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**ORIGINAL ARTICLE**

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

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**Abstract**
The use of olive oil mill wastewaters (OMW) as an organic fertilizer is limited by their 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 with the ability to decrease the chemical oxygen demand (COD) and color 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 $\geq$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 $>50^{\circ}$C, whereas the immobilized enzyme was very stable up to 70°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.

**Keywords:** *Olive oil, wastewaters, fungi, immobilized enzyme, phenoloxidases*

## Introduction

Large amounts of dark effluents ($>3.0\times10^7$ $m^3$ $year^{-1}$ in the Mediterranean Sea alone) are generated during the extraction of olive oil (olive oil mill wastewaters; OMW) (D'Annibale et al. 2000). These effluents contain a 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 the microbial inhibitory effect when used as organic fertilizers (Martínez et al. 1998; García et al. 2000). These compounds are also responsible for the color of OMW, which is a variable red–brown color depending on the age and the type of olive oil extraction process used (Zouari & 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 potential because of their ability to degrade lignin and other aromatic compounds (Aust & 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 2007). In most cases, the oxidation of phenols or other laccase 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

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reported using fungal liquid cultures and purified enzymes (Tsioulpas et al. 2002; Aggelis et al. 2003; D'Annibale et al. 2004; Jaouani et al. 2005).

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 & 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 color of OMW (Jaouani et al. 2003), and the role of laccase in the process has recently been reported (Jaouani et al. 2005). In this work, we investigated the immobilization of this enzyme on Eupergit® C and Eupergit® C 250L (which have different porosities), the properties of the immobilized biocatalysts, and their application in OMW treatment.

## Material and methods

### *Fungal strain and culture conditions*

The *P. coccineus* strain (MUCL38527) was grown in Erlenmeyer flasks (1 L) 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 solution contained per liter: $Na_2B_4O_7 \cdot 10H_2O$ (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 were used as preinocula (approximately 3.5 mg dry weight $mL^{-1}$) and the cultures were grown at 28°C and 180 rpm for 25 days.

### *Enzyme activity, protein and reducing sugar 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 minute. 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 the Bio-Rad protein assay and bovine serum albumin as standard.

### *Laccase preparation*

The *P. coccineus* laccase preparation was obtained from 25-day-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).

### *Immobilization procedure*

The acrylic epoxy-activated resins Eupergit C and Eupergit C 250L (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.

### *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°C, at pH 5.0, using the same buffer.

### *OMW treatment with immobilized laccase*

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

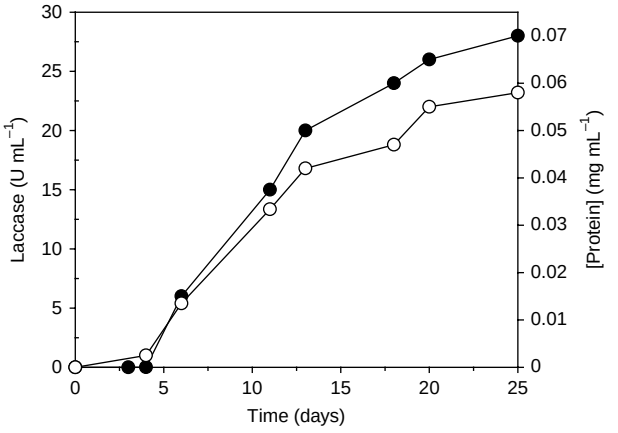


Figure 1. Profile of laccase activity (●) and total protein (○) in the *P. coccineus* culture growing in glucose-peptone medium with $CuSO_4$ and ethanol as enzyme inducers.

## Results and discussion

### *Laccase production and optimization of the immobilization process*

The laccase preparation was obtained from *P. coccineus* filtrates with high laccase activity after 25 days (Figure 1). The protein profile was similar to the laccase activity profile, suggesting that this enzyme was the major protein (Figure 1). Laccase is the only ligninolytic enzyme secreted by the fungus under 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 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 binding capacity may reach 100 mg of protein per gram of resin (dry weight).

Since it is well known that ionic strength can 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 with increasing buffer concentration (data not shown), and, therefore, 0.5 M sodium phosphate buffer (pH 8.0) was used. The immobilization process using different enzyme loadings (0.45, 0.9 and 1.8 U mg$^{-1}$ support) with both Eupergit® C and Eupergit® C 250L showed no significant increase in the amount of protein immobilized after 48 h (Table I). 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.

### *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 (Figure 2) and temperature (Figure 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 was 80% between pH 3.0 and 6.0, and 100% between pH 7.0 and 10.0.

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

| | Bound protein (%)[a] | | | | | | Activity (U g$^{-1}$ biocatalyst)[b] | |
|---|---|---|---|---|---|---|---|---|
| | Eupergit C | | | Eupergit C 250L | | | | |
| Added laccase (U g$^{-1}$ support) | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h | Eupergit C | Eupergit C 250 L |
| 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.

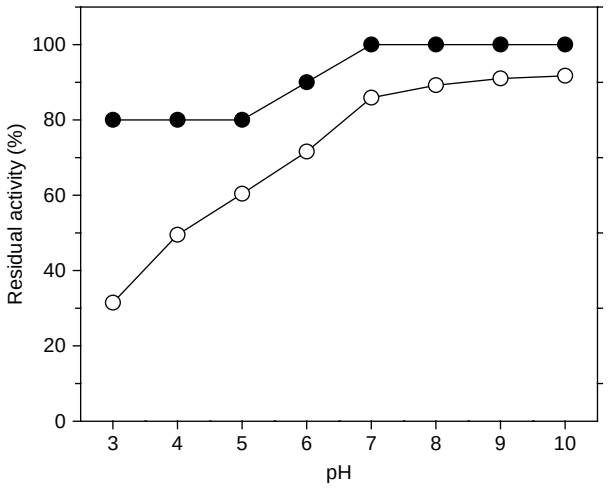


Figure 2. Residual activity of soluble (○) and immobilized on Eupergit C 250L (●) *P. coccineus* laccase after 24 h incubation at room temperature in 100 mM citrate–borate–phosphate buffer of different pH values.

Regarding the thermal stability, the soluble enzyme was rapidly inactivated between 50 and 80°C, while the immobilized enzyme showed significant stability in the range 50–70°C. At 80°C, the half-life of the immobilized enzyme was approximately 7 h.

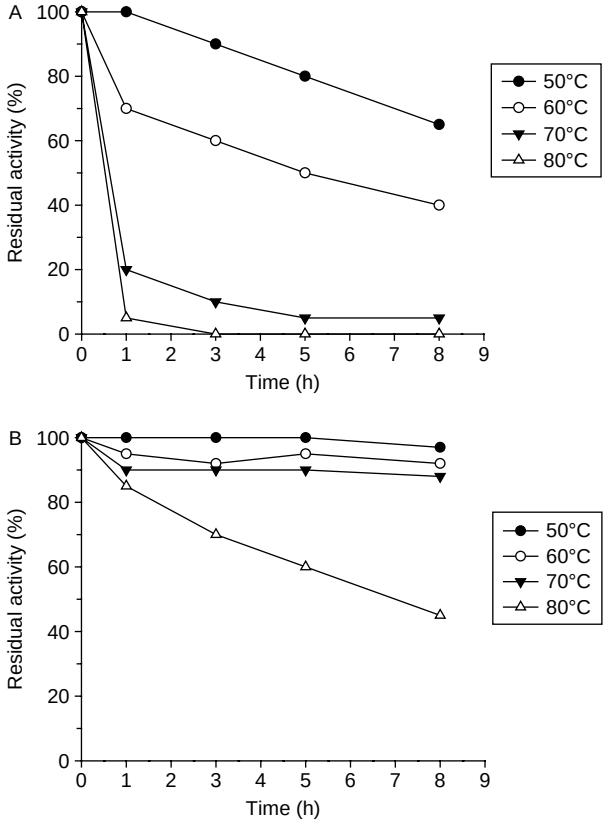


Figure 3. Thermostability of soluble (A) and immobilized on Eupergit C 250L (B) *P. coccineus* laccase at pH 5.0.

*OMW degradation by immobilized* P. coccineus *laccase*

The treatment of OMW with laccase immobilized on Eupergit C 250L (with a specific activity of 110 U $g^{-1}$) was carried out at room temperature for 8 h to check the efficiency of the immobilized enzyme. The 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 used for waste water treatment. The degradation of OMW has also been investigated using other white-rot fungi (Sayadi & Ellouz 1993; Martirani et al. 1996; Martínez et al. 1998). 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 evident by the appearance of a high molecular mass peak in gel filtration experiments, and a decrease in the peak corresponding to the phenolic compounds (Figure 4). These results are similar to those reported for 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 germination, 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

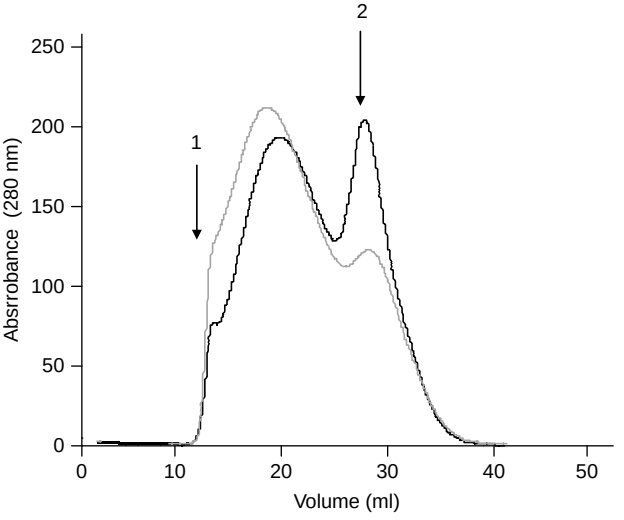


Figure 4. Molecular distribution of OMW on Sephadex G-100 before (——) and after (——) enzymatic treatment with immobilized laccase from *P. coccineus*. The mobile phase was 50 mM NaOH/25 mM LiCl, and the flow rate 0.4 mL $min^{-1}$. Blue dextran (average $M_r$ $2.10^6$, arrow 1) and syringic acid (arrow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

laccase from *P. coccineus* for this and other environmental applications are the high volumetric activity obtained in 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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