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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 mycelial colour removal (MyCoR) 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, Swedish Pulp and Paper Research Institute, 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; Stonehouse, Gloucester, UK) 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, Canterbury, Kent, UK) 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-

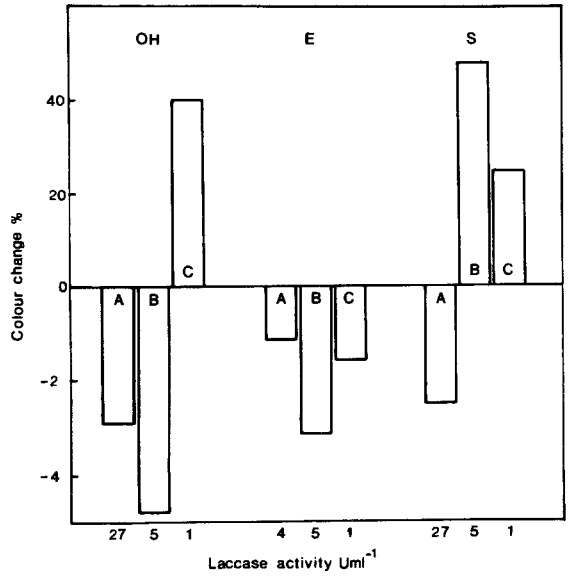

Fig. 1. Colour change in hydroxide (OH), pulp mill (E) and sulphide (S) effluents by laccase after 3 days: *A*, soluble laccase; *B*, alginate-entrapped laccase; *C*, alginate-entrapped laccase-tyrosine co-polymer. Initial colour density 4×10^3 colorimetric units (CU); U = units

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

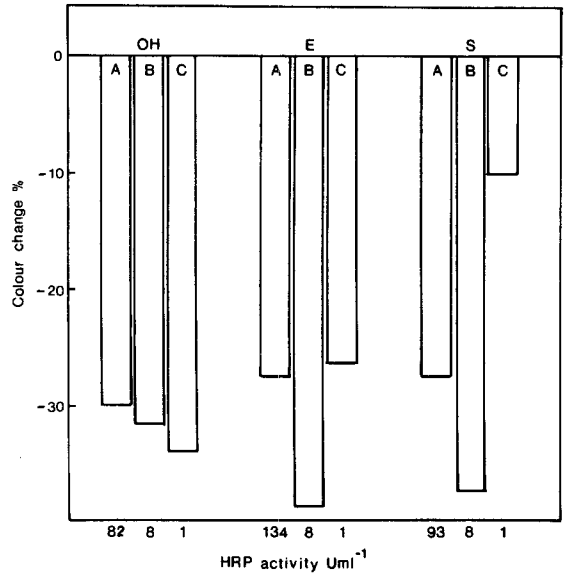

Fig. 2. Colour change in OH, E, S effluents by horseradish peroxidase (HRP) with 50 m*M* H_2O_2 after 3 days: *A*, soluble HRP; *B*, alginate-entrapped HRP; *C*, alginate-entrapped HRP-tyrosine copolymer. Initial colour density 4×10^3 CU

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 with the standard deviation. CU = colorimetric units

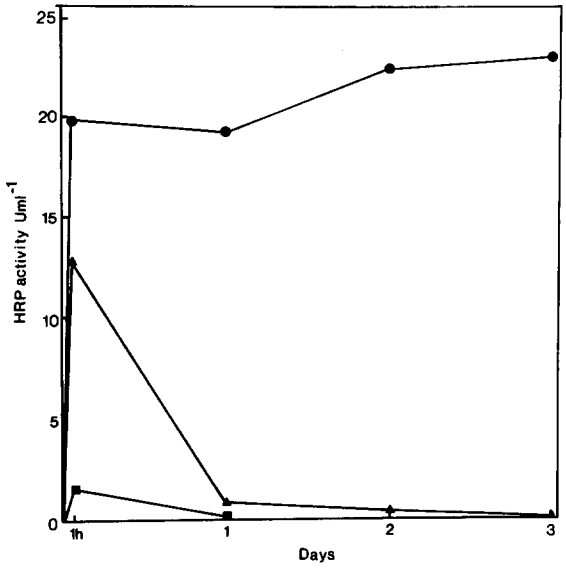

Fig. 3. Effect of H_2O_2 concentration on the activity of released HRP in OH effluent containing alginate-entrapped HRP: ●, 7 m*M* H_2O_2; ▲, 70 m*M* H_2O_2; ■, 700 m*M* H_2O_2. Initial activity was 22.5 U ml^{-1} as entrapped soluble HRP

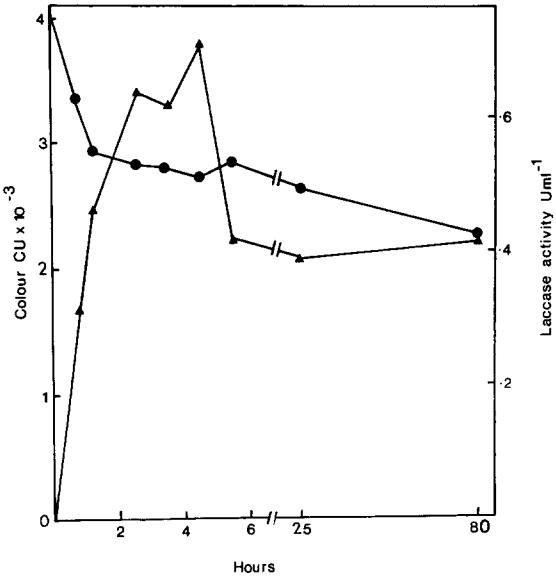

Fig. 4. Diffusion of laccase from alginate-entrapped laccase-tyrosine co-polymers in E effluent: ▲, laccase activity in effluent; ●, colour. Initial colour density was 4.0×10^3 CU

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, Bjerkandera adusta, Pseudotrametes gibbosa*), *C. versicolor* strains were the most efficient in decolorizing the effluents, removing a

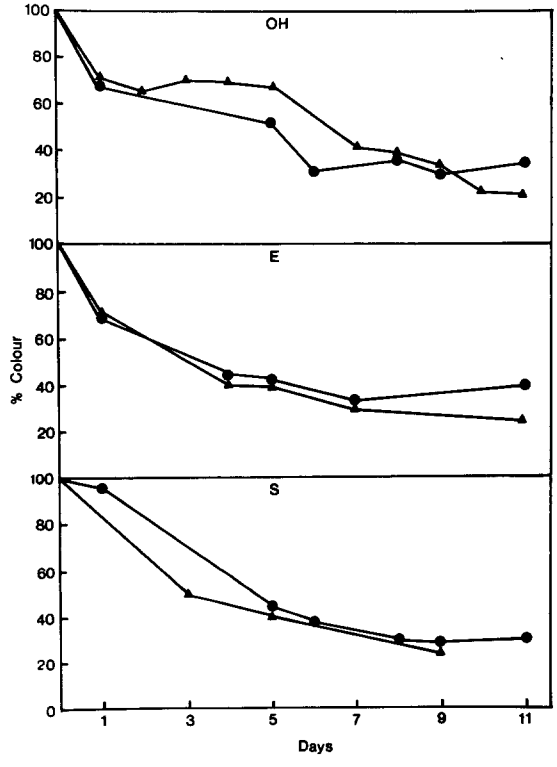

Fig. 5. Decolorization of phenolic effluents by white-rot fungi in shaken culture with glucose as an additional carbon source. Initial colour densities in CU: OH, 1×10^3; E, 1×10^3; S, 5×10^3. ●, *Coriolus versicolor* (Bath); ▲, *C. versicolor* (UKC)

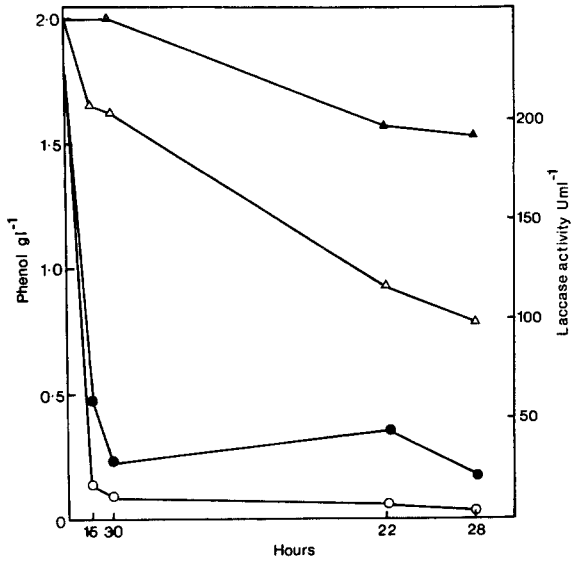

Fig. 6. Phenol removal from artificial coal converison effluent by soluble laccase at pH 6.0: △, phenol; ○, laccase; at pH 8.5: ▲, phenol; ●, laccase

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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