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Wastewater Benzenediol Removal Catalyzed by Crude Arugula Peroxidase

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ABSTRACT

This study explores the potential of arugula (Eruca sativa) as a novel source of peroxidase enzymes for the bioremediation of wastewater containing benzenediol (BZOL) pollutants. The peroxidase was extracted and partially purified from arugula leaves, and its catalytic efficacy was evaluated in the enzymatic degradation of two representative BZOL compounds, resorcinol and catechol. Crude arugula peroxidase (AP) demonstrated significant activity under mild reaction conditions, with optimal pH values identified as 6.1 for resorcinol and 5.7 for catechol removal. Remarkably, near-complete elimination (up to 95%) of both compounds was achieved using minimal reagent concentrations: 0.15% hydrogen peroxide and 0.072 $U \cdot mL^{-1}$ enzyme activity over a 3-hour reaction period. Kinetic analyses revealed that the degradation process adhered to pseudo-first-order kinetics, with catechol exhibiting a faster reaction rate compared to resorcinol. These results underscore the efficiency and eco-compatibility of arugula-derived peroxidase as a low-cost, plant-based catalyst for the treatment of aromatic pollutants in wastewater. The findings hold promise for scalable, sustainable bioremediation strategies in environmental engineering.

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, which is also known as dihydroxybenzene. BZOL belongs to the class of organic compounds commonly known as phenols owing to the presence of hydroxyl groups (Venkatesh & 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 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 from wastewater is crucial prior to their release into the environment. Catechol and hydroquinone also 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 following chronic exposure (Bachwenkizi et al. 2024). BZOL is 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 environmental contamination (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 issues related to phenol pollution (Sun et al. 2022). Among these, biological treatment has emerged as an effective solution that relies on microorganisms (bacteria and fungi) to degrade phenol molecules into less harmful materials. Although 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 concentrations can inhibit microbial activity. This 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 mixed or highly toxic industrial wastewater and requires significant space, continuous monitoring, and maintenance, which adds operational complexity and cost (Henze & Comeau 2008).

Chemical oxidation is another widely used method, involving strong oxidants such as ozone, hydrogen peroxide, and 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 industrial setting when dealing with high concentrations of phenol (Oturan & 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 low concentrations of phenol (Herrmann 1999). However, chemical oxidation has some disadvantages, including high operating costs due to the use of strong oxidants, 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 requires 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 to purify wastewater and potable water by removing organic contaminants (Ioannidou & Zabaniotou 2007). In addition, membrane filtration uses semi-permeable membranes to block phenol molecules from passing through the membrane pores. This method is widely used for water purification, particularly in drinking water and recycled water systems (Wiesner & Chellam 1999).

Finally, 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 to industrial toxic waste, especially for degrading compounds that are difficult to eliminate using traditional methods (Fernandez et al. 2023).

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

Arugula peroxidase (Cordeiro-Araújo et al. 2015b) is an enzyme found in arugula (*Eruca sativa*) and is known for its action as an oxidase with hydrogen peroxide as the oxidizing agent. Industrial effluents are well-known sources of toxic pollutants, such as phenols, dyes, and various organic compounds, and this enzyme shows potential for degrading such contaminants. The peroxidase enzyme uses a heme group to receive electrons from organic compounds and transfer them 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 these 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 & Mahmoudi 2009, Svetozarević et al. 2019).

AP is superior to soybean and horseradish peroxidase because of 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 the 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 limited. This gap highlights the relevance of conducting a study to evaluate the capacity of AP enzymes to treat organic contaminants. These enzymes can decompose toxic compounds and transform them into less toxic substances, providing an environmentally safe alternative to chemical treatment methods. The overall goal

of the present study 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.

MATERIALS AND METHODS

Materials

Crude dry peroxidase enzyme extracted from arugula (Eruca sativa) (EC 1.11.1.11) (Cordeiro-Araújo et 2015a) was prepared in-house with an estimated activity of 0.6 $U.mg^{-1}$. The enzyme was stored at 4°C to maintain its stability throughout the experiment. The phenolic compounds used in this 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 obtained from a Milli-Q water purification system (Millipore, Burlington, MA, USA).

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

Methods

Arugula Peroxidase Extraction, Purification, and Activity Assay

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

AP was obtained using ion-exchange column chromatography with a pH gradient. The enzyme was characterized in terms of its pH and temperature optima and kinetic parameters. The pH activity profiles were determined in 0.067 M phosphate buffers at different pH values ranging from4.0 to 9.0. At the optimum pH, enzyme activity was determined as a function of temperature, ranging from 10 to 70°C. The substrate specificity and kinetic parameters of the peroxidase enzyme were determined using guaiacol, applying either the Michaelis-Menten or Lineweaver–Burk plot.

Free AP activity was measured using spectrophotometry. 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 s. Sample dilution was adjusted according to the expected enzyme activity to yield an estimated increase in absorbance of 0.2 within 30 s (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 the test conditions described (Feng et al. 2013).

Buffer Preparation

Buffer preparation was performed following the methodology described by. To achieve appropriate pH control, acetate (pH range: 3.6–5.6), phosphate (pH range: 5.7–8.0), and carbonate-bicarbonate (pH range: 9.2-10.7) buffers were utilized.

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 the optimal conditions for peroxyacid production and quantification, a design–of–experiments approach was used, allowing for the 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

The 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 approximately 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 an appropriate concentration, and hydrogen peroxide to an appropriate concentration to initiate the reaction. The

batch reactors were stirred gently for 3 h and opened to the atmosphere. Subsequently, the samples were quenched with excess catalase to a concentration of 62·5 $U.mL^{-1}$ to quickly consume any residual hydrogen peroxide, microfiltered, and then tested for the residual concentration using HPLC-UV.

Based on earlier research, the reaction time was selected to ensure a notable decrease in the BZOL compounds (Al-Ansari et al. 2010, Altahir et al. 2016, Steevensz et al. 2013). The optimal conditions for achieving 95% phenol removal were determined, as this threshold allowed for the precise detection of the remaining sub while minimizing interference near the detection limits. The reaction mixtures were prepared using distilled water, buffer, BZOL substrate, peroxidase enzyme, and hydrogen peroxide. To terminate the reaction, excess catalase (17 $U.mL^{-1}$ final concentration) was added to rapidly decompose any residual hydrogen peroxide (Mazloum et al. 2016).

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

Under ideal conditions for pH, enzyme concentration, and hydrogen peroxide concentration, time-course investigations were carried out in 200 mL batch reactors. Using a micropipette, aliquots (5 mL) were collected 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).

RESULTS

Purification and Characterization of AP

In this study, simple purification procedures were employed for the separation of arugula peroxidase (AP) from the crude extract. Several factors, including temperature, pH, storage duration, ions in buffer solutions, and the presence of detergents and protective agents, influence enzyme stability (Table 1). Therefore, eliminating or inactivating secondary metabolites from plant cells that hinder enzyme recovery and significantly reduce yield is crucial when developing a purification process from a plant homogenate.

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

DEAE ion-exchange chromatography was used to eliminate contaminating proteins and purify the oxidative enzymes from arugula. Enzyme purification was accomplished by contacting DEAE with an impure liquid containing the enzyme and soluble contaminants. This allowed the removal of contaminants that hindered enzyme recovery and reduced the 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 before further purification steps.

Peroxidases are considered to play an important role 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 corresponding to the activity.

AP's optimum pH of AP was observed to fall between 6.2 and 8.3. The existence of isoenzymes with various pH optima most likely causes this 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 of 20-100°C. The optimal temperature for AP was between 20°C and 60°C. AP activity significantly decreased when heated to 85°C, but 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 is denatured. This is known as the thermal stability of a protein. It is important to understand the thermal stability of proteins because some applications in biotechnology and food science, among others, require proteins to be exposed to changes in temperature.

Table 1: Partial purification of AP enzyme.

Purification steps	Total activity/U	m(total protein).mg^{-1}	Specific activity [$U.mg^{-1}$]	Recovery/%	Purity/fold
Crude extract	3.12	466	0.007	100	
AP	0.081	8.2	9.87	2.86	1646.3

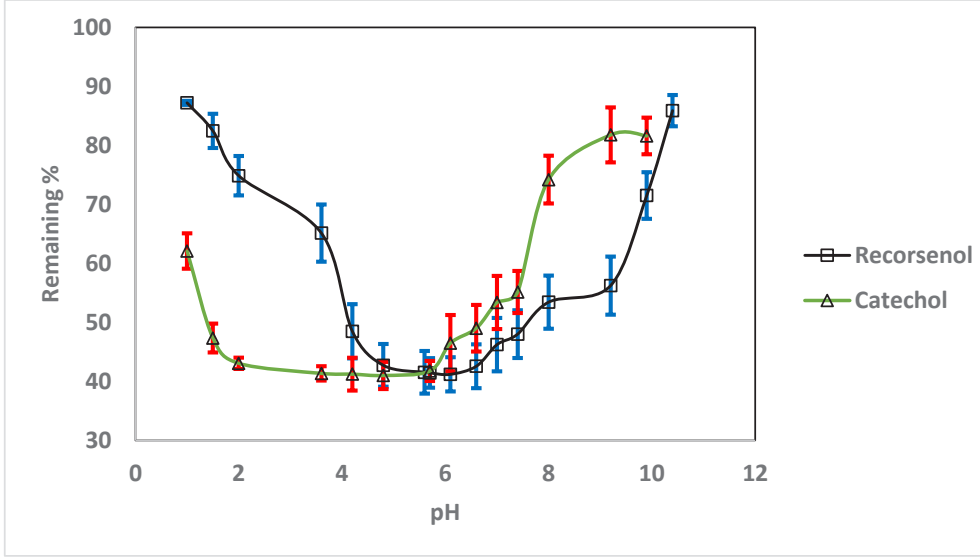

Fig. 1: pH optimization for resorcinol and catechol analysis. Conditions: The enzyme activity was 0.05 $U.mL^{-1}$, the hydrogen peroxide and substrates were equimolar (0.05 mM), and the reaction lasted for three hours.

Kinetic Analysis of Isolated AP

Guaiacol was used as the reducing substrate to determine the kinetic parameters for AP. At varying H2O2 concentrations, AP activity followed a Michaelis-Menten relationship.

The Km and Vmax values for AP were 0.109 mM and 0.0162 $mmoL.min^{-1}$, respectively (Table 2). 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 surface and the heme group located at the enzyme active site.

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 solution. The catechol solution transitioned from pale brown to colorless, accompanied by the formation of larger brownish precipitate particles, whereas the resorcinol solution changed from light yellow to nearly colorless with smaller precipitate particles. Prior to HPLC analysis, all samples were centrifuged and microfiltered to remove any potential interference with the measurements.

Table 2: Kinetic study of AP, Km, Vmax, and reaction order range.

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

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 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.

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 shown in Fig. 1. The results indicated that the optimal pH for the enzymatic treatment of resorcinol was 6.1, which is within the mildly acidic range. Similarly, the optimal pH for catechol removal was 5.7, suggesting that the enzyme exhibits a preference for slightly acidic to neutral conditions during these reactions.

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 three hours, to achieve 95% removal under the previously determined optimal enzyme activity and pH conditions (Fig. 2). Within the same peroxide concentration range, chemical oxidation (without the enzyme) exhibited negligible efficacy, as illustrated by the lines in Fig. 2. The results indicated that for both resorcinol and catechol,

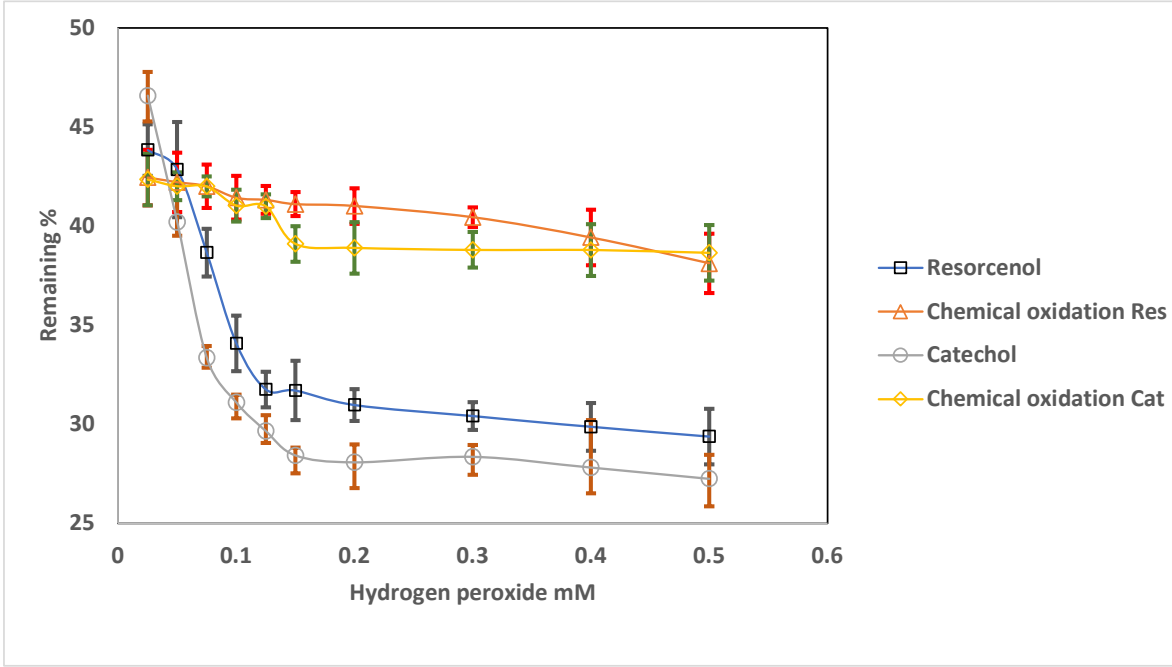

Fig. 2: Optimization of H2O2 concentration. Conditions: 0.05 mM catechol and resorcinol. The enzyme activity was 0.05 U.mL^{-1} at pH 6.1 for resorcinol and pH 5.7 for catechol, with a three-hour reaction time.

a minimum H_2O_2 concentration of 0.15% was necessary to achieve 95% removal. This concentration was selected as the optimal H_2O_2 level for subsequent experiments.

Enzyme Activity

The effect of increasing enzyme concentration at the corresponding optimal pH levels was investigated for three hours (Fig.3). The goal was to achieve a 95% removal of phenolic compounds (resorcinol and catechol). As shown, lower enzyme concentrations were insufficient to achieve 95% removal within the given timeframe. The results indicated that the minimum enzyme concentration required to effectively remove both catechol and resorcinol was 0.072 U.mL^{-1}. This concentration was selected as

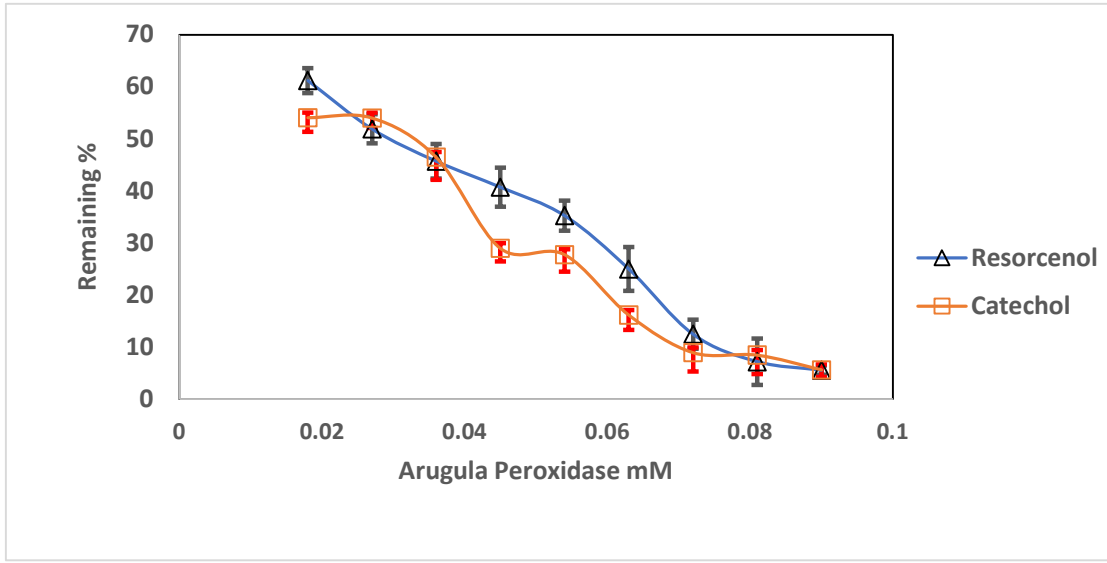

Fig. 3: Enzyme activity. Conditions: resorcinol and catechol 0.05 mM, pH 6.1 for resorcinol, pH 5.7 for catechol, H2O2 concentration 0.15 mM, three hours reaction time.

the lowest effective enzyme activity for the subsequent experiments.

Effect of Reaction Time

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 wastewaters were determined. As shown by the blue and red trends in Figs. 4 and 5, approximately 95% removal of both substrates was achieved within 3 h

Synthetic Wastewater

In synthetic wastewater, a 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 a quantitative analysis. The equation used was: Percentage remaining = (Initial percentage) × e^{-kt} Where k is the apparent first-order rate constant, and the initial 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 did. However, both substrates reached near-complete degradation within a 3-h observation period.

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

Natural Wastewater

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

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

	K value	Half-life, min	Initial percentage %	R^2	Represented in Fig. 4
		30 min			
Resorcenol	$(4\pm0.2)\times10^{-2}$	17.48675	102 ± 2.2	0.98894	Solid blue line
Catechol	$(3.0\pm0.1)\times10^{-2}$	22.94702	102 ± 2	0.99003	Solid red line
		180 min			
Resorcenol	$(3.5\pm0.3)\times10^{-2}$	19.76612	99 ± 4.5	0.96237	Light blue line
Catechol	$(2.5\pm0.2)\times10^{-2}$	27.78669	97± 3.1	0.97815	Light red line

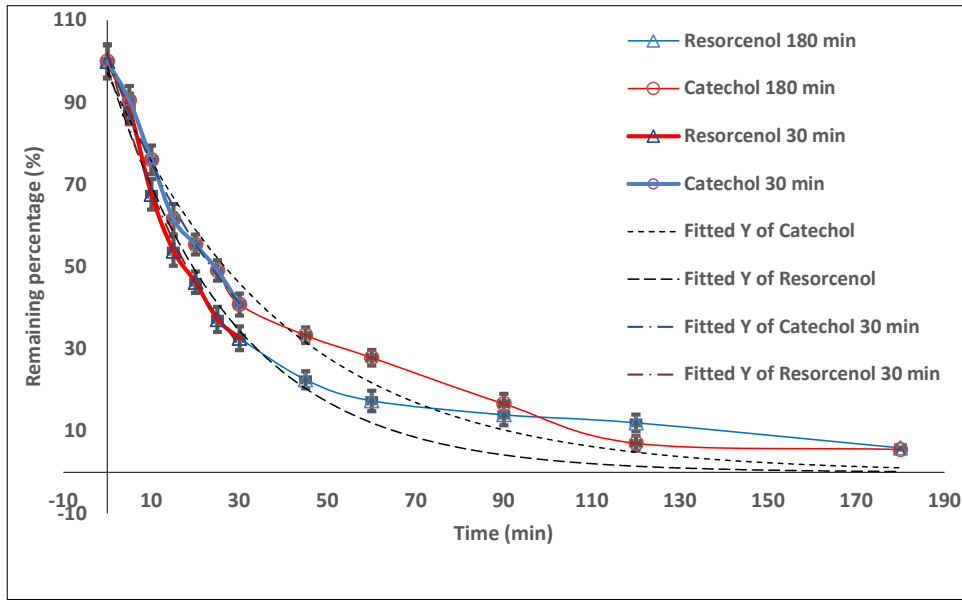

Fig. 4: Removal progress curves for synthetic wastewater. Under optimal conditions, the concentration of resorcinol (blue trend line) and catechol(red trend line) was 0.05 mM, the pH was 6.1 for resorcinol and 5.7 for catechol, the H_2O_2 concentration was 0.15 mM, and the enzyme activity was 0.072 $U.mL^{-1}$ for both resorcinol and catechol.

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	$(3.3\pm0.1)x10^{-2}$	20.4	102 ± 2.1	0.98729	Solid blue line
Catechol	$(2.8\pm0.2)x10^{-2}$	24.5	107 ± 3.8	0.95293	Solid red line
		180 min			
Resorcenol	$(2.5\pm0.3)x10^{-2}$	27	95 ± 6.2	0.89846	Narrow blue line
Catechol	$(2.1\pm0.2)x10^{-2}$	32	100 ± 5.5	0.92234	Narrow red line

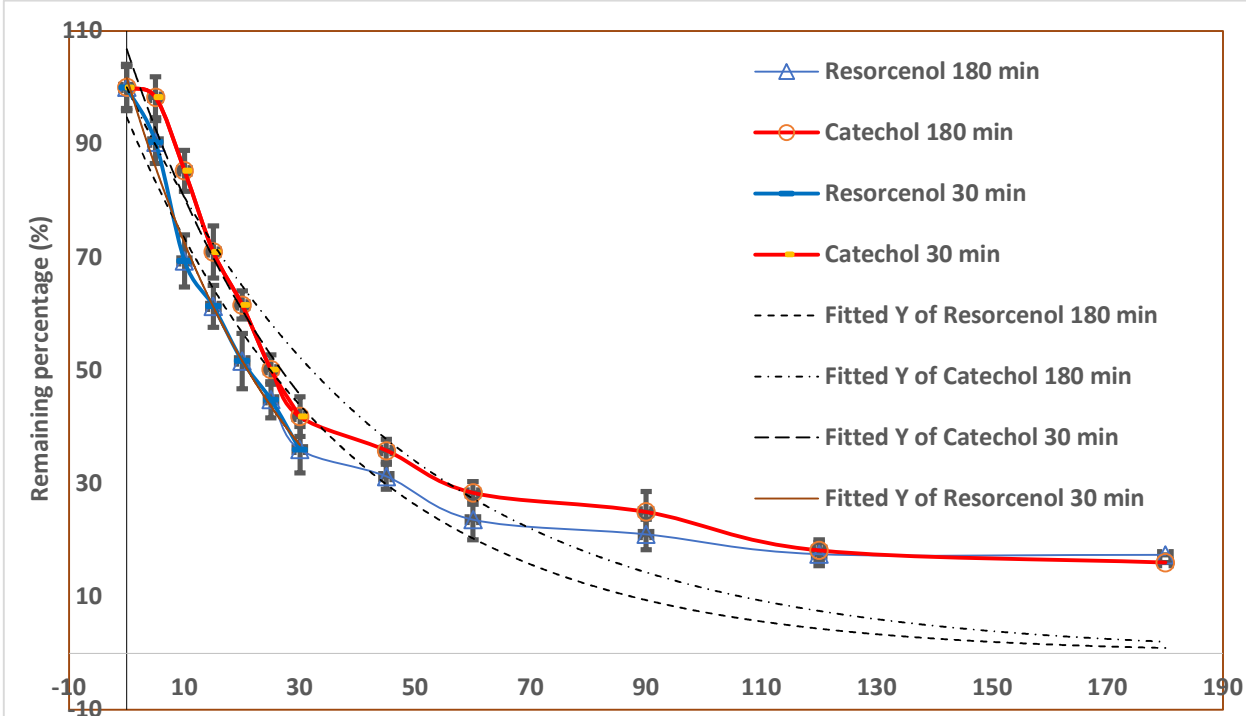

Fig. 5: Removal progress curves for natural wastewater. Under optimal conditions, resorcinol (blue trend line) and catechol(red trend line) concentrations were 0.05 mM, pH 6.1 for resorcinol, pH 5.7 for catechol, H_2O_2 concentration was 0.15 mM, and enzyme activity was 0.072 $U.mL^{-1}$ for both resorcinol and catechol.

Similar to the synthetic wastewater results, the data show that resorcinol exhibited a slightly faster reaction rate, achieving significant removal earlier than that of 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 exhibiting a shorter half-life than catechol. This suggests a higher affinity or catalytic efficiency of the enzyme towards resorcinol under the tested conditions.

DISCUSSION

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

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. These pH optima are consistent with those reported for other peroxidases, such as horseradish and soybean peroxidases (Altahir et al. 2020), emphasizing the role of the ionization state of both the enzyme and substrate.

Table 5: Comparison of the proposed method with oxidation using SBP.

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

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

Method	pH	$[H_2O_2]$ mM/[substrate] mM^{-1}	Rate constant k min^{-1}	Reference
Resorcinol				
Fenton	3.0	4/1	-	(Mijangos et al. 2006)
Ozonation	11.8	---	3.7×10^{-2}	(Parisheva & 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	$(4\pm0.2)x10^{-2}$	
Catechol				
Fenton	3.0	4/1	-	(Mijangos et al. 2006)
Ozonation	11.8	---	2.9×10^{-2}	(Parisheva & 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	$(3.0\pm0.1)x10^{-2}$	

Enzyme Activity

The minimum enzyme activity required to achieve 95% removal of phenolic compounds was 0.072 $U.mL^{-1.}$ This finding is consistent with previous studies that demonstrated a correlation between enzyme activity and substrate conversion efficiency. The observed dependence of the reaction rate on enzyme activity further suggests that AP maintains its catalytic efficiency even at low activity levels, indicating its cost-effectiveness for wastewater treatment applications.

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 can lead to secondary reactions, such as the formation of oligomeric or polymeric byproducts. These possibilities warrant further investigation in future studies to confirm these findings. Table 5 compares the proposed method with oxidation using soybean peroxidase (SBP).

Reaction Time

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

Comparative Analysis

These 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 easily extracted with relative ease. Table 6 shows the comparison of the current method with other chemical and physical treatments of BZOL.

Statistical analysis using t-tests showed significant differences between the synthetic and natural wastewater

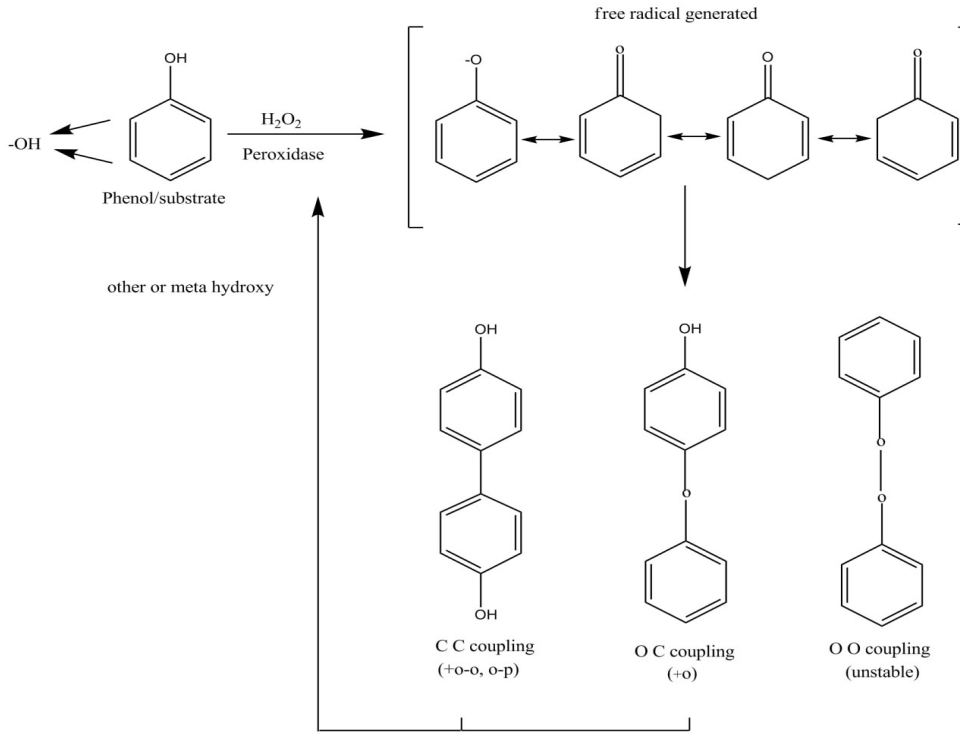

Fig. 6: Proposed mechanism of BZOL oxidation and dimer formation.

removal rates. The p-value for resorcinol was 1.14×10^{-4} (t = -5.83207), and that for catechol was 8.5×10^{-4} (t = -4.53334), confirming a statistically significant difference and rejecting the null hypothesis.

Practical Implications

This study provides valuable data for designing wastewater treatment systems that employ arugula peroxidase. These results support the industrial feasibility of AP for the effective degradation of toxic phenolic compounds. Further work should focus on scaling up 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 various chemicals in laboratories and industries and are known for their high toxicity. This method offers a viable and environmentally friendly alternative to traditional chemical treatments.

Mechanism of Action

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

The steps of this mechanism include the reaction of H_2O_2 with the enzyme. The enzyme has a ferric (Fe^{3+}) heme, which forms compound I. It is a highly oxidizing intermediate with a porphyrin radical cation.

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

The second step involves the oxidation of phenol and the formation of a phenoxyl radical. Compound I oxidizes phenol (ArOH) by removing one electron. Phenoxyl radicals (ArO•) are key intermediates in phenol oxidation (Frey 2001). This step forms an enzyme-substrate complex, enabling electron transfer.

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

In the subsequent 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, which are important in plant defense, lignin biosynthesis, and wound sealing (Shibata & Toraya 2015, de Oliveira et al. 2021). Fig. 6 shows the proposed mechanism for BZOL oxidation and dimer formation.

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

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

CONCLUSIONS

This study demonstrated the potential of arugula peroxidase (AP) for the enzymatic treatment of hydroxybenzene compounds, specifically resorcinol and catechol, in wastewater. The findings revealed that the enzyme effectively removed 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, demonstrating the enzyme's adaptability to slightly acidic environments.

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

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

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

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