

Impact of Dissolved Wastewater Constituents on Laccase-Catalyzed Treatment of Bisphenol A

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ABSTRACT

The impact of dissolved wastewater constituents on the treatment of synthetic bisphenol A (BPA) solutions was investigated under a variety of reaction conditions. The laccase enzyme from *Trametes vesicolor* was used for the BPA treatment. The constituents studied included various inorganic salts, organic compounds and heavy metal ions. BPA degradation was inhibited by sulfate, thiosulfate, sulfide, nitrite, and cyanide ions at 25 mg/l, 100 mg/l, 25 mg/l, 150 mg/l, and 2.5 mg/l, respectively. However, the inhibitive effects of sulfide and sulfite on BPA degradation were diminished by additional aeration to oxidize them. Formaldehyde significantly reduced the rate of BPA degradation at 1.0% among organic compounds studied. Among heavy metal ions tested, Fe(III) substantially suppressed BPA removal at 1 mM. MgCl₂ and CaCl₂ exhibited great inhibition of BPA degradation at 25 mM.

Keywords: laccase, bisphenol A, 2,2-bis(4-hydroxyphenyl)propane, *Trametes vesicolor*, endocrine disrupting chemical

I. Introduction

Bisphenol A (BPA), 2,2-bis(4-hydroxyphenyl)propane, is a common raw material used in the manufacture of polycarbonate and epoxy resins, and is also used as stabilizer or antioxidants for many types of plastics such as polyvinyl chloride.¹⁾ This chemical has been demonstrated to have endocrine disrupting effects as a synthetic compound with estrogenic activities.^{2,3)}

Laccase (polyphenoloxidase, EC 1.10.3.2) is a multi-copper-containing oxidase which can catalyze the four electron reduction of molecular oxygen to water. This enzyme has a broad substrate specificity towards aromatic compounds containing hydroxyl and amine groups.⁴⁻⁶⁾ Currently, the laccase-based process is being investigated for its potential to degrade and detoxify recalcitrant environmental pollutants from industrial wastewater.^{7,8)}

The effectiveness of the laccase-based process for the treatment synthetic wastewater consisting of buffer solutions of selected aromatics has been

demonstrated in numerous studies.^{9,10)} However, real wastewaters could contain a variety of chemical species, which have potential to affect enzyme-catalyzed reactions through inactivation or inhibition of the enzyme or by influencing other steps of the treatment process. Therefore, the objective of this study was to assess the influence of several wastewater constituents on treatment effectiveness and enzymatic activity of laccase-catalyzed process.

II. Materials and Methods

1. Materials

Bisphenol A was purchased from TCI (Tokyo Chemical Industry, USA). Laccase (*Trametes vesicolor*), ϵ -caprolactam, isoprene, and phenol were purchased from Sigma-aldrich (Canada). Methanol and formaldehyde were purchased from Fisher (Canada). Other chemicals, obtained from various commercial suppliers, were of analytical grade purity.

2. Enzymatic assay

Laccase activity was determined by oxidation of ABTS (2,2'-azinobis-(3-ethyl benzthiazoline-6-sulfonic acid). The reaction mixture consisting of

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2.5 mM ABTS, 100 mM citrate/phosphate buffer (pH 4.5) and a suitable amount of enzyme in a total volume of 1 ml was incubated at 25°C. Oxidation of ABTS was followed by absorbance increase at 420 nm ($\epsilon_{420}=3.67 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$).¹¹⁾ One unit of enzyme activity was defined as the amount of enzyme using to oxidize 1 μM of ABTS per min.

3. Analytical method

Samples of the reaction mixture were withdrawn and a small amount of concentrated acetic acid was added to stop enzymatic reaction. The acidified samples were filtered with 0.45 μm filter (Millipore, Canada) to remove insoluble products. BPA concentration in the filtrate was assayed by HPLC 1100 (Agilent, USA) using a ZORBAX SB-C18 column (Agilent, USA) and detected at 277 nm with isocratic elution at 1.0 ml/min with a mobile phase composed of water and acetonitrile (40:60; v:v).

4. Experimental procedures

The reactions were conducted in 20 ml batch reactor at 25°C. The medium was mixed with a magnetic stirrer during reaction. BPA (120 μM final concentration) was incubated for 1 h in 25 mM acetate buffer (pH 5.0) using laccase (0.15U/ml final activity). Before the reaction was started, bisphenol A and oxygen were dissolved by vigorous shaking in the buffer solution.

For experiments involving iodide, nitrite, sulfite, thiosulfate and sulfide, the respective sodium salts were prepared in deionized water. The supplied concentrations to reaction mixtures ranged from 10 to 200 mg/l in terms of the concentration of anions.

For experiments involving reaction in the presence of cyanide and urea, the concentrations to reaction mixtures ranged from 0.1 to 50 mg/l.

For the preparation of reaction mixtures containing organic compounds, stock solutions of formaldehyde (10% in deionized water), ϵ -caprolactam (200 mM in 100% methanol), isoprene (200 mM in 100% methanol) and phenol (200 mM in deionized water) were used. The concentrations to reaction mixtures ranged from 1 to 1000 μM for ϵ -caprolactam, isoprene and phenol, from 0.05 to 1.0% (v/v) for

formaldehyde, and from 0.5 to 10.0% for methanol.

For experiments involving reaction in the presence of metal ions, stock solutions of Zn(II), Ni(II), Mn(II), Cu(II) were prepared using their sulfate salts and stock solutions of Fe(III) and Co(II) were prepared using nitrate salts. The concentrations to reaction mixtures ranged from 0.25 to 5.0 mM in terms of the concentration of metal ion.

Samples containing NaCl, CaCl_2 , MgCl_2 , NH_4Cl and $(\text{NH}_4)_2\text{SO}_4$ were prepared by dissolving the salts in deionized water. The effects of these salts were investigated in the range of 2.5-200 mM.

Before the stock solutions were added to the reaction mixtures containing BPA, they were diluted with 25 mM acetate buffer (pH 5.0).

III. Results and Discussion

1. Reducing anions

Batch experiments were carried out in order to examine the impact of the reducing anions such as iodide, thiosulfate, sulfite, nitrite and sulfide on the treatment of BPA. These ions could consume dissolved oxygen and potentially competitively inhibit BPA oxidation. The results for them are shown in Fig. 1.

BPA degradation did not change when treatment was conducted at 200 mg/l of iodide. Since iodide needs oxygen to be oxidized, its oxidation could also lead to dissolved oxygen consumption. This indicates that the rate constants of BPA removal are much higher than those of iodide. Nitrite did

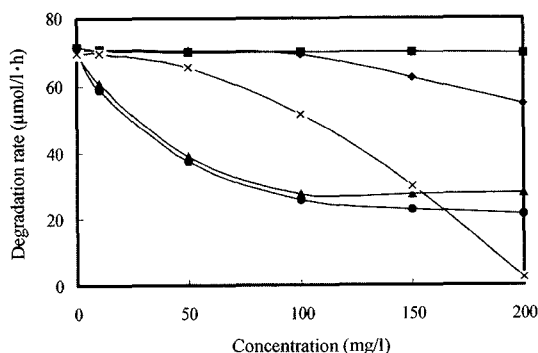


Fig. 1. BPA degradation on the concentration of reducing anions (▲: sulfite, ◆: nitrite, ■: iodide, ●: sulfide, ×: thiosulfate) : Reaction conditions ; 25°C, pH 5.0, 120 μM BPA, 0.15U/ml laccase.

Table 1. The effect of aeration on BPA degradation

Reducing anion	Control ($\mu\text{mol/l} \cdot \text{h}$)	BPA degradation before additional aeration ($\mu\text{mol/l} \cdot \text{h}$)	BPA degradation after additional aeration ($\mu\text{mol/l} \cdot \text{h}$)
nitrite	71.0	54.7	59.4
thiosulfate	69.8	2.6	2.0
sulfite	70.0	27.8	60.3
sulfide	71.5	21.6	70.4

Reaction conditions : 25°C, pH 5.0, 120 μM BPA, 0.15U/ml laccase, 200 mg/l anion.

not affect BPA degradation at 50 mg/l, but it showed the inhibitive effect on BPA removal from about 100 mg/l.

Thiosulfate, sulfide, and sulfate greatly suppressed BPA degradation at 200 mg/l. The enzymatic BPA degradation of sulfide and sulfate occurred when these anions were oxidized by additional aeration of reaction mixture. However, the inhibition of thiosulfate and nitrite was not achieved by additional aeration (Table 1). Therefore, the inhibition of sulfide and sulfite in the wastewaters could be overcome by sufficient oxygen supply.¹²⁾

2. Cyanide and Urea

Cyanide and urea are used as catalysts in polymerization and condensation reaction of plastic industries.¹³⁾ Catalysts would influence the solubility and/or the stability of reaction products and would lead to enzyme inactivation. Therefore, the effects of these catalysts were also investigated under the various concentrations. The results are shown in Fig. 2.

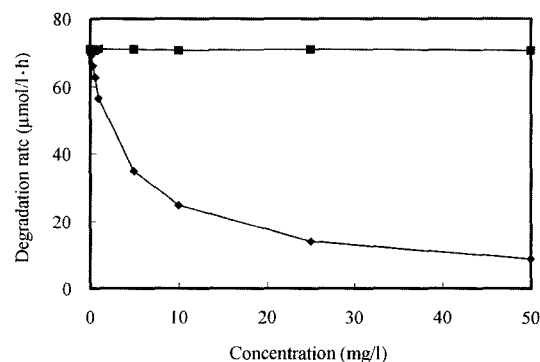


Fig. 2. BPA degradation on the concentration of cyanide and urea (◆: cyanide, ■: urea) : Reaction conditions ; 25°C, pH 5.0, 120 μM BPA, 0.15U/ml laccase.

When cyanide was added in the form of its sodium salt, the degree of BPA removal decreased in proportion to the increase of cyanide concentration. For example, 5 mg/l of cyanide was sufficient to reduce the treatment velocity by 50%. Cyanide could dissociate copper ion from the electron center of laccase and cause the loss of enzymatic activity.^{14,15)} It also inhibited and deactivated the enzyme activity (data not shown). However, urea did not affect BPA degradation when the treatment was conducted at 50 mg/l.

3. Organic compounds

Wastewaters often contain a variety of organic compounds, many of which will not be the substrates of the enzyme. Experiments were conducted to determine whether the presence of methanol and formaldehyde would have an impact on the BPA removal. Phenol, ϵ -caprolactam and isoprene were selected as the organic compounds because

Table 2. BPA degradation as a function of the concentration of basic materials

Organic compound	Concentration (μM)	Degradation rate ($\mu\text{mol/l} \cdot \text{h}$)
Control	-	70.9
Phenol	10	70.7
	100	70.4
	1,000	68.0
ϵ -Caprolactam	10	70.9
	100	70.6
	1,000	70.1
Isoprene	10	70.7
	100	70.5
	1,000	69.9

Reaction conditions : 25°C, pH 5.0, 120 μM BPA, 0.15U/ml laccase.

Table 3. BPA degradation as a function of the concentration of organic solvents

Organic solvent	Concentration (%)	Degradation rate ($\mu\text{mol/l} \cdot \text{h}$)
Methanol	0.0	70.4
	0.5	69.9
	1.0	69.3
	5.0	63.4
	10.0	57.9
Formaldehyde	0.00	70.9
	0.05	69.9
	0.10	69.0
	0.50	61.7
	1.00	54.9

Reaction conditions : 25°C, pH 5.0, 120 μM BPA, 0.15U/ml laccase.

they are also found in wastewaters produced in plastic industries.¹³⁾

The BPA degradation rates in the presence of phenol, ϵ -caprolactam and isoprene the did not significantly differ from the rate of the control sample (Table 2). Thus, these organic compounds did not interfere with BPA removal. Phenol is also a substrate for laccases, but it did not affect BPA degradation. This indicates that laccase from *Trametes vesicolor* is specific to BPA more than phenol at pH 5.¹⁶⁾

Experiments were conducted in order to test whether methanol and formaldehyde would interfere with BPA degradation at various concentrations. The results in Table 3 show that there was inhibition of organic solvents on BPA degradation. Methanol inhibited BPA degradation a little, but formaldehyde exerted a substantial inhibition effect on BPA removal. The inhibiting effect of the solvents on laccase could be caused by the fact that the binding site of organic substrate in this enzyme would be closed to the surface of enzyme globule and be readily accessible for the solvent.¹⁷⁾ Depending on the hydrophobic properties, formaldehyde might be more competitive than methanol for the binding site.¹⁸⁾ However, the nature of inhibition of BPA removal by these organic solvents needs more detailed study.

4. Heavy metal ions

Metal ions are also the important groups of

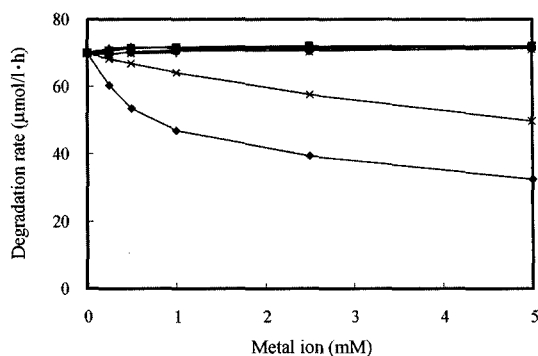


Fig. 3. BPA degradation on the concentration of metal ions (▲ : Co²⁺, ◆: Fe³⁺, ■: Zn²⁺, ●: Ni²⁺, ×: Cu²⁺, +: Mn²⁺) : Reaction conditions ; 25°C, pH 5.0, 120 μM BPA, 0.15U/ml laccase.

enzyme activity modulators. The abundance of heavy metal ions in industrial wastewaters and their potential effects on enzymatic catalysis make it crucial to test the possible impacts of these substances on laccase-catalyzed BPA removal. Manganese(II), iron(III), cobalt(II), nickel(II), copper(II) and zinc(II) ions were investigated in this study. The results of these experiments are shown in Fig. 3.

Solutions treated in the presence of Cu(II) and Fe(III) exhibited inhibition of BPA degradation at low concentration. It is known that most of heavy metal ions do not inhibit the enzymatic activities of laccases in vitro. However, they could reduce substrate transformation.¹⁹⁻²¹⁾ The ions of copper and iron studied did not also inhibit laccase activity in vitro when they were present at 1 mM (data not shown). However, these metal ions might interrupt electron transport system of laccase and inhibited BPA degradation.²²⁻²⁴⁾

Compared with the effect of Cu(II) and Fe(III), solutions treated in the presence of Co(II), Zn(II), Ni(II) and Mn(II) exhibited elevated BPA degradation insignificantly. Therefore, these ions could not be major problems for the treatment of real wastewaters. However, the mechanisms of how metallic ions interfere with BPA degradation or inhibit enzyme are not fully understood.

5. Salinity

Wastewaters can vary substantially in terms of their salinity. The presence of high concentrations

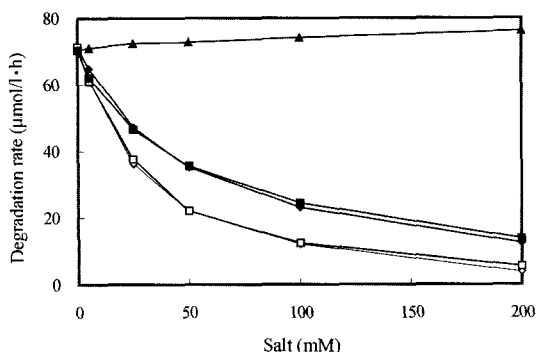


Fig. 4. BPA degradation on the concentration of salts (▲ : $(\text{NH}_4)_2\text{SO}_4$, ◆ : NaCl, ■ : NH_4Cl , □ : MgCl_2 , ◇ : CaCl_2): Reaction conditions ; 25°C, pH 5.0, 100 μM BPA, 0.15U/ml laccase.

of inorganic salts could affect enzymatic BPA degradation through the changes in intrinsic enzyme rate constants. The salinity could also influence the solubility and/or the stability of reaction products, which could lead to changes in the rate of enzyme inactivation. Therefore, the effects of high concentration of inorganic salts were also investigated.

Ammonium sulfate $((\text{NH}_4)_2\text{SO}_4)$, ammonium chloride (NH_4Cl) , sodium chloride (NaCl) , calcium chloride (CaCl_2) and magnesium chloride (MgCl_2) were tested for their possible impacts on laccase-catalyzed BPA degradation. The results of these experiments are presented in Fig. 4.

Neither cations (Na^+ , Ca^{2+} , Mg^{2+} , and NH_4^+) nor anion (SO_4^{2-}) exerted a specific effect on enzymatic BPA removal. However, the chloride compounds such as NaCl, NH_4Cl , CaCl_2 , and MgCl_2 substantially inhibited BPA degradation. At 50 mM of NaCl and CaCl_2 , 29% and 60% of laccase activity were observed respectively. This phenomenon might attribute to the increase of chloride concentration.²⁵⁻²⁷⁾

IV. Conclusion

This study has shown that the laccase-based BPA degradation process can be carried out in the presence of a variety of dissolved species that could be encountered in industrial wastewater.

Treatment of wastewaters containing reduced compounds such as sulfite and sulfide requires a large amount of dissolved oxygen due to oxidation

reactions that compete with the enzymatic BPA oxidation for the dissolved oxygen. The inhibition of sulfide and sulfite can be overcome by supplying extra dissolved oxygen to oxidize the sulfide to sulfur and the sulfite to sulfate. Thus, when studying the enzymatic treatment of real wastewater the inherent oxygen demand of these components needs to be evaluated in order to ensure that the extent of BPA removal is not limited by insufficient dissolved oxygen.

Certain wastewater components have negative effects on the enzymatic reaction. Specially, BPA oxidation is inhibited in the presence of nitrite, thiosulfate, cyanide, formaldehyde, Cu(II) , Fe(III) and chloride compounds. It is recommended that their concentrations be monitored in wastewaters that are to be treated with BPA, in order to avoid inhibition and inactivation of laccase from *Trametes vesicolor*.

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