TREATMENT OF PHENOLIC EFFLUENTS WITH IMMOBILISED ENZYMES

A thesis submitted to the University of Kent for the degree of Doctor of Philosophy in the faculty of Natural Sciences.

Susan Davis, Biological Laboratory, October 1989.

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or institute of learning.

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TO MY FAMILY

CONTENTS

ABBREVIATIONS

AP	coloured precipitate from acidified OH effluent
APPL	acid precipitable polymeric lignin
BOD	Biochemical Oxygen Demand
BOD_5	5-day Biochemical Oxygen Demand
CIL	carbon immobilised laccase
COD	Chemical Oxygen Demand
CPG	controlled pore glass
conc.	concentrated
CU	Colorimetric Units
D	Daltons
d	days
DHP	dehydropolymerisate, lignin model compound
2,6-DMP	2,6-Dimethoxyphenol
DTT	dithiothreitol
h	hours
HRP	horseradish peroxidase
kD	kiloDaltons
l	litres
mM	millimolar concentration or millimoles when used in rate expressions
min	minutes
MWL	milled wood lignin
NP	coloured supernatant from acidified OH effluent
PAAH	polyacrylamide hydrazide
PCB	polychlorinated biphenyl
SDS	Sodium dodecyl sulphate
TEMED	N,N,N',N'-Tetramethylethylenediamine

ABSTRACT

Phenolic effluents are produced by many industries including pulp and paper processing, coal conversion and dyeing and textiles. Such effluents pollute receiving waters due to toxicity, BOD and high colour. Because present treatment methods are expensive and often inefficient, the use of immobilised microbial cells and enzymes as alternative treatment methods were investigated. The effect of white-rot fungi and phenol oxidase enzymes on 3 industrial effluents were compared. The effluents were two from a cotton cleaning mill, hydroxide (OH) and sulphide (S); the fraction >1000 D from the E_1 stage of a kraft pulp mill. An artificial coal conversion effluent was also studied. Effluents added to batch cultures of white-rot fungi with glucose as a carbon source, were decolorised by 75-85% in 6 d (Coriolus versicolor K) and by 70-75% (Stereum hirsutum, Coriolus versicolor B). Successive additions of OH (1% v/v) to batch cultures of C.versicolor(K) at days 0, 8 and 15 were decolorised by 80-85% but at gradually reduced rates. OH diluted to 2% (v/v) and 10% was decolorised by C.versicolor(K) but 20% OH was toxic. Laccase production was induced by both high and low molecular weight phenolic compounds. The enzyme was routinely produced in 1 litre shake flasks (with a 2 cm glass bead) and was induced by the addition of 2,5-xylidine after 7 d growth of C.versicolor(K). Soluble laccase and horseradish peroxidase (HRP) removed colour from OH, E and S effluents. Colour removed by HRP was 61% (OH), 36% (E) and 51% (S), colour removed by laccase was 36% (OH), 40% (E) and 30% (S), in 2-4 d. Soluble laccase, 222 U/ml, could also precipitate 51 mg/l/h phenol from artificial coal conversion effluent at pH 6.0, although the optimum for activity was pH 4-5. However in all cases rapid and irreversible enzyme inactivation occurred. Entrapment of laccase in alginate beads improved decolorisation by factors of 2.1 (OH) and 1.5 (E), entrapment of HRP improved decolorisation by 1.1 (OH), 1.5 (E) and 0 (S). Copolymerisation of laccase or HRP with tyrosine produced insoluble polymers with enzyme activity. Entrapment of these copolymers in gel beads further increased the efficiency of decolorisation of E effluent by laccase and HRP. Decolorisation by entrapped enzymes increased with increasing bead:effluent ratios (v/v) and the maximum of 86% decolorisation of OH effluent was achieved with an HRP bead:effluent ratio of 1:2.5. However, all enzyme preparations were released from the alginate beads at such a rate that beads could not be used to decolorise more than one batch of effluent. Laccase could be immobilised on activated carbon by covalent coupling using a water soluble diimide. Up to 50 mg of laccase protein per g support was bound, but at the highest protein levels the expressed enzyme activity decreased. The maximum bound activity was obtained at 11.5 mg laccase/g support. Bound laccase (CIL) was not eluted from carbon by washing with 10 mM buffer (pH 4-9) and was stable to salt concentrations up to 1M. The pH profile was unchanged but the temperature range of activity was broadened. The activation energy of CIL (17.61 kJ/M O_2) was decreased compared with soluble laccase (22.28 kJ/M O_2) indicating the possibility of conformational changes during binding of laccase to carbon. Laccase bound to carbon retained 20% of its initial activity after oxidation of 7 batches of 2,6-dimethoxyphenol (DMP) and 55% of activity was retained after continuous oxidation of 9 l of DMP in a fluidised bed reactor. In batch incubations with E effluent, CIL removed 57% of colour at an average rate of 94 CU/h/U enzyme. In the continuous fluidised bed system the removal was higher, 115 CU/h/U enzyme, however, continuous recirculation of effluent through the column for 5-8 h was necessary to achieve high colour removal by the immobilised laccase.

CHAPTER ONE

INTRODUCTION

1.1. TYPES AND SOURCES OF PHENOLIC EFFLUENTS

1.1.1. Pulp mill effluents

The release of spent liquors from the chemical bleaching of wood pulps into receiving waters represents the most important environmental problem facing the pulp and paper industry. This is because effluents contain many chlorinated compounds, have high BOD and COD and discolour receiving waters. Compounds with a relative molecular mass less than 1000 D are of the greatest concern, since they are acutely toxic to aquatic organisms and show resistance to biological and chemical degradation (Boman et al. 1988). High molecular mass (> 1000 D) chlorinated materials in the effluents, previously thought to be recalcitrant and not an environmental problem, have been shown to be degraded slowly to toxic products. For example, Neilson et al. (1988) have shown that high molecular weight phenolic compounds can be slowly degraded by microorganisms to chlorinated catechols and guaiacols, which can then be converted to their corresponding chlorinated veratroles. The chlorinated veratroles are more persistent and more hydrophobic than the high molecular weight compounds.

In addition to acutely toxic compounds, pulp mills produce mutagenic materials which have been structurally identified (Kringstad & Lindstrom, 1984). Techniques presently used for treating wastewaters include aerated

lagoons and activated sludge plants (1.1.4), but these are less efficient during cold weather (e.g. in Canada and Sweden) and do not remove high molecular mass chlorinated lignins. Thus an effective purification process for bleach plant effluents must involve elimination of both low and high molecular mass chlorinated compounds under all prevailing climatic conditions.

Wood is the most important raw material for the production of chemical pulp. Its main components are: cellulose, hemicelluloses, lignin and extractives (organic solvent extractable compounds, e.g. saturated acids, monoterpenes and resin acids). Lignin is an aromatic polymer, formed in wood by an enzyme-initiated dehydrogenative polymerisation of a mixture of three different 4-hydroxyarylpropenyl alcohols (Fig. 1.1). The proportion of these alcohols vary with different wood species: gymnosperm lignin is composed mainly of coniferyl alcohol; angiosperm lignin is a mixed dehydrogenative polymer of conyferyl and sinapyl alcohols; and grass lignin contains coniferyl, sinapyl and p-coumaryl alcohols. Lignin, which contributes between 17% and 33% by weight of mature wood, is a branched molecule in which 50% of the phenyl propane based units are linked by β-O-4 ether links (e.g. between rings 7 and 8 in Fig. 1.1), but also by ether bonds of alkyl-alkyl and aryl-aryl configurations. The relative molecular mass of native lignin is considered infinite, and the aromatic content (as monomeric phenol) is approximately 51%. Characteristic functional groups include primary and secondary hydroxyl, methoxyl, free phenolic hydroxyl and carbonyl groups (Kringstad & Lindstrom 1984).

Most chemical pulping is carried out either by the kraft (sulphate) process or the sulphite process. The purpose is to remove lignin to

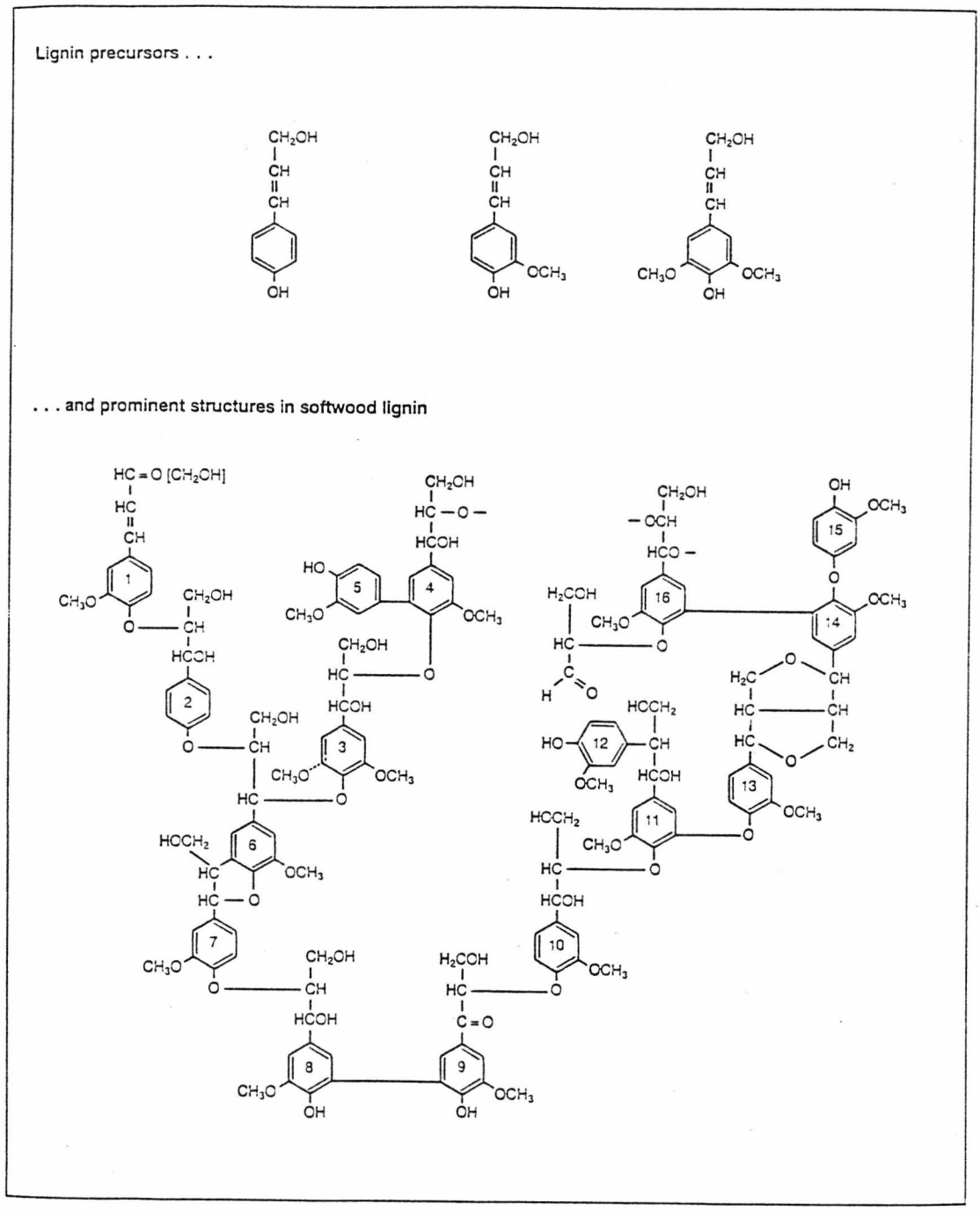


Fig. 1.1. Structural details of lignin, showing carbon-carbon bonds and characteristic functional groups (Source: Kringstad & Lindstrom 1984).

improve paper making properties of the polysaccharide fibres. The kraft process, which involves treating wood chips at 160-180°C with a solution of sodium hydroxide and sodium sulphide is the most important, providing 85% of world pulp. This treatment promotes cleavage of ether bonds in the lignin, removes 90-95% of the lignin as soluble products dissolved in the pulping liquor, and reduces the weight of the wood by more than 55%. The liquor is then evaporated to high concentration and burned to recover energy and inorganic chemicals. The sulphite process solubilises most of the lignin through sulfation at elevated temperatures, producing a liquor containing sulphur dioxide and Na, Ca or Mg oxide.

The residual lignin (5-10%) from both processes, cannot be removed by further chemical pulping without damaging the polysaccharide fraction of the wood. Instead, a multistage bleaching process is used. The sequence commonly involved is: chlorine (C_1), alkali (E_1), chlorine dioxide (D_1), alkali (E_2) and chlorine dioxide (D_2). Some processes include a hypochlorite stage (H) between the E_1 and D_1 stages. Most lignin is dissolved from the pulp during C and E_1 stages, this is known as prebleaching. The dissolved lignin is substantially modified by the bleaching process, undergoing depolymerization and the introduction of chlorine and acidic groups into its structure in the C stage (Lindgren 1979). In the E stage acidic groups (introduced during the C stage) are ionized, much organically bound chlorine is removed, and a marked decrease in carbonyl groups occurs (Larrson et al. 1975). The final composition of combined spent liquor from all the stages is extremely complex and it is these effluents which provide the industry with its most pressing pollution problems.

Problem compounds in bleaching effluents can be divided into high

(>1000 D) and low (<1000 D) molecular mass fractions. The structures of high molecular mass materials are difficult to determine and only a few attempts have been made (Bennet et al. 1971; Lindstrom & Osterberg 1984; Sarkanen & Strauss 1961). Some representative formulae in C-stage liquor are $C_9H_9O_4Cl$, $C_{10}H_{14}O_7Cl$ and $C_9H_{10}O_8Cl$ whilst in alkali extraction liquors, formulae such as $C_{14}H_{15}O_8Cl$ and $C_{14}H_{10}O_9Cl$ have been proposed. Previously, polymeric compounds were considered to be biologically inactive because they are not degraded by conventional treatment systems, however a number of reports of their degradation or modification by mixed bacterial cultures have been published (Neilson et al. 1983; 1988; Pellinen et al. 1987). Synthesis of tri- and tetrachloroveratroles was clearly demonstrated in cultures of bacterial strains incubated with high molecular weight fractions of chlorinated lignin from C and E bleaching stages (Neilson et al. 1983). These compounds were not further degraded by the same cultures and are highly lipophilic. Subsequent work (Neilson et al. 1988) prompted the hypothesis that the O-methylation of halogenated phenols, guaiacols, catechols and thiophenols, arising from the breakdown of high molecular mass material, may be a significant alternative to biodegradation in the environment. Therefore, high molecular mass materials, which may be degraded slowly to compounds which could accumulate in the environment, should not be released with effluents.

About 30% of the organically bound chlorine in C stage effluents and about 5% in E stage liquor is associated with the low molecular mass compounds. These include aliphatic acids such as formic and acetic, neutral compounds such as methanol and hemicelluloses, and phenolic compounds. Phenolics include phenols, guaiacols, vanillins, catechols,

syringols and syringaldehydes, which may have up to four chlorine substituents on the ring. The relative amounts of these compounds vary depending on the type of wood (hard or soft) and on variations in the bleaching process.

The toxicity of alkali extraction liquor to invertebrates and fish is mainly ascribed to the presence of 3,4,5-trichloroguaiacol, tetrachloroguaiacol, and mono- and dichlorodehydroabietic acid (Leach et al. 1978). Tetrachloroguaiacol is the most toxic of these and was recovered from E stage effluent at a concentration of 137 µg/l by Lindstrom & Nordin (1976). They also found high concentrations of trichlorophenol and trichloroguaiacol: 115 and 168 µg/l respectively. Concentrations in C stage effluent were much lower, with the exception of trichlorocatechol (102 µg/l). Therefore the toxicity of alkali extraction liquor is greater than chlorination liquor, although the latter is released in much larger volumes, resulting in greater total toxic loading. Toxicity of chlorination liquors is due to the chlorinated catechols, and increases with number of chlorine substituents. Rannug et al. (1981) using the Ames test, found the greatest mutagenic effect connected with spent chlorination liquor from bleaching of soft wood kraft pulp. Mutagenicity was almost entirely restricted to the low molecular mass fraction. Several compounds found in effluents, including carbon tetrachloride and chlorinated phenols and benzenes, have been classified as carcinogens on the basis of standard animal testing, but there is a need for further investigation of such compounds (Kringstad & Lindstrom 1984).

1.1.2. Coal conversion effluents

Coal conversion effluents typically contain high levels of phenol, ammonia and chloride with low levels of other organic compounds. They present different requirements for treatment compared with pulp mill effluents. Klibanov et al. (1983) gives a typical coal conversion waste water composition as phenol 2 g/l, together with ammonia 5 g/l, chloride 19 g/l, cyanide 0.1 g/l and thiocyanate 1 g/l, pH 9.0. Thicken et al. (1981) analysed waste streams from a coal gasification plant and found 99% of the mass was primary phenols and alkyl phenols. However, storage at 0-5°C led to approximately 75% decrease in dimethyl phenols after two weeks. This necessitates direct on-site treatment studies with fresh effluent or the use of an 'artificial' waste water as a model.

1.1.3. Other phenolic effluents

Department of the Environment (DOE) waste management bulletins (DOE, 1976, 1978, 1980) list categories of compounds which require special treatment before release to the domestic sewage system. One such category is halogenated organic compounds which are themselves subdivided: Type 5, hydrophobic aromatic compounds which are slightly water soluble with a low volatility and are slowly degradable. e.g. chlorinated benzenes, toluenes, xylenes and naphthalenes; and Type 6, aromatic compounds, frequently water soluble, non-volatile and resistant to degradation, possibly toxic to microorganisms. e.g. chlorinated phenols, cresols, xylenols and anilines (DOE 1978). Wood preserving wastes for example are sources of phenols, chlorinated phenols and their derivatives. They arise

from creosote as tar acids and occur in organic solvent-based preservatives such as PCB, PCB laurate and 2-phenylphenate. Such wastes are produced during manufacturing, formulating and treatment stages (DOE 1980).

1.1.4. Treatment of phenolic effluents

A major problem arising from the disinfection of potable water is the production of chlorinated phenols: these spoil the taste at very low concentrations and may have accumulative toxic effects due to their lipophilic nature. In the aquatic environment phenols have far reaching effects as they are directly toxic to fish and invertebrates, build up in food chains, and taint commercial fish flesh. According to the World Health Organisation regulations (WHO 1969) phenolic compounds in potable water must be below 0.001 mg/l as phenol due to their taste and odour producing properties. The EEC raw water directive (Water Act, 1989) sets limits of 0.001-0.1 mg/l in untreated water depending on the subsequent degree of treatment of the water and the subsequent usage, and limits of 0.5 µg/l as phenol in drinking water.

Statutory requirements set conditions for pretreatment of industrial effluents before discharge to the public sewer and subsequent conventional sewage treatment. The temperature must be less than 40°C, the pH between 6.0-10.0, BOD, COD and suspended solids are limited to that of strong sewage (i.e. 300, 400 and 200 mg/l respectively). In addition, specific limits are set for phenols, organic halogen and phosphorus compounds depending on the particular case. Phenol is readily biodegraded under aerobic conditions and up to 1.5 g/l can be

anaerobically degraded with domestic waste to CO_2 and CH_4 by methanogenic bacteria. However, other compounds in the wastes may be toxic and phenol degrading sludges require acclimation to the whole effluent before they will degrade high levels of phenol (Jones et al. 1973).

Methods vary but most effluents are pretreated on site (e.g. by activated sludge) before discharge to public sewers although this treatment may be incomplete where mixtures of phenolics are involved or because chlorination makes compounds more recalcitrant.

Pulp mill effluents are treated using a combination of physical, chemical and biological methods. Oxidative biological stabilisation is widely used at present because it is the most effective and economically viable technique for detoxifying pulp mill effluents. Results from a survey of biological treatments suggest that correctly operated treatment plants at bleached kraft mills should be capable of eliminating extractives, but there may be only limited removal capacity for the typical levels of dichlorodehydroabietic acid and trichloroguaiacol estimated to occur in bleached kraft effluents (Leach et al. 1978). Breakthrough of toxic compounds from chlorination stage effluents was also detected during suboptimal operation. e.g. during cold winters. This problem is highlighted by the work of Boman et al. (1988), who stated that in aerated lagoons reduction of chlorinated organic compounds is low, and overall efficiency of treatment decreases by approximately 25% in winter. Activated sludge plants are more effective than aerated lagoons in reducing low molecular mass chlorinated materials, but neither of these treatments remove high molecular mass chlorinated materials. Salkinoja-Salonen & Sundman (1980) reported that concentrations of chlorophenols in the Baltic Sea in winter were equivalent to releasing untreated effluent,

so little or no biodegradation had taken place in cold weather. The fate of high molecular mass materials in receiving waters has not been studied so this fraction of the effluents should not be released untreated until the full effects have been established.

Biological treatment does reduce the total organic load of bleached kraft mill effluents and reduces the content of organically bound chlorine. However, although chlorinated phenols are biodegradable, the rate of removal depends on the efficiency of the treatment process and can sometimes be slow. Chlorinated phenols and guaiacols are frequently detected in biologically treated kraft mill effluents and in receiving waters. (Kringstad & Lindstrom 1984)

Current methods for removing phenols from coal conversion and other industrial waters include solvent extraction, microbial degradation, adsorption on activated carbon, and chemical oxidation (Klein & Lee 1978; Singer et al. 1977). These methods have several shortcomings such as high cost and incomplete purification, formation of hazardous by-products and applicability to a limited concentration range. The use of biological oxidation for eliminating low levels of phenol from coal processing wastewaters has been extensively studied. The general approach is to use well evolved symbiotic mixed populations for a one step treatment (usually activated sludge) followed by a clarifying step. Holladay et al. (1978) compared different reactor designs for coal wastewater treatment but all types had disadvantages associated with their operation and were not successful in removing cyanides, nitrates and ammonia. Therefore cheaper and more efficient technologies are needed and whilst improving existing biological treatment may prove valuable, new technologies such as immobilized enzymes and cells are now being investigated.

1.2. BACTERIAL TREATMENT OF PHENOLIC EFFLUENTS

1.2.1. Bacterial metabolism of aromatic compounds

Many bacterial species are able to use monomeric or dimeric phenolic compounds as sole carbon sources, whilst others co-metabolize them. Generally, phenolcarboxylic acids are converted by mono- and dioxygenases to protocatechuic acid or gentisic acid followed by ring cleavage. Studies with pseudomonads showed that rapid aromatic ring rupture required the presence of a carboxyl group (or group easily oxidized to a carboxyl) on the aromatic ring plus a free phenolic group at the para position (Kawakami 1980). Thus the cleavage of the guaiacyl nucleus in C_1-C_6 compounds such as vanillin and vanillic acid takes place easily. However, compounds with a methoxyl group at the para position (e.g. veratric acid) are not cleaved (Cain 1980). In nearly every case aerobic degradation of the aromatic ring involves the cleavage of an ortho- or para-substituted dihydric phenol. Therefore these compounds provide the foci for peripheral pathways of metabolism from a wide range of aromatic compounds.

In the case of lignin related compounds, primary methoxyl or hydroxyl groups and phenylpropanoid chains are either intact or oxidised to a carboxyl group in the 1, 3, 4, or 5 positions. Dimeric compounds are also converted to phenylpropanoids and monoaromatics before ring cleavage. e.g. Pseudomonas putida (Fukuzumi 1980), P.ovalis (Kawakami 1980) and P.multivorans (Toms & Wood 1970) all cleaved dilignols. Three general stages are observed in the breakdown of these dilignols: 1. oxidation of C_3 side chain in phenyl propanoids; 2. oxidation of alcohol and aldehyde

groups at C_1 of ring to carboxyl; and 3. hydroxylation of monophenols to the corresponding diphenol (Fig. 1.2). Ring fission is very well documented (Cain 1980; Stanier & Ornston 1973) and takes place by either ortho cleavage (intradiol) via catechol or protocatechuate to 3-oxoadipate, acetyl CoA and succinate; or meta cleavage (extradiol) via gallate or 3-o-methylgallate to pyruvate and oxaloacetate. Ortho cleavage to 3-oxoadipate is effected by many bacteria and fungi, but has different converging pathways and different sets of enzymes for bacteria and fungi (Fig. 1.3). To date meta cleavage has only been observed in bacteria.

Certain bacteria can degrade lignin, albeit very slowly, and further degrade low molecular weight products arising from the decomposition of lignin by fungi (Crawford et al. 1983: Kawakami 1980: Odier et al. 1981). Actinomycetes have been particularly well studied and are discussed in 1.2.2. Degradation of tetrameric lignin by a non-defined mixed bacterial culture has been demonstrated by Jokela et al. (1987), who used tetrameric model compounds (instead of monomers or dimers) because they have a better structural analogy to lignin. The value of using model compounds is challenged by Pellinen et al. (1987) who reported that although a mixed bacterial culture degraded model compounds with interunit linkages typical of lignin, natural lignins with a similar molecular weight were not degraded. In fact both kraft lignin and chlorinated lignin from the alkali stage of bleaching were degraded to a lesser extent than native lignin or the model compounds. Gonzalez et al. (1986) studied the growth of bacterial isolates on monomers, a dimer and a trimer and the ether-soluble and ether-insoluble fractions of kraft lignin. They concluded that the metabolic properties of isolated strains depended on the carbon source used for initial isolation. Therefore using

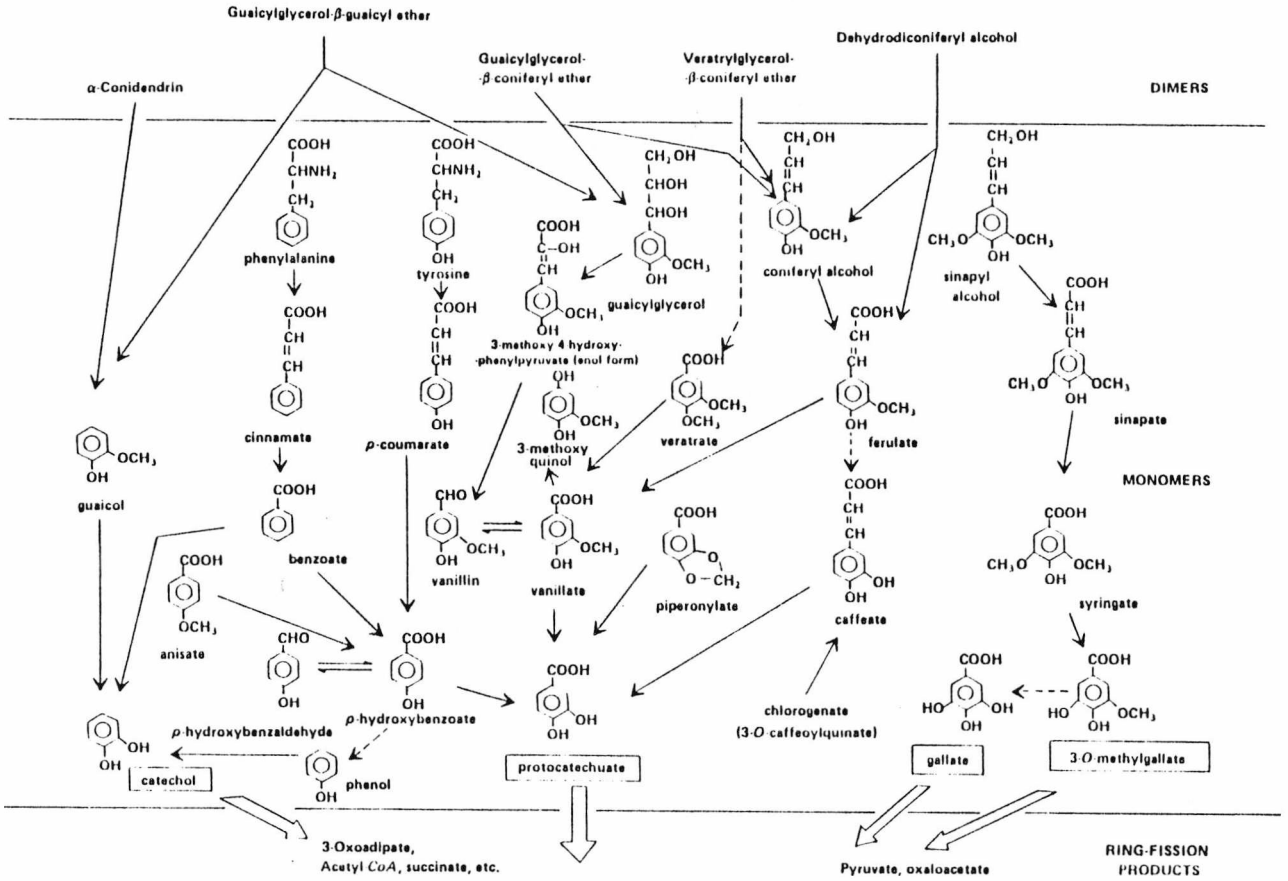


Fig. 1.2. Conversions of lignin-related aromatic compounds to diphenolic compounds as foci for ring-cleavage reactions (Source: Cain 1980).

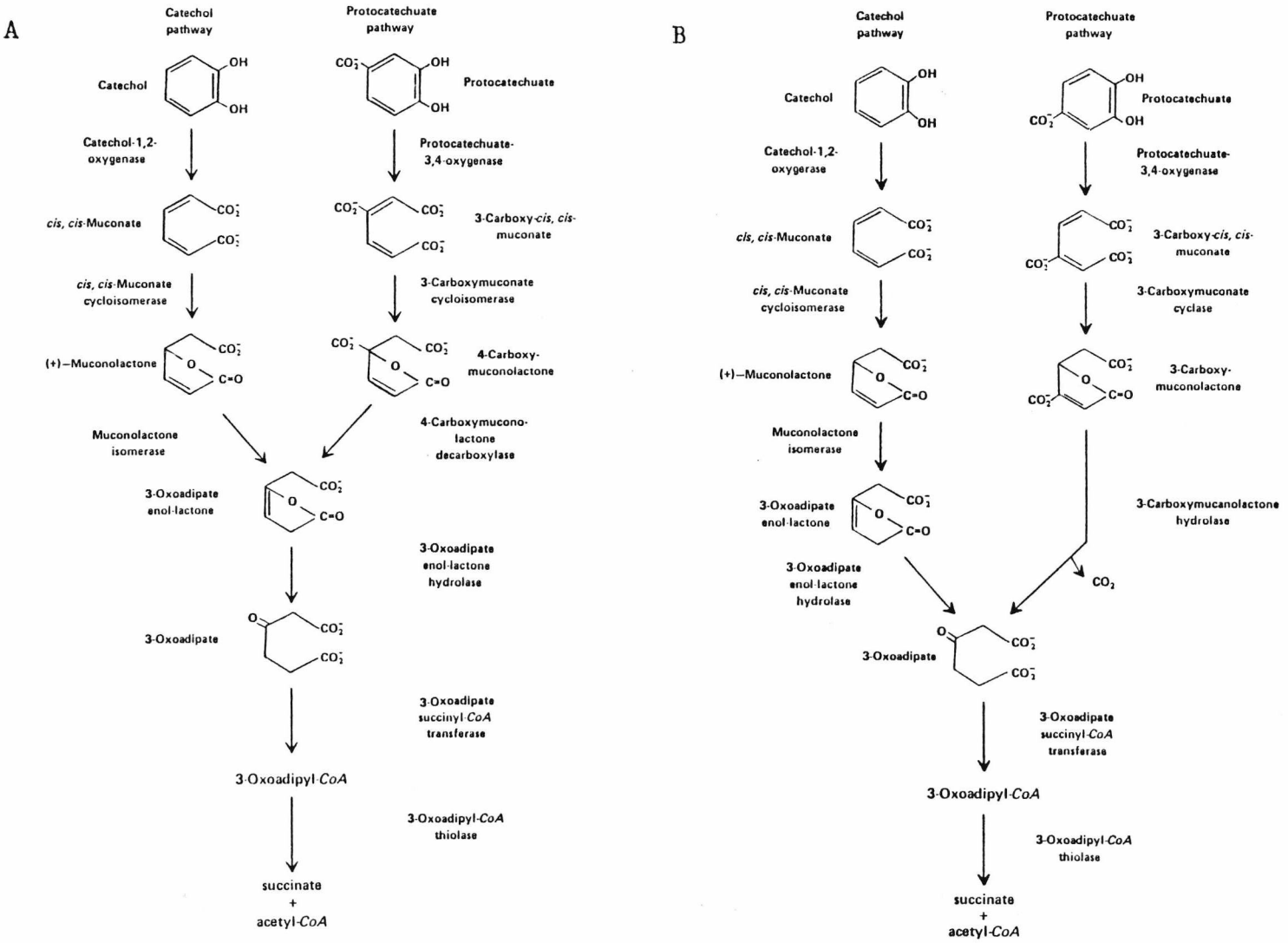


Fig. 1.3. The 3-oxoadipate pathway in (A) bacteria and (B) fungi (Source: Cain 1980).

monomers or dimers as models for lignin degradation by bacteria may not reveal the true metabolic capabilities of the population. However, Kern (1984) reported that as much as 35% of applied ^{14}C was released as $^{14}CO_2$ in 14 days from ring labelled dehydropolymerisate (DHP) by a culture of a Xanthomonas species.

In all studies, biodegradation of lignin and modified lignins takes place extremely slowly, and requires mixed bacterial cultures to substantially degrade the lignin. After 60 days stationary incubation with Pseudomonas ovalis the extent of degradation could be placed in the following order: lignin sulfonate < kraft lignin < hydrotropic lignin < oxygen-alkali lignin, (Kawakami 1980). C_6-C_1 compounds and phenylpropanoids were degraded more easily than C_6-C_2 and C_6 compounds. Lignin compounds were not degraded completely, but some changes in functional groups, e.g. demethoxylation of milled wood lignin (MWL) took place. Kuwahara (1980) studied the metabolism of lignin-related compounds by bacteria. Single C-units were easy to degrade but high molecular mass compounds were more difficult to degrade, particularly when associated with chloride.

In conclusion, bacteria may be able to degrade high molecular mass lignins but degradation is likely to be too slow to be of use in wastewater treatment. In fact Boman et al. (1987) suggest the use of ultrafiltration as the best method currently available for removing high molecular weight compounds from pulping waste liquors, and only then should the bacterial degradation of low molecular weight compounds in aerobic or anaerobic systems be used.

1.2.2. Metabolism of lignin related compounds by actinomycetes

Actinomycetes have not been investigated specifically for use in wastewater treatment although they may be present in mixed culture systems. This is surprising because actinomycetes are known to metabolise aromatic compounds and there is a large body of knowledge concerning their ligninolytic activity (Adhi et al. 1989; McCarthy & Broda 1984; Phelan et al. 1979).

Crawford & Sutherland (1980), isolated lignocellulolytic strains of actinomycetes from natural habitats rich in decomposing plant materials. Strains were of three degradative types: those degrading mostly cellulose; those degrading both lignin and cellulose equally; and those which extensively solubilized lignin but did not degrade the soluble components to carbon dioxide. Mixed isolates (mostly Streptomyces species) could also decompose [^{14}C]-labelled (MWL) and kraft lignins to CO_2. An important feature of this work is the ability of the actinomycetes to attack kraft lignin which is generally more difficult to decompose than lignocellulose. However, maximum degradation of lignocellulose was only 14% based on the labelled carbon dioxide recovered from [^{14}C]-lignin in lignocellulose. This is a low rate and of limited value where a commercial application is required, although the authors consider that it may be improved by optimal culture conditions and possibly strain improvement.

Mixed cultures of bacteria have been shown to grow on lignin as the sole carbon source and reduce the methoxyl content by 10-30%, but there are very few reports of single species able to grow on lignin. Trojanowski et al. (1977) describe a Nocardia species able to grow on lignin as the sole

carbon source and release $^{14}CO_2$ from [^{14}C]-labelled methoxyl groups, side chains, or ring carbons of conyferyl alcohol DHP. The rate of decomposition compared with that of brown-rot fungi, but was lower than that of white-rot fungi such as Coriolus versicolor. The Nocardia showed the ability to split ether linkages and should therefore have the ability to degrade phenolic groups with alkylated hydroxyl groups, which may be produced during fungal degradation of the lignin molecule. A Nocardia corallina strain isolated by Crawford et al. (1973) demethylated p-anisic acid and veratric acid leading to ring cleavage. However, ring cleavage could be blocked by chlorosubstituents in the ring. Vanillic and veratric acids are of interest because they have the ring substitution patterns, respectively, of nonetherified and etherified structural elements of conifer lignin. The modified lignin produced by Streptomyces strains is a water soluble polymer, termed acid precipitable polymeric lignin (APPL) (Adhi et al. 1989). The fastest production has been achieved in solid state fermentations over 6-8 weeks at 37°C, producing a 36% lignocellulose weight loss and 98 mg APPL per g initial lignocellulose (Pometto & Crawford 1986b). However although APPL has some commercial value, it contains some carbohydrate and is resistant to further degradation by the producing strains (Pometto & Crawford 1986a). Thus actinomycetes play an important role in the degradation of lignin and lignin related aromatics, but degradation rates would be very limiting for effluent treatment and the formation of filamentous growth forms could lead to problems such as those encountered with filamentous fungi (3.2).

1.2.3. Treatment of phenolic effluents with free and immobilized bacteria

Biological treatment of phenolic effluents with microbial communities in continuous culture has been investigated by several authors (Morsen & Rehm 1987; Wisecarver & Fan 1989; Yang & Humphrey 1975; Zache & Rehm 1989). Research has mainly involved coal conversion effluents, which may contain many different compounds, some of which are not biodegradable (Singer et al. 1977). Sudden high concentrations of phenol or other toxic substances often cause a wash out of microbial cells, thus leading to a breakdown of a continuous culture, (Hill & Robinson 1975; Wase & Hough 1966). Defining active species and routes of biological degradation within the complex microbial community is further complicated because the effluent is likely to contain a mixture of alternative carbon sources (Rozich & Colvin 1985) and a variety of physiological interactions such as diauxie, cometabolism or preference for one substrate over another may occur.

Studies with non-defined mixed cultures show that even after extensive acclimation to phenol, inhibition by alternative substrates and toxicity due to high phenol concentrations occurs. Rozich & Colvin (1985) investigated the effects of glucose on phenol degradation by mixed cultures and concluded that the removal of phenol was significantly influenced by the acclimation characteristics of the culture. During acclimation to a mixed waste, microbes rapidly removed glucose with a slow concomitant utilization of phenol, but phenol acclimated cells showed reduced phenol removal rate when subsequently challenged with phenol in the presence of glucose. In fact mixed effluents will often have several alternative carbon sources which may be used in preference

to the potential pollutant. In continuous treatment systems utilizing a biofilm composed of a mixed community, factors such as biofilm thickness, inlet phenol concentration, and oxygen concentration influence the phenol biodegradation rate. Hamoda et al. (1987) studied phenol biodegradation in a fixed film reactor, and for concentrations of 190-900 mg/l phenol, up to 99% removal was achieved, decreasing with increasing phenol concentration. Wisecarver & Fan (1989) used a gas-liquid-solid fluidised bed bioreactor with biofilm immobilised on activated carbon, which showed substrate inhibition above 800 mg/l phenol. Up to this concentration 100% phenol removal was obtained, but above this level biofilm thickness, oxygen tension and phenol concentration affected the degradation rate, which fell sharply.

One of the problems in studying undefined, mixed cultures is the difficulty in determining which biofilm components are degrading specific components of the effluent, and the factors affecting their performance. Therefore defined, mixed cultures may elucidate some of these mechanisms. Yang & Humphrey (1975) studied the microbial degradation of phenol in pure and mixed cultures. Pseudomonas putida and Trichosporon cutaneum were grown on phenol as the sole carbon source in both batch and continuous cultures. Both have inducible oxygenases, although P.putida uses the meta pathway and T.cutaneum the ortho pathway of ring cleavage (1.2.1). Both species were inhibited by substrate concentrations above 100 mg/l and the continuous system was not stable unless pH, temperature, dilution rate and inlet substrate concentrations were kept constant. Below 100 mg/l, 98-99% ofphenol was rapidly removed.

Immobilisation of cells can have advantages over free cells, because it allows a high cell density to be maintained in a bioreactor at any flow

rate. This can be important for treating waters contaminated with greater than 1-2 mg/l concentrations of xenobiotic compounds when microbial growth is slow (O'Reilly et al. 1988). Some immobilised cells can tolerate higher concentrations of toxic compounds than their free counterparts (Ehrhardt & Rehm 1985; Morsen & Rehm 1987).

Attempts to use Candida tropicalis cells entrapped in different polymeric networks for effluent treatment encountered problems of decreasing activity with increases in cells per bead. This was believed to be due to impairment of immobilised cell activity by a build up of toxic substances and substrate within the beads and to oxygen diffusional limitations (Klein et al. 1979). Activated carbon is presently used for wastewater treatment as a filter for phenolic substances, the carbon must then be regenerated by various biological, physical or chemical methods. Ehrhardt & Rehm (1985) used activated carbon to immobilise Pseudomonas and Candida cells and found that cells survived temporary high phenol concentrations of 15 g/l compared with the 1.5 g/l tolerated by free cells. In addition approximately 90% of the adsorbed phenol was degraded. They postulated that the activated carbon operated like a depot, adsorption of phenol prevented cells being in contact with high concentrations of phenol for too long and adsorbed phenol diffused out of the carbon to be used as a carbon source by the cells. Recently, degradation rates of 360 mg phenol/l/h were obtained using P.putida P8 adsorbed on activated carbon (Ehrhardt & Rehm 1989).

Bettman & Rehm (1985) also had success with Pseudomonas putida P8 entrapped within polyacrylamide hydrazide (PAAH). Under sterile conditions, 7.2 g/l/d of phenol was degraded but when non-sterile phenolic wastewater was used, only 3.12 g/l/d was reached with maximum

aeration. In both cases cells showed a consistent phenol degradative activity, but simultaneously fed cresols (100 mg/l) and 4-chlorophenol (130 mg/l) were only utilized completely when the phenol concentration was decreased to 1 g/l.

Alcaligenes sp A7-2 entrapped in calcium alginate beads was able to degrade 4-chlorophenol at increased concentrations (0.40-0.55 mM) and more rapidly than free cells in a sterile system (Westmeier & Rehm 1985). Low concentration additions (0.05 mM) of 4-chlorophenol fed with high frequency worked best, due to the toxicity to the cells of high concentrations. A defined mixed culture of P.putida P8 and Cryptococcus elinovii H1 was able to tolerate phenol concentrations up to 17 g/l but only degraded the phenol at 0.079 g/l/h (Morsen & Rehm 1987), however the same mixture entrapped in calcium alginate or chitosan alginate could degrade phenol at a rate of 0.410 g/l/h at a maximum concentration of 3.2 g/l (Zache & Rehm 1989).

In conclusion, although by using pure cultures and a single phenolic substrate in sterile conditions degradation may be efficient, industrial wastes present a far more complicated problem. Therefore, mixed cultures may be more efficient for total degradation of a range of different substances, providing toxic compound loadings are kept below inhibitory concentrations. Problems due to variation in toxic loadings, pH, temperature, etc. may all decrease the ability of a mixed population to degrade important compounds and much research is still required to understand and manipulate bacterial treatment systems. Therefore these problems have led to the investigation of immobilised cells and enzymes as more reliable and efficient treatment alternatives.

1.3. FUNGAL TREATMENT OF PHENOLIC EFFLUENTS

1.3.1. Fungal metabolism of lignin related aromatics

The mechanism of lignin degradation by fungi has been studied extensively and much of this work is appropriate to the study of the lignin derived compounds released in pulp mill effluents. Lignin degrading fungi comprise soft-rots, brown-rots and white-rots. Soft-rot fungi (Ascomycetes) such as Fusarium oxysporium, slowly attack lignin in the surface layer of wood, but are more successful in degrading the polysaccharides. Brown- and white-rot fungi (Basidiomycetes) penetrate the wood cells through openings or bore holes in the cell walls. The brown-rots such as Fomes pinicola, mainly degrade cellulose and hemicellulose but can degrade lignin. The white-rots for example Phanerochaete chrysosporium and Coriolus versicolor are a heterogeneous group characterised by the ability to degrade lignin and cellulose simultaneously, and also to produce extracellular enzymes which oxidise phenolic compounds.

Haider & Trojanowski (1980) compared the degradation of [^{14}C]-labelled cornstalk and DHP lignins by a range of microorganisms. White and brown rot fungi and soft rot fungi could degrade phenolcarboxylic and cinnamic acids as well as some phenolic compounds with completely alkylated phenolic hydroxyl groups. The fungi also introduced hydroxyl groups into benzoic and p-hydroxybenzoic acids before ring cleavage. White rots released CO_2 mostly from aromatic and side chain carbons of DHP and plant lignins, but brown rots released more CO_2 from methoxyl groups and were repressed by the addition of carbohydrate to cultures. Soft rot fungi

degraded lignin much more slowly than white-rot fungi. Bacteria (with the exception of some Nocardia species, 1.2.2), degraded labelled lignins very slowly but were more active in the metabolism of phenols. In conclusion, brown-rots cause limited aromatic hydroxylation and ring cleavage, but their major effect is demethylation of aromatic methoxyl groups within lignin to produce catechol moieties (o-diphenolic). Carbohydrates repress the oxidation of cleaved C_1 derivatives to CO_2 in brown-rot fungi so retarding the degradation of the whole lignin.

Degradative reactions of high molecular mass compounds by white-rot fungi are mainly oxidative with the formation of substantial amounts of aromatic and aliphatic carboxylic acids (Chang et al. 1980). Mechanisms include: oxidative splitting of side chains and the formation of aromatic carboxylic functions; cleavage of β-aryl ethers; oxidative modification of side chain structures; and probable oxidative ring cleavage to carboxylic acid groups. These mechanisms are all consistent with the involvement of oxygenases in the degradation of the lignin macromolecule. The likely mechanism is that the lignin is oxidatively attacked in the side chain structures to form aromatic acids and phenolic hydroxyl groups. The aromatic structures are then oxidatively cleaved and gradually 'eroded' from the lignin polymer as aliphatic fragments. The biochemistry of lignin degradation by white-rot fungi has been extensively studied and reviewed by Kirk & Farrell (1987).

Cain et al. (1968) studied the metabolism of low molecular mass aromatics by white-rot fungi. Those of the genera Polyporus, Poria, Fomes and Trametes were particularly effective at degrading lignin, producing aromatic intermediates including p-hydroxybenzoate, p-coumarate, vanillate, ferulate, 4-hydroxy-3-methoxyphenylpyruvate and the aldehydes,

vanillin, dehydrodivanillin, syringaldehyde and coniferaldehyde. Only ortho cleavage of the aromatic ring took place and Cain (1980) found that there was no evidence of meta cleavage in fungi. In fungi the aromatic ring is cleaved from catechol and protocatechuate eventually to 3-oxoadipate (Fig. 1.3). The fungal and bacterial pathways are closely related but the enzymes of the two types of pathway have slightly different substrate specificities.

The regulation of the 3-oxoadipate pathway has two features: aromatic growth substrates act as inducers for all the enzymes of the catechol and protocatechuate branches and the extent of coordinate induction (i.e. simultaneous induction of pathway enzymes) varies depending on the species. Uptake is by facilitated diffusion for p-hydroxybenzoate and protocatechuate, but intermediates of the ring fission pathway are not taken up. This is probably an ecological adaptation by the white-rot fungi which require the aromatic degrading system when high concentrations of aromatics are produced by extracellular ligninolytic activity. The ring fission intermediates are unlikely to be in high external concentration because the pathway enzymes are intracellular. White-rot fungi should therefore be capable of degrading many of the compounds present in phenolic effluents, particularly those from the paper industry which contain a variety of low molecular mass compounds derived from lignin.

It follows from this that the optimum culture conditions for lignin metabolism should also support the degradation of compounds derived from lignin in phenolic wastes, thus the regulation and physiological conditions providing optimum ligninolytic activity must be clearly understood. Most of the research in this area has involved Phanerochaete

chrysosporium, although a few experiments with Coriolus versicolor have produced similar results (Kirk 1980). Lignin metabolism is promoted in cultures grown under an atmosphere of elevated oxygen concentration and is triggered by secondary metabolism and carbon, sulphur or nitrogen limitation. A cosubstrate such as glucose or cellulose is required and the ligninolytic system has a narrow pH optimum between 4-5. Under optimal conditions, ligninolytic activity appears in cultures following the cessation of exponential growth and the onset of nitrogen starvation, and is not influenced by the presence of lignin. It is therefore apparent that if fungi are to be used for phenolic waste treatment, careful control of the culture conditions (e.g. nutrients, oxygenation, pH) will be necessary to ensure optimal degradative activity.

1.3.2. Fungal treatment of effluents

Although there has been much research in recent years on the fungal decomposition and metabolism of lignin and lignin-related aromatics (1.3.1), there has been much less emphasis on the use of fungi in cleaning up waste effluents. White rot fungi are only now finding technical use for degrading aromatic compounds in bleach plant effluents, primarily because they have exacting physiological demands in this context. For example, an immobilised cell system is required for efficient treatment of phenolic wastes because the use of free cells of filamentous fungi results in viscous cultures with consequent aeration and mixing problems.

Colour removal from pulp mill effluents can be measured as an indication of removal or degradation of high molecular mass materials. Fukuzumi

(1980) measured decolorisation of the first alkaline bleaching stage after extraction. Tinctoporia borbonica caused 99% decolorisation after four days in effluent diluted to approx 2.6 x 10^4 CU and supplemented with a carbon source. Trametes and Tyromyces species were also able to decolorise the effluent by 70-90% in the same time. However, the majority of research has concentrated on two species of white rot fungi, Phanerochaete chrysosporium and Coriolus versicolor.

The most successful method to date is known as the Forest Products Laboratory/North Carolina State University Mycelial Color Removal Process (FPL/NCSU MyCoR) which has been studied since the late 1970's and was patented in 1984 (Huynh et al. 1985). It is based on the dechlorination and decolorisation of waste effluents using P.chrysosporium (Eaton et al. 1982; Huynh et al. 1985). The mycelium of the white-rot fungus is immobilised on vertically rotating disks which are alternately aerated above the effluent and turned through the effluent. Although this removes about 60% of the colour in alkaline stage spent liquor and degrades 70% of organically bound chlorine into chloride, the hydraulic retention time needed to achieve these results is two days. Nevertheless, mycelium on the discs has been used continuously for 35 days with no apparent loss in decolorising ability. In a detailed investigation of the MyCoR process, Sundman et al. (1981) isolated the chromophoric compounds from the first alkaline stage of bleach plant effluent (1.1.1) and studied their decolorisation. They showed that conditions enabling colour reduction by mycelium were broader than those required for growth, although decolorisation was maximal over a low pH range (3.0-4.8). They also showed that the isolated chromophoric material which was decolorised by P.chrysosporium was removed by destruction of the lignin units and

phenolic hydroxyl groups to low molecular mass colourless, soluble or volatile products. Other attempts to immobilise P.chrysosporium have been directed towards continuous production of ligninase, although they may prove useful for waste treatment (1.4.4).

Various methods of immobilisation have also been applied to Coriolus versicolor, which is known to decolorise pulp mill effluents. Livernoche et al. (1983) measured decolorisation of both the combined bleached kraft mill effluent and caustic extraction (E_1) liquor with different species of white-rot fungi. C.versicolor showed the most efficient decolorisation of 15 species screened on agar medium containing bleached kraft mill effluent. In batch liquid culture C.versicolor removed over 60% of the colour of combined mill effluent in 6 days with sucrose as the additional carbon source. When the mycelium of C.versicolor was immobilised in calcium alginate gel beads, 80% decolorisation was achieved from both types of effluent in 3 days in batch liquid culture and decolorisation was also more rapid at pH 5.0 than pH 7.0 Thus immobilised mycelium was more efficient than free mycelium, and in addition, recycled beads retained their ability to decolorise effluent after several cycles.

Continuous decolorisation of kraft mill effluent has been achieved with C.versicolor using calcium alginate immobilised mycelium (Royer et al. 1983). Decolorisation was measured in four air-lift reactors connected in series and at steady state, averages over 5 days were 48%, 60%, 73% and 83% colour removed. In this system sucrose levels were not limiting therefore carbon limitation was not required to induce the decolorising enzyme system of C.versicolor.

Continuous decolorisation was further investigated using mycelial pellets instead of mycelium immobilised in calcium alginate, a process which was

not deemed commercially applicable due to the high cost (Royer et al. 1985). Colour removal could be divided into two phases, adsorption of chromophores to the mycelial surface, which was a physical process independent of additional carbon, and subsequent oxidation of chromophores which required additional carbon such as glucose or sucrose. Royer et al. (1985) also found that magnesium ions accelerated the oxidation process and a rate of decolorisation of 30 CU/g mycelium/h was attained, with pellets showing a loss of activity of 1% per day, but no mechanical damage. This is a promising area of research if the problems of immobilising fungal mycelium, reinoculation and the provision of an economic additional carbon source can be solved.

Other types of fungi have been immobilised for treatment of less complex phenolic wastes e.g. coal conversion effluents (1.1.2). Aureobasidium pullulans adsorbed to fibrous asbestos in a glass column removed 50 mg phenol/l/h (from 1.2 g/l artificial waste solution) under stable conditions, but higher phenol concentrations were totally inhibitory (Takahashi et al. 1981). Various methods of immobilising Fusarium flocciferum have shown improved degradation of phenol together with retention of viability in the presence of potentially inhibitory levels of phenol (Anselmo et al. 1985). Cells immobilised by entrapment and adsorption (in polyurethane) were able to degrade phenol up to 4 g/l and 2.5 g/l respectively compared with total inhibition of free cells at 1.3 g/l. However the rate of degradation at these level was only 1.7 mg/h compared to the maximum of 7 mg/h at 1.0 and 0.75 g/l respectively. Immobilised cells showed no loss of activity under repeated use for more than nine weeks.

Based on these and other studies it is obvious that fungi can degrade

complex phenolic wastes and are good candidates for phenolic effluent treatment. However, problems of biomass production, complicated physiological requirements, and the need for immobilisation of mycelial forms have prevented them from being widely exploited in the waste treatment industry.

1.4. ENZYMIC TREATMENT OF PHENOLIC EFFLUENTS

The search for new or alternative methods of wastewater treatment has recently concentrated on phenoloxidases for the removal of phenolics and related compounds (Atlow et al. 1983; Klibanov et al. 1983; Shuttleworth & Bollag 1986). Three enzymes in particular have been studied, peroxidase, laccase and tyrosinase.

1.4.1. Peroxidases

Peroxidases (donor: H_2O_2 oxidoreductase, EC 1.11.1.7) are a complex group of haemoproteins which oxidise phenols and aromatic amines in the presence of hydrogen peroxide, alkylperoxides or aromatic peracids. They are found in most higher plants, fungi, and bacteria. Commercial preparations of peroxidase are obtained from horseradish roots (Cochlearia armoracia) which are particularly rich in the enzyme. In woody plants, peroxidases are located in the regions of lignin formation and are thought to take part in the polymerisation of phenolic compounds. However, during attack on wood by white-rot fungi phenoloxidases are released as extracellular enzymes and may serve to detoxify the phenolic

units produced during the breakdown of lignin. Ander et al. (1980) showed that a phenoloxidase-less mutant did not degrade kraft lignin or lignin in wood but a revertant degraded lignin as well as the wild type. Therefore they concluded that peroxidase was necessary for lignin degradation.

Interest in the use of phenol oxidases for removal of phenols from waste waters is relatively recent. Klibanov & Morris (1981) proposed the use of horseradish peroxidase (HRP) for the removal of carcinogenic aromatic amines from water. This is based on the knowledge that HRP catalyses the oxidation of amines and phenols by hydrogen peroxide (to generate the corresponding aromatic amine and phenoxy radicals) which then polymerize to form insoluble polyaromatic products. These polymers can be removed by filtration or sedimentation. Using this method Klibanov & Morris (1981) removed ten carcinogenic aromatic amines from aqueous solution with efficiencies from 95.4-99.9%, using 100-1000 U/l HRP and 1-5 mM H_2O_2. This treatment had advantages over conventional peroxide only treatment in that it was effective over a broad pH range, and needed only a 2-fold molar excess of H_2O_2 over pollutant compared to 14-fold molar excess required by conventional H_2O_2 iron catalyst treatment. Removal efficiency increased with peroxidase concentration, but not pollutant concentration, and at low enzyme concentrations (1 U/l), increase in treatment time can increase the removal of amines. HRP has been chosen because it has a very high specific activity and need not be highly purified to be used in wastewater treatment.

One significant property of peroxidase treatment was that easily removed aromatic amines and phenols enhance the enzymic precipitation of aromatic compounds which are poor substrates for HRP (Alberti & Klibanov 1981).

This is important for the treatment of real wastewaters which contain a mixture of compounds, some of which may be poor substrates for the enzyme concerned. For example 2,3-dimethylphenol forms a high molecular weight polymer on HRP treatment which easily precipitates, unlike phenol which only forms a soluble polymer. However, when the two are treated together in solution, a high molecular weight mixed polymer is formed which readily precipitates. This technique was applied by Klibanov et al. (1983) to coal conversion waste waters over a wide range of pH (3-12) and at low temperatures. PCBs such as 4,4-dichlorobiphenyl and 2,4,5-trichlorobiphenyl could not be removed from aqueous solution by HRP, but when dissolved in coal conversion waters they were coprecipitated with phenol and 91% and 86% removed. A model coal conversion waste water was used to show that although at low phenol concentrations (0.1 g/l) HRP is inhibited by other components of the effluent, at high phenol concentrations (2 g/l) inhibitory complexes are displaced from the enzyme, thus 97% phenol removal was achieved with both the model effluent and a real effluent from a coking plant (Klibanov et al. 1983). Very high concentrations of pollutants can be treated as long as proportions of enzyme and H_2O_2 are varied in relation to pollutant concentrations.

In conclusion, HRP is less sensitive than bacterial treatments to variation in pH, phenol concentration, other toxic pollutants and temperature. Also the precipitate can be burned to provide energy to reduce running costs. The major disadvantage with this method is the complete inactivation of the enzyme during the reaction, due to interaction of phenoxy radicals with the active centre of the enzyme.

HRP has also been used to decolorise the caustic extraction stage of

bleach plant effluents (1.1.1), (Paice & Jurasek 1984). HRP enhanced colour removal when compared with hydrogen peroxide only, and was optimum at 40°C, conveniently the temperature of the extraction stage effluent at outflow. Until recently it was thought that peroxidases only degraded monomeric and dimeric phenols or water soluble lignosulphonates (Lobarzewski et al. 1982) however Dordick et al. (1986) reported vigorous depolymerisation of both synthetic and natural lignins by peroxidase in organic media (dioxane with 5% aqueous buffer). They postulated that the organic solvent functioned to quench free radicals formed from lignin fragments, so preventing spontaneous repolymerisation from decreasing the apparent rate of degradation.

1.4.2. Laccase

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2.) was first found in the Japanese lacquer tree Rhus vernicifera by Yoshida in 1883 and first purified from the white-rot fungus Trametes versicolor by Malmstrom in 1958. Laccases are glycoproteins and have been found in many higher fungi e.g. Agaricus bisporus (Blaich & Esser 1975), Ascomycetes e.g. Neurospora crassa (Froehner & Eriksson 1974b) and actinomycetes e.g. Streptomyces spp (Kuster 1963). Lyr (1956) investigated 182 fungal species and found extracellular laccase activity in 75. Laccase is produced by most white-rot fungi during lignin degradation and many laccases from different microbial sources have similar molecular weights, around 60 000-80 000, but are induced by different compounds depending on the species of origin. e.g. 2,5-xylidine induces high laccase production in Coriolus versicolor (Malmstrom 1958), but Neurospora laccase is

induced by protein synthesis inhibitors such as cycloheximide (Froehner & Eriksson 1974a). Laccases from plants are larger, 110 000-140 000 from the Japanese lacquer tree, 88 000 in Alternaria.

Laccase is similar in activity to HRP, but uses molecular oxygen instead of hydrogen peroxide and catalyses a one electron oxidation of phenolic substrates (Sjoblad & Bollag 1981). Laccase oxidizes a wide range of phenolic substrates, which can be divided into three groups on the basis of their effect on the enzyme activity (Fahreus & Ljunggren 1961). Monophenols (e.g. phenol, o-,m-,p-cresols, p-chlorophenol and 2,4-dichlorophenol) inactivate laccase rapidly, but are oxidised in the presence of gelatin or Tween 80. Both o- and p-substituted diphenols (e.g. guaiacol, catechol, vanillin and pyrogallol); polyphenols (e.g. gallic acid); aminophenols (e.g. o-aminophenol); and diamines (e.g. p-phenylenediamine) do not inactivate laccase. M-substituted phenols (e.g. resorcinol); and monoamines (e.g. aniline and 2,5-xylidine), do not or only partially inactivate laccase. Oxidation of these compounds is also stimulated by gelatin. Results from competitive binding with salicylic acid (Fahreus 1961) showed that all the different laccase substrates have a common binding centre on the laccase molecule, not two separate centres for catecholase and cresolase activity as was originally thought.

There has been much debate on the function of laccase in white-rot fungi. Arora & Sandhu (1985) proposed a role for laccase in lignin degradation by Daedalea flavida because the enzyme was produced constitutively but could be induced to higher levels by adding lignin preparations such as Indulin AT or Polyfon, or other phenolic compounds e.g. gallic, tannic acids. However Haars & Hutterman (1980) inhibited extracellular activity

of laccase with thioglycolic acid but still showed cleavage of high molecular weight lignosulfonate. In the absence of the copper chelating agent the polymerising activity of laccase prevented detection of lignosulfonate breakdown products. Westermark & Eriksson (1974a,b) concluded that laccase functions in the delivery of hydrogen acceptors for the oxidative breakdown of cellulose. This theory is consistent with the ideas of Green (1977), who proposed a laccase-glucose:quinone oxidoreductase cycle for the detoxification of phenol and quinone intermediates produced during lignin breakdown. This was also confirmed by Szklarz & Leonowicz (1986) who measured the depolymerising activity of laccase on the high molecular weight fractions of lignosulphonate (Peritan NA 97 000 D). The glucose oxidase activity of reducing quinones improved the efficiency of lignin depolymerisation compared with laccase alone.

The effect of laccase on different lignin preparations has been investigated by several authors. Konishi & Inoue (1971), showed that the laccase of C.versicolor decreased the molecular mass of MWL and increased its water solubility but Trojanowski et al. (1966) found that laccase purified from C.versicolor cultures and incubated with MWL caused an increase in molecular weight and insolubility, but also a small amount of degradation to low molecular weight products e.g. 2,6-dimethoxy-p-benzoquinone was also isolated. The enzyme was also suggested as responsible for demethylation of methoxy aromatic acids.

In conclusion, laccase can cause both polymerisation and depolymerisation of phenolic compounds, although in vivo polymerisation may be suppressed when lignin and carbohydrates are present. Laccase also has demethylating action which is thought to be an initial step in biodegradation.

Demethylation produces o-quinones which can be reduced to catechol compounds, providing substrates for ring cleaving dioxygenases. Side chain elimination through Cα-oxidation and aryl-Cα cleavage by laccase leaves p-quinone moieties which can again be reduced to hydroquinone type compounds and subjected to oxygenase catalysed ring cleavage. Thus laccase plays an important part in lignin degradation but cannot act alone. One major obstacle to laccase is provided by etherified groups in native lignin which constitute about 80% of functional side groups and which are not attacked by laccase, (Ishihara 1980). However, the various pulping methods alter these groups (1.1.1) making modified lignins such as kraft lignin more susceptible to attack by laccase and other phenol oxidases.

The action of laccase on high molecular mass lignosulfonates from the sulphite pulping process and on lignin model compounds has also been investigated, to find the range of substrates treatable, and to elicudate the mechanism of action of laccase. Lobarzewski (1984), preincubated lignin and lignosulfonates with mycelium of higher fungi, (e.g. Trametes versicolor) and showed changes in molecular weight. With a phenol oxidase preparation from the mycelium of T.versicolor an increase in the molecular weight of sodium lignosulphonate was observed. Forss et al. (1987) screened 24 fungal species for polymerisation of lignosulphonates in shake-flask culture, and found 16 able to substantially increase the molecular mass of such compounds. They showed that laccase was responsible for the polymerisation of lignosulphonate, and that laccase could also polymerise low molecular weight phenols (<1000 D) from paper mill debarking wastewaters.

Shuttleworth & Bollag (1986) quantified the action of Rhizoctonia

praticola laccase on various substituted phenols, and assessed the feasibility of using an immobilised laccase to precipitate these compounds through oxidative coupling. Their major finding was that there were no substantial differences between the relative activities of free enzyme and that covalently coupled to Celite (a diatomaceous silica product). Addition of BSA protected the enzyme from the inactivation effects of phenoxy radicals produced from alpha-naphthol and 2,6-dimethoxyphenol, having the same effect as gelatin.

1.4.3. Tyrosinase

The third phenol oxidase studied for industrial applications is tyrosinase, EC 1.14.18.1 (also known as monophenol monooxygenase, polyphenol oxidase, catechol oxidase, and O_2: o-diphenol oxidoreductase). Many species of fungi such as Agaricus campestris (Keilin & Mann 1938), Aspergillus nidulans (Bull & Carter 1973) and Neurospora crassa (Gutteridge & Robb 1975) produce tyrosinases, they are also found in actinomycetes such as Streptomyces scabies (Douglas & San Clemente 1956). The molecular weights of tyrosinases from different microbial sources vary from 29 100 to 130 000 and the copper content is between 1 and 4 atoms per molecule (Sjoblad & Bollag, 1981). The reactions catalysed by tyrosinase can be divided into two stages: cresolase activity, the ortho hydroxylation of tyrosine and other monophenols to form o-diphenols, and catecholase activity, the subsequent dehydrogenation of o-diphenols to o-quinones. The activities are associated with two types of active centres and the cresolase activity is more easily inactivated, also the ratios of the two activities vary in enzymes from different sources.

Coupling of the quinones formed in the reaction can lead to the formation of polymeric structures, e.g. a heterocyclic red compound is formed from tyrosine.

Tyrosinase has also been investigated for effluent treatment by Atlow *et al*. (1983) who showed that it could almost completely (98-99%) dephenolize phenolic solutions in the concentration range of 0.01-1.0 g/l. A crude preparation from mushrooms (*Agaricus bispora*) was as efficient as the commercial preparation. As with HRP treatment aromatic amines which are poor substrates e.g. aniline (39% removed alone) could be co-precipitated with phenol (97% of aniline removed).

1.4.4. Ligninase

Recent research has discovered a new enzyme which has an important role in lignin degradation. Ligninase was first reported in 1983 as an extracellular enzyme requiring H_2O_2 and catalysing some of the reactions seen in cultures of *Phanerochaete chrysosporium* (Gold *et al*. 1983; Kirk & Tien 1983). Ligninase is now known to be a heme containing glycosylated protein of 41-42 kD molecular mass (Gold *et al*. 1984; Kuwahara *et al*. 1984; Tien & Kirk 1984). The mechanism of action is essentially peroxidative requiring H_2O_2, susceptible aromatic nuclei are oxidised by one electron to produce unstable cation radicals which undergo a variety of nonenzymic reactions (Hammel *et al*. 1986) and the reactions catalysed by ligninase have been extensively reviewed by Kirk & Farrell (1987). Since ligninase catalyses similar reactions to phenol oxidases it obviously has potential for use in phenolic effluent treatment. However, ligninase is produced in very low amounts by wild type *Phanerochaete*

cultures and only recently has research into strain improvement and culture conditions produced higher yields.

Original activities of approximately 5 U/l (by veratryl alcohol oxidation Tien & Kirk 1984) have been increased to 400 U/l using a mutant strain grown on glycerol (Buswell et al. 1984). Immobilisation of spores has also been investigated to continuously produce ligninolytic enzymes (Kirkpatrick & Palmer 1987; Linko et al. 1986). Spores immobilised in agarose and agar gel beads germinated and could be used for the production of lignin peroxidase up to 245 U/l for 12 batches of fresh medium with a gradual decrease in activity after the sixth batch. Mycelial pellets were also employed in vertical and horizontal column bioreactors and produced ligninase continuously over 20 days although conditions were not optimised. Such immobilisation methods may prove useful in waste treatment. Foam-immobilisation of P.chrysosporium spores facilitated increased yields of ligninase in batch and semi-continuous cultures (Kirkpatrick & Palmer 1987). This method also enabled foam cubes with P.chrysosporium spores to be stored and regenerated in 48 h. Foam immobilisation has advantages over mycelial pellets because clumping of pellets, prevented by the foam pore structure, causes diffusional limitations and variations in performance and results, These methods still suffer from limited viability and pellets require exacting conditions for production, therefore present ligninase production levels are not sufficient for use in industrial treatment systems.

1.4.5. Immobilisation of enzymes for effluent treatment

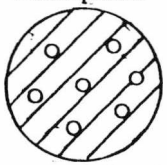
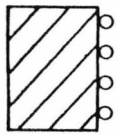
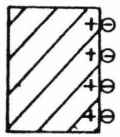
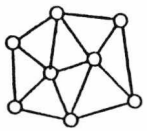
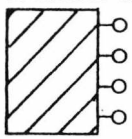
Over the past decade interest in immobilised enzymes and cells has grown and now the preparation and applications of many immobilised biocatalysts is very well documented (Kennedy et al. 1988; Klibanov 1983; Kricka & Thorpe 1986; Weetall 1985). One definition describes immobilised enzymes as catalysts that are physically confined, or localised in a microenvironment with retention of their activities and which can be used repeatedly and continuously (Kennedy et al. 1988). The use of enzymes for effluent treatment has advantages over conventional chemical processes: reactions can be catalysed in one step compared to many step chemical syntheses; more specific product formation can be achieved due to enzymes specific action; and lower temperatures and pressures can be employed in reactors resulting in savings in energy costs. However, common problems which may have contributed to the relatively low numbers of successful industrial applications of enzymes are due to a decreased stability during reaction compared to chemical catalysts and decreased temperature resistance (Weetall 1985) (Table 1.1). Thus immobilisation has been employed to try to reduce these and other problems and this has great commercial potential if stability and activity can be preserved at an economic cost.

No one method of immobilising enzymes finds universal application due to the vast differences in chemical characteristics and composition of enzymes and the properties of their substrates and products. The initial problem is to choose a support and immobilisation method which does not result in loss of activity of the enzyme (Table 1.2). Many immobilisation methods are also unsuitable for industrial application because the

Table 1.1. Commercial applications of immobilised enzymes

Enzyme	Immobilisation method	Reference
Amyloglucosidase (Hydrolysis of starch β-1-4 links releasing β-glucose units)	Precipitated with acetone and crosslinked with glutaraldehyde in the presence of bone char	Daniels & Farmer 1981
Glucose isomerase (glucose to fructose)	*Bacillus coagulans* cells, lysed, crosslinked with glutaraldehyde and granulated	Novo industries
β-galactosidase (raffinase to sucrose)	Pellet bound	Obara *et al*. 1977
β-galactosidase (lactose hydrolysed to glucose and galactose)	Entrapped in cellulose triacetate fibres	Pastore *et al*. 1974

Table 1.2. Advantages and disadvantages of different types of immobilisation methods

Immobilisation method	Advantages	Disadvantages
Entrapment 	High enzyme stability Protection against microbial attack Large variety of support shapes and sizes available	No regeneration of carrier Not stable to high ionic concentrations
Physical adsorption 	Simple coupling procedure and mild coupling conditions used Many supports available Regeneration possible	Weak binding forces, easy enzyme desorption by changes in temperature and ionic concentration
Ionic binding 	Simple procedure and mild coupling conditions used Regeneration possible High activity obtained	Low stability of immobilised enzyme Binding disrupted by changes in pH and ionic strength
Crosslinking 	Strong binding force High stability of immobilised enzyme	Can cause conformational changes leading to loss in activity No regeneration Low mechanical strength
Covalent attachment 	Strong binding force providing physical stability	No regeneration High cost Harsh coupling procedure can cause conformational changes and loss of activity

 Support o Enzyme molecule

carrier has low mechanical stability in operation, (e.g. immobilisation by crosslinking) or cannot be regenerated if enzyme activity ceases (e.g. covalent binding to organic supports). Entrapment methods also present diffusional problems when large molecular weight substrates and products are involved (Tanaka et al. 1984). As a result comparatively mild immobilisation procedures involving physical adsorption, chelation and metal binding are the most appropriate ensuring a high level of residual enzyme activity. Even these methods do not ensure retention of the catalyst on or within the carrier, but do have a good possibility of regeneration of carrier (Weetall 1985).

Microbial enzymes (rather than plant or animal) are the most economical in terms of cheapness of media, speed of growth and ease of large scale production. However, recent advances in tissue culture techniques may provide a new source of cheap enzymes. Although some immobilised enzymes have been successfully used industrially, e.g. glucose isomerase (Thompson et al. 1974) and aminoacylase (Chibata et al. 1972), the effects of immobilisation on enzyme properties are generally unpredictable.

Generally immobilisation of enzymes on charged supports causes a shift in the apparent pH optimum proportional to the charge on the support. This is due to the effect of the support on the microenvironment of the enzyme (Goldstein et al. 1964). For example, anionic supports shift pH optima towards the alkaline side, due to negatively charged groups of the matrix attracting H^+ ions, creating an enzyme microenvironment which is more acidic than the bulk solution in which pH is measured. Thermal stability of covalently bound enzymes is generally improved; this may be due to immobilisation increasing the enzyme rigidity and preventing changes in

the tertiary structure on heating (Weetall 1985). The apparent K_m of an immobilised enzyme is that obtained by measurement and represents the mean of the K_m values of all the enzyme molecules, which will vary. The apparent K_m is only applicable for a particular set of reaction conditions and can be increased or decreased in comparison with soluble enzyme due to such factors as diffusional resistances and the charge of substrate and carrier.

Interest in the use of phenol oxidases industrially has resulted in the search for a more stable preparation; various methods of immobilisation show good retention of activity with time and some increased resistance to reaction inactivation (Table 1.3). Laccase from Neurospora crassa has been immobilised on Concanavalin A Sepharose and cyanogen bromide activated Sepharose 4-B, retaining 100% and 60% of activity respectively (Froehner & Eriksson 1975) but these carriers are unsuitable for large scale operation due to low mechanical strength and susceptibility to microbial degradation. Lobarzewski et al. (1982) immobilised peroxidase from C.versicolor onto an affinity column using vanillin attached to a silanised glass carrier. The immobilised enzyme showed more stable activity than free peroxidase which was irreversibly inactivated by complex formation with lignosulfonate. Thus recent attempts to immobilise laccase have used more rigid and inert carriers such as Celite (a diatomaceous silica product, Shuttleworth & Bollag 1986) and porous glass (Leonowicz et al. 1988). On Celite 99% of activity was retained, but stability was not enhanced compared to the soluble enzyme. However, immobilisation on porous glass improved heat stability and reusability, together with high activity over a wide pH and temperature range. However, the laccase was still susceptible to inactivation by some

Table 1.3. Current methods of immobilising laccase

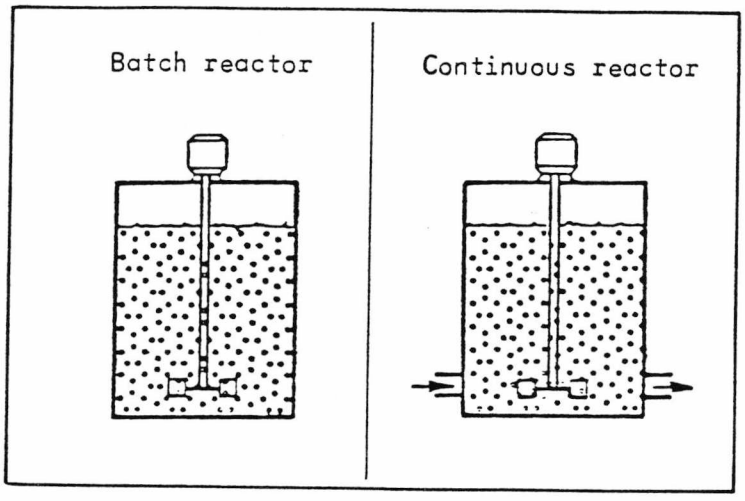
Immobilisation method	Activity bound (%)	Reference
Adsorption to Concanavalin A	100	Froehner & Eriksson 1975
Adsorption to cyanogen bromide treated Sepharose	60	"
Covalent coupling to Celite	99	Shuttleworth & Bollag 1986
Covalent coupling to Silanised clays and soils	Up to 100 depending on amount of clay	Sarkar *et al*. 1988
Covalent coupling to silanised controlled pore glass	99	Leonowicz *et al*. 1988

substrates, (e.g. ferulic acid, sinapic acid).

A number of authors have reported increased stability of enzymes in synthetic or isolated humic-enzyme complexes, (Nannipieri et al. 1982; Sarkar et al. 1980; Sarkar & Burns 1984). Biosynthesis of humic substances in the soil and metabolism of polyphenols in soil from lignin degradation or microbial synthesis are mediated by phenol oxidases (Martin & Haider 1976). These enzymes become stabilised by linkage to the humic colloids and can be extracted from soil as stable complexes (Ruggiero & Radogna 1984; Suflita & Bollag 1980). Therefore synthetic humic enzyme complexes have been evaluated for increased stability. Laccase binds to clays and soils (Sarkar et al. 1988) and retains increasing activity with increasing clay content, implying that the active site is not close to the binding site. The laccase is also protected from some inactivating factors, e.g. soluble laccase added to a soil suspension lost 100% of activity in 15 days, but immobilised laccase only lost 12% of activity. The disadvantage of synthetic analogues of humic acid-enzyme complexes is that high losses of enzyme activity occur when the copolymers are formed, but some improvements may be made by optimising the reaction methodology. Laccase, tyrosinase and peroxidase all bind strongly to bentonites and clay-humus complexes, but less strongly to kaolinite and quartz, implying that cation exchange capacity is important in the binding of phenoloxidases to clay minerals (Claus & Filip 1988). Adsorption reached a maximum in the range of the specific isoelectric points, but all activity, with one exception, was lost on adsorption to bentonite. With kaolinite all enzymes retained some activity, 20-50% for tyrosinase from Agaricus bisporus and about 15% for laccase from Polyporus versicolor.

Laccase may have greater potential than peroxidase for effluent treatment because it does not require hydrogen peroxide as an oxidant, and may therefore provide a cheaper treatment process. Furthermore peroxidase is inactivated by H_2O_2 at high concentrations (Paice & Jurasek 1984) therefore laccase will have a higher intrinsic stability in a treatment process. This fact is reflected in the relative absence of reports suggesting the use of peroxidase immobilisation for effluent treatment. Tyrosinase, which also uses molecular oxygen, has a narrower substrate range than laccase or peroxidase. This will be a disadvantage in the treatment of chemically heterogeneous wastes and may account for the little interest that tyrosinase has attracted for effluent treatment.

The reactor type used for an immobilised biocatalyst is important to provide increased mass transfer between the biocatalyst and the surrounding medium, so that external diffusional limitations play practically no role in the reaction. Internal mass transport cannot be directly influenced by the nature of the bioreactor. The simplest reactor, a stirred reactor, has disadvantages due to the small amount of biocatalyst used in a large volume (Fig. 1.4), also large shear forces are generated which may damage the biocatalyst (Hartmeier 1986). Packed bed reactors allow the maximum density of biocatalyst possible, however with supports such as activated carbon, the irregular shape of the particles may cause channel formation in the bed resulting in uneven flow. In a fluidised bed the loose suspension of the particles minimises stress and allows gases and insoluble particles to be removed from the bed.



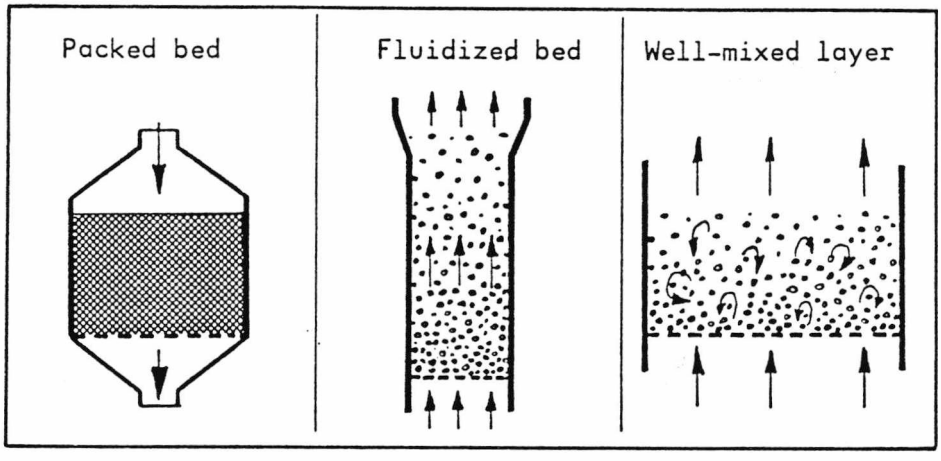


Fig. 1.4. Construction of simple stirred tank reactors (top) and types of bed reactors (bottom). ↑, direction of substrate flow; ⁘, biocatalyst particles.
(Source: Hartmeier 1986)

1.5. ANAEROBIC TREATMENT OF PHENOLIC EFFLUENTS

1.5.1. Anaerobic metabolism of aromatic compounds

The biochemistry of the anaerobic catabolism of aromatic compounds has been less well studied than aerobic breakdown and metabolism. However anaerobic treatments may be cheaper to run than aerobic equivalents because they require less mixing and the methane biproducts may offset energy costs. Therefore, in recent years the possibility of using anaerobic treatments for phenolic wastewaters has been researched.

Anaerobic catabolism of aromatic compounds related to lignin monomers has been most extensively studied. Many organisms can degrade benzoate and other monomers anaerobically there is also evidence that sulfate reducing bacteria can metabolise benzoate, phenylacetate and phenol (Widdel 1983). Work with lignin monomers (e.g. vanillin, ferulic, cinnamic acid and vanillic acids) showed these were metabolised to benzoate, a key intermediate, which is further metabolised to acetate and then cyclohexanone before ring cleavage (Fig. 1.5).

Most studies have looked at mixed cultures and methanogenic enrichments, fewer have isolated and studied mechanisms in pure cultures of anaerobes. A number of anaerobic reactions have been identified, which modify monomers by removing substituents: o-demethylation; hydrogenation of carbon-carbon double bonds in side chains; dehydroxylation; and decarboxylation. There are considered to be three modes of aromatic catabolism in the absence of oxygen (Zeikus 1980): 1. In respiration, dehydrogenation reactions of catabolism result in the hydrogenation of an electron acceptor e.g. sulphate or nitrate, producing reduced end

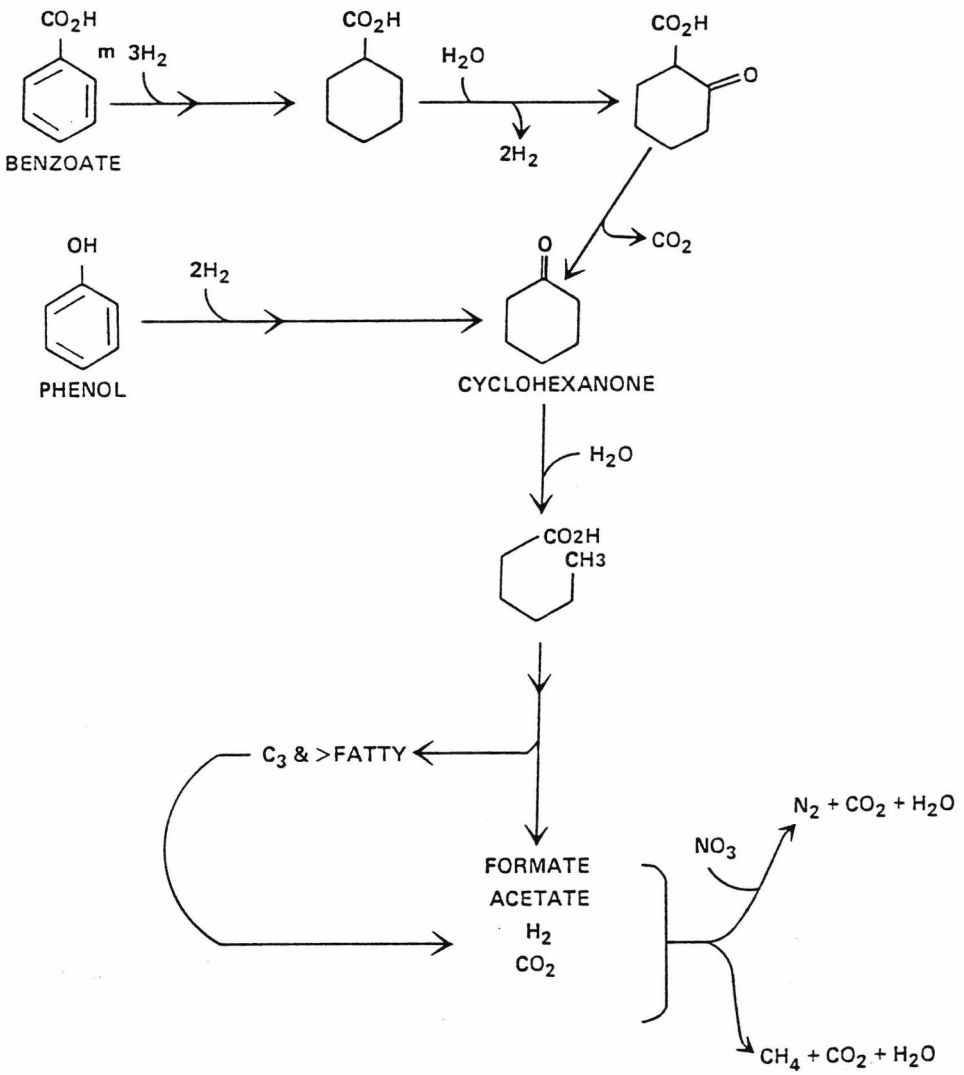


Fig. 1.5. Common reductive pathway for anaerobic catabolism of aromatic compounds by mixed cultures containing methanogenic or denitrifying bacteria (Source: Zeikus 1980).

products such as hydrogen sulphide and nitrogen. This can be performed by both pure and mixed cultures. e.g. nitrate respiration was shown by Taylor & Heeb (1972) using a pure culture of Pseudomonas PN-1 and by Williams & Evans (1975) for Moraxella species. 2. In fermentation, catabolic dehydrogenation is linked to hydrogenation of an original portion of the aromatic substrate being metabolised. Significant fermentation is only found in mixed cultures due to mechanistic and thermodynamic considerations, and the products are methane and carbon dioxide. For example, Evans (1977) reported that the benzene nucleus is first reduced and cleaved to aliphatic acids by facultative Gram-negative bacteria, then the acids are converted to substrates for final degradation by methanogens to methane and carbon dioxide. 3. Photometabolism also takes place using aromatics as a source of reducing equivalents for growth as illustrated by Rhodopseudomonas palustris (Whittle et al. 1976).

The anaerobic catabolism of lignin related dimers and oligomers is less well understood. Due to the difficulty of obtaining natural preparations of lignin oligomers (1.1.1), these have been studied using DHP's and radiolabelled natural lignins. Colberg & Young (1982), showed the release of $^{14}CH_4$ and $^{14}CO_2$ during incubations of soluble [^{14}C]-lignin preparations with mixed cultures, using lignin separated into different size fractions (MW 200, MW 600-700 and MW >1000). They found that the production of labelled gases was inversely proportional to the size of fractions, therefore degradation was limited by the molecular weight or size of the lignin fragments. They also showed that the release of lignin monomers could either be due to degradation of the double bond, or degradation of one of the rings to form a side chain, (Colberg & Young

1985). Degradation of lignin related dimers by rumen bacteria has been shown, but pathways and mechanisms have yet to be elucidated (Chen et al. 1985, 1986).

Several authors have concluded that the lignin polymer was not degraded anaerobically to any extent (Hackett et al. 1977: Odier & Monties 1983; Zeikus et al 1982). This contrasts with positive evidence from scanning electron microscopy studies of rumen bacteria which showed some delignification of woody plant material (Akin 1980). Also Benner et al. (1984), incubated [^{14}C]-lignin in wood with anaerobic sediment samples and reported 1.5-17% of the label released in CO_2 and CH_4 in 9 months. They also found increased mineralisation using kraft lignin (13-23%) which has smaller molecular weight subunits (Benner & Hodson 1985). Recently Young & Frazer (1987) extensively reviewed the literature regarding lignin derived compounds in anaerobic environments and came to four conclusions regarding the present state of knowledge. 1. Lignin monomers can be readily metabolised and completely mineralised. 2. Lignin oligomers are susceptible to anaerobic depolymerisation and metabolism. 3. The monomer substituent $-OCH_3$ can be used as a C_1 substrate for acetogens. 4. The lignin polymer can undergo a small but significant rate of degradation in several anaerobic environments. Environmental pollution due to accumulation and toxicity of aromatic compounds is therefore important in anaerobic environments, where they are more refractory (Zeikus 1980). In conclusion, the extent of anaerobic degradation of lignin and related compounds decreases with increasing molecular weight, but synthetic and chemically treated lignins are more susceptible to catabolism than native lignin.

1.5.2. Anaerobic treatment of phenolic effluents

Anaerobic treatment has not traditionally been applied to pulp mill wastewaters. However, increasing temperature and BOD_5 values of pulp mill wastewaters has begun to produce wastes better suited for anaerobic treatment. This is primarily due to increased internal recycling of used water to conserve costs and cut down the volume of effluent produced. Laboratory experiments from 1976 onwards have looked at these methods of treatment. Norrman (1982) concluded that it was feasible to treat wastewaters anaerobically but generally those free of lignin derived compounds were more suitable. This is due the recalcitrance of such compounds coupled with possible toxic effects to the anaerobic biomass. Also, BOD, COD and suspended solids were the only criteria measured to assess water quality and the presence of high molecular weight or chlorinated compounds was not taken into account. Frostell (1983), discussed five pilot scale applications of anaerobic treatment, but on waters containing aromatic compounds less COD and corresponding colour removal was achieved due to the recalcitrance of these compounds and problems were encountered with dilute wastewater streams, the minimum concentration required to maintain an effective biomass was 1.5-2.0 kg BOD_5/m^3 and this was not met by many pulp mill waters.

Anaerobic toxicity and biodegradability of sulfate evaporator condensate was studied by Benjamin et al. (1984). This liquor is produced during evaporative condensation of spent wood pulping liquors in the acid bisulfate process. It has a high soluble COD with predominant organics including the short chain acids, alcohols, furfural plus minor constituents including terpenes, aldehydes, ketones, phenols and sulphur

bearing compounds. The metabolism of toxicants added to mixed methanogenic cultures showed that three ranges of concentration could be identified for different compounds and they caused different effects: 1. no-effect; 2. inhibition characterised by a lag period and/or decreased rate of metabolism, leading ultimately to acclimation and renewed metabolic activity, sometimes including metabolism of the test compound; and 3. complete cessation of metabolic activity. They concluded that if an anaerobic reactor is likely to be exposed to occasional concentrated pulses of toxic compounds it may be advantageous to continuously add lower concentrations of some of those compounds to the reactor so the organisms become acclimated to them. However this would require a detailed analysis of individual wastes to fully identify the compounds present.

One possible drawback of anaerobic treatment has been pointed out by Neilson et al. (1987), who discovered the transformation of chloroguaiacols, chlorocatechols and chlorinated veratroles (which are common in pulp bleaching effluents), to more stable isomers. This occurred via de-o-methylation to chlorocatechols and subsequent dechlorination during cometabolism with non-chlorinated aromatic substrates e.g. methoxyaromatic acids and trihydroxybenzoates. However this took place under laboratory conditions with isolates which had been picked for dechlorinating ability, different isolation conditions could produce organisms able to degrade such compounds.

Therefore, anaerobic treatment may be easily applicable to pulp mill wastes which contain low levels of aromatic compounds, but for satisfactory purification of waters with a high phenolic content and potential toxicity further research and refinements are neccessary.

1.6. AIMS OF RESEARCH

The overall aim of the research described in this thesis is to design a biological process for the treatment of a range of phenolic effluents. These will include pulp mill effluents which are highly coloured, and contain high and low molecular weight compounds, and effluents such as coal conversion effluents in which high levels of monomeric phenols are the main problem.

White rot fungi such as Coriolus versicolor are prime candidates for evaluation of waste treatment capacity, because of their well known capacity to degrade lignin. Initial studies will concentrate on the degradation and decolorisation of effluents by identified strains of white rot fungi and by strains of fungi isolated from the environment. These studies will be directed towards a continuous treatment system and will attempt to define factors important for decolorisation such as pH, effluent concentration, carbon sources, using the fungal isolate most effective on all effluent types.

Treatment by fungi will be compared with that achievable using free and immobilised enzyme systems. In this context, phenoloxidases such as laccase and peroxidase will be studied for decolorisation and phenol removal from industrial and model effluents. The effects of pH, temperature and effluent concentration on the activity of enzymes on the effluents will be assessed. Effluent treatment will be compared by free enzymes and enzymes immobilised by different methods such as entrapment and covalent coupling. Suitable reactor designs for continuous effluent treatment by the most effective treatment will be considered.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Microbial isolates

The following fungi were screened for decolorisation of phenolic effluents: Coriolus versicolor (Strain B), Bjerkandera adusta, Bjerkandera fumosa, Chondrostereum purpureum, Coniophora putanea, Lenzites betulina, Pseudotrametes gibbosa, Stereum gausapatum, Stereum hirsutum. These isolates were provided by Dr A.D.M. Rayner, School of Biological Sciences, Bath University, Bath, UK. Coriolus versicolor (Strain K) was from UKC culture collection, originally isolated by Dr R.B. Cain.

2.2. Growth and maintenance of microorganisms

All fungi were maintained on malt extract agar slopes at 4°C and were subcultured every 6 months. White-rot fungi were also maintained at 4°C on wood chips moistened by autoclaving with malt extract broth and inoculated with plugs from agar plate cultures (Dr I. Berg, STFI, Stockholm, personal communication).

2.3. Media

Media were sterilised at 121°C for 15 min unless otherwise stated.

2.3.1. **Malt extract medium**

	g/l
Malt extract (Oxoid)	17
Mycological Peptone (Oxoid)	3
Glucose	5

Malt extract agar (MEA) was prepared by adding 15 g/l of Oxoid Agar No 3 to the above medium.

2.3.2. **Mineral medium** (Arora & Sandhu 1984)

	g/l	
KH_2PO_4	0.5	
$MgSO_4.7H_2O$	0.2	
$NaNO_3$	0.1	pH 4.8
KCl	0.1	
Fe_2SO_4	0.02	
$CaNO_3$	0.05	

2.3.3. **Basal medium for isolation** (Deschamps et al. 1980)

	g/l	
$(NH_4)_2SO_4$	2.6	
K_2HPO_4	1.0	
KH_2PO_4	0.5	pH 7.2
$MgSO_4$	0.2	
$CaCl_2$	0.01	
$FeSO_4$	0.001	
Yeast extract	0.1	

2.3.4. Carbon sources and phenolic compounds

Indulin AT and industrial effluents were sterilised with the medium, all sugars were filter sterilised separately. Phenolic substrates were added as alcoholic solutions in 95% (v/v) ethanol, after filter sterilisation.

2.4. Batch culture of white-rot fungi

2.4.1. Inoculum preparation

Malt extract medium, 100 ml in 250 ml Erlenmeyer flasks, was inoculated with four 1 cm diam plugs removed from the growing zone of fungi on MEA. Flasks were incubated at 30°C, 200 rpm with a 1 cm diam glass bead added to each flask to maintain cells as a homogeneous suspension. After 4-5 days, a dense mycelial suspension had formed, which was stored at 4°C until use.

2.4.2. Batch culture for laccase production

Laccase was purified from cultures of Coriolus versicolor (K) grown for 14 days as follows: 100 ml of mycelial suspension was added to 1 l of malt extract medium in 2 l Erlenmeyer flasks, and incubated at 30°C, 200 rpm. After 7 d, 2,5-xylidine, 0.5% in 50% ethanol, was added to give a final concentration of 0.2 mM. After a further 7 d laccase was harvested (2.8)

2.4.3. Preparation of mycelial pellets

The method of Royer et al. (1985) for the preparation of mycelial pellets was followed. One cm^3 plugs were cut from the growing zone of cultures on MEA and put into 200 ml malt extract medium in a 250 ml flask with a 1 cm

diam glass bead. Flasks were shaken at 200 rpm, 30°C for 24 h to produce a homogeneous mycelial suspension. The suspension was transferred to a 500 ml flask (without a glass bead). Every 24 h, the pellets were allowed to settle and the medium was replaced. This process was repeated until fungal pellets of the desired diameter were formed, then pellets were washed with sterile saline and stored at 4°C.

To inoculate experimental flasks, the pellets were poured onto a sterile metal screen with 4 mm diameter holes. Pellets just large enough to fit into the holes were transferred into subsequent media using sterile forceps. Therefore 4 mm diam pellets were routinely used.

2.4.4. Dry weight measurement

For the dry weight estimation of fungal biomass, Whatman no 541 filter papers, 5.5 cm diameter, were dried at 50°C then fungal cultures were filtered (Buchner filter 5.5 cm diam) and the filter re-dried to a constant weight.

2.5. Decolorisation of effluents by fungi

2.5.1. Isolation of fungi from soil

Fungi were isolated from soil samples obtained from the premises of a cotton cleaning mill. This soil had been exposed to effluent for approximately 100 years (Biomechanics Ltd. Ashford, U.K., personal communication).

Fresh soil, 0.5 g wet weight, was shaken in 20 ml of sterile distilled water, then loopfuls were streaked onto basal medium with 2% (v/v) OH

effluent or 2% (v/v) S effluent, with or without 0.05% (w/v) glucose (OHG, SG media); and also onto MEA containing 250 µg/ml streptomycin. Plates were incubated at 25°C and examined for growth, colour changes in the OHG and SG media and phenol oxidase production using the plate test (2.7.1).

2.5.2. Screening of isolates

Screening of isolates for the decolorisation of effluent was performed in stationary culture in mineral medium, 100 ml in 250 ml flasks, supplemented with 1% (w/v) glucose and industrial effluents to give a final concentration of 10^3 Colorimetric Units (CU) (2.9.2). Duplicate flasks were inoculated with eight 0.5 cm diam plugs from the growing zone on MEA, and incubated at 25°C. Samples were taken every 2-3 days and pH, laccase activity and colour density measured.

Screening in shaken culture used the same medium, inoculated with 10 ml of a mycelial suspension of isolates and triplicate flasks were incubated at 30°C, 200 rpm. Samples were taken as above.

2.5.3. Effect of pH temperature and effluent concentration on decolorisation by Coriolus versicolor(K)

Duplicate flasks containing 100 ml of mineral medium plus 0.5% (w/v) glucose and OH effluent at 2% (v/v) were shaken at 200 rpm. The pH was adjusted with 10 mM acetate buffer (pH 4.0, 4.5, 5.0) or 10 mM phosphate buffer (pH 6.0, 7.0) and flasks incubated at 25°C. To discover the optimum temperature for decolorisation medium was kept constant at pH 6.0 and incubated at 25°C, 30°C, 37°C or 45°C. Flasks were inoculated with 3 ml of mycelial suspension. The effect of effluent concentration was

examined using 2, 10 and 20% (v/v) OH effluent in 50 ml mineral medium with 1% (w/v) glucose (to promote greater growth in the more concentrated effluent), inoculated with 10 ml of mycelial suspension.

All cultures were assayed at various time intervals for colour and laccase activity, and initial and final dry weights were determined.

2.6. Induction of laccase in fungal cultures

2.6.1. Induction by lignin related compounds

Mineral medium, 50 ml in 250 ml flasks was supplemented with lignin related compounds singly or plus glucose or malt extract: Glucose 1% (w/v); vanillin, vanillic acid, ferulic acid, veratric acid, α-naphthol, gallic acid and tannic acid all at 0.1% (w/v); Indulin AT, malt extract, both at 0.2% (w/v); OH effluent 2% (w/v). Flasks were inoculated with 20 mycelial pellets of C.versicolor(K) prepared as in 2.4.3 and incubated at 25°C, 200 rpm. They were assayed every 2-3 days for laccase activity, colour (where appropriate) and any changes in the colour or morphology of the pellets recorded.

2.6.2. Culture conditions for maximum laccase production

Using 100 ml medium in 250 ml flasks shaken at 200 rpm, 30°C, four media were compared for laccase production by C.versicolor(K): Malt extract with or without a 1 cm diameter glass bead to compare homogenised and pelleted mycelial growth forms; mineral medium with 5 g/l sucrose or 5 g/l glucose. Flasks were inoculated with 2 ml of a mycelial suspension of C.versicolor(K) and assayed for protein, laccase activity and reducing sugar every 2-3 d. After 5 days incubation, 2,5-xylidine (Malmstrom,

1958) was added to a concentration of 0.2 mM.

To further compare the effects of glass beads and inducer on laccase production, 2-l flasks containing 1 l of malt extract medium were inoculated with 5 ml of mycelial suspension and incubated at 200 rpm, 30°C. Flasks with and without a 2 cm diameter glass bead and with and without inducer were compared. Two replicates of each treatment were prepared. Flasks were assayed for pH, laccase activity, protein and reducing sugar every 2-4 days. Inducer was added after 7 days incubation. Two flasks without either inducer or marbles were sampled in order to measure fungal dry weight.

2.7. Phenol oxidase enzymes

2.7.1. Qualitative detection in solid media

To detect phenol oxidase production by fungal cultures on MEA, alcoholic solutions of phenolic substrates were added dropwise to the growing edge of mycelium on MEA. Colour formation after 24 h incubation indicated positive phenol oxidase production.

2.7.2. Colorimetric enzyme assays

For each enzyme, one unit of activity is defined as the amount of enzyme that produces an increase in absorbance of 0.001/s at 25°C, activity was expressed as units per ml. All assays were performed in triplicate.

Peroxidase activity was measured by the method recommended by Sigma Chemical Co. (St. Louis, MO). Enzyme sample, 0.1 ml was added to a solution containing 42 mM pyrogallol, 7.8 mM H_2O_2 and 10 mM pH 6.0 Na_2HPO_4/NaH_2PO_4, in a final volume of 3 ml. Increase in absorbance at

420 nm was followed.

Laccase activity was assayed using a number of substrates.

(i) Guaiacol, 0.4 mM, in a 5 ml reaction volume with 1 ml of laccase solution and 10 mM acetate buffer pH 5.0 (Arora and Sandhu 1985). The increase in absorbance at 450 nm was measured.

(ii) 2,6-Dimethoxyphenol, 0.1 mM in a 2.9 ml reaction volume with 0.1 ml laccase solution and 10 mM citrate/phosphate buffer pH 4.0 (Shuttleworth & Bollag 1986), increase in absorbance at 470 nm was measured.

(iii) Syringaldazine, 0.037 mM, in a 3 ml reaction volume containing 1 ml of enzyme solution and 10 mM acetate buffer pH 5.0 (Harkin & Obst 1973). The substrate was added as a solution in ethanol which never exceeded 30% of the mixture. Increase in absorbance at 525 nm was followed.

This method was a modification of Atlow et al. (1983). One ml of enzyme sample was added to a solution containing 0.5 mM L-tyrosine in 50 mM Na_2HPO_4/NaH_2PO_4 pH 6.5 in a final volume of 5 ml. The increase in absorbance at 480 nm was measured.

2.7.3. Polarographic enzyme assays

Uptake of oxygen by laccase was measured in a Clark Type oxygen electrode with a digital readout meter (Rank Brothers, Cambridge, England), connected to a Kipp and Zonen BD 8 chart recorder. A 4 ml reaction volume was used and temperature was kept constant by a water jacket; water was pumped using an H.R. Flow Inducer (Watson-Marlow Ltd. Falmouth, Cornwall, England) from a constant temperature bath (Grant Instruments Ltd, Cambridge, England). For routine activity measurements 0.1 mM 2,6-DMP was the substrate, in 10 mM citrate/phosphate buffer pH 4.0. Measurements of activity with industrial effluents used effluents corrected to pH 5.0

with conc. HCl in a 4 ml reaction volume. Activity was expressed as μM O_2 taken up per min per ml of enzyme solution, effluent or per mg of carbon support at 30°C.

2.7.4. Decolorisation of industrial effluents by soluble enzymes

Colour removal by soluble enzymes was measured in duplicate flasks containing 50 ml effluent at 25°C, 200 rpm. Effluents were adjusted to pH 5.0 with 50 mM CH_3COONa/CH_3COOH for laccase treatment; pH 6.0 with 50 mM Na_2HPO_4/NaH_2PO_4 for HRP treatment with H_2O_2 50 mM; and pH 6.5 with 50 mM Na_2HPO_4/NaH_2PO_4 for tyrosinase treatment. Controls used enzyme denatured by boiling for 10 min. Colour and enzyme activity were measured every 24 h for 3 d.

2.8. Purification of enzymes

2.8.1. Preparation of crude extract of laccase

Laccase was routinely purified from the medium of C.versicolor(K) cultures grown as described in 4.2. and according to the modified method of Fahreus & Reinhammar (1967). The mycelial suspension was centrifuged for 20 min at 11 300 g using a Beckman J2-21 centrifuge with a JA-10 rotor. Laccase was precipitated from the supernatant, at 4°C, with 80% saturation $(NH_4)_2SO_4$ (51.6 g/100 ml). The precipitate, which rose to the top of the solution, was removed by centrifugation as above for 10 min. The pellet which formed a thick gel, was homogenised with distilled water in a Waring blender for 10 s. Altogether the precipitate from 6 2-l flasks was redissolved in 500 ml of distilled water. The redissolved precipitate was then dialysed against 10 l of distilled water at 4°C. To

remove polysaccharide which precipitated with the laccase, the solution was frozen at -20°C overnight then thawed. This changed the form of the polysaccharide from a gelatinous matrix to a sponge like matrix, which was removed by filtration through a double layer of muslin, laccase solution being squeezed out of the collected polysaccharide. The laccase solution obtained by this process was termed 'crude laccase' and was stored at -20°C until use.

2.8.2. Column chromatography of laccase

The crude laccase solution was subject to three types of purification to try to remove contaminating pigment and to increase the specific activity. Fractions from chromatography columns were collected using a Pharmacia FRAC-100 fraction collector and a Pharmacia P-1 peristaltic pump. Absorption at 280 nm was measured using an LKB Ultrospec 4050 equipped with a quartz cuvette. Laccase activity in fractions was measured using syringaldazine as substrate (2.7.2). All purifications were performed at 4°C. Fractions containing laccase were pooled and stored at -20°C until use.

Separation by gel filtration was performed using Sepharose 4B, 490 ml packed volume in a 100 cm long column, equilibrated with 10 mM CH_3COONa/CH_3COOH buffer, pH 5.0 and loaded with 10 ml of crude laccase extract. The column was eluted with the same buffer at 40 ml/h and 10 ml fractions were collected.

Separation using hydroxylapatite ion-exchange followed the method of Mosbach (1963) for laccase purification, modified as follows. Hydroxylapatite (Calbiochem Fast Flow) 20 ml in a 10 cm long column, was equilibrated with 20 mM Na_2HPO_4/NaH_2PO_4 buffer, pH 6.0. Laccase was

loaded onto the column in 10 mM CH_3COONa/CH_3COOH buffer pH 5.0. For isocratic elution the equilibration buffer was used at a flow rate of 20 ml/h, 3 ml fractions were collected.

For stepwise elution laccase was eluted with 20 ml of 5 mM Na_2HPO_4/NaH_2PO_4 buffer pH 6.0 followed by 20 ml of 20 mM buffer and then 20 ml of 40 mM buffer, at a flow rate of 38.5 ml/h and collecting 2 ml fractions.

Laccase was also eluted using a gradient of 10 mM to 100 mM Na_2HPO_4/NaH_2PO_4 buffer pH 6.0. The flow rate was 40 ml/h and 2.5 ml fractions were collected.

Separation by DEAE-Sephacel ion-exchange followed the method of Fahreus & Reinhammar (1967) for laccase purification, modified as follows. DEAE-Sephacel, 40 ml packed volume in a 20 cm long column, was equilibrated with 10 mM Tris/HCl buffer pH 7.5. Laccase solution was diluted with the same buffer and loaded onto the column at 20 ml/h, the column was then eluted with the starting buffer until the A_{280} was steady. Stepwise elution was performed with 40 ml of 50 mM Tris/HCl buffer pH 7.5, then with 40 ml of 100 mM Tris/HCl pH 7.5. The flow rate was 20 ml/h and 2 ml fractions were collected.

Gradient elution was performed using a gradient of 10-200 mM Tris/HCl pH 7.5 and 5 ml fractions were collected.

2.8.3. <u>One Dimensional Polyacrylamide Gel Electrophoresis (PAGE)</u>

The method of protein electrophoresis was developed by Laemmli (1970), and was carried out with the following alterations.

Electrophoretic gels of dimensions 17.5 cm x 15.0 cm x 1.5 mm consisted of two separate layers, the resolving gel and the stacking gel. These

gels were poured between two glass plates of dimensions 19.5 cm x 19.5 cm (rear plate) and 16.0 cm x 19.5 cm (front plate) separated by 1.5 mm plastic spacers and aligned so that the front plate was 3.5 cm short of the top of the rear plate. The resolving gel (12.5%) contained: 12.5 ml 40% acrylamide:1% bisacrylamide; 10.5 ml distilled water; 15 ml 1 M Tris/HCl pH 8.7 and 200 µl 20% (w/v) SDS. This suspension was mixed and degassed for 2 min before addition of 200 µl 10% (w/v) ammonium persulphate and 30 µl TEMED. The gel solution was poured between the glass plates to a distance of 3 cm from the top of the front plate, overlayed with a layer of distilled water and allowed to set.

The stacking gel (5%) contained; 2.5 ml 40% acrylamide:1% bisacrylamide; 14.84 ml distilled water; 2.5 ml 1 M Tris/HCl pH 6.8 and 100 µl 20% (w/v) SDS. This was again degassed for 2 min prior to the addition of 100 µl 10% (w/v) ammonium persulphate and 16 µl TEMED. The water overlay was decanted from the resolving gel and the stacking gel was poured to the top of the front plate, a plastic comb of 20 wells was inserted into the gel and this was left to set.

The complete gel was positioned in a Model V16-2 Vertical gel Electrophoresis System (BioRad Laboratories), which was then filled with running buffer (0.3% (w/v) Tris; 1.44% (w/v) glycine; 0.1% (w/v) SDS). Protein samples, 20 µg, were denatured by boiling for 10 min in 5 x loading buffer (0.5 M DTT; 0.4 M Tris/HCl pH 6.9; 10% (w/v) SDS; 50% (w/v) glycerol; 0.02% (w/v) bromophenol blue), prior to loading into wells. The gel was calibrated with molecular weight markers, prestained for molecular weights 27 000-180 000 (Sigma). Each gel was run for 18 h at 3.3 V/cm. When the bromophenol blue marker had run off the gel, electrophoresis was stopped. The gel plates were separated and the

resolving gel placed in stain (45% (v/v) methanol; 10% (v/v) acetic acid; 0.125% (w/v) Coomassie blue) for 60 min. The stain was decanted and destain was added (10% (v/v) methanol; 7% (v/v) acetic acid) and destaining continued until all the excess stain had been removed.

2.8.4. Preparation of crude extract of tyrosinase

The method of Atlow, et al. (1983) for tyrosinase extraction was followed. Common mushrooms (Agaricus bispora), 0.5 kg, were homogenised with 1 l of acetone at 4°C. The resulting pulp was filtered on a Buchner filter with Whatman filter paper no. 541. and then squeezed between sheets of absorbent paper to give dry discs of pulp. These were frozen over dry ice then broken up and dissolved in 5 ml of distilled water overnight at 4°C. A thick paste was formed which was centrifuged at 2000 g for 10 min. The supernatant was retained and stored at 4°C.

2.9. Industrial effluents

2.9.1. Sources and types of effluents

All three industrial effluents studied were very dense in colour, formed a precipitate upon acidification to pH 2.0 and had coloured material associated with molecular masses from <10 to >30 kD. Before addition to media, OH and E effluents were adjusted to pH 7.0 with 5 M HCl before use in experimental media.

Pulp mill effluent, (E), pH 7.5 (provided by Professor K.-E. Eriksson, STFI, Stockholm, Sweden) was a concentrate of soluble high molecular mass material (>1000 D) from the alkaline extraction stage in chlorine bleaching of softwood kraft pulp (Kringstad & Lindstrom 1984). The

effluent was obtained after it had been ultrafiltered using a Nitto membrane, NTR-7410, concentrated 22 times and washed twice with 12 volumes of water. 500 ml of concentrated effluent resulting from this treatment corresponded to about 10 l of effluent.

Cotton mill effluents were of two types and were provided by Biomechanics Ltd. Ashford, Kent, UK. Hydroxide type effluent (OH) was from a caustic treatment stage and was pH 14.0. GC-MS studies (Biomechanics Ltd. Ashford, U.K.) showed that in addition to high molecular mass coloured compounds, OH effluent contained 16 monomeric phenolic compounds similar to those often found in pulp mill effluents (in decreasing order of relative concentration): phenol, benzenepropanoic acid, dimethoxybenzaldehyde, trimethoxybenzaldehyde, C_4-alkyl phenol, benzyl alcohol, hydroxymethoxybenzoic acid, hydroxyacetophenone, hydroxymethoxyacetophenone, dimethoxyphenol, benzoic acid, benzeneacetic acid, methoxyphenol, hydroxybenzaldehyde, hydroxymethoxybenzaldehyde.. Sulphide type effluent (S) was high in sulphide and had a pH of 7.0. An analysis of the constituents of this effluent was not available.

2.9.2. Measurement of colour

Before colour measurement all solutions were centrifuged for 10 min at 13 400 g (Gallenkamp Micro Centaur centrifuge) to remove any suspended solids. Colour of supernatants was measured at 465 nm after correction of pH to 7.6 by 1:1 dilution 0.5 M Tris/HCl due to the variation in colour density with pH. Readings were converted to colorimetric units (CU) by the following equation: $CU = 500 \times A_1/A_2$ where A_1 is the optical density of the solution and $A_2 = 0.132$ (Paice & Jurasek 1984).

2.9.3. Ultrafiltration of effluents

Effluents were fractionated by ultrafiltration through Amicon PM membranes with cutoff at 10 kD and 30 kD. In each case, effluent was filtered through the PM 10 membrane and the filtrate kept at 4°C, then the retained solution was washed in the filter with 1.5 l of distilled water. The solution was then filtered through the PM 30 membrane, the filtrate stored as before and the retained portion washed. The fractions were designated <10, 10-30, and >30 kD.

2.9.4. Isolation of acid-precipitated fractions of effluent

To isolate the fraction of OH and S effluents precipitable by acid, conc. H_2SO_4 was added until the pH was 2.0. The mixture was centrifuged for 10 minutes at 2000 g in an MSE bench centrifuge and the supernatant and pellet separated. The pellet was washed with acid then redissolved in distilled water. The separate fractions were kept at 4°C until use.

2.9.5. Molecular weight profile of effluents

The molecular weight profile of effluents was measured using a Pharmacia FPLC system fitted with a Superose 6 column, 25 ml packed volume. Effluents were diluted with 0.2 M Glycine/NaOH pH 10.0 to give a final concentration of buffer of 0.1 M and absorption at 280 nm of 0.200, then filtered through a 0.2 µm cellulose-nitrate filter (Whatman) using a Millipore filter holder. Samples of 200 µl were injected onto the column and eluted with 0.1 M glycine/NaOH pH 10.0, using a flow rate of 0.5 ml/min. The absorption at 280 nm was recorded with a chart speed of 0.5 cm/ml.

2.9.6. Treatment of artificial coal conversion effluent

Artificial effluent was constructed according to Klibanov et al. (1983).

	g/l
Phenol	2.0
NH_4Cl	15.71
$MgCl_2$	24.60
NaSCN	2.02

The pH was corrected to 8.0 or 6.5 using 5M NaOH and the effluent stored at room temperature in a dark bottle.

Phenols were assayed by the method of Emerson (1943). A 50 µl sample was added to 0.5 ml of 0.1M glycine/NaOH pH 10 containing 1% $K_3Fe(CN)_6$, in a 3 ml cuvette. After mixing, 2 ml of 1% 4-aminoantipyrene in the same buffer was added, followed by 1 ml of buffer. The solution was mixed, left to stand for 10 minutes then the absorbance read at the following wavelengths; phenol 505 nm; guaiacol 475 nm; resorcinol 470 nm; catechol 433 nm; and pyrogallol 437 nm.

2.10. Protein assays

Three methods were compared, all using bovine serum albumin (BSA, Sigma, Fraction V) dissolved in 0.15 M NaCl, as a standard. Concentrations were determined spectrophotometrically based on ϵ_{280} 1% = 6.6. Appropriate concentrations of buffer were used as blanks where required.

Assay of protein by the binding of Coomassie Brilliant Blue G-250 was adapted from the method of Bradford (1976). To prepare the colour reagent, 100 ml of phosphoric acid 85% (w/v) was added to 100 mg of

Coomassie Blue G250 dissolved in 50 ml of 95% (v/v) ethanol and this was diluted to 1 l with distilled water. The assay consisted of adding 1 ml of a suitably diluted protein solution to 1 ml of reagent in a 2 ml plastic cuvette and mixing. Absorbance at 595 nm was read after 2 min and protein calculated from the standard curve covering 0-20 µg/ml.

Protein assay reagents were prepared for the Lowry method as given by Lowry et al. (1951), but the method was modified as follows: 0.1 ml of protein sample was added to 0.5 ml reagent C, mixed and allowed to stand for 15 min at 20°C. Then 0.05 ml of reagent D was added rapidly with immediate mixing and the solution allowed to stand for 30 min. Absorbance at 750 nm was read in 1 ml cuvettes, and protein calculated from a standard curve covering 0-500 µg/ml.

To assay protein by the Biuret method, the reagents for the assay were prepared as by Gornell et al. (1949). To 0.1 ml of protein solution, 0.7 ml of reagents was added, mixed and allowed to stand for 30 min at room temperature. Absorbance was read at 450 nm in a 1 ml cuvette and protein calculated from a standard curve covering 0-20 mg/ml.

2.11. Assay for reducing sugars

Glucose was measured according to Miller (1959), using dinitrosalicylic acid. The recommended modified reagent was prepared in distilled water as below.

	g/100 ml
Dinitrosalicylic acid	1.0
Phenol	0.2
Sodium sulphite	0.05

NaOH	1.0

Three ml aliquots of the reagent with 3 ml of glucose solution, were heated for 5 min in a boiling water bath, 0.1 ml of 40% (w/v) $KNaC_4H_4O_6.4H_2O$ was added to the hot solution which was then cooled to ambient temperature under a running tap. The absorbance at 575 nm was read and converted using a standard curve from 0-400 µg/ml of glucose.

2.12. Immobilisation of enzymes by entrapment

2.12.1. Copolymerisation of enzymes with phenolic compounds

The methods of Sarkar & Burns (1984) were adapted to copolymerise laccase or peroxidase with a number of phenolic compounds.

To copolymerise laccase with resorcinol, 250 mg of resorcinol in 100 ml of 0.1 M CH_3COONa/CH_3COOH pH 5.4, was mixed 1:1 with 1% (v/v) H_2O_2. This solution was mixed with a cooled peroxidase solution, 10 mg in 10 ml of the same buffer. At the time of quinone formation (approximately 3 min) 40 mg of commercial laccase in 40 ml of the above buffer was added dropwise for 5 h with stirring, the mixture was kept on ice and left to stir overnight. Alternatively 10 ml of crude laccase extract was added dropwise for 10 h and then stirred overnight. The copolymers formed were centrifuged at 13 400 g (Gallenkamp Micro centrifuge) and assayed for laccase activity using guaiacol as substrate (2.7.2).

To copolymerise laccase with pyrogallol, the above method was followed replacing resorcinol with 250 mg pyrogallol and adding dropwise for 10 h a total of 10 ml crude laccase extract.

For the copolymerisation of laccase or peroxidase with tyrosine, mushroom tyrosinase, 35 mg in 35 ml of 50 mM Na_2HPO_4/NaH_2PO_4 buffer pH 7.6 was

added to 0.2 g L-tyrosine dissolved in 500 ml of the same buffer and slowly stirred at 25°C. On quinone formation (appearance of a yellow colour after about 15 min), 10 ml of enzyme solution in the same buffer was added slowly. The mixture was stirred overnight at 25°C and resulting copolymers isolated as above then assayed for laccase activity using guaiacol as substrate or for peroxidase activity using pyrogallol as substrate (2.7.2)

2.12.2. Preparation of calcium alginate gel beads

Enzyme solution (laccase or peroxidase) was added to calcium alginate to give a 3% solution of alginate. This solution was pumped by a Watson-Marlow H-R Flow Inducer through a hypodermic needle so that drops fell into a solution of $CaCl_2$. When all the beads were formed, the $CaCl_2$ solution was poured off, replaced with fresh solution and the beads allowed to harden at 4°C for 1 h. They were then washed and stored covered with distilled water at 4°C until use. All beads were used within 6 h of hardening.

2.12.3. Measurement of bead size

The mean diameter of the beads was calculated by measuring the displacement volume of water caused by 100 beads, then substituting the volume of one bead, v, into the equation: $r = 3v/4\pi$ and multiplying by 2 to find the average diameter of one bead.

2.12.4. Hardening gel beads with glutaraldehyde

To harden calcium alginate gel beads by crosslinking the surface with glutaraldehyde, the method of Bashan (1986) was followed. 100 beads were

added to 20 ml of 0.1 M glutaraldehyde (aq) and gently stirred for 2.5 h at 4°C. Beads were then washed with distilled water to remove excess glutaraldehyde.

Beads treated with glutaraldehyde were either stored at 4°C or further treated with gelatin. To 100 treated beads, 100 mg of gelatin (225 bloom, Sigma) was added with an additional 20 ml of 0.2 M glutaraldehyde (aq) and the mixture was gently stirred for 1 h at 4°C then for 15 min bringing the temperature up to 30°C. Beads were washed with distilled water and stored as 2.12.2

2.12.5. Assay of enzyme activity in alginate gel beads

The enzyme assays given in 2.7.2 were used, with 5 beads per 3 ml reaction volume instead of enzyme solution. For beads with very low enzyme activity, 5 beads were shaken in 3 ml reaction mixture in 25 ml volume Erlenmeyer flasks at 25°C until absorption was greater than 0.100.

2.12.6. Decolorisation of effluents by entrapped laccase

To measure the decolorisation of effluent, 5 ml (by volume) of alginate beads containing entrapped laccase were added to 50 ml effluent, in 250 ml flasks. Effluents were adjusted to pH 5.0 with 10 mM CH_3COONa/CH_3COOH. Duplicate flasks were incubated at 25°C, 200 rpm and assayed periodically for colour and enzyme activity both in the beads and in the supernatant. In control flasks beads contained heat denatured enzyme.

The effect of temperature and pH on decolorisation was studied using hydroxide type effluent at a concentration of 4% (v/v). The pH was varied from 4.5-5.5 using 10 mM CH_3COONa/CH_3COOH and from 6.0-7.6 using 10 mM

Na_2HPO_4/NaH_2PO_4 otherwise flasks were as above. Flasks at the same pH as 2.14.6 were also incubated at 4, 25, 30 and 37°C.

The effect of effluent concentration on decolorisation was studied using duplicate flasks as above supplemented with the three types of effluent at concentrations of 2, 5 and 10% (v/v) and incubated as above.

The effect of bead:effluent volume on decolorisation by entrapped laccase was measured using duplicate flasks containing 50 ml OH effluent (5% v/v) buffered with pH 5.0, 10 mM CH_3COONa/CH_3COOH; to which was added 5, 10, 15, or 20 ml volumes of beads. The flasks were incubated as above.

2.12.7. Decolorisation of effluents by entrapped HRP

To measure decolorisation of effluents by alginate entrapped HRP, the methods given in 2.12.6 were followed but the effluents were corrected to pH 6.0 with 10 mM Na_2HPO_4/NaH_2PO_4 buffer and 50 mM H_2O_2 was added.

The effect of bead volume and H_2O_2 concentration on decolorisation was investigated using 50 ml aliquots of OH effluent (5% v/v) in 250 ml flasks, buffered with 10 mM Na_2HPO_4/NaH_2PO_4 pH 6.0; then 5, 10, 15, or 20 ml of beads were added with 40, 80, 120 or 160 mM H_2O_2 respectively. To investigate the effect of H_2O_2 concentration flasks as above were incubated with 5 ml beads and 7.2, 72.4 or 723.5 mM H_2O_2. All flasks were measured for colour, and enzyme activity was assayed in the beads and the effluent.

2.12.8. Decolorisation by entrapped enzyme-tyrosine copolymers

To measure decolorisation by entrapped enzyme-tyrosine copolymers, the procedures for entrapped laccase (2.12.6) and entrapped peroxidase (2.12.7) were followed, substituting the respective entrapped

enzyme-tyrosine copolymers for entrapped enzymes.

Decolorisation of several batches of effluent by one batch of beads was measured using beads containing HRP-tyrosine copolymers. 15 ml volumes of beads were added to 50 ml volumes of 10% (v/v) E effluent with 50 mM H_2O_2. Incubation was at 30°C, 200 rpm. At 0, 2, 3 or 4 hourly intervals beads were removed from the effluent, rinsed in distilled water and transferred to a fresh flask of the original effluent. Enzyme activity in the effluent and colour were measured approximately every hour.

2.12.9. Duration of enzyme activity in alginate beads

To compare the retention of activity by different types of beads, alginate entrapped laccase, peroxidase, laccase-tyrosine and peroxidase-tyrosine copolymers were used. With each type of bead the effects of two storage regimes were compared: 20 ml beads in 50 ml of 10 mM Na_2HPO_4/NaH_2PO_4 pH 6.0 for HRP; and 10 mM CH_3COONa/CH_3COOH pH 5.0 for laccase, flasks shaken at 200 rpm, 30°C; 20 ml beads in the minimum amount of buffer to simulate storage conditions, stored at 4°C. Enzyme activity was assayed in the beads and in the solutions daily for 20 d.

2.13. Covalent immobilisation of laccase

Covalent immobilisation of laccase was achieved by coupling to activated carbon using a variety of methods. Prior to derivatisation carbon was washed according to Cho & Bailey (1978). 20 g of carbon (Darco, 20-40 mesh) was refluxed with 1 l of 6N HCl at 80°C for 1 h, then washed with distilled water until the conductivity of the water was less than 20 µmho/cm. The carbon was dried overnight at 110°C.

The activity of laccase bound to carbon by all methods was measured polarographically as described in 2.7.3. Immobilised preparations were stored at 4°C as a wet cake.

2.13.1. Attachment of laccase to silanised carbon (Method A)

Carbon was silanised according to Cho & Bailey (1979) and then converted to the aldehyde derivative according to Leonowicz et al. (1988). Two g portions of carbon were refluxed for 24 h in 60 ml of 20% 3-aminopropyltriethoxysilane in toluene. The carbon was washed thoroughly in toluene and dried overnight at 50°C. 10 ml of 5% glutaraldehyde in 0.1 M Na_2HPO_4/NaH_2PO_4 pH 7.0 was added to the carbon and the suspension degassed for 1 h under vacuum. The carbon was washed 5 times with 20 ml distilled water and twice with 20 ml of buffer, then added to laccase in 30 ml of the same buffer, mixed gently for 24 h at room temperature, washed 5 times with buffer and twice with distilled water and assayed for laccase activity (2.7.3). Protein in the washings was measured by the Bradford assay (2.10.1) and the protein bound to the carbon calculated by subtraction.

2.13.2. Attachment of laccase to amino carbon (Method B)

Amino carbon was prepared according to Cho & Bailey (1979). Two g portions of carbon were mixed for 4 h at 50°C in 60 ml of mixed acid comprising equal volumes of 98% H_2SO_4 and 90% HNO_3. The carbon was rinsed, boiled in 80 ml 5% sodium dithionate for 1 h, rinsed in 100 ml acetone 50% (v/v) at 60°C, and dried at 40°C. The carbon was then treated with glutaraldehyde and coupled with laccase as in 2.13.1.

2.13.3. Attachment of laccase using a water soluble diimide

The method of Cho & Bailey (1978) for the derivatisation of carbon with diimide was followed. For each g (dry weight) of carbon 150 mg diimide (1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene sulphonate) in 5 ml, 10 mM CH_3COONa/CH_3COOH pH 5.5 was added and mixed gently for 15 min, the carbon particles were then washed 3 times with 30 ml buffer. Both amino carbon (Method C) and acid washed carbon (Method D) were derivatised in this way. Laccase was added to the wet cake in 35 ml buffer to 1 g of carbon and mixed gently for 24 h at room temperature. The carbon was rinsed 3 times with 30 ml 0.1 M CH_3COONa/CH_3COOH pH 5.5 and twice with 30 ml distilled water. Activity and washings were assayed as in 2.13.1.

2.13.4. Assessment of stability of laccase immobilised by diimide method

Laccase immobilised by diimide method D was chosen for further study, and a number of characteristics assessed. Laccase activity during storage at 4°C and -20°C as a moist cake, and after air drying was measured.

The effect of temperature and pH on activity of bound laccase was measured as follows. Laccase activity was assayed as in 2.7.3. using 10 mg portions of carbon. Citrate/phosphate buffer, 0.1 M was from pH 2.0-7.0 in steps of 0.5. The relative activity at different temperatures was measured as above with pH 4.0 citrate/phosphate buffer, 0.1 M. The temperature of the oxygen electrode vessel was varied from 10-60°C in steps of 10°C. A comparison was made using soluble laccase diluted to give the same activity reading at 30°C as that of the bound laccase so that reaction times would be equal.

The stability of immobilised laccase to fluctuations in pH and ionic

concentration was measured in two stages. The effect of pH was determined using citrate/phosphate buffer 0.01 M, at pH 4.0, 5.0, 6.0 and 7.0 and Tris/HCl 0.01 M at pH 8.0 and 9.0. Two ml of buffer was added to 0.5 g (wet weight) of carbon in 50 ml flasks and incubated at 30°C, 200 rpm for 30 min. The buffers were decanted, filtered through 0.2 μm filters (Whatman) to remove carbon fines, and assayed for protein (section 10.1). The effect of increasing ionic concentration was examined using 0.01 M pH 7.0 citrate/phosphate buffer containing 0.01, 0.05, 0.1, 0.5, 1.0 and 5.0 M NaCl and following the above method.

The effect of oxidation of batches of 2,6-DMP on immobilised laccase activity was measured by oxygen uptake (2.7.3) using 20 mg samples of carbon immobilised laccase (CIL). The initial oxygen uptake was measured, then the reaction continued until all the substrate was oxidised (i.e. no more oxygen taken up). The carbon sample was then washed 3 times in distilled water and the oxygen uptake measured using a fresh 4 ml aliquot of substrate. This process was repeated for up to 7 batches of substrate and replicated 4 times.

2.13.5. Batch decolorisation of E effluent by immobilised laccase

To measure decolorisation by CIL in a batch system, E effluent at $1x10^3$, $5x10^3$, $1x10^4$ and $5x10^4$ CU in 20 mM CH_3COONa/CH_3COOH pH 5.0 was added in 10 ml aliquots to 0.1 g (dry weight) samples of CIL and incubated at 30°C, 200 rpm for 3 d. Initial and final colour was measured (2.9.2) after filtration (2.13.5.2) and initial and final enzyme activities were also assayed.

2.13.6. Comparison of column systems for immobilised laccase

To compare column reactor systems for continuous treatment of effluent by CIL, a Pharmacia column, 2.5 cm diameter was used throughout. The column was connected to a Watson-Marlow H.R. Flow Inducer and the outflow to an LKB 4050 spectrophotometer using silicone tubing and a 1 ml glass cuvette. The substrate used was 2,6-DMP 0.1 mM in 10 mM CH_3COONa/CH_3COOH pH 5.0 at room temperature. The column was operated in two modes, firstly as a packed bed or secondly as a fluidized bed by reversing the flow of substrate through a 6 g (dry weight) sample of carbon. The degree of oxidation of the substrate flowing from the column was measured as absorption at 470 nm.

The optimum column flow rate with the above column operating as a fluidised bed was determined as follows, using DMP as above with 1 g (dry weight equivalent) of carbon. The flow rate was varied and the absorption of the oxidised substrate allowed to reach a steady state for each flow rate used. The absorption was recorded on a Kipp and Zonen BD 8 chart recorder connected to the spectrophotometer.

2.13.7. Continuous treatment of effluents in fluidised bed reactor

To measure the decolorisation of effluent by CIL in a fluidised bed reactor, effluents were diluted to required concentration with 20 mM CH_3COONa/CH_3COOH pH 5.0 and pumped through the column at a flow rate of 30 ml/min. The ratio of effluent:carbon was 100 ml:1 g (dry weight) and the colour of the effluent was measured after filtration (2.9.2). In this case the effluent was continuously recycled through the column and the colour of the effluent in the reservoir was measured with time.

2.14. Materials

Reagents were from Fisons or Aldrich Chemical Co., laccase (EC 1.10.3.2) from Pyricularia oryzae, peroxidase (EC 1.11.1.7.) Type II from horseradish and tyrosinase (EC 1.14.18.1) from mushroom, were all from Sigma.

2.15. Statistical analysis

The Students t-test was used to see if there was a significant difference between the means of two small samples.

Equation;

$$t = \frac{X_1 - X_2}{s\sqrt{\frac{1}{N_1} + \frac{1}{N_2}}}$$

The value of t was entered into tables using $N_1 + N_2 - 2$ degrees of freedom to test whether means were significant at the probability of 0.05.

In the figures, experimental data represents the mean of three replicates, and the bar indicates the standard error of the mean.

Equation;

$$S.E. = \frac{\sum X^2 - (\sum X/N)^2}{N(N-1)}$$

Where only one bar is shown beside the graph, it represents the mean S.E. for all the points, used when the S.E. of all points were within 5% of each other.

CHAPTER THREE

TREATMENT OF EFFLUENTS WITH WHITE ROT FUNGI AND OPTIMISATION OF EXTRACELLULAR LACCASE PRODUCTION

INTRODUCTION

Treatment of effluents with fungi has mainly been investigated using Phanerochaete chrysosporium and Coriolus versicolor. The use of P.chrysosporium has been constrained by the exacting physiological requirements for induction of the lignin degrading system, which is involved in effluent treatment. Induction requires amongst other factors, carbon, nitrogen or sulphur limitation, coupled with a high dissolved oxygen level and a narrow pH optimum. Surprisingly, culture agitation supresses lignin degradation unless mutant strains are used or detergent is added to wild type cultures (Kirk & Farrell 1987), this suppression has not been explained. However, other fungal species (e.g. Tinctoporia borbonica, Rhizoctonia praticola) will grow well in agitated cultures (Bollag & Leonowicz 1984). Coriolus versicolor has been extensively studied (Livernoche et al. 1983; Royer et al. 1983; Royer et al. 1985) and decolorised effluents in less stringent conditions as well as in agitated cultures: an obvious advantage when the provision of high oxygen levels is important.

Laccase is produced constitutively by many white rot fungi (1.4.2), which can then be induced to produce higher enzyme levels by aromatic compounds (e.g. 2,5-xylidine, Bollag & Leonowicz 1984; Malmstrom 1958) and protein synthesis inhibitors (e.g. cycloheximide, Arora & Sandhu 1985). Fungal laccases, regardless of whether they are constitutive, and

independent of species have been shown to be similar in substrate range (Bollag & Leonowicz 1984). Guaiacol, syringaldazine and 2,6-dimethoxyphenol (DMP) frequently used by others, were also used in this research as substrates in laccase assays because, in the absence of hydrogen peroxide, they are specific for the enzyme (Harkin & Obst, 1973) as well as showing different advantages depending upon the method used for assay (see Chapter Four).

The aims of the work described here were: (i) to measure decolorisation of three industrial effluents, using batch cultures of white-rot fungi and fungi isolated from soil contaminated with cotton mill effluent; (ii) to determine factors affecting the rate and total amount of decolorisation including temperature, pH and carbon source; and (iii) to study the regulation of fungal laccase production by aromatic compounds. Conditions inducing high laccase production by C.versicolor(K) in batch culture were also investigated in an attempt to provide a convenient and inexpensive supply of laccase.

RESULTS

3.1. Isolation of fungi from contaminated soil

Fungi were isolated in large numbers from soil exposed to cotton mill effluent for approximately 100 years (Biomechanics Ltd, Ashford, U.K., personal communication) using both malt extract agar (MEA) plus streptomycin (MEAS) and effluent agar (OHG) (Table 3.1). Total numbers of fungi were significantly higher on OHG plates. Visually different colonies were transferred from both media to MEAS and tested for phenol oxidase production using the plate test (2.7.1). From a total of 19 isolates, four isolated on OHG produced a positive reaction on MEAS (Table 3.2), but none produced clearing zones on OHG agar. Phenol

Table 3.1. Enumeration of fungi from soil contaminated with industrial effluent. Counts obtained after 3 days incubation at 25°C on either malt extract agar (MEAS) or effluent agar (OHG)

Dilution	MEAS No/g dry soil	OHG No/g dry soil
10^{-3}	$3.4 \pm 0.5 \times 10^5$	TNTC
10^{-4}	$9.7 \pm 2.9 \times 10^5$	$5.5 \pm 0.6 \times 10^6$
10^{-5}	ND	$1.2 \pm 0.2 \times 10^7$

TNTC = to numerous to count (i.e. > 300 colonies), ND = not determined.

Table 3.2. Oxidation of phenolics by selected fungal isolates after 5 days growth on MEAS. Oxidation detected by colour formation

Isolate	Syringaldazine	Guaiacol	2,6-Dimethoxyphenol
E37	-	-	+
E38	-	+	++
MF5B	-	+	-
F1	-	+	+
Stereum hirsutum	-	++	++
Coriolus versicolor(B)	-	++	++
Coriolus versicolor(K)	-	++	++
Chondrostereum purpureum	-	++	++
Lenzites betulina	-	++	++
Pseudotrametes gibbosa	+	+	+
Bjerkandera fumosa	+	+	+
Bjerkandera adusta	-	-	-
Coniophora putanea	-	-	-
Stereum gausapatum	+	++	++

E37, E38, MF5B and F1 were isolated from soil contaminated with industrial effluent, all others were from culture collections (2.1).
-, no colour formation; +, weak colour formation; ++, strong colour formation.

oxidase positive isolates all produced white mycelial growth on MEA, did not sporulate and were provisionally identified as Basidiomycetes. Plates 1 and 2 show examples of the phenol oxidase plate test and the clearing zones produced by fungi on OHG.

3.2. Decolorisation of industrial effluents by fungal isolates

All phenol oxidase positive isolates were screened for the rate of decolorisation of pulp mill E stage effluent (E) (Fig. 3.1). Decolorisation by Lenzites betulina, Stereum hirsutum, Coriolus versicolor (K) and (B), Bjerkandera adusta and Pseudotrametes gibbosa was linear. The maximum decolorisation of 57.5% was effected by Coriolus versicolor(B) although C.versicolor(K), Stereum hirsutum, Lenzites betulina, Pseudotrametes gibbosa and Bjerkandera adusta produced similar results. [The unexpected low colour values obtained on day 11 cannot be explained by reference to the literature and are attributed to technical errors.] In contrast, the fungal species isolated from soil did not achieve more than 20% decolorisation. This was attributed in part to adsorption of colour to the mycelium, which turned brown compared with that of the above isolates which remained cream in colour.

From this initial screen, 5 isolates were chosen for further study and the decolorisation of OH and S effluents in stationary culture was measured (Fig. 3.2). There was a 3 d lag before decolorisation of S began except for P.gibbosa which did not reduce colour until day 10. In all cases there was an increase in colour of OH effluent in the first 3 days of incubation (except for P.gibbosa cultures in which the colour increased for 5 d). The 3 effluents were unbuffered and the pH steadily decreased in all 3 experiments such that by day 17 pH had declined from 5.5 (E), 7.0 (S) and 6.0 (OH), to pH 4-5. However, despite differences in the initial pH, rates of decolorisation of each effluent were not

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Plate 1. Typical colour reactions produced by adding alcoholic solutions of phenolic substrates to fungal cultures on MEA. Phenolic substrates, clockwise from bottom left of each petri dish: guaiacol; syringaldazine; 2,6-dimethoxyphenol. Fungal isolates left to right, from top row: Lenzites betulina; Bjerkandera fumosa; E38; Stereum hirsutum; Pseudotrametes gibbosa; Stereum gausapatum; Coriolus versicolor(K); F1 and E37.

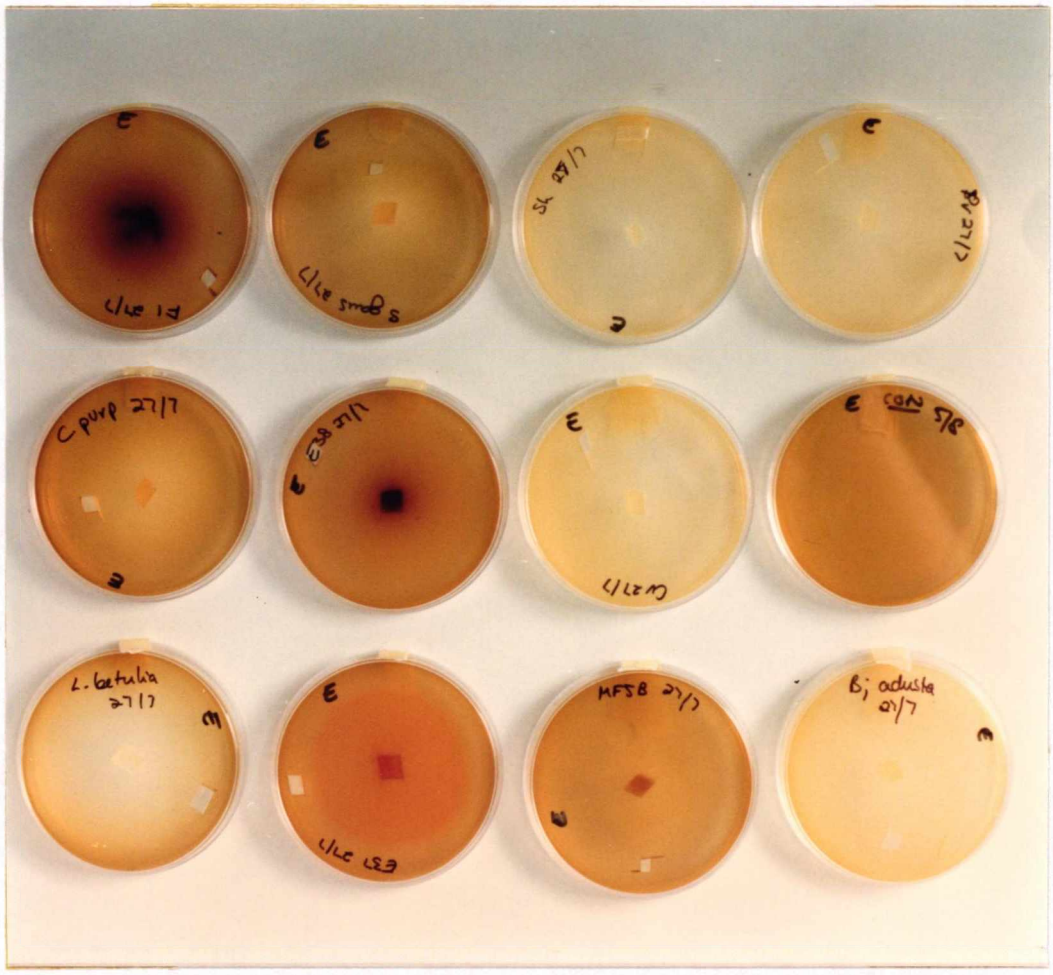

Plate 2. Clearing zones in effluent agar (5% v/v OH effluent in mineral medium plus 0.5% glucose) due to 5 days growth of fungal isolates at 25°C. Isolates left to right, from top row: F1; Stereum gausapatum; Stereum hirsutum; Coriolus versicolor(K); Chondrostereum purpureum; E38; Coriolus versicolor(B); uninoculated control; Lenzites betulina; E37; MF5B and Bjerkandera adusta.

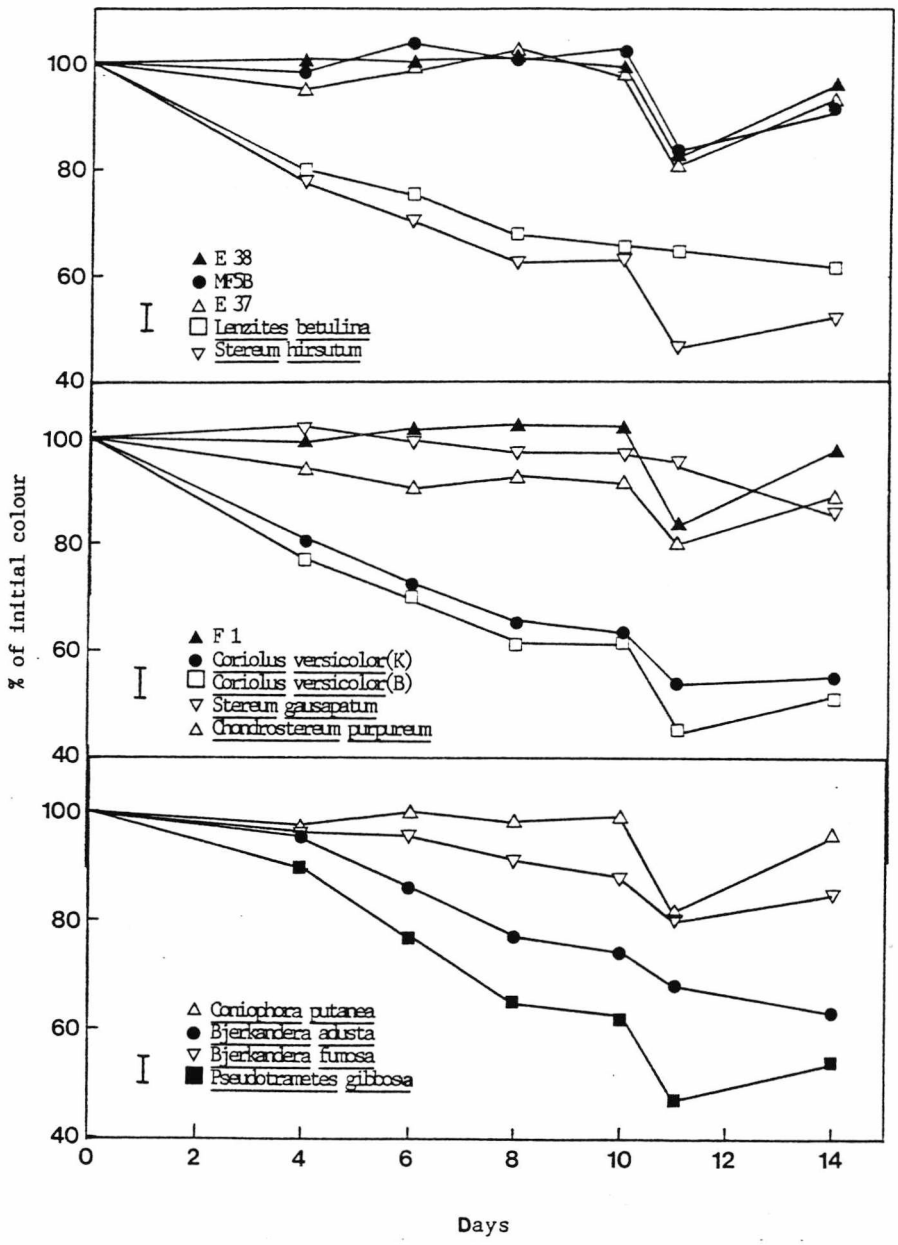

Fig. 3.1. Decolorisation of E effluent by fungal isolates in stationary culture, 25°C. Initial colour 1.7 x 10^3 CU.

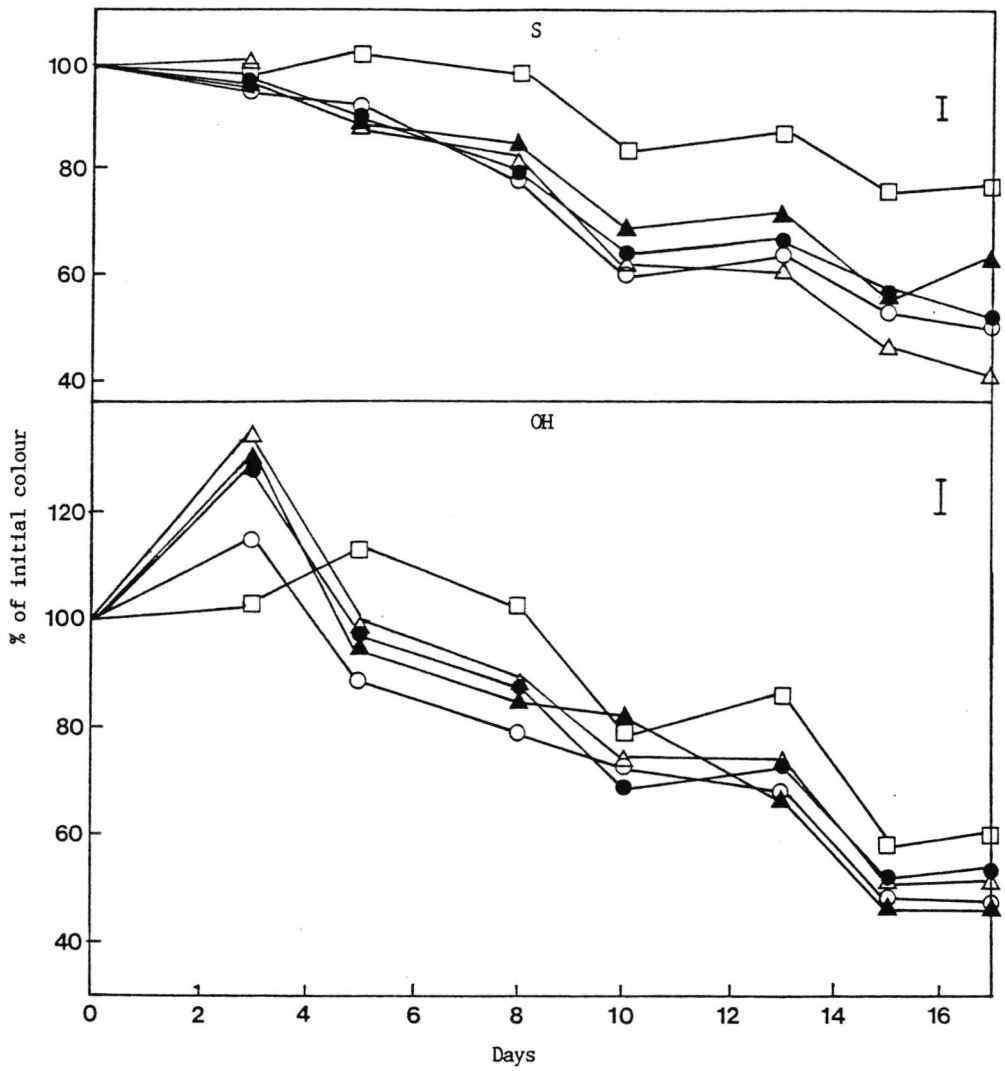

Fig. 3.2. Decolorisation of OH and S effluents by fungal isolates in stationary culture, 25°C. △, Coriolus versicolor(K); ○, Coriolus versicolor(B); □, Pseudotrametes gibbosa; ▲, Lenzites betulina; ●, Stereum hirsutum. Initial colour; S 1.86 x 10^3 CU; OH 2.74 x 10^3 CU.

greatly different. Colour of OH or S was not reduced by greater than 60% by any isolate and P.gibbosa was the least effective on both OH and S (maximum decolorisation was 20% S, 40% OH). C.versicolor (K) and (B), S.hirsutum and L.betulina were therefore chosen to measure decolorisation in shaken batch culture (Figs. 3.3; 3.4; Plate 3).

In shaken culture decolorisation by C.versicolor(K) and (B) and S.hirsutum was more rapid than in stationary cultures. In 11 days both C.versicolor isolates had decolorised E effluent by 70-75% compared with 45-55% in stationary culture. L.betulina was the least effective species studied and was particularly ineffective on E effluent (only 35% removed in 13 d).

In all cases laccase levels remained below 20 guaiacol units (GU)/ml except in the case of C.versicolor(K) on OH effluent in which laccase activity increased dramatically from 10 GU/ml on day 7 to 85 GU/ml by day 10. In the shaken cultures the pH was buffered at 5.0 which may have been a factor in increasing the rate of decolorisation over stationary cultures.

3.3. Decolorisation of different strengths of OH effluent by C.versicolor(K)

In previous experiments the effluents were added to media at a concentration of 2% (v/v), a large and probably unrealistic dilution to make prior to treatment. Therefore comparisons were made with higher concentrations of effluents on the rate and amount of decolorisation. At a concentration of OH of 3.91×10^3 CU (2%) laccase production was high and decolorisation rapid, 85% of the colour being removed in 6 days (Fig. 3.5). However, at a concentration of 10% (13.95×10^3 CU) laccase was not produced until 6 d after inoculation and only 65% of the colour was removed in 12 days. Nonetheless, the final levels of laccase were at

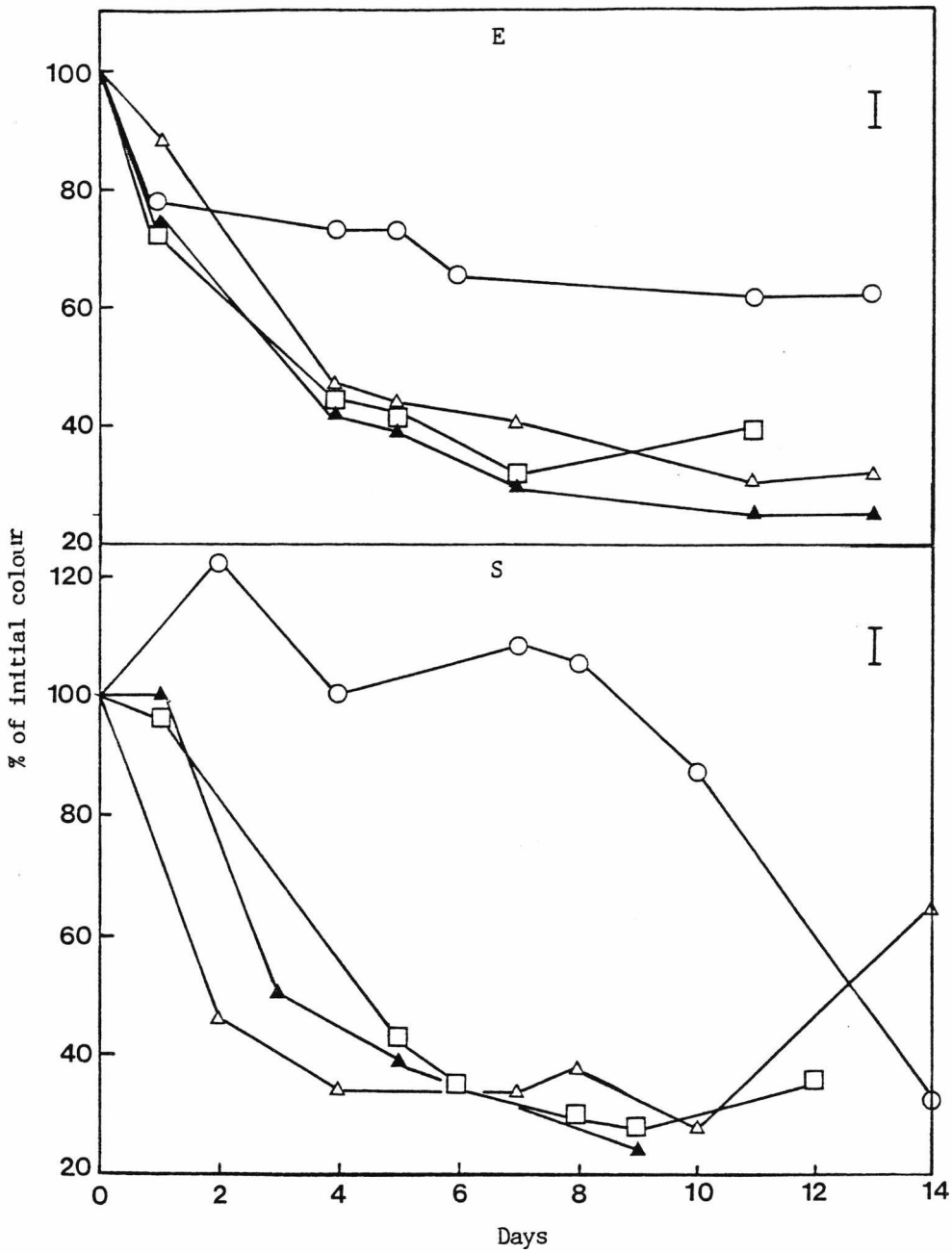

Fig. 3.3. Decolorisation of E and S effluents by white-rot fungi in shaken culture. ○, Lenzites betulina; ▲, Coriolus versicolor(K); □, Coriolus versicolor(B); △, Stereum hirsutum. Initial colour; E 5.7 x 10^3 CU; S 4.3 x 10^3 CU.

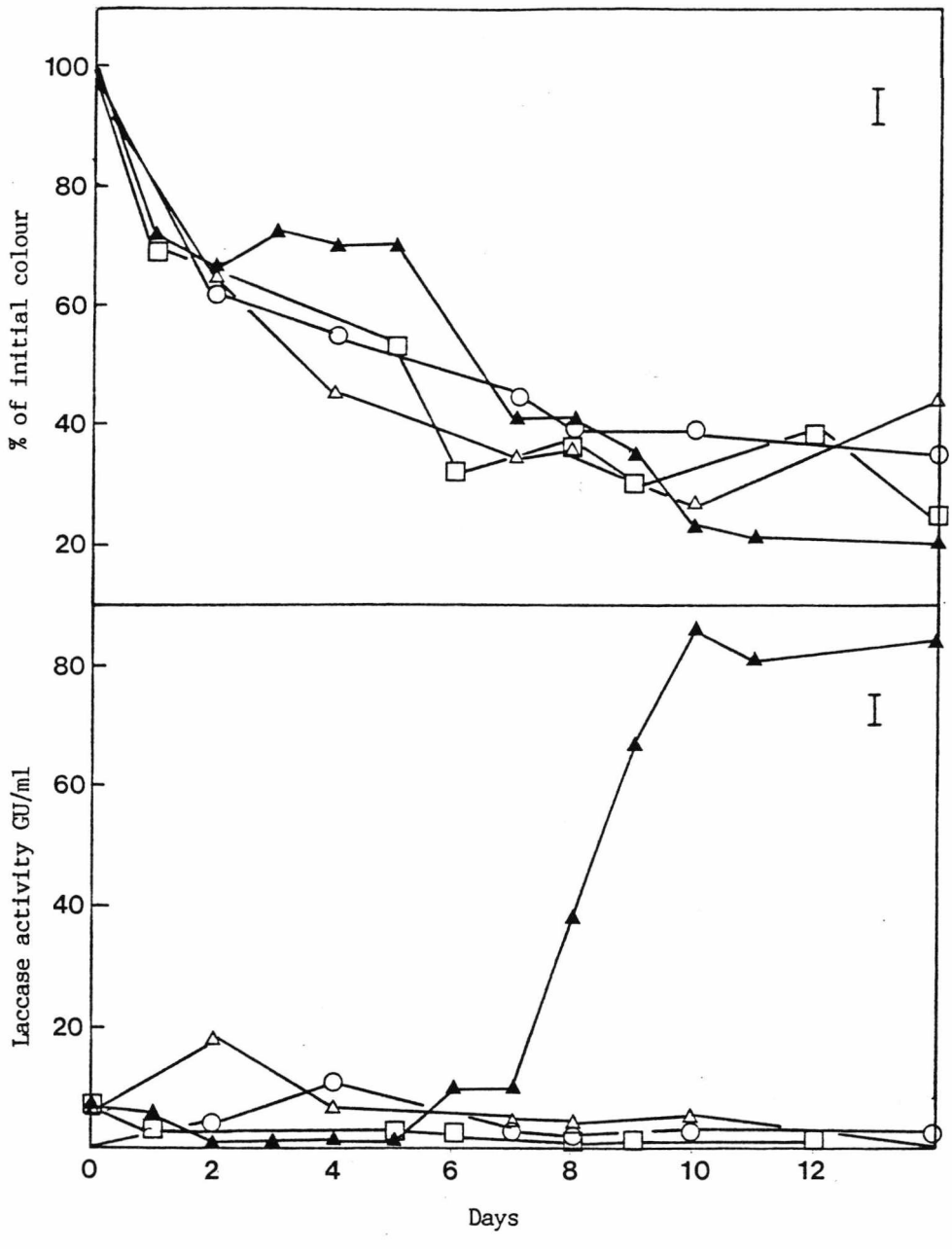

Fig. 3.4. Decolorisation of OH effluent and laccase production by white-rot fungi in shaken culture, 25°C. ○, Lenzites betulina; ▲, Coriolus versicolor(K); □, Coriolus versicolor(B); △, Stereum hirsutum. Initial colour 2.8 x 10^3 CU.

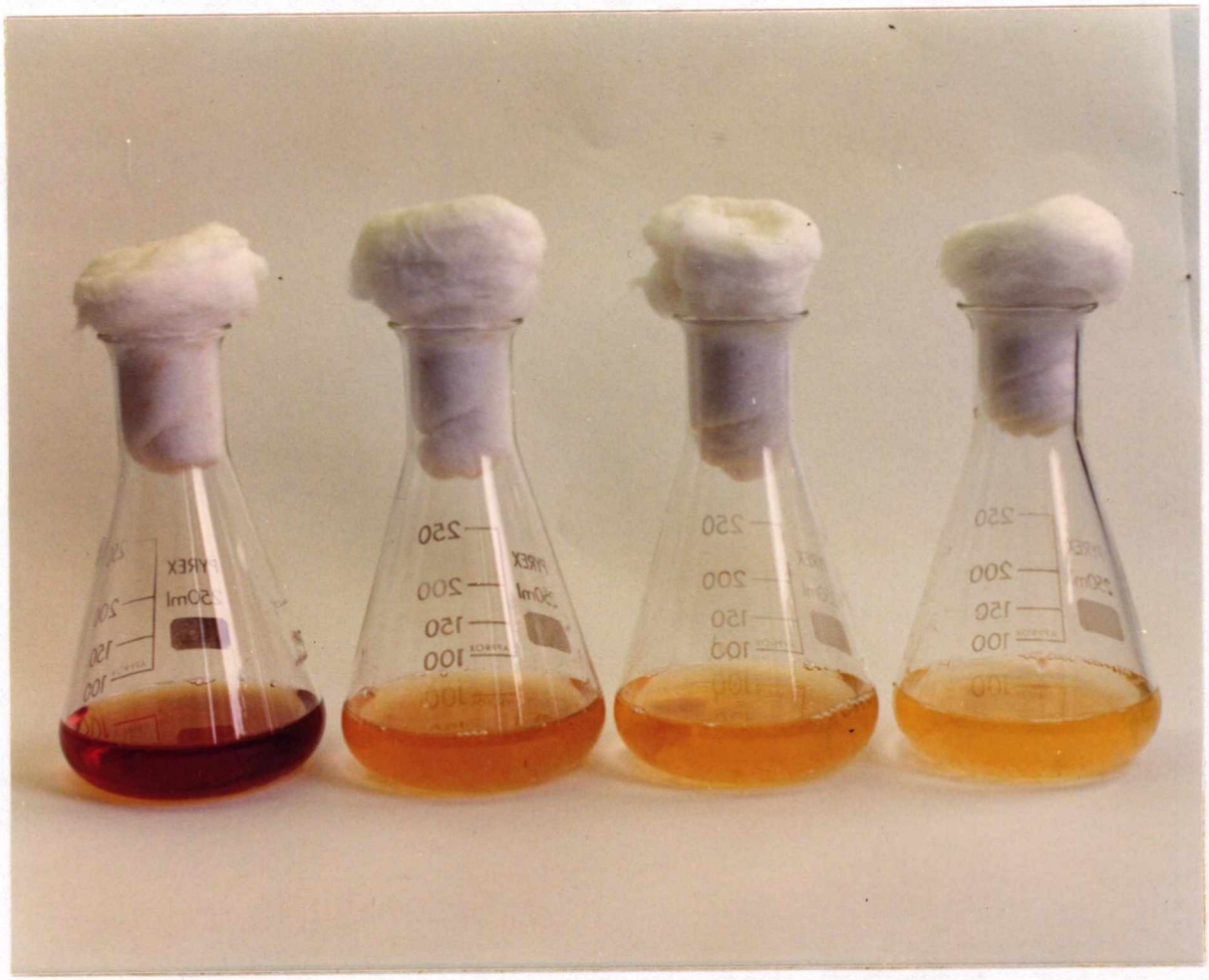

Plate 3. Decolorisation of 5% v/v E effluent (in mineral medium plus 1% w/v glucose) with time by Coriolus versicolor(K) in shaken culture, 25°C. From left to right: uninoculated control; 3 d incubation; 5 d incubation; 7 d incubation.

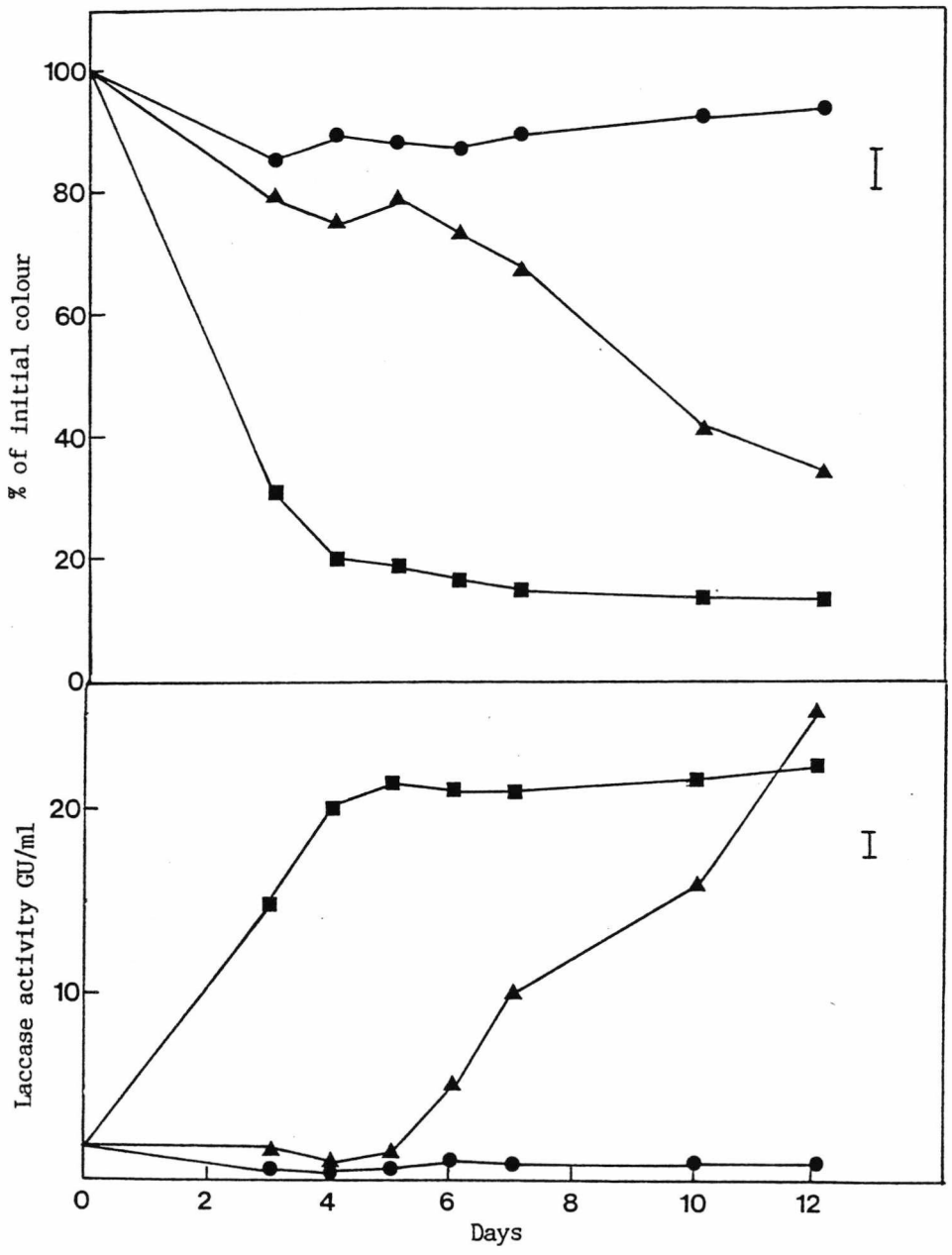

Fig. 3.5. Decolorisation of increasing concentrations of OH effluent and laccase production by Coriolus versicolor(K) in shaken culture, 25°C. Initial colour; ■, 3.91 x 10^3 CU; ▲, 13.95 x 10^3 CU; ●, 33.86 x 10^3 CU.

least as high at 25 GU/ml compared with 21 GU/ml in the 2% effluent. A 20% concentration of effluent (23.86 x 10^3 CU) was toxic to C.versicolor(K) which did not even grow. There was however a small amount (5%) of colour removed, but this was probably due to adsorption to the inoculum as the mycelium was seen to turn dark brown. In 2% effluent the inoculum established and grew and the mycelium remained a pale cream colour as decolorisation was rapid, and in 10% effluent the inoculum grew and the mycelium turned brown over the first 5 d and then turned cream in colour as the effluent was decolorised.

When repeated additions of OH effluent (1%) were made to one batch of C.versicolor(K) mycelium (Fig. 3.6), decolorisation of the first batch was rapid (7.34 CU/h) but decolorisation of the next 2 batches (day 8 and day 15) decreased to 6.9 CU/h and 5.65 CU/h respectively. Laccase activity was consistently below 10 GU/ml until the third batch of effluent was added (day 15) after which it rose steadily to 25 GU/ml.

3.4. Effect of temperature and pH on decolorisation of E effluent by C.versicolor(K)

Initial pH values in the range 4.0-7.0 did not result in significantly different colour removal from E effluent (Fig. 3.7) and the pH of the cultures decreased to 4-5 during the first 6 days incubation and remained at this level. However a starting pH of 3.0 resulted in a significantly lower amount of colour removal and the pH of the medium did not change during the incubation period. Decolorisation of E effluent was significantly lower at 37°C (Fig. 3.8) than at 25 or 30°C.

3.5. Decolorisation of fractions of OH effluent by C.versicolor(K)

Fractionation of the colour of phenolic effluents can be achieved by reducing the pH to 2.0, to produce a coloured precipitate and a coloured

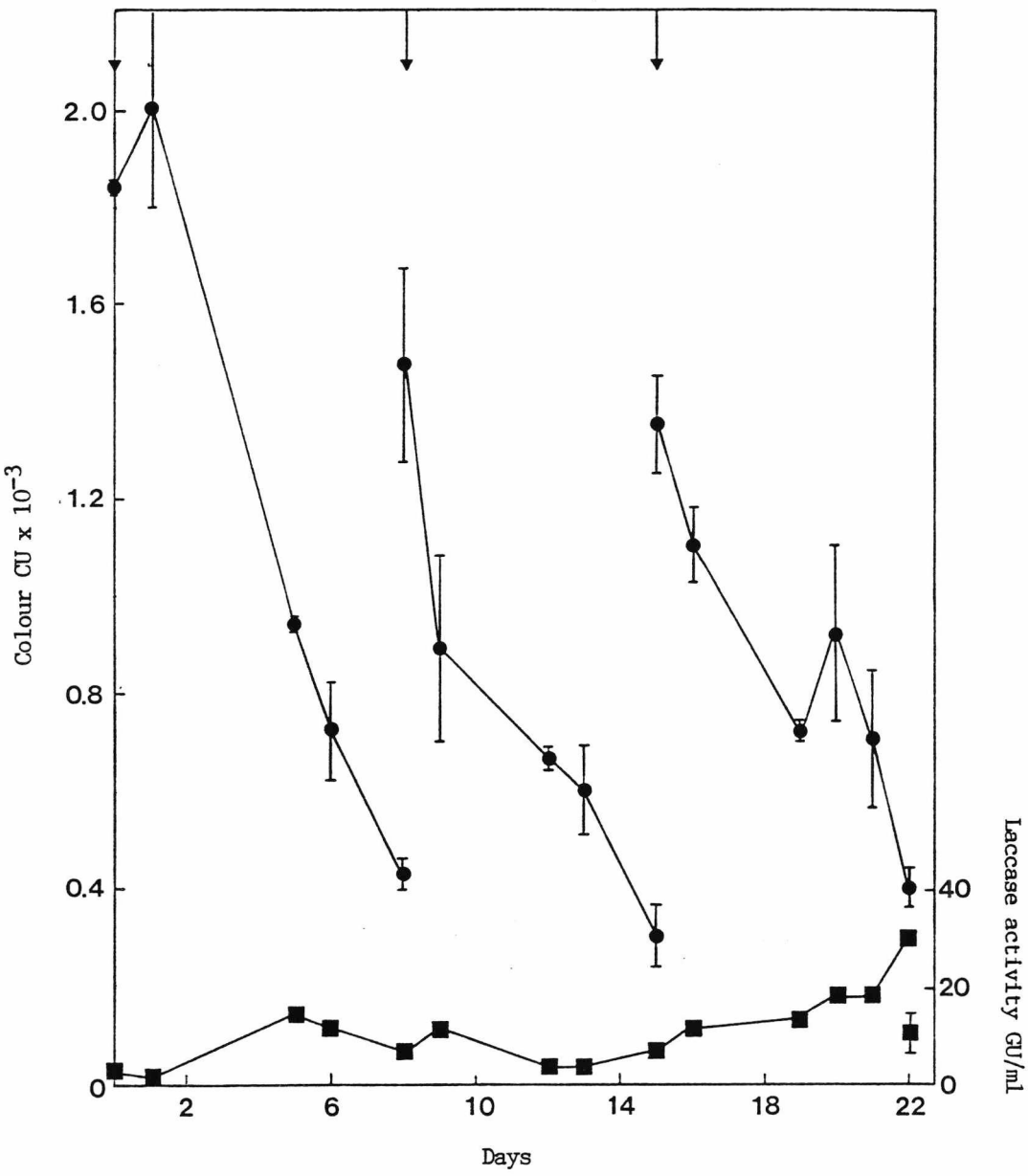

Fig. 3.6. Decolorisation of three successive additions of OH effluent at days 0, 8 and 15 by one batch of Coriolus versicolor(K) in shaken culture with 0.5% w/v glucose, 25°C. ↓, addition of fresh effluent 1% v/v; ●, colour of medium; ■, laccase activity.

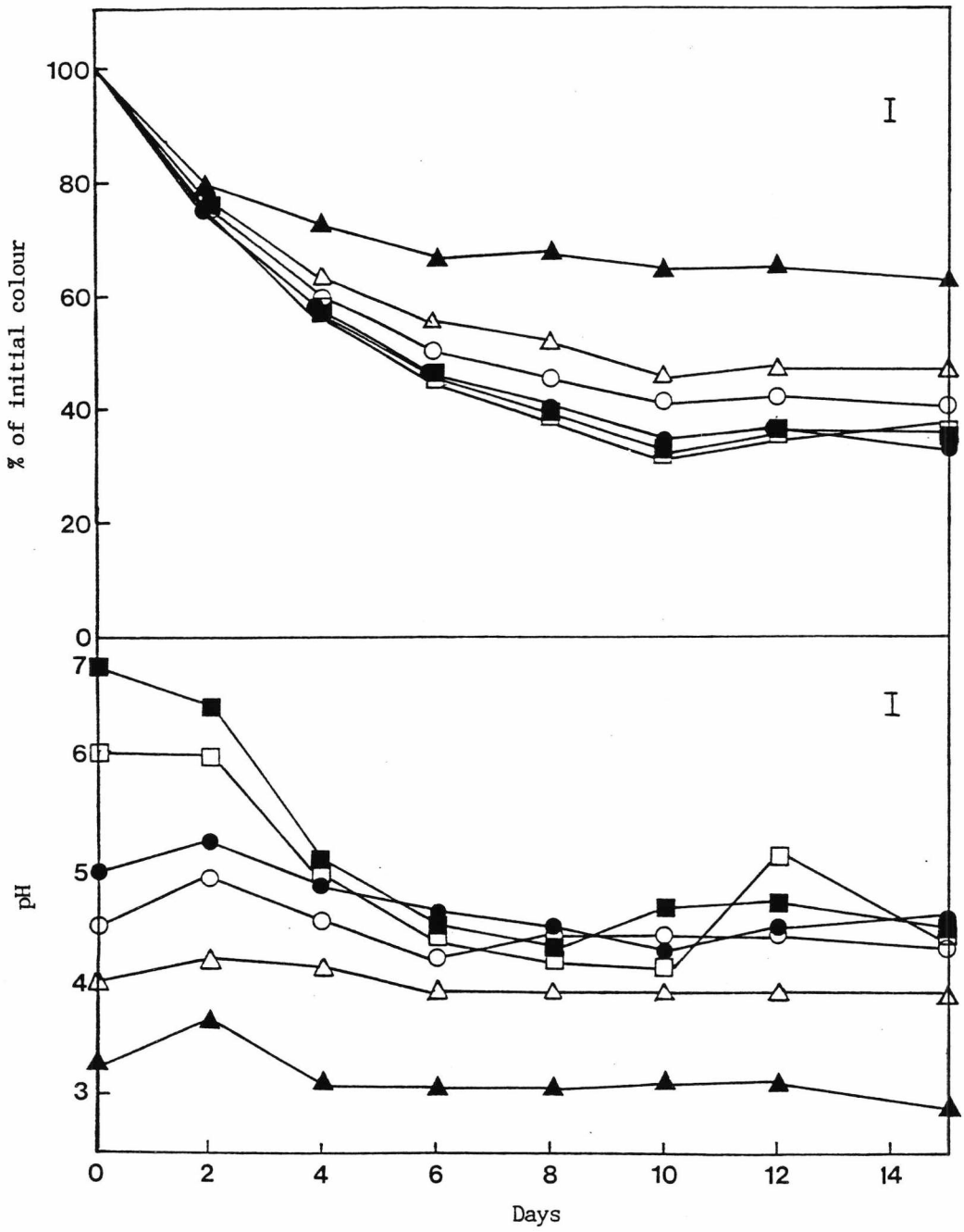

Fig. 3.7. Effect of initial pH of growth medium on decolorisation of E effluent by Coriolus versicolor(K). Initial colour 1.28 x 10^3 CU. ▲, pH 3.0; △, pH 4.0; ○, pH 4.5; ●, pH 5.0; □, pH 6.0; ■, pH 7.0.

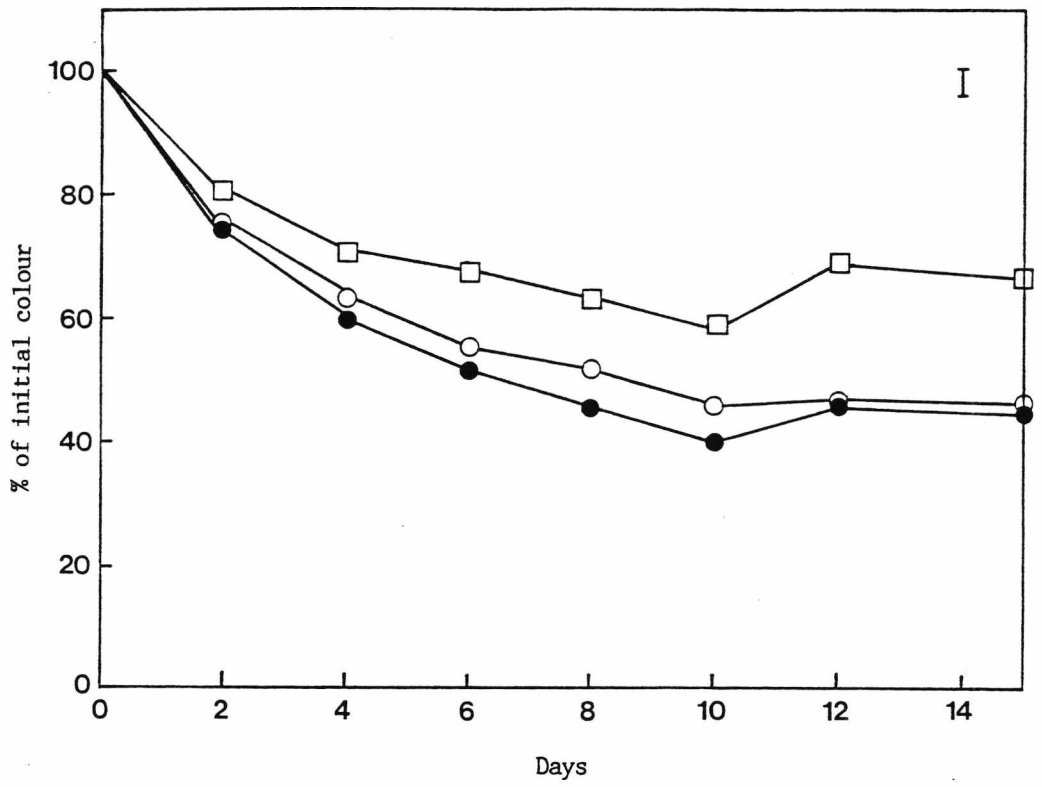

Fig. 3.8. Effect of temperature on decolorisation of E effluent by Coriolus versicolor(K). Initial colour 1.28 x 10^3 CU. □, 37°C; ○, 30°C; ●, 25°C.

supernatant. The precipitate redissolves on increasing the pH and by adjusting both fractions to pH 7.0 they can be treated as two types of effluents. The decolorisation profiles of acid-precipitated (AP) and non-precipitated (NP) fractions of OH were very similar (Fig. 3.9), the colour in both was reduced by 60% in the first 5-7 days after which it was constant. However laccase production was different: in the AP fraction laccase activity peaked at 55 GU/ml on day 4, falling steadily to 5 GU/ml after 12 days, whilst in the NP fraction laccase activity dropped to 8 GU/ml on day 6 then rose to 30 GU/ml by day 20. The low molecular weight fraction (<10 kD) responded similarly to the NP fraction in both colour removal and laccase production. In all cases initial laccase activity was high (45-50 GU/ml) due to a high activity in the inoculum.

3.6. Degradation of phenolic polymers by C.versicolor(K)

Phenolic polymers were considered as models of the polyphenolic compounds which occur in waste effluents (Kringstad & Lindstrom 1984) and therefore may show whether C.versicolor(K) is able to degrade such compounds. Caffeic acid and resorcinol polymers in mineral medium inoculated with C.versicolor(K) were assumed to be degraded because of observed decreases in the absorbance of the medium (Figs. 3.10; 3.11) but other polymers were either not degraded (guaiacol) or were further polymerised (vanillin, vanillic acid, guaiacol and ferulic acid) as shown by an increase in absorbance. Laccase activity increased to 7 GU/ml in medium containing guaiacol polymers and to 3.5 GU/ml in medium containing caffeic acid and ferulic acid polymers.

3.7. Induction of laccase by lignin related aromatics

The effect of aromatic compounds on laccase induction was investigated

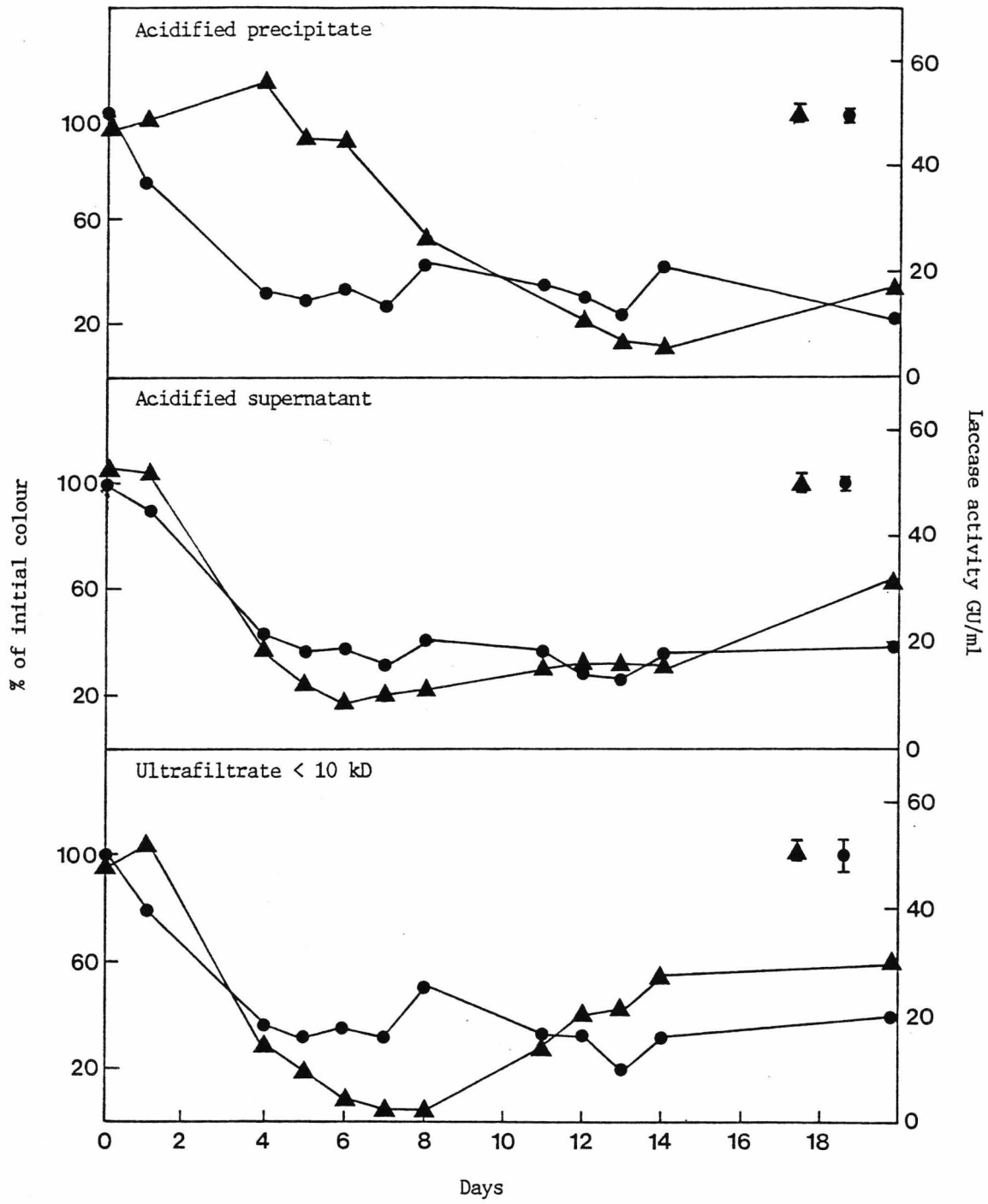

Fig. 3.9. Decolorisation of fractions of OH effluent and laccase production by Coriolus versicolor(K) in shaken culture. ●, colour; ▲, laccase activity.

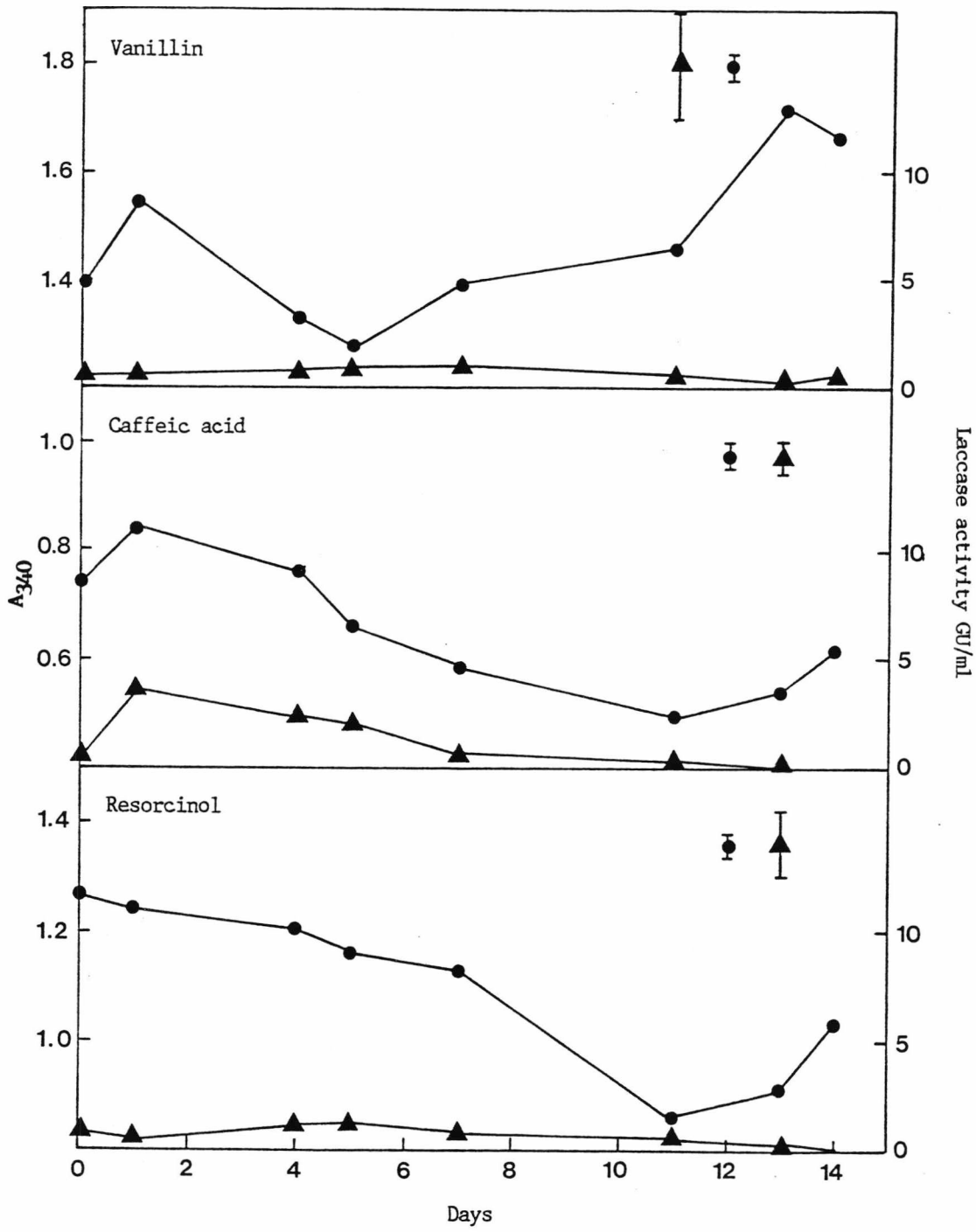

Fig. 3.10. Laccase production and absorbance changes by Coriolus versicolor(K) shaken cultures in mineral medium containing phenolic polymers. ●, A_{340} (polymer); ▲, laccase activity. Medium supplemented with 5% v/v polymer solution plus 1% w/v glucose.

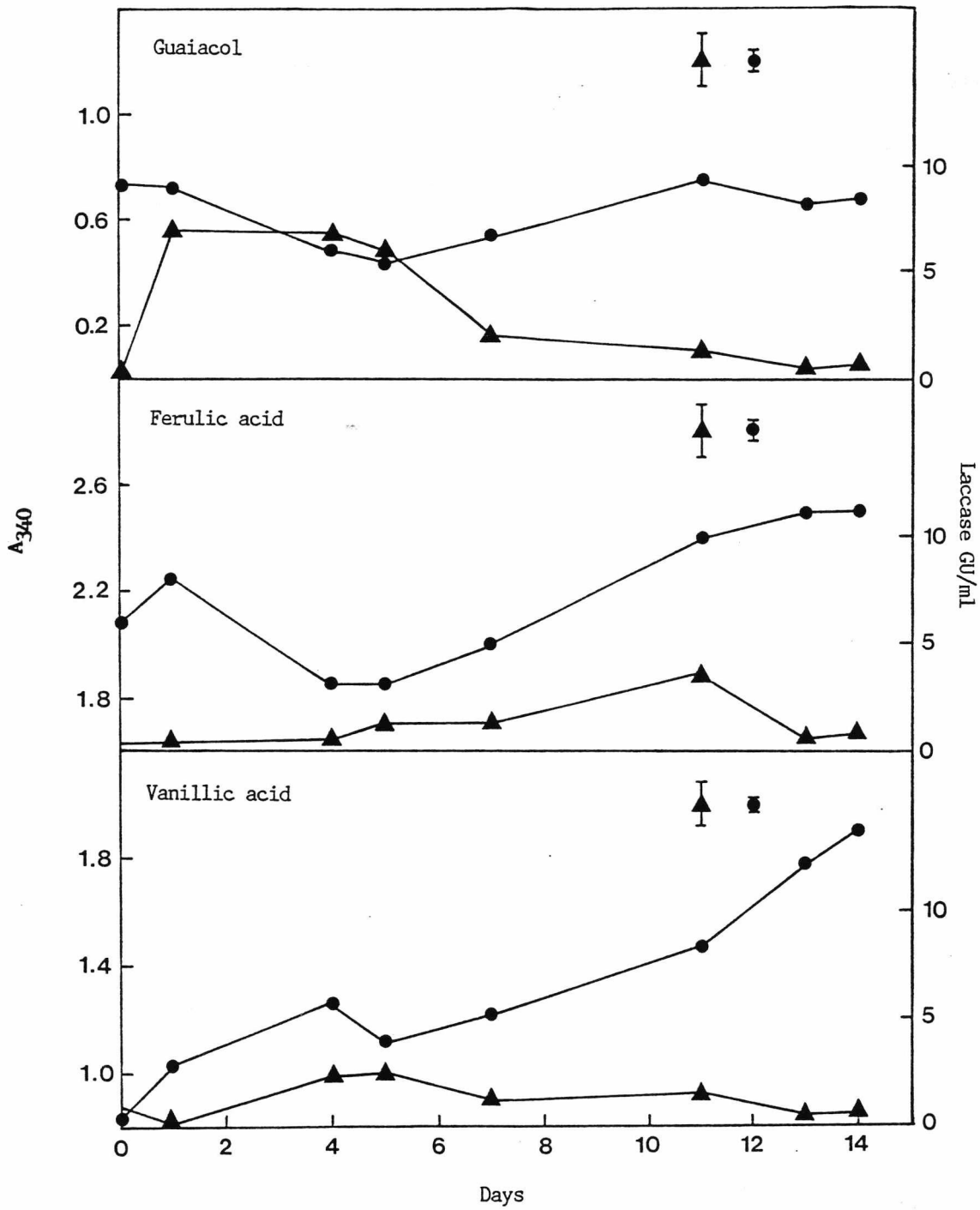

Fig. 3.11. Laccase production and absorbance changes by Coriolus versicolor(K) shaken cultures in mineral medium containing phenolic polymers. ●, A_{340} (polymer); ▲, laccase activity. Medium supplemented with 5% v/v polymer solution plus 1% w/v glucose.

using 4 mm diam mycelial pellets of Coriolus versicolor(K) to standardise the inoculum (Table 3.3). High molecular weight compounds related to lignin by structure (Indulin AT, tannic acid) had a stronger inducive effect than phenolic monomers e.g. tannic acid plus glucose induced 41.2 GU/ml and of the monomers gallic acid (22.6 GU/ml), vanillin (21.3 GU/ml) and vanillic acid (17.8 GU/ml) produced the greatest effect. The highest laccase levels (49 GU/ml) were produced in cultures with Indulin AT plus malt extract. Malt extract enhanced laccase production (7.6 GU/ml) compared with glucose (3.3 GU/ml).

3.8. Culture conditions for maximum laccase production

Laccase was chosen for further investigation in the context of enzymic treatment of phenolic effluent because phenol oxidases have previously been shown to precipitate phenols from aqueous effluents. To obtain large amounts of laccase, factors affecting its production by C.versicolor(K) in batch culture were investigated. The inducer 2,5-xylidine caused a dramatic increase in laccase production when added to cultures after an initial growth period of 5 days (Figs. 3.12; 3.13). In 100 ml cultures, malt extract broth supplemented with glucose (MGB) was the preferred medium in which C.versicolor(K) produced an average of 510 syringaldazine units (SU)/ml by day 9. On sucrose or glucose alone there was poor growth and consequently little enzyme production (60 and 35 SU/ml respectively, by day 10) and laccase was not detected at all until after inducer was added at day 6. The addition of a glass bead to MGB cultures appeared to increase laccase production to 800 SU/ml on day 9 but this was not significant compared with flasks without beads due to the large variation between replicate flasks. Increases in protein levels corresponded with laccase production.

When one litre volumes of MGB were used (Fig. 3.14) laccase activity

Table 3.3. Laccase production by mycelial pellets of C.versicolor(K) incubated in mineral medium supplemented with lignin-related aromatics for 5 days

Growth medium	Laccase, guaiacol units/ml
Glucose	3.3 ± 0.9
Malt extract	7.6 ± 1.4
Malt extract + Indulin AT	44.5 ± 2.0
Indulin AT	36.1 ± 4.9
Glucose + Indulin AT	49.1 ± 2.1
Glucose + vanillic acid	17.8 ± 2.5
Glucose + vanillin	16.8 ± 1.0
Vanillin	21.3 ± 3.6
Ferulic acid	1.6 ± 0.6
Glucose + ferulic acid	2.1 ± 0.1
Glucose + veratric acid	0.0
Glucose + gallic acid	22.6 ± 5.0
Glucose + tannic acid	41.2 ± 6.0
Glucose + α-naphthol	0.0
OH effluent	16.9 ± 0.9
Glucose + OH effluent	15.3 ± 9.0
Malt extract + OH effluent	20.4 ± 9.0

The following concentrations were used (w/v): OH 2%; glucose 1.0%; malt extract and Indulin AT 0.2%; vanillic acid, vanillin, ferulic acid, veratric acid, gallic acid, tannic acid and α-naphthol 0.1%.

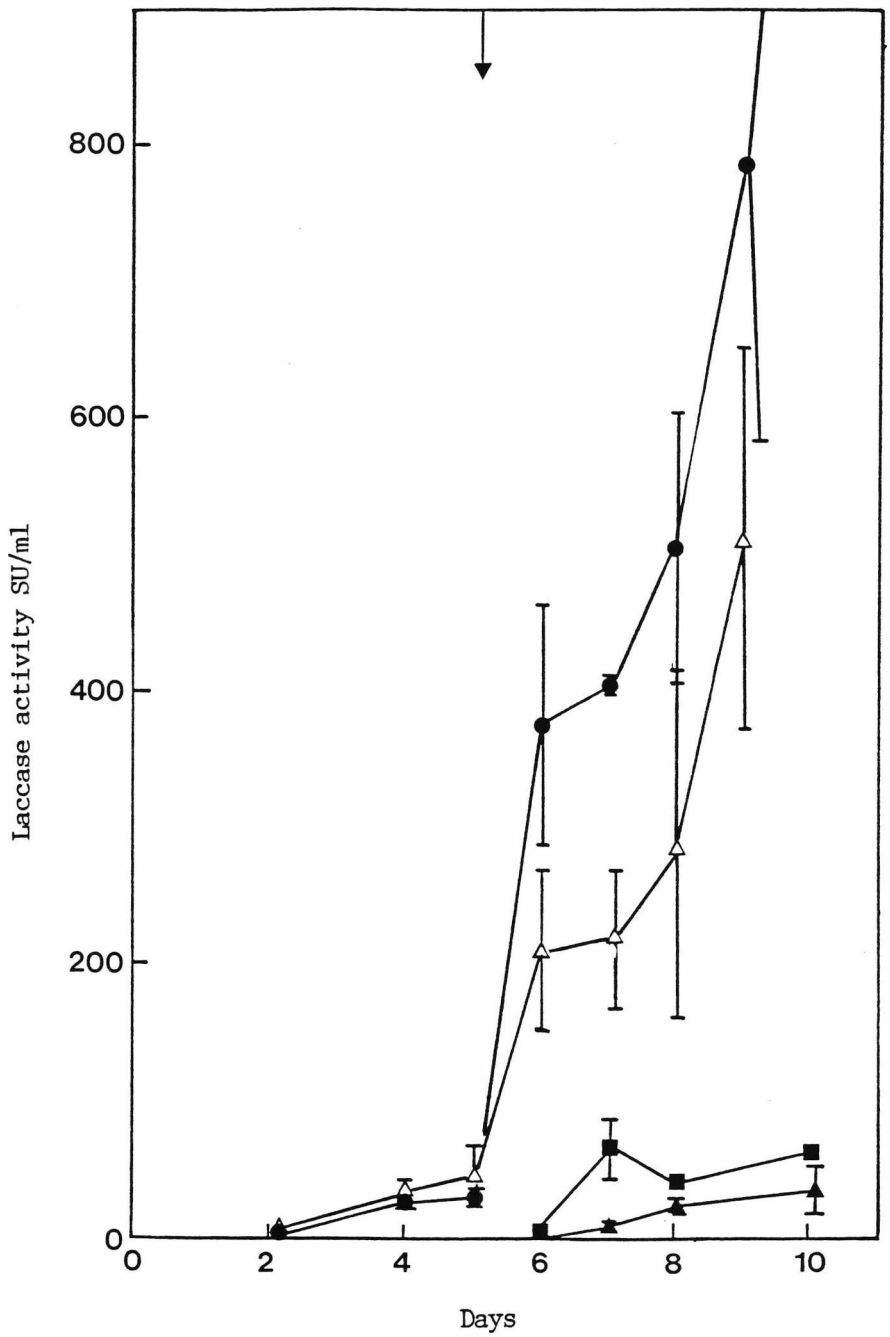

Fig. 3.12. Laccase production by C.versicolor(K) in 100 ml shaken batch cultures containing different carbon sources and following addition of inducer. ▲, glucose 5 g/l; ■, sucrose 5 g/l; △, MEA; ●, MEA plus 1 cm diam glass bead; ↓, inducer 2,5-xylidine added at day 5, 0.2 mM.

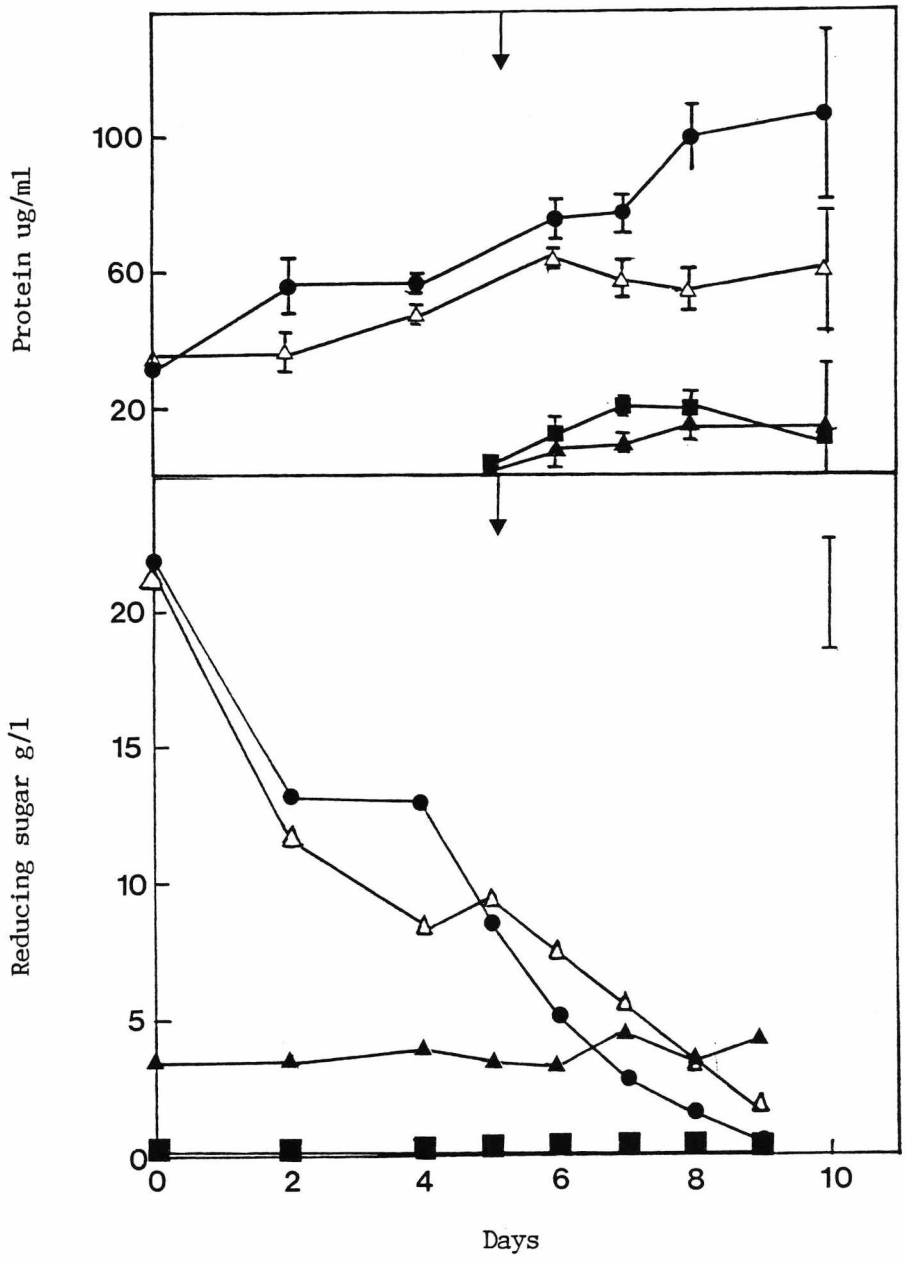

Fig. 3.13. Changes in protein and reducing sugar during laccase production by C.versicolor(K) in 100 ml shaken batch cultures containing different carbon sources and following addition of inducer. ▲, glucose 5 g/l; ■, sucrose 5 g/l; △, MEA; ●, MEA plus 1 cm diam glass bead; ↓, inducer 2,5-xylidine added at day 5, 0.2 mM.

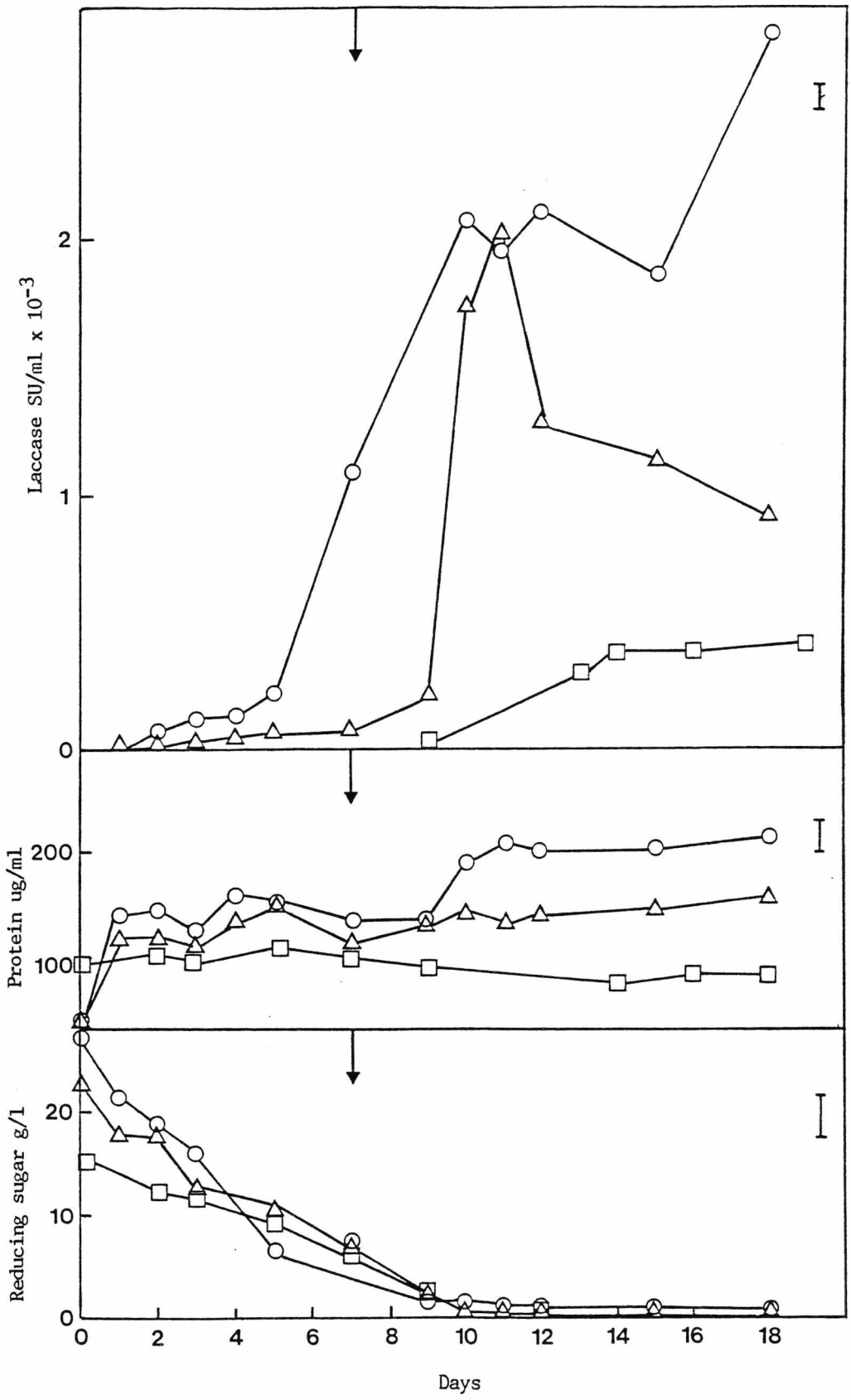

Fig. 3.14. Laccase production by Coriolus versicolor(K) in 1 l shaken cultures on malt extract broth. ↓, inducer (2,5-xylidine) added; □, no inducer; △, with inducer; ○, with inducer and 2 cm glass bead.

peaked at 2000 SU/ml on the 11th day in flasks without glass beads. The effect of glass beads was to significantly stimulate laccase production before inducer was added (1008 SU/ml) and to cause a prolonged increase in laccase production after inducer was added resulting in a maximum of 2800 SU/ml on day 18. Therefore laccase was routinely produced in one litre cultures of MGB inoculated with C.versicolor(K), plus a 2 cm glass bead. Inducer was added on day 7 and laccase was extracted after 14 days incubation.

DISCUSSION

Fungi were isolated from soil contaminated with cotton mill effluent in significantly higher numbers on effluent medium (OHG) than on MEAS. This difference may be due to the utilisation of effluent components as carbon sources by some of the fungi. Although four isolates produced phenol oxidases according to the plate test, none of the isolates produced clearing zones on the OHG. This was surprising because colour formation with phenolic substrates due to the production of phenol oxidases is regarded as a good indication of white rot fungi (Harkin & Obst 1973). In fact known white-rot fungi from culture collections which did produce phenol oxidases (e.g. C.versicolor, S.hirsutum) also produced clearing zones on OHG and decolorised effluents in liquid cultures. It is therefore possible that the fungi isolated from soil were brown rot fungi, producing phenol oxidases but which were unable to decolorise effluents due to a limited capacity to degrade lignin related compounds (1.3.1).

During stationary culture screening for decolorisation, the pH of the medium decreased in all cultures from an initial pH of 5-7 to between 4 and 5. In buffered effluent colour removal was not affected by initial

pH values between 4.0-7.0. However the pH in the medium had decreased rapidly during incubation, despite buffering, to between 4.0-4.5, therefore this range was taken as optimum for decolorisation.

The initial increase in colour of OH effluent that was observed in stationary culture but not shaken culture, cannot be explained without further experiments. A number of factors could account for the increased rates of decolorisation observed in shaken cultures, e.g. increased oxygen transfer rates to the cells and improved contact of wall bound enzymes with effluent components due to mixing. The buffering of the effluent to pH 5.0 would be expected to cause an increase in decolorisation rate because this is the optimum pH for that process. In shaken cultures the extent of decolorisation was greater than that in stationary culture, which could be due an increased growth rate at favourable pH 5.0 and increased dissolved oxygen, resulting in a greater production of biomass. Therefore adjustment of effluent pH to 4-5 prior to treatment would be required for the maximum rate of decolorisation, but high total decolorisation at a slower rate could be achieved in shaken cultures of effluent adjusted to a broader pH of 4.0-7.0, as the fungal metabolism rapidly reduces the pH of the medium.

Induction of laccase production by Coriolus versicolor(K) in OH cultures was attributed to two factors. Firstly, the presence of unknown phenolic compounds which are components of OH effluent or secondly compounds which are produced during breakdown of the effluent components. The latter is indicated by the fact that laccase was not induced until day 7 during shaken culture of C.versicolor with OH effluent.

The ligninolytic enzyme system is involved in decolorisation of phenolic effluents by P.chrysosporium and is synthesised during the secondary metabolic phase of growth (Eaton et al. 1982). This would explain the time lag seen here before decolorisation in stationary culture (3.2).

However in shaken culture the delay in the onset of decolorisation was shortened or absent (3.2; 3.3). This is not due to a change in rate of induction but to an initial period of colour removal from the medium by adsorption of phenolic compounds to fungal mycelium (the mycelium gets darker) followed by decolorisation of both adsorbed and free phenolics after a time lag for induction of the secondary metabolic system. Royer et al. (1985) showed that these two phases could be separated into adsorptive decolorisation (in the absence of nutrients and carbon) and oxidative decolorisation (by growing mycelium) in the presence of sucrose.

In the experiments reported here, several fungal species showed good growth and decolorisation in agitated culture conditions, in contrast to results found by Bollag & Leonowicz (1984) who found that Rhizoctonia praticola and Tinctoporia borbonica were the only isolates from 10 tested including C.versicolor, Botrytis cinerea and Fomes annosus, which would grow in shaken culture. However they used a sugar rich (4% w/v) medium compared to the 1% glucose used here, which may have influenced the mycelial growth. Also, inoculum size and form (e.g. plugs from agar plates, homogenised mycelium) are important factors influencing growth in submerged culture (Metz & Kossen 1977) but this information was not stated by Bollag & Leonowicz.

High concentrations of OH effluent (1.4×10^3 CU) were inhibitory to growth and correspondingly caused a delay in decolorisation. Undiluted OH effluent as produced from the cotton mill (1.4×10^5 CU), would therefore be totally inhibitory to growth and decolorisation by C.versicolor(K) and considerable dilution would be required prior to treatment. However the outflow concentration of E effluent is approximately 5×10^3 CU (Eaton et al. 1982; Livernoche et al. 1983; Royer et al. 1985) which should be treatable after amendment with an

additional carbon source and pH adjustment but without dilution. Nonetheless, a fungal treatment system would be susceptible to damage and inhibition of activity if pulses of concentrated effluent concentration occurred (e.g. during a mill malfunction), therefore monitoring and control of concentration would be important factors.

The aim of this work was to find a robust continuous treatment system for phenolic effluents. However, in our experiments, when one batch of C.versicolor(K) mycelium was used to treat three successive batches of effluent, the rate of decolorisation decreased with each batch. The subsequent rates might be improved by adding small amounts of sucrose with each batch of effluent as Royer et al. (1985) achieved good decolorisation in this way using mycelial pellets. Also the products of decolorisation from the previous batch of effluent were not removed from the cultures, therefore a build up of degradation products may have contributed to decreases in the decolorisation rate. This possibility was investigated by Royer et al. (1985) who inhibited the decolorisation of E effluent by C.versicolor cultures by adding back dialysed samples of decolorised effluent. This suggests that either untreatable compounds or products from the modification of coloured compounds can inhibit decolorisation.

Incubation of C.versicolor(K) at 37°C inhibited growth and decolorisation of E effluent compared with that at 25°C, agreeing with the report of Eaton et al. (1982) who found that C.versicolor would not grow at all at 39°C but would reduce colour of E effluent if grown at 27°C prior to raising the temperature. This colour removal by non-growing mycelium was almost certainly due to adsorption of colour to the mycelium, but incubation conditions were not stated. Royer et al. (1985) reported an optimum of 30°C for decolorisation by C.versicolor and complete inhibition of growth at 40°C. Therefore since pulp mill

effluent leaves the plant at about 40°C, pH adjustment and nutrient supplement stages should also reduce the temperature to around 30°C without additional cooling.

Synthetic phenolic polymers have been used as models of the humic acid type structures found in soil (Sarkar & Burns 1984). In our experiments polyphenols were used to determine the degradative effect of fungi on such polymeric compounds and to investigate the induction of laccase production by C.versicolor(K). However the effect of C.versicolor(K) on the polymers was inconsistent, only caffeic acid and resorcinol polymers were degraded to any extent whilst vanillin and guaiacol polymers were further polymerised. Therefore the capacity of C.versicolor to degrade lignin and decolorise phenolic effluents does not mean that all phenolic polymers are suitable substrates. Furthermore the degradation of some of the polymers (measured by colour reduction) may have been counteracted by the polymerising activity of laccase in the culture medium. Laccase was induced by the non-degraded polymers of guaiacol and ferulic acid but was not induced by ferulic acid monomers (guaiacol was toxic) (3.7). Laccase production (55 GU/ml) was also induced by the acid-precipitated fraction (AP) of OH effluent and this compared to a maximum of 30 GU/ml induced by the other fractions (i.e. non-precipitated, NP and ultrafiltrate < 10 kD). All three fractions were decolorised to the same extent and at the same rate, in accordance with the work of Sundman et al. (1981) who found that P.chrysosporium decolorisation of chromophores isolated from bleach plant effluent showed no preference for molecular size (compounds from 400-30 000 D were used). Laccase induction by the AP fraction could be caused by polymeric structures and polyphenolics such as vanillin copolymers have previously been shown to induce laccase production by C.versicolor(K).

Decolorisation of effluents is not necessarily dependent on high laccase

production by the fungi but laccase production in effluent media is a result of the initial presence of inducing compounds or their subsequent production by C.versicolor(K) during decolorisation of the effluent. To gain further information on laccase induction various lignin related compounds were added to C.versicolor(K) cultures. High molecular weight compounds such as Indulin AT (a commercial kraft lignin) induced laccase to greater levels than low molecular weight phenolics such as vanillin and gallic acid. High molecular weight compounds also induced laccase in Daedalea flavida to a greater extent than low molecular weight compounds (Arora & Sandhu 1985). Therefore it appears that laccase is more effectively induced by polymeric phenolic compounds than monomeric phenols, a fact which would be advantageous for fungal metabolism of high molecular weight lignin, in which laccase is thought to play a degradative role (Kirk & Farrell 1987).

Indulin AT was considered as an inducer for routine laccase production, but is difficult to work with due to its insoluble nature, and in any case it may cause concommitant induction of other lignin degrading enzymes, thereby complicating laccase purification. It has previously been shown with Coriolus versicolor cultures that almost all the 2,5-xylidine induced extracellular protein is laccase (Malmstrom 1958), therefore for routine laccase production in our experiments 2,5-xylidine was used which induced the same laccase levels as Indulin AT.

Sucrose and glucose have been shown to repress laccase production and growth by D.flavida (Arora & Sandhu 1985) and were shown to repress growth and laccase production by C.versicolor(K). However malt extract, which had an inductive effect, is a good medium for laccase production because it provides the complete pool of amino acids for enzyme synthesis. It was not possible to correlate protein levels in malt extract medium with laccase production because the medium has a high

protein content. Comparison of laccase levels with those obtained by Bollag & Leonowicz (1984) shows that shaken cultures of C.versicolor(K) produced 2800 SU/ml after 18 days whereas their C.versicolor isolate only produced 2333 SU/ml after 45 days in stationary culture, confirming that C.versicolor(K) is a high laccase producing isolate.

Therefore, as a source of cheap laccase for further experiments C.versicolor(K) laccase produced in batch culture as described here was sufficient. However, the development of a method of continuous enzyme production is necessary if laccase is to be a commercial alternative to inexpensive HRP or fungal treatments. Continuous production of fungal extracellular enzymes would depend on an immobilised system in order to reduce biomass production to a minimum, a problem which has been discussed by several authors (1.3.2). Immobilisation methods applied to mycelial fungi include the use of stable mycelial pellets which have similar properties to mycelium immobilised in calcium alginate beads (Royer et al. 1983; 1985). Recently the production of chloroperoxidase (CPO) by Caldariomyces fumago (Carmichael & Pickard 1989) used mycelial pellets in an airlift reactor, albeit for only 8 days, but achieving continuous production of CPO at 128 mg/l. This is similar to the method of Royer et al. (1983) who used C.versicolor(K) mycelial pellets to decolorise kraft mill effluent. Therefore with optimisation of medium and culture conditions continuous laccase production in an airlift reactor should be feasible and would be an important step in the development of an enzymic treatment system for phenolic wastes.

CHAPTER FOUR

PURIFICATION OF LACCASE FROM CORIOLUS VERSICOLOR(K) AND TREATMENT OF PHENOLIC EFFLUENTS WITH SOLUBLE ENZYMES

INTRODUCTION

There has been much recent interest in the enzymatic treatment of phenolic effluents. Horseradish peroxidase (HRP) in particular has been shown to precipitate low molecular weight phenolics from coal conversion effluents (Klibanov et al. 1983) and to remove colour from bleach plant extraction stage effluent (Paice & Jurasek 1984). The main disadvantage of HRP treatment is the rapid loss in enzyme activity during the reaction and the high cost of hydrogen peroxide. Laccase also has been studied in the context of detoxification of phenolic pollutants in soils and aqueous systems (Bollag et al 1988) and has advantages over HRP in that it uses less expensive molecular oxygen as the oxidant and is inherently more stable. Laccase can be purified in large quantities from the extracellular culture medium of white rot fungi such as Coriolus versicolor (Malmstrom 1958; Mosbach 1963; Fahreus & Reinhammar 1967). Another phenol oxidase enzyme, tyrosinase, has also been shown to dephenolise aqueous solutions (Atlow et al. 1983), but has not been investigated for effluent decolorisation.

The aims of the research reported in this chapter were: (i) to investigate the effects of soluble laccase, peroxidase and tyrosinase on effluent colour and phenol concentration; (ii) to compare laccase preparations of different purity for stability during storage and phenol oxidation. It was also necessary to compare laccase substrates because

they had individual properties suited to different methods of activity assay.

RESULTS

4.1. Decolorisation of effluents by commercial enzymes

When soluble enzymes were incubated with buffered effluents, laccase (Pyricularia oryzae) and tyrosinase (Agaricus bispora) caused 11% and 14% decolorisation of OH effluent, respectively (Fig. 4.1) after 7 d incubation and no further decolorisation took place. Horseradish peroxidase (HRP) and H_2O_2, (50 mM) caused 61% reduction of colour, 4% more than that caused by H_2O_2 alone, but this was not significant and again decolorisation ceased after 7 days. Decolorisation of S effluent followed a different pattern (Fig. 4.1): laccase and tyrosinase reduced the colour by 20% within the first 24 h, then colour increased gradually reaching 93% and 94% of the initial colour by day 8, not significantly different from the controls (without enzyme). HRP and H_2O_2 caused a decrease in S effluent colour (51%) which was significantly greater than that due to H_2O_2 alone (38%).

4.2. Decolorisation of effluents by culture supernatants

The previous experiment used commercial laccase (Sigma) from Pyricularia oryzae and this enzyme was found to have low activity on guaiacol during assay (2.7.2). Therefore the laccase from C.versicolor(K) which had high activity against guaiacol, was used. In a preliminary experiment the effect of C.versicolor(K) culture supernatant on the colour of phenolic effluents was investigated to find out if filtrates with high laccase activity caused high decolorisation.

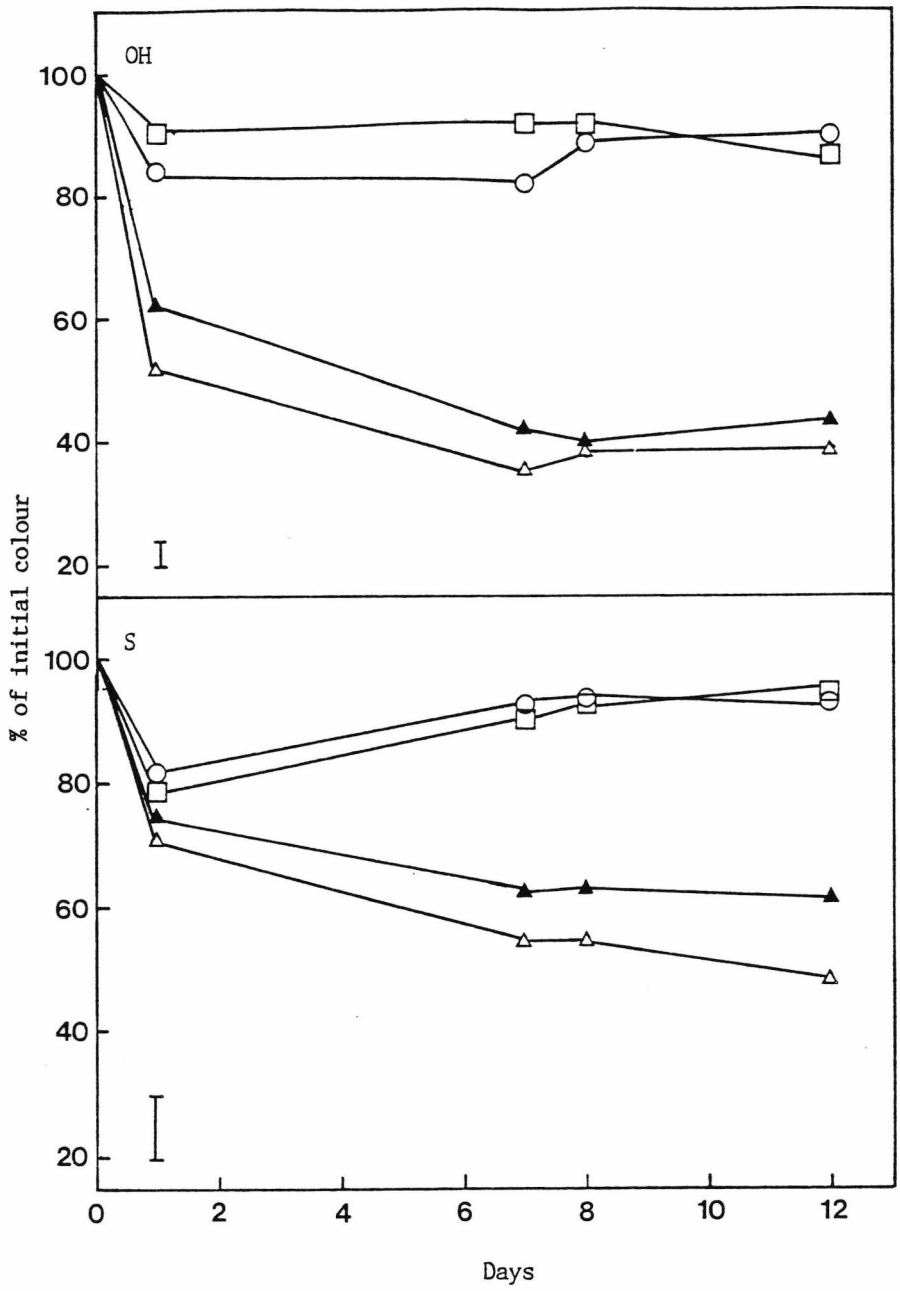

Fig. 4.1. Decolorisation of effluents by soluble enzymes. OH, initial colour 3.82 x 10^3 CU; S, initial colour 3.19 x 10^3 CU. ○, laccase (Pyricularia oryzae) 1.4 GU/ml; □, tyrosinase 1.0 U/ml; △, HRP 1.9 U/ml with 50 mM H_2O_2; △, 50 mM H_2O_2.

The maximum decolorisation (24% from OH effluent) was achieved by 6-day old MGB filtrate (Table 4.1), but decolorisation did not correlate with laccase levels. Filtrates from OH effluent cultures caused no decolorisation. The most effective cell extracts (CE) were from 6-day old MGB cultures which caused 30% decolorisation of S, and from 4-day old OH cultures which caused 31% decolorisation of OH. Overall there was no relationship between culture age and decolorising capacity of culture filtrates, except that filtrates from MGB removed more colour fom OH and S effluents than those from OH medium.

4.3. Decolorisation of effluents by soluble laccase from C.versicolor(K)

The dialysed ammonium sulphate precipitate of laccase from C.versicolor(K) (4.8) (designated crude laccase) was used in all further experiments. In buffered effluents (pH 5.0) soluble laccase (20 GU/ml equivalent to approximately 150 SU/ml) decolorised OH by 36% and E by 40% in 48 h (Fig. 4.2). Gelatin, Tween 80 and bovine serum albumin (BSA) were added in an attempt to protect laccase from inactivation by monophenols (Fahreus 1961; Shuttleworth & Bollag 1986) and thereby improve decolorisation, but they had no significant effect. Laccase caused S effluent to polymerise, shown by a 27% increase in colour over the first 2-3 hours incubation, but caused no significant decrease below the initial colour in 48 h. To improve colour removal from S effluent, 2,6-dimethoxyphenol (DMP) was added together with a higher laccase concentration (1156 SU/ml) to try to copolymerise components of the effluent with the DMP (Fig. 4.3) (Shuttleworth & Bollag 1986). The higher activity resulted in 32% decolorisation of S effluent in 90 h, however the DMP caused no significant difference until 90 h after incubation started, when the colour of S effluent with DMP rose and that without DMP

Table 4.1. Decolorisation of OH and S effluents in 72 h by C.versicolor(K) culture filtrates from cultures grown on malt extract broth (MGB) or mineral medium with 2% OH effluent and 1% glucose (OHG)

Growth medium	Culture age (days)	Effluent treated	Laccase (GU/ml)	Decolorisation (%)
OHG	4	OH	3.91 ± 0.41	0
	6		2.41 ± 0.45	2 ± 0.1
	12		9.06 ± 2.29	0
OHG	4	S	3.91 ± 0.41	0
	6		2.41 ± 0.45	0
	12		9.06 ± 2.29	0
MGB	4	OH	53.80 ± 0.93	22 ± 1.2
	6		39.41 ± 1.70	24 ± 1.4
	12		47.99 ± 3.47	14 ± 0.8
MGB	4	S	53.80 ± 0.93	18 ± 0.5
	6		39.41 ± 1.70	0
	12		47.99 ± 3.47	0
OHG Cell	4	OH	0.23 ± 0.03	31 ± 1.9
extract	6		0.60 ± 0.15	0
MGB Cell	4	S	17.36 ± 2.76	0
extract	6		27.20 ± 0.40	30 ± 2.1

Initial colour OH, 3.54×10^3 CU; S, 4.12×10^3 CU. GU = guaiacol units.

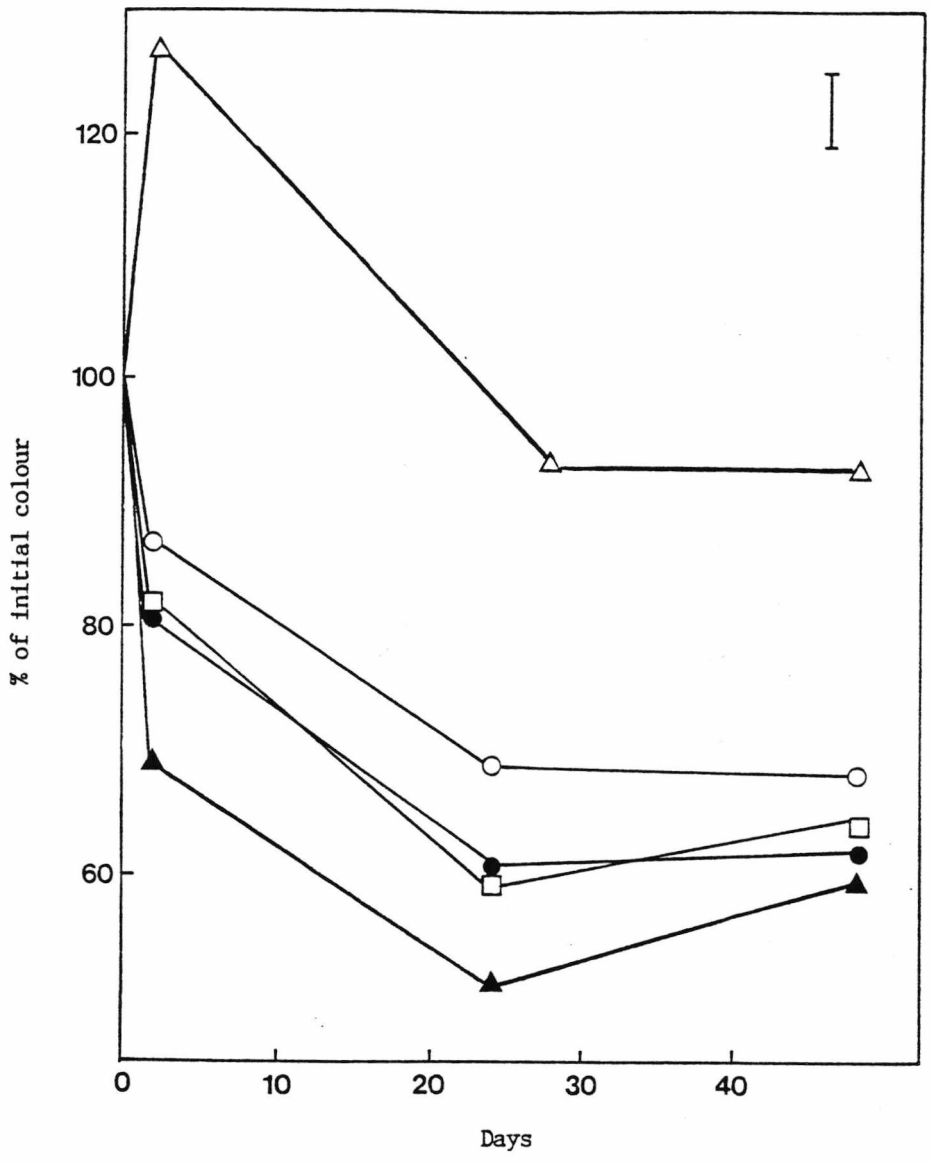

Fig. 4.2. Decolorisation of effluents, pH 5.0, by soluble laccase (C.versicolor(K)), 20 GU/ml. □—□, OH effluent, initial colour 2.78 x 10^3 CU; ○—○, OH effluent plus BSA 0.8 mg/ml; ●—●, OH effluent plus Tween 80 2 ul/ml; ▲—▲, E effluent, initial colour 4.04 x 10^3 CU; △—△, S effluent initial colour 4.2 x 10^3 CU.

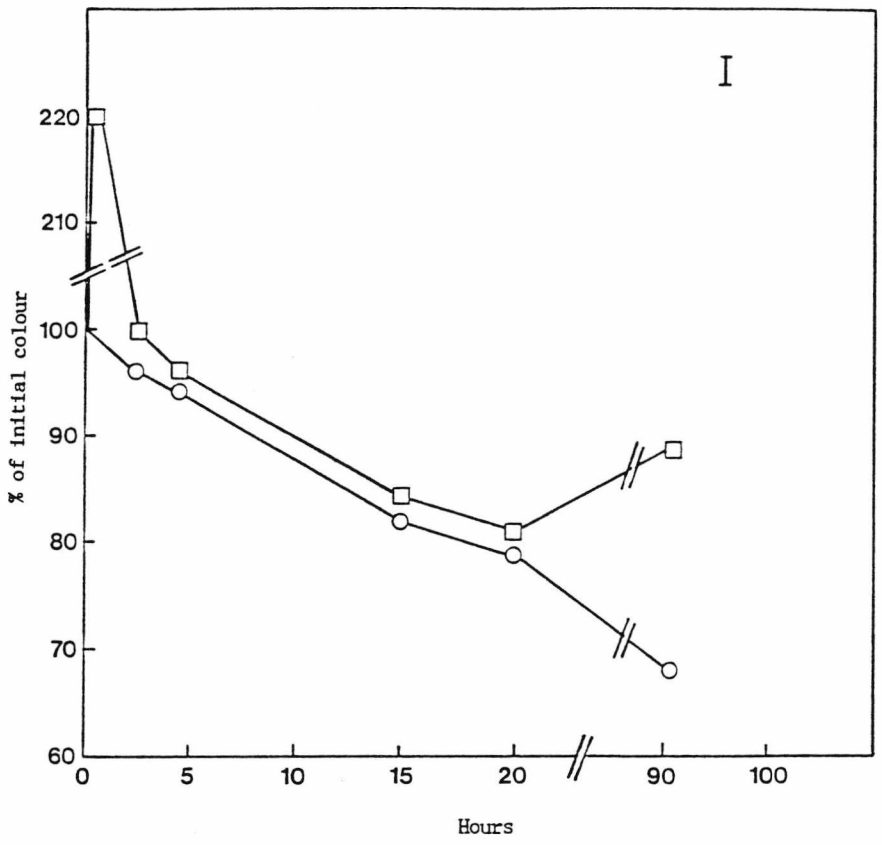

Fig. 4.3. Decolorisation of S effluent by soluble laccase 1156 SU/ml. Initial colour 3.37 x 10^3 CU. ○, laccase; □, laccase plus 2,6-dimethoxyphenol 0.5 mM

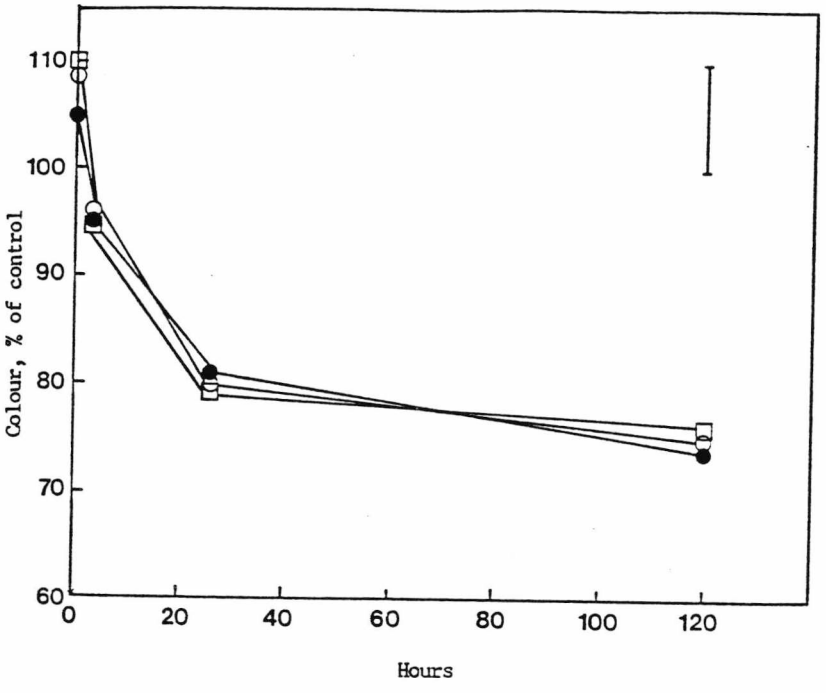

Fig. 4.4. Colour removal from E effluent by soluble laccase. ●, 98 SU/ml; ○, 197 SU/ml; □, 295 SU/ml. Initial colour 9.76 x 10^3 CU.

continued to fall.

Figure 4.4 shows the effect of increasing the amount of laccase added to E effluent over a small range of activity values (100-300 SU/ml). At this range (with a high concentration of E effluent, 9.76 x 10^3 CU) there was no significant difference between treatments. Table 4.2 shows the CU per enzyme unit per h removed in the different experiments. Decolorisation was more efficient in terms of CU/U when low laccase activities were added, implying that the laccase was substrate limited. This was confirmed by the fact that there was no linear relationship between enzyme added and total colour removed. The maximum decolorisation achieved by soluble laccase from C.versicolor(K) was 40% of E effluent using 20 GU/ml.

4.4. Decolorisation of effluents by peroxidase

Decolorisation by HRP is complicated by the bleaching effect of H_2O_2 and the inactivation of the enzyme by high H_2O_2 concentrations. Fig. 4.1 shows that both OH and S effluents were decolorised by HRP with 50 mM H_2O_2 but this was no different from H_2O_2 alone on OH effluent. However, by increasing the initial HRP activity (with 50 mM H_2O_2) decolorisation of OH was increased in a linear fashion from 47% by 50 U/ml to 59% by 200 U/ml, H_2O_2 accounting for a constant 43% colour removal in 4 d. Addition of 0.8 mg/ml BSA to OH effluent before treatment had no effect on colour removal. In these experiments HRP activity in the effluents declined rapidly and no activity could be detected after 3 d.

HRP also decolorised E effluent (42%) but to a lesser extent than OH (61%). Table 4.3 shows the CU/U enzyme removed from OH, E and S effluents with different levels of HRP. The rate of colour removal per enzyme unit decreased as the enzyme activity increased, implying that HRP is also

Table 4.2. Comparison of colour removal rates in 72 h from industrial effluents by laccase at different activity levels

Effluent type	Starting colour CU x 10^{-3}	Laccase SU/ml	CU/SU/h removed	pH of effluent
OH	2.8	150.0	0.317	5.0
E	4.0	150.0	0.550	
E	9.8	98.0	0.865	5.0
		197.0	0.515	
		295.0	0.374	
S	3.4	1156.0	0.029	5.0
	4.2	150.0	0.010	

Table 4.3. Comparison of colour removal rates in 72 h from industrial effluents by HRP plus 50 mM H_2O_2 at pH 6.0

Effluent type	Starting colour CU x 10^{-3}	HRP U/ml	CU/U/h removed
OH	3.9	8.2	2.255
		81.6	0.755
OH	3.4	50.0	0.802
		100.0	0.369
		200.0	0.331
E	4.2	134.4	1.538
		840.0	0.298
S	4.6	93.5	1.563
		840.0	0.193

substrate limited when oxidising these effluents if high initial enzyme activities are used (e.g. 200 U/ml).

4.5. Decolorisation of effluents by combined laccase and peroxidase

It was thought that by combining laccase and peroxidase, more colour could be removed from effluents, as the two enzymes have different substrate ranges. Colour removal from OH effluent by both enzymes (Fig. 4.5a) showed no significant difference from the effect of laccase alone on OH, HRP activity rapidly declined and could not be detected after 4 h whereas 90% of the initial laccase activity remained. On E effluent (Fig. 4.5b) laccase plus HRP caused a more rapid decrease in colour than either enzyme alone in the first 24 h but by 48 h the colour removal (12-18%) was not significantly different from that due to HRP or laccase alone. The low level of colour removal was due to low initial enzyme activities.

4.6. Effect of laccase on molecular weight profiles of effluents

Laccase was chosen for covalent immobilisation (Ch.6.) because it removed high levels of colour from industrial effluents, it uses molecular oxygen as the oxidant, and has a higher stability in effluents than HRP. The effect of soluble laccase on compounds of different molecular weight in the effluent was investigated more closely. Fig. 4.6 shows that effluent components of up to 20 kD were polymerised after 16 h incubation with laccase. This indicates that polymeric as well as monomeric phenolic compounds are substrates for laccase. This broad substrate range accounts for the colour removal from E effluent in which all components are larger than 1000 D (2.9.1).

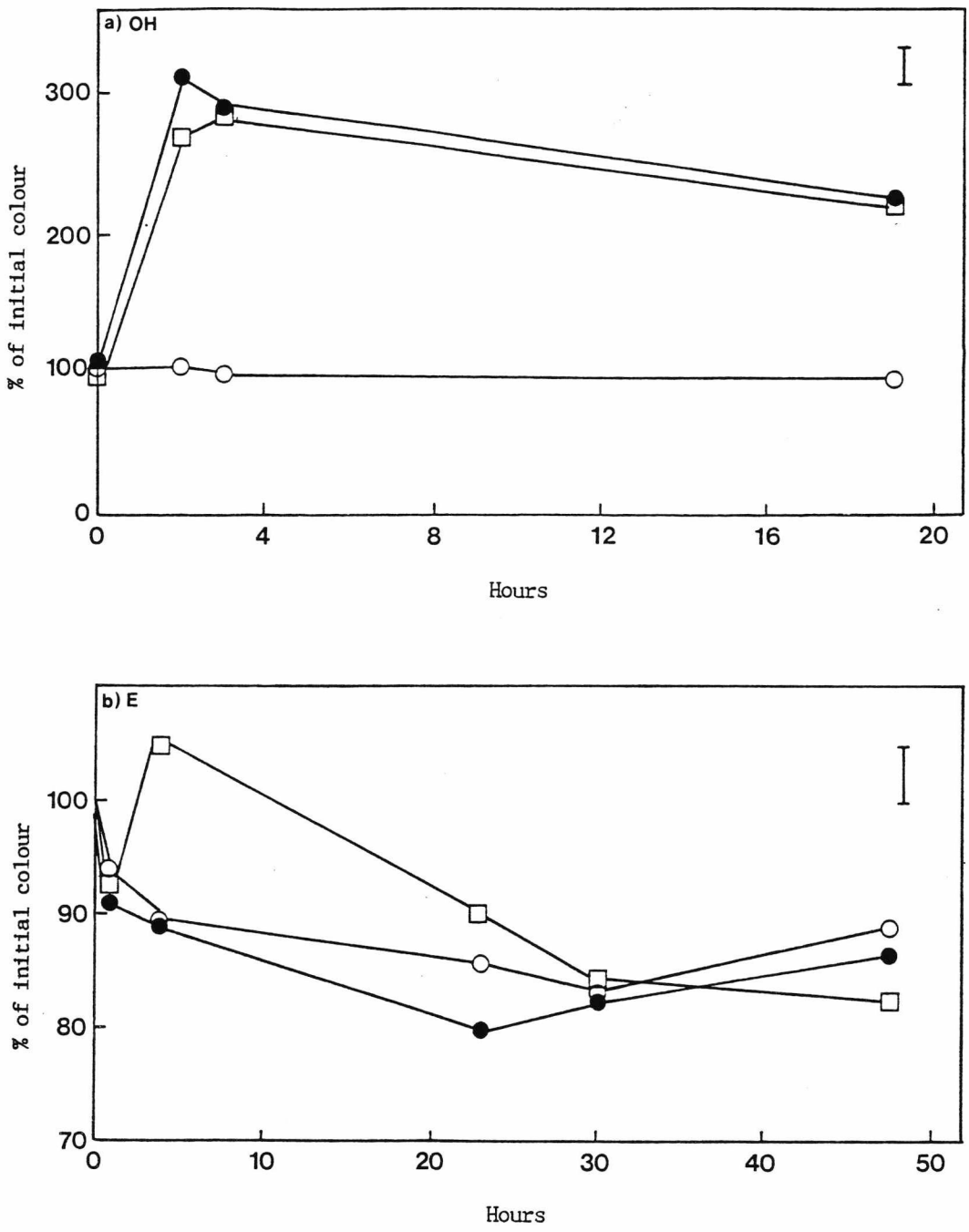

Fig. 4.5. Decolorisation of effluents using laccase and HRP combined. OH, initial colour 6.91 x 10^3 CU; E, initial colour 7.90 x 10^3 CU. ○, HRP 5.82 U/ml and 50 mM H_2O_2; □, laccase 3.86 SU/ml; ●, laccase 3.86 SU/ml, HRP 5.82 U/ml and H_2O_2 50 mM.

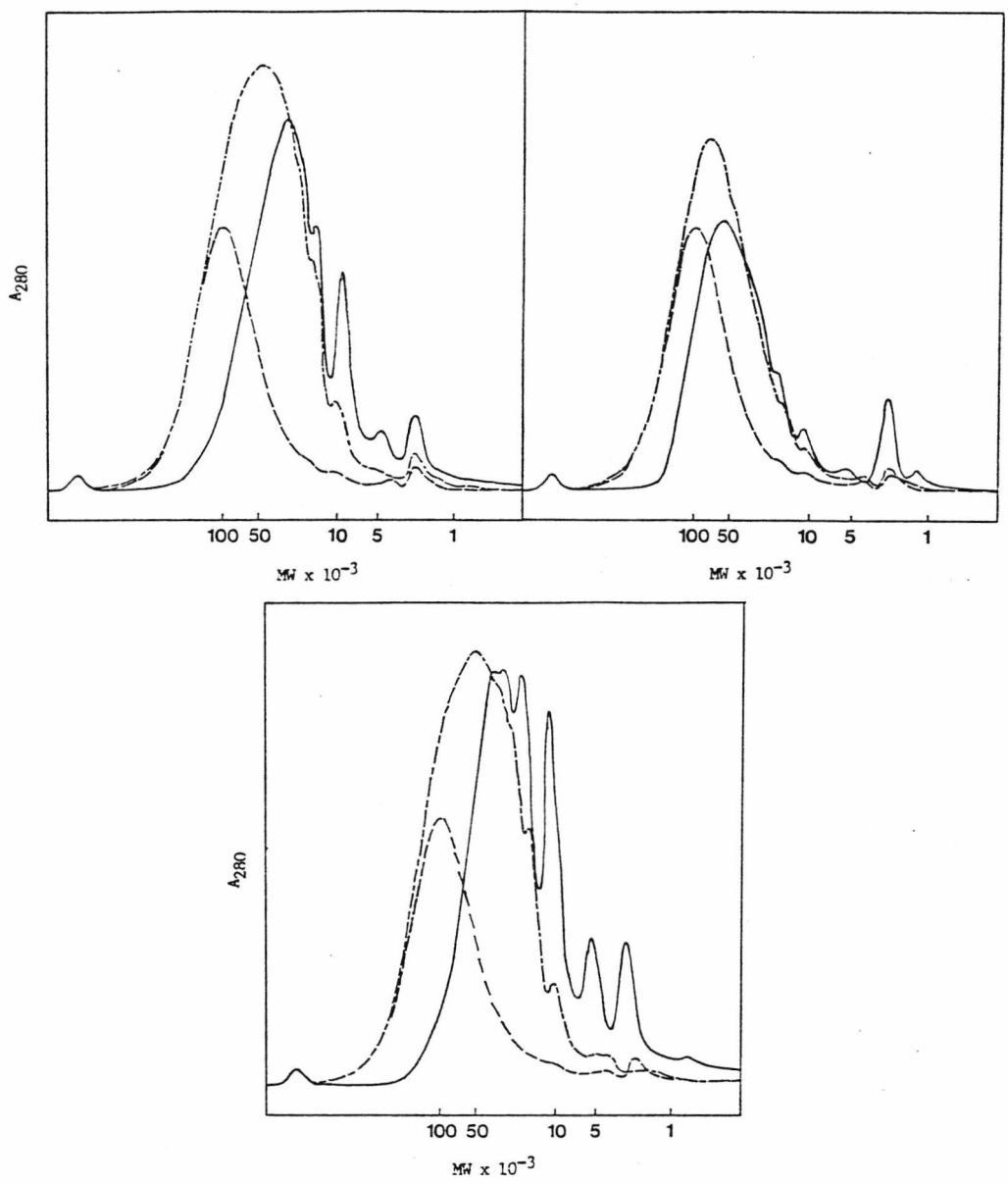

Fig. 4.6. Molecular weight profiles of effluents before _____ , and after _·_·_, incubation with laccase for 16 h (laccase – – –).

4.7. Comparison of substrates for laccase assay

Syringaldazine, guaiacol and 2,6-dimethoxyphenol (DMP) were compared as substrates for laccase with regard to colour formation and pH optima. They were all used at 0.5 mM with the same amount of enzyme in each assay. Fig. 4.7 shows that 2,6-DMP has a lower pH optimum (4.0) than the other two substrates (4.5), and that syringaldazine produced a much higher absorbance per unit enzyme than 2,6-DMP or guaiacol. For all substrates activity was negligible at pH 2.5 but a sharp rise occurred towards the optimum pH. Only activity against syringaldazine could be detected at pH 8.0.

4.8. Purification of laccase from C.versicolor(K) cultures

A rapid method of purifying large quantities of laccase was required in order to use the enzyme in further experiments. Also the properties of preparations of increasing purity were important as the cost of a treatment system would depend on the cost of laccase preparation. According to Mosbach (1963) laccase is precipitated with 80% saturation ammonium sulphate (561g/l) and this was confirmed (Fig. 4.8). However, some non-laccase protein was precipitated at 40% saturation (indicated by an increase in specific activity (SA) of the laccase remaining in supernatant) therefore preprecipitation at this point also would increase the specific activity of the final preparation. Table 4.4 shows a typical preparation from C.versicolor(K) culture filtrates, indicating that ammonium sulphate precipitation concentrated laccase approximately 10-fold, but did not increase the specific activity.

This crude laccase extract was orange in colour and was further purified by column chromatography. Using gel filtration through Sepharose 4B (Fig. 4.9) laccase was eluted as a single peak, with a specific activity

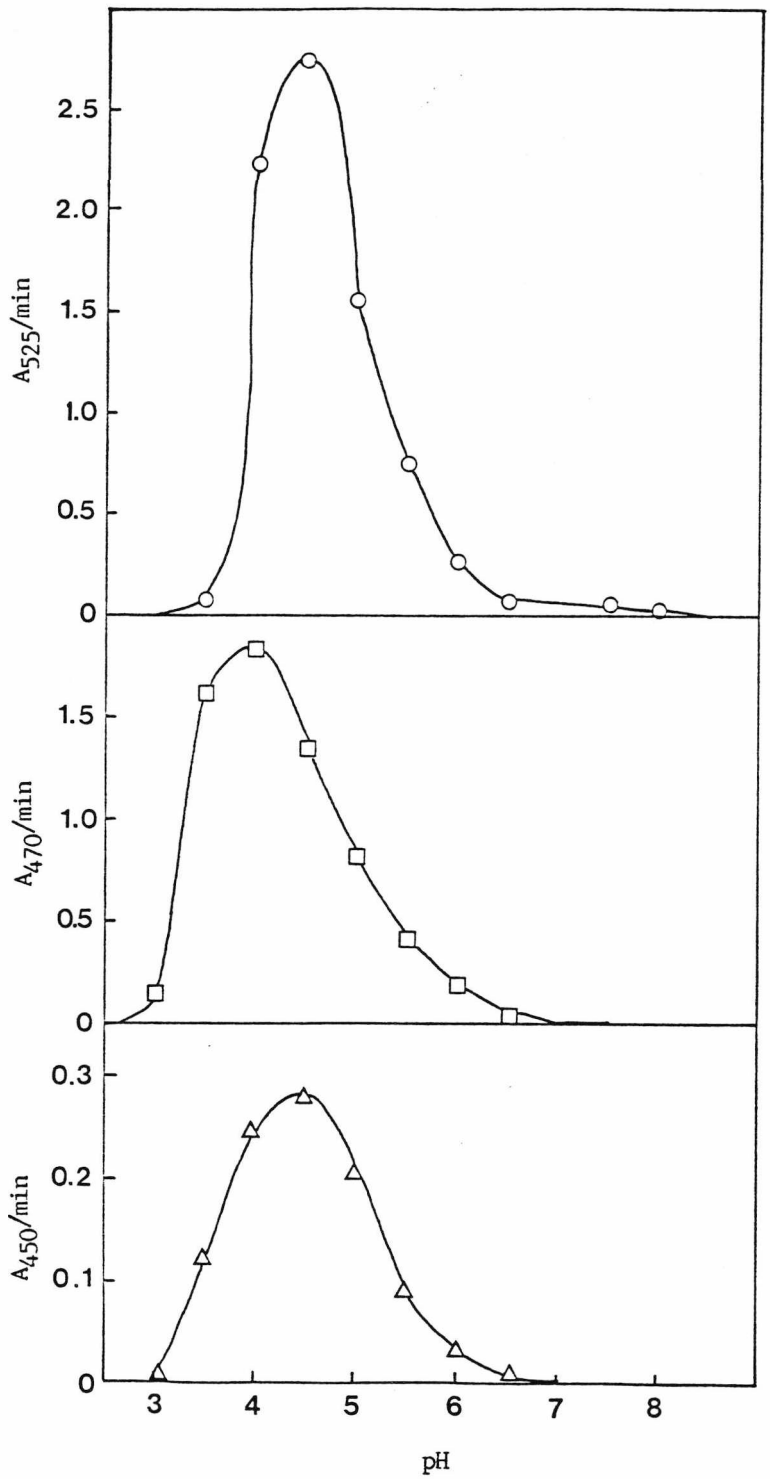

Fig. 4.7. Oxidation of substrates (0.5 mM) by laccase at different pH values. ○, syringaldazine; □, 2,6-dimethoxyphenol; △, guaiacol.

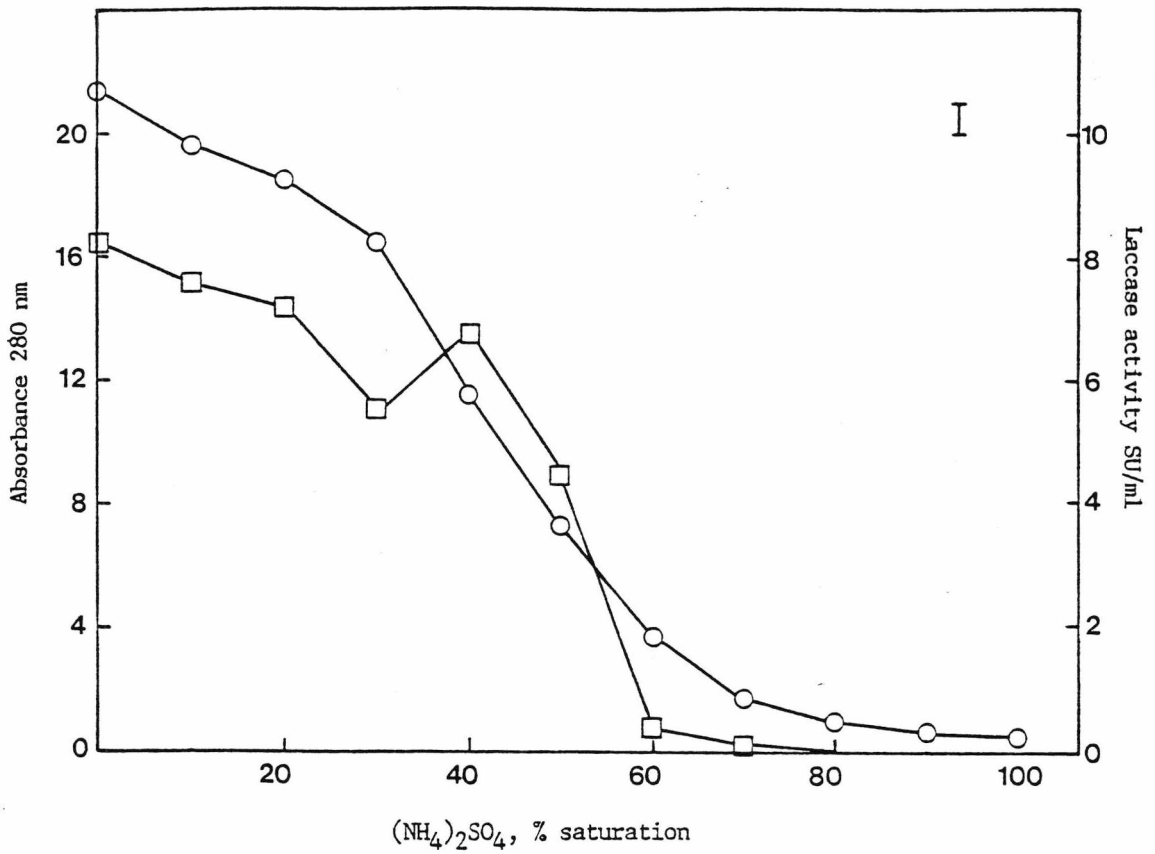

Fig. 4.8. Precipitation of laccase from culture filtrate by ammonium sulphate. □, laccase activity in filtrate; ○, A_{280} of filtrate.

Table 4.4. Laccase yield from C.versicolor (K) culture filtrate before and after precipitation with 80% ammonium sulphate and dialysis against distilled water

Purification stage	Laccase SU/ml	Protein mg/ml	Volume ml	Specific activity
Culture filtrate	236	0.038	700	6210
Dialysed	2363	0.395	70	5982

SU = syringaldazine units.

(SA) of 176 (i.e. enzyme activity divided by the absorbance at 280 nm). This preparation was still orange in colour, therefore the laccase had not been separated from the contaminating pigment. Laccase was then applied to hydroxylapatite ion-exchange resin and isocratic elution with 20 mM phosphate buffer resulted in a single peak of laccase protein (Fig. 4.10) and several peaks of protein without laccase activity. However the eluted laccase had an SA of 1259 which was lower than the initial SA of 1604. The laccase fractions were green in colour (pure laccase is blue, Fahreus & Reinhammar 1967; Morohoshi et al. 1987; Mosbach 1963), but much orange pigment remained bound to the column together with some laccase activity.

Gradient elution from hydroxylapatite produced a peak of laccase with a shoulder at 40 mM buffer (Fig. 4.11) this peak was eluted with much protein and had an SA of 649 (initial SA 1604). Stepwise elution from hydroxylapatite was then performed using a step from 20 mM to 40 mM buffer (Fig. 4.12). This produced two peaks of laccase, the first with an SA of 2041, an increase in SA of 27%, but the second was associated with large amounts of protein and pigment and the fractions were all dark orange in colour (the SA was not measured).

Purification of laccase on DEAE-Sephacel was used as an alternative ion-exchange method to hydroxylapatite. A preliminary test tube experiment showed that laccase did not bind to DEAE-Sephacel below pH 7.0 and incompletely at pH 7.0, therefore pH 7.5 Tris/HCl was used as the running buffer. Gradient elution of laccase (Fig. 4.13) produced two peaks corresponding to laccases A and B as determined by Mosbach (1963) and Fahreus & Reinhammar (1967). The first peak (laccase B) at 40 mM buffer had an SA of 1970, the second (laccase A), at 80 mM buffer had an SA of 2430, an improvement of 11% over the initial SA of 2189 and a 41%

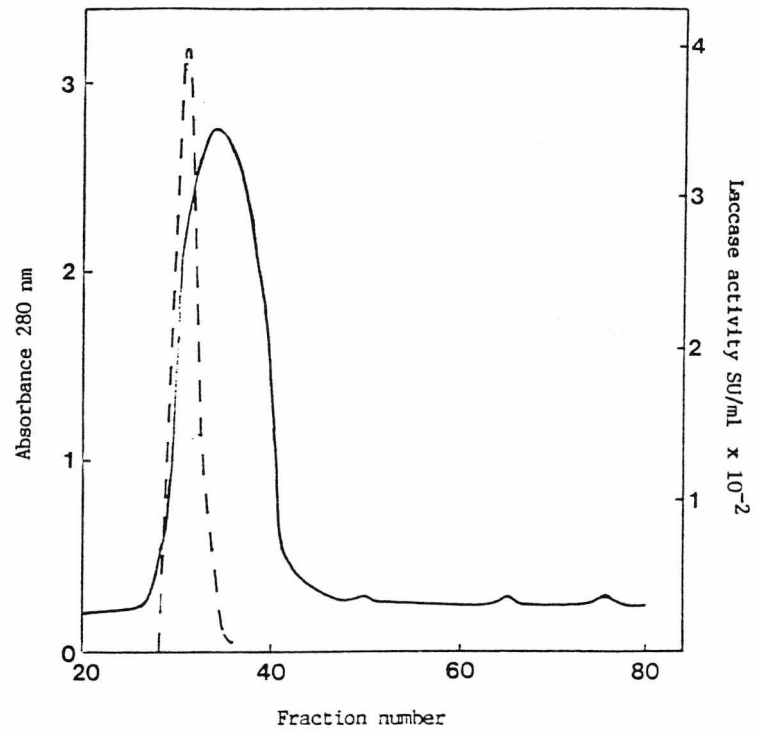

Fig. 4.9. Gel filtration of laccase with Sepharose 4B. Eluted with 10 mM acetate buffer, pH 5.0, 10 ml fractions. ——, A_{280}; – –, laccase activity.

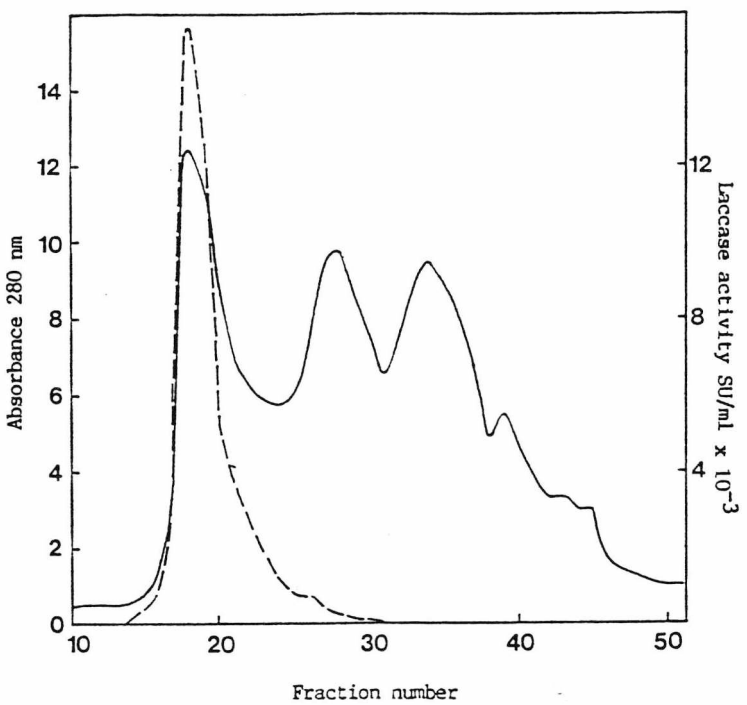

Fig. 4.10. Isocratic elution of laccase from hydroxylapatite at 25 ml/h, 2.5 ml fractions. Laccase eluted with 20 mM phosphate buffer, pH 6.0. ——, A_{280}; – –, laccase activity.

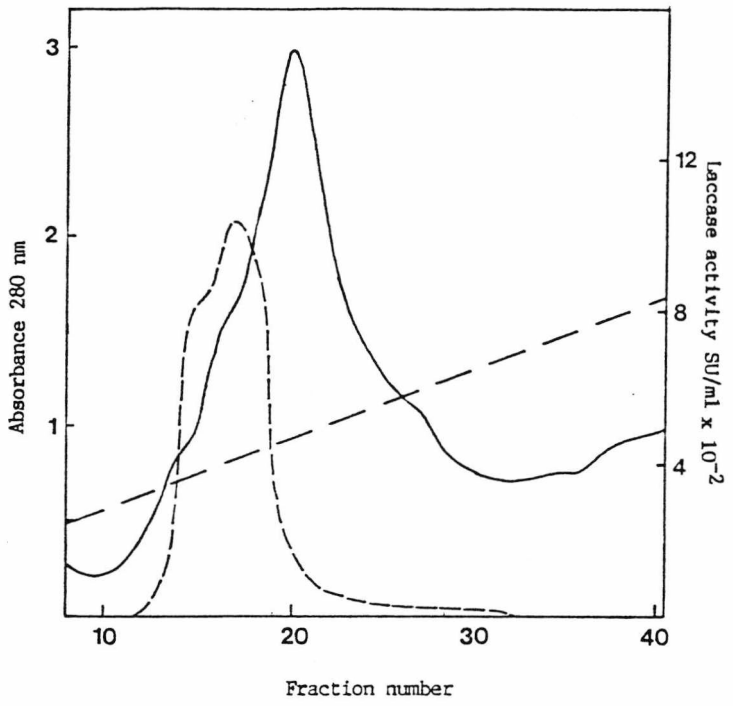

Fig. 4.11. Gradient elution of laccase from hydroxylapatite, pH 6.0, 1.6 ml fractions. ——, A_{280}; – – –, laccase activity; — —, gradient from 20–80 mM phosphate buffer.

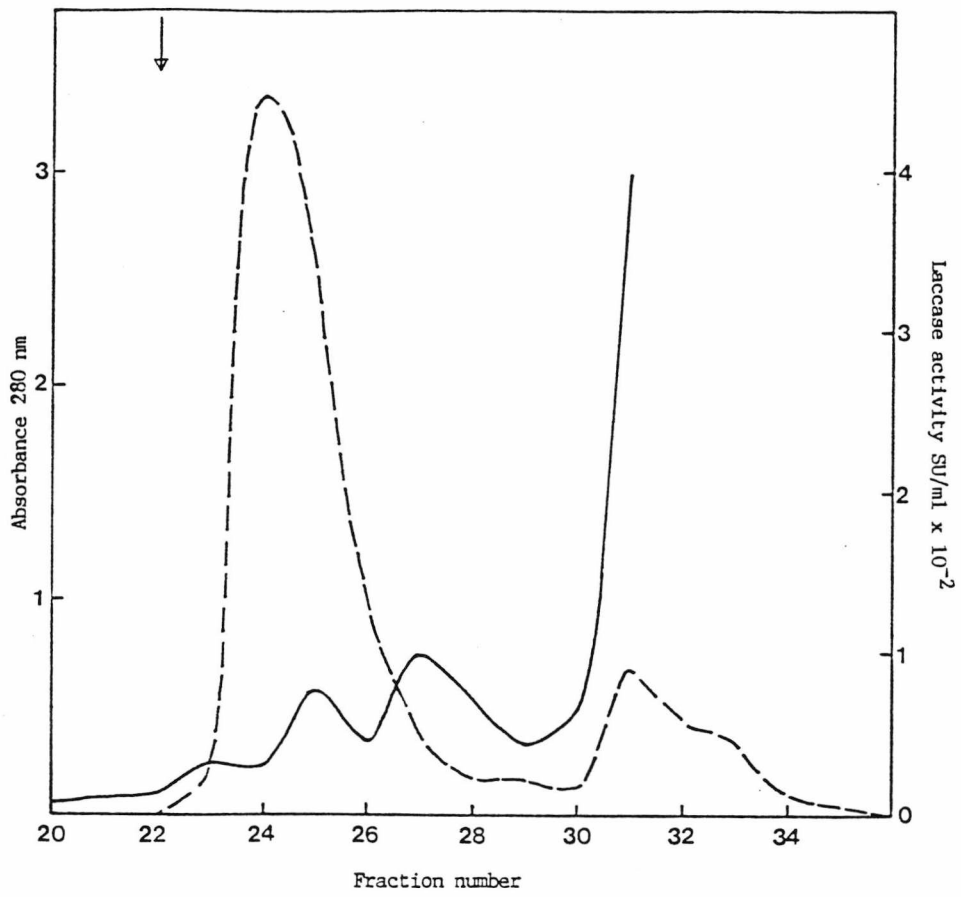

Fig. 4.12. Stepwise elution of laccase from hydroxylapatite 40 ml/h, 2 ml fractions. ——, A_{280}; – –, laccase activity; ↓, step from 0.02 M to 0.04 M phosphate buffer pH 6.0.

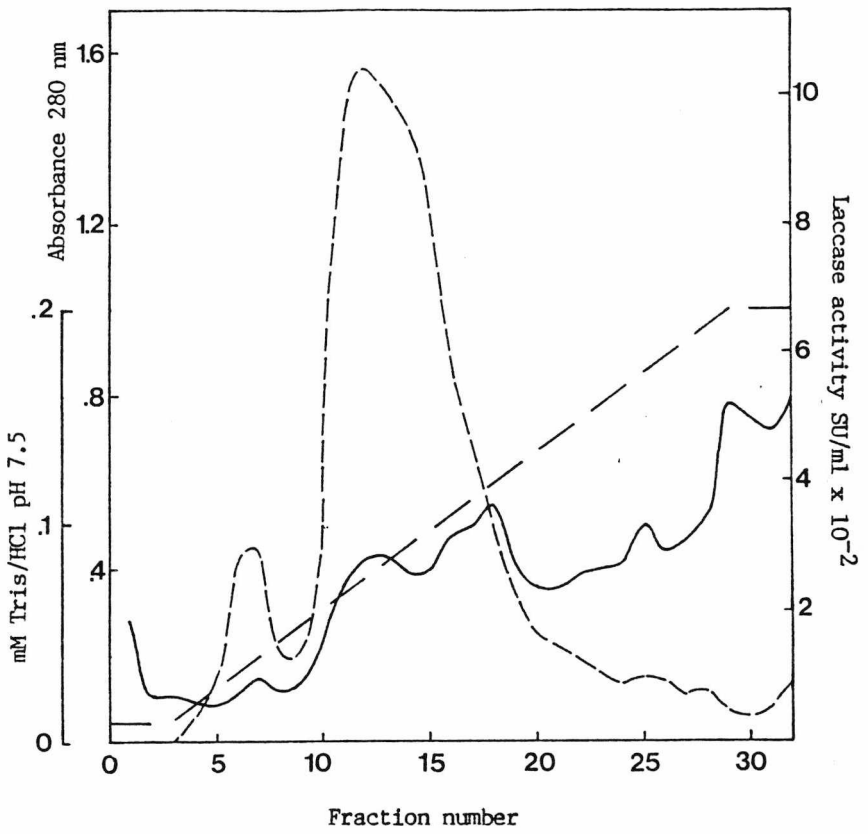

Fig. 4.13. Gradient elution of laccase from DEAE-Sephacel with pH 7.5 Tris/HCl buffer, 5 ml fractions, 20 ml/h. ——— , A_{280}; – – – , laccase activity; — - —, gradient molarity.

yield in laccase protein. Stepwise elution with 50 mM and 100 mM buffer (Fig. 4.14) produced the same peaks but this time with SA values of 2167 (B) and 2625 (A) compared to 2189. The laccase A peak was split into a high SA portion and a smaller peak with an SA of only 2005 and a total yield of 29% of added protein.

4.9. Comparison of laccase preparations of increasing purity

Three laccase preparations were compared: (i) culture filtrate; (ii) crude laccase (dialysed ammonium sulphate precipitate); and (iii) pooled laccase A from stepwise elution from DEAE-Sephacel (fractions 32-53). Some properties of the preparations are shown in Table 4.5. SDS-PAGE electrophoresis of the three preparations showed that (i) contained many bands staining for protein, (ii) contained one dense band at the appropriate molecular weight for laccase (58-64 kD) and two bands of lower molecular weight and (iii) contained a single dense protein band at the above molecular weight. Storage of the three preparations in aqueous solution (Fig. 4.15) at room temperature or at 4°C showed no significant differences in their stability with time. In all cases after 90 days storage at 4°C, 40-60% of the initial activity remained, showing that laccase has a high intrinsic stability. Laccase A lost activity at an average rate of 0.46%/d compared with a loss of 0.67%/d reported by Leonowicz et al. (1988) for a soluble laccase preparation.

Kinetics constants for laccase oxidation of syringaldazine (Fig. 4.16) show a higher K_m for laccase A (1.9×10^{-2} mM) than the crude laccase (1.26×10^{-2} mM) and a lower V_{max} value 37.7 mM/s compared to 65 mM/s.

4.10. Phenol removal from artificial effluent

The effect of laccase on artificial coal conversion effluent was also

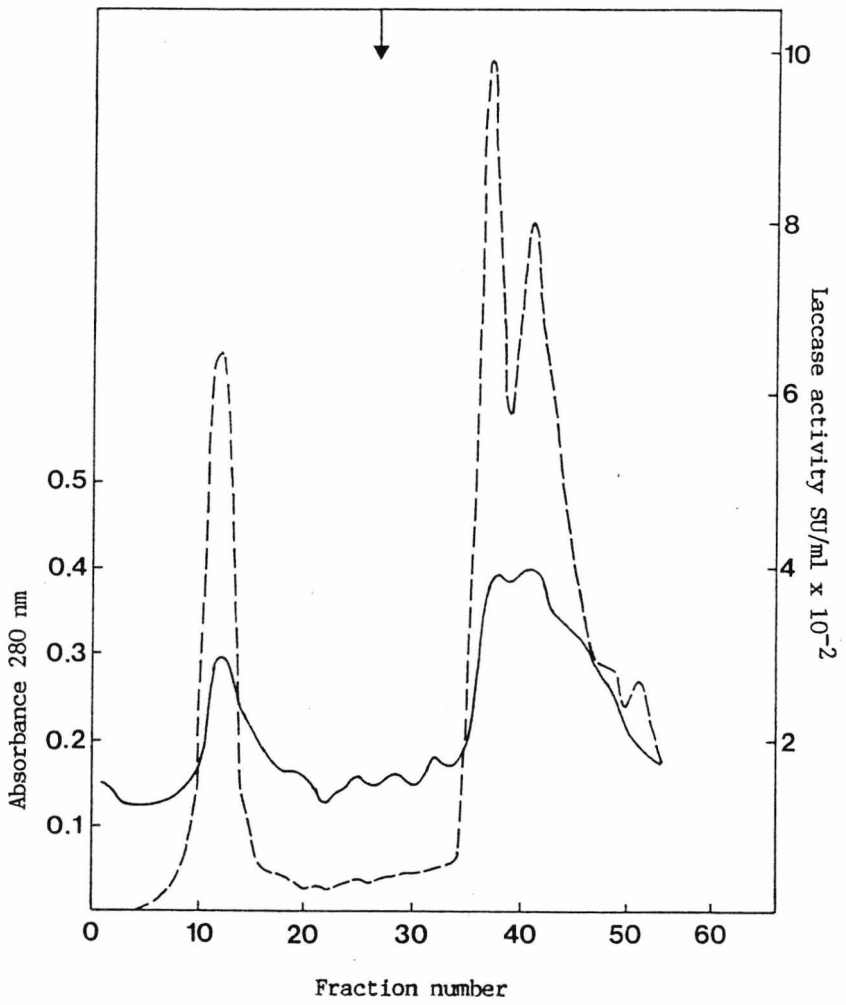

Fig. 4.14. Stepwise elution of laccase from DEAE-Sephacel, 2 ml fractions, 22 ml/h. ——, A_{280}; — —, laccase activity; ⇓, step from 0.05 M to 0.1 M Tris/HCl buffer, pH 7.5.

Table 4.5. Activity characteristics of laccase preparations of different purity

Laccase sample	Activity SU/ml	Protein mg/ml	Specific activity SU/mg
(i) culture filtrate	117.6	0.398	294.5
(ii) crude extract	250.3	0.504	496.6
(iii) laccase A	442.7	0.143	3095.8

SU = syringaldazine units.

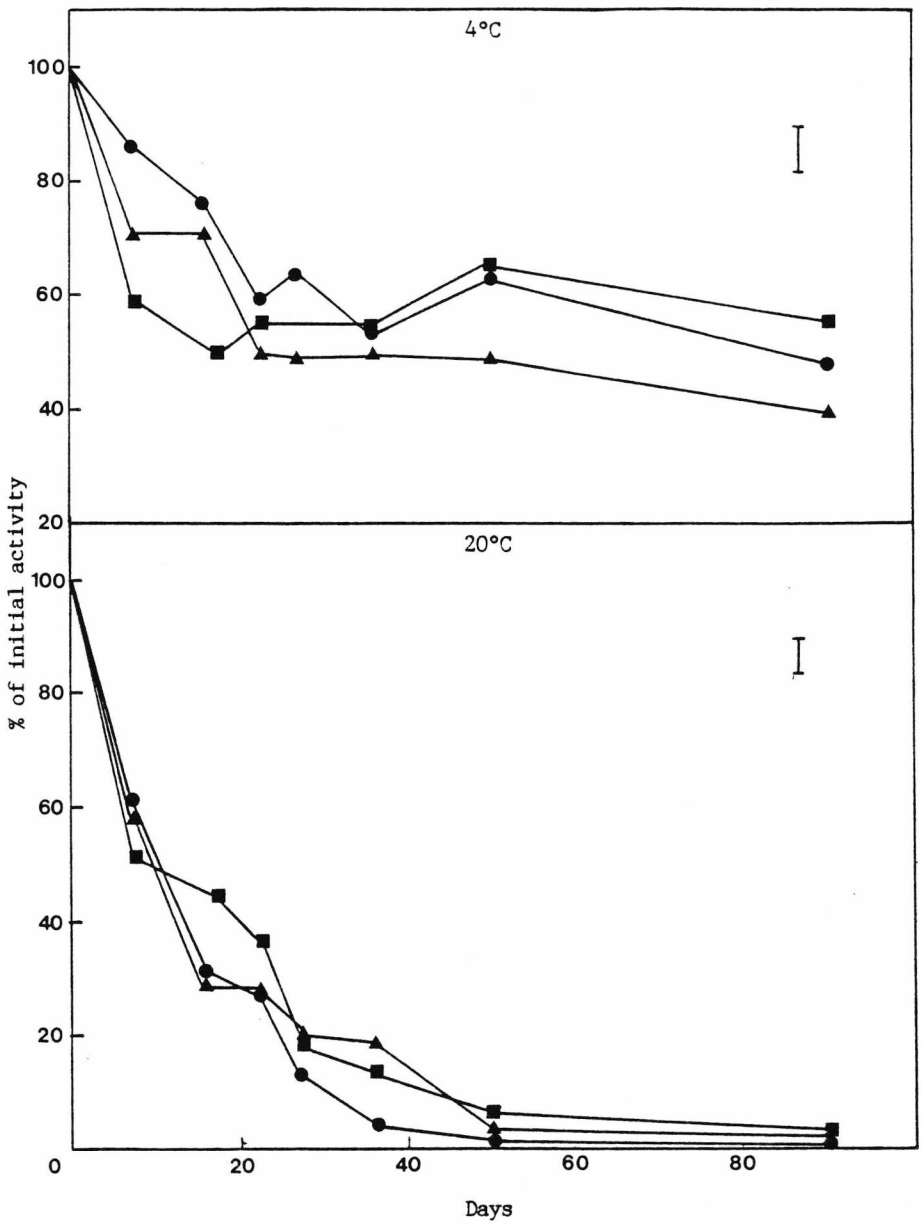

Fig. 4.15. Activity of soluble laccase preparations stored in aqueous solution. ▲, culture filtrate; ●, crude laccase; ■. laccase A.

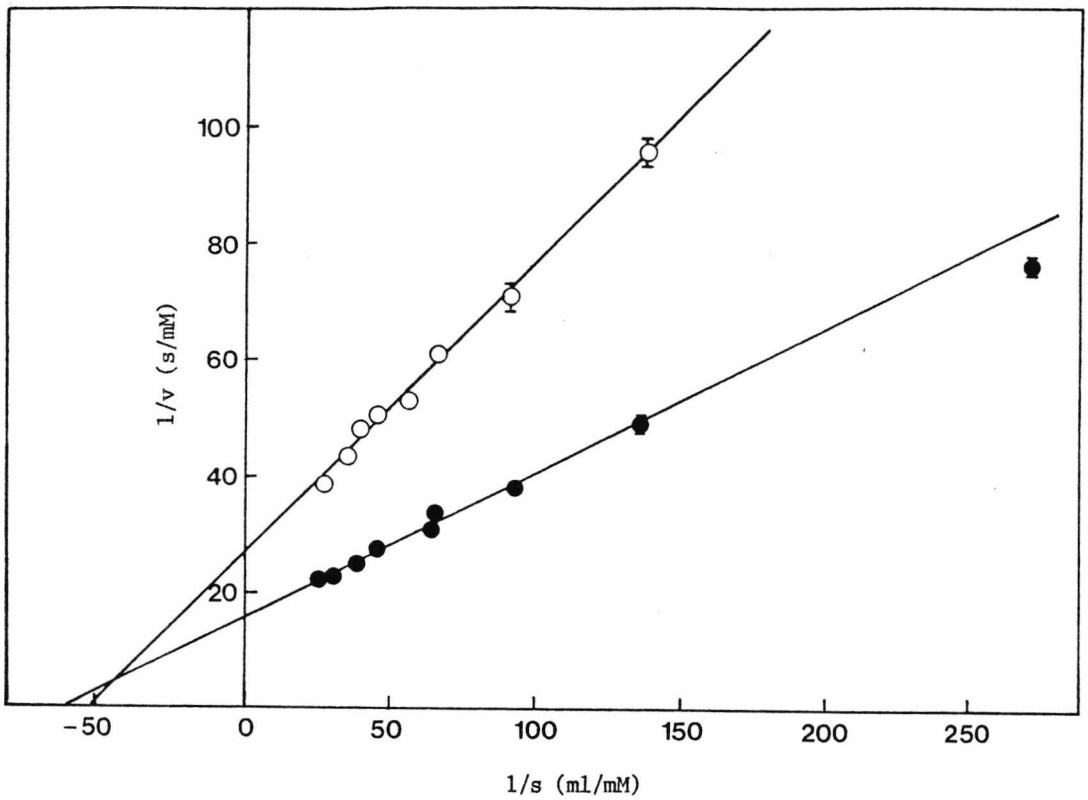

Fig. 4.16. Lineweaver-Burke plot of laccase activity on syringaldazine. ● , crude laccase preparation; ○, laccase A.

determined. This effluent had no colour, therefore phenol removal was used as a measure of treatment success (2.9.6). The pH of coal conversion effluents varied from 8.0-9.5 (Singer et al. 1977) so in our experiments artificial effluents were made up at pH 8.5 and pH 6.0 (closer to the optimum range for laccase activity of 4.0-5.0). With soluble laccase, the phenol removal was more rapid at pH 6.0 than at pH 8.5 (Fig. 4.17) but was accompanied by more rapid enzyme inactivation in solution. In both cases a dark purple precipitate with laccase activity was formed suggesting that laccase had coprecipitated with the phenol. Using an initial activity of 222 SU/ml at pH 6.0, 71% of the phenol in the artificial effluent was removed in 28 h, corresponding to a phenol removal rate of 51 mg/l/h. The rate of removal was linear from 1.5 h onwards and had not ceased after 28 h incubation.

Phenol removal from artificial effluent (pH 6.0) by equivalent amounts of laccase (i), (ii) or (iii) is shown in Fig. 4.18. Laccase in the culture filtrate (i) oxidised phenol most slowly in the first 10 h but after 30 h had removed 65% of phenol compared to 30% by laccase A (iii), with the crude laccase (ii) removing 52%. Therefore the culture filtrate had removed significantly more phenol than (ii) and (iii) after 30 h. Laccase activity in the culture filtrate was also the most stable. 100% of the initial laccase activity from (i) and (ii) was accounted for by the laccase activity of the coprecipitates formed from phenol and laccase but only 12% of the initial activity of laccase A was recovered. The maximum phenol removal rate was 23 mg/l/h in the first 30 h by the culture filtrate, lower than that achieved in the previous experiment because the initial enzyme activity added was kept low, so that differences between the laccase preparations would be apparent.

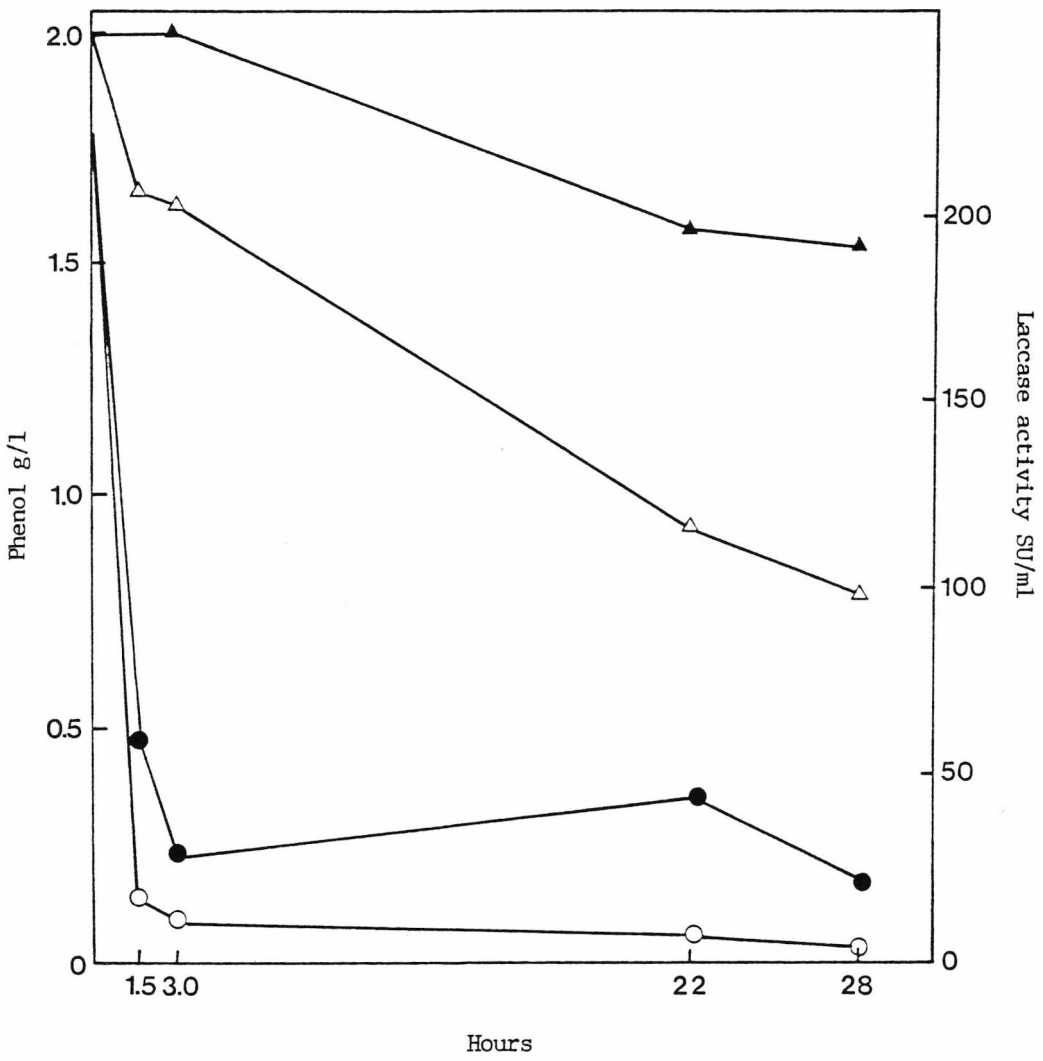

Fig. 4.17. Phenol removal from artificial coal conversion effluent by soluble laccase at pH 6.0: ○, laccase activity; △, phenol; and pH 8.5: ●, laccase activity; ▲, phenol.

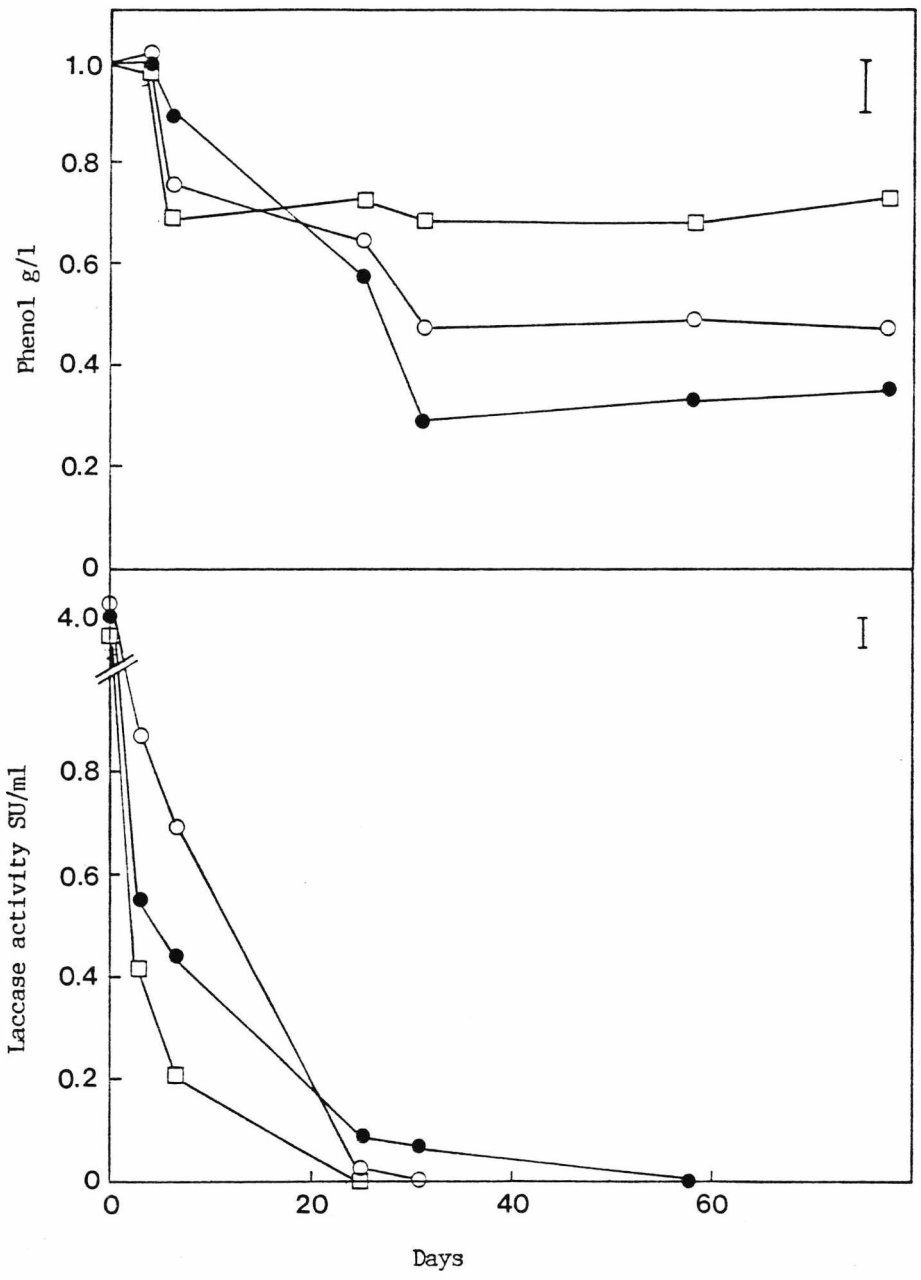

Fig. 4.18. Phenol removal from coal conversion effluent pH 6.0 by laccase preparations of increasing purity. ●, culture filtrate; ○, crude laccase preparation; □, laccase A.

DISCUSSION

Soluble phenol oxidases are commonly described as oxidisers of monomeric phenols, forming insoluble polymeric products (Alberti et al. 1981; Sjoblad & Bollag 1981) but there is little information on the effect of these enzymes on compounds with higher molecular weights, such as polymeric lignosulphonate material (Forss 1987). The maximum decolorisation of phenolic effluents achieved by soluble laccase was 36% of OH and 40% E after 2 days and 30% S after 4 days, at pH 5.0, 25 °C. This is the first report of colour removal from phenolic effluents by laccase. The mechanism of decolorisation is thought to be via oxidation of coloured compounds to phenoxy radicals, followed by spontaneous polymerisation to form insoluble precipitates, as precipitate formation was a characteristic feature of laccase treated effluents.

Crude laccase from C.versicolor(K) decolorised effluents much more effectively than the purified commercial laccase from P.oryzae. The reason for this is not known, but it could be due to the P.oryzae laccase either having a very low substrate range or a low stability. Crude laccase from C.versicolor was much more stable during phenol oxidation than purified forms (see 4.9) suggesting that the contaminating protein in the crude preparation exerted a protective effect on laccase during substrate oxidation. Increases in the colour of S effluent by laccase were attributed to a low enzyme activity that was sufficient to cause limited oxidation of phenolic compounds but not enough to give precipitate formation. Laccase was less effective at decolorising S effluent than OH or E effluents, possibly due to an inhibitory effect of the high sulphide content of S effluent.

Using increased tyrosinase concentration had no effect on reducing the

colour of the effluents. In fact OH and S effluent colour increased and therefore tyrosinase was not considered appropriate for further study. The inability of tyrosinase to decolorise the effluents studied here may be due to its relatively small substrate range compared with laccase and peroxidase (Sjoblad & Bollag 1981). This result was disappointing because tyrosinase has previously been shown to remove phenol from less complex industrial effluents (Atlow et al. 1984) and unlike laccase and peroxidase has not shown evidence for a reaction mechanism using free radicals (Sjoblad & Bollag 1981). Tyrosinase therefore should be more resistant to reaction inactivation.

Decolorisation of OH and S effluents by laccase containing cell extracts was 7% greater than that by culture filtrates, possibly due to the more complex mixture of intracellular enzymes released from the cells compared to those in the culture filtrate. There was no correlation of decolorisation with laccase activity, or with culture age. This is surprising because after several days growth and therefore depletion of nutrients such as carbon and nitrogen by C.versicolor, the lignolytic enzyme system should be induced, resulting in colour removal from phenolic effluents (Eriksson & Kirk 1985; Kirk & Farrell 1987; Sundman et al. 1981). However the time scale of the experiment may have been too short to reveal such a trend. Also, induction may have occurred in cultures grown on malt extract broth because malt extract was shown to induce laccase production by C.versicolor (Chapter Three), so may have induced other components of the lignolytic enzyme system which were not assayed. The lack of decolorisation of OH by filtrates from OH supplemented medium could be attributed to slower growth and enzyme synthesis on this medium, greater decolorisation might have been achieved using filtrates from longer incubations.

Despite reports that laccase can be protected from the inactivating effects of phenoxy radicals (produced during the oxidation of monomeric phenols) by the addition of BSA, gelatin or Tween 80 (Fahreus & Ljunggren 1961; Shuttleworth & Bollag 1986), in our experiments these compounds made no difference to the decolorisation of OH effluent by soluble laccase, nor to the loss in enzyme activity during decolorisation. Also the addition of an easily removable phenol (DMP) to the reaction mixture made no difference to the decolorisation of S effluent by laccase, in fact it caused an increase in the final colour. These observations lead to the conclusion that the effluents treated here have a very low monomeric phenol content and colour removal represents the oxidation of larger compounds such as polymeric phenols. Therefore, loss in laccase activity is not due to the coupling of phenoxy radicals to the protein. Also, DMP may not copolymerise readily with the compounds in the S effluent. As this seems to be the case, a range of phenolic substrates for laccase should be tested for more efficient copolymerisation with effluent components which would result in improved decolorisation of S effluent.

Increasing the concentration of laccase added to effluent by 2-3 fold resulted in little additional colour removal over the concentrations used, implying that either a much greater increase in initial laccase activity was required to produce a significant increase in colour removal or that maximum colour removal was achieved using the lower laccase activity. The latter is not thought likely because a high concentration of E effluent (9.8×10^3 CU) was used. Decolorisation was rapid over the first 24 h (99 CU/h) but then ceased. High effluent concentrations were not inhibitory to enzyme activity as increasing the E effluent from 4.0×10^3 to 9.8×10^3 CU did not reduce the rate of decolorisation. Evidence

that laccase was substrate limited at the concentrations of effluent used in these studies comes from the observation that increases in enzyme activity lead to a decrease in the rate of colour removal.

Provided that hydrogen peroxide concentrations were kept constant, a linear relationship of colour removal to added HRP activity was observed with OH effluent. The maximum decolorisation by HRP was 61% of OH in 7 d, 51% of S in 7 d and 36% of E in 3 d. Decolorisation of E effluent was less than the 58% maximum colour removal from E effluent reported by Paice & Jurasek (1984) but a comparison between initial enzyme units was prevented by the different substrate (guaiacol) which they used for HRP activity assays. Hydrogen peroxide functions both as a bleaching agent and as a cosubstrate for HRP, and at high peroxide:enzyme ratios all colour removal can be accounted for by the peroxide because the enzyme is very quickly inactivated. HRP also appeared to be substrate limited, because as enzyme levels increased the rate of colour removal per enzyme unit decreased. The work of Paice & Jurasek (1984) used a similar concentration of effluent to that used here (4.6-4.8 x 10^3 CU), however they did not examine the effect of changing the substrate concentration.

Combining laccase and peroxidase did not increase decolorisation, in fact the colour removed from OH or E was never more than that removed by one enzyme acting alone. This could be due to all the compounds being substrates for both enzymes, or possibly substrate limitation. However, very low levels of activity were used to avoid the latter possibility. GC-MS of OH effluent indicated the presence of 16 aromatic compounds (2.9.1) of which phenol, methoxycinnamic acid and hodroxymethoxybenzoic acid are known to be substrates for laccase.

The examination of molecular weight profiles of the three effluents before and after incubation with laccase confirmed that the effluents

contained relatively small amounts of monomeric phenols. Also laccase activity was clearly not limited to low molecular weight (<1000 D) compounds but the enzyme polymerised compounds up to 20 kD in size. Similar results were obtained by Forss et al. (1987) who showed that laccase could polymerise lignosulphonates (from spent sulphite liquor) of up to 500 kD. Furthermore, in debarking water laccase polymerised compounds of 1.5 kD to over 10 kD in size. When immobilised peroxidase from Trametes versicolor was incubated with lignosulphonates for 3-24 h, compounds of 4.2-4.5 kD were polymerised to 7, 8 and 45-50 kD compounds, and occasionally (depending upon the enzyme fraction used) small amounts of low molecular mass compounds (2.5-2.8 kD) were formed (Lobarzewski et al. 1982)

It was necessary to compare the different laccase substrates used by others for assay as they were found to have particular advantages and disadvantages in practical use. Guaiacol was used in this work when low activities were being measured as a longer incubation time could be used. Syringaldazine, although producing a higher absorbance per unit enzyme, has a high ethanol concentration in the reaction mixture (17-30%), which can cause variation in results during prolonged incubations. Syringaldazine is oxidised to tetramethoxyazo-p-methylenequinone (Harkin & Obst 1973) whereas guaiacol is oxidised to carbon-carbon and carbon-oxygen coupled dimers trimers and tetramers (Fig. 4.19) (Sjoblad & Bollag 1981). These show on an absorbance spectrum as two peaks of maximum absorbance in the visible wavelengths compared with one peak produced by DMP and syringaldazine. Dimethoxyphenol is oxidised to one product, tetramethoxydiphenoquinone, therefore it was used in oxygen uptake assays to replace syringaldazine and eliminate alcohol from the reaction mixture.

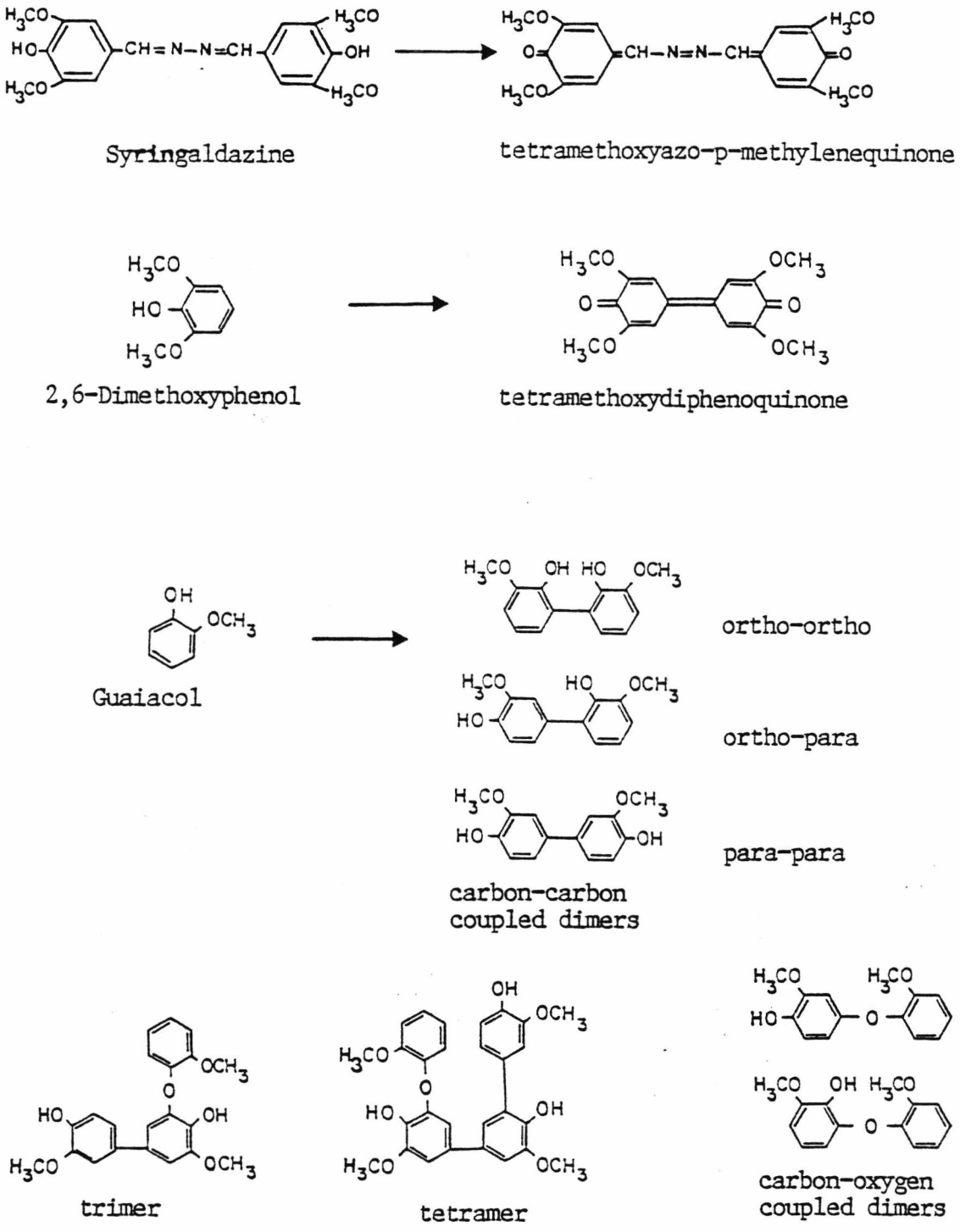

Fig. 4.19. Oxidation products of laccase assay substrates.

Laccase has previously been purified by a number of authors using a variety of methods (1.4.2). They were compared here for the production of a pure laccase sample for comparison with crude preparations. Precipitation with ammonium sulphate did not increase the specific activity of the preparation, but served to concentrate the laccase 10-fold from the culture filtrate and remove contaminating polysaccharide. This result is in conflict with a lone report by Fahreus & Reinhammar (1967) who obtained a 10-fold increase in specific activity of laccase by ammonium sulphate precipitation. The orange pigmentation of the crude laccase was thought to be caused by its association with the inducer 2,5-xylidine or the formation of polymerised high molecular weight xylidine oxidation products also associated with the enzyme. This pigmentation was also mentioned by Mosbach (1963) and Fahreus & Reinhammar (1967) who used xylidine to induce C.versicolor laccase, but not by Morohoshi et al. (1987) who did not use an inducer. In practise the pigment was very difficult to separate from the laccase.

Stepwise elution from hydroxylapatite was more successful than isocratic or gradient elution, but did not completely separate laccase from the pigment, contrary to reports by Mosbach (1963). Laccase from the hydroxylapatite was scanned for absorbance in the visible region and showed an increase in absorbance at 405 nm corresponding with that found by Mosbach (1963).

DEAE-cellulose has also been used to purify laccase with phosphate buffer at pH 6.0 (Fahreus & Reinhammar 1967) and pH 7.0 (Mosbach 1963). Using DEAE-Sephacel and Tris buffer (as recommended by Pharmacia), laccase did not bind to the matrix at pH 6.0 but bound strongly at pH 7.5, which was therefore used to provide the optimum conditions for laccase elution using ionic concentration, by providing strong binding to laccase at low

ionic concentrations. Using phosphate with an anion exchange resin can cause interference with binding. DEAE purification was found superior to hydroxylapatite as the laccase was separated into the A and B forms described by Mosbach (1963) and Fahreus & Reinhammar (1967). However this still did not remove all of the contaminating pigment although a high proportion of it remained firmly bound to the column. Therefore laccase A from DEAE purification was chosen for further study.

The stability of all three forms of laccase to long term storage at 4°C shows that even in a purified form laccase has a high intrinsic stability. However during phenol removal from artificial effluent it was clear that the laccase in the culture filtrate was significantly more stable during oxidation of substrates than laccase A. This could be due to the presence of other proteins in the filtrate protecting the enzyme from inactivation by the free radicals produced during the reaction. This would also explain why the addition of BSA to reaction mixtures had no effect on the stability of the crude laccase. The difference in kinetic properties of crude laccase and laccase A is the reverse of that usually encountered on enzyme purification. However, in comparison with the K_m of 0.03 mM (Milstein *et al.*, 1989) and of 17.2 mM (Leonowicz & Grzywnowicz 1981) both K_m values (0.019 mM, laccase A; 0.013 mM, crude laccase) are very much smaller indicating that the laccases studied here have a higher affinity for syringaldazine than the laccases from *Trametes versicolor*. A decrease in affinity due to purification can be caused by the removal of a cofactor such as a metal ion which enhances enzyme activity. However there are no reports in the literature of a cofactor for laccase, with the possible exception of the stimulation of laccase oxidation of p-cresol (but not other phenolic compounds) by Fe^{2+} ions observed by Fahreus & Ljunggren (1961). This work is also the only published

comparison of kinetic data for crude and purified forms of laccase.

In addition to precipitation of coloured phenolic compounds, phenol oxidase enzymes are known to precipitate a range of monomeric phenols from aqueous solutions including effluents in which colour is less of a problem (1.4) (Klibanov et al. 1983). This treatment is accompanied by enzyme inactivation therefore improvements in stability due to immobilisation would also be useful, e.g. for treating coal conversion effluents. The pH of real coal conversion effluents varies from 8.0-9.5 (Singer et al. 1977) so in our experiments with artificial effluents soluble laccase was added at pH 8.5 as well as at pH 6.0 which is closer to the optimum for its activity. Laccase removed phenol at pH 6.0 at a rate of 50.7 mg/l/h, a much greater removal than that of 3 mg/l/h obtained by Shuttleworth & Bollag (1986) with Rhizoctonia praticola laccase (using activity equivalent to 1000 U/ml). However by increasing laccase concentration in parallel with phenol concentration and temperature, this removal rate could be considerably improved.

Using HRP and tyrosinase did not remove any significant amount of phenol from artificial effluent after 48 h. This is in contrast with reports in the literature of good phenol removal from phenolic effluents by both HRP and tyrosinase (Alberti & Klibanov 1981; Atlow et al. 1983; Klibanov et al. 1983) using similar amounts of added enzyme.

In conclusion, soluble laccase and HRP showed good decolorising activity with industrial effluents. Nevertheless, decolorisation was restricted by enzyme inactivation and therefore immobilisation of the enzymes, which can result in increases in enzyme stability and would facilitate treatment of effluents in continuous systems, was selected as the next step in the development of an effluent tretment system. Different types of enzyme immobilisation are discussed in Chapters Five and Six.

CHAPTER FIVE

TREATMENT OF EFFLUENTS BY CALCIUM ALGINATE ENTRAPPED ENZYMES

INTRODUCTION

Chapter Four described how soluble enzymes were effective in removing colour from phenolic effluents, but showed that enzyme inactivation occurred during the reaction. Therefore immobilisation methods were investigated with the aim of increasing operational stability and longevity of the enzymes during effluent treatment. A successful immobilised enzyme treatment system would also significantly reduce the cost in comparison to the successive use of batches of soluble enzymes. Matrix entrapment, such as occurs in alginate beads, provides a rapid and simple method of immobilising enzymes and is well known to enhance stability and protect the enclosed enzyme against microbial attack (Kennedy et al. 1988). No chemical or physical bond is formed between the enzyme and the gel matrix, reducing the chance of inactivating the enzyme by altering its structure or by binding to the active site. The relevant property of a gel matrix is that it contains pores large enough for the substrate to penetrate and for the product to diffuse from the gel but small enough to prevent the enzyme from escaping. Gel filtration chromatography of the three industrial effluents (4.6) showed the presence of compounds from 1-150 kD in size, compared with the crude laccase which had a molecular weight of approximately 100 kD. Therefore by entrapping laccase in gel matrix, potential substrates above 100 kD would be excluded. However, since compounds < 20 kD were shown (4.6) to be polymerised by laccase to a greater extent than those of higher

molecular weight, a porous gel retaining laccase is likely to admit most of the substrate molecules.

Synthetic polyacrylamide gels have also been widely used for enzyme immobilisation, but have a much smaller pore size than alginate and can even limit the diffusion of low molecular weight compounds such as KCl and urea (White & Dorion 1961). Furthermore, the generation of free radicals during gel formation may affect the activity of the immobilised enzyme. Alginate gels have a mean pore size of 10 μm (Scherer et al. 1981) although the surface of the gel has smaller pores than the bulk phase, even so small molecules will not be retained without further crosslinking of the surface. Problems with alginate gel entrapment may be encountered due to gradual disintegration of the alginate if phosphate or citrate buffers are used as they withdraw the calcium from the alginate. Therefore Tris buffers are generally used. Alginate beads are soft and can be deformed by gentle pressure, but can be hardened by drying (using normal pressure and elevated temperature). The resulting shrunken beads do not swell or soften when rehydrated (Klein & Wagner 1978).

Alginic acid is a copolymer of β-D-mannuronic acid and α-L-guluronic acid linked by (1-4)-glycosidic linkages. It is produced by brown algae (Phaeophyceae) and commercial preparations are derived from Macrocystis, Laminaria and Ascophylum species. Ratios of the two acids vary according to the source and will influence gel formation, and most alginic acids consist of homopolymeric blocks of D-mannuronic acid and L-guluronic acid interspersed with mixed polymers (McDowell 1977). Most alginates are sold as the sodium salt but preparations can contain other cations such as calcium, magnesium and potassium. In our study sodium alginate from Laminaria hyperborea was used.

Enzymes can be immobilised by copolymerisation with phenolic compounds to

form synthetic phenolic-enzyme complexes (1.4.5). These complexes have shown increased stability against microbial attack and greater longevity than soluble enzymes (Sarkar & Burns 1984). Copolymerisation also results in an enzyme preparation with a greater molecular weight than the soluble enzyme and may increase the retention of the enzymes in calcium alginate gel.

The aims of the work described in this chapter were: (i) to copolymerise laccase and peroxidase with phenolic compounds; (ii) to immobilise laccase and peroxidase by entrapment in alginate; (iii) to assess the capacity of the immobilised enzymes for colour removal from industrial effluents; (iv) to assess the stability of the entrapped enzymes and the copolymers during effluent treatment and (v) to compare any colour removal from phenolic effluents achieved with the entrapped enzyme preparations with that shown with soluble enzymes in Chapter Four.

RESULTS

5.1. Copolymerisation of phenol oxidases with phenolic compounds

Initially an attempt was made to increase the stability of the enzymes by forming synthetic polyphenolic-enzyme complexes (Sarkar & Burns 1984) (2.12.1). Using HRP or tyrosinase as the polymerising enzyme, several combinations of enzyme with different phenolics were tried (Table 5.1). In all cases HRP-catalysed copolymerisation of laccase with resorcinol or pyrogallol resulted in a complete loss of activity. However, when tyrosinase and tyrosine were used, both laccase and HRP retained some activity in the resulting colloidal black copolymers. Unfortunately a considerable loss of activity occurred and only 12% (laccase) and 38%

Table 5.1. Copolymerisation of phenol oxidases with phenolic compounds and resulting phenol oxidase activity in copolymer

Catalyst	Phenolic compound	Enzyme to be copolymerised	Activity in copolymer %
HRP	Resorcinol	Laccase from *P.oryzae*	0
HRP	Resorcinol	Laccase from *C.versicolor*	0
HRP	Pyrogallol	Laccase from *C.versicolor*	0
Tyrosinase	Tyrosine	Laccase from *C.versicolor*	12
Tyrosinase	Tyrosine	HRP	38

(HRP) were measured in the copolymers. Nonetheless, the activity obtained and the possibility of it being more stable than the soluble enzymes warranted a further study of the tyrosine-enzyme copolymers. Due to the colloidal nature of the black copolymers formed, it was necessary to further immobilise them in alginate to prevent interference with colour measurements during effluent treatment.

5.2. Immobilisation of laccase or HRP in calcium alginate gel

Laccase and HRP could be entrapped in calcium alginate gel beads, but on incubation in effluent or even in buffer considerable amounts of enzyme were released. (Figs. 5.1; 5.2). Entrapped soluble enzyme and entrapped enzyme-tyrosine copolymers were released at the same rate. Also enzymes and copolymers released from beads retained activity equally well in solution except for released HRP-tyrosine copolymers which lost activity faster than HRP.

The activity measured on a per bead basis represented a very large initial loss in laccase (-97%) or HRP (-89%) activity on entrapment (Table 5.2). A more representative estimate of bead activity is obtained by calculating the total enzyme released from beads into solution, during incubation of beads in buffer (Figs. 5.1;5.2). The figures show that there is a rapid release of enzyme from the alginate, although the beads retained considerable activity after 20 h incubation. For example, HRP entrapped in alginate gave a total activity measured in the beads of 10 508 U; in agitated buffer a total activity of 88 320 U was measured as being released from the same beads (not correcting for any activity loss on incubation). Therefore the activity in the beads was underestimated by at least a factor of 8.4. In the same way the entrapped activity of HRP-tyrosine copolymers was understimated by 4.2, and that of entrapped

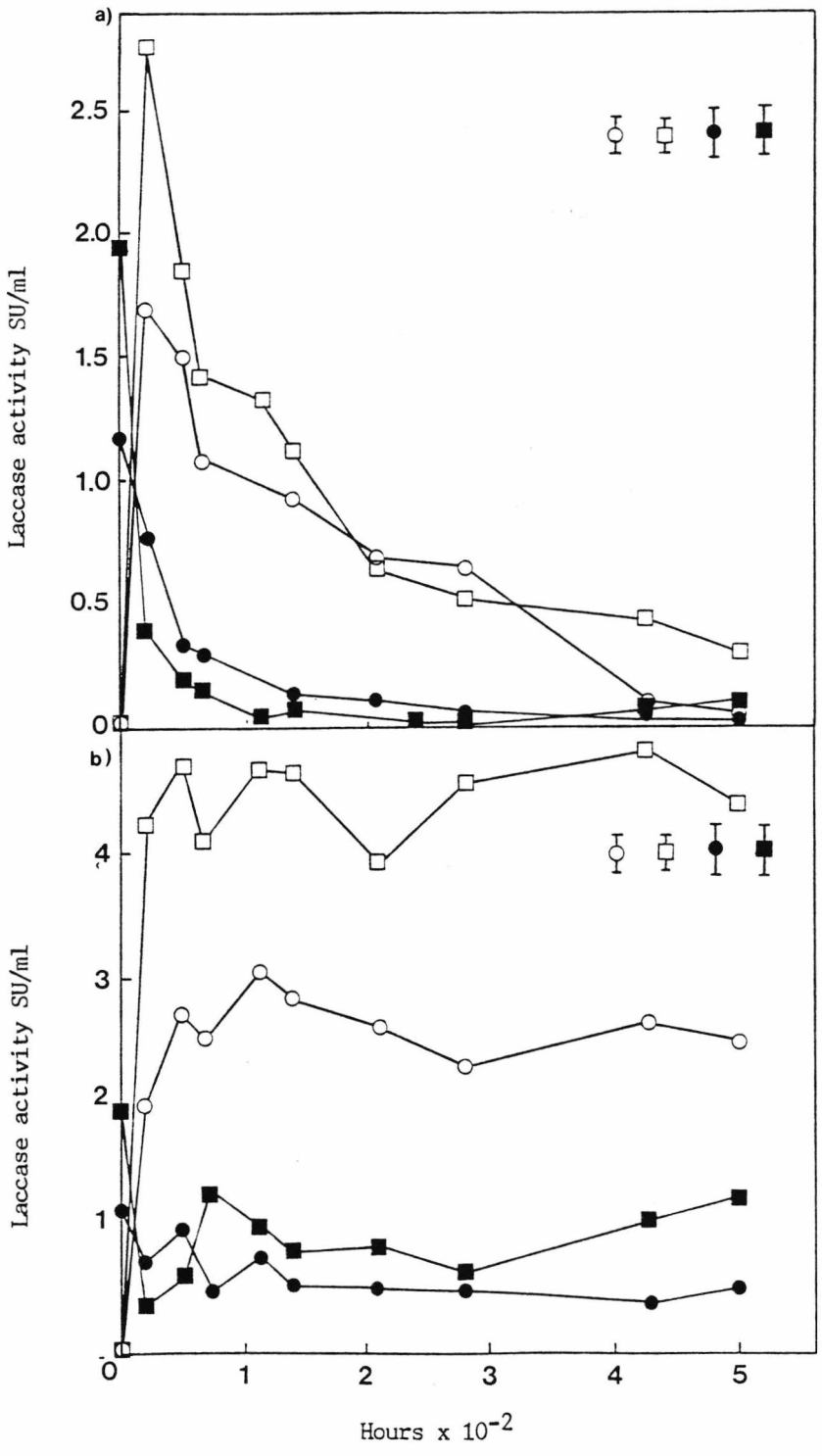

Fig. 5.1. Laccase release from alginate beads in buffer: a) 30°C, 200 rpm, 20 ml beads:50 ml buffer; b) 4°C 20 ml beads:10 ml buffer. □, entrapped laccase; ○, entrapped laccase-tyrosine copolymers. Open symbols represent SU/ml buffer, closed symbols represent SU/ml beads.

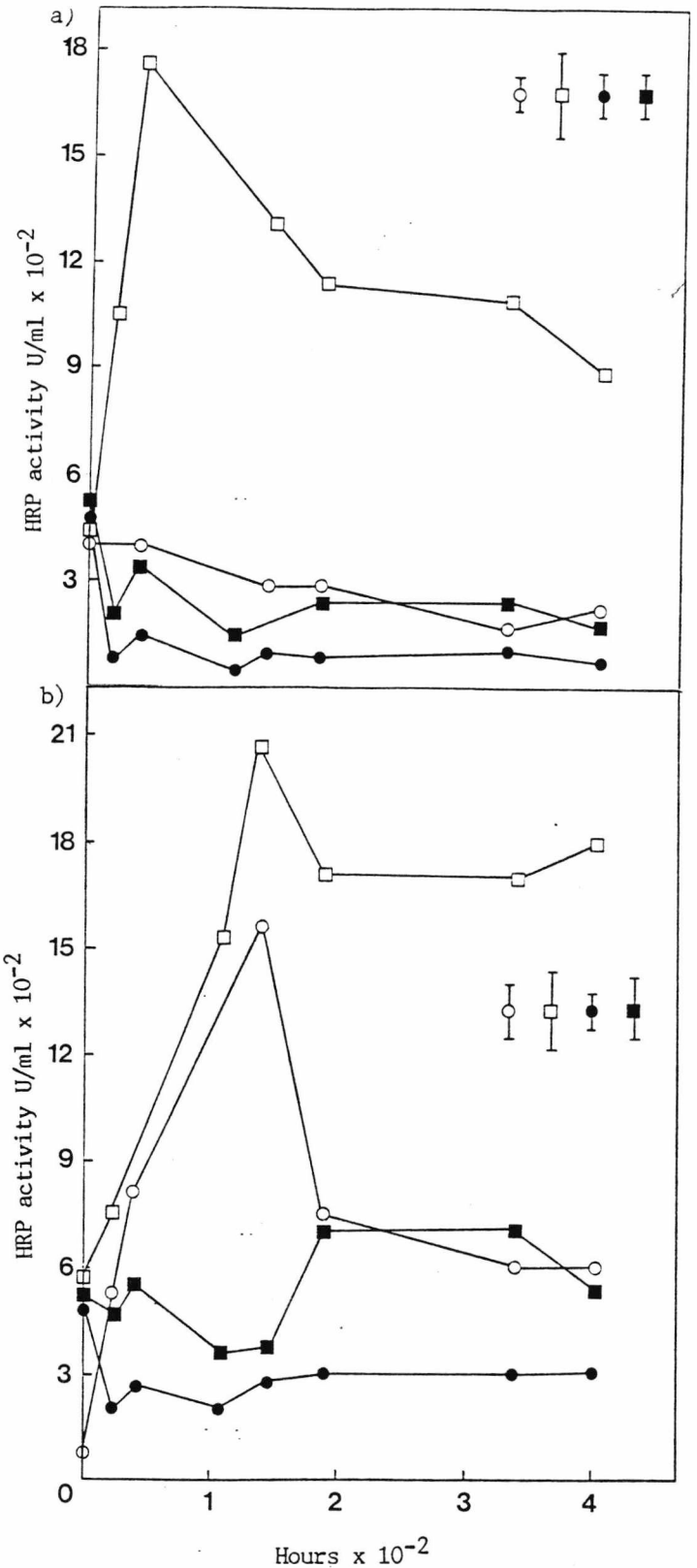

Fig. 5.2. HRP release from alginate beads in buffer: a) 30°C, 200 rpm, 20 ml beads:50 ml buffer; b) 4°C 20 ml beads:10 ml buffer. □ entrapped HRP; ○, entrapped HRP-tyrosine copolymers. Open symbols represent U/ml buffer, closed symbols represent U/ml beads.

Table 5.2. Activity losses on entrapment of enzymes and copolymers in alginate beads

Initial activity, U	Entrapped activity U/ml beads	Final activity, U	Loss of activity, %
Laccase			
30 260	2.84	880.4	97
Laccase-tyrosine copolymers			
16 668	7.31	365.5	98
HRP			
11 399	81.40	1221.4	89
HRP-tyrosine copolymers			
120 000	16.90	3380.0	97

laccase and entrapped laccase-tyrosine copolymers by 3.54 and 3.43 respectively. The amount of underestimation is unpredictable, therefore as far as possible experimental comparisons were made using one batch of beads.

It was thought that some colour was removed from the effluents by diffusion into alginate beads, because alginate beads made without enzyme absorbed 14-15% of the colour of OH, E and S effluents. However the experimental data presented here was not corrected for this because beads containing denatured enzyme or enzyme-phenolic copolymers did not absorb significant amounts of colour.

5.3. Colour removal from effluents by entrapped enzymes

Colour removal from effluent after 3 days incubation was measured with the different types of beads. Entrapped HRP removed colour from all three effluents (Fig. 5.3) and colour removal decreased with increasing OH concentration but not with E or S. The maximum colour removed was 68% of OH effluent with an initial colour of 1.28×10^3 CU (2% v/v original industrial effluent). Colour removal from 5% OH effluent by entrapped HRP increased with temperature from 4°C to a broad peak between 30-40°C. The effect of pH (4.5-7.5) was minimal with only a gradual increase in colour removal towards pH 7.5.

Decolorisation by entrapped HRP-tyrosine copolymers did not follow the same pattern (Fig. 5.4) as entrapped soluble HRP. Colour removal decreased with increasing S concentration but not with OH effluent, and overall decolorisation was lower almost certainly because of a lower initial enzyme activity (10.1 U/ml entrapped copolymers compared with 81.4 U/ml entrapped enzyme). Copolymers were much more sensitive to pH than entrapped soluble enzyme with a peak at pH 6.5 of 67% decolorisation

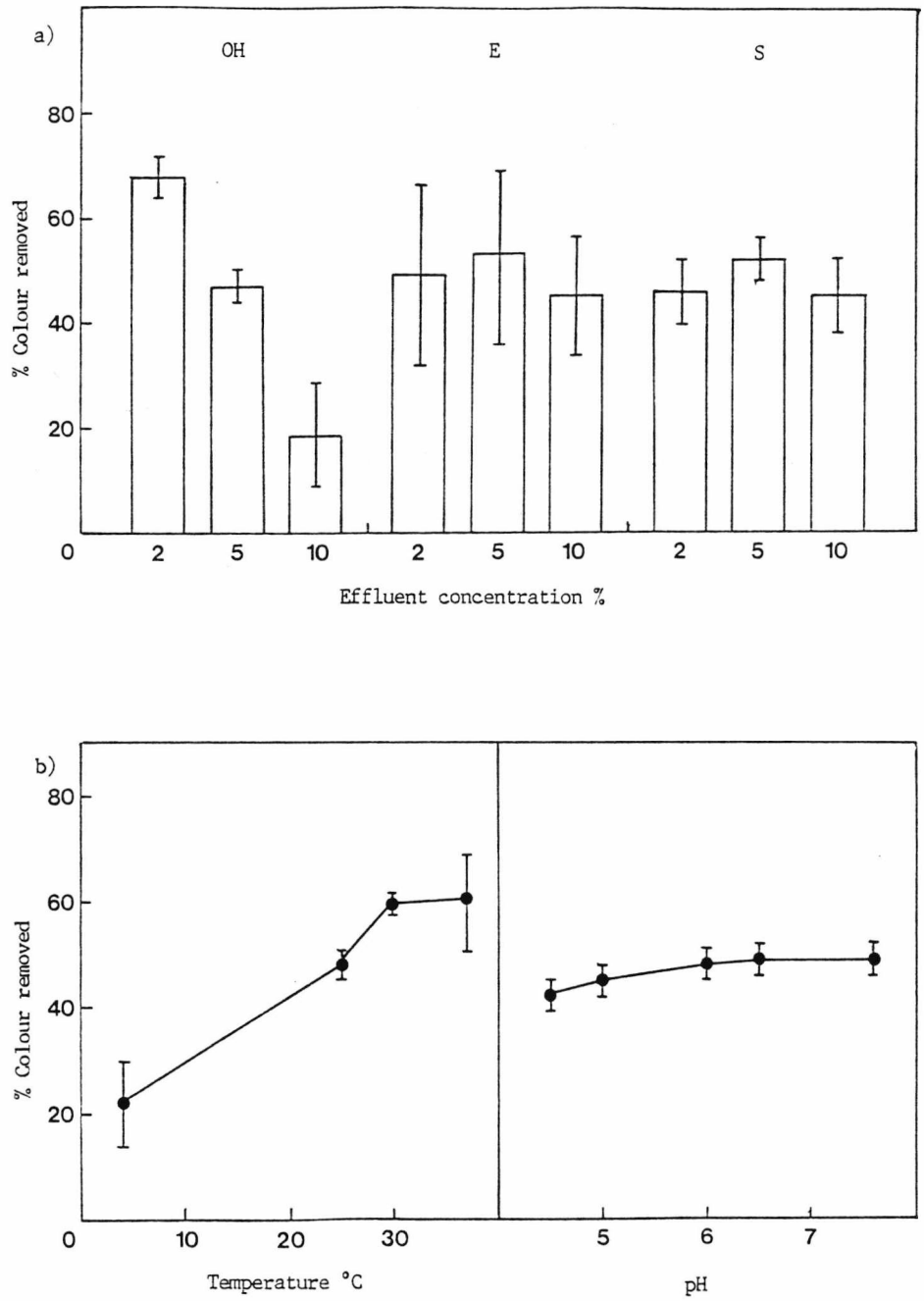

Fig. 5.3. Decolorisation of effluents by entrapped HRP: a) effect of concentration on decolorisation of effluents, pH 6.0; b) effect of temperature and pH on decolorisation of 5% OH (initial colour 3.66 x 10^3 CU). Initial activity 8.14 U/ml effluent.

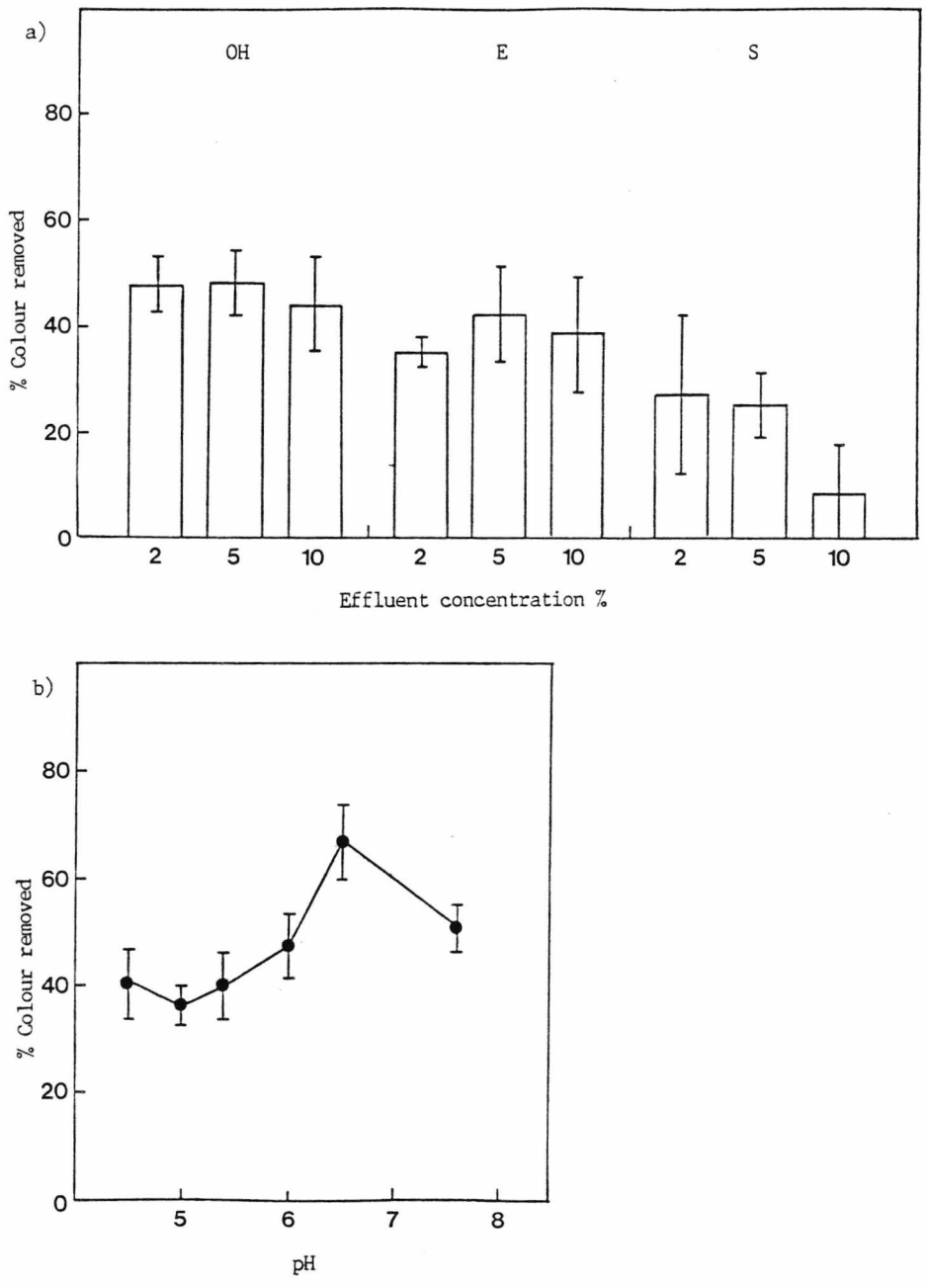

Fig. 5.4. Decolorisation of effluents by entrapped HRP-tyrosine copolymers: a) effect of concentration on decolorisation of effluents, pH 6.0; b) effect pH on decolorisation of 5% OH (initial colour 3.66 x 10^3 CU). Initial activity 1.012 U/ml effluent.

of OH effluent (initial colour 3.66 x 10^3 CU, 5% v/v), this was the maximum decolorisation achieved.

Colour removal by entrapped soluble laccase decreased with increasing OH concentration (Fig. 5.5) but not E concentration. In contrast, entrapped soluble laccase caused an increase (+ 70%) in colour of S effluent yet no precipitation occurred. The effect of pH on colour removal by laccase was variable with maximum decolorisation at pH 6.0, with a smaller peak at pH 5.0. Colour removal was not strongly temperature dependent but was maximal at 25°C. The maximum decolorisation achieved was 75% of OH effluent, with an initial colour of 3.66 x 10^3 CU, at 25°C, pH 6.0.

Laccase-tyrosine copolymers decolorised 5% effluents by 77% (OH, initial colour 3.66 x 10^3 CU), 31% (E, initial colour 4.09 x 10^3 CU) and increased the colour of S (initial colour 2.62 x 10^3 CU) by 24% (not shown). Therefore entrapped laccase-tyrosine copolymers removed 45% more colour from OH effluent than entrapped soluble laccase, but 31% less from E effluent despite having a similar initial activity (2.54 SU/ml beads) to entrapped soluble laccase (2.84 SU/ml beads). In this instance, the effect of pH and temperature were not investigated due to limited enzyme and effluent supplies.

The above experiments were performed with a ratio of 1ml beads to 10 ml effluent. Using three different types of immobilised enzyme (Fig. 5.6) it was shown that as the bead to effluent ratio increased relative colour removal declined, e.g. 70% colour was removed from OH by laccase beads at a ratio of 1:10, but only 82% decolorisation was achieved by a ratio of 1:3.3. With volumes of effluent giving a ratio less than 1:2.5 it became difficult to maintain mixing and prevent some beads from drying out. Therefore a ratio of beads:effluent of 1:10 was the most efficient with respect to decolorisation of OH effluent.

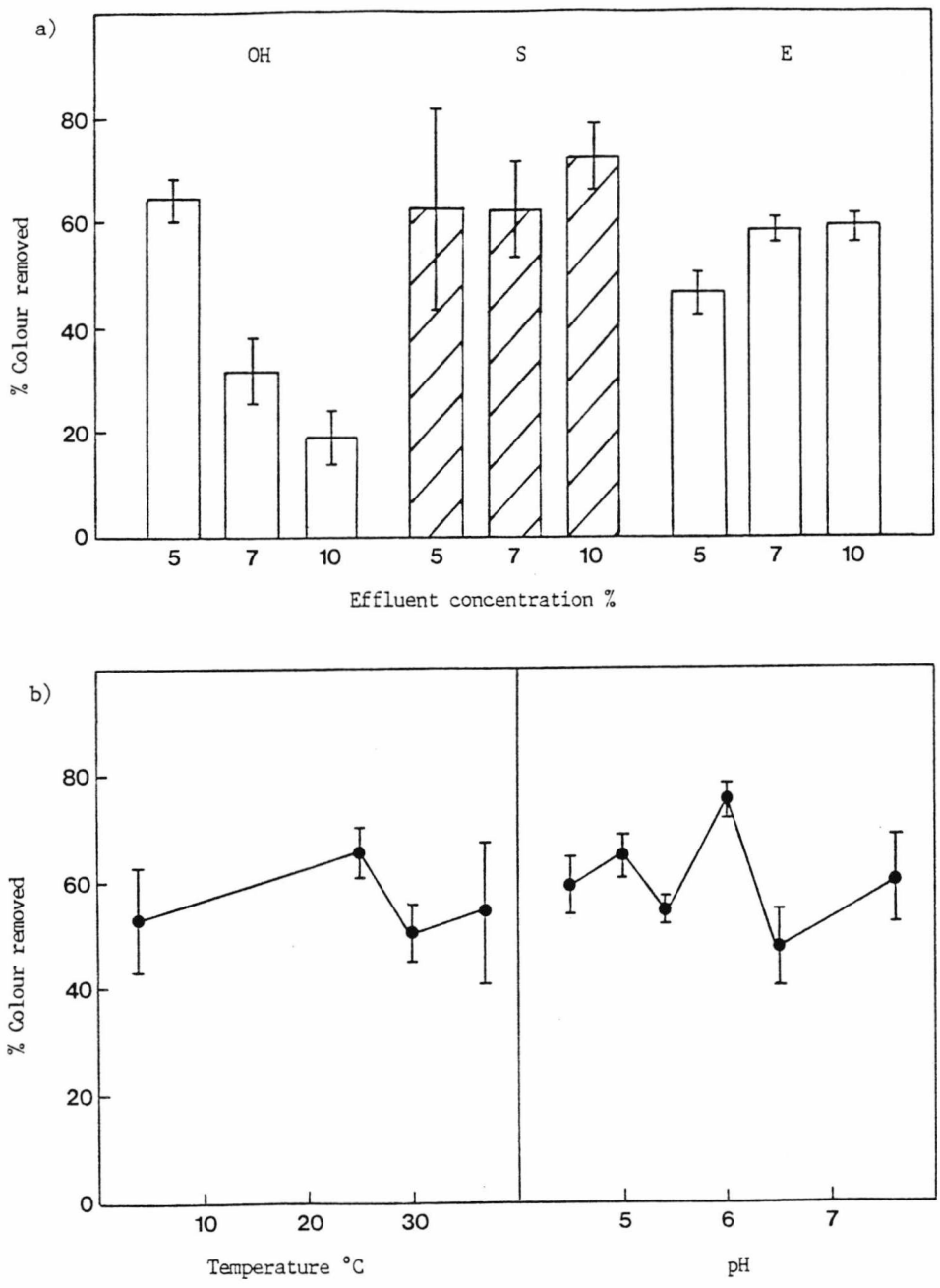

Fig. 5.5. Decolorisation of effluents by entrapped laccase: a) effect of concentration on decolorisation of effluents, pH 5.0. □, colour removed; ▨, colour increase; b) effect of temperature and pH on decolorisation of 5% OH (initial colour 3.66×10^3 CU). Initial activity 0.284 SU/ml effluent.

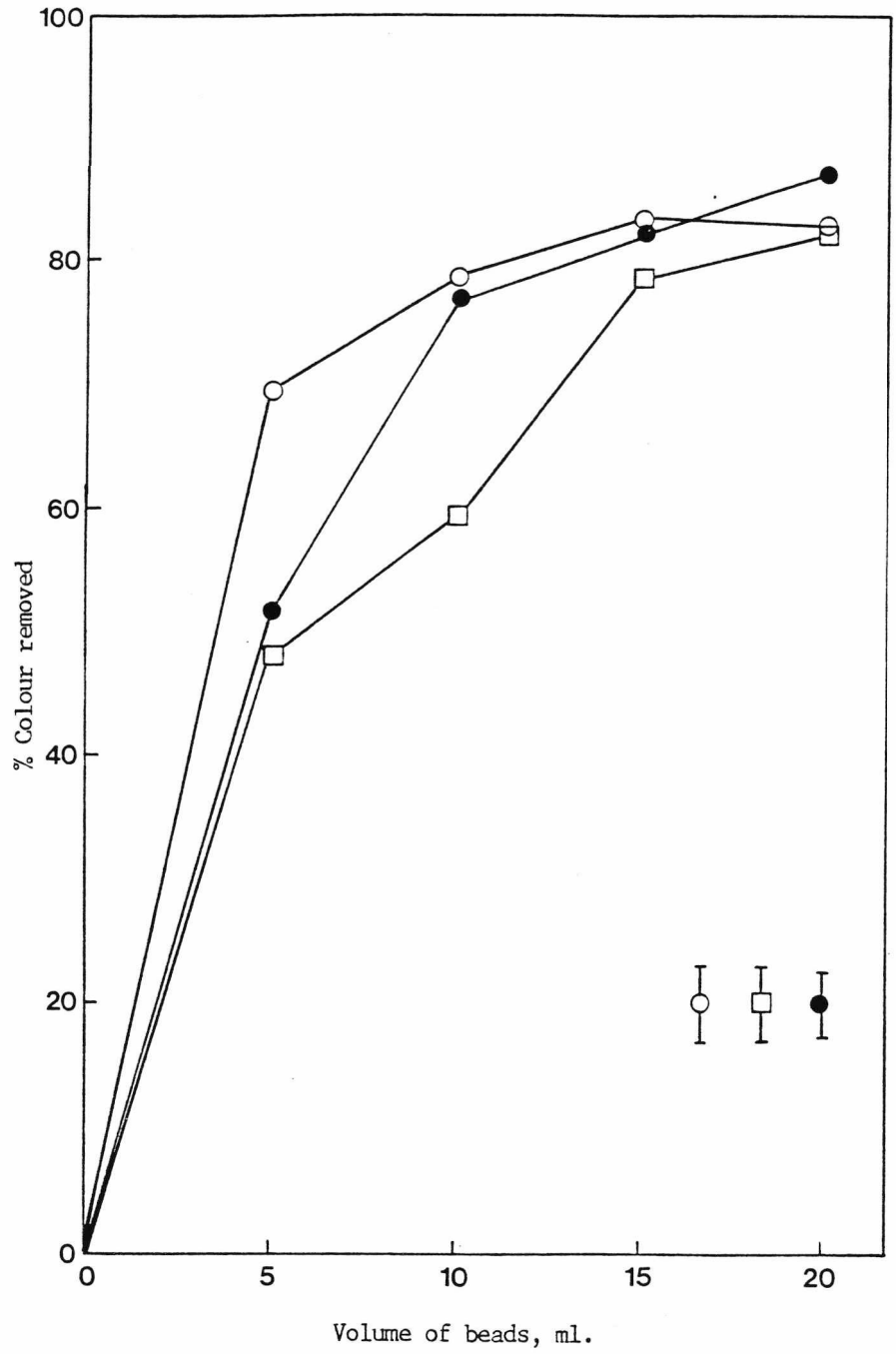

Fig. 5.6. Effect of bead volume on the decolorisation of 50 ml OH effluent by entrapped enzymes in 48 h. Initial colour 3.86 x 10^3 CU. ○, laccase 2.58 SU/ml beads; ●, HRP 80.14 U/ml beads; □, HRP-tyrosine copolymers 16.93 U/ml beads.

5.4. Effect of hydrogen peroxide on HRP activity and decolorisation

Hydrogen peroxide concentration had a marked effect on the decolorising ability of entrapped HRP due to its inactivation of the enzyme at high concentration (Fig. 5.7). At 7 mM H_2O_2, HRP activity increased in the buffered effluent due to release of enzyme from alginate beads. At 70 mM H_2O_2, however, rapid inactivation of the enzyme occurred after its release and with 700 mM H_2O_2 almost all the enzyme activity was destroyed in the first hour. In 72 h, 7 mM peroxide allowed 53% decolorisation to take place but with a concentration of 70 mM, colour removal declined to 49% (25% was due to peroxide alone as shown by controls). Colour removal was increased to 78% with 700 mM H_2O_2, which compensated for the HRP inactivation (40% colour removal was due to the H_2O_2). Fig. 5.8 shows the effect of a large range of H_2O_2 concentrations on decolorisation of OH by entrapped HRP-tyrosine copolymers, confirming that at very low H_2O_2 concentrations (31.6 μm) decolorisation is high (80%) due to enzymic activity, but decreases with higher levels of H_2O_2 (72% decolorisation with 6.31 mM) as the enzyme becomes more rapidly inactivated.

5.5. Decolorisation of batches of effluents by entrapped enzymes

Immobilisation of enzymes was aimed at increasing their reusability, however, when entrapped laccase-tyrosine copolymers were incubated with 3 batches of OH effluent (renewed every 2 h) a high initial decolorisation (60% in 2 h) was followed by a very low rate of decolorisation of successive batches (10% in 4 h and 7% in 4 h, respectively) (Fig. 5.9). Enzyme diffusion from beads was also progressively reduced. Fig. 5.9 also shows that decolorisation was rapid and complete in 4-6 h. However in previous experiments (section 5.3) longer incubations of 3 days were used to ensure that no more decolorisation took place after 4-6 h. Fig. 5.10

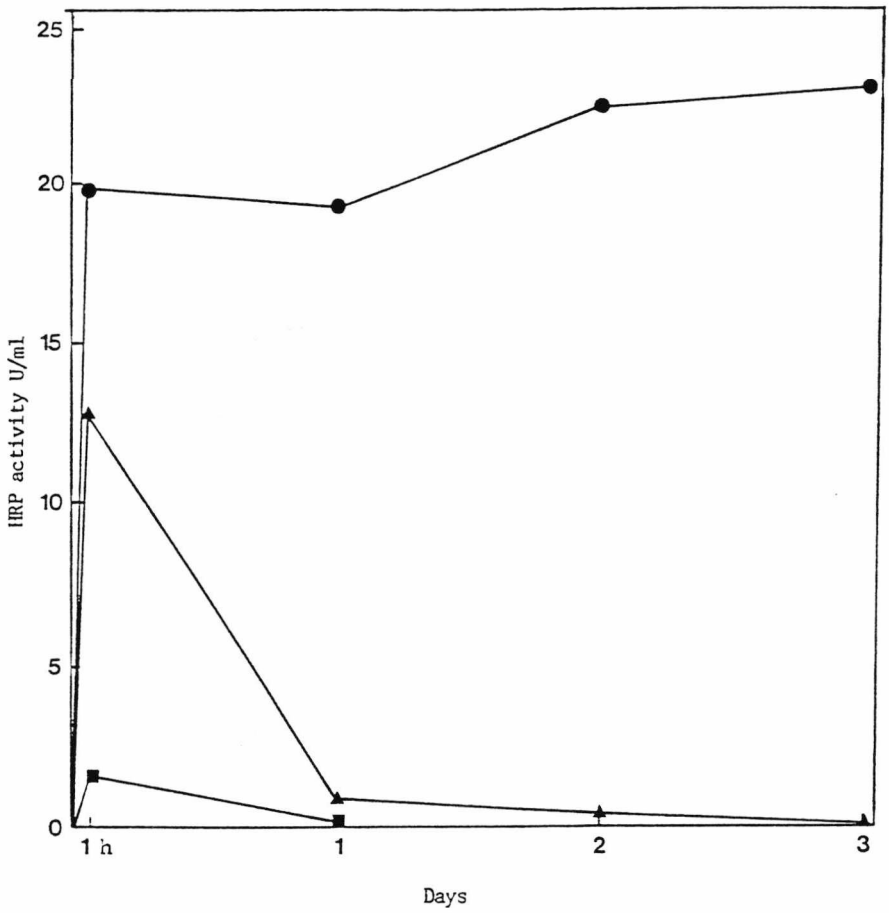

Fig. 5.7. Effect of H_2O_2 concentration on HRP activity in OH effluent containing alginate entrapped HRP. ●, 7 mM H_2O_2; ▲, 70 mM H_2O_2; ■, 700 mM H_2O_2. Initial HRP activity 22.5 U/ml as entrapped soluble HRP.

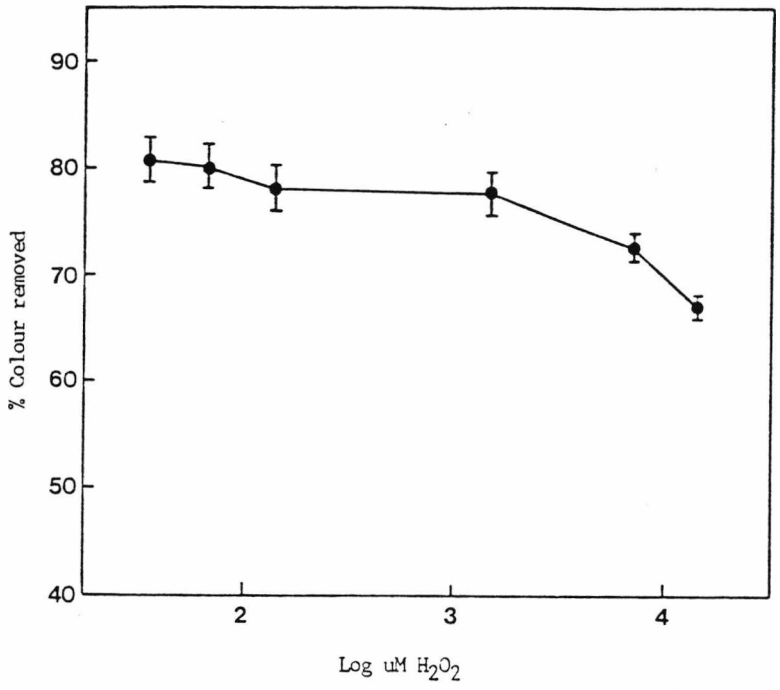

Fig. 5.8. Effect of H_2O_2 concentration on decolorisation of OH effluent by entrapped HRP-tyrosine copolymers in 72 h. Initial colour 3.98 x 10^3 CU. Initial activity 1.69 U/ml as entrapped HRP-tyrosine copolymers.

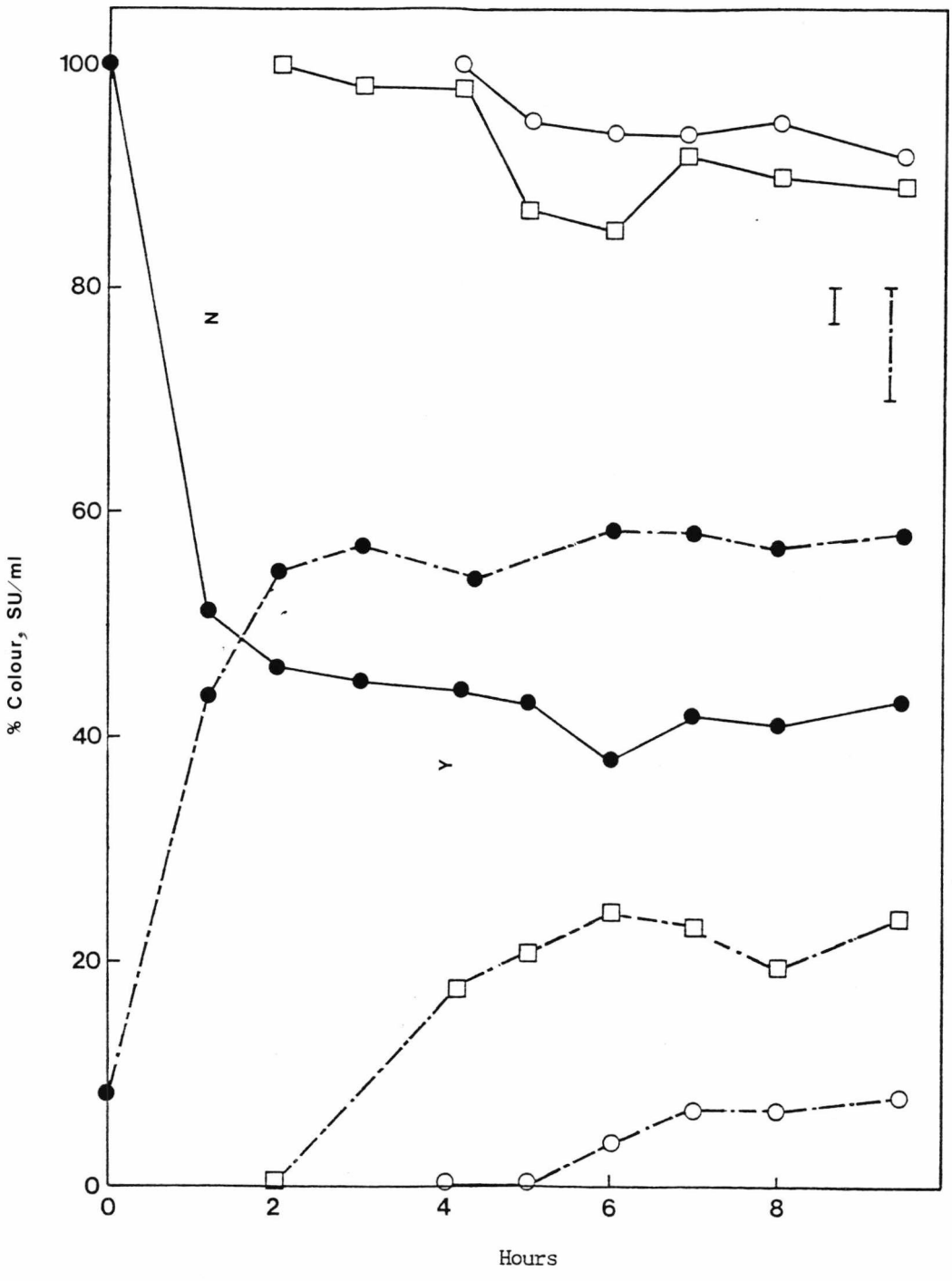

Fig. 5.9. Decolorisation of batches of effluent and enzyme release by one batch of entrapped laccase-tyrosine copolymers. Fresh effluent added every 2 h. Initial activity 0.19 SU/ml beads, 15 ml beads in 50 ml E effluent, initial colour 8.64 x 10^3 CU. ●, batch 1; □, batch 2; ○, batch 3; —, % colour; –·–, laccase in effluent SU/ml.

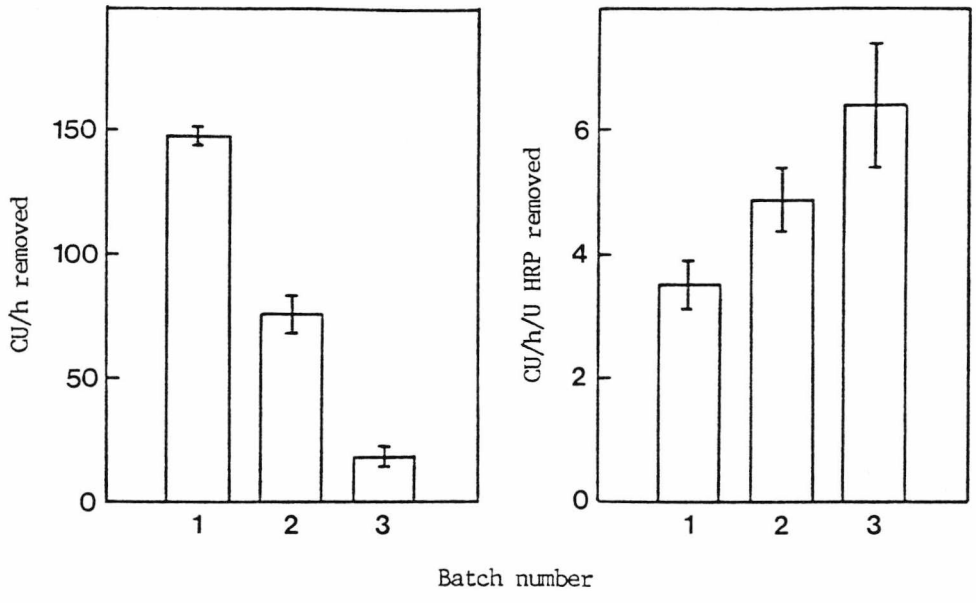

Fig. 5.10. Rate of decolorisation of 3 batches of OH effluent by 1 batch of HRP-tyrosine copolymers related to released enzyme activity. Batches changed every 24 h. Initial colour 3.98 x 10^3 CU, initial activity in beads 16.9 U/ml, 4.2 mM H_2O_2.

illustrates the successive decreases in colour removal from 3 batches of OH effluent by HRP-tyrosine copolymers, but here incubation was for 24 h before replacement with a fresh batch of effluent and a ratio of 1 ml beads to 3.3 ml effluent was used to increase the total amount of added enzyme. Not only the amount of colour removed, but also the rate of removal decreased with successive batches (149 CU/h from batch 1, decreasing to 20 CU/h from batch 3). However the CU removed per enzyme unit in supernatant increased from 3.52 CU/U/h to 6.38 CU/U/h), supporting the hypothesis that the enzymes are substrate limited in these effluents (4.3; 4.4).

5.6. Treatments to increase longevity of enzymes in beads

With the aim of slowing the rate of release of enzymes from beads, alginate beads containing entrapped soluble laccase were crosslinked with glutaraldehyde (G) and glutaraldehyde followed by gelatin (GG). Diffusion from GG beads was greater than from G beads (Fig. 5.11), but colour removal from 3% E effluent by both types of bead (GG, 35%; G, 27%) was much less than that removed from 5% E by entrapped soluble enzymes (62%), therefore these treatments were not successful in improving the longevity of the enzymes in the beads.

DISCUSSION

Many extracellular enzymes become immobilised naturally in soil through association with clays and humic colloids (Burns et al. 1972; Ceccanti et al. 1978). Phenolic copolymers have been synthesised (as analogues of soil humic matter) and show considerable stability when incorporated into soil (Burns & Martin 1984; Haider & Martin 1970). In assessing this

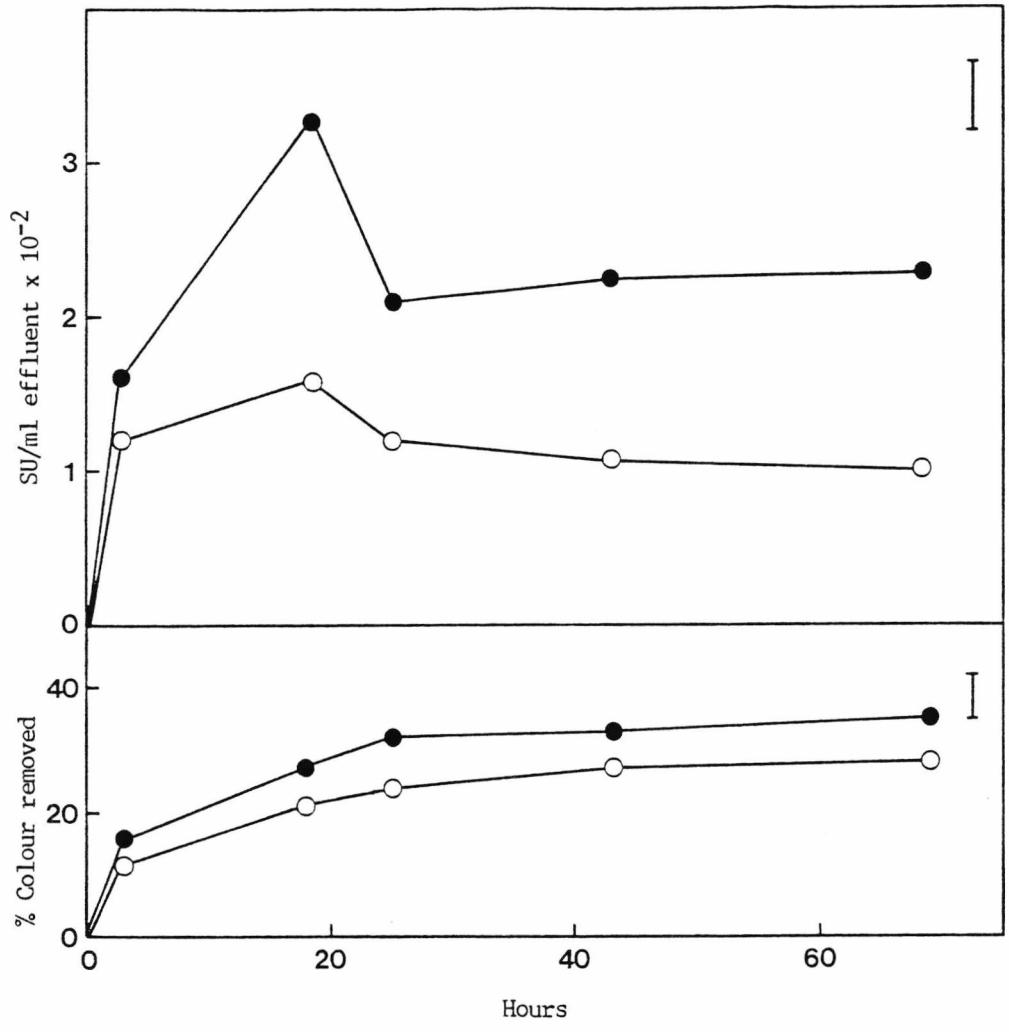

Fig. 5.11. Effect of crosslinking alginate beads containing soluble laccase on the release of enzymes into E effluent. ● , glutaraldehyde and gelatin treatment, initial activity 27.0 SU/ml; ○, glutaraldehyde treatment, initial activity 14.28 SU/ml. Initial colour 2.28 x 10^3 CU.

method to increase the stability of phenol oxidase enzymes for industrial waste treatment, a problem was encountered because phenol oxidases themselves are used in the polymerisation of some of the recommended phenolic supports (e.g. resorcinol, pyrogallol). Therefore the enzyme to be immobilised may lose activity by reacting with the phenolic substrate or by forming a bond at the active site. Tyrosine, however is not a substrate for laccase or peroxidase and there is no evidence that tyrosinase catalyses tyrosine oxidation via a free radical mechanism (Hamilton 1969) reducing the possibility of enzyme inactivation by this method. As predicted, copolymerisation with tyrosine was the only method which did not result in complete loss of laccase or HRP activity.

Nevertheless, considerable reduction in activity was encountered on copolymerisation, however by concentration of the copolymer (ultrafiltration or centrifugation) the resulting activity was considered sufficient to warrant further study of the stability and decolorising activity of the copolymers. One method of improving the activity yields would be to include a reversible (competitive) inhibitor of laccase or peroxidase in the reaction mixture to prevent coupling at the active sites of the enzymes, however there is no report of such an inhibitor for laccase in the literature.

Entrapment in calcium alginate was not a perfect method of immobilisation, and resulted in a slow release of enzyme from beads into the medium. However some activity was retained in the beads even after incubation for 500 hours. This release of enzyme corresponds with data for diffusion of different size compounds from alginate (Tanaka et al. 1984), showing that compounds with molecular weights less than 20 kD freely diffuse in and out of calcium alginate gel beads made from 2-4% alginate. However compounds of 69-341 kD diffuse from beads at a lower

rate than diffusion in water. Laccase from C.versicolor has been reported to have a molecular weight of 60-64 kD (Fahreus & Reinhammar 1967; Mosbach 1963) however during the determination of molecular weight changes in effluents (4.6), laccase eluted from the gel filtration column in the fractions corresponding to a molecular weight of 100 kD. If this is the case, then laccase diffusion from alginate beads should be slow compared to diffusion in water and would explain why some laccase activity is retained in the beads even after prolonged incubation (some laccase may also be retained by small pores on the surface of alginate beads (Scherer et al. 1981).

In conflict with the above data, Kierstan & Bucke (1977) reported that glucose oxidase (154 kD) and inulinase (> 100 kD) did not diffuse from alginate beads. In addition, by entrapping large molecular weight compounds in alginate gel the characteristics of the gel are changed in such way as to increase the diffusion of the compounds from the beads (Tanaka et al. 1984). To reduce such an effect it would be neccessary to coat the bead surface with another polymer or a mixture of alginate and polymer. Indeed such an approach has been taken by Fukushima et al. (1988) who immobilised several enzymes in colloidal silica mixed with alginate to produce beads with increased rigidity and decreased pore size, however this treatment decreased the molecular weight of compounds able to penetrate the gel and in particular, glycoproteins were not retained. In conclusion, substrates in the effluent which were polymerised by laccase would be able to diffuse in and out of alginate gel beads freely, therefore alginate entrapment of laccase and HRP must be considered as a slow release mechanism for supplying enzyme to the effluent.

The rate of diffusion of enzymes from alginate gel beads can be reduced

by increasing the gel strength in two ways (G. Vaughan 1988, personal communication). (1) Increasing the alginate concentration from 3-10% decreased enzyme losses from 30% to 17% in 3 h, however the final enzyme loss (80%) after 24 h was the same. (2) Increasing calcium chloride concentration from 0.01 M to 0.5 M resulted in the formation of stronger beads and better retention of enzymes, although increasing the concentration any further resulted in much faster release of enzyme. It appears that the enzyme cannot be completely prevented from diffusing from beads, so ultimately the useful life of the immobilised enzyme is limited by this factor. This confirms the report of Tanaka et al. (1984) who found that the rate of enzyme release was retarded more by increasing the content of alginate in the beads than by increasing the calcium chloride concentration in preparation of the beads.

Entrapped laccase was more effective than HRP at removing colour from OH effluent, however entrapped HRP could also decolorise S effluent. The maximum colour removal by entrapped laccase was 75% of OH effluent, and 75% of OH by entrapped laccase-tyrosine copolymers, both considerably greater than that achieved by soluble laccase (36% OH), decolorisation of E by entrapped laccase was also improved by 19%, but that by copolymers was 9% less than that by soluble laccase. However, entrapped soluble laccase or copolymers caused an increase in the colour of S effluent but no colour was removed. The polymerisation but not precipitation of OH effluent by entrapped laccase and entrapped laccase-tyrosine copolymers may be due to the comparatively low laccase activity obtained on bead formation, as low levels of soluble laccase activity were previously shown to increase the colour of S effluent (4.3). Entrapped HRP caused greater decolorisation (68%) of OH than soluble enzyme, (59%) of OH, but entrapped HRP-tyrosine copolymers were much less effective (48%) of OH.

Again this could be attributed to low enzyme activity, in this case that of the copolymers (10.12 U/ml beads), compared with the entrapped enzyme (81.40 U/ml beads).

For the same reason, pH may have had more effect on the decolorisation of OH effluent by HRP-tyrosine copolymers than that by entrapped soluble HRP. HRP has previously been shown to have activity over a broad range of pH values. Paice & Jurasek (1984) observed high colour removal from E effluent from pH 4.0-8.0 with only a 5% increase in colour removal at the optimum pH between 5.0-6.0. The two functions of H_2O_2 as cofactor and as bleaching agent were observed in decolorisation of OH effluent, and have been previously recorded with E effluent (Paice and Jurasek 1984). The most efficient enzymic decolorisation took place at hydrogen peroxide:HRP ratios of less than 3 mM:1 unit of HRP, although this is difficult to maintain in practise when entrapped HRP is slowly released from beads.

Laccase showed a different pattern of activity with pH, the optimum for oxidation of assay substrates was 4.0-4.5 (4.7) however laccase showed decolorising activity from pH 4.0-7.5. The variation over this range could be due to optimal removal of different components of the effluent at different pH values. For example there was a peak of decolorisation at pH 6.0 and a smaller peak at 5.0. In all cases comparable levels of decolorisation by soluble enzymes were only achieved using much greater activities than that added as entrapped enzymes.

The temperature of the effluent leaving the pulp mill is around 40°C (Boman et al. 1988) and the temperature optimum for HRP activity was in the range 30-40°C. Thus, HRP may be appropriate for treating effluent if contained in a reactor connected to the effluent outflow. However laccase decolorisation of OH effluent was not significantly different at 37°C from that at 4°C, with a peak of activity at 25°C. Therefore for more

efficient decolorisation a holding tank may be necessary to allow the temperature to drop to 30°C. Such an arrangement would also be beneficial to HRP decolorisation because by reducing the loss of activity due to thermal denaturation at high temperatures, a greater amount of substrate is oxidised per enzyme molecule with time (Hartmeier 1986).

Although colour removal could be increased by increasing the ratio of bead volume to effluent volume, the efficiency of decolorisation decreased. From the shape of the curve the implication is that the maximum possible colour removal was gradually reached, as 86% removal (by 20 ml entrapped HRP beads) was the maximum colour removal achieved throughout this study, and was equal to the maximum colour removed by fungi (85% of colour of OH effluent in 6 d by C.versicolor(K), 3.3), but was achieved after only 3 d incubation.

In view of the high decolorisation produced by a high ratio of beads to effluent, a suitable reactor would have to provide a large support:effluent ratio. A packed bed reactor provides the greatest ratio of packing to volume (1.4.5), however alginate beads have a low mechanical strength and would be subject to compression forces in such a reactor. An alternative would be a fluidised bed which would also be advantageous for the provision of oxygenated effluent to laccase, because gases are released into a packed bed as discrete bubbles due to the pressure drop across the bed. Fluidised bed reactors also have advantages for the removal of particulate products out of the top of the column, which would clog a packed bed reactor (Hartmeier 1986).

The incubation of one batch of beads with more than one batch of effluent showed that decolorisation was relatively fast, ceasing after 4-6 h, not after 2-3 days despite the longer incubations previously used. However some colour may still be removed after 4-6 h as copolymerisation is a

slow process, e.g. Sarkar & Burns (1984) incubated reaction mixtures for the formation of phenolic copolymers for up to 12 h. The additional colour removal, after the initial copolymerisation of phenolics, is likely to be due to increasing molecular weights of colloidal polymers already formed. The batch incubations also showed that even with a very high loading of enzyme in beads, or by decreasing the diffusion rate of enzyme from the beads, the number of batches of effluent which could be treated by one batch of beads would be limited and the colour removed from each batch would decrease rapidly.

Crosslinking the outside of the beads with glutaraldehyde and gelatin did not improve the decolorisation of E effluent by entrapped laccase, and increasing alginate or calcium chloride concentrations would only slow the diffusion rate and not prevent the enzymes from escaping from the beads. Therefore, the best possible method of using alginate beads would be to covalently link the enzymes to the alginate in such a way that laccase or HRP would be permanently attached to the porous bead, which would function as a solid support. One possible method in the case of laccase would be to include Concanavalin A (Con A) in the alginate to bind laccase into the beads. Con A is a lectin which binds to sugar residues and has previously been used to immobilise laccase without causing any change in the catalytic activity of the enzyme (Froehner & Eriksson 1975). It has also been used by Husain et al. (1985) to immobilise glucose oxidase in alginate. Dominguez et al.(1988) have immobilised β-galactosidase by covalent coupling to alginate using a water soluble carbodiimide. These and other methods may offer improved opportunities for using alginate to immobilise enzymes.

However in view of the poor mechanical properties of alginate and also its relatively high cost, a more suitable method of immobilisation might

be to covalently immobilise laccase or HRP onto a solid support, which could be used in different reactor types and which could be prepared cheaply. This will be discussed in the next chapter.

CHAPTER SIX

COVALENT IMMOBILISATION OF LACCASE ON ACTIVATED CARBON AND TREATMENT OF EFFLUENTS IN A FLUIDISED BED REACTOR

INTRODUCTION

Matrix entrapment proved to be an unsuitable immobilisation method for laccase or HRP, despite some enhancement of decolorising activity (Chapter Five). In view of the variety of types of phenolic effluents (1.1), encompassing a wide range of ionic concentration and pH values, an immobilisation method providing a versatile and resilient catalyst was required. One possibility is the covalent binding of enzymes to solid supports, a type of attachment known to provide stability against fluctuations in ionic concentration and pH (Kennedy et al. 1988). However, loss of catalytic activity can be caused during the immobilisation reaction by conformational changes to the enzyme molecule on binding to the support, and by binding involving the active site of the enzyme. To avoid this chemical reactions to activate functional groups are usually performed with the support, not the enzyme, and activating and coupling steps kept as separate as possible.

Laccase was chosen for this study because it was shown to have a greater inherent stability than HRP in effluents (4.3; 4.5), and also does not require a cosubstrate (peroxide) for activity. Previously, laccase has been covalently coupled to Celite (Shuttleworth & Bollag 1986) and to porous glass (CPG) (Leonowicz et al., 1988) retaining high levels of activity in both cases (99% and 90% respectively). However immobilisation on Celite did not result in any increase in stability compared to the

soluble enzyme, whereas immobilisation on CPG resulted in wider pH and temperature ranges of activity compared to the soluble enzyme and the ability to reuse the immobilised laccase. CPG is one of the most widely used supports for covalent coupling and the OH groups on the surface can be coupled to enzymes via silanisation. However few other methods of derivatisation have been reported.

Activated carbon (AC) has several advantages as a enzyme support. AC can be easily derivatised to produce many different functional groups (e.g. COOH, amino, phenolic OH) for enzyme coupling, it has good mechanical strength, and is available readily and cheaply because it is already used in the food and waste treatment industries (Cho & Bailey 1978). The pore structure of AC can be divided into three types: (i) micropores (0-0.4 nm diam), (ii) transitional pores (4-100 nm diam), and (iii) macropores (100-4000 nm diam) which are comparable in size to those in calcium alginate gel (10 μm diam). Cho & Bailey (1979) described several methods of derivatising carbon for enzyme immobilisation. One of the most successful used a water soluble diimide and proved to be a gentle method in which derivatisation and coupling steps were separated so that the enzyme was not in contact with the coupling chemicals, therefore the inactivating effect was minimised.

The reactor type used for an immobilised biocatalyst is important to provide increased mass transfer between the biocatalyst and the surrounding medium (1.4.5). To provide laccase with air saturated effluent a fluidised bed is likely to prove the most useful, because the formation of air bubbles in a packed bed disrupts the flow. In a fluidised bed reactor plug flow keeps the particles in a loose suspension, minimising mechanical stress and allowing insoluble products to be carried out of the top of the bed. In practise, if plug flow is not

achieved, or throughput of a gas is required the system approaches a well mixed layer in which back mixing takes place (Fig. 1.4).

The aims of the research reported in this chapter were: (i) to compare several methods of immobilising laccase to activated carbon; (ii) to measure colour and phenol removal from effluents by the immobilised laccase and compare it with that achieved by soluble enzyme (Chapter Four); and (iii) to evaluate reactor types in the treatment of phenolic effluents by immobilised laccase.

RESULTS

6.1. Immobilisation of laccase on activated carbon

Table 6.1 shows the amounts of laccase protein bound to carbon using four methods of carbon derivatisation. The total amount of protein bound to the carbon was not significantly different regardless of the method, but considerably higher bound activity was obtained using diimide coupling of laccase to the carboxyl groups on the carbon (method D) (maximum of 9.396 μMO_2/min/g carbon compared with a maximum of 4.053 μMO_2/min/g carbon on amino carbon, method C). Diimide coupling was used subsequently to prepare large batches of immobilised laccase. The activity of the immobilised laccase was measured by oxygen uptake using DMP as substrate, as this avoids the inaccuracies of spectrophotometric determinations in which adsorption of substrate or product by the carbon support occurs.

6.2. Characteristics of carbon immobilised laccase

When increasing amounts of laccase were applied to diimide activated carbon up to 43 mg of laccase per g of carbon was bound, but as Fig. 6.1 shows, the corresponding enzyme activity decreased as protein loading

Table 6.1. Protein loadings and enzyme activities obtained using different methods of covalently binding laccase to activated carbon

Binding method	Laccase added mg/g carbon	Laccase bound mg/g carbon	Bound activity μMO_2/min/g
A. Silanisation	10	9.04 ± 0.49	1.851 ± .048
of carbon.	30	23.91 ± 4.26	1.678 ± .099
	50	36.81 ± 1.62	2.150 ± .377
B. Crosslinking	10	4.48 ± 0.11	1.455 ± .374
after binding to	30	23.09 ± 1.39	2.418 ± .361
carbon amino groups.	50	37.58 ± 2.93	2.652 ± .315
C. Activation of	10	4.87 ± 0.36	2.179 ± .073
amino groups with	30	23.78 ± 3.64	2.995 ± .182
diimide.	50	36.30 ± 2.00	4.053 ± .201
D. Activation of	10	7.61 ± 0.61	9.396 ± .620
carboxyl groups	30	23.06 ± 1.84	5.536 ± .360
with diimide.	50	34.87 ± 0.72	7.692 ± .556

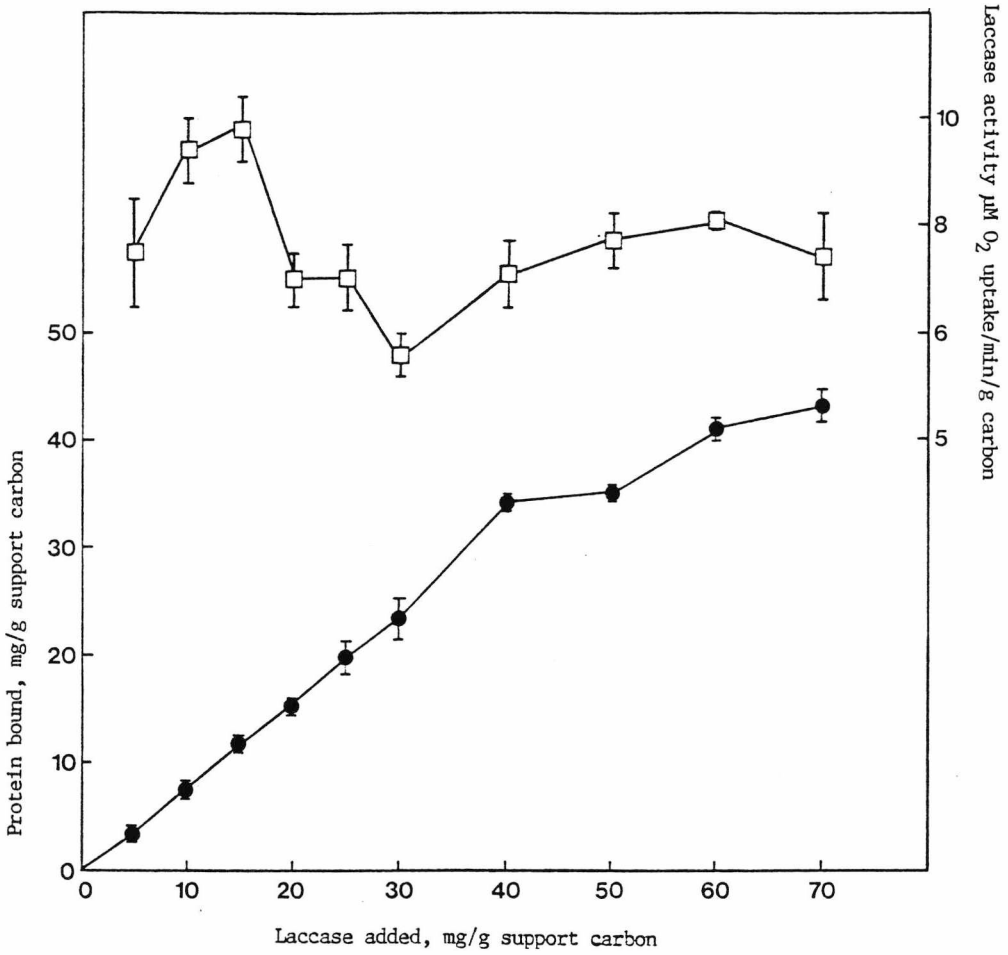

Fig. 6.1. Laccase binding and subsequent activity as a result of the covalent coupling of the enzyme to carbon activated with a water soluble diimide. ●, protein; □, laccase activity.

increased. Laccase binding was linear up to 35 mg/g bound, then curved towards a maximum of approximately 50 mg of protein bound/g carbon support (by extrapolation). In all cases less than 100% of the added protein was bound to the carbon, the highest amount being 77% at added concentrations of 10-40 mg of protein/g carbon. The maximum enzyme activity (9.802 μMO_2/min/g carbon) was reached at 15 mg laccase added/g carbon (11.5 mg laccase bound/g carbon) with another peak (8.068 μMO_2/min/g carbon) at 60 mg laccase added/g carbon (41 mg laccase bound/g carbon), with 82% of the maximum activity. Therefore 11.5 mg laccase bound/g carbon was chosen as the optimum level of laccase on carbon to provide the highest immobilised laccase activity.

Activated carbon has a water holding capacity (WHC) of 1 ml/g and it was maintained at this level throughout storage and assay of the immobilised preparations. Air dried carbon immobilised laccase (CIL) lost all of its activity, therefore care was taken to prevent the preparations from drying out. CIL was stable to washing with 10 mM citrate-phosphate buffer from pH 4.0-9.0 and to NaCl concentrations up to 0.1 M (Fig. 6.2). However even with 1 M salt only 1% of the bound protein was removed during a 30 minute wash.

The incomplete binding of laccase protein to carbon may have been due to residual ammonium sulphate in the extract, or to the orange pigment formed by inducing laccase with 2,5-xylidine (4.8). The binding of bovine serum albumin (BSA) to carbon using the diimide method (Fig. 6.3) corresponded with the binding curve shown by laccase (Fig. 6.1), but the addition of just 10 mM ammonium sulphate reduces the binding by 5%. Also laccase from non-induced cultures binds to carbon at the same rate as BSA, but induced laccase contaminated with orange pigment binds below the curve, and this sample too is affected by the addition of 10 mM ammonium

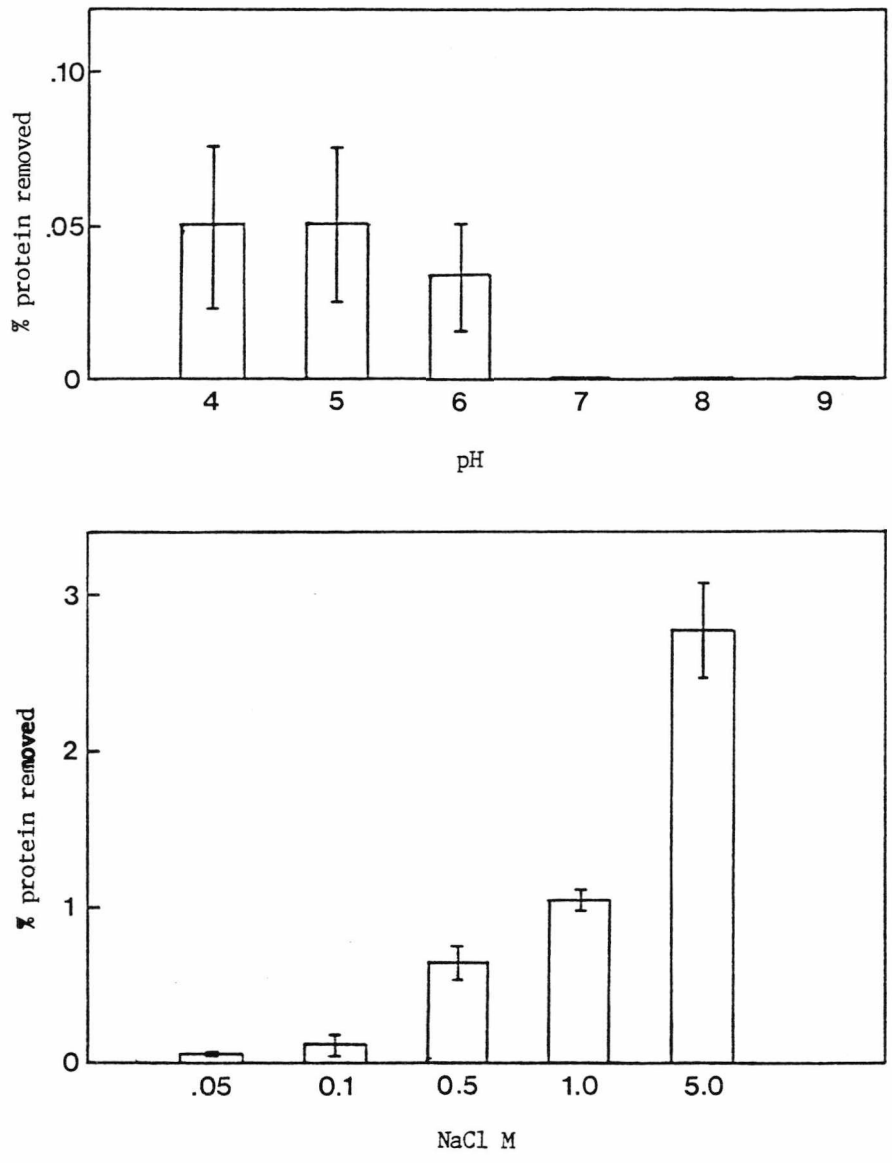

Fig. 6.2. Stability of laccase covalently bound to carbon to pH and salt concentration, after incubation at 30°C, 30 min, 200 rpm.

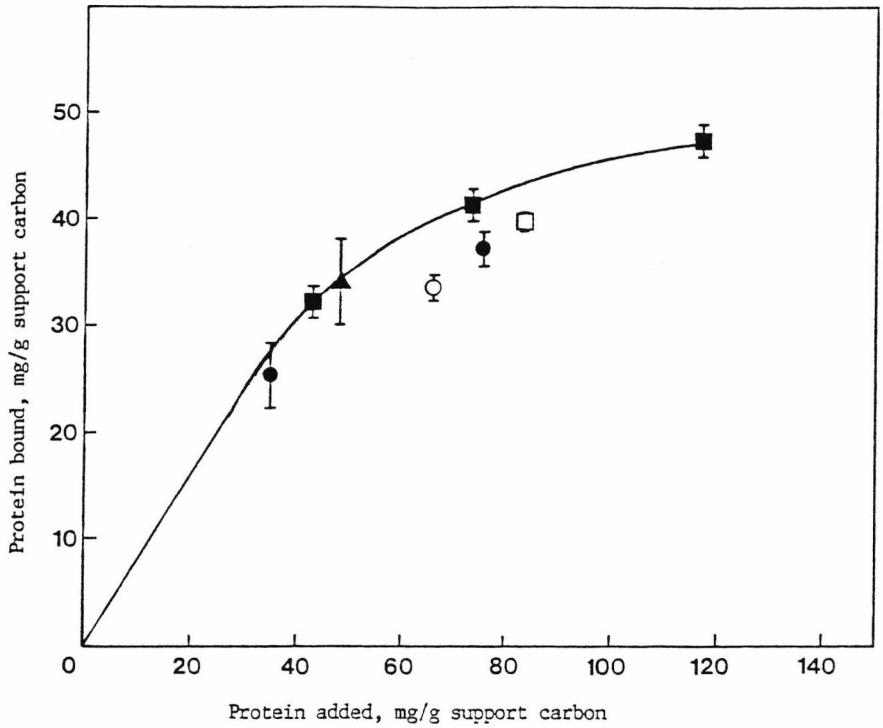

Fig. 6.3. Factors affecting protein binding to carbon activated with a water soluble diimide. ■, BSA; □, BSA plus 0.01 M $(NH_4)_2SO_4$; ●, induced laccase; ○, induced laccase plus 0.01 M $(NH_4)_2SO_4$; ▲, non-induced laccase.

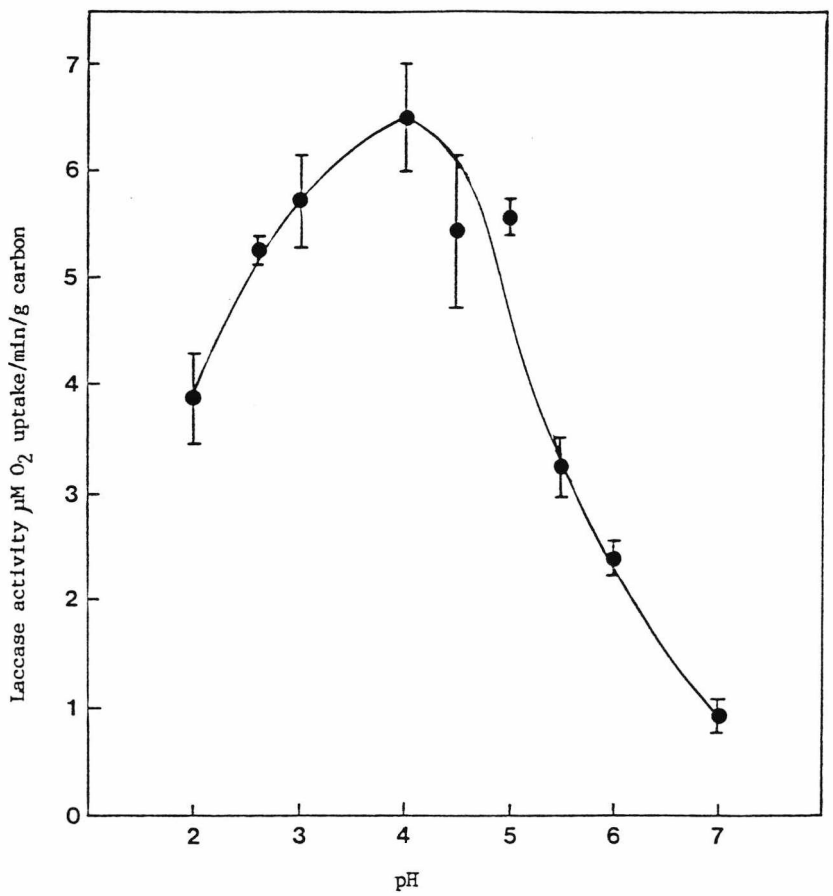

Fig. 6.4. Effect of pH on the activity of covalently immobilised laccase at 30°C, with 2,6-dimethoxyphenol substrate.

sulphate.

The pH curve of immobilised laccase, measured as oxygen uptake with 2,6-dimethoxyphenol (DMP) follows that of the free enzyme on DMP (Fig. 6.4) with the optimum at pH 4.0 and activity falling off more slowly on the alkaline side than on the acid side. In contrast, the effect of high temperature on activity is moderated by immobilising laccase on carbon (Fig. 6.5), but the effect was not significant even at 40 and 50°C. However the activation energy (Ea) of immobilised laccase calculated from an Arrhenius plot (17.61 $\pm$ 1.48 kJ/M O_2) was decreased compared with soluble laccase (22.28 $\pm$ 0.94 kJ/M O_2).

Laccase immobilised on activated carbon maintained activity very well during storage in nonsterile distilled water at 4°C (Fig. 6.6). During the first 4 d storage there was a 38% drop in activity, but then activity declined in a linear fashion losing another 22% in 126 d, equivalent to -0.17%/d over the linear region and a total loss of 60% in 130 d. Laccase stored at -20°C showed no decline in activity with time but storage in this form was unsuitable because freezing and thawing caused physical damage to the carbon and 54% loss of laccase activity on thawing. Therefore, immobilised laccase was routinely stored at 4°C. The antiseptic, Hibitane (0.1% v/v) was also added to stored laccase to inhibit microbial growth.

Enzymes immobilised on porous supports exhibit diffusion restricted kinetics as Fig. 6.7 shows, i.e. at low substrate concentrations (oxygen in this case) the reaction velocity is much reduced, but at high concentrations the velocity approaches that of the free enzyme. Thus kinetic constants calculated from the experimental data (K_m 0.219 μMO_2, V_{max} 0.162 μMO_2/min) are somewhat different from those of the free enzyme (K_m 0.113 μMO_2, V_{max} 0.210 μMO_2/min). The particle size of the carbon

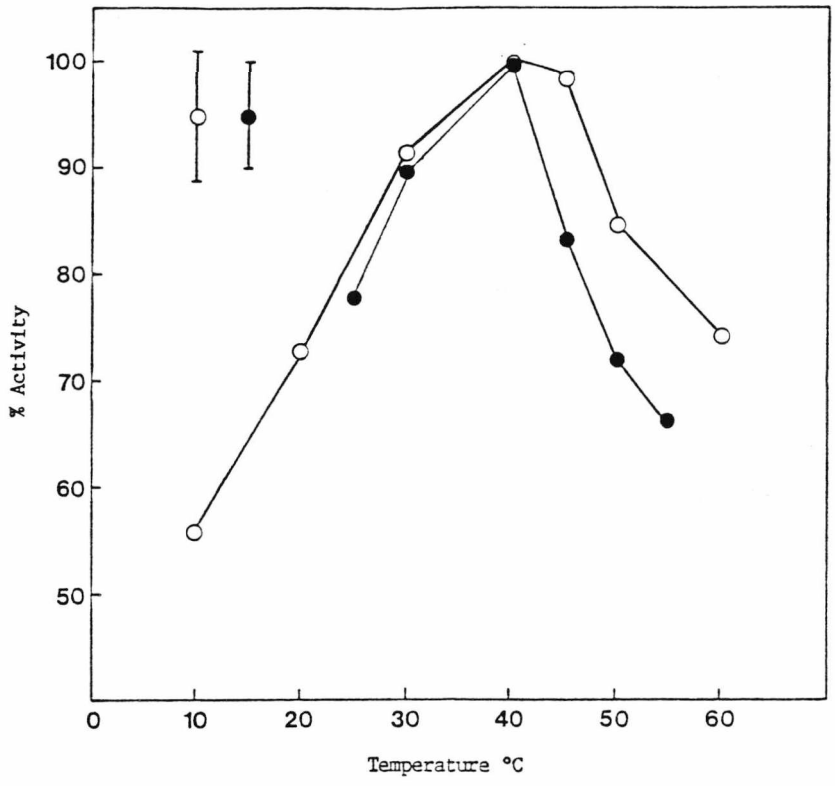

Fig. 6.5. Effect of reaction temperature on the activity of laccase measured by oxygen uptake with 2,6-dimethoxyphenol substrate. ● , soluble laccase; ○, covalently immobilised laccase.

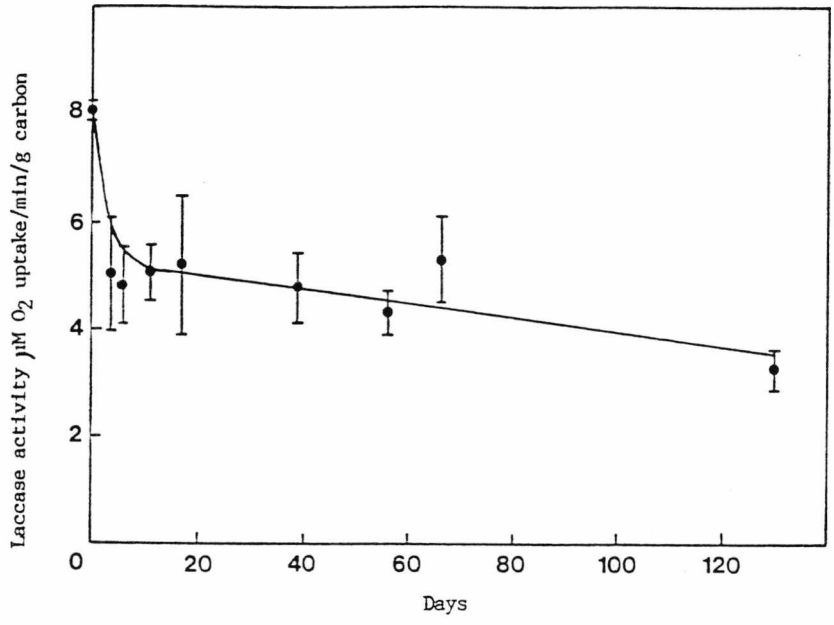

Fig. 6.6. Activity of covalently immobilised laccase during storage in distilled water (100% water holding capacity), 4°C.

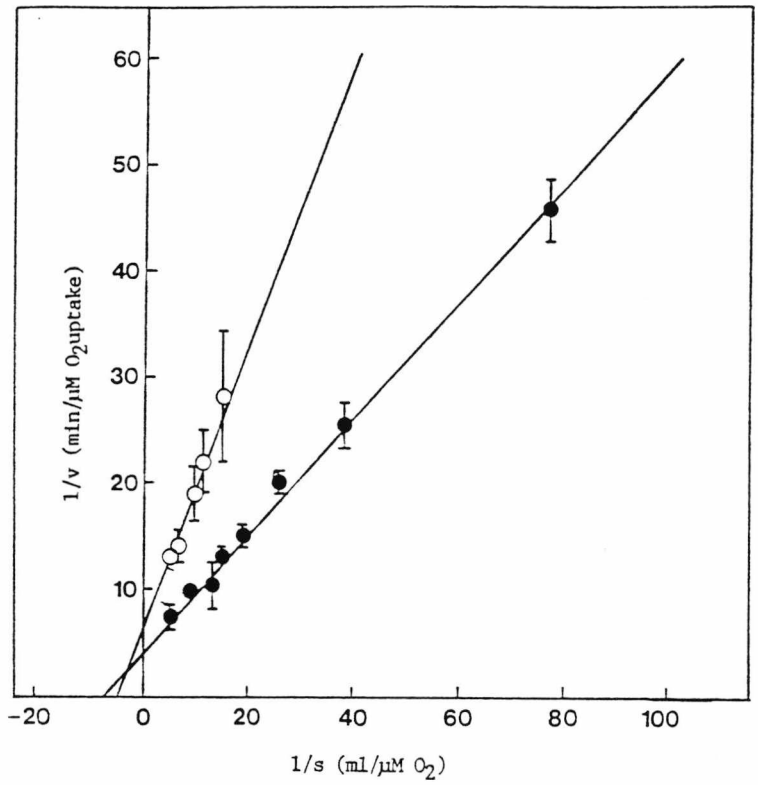

Fig. 6.7. Lineweaver-Burke plot of laccase activity measured as oxygen uptake with 2,6-dimethoxyphenol substrate. ●, soluble laccase; ○, laccase covalently immobilised on carbon (20-30 mesh).

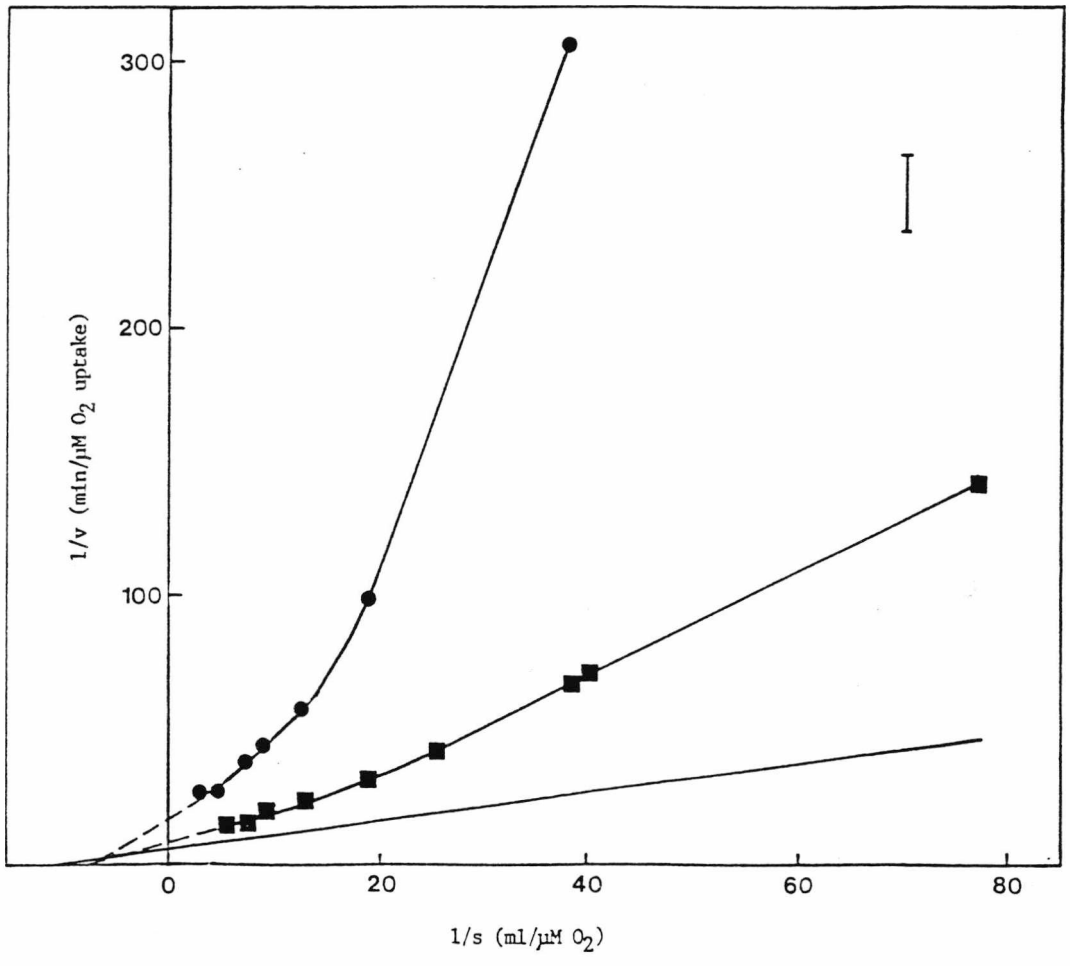

Fig. 6.8. Lineweaver-Burke plot showing the effect of support particle size on the activity of covalently immobilised laccase. ——, soluble laccase; laccase covalently immobilised on carbon: ■, 50-300 mesh; ●, 20-30 mesh.

also directly affects the degree of diffusional limitation (Fig. 6.8) by increasing the diffusional distance to the centre of the particle. Therefore apparent kinetic constants (i.e. experimental values with no correction for diffusion) will vary according to the substrate concentration throughout the reaction.

6.3. Treatment of effluents with immobilised laccase

An important factor in assessing the value of immobilised enzymes in any system is the reusability of the preparation in a batch or continuous system. Fig. 6.9 shows that carbon immobilised laccase can oxidise up to 7 batches of DMP and still retain 20% of the initial activity. The figure shows three separate batches with different initial activities.

The effect of CIL on effluent was first investigated using 30 ml batches of E effluent incubated with 0.1 g batches of carbon (Fig. 6.10). At low concentrations of effluent ($<1 \times 10^4$ CU) colour removal by immobilised laccase was the same as the control (heat denatured immobilised laccase), but as the concentration increased, removal by CIL increased compared with the control. The maximum colour removed occurred by day 3 and was 57% of E at 1.2×10^4 CU and 55% of E at 6.6×10^4 CU (both significantly greater than the control treatments).

Before comparing colour removal in batch with that by a column system, the optimum reactor configuration for columns had to be found. Fig. 6.11 shows the dramatic effect of using a fluidised bed compared to a packed bed (with identical batches of CIL), on the oxidation of DMP. The maximum rate of oxidation increased by 27% in the fluidised bed system over the packed bed system. In this experiment a low flow rate of 18 ml/min was used so that the extent of oxidation of DMP was low and differences between the two systems would be apparent. This accounts for the high

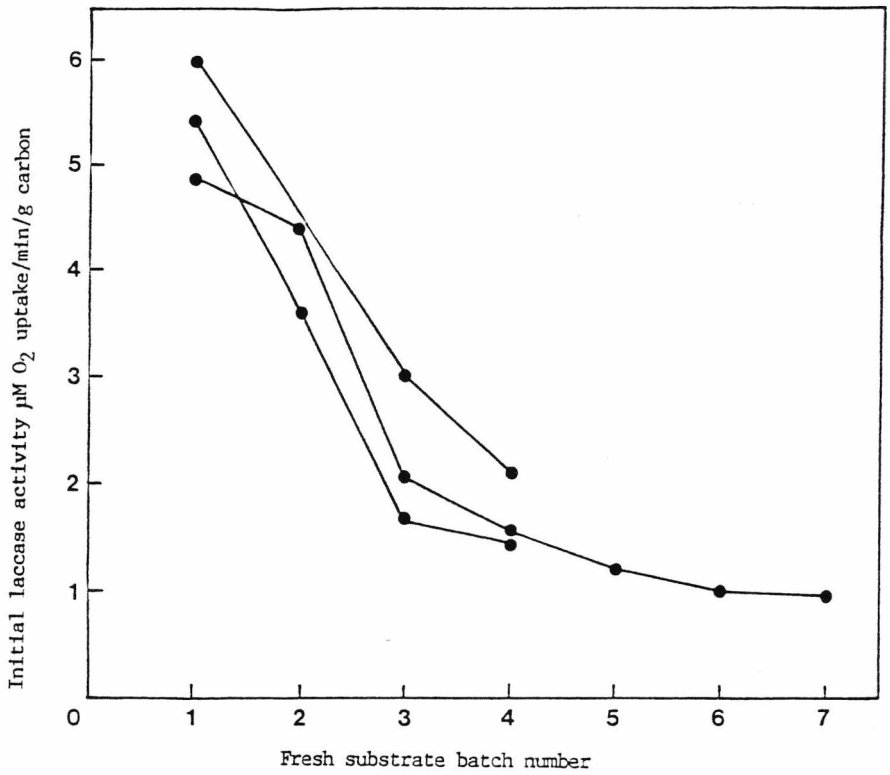

Fig. 6.9. Effect of reusing covalently immobilised laccase on initial enzyme activity, measured as oxygen uptake with 2,6-dimethoxyphenol substrate (0.4 mM oxidised completely in each batch). Three separate batches of immobilised laccase are shown with 4 or 7 separate additions of substrate.

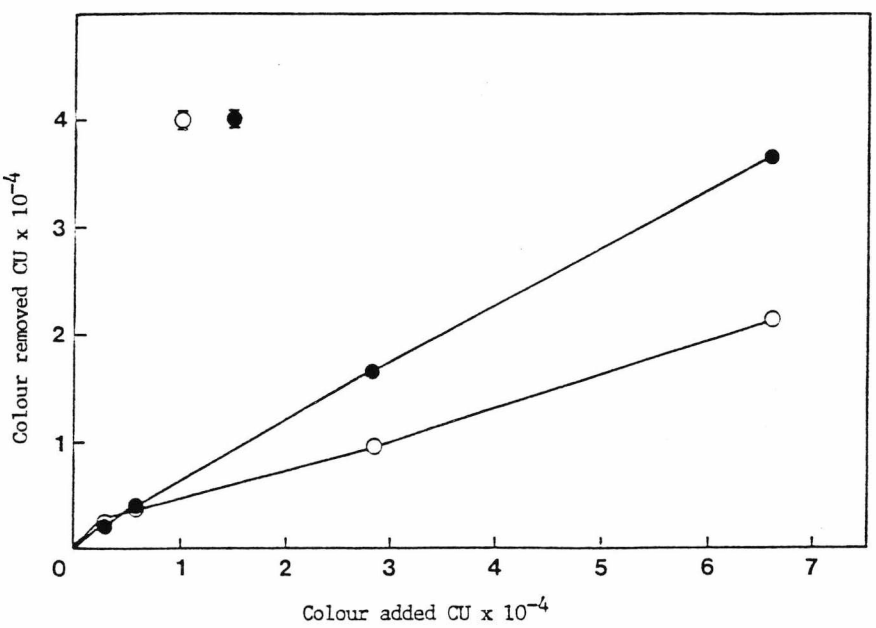

Fig. 6.10. Colour removal from E effluent by covalently immobilised laccase after 3 d, 0.1 g samples shaken in pH 5.0 effluent, 25°C, 200rpm. ●, active immobilised laccase; ○, heat denatured immobilised laccase. Initial laccase activity 10.374 uM O_2/min uptake/g support carbon.

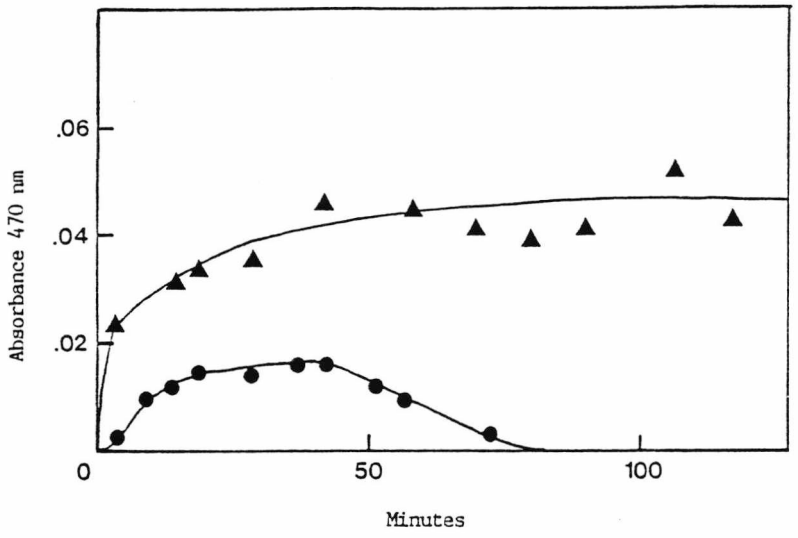

Fig. 6.11. Comparison of reactor types for the oxidation of 2,6-dimethoxyphenol by covalently immobilised laccase using identical conditions except for direction of flow. ●, packed bed reactor; ▲, fluidised bed reactor.

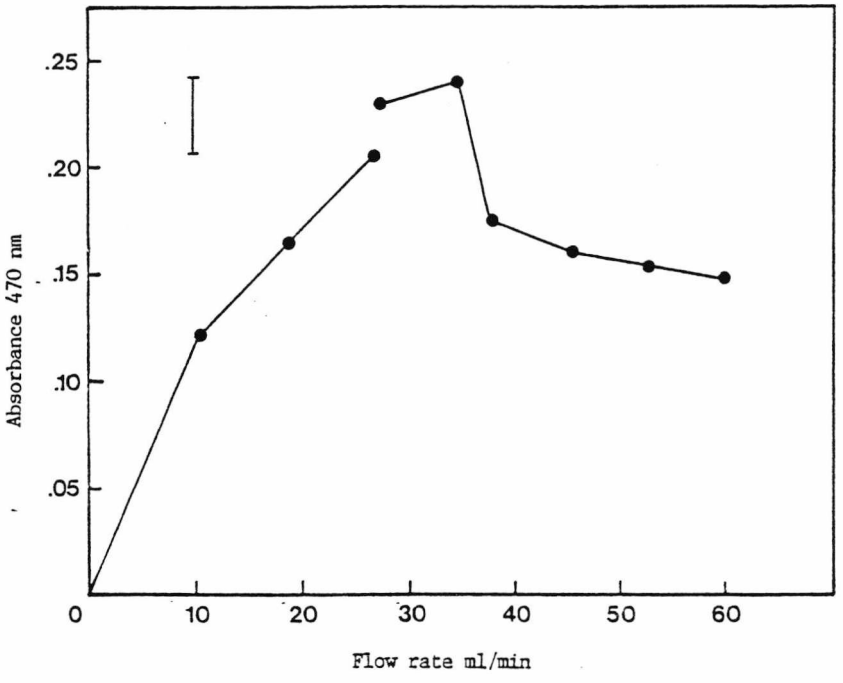

Fig. 6.12. Effect of flow rate on the degree of oxidation of 2,6-dimethoxyphenol by laccase immobilised on activated carbon. Two identical batches of covalently immobilised laccase used to counteract loss of activity with time.

fluctuation in readings. The initial activity was 7.15 μMO_2/min/g carbon and the final activity was 3.59 μMO_2/min/g carbon, therefore 55% of the initial activity remained after oxidation of 9 l of DMP. Thus the fluidised bed system was used in all further studies (Plate 4 shows DMP being oxidised by CIL in the fluidised bed system).

After selecting the fluidised bed system, the optimum flow rate for further studies was investigated using DMP as substrate. Fig. 6.12 shows that an area of maximum oxidation was reached between 27.5 and 35 ml/min (1.8 l/h) and therefore 30 ml/min was chosen for further measurements. Two identical batches of CIL were used, to reduce the effect of activity losses during the assay (as in Fig. 6.9), and the assay time was kept as short as possible to reduce the effect still further.

Oxygen uptake by immobilised laccase with the three industrial effluents (Fig. 6.13) shows that the immobilised laccase may be substrate limited (4.3) at the effluent concentrations used routinely (10^3-10^4 CU). CIL had a much higher oxygen uptake on OH than E and S effluents. The four points represent 5%, 10%, 15% and 20% dilutions of each effluent, and OH was originally at a lower concentration. Further measurements were not made due to limited effluent supplies.

Colour removal from effluents by CIL was measured in the fluidised bed using continuously recycled effluent, adjusted to pH 5.0 but not buffered. Fig. 6.14 shows that the average colour removal rate from E effluent was higher at a greater initial effluent concentration (594 $\pm$ 26 CU/h at initial colour of 10.2 x 10^3 CU; 531 $\pm$ 24 CU/h at initial colour of 6.8 x 10^3 CU). In all cases colour removal followed the pattern of a rapid decline in the first 2-4 h then a decreasing removal rate.

Table 6.2. shows the colour removal from all 3 types of industrial effluent by CIL. The most efficient removal was from E effluent (115.1

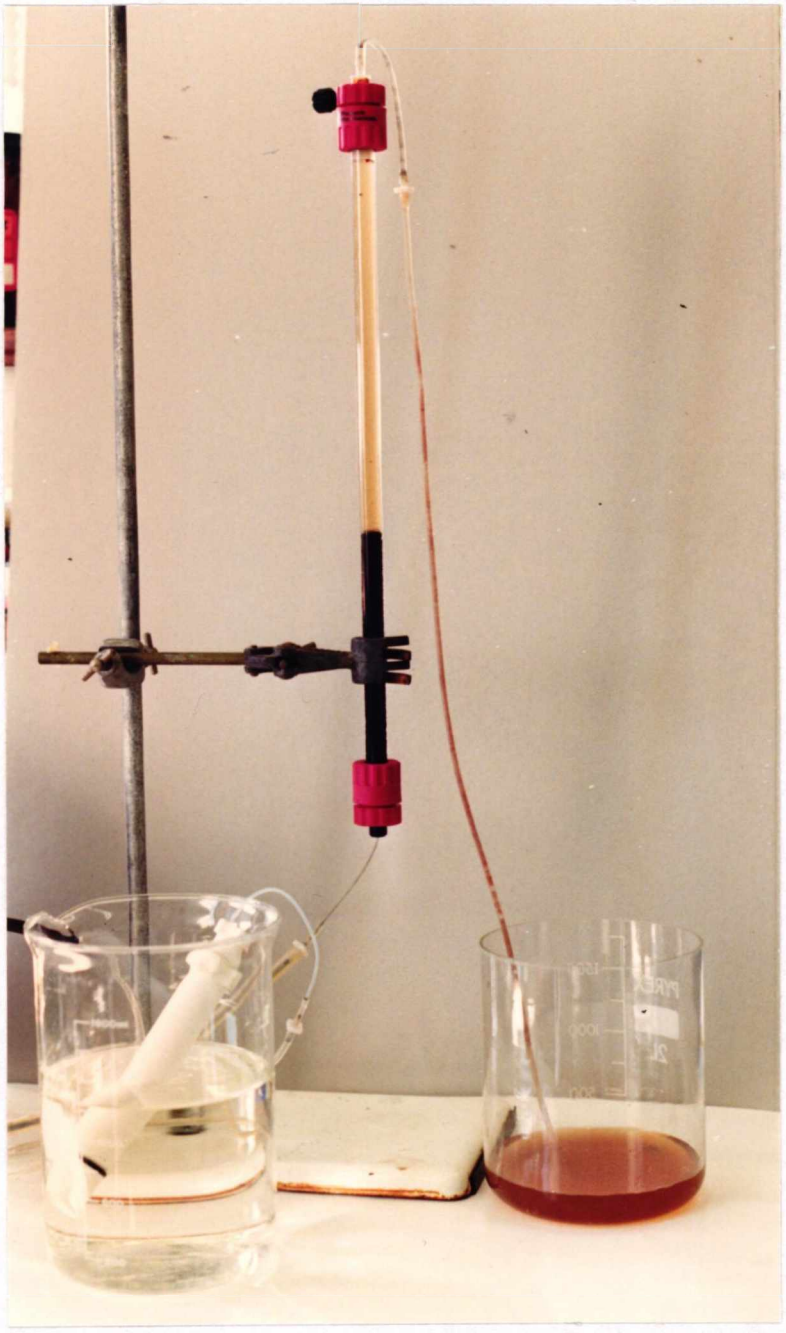

Plate 4. Oxidation of 0.1 mM 2,6-dimethoxyphenol by laccase covalently coupled to activated carbon (method D, 2.13.3) in a fluidised bed reactor, substrate flow from left to right of picture.

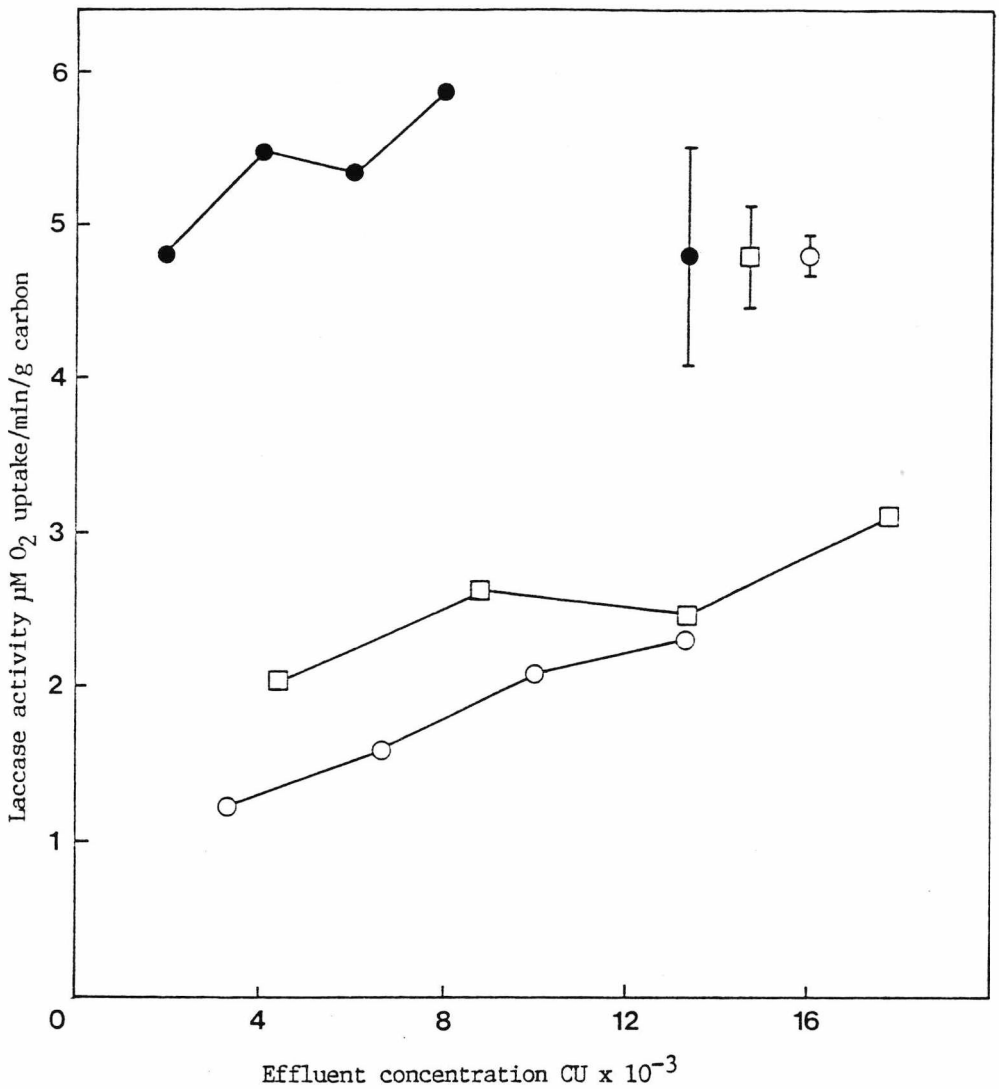

Fig. 6.13. Effect of effluent concentration, pH 5.0, on oxygen uptake by covalently immobilised laccase at 30 °C. ●, OH; □, S; ○, E.

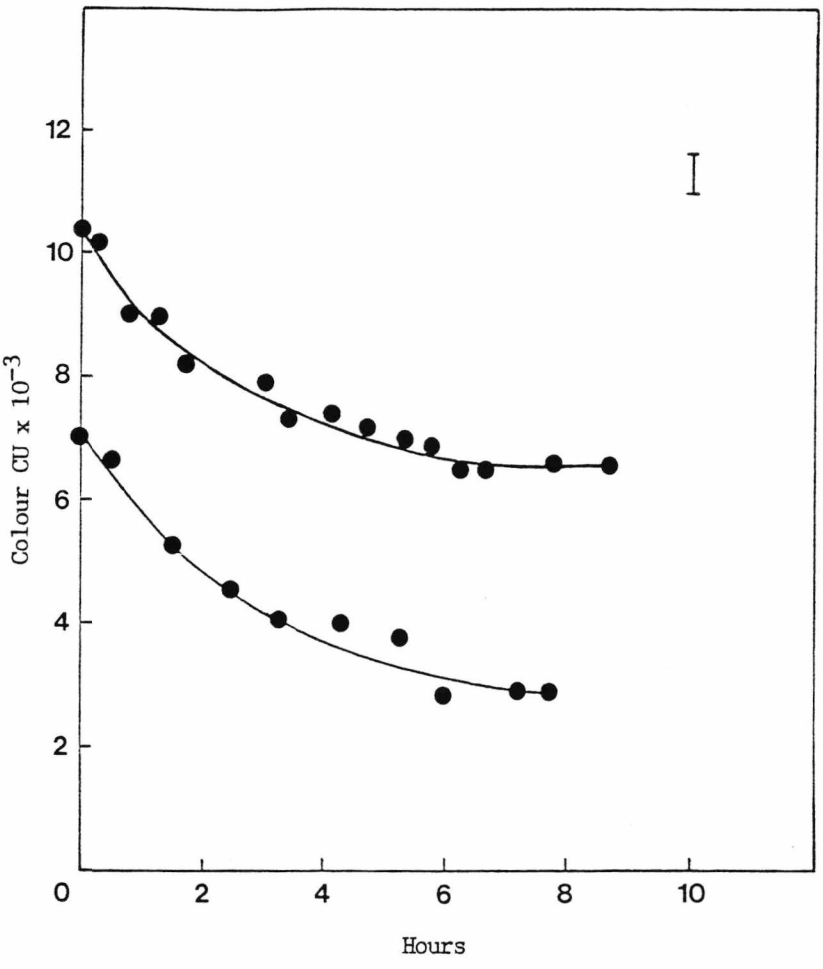

Fig. 6.14. Effect of initial effluent concentration on the decolorisation of E effluent, pH 5.0, by covalently immobilised laccase in a fluidised bed reactor with effluent continuously recycled through reactor. 1 g carbon support:100 ml effluent, flow rate 30 ml/min.

Table 6.2. Colour removal from industrial effluents by laccase covalently bound to activated carbon in fluidised bed. Effluent corrected to pH 5.0

Effluent type	Initial colour CU x 10^{-3}	Colour removed CU/h	Initial activity μMO_2/min/g	Decolorisation efficiency CU/U/h
OH	18.4	467	10.546	44.28
OH	11.4	971	10.546	92.10
E	10.2	594	5.161	115.10
E	6.8	531	5.161	102.90
S	2.25	287	7.663	37.50

CU/U/h, initial colour 10.2 x 10^3 CU). In contrast to the results with E effluent increasing the concentration of OH from 11.4 x 10^3 CU to 18.4 x 10^3 CU resulted in a decrease in colour removal rate from 92.1 CU/U/h to 44.3 CU/U/h.

Fig. 6.15 shows the colour removal from two successive batches of OH effluent by one batch of CIL, initial activity 10.546 $\pm$ 0.277 μMO_2/min/g carbon. The average rate of decolorisation was 633 CU/h for the first 12 h (60 CU/U/h), and the activity had decreased to 8.476 $\pm$ 0.539 μMO_2/min/g carbon (-20%) at the end of this period. The second batch of effluent was decolorised at a much lower average rate of 214 CU/h, (35.6 CU/U/h) and this time the enzyme activity had decreased to 6.011 $\pm$ 0.444 μMO_2/min/g carbon (-43%). Therefore decolorisation of more than one batch of effluent by CIL leads to a rapid decrease in enzyme activity.

DISCUSSION

Laccase has been successfully immobilised using covalent binding methods by a number of authors and in the research reported here was bound to activated carbon (AC) by four methods. Although similar amounts of laccase protein were bound to the carbon support by each method, the greatest immobilised activity was achieved following activation of the carboxyl groups on the carbon with a water soluble diimide which then formed a peptide bond with the amino groups of laccase. Loss of enzyme activity by two methods: coupling laccase directly to amino carbon and coupling via peptide bonds between the carboxyl group of the laccase and the amino groups of carbon (using the diimide), may have been due to coupling close to the active site of laccase or changes in the conformation of the enzyme. This was not expected because laccase is a

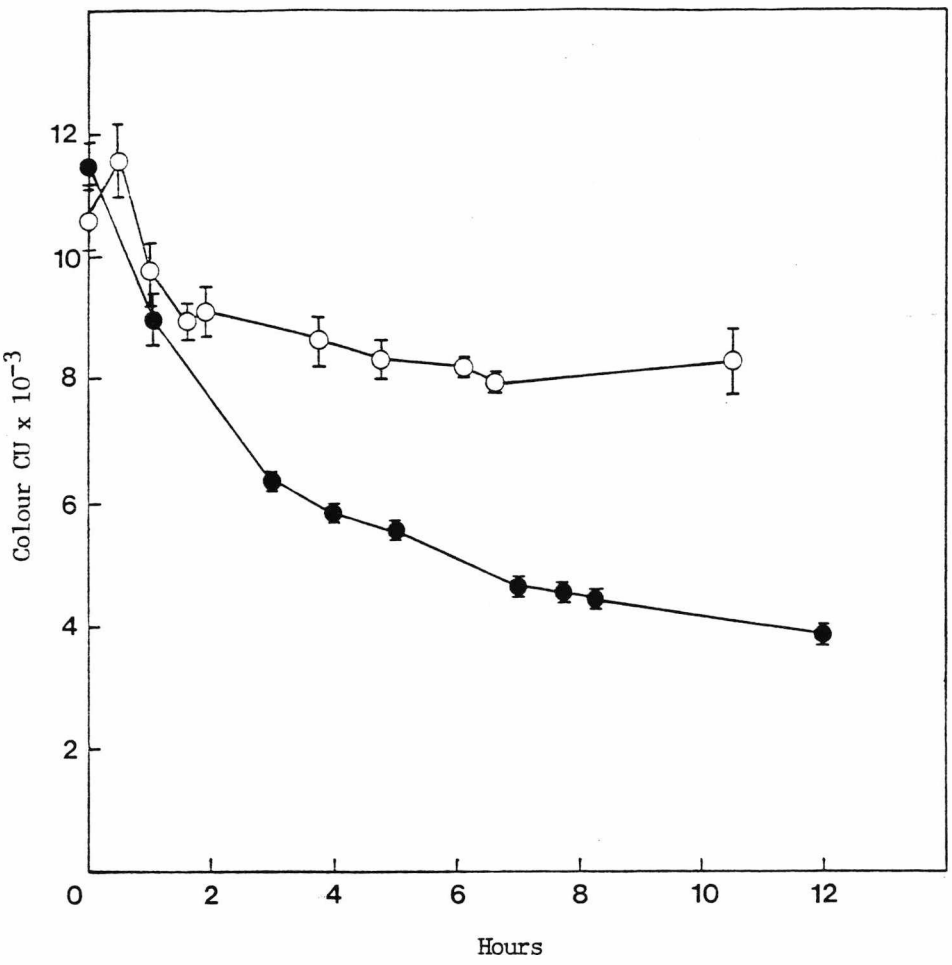

Fig. 6.15. Decolorisation of 2 batches of OH effluent, pH 5.0, by covalently immobilised laccase in a fluidised bed reactor with effluent continuously recycled through reactor. 1 g carbon support:100 ml effluent, flow rate 30 ml/min. ●, batch 1; ○, batch 2.

glycoprotein (1.4.2) and therefore coupling by carboxyl groups is likely to bind the enzyme through the carbohydrate region, away from the active site. Such binding was shown by Froehner & Eriksson (1975) who coupled laccase to Concanavalin-A-Sepharose 4B via the carbohydrate moiety with no change in the properties of the enzyme. However on binding to carbon laccase may form more than one bond per enzyme molecule compared with binding on Con-A-Sepharose where the active groups may be at a lower density on the support surface.

The decrease in activity with increasing laccase loading on AC may be due to laccase overloading which would block the pores in the carbon and thereby decrease access to active sites and increase diffusional restrictions to the substrate. A similar relationship between enzyme activity and support loading was observed when laccase was immobilised on silanised carbon. In contrast, the activity of laccase immobilised on amino carbon increased with the amount of protein bound. The latter relationship was reported by Leonowicz et al. (1988) for laccase covalently immobilised to CPG by silanisation. Therefore, the resulting activity must be influenced by the distribution of reactive groups on the support surface and on the orientation of the enzymes with respect to binding proximity to the active site, and any steric hindrances imposed by a high density of bound enzyme molecules. The activities obtained may be an indication of steric hindrances playing more part in the former two types of binding than if laccase is bound by carboxyl groups, resulting in a different orientation of the enzyme due to binding to the carbohydrate region of the molecule. However, insufficient information on the structure of laccase is available to confirm this theory.

The fact that only 77% of added laccase protein was bound to carbon, at first was attributed to either competitive binding by a nonprotein

contaminant or to residual ammonium sulphate in the extract which was shown to reduce binding. However, a pure sample of BSA did not bind by 100%, suggesting that an intrinsic property of the reaction caused incomplete binding of the added protein, but such data is not given by Cho & Bailey (1979) for the attachment of glucoamylase to carbon by the same method. The most efficient laccase binding was achieved with non induced laccase, but for economical reasons, increasing the added level of induced laccase to obtain the same final level of protein bound per g of support was found to be a suitable alternative due to the much higher levels of laccase produced in induced cultures compared to non induced cultures (3.10). A small scale binding assay to find the percent of added protein binding to the carbon was performed for each different batch of laccase so that the optimum bound level of 11.5 mg bound/g carbon could be obtained using different batches of laccase.

The stability of carbon immobilised laccase (CIL) at a range of pH values is a valuable property for treating a variety of effluents and also when the use of laccase at suboptimal pH values is necessary, e.g. when treating highly alkaline effluents. The pH optimum of immobilised enzymes may be altered if the support carries a charge (1.4.5). However, immobilisation caused no change in the pH profile of laccase compared with the soluble enzyme, contrary to reports by Cho & Bailey (1978) that glucoamylase and glucose oxidase immobilised to carbon by the same method showed a shift in the pH optimum towards alkalinity. This discrepancy may be due to the broad pH optimum of laccase so that a shift of 0.5 or 0.8 pH units (which Cho & Bailey observed) would not produce a marked change in the pH profile of laccase. The stability of laccase to high salt concentrations (losing only 1% protein after 30 min incubation in 1.0 M NaCl) is an advantage for the treatment of coal conversion effluents

where concentrations of chloride ions are as high as 0.5 M. In contrast, the removal of laccase from the carbon with high salt concentrations (e.g. 5 M) could be a useful way of regenerating the support if costs deemed it neccessary.

The inactivating effect of high temperature on laccase activity was reduced when laccase was immobilised on carbon. This must be a physical effect of the covalent binding, hindering conformational change of the protein at temperatures above 40°C. This property has been observed for other covalently immobilised enzymes, and was observed for laccase immobilised on controlled pore glass (CPG) (Leonowicz et al. (1988) but not for laccase immobilised on CNBr-Sepharose or Con-A-Sepharose (Froehner & Eriksson 1975). The increased stability of CIL to temperature is an advantage in view of the temperature of pulp mill effluents at outflow (40°C). The increase in activity on binding to carbon by the diimide method compared to the other three methods may have also been caused by conformational changes in the enzyme, as the activation energy of bound laccase was decreased compared to that of soluble laccase.

After a rapid initial drop in activity (-38%), laccase bound to carbon was very stable to storage in non-sterile conditions as a moist cake at 4°C and lost activity at a slower rate than sterile soluble laccase (4.9). The initial rapid decline in activity may have been due to leaching of unbound enzyme from the preparation, which was not removed by the washing sequence (2.13.3). An improvement in storage stability after immobilisation was reported after immobilisation of laccase or hydrogenase to porous glass (Deloggio & Graves 1988; Leonowicz et al. 1988). Furthermore CIL was stored in non-sterile conditions at 4°C, in which conditions soluble laccase very rapidly lost activity due to degradation by microbial contaminants.

Retention of laccase activity during oxidation of successive batches of DMP compared well with that achieved by laccase immobilised on CPG (Leonowicz et al. 1988). An exponential decline in enzyme activity (as observed in Fig. 6.9) is usually observed with soluble enzymes and immobilised enzymes subject to diffusional limitations show a linear decline in activity (Cheetham 1985). In this case the decrease in activity may have been accelerated by adsorption of oxidised DMP to the carbon thereby blocking the pores and decreasing the available surface area with active enzyme. Adsorption of oxidised DMP can be seen to take place because the carbon particles turn an orange colour and the orange precipitate can be removed by vigorous washing. Reuse of Celite immobilised laccase was assessed by Shuttleworth & Bollag (1986) who reported a gradual decrease in the oxidation rates of several phenolic compounds with successive batches of substrate. In our work, the 55% laccase activity remaining after oxidation of 9 l of DMP was greater than the 25% recorded by Leonowicz et al. (1988) after immobilised laccase oxidation of 6 l of DMP. Therefore our preparation of laccase, immobilised on activated carbon by the diimide method, is highly stable and compares very well with laccase covalently immobilised by other workers.

Since laccase was immobilised on a porous support, both internal and external diffusional (1.4.5) effects were observed. However, as long as sufficient mixing is achieved in the bulk solution then external diffusional limitations should be negligible. Reduction of external diffusional limitations (and therefore increases in activity) were achieved by increasing the flow rate of DMP substrate through CIL in the fluidised bed system. The decrease after the peak of activity was reached was due to back mixing (Cheetham 1985; Hartmeier 1986) in the reactor; at

high flow rates plug flow was not achieved and the efficiency of conversion decreased.

With respect to internal mass transfer, the particle size of the support and the substrate concentration are important factors in determining the rate of reaction. In our case a balance is required between a small particle size (to reduce the diffusion path within the particles) and a particle with sufficient density to drop from suspension at the top of the fluidised bed column. This means that a high initial effluent concentration is required since laccase may be substrate limited in diluted effluent (4.3). The apparent K_m quoted (6.2) represents the overall K_m value for all the enzyme molecules bound on and within the carbon particles, which will give different intrinsic values, and the kinetic constants will only apply to the particular set of conditions used in determination (a mathematical explanation of diffusional limitations in porous particles is given by Engasser & Horvath 1976). With the carbon-laccase system used here internal diffusional limitations were confirmed when the kinetic data, plotted according to Eadie-Hofstee, gave a sigmoidal curve rather than the linear plot given by soluble laccase.

Using effluents diluted to give low colour levels ($<< 1 \times 10^4$ CU), colour removal by adsorption to carbon alone was as great as that by CIL. However since laccase is likely to be substrate limited when treating such low concentrations of effluent more efficient decolorisation would be achieved using undiluted effluents. Maximum colour removal occurred from E effluent in batch incubations and was 57%, which represents an average removal rate of 64.63 CU/h/mg of laccase over the 3 day incubation period.

The advantages of a fluidised bed system compared with a packed bed

reactor were illustrated by the increased laccase activity. Therefore the fluidised bed reactor was used in all further experiments. The benefits of a fluidised bed system (1.4.5) were particularly well illustrated by comparing the laccase oxidation of DMP with that achieved in a packed bed system. Better oxygen transfer to the enzyme (essential for laccase activity) takes place and also problems associated with the retention of insoluble product on the bed particles are reduced as the particles are not packed down. In other words, in the packed bed the insoluble oxidised product of DMP fills the pores and interparticle spaces of the carbon creating severe diffusional restrictions, in the fluidised bed agitation of the particles reduces this effect.

Due to the long residence times required for decolorisation of industrial effluents (typically > 5 h) the fluidised bed system was operated using effluent recycled via a reservoir and the colour of the bulk effluent in this reservoir was measured. As with batch experiments the colour removal rate increased with the effluent concentration. The initial rapid colour removal in the first 1-2 h may be due to oxidation of low molecular weight substrates for which laccase has a higher affinity, succeeded by more slow removal of compounds for which laccase has a lower affinity. Colour removal from E effluent compared on an enzyme unit basis was slightly greater in the continuous system (115 CU/U/h from initial concentration of 10.2 x 10^3 CU) than in the batch system (94 CU/U/h from initial colour of 28.7 x 10^3 CU) despite the higher concentration of effluent used in the batch system.

Oxygen uptake measurements using CIL with effluents as substrates confirmed that CIL was oxidising components of the industrial effluents and that concentration was limiting to laccase activity. This was also shown with the exception of OH effluent by the colour removal per unit

enzyme by immobilised laccase treating all three industrial effluents. The great rate of oxygen uptake on OH effluent compared to E and S effluent may be accounted for by the low molecular weight (< 1000 D) compounds shown by FPLC molecular weight profiles (4.6). However, these low molecular weight fractions were also present in S effluent, so the reduced activity in this instance may be due to an inhibitory effect of sulphide on laccase. However the decreased rate of decolorisation caused by very high concentrations of OH effluent needs further investigation as no inhibition was previously encountered using soluble laccase.

The effect of increasing the ratio of support to effluent volume above 1:100 was not examined, although this would be an important consideration in scaling up the treatment system. Colour removal by enzymic oxidation, above that removed by adsorption to the carbon support, was again confirmed by the treatment of more than one batch of effluent using one batch of CIL, however the decrease in laccase activity after oxidation of just two batches of OH effluent was 43%, therefore limiting the number of batches of effluent which could be treated with one batch of CIL.

The research described here is the first report of colour removal from industrial effluents by immobilised phenol oxidases and therefore comparisons with published work are difficult to make. However, laccase covalently immobilised to CPG or Celite (Leonowicz et al. 1988; Shuttleworth & Bollag 1986) has shown promise for the removal of individual phenolic substrates from aqueous solutions and this work is a logical extension of that approach.

CHAPTER SEVEN

DISCUSSION AND CONCLUSIONS

In Chapter Three we demonstrated that industrial phenolic effluents could be decolorised by white-rot fungi, but that such effluents required considerable dilution (20-50 fold), amendment with nutrients and adjustment to pH 5-6. The requirement for aeration and an immobilisation method for the fungal mycelium would also add cost to the system and necessitate disposal of biomass at the end of its active life.

The maximum decolorisation achieved was 85% of OH effluent by C.versicolor(K) in 6 days, however, high concentrations of effluent (20% v/v) were toxic to the fungus. Consequently alternative decolorisation methods were investigated.

Phenolic industrial effluents were also decolorised by culture filtrates from C.versicolor(K), although no relationship between culture age and extent of decolorisation was found. C.versicolor(K) produced high laccase activities when grown in OH supplemented medium, and laccase was also induced by high molecular weight lignin-related compounds such as Indulin AT and also by some monomeric phenols including vanillin and gallic acid. However, for routine laccase production, 2,5-xylidine was as effective as Indulin AT when added to C.versicolor(K) cultures grown for 7 days on malt extract broth with added glucose. The induction of laccase by monomeric phenolic compounds has resulted in the proposal of a detoxifying role of laccase in lignin degradation by polymerising low molecular weight phenolic compounds toxic to the fungus (Haars & Hutterman 1980). However, laccase has a demonstrated capacity to depolymerise lignin in vivo (Kirk & Farrell 1987) and in this context the

induction of laccase production to high levels by high molecular weight phenolic compounds would be an advantage for rapid lignin degradation.

The addition of glass beads to C.versicolor(K) cultures dramatically increased laccase production by maintaining the fungal mycelium in a finely dispersed state, thus increasing the nutrient and oxygen availability to individual fungal cells. This illustrates the disadvantages of using pelleted or film forms of mycelium, in which the cells will be in different nutrient states.

Soluble laccase from C.versicolor(K) was shown to be as effective as HRP in removing colour from phenolic effluents and very effective at phenol removal from artificial coal conversion effluent, this is the first report of such properties of laccase. The maximum decolorisation achieved using laccase was 36% (OH), 40% (E) and 30% (S) in 2-4 days, and that by HRP was 61% (OH), 36% (E) and 51% (S) in 2 days. These levels are lower than that achieved using fungi, but also require less time for treatment. Laccase had advantages over HRP due to the use of molecular oxygen as the oxidant, and a higher intrinsic stability in the effluents. Laccase activity remained after colour removal had ceased, whereas HRP activity quickly declined due to the inactivating effect of hydrogen peroxide, and possible due to the more pure form of the enzyme. Laccase has further benefit, in that it can be produced cheaply in microbial culture and extensive purification is not necessary; the crude extract (obtained by ammonium sulphate precipitation) proved to be more stable and more effective than a purer preparation. This property was attributed to the protective effect of contaminants in the crude preparation which also accounts for the lack of enhancement of decolorisation by laccase when gelatin, BSA or Tween 80 were added to treatments. The crude laccase had a higher affinity for syringaldazine than laccase A from DEAE

purification, a property which is difficult to explain unless purification removed a loosely bound cofactor from the laccase molecule, such as a metal ion (e.g. Mg^{2+}, Fe^{2+}). However, no reports of such a cofactor were found in the literature.

Quantification of enzyme activity towards the industrial effuents was complicated by an absence of linearity between colour removal and initial enzyme activity in the case of laccase, possibly due to the enzyme acting on a range of substrates in the effluents for which the enzyme had different affinities. At concentrations of effluent suitable for fungal treatment, substrate limitation of both laccase and HRP was indicated by low rates of activity per enzyme unit. This factor together with the lack of inhibition of activity by high concentrations of effluent means that decolorisation by laccase or HRP will be more efficient with undiluted effluent, a considerable advantage for minimising adjustment of effluents before treatment. Both enzymes removed colour over a wide pH range, reducing the need for strict pH control: adjustment of effluent pH to within 1 pH unit of the optimum for decolorisation would be adequate for efficient colour removal. The effect of laccase on the molecular weight profile of E effluent after treatment confirmed the observation that laccase removed phenolic compounds from effluents by polymerisation and also that compounds up to 20 kD are substrates for laccase. However this does not clarify the role of laccase in lignin breakdown, as laccase causes depolymerisation of lignin related compounds (in vivo) (Kirk & Farrell 1987).

The success of the soluble enzyme decolorisation of phenolic effluents merited investigation of immobilisation methods to facilitate reuse of the enzymes for more than one batch of effluent and separation of enzyme from the effluents after treatment. Two types of immobilisation were

compared: (1) entrapment which uses mild immobilisation conditions and gave preparations with high enzyme activity; and (2) covalent immobilisation which gives a very stable preparation but the comparatively harsh methods of preparation can lead to substantial losses in enzyme activity.

In the case of phenol oxidases, the requirement was for an entrapment medium which would retain the enzymes, but allow free diffusion of a large range of potential substrates of different molecular weights to and from the entrapped enzymes. Alginate entrapment did not retain the enzymes completely in the matrix despite attempts to increase the size of the enzymes by copolymerisation with tyrosine. However, the gradual release of laccase and HRP into effluents did enhance the colour removal from industrial effluents compared to soluble enzymes. Entrapped laccase caused decreases in colour of 75% (OH), 59% (E) and no colour removal from S effluent. HRP entrapped in alginate caused decreases in colour of 68% (OH), 52% (E) and 51% (S). However the maximum colour removed overall was 86% OH effluent using entrapped HRP with a bead:effluent ratio (v/v) of 1:2.5. However, beads were only suitable for the decolorisation of one batch of effluent, and enzyme release from beads was not decreased by the copolymerisation of enzymes with tyrosine. Also it was very difficult to add the correct level of hydrogen peroxide to enhance HRP activity as it was released from the beads. Overall, alginate entrapment was found to be inadequate as an immobilisation method for laccase or HRP for industrial effluent treatment as the reusability of the enzyme was severely limited.

Laccase was successfully bound to activated carbon using a water soluble diimide and the resulting immobilised enzyme was stable to a wide range of pH and ionic concentration values. However the maximum immobilised activity obtained was low due to both inactivation during the

immobilisation procedure and diffusional restrictions imposed by the porous nature of the support. Such restrictions can be minimised by using high substrate concentrations and a reactor configuration providing rapid substrate flow through the support, so that there are negligible external diffusional limitations. Therefore as expected, a fluidised bed column reactor was found to be the most efficient for the oxidation of 2,6-dimethoxyphenol by immobilised laccase. Covalently immobilised laccase could continuously oxidise DMP retaining high levels of activity, comparing very well with reports in the literature for continuous oxidation of monomeric phenols by immobilised laccase. This process merits further investigation for the treatment of effluents such as coal conversion.

One disadvantage of activated carbon as a support was the adsorption of both substrate (DMP) and coloured compounds from the industrial effluents onto the carbon particles. However, at high effluent concentrations the adsorptive capacity of the carbon was saturated and additional colour removal was due to laccase activity. Also, decolorisation of effluents by immobilised laccase in the fluidised bed system was very slow and the effluent had to be continuously recycled for several hours to obtain significant decolorisation. Despite this, the maximum decolorisation achieved by this system (57% decolorisation of E effluent) was greater than that achieved by soluble laccase (40% of E effluent) using much lower immobilised enzyme activities, and with the retention of considerable laccase activity bound to the carbon. Further work in this area is required to investigate effluent:support ratios, alternative support materials, and improvements in laccase loading (using different carbon pretreatments) to increase the immobilised activity with the aim of improving the colour removal from industrial effluents. The

polymerisation of monomeric phenols by laccase could be useful for the detoxification of environmental pollutants and other effluents such as coal conversion effluents which contain a variety of low molecular weight phenolics.

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PUBLICATIONS

Davis, S., & Burns, R.G. (1988). Decolorization and degradation of phenolics in industrial effluents. Society for General Microbiology : Abstracts. 110th meeting.

Phenolic effluents are produced by many industries. They are pollutants of receiving waters due to their toxicity, BOD and high colour. Present treatment methods are expensive and often inefficient.
Colour and phenol removal from industrial effluents by white rot fungi and immobilized phenol oxidases were measured. Three types of phenolic effluent (pulp mill E-stage,[E], cotton mill hydroxide,[OH], and cotton mill sulphide,[S]) were added to batch cultures of white rot fungi with glucose as carbon source. After 6 days 2 000-5 000 colorimetric units,[CU], of each effluent was reduced by 70-75% (Stereum hirsutum. Coriolus versicolor) and by 75-85% (Polystictus versicolor). Concentrations of OH of 14 000 CU but not of 20 000 CU were decolourized by P.versicolor and three separate additions (at day 0,8,16) of 1200 CU were each decolourized by 80%.
Commercial horseradish peroxidase (HRP) or laccase from P.versicolor, removed 50% and 43% of colour from OH (3 000-4 000 CU) as free enzymes and 50% and 64% when immobilized in calcium alginate beads. Copolymerization of HRP or laccase with tyrosine, before entrapment, was a more efficient method of decolorizing OH effluent than entrapped HRP or
laccase. None of the enzyme treatments removed colour as well as the batch cultures of fungi.
Future work will concentrate on the removal of individual phenols and lignin breakdown compounds identified in the three effluents (e.g.vanillin, ferulic acid, veratric acid) using free and immobilized P.versicolor, S.hirsutum, C.versicolor and phenol oxidases.

Davis, S., & Burns, R.G. (1989). Immobilization of phenol oxidase enzymes for industrial effluent treatment. Society for General Microbiology : Abstracts. 113th meeting.

The effects of soluble and immobilised phenol oxidase enzymes on industrial phenolic effluents were compared. Soluble laccase and horseradish peroxidase (HRP) removed colour fromm pulp mill (E), cotton mill hydroxide (OH) and cotton mill sulphide (S) effluents, but rapid and irreversible enzyme inactivation took place. Soluble laccase (222 U ml^{-1}) precipitated 1.2 g l^{-1} phenol from artificial coal conversion effluent at pH 6.0, although the optimum for activity was 4.0-5.0.
Entrapment of laccase in alginate beads improved decolorization by factors of 3.5 (OH) and 2 (E); entrapment of HRP improved decolorization by 36 (OH), 20 (E) and 9 (S). Copolymerization of laccase or HRP with L-tyrosine gave insoluble polymers with enzyme activity. Entrapment of these copolymers in gel beads further increased the efficiency of decolorization of E effluent by 28 (laccase) and by 132 (HRP) compared with soluble enzymes. However, beads were unsuitable for continuous use because the enzymes were rapidly released into solution.
Laccase could be immobilized on activated carbon by covalent coupling using a water soluble diimide. Up to 50 mg/g carbon could be bound but at the highest protein levels the relative enzyme activity decreased. The immobilized enzyme was not eluted from carbon by washing with 10 mM buffer (pH 4.0-9.0) and was stable to salt concentrations up to 1M. The pH profile was unchanged but the temperature range of activity was broadened. Carbon immobilized laccase retained 20% of original activity after oxidation of seven batches of 2,6-dimethoxyphenol.

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Decolorization of phenolic effluents by soluble and immobilized phenol oxidases

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Summary. Colour removal from phenolic industrial effluents by phenol oxidase enzymes and white-rot fungi was compared. Soluble laccase and horseradish peroxidase (HRP) removed colour from pulp mill (E), cotton mill hydroxide (OH) and cotton mill sulphide (S) effluents, but rapid and irreversible enzyme inactivation took place. Entrapment of laccase in alginate beads improved decolorization by factors of 3.5 (OH) and 2 (E); entrapment of HRP improved decolorization by 36 (OH), 20 (E) and 9 (S). Beads were unsuitable for continuous use because the enzymes were rapidly released into solution. Co-polymerization of laccase or HRP with L-tyrosine gave insoluble polymers with enzyme activity. Entrapment of the co-polymers in gel beads further increased the efficiency of decolorization of E by 28 (laccase) and by 132 (HRP) compared with soluble enzymes. Maximum decolorization of all three effluents by batch cultures of *Coriolus versicolor* (70%–80% in 8 days) was greater than the maximum enzymic decolorization (48% of OH in 3 days by entrapped laccase). Soluble laccase (222 units ml^{-1}) precipitated 1.2 $g\,l^{-1}$ phenol from artificial coal conversion effluent at pH 6.0 and the rate of precipitation and enzyme inactivation was faster at pH 6.0 than at pH 8.5.

Introduction

Phenolic effluents, which colour receiving waters and are toxic to mammals and fish (Peyton 1984), are produced by pulp and paper, coal conversion, petrochemical, dyeing and textile industries. A major source of phenolic wastes is the alkaline-extraction-stage effluent from wood-pulp bleaching, which contains over 50% of the colour load (Eriksson and Kirk 1986). Conventional treatment methods, such as aerated lagoons and activated sludge plants are ineffective in removing this colour. However, physical and chemical treatment methods, including ultrafiltration, ion exchange and lime precipitation, are expensive and alternative biotreatment processes are now being considered (Boman et al. 1988).

Treatment of phenolic effluents with immobilized white-rot fungi removes colour and degrades toxic compounds (Eaton et al. 1982; Royer et al. 1985). The RBC process uses *Phanerochaete chrysosporium* mycelium immobilized on rotating biological contactors (Sundman et al. 1981; Eaton et al. 1982), and requires an initial growth period after inoculation prior to exposure to the effluent. *Coriolus versicolor* immobilized in calcium alginate beads (Royer et al. 1983) or as mycelial pellets (Royer et al. 1985) has been used in air-lift reactors, but there are diffusional limitations and alkaline effluents have to be adjusted to pH 4–5 before treatment.

Laccase and peroxidase oxidize phenolics to aryloxy radicals, which spontaneously polymerise to form insoluble complexes; these can be removed by precipitation, filtration or centrifugation (Alberti and Klibanov 1981). Treatment with horseradish peroxidase (HRP) facilitated the removal of aromatic amines and phenols from industrial effluents (Alberti and Klibanov 1981; Klibanov and Morris 1981) with concomitant removal of carcinogens. HRP is also active over a wide pH range and has been used to precipitate phenol from coal conversion effluents at pH 9.0 (Klibanov et al. 1983).

The use of tyrosinase was proposed as a cheaper alternative to HRP (Atlow et al. 1984) as it uses molecular oxygen as the oxidant instead of hydrogen peroxide. A crude preparation of tyrosinase from mushrooms was as effective as highly purified commercial enzyme in dephenolizing water from a coke plant. However the enzyme was rapidly inactivated during the reaction.

Laccase can oxidise a wide range of substituted phenols (Shuttleworth and Bollag 1986) and can easily be purified from the culture medium of *C. vesicolor*

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(Mosbach 1963). In aerated liquid medium, laccase polymerizes lignosulphonates from spent sulphite effluent (Forss et al. 1987). However, we have found no reports of laccase decolorization of industrial effluents.

Since a drawback to utilising phenol oxidases rather than white-rot fungi in effluent treatment is the cost of the enzymes, the operational stability of the catalyst is of paramount importance. We report here the effect of a simple immobilization method on enzymic removal of colour from three industrial effluents and an artificial coal conversion effluent. The method is compared with colour removal by white-rot fungi in batch culture.

Materials and methods

Enzymes and reagents. Horseradish peroxidase (Type II) (donor: H_2O_2 oxidoreductase, EC 1.11.1.7), mushroom tyrosinase (O_2: *o*-diphenol oxidoreductase, EC 1.14.18.1), pyrogallol (1,2,3-trihydroxybenzene) and guaiacol (*o*-methoxyphenol) were purchased from Sigma (St. Louis, Mo, USA), hydrogen peroxide (30% solution) from Fisons, Loughborough, Leics, UK, and sodium alginate (*Laminaria hyperborea*) from BDH, Poole, Dorset, UK. All other chemicals were standard reagent grade.

Effluents. Four types of phenolic effluents were examined: (i) pulp mill bleach plant effluent (E effluent) (provided by Professor K.-E. Eriksson, [illegible], Stockholm, Sweden); (ii) cotton cleaning mill effluent high in sulphide (S effluent); (iii) cotton cleaning mill effluent from a caustic treatment stage (OH effluent), both provided by Biomechanics, Ashford, Kent, UK; and (iv) artificial coal conversion effluent.

The E effluent (pH 7.5) was a concentrate of soluble high molecular mass material (>1000 daltons) from the alkaline extraction stage in chlorine bleaching of softwood kraft pulp (Kringstad and Lindstrom 1984). The colour density of OH effluent (pH 14.0) increased with decreasing pH and a precipitate was formed on acidification to pH 2–3. Gas chromatographic-mass spectrophotometric analysis (personal communication, Biomechanics) has shown that OH effluent contains low molecular weight compounds such as ferulic and vanillic acids, typical substrates for laccase and peroxidase. Ultrafiltration (Amicon PM 10 and 30 membranes; [illegible]) revealed that colour is associated with compounds from 1×10^3 to a 2×10^5 Daltons. The S effluent (pH 7.0) also formed a precipitate upon acidification. All effluents were adjusted to pH 7.0 before use in enzyme and microbial growth experiments. Artificial coal conversion effluent was composed of: phenol, 2 g/l; NH_4Cl, 15.7 g/l; $MgCl_2$, 24.6 g/l; and Na thiocyanate, 2 g/l (Singer et al. 1978). The pH was adjusted to 8.5 (5 *M* NaOH) or 6.0 (5 *M* HCl).

Culture of fungi and preparation of laccase. Coriolus versicolor (UKC Culture Collection, [illegible]) and *C. versicolor* (from Dr. A. D. M. Rayner, Bath University, UK) were grown in 100 ml malt extract medium in 250-ml erlenmeyer flasks, inoculated with four 1-cm diameter plugs from the growing zone of fungi on malt extract agar. Flasks were incubated at 30° C and 200 rpm with a 1-cm diameter glass bead. After 4–5 days a dense mycelial suspension had formed. Aliquots (5 ml) of this suspension were used as inocula for duplicate flasks of mineral medium (Arora and Sandhu 1985), supplemented with 5.0 g l^{-1} glucose plus separately sterilised effluent to give a final concentration of $1–5 \times 10^3$ colorimetric units (CU) and incubated at 25° C in shaken batch culture (200 rpm).

Laccase was prepared from 1 l shaken cultures of *C. versicolor* UKC, grown on malt extract medium plus 5.0 g l^{-1} glucose and induced at day 7 with 2,5-xylidine (Fahreus and Reinhammar 1967). After 14 days laccase was precipitated from the culture medium with 80% saturation $(NH_4)_2SO_4$, the precipitate redissolved in 0.01 *M* acetate buffer pH 5.0, and dialysed against buffer for 48 h at 4° C. The dialysate was frozen at −20° C, thawed, and laccase solution squeezed from the resulting polysaccharide matrix (Mosbach 1963). This crude laccase preparation was used in experiments without further purification.

Enzyme assays. Laccase activity was measured using 0.35 m*M* guaiacol as substrate at pH 5.0 (Arora and Sandhu 1985). Horseradish peroxidase (HRP) activity was measured by the method recommended by Sigma using 42 m*M* pyrogallol at pH 6.0. Tyrosinase activity was measured in a 5-ml volume containing 0.5 m*M* L-tyrosine in 50 m*M* phosphate buffer, pH 6.5. For each enzyme one unit of activity is defined as the amount of enzyme that produced an increase in absorbance (laccase 450 nm, HRP 420 nm, tyrosinase 480 nm) of 0.001/s at 25° C. Assay of enzyme activity in gel beads was as above, with five beads shaken in 3 ml reaction mixture in 25-ml volume flasks at 25° C until absorption was greater than 0.100. Rate of release of enzyme from the beads was measured as increase in enzyme activity in the supernatant with time. All assays were performed in triplicate and gave standard deviations of less than 10% of the mean.

Immobilization of enzymes. Laccase and HRP were co-polymerised with L-tyrosine (Sakar and Burns 1984), centrifuged (30 mins, 20000 *g*), the pellet washed with 0.01 *M* phosphate buffer, pH 6.0, and stored as a wet cake. Laccase, HRP and corresponding L-tyrosine-enzyme co-polymers were immobilized by entrapment in 3% w/v calcium alginate gel beads cured with 2% (w/v) $CaCl_2$ (Bashan 1986). Added enzyme or co-polymer constituted less than 5% (w/v) of the total beads formed. Beads were formed with an average diameter of 3 mm. All preparations were stored in a minimum amount of distilled water at 4° C until use.

Measurement of colour in industrial effluents. Colour removal by enzymes was measured in duplicate in 50 ml effluent at 25° C and 200 rpm. The effluents were diluted to give an initial colour density of 4×10^3 CU. Effluents were adjusted to pH 5.0 for laccase treatment, pH 6.0 for HPR treatment with 50 m*M* H_2O_2 unless otherwise stated, and pH 6.5 for tyrosinase treatment. Alginate beads were added to effluents at a concentration of 10% (v/v). Controls, to measure any sorption of colour by alginate beads per se, contained denatured enzyme. After treatment effluent solutions were centrifuged (20000 *g*, 10 min) to remove insoluble polyphenolics, adjusted to pH 7.6 (Atlow et al. 1984) with 0.1 *M* phosphate buffer, and colour density measured at 465 nm. Optical density readings were converted to CU by the equation: $CU = 500 \times A_{465}/0.132$ where 0.132 is the absorbance of 500 CU platinum-cobalt standard solution (Sundman et al. 1981).

Measurement of phenol concentration in artificial effluent. Phenol removal from artificial effluent was measured in triplicate by adding soluble laccase (4×10^3 units) to 20-ml volumes of artificial effluent in 100-ml flasks and incubating at 30° C and 200 rpm. After centrifugation (10 min, 20000 *g*) phenol in artificial effluent was measured colorimetrically at 505 nm (Emerson 1943). Standard deviation was less than 10% of the mean value.

Results

Colour removal by laccase

Soluble laccase polymerized phenolics and thereby permitted the removal of colour in both pulp mill (E) and hydroxide (OH) effluents (Fig. 1, Table 1). Entrapment of laccase in gel beads increased the efficiency of colour removal from OH effluent in comparison with sol-

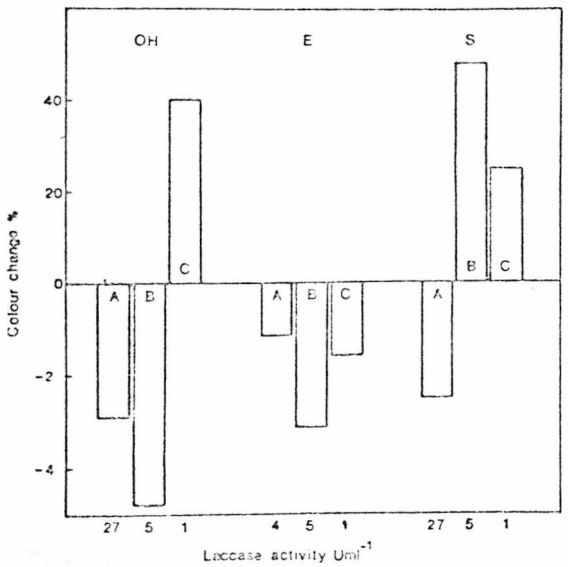

Fig. 1. Colour change in hydroxide (OH), pulp mill (E) and sulphide (S) effluents by laccase after 3 days: *A*, soluble laccase; *B*, alginate-entrapped laccase; *C*, alginate-entrapped laccase-tyrosine co-polymer. Initial colour density 4×10^3 colorimetric units (CU); U = units

uble enzyme, e.g. with OH effluent soluble laccase removed 111 CU per unit enzyme whilst entrapped laccase removed 387 CU per unit enzyme. Entrapped laccase-tyrosine co-polymers also polymerized OH effluent giving an increase in colour but without precipitate formation. Entrapment of laccase-tyrosine copolymers increased decolorisation of E effluent: 129 CU removed per unit free enzyme; 3630 CU removed per unit entrapped laccase-tyrosine co-polymer. In all cases decolorization was accompanied by precipitate formation. Free laccase removed 74 CU per unit enzyme from sulphide (S) effluent but although both forms of immobilized laccase increased the colour there was no precipitation.

Colour removal by peroxidase (HRP)

Soluble HRP removed colour from all three effluents (Table 1, Fig. 2) whilst enzyme entrapment increased removal efficiency by factors of 36 (OH), 20 (E) and 9 (S). Further improvements were seen when HRP-tyrosine co-polymers were entrapped, giving increases of $\times 274$, $\times 132$ and $\times 15$ respectively over soluble enzyme.

Colour removal by tyrosinase

Soluble tyrosinase significantly increased the colour of OH and S effluents in the first 72 h, but no insoluble product was formed. No colour was removed from E

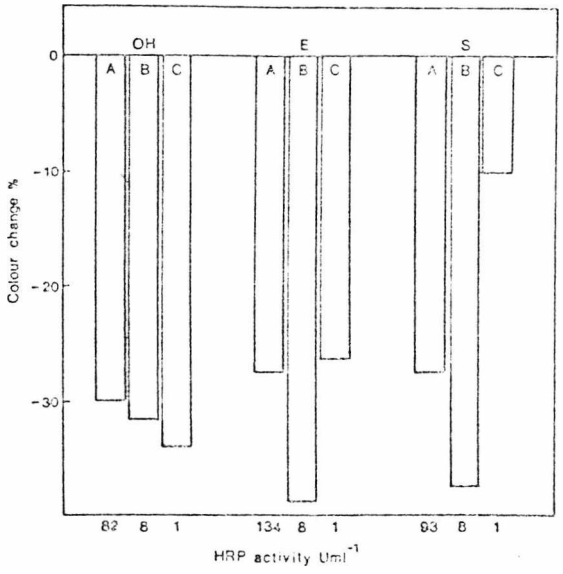

Fig. 2. Colour change in OH, E, S effluents by horseradish peroxidase (HRP) with 50 m*M* H_2O_2 after 3 days: *A*, soluble HRP; *B*, alginate-entrapped HRP; *C*, alginate-entrapped HRP-tyrosine co-polymer. Initial colour density 4×10^3 CU

Table 1. Effect of soluble and immobilized phenol oxidases on the colour of hydroxide (OH), sulphide (S), and pulp mill (E) effluents

Enzyme	Effluent type	Change in CU per enzyme unit after 3 days		
		Soluble enzyme	Entrapped enzyme	Entrapped enzyme-tyrosine co-polymers
Laccase	OH	−111+11	−387+34	+8674+1914
	E	−129+88	−251+91	−3630+1500
	S	− 74+ 6	+357+49	+6353+ 808
Peroxidase	OH	− 4+ 1	−145+13	−1097+ 200
	E	− 9+ 2	−184+80	−1185+ 366
	S	− 16+ 1	−140+15	− 239+ 143

Decrease in colour (−) indicates formation of insoluble polymers, removed by centrifugation; increase in colour (+) indicates formation of soluble polymers. Each value is mean of three replicates plus the standard deviation. CU = colorimetric units

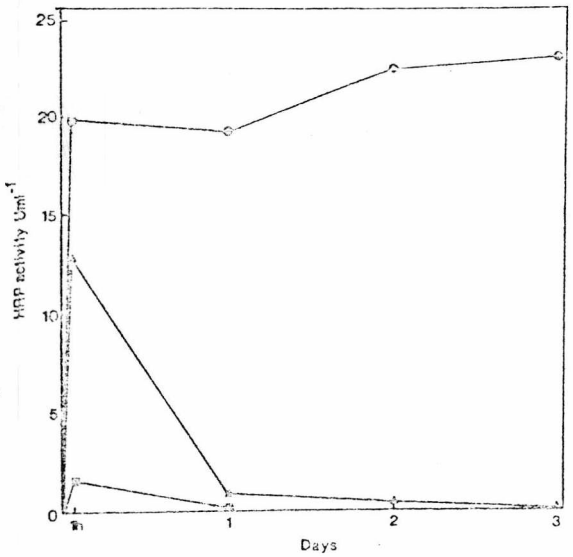

Fig. 3. Effect of H_2O_2 concentration on the activity of released HRP in OH effluent containing alginate-entrapped HRP: ○, 7 m*M* H_2O_2; ▲, 70 m*M* H_2O_2; □, 700 m*M* H_2O_2. Initial activity was 22.5 U ml^{-1} as entrapped soluble HRP

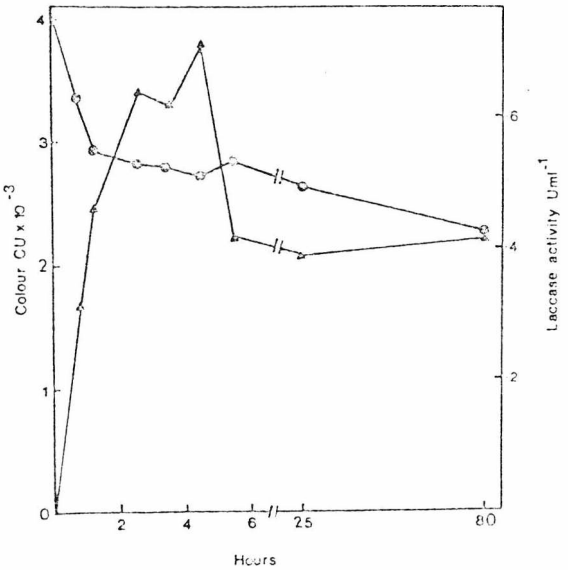

Fig. 4. Diffusion of laccase from alginate-entrapped laccase-tyrosine co-polymers in E effluent: ▲, laccase activity in effluent; ○, colour. Initial colour density was 4.0×10^3 CU

effluent and therefore tyrosinase was not immobilised in alginate or co-polymerised with phenolic compounds.

Effect of hydrogen peroxidase concentration on decolorization by HRP

Hydrogen peroxide concentration had a marked effect on the decolorizing ability of entrapped HRP due to inactivation of the released enzyme. At 7 m*M* hydrogen peroxide, HRP activity increased in the buffered effluent as it was released from the alginate beads (Fig. 3). At 70 m*M* hydrogen peroxide, however, inactivation of the enzyme took place after its release, and with 700 m*M* almost all the enzyme activity was destroyed in the first hour. In 3 days, 7 m*M* peroxide allowed 53% decolorization to take place, but at 70 m*M* peroxide, colour removal declined to 49% (25% was due to peroxide alone as shown by controls). Colour removal increased again to 78% in 3 days when the high peroxide concentration of 700 m*M* (causing 40% colour removal) compensated for the HRP inactivation.

Diffusion of enzymes from alginate beads

Diffusion of HRP and laccase from alginate beads was rapid in the first 2 h of incubation and correlated with colour removal (Fig. 4) followed by a slower rate of release. There was no difference in rates of release into buffered effluents or buffer alone, nor was there any significant difference between release of activity from entrapped enzyme and entrapped enzyme-tyrosine co-polymers. Fifty-seven percent of laccase remained active in the effluent after colour removal had ceased, indicating that some of the coloured components cannot be removed by enzymic treatment.

Decolorization by fungi

Of several white rot fungi screened (*Stereum hirsutum, S. gausapatum, Lenzites betulina, Pjerkandera adusta, Pseudotrametes gibbose*), *C. versicolor* strains were the most efficient in decolorizing the effluents, removing a

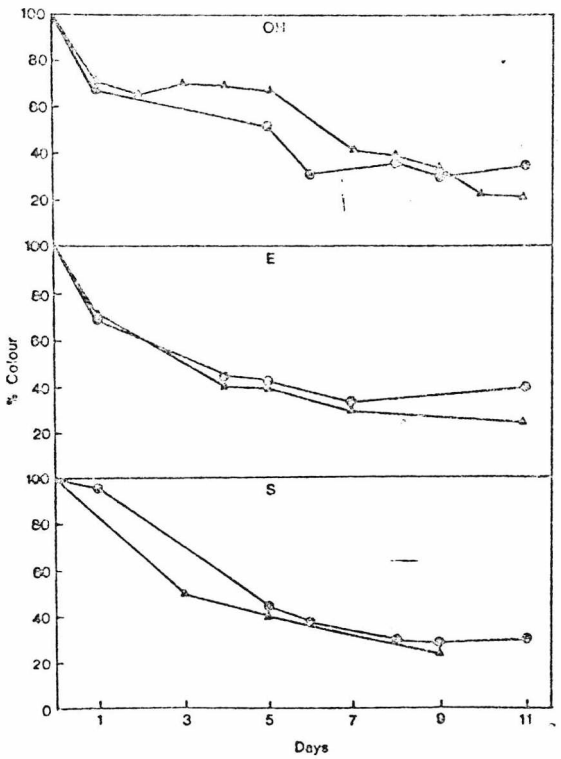

Fig. 5. Decolorization of phenolic effluents by white-rot fungi in shaken culture with glucose as an additional carbon source. Initial colour densities in CU: OH, 1×10^3; E, 1×10^3; S, 5×10^3. ○, *Coriolus versicolor* (Bath); ▲, *C. versicolor* (UKC)

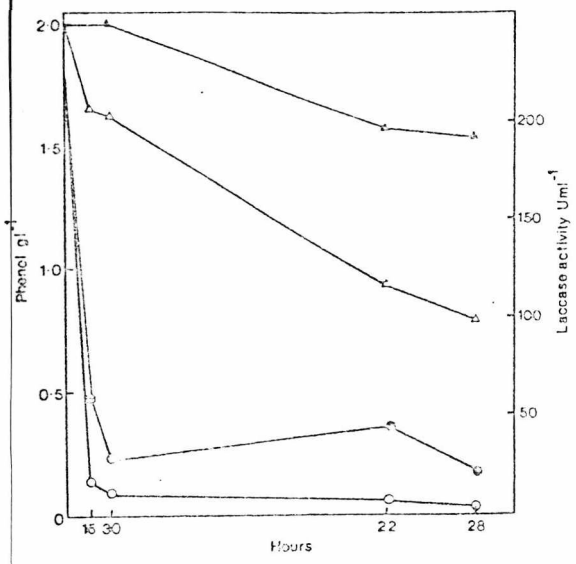

Fig. 6. Phenol removal from artificial coal conversion effluent by soluble laccase at pH 6.0: Δ, phenol; O, laccase; at pH 8.5: ▲, phenol; ●, laccase

maximum of 70%–80% of colour in 8 days (Fig. 5). Additional experiments showed that effluents with an initial colour density greater than 2×10^4 CU were toxic to *C. versicolor*, prevented growth and were not decolorized.

Phenol removal from artificial coal conversion effluent

At pH 6.0 the phenol removal was more rapid than at pH 8.5 (Fig. 6), but was accompanied by more rapid enzyme inactivation in solution, which might be expected. However, a dark purple precipitate was formed which had laccase activity, suggesting that phenol and laccase had co-polymerized. Using an initial activity of 222 laccase units/ml at pH 6.0, 71% of the phenol was removed in 28 h.

Discussion

Both soluble and immobilized laccase were more effective than HRP at removing colour from OH effluent; however, immobilized HRP could also decolorize S effluent. The polymerization, but not precipitation, of OH effluent by entrapped laccase-tyrosine co-polymers may be due to the comparatively low laccase activity obtained on co-polymer formation. A decrease in added activity would also account for colour removal from S by soluble but not by entrapped laccase.

The range of effluents treatable may be extended by combining the two enzymes, as some simple aromatic amines are substrates for HRP but not laccase, and laccase is inactivated less rapidly than HRP in reaction mixtures (Sjoblad and Bollag 1977; Alberti and Klibanov 1981). The differences in colour removal by each enzyme from the three types of effluent can be related to effluent composition. Removal of colour from E effluent shows that both laccase and HRP can polymerize and precipitate high molecular mass phenolics from bleach plant effluents.

The colour removed from OH effluent by the low molecular mass compounds may be enhanced by co-precipitation with higher molecular mass constituents which are poorer substrates for HRP and laccase. This property has been documented for HRP treatment of phenolic effluents (Alberti and Klibanov 1981; Klibanov and Morris 1981) in which the easily polymerized phenols and amines (e.g. benzidine, 2,3-dimethylphenol) enhanced the removal of more resistant aromatics (e.g. phenol and aniline). The S effluent is less well decolorized by laccase and this could be due to a number of factors such as molecular mass distribution, enzyme inhibition or enzyme inactivation.

The inability of tyrosinase to decolorize the effluents studied here may be due to its relatively small substrate range compared with laccase and peroxidase (Sjoblad and Bollag 1981). Tyrosinase has previously been shown to remove phenol from less complex industrial effluents (Atlow et al. 1984) and unlike laccase and peroxidase has not shown evidence for a reaction mechanism using free radicals (Sjoblad and Bollag 1981).

The two functions of H_2O_2 (as co-factor and as bleaching agent) were observed in the decolorization of OH effluent, and have been previously recorded with E effluent (Paice and Jurasek 1984). The most efficient enzymic decolorization took place at hydrogen peroxide:HRP ratios of less than 3 m*M*:1 unit of HRP, although this is difficult to maintain in practice when entrapped HRP is slowly released from the beads. The increase in colour removal on entrapment may be due to the continuous slow release of the enzymes into the effluent. This could be clarified by further experiments, adding soluble enzyme to effluents at a similar rate to their release from the beads.

Over the first 3 days of incubation fungal colour removal (30%–50%) compared favourably with that achieved by enzymes. However on prolonged incubation of fungi, a maximum of 70%–80% decolorization was achieved, some 20%–30% greater than the best enzyme treatment (48% of OH in 72 h by entrapped laccase). This confirms the findings of Paice and Jurasek (1984) who compared HRP-catalysed colour removal from bleach plant effluent with that by *C. versicolor*. The initial rate of descolorization was more rapid when using the enzyme but the total colour removed was greater using the fungus. However, oxidative treatment of effluent using fungi required glucose as an additional carbon source and adjustment of the pH to 4.5 (Royer et al. 1985). This would add to the cost of a treatment system. Also, dilution of added effluent was necessary before fungal treatment, in contrast to enzymic treatment in which the enzyme to substrate ratio determines the extent of decolorization and is independent of dilution (Klibanov et al. 1983).

In addition to colour removal, phenol oxidase enzymes are known to precipitate a range of monophe-

nols from effluents in which colour is less of a problem (Klibanov et al. 1983) and in this respect improvements due to immobilization would also be useful, e.g. for treating coal conversion effluents. The pH of real coal conversion effluents varies from 8.0–9.5 (Singer et al. 1978) so in our experiments with artificial effluents soluble laccase was added at pH 8.5 as well as at pH 6.0, which is closer to the optimum for its activity. Laccase removed phenol at pH 6.0 at a rate of 50.7 mg l^{-1} per hour. However, by increasing laccase concentration in parallel with phenol concentration and temperature, this removal rate could be considerably improved.

In conclusion, laccase and peroxidase have considerable potential for treating phenolic effluents, and we are currently studying more stable immobilization systems for this purpose.

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