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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%.

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