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Enzymatic Degradation of Micropollutants in Water: the Case of Tetracycline Degradation by Enzymes Immobilized on Monoliths

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Enzymatic monolithic reactors were applied for the degradation of micropollutants through flow-through reactor configuration. Silica monoliths with uniform macro-/mesoporous structures (20 µm and 20 nm macro- and mesopores diameters) high porosity (83%) and high surface area (370 m2 g^{-1}) were prepared. The monoliths were cladded in steel tubing and laccase from *Trametes versicolor* was immobilized by covalent grafting. Enzymatic monoliths presented a very good oxidation activity and were used for the degradation of tetracycline (TC) in aqueous solutions in a tubular plug reactor with recycling configuration. TC degradation efficiency was found to be 40-50 % after 5 h of reaction at pH 7. The immobilized laccase on silica monoliths exhibited high operational stability during 75 hours of sequential operation.

1. Introduction

Pharmaceuticals are currently used in human and animal medicine. Many of these molecules or their metabolites are currently discharged in wastewater but they are not completely removed by conventional wastewater treatment plants. Indeed, they end up in surface and underground waters (U. Szymańska et al (2019), Halling-Sørensen et al (2002), Watkinson et al (2007)). Among these molecules, antibiotics are a special family of pharmaceutical molecules that are causing increasingly frequent antibiotic resistance in patients. They have therefore become a real public health problem. Antibiotics, like tetracycline (TC) are currently found in wastewaters and natural water bodies increasing the ecotoxicity and anti-microbial resistance (Halling-Sørensen et al (2002)). There is a real need to develop alternative technologies techniques for the complete and efficient removal of these pharmaceuticals. Biodegradation of antibiotics is recently been explored using oxidoreductases enzymes as a bio-catalyst (Varga et al (2019). Laccase belongs to a group of polyphenol oxidases containing copper atoms in the catalytic center and usually called multicopper oxidases. In contrast to peroxidases, laccase does not require the presence of hydrogen peroxide or manganese for oxidation and needs only oxygen as the final electron receptor offering a green alternative in wastewater treatment (Primozic et al, 2019). Due to its biochemical structure, Laccase has ability to degrade large variety of micro pollutants at ambient temperatures and can be considered as good green catalysts for degradation of micro pollutants (Kües et al., 2015). Immobilizing laccases on different solid supports like membranes have been tested for TC degradation and resulted being more active than free laccases while showing a good stability (de Cazes et al., 2014). However, the degradation rates reported are limited by the small surface area available in membranes for enzymes immobilization. However, the amount of enzyme grafted on membranes is limited restraining the large scale application of this concept (Abejon et al, 2015).
Enzymatic monoliths are interesting candidates to be applied in continuous flow through plug reactors. In this operating mode, the flow is forced to pass exclusively through the internal monolith porosity where the biocatalyst is immobilized, intensifying the probability of contact between the reactants and the enzyme during the mass transfer process. Indeed, this configuration results in improved control of the reaction through the

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"micro-reactor concept" (Westermann, 2009). Silica monoliths featuring large surface area (400 - 700 m2/g), porosity and permeability have been successfully used for process intensification in catalytic reactions under continuous flow (Galarneau et al., 2016). Herein in this work we show that these new kind of monoliths are also promising supports to immobilize enzymes, as laccases, for improving the degradation rate of pollutants like TC. In this work silica monoliths with hierarchical porosity (macro-/mesoporosity) were prepared by a controlled sol-gel process and a Laccase form (trametes versicolor) has been grafted on the internal surface of the monoliths. Then, activated monoliths were immediately used for TC degradation in a plug flow reactor (PFR) with continuous recycling. Finally a model coupling the enzymatic kinetics with mass transfer was built in order to optimize the enzymatic reactor.

2. Experimental

2.1 Materials

Polyethylene glycol (PEG) (99%, 100 kDa), tetraethoxysilane (TEOS) (99%), (3-aminopropyl) triethoxysilane (APTES) (99%), commercial powder of laccase from *Trametes versicolor* (activity ≥0.5 U mg−1), tetracycline (TC) (≥98.0%), glutaraldehyde (25% v/v) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (≥98.0%,) were purchased from Sigma-Aldrich.

2.2 Silica monoliths synthesis, functionalization and characterization

Silica monoliths with hierarchical porosity (macro-/mesoporosity) were prepared by a controlled sol-gel process with TEOS and PEG (Galarneau et al, 2016). Prior to enzyme immobilization, the monoliths were pre-activated by grafting amino groups with APTES in ethanol under reflux (80 °C) overnight. Tubular reactors were built by cladding the monoliths inside Teflon™ heat shrinkable gains at 180 °C for 2 h to be finally connected to stainless-steel tubing. The enzymatic grafting of monoliths was carried out by a successive immersion of the pre-activated monoliths in different solutions prepared in a citrate phosphate buffer (pH 7, 0.1 M): a first solution of glutaraldehyde (4 % v/v) and a second solution of commercial laccase (*Trametes versicolor*) (5±1 U/mL). After the immersion in each activation solution the monolith was washed with the same buffer in order to deplete the rest of glutaraldehyde or non-grafted enzyme. Then, the activated monoliths were immediately used for the determination of activity (ABTS) in a stirred tank reactor or TC degradation in a plug flow reactor (PFR) with continuous recycling.
Monoliths were characterized structurally by scanning electron microscopy (SEM) (Hitachi S-4800 I FEG-SEM). Porosity was determined by mercury porosimetry (Micrometrics Autopore 9220) and nitrogen adsorption at 77 K (Micromeritics Tristar 3020). The quantitative determination of the number of amine functions on the surface of the silica monolith was determined by TGA analysis (PerkinElmer STA 6000).

2.3 Determination of immobilized enzymes activity

The activity ($U/mg_{monolith}$) was reassured using freshly activated monoliths which were gently crushed and a portion of the activated powder was used for the oxidation of ABTS (1mM) prepared in a citrate phosphate buffer at pH 4 on a stirred tank reactor. The activity was measured by the change in absorbance of a standard ABTS solution at 420nm (UV-VIS 2401 Shimadzu spectrometer). One unit of activity (U) corresponds to quantity of enzyme required for the oxidation of 1μmol of ABTS per one minute. The immobilization yield was calculated taking into account the activity of the enzymatic solution used for immobilization before and after activation process.

2.4 Determination of reaction kinetics parameters

Reactioz kinetics parameters (K_M and V_{MAX}) of ABTS oxidation by free and immobilized laccase were measured with a solution of ABTS (20-100μM) at 25°C in a citrate phosphate buffer 0.1M (pH 4) by the spectrometric methodology described above. Lineweaver-Burk plot was used to estimate kinetic parameters, K_M and V_{max} according to the following equation:

$$\frac{1}{V} = \frac{K_M}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \quad (1)$$

Where [S] is the substrate concentration (μmol/L), V and V_{MAX} are reactively the reaction rate and maximum reaction rate (μmol/min) and K_M the Michaelis constant (μmol/L).

2.5 Tetracycline degradation experiments

TC degradation tests were carried out with three laccase-activated monoliths connected in series (0.6 cm diameter and 0.5 cm length, each, equivalent to a total of 15.0 U ± 0.5 U of enzymatic activity). TC solution (20 mg/L) prepared in osmosed water pH 6, at 25°C was continuously flowed through the enzymatic monoliths (HPLC pump, Gibson model: 321, France) and constantly recycled from a reservoir. The flow rate was kept at 1 mL/min. These experiments were carried out also to determine the stability of laccase-activated monoliths under continuous operation for TC degradation. Indeed, degradation tests were carried out following a sequential procedure: after ~20 hours of reaction the pilot was emptied, rinsed with osmosed water and kept at room temperature overnight, then a new fresh TC solution was introduced and the run start again. The cycles were repeated for a total duration of 75 hours. Samples were taken from the reservoir and analysed by HPLC coupled to triple-quadrupole mass spectrometry (HPLC–MS). Samples were injected through a Macherey-Nagel C18 column (50 mm x 2 mm) with a Waters e2695 Separations Module, and the 410 m/z fragment was detected with a Micromass Quattro micro API device.

3. Results

3.1 Characterization of monoliths

The monoliths were characterized by SEM (Figure 1), mercury porosimetry, nitrogen adsorption at 77 K and thermogravimetric analysis. The monoliths presented homogeneous and interconnected flow-through macropores of 20~25 µm diameter, mesopores diameter of 20 nm, present a very high porosity (Vmacro = 3.4 mL g^{-1}, Vmeso = 0.8 mL g^{-1}, total porosity ε = 0.83) and a large surface area (370 m^2 g^{-1}). The thermogravimetric analysis allowed calculating the amount of amino functions of pre-activated monoliths by the loss of weight between 200°C to 900°C (1 mmol NH_2 g $monolith^{-1}$). After laccase immobilization some structural values changed. We observed a decrease of the specific surface (185 m2 g^{-1}), Vmeso (0.4 mL g^{-1}) and mesopores diameter (14 nm).

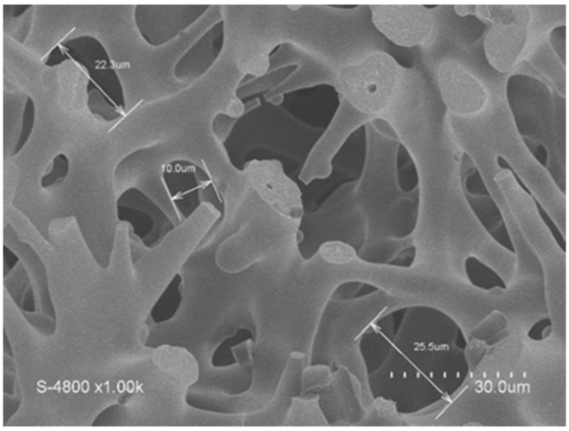

Figure 1: SEM of monoliths showing uniform macroporous structure.

3.2 Immobilization of enzymes and activity measurement

The measured activity of monoliths powder was 0.18 $U/mg_{monolith}$. The immobilization yield was equal to 80%, which was much higher than the immobilization yield observed in case of membranes as reported by de Cazes et al., (2014). This very high immobilization yield confirms that monoliths can graft large amount of active enzymes. The activity of a single monolith (6 mm diameter and 5 mm length, mass of 50 mg) was calculated from the measured activity of crushed monoliths explained above. The mean activity of a single monolith was of 7.8 ± 0.5 U.

3.3 Determination of reaction kinetic parameters

The kinetic parameters of the enzymatic reaction (K_M and V_{MAX}) are important factors to be measured because they describe the activity of enzymes towards a substrate conversion. Kinetic parameters for both free and immobilized enzymes were determined by the method described in the experimental section. Measured values are shown in Table1. We can notice that for the same concentration of free and immobilized enzymes:

(0.0085 U/mL) V_{MAX} is a little bit higher for immobilized enzymes. Likewise, K_M is lower for free enzymes. This result indicates that immobilized has a higher activity and a lower affinity towards the substrate than free enzyme. This result is surprising because generally structural conformation and diffusion limitations occur after immobilization (Salami et al., 2018). However, in some reported cases the immobilization of enzymes has resulted on a V_{MAX} enhancement. This particular behavior had been reported for laccase immobilized on membranes (De Cazes et al., 2014), or lipase immobilized on silica (Kumar et al., 2019).

Table 1: Reaction kinetic parameters for the oxidation of ABTS with free and immobilized laccase.

	V_{MAX} (μmol/min)	K_M (μmol/L)
Free	0.013	85
Immobilised	0.14	101

3.4 Tetracycline degradation experiments

TC (20 ppm) degradation results are displayed in Figure 2. Control experiments (blank tests) were carried out to study the effect of TC self-degradation or adsorption on the evolution of TC concentration. Blank experiments were carried out with enzymatic monoliths thermally deactivated by heating in oven at 100°C for 2 hours.

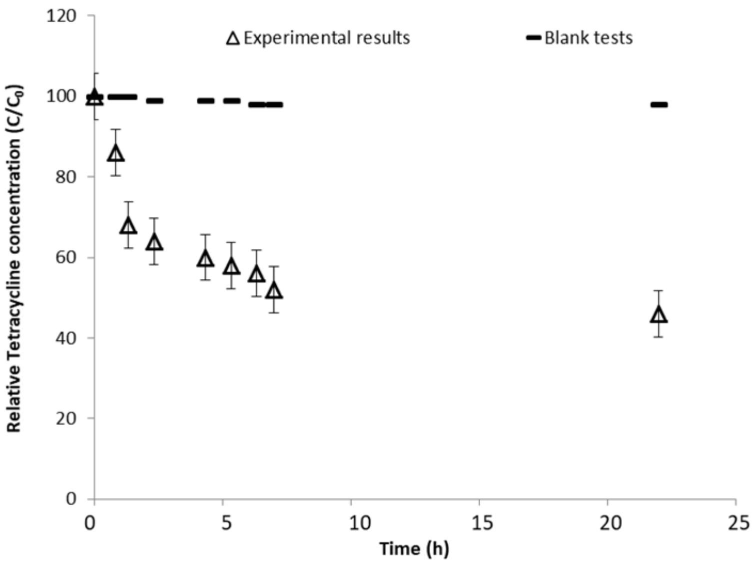

Figure 2: Evolution of % TC concentration (concentration/initial concentration).

The results of blank tests show that less than 2% of initial TC was depleted by adsorption or self-degradation. The evolution of TC concentration (percentage of the concentration/initial concentration) shows that laccase-activated monoliths were able to deplete 40% of initial TC during the first 5 hours and then the degradation rate decreases dramatically. This result can be explained by the decrease of substrate concentration, if the kinetics obeys to a Michaelis-Menten equation. However, the decrease of the oxygen content, or even the formation of inhibiting by-products can also be considered. Kurniawati and Nicell (2007) and Ortner et al (2015), have noticed that the velocity of oxidation of substrates by laccases can decrease very rapidly during the first hours of reaction because the oxygen concentration reduction near catalytic sites. Indeed, the conversion can be improved by stirring on air atmosphere or reach 100% by using pure oxygen (Ortner et al (2015)). In the case of experiments carried out in the monolithic system no stirring under air or oxygen was done. Then, it is conceivable that oxygen depletion nearby catalytic sites caused the degradation rate decrease.

In order to confirm the hypothesis of lack of oxygen a series of experiments were carried out in a stirred tank reactor with enzymatic monoliths previously crushed and using the same TC concentration and ratio of activated monolith/volume of TC solution. Two types of experiments were carried out: with and without air bubbling. The TC depletion in 7 hours of reaction was of 92% with air bubbling and only 40% without air bubbling. These results confirm that the relatively low percentage of TC degradation observed with the monoliths under continuous operation and recycling is certainly the result of the lack of oxygen as co-substrate of the reaction. Indeed, we can exclude the inhibition of the enzymatic reaction by oxidation products of TC. Moreover, for an efficient continuous operation in plug flow reactors with enzymatic monoliths it should be necessary to design a bubbling system in between each monolith or in the reservoir were the solution is recycled.

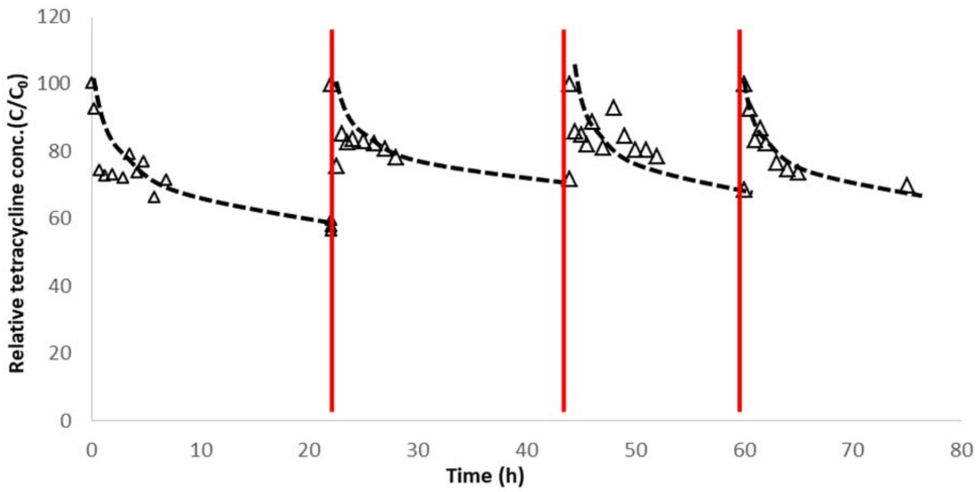

Figure 3: Evolution of TC degradation during 75 hours of sequential operation. Red lines indicate the time when the pilot was emptied, rinsed with osmosed water and filled again with a new fresh TC solution (20 mg/L).

The operational stability of enzymatic monoliths was studied during 75 hours (4 days) with the sequential operation procedure described in section 2.5; the results are displayed in Figure 3. We can observe that the decrease of TC concentration for one cycle is similar than the degradation rate observed in Figure 2: TC concentration decreases very quickly during the first hours of reaction and then the degradation rate go-slow. Moreover the evolution of TC concentration is almost similar for the four successive cycles. Furthermore, we can also notice that when TC solution is changed with a fresh one containing initial TC and oxygen concentration the same pattern of TC reduction is obtained. However, we can notice that the total depletion of TC is a little bit higher for the first cycle (between 40-50%) and then decreases and stabilizes for three other successive cycles of operation (35%). Indeed we can conclude that the enzyme immobilized in monoliths is stable during at least 75 hours of sequential operation. In a previous work with enzymatic membranes (De Cazes et al., 2014) it was demonstrated that this commercial laccase, in its immobilized form, is stable during at least 9 days of operation, it is obvious that longer experiments have to be done in order to define the long term stability of such system and to determine its industrial interest.

4. Conclusions

The preliminary results presented in this work show that the laccase immobilization in silica monoliths is very promising for tetracycline degradation. Silica monoliths with a homogenous mesoporous/macroporous structure featuring, high specific surface area and porosity were successfully prepared. These monoliths were pre-activated with APTES and glutaraldehyde in order to covalently immobilize laccase from *Trametes versicolor*. The activity and kinetic parameters were determined for ABTS as model molecule. From reaction kinetic parameters V_{MAX} and K_M values, it was concluded that after immobilization laccase was a little bit more active but presents lower affinity towards the substrate. Enzymatic monoliths were successfully implemented at laboratory scale for the degradation of tetracycline as model micropollutant in a continuous plug flow mode with recycling. Indeed, approximately 40-50% of TC in 20 ppm solutions was degraded in 5 hours. It was demonstrated that the rate observed is the result of a lack of oxygen a necessary co-substrate for the TC oxidation. Moreover, an aeration system has to be implemented in the continuous configuration with enzymatic monoliths. The immobilized laccase on silica monoliths exhibited high operational stability during 4 days of sequential operation this factor is fundamental for the applicability of such enzymatic reactors at large scale. Indeed, longer experiments have to be done in order to define the long term stability of such system and to determine its industrial interest.

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