

**EFFECTS OF BIOSURFACTANT AND CHEMICAL SURFACTANTS
ON THE ENZYMATIC REMEDIATION OF BISPHENOL A FROM
WASTEWATER**

BY

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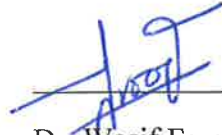
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This thesis is dedicated to my parents and family. A special dedication goes to my wife who supported me and motivated me through the last stages of my work.

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ppm, wavelength at 278 nm, injection volume: 3.3 μ L. The mobile phase
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LIST OF ABBREVIATIONS

17a-ethinylestradiol (EE2)

17b-estradiol (E2)

2,20-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)

2,3,4,5-Tetrachlorophenol (2,3,4,5-TTCP)

2,4,6-Trichlorophenol (2,4,6-TCP)

2,4-Dichlorophenol (2,4-DCP)

2,4-Dinitrophenol (2,4-DNP)

2,6-Dimethoxyphenol (2,6-DMEP)

2-Benzyl-4-chlorophenol (2-B-4-CP)

2-Chlorophenol (2-CP)

2-Methoxyphenol (2-MEP)

3-Chlorophenol (3-CP)

3-Methoxyphenol (3-MEP)

4-Acetamidophenol (4-AAP)

4-Chloro-3,5-dimethylphenol (4-C-3,5-DMP)

4-Chlorophenol (4-CP)

Bis(2-ethylhexyl) sulfosuccinate sodium (AOT)

Bisphenol A (BPA)

Bisphenol B (BPB)

Bisphenol C (BPC)

Bisphenol E (BPE)

Bisphenol F (BPF)

Bisphenol O (BPO)

Bisphenol T (BPT)

Bisphenol Z (BPZ)

Bovine serum albumin (BSA)

Carbamazepine (CBZ)

Chlorophenol (CP)

Critical micelle concentration (CMC)

Diclofenac (DIC)

Hexadecyltrimethyl ammonium bromide (CTAB)

Horseradish peroxidase (HRP)

Human serum albumin (HAS))

Hydrogen peroxide (H₂O₂)

Mesquite peroxidase (MPx)

Nonylphenol (NLP)

Paraconiothyrium variabile (PvL)

Part per million (ppm)

Pentachlorophenol (PCP)

Polyethylene glycol (PEG)

Polyethyleneimine (PEI)

Polyvinylpyrrolidone (PVP)

Rhamnolipid (Rhl)

Sodium dodecyl sulfonate (SDS)

Sodium dodecylbenzenesulfonate (SDBS)

Soybean peroxidase (SBP)

Sulfamethoxazole (SMZ),

Tetrachlorobisphenol A (TCBPA)

Toxic Substances Control Act (TSCA)

Triclosan (TC)

ABSTRACT

Full Name : Muntathir Ahmed Alshabib
Thesis Title : Effects Of Biosurfactant and Chemical Surfactants on the Enzymatic Remediation of Bisphenol A from Wastewater]
Major Field : [Chemical Engineering]
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The enzymatic remediation of synthetic wastewater containing Bisphenol A (BPA) as a model pollutant was examined in the absence and presence of rhamnolipid (Rhl), a common glycolipid biosurfactant. At room temperature, the concentration of Rhl was optimized in batch reactors. The results revealed that the addition of Rhl was only effective when utilized at pre-micellar concentrations. Most importantly, 1 ppm was found to be the optimum Rhl concentration (i.e. Rhl augmented the BPA removal extent by 22.81% when compared to the control reaction). Following the optimization of Rhl concentration, the impact of four variables, namely initial BPA concentration, pH value, temperature, and NaCl concentration, on BPA conversion in the absence and presence of 1 ppm Rhl was investigated. It was noticed that Rhl induced positive effects on BPA removal under the various selected conditions. In addition to this, a comparative study of Rhl and four selected additives (i.e. PEG, Triton X-100, SDBS, and CTAB) was carried out with the sake of assessing their impact on laccase activity and BPA conversion. It was observed that the nonionic additives (Rhl, PEG, Triton X-100) was more beneficial for improving the enzymatic degradation of BPA, while the addition of SDBS and CTAB resulted in negative effects. The biological surfactant. The findings obtained in this work reveal the superiority of Rhl, especially at low concentrations. In addition to such excellent performance, its biodegradability, sustainability and eco-friendly production might encourage its utilization in large-scale processes.

ملخص الرسالة

الاسم الكامل: منتظر أحمد الشيب

عنوان الرسالة: تأثير إضافة منشط سطحي حيوي ومنشطات السطح الكيميائية على المعالجة الإنزيمية لمركب البيسفينول أ

التخصص: هندسة كيميائية

تاريخ الدرجة العلمية: ديسمبر 2019

اهتم هذا العمل بدراسة المعالجة الإنزيمية لمركب البيسفينول أ في حالتي إضافة أو عدم إضافة المنشط السطحي الحيوي رهامانوليبيد. عند درجة حرارة الغرفة، تم اختبار تأثير تركيز المنشط السطحي الحيوي على إزالة البيسفينول أ في مفاعلات متقطعة. أشارت النتائج إلى أن الرهامانوليبيد كان فقط فعالاً عند استخدامه عند تراكيز أقل من تركيزه المذيلي الحرج. وقد وُجد أن أنسب تركيز للمنشط السطحي الحيوي هو 1 مجم/ لتر، حيث ساهم في تحسين معدل إزالة البيسفينول أ في وجود إنزيم اللاكيز بمقدار 22.81%. علاوةً على ذلك، تم فحص تأثير أربعة عوامل على المعالجة الإنزيمية، وهي تركيز مركب البيسفينول أ، الأس الهيدروجيني، درجة الحرارة، تركيز كلوريد الصوديوم في وجود وغياب ال 1 مجم/ لتر من الرهامانوليبيد. عند جميع الحالات، تم اكتشاف فعالية المنشط السطحي الحيوي على تحسين إزالة مركب البيسفينول أ من المحلول المائي. في الجزء الثاني من هذه الدراسة، تم مقارنة أداء الرهامانوليبيد مع أربعة مواد (غليكول بولي إيثيلين، تريتون أكس-100، دوديسيل بنزين سلفونات الصوديوم، ستريمونيوم بروميد) في تحسين نشاط إنزيم اللاكيز ومعدل إزالة البيسفينول أ من الماء. من خلال هذه المقارنة، أُستنتج أن إضافة الرهامانوليبيد، والغليكول بولي إيثيلين، والتريتون أكس-100 أدى إلى تحسين المعالجة الإنزيمية، بينما وجود الدوديسيل بنزين سلفونات الصوديوم و الستريمونيوم بروميد في التفاعل الإنزيمي كان له أثر سلبي على قدرة الإنزيم في إزالة البيسفينول أ.

CHAPTER 1

INTRODUCTION

1.1 Background

Environmental pollution is one of the most monumental issues which face the globe today. Such an issue has adversely intensified due to the rapid advances in urbanism and industrialization. The availability of fresh water, which is an essential resource in every aspect of life, is being threatened by the growing rate of human population, improper use, and chemical pollution [1].

A major source of chemical pollution is phenol and its derivatives that are produced from several chemical industries such as oil refining, petrochemicals, textiles, plastic and resin manufacturing. The release of such pollutants to the environment might dictate the contamination of water resources [2–5]. Such pollutants are hazardous to human health as the majority of phenolic compounds are toxic, mutagenic, carcinogenic, and teratogenic (see table 1-1) [3,6]. In addition to their impacts on human and animal health, phenolic pollutants represent a serious environmental hazard. For instance, the exposure of willow trees to wastewater containing 1000 parts-per million (ppm) phenols caused the death of the trees [7]. Therefore, proper and effective treatment of phenolic wastewaters before reuse or discharge is an imperative task.

Table 1-1: Noxious health effects of phenolic pollutants on human and aquatic creatures

Phenolic pollutants	Noxious effects	Ref(s)
Phenols	<ul style="list-style-type: none"> • Cause muscle fatigue, skin rashes, and diarrhea • Modify aquatic biota such as algae and other microorganisms • Lead to bronchoconstriction and adverse effects in rat and human lungs • Induce suicidal death in human red blood cells 	[6,8–11]
Bisphenols	<ul style="list-style-type: none"> • Lead to metabolic disorders and abnormalities in human babies • Cause cancer in breast and prostate glands • Induce mutations and disruption in reproduction systems in animals 	[12–16]
Chlorophenols	<ul style="list-style-type: none"> • Disturb organ and endocrine system in aquatic organisms • Adversely affect cell growth or induce genetic mutations in fish • Cause digestive tract infections, asthma, heart diseases, and sarcoma to humans • Lead to oxidative stress, deoxyribonucleic acid damage, and lung cancer in living organisms 	[17,18]
Alkylphenols	<ul style="list-style-type: none"> • Cause disturbance in testicular development, and damage of some sustentacular cells • Adversely impact the secretion of progesterone and androstenedione in males 	[19–21]
Triclosans	<ul style="list-style-type: none"> • Disturb immune system, reduce the production of reactive oxygen species, and lead to malfunctioning in cardiovascular system • Lead to uncoupling of mitochondria, and induce lethal effects on cells 	[22,23]
Cresols	<ul style="list-style-type: none"> • Lead to abnormalities in gap junction and adherens junction • Suppress the formation of blood clots • Reduce the production of Reactive Oxygen Species in humans, leading to bleeding disorders 	[24,25]
Nitrophenols	<ul style="list-style-type: none"> • Suppress the pathway of androgen receptor signaling • Induce changes in testicular tissues • Sharply decrease the plasma amounts in some hormones • Hinder transcription process, thereby affecting the level of genes in thyroid system 	[26–29]
Aminophenols	<ul style="list-style-type: none"> • Decrease the level of haemoglobin and volume percentage of red blood cells in fish • Lead to malfunctioning in both reproductive and respiratory systems in humans • Lead to premature death of cells in liver and also damage to kidney 	[30–32]

There are a number of conventional methods for treating wastewaters polluted with phenolic compounds such as adsorption [33,34], distillation [35,36], chemical oxidation [37,38] and extraction [39,40]. Additionally, some advanced techniques such as membrane separation [41,42] and photocatalytic oxidation [43,44] have been proposed as alternative methods for treating phenolic wastewaters. Besides these physico-chemical treatment methods, biological treatment using microorganisms has been exploited as well. In such a treatment, microorganisms degrade phenolic compounds by opening the aromatic rings while consuming energy and carbon from the targeted pollutants [45]. However, most of these treatment techniques are either costly or detrimental to the environment.

Enzymes, which are biocatalysts with several industrial applications [46–48], have been regarded as a possible feasible and environmentally-friendly approach for the treatment of phenolic wastewater [49–51]. Enzymatic treatment is believed to be selective and can be operated at even high pollutant loadings, and low residence times along with mild conditions. Ease of process control, no shock loading effects, and no biomass generation add huge advantages to the enzymatic process [45,49,51–53].

In the beginning of 1980s, the application of enzymes was firstly scrutinized by Klibanov and his group to remove aromatics from wastewater [6,52]. With a view to treating wastewater polluted with phenolic compounds, oxidoreductases (i.e. peroxidases and laccases), which are a main class of enzymes, have been tested to oxidize such pollutants [52]. These enzymes have the capability to attack phenolic compounds by oxidation into free radicals or quinones that tend to be either polymerized or partially precipitated. The formed precipitates can be in turn removed by separation techniques such as filtration [54,55].

1.2 Significance of the Problem

Albeit enzymatic treatment seems to be attractive and promising, the loss of enzyme activity encountered during the remediation is still a monumental issue, hindering the adoption of such a process in large scale applications [56–59]. In an attempt to suppress such inactivation, researchers have scrutinized the potential of surface active agents (i.e. polymers and surfactants) as a way to protect the active site of enzymes from the attack of free radicals or polymeric products [2,60]. However, these fossil-based additives, such as polyethylene glycol (PEG) and Triton X-100, are reported to be relatively toxic and in some cases otiose [61,62].

In the light of the aforementioned issues, this project was devoted to investigate the influence of biosurfactant as an eco-friendly additive on the removal of Bisphenol a from wastewater. This phenolic compound was selected as a target pollutant because it is ubiquitous and can induce deleterious effects in the environment. Most importantly, no study has yet uncovered the effect of bio-derived surfactants on the enzymatic treatment of bisphenolic wastewater.

1.3 Objectives of the Research

The main objective of this project was to examine the impact of biosurfactant addition on the laccase-catalyzed removal of BPA from wastewater. The scope of this work involved the following points:

- 1- Optimizing the biosurfactant concentration, which corresponds to the maximum enhancement in the degradation efficiency of BPA.

- 2- Evaluating the effect of initial BPA concentration on the removal efficiency of BPA in the absence and presence of biosurfactant.
- 3- Studying the effect of temperature on the removal efficiency of BPA in the absence and presence of biosurfactant.
- 4- Investigating the effect of pH on the removal efficiency of BPA in the absence and presence of biosurfactant.
- 5- Studying the effect of NaCl on the removal efficiency of BPA in the absence and presence of biosurfactant.
- 6- Monitoring the degradation rate of BPA over time under varying parameters in the absence and presence of biosurfactant.
- 7- Comparing the performance of biosurfactant with common surface-active additives.

CHAPTER 2

LITERATURE REVIEW

2.1 Enzymatic Treatment of Phenolic Wastewaters

Enzymatic reactions involve the utilization of enzymes in wastewater sample to catalyze the removal of phenolic pollutants. The most prevalent enzymes, which have been successfully used to eradicate phenolic pollutants, are peroxidases and laccases. Peroxidases have either a heme cofactor in their active sites or redox-active cysteine/selenocysteine residues [63]. Owing to the easy access to their active sites, the removal of several pollutants from wastewaters, including phenol and its derivatives, can be catalyzed by peroxidases.

The most widely utilized peroxidase for the removal of phenolic compounds from wastewaters is horseradish peroxidase (HRP) [64–67]. A recent study, for example, evaluated the use of HRP for the degradation of phenols from a biorefinery wastewater sample. The enzymatic system removed over 99% of phenols within 35 min of treatment [68]. Another study revealed that increasing HRP concentration to 7.5% enhanced Bisphenol A (BPA) degradation to more than 98% within 3 h of treatment [69]. This extent of BPA removal was achieved in the presence of hydrogen peroxide (H_2O_2), which is an essential electron acceptor additive for effective degradation of phenolic substrates using HRP. However, at high H_2O_2 dose, HRP was subjected to deactivation, resulting in the reduction of BPA removal [69].

The potential use of HRP on an industrial scale is severely limited by the high enzyme production cost, its high vulnerability to deactivation [45,55,70], and its limited availability as a result of the laborious cultivation and extraction processes [52]. An alternative to HRP is soybean peroxidase (SBP), which is more abundant [52,71], has the potential to be produced more cheaply than HRP, and it exhibits a lower vulnerability to irreversible deactivation by H₂O₂. In terms of catalytic power, it has been reported that SBP is more powerful than HRP in removing Triclosan (TC) [72], which is a recalcitrant phenolic pollutant, from wastewater. For example, SBP effectively removed 98% of this pollutant within 30 min while only 36.5% removal was obtained using HRP within the same time frame [72].

Although SBP might be produced from soybean hulls, most of other peroxidases, are mainly extracted from agricultural sources, which might divert crops and agricultural lands [70]. Thus, researchers are seeking alternative sources. Hairy roots extracts [73–75], white radish (*Raphanus sativus*) [76] and waste *Brassica oleracea* [77] are good non-food sources for peroxidases production, which also do not compete with crops for agricultural land use. Additionally, wastes produced from food processing plants can also be used for peroxidases production. Accordingly, Kurnik et al. [55] utilized potato pulp, a waste by-product produced in potato starch factories, to extract peroxidases and used the produced peroxidases for the removal of phenol from synthetic and real wastewater samples. The authors reported that more than 95% of phenol was removed from synthetic wastewater samples containing optimized phenol concentrations; the term “optimized phenol concentrations was used by the authors [55] to refer to the initial phenol concentrations in synthetic wastewater samples, which correspond to more than 95% phenol removal at the

end of the treatment process. Furthermore, over 90% of phenol was removed from industrial wastewater samples containing phenol concentrations in the range of 0.02 to 0.1 mM [55]. Another study tested the efficiency of peroxidases derived from potato waste in removing 2,4-dichlorophenol (2,4-DCP) from a synthetic wastewater sample [78]. At a pollutant concentration ranging from 1 to 3 mM, the removal extent reached 98%. The above studies demonstrate the potential production of peroxidases from food wastes and their utilization for the remediation of phenolic compounds from wastewaters.

Recently, scientists extracted a peroxidase from a mesquite tree (*Prosopis juliflor*) and compared this low purity peroxidase with HRP [70]. Mesquite peroxidase (MPx) provided 90-92% phenol and chlorophenol (CP) removal from wastewater while HRP could not remove more than 40–60% of these phenolic pollutants. In terms of activity, MPx possesses 6-fold oxidation power higher than that of the purified HRP. Moreover, the MPx exhibited a higher residual activity and more resistance to inhibition by the reaction products. In addition to its higher catalytic efficiency over HRP, MPx is also more sustainable enzyme since it is produced from a source that is widely and sustainably available in nature. Furthermore, the extraction of MPx is easier relative to HRP [70].

One important drawback of using peroxidases is the requirement of adding H₂O₂ to the reaction medium in order for these enzymes to function properly. However, even low concentrations of H₂O₂ can lower the enzymatic reaction rate [79] while high H₂O₂ concentrations could render these enzymes totally inactive [79,80]. Furthermore, it has been reported that the oxidation power of peroxidases highly depends on the type of phenolic substrates and also on the source of the utilized peroxidase [81]. Accordingly, alternatives have been sought by several researchers. Laccases represent good alternatives

to peroxidases since these enzymes can function well in the absence of H₂O₂. Unlike peroxidases which require H₂O₂ to function properly, the oxidant required in the case of laccases is oxygen, which is harmless, does not inhibit enzyme activity and can be easily obtained from free and abundant sources (e.g., air) [82].

Laccases, mainly produced by fungi, contain four copper atoms, which are categorized into three groups (i.e., type 1, type 2 and type 3) according to analysis using UV-visible and electron paramagnetic resonance spectroscopy (figure 2-1). The type 1 (T1) single copper atom is linked to two histidines, one cysteine in tandem with a methionine motif, which hugely contributes to stabilizing the enzyme structure. The T2 (i.e. two copper atoms) and T3 (i.e. one copper atom) sites which act together as a trinuclear cluster mainly comprise of histidines [83].

As depicted in figure 2-2, the laccase mechanism begins when electrons are extracted from a phenolic substrate by the type 1 (T1) copper site. These electrons are then transferred internally into T2 and T3 copper sites followed by the reduction of oxygen (i.e., externally transfer of electrons to oxygen) to form water [53,83,84]. The oxidation of phenolic compounds leads to the formation of phenoxy radicals, which in turn undergoes non-enzymatic coupling with each other to form dimers. Eventually, these dimers combine to produce polymers after some enzymatic reaction cycles [6].

Laccases have been extensively used to treat wastewaters contaminated with phenols. One of the key reasons for the popularity of laccases for the remediation of wastewaters contaminated with phenolic compounds is their non-specificity [85]. These multicopper oxidases [86] are capable of catalyzing a wide variety of substrates [6,49,63,87], including phenolic and nonphenolic compounds.

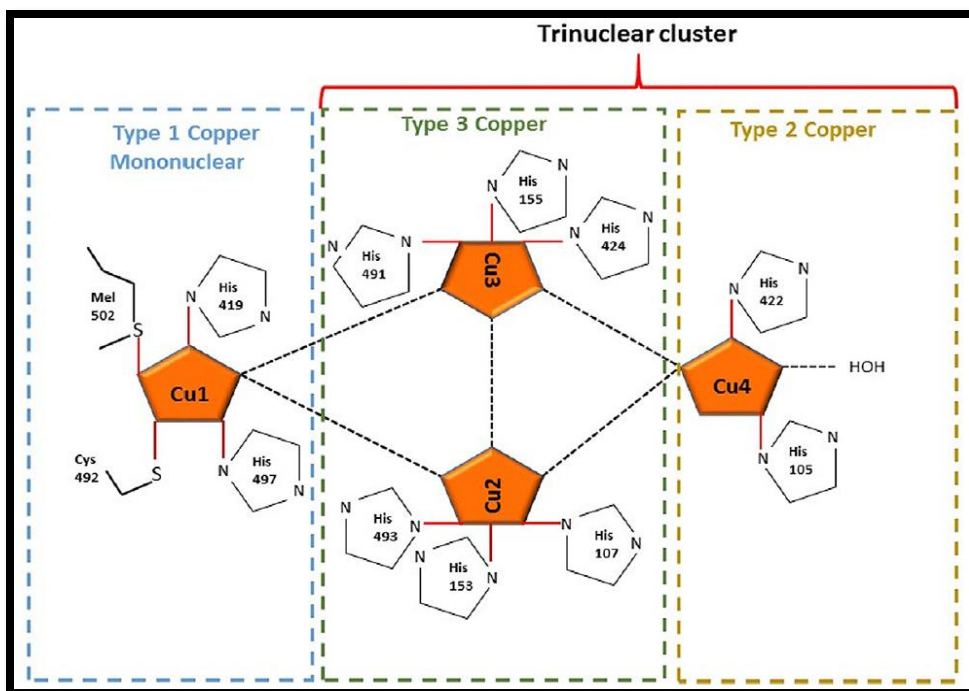


Figure 2-1 The four blue coppers and related ligands in the active site of laccase [83]

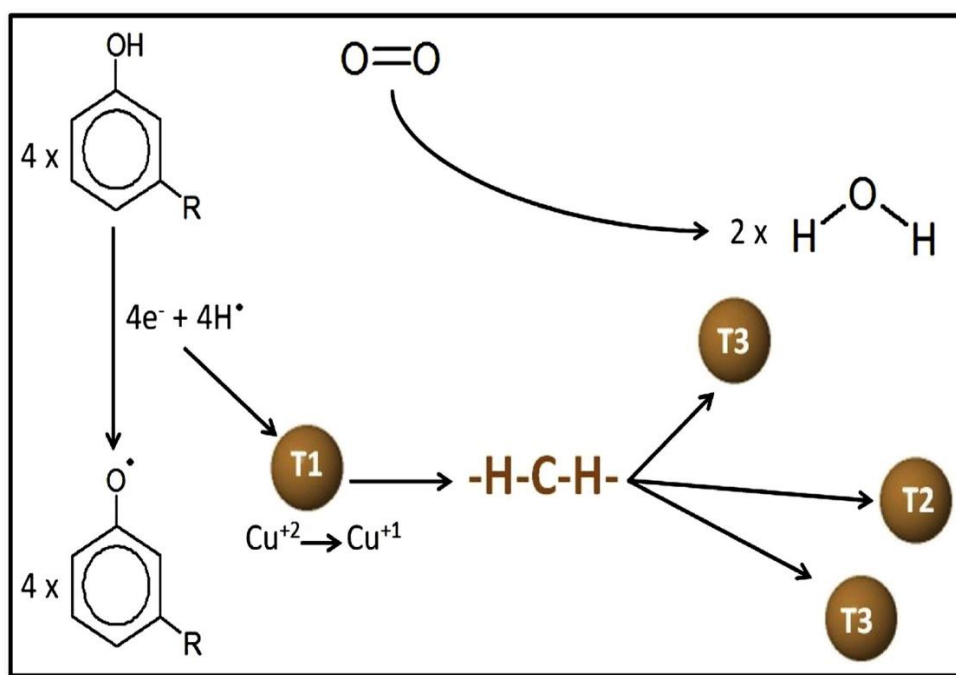


Figure 2-2 Laccase mechanism towards the oxidation of phenolic substrates [88]

There are several studies on utilizing homogeneous enzymatic reactions based on laccases for the removal of phenolic compounds from wastewaters. A study of such was conducted by Asadgol et al. [86] where phenol and BPA were separately removed from wastewater samples using laccase. The initial concentration of each pollutant was 4 mM and the treatment was carried out for 30 min. After 30 min incubation of each of these two pollutants in 5 U/mL concentration of the enzyme, the extents of phenol and BPA removal were 80 and 60%, respectively. With optimizing the conditions of the degradation of these two phenolic pollutants, the removals of phenol and BPA were increased to about 96% and 88%, respectively, within the same treatment duration [86]. The removal of BPA (44 μ M) from wastewater using a laccase cocktail obtained from *Pycnoporus sanguineus* CS43 at a concentration of 0.1 U/mL has been also studied by Garcia-Morales et al. [16]. The researchers found that about 90% of BPA was removed by the cocktail within about 5 h of treatment [16]. In the above two studies, almost the same extent of BPA removal was achieved; however, the laccase used by Asadgol et al. [86] was much faster in removing BPA. Despite that the initial BPA concentration used by Asadgol et al. [86] was more than 90 folds higher, those researchers also utilized a higher enzyme concentration, which might explain their observation of faster BPA degradation rate. Furthermore, the source and the purity of the utilized laccase, and the treatment conditions (i.e., pH, temperature, presence of organic/inorganic co-pollutants) might also play roles in the observed big difference in the treatment duration of BPA reported by these two studies.

Laccase has been also reported to be effective in removing phenol from a refinery wastewater sample [57]. However, although laccase was able to remove 90% of phenol from the above sample, the rate of phenol remediation was less than that obtained using

SBP [57]. The 90% removal of phenol from the refinery wastewater sample was achieved at the optimal (0.12 U/mL) laccase concentration. Although phenol is, supposedly, easier to degrade than BPA, it has been reported that a laccase concentration of 0.12 U/mL was sufficient to almost completely remove BPA from a synthetic wastewater sample within 3 h [69], relative to 90% removal of phenol from a refinery wastewater sample within the same treatment duration [57]. The presence of other contaminants and the complexity of the industrial wastewater sample (i.e., the refinery sample) is probably the reason for such higher remediation of BPA than phenol. In line with the complete removal of BPA from synthetic wastewater sample reported by Escalona et al. [69], a complete removal of BPA within 4 h of treatment was also reported by Daâssi et al. [89], who utilized a laccase produced by *Coriolopsis gallica* fungi. Laccase produced by other fungi (*Bjerkandera adusta* and *T. versicolor*) was less effective [89], demonstrating the effect of laccase source on the removal of phenolic pollutants from wastewaters.

2.2 Effect of Operating Temperature

Operating temperature is one of the most important operational and economic factors that must be considered in any enzymatic wastewater treatment process. It is known that the rate constant of enzyme-catalyzed reactions increases with increasing the reaction temperature, resulting in a higher rate of pollutants removal from wastewaters. However, this positive effect of temperature is counteracted by its negative effect on enzyme stability since enzymes might undergo some structural changes, especially at high temperatures, leading to a reduction in, or even a complete loss of, their activity [90,91] and, thus, their effectiveness in removing phenolic pollutants [15,92]. Accordingly, there is always an

optimal temperature where the enzyme activity is the highest, above which thermal deactivation becomes more pronounced. However, this optimum temperature can be very narrow or it might span over a relatively wider range depending on the characteristics of the utilized enzyme, substrate and the conditions of the reaction medium. Optimal temperature for different enzyme-phenolic substrate systems is summarized in table 2-1.

Several studies have reported the effect of reaction temperature on the removal of phenolic pollutants from wastewaters. For example, Dehghanifard et al. [92] investigated the effect of temperature on the removal of 2,4-DNP from a wastewater sample using laccase and observed that the optimal temperature is 40 °C, at which more than 90% 2,4-DNP removal was obtained within 3 h of treatment. However, when the reaction temperature was increased to 60 °C, 2,4-DNP removal dropped to 33% within the same treatment duration [92]. Phenol removal also dropped significantly from more than 96% at the optimal reaction temperature of 50 °C to about 47% when the temperature of the laccase-containing reaction medium was raised to 60 °C [86]. Similarly, the extent of BPA removal using laccase dropped from about 90% at 50 °C (the optimal temperature) to less than 50% with 10 °C increment in the reaction temperature [86]. The same adverse effect was also observed for phenol removal by peroxidase extract (obtained from jicama) where the increase in the reaction temperature to above 50 °C (the optimal temperature is in the range of 30 to 40 °C) led to a reduction in the extent of phenol removal to less than 50% [79]. Contrarily, a peroxidase derived from mesquite tree showed a good resistance to thermal deactivation, where the enzyme was able to remove 75% of chlorophenols even at high temperature (i.e., 80 °C) [70], despite that the highest chlorophenol removal (> 90%) was

obtained at 25 °C. This finding demonstrates the possibility of producing biocatalysts that have reasonably good thermal stability.

Table 2-1 Temperature optima for different enzymes utilized for the removal of various phenolic pollutants

Enzyme	Source	Substrate	Optimal Temp (°C)	Ref
Laccase	<i>Trametes versicolor</i>	2,4-DNP	40	[92]
Laccase	<i>Trametes versicolor</i>	BPA, BPB, BPC, BPE, BPF, BPO, BPT, BPZ	40	[93]
Laccase	<i>Trametes versicolor</i>	BPA	40	[54]
Laccase	<i>Pycnoporus sanguineus</i> CS43	BPA	25	[16]
Laccase	<i>Trametes versicolor</i>	Phenol	25	[50]
Laccase	<i>Trametes versicolor</i>	BPA	35	[15]
Laccase	<i>Trametes versicolor</i>	2,4-DCP	35	[94]
Laccase	<i>Paraconiothyrium variabile</i> (PvL)	Phenol, BPA	50	[86]
Laccase	<i>Trametes pubescens</i>	2-CP, 2,4-DCP, 2,4,6-TCP, PCP	40	[95]
Laccase	<i>Coriolus versicolor</i>	2,4-DCP	35	[96]
Laccase	<i>Trametes versicolor</i>	BPA	40	[93]
Laccase	<i>Trametes versicolor</i>	Phenol, 3-MEP, NLP, BPA, 2- CP, 3-CP, 4-CP, 2,4-DCP, 4- AAP,	40	[97]

		2-B-4-CP, 4-C-3,5-DMP		
Laccase	<i>Ascomycete Trichoderma atroviride</i>	2,6-DMEP	50	[98]
Laccase	<i>Trametes versicolor</i>	BPA	22 to 26	[99]
Peroxidase	Horse Radish	Phenol, 4-CP	25	[100]
Peroxidase	Horse Radish	2-MEP and other phenolic compounds	28	[68]
Peroxidase	Horse Radish	2,4-DCP	25	[101]
Peroxidase	Horse Radish	2-CP, 4-CP, 2,4-DCP	30	[66]
Peroxidase	Horse Radish	BPA	30	[90]
Peroxidase	Horse Radish	BPA	30	[102]
Peroxidase	Horse Radish	Phenol	37	[80]
Peroxidase	Horse Radish	Phenol	30	[103]
Peroxidase	Horse Radish	2,4-DCP	25	[104]
Peroxidase	Jicama Skin Peels	Phenol	30 to 40	[79]
Peroxidase	Soybean hulls	TC	25	[72]
Peroxidase	White Radish (<i>Raphanus sativus</i>)	α -naphthol and other phenolic compounds	40	[76]

2.3 Effect of Operating pH

In addition to the effect of temperature, the pH value of the enzyme-containing medium has also a great effect on the enzyme stability and activity [99,105]. Accordingly, there is

always a pH range (which might be relatively narrow or wide depending on the characteristics of the utilized enzyme and substrate) through which the enzyme activity is at its maxima (i.e., optimal pH). Table 2-2 illustrates the pH optima for different enzyme-phenolic substrate systems. The presence of pH optima is related to the degree of the ionization of the amino acids of the enzyme; such ionization is pH-dependant. Thus, changing the medium pH might drive the formation/breakdown of some intramolecular ionic bonds, leading to the alteration of the tertiary structure of the enzyme molecules. Such alteration could potentially alter the enzyme activity. For example, it has been reported that the ionic groups of laccase formed a strong electrostatic repulsion when the pH value was varied, leading to the destruction and the degeneration of the enzyme active site and, thus, the enzyme was almost completely inactive in alkaline media [106]. Furthermore, HRP has undergone a significant activity loss at low pH values as a result of the chemical modification in the heme group of the peroxidase [77], most likely due to the physical changes in the enzyme tertiary structure [68].

Besides the possible alteration of the enzyme ionization state and, thus, its tertiary structure, the change in pH could also alter the ionization state of the polar substrates, leading to a significant change in the enzyme affinity for those substrates [107] (i.e., a change in the enzyme-substrate interaction). Accordingly, the removal of phenol from a wastewater sample by peroxidase extracted from jicama skin peels has dropped from more than 80% at pH 7 (the optimal pH) to less than 20% at pH 8 due to the formation of phenol conjugated base [79]. Such phenol conjugate eliminates the hydrogen donor character of the phenol substrate, which has resulted in delaying the binding of this conjugate into the enzyme active site [79], and accordingly, the reduction in the rate and the extent of phenol

remediation. Similarly, the removal efficiency of α -naphthol by HRP significantly decreased at low and high pH values (>90% at pH 7 (the optimal value); ~10% at pH 2 and ~0% at pH 10). The substantial decrease in α -naphthol removal efficiency at pH 2 is correlated to the HRP deactivation due to the release of heme prosthetic group from the polypeptide chain of the enzyme. On the other hand, the null removal of α -naphthol at pH 10 might be attributed to the formation of α -naphthol conjugated base (the pKa of α -naphthol is 9.38), leading to a negligible binding of the substrate to the enzyme active site [76].

Table 2-2 Optimal pH values for the degradation of various phenolic pollutants by different enzymes

Enzyme	Source	Substrate	Optimal pH	Ref
Laccase	<i>Trametes versicolor</i>	2,4-DNP	5	[92]
Laccase	<i>Trametes versicolor</i>	BPA, BPB, BPC, BPE, BPF, BPO, BPT, BPZ	7	[93]
Laccase	<i>Trametes versicolor</i>	BPA	5	[54]
Laccase	<i>Pycnoporus sanguineus</i> CS43	BPA	5	[16]
Laccase	<i>Trametes versicolor</i>	Phenol	5	[50]
Laccase	<i>Trametes versicolor</i>	BPA	6	[15]
Laccase	<i>Trametes versicolor</i>	2,4-DCP	3	[94]
Laccase	<i>Paraconiothyrium variabile</i> (PvL)	Phenol, BPA	5	[86]

Laccase	<i>Trametes pubescens</i>	2-CP, 2,4-DCP, 2,4,6-TCP, PCP	6	[95]
Laccase	<i>Coriolus versicolor</i>	2,4-DCP	6	[96]
Laccase	<i>Trametes versicolor</i>	BPA	7	[93]
Laccase	<i>Trametes versicolor</i>	Phenol, 3-MEP, NLP, BPA, 2-CP, 3- CP, 4-CP, 2,4-DCP, 4-AAP, 2-B-4-CP, 4-C-3,5-DMP	4.6	[97]
Laccase	<i>Ascomycete</i> <i>Trichoderma atroviride</i>	2,6-DMEP	5	[98]
Laccase	<i>Trametes versicolor</i>	2, 6-DMEP	6	[107]
Laccase	<i>Trametes versicolor</i>	BPA	6	[99]
Peroxidase	Soybean hulls	Phenol	6 to 8	[57]
Peroxidase	Horse Radish	Phenol, 4-CP	7	[100]
Peroxidase	Horse Radish	2-MEP and other phenolic compounds	6.3	[68]
Peroxidase	Horse Radish	2,4-DCP	6	[101]
Peroxidase	Horse Radish	2-CP, 4-CP, 2,4-DCP	6.4	[66]
Peroxidase	Horse Radish	BPA	6	[90]
Peroxidase	Horse Radish	BPA	4	[102]
Peroxidase	Horse Radish	Phenol	7	[80]
Peroxidase	Horse Radish	Phenol	7.4	[103]
Peroxidase	Horse Radish	2,4-DCP	6.5	[104]
Peroxidase	Jicama skin peels	Phenol	7	[79]

Peroxidase	Soybean hulls	TC	7	[72]
Peroxidase	White Radish (Raphanus sativus)	α -naphthol and other phenolic compounds	6.5	[76]

2.4 Effect of Surface Active Additives

Along with the enzyme deactivation induced by pH and temperature variations, another source of deactivation might result from the free radical attack on the enzyme molecules and/or *via* the formation of inhibitory polymeric products [52,60,108]. The presence of organic and/or nonorganic contaminants in the enzymatically treated phenolic wastewaters might also contribute to the enzyme inhibition [109].

Different hypotheses have been proposed to explain such deactivations. For instance, it has been speculated that enzyme molecules possibly interact irreversibly with the formed intermediates (e.g., phenoxy radicals). This interaction induces a covalent bond between the enzyme and the oxidized radicals, leading to a loss in the enzyme activity [110]. Another proposed mechanism is that the enzyme molecules bind to the surface of charged micro-aggregates, which are formed during the biocatalytic reaction. As a result, a stagnant layer is formed around these micro-particles [110], causing diffusional limitations of phenolic substrates to the active site of the enzyme. In an attempt to eliminate or reduce enzyme deactivation caused by free radical attack and/or the formation of toxic polymeric products, researchers have utilized surface-active additives (i.e. polymers and surfactants).

2.4.1 Polymers

Enzymatic remediation of phenolic wastewaters in the presence of polymeric additives has been reported in a number of published studies (see Table 2-3). The most commonly used

polymeric additive is polyethylene glycol (PEG), mainly, due to its low cost [108] and effectiveness at low concentrations [111]. It has been reported that the addition of PEG at a concentration of 4 g/L has reduced the required amount of Horseradish peroxidase (HRP) by 200-fold [52,112]. Phenol removal enhancement in the presence of PEG (and other polymeric additives) has been attributed to the association of PEG with the polymeric products, preventing the enzyme molecules from being removed from the reaction medium *via* adsorption onto the polymeric products [55]. Another possible mechanism is the prevention of free radicals formed during the biocatalytic reaction from accessing and, thus, blocking the enzyme active site [52,55,90,113]. Both mechanisms would result in the presence of a higher level of active enzyme molecules in the reaction medium, leading to a higher enzymatic degradation rate of phenol pollutants. Since the formed free radicals and/or the polymeric products might vary according to the characteristics of the enzymatically treated phenol pollutant, the level of enzyme protection and, thus the removal enhancement, might depend on the type of the phenol pollutant being treated. Additionally, enzyme source has been also reported to impact the extent and the rate of phenol pollutant removal [73,74].

Diao et al. [81] have added PEG to peroxidases obtained from different plant sources (i.e., *Allium sativum*, *Ipomoea batatas*, *Raphanus sativus* and *Sorghum bicolor*). These researchers reported that the addition of PEG has largely increased the removal efficiency of various phenolic pollutants (i.e., gallic acid, ferulic acid, 4-hydroxybenzoic acid, pyrogallol and 1,4-tyrosol) from wastewater samples obtained from a leather processing plant by about 82% in the presence of 5 mM hydrogen peroxide (H₂O₂), which is an essential electron acceptor cofactor for effective degradation of phenolic substrates using

HRP. However, even low concentrations of H₂O₂ can lower the enzymatic reaction rate [79] while high H₂O₂ concentrations could render the peroxidase enzymes totally inactive [79,80]. Nonetheless, Diao et al. [81] did not assess the effect of H₂O₂ on the enzymatic activity in the presence of PEG.

Yamada et al. [90] have also utilized PEG for the enhancement of BPA removal from a wastewater sample in the presence of 0.3 mM hydrogen peroxide (H₂O₂). The addition of PEG (0.1 g/L) has facilitated the aggregation of the products formed during the enzymatic reaction, resulting in the preservation of the enzyme activity and thus the complete removal of BPA within 2 h. The addition of the same level of PEG (0.1 g/L) to a white radish peroxidase (from *Raphanus sativus*) has enhanced the removal of α -naphtholic from a synthetic wastewater sample by 2.7 folds [76]. Additionally, phenol removal using a peroxidase enzyme extracted from *Brassica oleracea* waste has been significantly improved in the presence of PEG from 35% to over 90% [77].

The addition of PEG to laccase-catalyzed reaction media has also resulted in a positive effect. For example, the amount of laccase required to achieve over 95% removal of 2,4-DCP has reduced to half in the presence of PEG [56]. Such a trend has been also reported in a recent study where the addition of PEG to laccase, obtained from *Trametes versicolor*, has reduced the required enzyme amount for the removal of BPA and its derivatives by 50-fold [114]. Such a huge reduction in the enzyme amount has been attributed to the PEG-driven protection of the enzyme against the entrapment of laccase molecules within the water-insoluble oligomer precipitates [114]. In line with this assertion, Kim and Nicell [115] proposed that water molecules bind to PEG, leading to the formation of a relatively large hydrated volume. PEG molecules have the ability to fold and, thus, entrap more water

molecules. The interaction of PEG with water molecules leads to the formation of a globular PEG structure, which is responsible for minimizing enzyme deactivation [59].

The beneficial effect of PEG, however, is dependant on its molecular weight. Kimura et al. [93] have observed that the removal of BPA by laccase in the presence of PEG increased with increasing the polymer molecular weight. Another study has also reported that the extent of BPA removal by laccase increased with increasing the molecular weight of PEG up to 10,000 g/mol, above which no further gain in BPA removal was obtained [115]. The extent of phenol removal has been also enhanced with increasing the molecular weight of PEG from 4000 to 10,000 [77]. Additionally, the level of peroxidase protection, and thus the enzyme activity, in the presence of PEG-10,000 was higher than in the presence of PEG-4000 [77]. Such enhancement of enzyme protection and phenol removal by using higher molecular weight PEG might be attributed to the more efficient formation of water-insoluble oligomers upon the interaction of high molecular weight PEG with the free radicals formed during the enzymatic degradation of phenolic pollutants [90].

Table 2-3 Polymeric additives utilized for the enhancement of phenolic pollutants removal from wastewaters using enzymes

Enzyme	Phenolic pollutant	Additive(s)	Level of removal enhancement	Removal extent (%)	Operating conditions	Enzyme activity	Ref
Laccase (from <i>Trametes villosa</i>) (0.001 U/mL)	Bisphenol A (BPA) (0.5 mM)	PEG (75 mg/L)	Reduced the required amount of laccase by 5.2 folds	Over 95 % (under optimized conditions)	Time: 3 h pH: 5.6 T: Not reported (NR)	20% increase in enzyme activity by PEG	[114]
Laccase (from <i>Trametes villosa</i>) (0.001 U/mL)	2,4-dimethylphenol (2,4-DMP) (1 mM)	PEG (1 mg/L)	Reduced the required amount of laccase by 2 folds	Over 95 % (under optimized conditions)	Time: 3h pH: 5.2 T: 20 °C	NR	[56]
Laccase (from <i>Trametes villosa</i>) (0.080 U/mL)	Phenol (1 mM)	PEG (200 mg/L)	No significant enhancement	Over 95 % (under optimized conditions)	Time: 3 h pH: 5.0–6.2 T: 22 °C	NR	[116]
Laccase (from <i>Trametes villosa</i>)	Phenol, o-, m-, p-cresols (treated separately with	PEG (5 mg/L), polyethyleneimine (PEI), and polyvinylpyrr-	Significant positive effect observed only for the case of cresols.	Over 95 % (under optimized conditions)	Time: 3 h pH: 5.6 T: Room temperature	The addition of PEG preserved the enzyme activity in the case of cresols, but no positive	[11]

(Different concentrations)	initial concentration of 1 mM each)	olidone (PVP) (used separately with 50 mg/L each)				effect was gained for in the case of phenol	
Laccase (from <i>Trametes versicolor</i>) (0.05 U/mL)	BPA, bisphenol B (BPB), bisphenol F (BPF), bisphenol E (BPE), bisphenol O (BPO), bisphenol T (BPT) (treated separately at initial concentration of 0.3 mM each), bisphenol C (BPC) (0.05	PEG (100 mg/L)	Reduced the required amount of laccase by 20%	100 % for BPA. Other bisphenols had differed removal percentages at various enzyme concentrations.	Time: 2 h pH: 5.0 T: 40 °C	NR	[93]

	mM),and bisphenol Z (BPZ) (0.02 mM)						
Laccase (from <i>Trametes versicolor</i>) (0.3 U/mL)	BPA (120 mM)	PEG (5 mg/L)	Reduced the required amount of laccase by 33%	Over 95 % (under optimized conditions)	Time: 2 h pH: 5.0 T: 25 °C	Enzyme activity after 1 h reaction was 90% of the original activity in the presence of PEG relative to 70% in its absence	[115]
Laccase (from <i>Trametes villosa</i>) (0.12 U/mL) and Soybean peroxidase (SBP) (1.5 U/mL)	Phenol (1 mM)	PEG (10–400 mg/L)	Reduced the required amount of SBP by 33% in the presence of 2 mM H ₂ O ₂ ; No effect in the case of laccase.	Over 95% (under optimized conditions)	Time: 3 h pH: 5.6–6.0 (laccase); 6.0-8.0 (SBP) T: NR	NR	[57]
HRP (extracted from	Phenol (2.0 mM)	PEG (300 mg/L)	Positive effect on the enzyme stability	About 95%	Time: 2 h pH: 7.2	NR	[117]

<i>Cochlearia armoracia</i>) (1.2 U/mL)					T: 20°C H ₂ O ₂ : 2.5 mM		
HRP (0.10 U/mL)	BPA (0.3 mM)	PEG (10 mg/mL)	Reduced the required dose of the enzyme	100%	Time: 2 h pH: 6.0, T: 30 ° C H ₂ O ₂ : 0.3 mM	NR	[90]
HRP (0.5 U/mL) and mushroom tyrosinase (50 U/mL)	Phenol , 2-methoxy-phenol (2-MEP), 3-methoxy-phenol (3-MEP), 4-methoxy-phenol (4-MEP), 2-chlorophenol (2-CP),	Polyallylamine -conjugated thermo-responsive polymers (1 g/L)	The remediation was enhanced for both enzymes in the presence of the polymer despite that the enhancement was more pronounced in the case of HRP	Almost complete removal of all pollutants in the case of HRP while only p-methoxy-phenol and p-cresol were completely removed in in the case of tyrosinase	Time: 12 h pH: 6.8 T: NR H ₂ O ₂ : 2.9 mM	NR	[118]

	<p>3-chlorophenol (3-CP), 4-chlorophenol (4-CP), 3-aminophenol (3-AP), 4-aminophenol (4-AP), 4-isopropyl-3- methylphenol (4-IP-3-MP) , and o-, m-, p-cresol (treated separately with initial concentration of 20 mg/L each) , BPA,</p>					
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	estrone, 17 β -estradiol (E2), estriol, and ethynylestradiol (ENE) (treated separately with initial concentration of 2 mg/L each)						
White radish peroxidase (extracted from <i>Raphanus sativus</i>) (1.0 U/mL)	α -naphthol and other phenolic pollutants (treated separately with initial concentration of 0.5 mM each)	PEG (0.1 mg/mL)	Significant removal enhancement for all pollutants	96% α -naphthol; the removal of other phenolic pollutants was lower	Time: 2 h pH: 6.5 T: 40 °C H ₂ O ₂ : 0.75 mM	NR	[76]
HRP (0.0396 U/mL)	A mixture of phenol (0.5 mM) and 2,4-	PEG (42 mg/L)	Enhanced the removal of both phenolic pollutants	94 % phenol; 89 % 2,4-DCP	Time: 3 h pH: 7.4 T: 25 °C	NR	[67]

	dichlorophenol (2,4-DCP) (0.5 mM)				H ₂ O ₂ :1.5 mM		
Peroxidases (extracted from tomato hairy roots) (0.2 U/L)	2,4-DCP (10 mg/L), phenol (10 mg/L)	PEG (100 mg/L)	Enhanced the removal of phenol, but not 2,4-DCP	78% 2,4-DCP; 98% phenol	Time: 1 h pH: 6-7 T: 25 °C H ₂ O ₂ : 1 mM (2,4-DCP); 0.1 mM (phenol)	Peroxidases activity increased by up to 13 folds upon PEG addition	[74]
Peroxidase (extracted from hairy roots) (200 U /mL)	2,4-DCP (25 mg/L)	PEG (100 mg/L)	Slight removal enhancement	83%	Time: 1 h pH: 6.5 H ₂ O ₂ : 0.5 mM	PEG addition preserved 40% of the original enzyme activity	[73]
Peroxidase from waste <i>Brassica oleracea</i> (0.6 U/ml)	Phenol (1 mM)	PEG (150 mg/L)	Significant enhancement	90%	Time: 3 h pH: 7.0 T: 30 °C H ₂ O ₂ : 2 mM	NR	[77]

2.4.2 Biological Polymers

Owing to the reported toxicity of PEG and the unsustainable route of PEG (and other polymers) production, few researchers have investigated the feasibility of replacing the chemical-based with bio-based polymeric additives (see Table 2-4). Among these researchers, Bratkovskaja et al. [110] have studied the peroxidase-catalyzed removal of 1-naphthol, 2-naphthol, and 4-hydroxybiphenyl using two different biopolymeric additives (bovine serum albumin (BSA) and human serum albumin (HAS)) and compared the efficacy of these biopolymers with those of chemically synthesized polymers (PEG and PEI). The isoelectric points of the peroxidase (obtained from *Coprinus cinereus*), BSA, HAS and PEI are, respectively, 3.5-3.8 [110], 4.7 [119,120], 4.7 [121] and 10.6 [122]. Since the removal of the above phenolic pollutants were carried out at pH 5.5 [110], the net charge on the enzyme, BSA and HAS is negative while PEI is positively charged. Accordingly, the investigators proposed that the biopolymeric additives (BSA and HAS) suppress peroxidase deactivation, most likely, due to the binding of naphthoyl radicals (which are positively charged) to the biopolymeric additives instead of the enzyme while in the case of PEI both the polymer and naphthoyl radicals bind to the enzyme, rendering it less effective [110].

The addition of other biopolymers (i.e. 10.08 vol% of dextran, 0.41 vol% of sodium alginate in 64 mM sodium acetate buffer) has been also investigated and reported to be beneficial [123]. These biopolymers have improved both the activity and stability of HRP, leading to an enhanced phenol degradation [123]. Carbohydrates (i.e. 18.25 vol% galactose, and 0.35 vol % guar gum in 20 mM sodium phosphate buffer) were also utilized as additives for the HRP-catalyzed removal of phenol from wastewater samples [103]. The

presence of these additives (individually) lowered the HRP dose required to achieve the same phenol degradation extent. A positive synergy was also observed, where the combination of guar gum and galactose provided higher removal of phenol as a result of more effective protection of the enzyme activity and stability in the presence of such a combination [103]. Further studies on the enzymatic remediation of different phenolic pollutants in the presence of other biopolymers and their mixtures are urgently needed in order to gain more insights into the effectiveness of these environmentally-friendly additives.

Unlike polymeric additives, no increase in the toxicity of the enzymatically treated phenolic wastewater in the presence of biopolymeric additives has been reported yet in the published literature. Contrarily, the enzymatic (using HRP) treatment of a wastewater sample containing phenol in the presence of chitosan biopolymer has resulted in a decrease in the toxicity of the treated wastewater [124]. Other researchers [125] reported a similar observation upon treating wastewater samples containing phenol and chlorophenols using mushroom tyrosinase in the presence of chitosan. Although the addition of chitosan to the enzyme-catalyzed phenol removal resulted in a decrease in the toxicity of the treated wastewaters, further studies are required to confirm that this is also the case for other biopolymers.

Table 2-4 Biopolymeric additives utilized for the enhancement of phenolic pollutants removal from wastewaters using enzymes

Enzyme	Phenolic pollutant	Additive(s)	Level of enhancement	Removal extent (%)	Operating conditions	Enzyme activity	Ref
Laccase (from <i>Trametes versicolor</i>) (0.3 U/mL)	BPA (0.12 mM)	Ficoll, (50 mg/L)	Increased the removal of BPA by 12%	78% in the absence of the biopolymer relative to 90% in its presence	Time: 1 h pH: 5 T: 25 °C	The remaining activity rose from 72 % to over 85 % after 1 h.	[115]
Laccase (from <i>Trametes villosa</i>) (0.002 U/mL)	o-cresol (1.0 to 5.0 mM)	Dextran (50.0 mg/L)	No enhancement	18 %	Time: 3 pH: 5.6–6.0 T: Room temperature	NR	[59]
Laccase (from <i>Trametes villosa</i>) (0.01 U/mL)	m-cresol (1.0 to 5.0 mM)	Dextran (50.0 mg/L)	No enhancement	45%	Time: 3 h pH: 5.6–6.0 T: Room temperature	NR	[59]

Laccase (from <i>Trametes villosa</i>) (0.001 U/mL)	p-cresol (1.0 to 5.0 mM)	Dextran (50.0 mg/L)	No enhancement	40%	Time: 3 h pH: 5.6–6.0 T: Room temperature	NR	[59]
HRP (1.8 U/mL)	Phenol (0.1 mM)	18.2% vol galactose, and 0.35 vol % guar gum in 20mM sodium phosphate buffer	Enhanced the removal of phenol by 30%	65% in the absence of biopolymeric additives relative to 95% in their presence	Time: 3 h pH: 7.4 T: 60 °C H ₂ O ₂ : 1.5 mM	NR	[103]
HRP (0.9 U/ml)	Phenol (0.1 mM)	10.08 vol % of dextran, 0.41 vol % of sodium alginate in 64 mM sodium acetate buffer	Enhanced the removal of phenol by 6 folds	10% in the absence of biopolymeric additives relative to 60% in their presence	Time: 2.5 h pH: 4.2 T: 30 °C H ₂ O ₂ :1.5 mM	NR	[123]
Recombinant peroxidase (from <i>Coprinus</i>)	1-naphthol (25 μM)	HAS (32 nM)	Enhanced the removal of 1-naphthol by 30%	60% in the absence of biopolymeric	Time: 10 min pH: 5.5 T: 25°C	NR	[110]

<i>cinereus fungus</i> (1 nM)				additive relative to 90% in its presence	H ₂ O ₂ : 0.1 mM .		
Recombinant peroxidase (from <i>Coprinus cinereus fungus</i>) (4 nM)	1-naphthol (50 μM)	BSA (20 nM)	Enhanced the removal of 1- naphthol by more than 50%	40% in the absence of biopolymeric additive relative to more than 90% in its presence	Time: 6 min pH: 5.5 T: 25°C H ₂ O ₂ : 0.1 mM	NR	[110]

2.4.3 Chemical Surfactants

Chemical surfactants have been also utilized for the enhancement of enzyme-catalyzed removal of phenolic pollutants from wastewater samples (see table 2-5). The enhancement of phenol removal in the presence of surfactants might stem from the entrapment (encapsulation) of some phenolic molecules within the surfactant micelle [2,3]. Another possible mechanism is *via* the formation of surfactant-pollutant insoluble complexes [113]. In the first mechanism, the concentration of the added surfactant must be at or above the critical micelle concentration (CMC) [2] while in the second mechanism, monomeric surfactant concentrations might be sufficient. In addition to surfactant-pollutant interactions, surfactants might also interact with the enzymatic reaction products/intermediates and, thus, reduce the interaction of such components with the enzyme molecules, leading to the suppression/minimization of the enzyme deactivation. However, undesirable surfactant-enzyme interactions, leading to a partial or complete enzyme denaturing, might be encountered in some systems, particularly for ionic surfactants [57,113].

The interaction of the added surfactant with the phenolic pollutants and/or their products is likely affected by the characteristics of the surfactant molecules. Broadly, there are four classes of surfactants (anionic, cationic, zwitterionic and nonionic). One of the most widely used surfactant for enhancing the enzyme-catalyzed removal of phenolic pollutants from wastewaters is Triton X-100, which is nonionic. For instance, Steevensz et al. [126] utilized Triton X-100 for the enhancement of phenol remedation from synthetic and real wastewater samples using SBP. The authors observed that the addition of Triton X-100 (125 to 645 mg/L) has reduced the required enzyme concentration for achieving more than 95% phenol removal from synthetic wastewater samples by more

than 10-fold. The addition of Triton X-100 to real wastewater samples has also resulted in a remarkable increase in the phenol removal extent [126].

Ji et al. [60] have also utilized Triton X-100 for the enhancement of BPA removal from synthetic wastewater samples using laccase from *Trametes versicolor* and reported an enhanced BPA degradation when the utilized Triton X-100 concentration was close to its CMC. However, above the CMC, the surfactant micelles entrapped some BPA molecules, shielding them from the contact with the enzyme, which has resulted in a lower BPA degradation. Despite the lower extent of BPA degradation in the micellar surfactant solutions, the enzyme stability was improved in both monomeric and micellar surfactant solutions [60]. In an effort to elucidate the mechanism of the improved enzyme stability in the presence of Triton X-100, Ji et al. [60] have conducted fluorescence studies and concluded that the interaction between the surfactant and enzyme played a significant role in the folding and, thus, the stabilization of laccase. The binding of Triton X-100 molecules to the laccase surface has contributed to the suppression of the enzyme deactivation caused by the free radicals and/or the polymeric reaction products [60]. In another study, Zhang et al. [2] used Triton X-100 at concentrations ranging from about 30 to 930 μM for the enhancement of phenol removal from a synthetic wastewater sample containing 50 ppm phenol using laccase, and reported that the highest phenol removal was obtained at 155 μM Triton X-100, which is below the CMC (310 μM) of this surfactant. Similar to the observation reported by Ji et al. [60], lower phenol removal extent was observed by Zhang et al. [2] in the presence of micellar Triton X-100 solutions. Such a decrease in the phenol removal in the presence of micellar Triton X-100 concentrations has been also attributed to the encapsulation of a fraction of phenol in the surfactant micelles.

In addition to Triton X-100, other surfactants have been also utilized for the enhancement of phenolic pollutants removal by enzymes. One of these surfactants is Dynol 604, which is acetylenic-based nonionic surfactant. Although the addition of Dynol 604 did not increase the initial degradation rate of phenolic pollutants (phenol, 1-naphthol, 2-naphthol, and 1-hydroxypyrene) by recombinant *Coprinus cinereus* peroxidase, the ultimate degradation extents of these phenolic pollutants have significantly increased [127]. For example, increasing the concentration of Dynol 604 from 1 to 10 ppm has doubled the extent of 1-naphthol removal. Additionally, no enzyme inhibition was observed in the presence of Dynol 604. Thus, the significant improvement of 1-naphthol removal upon the addition of an appropriate concentration of Dynol 604 might be correlated to the enzyme protection effect imparted by the surfactant molecules. Such a positive effect of Dynol 604 addition was also observed for the enzymatic removal of 2-naphthol [127]. However, in order to double the extent of 2-naphthol removal, Dynol 604 concentration has to be increased by 30-fold instead of 10-fold for the case of 1-naphthol. Additionally, marginal enhancement of 1-hydroxypyrene removal was observed even with increasing Dynol 604 from 20 to 70 ppm. The insignificant improvement of 1-hydroxypyrene removal with increasing Dynol 604 by almost 4-fold could, intuitively, be justified by the complexity of this phenolic pollutant. However, the presence of Dynol 604 did not provide any enhancement for phenol removal regardless of the utilized concentration of the surfactant. Such null improvement in phenol removal with the addition of Dynol 604 suggests that the improved removal of 1-naphthol and 2-naphthol in the presence of this surfactant is not merely due to the protection of the enzyme against inhibition but rather through other (and probably more complex) mechanisms, which worth further investigations.

Table 2-5 Chemical surfactants additives utilized for the enhancement of phenolic pollutants removal from wastewaters using enzymes

Enzyme	Phenolic pollutant	Additive(s)	Level of enhancement	Removal extent (%)	Operating conditions	Enzyme activity	Ref
Laccase (from <i>Trametes versicolor</i>) (0.37 U/mL)	BPA (0.3 mM)	Triton X-100 (50 mg/L)	More than 9 folds enhancement	10% in the absence of the surfactant relative to about 92% in its presence	Time: 0.5 h pH: 4.2 T: 25 °C	13% of the initial activity was retained without Triton X-100 in 0.5 h , but 70% was retained with Triton X-100 after 1 h.	[60]
Laccase (from <i>Trametes versicolor</i>) (1.155 U/mL)	Phenol (50 mg/L)	Triton X-100 (0.155 mM)	Enhanced phenol removal by 1.2 folds	Removal efficiency increased from 72 to 85%	Time: 6 h pH: 6.0 T: 25 °C	NR	[2]
Laccase (from <i>Trametes versicolor</i>) (1.155 U/ml)	Phenol (50 mg/L)	Sodium dodecyl sulfonate (SDS) (4 mM)	Reduced the removal of phenol by about 14%	74% in the absence of SDS relative to 60% in its presence	Time: 6 h pH: 6.0 T: 25 °C	NR	[3]

Laccase (from <i>Trametes versicolor</i>) (1.155 U/ml)	Phenol (50 mg/L)	Hexadecyltrimethylammonium bromide (CTAB) (0.5 mM)	Reduced the removal of phenol by about 9%	74% in the absence of CTAB relative to 65% in its presence	Time: 6 h pH: 6.0 T: 25 °C	NR	[3]
SBP (various concentrations)	Phenol (1–10 mM)	Triton X-100 (125–645 mg/L)	Reduced the required amount of SBP by at least 10 folds	Over 95 %	Time: 3 h pH: 7.0 T: 20°C H ₂ O ₂ : 1.5 times the phenol molar concentration	NR	[126]
Recombinant <i>Coprinus cinereus</i> peroxidase (38 nM)	Phenol (0.012 mM)	Dynol 604 (0–48 ppm)	Extents of phenol removal in the presence and the absence of the surfactant were similar	100%	Time: 10 min pH: 5.5 T: 25 °C H ₂ O ₂ : 0.1 mM	NR	[127]
Recombinant <i>Coprinus</i>	1-naphthol (0.024 mM)	Dynol 604 (0–10 ppm)	Varied levels of enhancement depending on the	NR	Time: 10 min pH: 5.5 T: 25 °C	NR	[127]

cinereus peroxidase (1 nM)			surfactant concentrations		H ₂ O ₂ : 0.1 mM		
Recombinant Coprinus cinereus peroxidase (1 nM)	2-naphthol (0.024 mM)	Dynol 604 (0–30 ppm)	Varied levels of enhancement depending on the surfactant concentrations	NR	Time: 10 min pH: 5.5 T: 25 °C H ₂ O ₂ : 0.1 mM	NR	[127]
Recombinant Coprinus cinereus peroxidase (32 pM)	1-hydroxypyrene (0.0073 mM)	Dynol 604 (0–73 ppm)	Varied levels of enhancement depending on the surfactant concentrations	NR	Time: 10 min pH: 5.5 T: 25 °C H ₂ O ₂ : 0.1 mM	NR	[127]

Another acetylenic-based nonionic surfactant that has been also proposed to boost enzyme-catalyzed removal of phenolic pollutants from wastewater is Surfynol 465. One of the proposed benefits of adding this surfactant to the enzymatically treated wastewater is the suppression of enzyme deactivation. Such benefit has been reported by Ruta and Juozas [61] who observed that when this surfactant was added (in a dose manner) to the medium of peroxidase-catalyzed removal of 2-naphthol, the enzyme inhibition was completely eliminated. Such enzyme protection has improved the removal extent of 2-naphthol, which increased with increasing Surfynol 465 concentration. However, no further removal enhancement was observed above the surfactant CMC [61].

Besides the above-mentioned nonionic surfactants, ionic surfactants have been also studied with the aim of revealing their potential as enzyme-catalyzed phenol removal enhancers. For example, Chhaya and Gupte [128] studied the removal of BPA using laccase in the presence of reversed micelles of bis(2-ethylhexyl) sulfosuccinate sodium (AOT), which is an anionic surfactant, and reported a complete degradation of BPA within 2 h [128]. The authors also observed that the AOT reversed micellar solutions improved the stability and activity of laccase due to the effective shielding of the enzyme molecules by a water layer and a surfactant shell. The solubility of the substrate and the enzyme might also be improved in the reversed micellar solutions of AOT, allowing easier access of BPA molecules to the enzyme active site and, thus, enhanced BPA removal [128]. In addition to AOT, the HRP-catalyzed removal of phenol in the presence of another anionic surfactant, sodium dodecylbenzenesulfonate (SDBS), has been also enhanced [129]. For instance, adding 0.45 g of SDBS to the reaction medium (~50 mL) has resulted in an extensive

polymerization of phenol (converted to phenylene and oxyphenylene), with more than 94% phenol conversion within 0.5 h relative to less than 5% in the absence of SDBS [129].

However, there are cases where the addition of ionic surfactants was not beneficial. For instance, the addition of sodium dodecyl sulfonate (SDS), which is an anionic surfactant with some similarities to SDBS, did not provide a significant improvement of phenol removal by laccase [3]. Similar observation was also reported for the same system but with replacing the anionic surfactant (SDS) with the cationic surfactant, hexadecyltrimethylammonium bromide (CTAB) [3]. These findings contradict those reported by Chhaya and Gupte [128] and Zhang et al. [2]. It is expected that phenol removal enhancement in the presence of a given surfactant will depend on the surfactant-enzyme, surfactant-products/pollutants, and enzyme-products/pollutants interactions; these interactions might significantly vary with the variations in the reaction conditions (e.g., type of the phenolic pollutant, the utilized enzyme, medium temperature and pH, presence of salt ions or other additives/contaminants). Therefore, to draw a clear and reliable conclusion on which surfactant(s) are more effective, variations in the reaction conditions, in the presence of surfactants, have to be minimized.

2.4.4 Biological Surfactants

Despite that the addition of synthetic surfactants proved useful in some cases, these fossil-based materials are usually non-biodegradable and might be toxic to aquatic life [130–132]. It was reported, for instance, that some chemical surfactants such as Triton X-100 and SDS are harmful to aquatic organisms and might pose long-term negative effects on the marine creatures [61]. To tackle the secondary pollution problems associated with the use of chemical surfactants, biosurfactants have been proposed as alternatives. Biosurfactants

are biodegradable [133–135] and biocompatible [134,136] and, thus, unlikely to pose environmental hazards. Additionally, biosurfactants are produced from sustainable sources and they are usually efficient even at low concentrations when compared to chemical surfactants [133].

Biosurfactants molecules possess hydrophobic and hydrophilic moieties, making them amphiphilic compounds. The hydrophilic portion of the biosurfactant molecule can be alcohol, carboxylic acid, carbohydrate, cyclic peptide, phosphate or amino acid while the hydrophobic moiety is based on long-chain or hydroxy fatty acids [137]. The presence of hydrophobic and hydrophilic moieties on every biosurfactant molecule promotes its self-assembly at fluid-fluid interfaces [138–144]. Additionally, this amphiphilic character leads to the formation of biosurfactant aggregates (i.e., micelles) in solutions when the biosurfactant concentration is equivalent or above its CMC. One of the appealing characteristics of biosurfactants is their relatively lower CMC compared to synthetic surfactants. The CMCs of biosurfactants are usually 10-40 times lower than those of common chemical surfactants [145]; lower CMC might be associated with the requirement of relatively less biosurfactant amount, which is an important economic factor. Furthermore, these bio-based surface active agents are usually effective even under extreme values of pH, temperature and salinity [146–148], making them an attractive option as additives for the enhancement of enzymatic remediation of phenolic wastewaters. Despite the attractiveness of biosurfactants, a limited number of studies have been published so far on their utilization for the enhancement of phenolic wastewaters remediation using enzymes. One of these studies used rhamnolipid, which is an anionic glycolipid biosurfactant, and reported that the addition of this biosurfactant provided 60%

enhancement of 2,4-DCP removal from wastewater samples using minced horseradish from *Armoracia rusticana* [58]. Interestingly, unlike chemical surfactants, micellar rhamnolipid concentration did not reduce the extent of 2,4-DCP removal [58]. In support of this observation, Liu et al. [3] have reported more than 4-fold enhancement of phenol removal from wastewater samples using laccase in the presence of rhamnolipid biosurfactant. Higher concentration of the biosurfactant (3 times above the CMC) did not reduce the extent of phenol removal but rather a slight improvement relative to the pre-micellar solution was observed. Comparing this performance with those of CTAB and SDS revealed the superiority of the biosurfactant at both pre-micellar and micellar concentrations. These chemical surfactants were, indeed, detrimental to phenol removal regardless of their concentrations. This is in line with the statement presented by Otzen [149] that biosurfactants are less aggressive towards enzymes and, thus, they usually do not denature/destabilize enzymes.

However, Ruta and Juozas [61] reported a contradicting observation, where pre-micellar concentrations of rhamnolipid enhanced the removal of 2-naphthol using a peroxidase enzyme obtained from *Coprinus cinereus*, while micellar solutions resulted in a reduction in this phenolic pollutant remediation. Additionally, Ruta and Juozas [61] observed that the rate of 2-naphthol removal in the presence of the biosurfactant was always lower than that in the presence of the nonionic chemical surfactant, Surfynol 465. It is unclear, however, whether such contradiction stems from the characteristics of the used biocatalyst/phenolic substrate or from other factors (e.g., experimental conditions). Regardless of the reason behind such contradiction, it is highly recommended to

eliminate/minimize the operational variabilities between conducted studies in order to draw meaningful and reliable conclusions.

Besides the positive effect of biosurfactant addition (particularly at pre-micellar concentrations) on the enzymatic remediation of phenolic wastewaters, their positive effect on the biological utilization of phenolic substrates has been also demonstrated in some published studies. For example, Zhou et al. [135] reported that the addition of saponin and rhamnolipid to the fermentation medium of *P. simplicissimum* has led to a higher microbial degradation of phenol substrate and also to a higher activity of the laccase produced from the fermentation process. In another study [150], the addition of rhamnolipid to the growth medium of *P. chrysosporium* has improved the activity of the produced lignin peroxidase, CMCase and xylanases enzymes but inhibited the activity of manganese peroxidase. Contrarily, the addition of SDS has rendered these four enzymes almost inactive. Liu et al. [151] also studied the effect of adding rhamnolipid and Tween-80 to the fermentation medium of *Trichoderma viride* on the production of Avicelase, CMCase and cellobiase enzymes. The researchers reported that despite the positive effect of both surface active agents on the activity of the produced enzymes, the biosurfactant was more effective. Similar observation was also reported by Jadhav et al. [152] who reported that the activities of lignin peroxidase and veratryl alcohol oxidase enzymes produced by *Bacillus* sp VUS NCIM 5342 were improved when rhamnolipid was added to the growth medium. Such observations encourage further in-depth studies to fill in the huge gap with respect to the assessment of biosurfactants as potentially effective additives that pose no environmental hazards for enhancing the enzymatic treatment of phenolic wastewaters.

2.5 BPA as a Model Pollutant

According to the literature, a too limited number of studies have been devoted to evaluate the effect of biological surfactants on the enzymatic treatment of phenolic wastewater. More specifically, no single paper has attempted yet to uncover the potential impact of biosurfactant on the removal efficiency of BPA from water by laccase. To justify the selection of BPA as a target chemical, brief information about BPA will be highlighted in the upcoming two sub-sections.

2.5.1 BPA Sources

BPA or (2,2-(4,4'-dihydroxydiphenyl)propane is formed by reacting phenol with acetone (figure 2-3) [153]. BPA is largely deployed in the synthesis of plastic polycarbonates and epoxy resins [69]. These materials participate in the manufacture of plastic bottles, water supply pipes, and electronic devices. The final products are also utilized as protective coatings for canned food and beverages [63]. The high production of BPA, which is estimated to be 3.8 million tons per year [14], along with the improper disposal of its final products might highly contribute to the contamination of water resources [154].

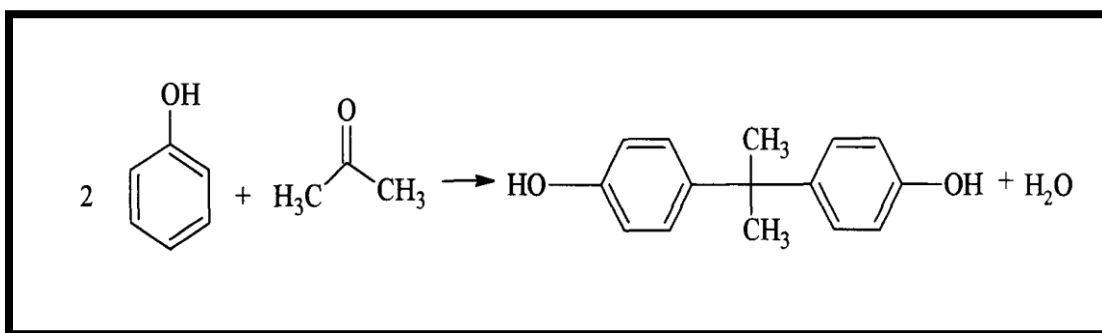


Figure 2-3 The formation of BPA by reacting phenol with acetone [155]

2.5.2 BPA in Effluents and Water Bodies

It has been reported that the concentrations of BPA in industrial effluents can range from 0.1 ppm to 2 ppm, according to the Toxic Substances Control Act (TSCA) [156]. In Saudi Arabia, a group of researchers revealed that BPA was detected in some treated wastewater samples from different wastewater plants at an average concentration of 4.37 $\mu\text{g/L}$ [157]. In addition to its availability in effluents, this endocrine disrupter can be found in surface and ground waters. For instance, the maximum detected levels of BPA are 4.23 $\mu\text{g/L}$ and 6.4 $\mu\text{g/L}$ in surface waters and ground waters, respectively [158]. The aforementioned values, however, exceed the safe concentration, which is set 1.5 $\mu\text{g/L}$ by the European Union [159]. Thus, it is imperative to develop effective techniques having the potential to treat the waters contaminated with BPA.

CHAPTER 3

EFFECT OF BIOSURFACTANT ON BPA REMOVAL

In this chapter, the enzymatic decontamination of bisphenol a (BPA) from aqueous solution by laccase was investigated in the absence and presence of biosurfactant rhamnolipid (Rhl). Optimization of Rhl concentration at 50 ppm BPA and 0.05 mg/L laccase over 2h of treatment period was carried out. At the optimal concentration of Rhl, the influence of various reaction parameters on the enzymatic treatment including initial BPA concentration, pH value, temperature, and NaCl concentration was evaluated. It was found that the presence of Rhl improved the removal efficiency of BPA over the tested range for each parameter.

3.1 Introduction

Bisphenol a (BPA), which is a common phenolic derivative, is regarded as an environmental endocrine disruptor, due to its estrogenic activity [155]. Such a pollutant, even at low concentrations, can lead to metabolic disorders and abnormalities in human babies and cause cancer in breast and prostate glands [160]. In addition to its health effects on humans, BPA has the capability to hinder the growth of plants [161] and disturb aquatic biota [161]. Due to these concerns, it has become of great significance to eradicate BPA from water systems. Accordingly, several techniques including adsorption [162], membrane separation [163], photo-oxidation [164], and biodegradation [63] have been

proposed to remediate wastewaters polluted by BPA. However, most of these methods are either expensive or environmentally deleterious [165].

Recently, researchers have been attracted by the bio-catalytic treatment (i.e. using extracellular enzymes such as peroxidases and laccases) as an eco-friendly alternative to the aforementioned techniques with the sake of treating phenolic wastewaters [165]. Laccases (EC 1.10.3.2) featured with four blue coppers can spur the oxidation of aromatics, primarily phenolic compounds, in the presence of oxygen [85]. Despite its promising potential for enhancing the degradation rate of phenols, undesirable interactions between laccase and reaction products might occur during the treatment, leading to enzyme inactivation [114]. In an attempt to mitigate the loss of activity, polymers and chemical surfactants such as polyethylene glycol (PEG) [56] and Triton X-100 [126] have shown great aptitude for protecting the active site of enzymes from the attack of radicals and polymeric products. However, these chemical additives are mostly non-biodegradable, and in some cases, could induce noxious effects upon the interaction with aquatic species [130,131].

Biosurfactants are thought to be a good substitute for the fossil-originated surface active materials due to their unique characteristics such as biodegradability [62,145], biocompatibility [137], and superior effectiveness at low concentrations [133,166]. The most prevalent type of biosurfactants is rhamnolipids (Rhl) which are commonly produced by *Pseudomonas aeruginosa chlororaphis* [167]. The structure of Rhl is formed when one or two molecules of rhamnose bound to hydroxydecanoic acid [166]. As reported by some researchers, Rhl tend to ameliorate the activity of many enzymes including peroxidases [152], laccases [135], and xylanases [150] when utilized in various applications.

Nonetheless, a limited number of studies [3,58,61] have evaluated their ability to enhance the enzymatic treatment of phenolic wastewaters. More specifically, no single study has yet attempted to scrutinize the utilization of Rhl in tandem with laccases for augmenting the removal rate of BPA from aqueous solution. Hence, the current study investigated the enhancement of BPA removal by Rhl addition in the presence of laccase. The performance of such a system was evaluated by varying some parameters such as biosurfactant concentration, initial BPA concentration, temperature, pH value, and salt concentration.

3.2 Materials

Laccase (EC 1.10.3.2) from *Trametes versicolor*, Rhl, BPA, 2,20-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) were all purchased from Sigma-Aldrich. The utilized RL is a mixture of mono- and di-rhamnolipid; the ratio of the former to the latter is 3:2. The purity of this Rhl is 90 w%; the remaining fraction represents non-canonical rhamnolipids (unsaturated rhamnolipid molecules and/or those with a lipid chain of 8 or 12 carbons rather than the standard 10 carbon atoms). The remaining reagents were of analytical grade. Britton-Robinson buffer, which is composed of 0.1 M phosphoric acid, 0.1 M boric acid, 0.1 M acetic acid, was used to study the effect of pH during the experiments. The value of pH was adjusted to the targeted values by the addition of 0.2 M sodium hydroxide.

3.3 Methods

3.3.1 Laccase Activity Assay

The activity of laccase was determined by conducting an assay which involved the oxidation of ABTS. The assay reaction mixture with a final volume of 4 mL was incubated in a cuvette containing 0.2 mM ABTS and a suitable amount of laccase in 0.2 M Acetate Buffer (pH 4.5). The reaction rate (i.e. absorbance increase at a wavelength of 420 nm) with the molar extinction coefficient of $36 \times 10^{-3} \text{ M}^{-1} \text{ cm}^{-1}$ at 25 °C [168] was monitored by using a UV- 1601PC spectrophotometer (Shimadzu, Japan). One unit (U) of laccase activity was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per minute.

3.3.2 BPA Concentration Measurement

The remaining amount of BPA in enzymatic samples was quantified by high-performance liquid chromatography system (HPLC, Agilent, USA) fitted with a ZORBAX Eclipse XDB-C18 column (Agilent, USA). A detection wavelength of 276 nm was used with isocratic elution rate of 1 mL/min. The mobile phase consisted of deionized water and acetonitrile (40%/60%, v/v).

3.3.3 Critical Micelle Concentration (CMC) Determination

To determine the CMC of RL, dynamic surface tension measurements were carried out as described elsewhere [139,169,170]. Briefly, a specific volume (~ 8 mL) of RL solution at a given concentration was placed in a cuvette and then a small air bubble (volume ~ 25 μL) was created in the RL solution. Then, the change in the surface tension of the air bubble

was measured as a function of time using KRUSS DSA 25S until an equilibrium surface tension value was obtained. The above procedure was repeated using different RL concentrations. Each experiment was carried out at least twice and the reproducibility was quite good (i.e., error was less than 5%). The collected values of the equilibrium surface tension were plotted against the logarithmic values of RL concentrations, enabling the determination of the RL CMC.

3.3.4 Experimental Protocol

Degradation experiments were performed at room temperature in 50-mL beakers containing BPA and RhI as an additive where appropriate. A certain dose of laccase solution was added to initiate the reaction. The batch reactors were vigorously stirred via magnetic stirrers and Teflon-coated stir bars. To halt the reaction at the targeted time, 3 ml from reaction medium was drawn and quenched with 1 ml of 0.5 M Hydrochloric Acid. The collected samples were then filtered through 0.2 micron polyvinylidene fluoride (PVDF) membrane filters to remove insoluble particles prior to HPLC analysis.

For the purpose of studying the effect of pH on degradation efficiency with or without RhI, Britton-Robinson buffer was used to adjust pH values in reaction solutions from 3 to 9 by adding an appropriate volume of 0.2 M sodium hydroxide. The effect of temperature was also studied at a fixed concentration of laccase and BPA in the absence and presence of RhI. The temperature of reaction solutions (i.e. 22, 40, 50, 60, 70 °C) was adjusted by a stirring hot plate. To uncover the impact of salt on BPA oxidation with or without RhI, a stock of 2 M sodium chloride was prepared in deionized water, and then utilized to obtain the desired salt concentrations (i.e. from 20 to 500 mM). All experiments were performed at least twice. All data in figures are expressed as averages of the collected data.

3.4 Results and Discussion

3.4.1 Effect of Initial BPA Concentration and Laccase Dosage

The influence of initial BPA concentrations on the removal efficiency of BPA was studied. At 22 °C, pH 5.8 and laccase concentration of 0.05 mg/mL, the initial pollutant concentration was varied from 5 to 85 ppm. As depicted in Figure 3-1, the removal efficiency decreased with increasing the initial BPA concentration during the 2-h treatment. Specifically, the conversion rate was slightly dropped when raising BPA concentration from 5 to 25 ppm. However, doubling the initial pollutant concentration to 50 ppm led to a sharp drop in removal efficiency from 70.73 to 53.26 %. Further addition of BPA, beyond that point, did not alter the removal efficiency, implying that enzymatic reaction might have reached to the equilibrium state. The same pattern was also observed by Kalaiarasan and Palvannan [103], who reported a decline in the phenol removal at higher pollutant loadings. Such poor degradation efficiencies could be attributed to the accumulation of more free radicals, which are thought to be intuitively responsible for enzyme deactivation [171].

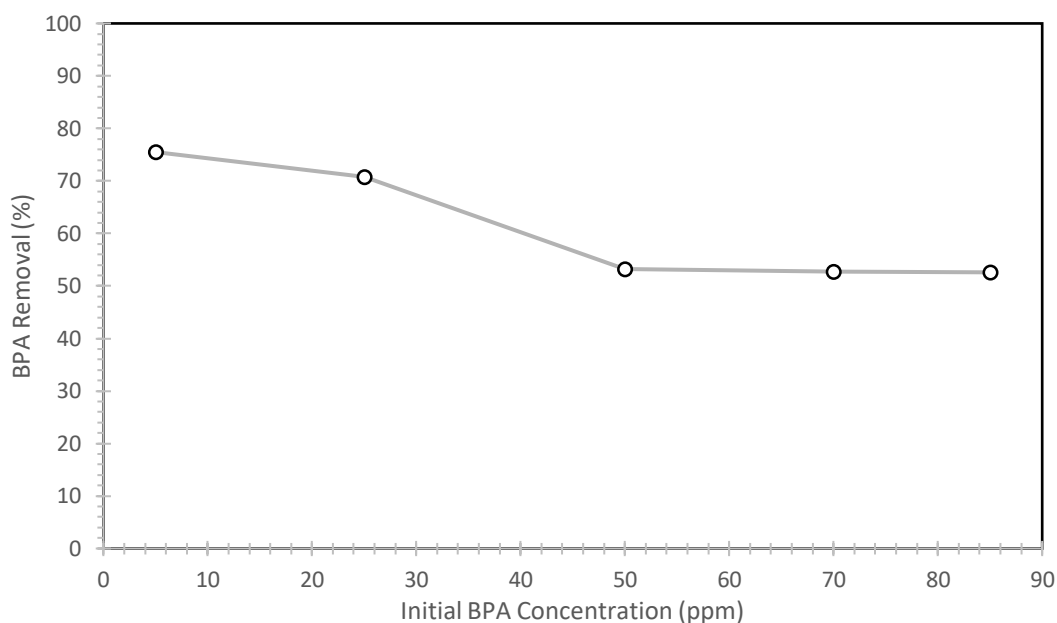


Figure 3-1 Effect of initial BPA concentration on the removal efficiency. Conditions: 0.05 mg/mL laccase, pH 5.8 and 22°C after 2 h reaction

At 22 °C, pH 5.8 and BPA concentration of 50 ppm, five different laccase doses (i.e. from 0.01 to 0.4 mg/mL) were introduced into batch reactors to examine their effect on the removal efficiency for 2 h. As it is clear from figure 3-2, increasing the amount of laccase augmented the conversion rate of BPA. More specifically, a linear increase in the removal efficiency from 27.86 to 73.24 % was witnessed until 0.1 mg/mL of laccase. However, the degradation efficiency started to slowly increase beyond this point, reaching to a removal of 97.61 % using 0.4 mg/ml of laccase. The same trend was also reported by Asadgol et al. [86] who found that raising laccase concentration from 1 to 20 U/mL resulted in over 90% removal of BPA after 0.5 h. Such findings are in a good agreement with other studies as well [69,172].

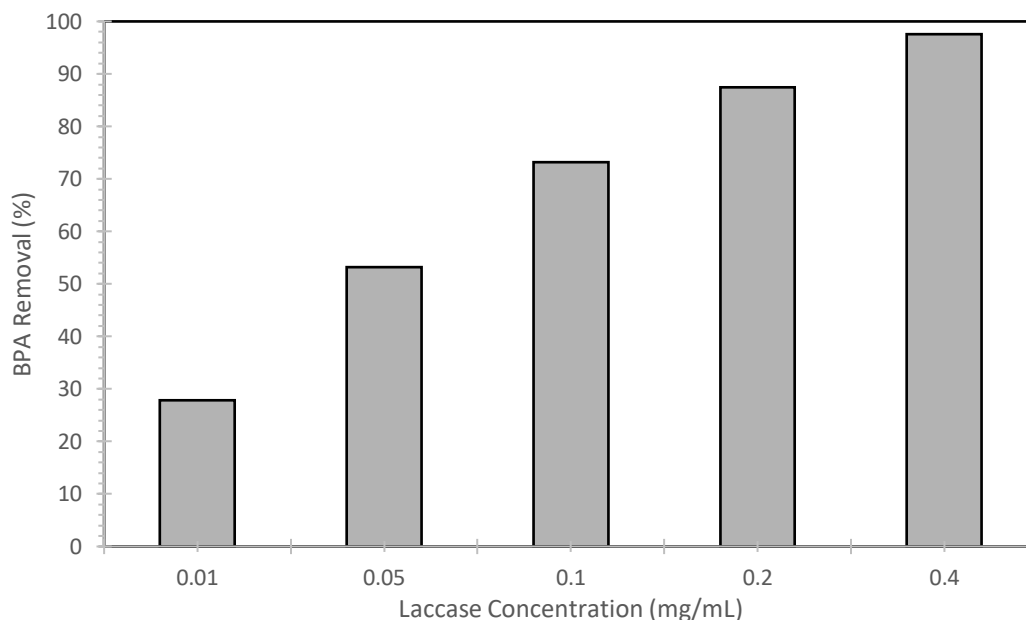


Figure 3-2 Effect of laccase concentration on the removal efficiency. Conditions: 50 mg/L BPA, pH 5.8 and 22°C after 2 h reaction

3.4.2 Effect of Rhl Concentration

Surfactants possess both hydrophobic groups (their tails) and hydrophilic groups (their heads). At low concentrations, they exist as monomers (below CMC) while they tend to form aggregates or micelles at high concentrations (at or above CMC) [165]. Thereby, their effect on the enzymatic degradation of BPA will most likely depend on the used concentration. In this work, the impact of Rhl at concentrations below and above its CMC (i.e. 50 ppm) on the removal of BPA catalyzed by laccase was examined (see figure 3-3). The results revealed that a positive effect upon the addition of Biosurfactant was only attained below its CMC, particularly at low concentrations. The optimal concentration of Rhl was 1 ppm, which enabled a 22.81% enhancement in BPA conversion after a 2 h-

treatment. This positive impact might be due to the positive interactions between laccase and RhI, leading to a 13%-increase in laccase activity (i.e. Determined in this study by laccase activity assay). However, the stimulative effect of RhI at higher concentrations diminished, showing an opposite impact at concentrations equal and above CMC. Similar scenarios have been also observed when increasing the concentration of some additives including Triton X-100 [2,60], and Tween 80 [173]. For example, raising the concentrations of both rhamnolipid JBR425 (i.e. from 0.25 μM to 3 mM, CMC = 1 mM) and Surfynol465 (i.e. from 0.25 μM to 30 mM, CMC = 11 mM) resulted in lower removal rates of 2-naphthol in the presence of 1 nM peroxidase [61].

However, our study noticed a negative impact on BPA degradation in micellar solutions of RhI with relative to the control reaction. The same result was also observed when the addition of sodium dodecyl sulfonate (SDS) and hexadecyltrimethylammonium bromide (CTAB), above their CMC, lowered the phenol conversion rate by almost 14% and 9%, respectively [3]. It can be speculated that the enhancing effect below CMC is correlated to the changes in enzyme conformations induced by its interaction with surfactant monomers [174]. Above CMC, biological aggregates or micelles might have entrapped BPA molecules, preventing their access to the active site of enzyme, and thus, lowering the removal efficiency [174].

Unlike our findings and other results including [2,60,61], some studies reported positive effects with increasing surfactant concentrations. In this regard, one group of researchers found that the addition of dirhamnolipid, even above CMC, augmented the conversion rate of phenol by laccase [3]. Similarly, a three-fold increase in the removal efficiency of 2,4-dichlorophenol (2,4-DCP) by HRP was attained in the presence of micellar RhI (10 CMC)

[58]. Such contradicting results arising from the aforementioned studies can be justified by the differences in the nature of enzymes and/or structure of phenolic compounds. Most importantly, the characteristics of surfactants (i.e. the source, degree of purity, and properties of micelles) along with the reaction environment could have contributed to such variations.

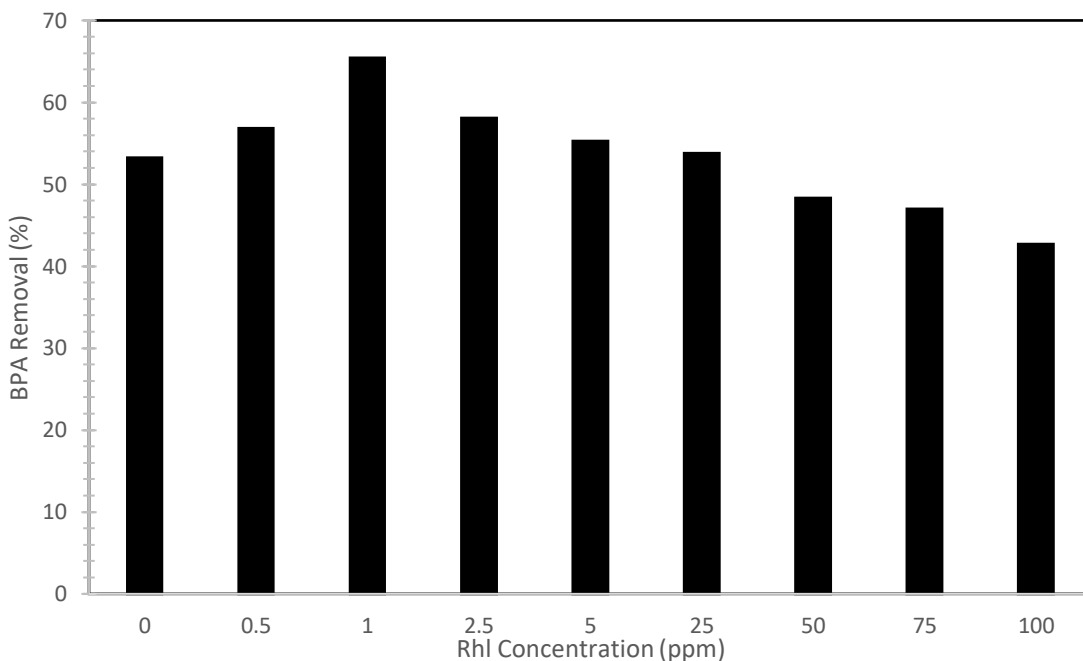


Figure 3-3 Effect of Rhl concentration on the removal efficiency. Conditions: 50 mg/L BPA, 0.05 mg/mL laccase, pH 5.8 and 22°C after 2 h reaction

3.4.3 Effect of BPA Concentration in the Presence of Rhl

Figure 3-4 shows the level of enhancement in the enzymatic removal of BPA upon the addition of Rhl over various initial BPA concentrations (i.e. from 5 to 85 ppm) after 2h of treatment. As it is clear from the results, the presence of 1 ppm Rhl enhanced the removal of BPA catalyzed by 0.05 mg/mL laccase. The stimulative effect of Rhl on the removal

efficiency was increasing up to a BPA/Rhl molar ratio of 50, followed by a decrease in the enhancement rate when introducing more BPA into the reaction. For instance, the level of enhancement rose from 10.24 to 22.81% after increasing BPA concentration from 5 to 50 ppm. However, beyond that point, a decline in the enhancement level was attained. Similarly, Ji et al. [60] reported that the addition of Triton X-100 at different BPA concentrations improved the BPA conversion, even though the level of enhancement in BPA degradation started to decrease beyond a BPA concentration of 0.3 mM (or 68.49 ppm).

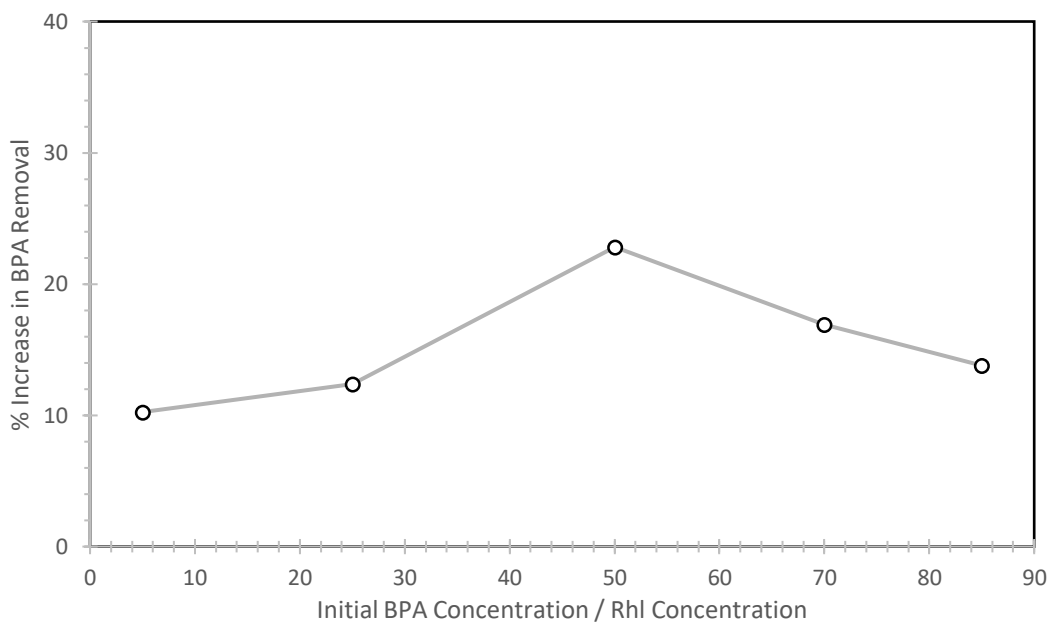


Figure 3-4 The percentage increase in BPA removal as a function of initial BPA concentration / Rhl concentration ratios. Conditions: 1 mg/L Rhl, 0.05 mg/mL laccase, pH 5.8 and 22°C after 2 h reaction

The reaction progress over time was studied as well in the absence and presence of 1 ppm Rhl (figures 3-5 and 3-6) at two different BPA concentrations. It can be judged from the shape of the curves that the effect of Rhl generally became more intense as the enzymatic reaction proceeded. Consequently, laccase was able to degrade more BPA, thanks to the interactions between Rhl and laccase and /or reaction products [175]. Similar findings were also obtained by Liu et al. [3] who reported that a more positive effect induced by dirhamnolipid on the removal efficiency as the reaction time increased over different phenol concentrations. Comparing their results with our study at a pollutant concentration of 50 ppm, almost the same enhancement level was obtained in the presence of 0.05 mg/mL laccase.

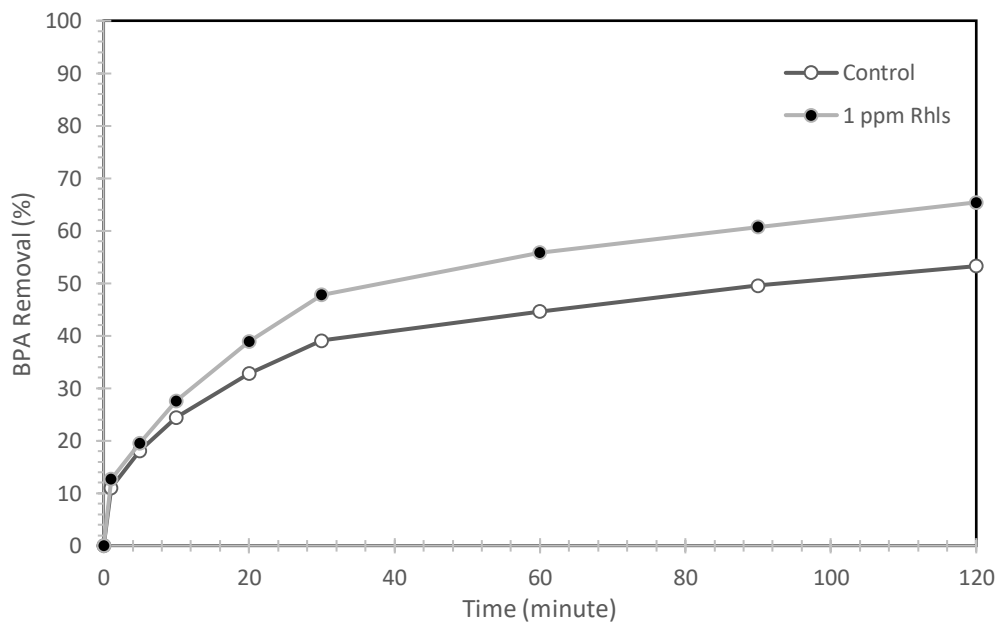


Figure 3-5 Time course of BPA removal in the absence and presence of Rhl. Conditions: 50 mg/L BPA, 0.05 mg/mL laccase, pH 5.8 and 22°C after 2 h reaction

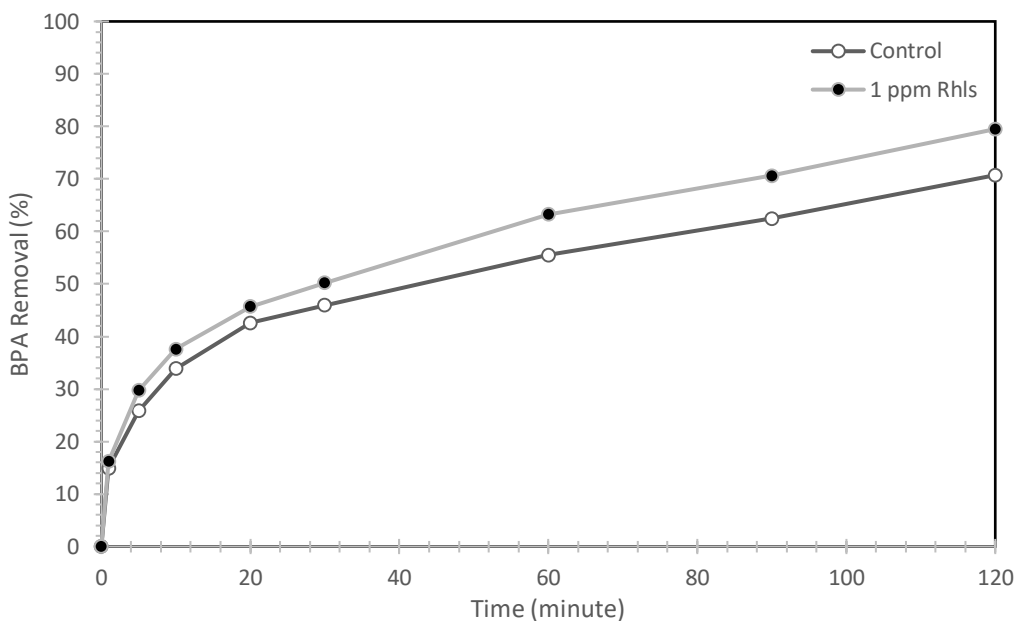


Figure 3-6 Time course of BPA removal in the absence and presence of RhI. Conditions: 25 mg/L BPA, 0.05 mg/mL laccase, pH 5.8 and 22°C after 2 h reaction

3.4.4 Effect of pH in the Absence and Presence of RhI

An important operational parameter to consider in enzymatic reactions is pH as the variation of its value has a robust influence on the catalytic activity of enzymes [160]. In this work, the removal efficiency of BPA was assessed at pH values ranging from 3.12 to 8.6 in the absence and presence of 1 ppm RhI for 2 h of laccase treatment. As depicted in figure 3-7, laccase was more effective under the acidic environment with an optimal pH of 5.8, which is close to the values reported by [3,56,114]. The low removal efficiencies (i.e. over a 50% reduction in the BPA conversion after increasing pH from 5.8 to 8.6) at the non-optimal pH values, particularly at alkaline conditions, could be correlated to the formation of robust electrostatic repulsion by laccase, which resulted in the destruction of the active site [106]. Another possible reason is that the affinity of BPA towards laccase

might have been decreased due to the formation of BPA conjugate base at higher values of pH [76], leading to less degradation rates.

The removal efficiency in the presence of Rhl was increased by 1.20- to 1.32- fold compared to the control reactions over the tested pH range. Nonetheless, the addition of Rhl did not alter the optimal pH for the system. Similarly, Liu et al. [3] examined the influence of pH on phenol conversion catalyzed by laccase in the presence of dirhamnolipid. The researchers reported that the biosurfactant was very tolerant to pH effects, showing almost 1.1- to 2.4- fold increase in phenol conversion with relative to the control samples. This is in line with the results obtained by Abdel-Mawgoud et al. [176] who found that Rhl exhibited high surface activities under acidic and basic conditions (i.e. pH from 2-13).

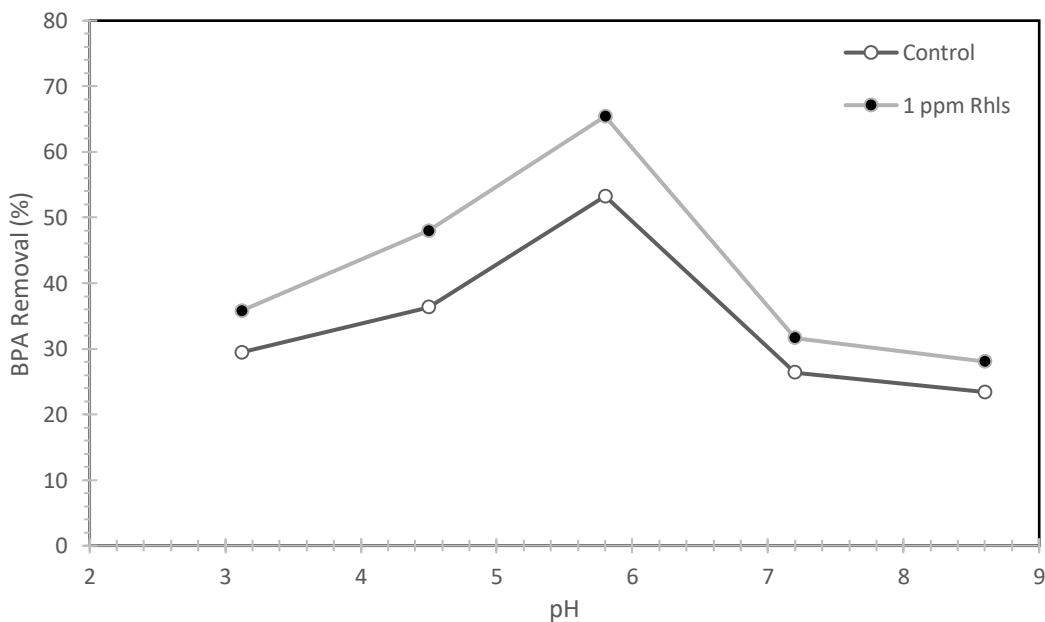


Figure 3-7 Effect of pH on the removal efficiency in the absence and presence of Rhl.

Conditions: 50 mg/L BPA, 0.05 mg/mL laccase, and 22°C after 2 h reaction

The effect of time on the degradation efficiency of BPA was also monitored in the absence and presence of 1 ppm RhI (see figures 3-8 and 3-9). For example, at pH 3, it can be noticed that the addition of RhI provided a higher removal rate than the control reaction. Initially, the biosurfactant did not induce a noticeable change on the degradation rate. However, the level of enhancement was augmented as the enzymatic reaction evolved. Such behavior is potentially linked to the ability of RhI to combat the rate of laccase deactivation which is caused by the attack of free radicals/ polymeric products [165].

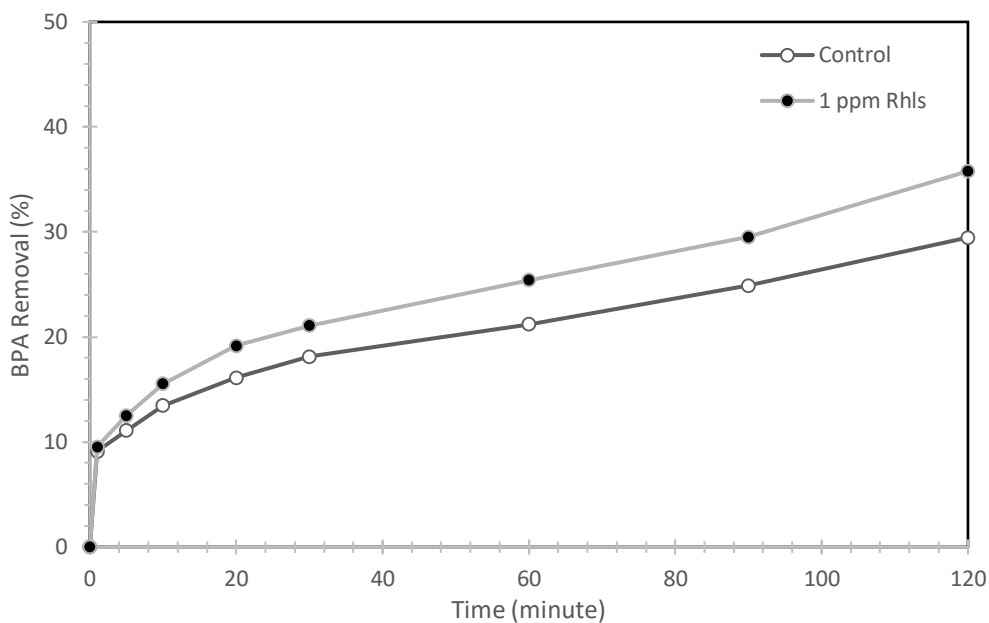


Figure 3-8 Time course of BPA removal in the absence and presence of RhI. Conditions: 50 mg/L BPA, 0.05 mg/mL laccase, pH 3.11 and 22°C after 2 h reaction

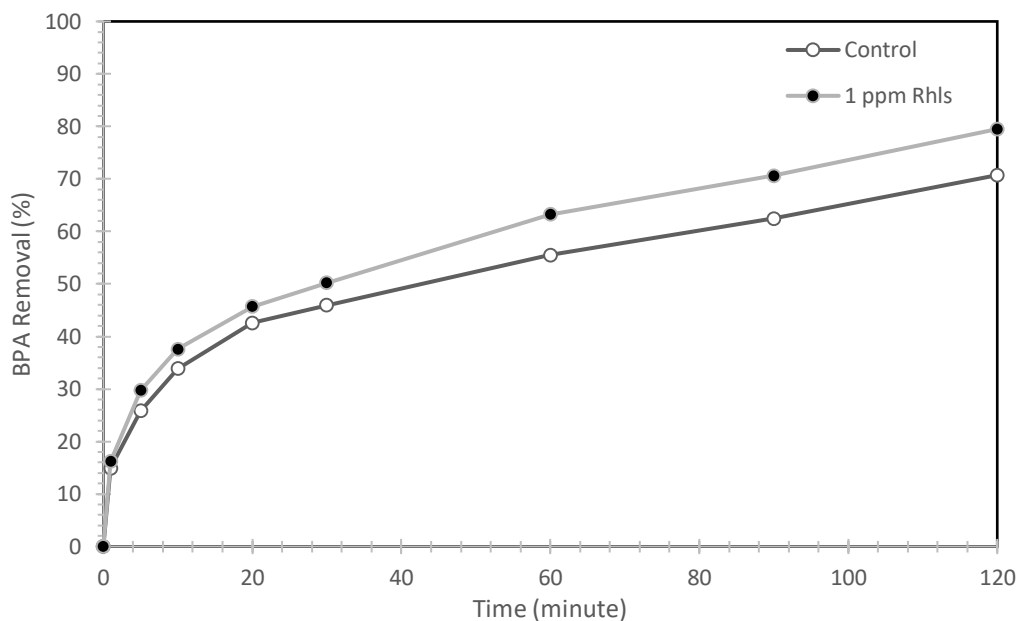


Figure 3-9 Time course of BPA removal in the absence and presence of RhI. Conditions: 50 mg/L BPA, 0.05 mg/mL laccase, pH 5.8 and 22°C after 2 h reaction

3.4.5 Effect of Temperature in the Absence and Presence of RhI

At pH 5.8, the removal efficiency of BPA was studied under different temperatures (from 22 to 70 °C) in the absence and presence of 1 ppm RhI after a treatment time of 2 h (figure 3-10). As it is clear from the figure, the removal efficiency increased with the rise of temperature from 22 to 50 °C. However, a further increase in the reaction temperature led to a sharp decline in the removal efficiency. These results are a par with the findings of Asadgol et al. [86], who found that 50 °C was the optimal temperature for BPA removal, and above this value, a sharp reduction in BPA degradation was observed. This phenomena can be explained by the increase in the rate constant of the enzymatic reaction below the optimal temperature, while the drop in the removal efficiency is highly linked to the adverse effects induced on laccase conformation at high temperatures [106].

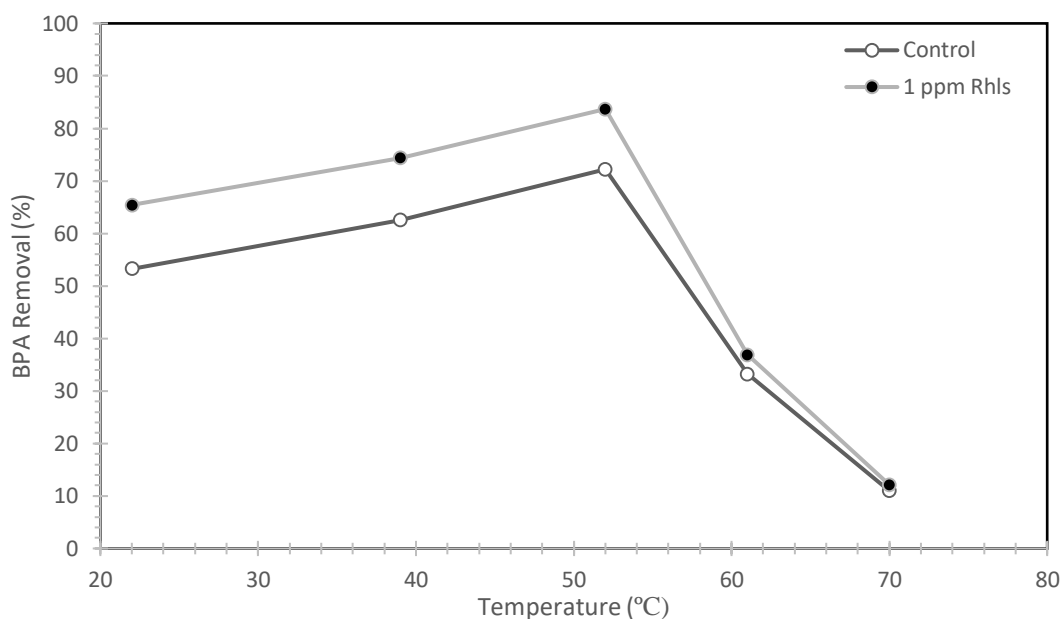


Figure 3-10 Effect of temperature on the removal efficiency in the absence and presence of Rhl. Conditions: 50 mg/L BPA, 0.05 mg/mL laccase, and pH 5.8 after 2 h reaction

The same behavior was also observed in the presence of Rhl. However, even though the biosurfactant became less tolerant with the rise of temperature, it was still able to induce a positive effect at elevated temperatures. For example, an increase of 11.01% in the removal efficiency at 60 °C was attained after the addition of Rhl. Our results can be underpinned by the findings of Udoh and Vinogradov [177] who found that the Rhl exhibited a stable surface activity up to a temperature of 70 °C. This remarkable performance at high temperatures might be attributed to the robust binding between the hydroxyl groups of biosurfactant and water molecules, which prohibited the dehydration of headgroup [178].

The removal efficiency of BPA against time was also evaluated in the absence and presence of 1 ppm Rhl (see figures 3-11 and 3-12). At the same temperature, it is obvious from the figures that Rhl-assisted reaction had a higher degradation rate than the control reaction.

Within the first five minutes, the biosurfactant did not highly alter the degradation rate. However, its stimulative effect became stronger as the reaction progressed over time. Such phenomena might be intuitively correlated to the protection of laccase by RhI from the attack of radicals/ polymeric products [165]. Thus, it can be inferred from these results that the longer treatment time is the more RhI enhances the enzymatic rate. Nonetheless, a compromise between the treatment time and cost along with other parameters should be considered.

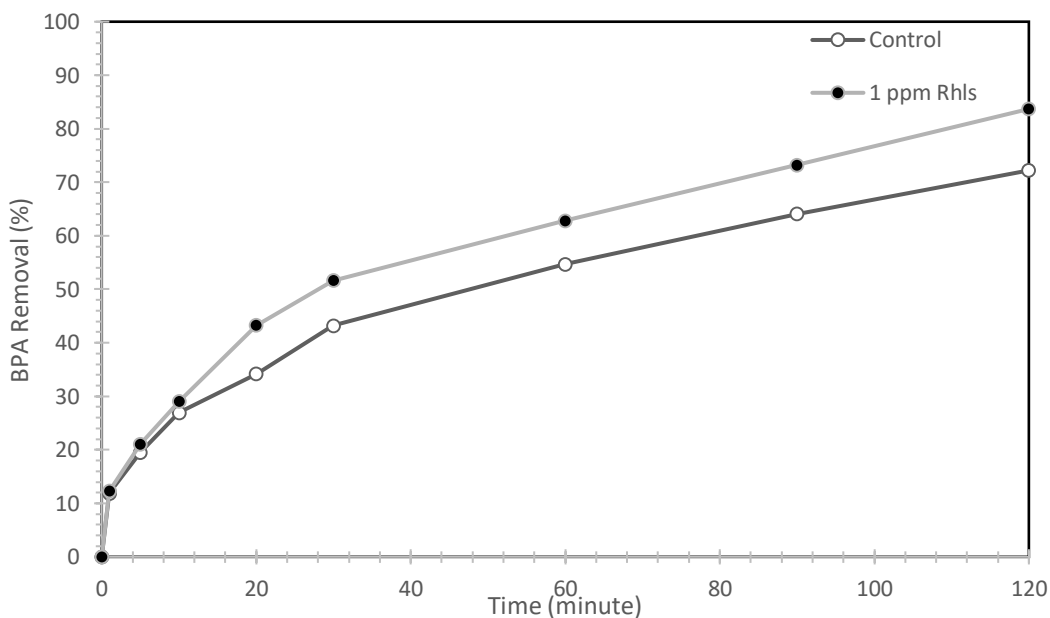


Figure 3-11 Time course of BPA removal in the absence and presence of RhI. Conditions: 50 mg/L BPA, 0.05 mg/mL laccase, pH 5.8 and 50 °C after 2 h reaction

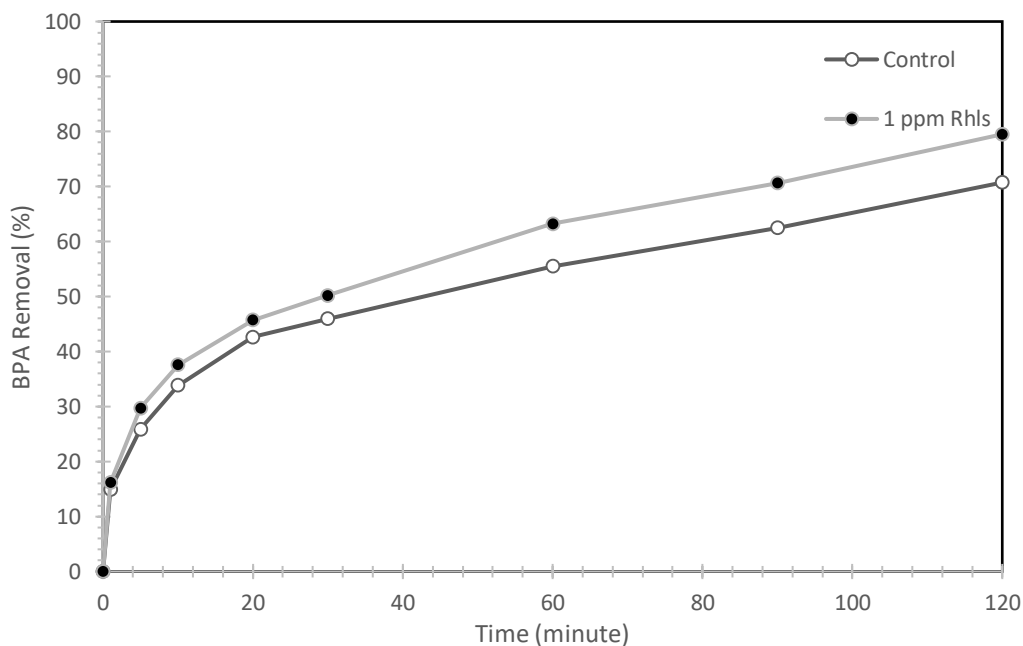


Figure 3-12 Time course of BPA removal in the absence and presence of RhI. Conditions: 50 mg/L BPA, 0.05 mg/mL laccase, pH 5.8 and 22°C after 2 h reaction

3.4.6 Effect of NaCl in the Absence and Presence of RhI

Most wastewater streams contain appreciable levels of inorganic salts. It was reported that the presence of salts, especially chlorides, induces inhibitory effects on enzymes, which in turn, reduces their effectiveness towards the removal of substrates [179]. Thus, the influence of sodium chloride (NaCl) on the enzymatic degradation of BPA was evaluated (see figure 3-13). The results showed that the removal efficiency of BPA decreased when the concentration of NaCl was raised from 0 mM to 500 mM. More importantly, the presence of 40 mM NaCl was sufficient to reduce the removal efficiency of BPA by almost 50% as compared to the control reaction (i.e. from 53.26% to 26.05%). Similar phenomena have been also observed in the previous investigations. For instance, Kim and Nicell [105] revealed that the addition of 50 mM NaCl led to a 50% reduction in the degradation

efficiency of BPA. Such negative effects can be justified by the fact that chloride ions tend to bind near the T1 copper site, preventing the access of BPA to the active site of laccase [179,180].

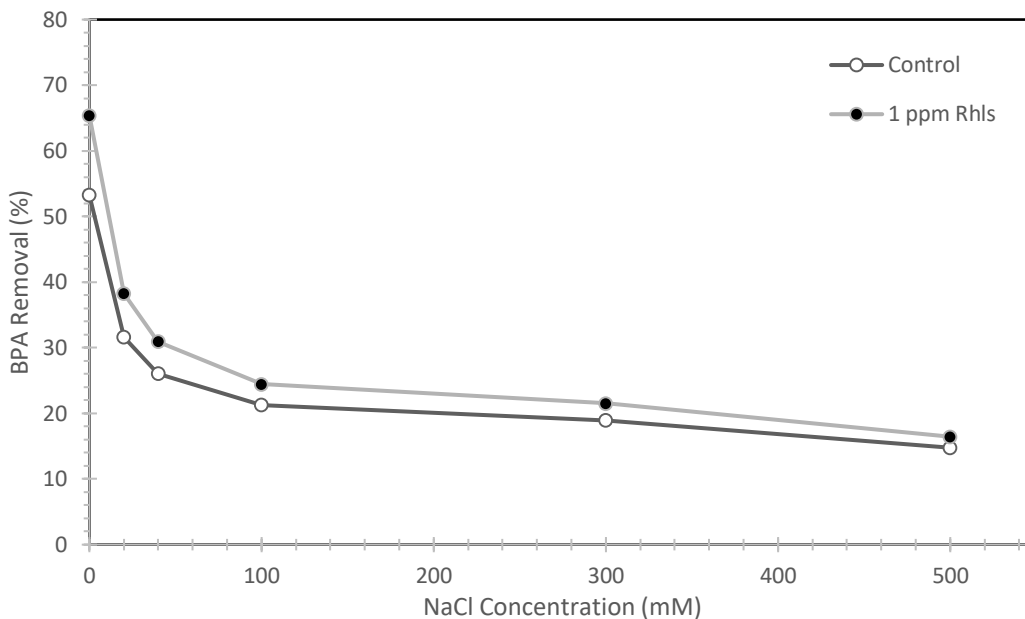


Figure 3-13 Effect of NaCl Concentration on the removal efficiency in the absence and presence of RhI. Conditions: 50 mg/L BPA, 0.05 mg/mL laccase, pH 5.8 and 22°C after 2 h reaction

At the same conditions, the effect of NaCl concentration on the degradation efficiency of BPA was studied in the presence of 1 ppm RhI as well (see figure 3-13). As shown in the figure, the addition of 1 ppm RhI to the reaction media enabled a 1.11- to 1.21- fold increase in the removal efficiency compared to the control reactions over the tested NaCl concentration range. In addition to this, the effect of reaction time on BPA degradation in the presence of 20 mM NaCl was monitored (see figure 3-14). The results revealed that the level of enhancement in the removal efficiency, which was imposed by the biosurfactant,

became more obvious as the reaction time progressed. Such positive impacts can be elucidated by the potent stability or surface activity of RhI against salty environments as noted by Abdel-Mawgoud et al. (2009).

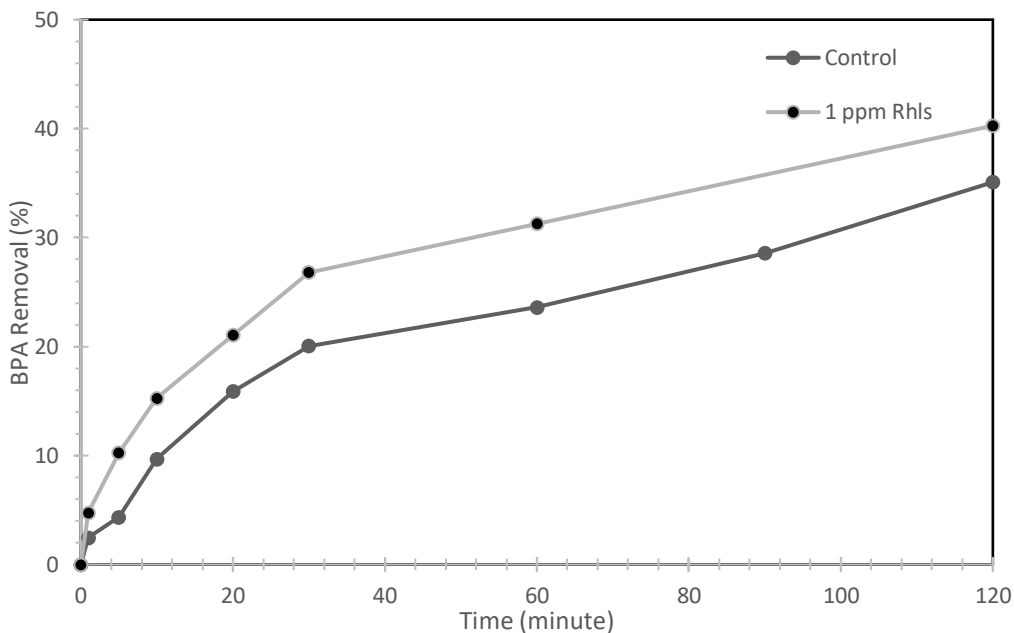


Figure 3-14 Time course of BPA removal in the absence and presence of RhI. Conditions: 50 mg/L BPA, 0.05 mg/mL laccase, 20 mM NaCl ,pH 5.8 and 22°C after 2 h reaction

3.5 Conclusion

The utilization of RhI, as a surface-active additive, enhanced the biocatalytic degradation of BPA by laccase within a treatment time of 2 h. At room temperature, the maximum level of enhancement in the removal efficiency of BPA was achieved when the RhI concentration was 1 ppm (i.e. a 22.81% increase as compared to the control reaction). This concentration was selected for evaluating the performance of RhI under different process parameters. The biosurfactant exhibited stimulating effects on the removal efficiency of BPA over the tested

conditions. It can be concluded that RhI has great aptitude in enhancing the enzymatic treatment and might pave the way, when integrated with laccase, towards a sustainable hybrid technology for the treatment of bisphenolic wastewaters.

CHAPTER 4

COMPARISON OF BIOSURFACTANT WITH CHEMICAL ADDITIVES

As discussed in the previous work (see chapter 3), Rh1 exhibited stimulating effects on the activity of laccase and improved the bio-catalytic degradation of BPA in aqueous solution below its CMC. More importantly, the biosurfactant at 1 ppm was able to enhance the enzymatic reaction rate under varying process conditions (i.e. BPA concentration, temperature, pH, and salt concentration). For the sake of having a more comprehensive work, this chapter was dedicated to discuss the comparison of Rh1 with common surface-active additives (i.e. Polyethylene glycol (PEG), Triton X-100, Cetyl trimethylammonium bromide (CTAB), and Sodium dodecylbenzenesulfonate (SDBS)) at concentrations of 1 ppm and 25 ppm in enhancing the enzymatic removal of BPA from water.

4.1 Introduction

Water pollution has become one of the most pressing issues that the globe is facing today. A major cause of this contamination is the continuous release of chemicals to the environment. One of such toxic chemicals is BPA, which is a widespread phenolic derivative [181]. This endocrine disruptor plays a big role in making polycarbonates and epoxy resins [105]. Other BPA-containing materials include, but are not limited to, pipe linings, papers, optical lights, plastic packings, and paints [63,182]. Despite the ubiquitous

uses, BPA was found to produce noxious effects in humans and other organisms. For instance, the exposure to BPA can lead to excessive malfunctions in hormonal systems [183] and induce adverse effects on reproduction organs [160]. As reported by [184], BPA can hinder the protective action of white blood cells in fish against pathogens and foreign substances.

Due to these concerns, various methods including adsorption [185], photo-oxidation [164], membrane separation [163], and biological degradation [63] have been extensively exploited to treat bisphenolic wastewater streams. However, such techniques are plagued by the high cost, time-consuming operation, or toxicity of by-products [89]. As a substitute, the utilization of extracellular enzymes has received a huge attention over the recent decades [51,82]. One of these enzymes is laccase (EC 1.10.3.2), which has been reported to be capable of degrading a wide range of chemicals including BPA [53]. However, it has been reported that laccase is highly susceptible to lose its activity due to the interactions with free radicals or polymeric products [186].

Even though the mechanism of enzyme deactivation is still ambiguous, some theories have attempted to elucidate the causes of such deactivations. For example, it has been hypothesized that some free radicals, which are generated as intermediates during the enzymatic reaction cycles, tend to attack laccase molecules. Due to such interactions, a covalent bond is formed between the free radicals and enzyme, resulting in the loss of enzyme activity [110]. Another possible scenario is the adsorption of laccase molecules on the surface of charged micro-particles. As a result, a diffusion layer around the micro-aggregates is established, which hinders the access of BPA to the active site of laccase, rendering the enzyme inactive/less active [165].

With a view to minimizing the rate of enzyme deactivation, surface-active additives including PEG [93,114] and Triton X-100 [108,113] have been used to enhance the enzymatic degradation of various phenols. Nonetheless, a handful of work [110,113,115] have been published yet on comparing the effect of different additives on the enzymatic degradation of phenols. To the best of our knowledge, there is no single study in the literature, which has attempted to evaluate the effect of various additives on the removal of BPA catalyzed by laccase.

Therefore, this work aimed to study the enzymatic degradation of BPA catalyzed by laccase in the presence of five additives (Rhl, PEG, Triton X-100, SDBS, and CTAB). More precisely, the performance of Rhl, a nonionic biosurfactant was compared with the aforementioned chemical additives. PEG and Triton X-100 were selected due to the previous investigations showing their positive impact on the enzymatic removal of BPA. SDBS and CTAB were considered in this study as no work has been yet devoted to uncover their effect on the removal of BPA in the presence of laccase.

4.2 Materials

BPA as a model pollutant, laccase (EC 1.10.3.2) from *Trametes versicolor*, 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), Rhl and other additives (CTAB, Triton X-100, and SDBS) were all purchased from Sigma-Aldrich. PEG-2025 was purchased from BDH Chemicals. The remaining reagents were of analytical grade.

4.3 Methods

4.3.1 Laccase Activity Assay

The activity of laccase was spectrophotometrically measured at room temperature by following the oxidation of ABTS as a substrate for 4.5 minutes. 2 mL of 0.4 mM ABTS (prepared in 0.2 M acetate buffer, pH 4.5), 1 mL of 0.1 mg/mL laccase (prepared in a deionized water), and a suitable amount of additive, when required, were incubated in a cuvette with a final reaction volume of 4 mL. The enzymatic reaction rate (i.e. absorbance increase at a wavelength of 420 nm) with the molar extinction coefficient of $36 \times 10^{-3} \text{ M}^{-1} \text{ cm}^{-1}$ at 25 °C [168] was monitored by using a UV- 1601PC spectrophotometer (Shimadzu, Japan). One unit (U) of laccase activity was defined as the amount of laccase required to oxidize 1 μmol of ABTS per minute.

4.3.2 BPA Concentration Measurement

A high-performance liquid chromatography instrument (HPLC, Agilent, USA) equipped with a ZORBAX Eclipse XDB-C18 column (Agilent, USA), which was thermostated at 40 °C, was used to measure the residual concentration of BPA in the reaction samples. A detection wavelength of 276 nm was utilized with a flow rate of 1 mL/min (isocratic elution). The retention time of BPA and injection volume of filtered samples were 3.8 minutes and 3 μL , respectively. The mobile phase consisted of ultra-pure water and acetonitrile (40%/60%, v/v). The degradation percentage of BPA was calculated based on the peak area and the slope of the calibration curve.

4.3.3 BPA Removal Studies

In aqueous solution, BPA at a concentration of 50 ppm was enzymatically treated with laccase (0.05 mg/mL) in the absence and presence of the surface-active additives (i.e. at 1 ppm or 25 ppm of additive concentration). During the 2 h of laccase treatment, samples were collected at the desired times to study the reaction progress with/out the additives. For the sake of halting the enzymatic reaction, 3 ml from the reaction mixture was quenched with 1 ml of 0.5 M hydrochloric acid. Before analyzing the samples in HPLC, 0.2 micron polyvinylidene fluoride (PVDF) membrane filters were used to filter the collected samples. It is noteworthy to mention that all activity assay and decontamination runs were conducted twice. All data in figures are expressed as averages of the collected data.

4.4 Results and Discussion

Prior to discussing the results, it is noteworthy to mention here that the CMC of Rhl (i.e. 50 mg/L) was determined in this study as described in sub-section 3.3.3. However, the CMCs of Triton X-100, SDBS, and CTAB in mg/L were reported to be 150 to 200 [2,126], 976 to 1010 [187], 364.45 [3], respectively. In the subsequent sections, the kinetics and extents of BPA removal by BPA in the absence and presence of surface active additives will be discussed.

4.4.1 Effect of Rhl on BPA Conversion

At room temperature, the degradation reaction of BPA (50 ppm) catalyzed by laccase was monitored as a function of time in the absence and presence of Rhl. At all the tested times, it can be observed from figure 4-2 that the reactions supplemented with 1 ppm and 25 ppm

of Rhl had higher conversion rates of BPA than the control reaction. At a Rhl concentration of 1 ppm, the biosurfactant was able to boost the degradation rate of BPA within the first ten minutes, allowing laccase to remove over 30% of the initial BPA amount while 21.11% was only degraded in the absence of Rhl. Further laccase treatment of BPA in the presence of Rhl (up to 2 h) led to almost 23% enhancement in the removal efficiency of BPA (65% for Rhl- supplemented reaction as compared with 52.71 % for the control. The positive impact of Rhl might be linked to its high capability to mitigate the loss of enzyme activity by preventing the adsorption of free radicals/polymeric products into the active site of laccase [165]. This is also in line with the findings of [61], who found that the addition of Rhl (0.1 to 2 ppm) enhanced the oxidation rate of 2-naphthol by almost 1.5 fold as compared to the control reaction after 10 minutes of peroxidase treatment. In support to these observations, our laccase activity measurements showed that the addition of 1 ppm Rhl increased the activity of laccase by almost 13% (see figure 4-1).

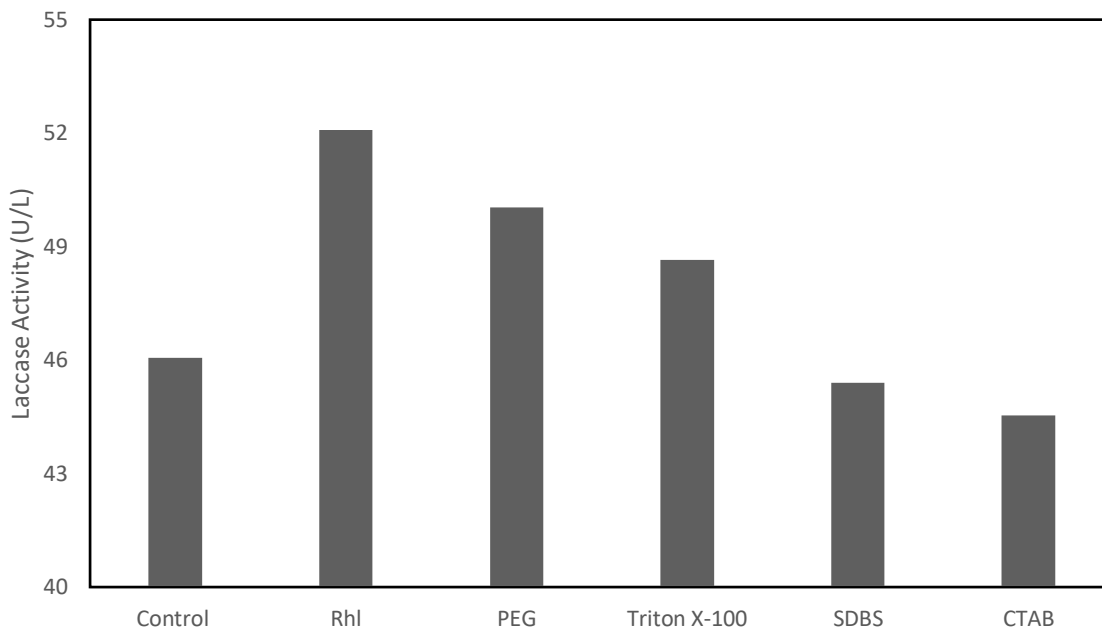


Figure 4-1 Laccase activity in the absence and presence of additives. Conditions: 0.025 mg/mL laccase, 0.2 mM ABTS, pH 5 (0.1 M acetate buffer) and 22 °C, with a reaction time of 4.5 minutes.

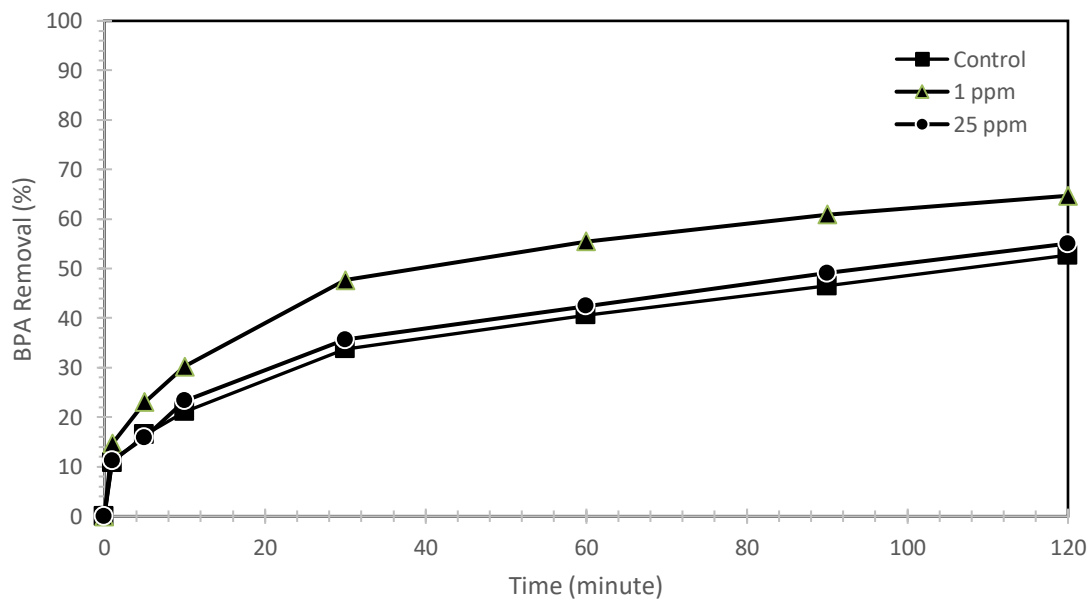


Figure 4-2 Time course of BPA removal in the absence and presence of Rhl. Conditions: 50 mg/L BPA, 0.05 mg/mL laccase, pH 5.8 and 22°C after 2 h reaction

However, RhI at a concentration of 25 ppm imposed inconsiderable effects on the enzymatic system even though it had a similar trend to the reaction supplemented with 1 ppm RhI. As it is clear from figure, 55% of the original BPA amount was only removed from the mixture, which is lower than the removal efficiency obtained with 1 ppm RhI at the same conditions. This can be explained by the phenomenon that some BPA molecules at higher RhI concentrations were sequestered into the micelle pseudophase and therefore prohibited from the interaction with laccase, leading to lower BPA degradation [60]. These results indicate that RhI in its pre-micellar form, particularly at low concentrations, is sufficient to induce enhancing effects on the enzymatic rate of BPA.

4.4.2 Effect of PEG on BPA Conversion

Figure 4-3 shows the time profile of BPA conversion in the absence and presence of PEG. At a fixed laccase concentration and all tested reaction times, the polymeric additive improved the removal efficiency of BPA. It can be noted from the figure that the addition of PEG at a concentration of 1 ppm did not alter the degradation rate during the first 10 minutes; however, a negligible improvement on the removal efficiency was attained as the reaction proceeded. More specifically, 55.2% of BPA was removed in the presence of 1 ppm PEG after a 2h-treatment, which is slightly higher than the control reaction (52.71%). Increasing the concentration of PEG to 25 ppm augmented the BPA removal by a larger extent than that with 1 ppm of PEG. For example, the degradation efficiencies of BPA in the presence of 1 ppm and 25 ppm PEG after 1 h of laccase treatment were 43.00% and 50.83%, respectively. The enhancing effect of PEG on BPA removal catalyzed by laccase was also studied by [115]. These researchers reported that PEG at 5 ppm induced a positive

impact on the enzymatic treatment and was able, under optimized conditions, to remove over 95% of the initial BPA amount.

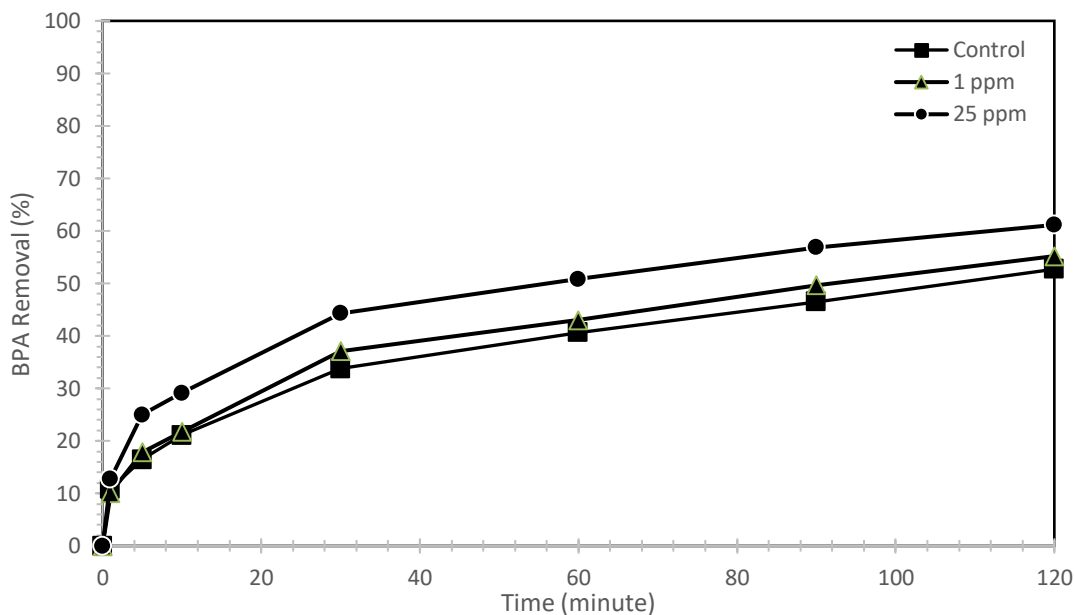


Figure 4-3 Time course of BPA removal in the absence and presence of PEG. Conditions: 50 mg/L BPA, 0.05 mg/mL laccase, pH 5.8 and 22°C after 2 h reaction

Such enhancement in BPA removal after the addition of PEG is most likely correlated to its protective effects on laccase activity [165]. This was confirmed in our study which showed that the presence of PEG enhanced the activity of laccase by 8.64% (see figure 4-1). This result also agrees with [56], who mentioned that PEG at a concentration of 1 ppm combated the deactivation of laccase, lowering the required enzyme amount by over 2-fold to achieve the same removal extent of 2,4-dimethylphenol in the absence of PEG. It was postulated that PEG tends to preclude the entrapment of enzyme molecules within oligomer products which are generated during the reaction [93]. In consonance with this statement, PEG was found to bind with water, which results in the formation of a relatively bulky

hydrated volume [77]. PEG molecules, as stated by [59], have the tendency to fold and capture more water molecules, creating a rounded PEG structure. Such an interaction primarily contributes to the protection of laccase against inhibitory products [115].

4.4.3 Effect of Triton X-100 on BPA Conversion

The time course of BPA removal catalyzed by laccase was assessed in the absence and presence of the nonionic surfactant Triton X-100 (figure 4-4). As the figure indicates, a positive impact on the BPA degradation extent was gained upon the addition of Triton X-100 at any tested reaction time. In the absence of Triton X-100, laccase degraded 10.86% of the initial BPA in 1 minute, and 60 minutes later, it was able to remove 40.62%. However, when Triton X-100 at 25 ppm was introduced into the reaction, 13.85% and 47.89% of the initial BPA amount were removed after 1 minute and 60 minutes of laccase treatment, respectively. Similar to PEG, this surfactant was found to be more effective at a concentration of 25 ppm than 1 ppm. In this regard, the results revealed that a 13.88% increase in BPA removal was attained in the presence of 25 ppm Triton X-100, while almost a 3% increase in BPA removal was only achieved in the case of 1 ppm Triton X-100 after 2 h. This is consistent with the findings of [60], who observed that increasing the concentration of Triton X-100 (i.e. when present in a monomeric phase) enhanced the BPA conversion catalyzed by laccase. The same pattern was also reported in the other investigations [2,126].

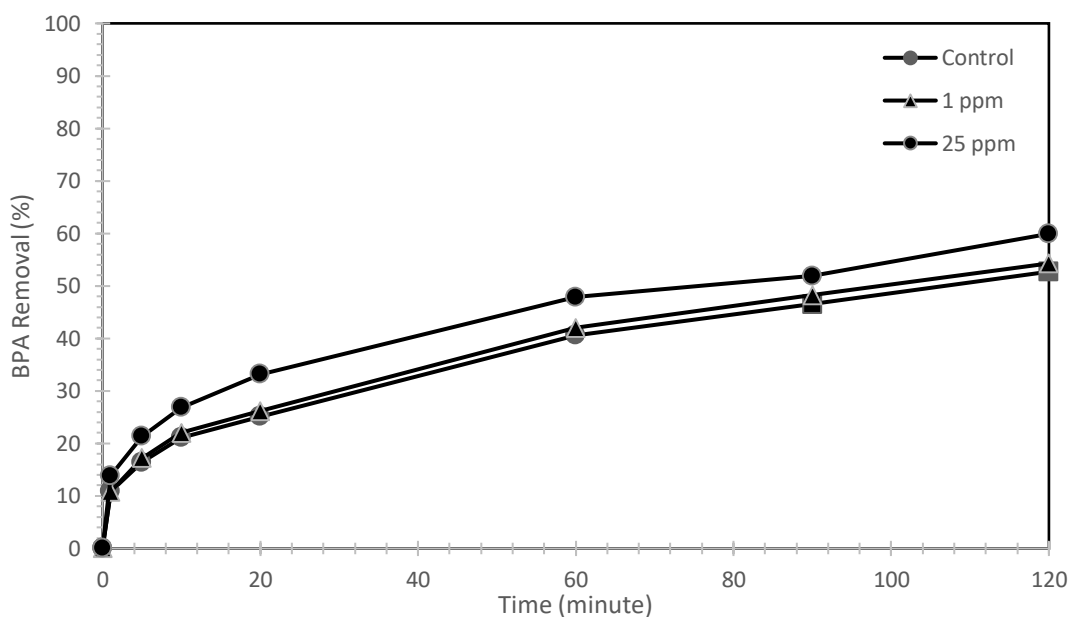


Figure 4-4 Time course of BPA removal in the absence and presence of Triton X-100. Conditions: 50 mg/L BPA, 0.05 mg/mL laccase, pH 5.8 and 22°C after 2 h reaction

It is believed that the interaction between laccase and Triton X-100 is highly responsible for stabilizing laccase, and thereby enhancing the BPA conversion [174]. In this regard, our study showed that Triton X-100 in a buffer solution increased the activity of laccase by **5.62%** when compared to the control at the same conditions (see figure 4-1). Similar results demonstrating the protective effects on laccase upon the addition of Triton X-100 were also reported [2,60]. This positive impact can be supported by the molecular analysis which revealed that the surfactant induced some changes in laccase structure, and as a result, laccase was converted into a more active/stabilized form [2]. Such interactions allowed the hydrophobic amino acid residues of laccase to be entrapped within the inner part of laccase conformation. This action is thought to be useful for minimizing the rate of

enzyme deactivation due to the attack of free radicals or the attachment of polymeric products to the surface of laccase.

Comparing the performance of Triton X-100 with PEG, it can be observed that the nonionic surfactant had almost comparable effects with PEG at both concentrations. In another study, [113] declared that the addition of PEG and Triton X-100 into a reaction catalyzed by peroxidase enhanced the phenol removal efficiency by almost the same extent. This might be associated to the similarities in their chemical structure (i.e. the hydrophilic group in both additives is comprised of poly(oxyethene) residues) as shown in **figure 4-1**. In contrast to Triton X-100 and PEG, the biosurfactant at a concentration of 1 ppm exhibited a more positive impact on the enzymatic degradation of BPA. The superior effectiveness of Rhl at a low concentration could be explained by the low CMC [160], and more importantly, its complex structure as noted by [149].

4.4.4 Effect of SDBS on BPA Conversion

The influence of SDBS on BPA removal catalyzed by 0.05 mg/mL laccase was monitored as a function of time. As depicted in figure 4-5, the presence of the anionic surfactant in the enzymatic system imposed a negative impact on BPA degradation. More specifically, SDBS at a concentration of 1 ppm induced an imperceptible decrement on BPA conversion. However, a more obvious decrease in BPA removal was observed after raising the SDBS concentration into 25 ppm. In the SDBS-free reaction, 10.86% of the initial BPA amount was enzymatically degraded in the first minute, and two hour later, laccase was capable to remove 52.71% of BPA. However, when SDBS was added at a concentration of 25 ppm, 7.66% and 48.75% of the initial BPA amount were only eliminated after 1 minute and 120 minutes of laccase treatment, respectively. This is in consistency with the

findings of [3], who figured out that the addition of SDS, having almost similar characteristics to SDBS, at a concentration of 0.5 mM led to a 13% reduction in the removal efficiency of phenol by laccase.

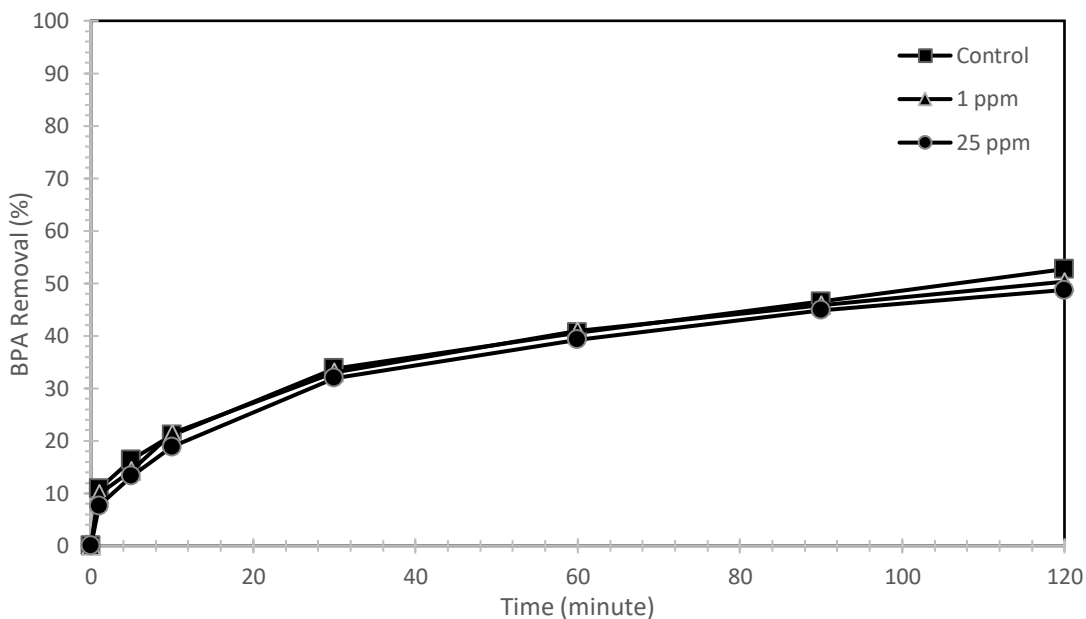


Figure 4-5 Time course of BPA removal in the absence and presence of SDBS. Conditions: 50 mg/L BPA, 0.05 mg/mL laccase, pH 5.8 and 22°C after 2 h reaction

It has been reported in previous investigations that anionic surfactants can act as inhibitors for laccases. For instance, it was found that 0.1 mM (or 28.84 ppm) of SDS was enough to lower the activity of laccase by almost 20% when compared to the control reaction [188]. Another study revealed also that the addition of SDS at the same concentration caused a 25.7% inhibition in the activity of laccase [189]. These results are in support of our work, which observed a 1.42% reduction in laccase activity after the addition of 1 ppm SDBS (see figure 4-1). This might be associated to the fact that anionic surfactants, thanks to their hydrophobic tail, can easily penetrate through the tertiary conformation of laccase [189].

Such a mechanism allows the sulfate group in SDS or SDBS to interact with the positively charged side chain on amino acids, leading to the destruction of enzyme [190]. This behavior might elucidate the decrement in BPA removal extent after the addition of SDBS to the enzymatic system.

4.4.5 Effect of CTAB on BPA Conversion

The time course of BPA removal catalyzed by laccase in the absence and presence of CTAB was studied as well. It can be deduced from figure 4-6 that the degradation extent of BPA was negatively affected upon the addition of CTAB. Similar to SDBS, a very slight decrease in BPA conversion was noticed at a SDBS concentration of 1 ppm. However, at all tested times, the addition of 25 ppm CTAB to the enzymatic reaction led to a larger drop in BPA removal than SDBS at the same concentration. After 2 h of laccase treatment, the removal efficiency of BPA was reduced from 52.71% to 48.75% and 43.77% when SDBS and CTAB at 25 ppm was present in the reaction, respectively. These results match with a recent study [3] reporting that CTAB was more detrimental to the removal efficiency of phenol than SDS. As the authors mentioned, SDS at 0.1 mM (or 28.84 ppm) lowered the phenol removal extent by almost 13% while a 19% decrease in the phenol removal was obtained in the case of CTAB at the same concentration.

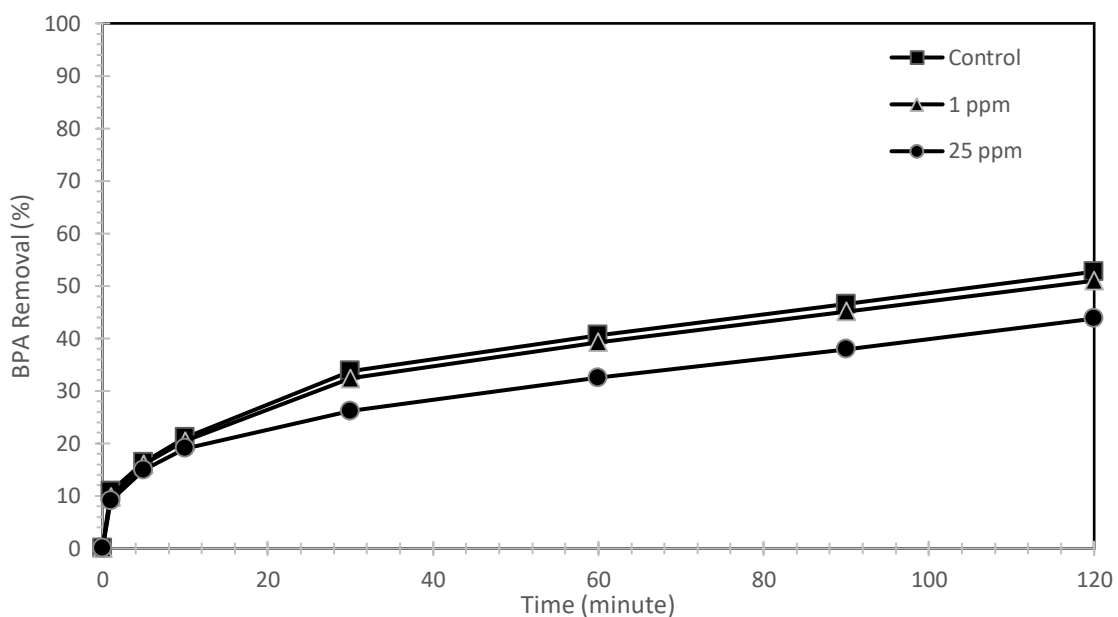


Figure 4-6 Time course of BPA removal in the absence and presence of CTAB. Conditions: 50 mg/L BPA, 0.05 mg/mL laccase, pH 5.8 and 22°C after 2 h reaction

The decline in pollutant removal observed after the addition of CTAB to the enzymatic reaction might be interpreted by its unfavourable binding with laccase. In this regard, it is postulated that the hydrophilic group of CTAB consisting of ammonium might strongly interact with the amino acid residues of laccase [191]. As a result, undesirable alterations in laccase structure occur, which in turn, render the activity of laccase otiose. In line to this fact, laccase activity assay results (see figure 4-1) revealed that the presence of 1 ppm CTAB enabled a 3.82% reduction in laccase activity. A higher concentration of CTAB (i.e. 182.23 ppm) lowered the activity of laccase by almost 40% when compared to the control reaction as reported by [191].

4.5 Conclusion

The effect of surface-active additives (i.e. Rh1, PEG, Triton X-100, SDBS, and CTAB) on the degradation of BPA by laccase in aqueous solution was investigated. It was shown that the Rh1 was the most powerful at augmenting the BPA removal and in protecting the activity of laccase. In addition to this, comparable positive effects on BPA removal were observed upon the addition of PEG and Triton X-100. However, the presence of SDBS or CTAB, especially at a higher concentration, caused a noticeable reduction in the removal efficiency of BPA. It can be deduced from these findings that the interactions between the additive and laccase played a big role in enhancing the enzymatic degradation of BPA. In this regard, the uncharged additives (Rh1, PEG, and Triton X-100) tended to be more beneficial for improving the removal extent of BPA, whereas the ionic additives (SDBS and CTAB) were shown to be detrimental due to their inhibitory effects on laccase. Compared to the other tested additives, the biosurfactant is eco-friendly and proved to be more efficient at low concentrations. However, more work should be devoted to assess its effectiveness in enhancing the enzymatic removal of other phenolic pollutants from wastewater.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Based on the results of this work, which was dedicated to study the enzymatic removal of BPA from aqueous solution, the following points were drawn:

- The addition of RhI to the enzymatic reaction catalysed by laccase provided enhancing effects on BPA removal when it was used at concentrations below its CMC.
- The optimal concentration of RhI was found to be 1 ppm, leading to a 22.81% increase in BPA removal when compared to the RhI-free reaction.
- The presence of RhI enhanced the BPA conversion at all tested initial BPA concentrations. However, the maximum level of enhancement was attained at a BPA/RhI molar ratio of 50.
- The optimum pH for BPA removal by laccase in the absence and presence of RhI was 5.8. Upon the addition of RhI, the removal efficiency of BPA was raised by 1.20- to 1.32- fold compared to the control reactions over the tested pH range. Laccase was found to be more active in the acidic environment and its tolerance to acidity was amplified in the presence of RhI.

- In the absence and presence of Rhl, the optimum temperature for BPA degradation was 50 °C. Thanks to its high thermal stability, Rhl was capable to persuade the enzymatic reaction even after increasing the temperature up to 70 °C.
- Over the tested NaCl concentration range, the addition of 1 ppm Rhl to the enzymatic system allowed a 1.11- to 1.21- fold increase in the removal extent of BPA when compared to the Rhl- unsupplemented reactions.
- Under various process conditions, the kinetic results revealed that the presence of Rhl in the enzymatic reaction enhanced the BPA degradation extent at all tested reaction times.

In addition to this, a comparative study was conducted to examine the influence of Rhl and four chemical surface-active additives (i.e. PEG, Triton X-100, SDBS, and CTAB) on BPA removal by laccase. The findings of this part are summarized as follows:

- Comparing the performance of Rhl with these additives revealed that the biosurfactant was the most influential at augmenting the BPA removal extent.
- Owing to the similarity in their structure, the addition of PEG and Triton X-100 at concentrations of 1 ppm and 25 ppm to the enzymatic reaction had almost comparable impacts on BPA removal extents.
- The interaction of SDBS or CTAB with laccase led to a drop in BPA removal rates, and their negative impacts were more pronounced at a higher concentration (25 ppm).

- Compared to the control reaction, the non-ionic additives (Rhl, PEG, and Triton X-100) had the tendency to increase the activity of laccase by 13.08%, 8.64%, and 5.62%, respectively.
- The ionic additives (SDBS and CTAB) were found to be aggressive towards laccase, and as a results, the activity of laccase was inhibited by 1.42% and 3.28%, respectively.

5.2 Recommendations

The target application of enzymatic process technology is to eradicate phenolic pollutants from industrial wastewater streams at the source of production or incorporate it into the secondary stage of municipal wastewater treatment plants. However, developmental efforts need to be dedicated to make the enzymatic process economically feasible for large-scale purposes. These mainly involve the development of strategies for maintaining the activity of laccase and assessing the environmental impacts.

Due to their tedious methods of extraction and purification, the production of laccases has become expensive, making the enzymatic treatment less competitive with the existing treatment techniques. However, this issue can be alleviated by maintaining the operational activity of laccases. One way is to stabilize laccases by the use of surface-active additives having the potential to suppress enzyme deactivation and pose no adverse effects on environment. As shown in our work, the integration of Rhl with laccase provided a positive impact on the removal of BPA. However, this technology needs to be applied for the treatment of other phenolic pollutants. Most importantly, the effectiveness of such a process should be tested for treating a real sample of wastewater containing a mixture of

phenolic compounds and co-pollutants. Another important way for maintaining the activity of laccase is immobilization, where laccase molecules are attached to an insoluble support. Doing so improves the stability of laccases and increases their resistance to hostile conditions and denaturing agents.

Knowing the fact that aromatic polymers are generated as by-products during the enzymatic degradation of BPA or any phenolic derivative, these products might be more hazardous than the pollutants being treated. Thereby, it is imperative to assess the nature of reaction products by performing in-depth structural characterization analysis. Last but not least, one should also consider evaluating the fate and toxicity of the reaction products, and select suitable techniques to safely dispose any polymeric precipitates.

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APPENDIX A: BPA ASSAY USING HPLC

The Standard Curve of BPA Using HPLC Peak Area

For the aim of establishing a standard curve, various concentrations of BPA ranging from 5 to 100 ppm were used. The samples were prepared as per the subsequent procedure. The detection wavelength was set at 278 nm for BPA. The results (peak area vs BPA concentration) are plotted in figure A-1. There is a linear relationship between BPA concentration and the peak area from HPLC. In the analysis, 3 μ L from HPLC vials were injected in to the instrument with a flow rate of 1 mL/min. The mobile phase consisted of 60% acetonitrile and 40 % water. The average retention time of BPA for all standard and authentic runs was 3.80 minutes. Using the peak area results, the remaining amount of BPA in enzymatic solutions was determined.

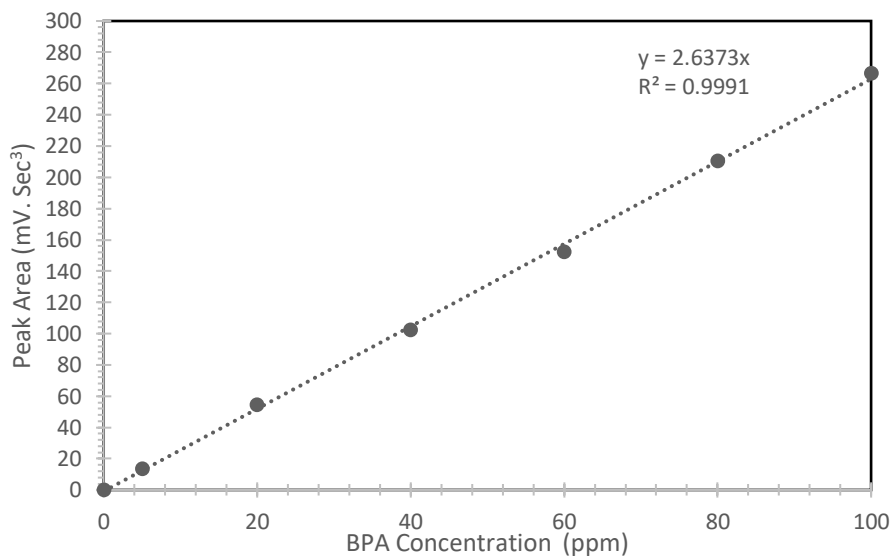


Figure 0-1 BPA standard curve by HPLC. Condition: BPA Concentrations from 5 to 100 ppm, wavelength at 278 nm, injection volume: 3.3 μ L. The mobile phase consisted of 60% acetonitrile and 40 % water

APPENDIX B: LACCASE ASSAY USING UV-VIS SPECTROPHOTOMETER

The activity of laccase in the absence and presence of additives was determined by conducting an assay where 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS was utilized as a substrate. The initial rate was obtained by observing the rate of color formation in a solution for 4.5 minutes. 0.2 mM of ABTS was oxidized to its corresponding quinone. The products of the enzymatic reaction absorbed light at a peak wavelength of 420 nm.

Calculations

The laccase activity in the cuvette in terms of micromoles of ABTS converted per minute:

$$\text{Laccase Activity in the cuvette (U/mL)} = (\Delta A * 4.0 \text{ mL} * 10^6) / (36000 * 1.0 \text{ mL} * 10^3)$$

Where:

ΔA = Change in absorbance per minute: $A_{4.5 \text{ min}} - A_{0 \text{ min}}$

4.0 = Total volume in cuvette (mL)

1.0 = The volume of laccase added to the cuvette (mL)

10^6 = Conversion factor from mol/L/min to $\mu\text{mol/L/min}$

36×10^{-3} = the molar extinction coefficient ($\text{M}^{-1} \text{ cm}^{-1}$)

10^3 = Conversion factor from U/L to U/mL

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1. **M. Alshabib**, S.A. Onaizi, A review on phenolic wastewater remediation using homogeneous and heterogeneous enzymatic processes: Current status and potential challenges, *Sep. Purif. Technol.* 219 (2019) 186–207. doi:10.1016/j.seppur.2019.03.028.
2. **M. Alshabib**, S.A. Onaizi, Effects of Surface Active Additives on the Enzymatic Treatment of Phenol and Its Derivatives: a Mini Review, *Curr. Pollut. Reports.* 5 (2019) 52–65. doi:10.1007/s40726-019-00105-8.
3. **M. Alshabib**, S.A. Onaizi, Effect of Biosurfactant on the Degradation of Bisphenol A in Aqueous Solution Catalyzed by Laccase (2019). *To be submitted.*
4. **M. Alshabib**, S.A. Onaizi, Enzymatic Remediation of Bisphenol A from Wastewaters: Benchmarking the Effects of Biosurfactant, Chemical Surfactants and Polymeric Additives (2019). *To be submitted.*