Enzyme and Microbial Technology 45 (2009) 253–260

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

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

Soybean peroxidase-catalyzed removal of phenylenediamines and benzenediols from water

Mohammad Mousa Al-Ansari [a], A. Steevensz [a], N. Al-Aasm [a], K.E. Taylor [a,*], J.K. Bewtra [b], N. Biswas [b]

[a] Department of Chemistry and Biochemistry, University of Windsor, 401 Sunset Avenue, Windsor, ON, Canada N9B 3P4
[b] Department of Civil and Environmental Engineering, University of Windsor, 401 Sunset Avenue, Windsor, ON, Canada N9B 3P4

ARTICLE INFO

Article history:
Received 9 February 2009
Received in revised form 22 June 2009
Accepted 4 July 2009

Keywords:
Soybean peroxidase
Phenylenediamines
Catechol
Resorcinol
Hydroquinone
Sodium dodecyl sulfate
Aluminum sulfate
Wastewater
Hazardous waste

ABSTRACT

Crude soybean peroxidase (SBP), isolated from soybean seed coats (hulls), catalyzes the oxidative polymerization of hazardous aqueous phenylenediamines and benzenediols in the presence of hydrogen peroxide. Experiments were conducted to investigate the optimum operating conditions including pH, hydrogen peroxide-to-substrate concentration ratio and the minimum SBP concentration required to achieve at least 95% conversion of these pollutants in synthetic wastewaters. The substrate conversion and hydrogen peroxide consumption were monitored over the period of the reactions. Polyethylene glycol (PEG) was ineffective as an additive in enhancing the conversion efficiency. The enzymatically generated polymeric products from phenylenediamines could be removed with the aid of a surfactant, sodium dodecyl sulfate (SDS), whereas the polyvalent metal cation salt, aluminum sulfate (alum), was able to remove the products from benzenediols, except hydroquinone. Enzyme-catalyzed polymerization with SBP and subsequent removal of the polymeric products generated can provide an alternative means to the conventional methods for treating many aromatic wastewater pollutants, including the title compounds.

1. Introduction

Phenylenediamines (*o*-, *m*-, *p*-phenylenediamines or PD) and benzenediols (catechol, resorcinol, hydroquinone) are widely used as commodity synthetic intermediates and monomers. They occur variously in the process water of many industries including those producing polymers, resins, coatings, paints, dyestuffs, photographic developing agents, agrochemicals, and pharmaceuticals [1,2]. Those compounds have been shown to have an adverse on human health upon ingestion and inhalation, causing severe infections and even death [1]. The health effect extends to animals and aquatic life [1]. Thus, the discharge of these compounds, except resorcinol, into surface waters is regulated and monitored [2]. Insofar as the affected industries are in compliance with discharge limits, these compounds are not of special environmental and/or public health importance. However, their treatment is a matter of substantial activity, since the total on- and off-site disposal in the U.S. in 2007 for catechol, hydroquinone, *o*-PD, *m*-PD and *p*-PD were 2121, 406,203, 10,330, 112,291 and 35,208 pounds, respectively [2] (these statistics exclude air emissions and surface-water discharges, thus representing amounts treated).

Conventional treatment methods for phenylenediamines and benzenediols include various microbiological approaches, adsorption methods and advanced oxidation processes. While these compounds are not particularly bio-refractory, these methods suffer from various drawbacks such as high cost, incomplete removal, formation of hazardous byproducts, low efficiency, high energy requirements and/or applicability only in a low concentration range [3]. Our long-term goal is to determine if, and if so, under what circumstances, enzymatic treatment with a peroxidase or laccase may overcome these drawbacks in a cost-effective manner [4,5].

These enzyme classes catalyze the oxidization of phenols and anilines to aromatic radicals in the presence of hydrogen peroxide (for peroxidases) or oxygen (for laccases). Those radicals diffuse from the active site of the enzyme into solution where they couple non-enzymatically to form dimers. If the dimers are soluble and still phenolic or anilino, they become substrates for another enzymatic cycle, forming higher oligomers. The cycle continues until the polymer generated reaches its solubility limit and precipitates out of solution, later to be removed by filtration or sedimentation [6].

Horseradish peroxidase (HRP) has been found to be effective in removal of phenols and aromatic amines with 95% or higher efficiency in wastewater [7]. The main drawback in using HRP is its unavailability in large quantity at a price appropriate for waste treatment. As a result, a number of other peroxidases have

* Corresponding author. Tel.: +1 519 253 3000x5031; fax: +1 519 973 7098.
E-mail addresses: mousa@uwindsor.ca (M.M. Al-Ansari), steeve2@uwindsor.ca (A. Steevensz), alaasmna@students.udmercy.edu (N. Al-Aasm), taylor@uwindsor.ca (K.E. Taylor), bewtra@uwindsor.ca (J.K. Bewtra), biswas@uwindsor.ca (N. Biswas).

0141-0229/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2009.07.004

been investigated such as *Coprinus cinereus* peroxidase (CCP), *Coprinus macrorhizus* peroxidase (CMP), *Arthromyces ramosus* peroxidase (ARP; it has been suggested that these three microbial peroxidases are essentially the same [8]) and soybean peroxidase (SBP) [4,9–14]. Laccases have also been investigated because of their ability to oxidize phenols and anilines, analogous to peroxidases [4,15,16].

SBP, an oxidoreductase extracted from the soybean seed coat [6], has several features that recommend it for wastewater treatment. Above all, SBP can be cheaper (certainly than HRP, arguably than the microbial peroxidases), since it is easily extracted from the soybean seed coats which are themselves a byproduct of the soybean processing industry [14]. A crude SBP extract has been found to be more efficient than the purified one [17] (as are the peroxidases cited above) and it is active over a broad range of pH (3.5–8.0) [9,11,18,19]. SBP has a higher thermal stability (being active at 70 °C) than other peroxidases [20] and it is less susceptible to irreversible inactivation by hydrogen peroxide compared to HRP [19] and ARP [9]. On the one hand, its catalytic efficiency (k_{cat}/K_M) was found to be about 20-fold higher than that of HRP for the oxidation of the assay substrate ABTS [18], on the other hand it has been reported to have slower catalytic activity and lower phenol removal efficiency than HRP [19].

Nonetheless, inactivation represents one of the drawbacks in SBP's application as with other peroxidases. There are three possible pathways by which peroxidases are deactivated: irreversible inactivation, a form of suicide inhibition, due to the free radicals generated during the catalytic process [21]; inactivation due to end-product polymers formed during the catalytic process [22] which may adsorb the enzyme and co-precipitate it when they exceed their solubility limit; inactivation can be due to excess hydrogen peroxide which is another form of suicide inhibition [23,24]. Additives such as polyethylene glycol (PEG), gelatin and certain polyelectrolytes [25] have been found to limit the inactivation, with PEG better than others in terms of the minimum effective concentration required, lack of interference with the removal efficiency, being easily separated from solution as a co-precipitate with the enzymatic products formed, non-toxic and more cost-effective [22,25,26]. The mechanism by which PEG protects the enzyme is not fully understood but it is believed to follow the "sacrificial polymer" theory by reacting with the free radicals generated during the catalytic process and/or the polymeric products of radical coupling, instead of these products' reacting/associating with the enzyme and precipitating it [22].

The polymeric products normally reach their solubility limit and are separated from solution by filtration or sedimentation. However, in some cases the polymers remain soluble, in which case coagulating/flocculating agents can be employed to induce removal of such (usually colored) products. In the most common approach, hydrolyzing coagulants such as aluminum sulfate (alum), polyaluminum chloride (PAC), ferric sulfate and ferric chloride are used in removing a wide range of impurities such as colloidal particles, natural organic matter (NOM) and dissolved organic substances (DOC) [27–29].

Surfactant-mediated separation methods have been used for removal of organic species in solution. In particular, adsorptive micellar flocculation (AMF) with sodium dodecyl sulfate (SDS) and alum combined [30] was used to remove colored polymeric products after the enzymatic treatment of diphenylamine and of diaminotoluenes, while neither SDS nor alum alone were effective in removing these polymeric products [31,32].

In keeping with our long-term goal given at the outset, the hypothesis to be tested here is that SBP is an efficient catalyst for treatment of the target compounds. Preliminary optimization of SBP-catalyzed polymerization of three phenylenediamines (*o*-, *m*- and *p*-phenylenediamines) and three benzenediols (catechol, resorcinol and hydroquinone) is carried out in order to determine the best removal of those polymers from solution.

2. Materials, analytical equipment and methods

2.1. Materials

Crude dry solid SBP (E.C. 1.11.7, Industrial Grade lot #18541NX); (Rz value of 0.75 ± 0.10) was obtained from Organic Technologies (Coshocton, OH). Liquid ARP (SP-502, activity 2000 U/mL) was a developmental preparation of Novozymes Inc. (Franklinton, NC). Dry solid bovine liver catalase (E.C. 1.11.1.6, lot #120H7060, 19,900 U/mg solid) was purchased from Sigma Chemical Company Inc. (St. Louis, MO). Polyethylene glycol (PEG), average molecular mass of 3350 g/mole, was obtained from Sigma Chemical Company Inc. The aromatic compounds (all having a purity ≥98.0%) were obtained from Aldrich Chemical Corporation (Milwaukee, WI). All other chemicals were of analytical grade and purchased either from Sigma Chemical Company Inc., Aldrich Chemical Corporation or BDH Inc. (Toronto, ON).

2.2. Analytical equipment

Aromatic compound concentrations were analyzed with a high performance liquid chromatography (HPLC) System obtained from Waters Corporation (Milford, MA). The system consisted of binary HPLC pump, autosampler, dual λ absorbance detector and C_{18} reverse phase column (5 μM, 4.6 mm × 150 mm) operated by Breeze software. Elutions were isocratic with a mobile phase consisting of methanol and 50 mM phosphate buffer pH 6.7 for phenylenediamines (60:40 for *o*-PD (at 290 nm), 40:60 for *m*- (at 290 nm), and *p*-PD (at 310 nm)). The mobile phase for benzenediols consisted of acetonitrile and 0.1% acetic acid (40:60 for catechol (at 276 nm), 30:70 for resorcinol (at 274 nm), 20:80 for hydroquinone (at 290 nm), and 20:80 for *p*-benzoquinone (at 248 nm)). The injection volume was 10 μL and flow-rate was 1.0 mL/min.

A Hewlett Packard Diode Array Spectrometer (Model 8452A), with wavelength range between 190 and 820 nm and 2 nm resolution and controlled by Hewlett Packard Vectra ES/12 computer, was used to measure sample absorbance. Quartz semi-micro-spectrometer cells with 1 cm optical path length were supplied by Hellma Canada Limited (Concord, ON).

A Shimadzu TOC-V CSH Total Carbon Analyzer (TOC), supplied by Shimadzu Scientific Instruments (Columbia, MD), was used to measure the total organic carbon in the samples (corrected for inorganic carbon (TIC), if necessary). The microfiltered (0.2 μm) samples were purged by using nitrogen gas; acidified with phosphoric acid, oxidized with oxygen, and detected by using a non-dispersive infrared spectrophotometer (NDIR). The combustion chamber had a temperature between 680 and 700 °C.

2.3. Analytical methods

2.3.1. Colorimetric assay for SBP activity

Enzyme catalytic activity (U) is defined as the number of micromoles of hydrogen peroxide converted per minute at pH 7.4 and 23 °C. The enzyme activity was determined by monitoring the initial rate of color formation at 510 nm resulting from the oxidative coupling of phenol and 4-aminoantipyrine (AAP) in the presence of hydrogen peroxide when using SBP as catalyst [4].

2.3.2. Colorimetric assay for aromatic amines

The phenylenediamine concentrations were determined by measuring the color intensity at ~430 nm resulting from the nucleophilic substitution on trinitrobenzenesulfonic acid (TNBS) by the