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Characterization of combined cross-linked enzyme aggregates from laccase, versatile peroxidase and glucose oxidase, and their utilization for the elimination of pharmaceuticals

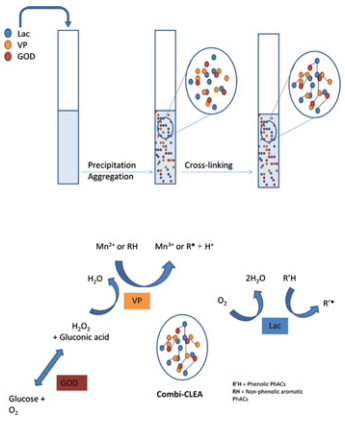
Imad E. Touahar [a], Lounès Haroune [b], Sidy Ba [c], Jean-Phillipe Bellenger [b], Hubert Cabana [a,*,1]

[a] Department of Civil Engineering, Université de Sherbrooke, 2500 Boulevard de l'Université, Sherbrooke, Québec J1K 2R1, Canada
[b] Department of Chemistry, Université de Sherbrooke, 2500 Boulevard de l'Université, Sherbrooke, Québec J1K 2R1, Canada
[c] Department of Chemical Engineering, Université de Sherbrooke, 2500 Boulevard de l'Université, Sherbrooke, Québec J1K 2R1, Canada

HIGHLIGHTS

- An active combi-CLEA of lac, VP and GOD was successfully produced.
- Residual activities of the combi-CLEA were higher than their free counterparts.
- In presence of its co-substrates and GOD, VP could remove more PhACs than Lac.
- Higher removal of more PhACs in different conditions proves combi-CLEA versatile.
- Combi-CLEA has shown versatility by removing more PhACs under different conditions.
- Combi-CLEA exhibited eliminative activity *vs.* acetaminophen in urban wastewater.

GRAPHICAL ABSTRACT



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ABSTRACT

In order to transform a wide range of pharmaceutically active compounds (PhACs), the three oxidative enzymes laccase (Lac) from *Trametes versicolor*, versatile peroxidase (VP) from *Bjerkandera adusta* and glucose oxidase (GOD) from *Aspergillus niger* were concomitantly cross-linked after aggregation, thus, making a combined cross-linked enzyme aggregate (combi-CLEA) that was versatile and involved in an enzymatic cascade reaction. From the initial enzymes about 30% of initial laccase activity was recovered along with 40% for each of VP and GOD. The combi-CLEA showed good results in conditions close to those of real wastewater (neutral pH and medium temperature) as well as a good ability to resist to denaturing conditions such as high temperature (60 °C) and low pH (3). Batch experiments were realized to test the free enzyme's ability to degrade, a PhACs cocktail, mainly in a synthetic wastewater containing acetaminophen, naproxen, mefenamic acid, indometacin, diclofenac, ketoprofen, caffeine, diazepam, ciprofloxacin, trimethoprim, fenofibrate and bezafibrate, carbamazepine and its by-product 10–11 epoxy-carbamazepine. High removal was achieved (more than 80%) for the five first compounds. Then, the elimination ability of the combi-CLEA with or without hydrogen peroxide, glucose or manganese sulfate was determined. Globally, our results demonstrated that VP has a wider removal spectrum than Lac. These removal features are enhanced under more specific conditions, whereas the combi-CLEA

* Corresponding author at: Environmental Engineering Laboratory, Department of Civil Engineering, Université de Sherbrooke, 2500 boul. de l'Université, Sherbrooke, Québec J1K 2R1, Canada. Tel.: +1 819 821 8000x65457; fax: +1 819 821 7974.
E-mail address: Hubert.Cabana@USherbrooke.ca (H. Cabana).
[1] Etienne-Le Bel Centre de Recherche Clinique, Centre Hospitalier Universitaire de Sherbrooke, 3001, 12e Avenue Nord, Sherbrooke, Québec J1K 2R1, Canada.

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combined advantages of both VP and laccase. Finally, the elimination of PhACs in a municipal wastewater treatment plant effluent using the combi-CLEA was marginally investigated. Concentrations of most of the selected PhACs were below the limit of quantification (lower than 20 ng/L) except for acetaminophen. Its combi-CLEA-mediated removal reached up to 25%.

1. Introduction

In the last decades, the aging populations of western countries have induced a significant increase in the consumption of pharmaceutically active compounds (PhACs). These PhACs represent a new challenge to environmental quality, as many of them are found in wastewater treatment plants (WWTPs) at concentrations ranging from ng/L to μg/L and are readily transferred to the environment (Verlicchi et al., 2012; Khetan and Collins, 2007; Celiz et al., 2009). The general conclusion is that since WWTPs fail to remove most PhACs from wastewaters, these molecules easily enter the environment through treated water discharges (Khetan and Collins, 2007; Verlicchi et al., 2012). Major efforts have been deployed to characterize the dynamics (e.g. transfer, interactions with natural matrices) and fate of PhACs in the environment and to evaluate their potential side effects on the biosphere (ecotoxicology) and human health (Pomati et al., 2008; Celiz et al., 2009).

Enzymes have attracted interest as a way to deal with the PhACs removal issue. Oxidative enzymes, namely laccase (Lac, EC 1.10.3.2), manganese peroxidase (MnP, EC 1.11.1.13), lignin peroxidase (LiP, EC 1.11.1.14) and versatile peroxidase (VP, EC 1.11.1.16), secreted, among others, by wood degrading fungi, have shown great potential for the transformation of a wide range of PhACs and other organic contaminants, such as pesticides and chemicals with known or suspected endocrine disrupting properties (Davila-Vazquez et al., 2005; Taboada-Puig et al., 2011). Both Lac and VP belong to the lignin modifying enzymes (LME) family. Lac is a multi-copper containing enzyme that can oxidize phenolic substrates in the presence of dioxygen (Wong, 2009), and non-phenolic substrates in the presence of mediators such as 1-hydroxibenzotriazole (HOBt) (Srebotnik and Hammel, 2000). In contrast, LiP, MnP and VP belong to the heme peroxidases family requiring the presence of hydrogen peroxide (H_2O_2) for their oxidation of substrates (Wong, 2009). In the presence of hydrogen peroxide, LiP oxidizes substrates containing methoxybenzene groups such as veratryl alcohol generating cation radicals involved in the carbon-carbon and ether bonds cleavage in lignin model compounds (Tien et al., 1988). In contact with hydrogen peroxide and Mn(II), MnP is able to oxidize a wide range of organic compounds (Glenn et al., 1986). The transformation process is based on the conversion of Mn(II) into Mn(III). The Mn(III) form complexes with α-hydroxy acids and oxidizes lignin-like substrates (Glenn et al., 1986). Finally, VP shows the potential to oxidize phenolic and non-phenolic substrates, exhibiting both LiP and MnP oxidative properties. These oxidative properties can be expressed either in the presence or in the absence of manganese but require the presence of hydrogen peroxide in both cases (Rodríguez et al., 2004).

Lac has shown the ability to transform PhACs such as diclofenac (Sathishkumar et al., 2012), tetracycline (Suda et al., 2012) and mefenamic acid (Margot et al., 2013). Since the discovery of VP (Martinez et al., 1996), interest towards enzymes as elimination enhancing agents has emerged recently, hence, to date, only few studies have considered VP as an eliminative agent for PhACs. Meanwhile, a recent study has shown that sulfamethoxazole and naproxen could be removed using VP (Eibes et al., 2011). Moreover, since VP shows the oxidative activities of both LiP and MnP, it is hypothesized that VP might transform the same compounds as these enzymes. For instance, LiP was responsible for complete removal of diclofenac (Zhang and Geissen, 2010) and according to Wen et al. (2009, 2010), MnP and Lip were both able to efficiently remove tetracycline.

Complementarily to the presence of VP, glucose oxidase (GOD, EC 1.1.3.4) is required to provide hydrogen peroxide that allows the expression of both LiP and MnP activities. GOD is able to transform glucose into gluconic acid and hydrogen peroxide (Bankar et al., 2009).

The transformation of phenolic or non-phenolic substrates by Lac and VP leads to the formation of oligomers or quinones (Wong, 2009). The transformation products generated by the action of these enzymes are typically less toxic to organisms or show lower biological activity (e.g. endocrine disruptions) than the parent compounds (Arboleda et al., 2013; Cabana et al., 2007).

Due to substrate limitations, changing operational conditions (e.g. presence/absence of manganese) and the synergistic action that results from the combination of different enzymes, it is preferable to use multiple enzymes to eliminate a wide range of PhACs in municipal wastewaters (Rodríguez et al., 2004). It is therefore necessary to develop biocatalysts that combine the enzymes involved in the transformation of contaminants with different chemical structures and under variable operating conditions (Ammann et al., 2013; Van Aken et al., 2000; Taboada-Puig et al., 2011). These versatile biocatalysts can be constituted of oxidative (e.g. laccase, peroxidases) and supporting enzymes (e.g. glucose oxidase) that interact in a cascade of reactions (Sheldon., 2011, 2012; Van Acken et al., 2000).

Even though free enzymes may be efficient biocatalysts for the removal of PhACs, their utilization involves some limitations. The solubility of the enzymes in the aqueous phase makes them difficult to maintain in a reactor. In addition, their lack of stability towards chemicals or thermal denaturation reduces their potential for the development of bioprocesses. The latter greatly affects the cost of such enzyme-based processes (Strong and Claus, 2011). The use of insolubilized laccase facilitates the recovery of both enzyme and product and allows multiple reuse of the enzyme (Park et al., 2012; Sangeetha and Abraham, 2008), continuous operation of enzymatic processes (Cabana et al., 2009), rapid termination of reactions and greater variety of bioreactor designs (Cabana et al., 2009; Taboada-Puig et al., 2011).

The formation of cross-linked enzyme aggregates (CLEAs) is a rapid, gentle and cost effective method for the production of carrier free insolubilized catalytically active enzymes (Sheldon, 2011; Cao et al., 2000, 2003). The insolubilization of enzymes as CLEAs consists of a two step process: the precipitation of the active protein followed by its cross-linking. The resulting insolubilized biocatalysts have shown a high enzyme activity per unit volume (Sheldon, 2007) with improved properties (eg. stability, kinetics, reusability, etc.) compared to their free counterparts (Ju et al., 2013; Reshmi and Sugunan, 2013).

In addition to the production of CLEAs composed of single enzymes, a new technique has been recently developed, involving concomitant insolubilization and cross-linking of two or more enzymes/proteins producing a new type of biocatalysts called combined cross-linked enzyme aggregates (combi-CLEAs) (Mateo et al., 2006). This innovative approach adds two important arguments for the use of CLEA-based technology. Firstly, one-pot multistep catalytic cascade process is made easier thanks to combi-CLEAs. Individual CLEAs of these enzymes show improved conversion yields than their free counterparts, and the combi-CLEAs demonstrate even better catalytic properties (Mateo et al., 2006). Secondly, catalysis of multiple non-cascade reactions has also been reported recently. The aim of such combination is the production of multi-purpose biocatalyst (Dalal et al., 2007). Versatility against environmental conditions (pH, temperature, etc.) may also be improved