Immobilization of *Pycnoporus coccineus* laccase on Eupergit C: stabilization and treatment of olive oil mill wastewaters

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Running title: Degradation of OMW by immobilized *Pycnoporus coccineus* laccase

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Abstract

The use of olive oil mill wastewaters (OMW) as organic fertilizer is limited by its phytotoxic effect, due to the high concentration of phenolic compounds. As an alternative to physico-chemical methods for OMW detoxification, the laccase from *Pycnoporus coccineus*, a white-rot fungus that is able to decrease the chemical oxygen demand (COD) and colour of the industrial effluent, is being studied. In this work, the *P. coccineus* laccase was immobilized on two acrylic epoxy-activated resins, Eupergit C and Eupergit C 250L. The highest activity was obtained with the macroporous Eupergit C 250L, reaching 110 *U* g^{-1} biocatalyst. A substantial stabilization effect against pH and temperature was obtained upon immobilization. The soluble enzyme maintained ≥80% of its initial activity after 24 h at pH 7.0-10.0, whereas the immobilized laccase kept the activity in the pH range 3.0-10.0. The free enzyme was quickly inactivated at temperatures above 50 ^{o}C, whereas the immobilized enzyme was very stable up to 70 ^{o}C. Gel filtration profiles of the OMW treated with the immobilized enzyme (for 8 h at room temperature) showed both degradation and polymerization of the phenolic compounds.

Key words*:* Olive oil, wastewaters, fungi, immobilized enzyme, phenoloxidases.

1. INTRODUCTION

Large amounts of dark effluents (>$3.0 \cdot 10^7$ m^3 $year^{-1}$ only in the Mediterranean Sea) are generated during the extraction of olive oil (olive oil mill wastewaters, OMW) (D'Annibale et al., 2000). These effluents contain high organic load, including lipids, pectin, polysaccharides and phenols (Paredes et al., 1999; Sayadi et al., 2000). The large concentration of phenolic compounds seems to be responsible for the OMW phytotoxicity and microbial inhibitory effect when used as organic fertilizers (García et al., 2000; Martínez et al., 1998). These compounds are also responsible for the colour of OMW, which show variable red-brown colour depending on the age and the type of olive oil extraction process used (Zouari and Ellouz, 1996).

As an alternative to conventional physico-chemical processes for OMW detoxification, treatments with different microorganisms and their enzymes are being studied. Among them, white-rot fungi have a high potentiality because of their ability to degrade lignin and other aromatic compounds (Aust and Benson, 1993; Pointing, 2001). The ligninolytic enzymes secreted by these fungi, laccases (EC 1.10.3.2) and peroxidases –including lignin peroxidase (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13) and versatile peroxidase (EC 1.11.1.16)- catalyze the one-electron oxidation of aromatic compounds, resulting in free radicals that produce different non-enzymatic reactions (Higuchi, 2004). Some peroxidases are stronger oxidants than laccases but they need hydrogen peroxide for their catalytic activity. The advantages of laccases for industrial and environmental application are their broad substrate specificity and the use of oxygen, a non-limited electron acceptor, which is reduced to water (Alcalde, 2006). In most cases, the oxidation of phenols or other laccases substrates leads to polymerization of the

formed radicals through oxidative coupling, which can result in detoxification of these aromatic contaminants (Martirani et al., 1996). The effect of laccases on OMW has been reported using fungal liquid cultures and purified enzyme (Aggelis et al., 2003; D'Annibale et al., 2004; Jaouani et al., 2005; Tsioulpas et al., 2002).

The use of laccases in OMW detoxification could be enhanced by enzyme immobilization. This process usually increases pH and temperature stability and allows the reuse of the biocatalyst (Gianfreda et al., 2003). Eupergit® C is a carrier (100-250 μm), made by copolymerization of *N,N'*-methylene-bis-methacrylamide, glycidyl methacrylate, allyl glycidyl ether and methacrylamide (Katchalski-Katzir and Kraemer, 2000). This support is chemically and mechanically stable in the pH range from 1 to 12.

Previous studies have shown that the white-rot fungus *Pycnoporus coccineus* can decrease the phenolic content, chemical oxygen demand (COD) and colour of OMW (Jaouani et al., 2003), and the role of laccase in the process has been recently reported (Jaouani et al., 2005). In this work we have investigated the immobilization of this enzyme on Eupergit® C and Eupergit® C 250L (which have different porosity), the properties of the immobilized biocatalysts and their application in OMW treatment.

2. MATERIAL AND METHODS

2.1. Fungal strain and culture conditions

The *P. coccineus* strain (MUCL38527) was grown in 1 L Erlenmeyer containing 200 mL of the following medium: 10 g glucose, 2 g ammonium tartrate, 1 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 1 g yeast extract, 1 mL trace elements solution and 1 L of distilled water, supplemented with 150 μM $CuSO_4$ and 500 μM ethanol to induce laccase production (Jaouani et al., 2005). The trace elements solutions contained per litre: $Na_2B_4O_7 \cdot 10H_2O$ (100 mg), $ZnSO_4 \cdot 7H_2O$ (70 mg), $FeSO_4 \cdot 7H_2O$ (50 mg), $CuSO_4 \cdot 5H_2O$ (10 mg), $MnSO_4 \cdot 4H_2O$ (10 mg) and $(NH_4)Mo_7O_{24} \cdot 4H_2O$ (10 mg). Homogenized mycelium from 5-day-old shaken cultures was used as preinocula (approx. 3.5 mg dry weight mL^{-1}) and the cultures were grown at 28°C and 180 rpm for 25 days.

2.2. Enzyme activity, protein and reducing sugars analysis

Laccase activity was determined using 10 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate) (ABTS, Sigma) as substrate in 100 mM sodium acetate buffer, pH 5.0 ($\varepsilon_{346}^{\bullet+}$ = 29,300 M^{-1} cm^{-1}). One unit of enzyme activity was defined as that corresponding to the oxidation of 1 μmol of substrate per min. Reducing sugars were assayed by the Somogyi and Nelson method (Somogyi, 1945) using glucose as standard. Protein concentration was determined by the method of Bradford, using Bio-Rad protein assay and bovine serum albumin as standard.

2.3. Laccase preparation

The *P. coccineus* laccase preparation was obtained from 25 days-old cultures when laccase activity (the sole ligninolytic enzyme present in the cultures) reached its maximum. After removing the mycelium by centrifugation (13,000 rpm), the culture liquid was concentrated and dialyzed against 10 mM sodium phosphate buffer, pH 5.0, by ultrafiltration (Filtron, 5-kDa cutoff membrane).

2.4. Immobilization procedure

The acrylic epoxy-activated resins Eupergit C and Eupergit C 250 L (Degussa) were used to immobilize *P. coccineus* laccase. Different amounts of laccase (45, 90 and 180 laccase units) were mixed with 100 mg of the carrier in 0.5 M sodium phosphate buffer (pH 8.0). The mixture was incubated for 48 h at 4°C with roller shaking, and samples of supernatant were taken periodically for assay of protein. The biocatalyst was then filtered using a glass filter (Whatman), washed with water and subsequently dried under vacuum and stored at 4°C.

2.5. Determination of optimum pH and stability

The effect of pH on the activity and stability of soluble and immobilized *P. coccineus* laccase was investigated in 100 mM Britton and Robinson buffer (citrate-borate-phosphate), pH 3.0-8.0. For the stabilization assays, samples were incubated for 24 h and residual activity measured with ABTS under the standard conditions. The thermostability of soluble and immobilized laccase was determined over the range 50-80 ^{o}C, at pH 5.0, using the same buffer.

2.6. OMW treatment with immobilized laccase

Lyophilized OMW was reconstituted in distilled water to get a solution of 10 g L^{-1}. The enzymatic treatment was carried out on 2 mL of the OMW solution, using 20 mg of immobilized (on Eupergit® C 250L) laccase from *P. coccineus*. The incubation was carried out for 8 h at 4°C with gentle shaking. A blank control with the support was also performed.

Changes in the molecular mass distribution of the OMW after the enzymatic treatments were analyzed by gel filtration on Sephadex G-100. 200 μL of samples were applied to a column (1 x 48 cm) equilibrated with 50 mM NaOH and 25 mM $LiCl_2$, at a flow rate of 0.4 mL min^{-1}. The absorbance of the eluted fractions was monitored at 280 nm.

3. RESULTS AND DISCUSSION

3.1. Laccase production and optimization of immobilization process

The laccase preparation was obtained from *P. coccineus* filtrates with high laccase activity after 25 days (Fig. 1). The protein profile was similar to the laccase activity profile, suggesting that this enzyme was the major protein (Fig. 1). Laccase is the only ligninolytic enzyme secreted by the fungus in these culture conditions, as previously reported (Jaouani et al., 2005). A crude preparation (containing 180 U mL^{-1} and 0.19 mg mL^{-1}) was obtained by ultrafiltration and used for immobilization.

The immobilization process was carried out at pH 8.0 and 4°C, since *P. coccineus* laccase was stable under these conditions for at least 24 h (Jaouani et al., 2005). Eupergit® C binds proteins via their epoxide groups, which may react with different nucleophiles on the protein as a function of pH. At neutral pH, the amino acid side chain groups involved in covalent bonding are the thiol groups, at pH > 8 the amine groups, at pH > 11 the phenolic groups, and at slightly acidic pH the carboxyl groups (Boller et al., 2002; Gomez de Segura et al. 2004). Due to the high content in oxirane groups (0.93% for Eupergit C and 0.36% for Eupergit C 250L), the bonding capacity may reach 100 mg of protein per g of resin (dry weight).

Since it is well known that ionic strength may affect the efficiency of the immobilization (Grabski et al., 1995), different buffer concentrations (0.5, 1.0 and 1.5 M) were assayed. In this case the yield of immobilized enzyme decreased when increasing buffer concentration (data not shown), and therefore 0.5 M sodium phosphate buffer (pH 8.0) was further used. The immobilization process using different enzyme loadings (0.45,

0.9 and 1.8 U per mg support) with both Eupergit® C and Eupergit® C 250L showed no significant increase of the amount of protein immobilized after 48 h (Table 1). Although the amount of retained protein was higher with Eupergit® C under all experimental conditions, Eupergit® C 250L yielded biocatalysts with higher specific activity (the maximum value obtained was 110 U g^{-1} biocatalyst). Eupergit C 250L has the same composition and reactive groups as Eupergit C, but larger pores (Gomez de Segura et al., 2004), which may explain the higher catalytic efficiency of the resulting biocatalysts.

3.2. Characterization of the immobilized biocatalysts

Comparative studies with free and immobilized laccase showed the same optimum pH (3.5) using ABTS as substrate. However, immobilization of *P. coccineus* laccase increased stability against both pH (Fig 2) and temperature (Fig 3). The study on pH stability, carried out at room temperature, showed a substantial inactivation of free laccase in the pH range 3.0-5.0 after 24 h, whereas ≥80% of the initial activity remained at pH 7.0-10.0. In the case of the immobilized laccase the remaining activity reached 80% between pH 3.0 and 6.0 and 100% between pH 7.0 and 10.0.

Regarding the thermal stability, the soluble enzyme was swiftly inactivated between 50 and 80 ^{o}C, and the immobilized enzyme was significantly stable in the range 50-70 ^{o}C. At 80 ^{o}C, the half-life of the immobilized enzyme was approx. 7 h.

3.3. OMW degradation by immobilized P. coccineus laccase

The treatment of OMW with the immobilized laccase on Eupergit C 250L (with a specific activity of 110 U g^{-1}) was carried out at room temperature during 8 h to check the efficiency of immobilized enzyme. The obtained results were similar to those reported with the whole fungus (Jaouani et al., 2003) and the purified enzyme in solution (Jaouani et al., 2005)**,** suggesting that laccase plays an important role in the degradation of phenolic compounds present in OMW, and that immobilized enzyme could be use for the waste water treatment. The degradation of OMW has been also investigated by other white-rot fungi (Martínez et al., 1998; Martirani et al., 1996; Sayadi and Ellouz, 1993). The advantage of the enzymatic treatment is a shorter effluent treatment period. Oxidation of simple phenolic compounds in OMW by immobilized *P. coccineus* laccase produced radicals leading to polymerization. This was evidenced by the appearance in the gel filtration experiments of a high molecular mass peak, and a decrease of the peak corresponding to phenolic compounds (Fig. 4). These results are similar to those reported with the immobilized *L. edodes* laccase from solid state fermentation cultures (d'Annibale et al., 2000). A recent study with this purified laccase showed that OMW treatment increased wheat germinability, suggesting that the phenolic fraction was detoxified either by degradation and/or polymerization (Casa et al., 2003). These findings are similar to those reported after fungal treatment or treatment with enzyme in suspension. The main advantages of the laccase from *P. coccineus* for this and other environmental applications, are the high volumetric activity obtained in the liquid cultures, as well as its high thermal and pH stability when used in its immobilized form. Studies to analyze the degradation/detoxification degree of the treated effluent are currently in progress.

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Table I. Recovered protein and activity in the immobilization of *P. coccineus* lacasse on Eupergit® C and Eupergit® C 250L.

Added laccase (U per g support)	Bound protein (%) [a]						Activity (U g^{-1} biocatalyst) [b]	
	Eupergit C			Eupergit C 250L				
	24 h	48 h	72 h	24 h	48 h	72 h	Eupergit C	Eupergit C 250L
450	60.8	66.4	66.5	27.5	34.2	34.0	20.5	61.5
900	47.4	55.5	55.2	17.2	28.1	29.0	27.0	81.0
1800	10.3	14.7	15.0	10.2	12.4	12.5	22.5	110

[a] The amount of immobilized protein was calculated from the difference between the protein loaded and that remaining in solution.

[b] Assay conditions: 10 mM ABTS, 100 mM sodium acetate buffer, pH 5.0, 0.5 mg mL^{-1} biocatalyst. Measured with the immobilized biocatalyst obtained after 48 h.

Figure legends

Figure 1. Profile of laccase activity (●) and total protein (○) in the *P. coccineus* culture growing in glucose-peptone medium with $CuSO_4$ and ethanol as enzyme inducers.

Figure 2. Residual activity of soluble (○) and immobilized on Eupergit C 250L (●) *P. coccineus* laccase after 24 h incubation at room temperature in 100 mM citrate-borate-phosphate buffer of different pH values.

Figure 3. Thermostability of soluble (**A**) and immobilized on Eupergit C 250L (**B**) *P. coccineus* laccase at pH 5.0.

Figure 4. Molecular distribution of OMW in Sephadex G-100 before (—) and after (—) enzymatic treatment with immobilized laccase from *P. coccineus.* The mobile phase was 50 mM NaOH/ 25 mM LiCl, and the flow rate 0.4 mL min^{-1}. Blue dextran (average M_r 2.10^6, arrow 1) and syringic acid (arrow 2) were used as high and low molecular weight standards, respectively.

Fig. 1

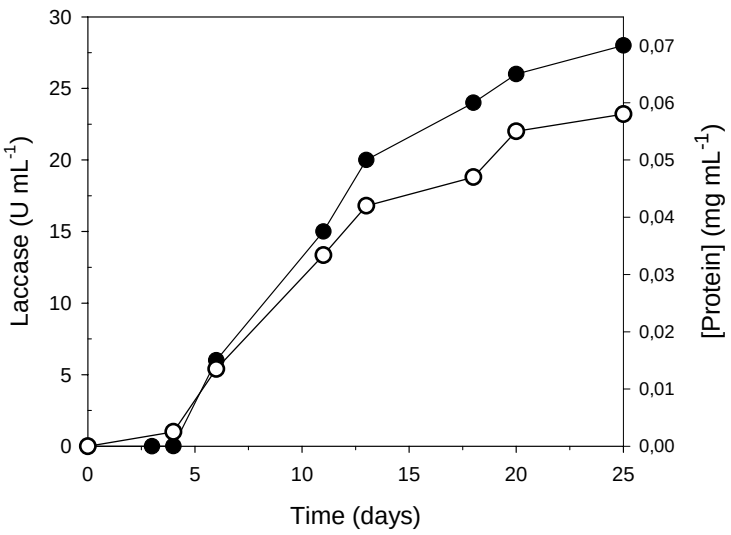


Fig. 2

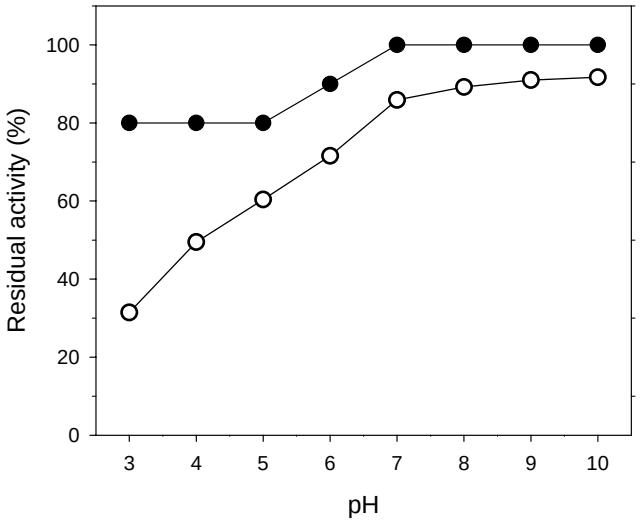

Residual activity (%)
pH
0
20
40
60
80
100
3
4
5
6
7
8
9
10

Fig. 3

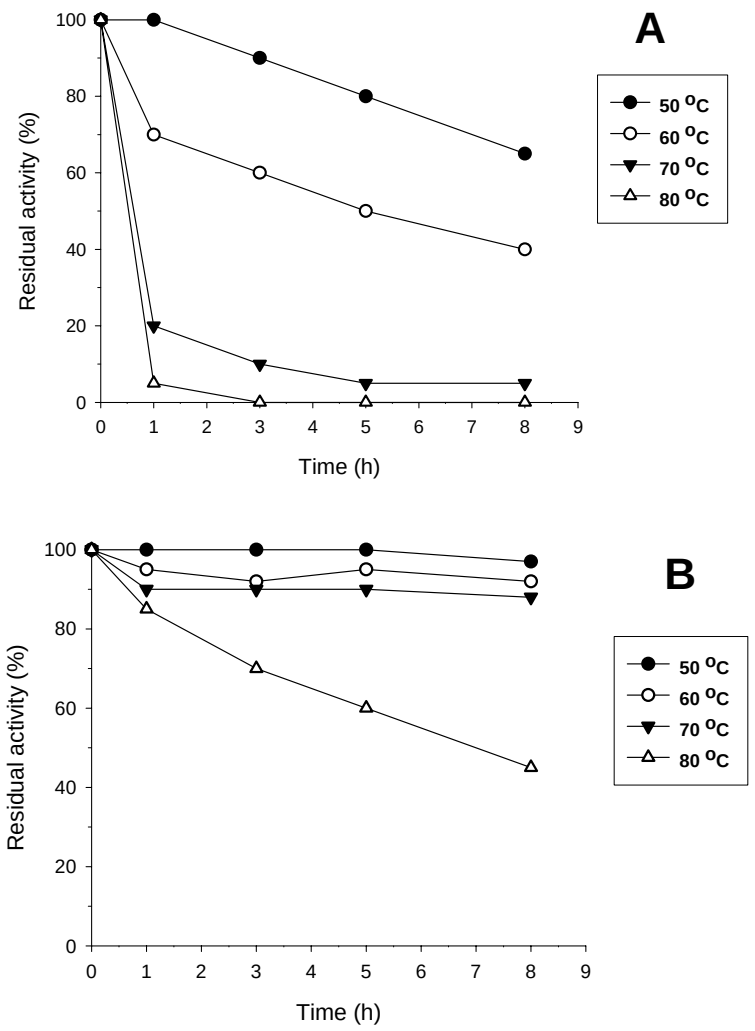
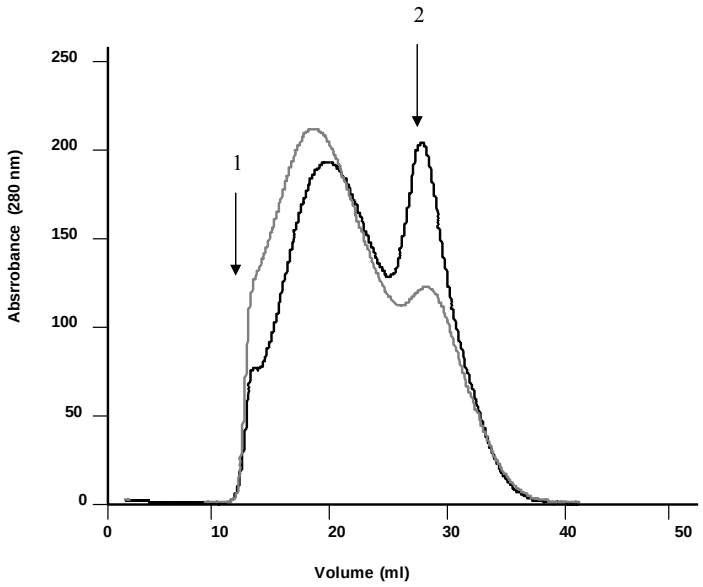

A
Residual activity (%)
Time (h)
50 °C
60 °C
70 °C
80 °C
B
Residual activity (%)
Time (h)
50 °C
60 °C
70 °C
80 °C

Fig. 4


2
1
250
200
150
100
50
0
Absrrobance (280 nm)
0
10
20
30
40
50
Volume (ml)