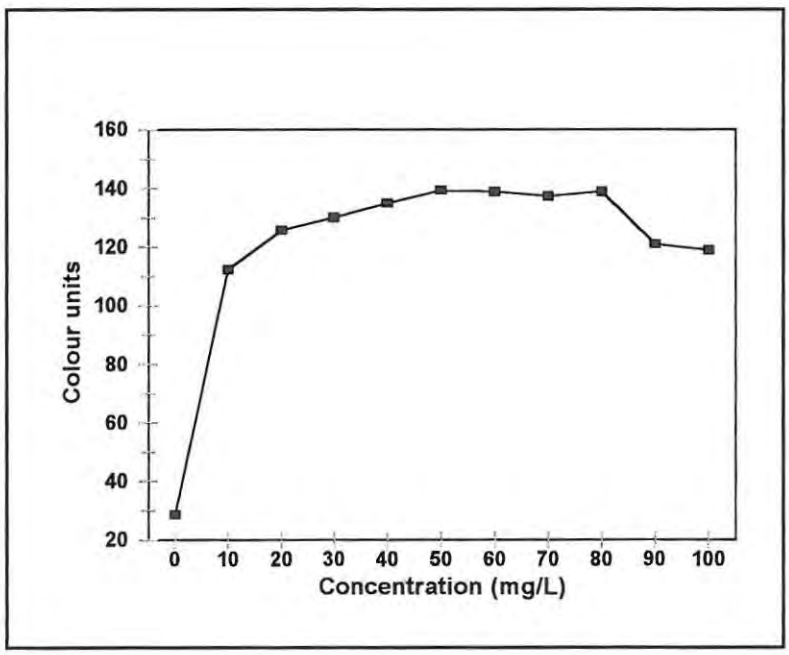
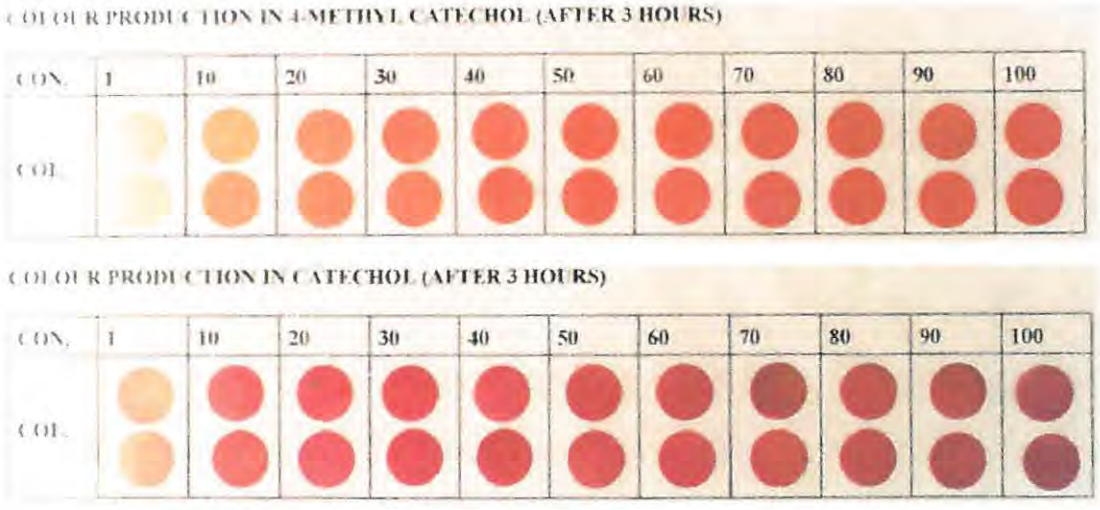


Figure 5.2. Numerical representation of the colours produced by the bioprobe (given in Adobe colour units) in 0-100 mg/L substrate solutions containing equimolar amounts of phenol, *p*-cresol and *p*-chlorophenol (after 3 hours)

It can be seen from Figures 5.1 and 5.2 that the colours produced by the bioprobe in the mixture of phenolic substrates were not proportional to the substrate concentrations after 3 hours. This result confirmed that it was necessary to find the response time required by the bioprobe to produce colour intensities proportional to the substrate concentrations.

5.3.2 Observation of the colours produced by the bioprobe in other phenolic substrates

The colours produced by the bioprobe in the range of catechol concentrations used were visually the same as the maroon colours produced in phenol while the colours produced in the range of 4-methylcatechol concentrations used were visually the same as the orange colours produced in *p*-cresol. This is to be expected, since catechol and 4-methylcatechol are intermediates in the reactions of phenol and *p*-cresol with polyphenol oxidase, respectively. The products of the reactions with phenol and *p*-cresol and their respective catechols would be the same, and therefore the coloured products would also be the same. Photographic representations of the colours produced by the bioprobe after 3 hours in 1-100 mg/L catechol and 4-methylcatechol solutions are shown in Figure 5.3. The numerical representations of the colours produced are presented graphically in Figure 5.4.



CON. = Concentration (mg/L)
COL. = Colours produced

Figure 5.3. Photograph showing the colours produced by the bioprobe (after 3 hours) in 1-100 mg/L catechol and 4-methylcatechol solutions

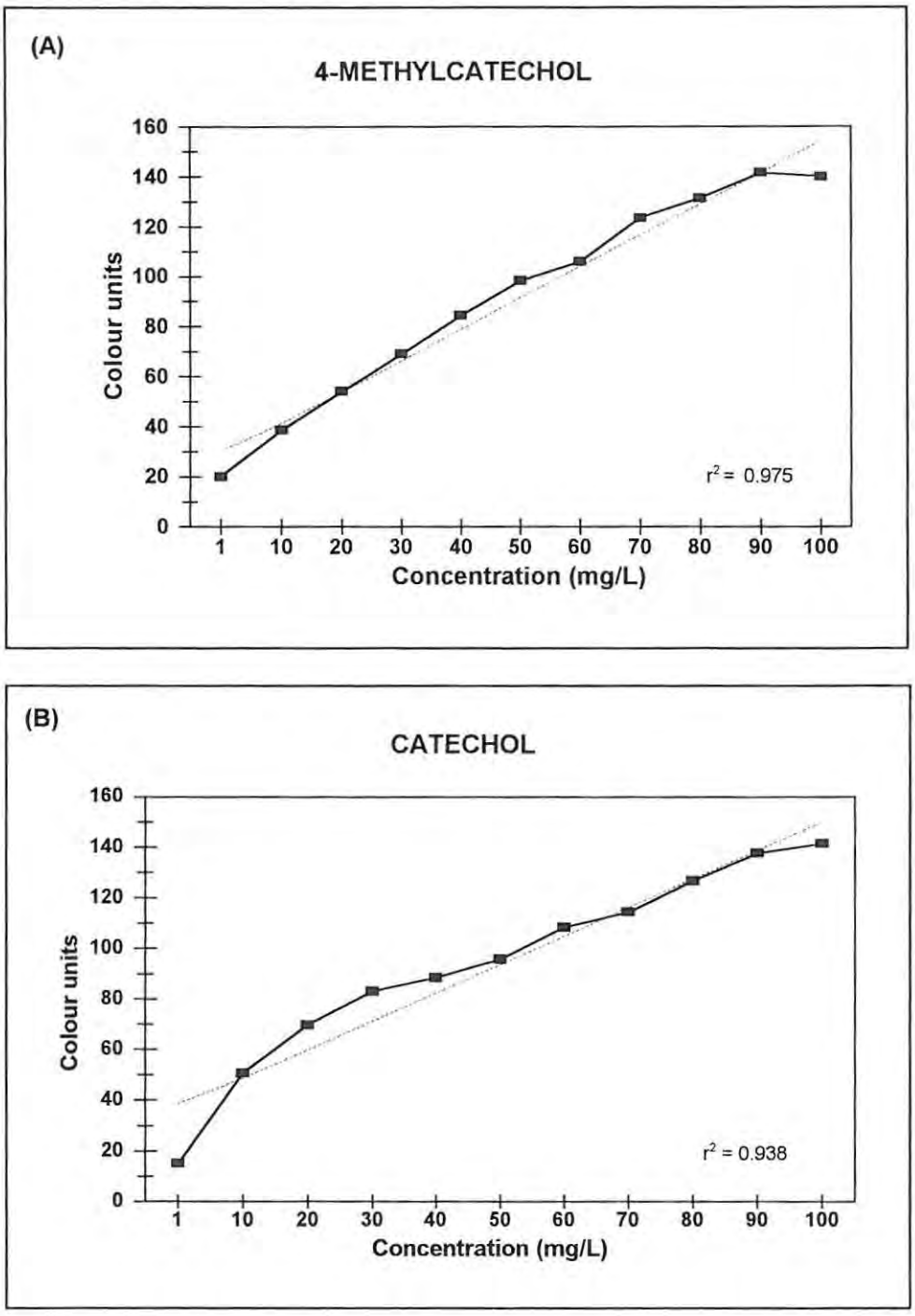


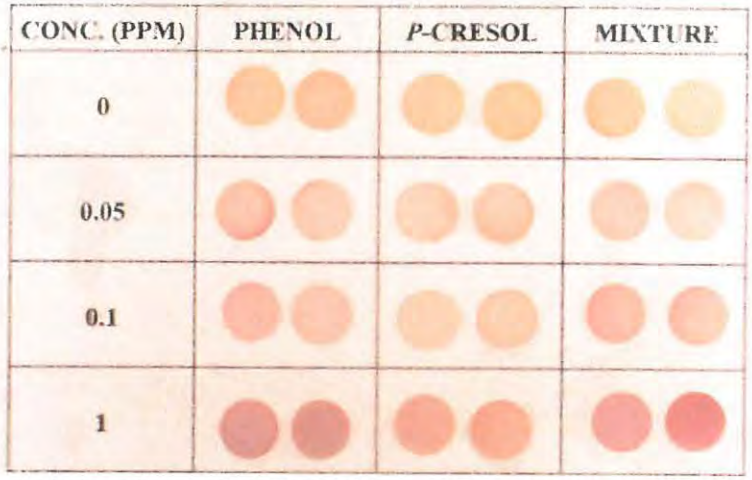
Figure 5.4. Numerical representations of the colours produced by the bioprobe (given in Adobe colour units) in 1-100 mg/L 4-methylcatechol (A) and catechol (B) solutions (after 3 hours)

In contrast to the colours produced by the bioprobe in the mixture of phenolic substrates, the colours produced in catechol and 4-methylcatechol were found to be proportional to the substrate concentrations after 3 hours (Figures 5.3 and 5.4). This indicated that the response time required by the bioprobe might vary for different phenolic substrates.

5.3.3 Bioprobe response to low substrate concentrations (lower than 1 mg/L)

The bioprobe produced colour gradations after 3 hours which could visibly distinguish between 0, 0.05, 0.1 and 1 mg/L concentrations of phenol, *p*-cresol and the mixture of phenolic substrates. The photographic and numerical representations of the colours produced are shown in Figures 5.5 and 5.6, respectively. The colours produced by the bioprobe in 0.1, 0.5 and 1 mg/L concentrations of phenol over 24 hours are shown in Figure 5.7. The numerical representation of these colours is shown in Figure 5.8. The colours produced on the set of discs shown in Figure 5.7 were uneven indicating an uneven immobilisation of the enzyme and MBTH. This uneven colouring was confirmed by the numerical representation of the colours shown in Figure 5.8. It could be seen, however, that the colours did not intensify significantly after the initial 2 hour time period indicating that most of the substrate had reacted with the enzyme after this time interval. It could be deduced from this that a longer response time would not be useful for the detection of lower concentrations of phenolic substrates.

BIOPROBE RESPONSE TO LOW PHENOL CONCENTRATIONS



CONC. = Concentration (mg/L)

Figure 5.5. Photograph showing the colours produced by the bioprobe (after 3 hours) in phenol, *p*-cresol and the mixture of phenolic substrates at 0, 0.05, 0.1 and 1 mg/L concentrations

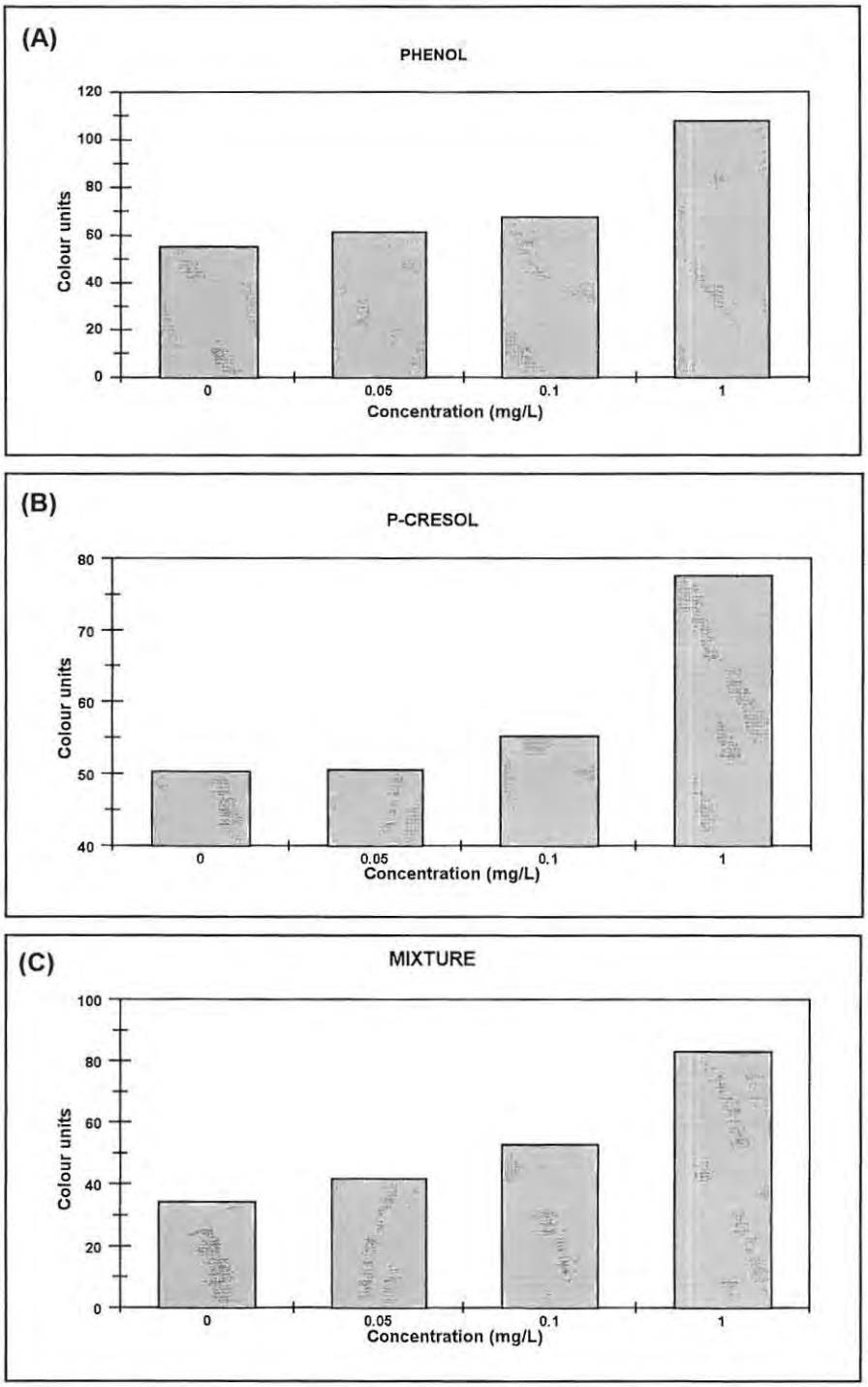


Figure 5.6. Numerical representations of the colours produced by the bioprobe (given in Adobe colour units) in phenol (A), *p*-cresol (B) and the mixture of phenolic substrates (C) at 0, 0.05, 0.1 and 1 mg/L concentrations (after 3 hours)

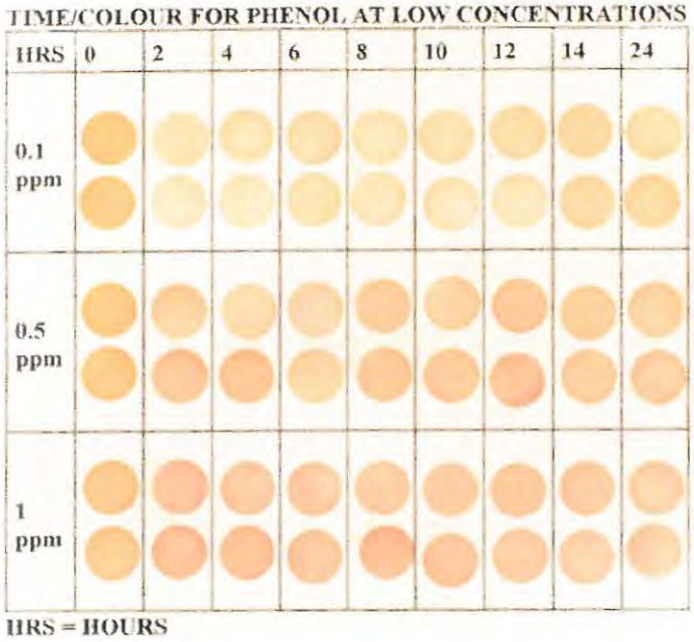


Figure 5.7. Photograph showing the colours produced by the bioprobe in phenol at 0.1, 0.5 and 1 mg/L concentrations over 24 hours

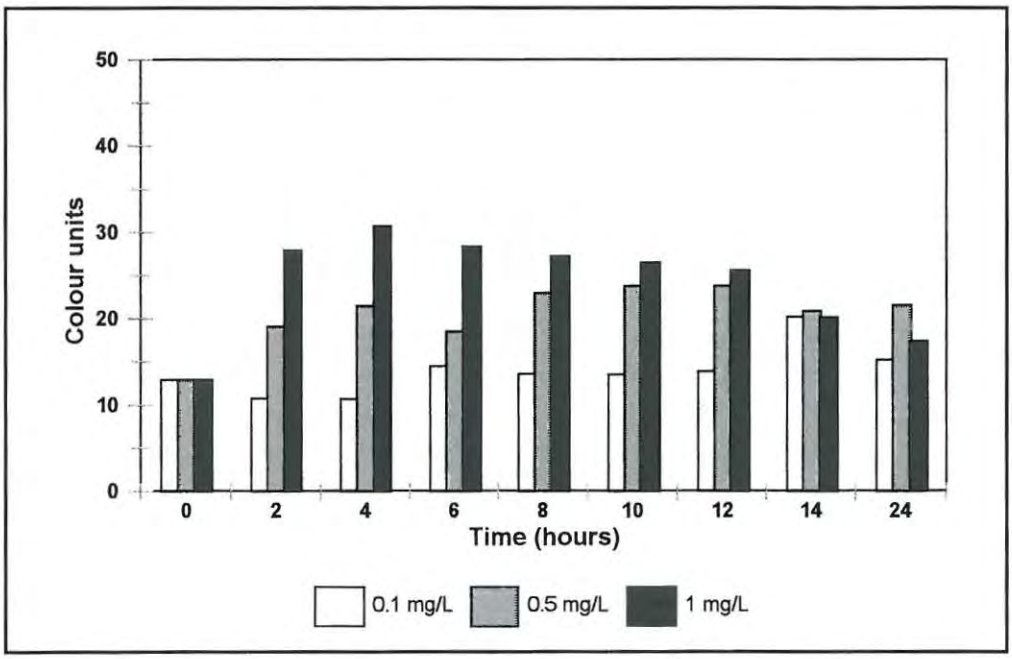


Figure 5.8. Numerical representation of the colours produced by the bioprobe (given in Adobe colour units) in phenol at 0.1, 0.5 and 1 mg/L concentrations over 24 hours

5.3.4 The effect of pH on the bioprobe response

The colours produced by the bioprobe in 100 mg/L phenol solutions at different pH's after 3 hours are shown in Figure 5.9. The numerical representation of the colours is shown in Figure 5.10. The pH optimum for the bioprobe appeared to be pH 9 from the numerical representation of the colours produced (Figure 5.10). However, no significant differences in the colour intensities produced in the pH range from 4 to10 were visible on the bioprobe discs (Figure 5.9) showing that the bioprobe response was not impaired in this pH range. At pH's below or above this range the colours produced were less intense or were not produced at all (Figure 5.9). The bioprobe would therefore be effective in a pH range from 4 to 10.

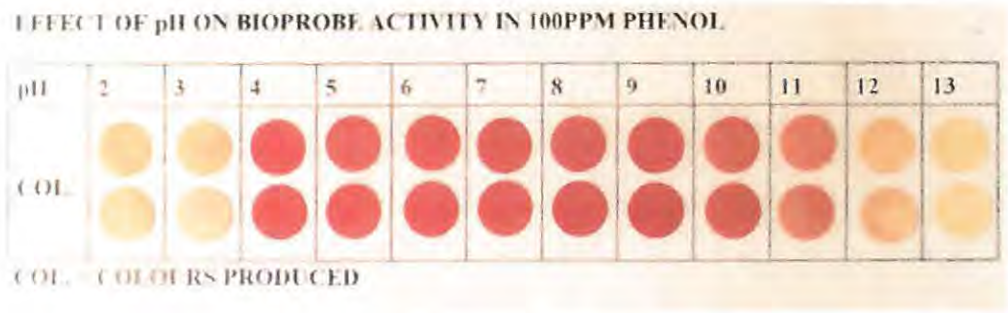


Figure 5.9. Photograph showing the colours produced by the bioprobe (after 3 hours) in 100 mg/L phenol solutions of varying pH

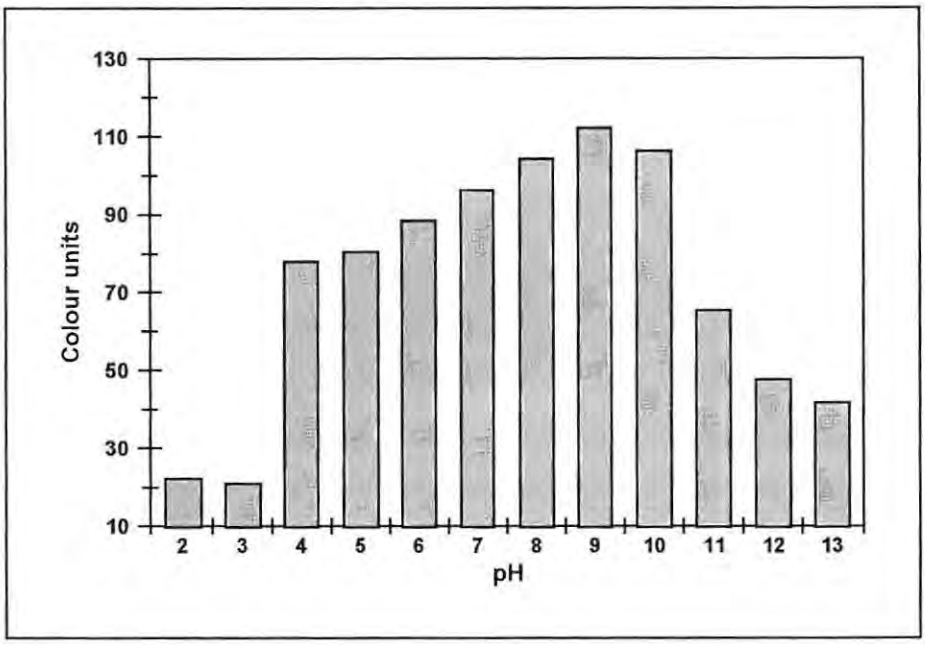


Figure 5.10. Numerical representation of the colours produced by the bioprobe (given in Adobe colour units) in 100 mg/L phenol solutions of varying pH (after 3 hours)

5.3.5 The effect of temperature on the bioprobe response

The colour production by the bioprobe was not markedly affected in the temperature range from 5-40^{0}C. The rate of the colour producing reaction, however, was slightly affected at the extremes of this temperature range. The colour produced in 100 mg/L phenol at 40^{0}C after 3 hours was slightly more intense than the colour produced after this time interval at 5^{0}C. This was not an unexpected result since it is well established that temperature affects the rate of chemical reactions (Atkins *et al.*, 1988). This would also not be a problem for the use of the bioprobe, since colour references could be made at the same temperature as the samples. The colours produced by the bioprobe after 3 hours in 100 mg/L phenol at the different temperatures are shown in Figure 5.11. The numerical representation of the colours is shown in Figure 5.12.

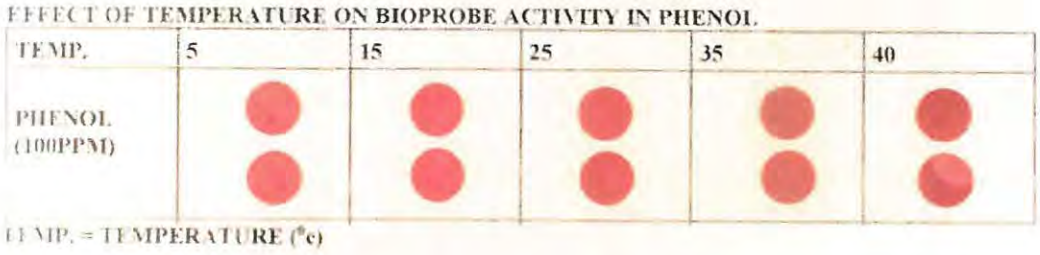


Figure 5.11. Photograph showing the colours produced by the bioprobe (after 3 hours) in 100 mg/L phenol solutions at different temperatures

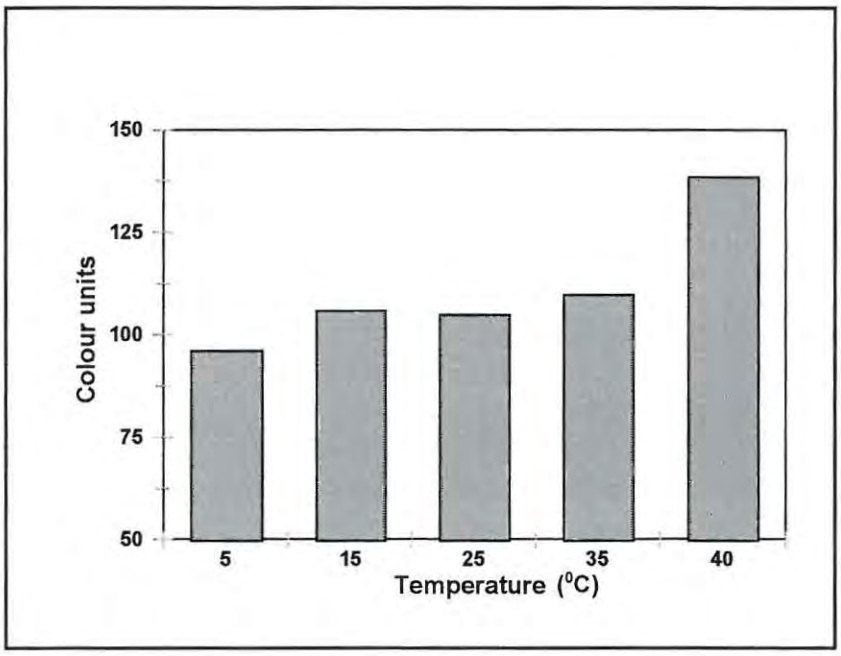
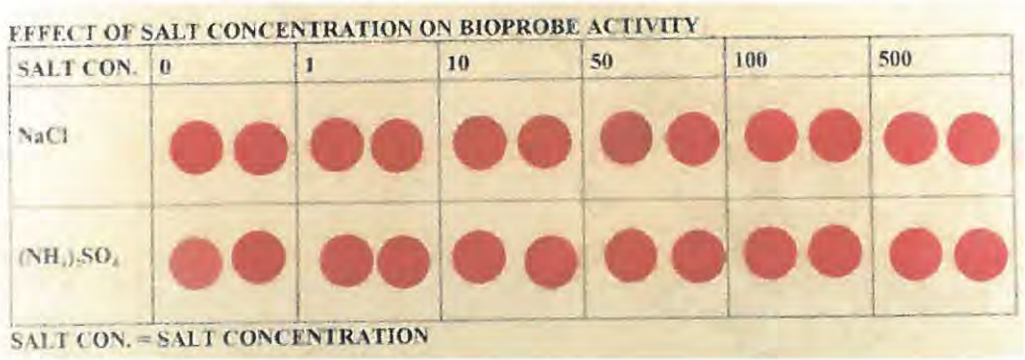


Figure 5.12. Numerical representation of the colours produced by the bioprobe (given in Adobe colour units) in 100 mg/L phenol solutions at different temperatures (after 3 hours)

5.3.6 The effect of salt and metal ion concentration on the bioprobe response

The activity of the bioprobe in 50 mg/L phenol and 50 mg/L *p*-cresol was not affected by NaCl or ammonium sulphate concentrations of 1, 10, 50, 100 and 500 mg/L and therefore the colours produced were also not affected. The colours produced in the presence of these ions in phenol after 3 hours are shown in Figure 5.13 and those produced in *p*-cresol after 3 hours are shown in Figure 5.15. Numerical representations of the colours produced in phenol and *p*-cresol in the presence of these ions after 3 hours are shown in Figures 5.14 and 5.16, respectively. The presence of magnesium, copper, calcium, iron and zinc ions, as well as nitrate, phosphate and sulphate ions did not affect the bioprobe activity or colour production in 50 mg/L phenol. The photographic and numerical representations of the colours produced in 50 mg/L phenol solutions containing these ions after 3 hours are shown in Figures 5.17 and 5.18, respectively.



SALT CON. = Salt concentration (mg/L)

Figure 5.13. Photograph showing the colours produced by the bioprobe (after 3 hours) in 50 mg/L phenol solutions containing various concentrations of sodium chloride and ammonium sulphate

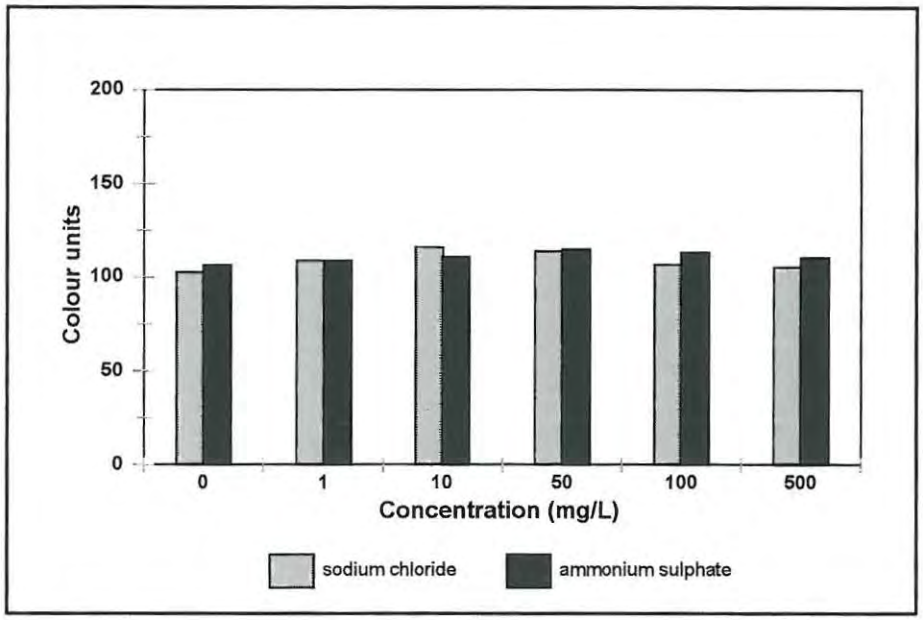
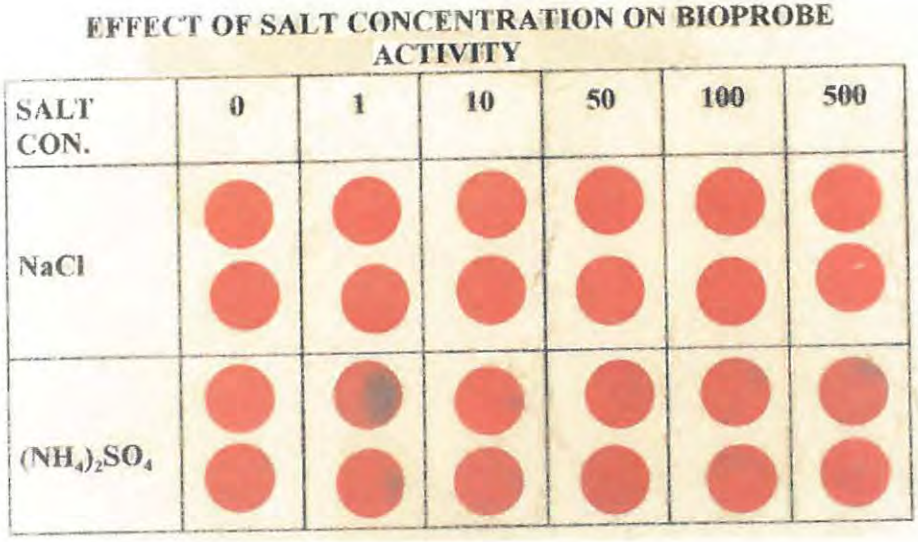


Figure 5.14. Numerical representation of the colours produced by the bioprobe (given in Adobe colour units) in 50 mg/L phenol solutions containing various concentrations of sodium chloride and ammonium sulphate (after 3 hours)



SALT CON. = Salt concentration (mg/L)

Figure 5.15. Photograph showing the colours produced by the bioprobe (after 3 hours) in 50 mg/L *p*-cresol solutions containing various concentrations of sodium chloride and ammonium sulphate

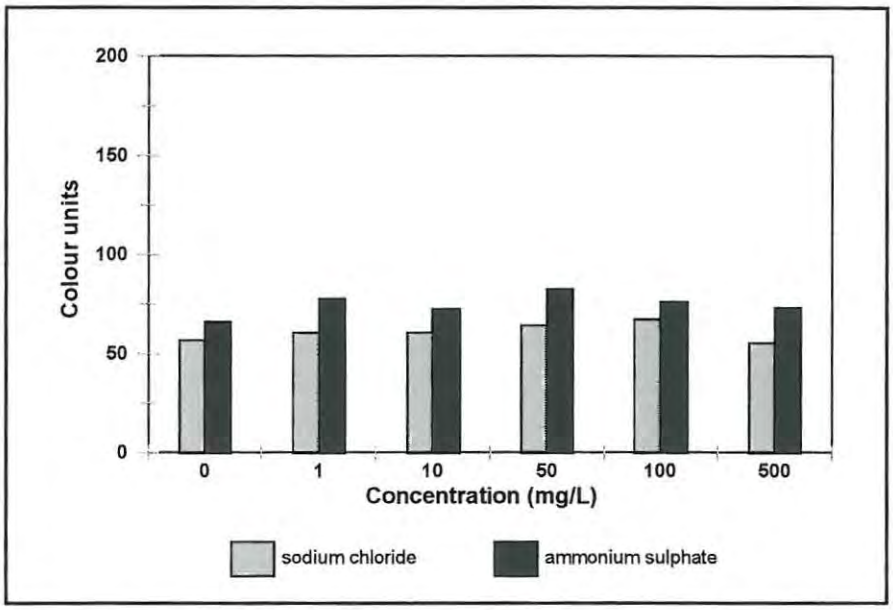


Figure 5.16. Numerical representation of the colours produced by the bioprobe (given in Adobe colour units) in 50 mg/L *p*-cresol solutions containing various concentrations of sodium chloride and ammonium sulphate (after 3 hours)

EFFECT OF VARIOUS IONS ON THE BIOPROBE ACTIVITY IN PHENOL

ION	[ION]	COLOURS PRODUCED
SODIUM SULPHATE	100	
MAGNESIUM SULPHATE	50	
COPPER SULPHATE	10	
CALCIUM CHLORIDE	50	
IRON SULPHATE	10	
ZINC ACETATE	10	
NITRATE	50	
PHOSPHATE	100	

[ION] = Ion concentration (mg/L)

Figure 5.17. Photograph showing the colours produced by the bioprobe (after 3 hours) in 50 mg/L phenol solutions containing different concentrations of various ions

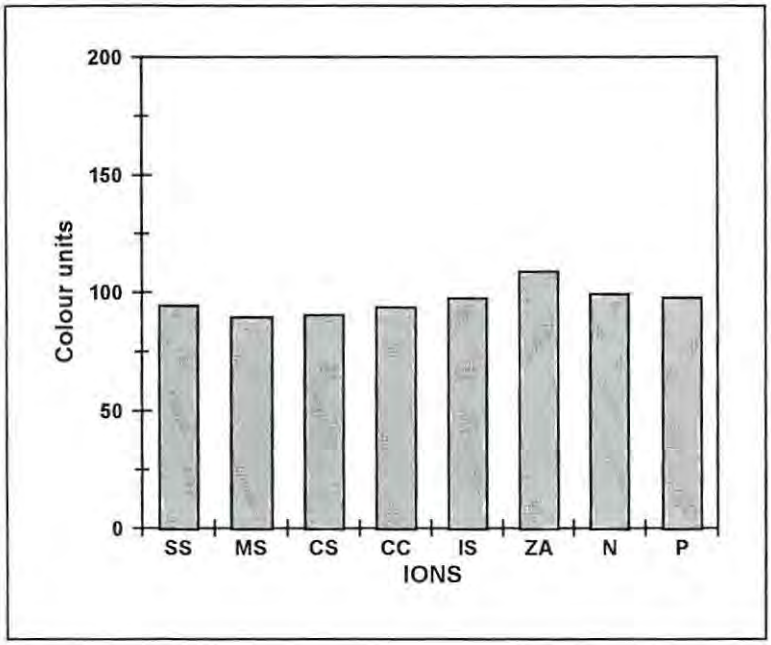


Figure 5.18. Numerical representation of the colours produced by the bioprobe (given in Adobe colour units) in 50 mg/L phenol solutions containing different concentrations of various ions (after 3 hours)

5.3.7 Investigation of the bioprobe activity after storage

The samples of the bioprobe stored in N_2 and air at 4^0C and 25^0C showed a visible loss of activity after 1 week of storage. The samples of the bioprobe stored in a vacuum desiccator showed a visible gradual loss of activity over a one-month storage period. The colours produced by the bioprobe (after 3 hours) in 10 and 100 mg/L phenol solutions after storage in the vacuum desiccator are shown in Figure 5.19. The numerical representation of these colours is shown in Figure 5.20. Storage of the bioprobe between sheets of foil with air bubbles excluded was found to be the most successful storage method for preserving the bioprobe activity. The bioprobe showed no visible loss of activity over a one-month storage period using this method. The numerical representation of the colours produced showed that the bioprobe had, in fact, gained activity over the storage period. Storage between sheets of plastic with air bubbles excluded was also successful, although slight activity was lost using this method. This may have been due to exposure to light. The colours produced by the bioprobe in 100 mg/L phenol solutions after storage using these methods are shown in Figure 5.21. The numerical representation of these colours is shown in Figure 5.22.

Of the four coating layers tested, gelatin was the only one which did not adversely affect the bioprobe activity. The colours produced by the gelatin coated bioprobe after 3 hours, however, were slightly less intense than the colours produced by the uncoated bioprobe. The effects of the coatings on the bioprobe activity in 100 mg/L phenol and 100 mg/L *p*-cresol are shown in Figure 5.23 and the numerical representation of the colours produced is shown in Figure 5.24.

The gelatin coating slightly improved the preservation of the bioprobe activity over a one-month storage period in a vacuum desiccator but it was not able to preserve the MBTH (Figures 5.25 and 5.26). On repeating the coating experiment with gelatin, the development of a pink colour was observed on the bioprobe discs. Various combinations of gelatin, polyphenol oxidase, nylon membranes and MBTH were tested in order to find the source of the pink colour. None of these tests, however, were able to identify the source and therefore, further testing of the bioprobe with a gelatin coating was discontinued.

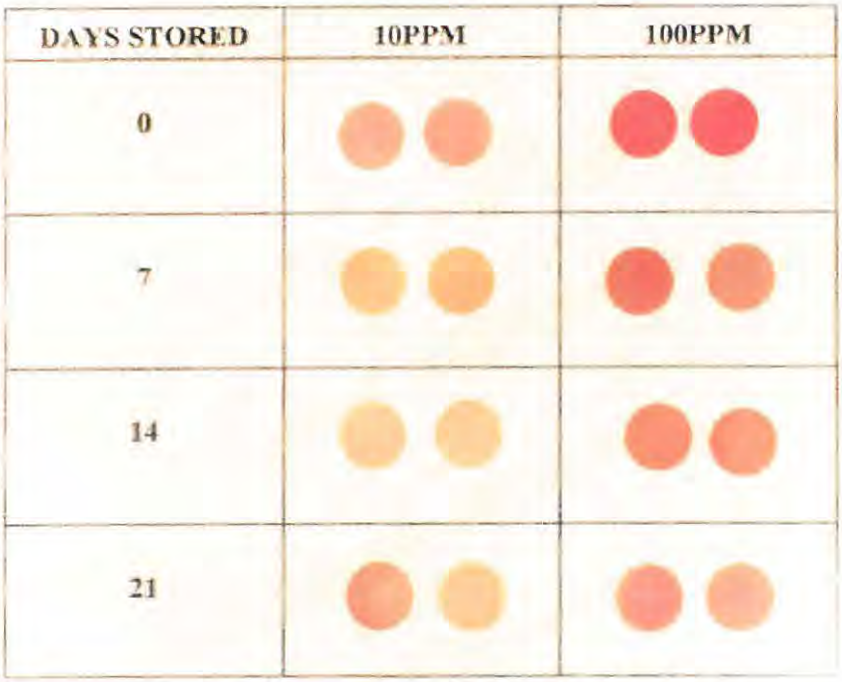


Figure 5.19. Photograph showing the colours produced by the bioprobe (after 3 hours) in 10 mg/L and 100 mg/L phenol solutions after storage in a vacuum desiccator

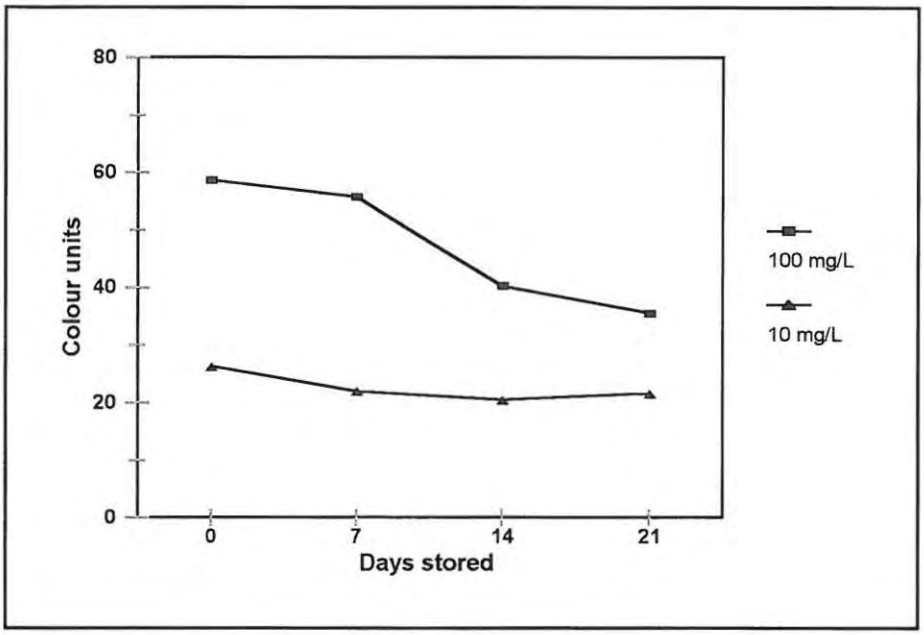


Figure 5.20. Numerical representation of the colours produced by the bioprobe after 3 hours in 10 mg/L and 100 mg/L phenol solutions (given in Adobe colour units) after storage in a vacuum desiccator

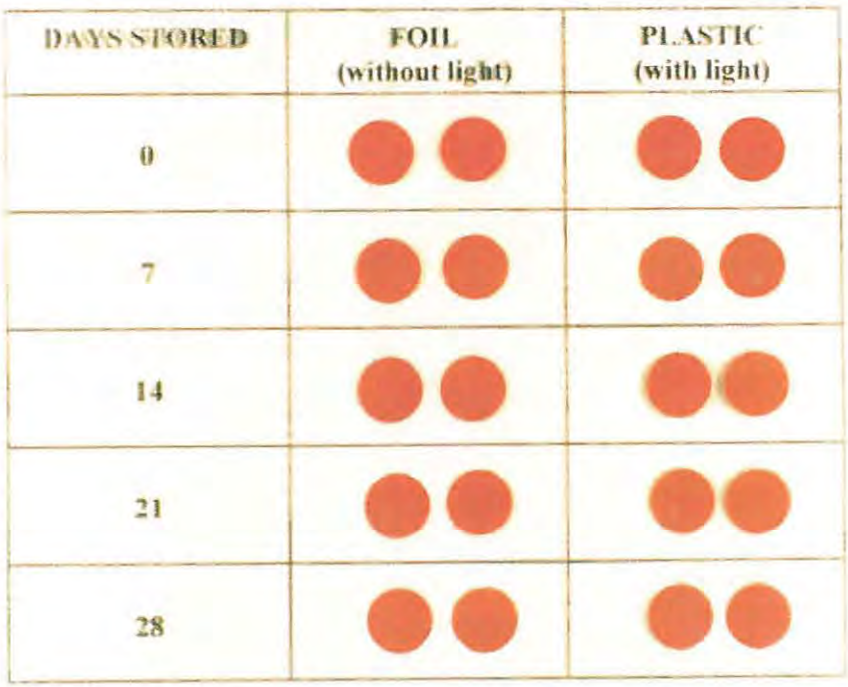


Figure 5.21. Photograph showing the colours produced by the bioprobe (after 3 hours) in 100 mg/L phenol solutions after storage sealed between sheets of foil and plastic

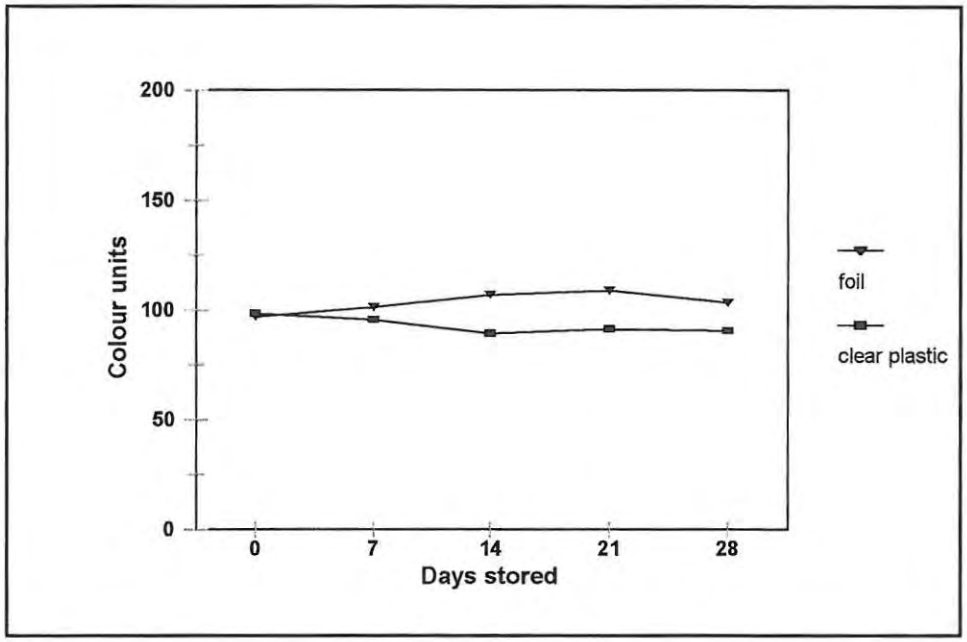


Figure 5.22. Numerical representation of the colours produced by the bioprobe after 3 hours in 100 mg/L phenol solutions (given in Adobe colour units) after storage sealed between sheets of foil and plastic

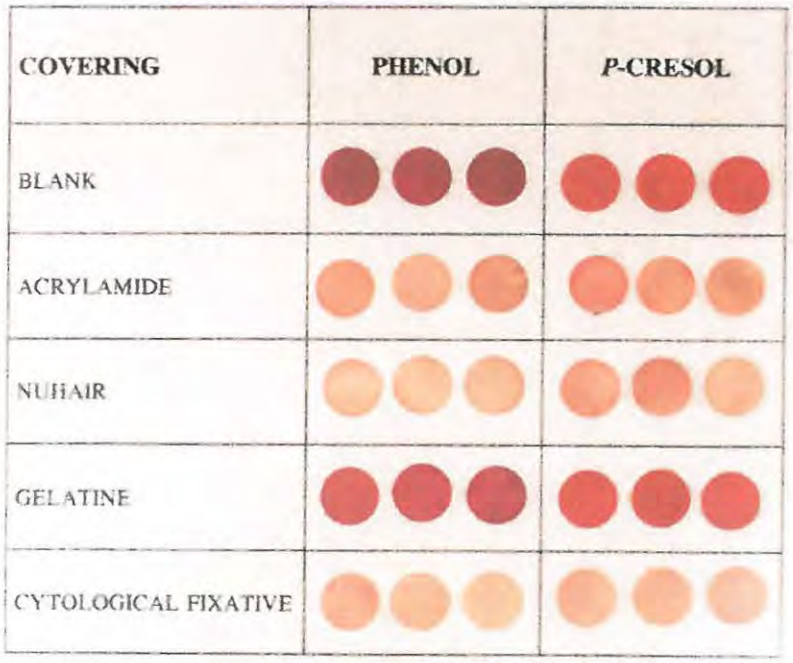


Figure 5.23. Photograph showing the colours produced by the bioprobe (after 3 hours) with various covering layers in 100 mg/L phenol and *p*-cresol solutions

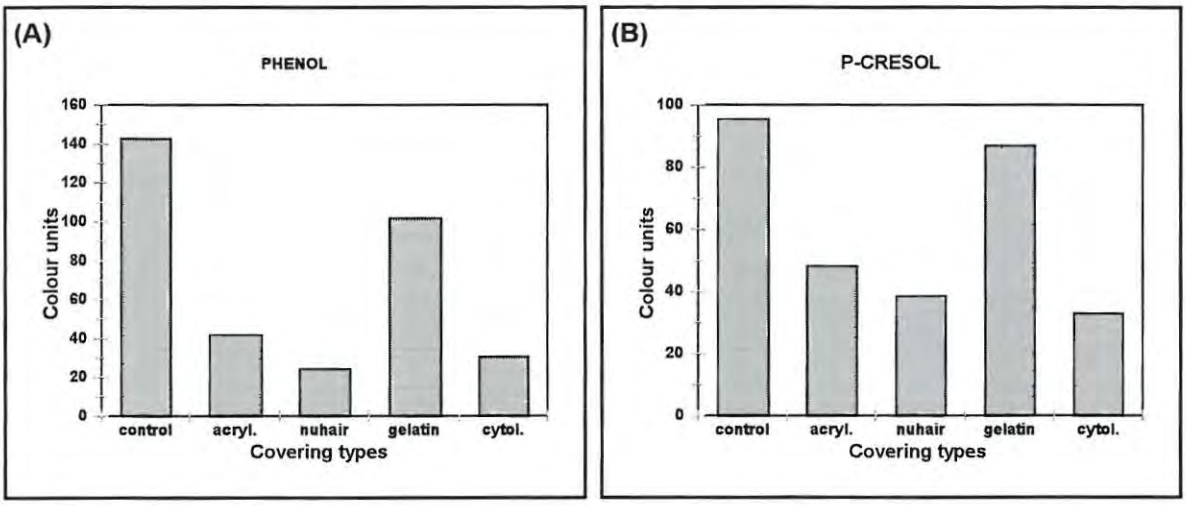


Figure 5.24. Numerical representations of the colours produced by the bioprobe with various covering layers (given in Adobe colour units) in 100 mg/L phenol (A) and *p*-cresol (B) solutions after 3 hours (acryl.=acrylamide; cytol.=cytological fixative)

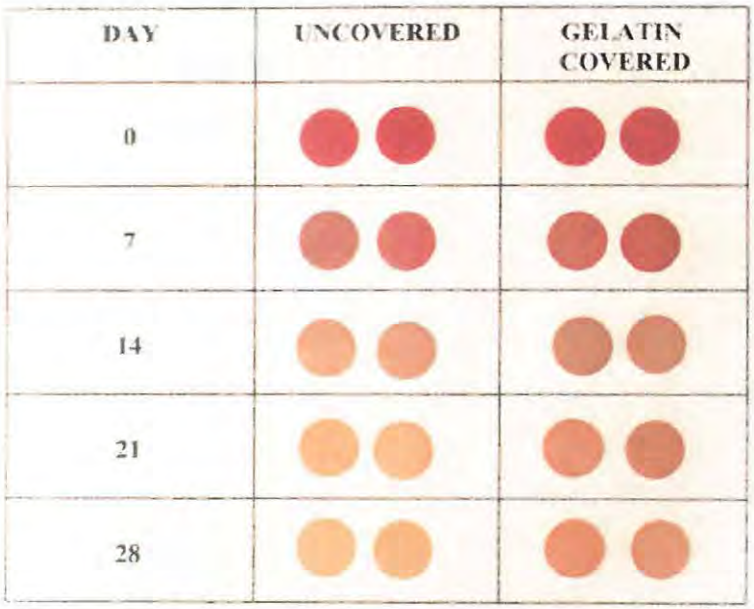


Figure 5.25. Photograph showing the colours produced (after 3 hours) in 100 mg/L phenol solutions by the bioprobe, with and without a gelatin covering, after storage

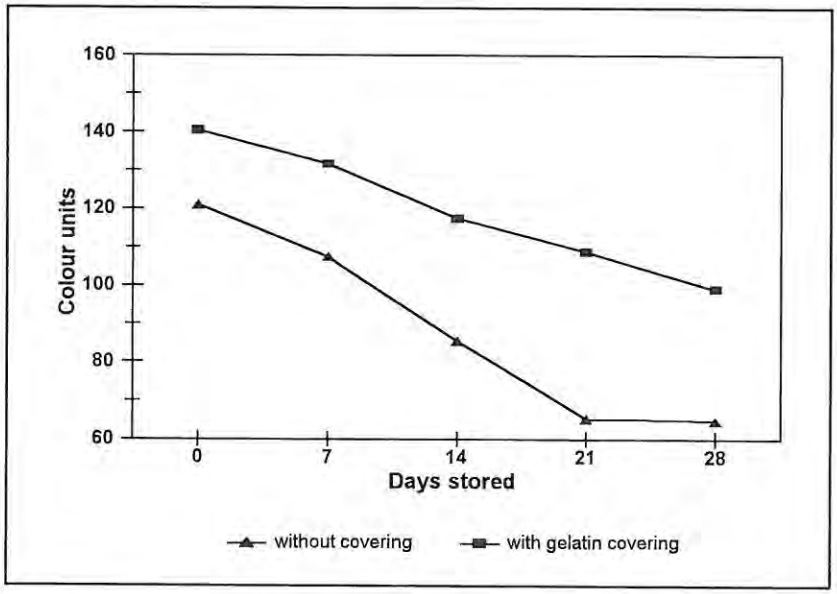


Figure 5.26. Numerical representation of the colours produced after 3 hours (given in Adobe colour units) by the bioprobe, with and without a gelatin covering, in 100 mg/L phenol solutions, after storage

5.3.8 Observation of the colours produced by the bioprobe in effluents

The phenol concentrations of the undiluted effluents as indicated by the bioprobe by the visual comparison of the colours produced in the effluents with the colours produced in pure phenol solutions (Figure 4.10) are given in Table 5.1. The phenol concentrations and pH of the effluents (analysed by the supplier) are also given.

Table 5.1. Phenol determination by the bioprobe in undiluted effluents

Effluent	Phenol concentration (mg/L)	pH	Bioprobe reading (mg/L)
RW	137	3.1	less than 1
SGL-BD	110	6.8	≈100
SGL-RW blow down	110	6.7	≈90

Very little colour change took place in the Fisher-Tropsch acid water (RW). This was to be expected, since the pH of this effluent was 3.1, and the bioprobe was shown to be active only

in a pH range from 4 to 10 (section 5.3.4). The phenol concentrations measured by the bioprobe for the SGL-BD and the SGL+RW blow down effluents were similar to those given in the analysis by the supplier. The bioprobe was therefore effective in semi-quantifying the phenol concentrations in these effluents. The pH's of these effluents were near neutral and therefore the activity of the bioprobe was not affected by the pH of these samples.

The concentration of the phenols and cresols in the diluted phenolic and cresylic industrial effluents, respectively, as indicated by the bioprobe are shown in Table 5.2. The concentrations of the phenolic substrates (given by the supplier) in the effluents after dilution are also given. The concentrations measured by the bioprobe were obtained by visually comparing the colours produced in the different effluent samples after 3 hours with the colours produced in 1-100 mg/L pure phenol and 1-100 mg/L pure *p*-cresol solutions after 3 hours (Figure 4.10). From Table 5.2 it can be seen that the bioprobe was able to give a semi-quantitative measurement of the phenolic substrate concentrations present in these effluent samples. The colours produced by the bioprobe in the diluted phenolic and cresylic effluents are shown in Figure 5.27.

Table 5.2. Phenol determination by the bioprobe in diluted effluents

Effluent	Concentration after dilution (mg/L)	Bioprobe reading (mg/L)
Phenolic	200	much greater than 100
	20	between 10 and 30
	2	≈5
Cresylic	120	over 100
	48	between 40 and 60
	12	≈10

(A)

BIOPROBE ACTIVITY IN DILUTED PHENOLIC EFFLUENT (APPROXIMATELY 2000 PPM)

DILUTION	COLOURS PRODUCED
1:10	
1:100	
1:1000	

(B)

BIOPROBE ACTIVITY IN DILUTED CRESYLIC EFFLUENT (APPROXIMATELY 12000 PPM)

DILUTION	COLOURS PRODUCED
1:100	
1:250	
1:1000	

Figure 5.27. Photograph showing the measurement of phenolic substrate concentrations in effluents by the bioprobe (A) Measurement of phenolic substrates in phenolic effluent (1:10, 1:100 and 1:1000 dilutions are equivalent to 200, 20 and 2 mg/L, respectively) (B) Measurement of phenolic substrates in cresylic effluent (1:100, 1:250 and 1:1000 dilutions are equivalent to 120, 48 and 12 mg/L, respectively)

5.3.9 Determination of the response time for the bioprobe

The colours produced by the bioprobe in 10 and 100 mg/L phenol, *p*-cresol and the mixture of phenolic substrates at 2 hour intervals over 24 hours are shown in Figures 5.28, 5.30 and 5.32, respectively. The numerical representations of these colours are shown in Figures 5.29, 5.31 and 5.33 for phenol, *p*-cresol and the mixture of phenolic substrates, respectively. The photographic and numerical representations of the colours produced by the bioprobe at 30 minute intervals over 3 hours are shown in Figures 5.34 and 5.35 for phenol, Figures 5.36 and 5.37 for *p*-cresol and Figures 5.38 and 5.39 for the mixture of phenolic substrates, respectively. The results of these experiments showed that the bioprobe required only 30 minutes in order to produce colours with intensities which could visibly distinguish between the two concentrations tested.

The photographic and numerical representations of the colour gradations produced by the bioprobe in 0-100 mg/L substrate concentrations after 30 minutes are shown in Figures 5.40 and 5.41 for phenol and in Figures 5.42 and 5.43 for *p*-cresol, respectively. The photographic and numerical representations of the colour gradations produced by the bioprobe in these same phenol and *p*-cresol solutions after 5 minutes are shown in Figures 5.44 and 5.45 for phenol and in Figures 5.46 and 5.47 for *p*-cresol, respectively. Figures 5.40 and 5.41 show that the colour intensities produced by the bioprobe after 30 minutes for the range of phenol concentrations were not proportional to the corresponding substrate concentrations. Figures 5.42 and 5.43 show that the colour intensities observed after 30 minutes in the range of *p*-cresol concentrations were also not proportional to the substrate concentrations. The colour intensities produced by the bioprobe in these same ranges of phenol and *p*-cresol concentrations after 5 minutes, however, were clearly proportional to the corresponding substrate concentrations (Figures 5.44-5.47). It should be noted here that by extrapolating to the Y-axis in Figures 5.45 and 5.47, "membrane blank" readings could be obtained, giving the readings for the membrane in the absence of substrate. This data would be valuable in confirming the applicability of the bioprobe for semiquantitative assay of phenolics. The results showed that, in contrast to the 3 hour response time required by the free enzyme, the response time required by the bioprobe was only 5 minutes.

Differences in the colour intensities of the bioprobe discs from one immobilisation to another was observed in a number of the experiments. This was probably due to the varying specific activities of the enzyme extracts used. Because the enzyme extracts had different specific activities, immobilisation of consistent amounts of protein would not have produced consistent immobilisation of enzyme activity. This was not a problem, however, for the purposes of these experiments, since the batch of discs used for each experiment had been treated identically, allowing for the various comparisons to be made. Uneven colouring of the bioprobe discs was also occasionally observed in some of the experiments. This problem would need to be further addressed with the commercialisation of the bioprobe.

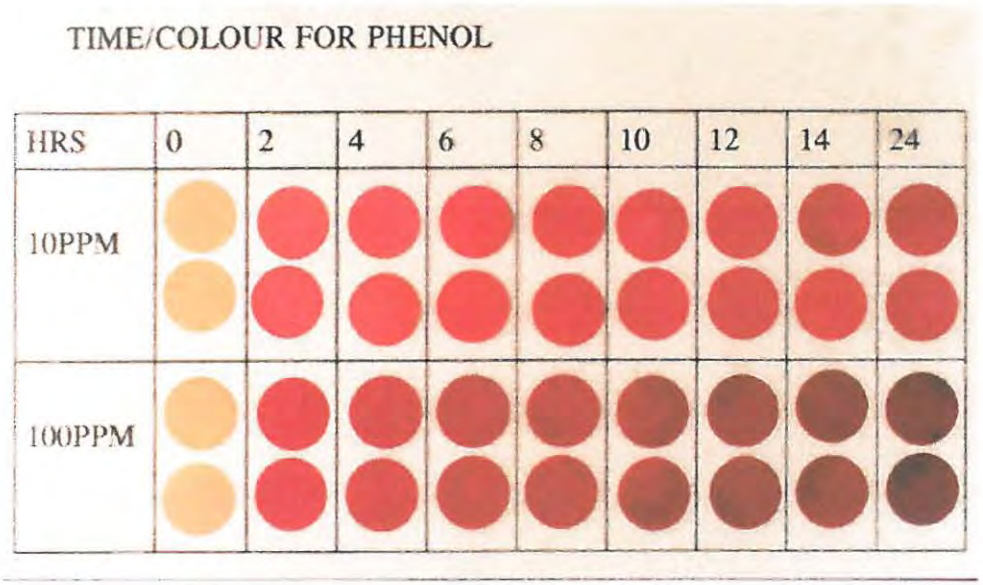


Figure 5.28. Photograph showing the colours produced by the bioprobe in 10 mg/L and 100 mg/L phenol solutions over 24 hours

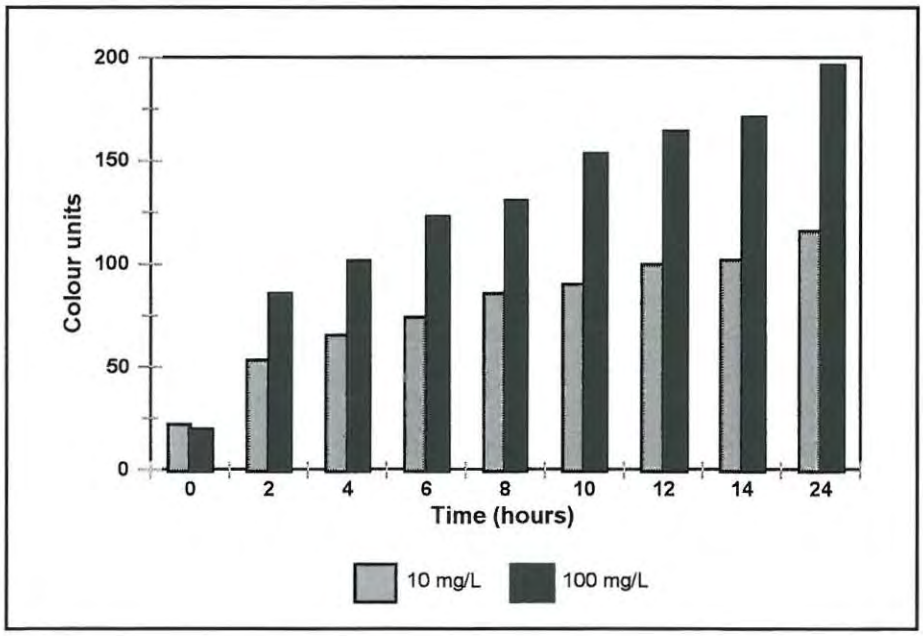


Figure 5.29. Numerical representation of the colours produced by the bioprobe (given in Adobe colour units) in 10 mg/L and 100 mg/L phenol solutions over 24 hours

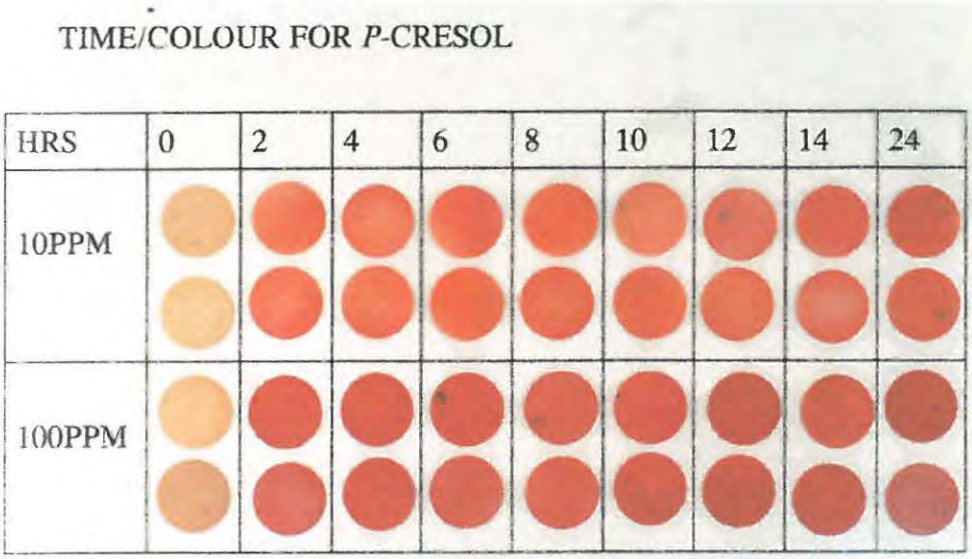


Figure 5.30. Photograph showing the colours produced by the bioprobe in 10 mg/L and 100 mg/L *p*-cresol solutions over 24 hours

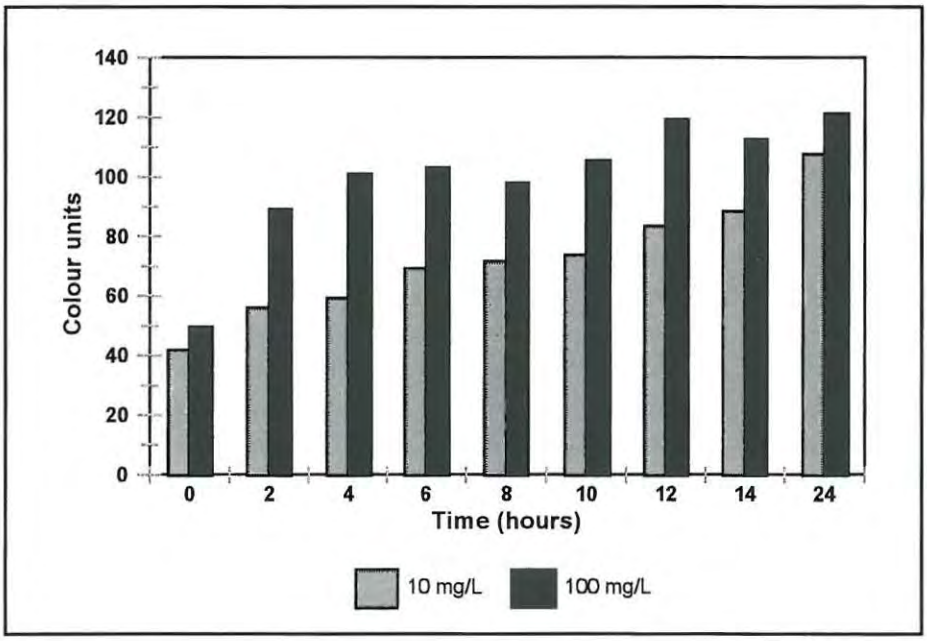


Figure 5.31. Numerical representation of the colours produced by the bioprobe (given in Adobe colour units) in 10 mg/L and 100 mg/L *p*-cresol solutions over 24 hours

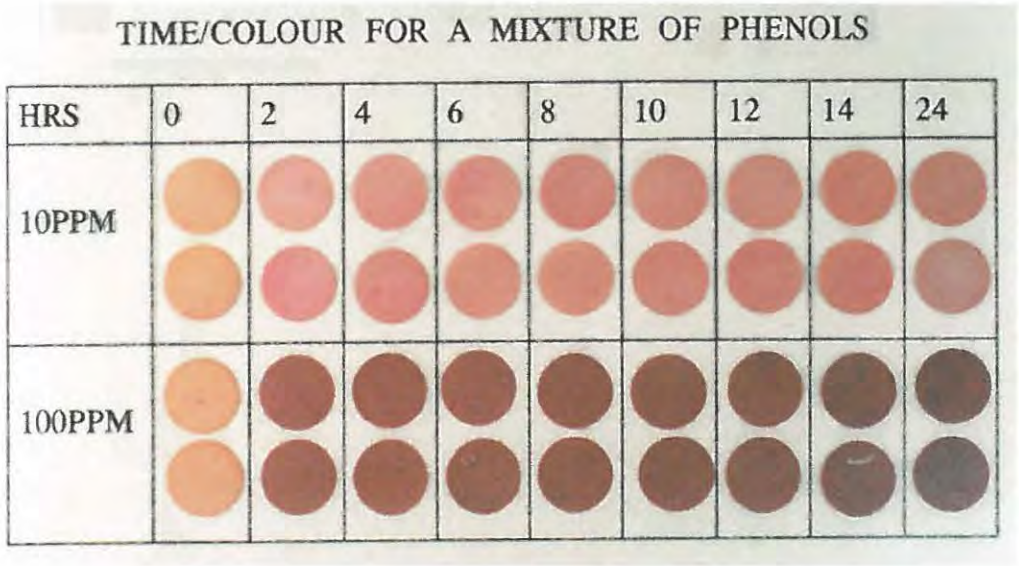


Figure 5.32. Photograph showing the colours produced by the bioprobe in a 10 mg/L and 100 mg/L mixture of phenolic substrates over 24 hours

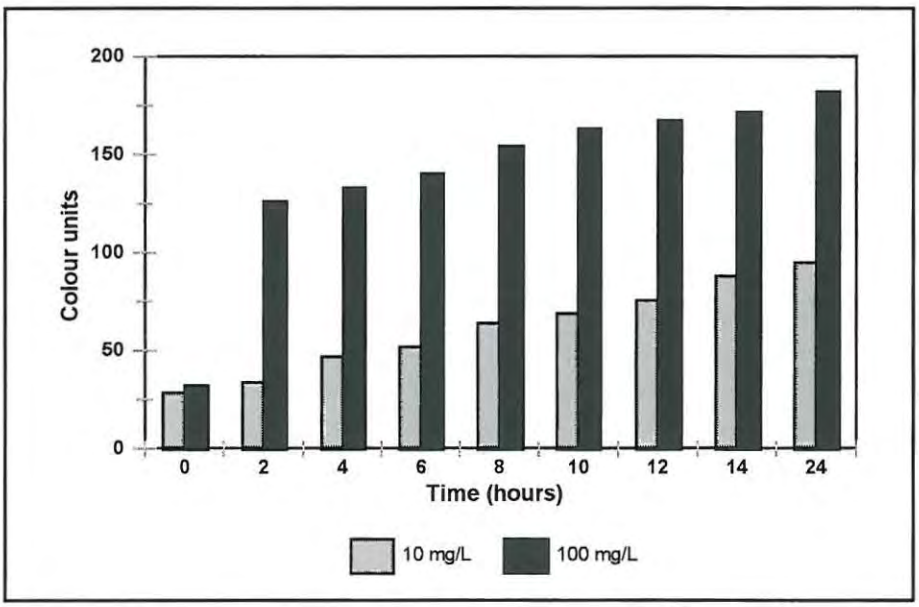


Figure 5.33. Numerical representation of the colours produced by the bioprobe (given in Adobe colour units) in a 10 mg/L and 100 mg/L mixture of phenolic substrates over 24 hours

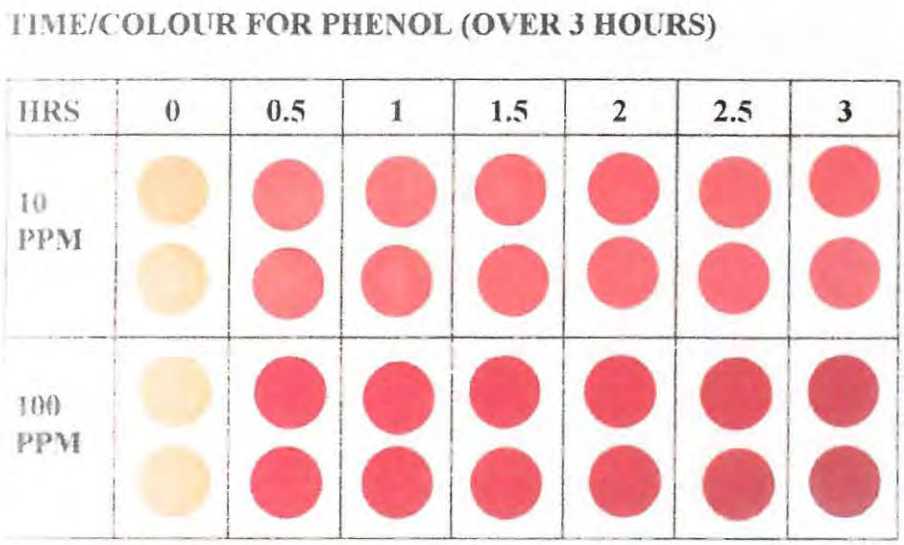


Figure 5.34. Photograph showing the colours produced by the bioprobe in 10 mg/L and 100 mg/L phenol solutions over 3 hours

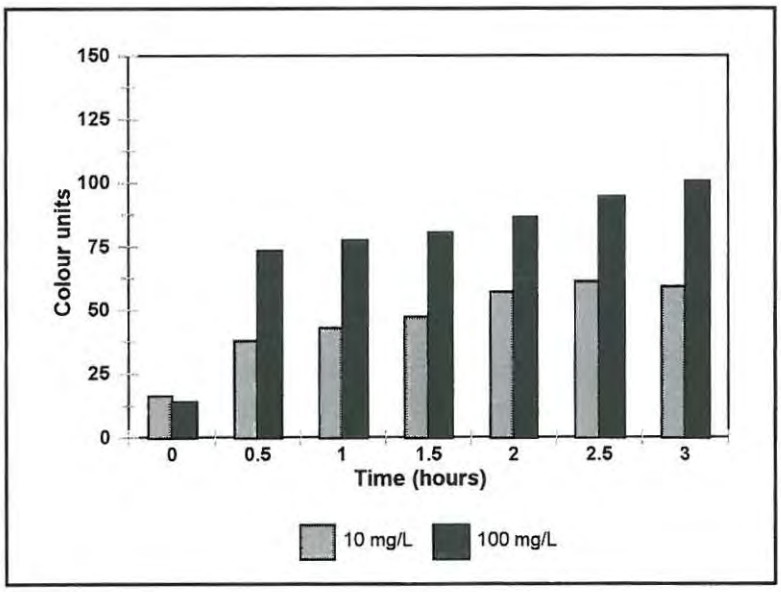


Figure 5.35. Numerical representation of the colours produced by the bioprobe (given in Adobe colour units) in 10 mg/L and 100 mg/L phenol solutions over 3 hours

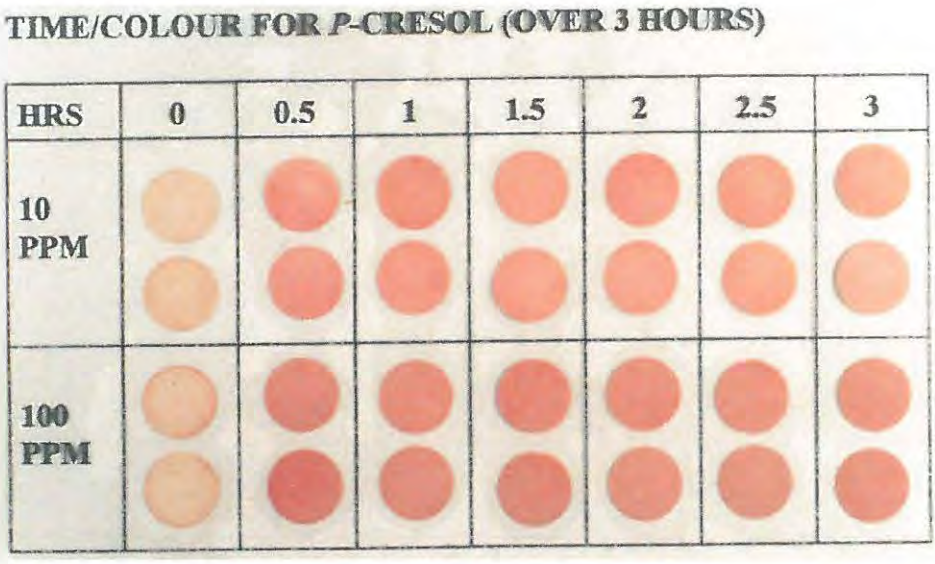


Figure 5.36. Photograph showing the colours produced by the bioprobe in 10 mg/L and 100 mg/L *p*-cresol solutions over 3 hours

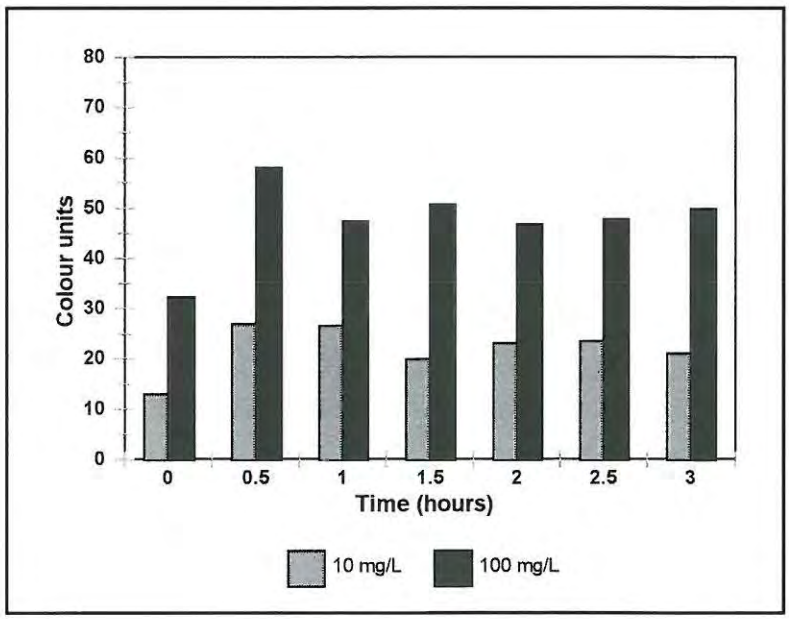


Figure 5.37. Numerical representation of the colours produced by the bioprobe (given in Adobe colour units) in 10 mg/L and 100 mg/L *p*-cresol solutions over 3 hours

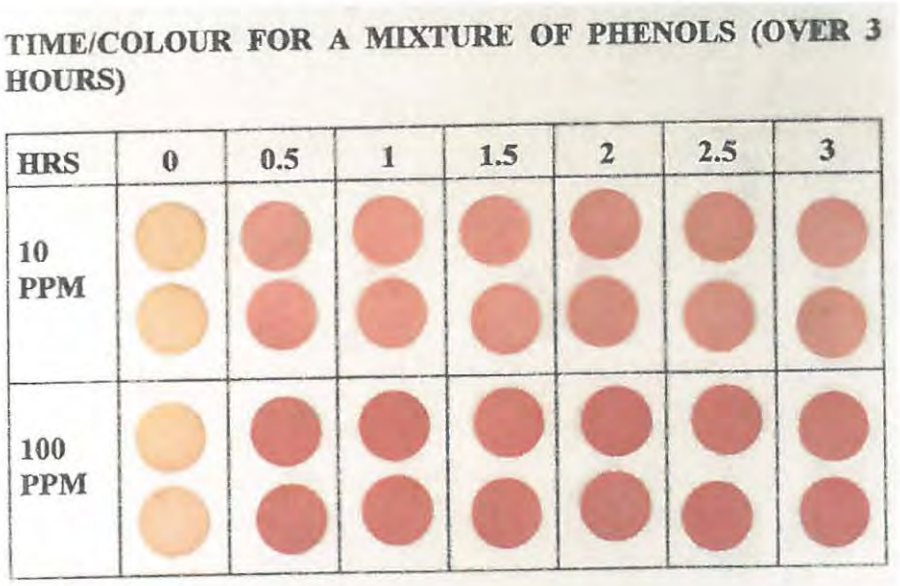


Figure 5.38. Photograph showing the colours produced by the bioprobe in a 10 mg/L and 100 mg/L mixture of phenolic substrates over 3 hours

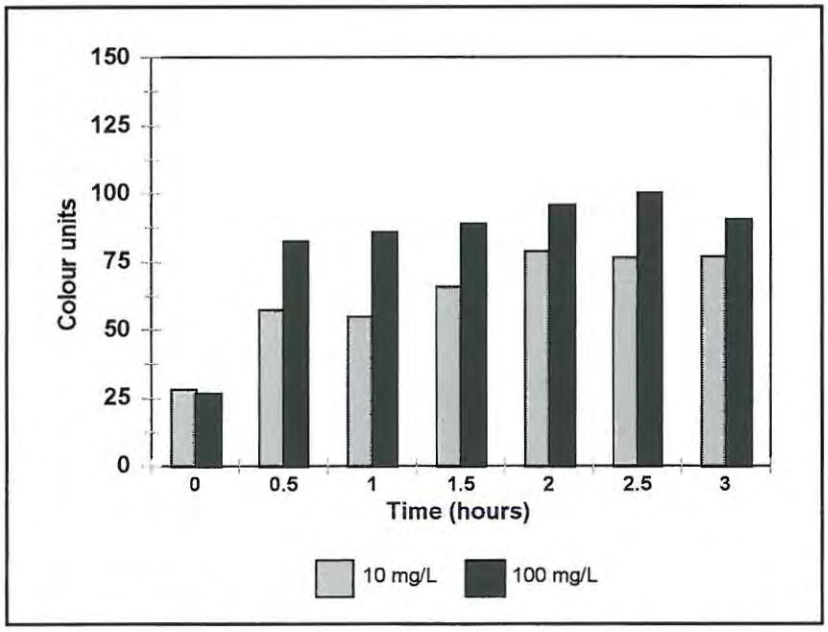


Figure 5.39. Numerical representation of the colours produced by the bioprobe (given in Adobe colour units) in a 10 mg/L and 100 mg/L mixture of phenolic substrates over 3 hours

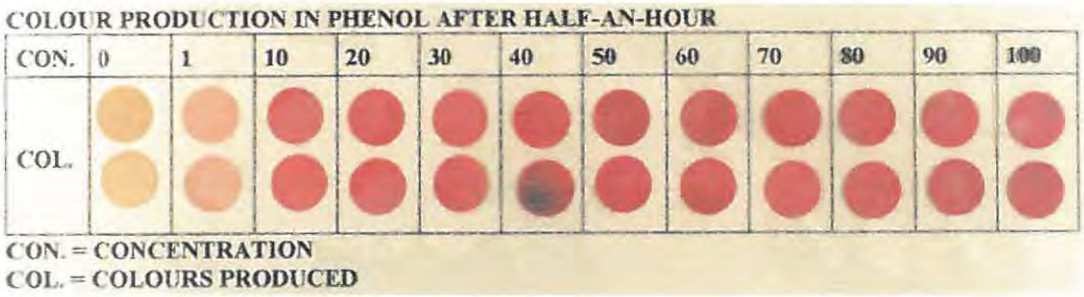


Figure 5.40. Photograph showing the colour gradations produced by the bioprobe in 0-100 mg/L phenol solutions after 30 minutes

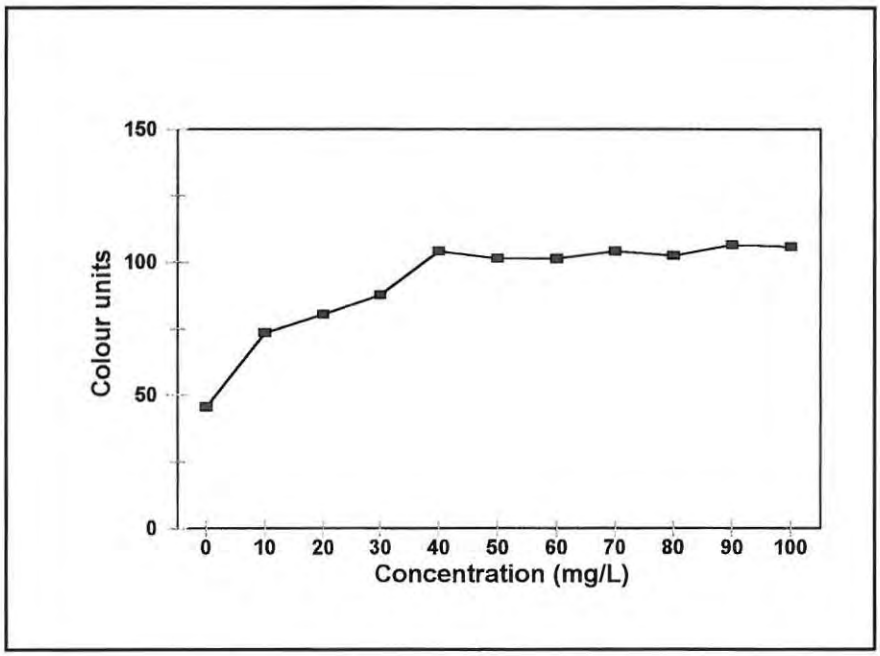


Figure 5.41. Numerical representation of the colour gradations produced by the bioprobe (given in Adobe colour units) in 0-100 mg/L phenol solutions after 30 minutes

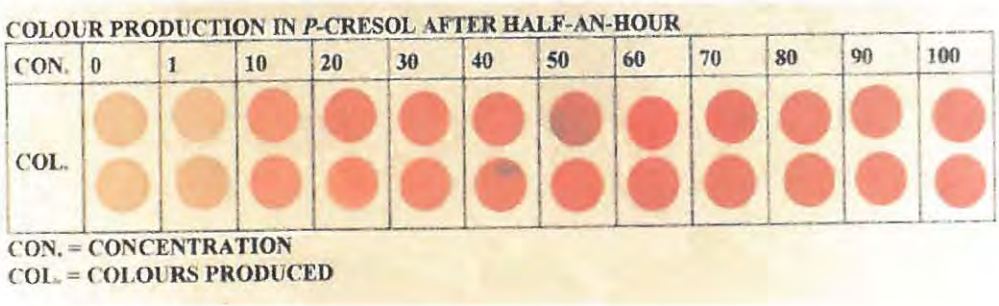


Figure 5.42. Photograph showing the colour gradations produced by the bioprobe in 0-100 mg/L *p*-cresol solutions after 30 minutes

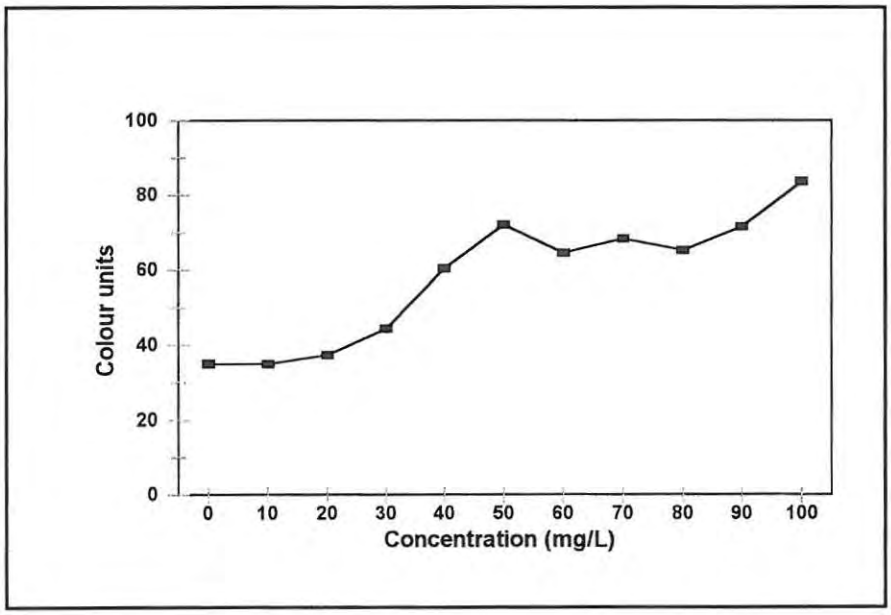


Figure 5.43. Numerical representation of the colour gradations produced by the bioprobe (given in Adobe colour units) in 0-100 mg/L *p*-cresol solutions after 30 minutes

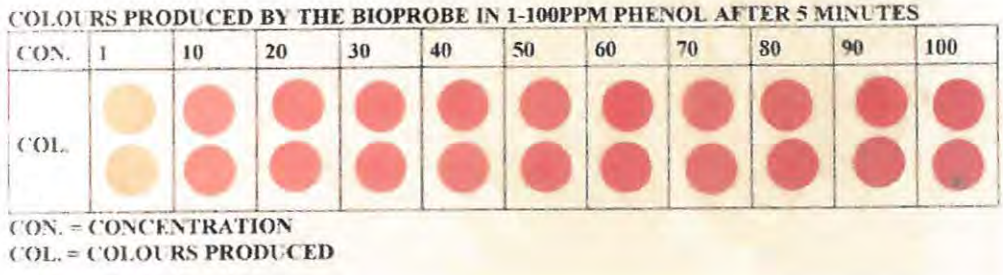


Figure 5.44. Photograph showing the colour gradations produced by the bioprobe in 1-100 mg/L phenol solutions after 5 minutes

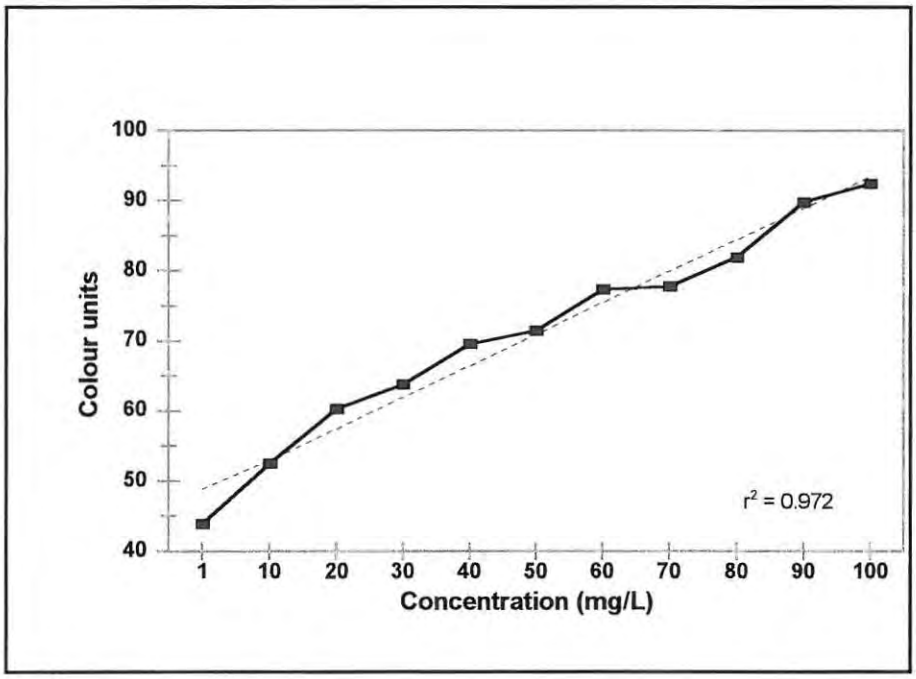


Figure 5.45. Numerical representation of the colour gradations produced by the bioprobe (given in Adobe colour units) in 1-100 mg/L phenol solutions after 5 minutes

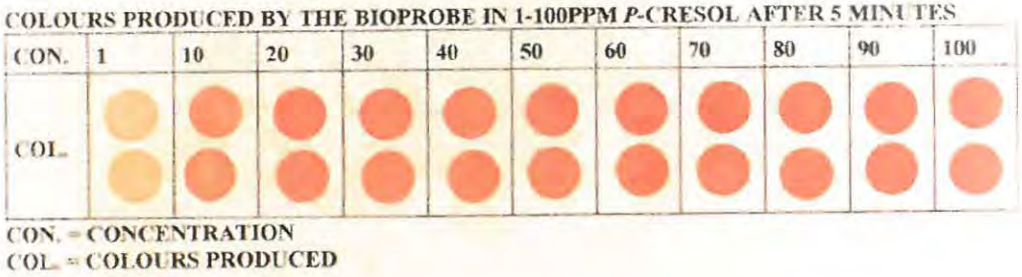


Figure 5.46. Photograph showing the colour gradations produced by the bioprobe in 1-100 mg/L *p*-cresol solutions after 5 minutes

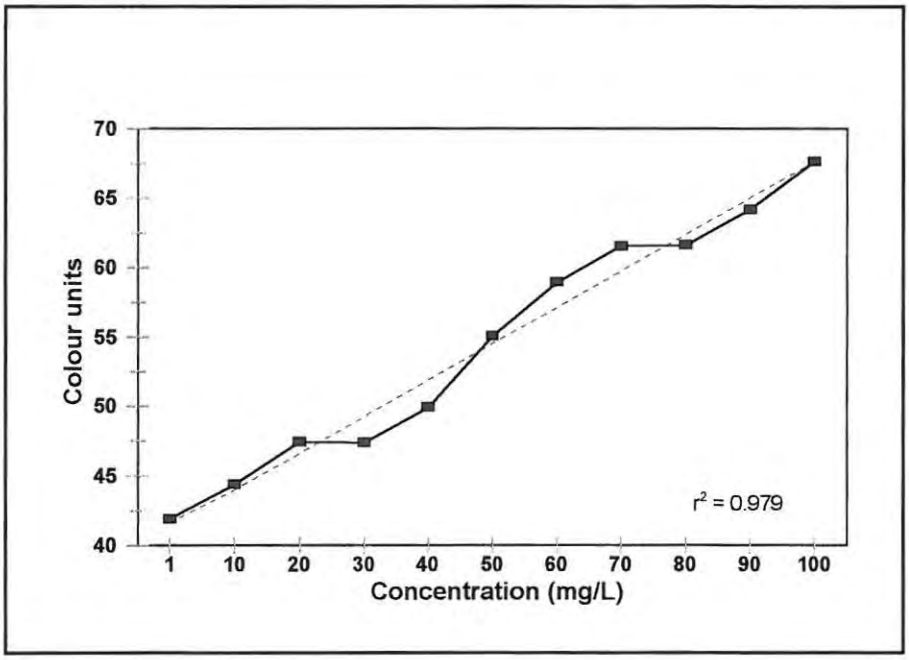


Figure 5.47. Numerical representation of the colour gradations produced by the bioprobe (given in Adobe colour units) in 1-100 mg/L *p*-cresol solutions after 5 minutes

5.4 CONCLUSION

The bioprobe produced a range of aesthetically pleasing maroon colours in a mixture of phenolic substrates. The bioprobe was also able to detect catechol and 4-methylcatechol, producing ranges of maroon and orange colours, respectively. The lower detection limit of the bioprobe for phenol and *p*-cresol was 0.05 mg/L. This value falls in the range of lower detection limits for other phenol detecting devices which range from 0.008 μg/L (Makower *et al.*, 1996) to 94 mg/L (Canofeni *et al.*, 1994). The bioprobe was shown to function in broad pH and temperature ranges and its activity was not affected by the presence of a variety of salt and metal ions in solution. No significant loss of activity was observed after a one-month storage period in the absence of light and air at room temperature. The phenol concentration readings given by the bioprobe in a number of effluents (by visual comparison with the colours produced in the pure substrates) compared favourably with the phenol concentrations given in the analyses of the effluents (by the supplier), indicating that the bioprobe was able to detect and semi-quantify phenolic substrates in these effluents. Finally, the bioprobe was able to detect phenol and *p*-cresol after a response time of 5 minutes, producing colours proportional to the substrate concentrations. These results show that the bioprobe has the potential for detecting and semi-quantifying phenolic substrates in a broad range of water samples including effluents.

CHAPTER 6:
Description of the bioprobe kit

The results obtained showed that the bioprobe was capable of detecting phenolic substrates in synthetic water samples under a broad range of conditions. Furthermore, testing of the bioprobe in some phenolic and cresylic industrial effluent samples, showed that the bioprobe was also able to detect and semi-quantify phenolic substrates in authentic samples. These results indicate that the commercialisation of such a bioprobe is feasible. The next step in the investigation, therefore, was to establish how the bioprobe would be used for the detection and semi-quantification of phenolic substrates in the field. This chapter explains how the bioprobe could potentially be used in a kit form using phenol as the test substrate. This kit would be suitable for field use and could be used by a "non-expert" for detecting phenol in water samples where phenol is the predominant substrate. The kit would be modified in order to allow for the additional measurement of phenolic substrates in samples where *p*-cresol is predominant.

6.1 FORMULATION OF THE BIOPROBE KIT

One of the problems experienced with the immobilisation of polyphenol oxidase on the nylon membranes was differences in the number of enzyme units immobilised on each batch of bioprobe discs. These differences between batches of discs resulted in differences in the colour intensities from one immobilisation to another (e.g. see colour intensities produced after 2 hours in Figures 5.30 and 5.36). This was due to differences in the specific activities of the enzyme extracts used (explained in section 5.3.9). To overcome differences between batches of discs, the use of the bioprobe would involve a kit containing a set of standard phenol solutions of known concentration so that a colour reference chart could be made for each individual kit. This would be done by simply immersing a bioprobe disc into each of the standard phenol solutions and allowing the colour to develop for each one. The phenol concentration in a sample would therefore be measured by comparing the colour produced by the bioprobe in the sample with the colours produced in the standard phenol concentrations (colour reference chart).

Visual comparisons of the colours produced by the bioprobe in the phenolic industrial effluent samples (section 5.3.8) with the colours produced in pure phenolic substrates indicated that the bioprobe would give a semi-quantitative measurement of the phenolic substrate concentration present in a sample. The measurement would be semi-quantitative because obvious visible colour differences were only observed between phenol and *p*-cresol concentrations of 1, 10, 20, 50 and 100 mg/L after 5 minutes (Figures 5.44 and 5.46). Standard phenol solutions at these concentrations would therefore be included in the kit to make up the colour reference chart. The concentration readings obtained by comparing the colours produced in various samples with the colour reference chart would therefore fall into certain concentration ranges e.g. less than 1mg/L; between 10 and 20 mg/L or greater than 100 mg/L. Samples with phenolic substrate concentrations greater than 100 mg/L would be diluted in order to obtain a more accurate reading.

A set of instructions would be included in the kit explaining to the user any procedures which would be necessary for adjusting a sample before using the bioprobe. Any solutions which would be required in order to follow the instructions would also be included in the kit e.g. the instructions and solutions required for carrying out dilution and pH alteration of samples would be given.

6.2 CONTENTS OF THE BIOPROBE KIT

1. Bioprobe discs (indicator discs) vacuum sealed in aluminium foil
2. Two sample bottles with markings for a 1:5 dilution
3. Small water bottle
4. Pair of plastic tweezers for handling the discs
5. Set of standard phenol solutions marked 1, 10, 20, 50 and 100 mg/L
6. Plastic droppers containing dilute solutions of sodium hydroxide (labelled 1) and hydrochloric acid (labelled 2)
7. pH paper with colour comparison chart
8. A piece of laminated or waxed white cardboard on which to place the bioprobe discs for comparison of the colours. (A hydrophobic surface would be required in order to prevent

drying out of the discs after use which results in colour loss).

9. A set of instructions

6.3 INSTRUCTIONS

1. Prepare water sample for testing by half filling sample bottle with water to be tested.

2. Dip a piece of pH paper into sample, remove it, and read pH using comparison chart. If pH reading falls between 3 and 11, continue with steps 3-7. If the pH reads outside these values, carry out instruction A (below).

3. Remove indicator discs from packaging and, using tweezers, place one disc into the water sample to be tested and one disc into each of the five standard solutions marked 1,10,20,50 and 100 mg/L.

4. Wait five minutes.

5. Using tweezers, remove discs from the standard solutions and place on the positions marked with the corresponding concentrations on the white cardboard.

6. Remove disc from sample bottle and compare the colour produced on the disc with the colours produced in the standard solutions. By lining the sample disc up with the closest matching colour, an approximate concentration for phenolic substrates can be obtained for the sample.

7. If the colour obtained on the sample disc is much darker than the 100 mg/L standard disc, carry out instruction B (below).

A. *pH alteration:*

If pH reading in step 2 was below 4, squeeze a drop of solution 1 into the sample bottle and swirl gently. Read pH as described above. Repeat until the pH reading falls between 3 and 11. Continue with steps 3-7.

If the pH reading in step 2 was above 10, squeeze a drop of solution 2 into the sample bottle and swirl gently. Read pH as described above. Repeat until the pH reading falls between 3 and 11. Continue with steps 3-7.

B. *Dilution:*

a. Fill sample bottle to the first marked volume with water to be tested.

b. Fill sample bottle to the second marked volume with water from the supplied water bottle and swirl gently.

c. Continue with steps 3-7.

d. If instruction B has to be repeated more than once, a second sample bottle should be filled to the first marked volume using the solution from the first sample bottle.

e. Once a concentration reading has been obtained which is below 100 mg/L, the reading must be multiplied by 5 for each time instruction B was carried out. e.g. if the concentration reading obtained was 20 mg/L after instruction B had been carried out 3 times, the concentration of phenolic substrates in the original water sample would be 20 x 5 x 5 x 5 = 2 500 mg/L.

A photograph of what the proposed kit would look like is shown in Figure 6.1. Figure 6.2 shows the colour reference chart produced from a set of standard phenol solutions as described.

Figure 6.1. Photograph of the proposed bioprobe kit

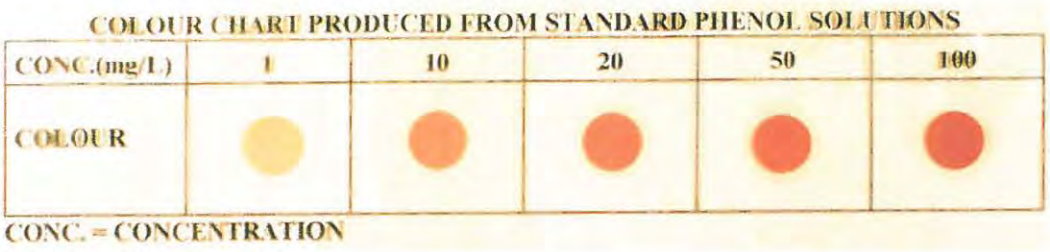


Figure 6.2. Colour reference chart produced by reacting the bioprobe with standard phenol concentrations

CHAPTER 7:
Discussion

The results obtained throughout this project show that the commercialisation of a bioprobe based on polyphenol oxidase for the detection of phenolic compounds in water is feasible. It was found that polyphenol oxidase could be relatively simply and cheaply extracted from frozen brown mushrooms. PAGE separations of the various extracts showed that their protein contents were very similar and compared favourably with the commercial enzyme extract. The units of polyphenol oxidase activity present per mg of each extract, however, varied significantly. These differences were taken into account with the use of the extracts in subsequent experiments by working with units of enzyme activity in place of masses of enzyme extracts used. In this way, uniform amounts of enzyme could be used and relevant comparisons could be made.

One of the most important results obtained was the confirmation of the linear relationship between substrate concentration and the colour production by the enzyme. The development of the bioprobe would be based on this and it was therefore important, first and foremost, to establish this relationship. The finding that the linear relationship between substrate concentration and colour production was dependent on time was also significant. It took 3 hours for the colour intensities produced in solution to become proportional to the corresponding substrate concentrations for the three substrates used (*p*-cresol, *p*-chlorophenol and phenol). This showed that a bioprobe comprising polyphenol oxidase in solution would need 3 hours in order to accurately quantify phenolic substrates in a sample. The response time for the immobilised enzyme was found to be significantly shorter due to the presence of the colour reagent, MBTH, which has been shown to decrease the lag time of the reaction. This short response time of only 5 minutes would be very favourable for the development of a "dipstick"-type test such as proposed for the bioprobe.

Polyphenol oxidase was shown to remain catalytically active after immobilisation on filter

paper, glass and three different membranes. The most successful immobilisation of the enzyme (in terms of quantity immobilised and colour production) was obtained with nylon membranes in the presence of MBTH which produced aesthetically pleasing ranges of colours in the different phenolic substrates. The amount of polyphenol oxidase immobilised in each case was significantly higher in the presence of the cross-linker, glutaraldehyde. Interference between the glutaraldehyde and the MBTH, however, resulted in a loss of the maroon and orange colours produced, and therefore the enzyme and MBTH had to be immobilised by simple adsorption in subsequent experiments. As a result of this, some leaching of the enzyme and/or colour reagent was observed when the bioprobe discs were placed in the substrate solutions. This would be an undesirable characteristic of the bioprobe since MBTH is toxic. Further refining of the bioprobe could therefore involve identifying another cross-linking agent which would not interfere with the MBTH and which, by covalently immobilising the enzyme and MBTH, would prevent leaching.

Some problems, which would need to be addressed with the commercialisation of the bioprobe, were experienced with the immobilisation of the MBTH and enzyme on the membranes. Even distribution of the MBTH was not always obtained, resulting in uneven colouring of the membranes in the presence of the substrates. A few of the bioprobe discs developed small dark blue stains (visible in Figures 5.15, 5.30, 5.40 and 5.42) after reacting with the phenolic substrates. The origin of these stains was not investigated but they are thought to have been caused by the presence of some undissolved MBTH in the immobilisation mix. This problem may therefore be easily overcome by ensuring that the MBTH is completely dissolved before adding it to the immobilisation mix. The same number of enzyme units could not always be immobilised on each batch of discs, resulting in differences in the colour intensities from one immobilisation to another. As explained in chapter 5, this was due to differences in the specific activities of the enzyme extracts used and was not a particular problem for the purposes of this study. Ideally, one would aim to produce bioprobe discs, each with identical enzyme activities, by standardising the enzyme extraction to produce enzyme extracts with identical specific activities. This investigation has shown this to be a difficult task. The differences between batches of discs, however, would be overcome with the use of the bioprobe kit by using a set

of standard phenol solutions and a colour reference chart as described in chapter 6.

The bioprobe activity was not adversely affected by a wide variety of different conditions. It was able to identify a number of phenolic substrates, producing maroon colours in the presence of phenolic substrates and orange colours in the presence of cresylic substrates. The bioprobe would give a measure of the total phenolic content of a sample rather than the concentration of one specific substrate. Most phenolic substrates are toxic and therefore such a reading would still be of interest. The lower detection limit for the bioprobe was found to be 0.05 mg/L for phenol, *p*-cresol and the mixture of phenolic substrates tested. This lower detection limit is well within the range of lower detection limits for other phenol detecting electrodes described in the literature. The bioprobe functioned in a broad pH and temperature range, indicating its usefulness for the detection of phenolic substrates in a wide variety of samples. It was also unaffected by the presence of a variety of salt and metal ions which may be present in water samples. The bioprobe was stored at room temperature for a month (in the absence of air and light) without loss of activity. This storage stability compares favourably with the storage stability of other enzyme-based phenol detectors described in the literature which have been shown to be stable for between one (Canofeni *et al.*, 1994) and five months (Hu, 1995). The bioprobe was able to detect phenolic substrates in industrial effluent samples confirming its ability to detect and semi-quantify phenolic substrates in authentic samples.

Research on polyphenol oxidase has shown that the enzyme is able to function successfully in organic systems provided that a small quantity of water is present (Burton *et al.*, 1993) and therefore, the bioprobe could theoretically be applied to the detection of phenolic substrates in organic systems. Nylon is generally stable in organic solvents and would therefore remain a suitable support for the enzyme in such systems. This area of application of the bioprobe was not addressed in this study but further research could involve work in this area.

The colours intensities produced by the bioprobe in phenol and *p*-cresol after 5 minutes were visibly proportional to the corresponding substrate concentrations ranging from 0.05-100 mg/L. The numerical analysis of the colours confirmed this linear relationship. Although the

differences in colour intensities at 10 mg/L concentration intervals were apparent in the numerical analyses of the colours, these differences were not obvious to the naked eye. Since the bioprobe test would measure phenolic substrates by a visual comparison of the colours produced in a sample with a colour reference chart, the differences in colour intensity revealed by the numerical analyses of the colours were not always relevant. The numerical analyses, however, were useful in confirming what was visible on the bioprobe discs, and in working out the relationships between the colour intensities produced by the bioprobe and the substrate concentrations. This form of analysis also provided a more satisfactory method of reporting the results.

Visual comparisons of the colours produced indicated that the bioprobe only produced colour intensities which could be easily distinguished at phenol and *p*-cresol concentrations of 1, 10, 20, 50 and 100 mg/L. As described in chapter 6, the colour reference chart for each bioprobe kit would therefore comprise the colours produced by the bioprobe at these concentrations. As a result, the bioprobe would give a semi-quantitative measurement of the phenolic substrate concentration present in a sample. This measurement would fall into certain concentration ranges e.g. less than 1 mg/L, between 10 and 20 mg/L or greater than 100 mg/L. In order to accommodate for the colours produced in both samples where phenol (maroon) or *p*-cresol (orange) are the predominant substrates, the bioprobe kit would need to include maroon and orange colour reference charts. Further refining of the bioprobe kit would need to be done before the bioprobe could be commercialised. This work was not within the scope of this study due to time constraints but future work could be done in this area.

PATENTING OF THE BIOPROBE TECHNOLOGY

The bioprobe was shown to be useful for the sensitive detection and semi-quantification of phenolic substrates in a broad range of samples of varying pH, temperature, ionic strength and substrate concentration. It therefore has the potential for commercialisation and marketing and the technology has been patented (Patent No. SA 97/0227). The complete patent specification is given in Appendix F. The title and claims of the patent are as follows:

PATENT - in the name of Water Research Commission

An immobilised-enzyme bioprobe for the detection of phenolic pollutants in water

Stephanie Gail Burton and Ingrid Margaret Russell, Rhodes University

CLAIMS

A *novel* means of detecting the presence and quantifying the concentration of phenolic substances in solutions including industrial effluents

The phenols can be *detected* in a concentration range approximately 0.1 - 200 ppm

The response of the bioprobe is linear with phenol concentration within this concentration range

The bioprobe uses an immobilised enzyme which requires no cofactor other than atmospheric oxygen

The enzyme is readily obtainable from certain sources and its activity can be standardised

The bioprobe can be used in aqueous or organic media

The bioprobe is portable and "low-tech" - requires no sophisticated instrumentation and can therefore be applied in the field by "non-experts"

The bioprobe is durable and tolerant of a range of pH and salt concentrations

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APPENDIX A

EQUATIONS USED FOR THE CALCULATION OF ENZYME UNITS AND DATA PRESENTED IN TABLES 2.1, 2.2 AND 2.3

1 Unit of polyphenol oxidase activity = 1 μmol quinone produced/min (μmol/min)

Beers Lambert Law states: $A = \varepsilon.l.c$ where

A = absorbance; ε = molar extinction coefficient ($M^{-1}cm^{-1}$); 1 = optical path length (cm); c = concentration (mol/L)

ε_{DOPA} = 3 600 L/mol.1/cm

Therefore:

A/min = ε.(c/min).1 cm

$$c/min = \frac{A/min}{\varepsilon.1\ cm}$$

$$= \frac{A/min}{3\ 600\ L/mol.1/cm.1\ cm}$$

$$= 2.777 \times 10^{-4}\ (A/min) mol/L$$

$$= 2.777 \times 10^{-4}\ (A/min) \times 10^{6}\ \mu mols/1000\ ml$$

$$= (A/min) 0.278\ \mu mols/ml$$

DOPA volume used for activity assays = 3 ml, therefore

Activity (c/min) in 3 ml = (A/min) x 0.278 μmol/ml x 3

But, 100 μl enzyme solution was used, therefore

Enzyme activity (Units/ml enzyme solution) = (A/min) x 0.278 μmol/ml x 3 x 10

Specific activity (Units/mg) = Activity (Units/ml)/Protein (mg/ml)

Total activity (Units) = Activity (Units/ml) x Volume (ml)

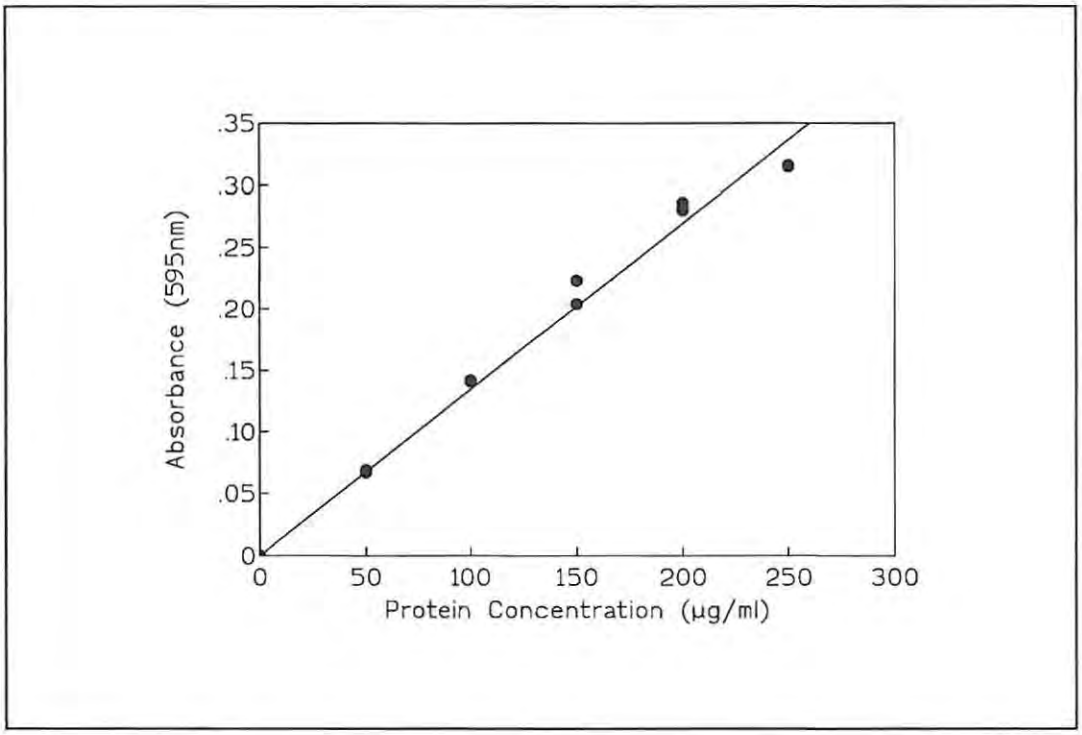
Total protein (mg) = Protein (mg/ml) x Volume (ml)

$$\text{Yield (\%)} = \frac{\text{Total activity (sample)}}{\text{Total activity (crude)}} \times 100$$

$$\text{Fold purification} = \frac{\text{Specific activity (sample)}}{\text{Specific activity (crude)}}$$

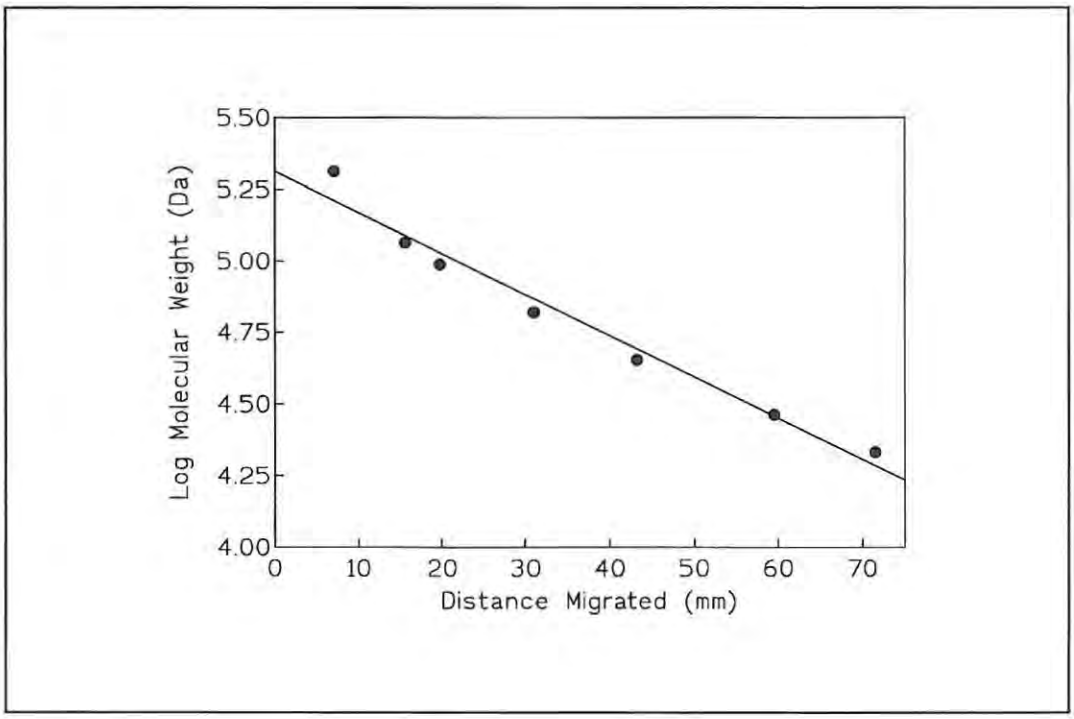
APPENDIX B

PROTEIN STANDARD CURVE



APPENDIX C

LOG OF MOLECULAR WEIGHT VERSUS DISTANCE MIGRATED FOR MARKER PROTEINS



The averages of the distances migrated for the marker proteins were calculated and used for this graph.

APPENDIX D

ANALYSIS OF EFFLUENTS FROM A LOCAL COAL GASIFICATION PLANT

BD = Average composition of samples taken from two blow down samples (Stripped gas liquor (SGL) blow down and SGL + RW blow down).

FISCHER TROPSCH ACID WATER (RW)	
COMPOUND	**CONCENTRATION (mg/L)**
Phenol	6
Catechol	52
Resorcinol	37
Hydroquinone	8
Total phenols	110

BD	
COMPOUND	**CONCENTRATION (mg/L)**
Phenol	32
o-Cresol	62
m-Cresol	27
p-Cresol	16
Total phenols	137

APPENDIX E

ORGANIC COMPOSITION OF PHENOLIC AND CRESYLIC INDUSTRIAL EFFLUENTS

PHENOLIC EFFLUENT	
COMPOUND	**CONCENTRATION (mass%)**
Acetone	0.0047
Acetonitrile	0.0418
Methanol	0.0542
Ethanol	0.1236
1-Propanol	0.0186
iso-Butanol	0.0024
n-Butanol	0.0062
Phenol	0.1855
Cresol	0.0497
2-Propanone	0.0236

CRESYLIC EFFLUENT	
COMPOUND	**CONCENTRATION (mass%)**
Phenol	0.211
o-Cresol	0.981
m+*p*-Cresol	1.139
2.6-Xylenol	0.009
Total organics	2.481

APPENDIX F

PATENT SPECIFICATION

THIS INVENTION relates to a bioprobe. In particular, the invention relates to such a bioprobe that has particular application for waste waters and to a monitoring kit.

According to the invention there is provided a bioprobe, which includes

a support means; and

indicator means comprising polyphenol oxidase immobilized on the support means for displaying the presence of at least one phenolic substance or compound in a solution in contact therewith by means of a colour change.

By bioprobe is meant an immobilized biological molecule, such as an enzyme, which interacts with a substance or compound of interest to produce a detectable chemical or physical signal which does not require electronic amplification of the signal.

By phenolic substance or compound is meant phenol or phenol derivatives, e.g. *p*-cresol, catechol, pentachlorophenol, *p*-chlorophenol, etc.

Polyphenol oxidase is also known as tyrosinase, phenol oxidase or polyphenolase. Polyphenol oxidase is a copper containing enzyme which catalyses two types of oxidation reactions. Polyphenol oxidase can act as a monooxygenase (incorporation of one oxygen atom from molecular oxygen into an organic substrate (phenol, *p*-cresol and *p*-chlorophenol)) by hydroxylating monophenols to form ortho-dihydroxyphenols (catechols). Polyphenol oxidase does not require a reducing cofactor such as NADH in order to carry out the reaction. The polyphenol oxidase can also act as an oxidase (oxidation of a substrate (phenol, *p*-cresol and *p*-chlorophenol) by molecular oxygen without incorporating additional oxygen into the product) by further oxidising the catechol to an *ortho*-quinone. *Ortho*-quinones are generally unstable and undergo a non-enzymatic polymerisation to form water-insoluble pigments called melanins. This process is responsible for the well-known phenomenon of browning of fruits and vegetable at open surfaces. Eumelanin, a black skin

pigment, and phaeomelanins, pigments responsible for red and yellow colours in hair, feathers and fur, are examples of melanins formed in this way. These coloured pigments or coloured compounds are produced as a result of polyphenol oxidase oxidizing catechols to form *ortho*-quinones and are an indication as to the presence of phenol or phenol derivatives.

The solution may be an aqueous or an organic solution. The water may be that of a body of water such as a reservoir, a river, a dam, an industrial effluent or effluents or the like. The organic solution may be an organic solution reservoir, an industrial effluent or effluents or the like.

The indicator means may also quantify or measure the approximate concentration of phenol and phenol derivatives in the solution. Concentrations of 0.05 - 500 ppm, e.g. 0.1 - 200 ppm phenol or phenol derivatives may be measured by the bioprobe.

The indicator means may comprise, in addition to the polyphenol oxidase, one or more indicator chemicals, reagents or substances for enhancing or amplifying the colour change, such as 3-methyl-2-benzothiazolianone hydrazone (MBTH) which results in a red/maroon colour change.

The indicator may be preserved by addition of a covering layer such as gelatin which is incorporated with the colour range.

The support means may be any suitable support means such as a membrane, e.g. in the form of discs or strips, filter paper, a body, e.g. a glass or plastics rod for use in dipstick fashion, or the like. The support means may be of a suitable size, so that it is easily portable and easy to manipulate or handle, while providing the required surface area to support the indicator means. The body may include a gripping member for handling the bioprobe in use. The body may be buoyant or may be constructed for fixing to a support such as a dam or reservoir wall.

The support means may be in the form of a membrane, e.g. a polyethersulphone membrane, a nitrocellulose membrane, or a nylon membrane. In a particular embodiment

of the invention, the support means comprises a membrane disc. In another particular embodiment of the invention, the support means comprises a membrane strip and a body, the polyphenol oxidase being immobilized to or on the membrane strip and the membrane strip being mounted on or secured to the body. Accordingly, the membrane may be provided as one or more discs or strips secured to or incorporated as an integral part of the body.

When buoyant, the body may be solid, or may be a hollow or foamed body of synthetic plastics material, or may be of any other suitable material that is buoyant in the solution. In other words, the body may be hollow, or made from a foamed plastics material or may be made from a plastics material which has a density less than that of the solution. When the body is a buoyant body, the body will be of a suitable size to provide the required buoyancy to support the indicator means.

Naturally, the indicator means should be of such nature and be mounted on the buoyant body in such fashion that, when the body floats in the solution, the indicator means is in contact with the solution, while the bioprobe as a whole remains buoyant.

The indicator means may replaceably or permanently be attached to the body.

The indicator means may, as mentioned above, undergo changes in colour in response to the presence and concentration of phenol or phenol derivatives in the solution.

Preferably, the polyphenol oxidase, the indicator chemical and the coating are non-toxic and/or non-leachable from the indicator means. The indicator means may take a certain time to change colour (i.e. a colour change response time) depending on the type of phenol or phenol derivatives present in the solution. In the case of phenol, *p*-cresol and chlorophenol, the indicator means may have a colour change response time of up to three hours.

According to another aspect of the invention there is provided a monitoring kit comprising

a bioprobe as described above, and

a set of instructions.

The monitoring kit may include a colour reference chart against which the bioprobe can be compared to indicate the absence or presence and approximate concentration of phenol or phenol derivatives in a solution.

The instructions may set out how the bioprobe is to be used in a solution, e.g. water body, and, by comparing the colour change of the indicator means on the bioprobe to the colour reference chart, an indication as to the presence and approximate concentration of phenol or phenol derivatives in the solution can be obtained.

In use, the bioprobe is placed in a solution such as a water sample taken from industrial effluents. The body, if buoyant, may float on the surface of the solution with the indicator means suspended thereunder, being at least partially submerged. The indicator means will then react and/or respond to the presence of phenol or phenol derivatives in the solution. A user need merely visually inspect the bioprobe after lifting it from the solution, to see at a glance whether or not phenol or phenol derivatives are present in the solution. By comparing the colour change to the colour reference chart, the actual concentration range of the phenol or phenol derivatives in the solution can be ascertained. The instructions supplied with the kit informs the user as to how to use the bioprobe.

The invention is now described, by way of non-limiting example, with reference to the following Figures, tests and examples of the development, manufacture and use of bioprobes in accordance with the invention.

Figures 1 - 4 indicate consistence of colour change in relation to phenol concentrations for the non-immobilized enzyme:

Figure 1 shows a plot of colour change measured as absorbance against concentration of phenolic substances or compounds in solution;

Figure 2 shows a plot of colour change measured as absorbance against concentration with time for *p*-cresol;

Figure 3 shows a plot of colour change measured as absorbance against

concentration with time for *p*-chlorophenol;

Figure 4 shows a plot of colour change measured as absorbance against concentration with time for phenol.

1. Extraction of polyphenol oxidase

Polyphenol oxidase was extracted from fresh open brown mushrooms (*Agaricus bisporus*) which were stored frozen as described by S. Burton, 1993 - References 1 and 2. 500 g of the mushrooms were homogenised with approximately 1 ℓ cold acetone in a Waring blender. The resulting homogenate was then filtered on a Buchner funnel while a vacuum was applied to obtain a residue. The residue was dried between sheets of filter paper and was then frozen with liquid nitrogen. 500 ml of cold water was added to the frozen residue along with 40 g of polyvinylpolypyrrolidone (PVPP) to absorb any phenolic substrate (phenol, *p*-cresol, and *p*-chlorophenol)s present in the residue or extract. The mixture was mixed thoroughly and allowed to stand overnight at a temperature of 4°C. The mixture was then filtered through a cloth placed over a funnel to obtain a filtrate and the filtrate was flushed of residual acetone by pumping nitrogen through it for two hours to obtain a crude polyphenol oxidase filtrate. The polyphenol oxidase filtrate or extract was further purified by performing a number of ammonium sulphate, $(NH_4)_2SO_4$, precipitations on the filtrate. A 40% ammonium sulphate precipitation was carried out by adding 22.6 grams $(NH_4)_2SO_4$/100 ml of solution which was left, stirring gently, overnight in a cold room. The solution was then centrifuged at 10 000 g for 10 minutes. A 52% ammonium sulphate precipitation was carried out on the resulting supernatant (40% supernatant) by slowly adding 7.35 g $(NH_4)_2SO_4$/100 ml of supernatant and leaving the supernatant overnight in a cold room. In other words, the resulting supernatant was slowly brought to a concentration of ammonium sulphate equal to 52% saturation of ammonium sulphate. The supernatant was then centrifuged at 10 000 g for 25 minutes and a pellet removed from the centrifuge. The pellet (52% pellet) was resuspended in 30 ml water and stored at a temperature of 4°C. All of the above procedures were carried out on ice or in a cold room as far as possible to minimize or avoid denaturing of the polyphenol oxidase. Protein and enzyme activity assays were performed on 1 ml samples of the crude polyphenol oxidase extract, the 40% supernatant and the 52% pellet which assays are described in more detail hereunder (see paragraph 2). The samples containing ammonium sulphate were

dialysed against water for one hour before performing these assays to remove any salt which may have interfered with the assay results. The 52% pellet was dialysed against phosphate buffer (50 mM, pH 7) overnight and then freeze-dried. Protein determinations and enzyme activity assays were carried out on the freeze-dried extracts to determine specific activities and percentage protein present in each. All centrifuging was carried out using a Beckman model J2-21 centrifuge.

2. Enzyme activity and protein assays

Enzyme activity in the samples obtained as described above was measured using a "Dopachrome assay" (Gardner and Cadman, 1990 - Reference 3). This assay measures the catecholase activity of enzyme samples using dihydroxyphenylalanine (DOPA) as the substrate in a phosphate buffer at a pH of 6. The DOPA is converted to dopachrome which has a molar extinction of 3 600 M^{-1} cm^{-1} at 475 nm (Burton, 1993 - Reference 1). A fresh DOPA solution (10 mM DOPA in 50 mM phosphate buffer, pH 6) was made up and 0.1 ml enzyme solution was added to 3 ml of the DOPA solution in a cuvette. The change in absorbance over 180s was then measured spectrophotometrically at 475 nm.

Protein determinations were carried out on the samples using "Bradford's assay" (Clark and Switzer, 1977 - Reference 4).

3. Establishing the relationship between colour change and phenol concentration

A series of solutions (concentrations from 1 - 100 parts per million (ppm)) of *p*-cresol, *p*-chlorophenol and phenol were made up as substrates for the extracted polyphenol oxidase. These units of concentration were used since these units of concentration are the units most commonly mentioned in literature dealing with phenolic pollutants in water bodies or effluents. Equal amounts (1.032 units) of the extracted and purified polyphenol oxidase were added to 3 ml of each substrate (phenol, *p*-cresol and *p*-chlorophenol) concentration. A colour change in each of the solutions was allowed to develop. The relationship between the colour change and the phenol concentration was then determined by measuring the absorbance of the solution spectrophotometrically and plotting a graph of absorbance readings against substrate (phenol, *p*-cresol and *p*-chlorophenol)

concentration. The experiment was carried out in duplicate and mean values were calculated.

The relationship between colour change and phenol concentration is illustrated graphically in Figure 1. Figure 1 shows that the relationship between colour change and phenol concentration is substantially linear. This is important as colours produced when the bioprobe is used in practice need to be proportional to the concentrations of the phenolic substrate (phenol, *p*-cresol and *p*-chlorophenol) present in water so that an approximate concentration of phenol or phenol derivatives in solution, e.g. in water, can be measured.

4. Investigation of the relationship between linearity and time

This experiment was conducted using the same procedure described above, in paragraph 3, except absorbance was read for each substrate (phenol, *p*-cresol and *p*-chlorophenol) concentration after regular time intervals until the colour changes or changes in colour intensity which occurred were substantially proportional to substrate (phenol, *p*-cresol and *p*-chlorophenol) concentrations.

The absorbance readings taken at 10 minutes, 1 hour and 3 hours for different concentrations of *p*-cresol, *p*-chlorophenol and phenol are illustrated graphically in Figures 2, 3 and 4 respectively. It was found that the time taken for the colour change to become proportional to the phenol concentrations varied for *p*-cresol, *p*-chlorophenol and phenol. The colours in the *p*-cresol solutions became proportional to the substrate concentrations after one hour, while the colours in the *p*-chlorophenol and phenol solutions only became proportional to the substrate concentrations after three hours. This indicates that *p*-cresol has a higher affinity for polyphenol oxidase than *p*-chlorophenol and phenol as it reacted more quickly with the polyphenol oxidase. The relationship between colour change produced by polyphenol oxidase and the concentration of phenol or phenol derivatives present in water samples was found to be linear after three hours (for *p*-cresol, *p*-chlorophenol and phenol). The results indicate that, in the case of phenol, *p*-cresol and *p*-chlorophenol, the bioprobe needs to be exposed to these substrates in solution for at least three hours in order to be able to quantify at least one of phenol, *p*-cresol and *p*-

chlorophenol in solution since the colour change only becomes proportional to the concentration of these substrates after at least three hours. Other phenol derivatives or phenolic substrates in a solution to be tested may require longer or shorter periods of exposure of the bioprobe to the solution to detect and quantify the particular phenol derivatives or phenol substrates present in the solution.

5. Method of manufacturing bioprobes in accordance with the invention

5.1. Immobilization of polyphenol oxidase on membranes

Polyphenol oxidase was immobilised onto three membrane types: polyethersulphone, nitrocellulose and nylon under various conditions. The combination of membrane and immobilisation conditions giving the best results was then decided on and developed into the bioprobe.

10 ml of a 4 mg/ml solution of polyphenol oxidase was made up. The polyphenol oxidase solution was added to 20 ml phosphate buffer (0.1M, pH 7) and thirty-three equal sized membrane discs were introduced into the solution. The membrane discs were incubated in the solution overnight at a temperature of 4°C with gentle stirring/agitation of the solution to ensure even distribution of the polyphenol oxidase in the solution. The membrane discs were then removed from the polyphenol oxidase solution and rinsed with phosphate buffer before being left to air dry. A protein determination and activity assay was conducted on the polyphenol oxidase solution before and after immobilization of the polyphenol oxidase on the discs in order to calculate the average amount of polyphenol oxidase immobilized onto each membrane disc. Once dry, the membrane discs with the polyphenol oxidase immobilized thereon were placed into 1 - 100 ppm solutions of *p*-cresol, *p*-chlorophenol and phenol for three hours and the resultant colour changes of the discs were noted and photographed.

Polyphenol oxidase was also immobilized onto membrane discs using the same procedure as described above except that glutaraldehyde was added to the solution prior to the membrane pieces being introduced into the solution to obtain a concentration of 2.5% w/v glutaraldehyde in solution.

Before immobilisation onto nylon discs, the nylon discs were pre-treated with hydrochloric acid (HCl). The discs were placed in a 3M HCl solution for ten minutes and then rinsed thoroughly in distilled water.

In another variation of the immobilisation procedure with nylon membranes, 3-methyl-2-benzothiazolinone hydrazone (MBTH) was added to the above-mentioned polyphenol oxidase solutions so that the MBTH had a final concentration of 5 mM MBTH in the solution. The MBTH was dissolved in 20 ml distilled water in place of the 20 ml phosphate buffer and this solution was then added to the 10 ml enzyme solution and the nylon discs were added.

5.2. Results

The amount of polyphenol oxidase immobilized per membrane disc for the different types of membranes tested are illustrated in Table 1 below:

Table 1: Immobilization of Polyphenol Oxidase onto Membranes

Membrane type	Protein immobilized per membrane disc (mg)	
	with glut.	without glut.
Polyethersulphone	0.027	0.012
Nitrocellulose	0.066	-
Nylon	0.066	0.031
Nylon with MBTH	0.056	0.036

glut. = glutaraldehyde

It was found that polyphenol oxidase could be immobilized evenly and in greater amounts on nitrocellulose membranes than on polyethersulphone membranes. It was also found that similar amounts of polyphenol oxidase could be immobilized on nylon membranes as on nitrocellulose membranes, the polyphenol oxidase being immobilized on the nylon and nitrocellulose membranes more evenly than on the polyethersulphone membranes tested.

Polyphenol oxidase appeared to be immobilized on the membranes more evenly when glutaraldehyde was added to the solution than without glutaraldehyde. It was also evident from the results that more polyphenol oxidase could be immobilized on the various membranes when glutaraldehyde was added to the solution and the glutaraldehyde did not appear to significantly affect the activity of the polyphenol oxidase. Since the polyphenol oxidase appeared to be more evenly distributed on the membrane discs when immobilized in the presence of the glutaraldehyde (cross-linker), this method of immobilization was more successful than adsorption.

The change of colours produced by polyphenol oxidase immobilized with MBTH in the presence of glutaraldehyde was similar to the colours produced by the polyphenol oxidase immobilised without MBTH in the presence of glutaraldehyde. The polyphenol oxidase with MBTH immobilized without glutaraldehyde produced a range of maroon colours for phenol and for a mixture of phenols and produced a range of orange colours for *p*-cresol. These results indicated that the most suitable membrane for immobilized polyphenol oxidase was the nylon membrane as more polyphenol oxidase could be immobilized on the nylon membrane than on the other membranes tested. The immobilization of the polyphenol oxidase in the presence of MBTH with glutaraldehyde failed to produce the maroon colours observed in the absence of the glutaraldehyde thereby indicating interference between the glutaraldehyde and MBTH. Immobilisation of the enzyme on nylon with MBTH without glutaraldehyde was therefore used to produce the bioprobe, resulting in ranges of orange or maroon colours for the phenolic substrates (phenol, *p*-cresol and *p*-chlorophenol) tested. (It should be noted that the immobilization of the polyphenol oxidase with MBTH without glutaraldehyde was done without rinsing the membrane discs before drying). Colour gradations obtained were good for most of the experiments but in some cases the colour gradations did not appear to match increasing concentrations of the substrate (phenol, *p*-cresol and *p*-chlorophenol). This may have been due to the polyphenol oxidase not being immobilized in equivalent amounts onto the membrane discs or due to saturation of the enzyme at higher concentrations. As a result of these observations it is suggested that phenol concentrations may be measured as falling into certain concentration ranges. These concentration ranges being narrow for low concentrations and wider for higher concentrations e.g. 1-5 ppm or 50-100 ppm.

Immobilization of polyphenol oxidase onto different surfaces or bodies as described above did not appear to significantly affect its catalytic activities. Polyphenol oxidase was shown to remain catalytically active after immobilization on various membranes. The immobilization methods used were adsorption and cross-linking of the polyphenol oxidase to the membrane surfaces. Adsorption relies on immobilization of the polyphenol oxidase to the membrane by an electrostatic attraction of opposite charges on the surface of the polyphenol oxidase and the membrane. Cross-linking involves covalent attachment of the polyphenol oxidase to the membrane by the formation of covalent bonds between the membrane and the cross-linking agent and between the cross-linking agent and the polyphenol oxidase. The polyphenol oxidase is more strongly immobilized to the membrane by means of the cross-linking method than by adsorption. It is believed that the cross-linking inhibits leaching of the polyphenol oxidase from the membrane. Cross-linking may also inhibit or reduce any toxicity associated with polyphenol oxidase or any indicator chemical, e.g. MBTH, forming part of the indicator means.

6. Determination of response time for the bioprobe

The bioprobe was tested in 10 ppm and 100 ppm phenol, *p*-cresol and a mixture of phenols over 24 hours at 2 hour intervals and over three hours at half hour intervals. The colours produced were observed and the response time required by the bioprobe to give a measure of the phenol concentrations was determined.

The colours produced by the bioprobe after 24 hours and after 3 hours for phenol are shown in Figures 5 and 6, respectively. The results obtained showed that the bioprobe required a half hour response time for the phenolic substrates at the concentrations tested.

7. Observation of colours produced by the bioprobe in a mixture of phenol, *p*-cresol and *p*-chlorophenol and in other phenolic substrates

The bioprobe was placed into 1 - 100 ppm solutions made up of a mixture of *p*-cresol, *p*-chlorophenol and phenol and the colours of the discs were observed after three hours (shown in Figure 7). The bioprobe was also placed into 1-100 ppm solutions of 4-methylcatechol and catechol and the colours were observed after three hours (shown in

Figure 8).

The colours produced by the bioprobe in a mixture of phenol, *p*-cresol and *p*-chlorophenol were similar to the maroon colours obtained in phenol solutions. 4-Methylcatechol and catechol produced orange and maroon colour ranges, respectively.

8. Ability of the bioprobe to detect low phenol concentrations

The bioprobe was tested in phenol, *p*-cresol and a mixture of phenols (phenol, *p*-cresol and *p*-chlorophenol) at concentrations of 0, 0.05, 0.1 and 1 ppm. The colours were observed after three hours.

The bioprobe was able to produce colour gradations at the concentrations tested after three hours (shown in Figure 9).

9. The effect of pH on the bioprobe

The effect of pH on the activity of the bioprobe was investigated by observing the colour changes produced in 100 ppm phenol at various pH. The phenol solutions were adjusted to a range of pH from pH 2 to pH 13 using a pH meter and HCl and NaOH. The bioprobe was then tested in the various phenol solutions and the colour changes produced were observed after three hours.

The activity of the bioprobe only appeared to be affected by extremes of pH. At pH 2 and 3 and at pH 12 and 13 the bioprobe failed to produce a colour change while at pH 10 and 11 the colours produced were not as intense as those produced from pH 4 to pH 9. This indicated that the bioprobe activity was impaired to some degree at these pHs. The bioprobe was effective at a pH of 4 - 9 (shown in Figure 10).

10. Observation of the colours produced in effluents

The bioprobe was tested in six different effluents. The colour changes produced were observed after three hours and compared with the colour changes produced in the

solutions of pure phenol substrates (phenol, *p*-cresol and *p*-chlorophenol). The first four effluent samples were:

a. Fischer Tropsch acid water (RW) before concentration.

b. Stripped gas liquor blow down (SGL-BD).

c. Stripped gas liqor blow down and Fischer Tropsch acid water (SGL _ RW)

d. Cooling Tower Effluent

The phenol concentration and pH of the these effluents as well as the bioprobe readings are shown in Table 2 below.

Table 2: Phenol concentrations and pH of effluents and corresponding bioprobe readings

Effluent	Phenol concentration (ppm)	pH	Bioprobe reading (ppm)
RW	137	very acidic	less than 1
SGL-BD	103	6.8	± 100
SGL-RW blow down	100-120	6.7	± 90
Cooling Tower Effluent	-	-	± 20-30

The colours produced by the bioprobe after being tested in these four effluent samples were compared to the colours produced by the bioprobe after being introduced into the mixture of phenol, *p*-cresol and *p*-chlorophenol in solution. Very little colour change took place in the Fischer Tropsch acid water (RW). The pH of the Fischer Tropsch acid water effluent was not available but it was believed to have a very low pH and contained 11 091 ppm acids. The small colour change produced by the bioprobe in the Fischer Tropsch acid water effluent therefore appears to have been due to the very low pH of this effluent. As stated above, extremes of pH effect the bioprobe activity. The colour changes obtained for the stripped gas liquor blow down and the mixture of stripped gas liquor blow down and Fischer Tropsch acid water allowed for the approximate quantification of phenol concentrations of these effluents. The pH of these effluents was nearly neutral and the activity of the bioprobe was therefore not effected by the pH of

these effluent samples. The cooling tower effluent gave a lower phenol concentration reading than the other effluents.

The other two effluents tested were a phenolic and a cresylic effluent. The bioprobe was active in various concentrations of these effluents and the maroon and orange colours produced by the bioprobe in these effluents are shown in Figure 11.

11. Investigation of bioprobe durability

The bioprobe was stored in a desiccator for a month at room temperature. After this storage period, the activity of the polyphenol oxidase was tested in 100 ppm phenol by observing the colour change produced after three hours and comparing it with the colour change produced after three hours using a freshly prepared bioprobe.

The colour changes produced by the bioprobe (with and without glutaraldehyde) in 100 ppm phenol after a 1 month storage period are described in Table 3 below:

Table 3: Colours produced by the bioprobe after storage

Support Substrate	Colour changes observed in 100 ppm phenol
bioprobe without glut.	membrane turned light pink
bioprobe with glut.	membrane turned light brown

glut. = glutaraldehyde

In each case, the colours produced were not as intense as the colours produced by freshly immobilized polyphenol oxidase, indicating that the polyphenol oxidase was less active after the one month storage period.

In order to more accurately assess the durability of the bioprobe, it was stored under various conditions and its activity was tested at weekly intervals. These conditions are listed below:

a. The possibility of covering the bioprobe with a protective layer in order to preserve the enzyme activity was tested. The effects of gelatin, acrylamide, cytological fixative and a hair spray on the bioprobe activity in 100 ppm phenol and *p*-cresol were tested. The bioprobe was stored with a gelatin covering.

b. storage in a vacuum

c. storage under N_2 at room temperature and at 4°C

d. in air at 4°C

Of the various coverings tested, gelatin was the only covering which did not adversely affect the bioprobe activity (Figure 12). The effect of gelatin on the durability of the bioprobe is shown in Figure 13. The gelatin was able to preserve the enzyme activity but not the MBTH activity. This problem was solved by storage in a vacuum which was able to preserve both the enzyme activity and the MBTH activity.

The bioprobe can also be used to test organic solutions as polyphenol oxidase also functions in organic media.

It is an advantage of the invention that the bioprobe provides a simple, fairly rapid and convenient means of indicating the presence of phenol or phenol derivatives and an approximation of the phenol and phenol derivative concentrations in solutions such as water. Phenol or phenol derivatives at concentrations of 0.05 - 500 ppm can be detected. The bioprobe in accordance with the invention can be used to measure phenol or phenol derivative pollutants in sea water, waste water, rivers, reservoirs and water supplies, organic solutions or the like. Further, the bioprobe is relatively easy to manufacture and simple to use even by uneducated users. Polyphenol oxidase is readily obtainable and activity of the polyphenol oxidase can be standardised. The bioprobe can be manufactured relatively inexpensively. The bioprobe is disposable. The bioprobe also lends itself to being sold in a disposable kit form, e.g. portable form. No cofactors (other than atmospheric oxygen) are required for polyphenol oxidase to function. No electronic amplification is required for the bioprobe such as is the case with biosensors. The colour change can easily be seen and compared to a colour reference chart to give the user an approximate indication of the concentration of phenol or phenol derivatives in the solution being tested. Furthermore, the bioprobe is fairly durable and can be used for solutions having a fairly wide range of pH and salt concentrations. The bioprobe kit would contain a set of

instructions where details of possible dilution of very concentrated samples would be given. In the case of samples at extremes of pH, the kit would contain instructions as to the alteration of pH before using the bioprobe.

References: (These References are incorporated herein by reference thereto).

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3. Gardner, A.R., Cadman, T.W. (1990). Product deactivation in recombinant *Streptomyces*. *Biotechnol. Bioeng.* **36**: 243-251.

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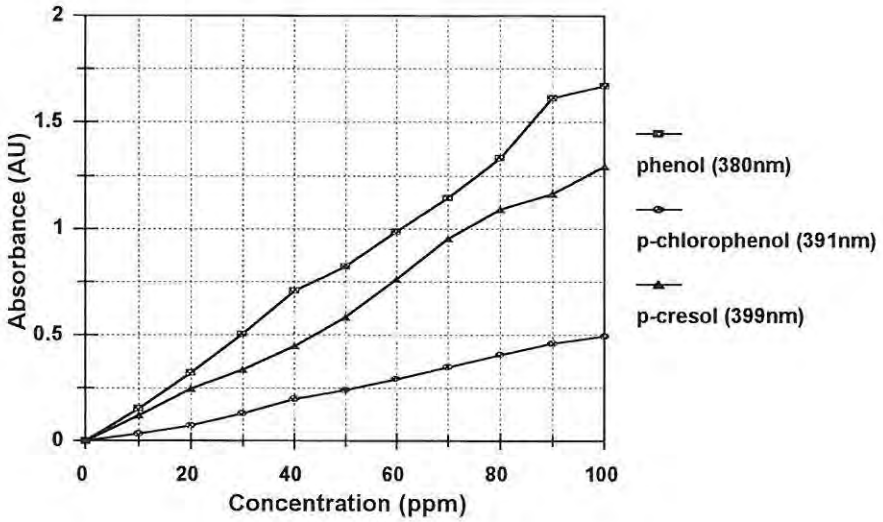

Figure 1. Absorbance versus substrate concentration for phenolic substrates

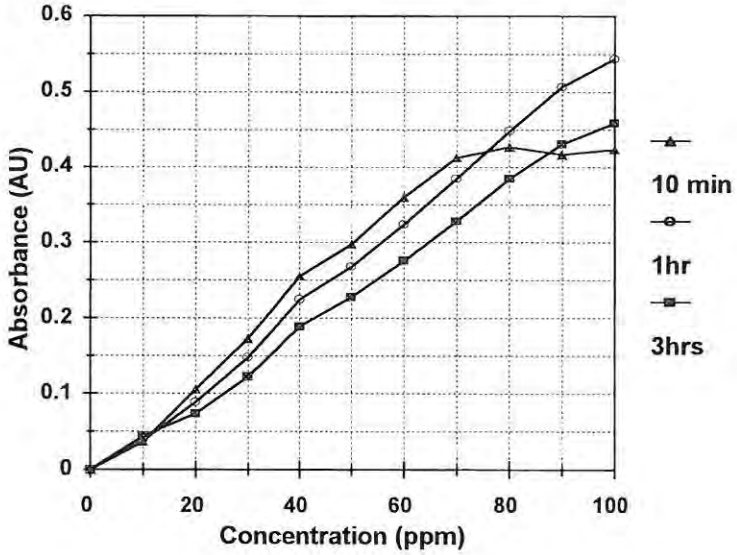

Figure 2. Colour versus *p*-cresol concentration with time

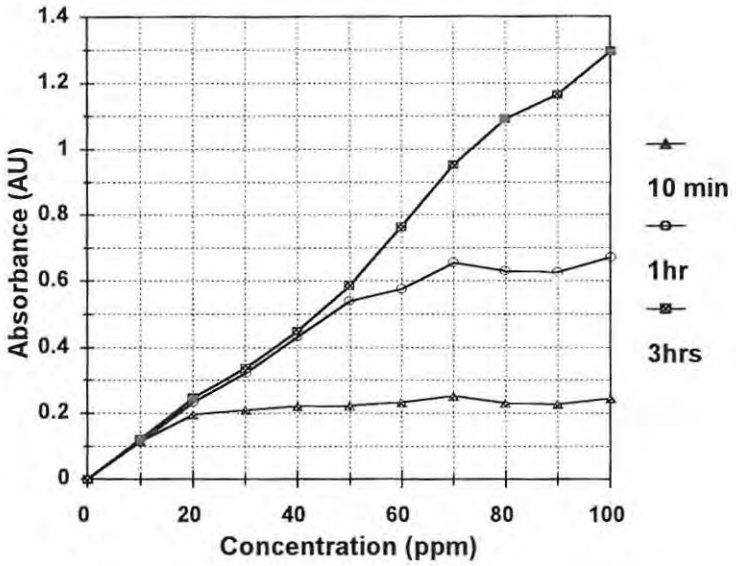

Figure 3. Colour versus *p*-chlorophenol concentration with time

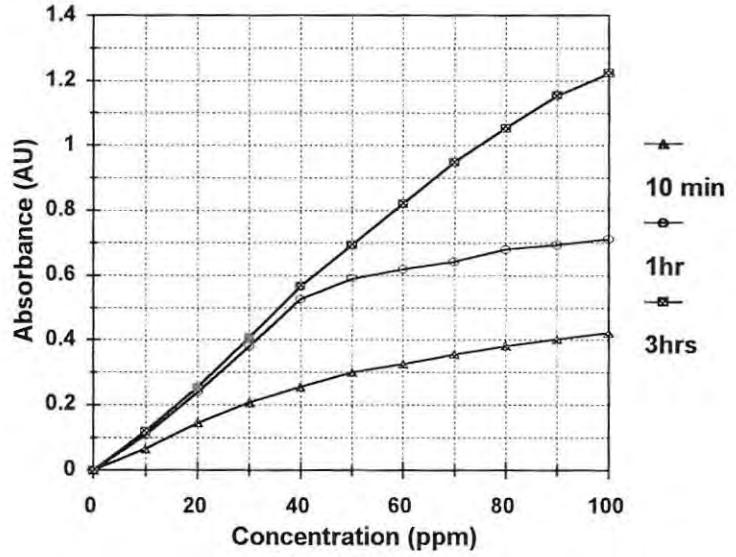

Figure 4. Colour versus phenol concentration with time

Figure 5. Colours produced by the bioprobe in phenol over 24 hours (refer to Figure 5.28)

Figure 6. Colours produced by the bioprobe in phenol over 3 hours (refer to Figure 5.34)

Figure 7. Colours produced by the bioprobe in a mixture of phenols (refer to Figure 5.1)

Figure 8. Colours produced by the bioprobe in 4-methylcatechol and in catechol (refer to Figure 5.3)

Figure 9. Bioprobe ability to detect low phenol concentrations (refer to Figure 5.5)

Figure 10. The effect of pH on the bioprobe activity in phenol (refer to Figure 5.9)

Figure 11. Bioprobe performance in effluents (refer to Figure 5.27)

Figure 12. The effect of various coverings on the bioprobe activity (refer to Figure 5.23)

Figure 13. Colours produced after storage in presence and absence of gelatin covering (refer to Figure 5.25)