Immobilisation of laccase on Eupergit supports and its application for the removal of endocrine disrupting chemicals in a packed-bed reactor

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Abstract

Laccase from *Myceliophthora thermophila* was covalently immobilised on Eupergit C and Eupergit C 250L yielding specific activities of up to 17 U/g and 80 U/g, respectively. Due to its superior activity, Eupergit C 250L was chosen for further research. The somewhat lower catalytic efficiency (k_{cat}/K_M) of the immobilised enzyme in comparison with that of the free enzyme was balanced by its increased stability and broader operational window related to temperature and pH. The feasibility of the immobilised laccase was tested by using a packed bed reactor (PBR) operating in consecutive cycles for the removal of Acid Green 27 dye as model substrate. High degrees of elimination were achieved (88, 79, 69 and 57% in 4 consecutive cycles), while the levels of adsorption on the support varied from 18% to 6%, proving that dye removal took place mainly due to the action of the enzyme. Finally, a continuous PBR with the solid biocatalyst was applied for the treatment of a solution containing the following endocrine disrupting chemicals: estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2). At steady-state operation, E1 was degraded by 65% and E2 and EE2 were removed up to 80% and only limited adsorption of these compounds on the support, between 12% and 22%, was detected. In addition, a 79% decrease in estrogenic activity was detected in the effluent of the enzymatic reactor while only 14% was attained by inactivated laccase.

Keywords: Estrone; 17β-estradiol; 17α-ethinylestradiol; Laccase; Covalent immobilisation; Eupergit

1. Introduction

Estrogenic compounds, frequently present in personal care products (PCPs), are either known or suspected endocrine disrupting chemicals (EDCs) (Sei et al. 2008; Ternes et al. 1999). These compounds are environmental pollutants that may cause dramatic environmental effects such as the feminisation of entire fish populations in contaminated streams even in trace concentrations (Auriol et al. 2006; Blázquez and Guieysse, 2007). EDCs have been detected in wastewater treatment plants (WWTPs), rivers, groundwaters and landfill leachates at concentrations ranging from nanograms to micrograms per litre (Carballa et al. 2005; Joss et al. 2006). Other studies reported that natural steroid estrogens (e.g. estrone and 17β-estradiol) and synthetic steroid estrogen (17α-ethinylestradiol) are the major contributors to the estrogenic activity detected in WWTP effluents (Leusch et al. 2005). Moreover, steroid estrogens have been found to be biologically active at concentrations as low as 0.1 ng/L (Auriol et al. 2006).

The degradation of this type of compounds is still an important ecological challenge since conventional physicochemical or biological treatment can only attain partial degradation while advanced oxidation processes such as UV exposure or ozonation render variable degradation yields (Esplugas et al. 2007). Additionally, these recent processes (e.g. chemical oxidation and adsorption on activated supports) usually present a number of disadvantages, such as high costs, prolonged detoxification periods and production of toxic residues (Auriol et al. 2006; Kim and Nicell, 2006). A biological alternative may be based on the use of white rot fungi. These microorganisms have been reported to degrade a wide range of xenobiotics by the action of their oxidative enzymes, e.g. manganese peroxidase, lignin peroxidase, versatile peroxidase or laccase (Durán and Esposito, 2000; Kim and Nicell, 2006).

70 Laccase (E.C.1.10.3.2., benzenediol: oxygen oxidoreductase) is a multi-copper protein
71 which is able to oxidise phenolic substrates by reducing molecular oxygen to water
72 (Wesemberg et al. 2003). This enzyme was reported to be a potential catalyst for the
73 biodegradation of recalcitrant compounds: dyes, aromatic hydrocarbons compounds,
74 olive oil mill wastewater and pulp delignification (Camarero et al. 2007; Yamak et al.
75 2009). In recent years, several research groups have explored the ability of free fungal
76 laccases to remove a wide range of hormones: genistein, estrone, 17β-estradiol, estriol,
77 and 17α-ethinylestradiol (Auriol et al. 2008*;* Nicotra et al. 2004; Tamagawa et al. 2006;
78 Lloret et al. 2010). Crude and purified laccase from *Trametes versicolor* and *Trametes*
79 *villosa* were used to degrade bisphenol A (Kim and Nicell, 2006) and nonylphenol
80 (Tsutsumi et al. 2001).
81 The advantages of laccases for environmental applications are their broad substrate
82 specificity and the use of oxygen, a non-limited electron acceptor (Berrio et al. 2007).
83 However, although high degradation levels were attained in those previous studies with
84 free laccase, its use in wastewater treatment may be limited due to the potential
85 inactivation of the soluble enzyme in presence of some inhibitor compounds, extreme
86 pH values or high temperatures. In order to overcome these limitations and provide
87 steady systems, different methods (e.g. the use of stabilising additives, derivatisation,
88 chemical modification of protein structure and immobilisation) could be applied
89 (Illanes, 1999). Immobilisation is the most commonly used method for enzyme
90 stabilisation, because it provides many other benefits, such as the physical separation
91 from the reaction medium, the reduction of enzyme replacement and the potential reuse
92 of the catalyst (Kunamneni et al. 2008; Osma *et al.* 2010). Furthermore, immobilisation
93 may shift the enzyme properties such as the kinetic parameters and the optimum values

of pH and temperature. It may also strengthen the protein structure (Cabana et al. 2009; Durán et al. 2002).

Enzyme immobilisation by covalent binding onto a support has the advantage that the enzyme is tightly fixed, which minimizes the leaching of the enzyme from the support. Moreover, the formation of multiple covalent bonds between the enzyme and the support reduces conformational flexibility and thermal vibrations, thus preventing protein unfolding and denaturation (Hanefeld et al. 2009). The application of covalent immobilisation techniques considers two different possibilities: either the use of inert carriers which can be properly activated or even the use of active supports commercially available. Among the latter, Eupergit carriers have been reported to be effective supports for immobilisation of laccase (Hublik and Schinner, 2000; Katchalski-Katzir and Kraemer, 2000; Knezevic et al. 2006; Russo et al. 2008). Eupergit supports (epoxy-activated acrylic polymers) have been developed between 1974 and 1980 by Röhm, Darmstadt, Germany. The carriers are very stable and have several good chemical and mechanical properties, being considered suitable for covalent immobilisation of enzymes for industrial applications (Kraemer et al. 1985).

Laccase, covalently immobilised on various supports, has been evaluated for the treatment of pollutants such as phenols and dyes (D'Annibale et al. 2000; Durán et al. 2002; Kunamneni et al. 2008; Russo et al. 2008). However, to our knowledge, immobilised laccases on Eupergit supports have not yet been evaluated for the oxidative degradation of endocrine disruptor chemicals (EDCs), specifically, estrone, 17β-estradiol or 17α-ethinylestradiol.

The main purpose of this study was to evaluate the ability of laccase from *Myceliophthora thermophila* immobilised on Eupergit to oxidize EDCs in a continuous packed-bed reactor. To attain this goal, specific objectives were proposed: (i)

119 immobilisation of laccase on two epoxy-activated acrylic polymers (Eupergit C and
120 Eupergit C 250L) to select the support with the highest immobilisation efficiency; (ii)
121 characterisation of the free and immobilised laccases (stability at different pH and
122 temperature, exposure to inactivating agents, kinetic parameters, reusability of
123 immobilised enzyme and storage stability); (iii) development and operation of a packed
124 bed reactor for the removal of a synthetic dye as a model compound and the targeted
125 EDCs: estrone, 17β-estradiol and 17α-ethinylestradiol.
126

127 **2. Materials and methods**

128 **2.1. Chemical reagents and enzyme**

129 The dye Acid Green 27 (AG27, purity 65%) and the endocrine disrupting chemicals
130 (EDCs, ≥99%): estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) were
131 obtained from Sigma-Aldrich (USA). A stock solution (2,000 mg/L) of the EDCs was
132 prepared in methanol for its use in the removal experiments. 2,2′-azinobis-(3-
133 ethylbenzothiazoline-6-sulfonate) (ABTS, ≥99%) was supplied by Fluka (USA). All
134 solvents were HPLC grade (Sigma-Aldrich, Germany). Epoxy-activated acrylic beads,
135 Eupergit C and Eupergit C 250L, were purchased from Rohm GmbH (Germany).
136 Commercial laccase (Novozym 51003) from *Myceliophthora thermophila* was supplied
137 by Novozymes (Denmark). This enzyme (molecular weight of 56,000 Da) was
138 produced by submerged fermentation of a genetically modified *Aspergillus* sp.
139

140 **2.2 Enzyme assay and protein estimation**

141 Laccase activity was determined by monitoring the oxidation rate of 5 mM ABTS to its
142 cation radical ($ABTS^{\cdot+}$) at 436 nm (ε_{436} = 29,300 $M^{-1}cm^{-1}$) in 0.1 M sodium acetate
143 buffer (pH 5) at 30ºC. One unit (U) of activity was defined as the amount of enzyme

forming 1 μmol of $ABTS^{\cdot+}$ per min. All spectrophotometric measurements were carried out on a Shimadzu UV-1603. Free laccase activity was expressed in U/L.

Immobilised laccase activity was assayed by incubating 20 mg/mL of biocatalyst preparation in a solution of 5 mM ABTS (in 0.1 M phosphate buffer, pH 5) previously tempered at 30ºC with continuous stirring at 150 rpm. The absorbance was monitored every minute over a 10 min period, by withdrawing 1 mL of solution. After measurement, the solution was remixed with the immobilised laccase. Immobilised laccase activity was expressed in U/g biocatalyst.

Protein estimation was performed by the method of Pierce® 660 nm Protein Assay Kit (Thermo Scientific), using bovine serum albumin as a standard. The quantity of protein bound to the supports was calculated from the difference between the protein loaded and that remaining in solution.

2.3. Laccase immobilisation on Eupergit supports

Laccase from *Myceliophthora thermophila* was immobilised on Eupergit supports by the method described by Katchalski-Katzir and Kraemer (2000). The enzyme was diluted in 0.1 M phosphate buffer (pH 7), centrifuged and filtered to a final concentration of 90 mg/mL or 80 U/mL. Different amounts of laccase: 11, 22, 55, 110 and 220 mg were mixed with 0.5 g of the carrier in 1 M phosphate buffer (pH 7) in a final volume of 5 mL. The reaction mixture was vortexed for 2 min and then the suspension was incubated at room temperature (24ºC±2ºC) with gentle shaking for 24 h. The decrease of activity due to the operational conditions was determined by monitoring the activity of free laccase subjected to identical conditions. The biocatalyst was then filtered using a glass filter (Whatman), washed three times with 5 mL of 0.1 M phosphate buffer (pH 7), dried under vacuum and stored at 4ºC. The immobilisation

process was carried out at pH 7 and room temperature since laccase from *Myceliophthora thermophila* retained 100% of initial activity under these conditions for at least 24 h (Lloret et al. 2010).

Bound protein was calculated as the difference between the protein content in the control and the protein remaining in the supernatant of the immobilised assay. The recovered activity was calculated as the ratio of the measured activity in the solid biocatalyst and the laccase activity theoretically immobilised onto the support. This theoretical activity was defined as the difference between the laccase activity in the controls and the remaining in the supernatant of the immobilised assay.

2.4. Characterisation of free enzyme and immobilised laccase

2.4.1. Optimum pH and pH stability

The pH of maximum laccase activity (free and immobilised) was investigated using 50 µM ABTS in a 0.1 M citrate–phosphate–borate buffer (pH 2-8). The relative activity was calculated as the ratio between the activity at each pH and the maximum attained.

The effect of the pH on the enzyme stability was studied by incubating laccase in 0.1 M citrate–phosphate–borate buffer (pH 2, 4, 6 and 8) at room temperature (24ºC±2ºC) during 24 h. Samples were transferred to standard reaction mixtures in order to determine the laccase activity with 50 µM ABTS. The residual activity was calculated referred to the value of the initial activity at each pH.

2.4.2. Optimum temperature and thermostability

The effect of temperature (20-80ºC) on laccase activity was determined by measuring activity at the corresponding temperature under standard conditions. The relative

activity was calculated as the ratio between the activity at each temperature and the maximum attained.

Thermal stability was determined by incubating enzymes in 0.1 M phosphate buffer (pH 7) at selected temperatures: 20, 40, 50, 60, 70 and 80ºC for different periods. Samples were transferred to standard conditions to determine the laccase activity with 50 µM ABTS at 30ºC.

2.4.3. Stability against chemical inactivation

The stability of laccase against different inactivating agents was tested using 1 g (10 U/g) of support (immobilised enzyme) or 10 U (free enzyme) in a volume of 3 mL. Enzyme inhibitors were added individually: 30 µM NaN_3, 10 µM $ZnCl_2$, 10 µM $CoCl_2$, 10 µM $CaCl_2$, 25% (v/v) methanol and 25% (v/v) acetone in 0.1 M phosphate buffer (pH 7) at room temperature (24±2ºC). The incubations lasted for 30 min and then residual activities were measured by ABTS (50 µM) standard assay.

2.4.4. Determination of apparent kinetic parameters

Apparent kinetic parameters of free and immobilised laccase were determined by measuring the laccase activity under standard conditions using ABTS as substrate in the range 25-1500 µM. The parameter values were obtained by fitting the data to the Michaelis-Menten equation using a non-linear regression (Sigma Plot 7.0, SPSS Inc., USA).

2.4.5. Reusability of the immobilised laccase

The reusability of the immobilised laccase was investigated in repeated batch experiments using ABTS as a substrate. The enzyme was incubated with 50 µM ABTS

for 5 h (1 cycle) in 0.1 M phosphate buffer (pH 7) at room temperature (24±2ºC). At the end of each oxidative cycle, the immobilised laccase was washed three times with 0.1 M phosphate buffer (pH 7) and spectrophotometrically measured with 50 µM ABTS. Thereafter, the procedure was repeated with a fresh solution of substrate. The activity measured in the first cycle was considered as the control (100%) for the calculation of the remaining percent activity after each cycle.

2.4.6. Storage stability

The storage stabilities of free and immobilised laccase were investigated at 4ºC and at room temperature (24±2ºC). Free enzyme was stored in a 0.1 M phosphate buffer solution (pH 7), whereas immobilised laccase was stored in a closed glass tube. The residual activities were measured after 1, 2 and 3 months by measuring the remaining laccase activity with 50 µM ABTS under standard conditions.

2.5. Decolourisation of a model synthetic dye by immobilised laccase

A glass column (40 cm height, 2 cm internal diameter, 50 mL of working volume) with immobilised laccase on Eupergit C 250L was used to remove the dye AG27 in a continuous operation of a packed bed reactor (PBR). Continuous operation conditions were the following: 10 g (9.7 U/g) of the biocatalyst, dye flow rate of 1.5 mL/min, hydraulic retention time of 30 min, 50 mg/L of the stock solution of the dye in the feeding tank, room temperature (24ºC±2ºC) and 0.1 M phosphate buffer (pH 7). After attaining the maximum decolourisation, the reactor was stopped and the decolourised solution drained. The packed bed was then washed with 20 mL of 0.1 M phosphate buffer (pH 7). The reactor was again filled with fresh dye solution and the process was then repeated for several cycles of identical duration. Samples from the reactor outlet

were collected to determine the percentage of degradation. Before adding the biocatalyst and operating as described above, the reactor was fed with AG27 solution at a flow of 1.5 mL/min until the absorbance measured in samples collected from the outlet was similar to that of the inlet stream.

Moreover, aiming to evaluate the potential leaching of the immobilised enzyme into the reactor, samples from the liquid phase were withdrawn during each cycle. The absorbance of the collected samples was measured after 2, 5 and 15 min in order to determine if there was dye removal by the potentially leached laccase. To quantify any potential adsorption of the dye on the support, a column was run in parallel with the same amount of support and inactivated laccase (incubated at 105°C for 4 h) under the same conditions applied for the PBR.

2.6. Removal of endocrine disrupting chemicals by immobilised laccase

A PBR was used for the continuous removal of selected EDCs (E1, E2 and EE2) from an aqueous solution. The operational conditions were identical to the ones mentioned previously with a concentration of the estrogens in the feeding tank of 5 mg/L of each EDC. The reactor was initially charged with the estrogens solution before adding the biocatalyst. The continuous operation was carried out for 8 h at room temperature (24±2ºC).

To quantify any potential adsorption for EDCs on the support with immobilised laccase, the same amount of Eupergit C 250L with previously inactivated laccase (incubated at 105°C for 4 h) was used in the operation of the PBR. Moreover, the potential leaching was evaluated by the same procedure described previously for the dye removal.

2.7. Analyses of Acid Green 27 dye

The efficiency of the enzymatic treatment was evaluated by monitoring the AG27 absorbance at its maximum absorption wavelength (650 nm) with a Shimadzu UV-1603 spectrophotometer. The percentage of decolourisation was calculated with respect to the initial measured absorbance.

2.8. High-performance liquid chromatography analyses

Determination of EDCs concentration was carried out through high performance liquid chromatography (HPLC) in a HP-1090 system equipped with a diode array detector and a Water Xterra-C18 column (4.6x150 mm, particle size 5 μm). The operational conditions for the analysis were: 10 μL injection volume, λ 210 nm, acetonitrile: phosphate buffer (50 mM, pH 4.5) as eluent in isocratic conditions of 40:60% at a flow rate of 1 mL/min.

2.9. Evaluation of estrogenic activity

The estrogenic activity was measured by the LYES (lyticase yeast estrogen screen-assay assisted by enzymatic digestion) assay previously described by Taboada-Puig et al. (2011), adapted from Schultis and Metzger (2004). The recombinant yeast *Saccharomyces cerevisiae* was kindly provided by the Laboratory of Microbial Ecology and Technology (Labmet, Ghent University, Belgium).

3. Results and discussion

3.1. Immobilisation of laccase

The effect of the amount of laccase on the immobilisation efficiency was evaluated in the range between 22 and 880 mg of protein/g support (Table 1). Specific activities of

293 up to 17 U/g and 80 U/g were measured for Eupergit C and Eupergit C 250L
294 respectively. It is evident that at increasing amounts of the enzyme, the activity recovery
295 decreased probably due to the saturation of the support. For example, the increase in
296 laccase load from 22 to 440 mg/g resulted in the decrease of activity recovery: from
297 36.1% to 21.1% for Eupergit C and more so, from 88.4% to 43.8%, in the case of
298 Eupergit C 250L. It is generally acknowledged that the immobilisation efficiency of
299 enzymes on solid supports decreases when enzyme loading exceeds a certain value
300 (Knezevic et al. 2006; Li et al. 2007). In fact, it was observed that Eupergit carriers
301 presented a saturation value of laccase of 440 mg protein/g support. The obtained
302 results were higher than those presented by Katchalski-Katzir and Kraemer (2000), who
303 reported that the binding capacity of an enzyme is about 100 mg protein/g Eupergit C.
304 The same effect was observed regarding bound protein (79.1-43.8% and 88.4-43.8% for
305 Eupergit C and Eupergit C 250L in the range 22-440 mg protein/g support).
306 Among both supports, Eupergit C 250L yielded the highest specific activity (80 U/g
307 biocatalyst) and retained the highest amount of protein. Eupergit C 250L presents lower
308 concentration of reactive groups than Eupergit C, but larger pores, which may explain
309 the higher immobilisation efficiency (Gomez de Segura et al. 2004; Berrio et al. 2007).
310 The activity yields related to bound protein and enzyme activity achieved in the present
311 study were in the same range of magnitude than the ones previously reported for
312 laccases immobilisation by covalent methods. For example, Kunamneni et al. (2008)
313 immobilised laccase from *Myceliophthora thermophila* on Sepabeads EC-EP3 and
314 Dilbeads NK supports with bound protein yields of 32.6 and 17.8%, respectively. Berrio
315 et al. (2007) attained levels of bound protein between 10.3-66.5% and 10.2-34.2% for
316 *Pycnoporus coccineus* laccase immobilised on Eupergit C and Eupergit C 250L,
317 respectively.

318 Due to its superior activity, only laccase immobilised on Eupergit C 250L was used for
319 further experiments: characterisation of the biocatalyst, decolourisation of a model dye
320 and removal of endocrine disrupting chemicals.
321
322 **3.2. Characterisation of free enzyme and immobilised laccase**
323 **3.2.1. Optimum pH and pH stability**
324 The pH-dependence of activity for free and immobilised laccases was analysed in a pH
325 range from 2 to 8, in a 0.1 M citrate–phosphate–borate buffer. Both free and
326 immobilised laccase showed that the optimal pH was 3 and the relative activity for the
327 immobilised laccase was slightly higher (10%) than that of free laccase in the range of
328 pH 4-7 (Figure 1A).
329 The effect of the pH on the enzyme stability was studied by incubating both laccase
330 formulations at pH 2, 4, 6 and 8 and room temperature for 24 h. Figures 2A and C
331 depict the profiles of pH deactivation for free and immobilised laccase, respectively.
332 While results were similar at neutral pH range, immobilised laccase presented higher
333 stability at acid pH. For example, free laccase was completely inactivated after 1 h of
334 incubation at pH 2, and retained only 15% of initial activity at pH 4. However, the
335 residual activity of immobilised laccase after 1 h amounted to 70 and 80% at pH 2 and
336 4, respectively, and 10 and 30% of the initial activity after 24 h.
337 Alptekin et al. (2010) reported that the multiple-covalent bonding of laccase to the resin
338 resulting in more rigid enzyme less prone to pH-induced conformational changes, that
339 could cause higher pH-stability. Kunamneni et al. (2008) immobilised laccase from
340 *Myceliophthora thermophila* by covalent binding with epoxy groups of
341 polymethacrylate-based polymers and achieved similar results for optimal pH.
342

3.2.2. Optimum temperature and thermostability

The effect of temperature on laccase activity of free and immobilised enzyme was determined in the range 20-80ºC under standard conditions (0.1 M sodium acetate buffer, pH 5). The optimum temperature was 60ºC for free and immobilised laccase, but the latter showed a broader profile and its relative activity was 10% higher than the free laccase in the range 20-50ºC (Figure 1B).

Thermal stability was tested by incubating both laccases in 0.1 M phosphate buffer (pH 7) at selected temperatures: 20, 40, 50, 60, 70 and 80ºC for 24 h. It was observed that the activity of the free laccase (Figure 2B) dropped more rapidly than that of immobilised laccase (Figure 2D). After incubation at 80ºC for 2 h, free laccase was completely inactivated. However, immobilised laccase retained 50% of its initial activity after 2 h, and 10% after 24 h. Similar results were achieved by incubating both laccases at 60 and 70ºC. Both free and immobilised enzyme retained 100 and 70% of initial activity after 24 h incubation at 20 and 40ºC, respectively. These results agreed with the study carried out by Osma et al. (2010), who reported that the immobilisation affects the conformational flexibility of the enzyme since it causes an increase in enzyme rigidity and stability towards denaturation by high temperatures. The improvement on stability attained in the present study was similar to that achieved in previous investigations. Kunamneni et al. (2008) and Forde et al. (2010), who carried out the immobilisation of the *Myceliophthora thermophila* laccase on Sepabeads EC-EP3 and Dilbeads NK polymers and on mesoporous silicates particles, attained an enhancement of 10-15% after immobilisation for the tested temperature range.

3.2.3. Stability against chemical inactivation

The stability of the immobilised and free enzymes against different inactivating agents was tested to anticipate their behaviour in applications where such conditions may be present, such as real effluents with variable composition of inhibitors, such as azides, chloride salts and organic solvents. The residual activity of both laccases after incubation with several chemical agents (NaN_3, $ZnCl_2$, $CoCl_2$, $CaCl_2$, methanol, acetone) after 30 min was measured.

It was observed that laccase immobilised on Eupergit C 250L had only a slightly higher stability (2-3%) than free laccase against $ZnCl_2$, $CoCl_2$, $CaCl_2$ salts (residual activity from 40-45% to 41-46%). This was probably due to the small dimension of the ions that allowed them to easily migrate into the structure of the biocatalyst and inactivate laccase to a similar extent (Cabana et al. 2007). The improvement of the stability against organic solvents by immobilisation was more significant (the residual activity in methanol varied from 41.6 to 55.9% and acetone from 40.4 to 46.3% between free and immobilised laccase, respectively). A stronger effect was observed in the presence of NaN_3. In this case, the immobilised laccase displayed a residual activity twice as high as the free laccase activity (31.9 and 15.1%). Similar conclusions were attained with *Coriolopsis polyzona* laccase immobilised through the formation of cross-linked aggregates (Cabana et al. 2007) and covalently immobilised on Celite R-33 support (Cabana et al. 2009) and with *Trametes pubescens* laccase immobilised on alumina pellets (Osma et al. 2010).

3.2.4. Determination of apparent kinetic parameters

Michaelis-Menten kinetic parameters of free and immobilised laccase were obtained by using ABTS as the substrate. The value of K_M for the immobilised laccase (150 ± 10

393 μmol/L) was approximately 3-fold higher than that of the free enzyme (56 ± 2 μmol/L),
394 and v_{max} of the immobilised enzyme was 5-fold lower (693 ± 13 and 3330 ± 42
395 μmol/min·L, respectively). The efficiency of the immobilised laccase (2.75 ± 0.05
396 L/μmol·min), based on k_{cat}/K_M value, was approximately twice as low as that of the free
397 enzyme (6.01 ± 0.40L/μmol·min). Lower efficiencies for immobilised laccase and
398 catalase from *Lentinus edodes* on Eupergit C were also reported by D'Annibale et al.
399 (2000) and Alptekin et al. (2010), respectively. Several authors have suggested that this
400 effect could be related to diffusion substrate limitations, substrate partitioning, lower
401 accessibility of the substrate to the active site, protein conformational changes, internal
402 mass transport constraint and/or substrate/product adsorption onto the carrier surface
403 (Davis and Burns, 1992; Hernaiz and Crout, 2000; Rekuc et al. 2010; Yamak et al.
404 2009).
405
406 **3.2.5. Reusability of the immobilised laccase**
407 The reusability of the immobilised laccase was also evaluated under an efficiency
408 perspective. The enzyme was incubated with ABTS in a 0.1 M phosphate buffer (pH 7)
409 at room temperature, for 10 cycles of 5 h. As expected, the activity of the immobilised
410 enzyme fell steadily as the number of cycles increased (Figure 3). However,
411 immobilised laccase retained 65% of initial activity after 10 batches. In literature,
412 laccase immobilised on activated poly(vinyl alcohol) retained 60% of the initial activity
413 values after 10 cycles (Yingui et al. 2002), while laccase immobilised on amine-
414 terminated magnetic nanocomposites, 80% after 5 consecutive operations (Xiao et al.
415 2006).
416
417

3.2.6. Storage stability

Free and immobilised laccases were stored at 4ºC and at room temperature, with periodical sampling and monitoring. After 3 months, the residual activity of both free and immobilised laccase stored at 4ºC was around 98%. However, at room temperature, the residual activity of free laccase amounted to 95%, while immobilised laccase maintained significant activity with a negligible loss of activity (1.5%) after 1 month and only 0.5% additional after two months. In previous research, covalently immobilised laccase from *Myceliophthora thermophila* on Sepabeads EC-EP3 carriers retained 96% of its initial activity after storage at 4ºC for 4 months (Kunamneni et al. 2008). Alptekin et al. (2010) reported residual activities of immobilised catalase on Eupergit C of 68 and 79% after storage for 28 days at 5ºC and room temperature, respectively.

3.3. Decolourisation of a model synthetic dye by immobilised laccase

With the aim of testing the feasibility of the immobilised laccase in a packed bed reactor (PBR), the biocatalyst was used for the continuous elimination of the dye AG27 as a model substrate. The reactor was operated continuously for short periods of 4 h until steady-state conditions were achieved.

As shown in Figure 4, the percentage of dye removal significantly decreased after each operation with the corresponding decrease of the adsorption of the dye on the support. The extent of this combined removal was well conserved over the treatment cycles, achieving high degrees of elimination: 88, 79, 69 and 57% for the different cycles. The levels of removal related to adsorption of AG27 on the support varied from 18% to 6% between the first and the fourth cycle. Under these circumstances, the operation time of the reactor might be increased in order to assure high levels of dye removal. This

443 decrease on the removal percentages may be related to an enzyme inactivation produced
444 by operational conditions, diffusion problems caused by dye or products adsorption
445 onto the carrier surface and the potential clogging phenomena caused by insoluble
446 products formed (Hernaiz and Crout, 2000; Rekuc et al. 2010). In order to verify that
447 assumption, the residual activity of the biocatalyst after the fourth cycle was determined
448 after washing the biocatalyst with 0.1 M phosphate buffer (pH 7). It was observed an
449 inactivation of 24% (residual activity 76%).
450 Enzymatic effect was observed as the major contribution in the removal of the dye. In
451 addition, negligible leaching of the immobilised enzyme was detected. Thus**,**
452 immobilised laccase on Eupergit C 250L showed high efficiency in the enzymatic
453 oxidation of AG27 dye and the reusability of the proposed catalyst was confirmed,
454 which is of remarkable interest regarding the potential use of immobilised laccase, in
455 spite of the decreasing in the decolourisation efficiency over the consecutive cycles.
456 This high efficiency and reusability of immobilised laccase on the oxidation of the
457 anthraquinonic dye used was reported by Kunamneni et al. (2008) with
458 *Myceliophthotra thermophila* laccase immobilised on Sepabeads EC-EP3 carriers in a
459 PBR, although the physical adsorption on the support was not taken into account. On
460 the contrary, Zille et al. (2003) reported that the decolourisation of Reactive black 5
461 dye, an azo dye, by laccase immobilised on silica beads was due to adsorption (79%)
462 and a minor amount (4%) to enzymatic activity. Soares et al. (2001) also demonstrated
463 the removal of an anthraquinonic dye (Remazol Brilliant Blue R) by a free commercial
464 laccase formulation, but in the presence of the mediator HBT and a non-ionic surfactant.
465
466
467

468 **3.4. Elimination of endocrine disrupting chemicals (EDCs) by immobilised laccase**

469 The packed bed reactor with the solid biocatalyst was also used for the removal of three
470 EDCs from an aqueous solution of 5 mg/L of each compound. The results of enzymatic
471 and adsorption-based removal of the selected estrogens are shown in Figure 5. The
472 maximum elimination of E1 (65%) was attained after 4 h of operation time (Figure 5A).
473 However, after a short period (2 h) the steady state removal of E2 and EE2 was
474 achieved (Figure 5B and C, respectively). This higher affinity for E2 and EE2 was also
475 observed with free enzyme in batch experiments, although higher levels of degradation
476 were attained by free laccase (Lloret et al. 2010). The degrees of elimination due to
477 adsorption to the support with inactivated laccase were approximately 22% for E2, less
478 than 20% for EE2 and 11% for E1. The adsorption seemed to be correlated with the
479 hydrophobicity of each chemical. The highest adsorption was seen for E2, which has the
480 highest octanol-water partition coefficient ($logk_{ow}$ 3.9-4.0) and the lowest solubility (3.6
481 mg/L). The slightly $logk_{ow}$ and water solubility of EE2 are 2.8-4.2 and 11.3 mg/mL and
482 of E1 3.1-3.4 and 30 mg/L (Suarez et al. 2008). Even if part of the elimination observed
483 was associated with the adsorption of these chemicals on the support, the enzymatic
484 action was clear in the removal of these EDCs (represented 80% of the total removal).
485 This oxidative ability may open the possibility of using *Myceliophthora thermophila*
486 laccase immobilised on Eupergit C 250L support to treat effluents containing this kind
487 of estrogenic compounds. Additionally, the biocatalyst retained 84% of its initial
488 activity after the treatment and negligible leaching of the immobilised enzyme was
489 detected during the operation. The same effect was demonstrated by Cabana et al.
490 (2009) during the elimination of 5 mg/L of nonylphenol, bisphenol A and triclosan in a
491 PBR by laccase covalently immobilised on Celite R-633 supports. The adsorption of
492 those compounds on the support with inactivated laccase varied from 40 to 60% and

493 this physical removal was also linked to the solubility and hydrophobicity of the
494 different EDCs. Russo et al. (2008) also observed the adsorption of an anthraquinone-
495 dye on Eupergit supports with immobilised laccase. Those authors reported two
496 different steps: during the first step, dye adsorption on the carrier was extensive and
497 enzymatic conversion was negligible; during the second step and under steady state
498 conditions, the removal of the dye was attributed to enzymatic conversion and an
499 adsorption/desorption equilibrium was attained. According with the results presented in
500 Figure 5, a similar interaction between the EDCs and the Eupergit support could take
501 place. Furthermore, adsorption is expected to be a determining factor during the
502 elimination of estrogens at lower concentrations (from μg/L to ng/L) since it has been
503 reported by previous studies. For instance, Carballa et al. (2004) observed a removal of
504 20% of E2 in the primary treatment of a Spanish STP, whereas in a German STP, EE2
505 and E2 were eliminated up to 35 and 29%, respectively (Andersen et al. 2003). Snyder
506 et al. (2007) reported higher adsorption percentages (75, 78 and 85% for E1, EE2 and
507 E2, respectively), on activated carbon. However, the main advantage of the processes
508 presented in the current work is that estrogens were eliminated mainly by enzymatic
509 transformation, therefore the compounds were transformed and their estrogenic activity
510 reduced.
511 Several previous studies reported high levels of enzymatic oxidation of these
512 compounds with free enzyme. For example, Tanaka et al. (2001) degraded EE2 by 90%
513 within 48 h using 0.8 U/mL of laccase from *Trametes* sp. and *Pycnoporus coccineus*.
514 Auriol et al. (2008) degraded completely E1, EE2 and E2 after 1 h-treatment using 20
515 U/L and Suzuki et al. (2003) transformed E2 and EE2 by 100% in 1 h by the laccase-
516 HBT system. In addition, our research group (Lloret et al. 2010) has recently used free
517 *Myceliophthora thermophila* laccase for the elimination of these compounds, with a

518 higher efficiency (total removal of E2 was attained in only 2 h, with less initial laccase
519 activity and no mediator was used). In the current study, we propose the immobilisation
520 of laccase into Eupergit supports for EDCs removal. Immobilisation is probably the best
521 way to attain enzyme stabilisation, since it provides increasing operational stability,
522 easy of separation and catalyst reuse (Kunamneni et al. 2008; Osma et al. 2010).
523 Moreover, the main advantage of the immobilisation of laccase on Eupergit supports is
524 the extremely easy technique procedure, comprising mixing of the components,
525 incubation and washing of the product. The continuous elimination of EDCs by
526 immobilised laccase has been reported for bisphenol A using laccase carried on nylon
527 membrane (Diano et al. 2007) and covalently bound onto alkylaminated controlled
528 porosity glass (Iida et al. 2003). Cabana et al. (2007, 2009) tested the elimination of
529 bisphenol A, triclosan and nonylphenol by laccase immobilised through the formation
530 of cross-linked enzyme aggregates and on support Celite R-633. In our study, E1, E2
531 and EE2 were degraded for the first time by *Myceliophthora thermophila* laccase
532 immobilised on Eupergit C 250L support. However, the use of PBRs with immobilised
533 enzymes presents several disadvantages such as: passive or poor aeration, slow mass
534 transfer, compaction of the bed and hence biocatalyst damage due to a high pressure
535 drop, the possible formation of insoluble products which would lead to clogging
536 phenomena, limitation in substrate's diffusion, intra particle diffusion limitations on
537 reaction rates and possible leakage of the biocatalyst from the support (Gómez et al.
538 2007; Murry et al. 2002; O'Neill et al. 1971; Osma et al. 2010; Rekuc et al. 2009).
539 Additionally, long-term operation of packed bed reactor is still a challenge. The
540 drawback is the requirement for periodical regeneration of the biocatalyst by some
541 cleaning solution (Watanabe et al. 2001). The future work will be focused on the

overcoming of those limitations and the study of the effect of compounds presented in real wastewaters on the feasibility of the degradation system.

Although the main objective was not to determine degradation products, an important effort has been devoted to the identification of products. Unfortunately, we have not identified any reaction products by the HPLC protocol used, suggesting that a polymerisation reaction could have occurred or that the products reacted with either the laccase or the carrier. However, regarding the toxicity of estrogens treated by laccase, several reports have shown that the removal of estrogenic activity was correlated with the removal of the parent compound, indicating that the products of the degradation exhibited lower or no estrogenic activity (Auriol et al. 2008). The estrogenic activity of the effluent of the continuous reactor was determined by the LYES assay. A decrease of 79% was measured in the enzymatic reactor while only 14% corresponded to inactivated laccase.

4. Conclusions

Myceliophthora thermophila laccase was covalently immobilised to Eupergit C 250L support. Although the immobilised biocatalyst displayed a lower substrate affinity than the free enzyme, the immobilisation improved enzyme stability for storage, pH, and temperature, as well as in the presence of different inactivating chemicals. It was also shown that immobilised laccase on Eupergit C 250L achieved high decolourisation of Acid Green 27 by enzymatic degradation and reusability. These important properties of immobilised laccase could be a potential advantage for various biotechnological and industrial applications. In addition, immobilised laccase on a solid support was applied for the first time for the continuous elimination of the EDCs E1, E2, and EE2 in a PBR.

566 Overall, our results showed that laccase immobilised on Eupergit C 250L is a promising
567 tool to increase the applicability of laccase in bioremediation processes.
568
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CAPTIONS TO FIGURES

Figure 1. Effect of pH on free (●) and immobilized laccase on Eupergit 250L (□). pH in the range 2-12 tested with 50 µM ABTS in 0.1 M citrate-phosphate-borate. 100% values corresponded to 11.36 U/g (immobilized laccase) and 100 U/L (free laccase). Data show the mean value of two independent experiments, with standard deviations.

Figure 2. pH stability profiles of free laccase (A) and immobilized laccase on Eupergit 250L (B). pH values tested: 2 (●), 4 (□), 6 (´) and 8 (_) by incubating laccase in 0.1 M citrate-phosphate-borate buffer at room temperature.

Figure 3. Effect of temperature on free (●) and immobilized laccase on Eupergit 250L (□). Various temperatures in the range 20-80ºC were tested with 50 µM ABTS. 100% values correspond to 32.91 U/g (immobilized) and 220 U/L (free laccase).

Figure 4. Thermostability profiles of free laccase (A) and immobilized laccase on Eupergit 250L (B). Temperatures tested: 50 ºC (●), 60 ºC (□), 70 ºC (´) and 80 ºC (_) by incubating laccase in 0.1 M phosphate buffer (pH 7).

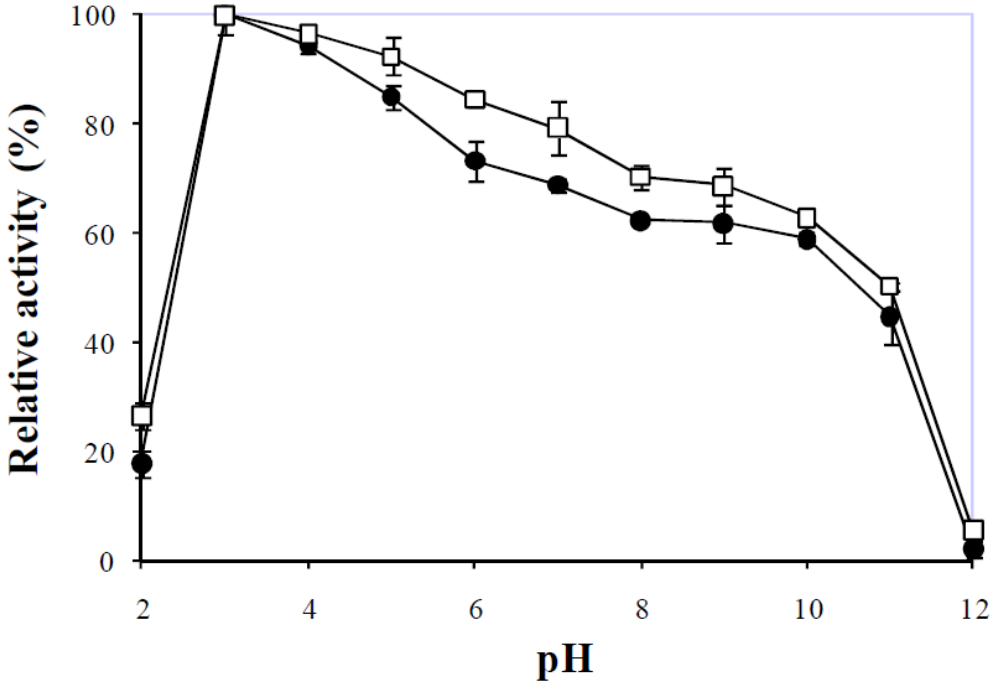
Figure 5. Effect of the reusability on activity of immobilized laccase on Eupergit 250L after cycles (5 h) of 50 µM ABTS oxidation in batch reaction.

Figure 6. Removal of AG27 dye by immobilized laccase (2.5 g biocatalyst, 24.3 U/g) in a PBD operating in a repeated batch mode, at room temperature and pH 7. The open symbols represent the removal accounted by the adsorption of the AG27 dye on the support with inactivated laccase. Measures were carried out by duplicate, standard desviations are shown.

Figure 7. SEM pictures of the solid biocatalyst before (A) and after (B) 4 cycles of AG27 dye treatment.

Figure 8. Removal of E1 (A), E2 (B) and EE2 (C) by immobilized laccase (2.5 g biocatalyst, 24.3 U/g) in a PBR at room temperature and pH 7. The open symbols

773 represent the removal accounted by the adsorption of the EDCs on the support with
774 inactivated laccase.
775



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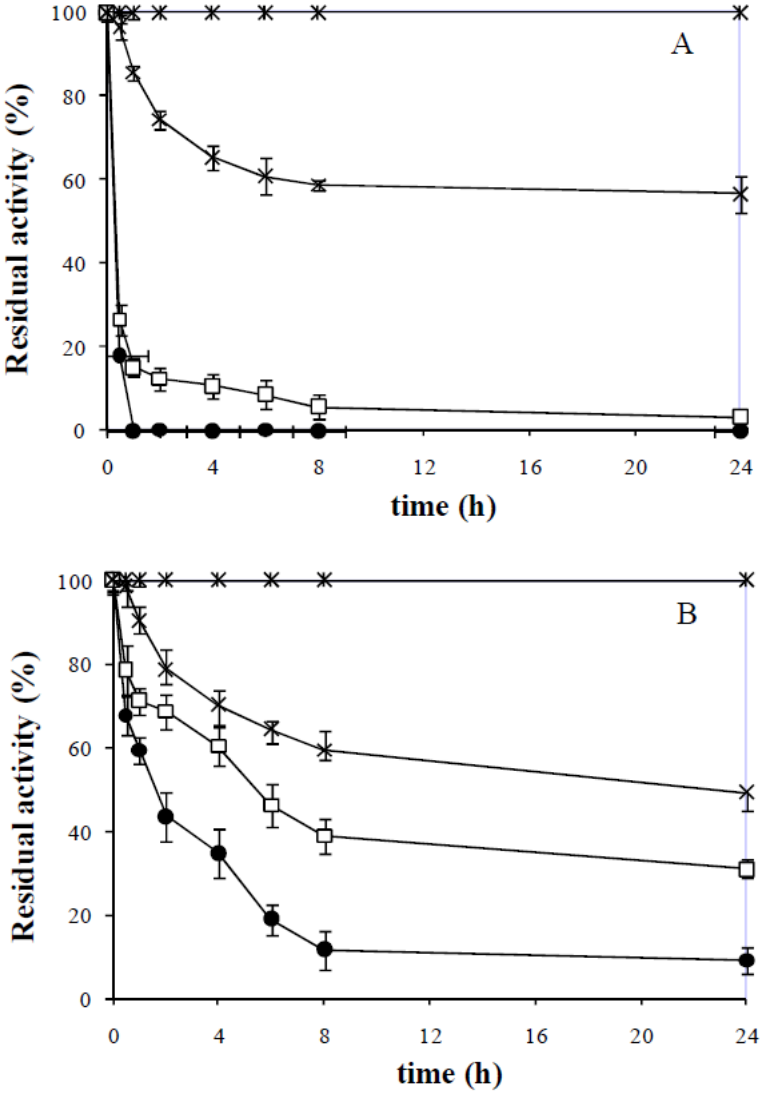

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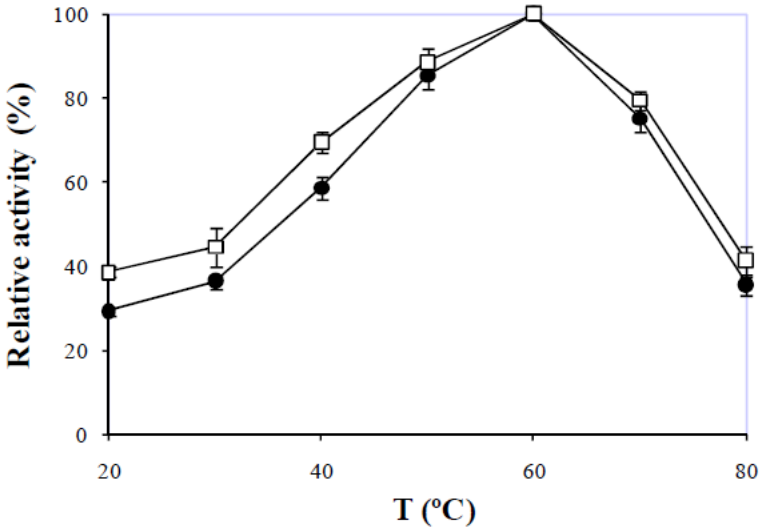

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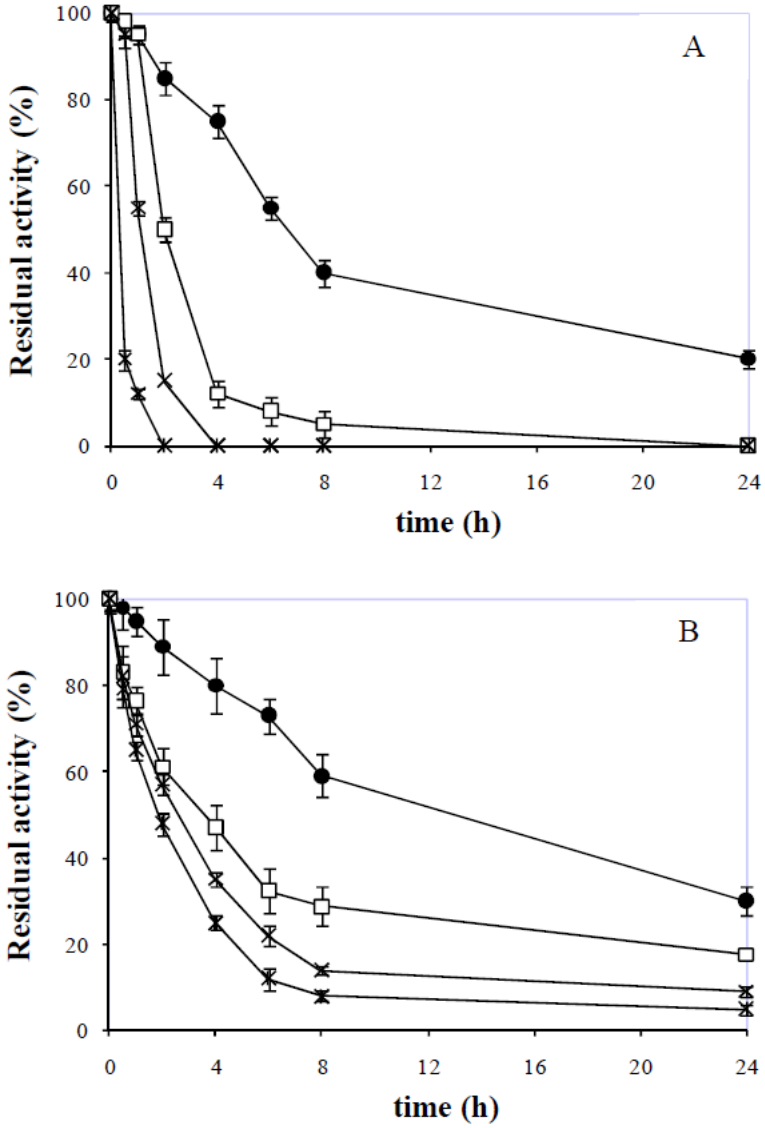
A
Residual activity (%)
time (h)
0
20
40
60
80
100
0
4
8
12
16
20
24

B
Residual activity (%)
time (h)
0
20
40
60
80
100
0
4
8
12
16
20
24

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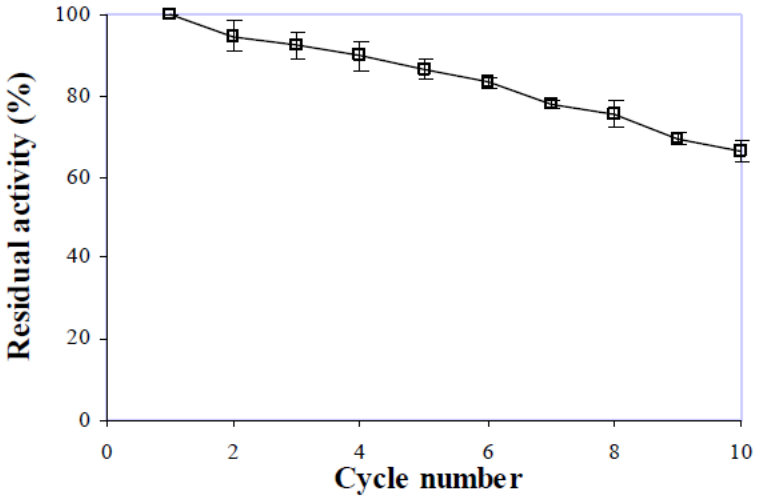

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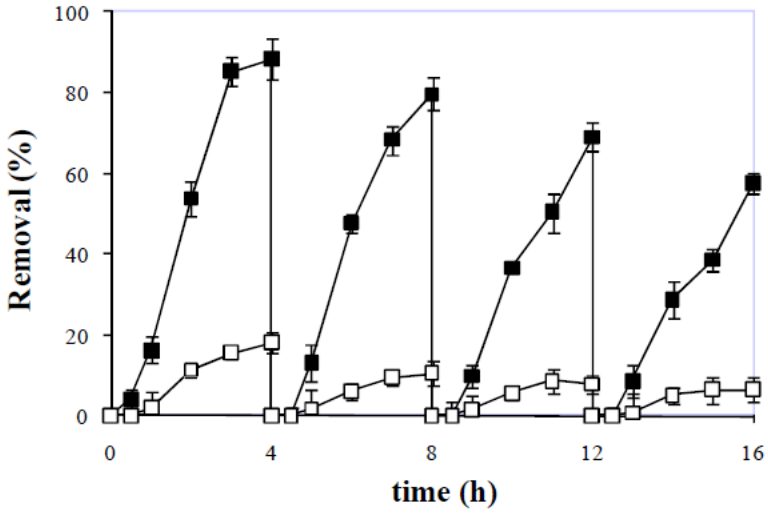

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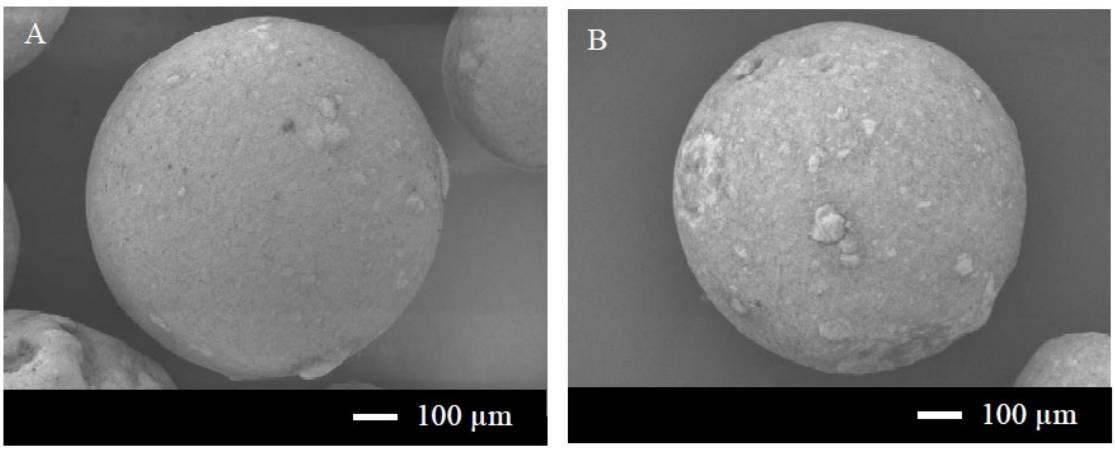

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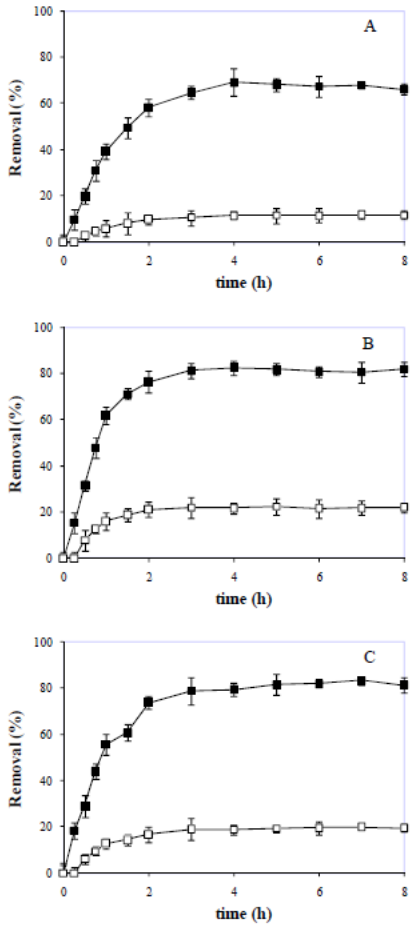

783

Table 1. Recovery protein and activity in the immobilization of *M. thermophila* laccase on Eupergit C supports.

Added protein (mg/g support)	Bound protein[a] (%)		Activity[b] (U/g biocatalyst)		Activity recovery[c] (%)	
	Eupergit C	Eupergit C 250L	Eupergit C	Eupergit C 250L	Eupergit C	Eupergit C 250L
22	79.1 ± 2.3	99.3 ± 2.8	1.37 ± 0.06	9.66 ± 0.45	36.1 ± 1.8	88.4 ± 4.1
44	75.9 ± 5.1	89.1 ± 1.3	2.35 ± 0.12	15.95 ± 0.74	28.4 ± 1.1	86.9 ±3.9
110	74.2 ± 4.2	84.2 ± 0.5	7.44 ± 0.19	24.30 ± 0.32	26.9 ± 0.9	50.9 ± 2.5
220	61.3 ± 0.2	75.1 ± 2.4	10.12 ± 0.29	53.80 ± 1.06	23.9 ± 2.1	63.8 ± 3.0
440	45.1 ± 0.9	61.9 ± 4.6	16.00 ± 0.19	72.85 ± 1.12	21.1 ± 1.3	43.8 ± 2.1
880	43.8 ± 1.1	56.7 ± 2.5	17.09 ± 0.25	80.01 ± 0.98	19.2 ± 1.1	39.5 ± 0.9

[a]The amount of immobilized protein was calculated from the difference between the protein in the control and protein remaining in the supernatant of immobilized assay.

[b]Assay conditions: 50 µM ABTS, 100 mM socium acetate buffer, pH 5, 20 mg/mL biocatalyst, in a final volume of 3 mL. Measured with immobilized biocatalyst over 10 min period.

[c]Activity recovered was calculated as the ratio of measured activity to theoretic bound activity (difference between the laccase activity in the controls and the remaining in the supernatant of immobilized assay).

Table 2. Residual activity (%) of free laccase and immobilized laccase after incubation during 30 min with different denaturants in 0.1 M phosphate buffer (pH 7) at room temperature.

Denaturant	Free laccase	Laccase immobilized on Eupergit C 250L
NaN_3 (30 µM)	15.1 ± 4.1	31.9 ± 2.1
$ZnCl_2$ (10 µM)	40.4 ± 2.3	41.1 ± 2.6
$CoCl_2$ (10 µM)	39.6 ± 2.1	42.8 ± 0.8
$CaCl_2$ (10 µM)	44.7 ± 2.1	45. 8 ± 1.5
Methanol (25%, v/v)	41.6 ± 4.2	55.9 ± 1.5
Acetone (25%, v/v)	40.4 ± 1.1	46.3 ± 2.5

Table 3. Michaelis-Menten kinetic parameters of free laccase and immobilized laccase for the oxidation of ABTS at pH 5 (sodium acetate buffer, 0.1 M) at room temperature.

Enzyme form	K_M (µM)	v_{max} (µM/min)	k_{cat}/K_M (1/(µM·min))	R^2
Free	56 ± 2	3330 ± 42	6.01 ± 0.40	0.998
Immobilized on Eupergit C 250L	150 ± 10	693 ± 13	2.75 ± 0.05	0.989