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# Removal of phenols from mixtures by co-immobilized laccase/tyrosinase and Polyclar adsorption

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**An enzymatic method for removal of phenols from their mixtures was investigated. Phenols in an aqueous solution were removed after a two-step treatment with co-immobilized laccase and tyrosinase and Polyclar (polyvinylpolypyrrolidone). A laccase from *Pyricularia oryzae* and mushroom tyrosinase were co-immobilized on Mikroperl in a fixed-bed tubular bioreactor by a rapid and simple method. The support immobilized 95% of the total laccase units and 35% of the total tyrosinase units. Different mixtures of phenols were passed through the column with co-immobilized laccase and tyrosinase. This method removed 42–90% of different phenolic substances by a single passage through the bioreactor. The second step employed Polyclar for additional removal of phenolic substances from mixtures. The degree of removal depends on the nature of the phenols. Complete removal was achieved for $\alpha$-naphthol, 2,4-dichlorophenol, 4-methoxyphenol, $\beta$-naphthol, 4-chloro-3-methylphenol and catehin. The operational stability of the immobilized system was 10–90 h depending on the substrate. The biocatalyst was capable of continuous transformation of different phenols in mixtures.** *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 383–388.

**Keywords:** laccase; tyrosinase; Polyclar; enzyme immobilization; phenol removal; bioremediation; waste treatment

## Introduction

Removal of phenols from industrial aqueous effluents is an important practical problem, because virtually all phenols are toxic and their presence in a number of industrial wastewaters is a health hazard [1].

Different approaches to the purification of phenolic effluents have been developed. Many factors limit the direct use of microorganisms and conventional physical and chemical technologies in the degradation of phenols [2,13]. Treatments with cell free enzymes have been proposed as an alternative [3,4]. Oxidative enzymes like laccase, tyrosinase and peroxidase catalyze oxidative coupling of phenolic compounds resulting in the formation of water-insoluble oligomeric and polymeric products. The resulting precipitate can be removed by sedimentation or filtration [10,12].

Wada and coworkers [19] suggested that treatment with tyrosinase immobilized on a cation exchange resin is much more effective compared with tyrosinase alone and the system can be used repeatedly. A capillary membrane bioreactor using immobilized tyrosinase has been developed and tested for removal of phenolic compounds from synthetic and industrial effluents [11]. A packed column containing chitosan was integrated into the system and almost complete removal of the colored quinones and associated polymers from the permeate was observed. Use of chitosan to bind the products of the tyrosinase-catalyzed reaction of phenols was also proposed by Payne *et al.* [16].

The ability of a laccase to transform phenolic substrates and detoxify phenolic pollutants has been examined [5,18]. Shuttleworth and Bollag [18] proposed immobilization of enzyme on Celite so that it is stable and reusable. Davis and Burns [8,9] immobilized laccase in alginate beads and bonded them covalently to activated carbon using four derivatization methods. A laccase of the basidiomycete *Trametes versicolor* was immobilized on porous glass beads [15]. The immobilized enzyme was reusable in treating different substrates. Ruggiero *et al.* [17] investigated the ability of an immobilized fungal laccase on soil or clay to transform 2,4-dichlorophenol, an intermediate of pesticide degradation. Finally, Crecchio *et al.* [7] ascertained that gel-entrapped tyrosinase or laccase were capable of removing naturally occurring and xenobiotic aromatic compounds from aqueous suspensions with different degrees of efficiency.

Generally, one of the main problems is that wastewater of different origins contains a mixture of many phenols and it is impossible to remove all phenols using one enzyme in one process. In this study, we propose to examine an enzymatic strategy for effective removal of mixtures of phenols in a two-step process. The first step employs co-immobilized laccase and tyrosinase to convert phenols to *o*-quinones in a continuous process. The second step employs treating mixtures with Polyclar (polyvinylpolypyrrolidone) — a cross-linked polymer, capable of adsorbing many phenols [20].

A further motivation for this work is that phenols are common industrial reactants and by-products, and it is often necessary to remove them from intermediate process streams.

## Materials and methods

### Reagents

Tyrosinase (EC 1.14.18.1) from mushroom and laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) from *Pyricularia oryzae* was obtained from Sigma Chemical (St. Louis, MO) and had specific activities of 2200 and 260 U $mg^{-1}$ of solid respectively. Siringaldazine (3,5-dimethoxy-4-hydroxybenzaldehydazin) and all other phenols were obtained from Aldrich (Milwaukee, WI) and were used without further purification.

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### Assay of enzyme activity and protein

Laccase activity was determined from a change in optical density ($A_{530\ nm}$) in a reaction mixture containing syringaldazine as substrate, according to Leonowicz and Grzywnowicz [14].

Tyrosinase activity was determined from a change in optical density ($A_{280\ nm}$) in a reaction mixture containing L-tyrosine.

Protein concentration was determined by the method of Bradford [6] using Biorad protein assay reagent (Biorad Laboratories, Richmond, CA) with bovine serum albumin as the protein standard.

### Immobilization

Solutions of laccase with a specific activity of 20 U $ml^{-1}$ and tyrosinase with a specific activity of 200 U $ml^{-1}$ were prepared in distilled water and then buffered with 0.1 M phosphate buffer (pH 6.5). For the immobilization of enzymes, a tubular reactor was used with a fixed layer of biocatalyst (200 mm high, 10 mm diameter). The reactor was equipped with a jacket connected to a water-bath circulator for temperature control. The reactor was tightly filled with KC-Mikroperl MP100 (ca. 100–200 $\mu$m) purchased from Kalichemie (München, Germany), washed and soaked in 0.1 M phosphate buffer (pH 6.5). Laccase and tyrosinase solutions (separately) were then passed simultaneously through the reactor (the solutions were mixed just before applying them to the column) at a rate of 2.0 ml $min^{-1}$. The effluent was continuously recirculated into a reservoir and back through the column. After the end of the procedure, the biocatalyst was washed with the same buffer until no enzyme activity could be detected in the wash water.

### Substrate specificity and removal of phenols by co-immobilized laccase/tyrosinase following treatment with Polyclar

Phenolic substances were solubilized in water at a final concentration of 0.1 mg $ml^{-1}$. The ability of the free enzymes to transform substituted phenols was tested with dissolved laccase and tyrosinase (see above) for 15 min at 25°C. After the reaction samples were filtered through Millex®-GS 0.22-$\mu$m filter units and analyzed.

Mixtures of phenols were prepared at a concentration of 0.1 mg $ml^{-1}$ and were passed through the reactor at a flow rate of 0.6 ml $min^{-1}$ and at 30°C. The amount of each substrate removed by the co-immobilized enzymes was calculated from the difference between the substrate measured (after filtration by Millex®-GS) in inlet and outlet solution. Samples were analyzed using a Bio-Rad HPLC system consisting of a model 2700 solvent delivery system and a model UV-1806 detector. Separation of compounds was achieved with Supelcosil LC-18 (5 $\mu$m), 25.0 cm×4.6 mm ID column (Supelco)(Bellefonle, PA), mobile phase methanol:water (66:34) and a flow rate of 1.0 ml $min^{-1}$.

After column 500 mg Polyclar were added to 50 ml of solution. Reactions were conducted at 25°C in bottles, which were agitated by shaking or by using a stir bar. Phenols were analyzed after filtration by HPLC to assess phenol removal.

## Results

### Free enzymes

Table 1 shows the percentage of the transformed and removed phenolic substrates after treatment with both enzymes separately.

Laccase and tyrosinase oxidized most of the phenolic substrates tested. Therefore we expected that simultaneous treatment of mixtures of phenolic compounds with laccase and tyronsinase would be more effective in transforming and separating undesired toxic products.

### Immobilization

The effect of the contact time between laccase and tyrosinase and Mikroperl inside the column is shown in Figure 1. After the first flow of laccase solution through the column (after 50 min) 95% of the total laccase units were immobilized. After 100 min the percentage of immobilization increased to 97%; afterward there

**Table 1** Removal of substituted phenolic substrates by free laccase and tyrosinase

| Phenols | Laccase | | Tyrosinase | |
|---|---|---|---|---|
| | Substrate removal (%) | Colored product | Substrate removal (%) | Colored product |
| 4-Methoxyphenol | 0 | – | 71 | + |
| 2,6-Dimethoxyphenol | 13 | + | 1 | + |
| 2,4-Dichlorophenol | 48 | + | 8 | – |
| 4-Chloro-3-methylphenol | 23 | – | 65 | + |
| 4-Chlorophenol | 13 | – | 30 | + |
| $\beta$-Naphthol | 0 | – | 12 | – |
| $\alpha$-Naphthol | 0 | – | 0 | – |
| Chlorogenic acid | 4 | – | 7 | + |
| Caffeic acid | 4 | – | 4 | + |
| 3-Methoxyphenol | 0 | – | 4 | – |
| 2-Chlorophenol | 8 | – | 4 | – |
| Guaiacol | 4 | – | 5 | – |
| *m*-Cresol | 16 | – | 44 | + |
| *p*-Cresol | 7 | – | 44 | + |
| *o*-Cresol | 4 | – | 9 | – |
| 3-Chlorophenol | 4 | – | 0 | – |
| Phenol | 16 | – | 20 | + |
| Catechol | 0 | – | 97 | + |
| Catechin | 10 | – | 66 | + |
| $\alpha$-DOPA | 0 | – | 88 | + |

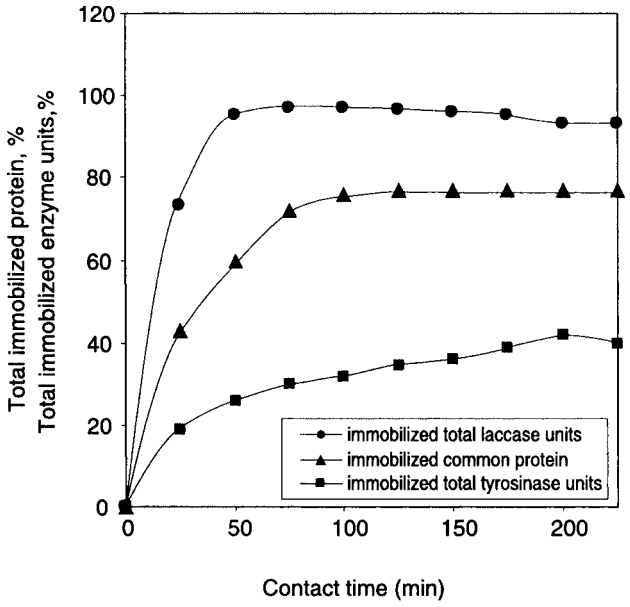


**Figure 1** Immobilization of laccase, tyrosinase and total protein on Mikroperl in a fixed-bed tubular bioreactor.

was a decline which is probably due to enzyme inactivation, because the quantity of immobilized protein was not observed.

A maximum of 42% immobilized tyrosinase was reached after 200 min, after which an equilibrium was observed.

Washing of the column removed only 1–2% of laccase and 7% of tyrosinase. So, on the column we had 95% of the total laccase and 35% of the total tyrosinase immobilized on Mikroperl.

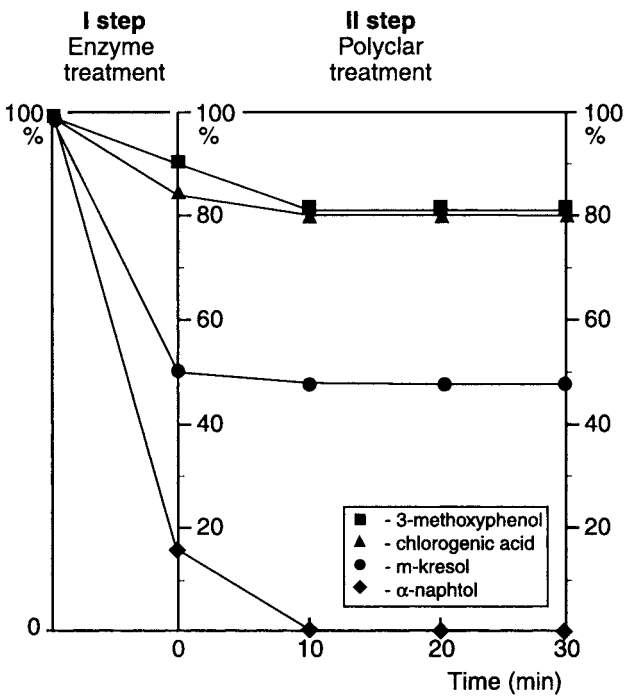


**Figure 2** Removal of chlorogenic acid, 3-methoxyphenol, *m*-cresol and $\alpha$-naphthol from mixtures by co-immobilized laccase and tyrosinase followed by treatment with Polyclar.

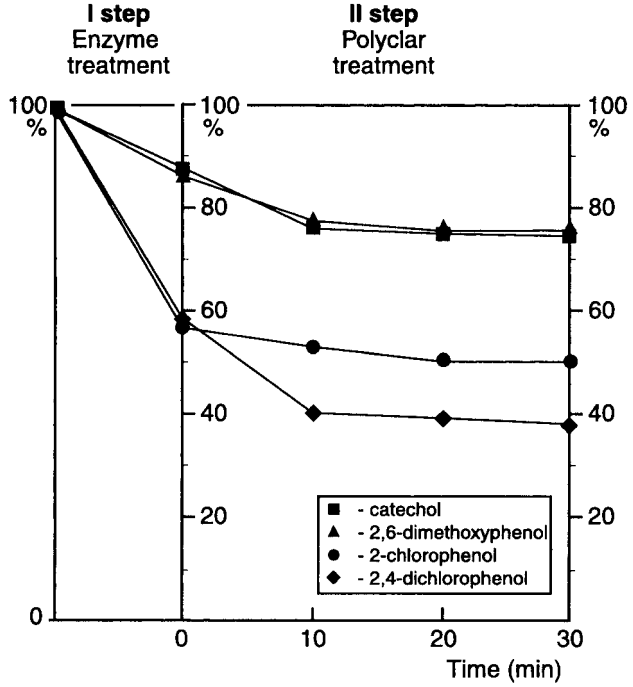


**Figure 3** Removal of catechol, 2,6-dimethoxyphenol, 2-chlorophenol and 2,4-dichlorophenol from mixtures by co-immobilized laccase and tyrosinase followed by treatment with Polyclar.

## *Removal of phenols*

The aim of this study was to demonstrate continuous removal of phenols from mixtures by co-immobilized laccase and tyrosinase

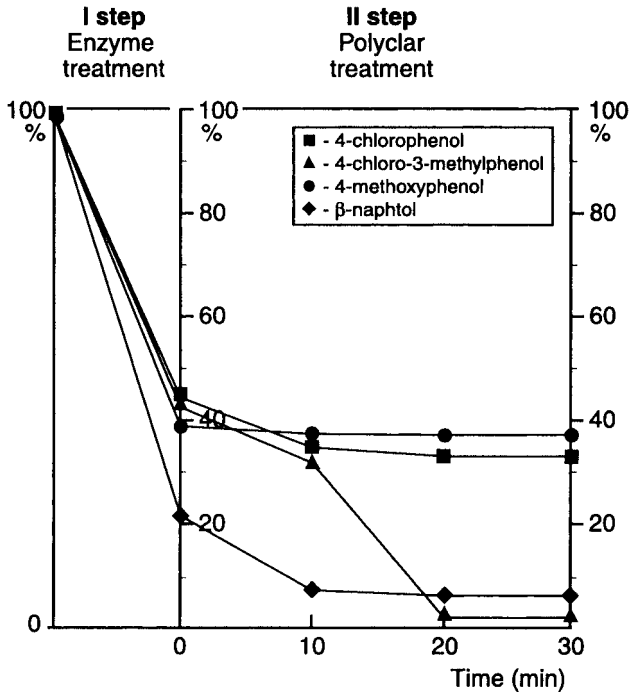


**Figure 4** Removal of 4-methoxyphenol, 4-chlorophenol, $\beta$-naphthol, and 4-chloro-3-methylphenol from mixtures by co-immobilized laccase and tyrosinase followed by treatment with Polyclar.

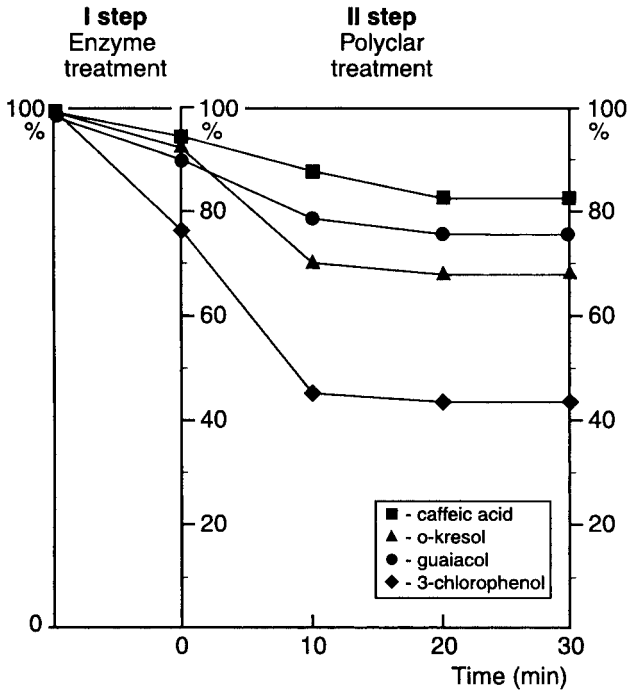


**Figure 5** Removal of caffeic acid, guaiacol, *o*-cresol and 3-chlorophenol from mixtures by co-immobilized laccase and tyrosinase followed by treatment with Polyclar.

in a tubular fixed-bed reactor and subsequent treatment with Polyclar. The results of these investigations are shown in Figures 2–6, for five mixtures of phenols. The figures show two phases—Phase I involves reduction of the respective phenolic compounds

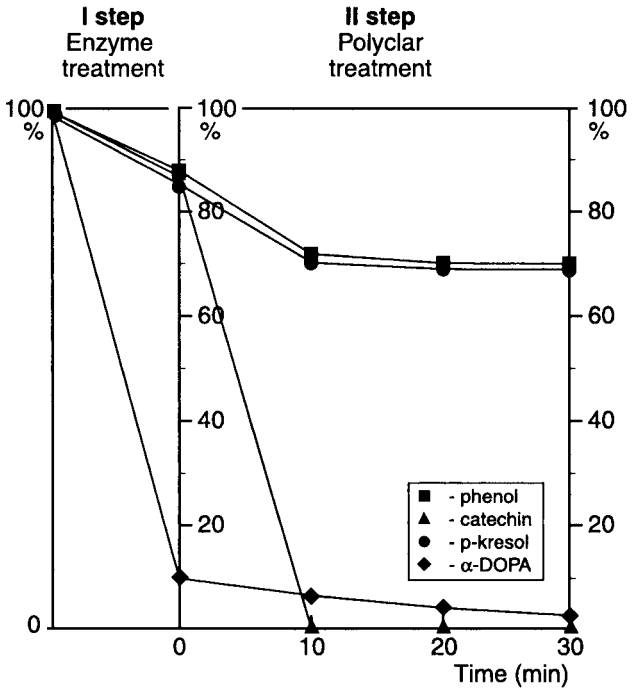


**Figure 6** Removal of catechin, phenol, *p*-cresol and $\alpha$-DOPA from mixtures by co-immobilized laccase and tyrosinase followed by treatment with Polyclar.

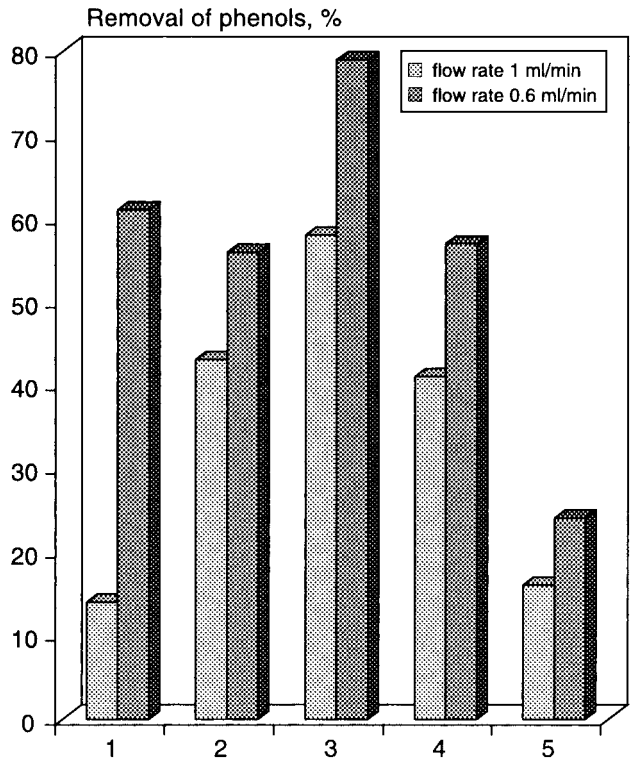


**Figure 7** Effect of substrate flow rate on removal of different phenols(1, 4-methoxyphenol; 2, 4-chlorophenol; 3, $\beta$-naphthol; 4, 4-chloro-3-methylphenol; 5, 2,4-dichlorophenol).

after passing the mixture through the column. Phase II involves reduction of the phenol content in the mixture as a result of Polycar processing.

Passing the solution through the column once resulted in simultaneous removal (42–90%) of *m*-cresol and $\alpha$-naphthol (Figure 2), 2-chlorophenol and 2,4-dichlorophenol (Figure 3),

**Table 2** The removal of phenols by both enzyme and Polyclar treatment and for the phenols remaining in the treated mixtures after 60 h storage

| Phenols | Removal of phenols (%) | Remaining phenols after 60 h |
|---|---|---|
| 4-Methoxyphenol | 62 | 0 |
| 2,6-Dimethoxyphenol | 23 | 50 |
| 2,4-Dichlorophenol | 60 | 0 |
| 4-Chloro-3-methylphenol | 97 | 0 |
| 4-Chlorophenol | 66 | 34 |
| $\beta$-Naphthol | 94 | 0 |
| $\alpha$-Naphthol | 100 | 0 |
| Chlorogenic acid | 20 | 80 |
| Caffeic acid | 18 | 26 |
| 3-Methoxyphenol | 20 | 70 |
| 2-Chlorophenol | 50 | 50 |
| Guaiacol | 24 | 76 |
| *m*-Cresol | 52 | 48 |
| *p*-Cresol | 28 | 72 |
| *o*-Cresol | 32 | 68 |
| 3-Chlorophenol | 57 | 43 |
| Phenol | 28 | 72 |
| Catechol | 24 | 76 |
| Catechin | 100 | 0 |
| $\alpha$-DOPA | 97 | 0 |

4-methoxyphenol, 4-chlorophenol, β-naphthol and 4-chloro-3-methylphenol (Figure 4) and α-DOPA (Figure 6). Other phenolic compound were removed from the mixtures in the range of 5–25%.

Treatment of the phenolic mixtures with Polyclar after their passage through the column effected an additional removal of considerable quantities depending on the compounds. Thus, we achieved complete removal of α-naphthol (Figure 2), catechin and α-DOPA (Figure 6) and 94–97% removal of β-naphthol and 4-chloro-3-methylphenol (Figure 4). In all other cases we obtained 10–20% decrease of the contents of phenolic compounds after processing with Polyclar. In all other cases except 4-chloro-3-methylphenol (Figure 4), a 10- to 15-min treatment with Polyclar was sufficient to reach a maximum level of adsorption and removal of the toxic phenolic compounds and some polyphenols.

With enzyme reactors of the column type with a fixed layer of biocatalyst, transformation of substrate depends on the duration of contact with the enzyme. This could be regulated by flow rate or by the biocatalyst layer's height. While the latter is fixed, the flow rate has a crucial influence on it. This was investigated using a mixture of five phenolic compounds. An increase of flow rate from 0.6 to 1.0 ml $min^{-1}$ decreased the transformation of phenols, but to different degrees (Figure 7). For instance, for 4-methoxyphenol, the decrease was the greatest, 47%, and for 4-chlorophenol it was the smallest, 13%. A flow rate less than 0.5–0.6 ml $min^{-1}$ is inefficient, thus all experiments were performed at a flow rate of 0.6 ml $min^{-1}$.

Table 2 summarizes removal data for phenolic compounds through the two-step process. For some phenolic compounds formation of insoluble polymer products continues. After 60 h the residual quantities of α-naphthol, 2,4-dichlorophenol, 4-methoxyphenol, β-naphthol, 4-chloro-3-methylphenol and catechin were not determined (Table 2). Decrease of 3-methoxyphenol by 10%, 2,6-dimethoxyphenol by 27% and caffeic acid by 50% was observed.

An important operational parameter from the point of view of full-scale continuous transformation of phenolic substances with immobilized enzymes is the long-term stability (operational stability) of the enzymes. This was examined for different phenolic compounds. The results are shown in Table 3. The immobilized enzymes demonstrate a very good operational stability (70–90 h) for 2,4-dichlorophenol and guaiacol and lowest (10–12 h) at a transformation of 2,6-dimethoxyphenol, 3-chlorophenol and 4-chlorophenol.

**Table 3** Operational stability of the immobilized laccase/tyrosinase system ($\tau_{1/2}$ — the time necessary for 50% decrease of the transformation of the substrates)

| Phenols | $\tau_{1/2}$ (h) |
|---|---|
| 2,6-Dimethoxyphenol | 10 |
| 2,4-Dichlorophenol | 90 |
| 4-Chloro-3-methylphenol | 15 |
| β-Naphthol | 15 |
| 4-Chlorophenol | 12 |
| *m*-Cresol | 22 |
| 3-Methoxyphenol | 23 |
| Guaiacol | 70 |
| Phenol | 50 |
| 3-Chlorophenol | 11 |
| *p*-Cresol | 20 |

## Discussion

Removal of phenols from wastewater and other solutions with soluble and immobilized oxidoreductases has been demonstrated by many authors [3–5,7–11,16–19]. In all cases, the transformation of individual phenolic compounds (in model solutions) by laccase or tyrosinase were investigated. However, in wastewater phenolic compounds are present as mixtures. Because of the different substrate specificities of the enzymes, it is not possible to achieve effective removal of phenolic compounds from mixtures with only one enzyme. We report for the first time enzymatic degradation of mixtures of phenols by co-immobilized laccase and tyrosinase on Microperl in a fixed-bed bioreactor with continuous use.

The results show (Figures 2–6, Table 1), that when the enzymes are co-immobilized their activities complement one another and can be used for treatment of phenolic mixtures. The proposed method for immobilization is simple and easily executed without additional agents. The conditions required are mild and the degree of immobilization is high, especially for the laccase, where almost complete immobilization is reached in a short time. The advantage of this method is the high degree of protein immobilization obtained (Figure 1), because, as was assumed by Wada *et al.* [19], other proteins remaining crude enzyme preparations play an important role in forming precipitates.

As addition to the enzymatic transformation of phenolic compounds, Wada *et al.* [19] and Payne *et al.* [16] investigated chitosan for effective removal of phenols. Our attention was drawn to another polymer, Polyclar (fine grade PVPP, combining high selectivity towards haze-forming polyphenols with increased efficiency of adsorption), successfully used for stabilization of beverages. This polymer was based on the application of co-immobilized laccase and tyrosinase and processing with Polyclar in a treatment of fruit juices, white wines and beer for production of beverages with stable turbidity and color.

Our results show that significant reduction in the levels of phenols in mixtures can be achieved using co-immobilized laccase and tyrosinase in a fixed-bed tubular bioreactor followed by treatment with Polyclar. The fixed-bed enzyme reactor technology employed here is based on the application of co-immobilized laccase and tyrosinase in a continuous process, whereas studies using other immobilization matrices have demonstrated the use of polyphenol oxidase by treating small volumes of effluent operating as batch systems [7,8,16,19]. Only Edwards *et al.* [11] investigated a capillary membrane bioreactor for removal of phenolic compounds from synthetic and industrial effluents in a continuous process. Our system for the removal of phenolic compounds is comparable to that of Edwards *et al.*, but the reaction time is shorter. Its advantages include the treatment of complex mixtures of phenols and their effective removal, and the combination of the effects of enzyme action and adsorption. This makes the system suitable for simultaneous purification and reduction of the contents of phenolic compounds in wastewater. The results for halogenated phenols are encouraging. As reported by Dec and Bollag [10], enzymatic polymerization of chlorophenols by oxidoreductases resulted in dechlorination. It can be expected that related pollutants are also dehalogenated if subjected to the same

enzymatic treatment. Dehalogenated products are usually less toxic and more susceptible to biodegradation than the halogenated parent compounds.

Another advantage is a good operational stability of the proposed co-immobilised enzyme system. It is interesting that the operational stability depends on the substrate type. This may be explained by different coating of the carrier by the various products, and consequently the enzyme is less accessible to the substrate (Table 3 *vs.* Table 1). Such a dependence was also noted by Leonowicz *et al.* [15]. This is important in biotechnology, where a biocatalyst's reusability determines its economic value. Further investigations are needed to examine the operational stability of the enzymes under realistic conditions, where mixtures of different phenols are treated. In this case certain substrates may act to cause decreased removal of other phenolic compounds. Also, industrial waste streams may have extreme pH values and may contain other constituents such as salts and heavy metals that could poison the enzymes. Alternatively, wastewater may be supplemented with a substance to protect the enzyme. Future experiments will be conducted to establish the conditions under which this kind of treatment will succeed.

In conclusion, the technique suggested here is simple, rapid and inexpensive on laboratory scale. The extent of transformation, the operational stability of enzyme activity, and the mechanical stability of the biocatalyst are feasible for successful full-scale operation of the biocatalyst. The system still needs to be further developed and perfected before it can be employed. Future studies should concentrate on the determination of factors which affect activity and stability of the immobilized enzymes. Nevertheless, considering the complexity of the problem, co-immobilized enzymes on organic or inorganic supports could represent an innovative and valuable technology for bioremediation of contaminated aqueous environments.

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