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## Design and optimization of an enzymatic membrane reactor for tetracycline degradation

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### ABSTRACT

The tetracycline, antibiotic considered as a recalcitrant pollutant, was successfully depleted from model aqueous solutions by immobilized laccase from *Trametes versicolor* in an enzymatic membrane reactor. The results obtained show that tetracycline is depleted from water solutions at room temperature and without adding any extra chemicals. The degradation of tetracycline in aqueous solutions at 20 mg L<sup>-1</sup> during 24 h, with equivalent amounts of free or immobilized biocatalyst, allowed reaching a tetracycline degradation yield of 56% with an enzymatic membrane whereas it was only of 30% with free laccase. This result highlights the good reactivity and stability of the immobilized enzyme for the degradation of tetracycline. Moreover, the enzymatic membrane reactor was able to reach a constant degradation rate of 0.34 mg of tetracycline per hour during 10 days.

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### 1. Introduction

With the background of an aging population and increasing urbanization, pharmaceutical products (PPs) and endocrine disrupting chemicals (EDCs) have been continuously released in the environment for a long time without being considered as priority pollutants to target.

As conventional wastewater treatment technologies are not efficient enough to completely remove pharmaceuticals from water, such products are currently found in water effluents from sewage facilities, as well as in surface water, in groundwater, adsorbed on sediments and even in drinking water [1–3]. Furthermore, ecotoxicity studies have demonstrated that PPs could affect the growth, reproduction and behavior of birds, fishes, invertebrates, plants and bacteria [4–6]. In particular, the presence of low concentrations of antibiotics in wastewaters could cause the development of

antibiotic resistance by bacteria and then be an important source of public health problems in the future [7].

Indeed, lately important research efforts have been done in order to find a system to eliminate the PPs before rejecting the effluents to the environment. Among the different processes tested (physical adsorption, chemical or biological reactions) for the depletion of certain groups of pollutants [8–12], the use of biocatalysts such as laccases, glycosylases, proteases and lipases have been found to be particularly efficient [13]. In particular laccases are able to oxidize a wide range of pollutants at room temperature within a large range of pH using as oxidant the oxygen dissolved in water. Consequently, some reports have noticed the potential of laccase-catalyzed reactions for the removal of a large spectrum of pollutants [14–19].

To overcome the drawbacks related to enzymes cost, biocatalysts can be immobilized on a large variety of supports [16,18] as well as in membranes and used in enzymatic membrane reactors (EMRs) [20–22]. In EMRs the substrate solution flows through the membrane to the biocatalyst as a result of transmembrane pressure. Then the reaction takes place simultaneously with the mass transfer process through the membrane and the product is recovered in the permeate. Thus a precise control of the reaction with

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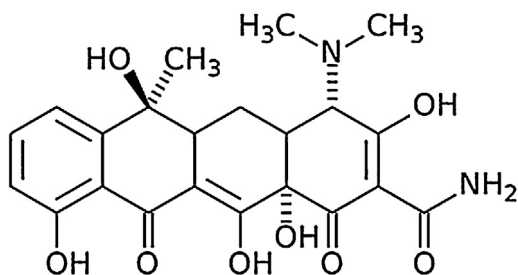


Fig. 1. Structure of tetracycline molecule.

minimized substrate and catalyst losses, faster reactions, higher yields and cleaner products can be expected [23].

This work describes the study of the potential enzymatic degradation of tetracycline (TC), a recalcitrant antibiotic present in some wastewaters. The laccase from *Trametes versicolor* was chosen as the biocatalyst because this biocatalyst has already demonstrated its activity for the degradation of this antibiotic [24]. For this purpose the enzyme was covalently linked onto a ceramic membrane previously coated with a polymer layer (i.e. gelatin). A comparison between free and immobilized enzymes was also carried out to evaluate the performances of the EMR and the stability of grafted enzymes. In addition different operating parameters like pH and temperature were studied in order to determine the optimal operating conditions for the tetracycline depletion. To our knowledge it is the first work reporting tetracycline degradation with immobilized laccase on ceramic membranes.

## 2. Experimental

### 2.1. Products

Commercial powder of laccase from *T. versicolor* (activity  $\geq 10 \text{ U mg}^{-1}$ , ref. 51639), tetracycline ( $\geq 98.0\%$ , ref. 87128) (Fig. 1), gelatin, glutaraldehyde and ABTS ( $\geq 98.0\%$ , ref. 11557) were purchased from Sigma-Aldrich. Mono-channel ceramic membranes ( $\alpha$ -alumina) were supplied by Pall-Exekia (pore diameter of  $0.2 \mu\text{m}$ , 15 cm long, external/internal diameter of 1 and 0.7 cm, effective area of  $28.6 \times 10^{-4} \text{ m}^2$ ). Tetracycline solutions were prepared at  $20 \text{ mg L}^{-1}$  in osmosed water (pH=6) or in different 50 mM citrate/phosphate buffers (pH 3 to 7). The TC is usually encountered in wastewaters (hospital and wastewaters treatment plants) in a concentration range from 1 to  $900 \mu\text{g L}^{-1}$  [25]. The model solutions tested in this work were prepared with buffers or osmosed water and pure TC at  $20 \text{ mg L}^{-1}$ . This concentration is high compared to the actual concentrations noted above. It was arbitrarily chosen in order to have a good precision on depletion rates while allowing the identification of the degradation products.

### 2.2. Immobilization protocol

Active membranes were prepared according to a 3-step procedure developed by Belleville et al. [26] and adapted for laccase immobilization by Chea et al. [14]. First, the wet ceramic supports ( $\alpha$ -alumina tubular membranes from Pall-Exekia, France (15 cm of length, 1 cm of external diameter and 0.7 cm of internal diameter,  $0.2 \mu\text{m}$  of mean pore size) were coated with a gelatin layer by filtering a gelatin solution at  $1 \text{ g L}^{-1}$ . Then the bio-polymer layer was activated by a glutaraldehyde solution (4% (w/v) for 1 h and finally, the laccase ( $10 \text{ g L}^{-1}$  solution) was let to react with free aldehyde groups of glutaraldehyde during 2 h. All the solutions were prepared in a 50 mM phosphate buffer pH 7 and after each step, the excess solution was removed by rinsing 4 times the membrane with the same phosphate buffer. The active membranes were then stored in a desiccator with  $\text{P}_2\text{O}_5$  until being used. Blank membranes were prepared with the same method but without enzymes.

### 2.3. Pilot unit

The pilot unit used for EMR runs is shown in Fig. 2. It was built with stainless steel and PTFE in order to avoid adsorption problems. The EMR can be operated with or without recirculating the retentate (Fig. 2). In this exploratory work we carried out experiments in batch configuration (the permeate valve was kept close except during sample withdrawing and permeate flux measurements). The temperature, the trans-membrane pressure and the flow rate can be measured and controlled with sensors and a cryothermostat. Since the feeding tank was open to atmospheric pressure, there was no need to add extra oxygen in the water. It was indeed proved that adding oxygen by sending pressurized air into water had no significant impact on dissolved oxygen concentration level.

### 2.4. Enzymatic degradation

In order to determine the optimal operating conditions and to compare the activity of free and immobilized enzymes, several experiments were carried out with 100 mL of tetracycline solutions ( $20 \text{ mg L}^{-1}$ ) prepared either in 50 mM citrate-phosphate buffer (pH 3 to 7) or in osmosed water (pH 6) at  $25^\circ\text{C}$  for 24 h in a stirred tank bioreactor. In the case of free enzyme experiments an amount of commercial powder was added to the tetracycline solution, whereas for immobilized enzymes we used small pieces of a crushed enzymatic membrane (EM). The amount of crushed EM used for the reaction corresponds to the same concentration of enzymes used for free enzymes experiments. Before degradation experiments the small portions of the crushed membrane were washed with osmosed water in order to eliminate potential free laccases that might not have been rinsed properly during the grafting step. Tetracycline auto-degradation and adsorption were estimated

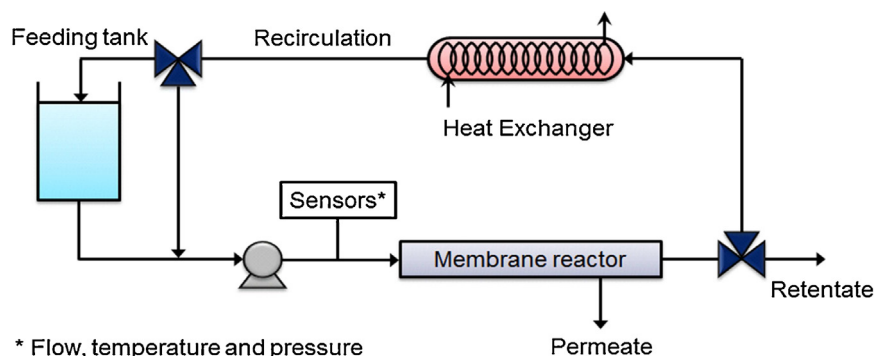


Fig. 2. Enzymatic membrane bioreactor and pilot unit.

with blanks experiments carried out under identical conditions: one containing only a solution of tetracycline (blank free) and the other with a portion of a blank membrane crushed in small pieces (blank immobilized).

Tetracycline degradation in the EMR was carried out in a batch configuration where the retentate was continuously recycled and the permeate exit was closed. Before each run, membranes were first hydrated with osmosed water in the pilot unit in order to eliminate potential free laccases that might not have been rinsed properly during the grafting step. Reactions were run with 20 mg L<sup>-1</sup> of TC in osmosed water with a total volume of 2 L. The experiment was carried out during 10 days but the substrate solution was periodically replaced by a fresh one. The residual TC percentage contained in each sample was calculated using the following equation (Eq. (1)):

$$\text{Residual tetracycline (\%)} = \left( \frac{C_{\text{TC, sample}}}{C_{\text{TC, feed}}} \right) \times 100 \quad (1)$$

where  $C_{\text{TC, feed}}$  corresponds to the TC concentration in the initial solution (mg L<sup>-1</sup>) and  $C_{\text{TC, sample}}$  is the TC concentration measured in the retentate (mg L<sup>-1</sup>).

The results obtained and presented in this study are the mean values of several experiments realized under similar conditions. After being used the ceramic supports were washed using the standard cleaning procedure and their permeability was measured to determine if they were reusable [14].

### 2.5. Determination of the apparent kinetics parameters

The apparent kinetics parameters of Michaelis–Menten equation ( $K_m$  and  $V_{\text{max}}$ ) of free and immobilized laccase were estimated from initial reaction rates determined in batch experiments using either TC or ABTS as substrate. All the reactions were carried out at 25 °C, with a concentration equivalent to 35 U mL<sup>-1</sup> of free enzymes or to 20 U mL<sup>-1</sup> of immobilized enzymes. TC solutions were prepared in osmosed water (pH 6) varying the concentration from 22.5 to 225 μM whereas ABTS solutions were prepared at 10, 15, 25, 50 and 100 μM in a 50 mM citrate–phosphate buffer pH 4.

### 2.6. Analytical methods

The structure of ceramic support and active membrane was observed using scanning electron microscopy (SEM) (Hitachi S4500).

Tetracycline concentration was determined by high-performance liquid chromatography coupled to triple-quadrupole mass spectrometry (HPLC–MS). Samples were injected through an Onyx® C18 column (25 mm × 4.6 mm) with a Waters e2695 Separations Module, and the 410  $m/z$  fragment was detected with a Micromass Quattro micro API device at 120 °C. The isocratic mobile phase used was made of HPLC grade water, 99% formic acid, and acetonitrile (74.9:0.1:25) at a flow rate of 2 mL min<sup>-1</sup>. The amount of TC was quantified with calibration standards prepared by dilution of the TC stock solution with 20% of ethanol in osmosed water.

The activity of free and immobilized laccase was determined at different pH with ABTS as substrate as previously reported [14]. One activity unit (U) corresponds to the quantity of enzymes needed to oxidize 1 μmol of ABTS per minute.

The immobilization yield was determined from the difference in enzyme activity (U) of the laccase solution before and after the immobilization step, taking into account the enzyme activity measured in the solution after the immobilization and the totality of washing solutions (Eq. (2)). It was calculated with the values of the

initial mass of enzymes in the grafting solution and the mass of immobilized enzymes (Eq. (3)).

$$A_{\text{grafted}} = A_{\text{initial}} = (A_{\text{left}} + \Sigma A_{\text{rinsing}} + \Sigma A_{\text{conditioning}}) \quad (2)$$

$$\rho_{\text{immobilization}} = \frac{A_{\text{grafted}}}{A_{\text{initial}}} \times 100 \quad (3)$$

where  $A_{\text{grafted}}$  corresponds to the activity of enzymes immobilized on the membrane,  $A_{\text{initial}}$  is the activity of enzymes in an aliquot of the initial solution left at room temperature and analyzed with other solutions after the immobilization process,  $A_{\text{left}}$  is the enzymatic activity of the solution used for grafting after enzymes immobilization,  $A_{\text{rinsing}}$  is the activity of enzymes in the rinsing solutions and  $A_{\text{conditioning}}$  the enzymatic activity in the rinsing solution used for the membrane hydration before using the enzymatic membrane for degradation.  $\rho_{\text{immobilization}}$  corresponds to the immobilization yield of laccase on the ceramic membrane (%).

The characterization of transformation products (TPs) generated during the treatment was performed by liquid chromatography coupled to a high resolution Orbitrap mass spectrometer (LC–HRMS). Samples were prepared and eluted as described by Gros et al. [27]. Chromatographic separation was achieved by using an Aquity HSS T3 column (50 mm × 2.1 mm i.d., 1.7 μm particle size) in a Turboflow™ system coupled to an Accela™ UHPLC system (Thermo Scientific). The MS analysis was performed with an electrospray ionization (ESI) interface coupled to a linear ion trap-orbitrap mass spectrometer (LTQ Orbitrap Velos™, Thermo Scientific) under positive and negative ionization conditions as described in Llorca et al. (under preparation). The mass accuracy accepted for the experiments was always within ±5 ppm. The MS acquisition was performed in full scan mode [100–1000 Da] at resolving power of 60,000 FWHM. In parallel, data-dependent MS/MS acquisition was carried out according to mass-to-charge ( $m/z$ ) ion intensity higher than 100.

## 3. Results and discussion

### 3.1. Characterization of the active membrane

SEM micrographs (Fig. 3) show that the gelatin is only deposited on the ceramic membrane surface, the gelatin layer thickness is 0.6 μm. The average immobilization yield determined as explained above (Section 2.5) was equal to 13%. According to this value, a 3 cm long piece of active membrane crushed in small parts into 100 mL of water corresponds to a concentration of 0.01 g L<sup>-1</sup> of Sigma product containing laccase from *T. versicolor*.

The kinetics parameters of Michaelis–Menten equation ( $K_m$  and  $V_{\text{max}}$ ) of both free and immobilized enzymes were estimated for two substrates (ABTS and TC). The calculated values obtained thanks to Lineweaver–Burk equation are reported in Table 1.

It is known that the enzymatic activity is better with high  $V_{\text{max}}$  and low  $K_m$  values. For both substrates, the  $K_m$  value is 5 about times lower with grafted enzymes. Therefore the bounded enzymes have a higher apparent affinity with the substrate and in principle have to be more efficient to degrade ABTS and TC than free enzymes. The literature confirms these results because it was proved that grafting enhanced the enzyme stability thanks to the bonding and the accessibility of the active site by the substrate [28,29]. However regarding the  $V_{\text{max}}$  values, although the concentration of free enzyme was higher (35 U mL<sup>-1</sup> and 20 U mL<sup>-1</sup> for free and immobilized enzyme, respectively) the  $V_{\text{max}}$  value observed for grafted enzymes was always higher. The rate of reaction would be thus higher with immobilized laccase compared to the free laccase.

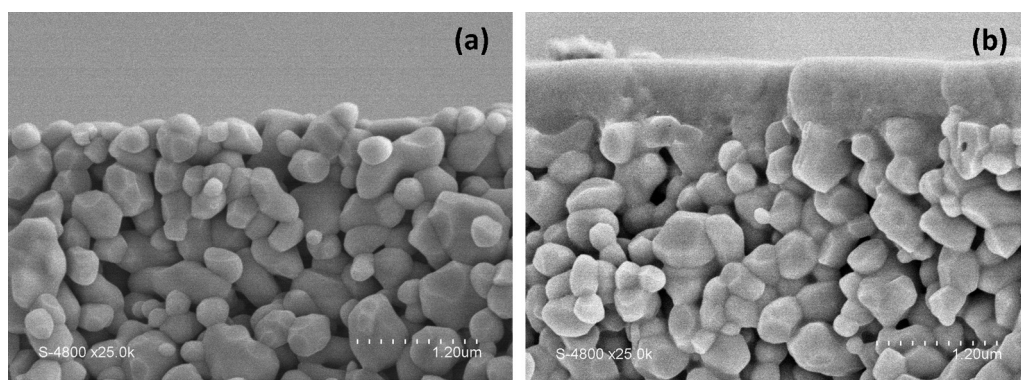


Fig. 3. Membrane characterization by scanning electron microscopy: ceramic support (a) and active membrane (b).

**Table 1**  
Kinetics constants for the degradation of ABTS and TC with free and immobilized laccase.

Experiment	Substrate	$V_{max}$ ( $\mu\text{mol min}^{-1}$ )	$K_m$ ( $\mu\text{mol L}^{-1}$ )
Free enzymes	ABTS	0.13	127
Grafted enzymes		0.58	28
Free enzymes	Tetracycline	0.60	282
Grafted enzymes		0.67	57

### 3.2. Impact of the pH and enzyme concentration on tetracycline degradation

As far as enzymatic activity toward a substrate is a complex reaction which depends on many parameters like specific enzyme-substrate interactions at a specific pH, the free laccase activity toward tetracycline was studied at different pH conditions. Laccases usually present their best degradation activity at low pHs [14,18]. In order to determine the optimal degradation conditions, we studied ABTS and TC oxidation with free laccase ( $0.1 \text{ g L}^{-1}$ ) in a range of pH from 3 to 7. Preliminary experiments with ABTS confirm the literature results. In fact the maximum of laccase activity ( $6.4 \text{ U mg}^{-1}$ ) was reached at pH 3 when experiments were carried out with this substrate (results not shown). The results for TC depletion at 24 h of reaction are presented in Table 2. We can observe that with TC the best results were obtained when the pH of the solution was around neutrality (i.e. pH 6–7). This result differs from experiments with ABTS and with the previous works of Plagemann et al. [18] and Chea et al. [14] who reported that the same laccase from *T. versicolor* was more active at pH 4 for the degradation of a dye and phenolic compounds, respectively. This result confirms that optimal pH value strongly depends on enzyme-substrate interactions. Moreover, it is important to notice that when TC solutions were prepared in osmosed water as solvent (pH=6) 78% of

**Table 2**  
Degradation of a  $20 \text{ mg L}^{-1}$  tetracycline solution by free laccase after 24 h of reaction at different pH or enzyme concentrations.

	Solvent	Residual tetracycline (%)
pH value <sup>a</sup>	3 Citrate-phosphate buffer	88
	4 Citrate-phosphate buffer	67
	5 Citrate-phosphate buffer	59
	6 Citrate-phosphate buffer	36
	7 Citrate-phosphate buffer	28
	6 Osmosed water	22
	Enzyme concentration <sup>b</sup> (g sigma product L <sup>-1</sup> )	0.01 Osmosed water
0.05 Osmosed water	45	
0.1 Osmosed water	22	

<sup>a</sup> Enzyme concentration  $0.1 \text{ g L}^{-1}$ .

<sup>b</sup> pH 6.

tetracycline was removed from the solution after 24 h of reaction. This last result is very interesting since the use of osmosed water enables to match with targeted conditions because the pH of wastewater effluents is supposed to be close to 7. In consequence, all the further experiments were carried out with TC solutions prepared in osmosed water.

Concerning the impact of enzyme concentration, it can be observed in Table 2 that 78% of the tetracycline was degraded at a laccase concentration of  $0.1 \text{ g L}^{-1}$  while only 30% was degraded when the laccase concentration was ten times lower. The use of a relatively high concentration of free laccase seems very efficient for tetracycline degradation. However as it was explained above, the use of free enzymes generates high process costs, especially concerning enzymes loss. Therefore it is preferable to graft enzymes on a carrier, even if the quantities are lower. The costs of process will thereby be lower.

### 3.3. Comparison between free and immobilized laccase

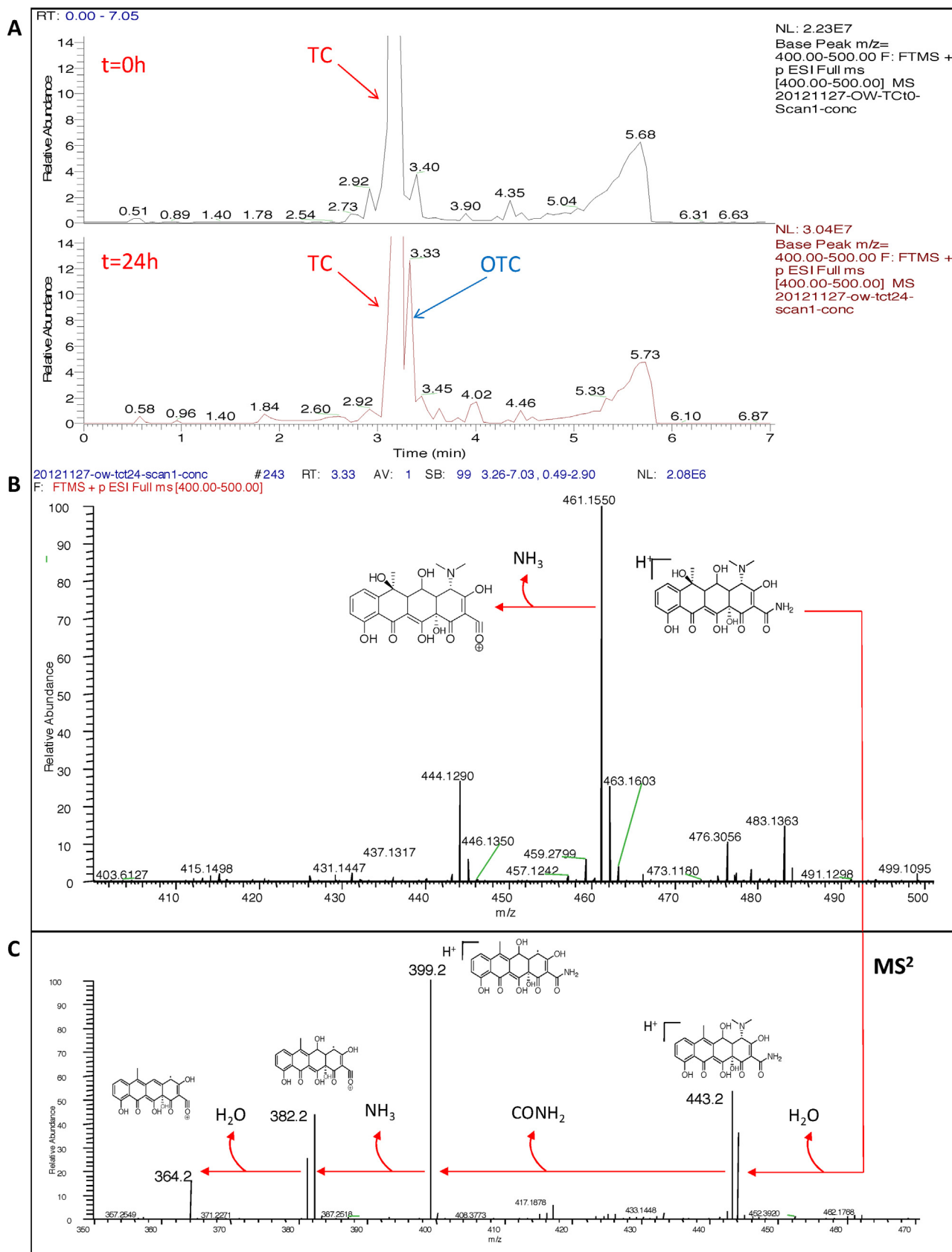
Preliminary experiments were carried out in order to determine the optimal pH for TC degradation with immobilized enzymes. For these experiments carried out in a stirred tank reactor with crushed EM, we worked under the same conditions and pHs described above in Section 3.2 but at a lower enzyme concentration ( $0.01 \text{ g L}^{-1}$ ). In such case the optimum of degradation was reached at pH 6 (49% of residual TC) whereas for pH 3 and 7 the residual TC was, respectively, of 80% and 71%, these results show that the optimal pH for both free and immobilized enzymes for TC degradation is quite similar.

In order to compare the TC degradation by free and immobilized enzymes in similar conditions (pH 6 and  $25^\circ\text{C}$ ) we used equivalent quantities of free or immobilized enzymes with  $100 \text{ mL}$  of a  $20 \text{ mg L}^{-1}$  tetracycline solution (see Section 2.4). The results presented in Table 3 show that after 24 h of reaction, a  $0.01 \text{ g L}^{-1}$  enzymatic solution enables the elimination of 30% of the tetracycline whereas the degradation yield can reach 56% with the equivalent quantity of grafted enzymes. The difference of tetracycline degradation obtained for the blanks shows that some tetracycline could be auto degraded and/or adsorbed on the membrane.

**Table 3**  
TC degradation yield after 24 h of reaction with free and immobilized laccase<sup>a</sup>.

Experiment	Degradation yield (%)
Free enzymes	30 ( $\pm 7$ )
Immobilized enzymes	56 ( $\pm 8$ )
Blank (free)	5 ( $\pm 5$ )
Blank (immobilized)	9 ( $\pm 5$ )

<sup>a</sup> Enzyme concentration:  $0.01 \text{ g L}^{-1}$  in osmosed water at  $25^\circ\text{C}$ .



**Fig. 4.** Chromatograms corresponding to sample at 0 h and after the treatment (A), MS spectra (B) and MS/MS spectra (C) corresponding to OTC TP generated during enzymatic treatment.

**Table 4**  
Literature results for tetracycline (TC) degradation with free enzymes.

Reference	Suda et al. [19]	Wen et al. [30]	Wen et al. [31]
Enzyme	Laccase ( <i>Trametes versicolor</i> )	Lignin peroxidase ( <i>Phanerochaete chrysosporium</i> )	Manganese peroxidase ( <i>Phanerochaete chrysosporium</i> )
TC (mg L <sup>-1</sup> )	10 <sup>-4</sup> mol L <sup>-1</sup> (≈6.4 g L <sup>-1</sup> )	40 U L <sup>-1</sup>	40 U L <sup>-1</sup>
Extra-chemicals	pH 4.5	pH 4.2	pH 4.8
Degradation yield	0.2	50	50
	1-Hydroxybenzotriazole	H <sub>2</sub> O <sub>2</sub>	Mn(II) and H <sub>2</sub> O <sub>2</sub>
	99% in 1 h	95% in 5 min	73% in 9 h

The degradation enhancement obtained with grafted enzymes could be the result of the stability improvement reached with the immobilization. Even though, concerning the beneficial effect of the immobilization the results reported in the literature are sometimes contradictory in terms of enzymes activity and stability. In some cases, there are no conformational changes and the active sites are still accessible thanks to the length of the covalent grafting bond. The grafted biocatalyst can either keep the same activity as the free enzyme by maintaining a good process efficiency [23] or its activity can be improved compared to free enzymes as it has been shown by other researchers [28,29]. In this work we can notice that the hydrophilic character of the gelatin layer and its nature (protein) could maintain a favorable environment around the enzymes and probably plays a role on the activity enhancement observed.

If we compare our results with those reported in the literature (Table 4) we can notice that tetracycline degradation with free enzymes is very efficient under some particular conditions. However in such previous works the enzyme concentrations used were much higher than the concentrations used in this study. In addition in all of these processes the TC degradation needs the use of supplementary chemicals and additives which is a major drawback in terms of cost mainly if we consider the treatment of wastewaters.

### 3.4. Effect of the temperature

The effect of the temperature on the TC degradation for free and grafted laccase is shown in Table 5. Three temperatures were studied 15, 25 and 35 °C, higher temperatures were not considered as relevant because the global goal of this study is to degrade TC in wastewaters. We can notice that for all of the temperatures studied the activity of grafted laccase is always higher than the free laccase activity. Moreover, for both enzyme forms the TC degradation increases from 15 °C to 25 °C and then remains stable up to 35 °C.

### 3.5. Study of transformation products (TPs)

According to the chromatograms presented in Fig. 4, two different compounds were detected as major TPs after the enzymatic treatment, the major TP was oxytetracycline (OTC) ( $m/z=461.1550$  corresponding to  $[M+H]^+$ ) followed by anhydrotetracycline ( $m/z=427.1501$  corresponding to  $[M+H]^+$ ). These two products have been previously reported in the literature as

**Table 5**  
Degradation of a 20 ppm TC solution by laccase at different temperatures after 24 h of reaction<sup>a</sup>.

T (°C)	Residual tetracycline (%)	
	Free enzymes	Grafted enzymes
15	89	87
25	65	50
35	64	47

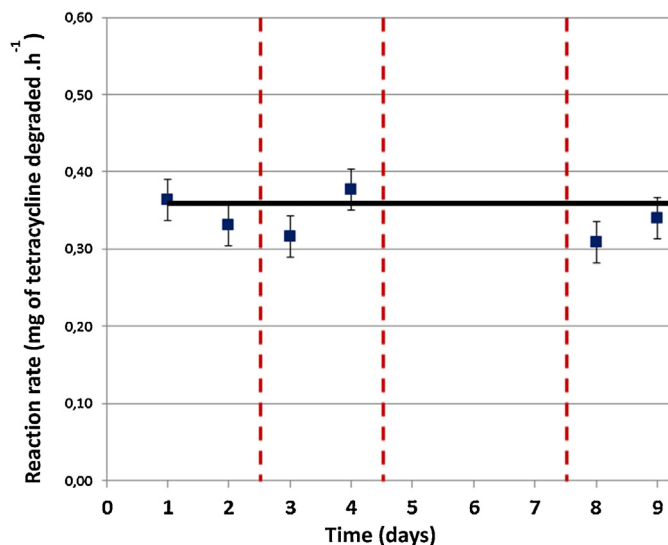
<sup>a</sup> Enzyme concentration: 0.01 g L<sup>-1</sup> in osmosed water.

by-products of tetracycline degradation by ozonation [32] or by sludge bacteria [33].

### 3.6. Degradation in the enzymatic membrane reactor

The enzymatic degradation in the membrane reactor was tested in order to assess the preservation of enzymatic activity after several cycles of newly prepared TC solution degradation. As explained above, the reaction was run at 25 °C with 20 mg L<sup>-1</sup> of tetracycline in osmosed water with a total volume of 2 L in batch configuration. This configuration was used in this work because we took the preliminary assumption that the reaction takes place at the surface of the membrane. As it was seen in Fig. 3, the gelatin only coated the membrane surface as a thin layer but not the inner pores. Then it can be reasonably concluded that enzymes were grafted only on the surface of the gelatin layer. During the runs duration (10 days, more than 200 h) the substrate solution was regularly replaced by a fresh one. Samples were taken from permeate and retentate and the tetracycline concentration was measured as previously. The percentage of adsorption within the whole pilot unit was measured; it was less than 1%. The results are shown in Fig. 5.

We can notice that the reaction rate is almost constant and equal to 0.34 (±0.01) mg of tetracycline degraded per hour, even after 4 cycles with feed of fresh tetracycline solution. As it can be seen, the enzymatic membrane is still active after 200 h of reaction. From this result we can notice that probably the gelatin layer ensures a high resistance to environmental conditions of the enzymes and prevents them from being stressed by the flow. Thus the active site of the biocatalyst can still react with the substrate without leaching or conformational changes. It is important to notice that the degradation rate reached in the membrane reactor is much higher than the degradation rate in the stirred tank (Section 3.3) which

**Fig. 5.** Tetracycline degradation by immobilized laccase in the enzymatic membrane reactor.

was only of 0.047 mg of tetracycline degraded per hour. We can advance some hypothesis about this conversion enhancement. First the ratio substrate amount/biocatalyst is much higher in the membrane reactor; in fact the initial substrate concentration did not change ( $20 \text{ mg L}^{-1}$ ) but the enzyme concentration is much lower (i.e. equivalent to  $0.002 \text{ g L}^{-1}$  instead of  $0.01 \text{ g L}^{-1}$ ) because of the higher volume of reaction (i.e. 2 L instead of 0.1 L). It is well known that reaction rates are lower under substrate limiting conditions. Second, it is possible that the reactor configuration allowed a better contact between the biocatalyst and the substrate because we are continuously sweeping the solution on the membrane surface and unlike the stirred tank where the flow-lines are not necessarily having an effect on a possible boundary layer around the membrane pieces where the enzymes are grafted.

#### 4. Conclusions

Laccase from *T. versicolor* was immobilized on ceramic membranes and successfully used for tetracycline degradation under the targeted working conditions (osmosed water at  $25^\circ\text{C}$ ) without the presence of any extra chemicals. The grafting process allowed avoiding enzyme leaching by the covalent bond created between the biocatalyst and the carrier. Among the parameters studied for the tetracycline degradation the pH resulted to be one of the most important. We demonstrated that on the contrary of other substrates degradation by laccase from *T. versicolor* which are depleted in acidic conditions, the optimum the tetracycline degradation was reached at a pH of 6, this is a very important result because this pH approaches wastewaters pH. Moreover, the enzymatic concentration has also an impact on the degradation rate but it is limited by the immobilization yield in the enzymatic membrane reactor.

Covalently grafted enzymes proved to be more efficient for tetracycline degradation than free enzymes. This stability enhancement is partly due to the protecting effect of the gelatin. The covalent bonds also prevent from conformational changes in the protein structure, making probably the active site always accessible.

The identification of the degradation products shows that laccase allows the transformation of tetracycline mainly on oxy-tetracycline and anhydrotetracycline products which have been previously reported in the literature as by-products of tetracycline degradation by ozonation or by sludge bacteria.

The results obtained show that active membranes can be used for several cycles of tetracycline degradation and during 10 days without enzymatic activity loss.

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