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Degradation of estrogens by laccase from *Myceliophthora thermophila* in fed-batch and enzymatic membrane reactors

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ABSTRACT

Several studies reported that natural and synthetic estrogens are the major contributors to the estrogenic activity associated with the effluents of wastewater treatment plants. The ability of the enzyme laccase to degrade these compounds in batch experiments has been demonstrated in previous studies. Nevertheless, information is scarce regarding in vitro degradation of estrogens in continuous enzymatic bioreactors. The present work constitutes an important step forward for the implementation of an enzymatic reactor for the continuous removal of estrone (E1) and estradiol (E2) by free laccase from *Myceliophthora thermophila*. In a first step, the effect of the main process parameters (pH, enzyme level, gas composition (air or oxygen) and estrogen feeding rate) were evaluated in fed-batch bioreactors. E1 and E2 were oxidized by 94.1 and 95.5%, respectively, under the best conditions evaluated. Thereafter, an enzymatic membrane reactor (EMR) was developed to perform the continuous degradation of the estrogens. The configuration consisted of a stirred tank reactor coupled with an ultrafiltration membrane, which allowed the recovery of enzyme while both estrogens and degradation products could pass through it. The highest removal rates at steady state conditions were up to 95% for E1 and nearly complete degradation for E2. Furthermore, the residual estrogenic activity of the effluent was largely reduced up to 97%.

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1. Introduction

Over the past decades water pollution by recalcitrant organic compounds has become one of the most serious problems in environmental engineering. The estrogenic activity of endocrine disrupting chemicals (EDCs) in municipal/industrial wastewaters and landfill leachates has been one the main concerns due to their adverse potential effect on human health and wildlife [1–3]. Hormones such as estrone (E1), estradiol (E2) and ethynilestradiol (EE2) are the major contributors to the estrogenic activity in sewage effluents and their presence in water can interfere with animal endogenous hormones even in concentrations as low as 0.1 ng/L [4].

The degradation of this type of compounds implies an important ecological challenge due to their complex structures and low bioavailability [5]. They have all been detected in effluents of wastewater treatment plants since conventional physicochemical or biological treatment can only attain a partial degradation [6]. Certain advanced treatment processes (e.g. ozonation, advanced oxidation processes (AOPs) and reverse osmosis) remove estrogens from wastewater effectively; however, these technologies present several important disadvantages such as high costs, time-consuming methodologies and formation of toxic residues [7–9]. Thus, novel processes are required to treat EDCs in a cost-effective manner.

Enzymatic treatment can be an attractive alternative for the removal of estrogens since these systems potentially have low energetic requirements and can operate at high target compounds concentrations [7,10]. Fungal oxidative enzymes, i.e. manganese peroxidase, lignin peroxidase, versatile peroxidase or laccase, were reported to degrade a wide range of xenobiotics [11]. Laccase (E.C. 1.10.3.2., benzenodiol: oxygen oxidoreductase) is a multi-copper protein which is able to oxidize phenolic substrates by reducing molecular oxygen to water [12]. This enzyme was reported to be a powerful biocatalyst for the biodegradation of recalcitrant compounds such as dyes, aromatic hydrocarbons and pulp delignification [13,14]. Moreover, the use of oxygen as the final electron acceptor represents a considerable advantage for the application of laccase compared with peroxidases, which require the supply of H_2O_2 [15].

An ample review of estrogen removal by microorganisms and enzymes is provided by Cajthaml et al. [16]. Auriol et al. [3] reviewed the most important data published on estrogenicity removal by ligninolytic enzymes. Some other previous investigations reported the ability of different free laccases to degrade EDCs. For example, Tanaka et al. [17] reported the degradation of EE2 by

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90% within 48 h using 800 U/L of laccase from *Trametes* sp. and *Pycnoporus coccineus*. Auriol et al. [9] attained complete degradation of E1, E2 and EE2 after 1 h treatment using 20,000 U/L and Suzuki et al. [18] transformed E2 and EE2 by 100% within 1 h incubation using the laccase-HBT system. We recently demonstrated the capability of laccase from *Myceliohpthora thermophila* to degrade E1, E2 and EE2 within a very short incubation period (30 min), lower enzyme activity (2000 U/L) and no laccase mediator [5].

However, once the capacity of laccase for the removal of estrogens has been demonstrated, technology must be developed for the efficient application of the biocatalyst. Although several authors have dealt with the capability of enzymes to degrade certain EDCs, relatively low effort has been devoted to the development of the technology for its application. Important issues to be considered for the implementation of the system are the stability of the enzyme, the non-use of a toxic mediator, the effective reduction of the estrogenic activity after treatment or the stability of the bioreactor operation.

In addition, the major drawback when operating with free enzymes in a conventional reactor is the large consumption of enzyme, which is washed out with the treated effluent. Thus, the recovery of the enzyme and its reusability for the continuous operation of an enzymatic reactor are key factors because the cost of the biocatalyst may limit its application. This limitation could be overcome by connecting the bioreactor with an ultrafiltration membrane enabling the recovery of the enzyme back to the reaction vessel. The main characteristic of this enzymatic membrane reactor (EMR) is the separation of biocatalysts from products and/or other substrates by a semi permeable membrane that creates a selective physical/chemical barrier [19]. That type of reactor present several advantages such as high enzyme loads, prolonged enzyme activity, high flow rates, reduced energy requirements, simple operation and control of the reactor and straightforward scale-up [19,20]. Although the feasibility of EMRs for the removal of different dyes by laccases has been demonstrated [21,22], the utility of this configuration for the removal of other recalcitrant compounds has not been evaluated. Those positive aspects present EMR as a possible sustainable technology for the EDCs removal.

The key goal of the present work was to develop a bioreactor for the degradation of estrogens with maximum efficiency and minimal enzyme requirements. The first objective was to determine the effect of the main variables which could affect the efficiency of the system (pH, aeration/oxygenation, estrogen feeding rate and enzymatic activity). These assays were carried out in fed-batch reactors where the estrogens were added in pulses. A second goal was to implement an EMR for the continuous elimination of estrogens. Moreover, oxygenation and hydraulic residence time were evaluated in the continuous treatment. The reduction in the estrogenic activity was also demonstrated.

2. Materials and methods

2.1. Chemicals and enzyme

All chemicals were of analytical grade. Both estrogens, estrone (E1) and 17β-estradiol (E2), were obtained from Sigma–Aldrich (USA). 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) was supplied from Fluka (USA).

Commercial laccase (Novozym 51003) from *Myceliophthora thermophila* was supplied by Novozymes (Denmark). This enzyme (molecular weight of 56,000 Da) was produced by submerged fermentation of genetically modified *Aspergillus* sp.

2.2. Enzymatic activity and estrogens analysis

A colorimetric assay was used to quantify the activity of the enzyme by the use of ABTS as substrate, and the concentration of the estrogens was determined by high performance liquid chromatography (HPLC). Both equipments and methodologies used were described in a previous work by Lloret et al. [5].

2.3. Degradation of estrogens by laccase

2.3.1. Fed-batch degradation

The oxidation of E1 and E2 in fed-batch mode was carried out in a 250 mL reactor, equipped with pH, temperature and pO_2 sensors and coupled with an online data collection and acquisition system. The reaction medium consisted of a mixture of E1 and E2 (5 mg/L each) and a single initial pulse of laccase (500 or 2000 U/L). Fed-batch addition of the estrogens (625 μL of a stock solution of 2000 mg/L of each compound, prepared in methanol) was carried out during the course of the reaction to add 5 mg/L of each estrogen into the reactor. Temperature was controlled at 26 °C and continuous magnetic stirring at 250 rpm.

Several experiments were conducted in order to evaluate the effect of different process parameters: pH, aeration and oxygenation, frequency of estrogen pulses and enzymatic activity. These conditions are summarized in Table 1.

It was demonstrated that the commercial laccase used presented its highest activity at acid pH, although it is quite unstable under these conditions. On the other hand, the enzyme presents a great stability at pH 7, although its relative activity decreases significantly at basic pH [5]. Therefore, pH values of 4 and 7 were selected to carry out the experiments in order to investigate the effect of the pH on the degradation as well as on the laccase stability under operational conditions. The effect of aeration/oxygenation was studied in an attempt to improve the enzymatic catalysis action since it is well known that laccases use oxygen as electron acceptor [12]. In previous batch experiments the capability of the commercial laccase to degrade the target compounds at an initial activity of 2000 U/L, has been demonstrated [5]. In the current study the initial amount of enzyme used was reduced in order to minimize its consumption. Finally, the frequency of pulses addition was reduced aiming to increase the efficiency by a longer contact time between the substrates and the enzyme.

The experimental design is detailed below. In a first step, two different values of pH (4 with 0.1 M sodium acetate buffer and 7 with 0.1 phosphate buffer) were considered (Experiments 1–2). Initial activity laccase was 2000 U/L and pulses of estrogens were added every hour. Since pH 7 provided the highest degradation levels, it was selected for further assays. In the following step the effect of the aeration was analyzed by supplying the reactor with 0.5 mL/min of air (Experiment 3). No significant improvement on the removal yields was observed under air supply. Consequently, two different strategies were investigated with the aim of increasing the removal of E1 and E2: the change of the estrogens frequency pulses to 2 h and the gas supply by periodic pulses of pure oxygen (1 bar for 30 s, every 1 h). Both strategies were studied at two levels of laccase activity: 500 and 2000 U/L (Experiments 4–5 and 6–7) in order to study the effect of the initial activity as well as to attempt to reduce the enzyme required to attain high degradation percentages. Finally, the combination of the conditions which provided the best results (pH 7, oxygenation, pulses of estrogens every 2 h and initial activity laccase of 500 U/L) was evaluated (Experiment 8).

To verify that degradation took place only due to enzymatic oxidation, controls were run in parallel without laccase. Samples were withdrawn during the course of each experiment to determine the evolution of laccase activity and the concentration of the estrogens. Reactions were stopped with 0.25 M hydrochloric acid to

Table 1
Comparative parameters of different operation strategies for fed-batch experiments.

Experiment	pH	Aeration/oxygenation	Estrogens pulses frequency (h)	Initial laccase activity (U/L)	Estrone degradation (%)	Estradiol degradation (%)
1	4	–	1	2000	59.2	73.3
2	7	–	1	2000	70.9	91.5
3	7	Aeration	1	2000	71.2	91.6
4	7	–	2	2000	92.4	93.8
5	7	–	2	500	90.5	92.0
6	7	Oxygenation	1	2000	90.1	93.5
7	7	Oxygenation	1	500	83.7	90.2
8	7	Oxygenation	2	500	94.1	95.5

deactivate the enzyme and samples were frozen for further analysis. The degradation yields were calculated at the end of each experiment by taking into account the total amount of each estrogen added in the experiment as well as the amount degraded.

2.3.2. Continuous degradation in an enzymatic membrane reactor

The continuous enzymatic reactor consisted of the stirred tank reactor (250 mL) used in the previous fed-batch operation which was coupled to an ultrafiltration polyethersulfone membrane (Prep/Scale-TFF Millipore) with a nominal molecular weight cutoff of 10 kDa, 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. Therefore, the total volume of the reactor system and which was considered in terms of hydraulic residence times (HRT) and reaction rates calculations was 370 mL. 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.

The influent containing a mixture of the estrogens (4 mg/L of each in 100 mM sodium phosphate buffer, pH 7) was continuously fed into the vessel by a peristaltic pump, while the laccase was only added in a single initial pulse of 500 U/L. A second pump was used to circulate the vessel effluent into the membrane module and is also returning the concentrate (enzyme) back into the reactor. Then, a valve located in that module was used to control both the reactor effluent and the recycling flow rates. The enzyme was recycled in a recycling:feed flow ratio 12:1 and the flow rate of the effluent of the reactor system was maintained in the same value as the influent.

An electrovalve located at the end of a flexible membrane tube (FMT) controlled by a cyclic timer was used to inject oxygen with a pulsing flow of 1 bar for 30 s each pulse. Two oxygen supply strategies were assayed: (i) a less regular addition every 1 h and (ii) a more frequent addition every 30 min. The lowest oxygenation frequency was assayed at a HRT of 2 h (which corresponded to a feed rate addition of 2 mg/L h). In order to investigate the effect of the HRT and to improve the degradation, the highest oxygenation frequency was evaluated at two different values of HRT: 2 and 4 h (feed addition rate 1 mg/L h). The conditions of these assays are detailed in Table 2.

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. In order to evaluate the potential stripping of the estrogens by gas flushing, a control lacking laccase was run in parallel under the highest oxygenation conditions. No removal of the compounds was observed after 10 h of continuous operation so it is assumed that the elimination occurred completely due to the enzymatic effect.

2.3.2.1. Membrane efficiency analysis. The suitability of the selected membrane was demonstrated by circulating a solution of laccase through the membrane under the operational conditions described above, and measuring enzyme activity in permeate and retentate. No loss of activity in the permeate was observed, concluding that the membrane retained the enzyme efficiently. Moreover, it was proved that no physical adsorption of the estrogens onto the membrane took place.

2.4. Evaluation of estrogenic activity

The estrogenic activity was measured by the LYES (lyticase yeast estrogen screen) assay assisted by enzymatic digestion with lyticase previously described by Routledge and Sumpter [23]. The recombinant yeast *Saccharomyces cerevisiae* was kindly provided by the Laboratory of Microbial Ecology and Technology (Labmet, Ghent University, Belgium). Yeast pre-cultures were inoculated in yeast-peptone-dextrose medium (yeast extract 10 g/L, casein peptone 20 g/L, dextrose 20 g/L in distilled water) and incubated at 30 °C for 48 h. Standards and samples withdrawals of 50 μL were delivered into a spectrophotometer cell. Distilled water was used as control. Each well was inoculated with 450 μL of yeast suspension. The plate was sealed and incubated at 37 °C. After 24 h, 200 μL of a lyticase solution diluted in Z-buffer containing 10× (60 mM $Na_2HPO_4{\cdot}7H_2O$, 40 mM NaCl, 1 mM $MgSO_4{\cdot}7H_2O$, 50 mM 2-mercaptoethanol) was added. The solution was incubated for 45 min at room temperature and then 175 μL Tween 80 (0.1% v/v) was added. After 20 min of incubation (room temperature), 125 μL chlorophenol red galactopyranoside (1/g L) was added. Finally, absorbances at 550 nm and 630 nm were measured after 2 h. The estrogenic activity was calculated as shown below:

$$\text{Response} = (A^{X}_{550\,\text{nm}} - A^{blk}_{550\,\text{nm}}) - (A^{X}_{630\,\text{nm}} - A^{blk}_{630\,\text{nm}}) \quad (1)$$

where X corresponded to the sample and blk to the control.

Biotic controls incubated without estrogens showed no estrogenic activity.

2.5. Identification of biodegradation products

The monitoring of degradation products from each target compound was attempted in batch experiments performed at pH 7 with an initial concentration of each estrogen of 5 mg/L and 2000 U/L of laccase in a final volume of 100 mL. The reaction was conducted for 8 h and then the mixture was acidified to a final pH of 2 to inactivate the enzyme. Acidified samples (20 mL) corresponding to 0 and 8 h were diluted in 100 mL of water. The Solid Phase Extraction (SPE) was carried out with 60 mg OASIS HLB cartridges (Water closet, Milford, MA, USA) previously supplemented with 3 mL of ethyl acetate, 3 mL of methanol and 3 mL of distilled water (pH 2) adjusted to pH 2 by hydrochloric acid (1 M). The cartridges were then dried with nitrogen stream for 45 min and eluted with 3 mL of ethyl acetate. An aliquot of 800 μL of the extract was withdrawn and 200 μL of BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) was added for the derivatization of the species. Afterwards, samples were analyzed by Gas Chromatography–Mass Spectrometry (GC–MS) (Saturn 2100T, Varian, USA).

Table 2
Estrone and estradiol degradation (%) after continuous operation in the enzymatic membrane reactor.

Experiment	pH	Estrogens addition rate (mg/L h)	Hydraulic residence time (h)	Initial laccase activity (U/L)	Oxygenation pulses frequency	Estrone degradation (%)	Estradiol degradation (%)
1	7	2	2	500	1 h	58.9	65.9
2	7	2	2	500	30 min	68.4	80.6
3	7	1	4	500	30 min	95.6	>98[a]

[a] Concentration of estrogens was below the detection limit.

3. Results and discussion

3.1. Fed-batch degradation of estrogens

Different operational conditions, such as the most relevant operational parameters in an enzymatic treatment: substrate feeding rate, pH and enzyme activity [24], were tested in order to obtain the optimal conditions for the transformation of the estrogens in fed-batch reactors. The degradation yields calculated after each assay are summarized in Table 1.

3.1.1. Effect of pH

Two different values of pH were considered according to previous results carried out in batch experiments [5]. In this report, pH 4 implied the maximal enzyme activity although enzyme stability was very low. On the contrary, the activity of enzyme was lower at pH 7 but remained highly stable for hours [5].

Fig. 1 shows the profiles of estrogens and laccase activity for this set of experiments. As expected, remarkable inactivation at pH 4 was observed during the initial minutes of the reaction. Laccase activity immediately decreased from 2000 U/L to 500 U/L and reached values close to 80 U/L after 6 h. Conversely, no initial inactivation was observed at pH 7, and enzyme was stable during the 6 h of operation. This difference in enzymatic activity was probably the reason behind the different removal percentages (Table 1 – Experiments 1 and 2). Both E1 and E2 were degraded more efficiently at pH 7 (70.9 and 91.5%, respectively) than at pH 4 (59.2 and 73.3%).

The same effect was observed by Kim and Nicell [25] who attained the maximum conversion bisphenol A by laccase from *Trametes versicolor* in batch experiments at pH 6, which corresponds to a value of pH that implies higher stability of the enzyme. They explained this phenomenon due to two possible reasons: (1) lower loss in activity due to inactivation and/or (2) increased rate of interaction between the substrate and enzyme.

Envisaging the operation of a continuous reactor, the stability of the enzyme is a key parameter. It was observed that the operation at pH 7 was beneficial for enzyme stability and removal percentages. Neutral values of pH are also favorable when working with real effluents, avoiding their acidification for the laccase treatment. Auriol et al. [9] evaluated the removal of estrogens from synthetic wastewaters by laccase and they observed that the optimal pH was 6–7, which was related to the enzyme stability; additionally no significant effect on the catalytic performance of the enzyme was derived from the use of the municipal wastewater.

It is also interesting to remark that apparently the degradation rates attained after each estrogens pulse increased with the subsequent batches in spite of the enzyme inactivation at pH 4. For instance, 2 mg/L of estrone was degraded during the first batch, while the elimination increased to 3 mg/L after the two next additions. It can be explained by the higher initial estrogens concentration due to their accumulation into the reactor, which may imply greater reaction rates. Nevertheless, the values of reaction constants calculated by fitting the experimental data of each batch to first order reaction kinetics (data not shown) evidenced the correlation between the initial enzyme activity and the degradation rates: the reaction constants decreased progressively from the first to the sixth batch.

3.1.2. Effect of aeration/oxygenation

Oxygen actively participates in the catalytic cycle of laccase. It acts as the electron acceptor, which in turn is reduced to water while the oxidation of the laccase takes place for the subsequent oxidation of the substrate. Thus, the degradation yields are expected to be improved when the reaction rates between the laccase and the oxygen increase due to high oxygen concentrations. Two strategies were evaluated aiming to raise the oxygen concentration into the reactor: continuous aeration and oxygenation by pulses (every 1 h).

When the experiment was carried out with continuous air supply, no differences were observed in terms of estrogens degradation (Table 1 – Experiment 3) in comparison with non-aerated experiment (Table 1 – Experiment 2). However, under oxygen supply, the removal percentages increased (Fig. 2), especially for E1 reaching an elimination of 90.1% (Table 1 – Experiment 6).

The supply of extra air in a continuous flow did not exert any significant effect because agitation was sufficient to maintain the concentration of dissolved oxygen close to the saturation values.

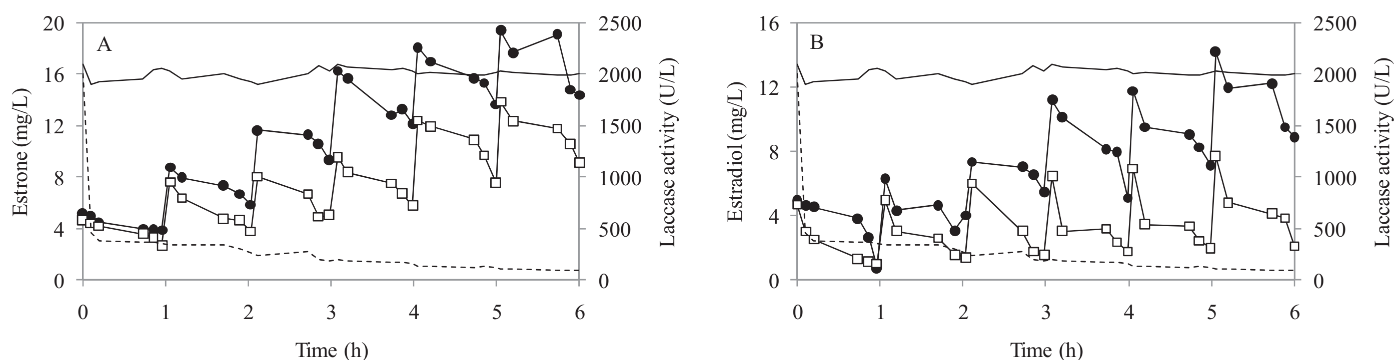

Fig. 1. Estrone (A) and estradiol (B) concentration during the treatment by laccase with fed-batch estrogens addition: experiment 1 (●) and 2 (□) from Table 1. Laccase activity profiles are also shown: experiment 1 (- - -) and 2 (—).

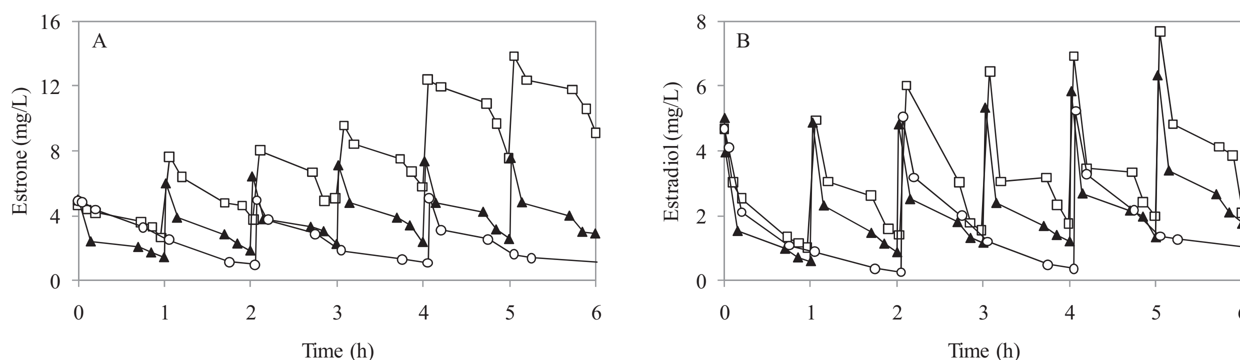

Fig. 2. Estrone (A) and estradiol (B) concentration during the treatment by laccase with fed-batch estrogens addition: experiment 2 (□), 4 (○) and 6 (▲) from Table 1.

However, when oxygen was supplied, the concentration of dissolved oxygen reached maximum values of 30 mg/L and it did not decrease below 13 mg/L. There are only a few reports evaluating its effect on the biobleaching of eucalyptus kraft pulp [26–28]. Fillat and Roncero [26] studied the influence of aeration/oxygenation, observing that kinetics of biobleaching was enhanced in both cases, especially when oxygen was supplied and reached concentrations above 20 mg/L. Ghosh et al. [29] reported improvements in the removal of phenol by laccase by means of the increase of dissolved oxygen concentration in the reactor. These authors investigated different alternatives to increase the concentration of dissolved oxygen: agitation, addition of oxidizing chemicals and air sparging, and they concluded that the highest efficiency was achieved by air bubbling.

3.1.3. Effect of frequency of estrogens addition

Due to the evidence of accumulation of E1 and E2 in the previous experiments, another strategy was proposed in order to enhance the degradation percentages: reduce the estrogen addition frequency (2 h) with the objective of increasing the time of laccase action (Fig. 2). As expected, this time was adequate for the enzyme to transform both compounds at percentages higher than 90% (Table 1 – Experiment 4). Hence, the change in the estrogen addition frequency led to a progressive elimination of the estrogens present in the effluent. Indeed, the degradation of both estrogens was quite similar, reaching similar removal percentages (92.4 and 93.8 for E1 and E2, respectively).

3.1.4. Effect of enzyme activity

One of the limitations of enzymatic treatment is the amount of enzyme required to achieve high removal efficiency due to the susceptibility of the enzyme to inactivation [28]. The primary objective of any engineering design should be to minimize the cost to make the process feasible. In an enzymatic treatment, the enzyme is the most expensive component and it should be minimized [30]. Consequently, a 4-fold reduction of the initial enzyme activity was considered.

An initial activity of 500 U/L was evaluated for non-oxygenated experiments with estrogens pulses every 2 h and for oxygen experiments with estrogens pulses every 1 h (Table 1 – Experiments 5 and 7). In both cases, the degradation percentages were slightly lower compared with their corresponding experiments with 2000 U/L. Thus, the effect of initial laccase activity can be analyzed by comparing two pairs of experiments: Experiments 4–5 and 6–7. From both pairs of operations the conclusions were similar: the removal was lower when the initial laccase activity was 4-fold lower, but this reduction was not as significant as expected. For example, E1 and E2 removal decreased from 92.4 to 90.5% and from 93.8 to 92.0%, respectively, by 2000 and 500 U/L, for non-aerated/oxygenated assays (Experiments 4–5). The same phenomenon was observed for the oxygenated treatments: reductions from 90.1 to 83.7% and from 93.5 to 90.2% were found (Experiments 6–7). On the other hand, the degradation percentages attained in Experiment 5 with 500 U/L seemed to be better than those obtained in Experiment 2 with 2000 U/L. However, the global amount of estrogens treated was significantly lower: 30 mg/L (estrogens pulses of 5 mg/L every hour) and approximately 15 mg/L when the additions were conducted every 2 h.

Auriol et al. [9] studied the removal of estrogens by laccase at different enzyme activities in the range 2000 U/L to 20,000 U/L. They found that when activity was increased, a complete elimination was achieved, whereas at the lowest activity, the removal percentages varied in the range 50–80% for the different estrogens. We lowered the enzyme activity down to 500 U/L, without compromising the efficiency of the system. Furthermore, no mediator of the laccase was needed in order to enhance the catalytic action, while some previous investigations reported the use of natural or synthetic mediators when the initial enzyme activity used was low. For example, Sei et al. [1] reported complete degradation of E1, E2 and EE2 by 100 U/L of a commercial purified laccase in only 1 h, but 1 mM of HBT or ABTS were used as mediators.

3.1.5. Fed-batch operated under the best conditions

Finally the strategies selected throughout this study were combined in order to obtain an efficient operation of the fed-batch reactor. The operational conditions were: pH 7, pulses every 2 h, oxygen supply and 500 U/L of laccase (Fig. 3). With this strategy, high degradation extent of E1 and E2 was observed during 8 h of operation, with degradation percentages of 94.1 and 95.5%, respectively (Table 1 – Experiment 8).

3.2. Continuous degradation of estrogens

In the current study, we proposed the use of an EMR operated in continuous for the removal of E1 and E2. Once the operational conditions were selected in the fed-batch system, these conditions were applied in the continuous reactor.

HRT is related to the desired conversion (degradation) of pollutants, which is directly affected by the pollutant concentration. Thus, a higher loading rate could lead to a faster but less efficient process. Therefore, a HRT of 2 h (feed addition rate of 2 mg/L h) and 4 h (feed addition rate 1 mg/L h) were selected in order to study its effect. Although previous experiments showed an important effect of the oxygen supply, the frequency of oxygenation was not evaluated. Hence, two different strategies for the oxygenation were assayed: oxygen supply every 1 h (at HRT of 2 h) and oxygen supply every 30 min (at HRT 2 and 4 h). Although O_2 concentration in the reaction medium varied in both cases in the range 13–30 mg/L, different average concentrations of dissolved oxygen were observed:

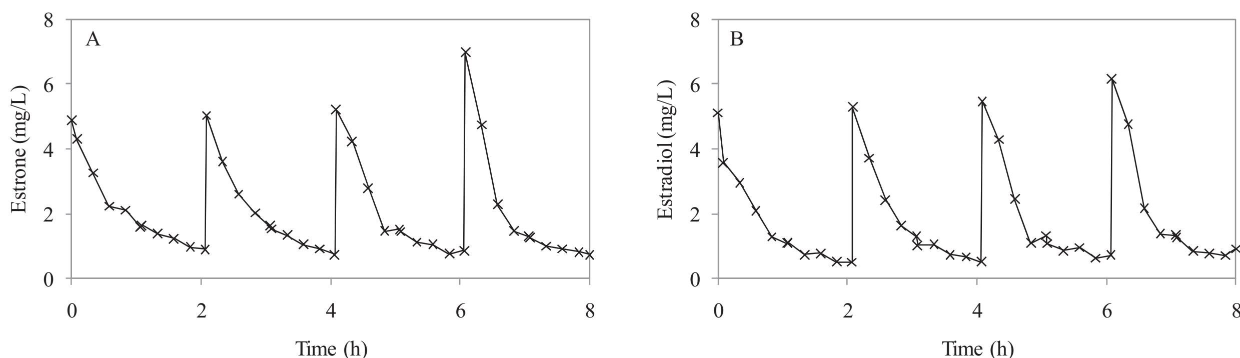

Fig. 3. Estrone (A) and estradiol (B) concentration during the treatment by laccase with fed-batch estrogens addition under the selected conditions (experiment 8 from Table 1).

20 and 26 mg/L for the less and more frequent O_2 supply, respectively. These conditions and results are summarized in Table 2.

Fig. 4 shows the E1 and E2 degradation rates during 10 h of continuous operation. It can be observed that steady state was reached earlier when oxygenation was performed more frequently (less than 1 h for 30 min O_2 pulses and about 2–3 h for 1 h O_2 pulses). Furthermore, when the HRT was 2 h, the removal percentages at steady state conditions were superior in the case of the higher oxygenation (68.4% for E1 and 80.6% for E2). Whereas when lower oxygenation was performed, only 58.9% of E1 and 65.9% of E2 were removed at stationary conditions. It is important to highlight that enzymatic activity did not decrease throughout the experiment, and it was not affected by the oxygen supply.

With the aim of improving the removal percentages and in order to study the effect of the HRT, a third assay was conducted at the optimum oxygenation conditions (pulses every 30 min) and at a higher HRT of 4 h (feed addition rate 1 mg/L h). Under those conditions, E1 was removed up to 95% and no E2 was detected in the effluent. Therefore, as can be observed in Fig. 4, a degradation rate of 0.95 mg/L h and 1 mg/L h of E1 and E2 was attained, respectively, under steady-state conditions.

There is no extensive information concerning in vitro degradation of estrogens in continuous bioreactors. The biodegradation of E2 and EE2 by *Trametes versicolor* cultures has been reported, and evidences of laccase involvement were found. For instance, Blánquez and Guieysse obtained a removal rate of E2 (0.16 mg/L h) in the same order of magnitude as those achieved by fungal enzymes, bacterial communities or bacterial isolates [31]. However, in the present article, we operated the continuous bioreactor reaching a degradation rate of E2 almost 10 times higher (1.5 mg/L h).

Other investigations dealt with the removal of other estrogenic compounds in continuous processes by immobilized laccases on solid supports. For example, Nicolucci et al. [32] removed almost 100% of 1 mM of bisphenol A by laccase immobilized on polyacrylonitrile beads after operating a fluidized bed reactor for 90 min. Cabana et al. [33] attained similar removal levels by laccase immobilized on Celite carriers by the use of a packed bed reactor. However, the technology proposed in the current work (continuous enzymatic treatment by free laccase retained by means of an ultrafiltration membrane) presents several advantages: (i) operation with free enzyme, avoiding limitations of mass transfer and, consequently, low kinetic rates; (ii) minimized loss of enzyme activity; (iii) lower inhibition by products; (iv) simple operation; (v) lower variability in the quality of the end-products; (vi) retention of non-biodegradable molecules with high molecular weight; etc. [24,30,34]. In addition, one of the main advantages of EMR is that fresh enzyme can be easily added to maintain the productivity [35].

3.3. Determination of estrogenic activity reduction

Some investigations reported the removal of estrogens by enzymatic treatment; however they have no verified the residual estrogenicity after the process. In this work, the estrogenic activity of the effluent was assessed by the LYES assay and compared with that of the influent. A 97% reduction of toxicity was reached in the continuous enzymatic membrane reactor, supplying O_2 every 30 min. A low estrogenic activity which may still be present in the treated aqueous solution could be attributed to residual traces of estrogens and to the synergic phenomena between the compounds that remained in solution. These results are consistent which previous studies [3,18,36], which reported good removal of estrogenicity

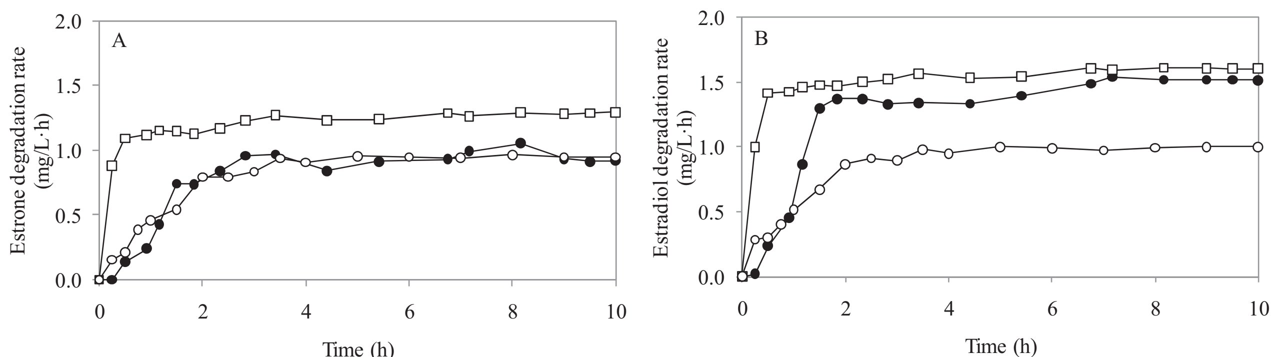

Fig. 4. Estrone (A) and estradiol (B) degradation rates by laccase in the enzymatic membrane reactor: experiment 1 (●), 2 (□) and 3 (○) from Table 2.

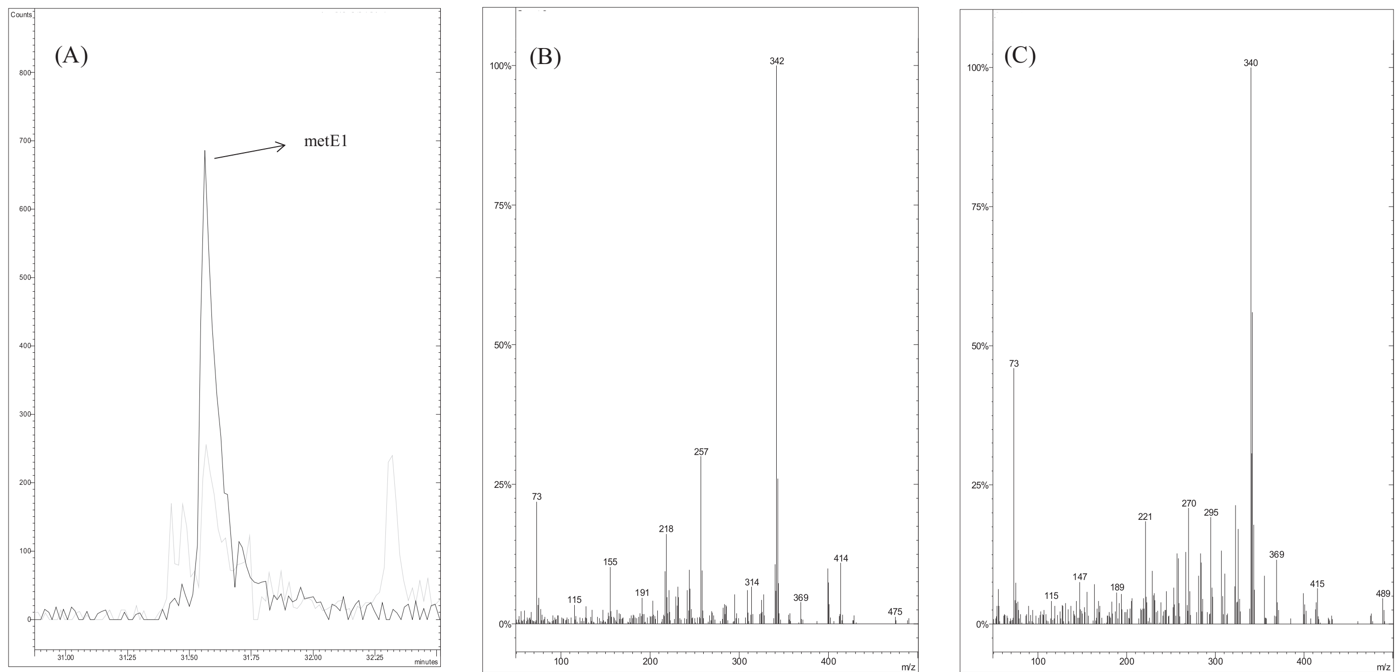

Fig. 5. Chromatogram of E1 removal samples at times 0 (grey line) and 8 h (black line) for m/z:340 (A) and mass spectra of E1 (B) and metE1 (C).

associated with E1 and E2 by manganese peroxidase and laccase-catalyzed treatment in batch experiments.

3.4. Identification of biodegradation products

In order to identify transformation products of E1 and E2, batch assays with 2000 U/L of laccase were carried out in Erlenmeyer flasks. Samples at initial time and after 8 h of enzymatic treatment were analyzed by GC–MS.

In the case of E1, a major metabolite with a quantification ion of m/z:340 was found. The GC–MS chromatograms of samples at 0 and 8 h are shown in Fig. 5A. The peak at a retention time of 31.6 min for the sample of 8 h may correspond to a new metabolite of E1 (metE1). Although the retention time was quite similar to that of E1, the mass spectrum was different. The mass spectra of E1 and metE1, after derivatization, are shown in Fig. 5B and C, respectively. With regard to E2, two different bioproducts (metE2-1 and metE2-2) were observed by analyzing the sample after 8 h of batch treatment (Fig. 6A). None of these peaks were detected in samples either at time 0 or in blanks. Thus, those new peaks may be considered as bioproducts formed by the laccase-catalyzed treatment of E2. The mass spectra of those metabolites are presented in Fig. 6B and C. In spite of the great effort, the identification of the metabolites previously detailed was not possible. However, a more exhaustive

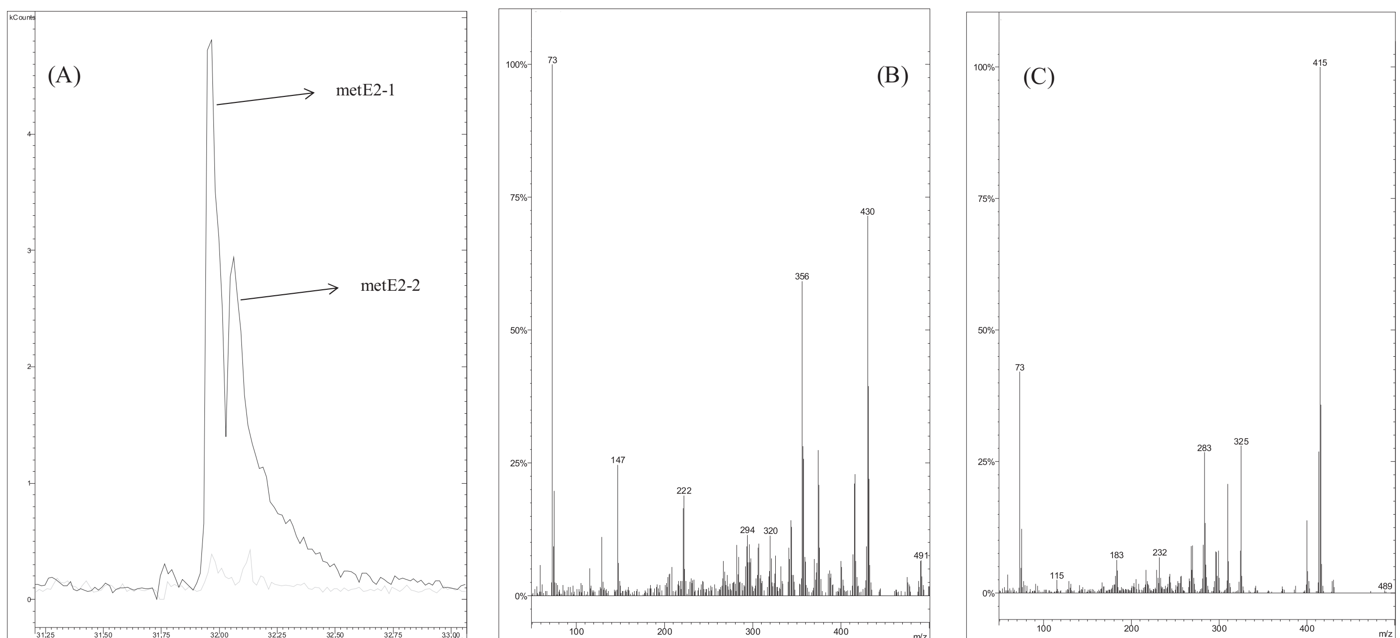

Fig. 6. Chromatogram of E2 removal samples at time 0 (grey line) and 8 h (black line) for m/z:430 + 356 (A) and mass spectra of metE2-1 (B) and metE2-2 (C).

study will be done aiming to identify those biodegradation products by other techniques and the comparison with standards to ensure their identification.

Some previous authors reported different outcomes regarding the identification of degradation products from estrogens. Suzuki et al. [18] obtained high removal yields of E2 by the laccase-mediator system in batch operation although they found no metabolites by HPLC analyses and they assumed the cleavage of the aromatic rings of the compounds. This assumption was confirmed by Nicotra et al. [37] by NMR analyses. On the other hand, the removal of E1 and E2 could be due to polymerization brought about by enzymatic oxidation since those compounds have a *para*-substituted phenol structure. Indeed, the enzymatic-catalyzed oxidative coupling of phenolic compounds has been studied by several authors [7,38], and they suggested that laccase oxidizes organic substrates to free radicals, which can undergo oxidative coupling reactions, producing dimers, oligomers and polymers. Other authors reported that E2 is oxidized to E1 which is further eliminated in aerobic batch experiments in a STP [2]. Other different results were found by the removal of estrogens by other mechanisms. For instance, Bila et al. [39] reported the transformation of E2 to hydroxi-estradiol by ozonation, while Mazellier et al. [40] demonstrated the formation of quinone methide and quinone derivates after photodegradation treatment.

4. Conclusions

In the present work, fed-batch experiments have been carried out in order to evaluate the influence of the main operational parameters: pH, laccase activity, substrate frequency addition and aeration/oxygenation, on the removal of E1 and E2 by a commercial laccase. The influence of aeration was negligible, while the oxygen supply by pulses allowed enhancing the degradation efficiency attained. On the other hand, initial laccase activity was successfully reduced in an attempt to decrease the treatment cost. Moreover, free laccase was applied for the first time for the continuous removal of E1 and E2 in an enzymatic membrane bioreactor. Estrone was degraded up to 95% and estradiol was not detected in the effluent under steady state conditions. Additionally, the residual estrogenic activity was significantly reduced by more than 95%. Overall, our results showed that the technology proposed is a promising tool to increase the applicability of laccases in bioremediation processes. Currently, on-going efforts are focused to investigate the transformation products as well as the removal of estrogens at environmental concentrations in real matrices and for longer treatment periods.

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References

[1] K. Sei, T. Takeda, S.O. Soda, M. Fujita, M. Ike, Removal characteristics of endocrine-disrupting chemicals by laccase from white-rot fungi, J. Environ. Sci. Health, Part A: Toxic/Hazard. Subst. Environ. Eng. 43 (2008) 53–60.
[2] T.A. Ternes, P. Kreckel, J. Mueller, Behaviour and occurrence of estrogens in municipal sewage treatment plants – II. Aerobic batch experiments with inactivated sludge, Sci. Total Environ. 225 (1999) 91–99.
[3] M. Auriol, Y. Filali-Meknassi, C.D. Adams, R.D. Tyagi, T.-N. Noguerol, B. Piña, Removal of estrogenic activity of natural and synthetic hormones from a municipal wastewater: efficiency of horseradish peroxidase and laccase from *Trametes versicolor*, Chemosphere 70 (2008) 445–452.
[4] M. Auriol, Y. Filali-Meknassi, C.D. Adams, R.Y. Surampalli, Endocrine disrupting compounds removal from wastewater, a new challenge, Process Biochem. 41 (2006) 525–539.
[5] L. Lloret, G. Eibes, T.A. Lú-Chau, M.T. Moreira, G. Feijoo, J.M. Lema, Laccase-catalyzed degradation of anti-inflammatories and estrogens, Biochem. Eng. J. 51 (2010) 124–131.
[6] S. Esplugas, D.M. Bila, L.G.T. Krause, M. Dezotti, Ozonation and advanced oxidation technologies to remove endocrine disrupting chemicals (EDCs) and pharmaceuticals and personal care products (PPCPs) in water effluents, J. Hazard. Mater. 149 (2007) 631–642.
[7] H.A. Garcia, C.M. Hoffmann, K.A. Kinney, D.F. Lawler, Laccase-catalyzed oxidation of oxybenzone in municipal wastewater primary effluent, Water Res. 45 (2011) 1921–1932.
[8] S. Snyder, S. Adham, A. Redding, F. Cannon, J. DeCarolis, J. Oppenheimer, E. Wert, Y. Yoon, Role of membranes and activated carbon in the removal of endocrine disruptors and pharmaceuticals, Desalination 202 (2007) 156–181.
[9] M. Auriol, Y. Filali-Meknassi, R.D. Tyagi, C.D. Adams, Laccase-catalyzed conversion of natural and synthetic hormones from municipal wastewater, Water Res. 41 (2007) 3281–3288.
[10] H. Cabana, J. Jones, S. Agathos, Elimination of endocrine disrupting chemicals using white rot fungi and their lignin modifying enzymes: a review, Eng. Life Sci. 5 (2007) 429–456.
[11] N. Durán, E. Esposito, Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: a review, Appl. Catal., B 28 (2000) 83–99.
[12] D. Wesemberg, I. Kyriakides, S.N. Agathos, White-rot fungi and their enzymes for the treatment of industrial dye effluents, Biotechnol. Adv. 22 (2003) 161–187.
[13] S. Camarero, D. Ibarra, A.T. Martinez, J. Romero, J.C. del Rio, Paper pulp delignification using laccase and natural mediators, Enzyme Microb. Technol. 40 (2007) 1264–1271.
[14] O. Yamak, N.A. Kalkan, S. Aksoy, H. Altinok, N. Hasirci, Semi-interprenating polymer networks (semi-IPNs) for entrapment of laccase and their use in Acid Orange 52 decolorization, Process Biochem. 44 (2009) 440–445.
[15] A.T. Martinez, M. Speranza, F.J. Ruiz-Duenas, P. Ferreira, S. Camarero, F. Guillen, M.J. Martinez, A. Gutierrez, J.C. del Rio, Biodegradation of lignocellulosics: microbial chemical and enzymatic aspects of the fungal attack of lignin, Int. Microbiol. 8 (2005) 195–204.
[16] T. Cajthaml, Z. Kresinová, K. Svobodová, K. Sigler, T. Rezanka, Microbial transformation of synthetic estrogen 17[α]-ethinylestradiol, Environ. Pollut. 157 (2009) 3325–3335.
[17] T. Tanaka, T. Tonosaki, M. Nose, N. Tomidokoro, N. Kadomura, T. Fujii, M. Taniguchi, Treatment of model soils contaminated with phenolic endocrine-disrupting chemicals with laccase from *Trametes* sp. in a rotating reactor, J. Biosci. Bioeng. 92 (2001) 312–316.
[18] K. Suzuki, H. Hiraia, H. Muratab, T. Nishida, Removal of estrogenic activities of 17β-estradiol and ethinylestradiol by ligninolytic enzymes from white rot fungi, Water Res. 37 (2003) 1972–1975.
[19] C. López, I. Mielgo, M.T. Moreira, G. Feijoo, J.M. Lema, Enzymatic membrane reactor for the biodegradation of recalcitrant compounds. Application to dye decolourisation, J. Biotechnol. 99 (2002) 249–257.
[20] E. Katchalski-Katzir, Immobilized enzymes-learning from past success and failures, Trends Biotechnol. 11 (1993) 471–478.
[21] M. Chhabra, S. Mishra, T.R. Sreekrishnan, Laccase/mediator assisted degradation of triarylmethane dyes in a continuous membrane reactor, J. Biotechnol. 143 (2008) 69–78.
[22] K.P. Katuri, S.V. Mohan, S. Sridhar, B.R. Pati, P.N. Sarma, Laccase-membrane reactors for decolourization of an acid azo dye in aqueous phase: process optimization, Water Res. 43 (2009) 3647–3658.
[23] E.J. Routledge, J.P. Sumpter, Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen, Environ. Toxicol. Chem. 15 (1996) 241–248.
[24] G. Eibes, C. López, M.T. Moreira, G. Feijoo, J.M. Lema, Strategies for the design and operation of enzymatic reactors for the degradation of highly and poorly soluble recalcitrant compounds, Biocatal. Biotransform. 25 (2007) 260–268.
[25] Y.-J. Kim, J.A. Nicell, Impact of reaction conditions on the laccase-catalyzed conversion of bisphenol A, Bioresour. Technol. 97 (2006) 1431–1442.
[26] U. Fillat, M.B. Roncero, Biobleaching of high quality pulps with laccase mediator system: influence of treatment time and oxygen supply, Biochem. Eng. J. 44 (2009) 193–198.
[27] A. Oudia, J. Queiroz, R. Simões, The influence of operating parameters on the biodelignification of *Eucalyptus globulus* kraft pulps in a laccase–violuric acid system, Appl. Biochem. Biotechnol. 149 (2008) 23–32.
[28] D. Moldes, T. Vidal, Laccase-HBT bleaching of eucalyptus kraft pulp: influence of the operating conditions, Bioresour. Technol. 99 (2008) 8565–8570.
[29] J.P. Ghosh, K.E. Taylor, J.K. Bewtra, N. Biswas, Laccase-catalyzed removal of 2,4-dimethylphenol from synthetic wastewater: effect of polyethylene glycol and dissolved oxygen, Chemosphere 71 (2008) 1709–1717.
[30] M.S.A. Ibrahim, H.I. Ali, K.E. Taylor, N. Biswas, J.K. Bewtra, Enzyme-catalyzed removal of phenol from refinery wastewater: feasibility studies, Water Environ. Res. 73 (2001) 165–172.
[31] P. Blánquez, B. Guieysse, Continuous biodegradation of 17beta-estradiol and 17alpha-ethynylestradiol by *Trametes versicolor*, J. Hazard. Mater. 150 (2008) 459–462.

[32] C. Nicolucci, S. Rossi, C. Menale, T. Godjevargova, Y. Ivanov, M. Bianco, L. Mita, U. Bercivenga, D.G. Mita, N. Diano, Biodegradation of bisphenols with immobilized laccase or tyrosine on polyacrylonitrile beads, Biodegradation 22 (2010) 673–683.
[33] H. Cabana, C. Alexandre, S.N. Agathos, P.J. Jones, Immobilization of laccase from the white rot fungus *Coriolopsis polyzona* and use of the immobilized biocatalyst for the continuous elimination of endocrine disrupting chemicals, Bioresour. Technol. 100 (2009) 3447–3458.
[34] G.M. Rios, M.P. Belleville, D. Paolucci, J. Sanchez, Progress in enzymatic membrane reactors – a review, J. Membr. Sci. 242 (2004) 189–196.
[35] A. Bódalo, J.L. Gómez, J. Bastida, M.F. Máximo, M.C. Montiel, Ultrafiltration membrane reactors for enzymatic resolution of amino acids: design model and optimization, Enzyme Microb. Technol. 28 (2001) 355–361.
[36] Y. Tamagawa, R. Yamaki, H. Hirai, S. Kawai, T. Nishida, Removal of estrogenic activity of natural and steroidal hormone estrone by ligninolytic enzymes from white rot fungi, Chemosphere 65 (2006) 97–101.
[37] S. Nicotra, A. Intra, G. Ottolina, S. Riva, B. Daniele, Laccase-mediated oxidation of the steroid hormone 17β-estradiol in organic solvents, Tetrahedron: Asymmetry 15 (2004) 2927–2931.
[38] K. Kunamneni, S. Camarero, C. Garcia-Burgos, F. Plou, A. Ballesteros, M. Alcalde, Review engineering and applications of fungal laccases for organic synthesis, Microb. Cell Fact. 7 (2008) 32.
[39] D. Bila, A.F. Montalvão, D.A. Azevedo, M. Dezotti, Estrogenic activity removal of 17beta-estradiol by ozonation and identification of by-products, Chemosphere 69 (2007) 736–746.
[40] P. Mazellier, L. Méité, J.D. Laat, Photodegradation of the steroid hormones 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) in dilute aqueous solution, Chemosphere 73 (2008) 1216–1223.