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RESEARCH ARTICLE

Recyclable cross-linked laccase aggregates coupled to magnetic silica microbeads for elimination of pharmaceuticals from municipal wastewater

A. Arca-Ramos[1] · V. V Kumar[2] · G. Eibes[1] · M. T. Moreira[1] · H. Cabana[3,4]

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Abstract In the present work, the use of magnetic mesoporous silica microbeads (MMSMB) as supports was proposed to produce magnetically-separable cross-linked enzyme aggregates (MCLEAs). The effects of cross linking time, addition of bovine serum albumin as protein feeder, pH, glutaraldehyde concentration, and laccase:MMSMB mass ratio on the immobilization yield and enzyme load were investigated. The best conditions allowed the rapid preparation of MCLEAs with high enzyme load, i.e., 1.53 U laccase/mg MCLEAs. The stability of MCLEAs was improved with regard to low pH, presence of chemical denaturants, and real wastewater matrix, compared to free laccase. In addition, the novel biocatalyst exhibited good operational stability, maintaining up to 70 % of its initial activity after 10 successive batch reactions. Finally, MCLEAs demonstrated its catalytic potential to transform acetaminophen and various non-phenolic pharmaceutical active compounds as mefenamic acid, fenofibrate, and indomethacin from biologically treated wastewater effluent, with similar or even higher efficiency than free laccase.

Keywords Laccase · CLEAs · Enzymatic treatment · Magnetic silica microparticles · Micropollutants · Pharmaceuticals · Secondary effluent

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✉ H. Cabana
Hubert.Cabana@USherbrooke.ca

[1] Department of Chemical Engineering, Institute of Technology, University of Santiago de Compostela, 15782 Santiago de Compostela, Spain

[2] Department of Biotechnology, School of Bioengineering, SRM University, Kattankulathur, Chennai 603203, India

[3] Department of Civil Engineering, Université de Sherbrooke, 2500 Boulevard de l'Université, Sherbrooke, Québec J1K 2R1, Canada

[4] Environmental Engineering Laboratory, Department of Civil Engineering, Université de Sherbrooke, 2500 Boulevard de l'Université, Sherbrooke, Québec J1K 2R1, Canada

Introduction

Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) are a type of lignin-modifying enzymes (LME) which constitute promising biocatalysts in industrial and bioremediation processes due to their wide substrate range and simple requirements of catalysis (Majeau et al. 2010). Nevertheless, two major drawbacks limit their application in continuous bioprocess. On the one hand, the susceptibility of free enzymes to inactivation over time due to unfavorable conditions (i.e., unfavorable pH, chemical/biological denaturing agents, or temperature) (Gasser et al. 2014). Another major obstacle is related to the retention and reusability of the biocatalyst. In order to mitigate these drawbacks and enhance the economy of biocatalytic processes, immobilization and insolubilization techniques for enzymes have been lately investigated (Brady and Jordaan 2009).

In this sense, the insolubilization of enzymes as cross-linked enzyme aggregates (CLEAs) is a simple technique to increase the stability and reusability of biocatalyst (Sheldon 2011). The preparation of CLEAs usually involves the precipitation from solution followed by a cross-linking step using a bifunctional reagent, such as glutaraldehyde. However, CLEAs may suffer from some drawbacks such as softness or poor mechanical stability, which make them difficult to handle in conventional reactors and often causes leaching of enzyme in the reaction medium (Wilson et al. 2002). In addition, the processes commonly applied to recover CLEAs from reaction medium,

namely, centrifugation or filtration, lead to increase in CLEAs size and clusters formation (clumping), resulting in internal mass transfer limitations (López et al. 2014). Hence, for many applications, CLEAs may require a physical support to improve their mechanical properties (Brady and Jordaan 2009).

The use of magnetic supports may be an alternative to produce mechanical resistant and magnetically-separable CLEAs, which can be recovered easily using a magnet instead of centrifugation or filtration methods (López et al. 2014). For instance, magnetic CLEAs of α-amylase were successfully applied to hydrolyze starch (Talekar et al. 2012). More recently, López et al. (2014) produced magnetic CLEAs of lipase to obtain biodiesel and biosurfactants.

In recent works done by Kumar et al. (2014) and Kumar and Cabana (2016), this approach was applied for the first time to laccase. Stable and magnetically separable biocatalysts were obtained by immobilizing laccase CLEAs onto iron-based magnetic nanoparticles previously functionalized with 3-aminopropyltriethoxysilane (APTES).

On the other hand, although laccase-based treatments have been successfully applied towards the elimination of many xenobiotics, most of works deal with single-compound solutions comprising laccase substrates (i.e., mainly phenolic) in buffer solutions or distilled water. Moreover, the transformation of non-laccase substrates requires the presence of mediators. Recent studies reported that the substrate spectrum of laccase could be extended in mixtures when phenolic compounds are present (Touahar et al. 2014). The latter may involve cross-coupling reactions with non-phenolic compounds. Thereby, this effect would compensate the absence of specific laccase mediators (e.g., 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) or 1-hydroxybenzotriazole).

Therefore, the first aim of this work was to produce robust magnetically-separable MCLEAs using as support magnetic mesoporous silica microbeads (MMSMB) already containing amino groups, according to the scheme depicted in Fig. S1 (Electronic Supplementary Material, ESM). The characterization of the novel MCLEAs and the evaluation of their catalytic potential to remove a mixture of phenolic and non-phenolic pharmaceutical active compounds (PhACs) from the secondary effluent of a municipal wastewater treatment plant were additional objectives of this research.

Materials and methods

Chemicals and enzyme

Commercial laccase from *Trametes versicolor* (≥10 U/mg), bovine serum albumin (BSA), ABTS (≥98 %), glutaraldehyde (25 % *v*:*v*), acetaminophen (≥99 %), ketoprofen (≥98 %), cyclophosphamide (≥97 %), mefenamic acid (≥98 %), caffeine (≥99 %), indomethacin (≥99 %), naproxen (≥98.5 %), fenofibrate (≥99 %), trimethoprim (≥98 %), and ibuprofen (≥98 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethyl acetate, dichloromethane, formic acid, and methanol were of the analytical grade and were purchased from Fisher Scientific (Ottawa, ON, Canada). Magnetic mesoporous silica microbeads (MMSMB; MAT540-SMY-AV) were kindly provided by Materium Innovations (Granby, QC, Canada). These consisted in magnetite nanoparticles (40–50 nm) encapsulated in hollow mesoporous silica microcapsules with an average size around 20 μm. The material used is bearing both amino and vinyl groups on their surface (information provided by Materium Innovations).

Enzyme activity

Laccase activity was measured by monitoring the oxidation of ABTS to its cation radical ($ABTS^{\bullet+}$) at 420 nm ($\varepsilon_{max} = 3.6 \times 10^4\ M^{-1}\ cm^{-1}$). Fifty microliters of laccase or MCLEAs containing sample (diluted in 0.1 M acetate buffer, pH 4.0) were added to 150 μL of 0.6 mM ABTS (in 0.1 M acetate buffer, pH 4.0) in a 96-well plate. The ABTS oxidation was monitored by measuring the absorbance at 420 nm over 60 s (6-s intervals) at 25°C. One unit (U) of activity was defined as the amount of enzyme forming 1 μmol of $ABTS^{\bullet+}$ per min. Activity measurements were carried in a 96-well plate using a double-beam UV–Vis spectrophotometer (SpectraMax Plus 384, Molecular Devices Corp., Sunnyvale, CA).

Magnetic CLEAs preparation

The initial conditions for preliminary immobilization assays were established based on the work of Kumar et al. (2014) for the synthesis of magnetically separable CLEAs. According to these, pH value of 4, co-aggregation with BSA (laccase:BSA ratio of 1:1 *w*:*w*), propanol as precipitant agent, and glutaraldehyde (5 mM) as cross linking agent were initially assayed. The initial laccase:MMSMB ratio was 1:2 (*w*:*w*). One hundred microliters of stocks of laccase and BSA (10 mg/L of protein in 0.1 M sodium acetate buffer, pH 4), freshly prepared at the beginning of each experiment, were added to 200 μL of a solution of MMSMB (10 mg/mL in 0.1 M sodium acetate buffer, pH 4) on 1.5 mL centrifuge tubes and shaken for 90 min at room temperature (24 ± 2 °C). Then, 1 mL of chilled propanol was added and stirred for 90 min. When precipitation occurred, glutaraldehyde was added into the suspension to reach a final concentration of 5 mM and then shaken again for 2, 6, or 22 h at room temperature (24 ± 2 °C). After cross-linking, the MCLEAs were magnetically separated from the mixture and washed thrice with sodium acetate buffer (pH 4) to remove excess of glutaraldehyde and any unbound enzyme.

Subsequently, the effect of several parameters onto the MCLEAs formation was investigated: (i) a preliminary sorption step where laccase, BSA, and MMSMB solutions were incubated for 2 h at room temperature (24 ± 2 °C) before precipitation with propanol; (ii) presence or absence of BSA, (iii) pH (0.1 M acetate buffer, pH 4, or 0.1 M phosphate buffer, pH 7), (iv) concentration of glutaraldehyde (1, 2.5, 5, and 10 mM), or (v) laccase:MMSMB ratio (1:1; 1:2; 1:3, and 1:4 *w*:*w*).

The immobilization yield (*IY*) of the MCLEAs was estimated based on the apparent laccase activity of the MCLEAs formed (A_f) relative to the initial applied laccase activity (A_i) (Eq. 1):

$$IY(\%) = \frac{A_f}{A_i} \cdot 100 \quad (1)$$

At the same time, washing losses (WL) were determined as the relative difference between the apparent laccase activity of MCLEAs formed (A_f) and the apparent laccase activity after the cross linking step, just before washing (A_{bw}) (Eq. 2):

$$WL(\%) = \frac{A_{bw} - A_f}{A_{bw}} \cdot 100 \quad (2)$$

Finally, the term enzyme load was defined as the apparent laccase activity in unit per milligram of immobilized biocatalyst according to Eq. 3, where the dry weight (*DW*) of the MCLEAs was obtained by lyophilizing the MCLEAs:

$$\text{Enzyme load } \left(\text{U}/\text{mg}\right) = \frac{A_f}{DW} \quad (3)$$

Characterization of MCLEAs

Magnetization curves and FTIR spectra

Magnetization curves of MMSMB and MCLEAs were recorded on a SQUID magnetometer (MPMS-5S XL Quantum Design magnetometer). Fourier transform infrared (FTIR) analysis was performed on a Nicolet FT-IR spectrophotometer.

Optimum pH and temperature

The pH of maximum laccase activity (free enzyme and MCLEAs) was investigated using 0.45 mM ABTS in a 0.1 M citrate–phosphate buffer (pH 2.6–8). The relative activity was calculated as the ratio between the activity at each pH and the maximum activity attained.

The effect of temperature (30–60 °C) on the activity of free laccase and MCLEAs was tested by determining the activity at the corresponding temperature under standard conditions, i.e., 0.45 mM ABTS in 0.1 M acetate buffer, pH 4. The relative activity was determined as the ratio between the activity at each temperature and the maximum activity attained.

Determination of kinetic parameters

Apparent kinetic parameters of free laccase and MCLEAs were determined by measuring the laccase activity under standard conditions (i.e., 0.1 M acetate buffer, pH 4.0, at 25 °C) using ABTS as substrate in the range 1.5–600 μM. Experimental data were fitted to determine the Michaelis-Menten parameters by minimizing the sum of squared residuals.

Storage stability and leaching test

The storage stability was investigated by incubating free laccase and MCLEAs in 0.1 M phosphate buffer at pH 7 and 4 °C for 1 month and measuring the residual activity periodically.

In order to determine any leaching of laccase from MCLEAs, these were washed to remove traces of free enzymes in the prepared MCLEAs suspension and incubated in 0.1 M phosphate buffer, pH 7, for 6 h at 250 rpm and room temperature (24 ± 2 °C). After the incubation period, the MCLEAs were separated from the buffer with an external magnetic field. Subsequently, the laccase activity in the buffer was measured to determine the amount of laccase leached from MCLEAs biocatalyst.

Long term stability in wastewater

Stability of free laccase and MCLEAs in wastewater was evaluated by incubating 1000 U/L of the biocatalyst in 5 mL of secondary effluent collected from the municipal wastewater treatment plant (WWTP) in Magog (Qc, Canada) (Table S1, ESM). The collected sample was filtered through 0.45 μm and adjusted to pH 7 with acetic acid 1 M. Samples were taken over a 1 month period, and the residual enzymatic activity was measured.

MCLEAs reusability

Reusability of the MCLEAs was assayed by means of consecutive cycles of ABTS (0.9 M) oxidation in 0.1 M phosphate buffer at pH 7. At the end of each 8-min oxidation cycle, the immobilized enzyme was retained by applying a magnetic field and the absorbance of the supernatant was measured (420 nm). Subsequently, the supernatant was discharged, the MCLEAs were washed once with the phosphate buffer, and the procedure repeated with a fresh aliquot of ABTS. The activity of the immobilized enzyme was considered to be 100 % in the initial cycle. These experiments were performed in duplicate. A control assay with deactivated MCLEAs was

conducted in parallel to verify that no ABTS oxidation occurred in the absence of active laccase.

Stability of MCLEAs against denaturation

The effect of the pH on the enzyme stability was studied by incubating laccase in 0.1 M citrate-phosphate buffer (pH 3, 5, 7, and 8.5) at room temperature (24 ± 2 °C) during 24 h. Samples were transferred to standard reaction mixtures in order to determine the residual laccase activity as described in "Enzyme activity" section. The residual activity was calculated referred to the value of the initial activity at each pH.

The thermal stability study was carried out by incubating samples of the biocatalysts in 0.1 M citrate-phosphate buffer (pH 7) at selected temperatures (20, 45, and 60 °C) and enzyme activity was periodically measured. The results were expressed in relative form, assigning 100 % activity to the initial measurement.

The stability of free laccase and MCLEAS laccase against chemical denaturation was tested by incubating 500 U/L in the presence of deactivating reagents $CaCl_2$, $CoCl_2$, $ZnCl_2$ (25 mM), and hydrophilic organic solvents: acetone, acetonitrile, and methanol (50 %) at pH 7 and room temperature (24 ± 2 °C). The incubations lasted for 1 h and subsequently activities were measured. The results for stability were given in percentage, allocating 100 % activity to the value in phosphate buffer at pH 7.

Pharmaceuticals degradation in secondary effluent

A sample of biologically treated wastewater was collected on May 2015 from the effluent of the municipal WWTP in Magog (QC, Canada) and filtered through 0.45 μm. The aim of the filtration was to remove particulate solids and other microorganisms, which could contribute to the elimination of the target pollutants by adsorption or undefined biotransformation, and hence, the evaluation of the sole enzymatic transformation by laccase could be possible (Lloret et al. 2013). The pH (originally 7.4) was adjusted to pH 7 with acetic acid 1 M and then spiked with each of the following PhACs to attain initial concentrations in the range of 10–50 μg/L: acetaminophen, ketoprofen, cyclophosphamide, mefenamic acid, caffeine, indomethacin, naproxen, fenofibrate, trimethoprim, and ibuprofen. Treatment of the PhACs by both free laccases or MCLEAs was performed in batch mode at 30 °C in 50-mL Erlenmeyer flasks with orbital shaking at 150 rpm for 6 h. The final volume of samples was 10 mL. Biocatalysts (free laccase or MCLEA) were added to obtain a final laccase activity of 1000 U/L. For controls, the biocatalyst solutions were substituted by deactivated MCLEAS or phosphate buffer solution. For each compound, the enzymatic removal by MCLEAs and free laccase was calculated according to the concentration detected in controls with inactivated MCLEAs and without enzyme, respectively. The total removal of each compound by MCLEAs (including the possible sorption onto the biocatalyst) was calculated using as reference the control without enzyme. Tests were performed in duplicate. After 6 h of reaction, MCLEAs were magnetically separated and the pharmaceuticals were extracted before analysis by ultra performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) as described in ESM (Touahar et al. 2014).

Results and discussion

MCLEAs preparation

The preparation of traditional CLEAs consists of two main steps, the aggregation by precipitation followed by the cross linking of the enzymes. It is expected that in the presence of a support containing amino groups not only intermolecular cross-linking of the sorbed enzyme occurs but also cross-linking to the amino groups of particles. Starting with the conditions established by Kumar et al. (2014), a series of parameters were tested to maximize the enzyme load while maintaining a high immobilization yield. Several cross linking times were initially evaluated aiming at establishing the time required to obtain the highest enzyme load, as it has been demonstrated that, whereas a too short cross linking time could result insufficient and lead to poor immobilization yield and operational stability, prolonged cross linking may be detrimental to enzyme activity (Talekar et al. 2013). As hardly differences were observed at 2, 6, and 24 h, (Table 1, run 1–3), the lowest cross linking time was selected to design a rapid process for MCLEAs preparation. Omitting the sorption step led to a slight increase in the immobilization yield (9.3 ± 0.4 %) (Table 1, run 4), probably due the lower time of laccase exposure to acidic pH. Although several studies reported that the use of protein feeders rich in lysine amine residues, such as BSA or egg white, in the preparation of CLEAs resulted in higher activity recovery and stability (Jiang et al. 2014), it was observed that in absence of BSA, the immobilization yield was two-fold higher than with the albumin (run 4 and 5). This is in accordance with a previous study of Cabana et al. (2007), who stated the same negative effect of BSA on laccase from *Coriolopsis polyzona*. The inadequate exposure of the catalytic centers to ABTS owing to the shielding effect of BSA and mass transfer limitations from the substrate solution to the laccase active site (Cabana et al. 2007) may be behind this fact. Besides, in the presence of albumin, laccase and BSA compete for binding to the support, resulting in a lower available surface for laccase attachment.

Regarding pH, a considerable increase in enzyme load and immobilization yield was attained when the MCLEAs were produced at pH 7 (Table 1, run 6). The higher laccase stability

Table 1 Effects of experimental parameters on MCLEAs formation

Run	Sorption step (h)	pH	BSA:Lac	Glut (mM)	Lac:MMSMB	Cross linking time (h)	WL (%)	IY (%)	Enzyme load (U/mg MCLEAs)
1	2	4	1:1	5	1:2	2	27.7 ± 9.1	3.5 ± 1.5	0.11 ± 0.03
2	2	4	1:1	5	1:2	6	29.2 ± 0.9	2.2 ± 0.01	0.06 ± 0.001
3	2	4	1:1	5	1:2	22	10.9 ± 0.4	3.0 ± 0.03	0.12 ± 0.001
4	–	4	1:1	5	1:2	2	45.5 ± 3.6	9.3 ± 0.4	0.25 ± 0.001
5	–	4	–	5	1:2	2	31.7 ± 10.7	19.2 ± 1.7	0.59 ± 0.05
6	–	7	–	5	1:2	2	31.2 ± 5.0	39.0 ± 0.9	1.53 ± 0.04
7	–	7	–	1	1:2	2	51.2 ± 7.4	27.6 ± 4.4	0.89 ± 0.16
8	–	7	–	2.5	1:2	2	39.4 ± 3.0	36.6 ± 2.0	1.28 ± 0.11
9	–	7	–	10	1:2	2	28.0 ± 4.9	35.1 ± 3.0	1.19 ± 0.28
10	–	7	–	5	1:1	2	67.5 ± 3.2	16.9 ± 2.0	1.12 ± 0.03
11	–	7	–	5	1:3	2	22.8 ± 6.0	42.1 ± 2.0	0.94 ± 0.003
12	–	7	–	5	1:4	2	17.0 ± 0.9	41.5 ± 0.9	0.75 ± 0.001

and the more favorable reactivity of glutaraldehyde with amino groups at pH 7 are likely to enhance the immobilization yield (Migneault et al. 2004). In this sense, it was reported that the reaction between glutaraldehyde and amine groups is reversible at pH above 3, except in the range of pH 7 to 9 where only a little reversibility is observed and a high immobilization yield in CLEAs is expected (Talekar et al. 2013). Apart from reversibility issues, it was found that in aqueous solution at acidic pH, glutaraldehyde is mostly present in a monomeric form, whereas alkaline pH promotes the existence of a polymeric form. This one is preferable to obtain CLEAs with more space among the aggregates, preventing the occurrence of diffusional restrictions (Talekar et al. 2013).

Glutaraldehyde is a common cross-linking agent for CLEA synthesis as it is inexpensive and available in commercial quantities (Sheldon 2011). Cross linker concentration is a crucial parameter in CLEAs production, and it is evident from the previous works that there is an optimum value for glutaraldehyde concentration (Talekar et al. 2013). In the current study, the concentration of glutaraldehyde was fixed at 5 mM. Lower glutaraldehyde concentrations increased washing losses, leading to lower enzyme loads (0.89 ± 0.16 and 1.28 ± 0.11 U/mg biocatalyst for 1 and 2.5 mM of glutaraldehyde, respectively) due to insufficient cross-linking. On the other hand, increasing the glutaraldehyde concentration up to 10 mM had a negative impact on laccase activity (Table 1, run 7–9) as, despite the lower washing loss, the final enzyme load was considerably lower than for 5 mM glutaraldehyde (1.19 ± 0.28 versus 1.53 ± 0.04 U/mg biocatalyst). In this case, rigidification of the enzyme molecule due to excessive cross linking may render into loss of enzyme flexibility, which is essential for its activity. Rigidification may also impede the substrate to reach the active site of laccase due to steric hindrance (Talekar et al. 2013).

Finally, different laccase:microparticles (Lac:MMSMB) ratios were evaluated (Table 1, run 10–12) using the best conditions previously established. Decreasing the amount of microparticles to a ratio of 1:1 led to higher washing losses, which resulted into lower immobilization yield (16.9 ± 2.0 %). On the other hand, the increase in the amount of microparticles resulted into much lower enzyme load.

According to these results, the conditions assayed in run 6 were applied to produce a large amount of MCLEAs for the subsequent experiments (characterization of the MCLEAs formed and elimination of pharmaceuticals from wastewater).

Characterization

Microparticles and MCLEAs Properties

The FTIR spectra of the MMSMB and final MCLEAs confirmed the presence of both amine and vinyl groups (Fig. S2, ESM). The vibration bands around 450 and 600 cm^{-1} determine the characteristics of (Fe–O) vibrations. The presence of amine groups has been verified by stretching vibrations of amine in the region of around 3300 cm^{-1}. The silica-coated magnetite sample shows a band around 1050 cm^{-1} corresponding to the stretching vibrations of Si–O–Si, and it is associated with the motion of oxygen in Si–O–Si antisymmetric stretch due to the asymmetric stretching bonds of Si–O–Si in SiO_2. The band around 450 cm^{-1} is for Si–O–Si or Si–O–Si bending modes. The absorption bands around 1600 and 1400 cm^{-1} were ascribed to the stretching vibration of C=C and bending vibration of C–H in vinyl groups.

The saturation magnetization (Ms) was 8.0 and 8.6 emu/g for the MCLEAs and MMSMB, respectively, which resulted enough to show a rapid response (below 30 s) to an external magnetic field of 200 mT (Fig. S3, ESM). The slight decline in the magnetization moment of MMSMB after laccase attachment was the consequence of the decrease in the amount of the magnetic moment per unit weight of MCLEAs. The magnetization curves showed slight hysteresis, characterized by coercive fields (H_{ci}) around 285 Oer and remnant

magnetization (M_r) of 1.7 and 1.9 emu/g for the MCLEAs and MMSMB, respectively. This trend reveals a superparamagnetic behavior.

Optimum pH and Temperature

A comparative study between free laccase and MCLEAs was conducted aiming to study the effect of pH and temperature on the activity (Fig. S4, ESM). Free laccase showed the maximum activity at pH 3 whereas the optimal pH for MCLEAs was found around 2.6. In the range 3–6, free laccase showed a slightly higher relative activity (no more than 8 % of difference), whereas at pH above 6, the relative activity of both biocatalyst forms was identical. Regarding optimal temperature, both free and MCLEAs laccases displayed maximal catalytic activities in the range of 40–50 °C.

pH and Thermal Stability

One of the most important factors affecting the enzymatic stability is pH. For this reason, its effect on laccase stability was investigated by incubating the MCLEAs and free enzyme in citrate-phosphate buffer at pH 3, 5, 7, and 8.5 for 24 h (Fig. 1).

A drastic increase in the stability of laccase at pH 3 was observed after MCLEAs formation; at the end of the incubation period, MCLEAs exhibited a residual activity above 43 %, whereas free laccase was completely inactivated, which confirmed the usefulness of insolubilization as a way to protect the enzyme. This is in accordance with most of published studies, which report increase in acid stability after laccase immobilization (Songulashvili et al. 2012; Zhang et al. 2015). At higher pH values, no significant differences were found between free laccase and MCLEAs.

Regarding thermal stability, no improvement was observed for MCLEAs in comparison with free laccase and both were completely deactivated after 3 h of incubation at 60 °C and neutral pH (Fig. S5, ESM). The lower stability for the immobilized laccase in comparison with the free one at 45 °C is difficult to explain, although a similar behavior was previously reported by Masuda et al. (2013) for the enzyme formaldehyde dehydrogenase immobilized onto mesoporous silica (MPS). These authors hypothesized that the enzyme immobilized on the MPSs suffered a faster denaturalization owing to thermal energy transmitted from silica wall.

Resistance against Chemical Inhibitors

Figure 2 shows residual activities of free laccase and MCLEAs to high concentrations (25 mM) of halide salts, $ZnCl_2$, $CoCl_2$, $CaCl_2$, and organic solvents (50 % *v*:*v*) at neutral pH. It is evident that MCLEAs displayed higher resistance against organic solvents than free laccase. For instance, after 1 h of incubation in the presence of acetonitrile (50 % *v*:*v*), free laccase was nearly deactivated whereas MCLEAs exhibited around 12 % of residual activity. In acetone, the residual activity of MCLEAs was 1.7-fold higher than for free laccase. The higher stability of enzyme in the presence of organic cosolvents after CLEAs formation has been observed in several works, and it was attributed to the rigidity of the biocatalyst formed (Ba et al. 2014). In the same way, the MCLEAs showed higher residual activity than the free enzyme in the presence of the chaotropic salts $ZnCl_2$, $CoCl_2$, and $CaCl_2$. These results confirmed that MCLEAs formation increases laccase resistance against chemical inhibitors.

Storage Stability and Enzymes Leaching

Storage stability at 4 °C and neutral pH did not differ considerably for free laccase and MCLEAs (mean values are not significantly different at $p<0.05$; see Fig. S6, ESM), which maintained residual activities above 74 % after 30 days. In relation to the leaching test, negligible leaching of laccase from MCLEAs was detected after incubation for 6 h at 250 rpm.

Reusability of laccase

Apart from stability, the reusability of the biocatalyst is an important feature to be considered in view of its large scale application. The reusability of MCLEAs was investigated by applying consecutive cycles of ABTS oxidation. As it can be seen from Fig. 3, the activity of MCLEAs was above 100 % during the first 5 cycles, indicating that no significant deactivation or leaking occurred during oxidation or washing procedure. The increase in the activity up to third cycle could be explained considering the different forms that laccase can present: native intermediate, resting oxidized, and partly reduced (Shleev et al. 2006). For instance, Tavares et al. (2015) also observed the activation of laccase immobilized in multiwalled carbon nanotubes along the first four cycles of ABTS oxidation, and hypothesized that laccase molecule may be passing from the resting state to the catalytic state.

After the 6th cycle, a gradual loss of activity started, probably because of enzyme deactivation during the consecutive batches, and after 10 cycles of ABTS oxidation and separation, laccase MCLEAs retained 70 % of the initial activity. This is in the range reported by other studies evaluating the immobilization of laccase onto silica carriers. For instance, Patel et al. (2014) immobilized laccases onto SiO_2 nanocarriers and observed a residual activity of 93.7 and 82.5 % after 5 and 10 cycles of ABTS oxidation, respectively, whereas Rekuć et al. (2009) reported a residual activity of 65 % after 10 cycles of ABTS oxidation for different laccases immobilized onto silica carrier.

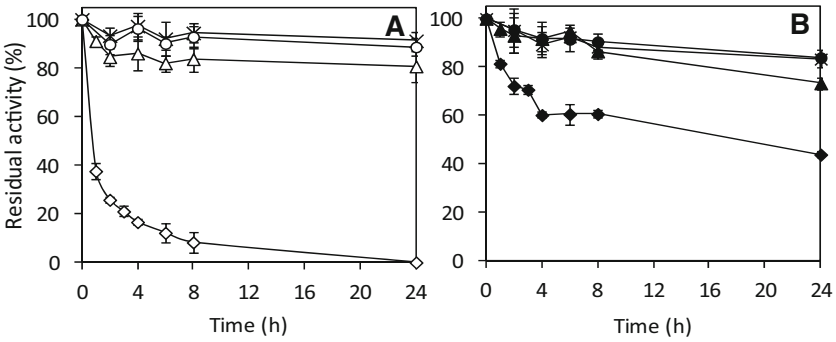

Fig. 1 Effect of pH on free laccase (**a**) and MCLEAs (**b**). Symbols pH 3 (*diamond*), pH 5 (*triangle*), pH 7 (*multiplication symbol*), and pH 8.5 (*circle*) (mean of triplicates ± standard deviation)

Kinetic Parameters

Table 2 presents the apparent Michaelis–Menten kinetic parameters for free and MCLEAs laccases using ABTS as substrate. In comparison to free laccase, a lower affinity of MCLEAs for ABTS was found as indicated by increase in the K_M value (0.038 ± 0.003 mM and 0.064 ± 0.006 mM, respectively). These results are in agreement with several studies reporting laccase immobilization (Fernández-Fernández et al. 2013) and could be attributed to several factors as diffusional limitations, conformational changes on the protein molecule after immobilization or steric hindrance (Talekar et al. 2013). Nevertheless, the affinity for the substrate was still higher than that of other enzymes immobilized onto porous supports, which have displayed higher K_M values (Fernández-Fernández et al. 2013, Songulashvili et al. 2012). In contrast, Cabana et al. (2007) did not find appreciable changes in K_M value after the insolubilization of laccase from *C. polyzona* in CLEAs, and Kumar et al. (2014) reported higher affinity of functionalized magnetic nanoparticles bonded to laccase CLEAs for ABTS, in comparison to the free laccase from *T. versicolor*. The turnover number (K_{cat}) for the free laccase and MCLEAs showed that the maximal rate for free laccase is slightly higher than for MCLEAs. As result, the catalytic efficiencies K_{cat}/K_M for MCLEAs laccase was 1.9-fold lower than for the free laccase.

Stability in Wastewater

The stability of the biocatalyst in a real environmental matrix must be evaluated in order to estimate the operational lifetime for bioremediation process. For this reason, the stability of the MCLEAs and free laccase in a municipal wastewater secondary effluent was tested by exposing the biocatalysts to a sample of biologically treated wastewater (characteristics are shown in Table S1, ESM). From Fig. 4, it is evident that MCLEAs are more resistant against real wastewater matrices than the free laccase.

Stabilization of laccases in wastewater matrix after immobilization or insolubilization has been reported in the previous works. For instance, laccase from *T. versicolor* immobilized onto fumed silica nanoparticles exhibited a residual activity above 40 % after 14 days of incubation in municipal wastewater secondary effluent, which resulted 4-fold higher than the activity retained by the free laccase (Arca-Ramos et al. 2015a).

Performance of MCLEAs for the Removal of Pharmaceuticals from Biologically Treated Wastewaters

In order to test the catalytic potential of the biocatalyst in real matrices, free laccase and MCLEAs were applied for the removal of a mixture of PhACs from spiked biologically treated wastewater effluent. Although laccase mainly targets phenolic, aromatic, and aliphatic amines, it was shown that the spectrum of laccase substrates can be extended in mixtures when reactive radicals (e.g., phenoxyl radicals) are generated (Jeon et al. 2012). For this reason, the solution of PhACs was designed to include phenolic (acetaminophen) and aniline (mefenamic acid) compounds, which are directly oxidizable by laccase, as well as non-laccase substrates (ketoprofen, cyclophosphamide, caffeine, indomethacin, naproxen, fenofibrate, trimethoprim, and ibuprofen). The PhACs concentrations in controls and reacting mixtures after the treatment are shown in Table S5 (ESM). The enzymatic activity at the end of the experiment of 6 h duration was 771 ± 29 U/L and 898 ± 63 U/L for free laccase and MCLEAs, respectively.

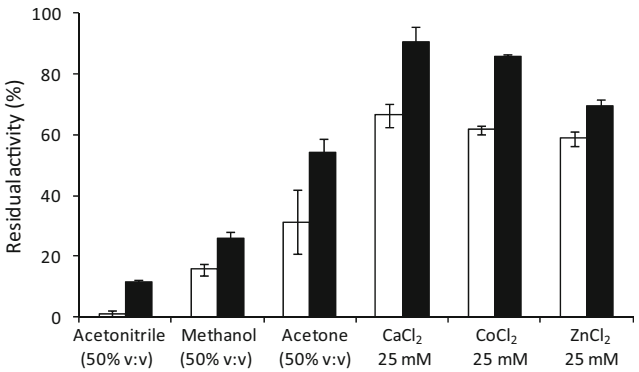

Fig. 2 Residual activity (A/A_0) of free laccase (*white bars*) and MCLEAs (*black bars*) after 1-h incubation with denaturants (mean of triplicates ± standard deviation)

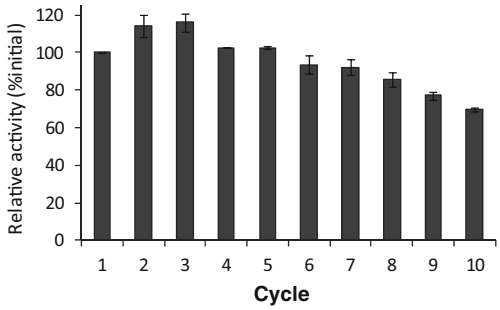

Fig. 3 Residual activity (%) of MCLEAs after batch oxidations of ABTS in phosphate buffer pH 7 (mean of replicates ± standard deviation)

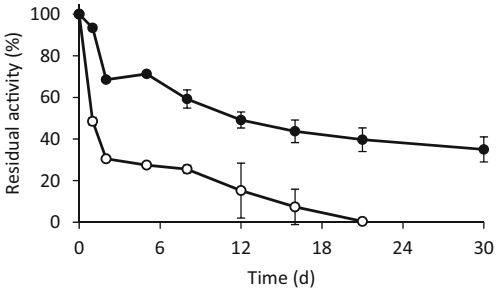

Fig. 4 Residual activity of free laccase (*white circle*) and MCLEAs (*black circle*) during 30 days of incubation in a wastewater effluent collected from the WWTP of Magog (Qc, Canada) (mean of triplicates ± standard deviation)

The removal of the selected PhACs by free and immobilized laccases after 6-h treatment is depicted in Fig. 5. For MCLEAs, the total removal percentage may result from two mechanisms, sorption on MCLEAs (grey bar, resulting from the experiment with inactivated MCLEAs) and enzymatic transformation (black bar, which results from the difference between the total PhAC removal by MCLEAs and the removal by inactivated MCLEAs). Nevertheless, the contribution of sorption was low or negligible for the majority of the PhACs. Significant removal of 4 out 10 of the investigated PhACs was observed. Free laccase was able to remove 80 % of acetaminophen after 6 h of contact time; whereas for the MCLEAs, the removal percentage was considerably lower, reaching 34 %. Acetaminophen is a phenolic compound susceptible to be degraded by laccase, as it has been demonstrated in the previous researches (Kumar and Cabana 2016; Lu et al. 2009; Touahar et al. 2014). Lu et al. (2009) found that the laccase-catalyzed transformation of acetaminophen followed second-order kinetics on the concentrations of both the substrate and the enzyme, and they reported a removal percentage of 73 % for an initial concentration of acetaminophen of 7.56 mg/L by 1000 U/L of laccase after 1-h treatment at pH 7. The extremely high concentration of acetaminophen used in such study likely explains its much faster transformation in comparison to the observed in our work. In addition, the real wastewater matrix may also be negatively affecting the transformation rate of the PhACs. In this sense, Kumar and Cabana (2016) evaluated the removal of 100 μg/L of acetaminophen from a mixture of PhACs in buffer solution by 1000 U/L of immobilized laccase. The much higher removal attained in such previous work (85 %) in comparison to the current one may be indicative of the negative impact of real wastewater matrix. In this sense, previous studies reported loss of removal efficiencies of bisphenol A and estrogens when comparing laccase-based treatments in wastewater and buffer, due to the presence of compounds which could compete with the target compounds for the enzyme or certain inorganic ions with inhibitory effect on laccase activity (Arca-Ramos et al. 2015b; Lloret et al. 2013).

The non-phenolic PhACs mefenamic acid, fenofibrate, and indomethacin were partially removed by laccase. This fact may be explained based on the chemical structures (Fig. S7, ESM). In general, electron donating groups (EDG) such as amine ($-NH_2$), hydroxyl (−OH), alkoxy (−OR), alkyl (−R), and acyl (−COR) are prone to oxidative attack whereas electron withdrawing groups (EWG) such as amide ($-CONR_2$), carboxylic (−COOH), halogen (−X), and nitro ($-NO_2$) group generates an electron deficiency and diminish the reactivity of the molecules (Yang et al. 2013). Mefenamic acid is an aniline compound and is known to be a substrate of laccase. Margot et al. (2013) studied the removal of mefenamic acid at high concentrations from buffered solutions and demonstrated the influence of initial activity and pH on the removal rate. For instance, for pH 7.2 and 888 U/L of laccase, the percentage

Table 2 Apparent kinetic parameters for free laccase and MCLEAs

	Free laccase	MCLEAs
K_M (μM)	38.5 ± 3.1	64.3 ± 6.7
K_{cat} (s^{-1})	153.7 ± 1.3	134.6 ± 6.7
K_{cat}/K_M (s^{-1} μM^{-1})	4.00 ± 0.29	2.10 ± 0.11

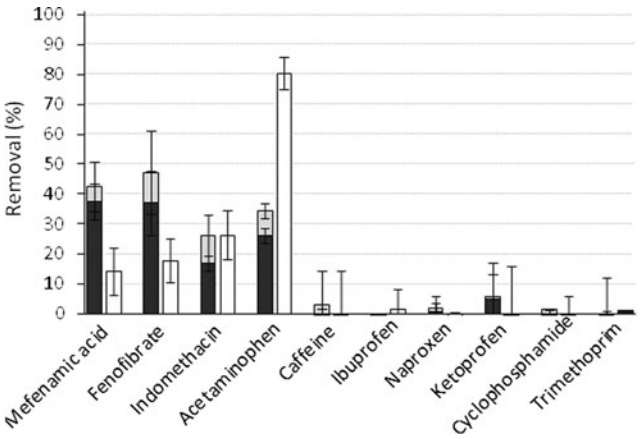

Fig. 5 Removal of PhACs by adsorption (*grey bar*) and enzymatic transformation (*black bar*) by MCLEAs; and free laccase (*white bar*) in filtered secondary effluent at pH 7 from Magog WWTP for a contact time of 6 h at 30 °C (mean of replicates ± standard deviation)

of removal was 39 %; whereas for pH 5.7 and 562 U/L, 98 % of mefenamic acid was transformed after 5.5 h. On the other hand, fenofibrate and indomethacin contain functional groups with electron donor properties in the laccase reaction as acyl in fenofibrate or alkoxy and alkyl in indomethacin (Tran et al. 2010; Yang et al. 2013). Radicals resulting from the laccase treatment of the phenolic PhAC such as acetaminophen could interact with electron donor groups present on non-phenolic PhACs, enhancing their transformation (Touahar et al. 2014). Removal of 20 % of fenofibrate (1 mg/L) after 14-h reaction at pH 5 and 750 U/L of free laccase was reported by the only previous work evaluating the enzymatic transformation of fenofibrate by free laccase in a mixture of pharmaceuticals (Touahar et al. 2014). Tran et al. (2010) reported the complete removal of indomethacin from a mixture of PhACs under much more favorable conditions: 6000 U/L of free laccase in acetate buffer at pH 4.5. To our knowledge, no studies were found evaluating the laccase catalyzed-transformation of fenofibrate or indomethacin as a single component.

Interestingly, the removal of mefenamic acid and fenofibrate was much higher for the MCLEAs (above 40 % for both compounds) than for the free laccase. A possible reason could be the creation of a concentration gradient of substrate onto the surface of the MCLEAs. In this sense, the high hydrophobicity of mefenamic acid (K_{OW} of 5.12) and fenofibrate (K_{OW} of 5.19) may be promoting their absorption onto MCLEAs due to the presence of free vinyl groups, making these PhACs more available for enzymatic attack. Nevertheless, this is not evident from Fig. 5, since sorption contribution for mefenamic acid and fenofibrate was similar to that for acetaminophen (which was removed faster by free laccase). Other possible explanation is related to conformational changes and/or chemical modifications of enzymes after immobilization. In this way, it is common to observe a certain decline in enzyme activity towards its natural substrate, as it was observed for ABTS ("Kinetic parameters" section). Nevertheless, in many cases, the target substrate is quite far from the physiological one and it is not unlikely to obtain biocatalysts with superior-specific activity towards a particular substrate (Rodrigues et al. 2013). Such random hyperactivation produced by a particular immobilization method towards a particular substrate may be based on the casual generation of a more active enzyme form, and it would be more likely to occur with enzymes having a flexible active center. For instance, it was reported that porous CLEAs of papain displayed higher catalytic efficiency than the free enzyme towards BSA, but for the substrates *N*-benzoyl-L-arginine ethyl ester (BAEE) and ovalbumin, free papain exhibited higher catalytic efficiency than the insolubilized enzyme (Wang et al. 2011). Therefore, the increased activity of MCLEAs towards mefenamic acid and fenofibrate may be justified based on this random hyperactivation. In the case of indomethacin, the percentage of transformation by free laccase and MCLEAs was almost identical, around 20 % for both.

The remaining PhACs showed low or negligible enzymatic degradation (≤5 %) after the 6-h treatment under the evaluated conditions. Whereas slight removal of naproxen by laccase was reported (Marco-Urrea et al. 2010a; Tran et al. 2010); ibuprofen and ketoprofen showed different behavior depending on their treatment as single pollutants, where no removal was appreciated (Marco-Urrea et al. 2010b; Marco-Urrea et al. 2009) or in a mixture, where partial removal was observed (Kumar and Cabana 2016; Tran et al. 2010). In the current study, the low concentration of PhACs (in the range of μg/L) may be diminishing the chance to have cross reaction between radicals and pollutants, and the synergistic effect of other compounds mediating transformation of non-phenolic molecules could be limited (Margot 2015). Furthermore, the wastewater matrix could also affect someway the laccase-catalyzed transformation of PhACs. For instance, the content of organic matter may hamper the transformation of PhACs.

Finally, caffeine and trimethoprim seem recalcitrant during the laccase treatment of a mixture of pharmaceuticals (Touahar et al. 2014). The in vitro degradation of cyclophosphamide by laccase had not been studied before but, in a recent work, this antineoplastic drug (10 mg/L) showed neither degradation nor sorption by *T. versicolor* pellets after 8-day treatment (Ferrando-Climent et al. 2015).

Conclusions

A rapid method to produce robust magnetically-separable CLEAs of laccase was developed. These showed higher stability against inhibitors, acidic pH, and wastewater than free laccases and can be reused after their recovery by a magnet. When applied for the elimination of a cocktail of PhACs from spiked real secondary effluent, the MCLEAs showed the ability to transform the phenolic compound acetaminophen and certain non-phenolic PhACs as mefenamic acid, fenofibrate, and indomethacin, with similar or even higher efficiency than free laccase. Therefore, MCLEAs could constitute interesting biocatalysts to remove target micropollutants from specific effluents after optimizing the removal conditions.

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Compliance with Ethical Standards The authors declare that they have no conflict of interests. The current research has not involved human participants or animals.

References

Arca-Ramos A, Ammann EM, Gasser CA, Nastold P, Eibes G, Feijoo G, Lema JM, Moreira MT, Corvini PFX (2015a) Assessing the use of nanoimmobilized laccases to remove micropollutants from wastewater. Environ Sci Pollut Res. doi:10.1007/s11356-015-5564-6 **(in press)**

Arca-Ramos A, Eibes G, Feijoo G, Lema JM, Moreira MT (2015b) Potentiality of a ceramic membrane reactor for the laccase-catalyzed removal of bisphenol A from secondary effluents. Appl Microbiol Biotechnol 99:9299–9308

Ba S, Haroune L, Cruz-Morató C, Jacquet C, Touahar IE, Bellenger J-P, Legault CY, Jones JP, Cabana H (2014) Synthesis and characterization of combined cross-linked laccase and tyrosinase aggregates transforming acetaminophen as a model phenolic compound in wastewaters. Sci Total Environ 487:748–755

Brady D, Jordaan J (2009) Advances in enzyme immobilisation. Biotechnol Lett 31:1639–1650

Cabana H, Jones JP, Agathos SN (2007) Preparation and characterization of cross-linked laccase aggregates and their application to the elimination of endocrine disrupting chemicals. J Biotechnol 132:23–31

Fernández-Fernández M, Sanromán MÁ, Moldes D (2013) Recent developments and applications of immobilized laccase. Biotechnol Adv 31:1808–1825

Ferrando-Climent L, Cruz-Morató C, Marco-Urrea E, Vicent T, Sarrà M, Rodriguez-Mozaz S, Barceló D (2015) Non conventional biological treatment based on *Trametes versicolor* for the elimination of recalcitrant anticancer drugs in hospital wastewater. Chemosphere 136:9–19

Gasser C, Ammann E, Shahgaldian P, Corvini PFX (2014) Laccases to take on the challenge of emerging organic contaminants in wastewater. Appl Microbiol Biotechnol 98:9931–9952

Jeon J-R, Baldrian P, Murugesan K, Chang Y-S (2012) Laccase-catalysed oxidations of naturally occurring phenols: from in vivo biosynthetic pathways to green synthetic applications. Microb Biotechnol 5:318–332

Jiang Y, Wang Q, He Y, Zhou L, Gao J (2014) Co-aggregation of laccase and nature egg white: a simple method to prepare stable and recyclable biocatalyst. Appl Biochem Biotechnol 172:2496–2506

Kumar VV, Sivanesan S, Cabana H (2014) Magnetic cross-linked laccase aggregates—bioremediation tool for decolorization of distinct classes of recalcitrant dyes. Sci Total Environ 487:830–839

Kumar VV, Cabana H (2016) Towards high potential magnetic biocatalysts for on-demand elimination of pharmaceuticals. Bioresour Technol 200:81–89

López C, Cruz-Izquierdo Á, Picó EA, García-Bárcena T, Villarroel N, Llama MJ, Serra JL (2014) Magnetic biocatalysts and their uses to obtain biodiesel and biosurfactants. Front Chem 2:72

Lu J, Huang Q, Mao L (2009) Removal of acetaminophen using enzyme-mediated oxidative coupling processes: I. Reaction rates and pathways. Environ Sci Technol 43:7062–7067

Lloret L, Eibes G, Moreira MT, Feijoo G, Lema JM (2013) Removal of estrogenic compounds from filtered secondary wastewater effluent in a continuous Enzymatic Membrane Reactor. Identification of biotransformation products. Environ Sci Technol 47:4536–4543

Majeau J-A, Brar SK, Tyagi RD (2010) Laccases for removal of recalcitrant and emerging pollutants. Bioresour Technol 101:2331–2350

Marco-Urrea E, Pérez-Trujillo M, Vicent T, Caminal G (2009) Ability of white-rot fungi to remove selected pharmaceuticals and identification of degradation products of ibuprofen by *Trametes versicolor*. Chemosphere 74:765–772

Marco-Urrea E, Perez-Trujillo M, Blanquez P, Vicent T, Caminal G (2010a) Biodegradation of the analgesic naproxen by Trametes versicolor and identification of intermediates using HPLC-DAD-MS and NMR. Bioresour Technol 101:2159–2166

Marco-Urrea E, Pérez-Trujillo M, Cruz-Morató C, Caminal G, Vicent T (2010b) White-rot fungus-mediated degradation of the analgesic ketoprofen and identification of intermediates by HPLC–DAD–MS and NMR. Chemosphere 78:474–481

Margot J, Maillard J, Rossi L, Barry D, Holliger C (2013) Influence of treatment conditions on the oxidation of micropollutants by *Trametes versicolor* laccase. New Biotechnol 30:803–813

Margot J (2015) Micropollutant removal from municipal wastewater—from conventional treatments to advanced biological processes. In: PhD Thesis. No 6505 Ecole Polytechnique Fédérale de Lausanne, Switzerland

Masuda Y, S-i K, Murai K, Hayashi A, Kato K (2013) Enhancement of activity and stability of the formaldehyde dehydrogenase by immobilizing onto phenyl-functionalized mesoporous silica. Colloids Surf B: Biointerfaces 101:26–33

Migneault I, Dartiguenave C, Bertrand MJ, Waldron KC (2004) Glutaraldehyde: behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking. BioTechniques 37:790–802

Patel S, Kalia V, Choi J, Haw J, Kim I, Lee J (2014) Immobilization of laccase on SiO_2 nanocarriers improves its stability and reusability. J Microbiol Biotechnol 24:639–647

Rekuć A, Bryjak J, Szymańska K, Jarzębski AB (2009) Laccase immobilization on mesostructured cellular foams affords preparations with ultra high activity. Process Biochem 44:191–198

Rodrigues RC, Ortiz C, Berenguer-Murcia A, Torres R, Fernandez-Lafuente R (2013) Modifying enzyme activity and selectivity by immobilization. Chem Soc Rev 42:6290–6307

Sheldon RA (2011) Characteristic features and biotechnological applications of cross-linked enzyme aggregates (CLEAs). Appl Microbiol Biotechnol 92:467–477

Shleev S, Reimann CT, Serezhenkov V, Burbaev D, Yaropolov AI, Gorton L, Ruzgas T (2006) Autoreduction and aggregation of fungal laccase in solution phase: possible correlation with a resting form of laccase. Biochimie 88:1275–1285

Songulashvili G, Jimenéz-Tobón GA, Jaspers C, Penninckx MJ (2012) Immobilized laccase of *Cerrena unicolor* for elimination of endocrine disruptor micropollutants. Fungal Biol 116:883–889

Talekar S, Ghodake V, Ghotage T, Rathod P, Deshmukh P, Nadar S, Mulla M, Ladole M (2012) Novel magnetic cross-linked enzyme aggregates (magnetic CLEAs) of alpha amylase. Bioresour Technol 123:542–547

Talekar S, Joshi A, Joshi G, Kamat P, Haripurkar R, Kambale S (2013) Parameters in preparation and characterization of cross linked enzyme aggregates (CLEAs). RSC Adv 3:12485–12511

Tavares APM, Silva CG, Dražić G, Silva AMT, Loureiro JM, Faria JL (2015) Laccase immobilization over multi-walled carbon nanotubes: kinetic, thermodynamic and stability studies. J Colloid Interface Sci 454:52–60

Touahar IE, Haroune L, Ba S, Bellenger J-P, Cabana H (2014) Characterization of combined cross-linked enzyme aggregates from laccase, versatile peroxidase and glucose oxidase, and their utilization for the elimination of pharmaceuticals. Sci Total Environ 481:90–99

Tran NH, Urase T, Kusakabe O (2010) Biodegradation characteristics of pharmaceutical substances by whole fungal culture *Trametes versicolor* and its laccase. J Water Environ Technol 8:125–140

Wang M, Jia C, Qi W, Yu Q, Peng X, Su R, He Z (2011) Porous-CLEAs of papain: application to enzymatic hydrolysis of macromolecules. Bioresour Technol 102:3541–3545

Wilson L, Illanes A, Abián O, Fernández-Lafuente R, Guisán JM (2002) Encapsulation of very soft crosslinked enzyme aggregates (CLEAs) in very rigid LentiKats®. Landbauforshung Volkenröde FAL Agric Res 241:121–125

Yang S, Hai FI, Nghiem LD, Price WE, Roddick F, Moreira MT, Magram SF (2013) Understanding the factors controlling the removal of trace organic contaminants by white-rot fungi and their lignin modifying enzymes: a critical review. Bioresour Technol 141:97–108

Zhang X, Hua M, Lv L, Pan B (2015) Ionic polymer-coated laccase with high activity and enhanced stability: application in the decolourisation of water containing AO7. Sci Rep 5:8253