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# Continuous Biotransformation of Estrogens by Laccase in an Enzymatic Membrane Reactor

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Although the removal of steroid estrogens by enzymatic treatment with free fungal enzymes has been already demonstrated in previous batch experiments, the main limitations associated to this system are the low productivity and degradation yield, typical of batch operations, as well as the unfeasibility of reusing the enzyme. With the aim of overcoming these drawbacks, an enzymatic membrane reactor (EMR) was designed for the continuous removal of estrone (E1) and estradiol (E2) by a commercial laccase from *Myceliophthora thermophila*. The use of an ultrafiltration membrane allowed the separation of the enzyme according to its high molecular weight while the degradation products could pass through it. Not only the continuous operation was proved, but also different operational parameters were assessed for their relevance on the efficiency of the process. Accordingly, the effects of laccase activity (500-1,000 U/L), hydraulic residence time (1-4 h) and oxygen frequency supply (2-8 pulses of oxygen each HRT) were investigated. Removal efficiencies between 64-100 % and degradation rates of 0.96-2.92 mg/(L·h) were attained. Furthermore, the residual estrogenic activity of the effluent was largely reduced up to 97 %. The present work proves the feasibility of using a laccase-mediated reactor for the continuous removal of estrogenic compounds.

## 1. Introduction

Over the last years, a large number of investigations concerning the effect that hormones may have on the environment and on living organisms have been carried out (Auriol et al., 2007). The endocrine disrupting chemicals (EDCs) are believed to disrupt the synthesis, secretion, transport, binding, action and elimination of the endogenous hormones which are responsible for maintaining homeostasis, reproduction, development and integrity in living organism and their progeny (Cabana et al., 2007). These natural and synthetic estrogens have been frequently detected at various levels in sewage effluents since existing wastewater plants are unable to remove this type of compounds completely (Auriol et al., 2007).
Several advanced treatment technologies (including ozone and Fenton treatment) have been evaluated with the objective of degrading these compounds (Ikehata et al., 2006). Nevertheless, they present a number of disadvantages such as high cost, potential formation of toxic products, long treatment periods, etc. These negative aspects make it necessary to search for alternative treatments (Kim and Nicell, 2006). A potential alternative may be based on the use of white-rot fungi and the enzymes that they secrete (Rodarte-Morales et al., 2010). Indeed, it has been recently demonstrated that laccases are effective in removing the estrogenic activities of endocrine disrupting chemicals such

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as bisphenol A, nonylphenol (Tsutsumi et al., 2001), estrone (E1), estradiol (E2) and ethinylestradiol (Auriol et al., 2008).
We recently demonstrated the ability of a commercial laccase from *Myceliophthora thermophila* to degrade E1 and E2 in batch mode (Lloret et al., 2010). However, the possibility of using continuous removal systems is sought because the efficiency of the processes could be significantly enhanced. Nevertheless, the retention of the biocatalyst in the reactor is imperative when a continuous prolonged operation is attempted. Therefore, an enzymatic membrane reactor (REM) was proposed in the current study for the continuous degradation of E1 and E2. This bioreactor was based on a stirred tank reactor coupled with an ultrafiltration membrane which allowed the retention of laccase and its subsequent reuse.

## 2. Materials and methods

### 2.1. Chemicals

Estrone (E1) and estradiol (E2) were purchased from Sigma-Aldrich (USA). 2,2´-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) was supplied by Fluka (USA). All solvents were HPLC grade (Sigma-Aldrich, Germany).

### 2.2. Enzyme

Commercial laccase (Novozym 51003) from *Myceliophthora thermophila* was supplied by Novozymes (Denmark). This enzyme (molecular weight of 56 kDalton) was produced by submerged fermentation of genetically modified *Aspergillus* sp.
Laccase activity was determined by the oxidation of ABTS (50 μM) to its cation radical ($ABTS^{\cdot+}$) in 1 mL sodium acetate buffer (0.1 M, pH 5) at 30 ºC. The monitoring of ABTS oxidation was carried out in a Shimadzu UV-1603 spectrophotometer at 436 nm (ε = 29300 L/(mol·cm)). One unit (U) of activity was defined as the amount of enzyme forming 1 μmol of $ABTS^{\cdot+}$ per min.

### 2.3. High-performance liquid chromatography analyses

Determination of the estrogen concentration was carried out through high performance liquid chromatography (HPLC) in a HP-1090 system equipped with a diode array detector and a reverse phase column Lichrocart 250-4 packed with Lichrosphere 100 RP-18 5 μm (Merck). The operation conditions for the analysis were 200 μL injection volume, wavelengths of 210 and 275 nm, 50 mM acetonitrile:phosphate buffer (pH 4.5) with the following gradient 50:50 and 90:10 and a flow rate of 0.8 mL/min.

### 2.4. Determination of estrogenic activity

The estrogenic activity was measured by the LYES (yeast estrogen screen-assay assisted by enzymatic digestion with lyticase) assay previously described by Routledge and Sumpter (1996). The recombinant yeast *Saccharomyces cerevisiae* was kindly provided by the Laboratory of Microbial Ecology and Technology (Labmet, Ghent University, Belgium).

### 2.5. Continuous removal of estrogens in an enzymatic membrane reactor

The enzymatic reactor consisted of a 250 mL stirred tank reactor operated in a continuous mode coupled to an ultrafiltration polyethersulfone membrane (Prep/Scale-TFF Millipore) with a nominal molecular weight cutoff of 10 kDalton, which permits the recycling of the enzyme to the reaction vessel. The additional volume held by the ultrafiltration unit and the interconnecting tubing was 120 mL. Temperature was maintained at 26 ºC by circulation of thermostated water and the agitation rate was 250 rpm. PTFE tubing was used to prevent adsorption of the compounds to the inner surface of tubing and the reaction mixture was continuously stirred using magnetic stirrers and Teflon-coated stir bars. A scheme of the reactor is shown in Figure 1.

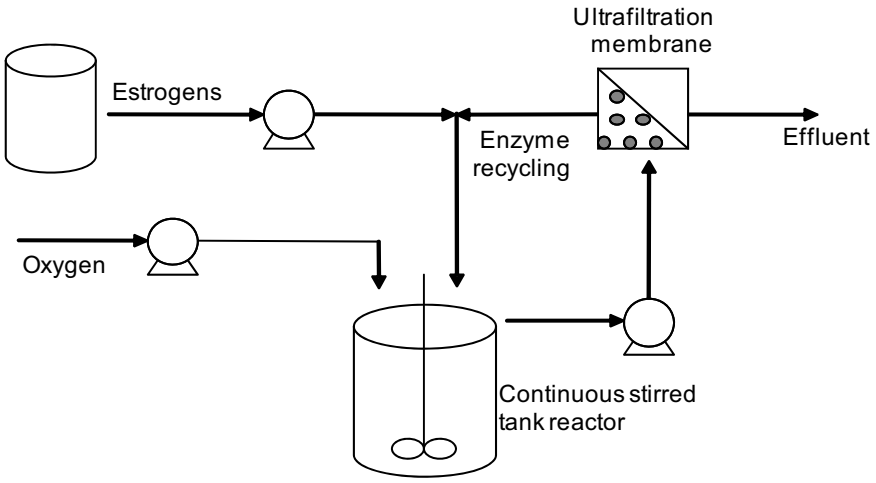


*Figure 1: Scheme of the EMR used*

The reactor was equipped with dissolved oxygen (DO), temperature, and pH sensors. A stock solution containing E1 and E2 (4 mg/L of each) in 100 mM sodium phosphate buffer (pH 7) was continuously fed into the reactor by a peristaltic pump. The feed addition rate was selected according to the HRT considered in each experiment (Table 1). Laccase was added in a single initial pulse and the enzyme was recycled in a recycling:feed flow ratio 12:1. At the start-up of the operation, the whole system (tank reactor, membrane module and pipes) was filled with the reaction solutions and at time zero, the reaction was initiated by the addition of the enzyme into the reactor. An electrovalve located at the end of a flexible membrane tube controlled by a cyclic timer was used to inject oxygen with a pulsing flow. Samples were withdrawn during the treatment time (8 h) to determine the concentration of the estrogens in the effluent and to measure laccase activity.

Table 1: Experimental conditions for E1 and E2 removal in the EMR

| Run | Inffluent concentration (mg/L) | Laccase activity (U/L) | HRT (h) | Oxygen supply (pulses/HRT) |
|---|---|---|---|---|
| 1 | | 1,000 | 2 | 2 |
| 2 | | 500 | 2 | 2 |
| 3 | 4 | 500 | 4 | 2 |
| 4 | | 500 | 4 | 8 |
| 5 | | 500 | 1 | 8 |

## 3. Results and Discussion

### 3.1. Membrane efficiency analysis

The suitability of the selected membrane was demonstrated by circulating a solution of laccase through the membrane and measuring the enzyme activity in permeate and retentate. No loss of activity in the permeate was observed, concluding that the membrane efficiently retained the enzyme. It was also proved that no physical adsorption of the estrogens onto the membrane took place.

In addition, the EMR was run under the maximum oxygen supply in order to evaluate the potential stripping of the estrogens by gas flushing. No removal of the compounds was observed indicating that the removal of the target compounds in the subsequent experiments could only be attributed to the oxidative action of laccase.

### 3.2. Continuous removal of estrogens in an enzymatic membrane reactor

The EMR used for the continuous removal of E1 and E2 was operated under the operational conditions presented in Table 1, with the aim of studying the effect of the main variables which affect the continuous enzymatic treatment: laccase activity, hydraulic residence time (HRT) and oxygen supply. The laccase concentration was the main factor evaluated in previous investigations (Khouni et al., 2010; Murugesan et al., 2007). Nevertheless, the HRT usually affects significantly the results since a higher HRT implies a longer contact between the compound and the biocatalyst. Therefore, a remarkable improvement in the percentage of degradation is expected when the system is working at a high HRT value, being defined as the ratio between the residual concentration of each estrogen in the effluent and the concentration in the influent (~4 mg/L). On the other hand, the degradation rate defined as the amount of the compound degraded per volume of reactor and time (mg/(L·h)) would be lower since the amount of compound treated per unit of time would decrease. Thus, the effect of the factors tested in the current study on both variables: percentage degradation (%) and degradation rate (mg/(L·h)), was investigated in an attempt to achieve a compromise solution. The results of percentage of degradation and degradation rates are shown in Figure 2A and 2B, respectively.

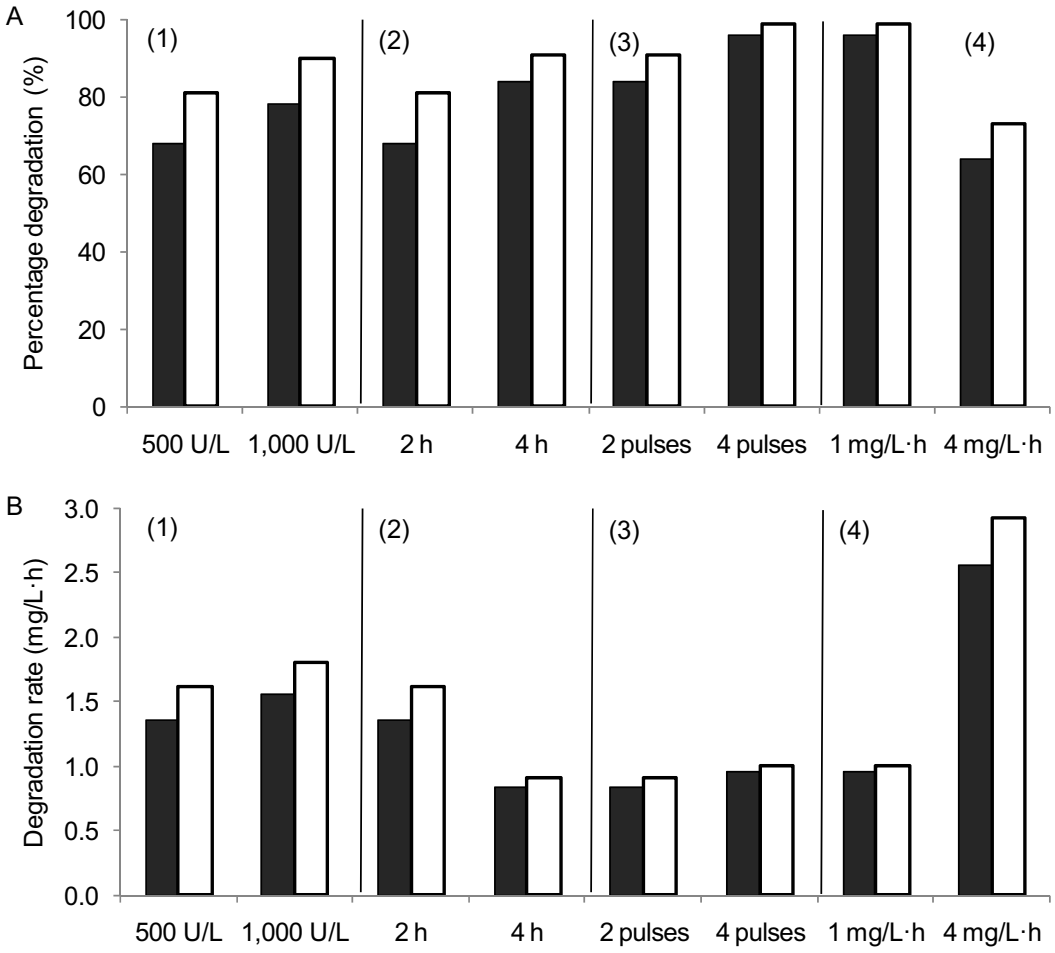


*Figure 2: Degradation percentages (A) and degradation rates (B) of E1 (black bars) and E2 (white bars). Effects of laccase activity (1), HRT (2), pulses of oxygen each HRT (3) and estrogen addition rate (4) were studied*

The effect of laccase activity was investigated at two levels: 1,000 and 500 U/L. The results indicated that the enzyme activity had a less significant effect on the removal than the expected (Figures 2A-1 and 2B-1). E1 and E2 were degraded by 78 and 90 % (1.56 and 1.80 mg/(L·h)), respectively, by 1,000 U/L of enzyme; while reducing the use of the enzyme to the half only implied a removal efficiency 10% lower. Tavares et al. (2009) studied the effect of the enzyme concentration on the batch decolourization of reactive yellow 15 and they also demonstrated that laccase activity did not affect the degradation in the range of activity tested. Thus, a laccase activity of 500 U/L was selected for the following experiments.

The influence of HRT was also evaluated: 2 and 4 h were tested. The results demonstrated that HRT had a greater effect on the removal of E1 since it was degraded up to 80 % for an HRT of 4 h, and only 68 % for 2 h of HRT (Figures 2A-2 and 2B-2). Nevertheless, the effect on E2 was negligible: it was oxidized up to 80 % for HRT 2 h and 90 % when HRT was maintained in 4 h.

The effect of the oxygen supply was studied with 2 and 4 pulses of oxygen each HRT. The higher oxygen frequency allowed improving the removal efficiencies (Figures 2A-3 and 2B-3). E2 was not detected in the effluent of the EMR while E1 was eliminated up to 95 % when the frequency of oxygenation was 4 pulses/HRT. Regarding the degradation rates, the compounds were removed at 1 mg/(L·h) under steady-state conditions. Fillat and Roncero (2009) also reported an enhancement on the enzymatic treatment applied on biocleaching processes in the presence of high concentration of oxygen. However, Dasgupta et al. (2007) reported no effect of dissolved oxygen concentration on the removal of phenolic compounds by laccase.

It is important to remark that although the percentages of removal were improved by means of increasing the contact time between the compounds and the biocatalyst, the degradation rates were significantly decreased due to the decrease in the addition rate. Thus, the effect of the addition rate was also studied by using two different values: 1 and 4 mg/(L·h). The results were promising in terms of degradation rates: values of 2.56 and 2.92 mg/(L·h) were found for E1 and E2, respectively, for a feed addition rate of 4 mg/(L·h); however, the percentages of degradation decreased to 64 and 73 % (Figures 2A-4 and 2B-4). Other authors reported the complete degradation of E1 after 1 h of treatment with laccase (Tamagawa et al., 2006), while Suzuki et al. (2003) indicated that the use of the mediator HBT was required to attain removal percentages over 80 % for E2 for an identical period.

### 3.3. Reduction in the estrogenic activity

In the present study the reduction in the estrogenic activity was calculated for the run 4, in order to investigate the potential estrogenic activity of the oxidation products. It was found that the residual estrogenic activity of the effluent was largely reduced up to 97 %. Therefore, the feasibility of the laccase and the EMR for the continuous degradation of estrogenic compounds was confirmed for the degradation of this type of compounds.

## 4. Conclusions

The feasibility of the laccase-catalyzed system to degraded estrogenic compounds was demonstrated in previously investigations. In the present work, an enzymatic membrane bioreactor was proposed for the continuous removal of estrogens in an attempt to implement the technology in the treatment of wastewaters. The effects of laccase activity, contact time between the target compounds and the biocatalyst and the oxygen supply on the degradation of estrone and estradiol were investigated with the aim of optimizing the treatment. Not only high degradation percentages and removal rates were attained but also an important reduction in the estrogenic activity was detected. Therefore, the technology proposed is a promising system in the application of laccases for the removal of potential endocrine disrupting compounds. The EMR designed allowed the reuse of the enzyme providing an important cost reduction as well as to avoid process stream contamination. A further study should be carried out in order to investigate the individual effect of the variables tested as well as the synergic effects by response surface methodology.

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