Science of the Total Environment 487 (2014) 748–755

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Synthesis and characterization of combined cross-linked laccase and tyrosinase aggregates transforming acetaminophen as a model phenolic compound in wastewaters

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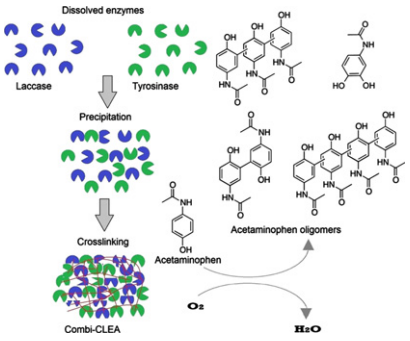
HIGHLIGHTS

- Laccase and tyrosinase were successfully insolubilized as combi-CLEA.
- The pH profiles of the combi-CLEA were broader than those of the individual free enzymes.
- The catalytic efficiencies K_{cat}/K_M were higher for the combi-CLEA than the free enzymes.
- The combi-CLEA displayed considerable denaturation-resistance to the various inhibitors compared to the free enzymes.
- The treatment of acetaminophen in real wastewaters showed high transformation of the drug.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 24 July 2013
Received in revised form 2 October 2013
Accepted 2 October 2013
Available online 26 October 2013

Editor: Damia Barcelo

Keywords:
Laccase
Tyrosinase
Combi-CLEA
Characterization
Acetaminophen
Wastewaters

ABSTRACT

Laccase (EC 1.10.3.2) and tyrosinases (EC 1.14.18.1) are ubiquitous enzymes present in nature as they are known to originate from bacteria, fungi, plants, etc. Both laccase and tyrosinase are copper-containing phenoloxidases requiring readily available O_2 without auxiliary cofactor for their catalytic transformation of numerous phenolic substrates. In the present study, laccase and tyrosinase have been insolubilized as combined crosslinked enzyme aggregates (combi-CLEA) using chitosan, a renewable and biodegradable polymer, as crosslinker. The combi-CLEA, with specific activity of 12.3 U/g for laccase and 167.4 U/g for tyrosinase, exhibited high enzymatic activity at pH 5–8 and temperature at 5–30 °C, significant resistance to denaturation and no diffusional restriction to its active site based upon the Michaelis–Menten kinetic parameters. Subsequently, the combi-CLEA was applied to the transformation of acetaminophen as a model phenolic compound in samples of real wastewaters in order to evaluate the potential efficiency of the biocatalyst. In batch mode the combi-CLEA transformed more than 80% to nearly 100% of acetaminophen from the municipal wastewater and more than 90% from the hospital wastewater. UPLC–MS analysis of acetaminophen metabolites showed the formation of its oligomers as dimers, trimers and tetramers due to the laccase and 3-hydroxyacetaminophen due to the tyrosinase.

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http://dx.doi.org/10.1016/j.scitotenv.2013.10.004

1. Introduction

Laccase (EC 1.10.3.2) and tyrosinase (EC 1.14.18.1), two oxidoreductases widely distributed in plants, fungi, and other organisms, have been proven to enzymatically oxidize phenolic and non-phenolic aromatic compounds (Duran et al., 2002). Both laccase and tyrosinase are non-substrate specific copper-containing phenoloxidases requiring readily available dioxygen as sole cofactor for the catalytic oxidation of phenolic substrates. Their oxidation reaction leads to a release of water as by-product and free reactive phenoxy radicals (for laccase) or quinones (for tyrosinase) (Flander et al., 2011) that subsequently polymerize and precipitate thus becoming easier to separate from the reaction solution. Moreover, the polymerization causes the inactivation of the reactive phenol or quinone functional groups to prevent them from reacting with living cells for instance. However, when applied in their free form for the treatment of contaminants present in solution, enzymes in general face major operational shortcomings such as rapid denaturation, lack of reusability, and requirement of large quantities which will impact the overall cost of their use (Cabana et al., 2007a; Sheldon, 2011). Insolubilization of enzymes as combined or simple crosslinked enzyme aggregates (combi-/CLEA) is of one the most effective techniques used to circumvent these drawbacks (Sheldon and Van Pelt, 2013). The technique consists of covalently binding the free enzymes between themselves with the aid of a crosslinking reagent (glutaraldehyde in most cases) to yield a stable and reusable biocatalyst. Lately, many enzymes have been insolubilized through this technique (Talekar et al., 2013). Yet, in the environmental field much investigation still remains to be performed for the use of combi-/CLEA to eliminate increasingly present micropollutants in wastewaters. As numerous of these micropollutants are aromatic or phenolic pharmaceuticals, they are oxidizable by either or both laccase and tyrosinase.

It is recognized that the most prevalent pharmaceuticals in wastewaters are molecules of drugs most frequently prescribed or purchased over-the-counter (Wu et al., 2012) including acetaminophen, an active agent used in the formulation of hundreds of medicines. Acetaminophen is a phenolic compound known under different brand names used worldwide as minor pain and fever reducer. Acetaminophen has widely been detected in wastewaters (one of its main sources of discharge) where it can surpass 150 μg/L (Wu et al., 2012). Although complete removal of acetaminophen from sewage treatment plants (STP) has been found in some studies (Verlicchi et al., 2012), there are several reported concentrations of this contaminant in both surface waters and outflows of STP in the range of 243 to 338 ng/L (Gros et al., 2012), and in the intake of raw surface water and groundwater used for public drinking water supply at 163 ng/L to 1.89 μg/L (Boleda et al., 2011; Fram and Belitz, 2011). Acetaminophen is an active chemical among the many pharmaceuticals whose immediate effects could escape detection if they are subtle (Daughton and Ternes, 1999). Also, it was suggested that for pharmaceuticals with molecules designed to be biologically active, it cannot be excluded that they affect sensitive aquatic organisms even at concentrations in the order of ng/L to μg/L (Huber et al., 2005). The continuous discharge of acetaminophen and its by-products to water bodies, where they can interact with aquatic organisms, deserves particular investigation on the basis of precautionary principle for effective treatment of this phenolic compound.

To our knowledge, no previous work has been published on the removal of acetaminophen from real wastewaters using combi-CLEA of laccase and tyrosinase. However, the removal of other non-pharmaceutical phenolics (bisphenol A and nonylphenol) in solution using combi-CLEA of versatile peroxidase and glucose oxidase was reported recently (Taboada-Puig et al., 2011). The objective of this work was to, first, insolubilize fungal laccase (active in acidic pH) and mushroom tyrosinase (active in neutral to alkaline pH) as combi-CLEA to form a stable biocatalyst with an expanded oxidative pH spectrum. A coupling of chitosan with *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC) was used as crosslinking agent due to the remarkable affinity of chitosan to proteins and to its biodegradability to innocuous products (Krajewska, 2004). The second objective was to characterize the combi-CLEA (pH, temperature, kinetics, stability). Finally, the biocatalyst was partially tested for the transformation of acetaminophen as a model phenolic compound in samples of wastewaters followed by an identification of the possible transformed products (metabolites). This final step of the study was aimed at presenting a proof of concept of the potential applicability of the combi-CLEAs to the treatment of real wastewaters.

2. Materials and methods

2.1. Materials

Trametes versicolor laccase (TvL) with a specific activity of 22.4 U/mg-solid, mushroom tyrosinase (Tyr) with a specific activity of 3610 U/mg-solid, chitosan from crab shells (65% deacetylation and molecular weight of 750 kDa), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 3,4-dihydroxy-L-phenylalanine (L-DOPA) and acetaminophen (≥99.0% purity) were purchased from Sigma-Aldrich Corporation (Saint-Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Preparation of Combi-CLEA and its yield estimation

Combination of 0.3 U of free TvL and 0.7 U of free Tyr was dissolved in deionized water to a total activity of 1 U/mL according to a proven procedure of previous work in our laboratory (Arsenault et al., 2011; Ba et al., 2012).

The yield of the combi-CLEA was estimated based on the activity balance of the amounts of free laccase and tyrosinase used and combi-CLEA produced according to the following equation:

$$Y(\%) = \frac{Total\,Unit\,of\,combi-CLEA\,produced}{Total\,Unit\,of\,free\,TvL\,and\,Tyr\,used} \times 100.$$

2.3. Enzyme activity assays

The activity measurements of free enzymes and combi-CLEA were conducted by measuring the initial reaction rate of substrate oxidation with a double-beam UV–Vis spectrophotometer (SpectraMax Plus 384, Molecular Devices Corporation, Sunnyvale, CA). Laccase activity was determined by monitoring the oxidation of 1 mM ABTS (Bourbonnais and Paice, 1990). Tyrosinase activity was determined by monitoring the oxidation of 5 mM L-DOPA (Edwards et al., 1999). Both substrates were mixed with 0.1 M citrate-phosphate pHs 3–6, 0.1 M sodium-phosphate pHs 7–8 and 0.1 M boric acid-hydroxide pH 9 buffers for activity measurement. One unit (U) of activity is defined as the amount of enzyme (TvL or Tyr) that catalyzes the conversion of substrate (ABTS or L-DOPA) into colored products (green for $ABTS^{\bullet+}$ or orange for dopachrome) causing an increase in absorbance at a rate of 1 μmol per min.

Throughout the paper, combi-CLEA-Lac refers to the combi-CLEA due to its laccase content and combi-CLEA-Tyr refers to the combi-CLEA due to its tyrosinase content.

2.4. Determination of pH and temperature optima

To determine the optimum pH of the free and insolubilized enzymes, their activities were measured in the pH range 3–9 at 20 °C following the activity assay described above. The effect of temperature on the biocatalysts was determined by measuring their activities in the temperature range 5–60 °C at pH 4 or 7 using ABTS or L-DOPA, respectively.