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Wastewater Benzenediol Removal Catalyzed by Crude Arugula Peroxidase

Hayder Saleem Saleh[1], Beadaa Abdalqader Mahdii [1] and Bahaa Malik Altahir [1†]
[1]Department of Biology, University of Baghdad, Baghdad, 10071, Iraq
†Corresponding author: Bahaa Malik Altahir; bahaa.malik@sc.uobaghdad.edu.iq, https://orcid.org/0000-0001-9002-7431

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ABSTRACT

This study involved the extraction and purification of peroxidase enzymes from arugula. The enzymatic removal of benzenediol (BZOL) compounds from wastewater was investigated using crude arugula peroxidase (AP). Based on the results, pH 6.1 and pH 5.7 were identified as the optimal pH values for the removal of resorcinol and catechol, respectively. The optimum enzyme activity and hydrogen peroxide were found to be at the lowest feasible limits (95% elimination) of 0.15% hydrogen peroxide and 0.072U/mL enzyme activity under reaction conditions (3 hours). Kinetic studies revealed that the reaction followed pseudo-first-order kinetics, with catechol degradation occurring faster than resorcinol degradation. These findings are promising for environmentally sustainable and robust bioremediation applications.

1. INTRODUCTION

The presence of hydroxyl (-OH) groups attached directly to the carbon atoms in the benzene ring (C_6H_6) is referred to as BZOL, also known as dihydroxybenzene. BZOL belongs to the class of organic compounds commonly known as phenols, due to the presence of these hydroxyl groups (Venkatesh and Singh 2007). BZOL has three structural isomers: catechol (1,2-benzenediol or ortho isomer of benzenediol), resorcinol (1,3-benzenediol), and hydroquinone (1,4-benzenediol) (Oliveira et al. 2020). These aromatic organic chemical compounds are used in chemical factories, medicines, paint production and pigments. BZOL is a fundamental structure in organic chemistry and serves as a key synthetic value derivative in many large-scale chemical processes (Oliveira et al. 2020). BZOL isomers are present in wastewater from agricultural runoff, industrial effluents and domestic sewage. Specifically, these compounds are considered emerging pollutants because of their: (i) toxicity; (ii) persistence and (iii) possible formation of carcinogenic by-products (Anku et al. 2017).

Therefore, the isolation and removal of BZOL isomers in wastewater is crucial prior to releasing into the environment. Catechol and hydroquinone also have the ability to auto-oxidize, producing reactive oxygen species (ROS), which are cytotoxic and linked to cancer and other diseases (Kayembe et al. 2013). Resorcinol has been reported to act as a thyroid function inhibitor, which increases the risk of developing hypothyroidism from chronic exposure (Bachwenkizi et al. 2024). BZOL are persistent in the environment and can cause ecosystem-level impacts. Their biodegradability is limited and they are highly toxic to aquatic organisms, potentially leading to long term contamination in the environment (Kayembe et al. 2013). Hydroxybenzene (phenol) is also highly toxic and has harmful effects on both the environment and human health, making its treatment in wastewater and industrial effluents essential. Various methods have been developed to address the issues related to phenol pollution (Sun et al. 2022). Among these, biological treatment has emerged as an effective solution, which relies on microorganisms (bacteria and fungi) to degrade phenol molecules into less harmful materials. While this method is widely used in industrial wastewater treatment plants, it has several drawbacks. It is sensitive to environmental factors such as temperature and pH, and excessive phenol concentration can inhibit microbial activity. It can be a slow process, may result in partial degradation of materials, and produces sludge that requires further management. It is also less effective on such mixed or highly toxic industrial wastewater and requires significant space, continuous monitoring, and maintenance, which adds operational complexity and cost (Henze and Comeau 2008).

Chemical oxidation is another widely used method; involving strong oxidant like ozone, hydrogen peroxide, or chlorine which can decompose phenol into harmless products such as carbon dioxide and water (Honarmandrad et al. 2021). This approach is especially useful in an industry setting when dealing with high concentrations of phenol (Oturan and Aaron 2014). Photocatalysis, which utilizes ultraviolet (UV) light and catalysts such as titanium dioxide, can enhance phenol degradation in light. This method is particularly effective for water with lower concentrations of phenol (Herrmann 1999). However, chemical oxidation has some disadvantages, including high operating cost due to the use of strong oxidants, the possible formation of toxic by-products, and the need for close control of the reaction conditions to avoid inefficiencies. It is less effective at very high or low concentrations of phenol and often needs complementary treatments for the complete elimination of pollutants (Villegas et al. 2016) .

Another possible treatment is adsorption using activated carbon, which relies on the large surface area of activated carbon to adsorb phenol molecules. It is commonly used in the purification of wastewater and potable water to remove organic contaminants (Ioannidou and Zabaniotou 2007). In addition, membrane filtration uses semi-permeable membranes to block the phenol molecules from passing through the membrane pores. This method is used widely for water purification, particularly in drinking water and recycled water systems (Wiesner and Chellam 1999).

Lastly, thermal treatment (pyrolysis) involves the decomposition of phenol waste at high temperatures, which converts organic compounds into simpler forms, including CO_2 and water vapor. This technique is primarily applied for the industrial toxic waste, especially for degrading hard-to-eliminate compounds by traditional methods (Fernandez et al. 2023).

These diverse treatment approaches contribute significantly to reducing phenol concentrations in wastewater, thereby aiding in environmental protection and public health preservation.

Arugula peroxidase (Cordeiro-Araújo et al. 2015b) is an enzyme found in the arugula plant (*Eruca sativa*), known for its action as an oxidase with hydrogen peroxide as the oxidizing agent. Industrial effluents are well-known sources of toxic pollutants like phenols, dyes, and various organic compounds, and this enzyme shows potential for the degrading such contaminants. The peroxidase enzyme uses a heme group to receive an electron from organic compounds and transfer it to hydrogen peroxide. The byproducts of these harmful organic substances are then transferred into less harmful substances, which can be further converted to water, carbon dioxide or compounds that can be treated later. The environmental footprint of arugula peroxidase (AP) is beneficial, as it provides a cost-effective method for industrial effluent treatment in bioremediation technologies, thereby reducing pollution and promoting environmental protection. Compared to typical chemical treatment methods, which usually generate harmful by-products and may require additional procedures for handling by-products, AP is a more sustainable option. Chemical methods also tend to lack sustainability and may have adverse effects on the environment, making them less "green" than bioremediation approaches (Chiong et al. 2016, Moussavi and Mahmoudi 2009, Svetozarević et al. 2019).

AP is superior to soybean and horseradish peroxidase due to its reliability, substrate specificity, catalytic performance, durability against inhibition, cost and accessibility, operational conditions, and lower environmental impact (Sellami et al. 2022, Cordeiro-Araújo et al. 2015a).

No studies have been published on the use of arugula peroxidase (AP) for the treatment of BZOL compounds (phenol), based on existing scientific literature of treating other BZOL compounds in the presence of AP, according to literature review. Although a few studies have explored the potential of peroxidase enzymes extracted from alternative plant sources, including horseradish, soybean and potato, for the remediation of phenolic-type contaminants in industrial effluents (Chiong et al. 2016), research on arugula peroxidase is very limited. This gap highlights the relevance of conducting a study to evaluate the capacity of AP enzymes for treating organic contaminants. Such enzymes have the potential to decompose toxic compounds and transform them into less toxic substances, which provides an environmentally safe alternative to chemical treatment methods. The overall goal of the present work was to define the optimal conditions for using AP as an efficient enzymatic bioremediation agent, promoting maximum degradation of contaminants under sustainable and low-cost conditions.

2. MATERIALS AND METHODS

2.1 Materials

Crude dry peroxidase enzyme extracted from arugula (Eruca sativa) (EC 1.11.1.11) (Cordeiro-Araújo et al. 2015a) was prepared in-house, with an estimated activity of 0.6 U/mg. The enzyme was stored at 4°C to maintain its stability throughout the experiment. The phenolic compounds used in the study, resorcinol (≥99% purity) and catechol (≥99% purity), were purchased from Sigma-Aldrich Chemical Company Inc. (Baghdad, Iraq). For buffer preparation, potassium chloride (KCl, ≥99% purity, BDH Chemicals, Toronto, ON, Canada), sodium acetate (≥99% purity, Merck KGaA, Darmstadt, Germany), and various chemicals from Sigma-Aldrich, including hydrochloric acid (HCl, 37%), acetic acid (≥99.8% purity), monobasic sodium phosphate (NaH_2PO_4, ≥99% purity), dibasic sodium phosphate (Na_2HPO_4, ≥99% purity), anhydrous sodium carbonate (Na_2CO_3, ≥99.5% purity), sodium bicarbonate ($NaHCO_3$, ≥99% purity), hydrogen peroxide (H_2O_2, 30% w/v), phenol (≥99% purity), and 4-aminoantipyrine (≥98% purity), were utilized. All solutions were prepared using distilled water sourced from a Milli-Q water purification system (Millipore, Burlington, MA, USA).

To assess the enzyme's activity, a phosphate buffer (10× concentration) was prepared using monobasic and dibasic sodium phosphate solutions. All compounds were of analytical quality and utilized without additional purification.

2.2 Methods

2.2.1 Arugula peroxidase extraction, purification and activity assay

In this study, arugula leaves were used as the raw materials. The leaves were trimmed, washed, and homogenized. The supernatant was collected after centrifugation. By using pH gradient elution from pH=4.0 to pH=9.0, AP were purified on a column filled with the positively charged diethylaminoethyl (DEAE) cellulose.

AP obtained by using ion-exchange column chromatography with a pH gradient. The enzyme was characterized in terms of its pH and temperature optima, as well as kinetic parameters. The pH activity profiles were determined in 0.067 M phosphate buffers at different pH values ranging from pH=4.0 to 9.0. At optimum pH, enzyme activities were also determined as a function of temperature ranging from 10 to 70 °C. Substrate specificity and kinetic parameters of the peroxidase enzyme were determined using guaiacol, applying either Michaelis-Menten or Lineweaver–Burk plot.

Free AP activity was measured using a spectrophotometric method. The rate at which a color developed at 510 nm was monitored from the quinone imine formed by the oxidative coupling of phenol and 4-aminoantipyrine in the presence of hydrogen peroxide (Steevensz et al. 2013). The assay was performed using a mixture of 10 mM phenol, 40 mM phosphate buffer (pH 7.4), 2.4 mM 4-aminoantipyrine, and 0.2 mM hydrogen peroxide (H_2O_2), with a total volume of 950 µL (Mazloum et al. 2016). The reaction was initiated by adding 50 µL of the diluted enzyme solution to 0.95 mL of the reagent, and the initial rate of color development at 510 nm was monitored for 30 seconds. Sample dilution was adjusted according to expected enzyme activity to yield an estimated increase in absorbance of 0.2 within 30 seconds (Altahir et al. 2016). One unit (U) of AP activity was defined as the amount of enzyme that catalyzes the conversion of 1 µmol of hydrogen peroxide per min under test conditions described (Feng et al. 2013).

2.2.2 Buffer preparation

The buffer preparation process was carried out following the methodology of (Stoll and Blanchard 2009). To achieve appropriate pH control, acetate buffer (pH range: 3.6–5.6), phosphate buffer (pH range: 5.7–8.0), and carbonate-bicarbonate buffer (pH range: 9.2-10.7) were utilized.

2.2.3 Experimental protocol

In this study, the peroxidase enzyme extracted from arugula (Eruca sativa) was investigated as a potential environmentally friendly catalyst for the removal of BZOL compounds, such as resorcinol and catechol, using batch reactors. To determine optimal conditions for peroxyacid production and quantification, a design–of–experiments approach was taken, allowing for identification of optimal reaction parameters (pH, enzyme concentration, hydrogen peroxide concentration, and reaction time) through screening and optimization strategies (Mazloum et al. 2016).

Experimental protocol:

Batch reactors consisted of 20 mL of a buffered mixture containing BZOL, hydrogen peroxide and SBP. The reactors were stirred using magnetic stirrers at an agitation rate of 500 rpm with Teflon-coated stir bars at a room temperature of about 21°C. The components of the sample mixture were added in the following order: water (distilled or tap water), 40 mM acetate, phosphate or carbonate buffer, BZOL as an aqueous stock solution, SBP to appropriate concentration and hydrogen peroxide to appropriate concentration to initiate the reaction. The batch reactors were stirred gently for 3 hours, open to the atmosphere. afterward, the samples were quenched with excess catalase to a concentration of 62·5 U/mL to quickly consume any residual hydrogen peroxide, microfiltered and then tested for the residual concentration by using a HPLC-UV

Based on earlier research, the reaction time was selected to ensure a notable decrease in BZOL compounds (Al-Ansari et al. 2010, Altahir et al. 2016, Steevensz et al. 2013). Optimal conditions for achieving 95%

removal of phenols were determined, as this threshold allows for precise detection of remaining substrate while minimizing interference near detection limits. The reaction mixtures were prepared in the following order: distilled water, buffer, BZOL substrate, peroxidase enzyme, and hydrogen peroxide. To terminate the reaction, excess catalase (17 U/mL final concentration) was added to decompose any residual hydrogen peroxide rapidly (Mazloum et al. 2016).

To ensure effluent clarity for analysis, the samples were then centrifuged and filtered through a 0.45-µm micro-syringe filter (highest pore-size) using HPLC (High-Performance Liquid Chromatography) once the reaction had terminated (Feng et al. 2013). Each experiment was performed three times, and the associated standard deviations were computed and shown as error bars in the appropriate figures.

Under ideal conditions for pH, enzyme concentrations, and hydrogen peroxide concentration, time-course investigations were carried out in 200 mL batch reactors. Using a micropipette, aliquots (5 mL) were taken out at different times, quenched with 0.25 mL of catalase solution, centrifuged, filtered, and spectrophotometrically examined to assess phenol degradation . Centrifugation ensured clear samples for accurate absorbance measurements (Altahir et al. 2016).

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Purification and characterization of AP

In this study, simple purification procedures were employed for the separation of arugula peroxidase (AP). Several factors, including temperature, pH, storage duration, ions in buffer solutions, and the presence of detergents and protective agents, were found to influence the stability of the enzyme. Therefore, eliminating or inactivating secondary metabolites from plant cells that hinder enzyme recovery and significantly reduce yield is a crucial consideration when developing a purification process from a plant homogenate.

Browning processes, which are triggered during tissue homogenization for the isolation of the AP enzyme, can lead to the formation of covalent bonds (e.g., Quinones), which may not be reversible. (Tilley et al. 2023). To prevent the undesirable effects of polyphenolic compound degradation, polyvinyl-polypyrrolidone (PVPP) was included during the homogenization of arugula tissue to obtain the crude enzyme extract.

For the elimination of contaminating proteins and purification of oxidative enzymes from arugula, DEAE ion-exchange chromatography was utilized. Enzyme purification was accomplished by contacting DEAE with an impure liquid containing the enzyme and soluble contaminants. This allowed for the removal of contaminants that hindered enzyme recovery and reduced yield. The DEAE column adsorbed soluble contaminants, while the

adsorbed enzyme was eluted from the column, leading to a purified liquid with greater enzyme activity prior to further purification.

Table 1: Partial purification of AP enzyme.

Purification steps	Total activity/U	m(total protein)/mg	Specific activity/(U/mg)	Recovery/%	Purity/fold
Crude extract	3.12	466	0.007	100	
AP	0.081	8.2	9.87	2.86	1646.3

Peroxidases, in general, are considered to have an important function in the oxidation of vacuolar and cell wall phenols as part of a sequence of metabolic processes linked to phenolic regeneration and degradation, as well as cell wall rigidification. Separate analyses were performed on the chromatographic peaks that had the corresponding activity.

AP's pH optimum was observed to fall between 6.2 and 8.3. The existence of isoenzymes with various pH optima most likely causes this comparatively wide range. These results were in line with earlier research on lettuce peroxidase (Altunkaya et al. 2011).

The effects of varying temperatures on AP activity were studied over a range from 20 °C to 100 °C. The optimal temperature for AP was found to be between 20 °C to 60 °C. AP activity significantly decreased when heated to 85 °C, yet it was not fully inactivated even at 95 °C, suggesting that AP may be heat-tolerant and may require additional treatments for complete inactivation.

For instance, at certain temperatures, the forces that hold a protein in its secondary, tertiary, or quaternary structure are overcome, and the protein becomes denatured. This is known as a protein's thermal stability. It is important to understand the thermal stability of proteins since some applications in biotechnology and food science, among others, require proteins to often be exposed to changes in temperature.

3.1.2 Kinetic analysis of isolated AP

Guaiacol was used as the reducing substrate to determine the kinetic parameters for AP. At varying concentrations of H_2O_2, the activity of AP followed a Michaelis-Menten relationship.

The Km and Vmax values for AP were found to be 0.109 mM and 0.0162 mmol/min, respectively. The low Km value for H_2O_2 indicates a strong affinity between the enzyme and substrate, suggesting the presence of a substantial number of hydrogen or hydrophobic bonds between the substrate's surface and the heme group located at the enzyme's active site.

Table 2: The information of kinetic study of AP Km, Vmax and reaction order range.

Km mM	Vmax mmol/min	Reaction order (range of H_2O_2 Con. mM)		
		1st	1st+ zero	zero
0.109	0.0162	(0-0.152)	(0.171-.247)	(0.266-0.38)

3.2 Optimization of BZOL removal from water

Qualitatively, the enzymatic reactions involving both BZOL compounds (resorcinol and catechol) led to the formation of precipitates and noticeable color changes in the solutions. The catechol solution transitioned from pale brown to colorless, accompanied by the formation of larger brownish precipitate particles, whereas the resorcinol solution changed from a light yellow to a nearly colorless with smaller precipitate particles. Prior to HPLC analysis, all samples were centrifuged and microfiltered to remove any potential interference with the measurements.

To optimize BZOL removal, experiments were conducted to evaluate key parameters such as pH, enzyme activity, hydrogen peroxide concentration, and reaction time in fully mixed batch reactors. These results provide crucial information for the development of a prototype reactor and a phenol treatment system. Due to the formation of solid byproducts during the reaction, enzyme immobilization was deemed impractical in this study, as it could hinder its application in continuous treatment systems.

3.2.1 pH

To assess the impact of pH, experiments were conducted under controlled conditions, focusing on enzyme activity levels that were insufficient to achieve complete substrate conversion, as illustrated in Fig. 1. The results indicated that the optimal pH for the enzymatic treatment of resorcinol was 6.1, which falls within the mildly acidic range. Similarly, the optimal pH for catechol removal was found to be 5.7, suggesting that the enzyme exhibits a preference for slightly acidic to neutral conditions in these reactions.

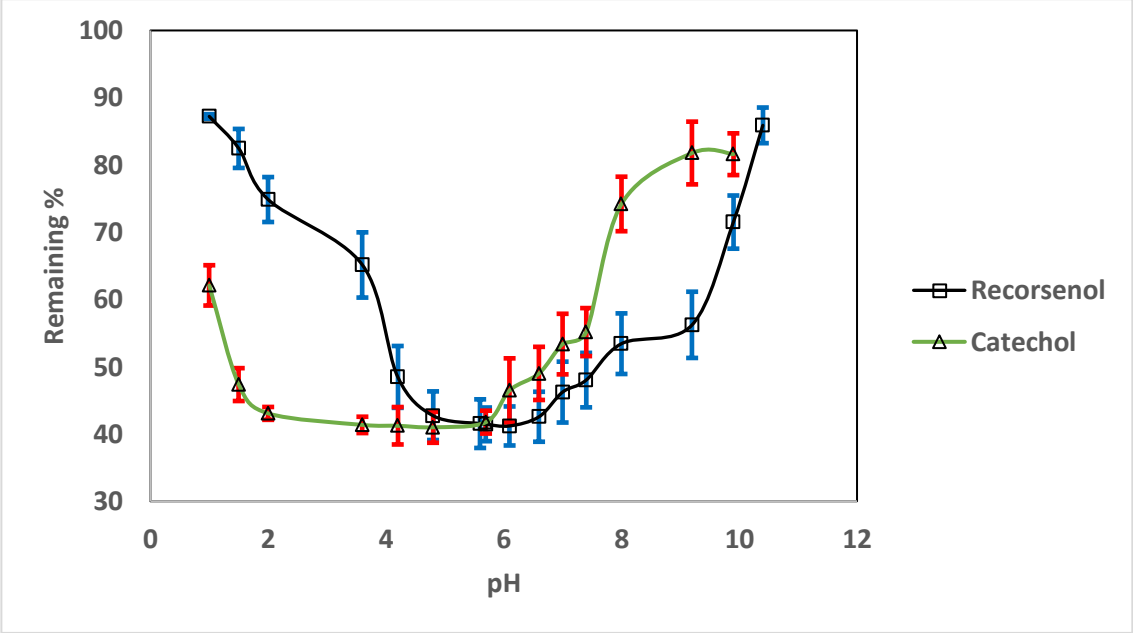

Fig. 1: pH optimization for resorcinol and catechol. Conditions: The enzyme activity was 0.05 U/mL, the hydrogen peroxide and substrates were equimolar (0.05 mM), and the reaction lasted three hours.

3.2.2 Optimum H_2O_2-to-substrate concentration ratio

Hydrogen peroxide (H_2O_2) concentrations between 0.05 mM and 0.5 mM were evaluated for the enzymatic treatment of resorcinol and catechol over a three-hour period, with the objective of achieving 95% removal under the previously determined optimal enzyme activity and pH conditions (Fig. 2). Within the same peroxide concentration range, chemical oxidation (without enzyme) exhibited negligible efficacy, as illustrated by the lines in Fig. 2. The results indicated that for both resorcinol and catechol, a minimum H_2O_2 concentration of 0.15% was necessary to attain 95% removal. This concentration was thus selected as the optimal H_2O_2 level for subsequent experiments.

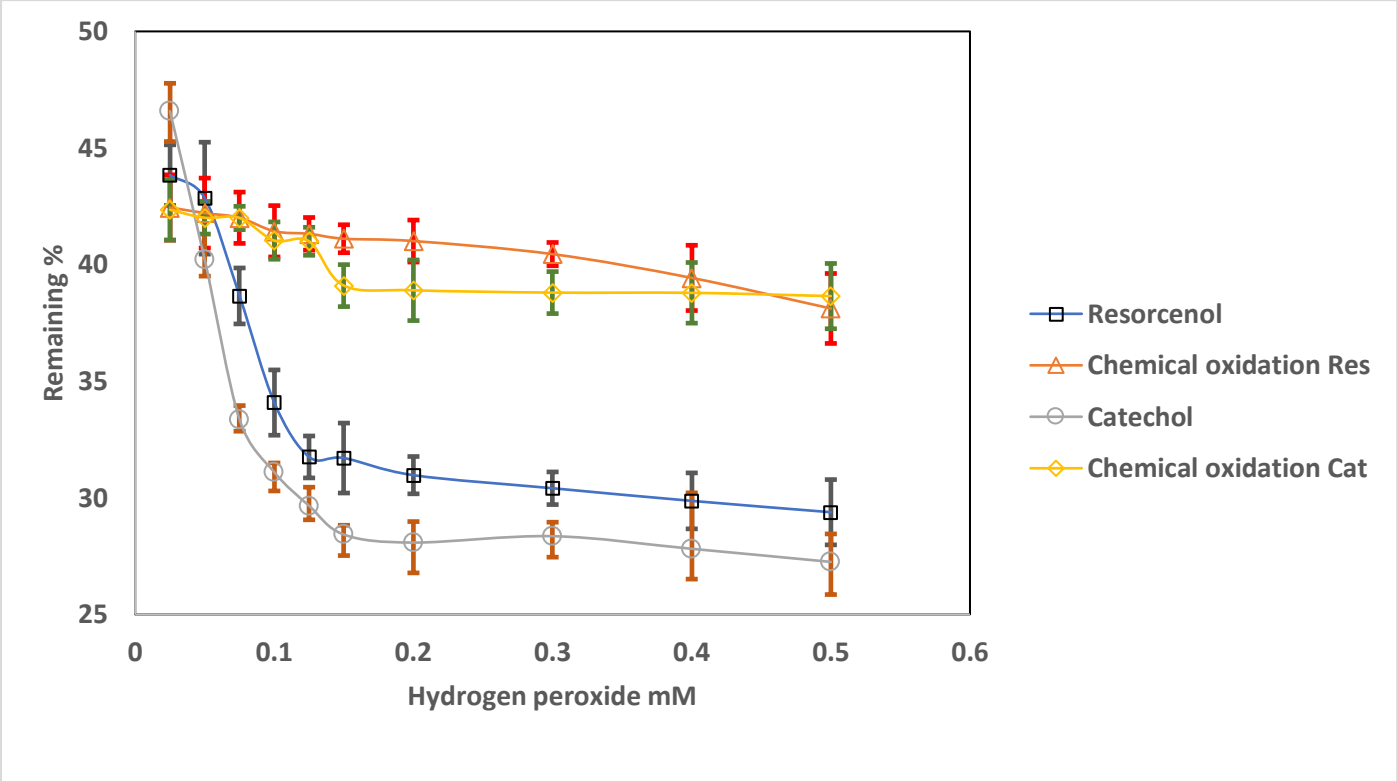

Fig. 2: Optimization of H2O2 concentration Conditions: 0.05 mM of catechol and resorcinol. The enzyme activity was 0.05 U/mL at pH 6.1 for resorcinol and pH 5.7 for catechol, with a three-hour reaction time.

3.2.3 Enzyme activity

The effect of increasing enzyme concentration at the corresponding optimal pH levels was investigated over a three-hour period (Fig. 3). The goal was to achieve 95% removal of phenolic compounds (resorcinol and catechol). As shown, lower concentrations of the enzyme were insufficient to reach 95% removal within the given time frame. The results indicated that the minimum enzyme concentration required to effectively remove both catechol and resorcinol, was 0.072 U/mL. This concentration was selected as the lowest effective enzyme activity for subsequent experiments.

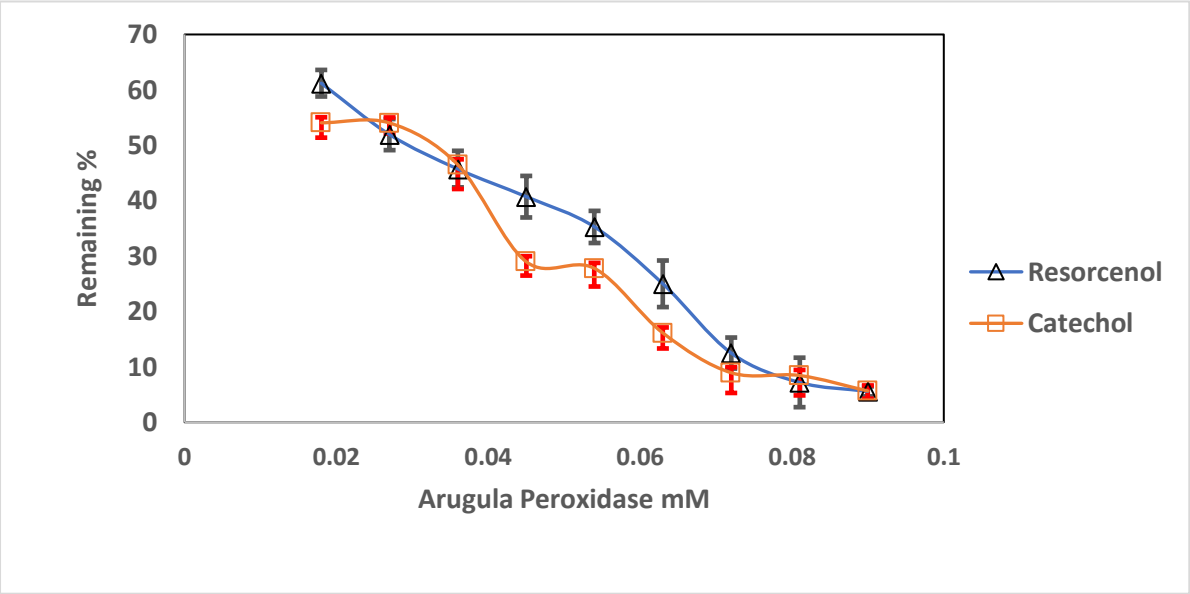

Fig. 3 Enzyme activity. Conditions: resorcinol and catechol 0.05 mM, pH 6.1 for resorcinol pH 5.7 for catechol, H2O2 concentration 0.15 mM, Three hours was the reaction time.

3.2.4 Reaction Time's Effect

Under optimal pH, enzyme activity, and hydrogen peroxide concentration, the time-course profiles for the enzymatic degradation of resorcinol and catechol in both synthetic and natural wastewater were determined. As shown by the blue and red trends in Figs. 4 and 5, approximately 95% removal of both substrates was achieved within three hours

3.2.4.1 Synthetic Wastewater

In synthetic wastewater, the BZOL (pure substrate) degradation curve was established. A pseudo-first-order kinetic model was applied to the data for both resorcinol and catechol to facilitate quantitative analysis. The equation used was:

Percentage remaining = (Initial percentage) × e^{-kt}

where, k is the apparent first-order rate constant and the starting percentage was nearly 100%.

In Fig. 4 and Table 3, the blue and red lines illustrate the removal progress over time, showing that resorcinol exhibited a slightly faster reaction rate, achieving significant removal earlier than catechol. However, both substrates reached near-complete degradation within the 3-hour observation period.

The results highlight the efficiency of enzymatic treatment for both phenolic compounds, with resorcinol showing a shorter half-life compared to catechol, indicating faster degradation kinetics. This observation suggests a higher affinity or catalytic efficiency of the enzyme towards resorcinol under the tested conditions.

Table 3: Parameter Fit of Progress Curves for Synthetic Wastewater BZOL Removal.

	K value	Half-life, min	Initial percentage %	R^2	Represented in Fig. 4
		30 min			
Resorcenol	$(4\pm0.2)x10^{-2}$	17.48675	102 ± 2.2	0.98894	Solid blue line
Catechol	$(3.0\pm0.1)x10^{-2}$	22.94702	102 ± 2	0.99003	Solid red line
		180 min			
Resorcenol	$(3.5\pm0.3)x10^{-2}$	19.76612	99 ± 4.5	0.96237	Light blue line
Catechol	$(2.5\pm0.2)x10^{-2}$	27.78669	97± 3.1	0.97815	Light red line

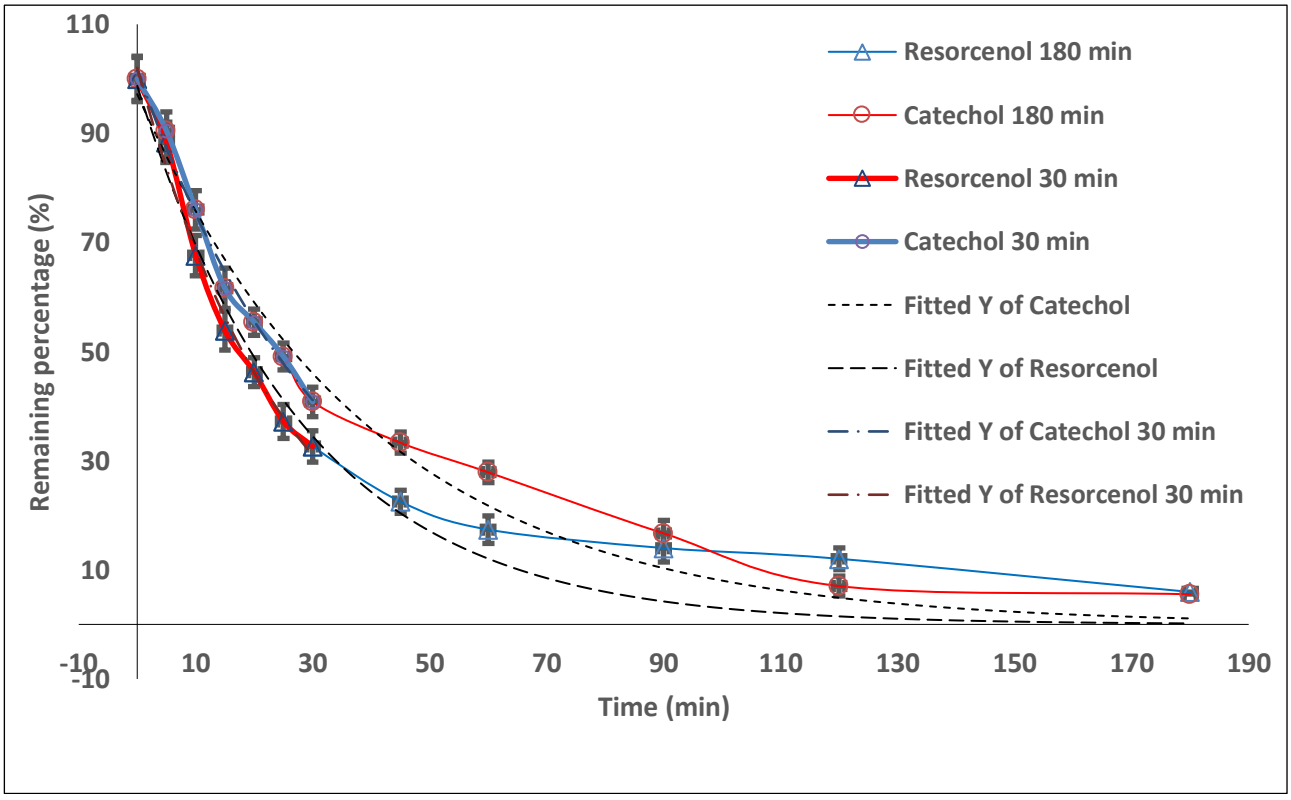

Fig. 4: Removal progress curves for synthetic wastewater. Under optimal conditions: resorcinol (blue trend line) and catechol(red trend line) 0.05 mM , pH 6.1 for resorcinol pH 5.7 for catechol, H2O2 concentration 0.15 mM, enzyme activity 0.072 U/mL of both resorcinol and catechol.

3.2.4.2 Natural waste water

In Fig. 5 and Table 4, the blue and red lines illustrate the removal progress over time of natural wastewater. Overall, the removal efficiency observed in natural wastewater was lower than that in synthetic wastewater, likely due to interference effects from other substances present in the matrix.

Similar to the synthetic wastewater results, the data show that resorcinol exhibited a slightly faster reaction rate, achieving significant removal earlier than catechol. However, both substrates reached near-complete degradation within the 3-hour time frame.

The results from both synthetic and natural wastewater highlight the effectiveness of enzymatic treatment for both BZOLs, with resorcinol showing a shorter half-life compared to catechol. This suggests a higher affinity or catalytic efficiency of the enzyme towards resorcinol under the tested conditions.

Table 4**:** Fit of progress curves by parameters for the removal of BZOL from natural wastewater.

	K value	**Half-life, min**	**Initial percentage %**	**R^2**	**Represented in Fig. 5 as**
		30 min			
Resorcenol	$(3.3\pm0.1)\times10^{-2}$	20.4	102 ± 2.1	0.98729	Solid blue line
Catechol	$(2.8\pm0.2)\times10^{-2}$	24.5	107 ± 3.8	0.95293	Solid red line
		180 min			
Resorcenol	$(2.5\pm0.3)\times10^{-2}$	27	95 ± 6.2	0.89846	Narrow blue line
Catechol	$(2.1\pm0.2)\times10^{-2}$	32	100 ± 5.5	0.92234	Narrow red line

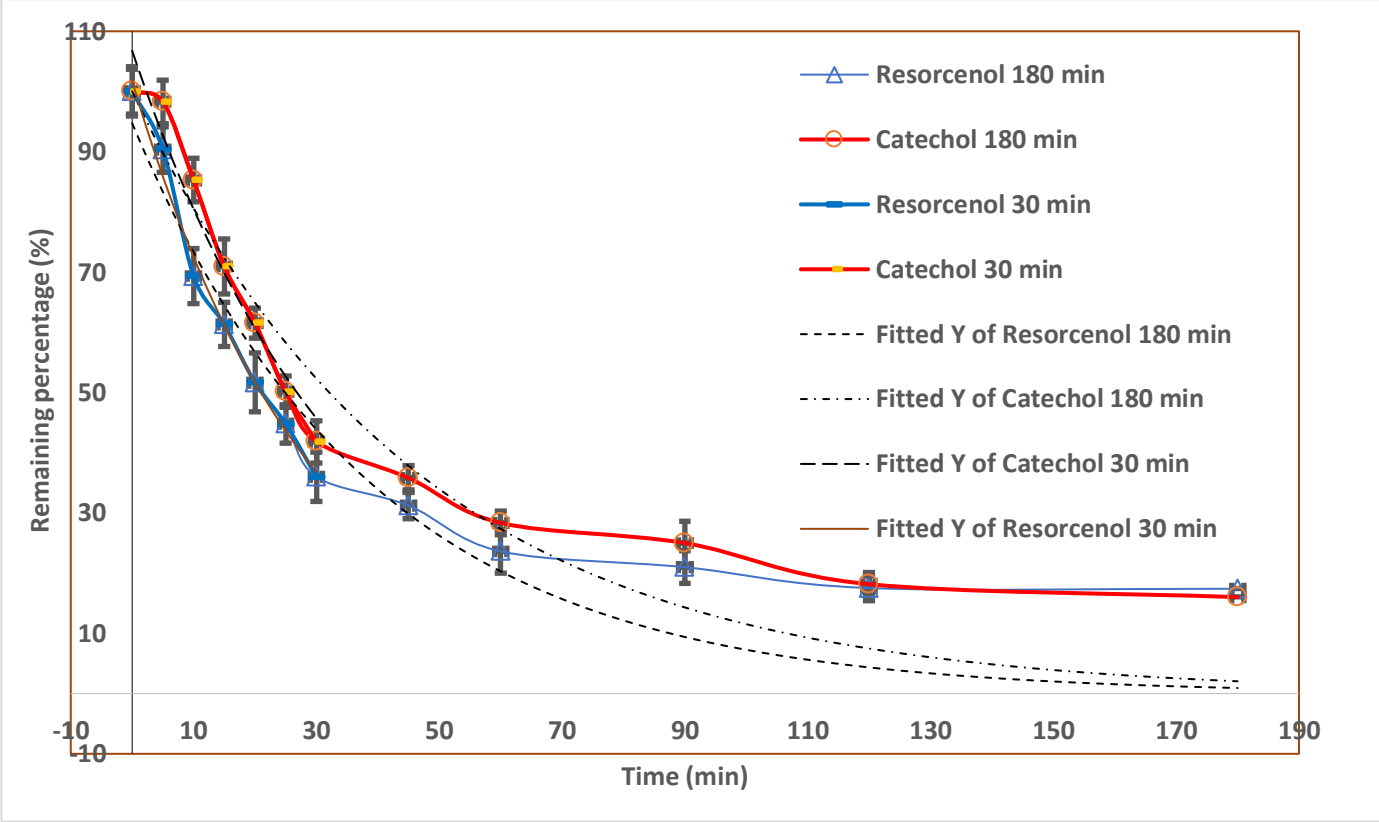

Fig. 5: Removal progress curves for natural wastewater. Under optimal conditions: resorcinol (blue trend line) and catechol(red trend line) 0.05 mM , pH 6.1 for resorcinol pH 5.7 for catechol, H_2O_2 concentration 0.15 mM , enzyme activity 0.072 U/mL of both resorcinol and catechol.

4 DISCUSSION

The enzymatic treatment approach presented in this study, shows significant potential for eliminating BZOL compounds (catechol and resorcinol) from wastewater. Comparison with existing enzymatic and non-enzymatic methods reveals several valuable insights.

4.1 Optimum pH

Catechol exhibited optimal degradation at a slightly acidic pH of 5.7), whereas the most favorable pH for resorcinol removal was 6.1. These observations align with the behavior of crude arugula peroxidase, which relies on the correct ionization of its catalytic residues for full activity. Such pH optima are consistent with earlier reports on other peroxidases such as horseradish and soybean peroxidases (Altahir et al. 2020), emphasizing the role of the ionization states of both enzyme and substrate.

4.2 Enzyme Activity

The minimum enzyme activity required to achieve 95% removal of phenolic compounds was found to be 0.072 U/mL This is in agreement with previous studies demonstrating a correlation between enzyme activity

and substrate conversion efficiency. The observed dependence of reaction rate on enzyme activity further suggests that the AP maintains its catalytic efficiency performance even at low activity levels, representing its cost-effectiveness for wastewater treatment applications.

4.3 Hydrogen Peroxide Concentration

The optimal H_2O_2 concentration for efficient substrate oxidation was determined to be 0.15% which aligns with theoretical expectations. However, excess peroxide could lead to secondary reactions, such as the formation of oligomeric or polymeric byproducts. These possibilities warrant further investigation in future studies. Table 5 compares the proposed method with oxidation using soybean peroxidase (SBP).

4.4 Reaction Time

Time-course study indicated nearly complete elimination of both resorcinol and catechol within 3 hours. The pseudo-first-order kinetic model provided accurate rate constants with resorcinol exhibiting faster degradation than catechol. This suggests a higher enzymatic affinity for resorcinol, likely due to differences in their molecular structures and reactivity (Theydan et al. 2024, Auied et al. 2024).

4.5 Comparative Analysis

The results are comparable to those reported for other pure enzymatic treatments, such as soybean and horseradish peroxidases. However, the use of AP provides a sustainable and environmentally friendly alternative, with the added benefit of utilizing a plant-based enzyme that can be extracted with relative ease.

Table 5: Comparison of Proposed Method with Oxidation Using SBP.

Method	**pH**	**[H_2O_2] mM /[substrate] mM**	**Minimum enzyme concentration Required for 95% conversion of**	**Reference**
		Resorcinol		
SBP	7.5-8.25	2 / 1	0.2 U/mLb	(Mousa et al. 2010)
Proposed method	**6.1**	**3 /1**	**0.072**	
		Catechol		
SBP	6.5-7.5	2.5 / 1	0.025 U/mLb	(Mousa et al. 2010)
Proposed method	**5.7**	**3 /1**	**0.072**	

Table 6: Comparison of current method with other chemical, physical treatment methods of BZOL.

Method	**pH**	**[H_2O_2] mM/[substrate] mM**	**Rate constant k min^{-1}**	**Reference**

Resorcinol				
Fenton	3.0	4/1	-	(Mijangos et al. 2006)
Ozonation	11.8	---	3.7×10^{-2}	(Parisheva and Demirev 2001)
Granular activated carbon	10.3			(Sundaramurthy et al. 2011)
Activated carbon cloth	6.55		0.23×10^{-2}	(Bayram et al. 2009)
Proposed method	6.1	3/1	$(4\pm0.2)\times10^{-2}$	
Catechol				
Fenton	3.0	4/1	-	(Mijangos et al. 2006)
Ozonation	11.8	---	2.9×10^{-2}	(Parisheva and Demirev 2001)
Ozone/ H_2O_2	6.5	------		(Kubesch et al. 2005)
Photocatalytic	2	----	----------	(Raeisivand et al. 2019)
Granular activated carbon	10.3			(Sundaramurthy et al. 2011)
Activated carbon cloth	6.44		0.56×10^{-2}	(Bayram et al. 2009)
Proposed method	5.7	3/1	$(3.0\pm0.1)\times10^{-2}$	

Statistical analysis using t-tests showed significant differences between the synthetic and natural wastewater removal data. The p-value for resorcinol was 1.14×10^{-4} (t = -5.83207) and for catechol was 8.5×10^{-4} (t = -4.53334), confirming a statistically significant difference and rejecting the null hypothesis.

4.6 Practical Implications

This study provides valuable data for designing wastewater treatment systems employing arugula peroxidase. These results support the industrial feasibility of the AP to degrade toxic phenolic compounds effectively. Further work should focus on scaling up of this approach and exploring enzyme immobilization strategies to improve stability and recyclability.

Overall, the results highlight the effectiveness of AP in removing hydroxyl benzene compounds, which are widely used in the synthesis of a variety of chemicals in the laboratory and industry, known for their high toxicity. This method offers a viable, environmentally friendly alternative to traditional chemical treatments.

4.7 Mechanism of action

Phenol peroxidase (such as horseradish peroxidase, HRP) catalyzes the oxidation of phenolic compounds using hydrogen peroxide (H_2O_2). The process involves formation of enzyme-substrate complexes and the generation of free radicals, especially phenoxyl radicals.

The steps of mechanism include the rection of H_2O_2 with enzyme. The enzyme has a ferric (Fe^{3+}) heme , forming Compound I. This is a highly oxidizing intermediate with a porphyrin radical cation.

$$Fe^{3+} + H_2O_2 \rightarrow \text{Compound I } (Fe^{4+} = O + \text{porphyrin}^{\bullet}+)$$

Second step includes oxidation of phenol and formation of phenoxyl radical. Compound I oxidizes a phenol (ArOH), removing one electron. The phenoxyl radical (ArO•) is the key intermediate in phenol oxidation (Frey 2001). This step forms the enzyme-substrate complex, enabling electron transfer.

$$\text{Compound I} + \text{ArOH} \rightarrow \text{Compound II} + \text{ArO}^{\bullet}$$

In further reaction, Compound II (Fe^{4+}=O) reacts with another phenol molecule. The resulting phenoxyl radicals are unstable and undergo non-enzymatic coupling reactions, leading to quinones, dimers, or polymers—important in plant defense, lignin biosynthesis, and wound sealing (Shibata & Toraya, 2015; de Oliveira et al., 2021). Fig. 6 shows the proposed mechanism of BZOL oxidation and dimer formation.

$$\text{Compound II} + \text{ArOH} \rightarrow \text{Resting Enzyme } (Fe^{3+}) + \text{ArO}^{\bullet}$$

$$2\text{ArO}^{\bullet} \rightarrow \text{Ar–O–Ar (dimer)}$$

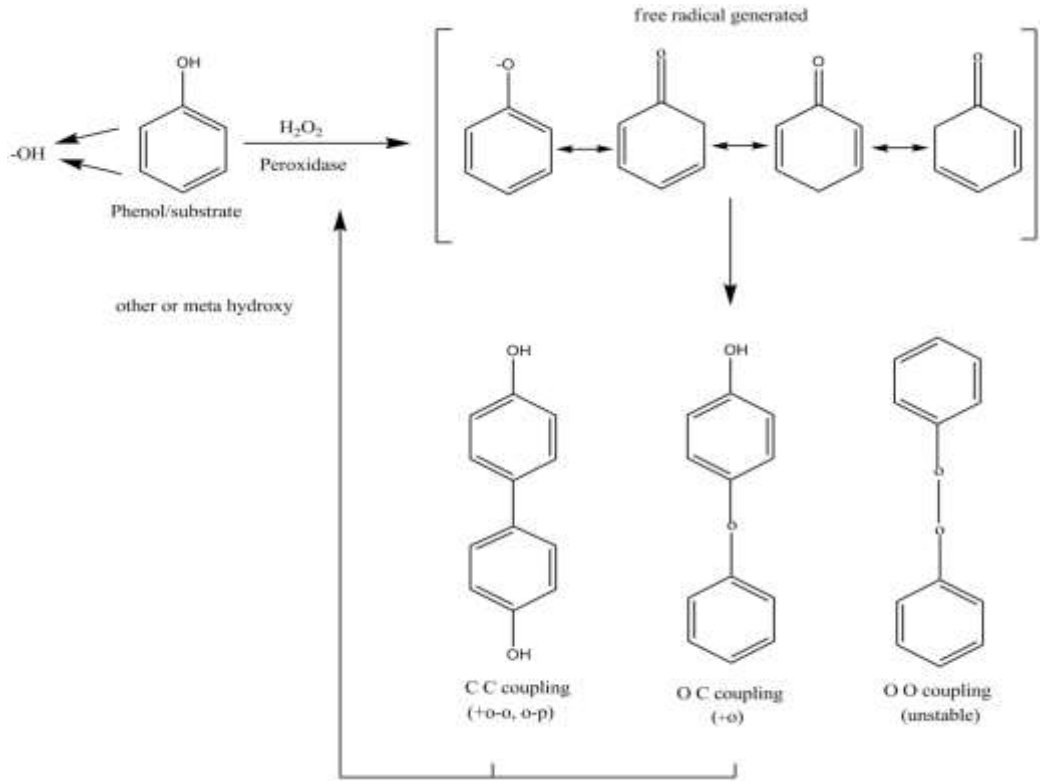

Fig. 6: The proposed mechanism of BZOL oxidation and dimer formation.

5. CONCLUSION

This study demonstrated the potential of arugula peroxidase (AP) in the enzymatic treatment of hydroxybenzene compounds, specifically resorcinol and catechol, in wastewater. The findings revealed that the enzyme effectively removes these toxic BZOL under optimized conditions, achieving up to 95% removal within three hours. The study identified pH 6.1 as optimal for resorcinol and pH 5.7 for catechol, showcasing the enzyme's adaptability to slightly acidic environments.

The analysis of enzyme activity showed that a minimum concentration of 0.072 U/mL is sufficient for effective treatment, emphasizing the cost-efficiency of this method. Additionally, hydrogen peroxide concentration of 0.15% was found to be optimal for maximum substrate degradation with minimal side reactions.

Kinetic studies confirmed that catechol degrades more rapidly than resorcinol, and the pseudo-first-order model was suitable for describing the reaction kinetics. These results underscore the enzyme's catalytic efficiency and specificity, making it a promising alternative for industrial wastewater management.

In conclusion, AP provides a sustainable and environmentally friendly solution for BZOL removal, with significant potential in designing scalable wastewater treatment systems. Future studies should explore enzyme immobilization techniques to enhance reusability and stability, further increasing the practical viability of this bioremediation approach. It can be accomplished through a variety of development stages, including scale-up potential, enzyme immobilization, and application in continuous systems.

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