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The potential of *Cerrena unicolor* laccase immobilized on mesoporous silica beads for removal of organic micropollutants in wastewaters

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ABSTRACT

Micropollutants (MPs) can be defined as inorganic and organic substances present at low concentrations (pg/L–ng/L) in the environment, however, having adverse consequences for living organisms even at these low concentrations. To date, an effective and sustainable global strategy against this insidious contamination of aquatic environments barely exists. Source controls and technical systems, such as wastewater treatment plants, function only as partial barriers or not at all. The enzyme laccase was identified as active in the degradation of different MPs. We present data showing that laccase from *Cerrena unicolor* immobilized on mesoporous silica beads controlled porosity carrier (CPC) in a continuous column reactor was suitable for elimination of MPs including bisphenol A(BPA), 4-nonylphenol (NP), and triclosan (TCS). A system equipped with a 3.5×0.7 cm column packed with immobilized laccase CPC beads (43 units/g of enzyme activity), and eluted at 1 mL/min with a solution containing BPA, NP, and TCS each at 50 μM, was able to totally remove the three MPs present in at least 1 L of the solution. These results open the possibility to use immobilized laccase in industrial and domestic processes for the elimination of harmful MPs.

Keywords: Micropollutants; Laccases; Water treatment; Biocatalyst

1. Introduction

A large amount of anthropogenic and natural chemicals has found their way into wastewater, surface water, and groundwater, many of which occur at very low concentrations and are able to disrupt the endocrine system of wildlife and humans with undesirable developmental, reproductive, neurological, and immune effects [1]. A wide variety of natural and man-made substances are thought to cause endocrine disruption. These include pharmaceuticals, dioxin and dioxin-like compounds, polychlorinated biphenyls, DDT, other pesticides, and plasticizers such as BPA. Endocrine disruptors are present in numerous in everyday products including plastic bottles, metal food cans, detergents, flame retardants, food, toys, cosmetics, and pesticides.

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The water systems are contaminated with these industrial and chemical compounds to such an extent, that it has been indicated as "one of the key environmental problems facing humanity". Most of these compounds, inorganic and organic substances, are present at low concentrations in a range of pg/L–ng/L and are therefore termed micropollutants (MPs) [2]. In recent years, an increasing number of studies have revealed severe toxicological effects of MPs [3]. Furthermore, metabolites or degradation products of MPs may also pose a threat to the aquatic environment. MPs such as bisphenol A (BPA: 4, 4′-dihydroxy-2, 2-diphenylpropane), nonylphenol (NP: 4-nonylphenol), and triclosan [TCS: 5-chloro-2-(2, 4-dichlorophenoxy) phenol] (Fig. 1) are widely used in a variety of industrial and residential applications and are suspected of having estrogenic (endocrine-disrupting) activity.

BPA is a raw material for the production of polycarbonates and epoxy resins and is also used as an inert ingredient in pesticides, fungicides, and as a rubber chemical. Most of the NP production goes into the manufacturing of nonylphenol ethoxylates (NPE). NPE are non-ionic surfactants used as cleaning, washing, and foaming agents. TCS is a compound which has antimicrobial properties and it is used in the formulation of soaps and a variety of other personal care products.

White-Rot Fungi are unique organisms possessing powerful oxidative ligninolytic enzyme systems including lignin peroxidase, manganese peroxidase, and laccase. These enzymes, naturally designed to cooperate in the destruction of lignin compartment of the lignocellulose biomass, may however also participate in the degradation of several natural or artificial compounds related or not to lignin. In particular, the extracellular isoenzymes of laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2.) which are multi-copper blue oxidases, widely distributed in many fungal species [4], may have interesting application prospects for bioremediation of hazardous chemicals. Laccases are considered to be non-specific to their substrates and are able to oxidize a wide range of aromatic compounds of interest including a broad range of chemicals such as pharmaceutical and personal care products with potential endocrine disrupting properties.

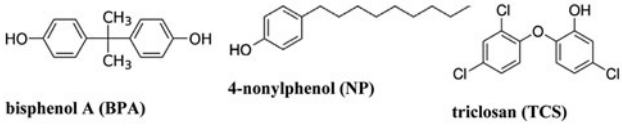

Fig. 1. Endocrine disrupting MPs.

The MPs BPA, NP, and TCS were found to be excellent phenolic substrates for degradation by laccases [5–7]. Therefore, the potential of laccases for solving an acute and insidious environmental pollution problem was explored.

The white rot fungus strain *Cerrena unicolor* emerged from a previous screening as a proficient laccase producer [8] *C. unicolor* C-139 produced 450,000 Units/L of laccase when cultivated in submerged fermentation of wheat bran [9]. This yield of enzyme production was extremely high when compared to yields reported in literature for other strains.

There are several papers describing the activity of free laccase towards MPs [6–8]. Fewer contributions were devoted to the use of immobilized forms of this enzyme, however in batch conditions [9]. Here, we present data showing that laccase from *C. unicolor* immobilized on mesoporous silica beads was suitable for continuous elimination in a column reactor of MPs in solution.

2. Material and methods

2.1. Continuous elimination and transformation of MPs by immobilized laccase

The transformation and elimination of the MPs BPA, NP, and TCS was performed with immobilized enzyme beads in defined conditions. Experiment was performed by using immobilized laccase beads containing 45 U/g laccase activity obtained as described in Songulashvili [9]. Laccase activity was determined by the oxidation of 2,2-azinobis(3-ethylbenzthiazoline)-6 sulphonate (ABTS) method [9]. One unit of the enzyme was defined as the amount of the laccase that oxidized 1 μmol of ABTS substrate per min. 0.82 g

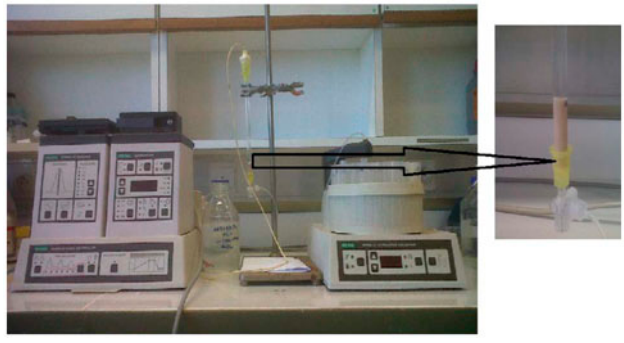

Fig. 2. System for continuous elimination of MPs, equipped with 3.5 × 0.7 cm column packed with laccase immobilized on CPC beads.

immobilized laccase controlled porosity carrier (CPC) beads were packed in a 3.5 × 0.7 cm column (Fig. 2). The three MPs diluted in a 50 mM citrate-phosphate buffer (pH-5.0), each at 50 μM concentration, were run continuously through the column. The flow rate of the mixture of MP's was 1 mL/min. In total, three litres of an aqueous solution of the three MPs were run for elimination and transformation. Samples were taken from each aliquot of 37.5 mL of treated mixture. For the determination of physi- and/or chemisorption of MPs on the CPC beads, the same experiment was done with boiled immobilized laccase CPC beads.

The concentrations of MPs were quantified on an Agilent 1200 HPLC-system (Agilent Technologies) equipped with a LiChrospher 100 RP 18-5 column (Merck KGaA) using a UV detector at a wavelength of 278 nm. Gradient elution started with 10% methanol in water, which was kept for 1 min, followed by an increase to 90% methanol within 4 min. This concentration was kept constant for 4 min and then linearly decreased back to 10% methanol in 1 min and kept constant for another 5 min. Flow rate was 1 mL/min. Temperature was fixed at 40˚C and the concentrations were quantified using external standards.

3. Results and discussion

3.1. Immobilization of laccase and design of a continuous column reactor

The application of enzymes in their native state is affected by significant operational barriers such as reusability, cost, and denaturation of the catalyst. Numerous efforts were devoted to immobilize the enzymes on solid carriers or insolubilize the enzymes, to overcome the drawbacks of using free enzymes [10]. New efficient biocatalysts developed within our research project were applied to different wastewater reactors allowing a continuous passage of effluents to be treated, while retaining the enzymes in the form of complexes. The biocatalyst production might be crucial to reach an economically viable treatment process for two reasons: (i) larger size of the biocatalyst (compared to the free enzyme) allows easier separation of the enzyme from the treated water, avoiding the loss of the enzyme, (ii) inclusion in the biocatalyst can enhance the stability of the enzyme, improving the lifetime of the treatment.

Previous data have shown that in batch conditions, free and immobilized *C. unicolor* laccase was particularly suitable for degradation of NP, BPA, and TCS [9].

We present here a new system of column reactor for continuous elimination of MPs (Fig. 2).

As detailed in material and methods, the MPs containing solution was pumped through a column containing the laccase immobilized on CPC beads. Outlet concentrations of the MPs were measured in fractions of the column eluate.

Immobilization of laccase on CPC beads was used in the present investigation. In this method, the enzyme was immobilized on silanized silica CPC beads, via a cross-linking reaction using glutaraldehyde (Fig. 3) following the procedure described in Songulashvili [9].

The activity of the immobilized laccase was approximately 45 U/g of silica beads. As reported in Songulashvili [9], the pH optimum of the immobilized enzyme was between 2.5 and 3.0, a similar range as determined for the free laccase. The immobilized biocatalyst showed significantly higher thermal stability compared to free enzyme. In the conditions used (citrate phosphate buffer 50 mM – pH 5.0), the immobilized biocatalyst had a half-life of 35 and 10 h compared to 5.5 and 0.43 h for free enzyme at 55 and 75˚C, respectively. In general, the immobilization of an enzyme and in particular, laccase [9] allows increasing its stability in a wide range of temperature, pH, and ionic strength. This is important in the case of use of enzyme technology to real situations where the physicochemical parameters of the medium to be treated can vary over a wide range.

3.2. Continuous elimination of BPA, NP, and TCS by the immobilized laccase

In batch experiments reported previously [9], the CPC immobilized laccase was able to eliminate in 60 min 80% of BPA, 40% of NP, and 60% of TCS from solutions containing 50 μM of each micropollutant separately. The experiments were run three times consecutively with the same batch of immobilized laccase without loss of enzyme activity.

The experiments reported here with the column reactor have shown that this process appears to be very efficient for the elimination of the three MPs.

A typical result of continuous degradation of the three considered MP in this setup is presented in

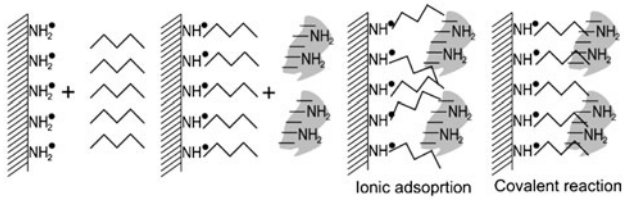

Fig. 3. Immobilization of laccase on CPC beads.

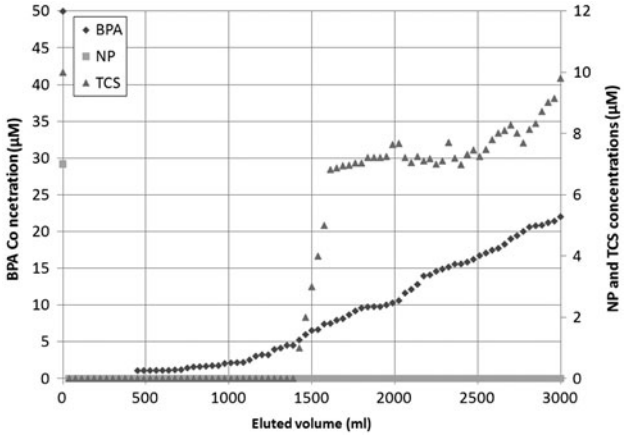

Fig. 4. Continuous elimination of BPA, NP and TCS by *C. unicolor* laccase immobilized on CPC beads.

Fig. 4, where the outlet concentration is shown for fluid samples charged at the inlet with 50 µM of BPA, NP, and TCS, and eluted in the column containing CPC beads with a 43 U/g activity. The results highlight the ability of the system to tackle for long duration for the considered MPs. NP outlet concentration remains undetectable for the whole experiment while BPA and TCS concentration are below detection limits (1 µM) at first, but progressively rises. For BPA, this phenomenon highlight a progressive inactivation of the enzyme while for TCS a more complex, yet to be fully understood, phenomenon takes place. Further investigations will be needed to better explain these data. Moreover, very high concentrations of 50 µM for the MPs were used in the experiments. This value is far from low MPs' concentrations encountered in real effluents [1,2]. Yet, the presently reported experiments should be considered in the framework of an indispensable validation procedure that has to be further adapted to real conditions.

4. Conclusion

C. unicolor laccase was very efficiently immobilized and stabilized on CPC-silica beads and operated in a continuous column reactor. This allowed rapid and efficient elimination of the endocrine disruptors BPA, NP, and TCS from solutions, and opens the possibility to use immobilized laccases in processes at industrial and domestic levels for elimination of harmful MPs. Scaling-up strategies leading to an applicable industrial scale installation of a bioreactor for NP, BPA, and TCS elimination in real industrial effluents has now to be considered. Moreover, the applicability of the system described here should be investigated with other types of MPs as for example organic UV filters in sunscreens, polycyclic musks used as perfumes in detergents (washing powders), and phthalates used in plastics [2].

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