



Effect of Polyethylene Glycol and Triton X-100 on the Enzymatic Treatment of Bisphenol A

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ABSTRACT

The current study includes the screening of indigenous extracellular laccase producing fungus from the soil, identified as *Phanerochaete chrysosporium* species. The selected strain was applied under the optimized condition for the production of laccase in the solid state fermentation. The effect of polyethylene glycol and Triton X-100 on the enhancement of enzyme activity and biocatalytic removal of bisphenol A using laccase produced by *Phanerochaete chrysosporium* was investigated at 40°C, initial laccase activity 3U/mL and initial bisphenol A concentration 2 mM after 3 h of treatment. Bisphenol A concentration was evaluated using a colorimetric assay in the presence of 4-AAP. The effect of PEG molecular weight on BPA removal was determined using PEG with a molecular weight between 1500 and 20000. The results showed that the addition of polyethylene glycol and Triton X-100 to the reaction mixture greatly increased the laccase activity and the residual enzyme activity, but no significant changes were found in the removal of bisphenol A. The maximum removal of BPA was obtained at pH 8.0 and 40° C in 90 mg/L of PEG-1500 and 50, 100 mg/L of PEG-6000, while laccase activity at all concentrations of PEG-1500 and 6000 increased.

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1. INTRODUCTION

Out of fifty compounds which have been identified as possible EDCs (endocrine disrupting chemicals), BPA 2,2-bis-(4-hydroxyphenyl)propane is an industrial complex that is synthesized by the condensation of two groups of phenol and one acetone molecule [1] and the use of bisphenol A (BPA), as a chemical compound in many products, has steadily grown over the past 58 years [2]. BPA contaminates water via the release of wastewater from plastics and household effluents, and its concentration in urban and industrial wastewater is about ng l^{-1} to μL^{-1} [3]. Unreacted BPA can exist in polycarbonate products, and polycarbonate in products is often degraded slowly by hydrolysis of the carbonate ester bonds at high temperatures or in alkaline pH environment [4].

Some conventional physicochemical methods such as advanced oxidation processes for the degradation of non-biodegradable contaminants [5], adsorption of phenolic compounds [6], and Fe/TiO₂ catalyst [7] were applied for degradation of non-biodegradable contaminants and phenolic compounds. Recently, enzymatic polymerization and precipitation methods have been investigated as a novel approach for the treatment of phenols in aqueous solutions. Various enzymes can be considered; among them, laccase is a good candidate as it has the ability to catalyze the oxidation of environmental pollutants such as endocrine disruptors [8]. Nevertheless, one of the main constraints in the development of laccase catalysis for industrial usages is the enzyme potential for inactivation [9].

The most significant sources of laccases are basidiomycetes, nevertheless, these enzymes, are found in plants, insects, and bacteria [10, 11]. Fungal laccases contain upper redox potential than plant or bacterial

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laccases (up to +800 mV) and their action seems to be pertinent in nature and they also find significant applications in biotechnology [12].

Once laccase is used for BPA removal, it is inactivated by reacting with BPA polymerization products [13]. Researchers have recently concentrated on the use of bio-catalysts in organic synthesis to improve the stability of the enzyme by modifying surfactants or molecular weight-based synthetic polymers[14].

The effects of several additives in enhancing the catalytic activity of oxidoreductases or reducing the amount of enzymes involved to treat phenolic compounds, for example, polyethylene glycol (PEG), gelatin, Triton X, dextran, and polyvinyl alcohol have been extensively investigated [15]. PEG is considered as an additive because treatment costs are significantly reduced and is a non-toxic compound approved for human consumption by the US Food and Drug Administration [11]. Adding PEG is an effectual compound for preserving laccase's activity for the catalytic conversion of phenolic compounds [13]. Asilian and coworkers [16] studied on the dechlorination and decomposition of Polychlorinated biphenyls (PCBs) in the real waste transformer oil by using PEG.

The presence of surfactant has been indicated to decrease the interaction between enzymes and oxidative polymerization products and protect them from inactivation [17]. Additionally, the interaction between the enzyme and the surfactant may lead to changes in the site or active site of the enzyme and consequently affect the activity and stability of the enzyme [18]. Guanglei et al. [17] indicated the positive effect of Triton X-100 on the enzymatic treatment of bisphenol A by laccase. It has been shown that the use of TX-100 at concentrations higher than CMC could increase the enzymatic conversion of BPA by increasing the enzyme stability and BPA solubility in water [17].

In this research, we studied the effects of PEG and Triton X-100 required for enzymatic treatment of BPA in aqueous solutions and its effect on the activity and stability of the enzyme.

2. MATERIALS AND METHODS

2. 1. Screening and Isolation for Potent Strain

Soil samples, including rotting wood and garden soil, were collected from forests and university campuses, for the isolation of lignolytic fungi. The laccase producing fungi were screened using a serial dilution and point inoculation techniques on potato dextrose agar plates. A volume of 1 mL of each sample was transferred to a PDA plate, including 0.01% guaiacol as a specific substrate of laccase. Moreover, chloramphenicol (0.01%

w/v) was added to each plate in order to avoid bacterial growth. Guaiacol was added to the culture media before autoclaving. Plates were incubated in dark at 30°C. Development of reddish halo zones around the colonies indicates the presence of laccase being produced by the fungus. One fungus with faster growth and bigger colored halo size was selected as potent strain [19].

2. 2. Enzyme Production

Solid-state fermentation (SSF) was performed for laccase production. SSF technology creates novel opportunities that permit the use of agricultural waste as a fermentation substrate without the necessity of using extensive pre-processing of the substrate [20]. Fermentation was done in a 250 mL Erlenmeyer flask containing 5g substrates Soybean, moistened with buffer solution of pH 5 (10mM Sodium- acetate buffer) (66% moisture) was autoclaved at 121°C for 15 min [21, 22].

The fungus was enhanced to the flask as a 2mL spore suspension (10^7 spore/mL) and was kept at 30°C in a temperature controlled oven for about 7 days. At the end of selected incubation time, 100 mL of water was added to the flask and put in a shaker at 200rpm (Labcon Shaking Incubator) for 2 hours. The contents were filtered through Whatman No.1 filter paper. The filtrates were centrifuged at $4000\times g$ for 15 min. The supernatants obtained were stored refrigerated to prevent contamination and were used as crude enzyme extracts [23].

2. 3. Enzyme Assay

Guaiacol has been commonly reported for its efficiency as a substrate for laccase assay. The laccase activity was measured by monitoring the oxidation of 1 mL of 10mM guaiacol buffered with 3 mL (10mM) sodium acetate buffer at 470nm spectrophotometrically [21]. Enzyme activity was indicated as International Units (IU), where 1IU is described as the amount of enzyme required to oxidize 1 micromole of Guaiacol per min. The laccase activity in U/mL is determined by the use of the Extinction Coefficient of Guaiacol ($26.6 \text{ m}^{-1}\text{M}^{-1}$) at 470 nm by Equation (1) [24]:

$$\text{Enzyme Activity (U/mL)} = (A \times V) / (t \times e \times v) \quad (1)$$

where: A = Absorbance at 470nm, V = Total volume of reaction mixture (mL), v = enzyme volume (mL), t = Incubation time (min) and e = Extinction Coefficient ($\text{M}^{-1}\text{cm}^{-1}$).

Briefly, the enzymatic reaction was carried out in the solution containing: 3mL acetate buffer, 1mL enzyme source, and, 1mL guaiacol as substrate. After 15min incubation at 30°C, absorbance was measured at 470 nm by spectrophotometer (Hach DR-5000).

Extinction coefficients of $26,600 (470 \text{ nm}) \text{ M}^{-1} \text{ cm}^{-1}$ was used for guaiacol [25].

2. 4. Determination of Bisphenol A Concentration

BPA concentration was evaluated using a colorimetric assay in alkaline conditions in the presence of 4-AAP [26]. In brief, the reagents were prepared in the following order: 700 μ L of phosphate buffer (0.1 M, pH 8), 300 μ L of aqueous BPA sample or enzyme-treated BPA sample, 10 μ L of 0.1 M 4-AAP (0.1 M), and 10 μ L of the 0.2 M potassium ferricyanide solution. After 15 minutes, absorbance was measured at 560 nm and the BPA concentration was determined by a standard curve.

2. 5. BPA Degradation Experiments by using Crude Extract of *Phanerochaete Chrysosporium*

A stock solution of BPA (10^{-2} M) in 30% (V/V) ethanol/water was prepared. Proper amounts of these solutions are used to adjust the concentration required for the experiments.

The reaction mixture (with a final volume of 3 mL) contained 1mL of BPA 2mM in 1mL of phosphate buffer solution (10mM) at pH=8 and a temperature of 40°C in the absence or presence of PEG and Triton X100. The reaction conditions were optimum, which was obtained in a previous study for Bis phenol A removal [8]. It is essential that the buffer is aerated for 24 hours before starting the reaction. The reaction was initiated by adding an aliquot of laccase with the initial concentration of 3 U/mL. The reaction mixture containing PEG was incubated at 40°C and 150 rpm. The control reactions verified that the existence of laccase was needed earlier than the conversion of BPA and the addition of PEG reasoned no BPA removal, and so, BPA elimination was due to the catalytic action of laccase. The reaction mixture containing Triton X100 was incubated without shaking in a water bath set at 40°C [27]. Medium without PEG and surfactant was used as the negative control experiment. Enzyme activity was checked before the reaction. The laccase-free medium under similar conditions was used to evaluate the evaporation and phenol spontaneous removal, which was ignored based on the test results.

The enzymatic reaction was stopped by boiling the solution for 10 minutes. The removal of BPA and the residual enzyme activity were determined by spectrophotometric methods.

2. 6. Effect of PEG and Surfactant Concentration on Enzyme Activity and BPA Removal

To evaluate the effects of PEG molecular weight on the removal of BPA, 50 mg/L PEG with a molecular weight between 1500 and 20000 was used. The minimum amount of PEG needed to convert BPA was determined using the reaction mixture containing 10 to 100 mg/L of PEG-1500 and 6000.

The influence of surfactant concentrations on enzyme activity and BPA elimination was determined at various concentrations of TX-100 (0.1–100 mM).

3. RESULTS AND DISCUSSION

3. 1. Screening and Identification of Laccase Producing Fungus

A total of 6 fungal colonies were isolated from 3 soil samples, designated as L1 to L6 and maintained on PDA for further experiments. The white rot fungus is the most common laccase producer among all fungi. Laccase has the ability to act on a number of substrates as a result of its potency. The quick color formation with guaiacol is an easy and reliable source for laccase screening [28]. The strain L5 exhibited fast growth and the biggest colored zone around its colony after 7 days of incubation. Other strains took more than 8 days of incubation for appropriate growth and oxidation of guaiacol.

Using this screening, a promising basidiomycete strain L5 was selected. The product of 18S rRNA gene sequencing was carried out and the strain was identified as *Phanerochaete chrysosporium*.

3. 2. Effects of Molecular Weight and Concentration of PEG on BPA Removal and Enzyme Activity

The effects of molecular weight and PEG concentration on the enzymatic treatment of BPA were evaluated at pH 8.0 and 40°C. Different molecular weights of PEG were added at a concentration of 50 mg/L. The results showed (Data not shown) changes in the BPA removal and residual activity of laccase after 3 h treatment with different molecular weights of PEG. The maximum BPA removal and residual activity of laccase were obtained for PEG-1500 and 6000. The laccase activity was determined in the reaction mixture in the presence and absence of 10 to 100 mg/L of PEG-1500 and 6000.

At pH 8.0 and 40°C, the maximum removal of BPA was obtained in 90 mg/L of PEG-1500 and 50, 100 mg/L of PEG-6000, while laccase activity at all concentrations of PEG-1500 and 6000-increased (Figure 1). The optimum molecular weight is dependent on the source of the enzyme [13]. Studies conducted by Kim, and Nicell [13] have shown a significant reduction in enzyme inactivation in the presence of additives such as PEG, Ficoll, and polyvinyl alcohol with a molecular weight of 40,000, 400,000 and 100,000. It has been shown that PEG with a molecular weight of more than 600 can be substantially bonded to more water [29]. It was found that the globular structure of the additive plays a significant function in preventing laccase inactivation [30].

Despite the increased activity of laccase in the presence of PEG, there was no positive effect on the removal of BPA. Reducing the effectiveness of enzymatic treatment of BPA by adding PEG is considered to be a major disadvantage in the catalytic treatment of BPA and is due to the capture of the laccase molecules in the final oligomers of the product [15].

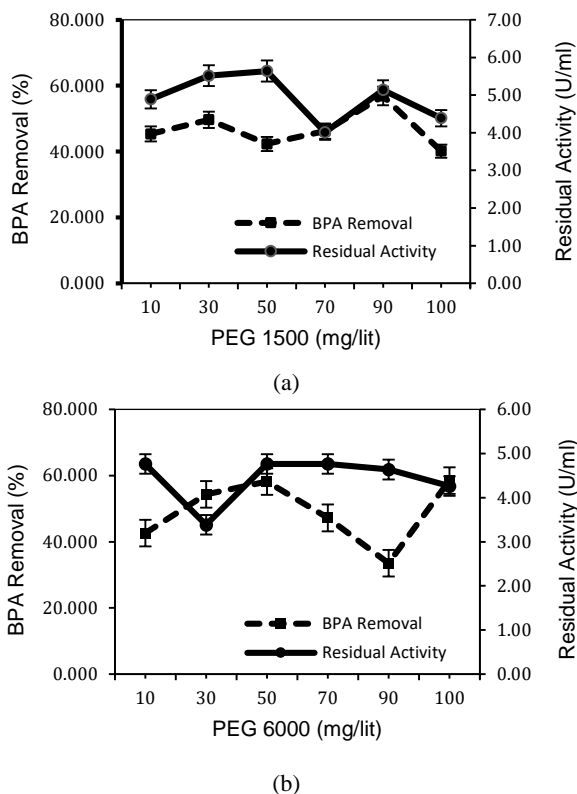


Figure 1. Effect of PEG-1500 (a) and PEG-6000 (b) on BPA removal and residual enzyme activity (pH 8, 40°C, initial laccase activity 3U/mL and Initial bisphenol A concentration 2 mM after 3 h of treatment)

To further investigate the effect of PEG on laccase activity, the enzyme was preincubated with 50 and 100 mg/L of PEG-6000 and 90 mg/L of PEG 1500 at 40°C for 15 min and 3 h, and enzyme activity was determined. Negative controls were used without PEG. The results showed no significant changes in laccase activity after 15 min, but the enzyme activity increased after 3 h of incubation at all concentrations. The enzyme activity in the 50 mg/L of PEG-6000 increased from 1.5 to 4.5 U/mL and is significant. In the next step, the

preincubated enzyme was used in the reaction mixture to compare the BPA removal by adding PEG and enzyme to the reaction mixture simultaneously. The results showed that in samples containing preincubated enzymes and PEG, the elimination of BPA did not decrease and was similar to non-PEG samples. On the other hand, in the presence of 50 and 100mg/L PEG-6000, the enzyme activity is significantly increased and about 74% of the initial enzyme activity (Table1).

Consequently, if PEG is added at the beginning of the reaction, enzyme activity increases, but the percentage of BPA elimination is reduced, which is a negative point, while the use of the preincubated enzyme and PEG in the reaction mixture have a significant positive effect on laccase activity without reducing the percentage of BPA removal. This may be due to the fact that laccase activity is increased by preincubating enzyme with PEG, and increasing the initial enzyme activity enhanced the BPA removal [8].

These results confirm that adding of PEG protects laccase from inhibition or deactivation. One more explanation for inactivation can be the return of the chemical produced by the enzyme to the active site [15]. Xia et al. [31] reported the creation of water-soluble complexes using enzymes with PEG through hydrogen bond between ether groups in PEG and carboxylic or phenolic OH groups in the enzyme. The blockage of enzyme molecules in the end-product oligomers reduces the effectiveness of enzymatic treatment of BPA, which is a major negative aspect of this process [15].

3. 3. Effect of Triton X-100 on BPA Removal and Enzyme Activity

The enzymatic elimination of BPA in different concentrations of Triton X-100 was investigated. Triton X-100 was added to the mixture at different concentrations to investigate the effect of surfactant concentration on the removal of bisphenol A and the residual enzyme activity. The results revealed that Triton X-100 had a negative effect on BPA transformation but laccase activity increases at all concentrations (Table 2).

TABLE 1. Effect of enzyme preincubation with optimal amounts of PEG on the removal of bisphenol A and residual enzyme activity (pH 8, 40°C, initial laccase activity 3U/mL and initial BPA concentration 2 mM after 3 h of treatment)

	Enzyme Activity (U/mL)	BPA Removal (%)	Residual Enzyme Activity (U/mL)
Preincubation (3 h)	PEG 6000, 50mg/L	4.511	79.083
	PEG 6000, 100mg/L	2.005	81.440
	PEG 1500, 90mg/L	2.256	81.872
	Without PEG	2.757	82.017
In situ	PEG 6000, 50mg/L	1.5	81.343
	PEG 6000, 100mg/L	1.5	78.698
	PEG1500, 90mg/L	1.5	81.824
	Without PEG	1.5	85.095

TABLE 2. Effect of different concentrations of Triton X-100 on BPA removal and residual enzyme activity

Options	Residual Enzyme Activity (U/mL)	BPA Removal (%)
	0.5mM	61.77764952
	0.9mM	17.62499098
	1 mM	42.34903710
	3 mM	40.7329929
	7 mM	14.8834868
	10 mM	10.69908376
	20 mM	34.21831037
Triton X100 concentration	30 mM	10.55479403
	40 mM	83.85397879
	50 mM	75.48517423
	60 mM	66.97207994
	70 mM	68.12639781
	80 mM	72.45508982
	90 mM	67.98210807
	100 mM	82.69966092
Without Triton X100 -	5.51	88.3456321

An interaction between Triton X-100 and laccase, performed by endogenous fluorescence emission of laccase, has been shown to be useful for folding and stabilizing of laccase[17].

The highest percentage of BPA removal was obtained at 40 mM Triton X-100. In aqueous solutions, Triton may be present as monomer (below CMC) or as micelles (higher than CMCs), so the effects of surfactants on the performance of enzymes can depend on their types of the organization [32]. Surfactants in aqueous solutions are able to influence the enzyme responses below or above CMC. This may be attributed to two reasons: 1) the interaction between the enzyme and the surfactant; 2) the substrate division between the micelles and the external medium [33]. Docking results by Zhang et al. [33] explained that phenol leads to the formation of hydrogen bonds and hydrophobic interactions with laccase, while the Triton X-100 created hydrophobic interactions with laccase which can increase the activity of laccase and increase phenol elimination.

3. 4. Total Organic Carbon (TOC) Remaining

The fate of PEG is significant in the treatment progression since of its possible environmental impacts on downstream [9]. The residual PEG value in the reaction mixture was detected by total organic carbon analysis (TOC). The instrument used for TOC analysis was ANA TOC Series 2000 to analyze the total organic carbon content using oxygen as an oxidizing agent. To avoid clogging of the tubes, the samples were filtered before the test.

Standard curves of polyethylene glycol, acetate buffer and bisphenol A were prepared separately. The difference between the determined TOC and the TOC

from bisphenol A and acetate buffer determined the TOC value of polyethylene glycol after reaction. The results indicate that the polyethylene glycol additive is eliminated in optimal concentrations, almost completely removed from the solution through sedimentation and do not have a negative environmental impact (Table 3).

TABLE 3. Fate of PEG after reaction

PEG concentration (mg/L)	6000, 50 mg/L	6000, 100 mg/L
Total TOC (mg/L)	459.9	461.26
TOC of remaining bisphenolA (mg/L)	20.89	12.10
TOC of buffer (mg/L)	439.19	439.19
TOC of PEG after reaction (mg/L)	0.18	9.97

4. CONCLUSIONS

Laccase production by isolating *P. chrysosporium* strain was performed under optimized conditions. In this study, the enzymatic treatment of BPA with the aid of polyethylene glycol and Triton X-100 was also investigated. Adding additives such as PEG and TX-100 is a solution to maintain the activity and stability of the laccase and, consequently, catalytic conversion of phenolic compounds. PEG with a molecular weight of 1500 and 6000 had the greatest effect on increasing laccase activity and protecting laccase from inactivation. In the presence of 50 and 100mg/L PEG-6000, the enzyme activity is significantly increased and about 74% of the initial enzyme activity.

Despite the increased activity of laccase in the presence of PEG, there was no positive effect on the removal of BPA. To further investigation the effect of PEG on laccase activity, the enzyme was preincubated with PEG at 40°C for 15 min and 3 h. The results showed laccase activity increased by preincubating enzyme with PEG, and increasing the initial enzyme activity enhanced the BPA removal.

The results also confirmed the use of the Triton X-100 as a way to improve laccase activity and stability. The results of measuring the TOC in the treated samples indicated that PEG almost completely removed from the solution and has no negative environmental effects.

Statistical optimization to reduce laccase dose by adding PEG and enhancing the residual enzyme activity is currently underway.

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Polyethylene Glycol

Triton X-100

مطالعه حاضر شامل غربالگری قارچ تولید کننده آنزیم لاکاز از خاک است که به عنوان گونه *Phanerochaete chrysosporium* شناخته شد. سویه انتخاب شده در شرایط بهینه برای تولید آنزیم لاکاز در تخمیر بستر جامد مورد استفاده قرار گرفت. اثر پلی اتیلن گلیکول و تریتون X-100 بر افزایش فعالیت آنزیم و حذف بیوکاتالیستی بیسفنول A با استفاده از لاکاز تولید شده توسط سویه *Phanerochaete chrysosporium* در دمای ۴۰ درجه سانتی گراد، فعالیت اولیه لاکاز ۳ U / mL و غلظت اولیه بیسفنول آ ۲ میلی مولار پس از ۳ ساعت درمان آنزیمی مورد مطالعه قرار گرفت. غلظت بیسفنول A با استفاده از آزمون رنگ سنجی در حضور ۴- آمینو آنتی پیرین مورد بررسی قرار گرفت. اثر وزن مولکولی پلی اتیلن گلیکول بر حذف بیسفنول آ با استفاده از پلی اتیلن گلیکول با وزن مولکولی بین ۱۵۰۰ و ۲۰۰۰۰ تعیین شد. نتایج نشان داد که افزودن پلی اتیلن گلیکول و تریتون X-100 به مخلوط واکنش، فعالیت لاکاز و فعالیت آنزیم باقی مانده را افزایش داد اما تغییرات قابل توجهی در حذف بیسفنول آ مشاهده نشد. در pH ۸ و ۴۰ درجه سانتیگراد، حداکثر حذف بیسفنول آ در ۹۰ میلی گرم در لیتر پلی اتیلن گلیکول ۱۵۰۰ و ۵۰، ۱۰۰ میلی گرم در لیتر پلی اتیلن گلیکول ۶۰۰۰ به دست آمد، در حالیکه فعالیت لاکاز در تمام غلظت های پلی اتیلن گلیکول ۱۵۰۰ و ۶۰۰۰ افزایش یافت.

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