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Characterization of *Trametes versicolor* laccase for the transformation of aqueous phenol

Selvia Kurniawati, James A. Nicell *

Department of Civil Engineering and Applied Mechanics, McGill University, 817 Sherbrooke Street West. Montreal, Quebec, Canada H3A 2K6

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Abstract

Laccase (oxygen oxidoreductase, EC 1.10.3.2) from *Trametes versicolor* was thoroughly characterized in terms of its catalytic stability and its effectiveness as a biocatalyst under various reaction conditions when using phenol as a model substrate. This enzyme demonstrated high or moderate degrees of stability at pHs from 5 to 8 at 25 °C and at temperatures from 10 to 30 °C at pH 6. Exponential decay expressions were successfully used to model laccase inactivation when incubated under various conditions of pH and temperature. Phenol transformation was optimum at pH 6, but significant transformation was observed over a pH range of 4–7, provided that sufficient laccase was present in the reacting solution. Partial inactivation of laccase was observed during the oxidation of phenol, even under conditions of optimal stability (pH 6 and 25 °C).

Keywords: Laccase; Inactivation; Phenol; Oxidation; Enzyme

1. Introduction

In recent years, numerous investigations have focussed on the development of processes to target toxic aromatic compounds for treatment based on the use of enzymes that have been isolated from their parent organisms (Karam and Nicell, 1997). It has been suggested that enzymatic processes could be used for various applications including: the treatment of specific chemicals in complex mixtures prior to treatment in either on-site or off-site processes; polishing of treated waters to meet regulatory quality standards; treatment of wastewaters at the source to permit wastewater re-use, facilitate product recovery, and/or remove pollutants before their release into the municipal wastewater systems; and to target the treatment of low concentration pollutants such as estrogenic compounds, pharmaceuticals, and trace quantities of persistent industrial pollutants (Kim and Nicell, 2006a,b; Nicell, 2003).

In particular, oxidoreductive enzymes such as horseradish peroxidase (HRP), lignin peroxidase, manganese peroxidase, tyrosinase and laccase have demonstrated their capabilities to transform a variety of aromatic compounds (Buchanan and Nicell, 1997; Ikehata and Nicell, 2000; Kinsley and Nicell, 2000; Klibanov and Alberti, 1981). Of these, HRP, which is the most widely studied of these enzymes, was used successfully to target phenols for treatment in foul-condensate from a kraft-pulping process, a petroleum refinery wastewater, and a foundry wastewater (Cooper and Nicell, 1996; Wagner and Nicell, 2001a,b).

The feasibility of using an enzyme in an industrial process will hinge on several important criteria including: (1) low costs of production; (2) inexpensive co-factors substrates (e.g., oxidants) and/or additives; (3) good stability when stored under moderate conditions; (4) ability to exert its catalytic activity at high reaction rates under desired reaction conditions; and (5) broad substrate specificity. A promising group of oxidoreductases that have the potential to meet these criteria are laccases produced by a variety of microorganisms and plants (Mayer and Staples, 2002), of which a number of fungal laccases are commercially avail-

* Corresponding author. Tel.: +1 514 398 6675; fax: +1 514 398 7361.
E-mail address: jim.nicell@mcgill.ca (J.A. Nicell).

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Nomenclature

a, c	constants describing biphasic inactivation of enzyme due to reaction environment (dimensionless)
b, d	constants describing biphasic inactivation of enzyme due to reaction environment (s^{-1})
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate)
A_{act}	concentration of active laccase expressed in activity units (U mL^{-1})
A_t	initial total enzyme activity (U mL^{-1})
ε	extinction coefficient (M cm^{-1})
k_{env}	first-order rate constant of inactivation due to reaction environment (s^{-1})
$k_{env\text{-}A}$	first-order rate constant of inactivation due to reaction environment for enzyme fraction A (s^{-1})
$k_{env\text{-}B}$	first-order rate constant of inactivation due to reaction environment for enzyme fraction B (s^{-1})
t	time (s)

able. These laccases exhibit high rates of catalysis and use oxygen as an inexpensive and readily available oxidant when oxidizing aromatic substrates. It has been reported that various fungal laccases are relatively stable when stored at or near neutral pH and below room temperature (Bonomo et al., 2001; Xu et al., 1996) and can exert their catalytic activities over relatively wide ranges of pH and temperature (Call and Mucke, 1997; Xu, 1997). Therefore, as a group, laccases demonstrate strong potential and are being considered for various environmental processes as well as other applications such as delignification, wood fiber modification, dye or stain bleaching, chemical/medicinal synthesis, biosensor development, drug analysis, wine clarification and immunoassays (Archibald et al., 1990; Bourbonnais et al., 1995; Call and Mucke, 1997; Duran and Esposito, 2000; Gianfreda et al., 1999; Johannes et al., 1998; Kang et al., 2002; Karam and Nicell, 1997; Mayer and Staples, 2002; Messerschmidt et al., 1997; Patel et al., 1994; Thurston, 1994; Yaropolov et al., 1994).

Many laccases from different sources could be considered for various industrial applications. Among these, laccase from fungus *Trametes versicolor* was reported to have the highest redox potential among laccases; i.e., 785 mV versus the standard hydrogen electrode (Call and Mucke, 1997; Nakamura, 1958; Reinhammar, 1972; Yaropolov et al., 1994), which makes this laccase particularly interesting since high redox potentials correlate with high laccase activity (Li et al., 1999; Xu, 1996). In addition, the ability of this laccase to exert its catalytic activity on many types of aromatic compounds has been demonstrated (Call and Mucke, 1997; Cantarella et al., 2003; Johannes and Majcherczyk, 2000; Kang et al., 2002; Keum and Li, 2004; Kulys et al., 2003; Roy-Arcand and Archibald, 1991; Tanaka et al., 2001; Tsutsumi et al., 2001; Ullah et al., 2000). These characteristics make laccase from *T. versicolor* particularly promising for industrial applications. However, the stability of laccase from *T. versicolor* and its ability to catalyze the transformation of substrates under various reaction conditions has not been well characterized. Therefore, there is a need to thoroughly investigate the robustness of laccase from *T. versicolor* as revealed through its abilities and limitations under a wide range of reaction conditions. Thus, the objectives of this study were to (1) characterize laccase with respect to its catalytic stability and (2) explore its effectiveness in catalyzing the transformation of a target substrate over wide ranges of pH and temperature. Phenol was selected as a model substrate for all reaction experiments since it is one of the most industrially-important chemicals.

2. Methods

2.1. Materials

Laccase from *T. versicolor* (EC 1.10.3.2), phenol (99.9%), [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] diammonium salt (ABTS), and buffer constituents were purchased from Sigma–Aldrich Co. (Oakville, ON). Acetonitrile (HPLC grade) was purchased from Caledon Laboratories (Georgetown, ON). Distilled-deionized water was prepared using a Nanopure–Ultrapure Water System D4741 (Barnstead-Thermolyne, Dubuque, IA).

Britton–Robinson buffer was chosen for the control of pH during incubation and reaction experiments since it is an effective buffer from pH 3 to 11 (Xu, 1996, 1997; Xu et al., 1996). The buffer consisted of 0.1-M phosphoric acid, 0.1-M boric acid, 0.1-M acetic acid, and the pH was adjusted to desired values by adding 2.0-M sodium hydroxide (Xu, 1996, 1997; Xu et al., 1996). Stock solutions of phenol were made at a concentration of 200 mM and stored until needed. The concentrations of phenol stock solutions were checked regularly using HPLC (see below). Stock solutions of laccase (10 mg mL^{-1}) were prepared in distilled-deionized water. The activity of the stock solution was measured before every experiment. All stock solutions were stored at 4 °C and allowed to equilibrate to 25 °C prior to use in experiments.

2.2. Enzyme assay

The activity of laccase was measured using a colorimetric assay with ABTS as a color-generating substrate, where the rate of color formation was proportional to enzyme activity. The formation of ABTS-cation radical ($ABTS^{+\cdot}$)