

Enzymatic green removal of anisidines from wastewater using partially purified *Phaseolus vulgaris* peroxidase

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ABSTRACT

Crude green bean (*Phaseolus vulgaris* L.) peroxidase (GBP) was extracted from fresh pods and partially purified. Following ammonium sulfate fractionation, dialysis, ion-exchange chromatography (DEAE-cellulose), and finally gel filtration, the specific activity obtained was 40.8 U mg⁻¹ corresponding to a purification fold of 32.9 and a yield of 49.35%. An enzyme activity assay was optimized, and maximal activity was reached in the dark, at pH 7.5 and 25 °C; kinetic studies based on substrate variation showed a K_m value of 0.064 mM, with a V_{max} of 4.91 U mL⁻¹. Anisidine removal by GBP was studied in batch reactor experiments. At H₂O₂ of 0.2 mM, and at enzyme activity of 3.1 U mL⁻¹, > 95% removal was achieved within 3 h for both p-anisidine (at pH = 4.5) and o-anisidine (at pH = 5.5). Pseudo-first-order kinetics demonstrated speedy degradation within the initial 30 minutes, exhibiting rate constants of 3.93 × 10⁻² min⁻¹ for p-anisidine and 3.54 × 10⁻² min⁻¹ for o-anisidine, with corresponding half-lives of 17.6 and 19.6 minutes. Subsequent to 180 minutes, the rates diminished, and the half-lives extended to 26.3 and 36.3 minutes. A 95 Eco-Scale score indicates a highly sustainable procedure utilizing plant-based peroxidase under moderate conditions, a solvent-free process for anisidine removal.

Keywords: anisidine; peroxidase; *Phaseolus vulgaris*; purification; wastewater.

INTRODUCTION

Anisidine compounds are aromatic amines widely used in industry and chemistry. For example, they are used to make azo dyes, triphenylmethane, pigments, drugs, perfumes, printing materials, corrosion inhibitors, and resin antioxidants (Desai, 2014; Stiborowa et al., 2005). Anisidine-related chemicals are primarily used in the printing industry, with smaller amounts employed in textile dyeing and paper production (Bilal et al., 2018). Additionally, anisidine has been detected in cigarette smoke, with reported concentrations ranging from less than 0.2 ng to 5.12 ng per cigarette, depending on the cigarette type (Stabbert et al., 2003). Methoxyaniline compounds enter the environment primarily through the waste generated by chemical and oil processing plants, as well as during the handling and disposal of dyes. This creates a major source of

water contamination (IARC, 1982). Due to their stability, high toxicity, and limited pathways of decomposition, these materials are considered a concern for human health and the environment. Exposure to these substances through inhalation, smoking, or direct contact can lead to blood damage, anemia, methemoglobinemia, and impaired oxygen transport in the blood, resulting in symptoms such as fatigue, headache, dizziness, cyanosis, respiratory distress, collapse, and even death (Stabbert et al., 2003). Several methods have been explored for anisidine removal, but each has a key limitation. Iron-assisted photocatalytic oxidation is highly sensitive to operating conditions, including UV intensity and mixing (Ghaly et al., 2001; Sharma et al., 2011). Biological treatment may lose efficiency due to compound toxicity (Shahrezaei et al., 2012). Activated carbon adsorption removes pollutants without degradation (Ioannidou and Zabaniotou,

2007). Membrane filtration alone shows limited effectiveness for anisidine removal (Wiesner & Chellam, 1999). Although several treatment methods have been investigated, their limitations have sparked interest in enzymatic degradation as a milder, more sustainable alternative. Conversely, enzymatic degradation provides a selective and efficient treatment approach under mild conditions, minimizing energy consumption and secondary emissions, therefore serving as a more sustainable alternative to traditional chemical procedures.

Plant-derived peroxidase has been reported as a low-cost biocatalyst for the removal of reactive dyes and bisphenol, with enzyme performance being strongly influenced by pH, temperature, and substrate conditions (Mohammed and M-Ridha, 2024). This benefit is especially pertinent when extracting and purifying plant-derived enzymes from green bean waste, as effective purification is crucial for enhancing production and minimizing expenses. Enzymes are specialized biocatalysts that operate via substrate binding at the active site, with their efficacy affected by substrate and enzyme concentration, pH, and temperature (Enachi et al., 2018). In comparison with other peroxidase enzymes, Horseradish peroxidase (HRP) demonstrates superior catalytic activity and sensitivity, rendering it optimal for analytical and diagnostic purposes. Soybean peroxidase (SBP) exhibits enhanced heat and pH stability, making it advantageous for industrial and environmental applications. Conversely, *Phaseolus vulgaris* peroxidase is economically viable and readily obtainable, rendering it appropriate for laboratory research and instructional applications, despite its relatively diminished stability and sensitivity (de Oliveira et al., 2021).

The present study aimed to extract peroxide enzymes specifically from green bean husks. This gap underscores the need to conduct a study evaluating the capacity of green bean enzymes to treat 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 optimal conditions for using green beans as an efficient enzymatic bioremediation agent, thereby promoting maximum degradation of contaminants under sustainable, low-cost conditions.

MATERIALS AND METHODS

Experimental protocol for enzyme extraction and purification

Fresh green bean (*Phaseolus vulgaris* L.) pods were washed with distilled water several times, cut into small pieces, homogenized for 15 min using a homogenizer, and the supernatant was separated and used as crude peroxidase extract. The homogenate was centrifuged at 6000 rpm for 15 min, and the supernatant was collected as crude enzyme extract. Ammonium sulfate precipitation at 60% saturation, followed by dialysis against phosphate buffer (pH 7.0) for 24 h at 4 °C, was used for partial purification. Ion-exchange chromatography on DEAE-cellulose was applied to the partially purified enzyme, which was further purified by gel filtration to obtain peroxidase. The purified enzyme extract was subsequently kept at 4 °C until its use.

Enzyme activity assay

Peroxidase activity can be quantified by a colorimetric technique that relies on the oxidation of phenol along with 4-aminoantipyrine (4-AAP) in the presence of hydrogen peroxide, thus resulting in the formation of a pink quinoneimine dye with peak absorbance at 510 nm. The reaction mixture generally comprises phosphate buffer (pH 6–7), phenol, 4-AAP, and the enzyme extract, initiated by the addition of H₂O₂. Absorbance is measured for 30 sec, and enzyme activity is determined from the alteration in absorbance per minute utilizing the molar extinction coefficient ($\epsilon = 6.58 \times 10^3 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$). One unit of peroxidase activity is referred to as the quantity of enzyme that generates 1 μmol of the quinoneimine dye per minute under specified test conditions.

Experimental protocol for anisidine treatment

Batch reactor studies are carried out to assess the anisidine removal from aqueous solution. The reaction mixture (20 ml total volume) comprised anisidine (0.05 mM), buffer solution (40 mM), at enzyme activity 3.1U/ml, and 0.2mM hydrogen peroxide to commence the reaction. The mixture was shaken at 500 rpm for 3 hours at 25 °C utilizing a magnetic stirrer. The reaction was stopped by adding an amount of catalase (final concentration

17 U/ml) to break down remaining hydrogen peroxide. Samples were subjected to centrifugation and subsequently filtered using a 0.45 µm micro syringe filter before analysis.

Data processing and statistical analysis

All experiments were done in triplicate ($n = 3$), and data are expressed as means $\pm$ standard deviation (SD). OriginLab software was used to perform the statistical analyses. Comparisons between two groups were performed with Student's t-test, and for comparisons among multiple groups, one-way ANOVA was used. Statistical significance was defined as $p < 0.05$.

RESULTS AND DISCUSSION

Purification step of peroxide enzyme

Purification of plant enzymes is essential for reliably evaluating the enzyme's catalytic and structural properties, evaluating its potential applications in processing and other fields, and separating it from damaged cell debris. Furthermore, peroxidase enzymes bind to several proteins in plant tissue as well as its polymorphic enzyme, emphasizing the need for purification.

Peroxidase from green bean pods was successfully purified through a multi-step procedure involving ammonium sulfate fractionation, dialysis, ion-exchange chromatography, and gel filtration (Table 1), and the crude extract exhibited a high total protein content accompanied by low specific activity (1.24 U mg^{-1}), reflecting substantial contamination by non-target proteins, which is typical for plant-derived enzyme extracts. Firstly, Ammonium sulfate was performed stepwise at 20%, 40%, and 60%

saturation to determine the most effective cut for peroxidase enrichment, the 60% saturation fraction showed the highest recovered peroxidase activity (Ahmad et al., 2024).

A marked enhancement in enzyme purity was observed following ammonium sulfate precipitation, as evidenced by an approximately four-fold increase in specific activity (4.15 U mg^{-1}), while 95.8% of the initial enzymatic activity was retained; these results indicate that the salting-out process effectively concentrated peroxidase while removing a substantial proportion of soluble impurities. Secondly, dialysis resulted in a moderate improvement in specific activity (5.6 U mg^{-1}), but this was accompanied by the expected reduction in total activity, which is commonly associated with desalting procedures and may be due to enzyme diffusion through the membrane or partial inactivation during prolonged buffer exchange (Boxi et al., 2020). After ammonium sulfate fractionation, the enzyme solution was concentrated using an Amicon® centrifugal ultrafiltration tube, reducing the volume from 55 to 40 ml and slightly increasing the activity from 5.4 to 5.8 U/ml. This resulted in total activity decreasing from 297 to 232 U (yield 74.84%), showing small losses during ultrafiltration, which could be related to non-specific adsorption to the membrane or stress related to centrifugation.

The concentration of total protein decreased from 71.5 to 44 mg, increasing specific activity from 4.15 to 5.27 U mg^{-1} , indicative of partial removal of non-target proteins and sample improvement before the dialysis and chromatographic steps. Also, the important purification steps were ion-exchange chromatography, which caused a dramatic increase in specific activity to (19.78 U mg^{-1}) (Gerni & Özdemir, 2024). Such a terrific enhancement highlights the strong electrostatic interaction “single point” between DEAE matrix

Table 1. Purification step of peroxide enzyme

Step	Volume (mL)	Activity (U/mL)	Total activity (U)	Protein concentration (mg/mL)	Total protein (mg)	Specific activity (U mg^{-1})	Purification fold	Yield (%)
Crude enzyme	100	3.1	310	2.5	250	1.24	1	100
Ammonium sulfate (60%)	55	5.4	297	1.3	71.5	4.15	3.35	95.8
Amicon centrifugal ultrafiltration	40	5.8	232	1.1	44	5.27	4.25	74.8
Dialysis	33	5.6	184.8	1	33	5.6	4.52	59.6
Ion exchange	20	8.9	178	0.45	9	19.78	15.95	57.4
Gel filtration	15	10.2	153	0.25	3.75	40.8	32.9	49.35

and peroxidase, and allows precision separation of the target enzyme with residual proteins that bear opposite character in terms of positive charge or negative charge.

Finally, gel filtration yielded a well-purified peroxidase with a specific activity of (40.8 U mg⁻¹), a 32.9-fold purification, and a final yield of 49.35%. Size-exclusion chromatography efficiently removed low- and high-molecular-weight contaminants (Rahim et al., 2025). In summary, the purification procedure was effective, with ammonium sulfate aiding enzyme concentration, ion-exchange chromatography providing the greatest improvement in purity, and gel filtration yielding a highly purified peroxidase with high specific activity and acceptable yield.

Study of optimum enzyme activity parameters: pH, temperature, and light

The enzyme activity reagent preparation was modified with optimum findings of pH, temperature, and light intensity. The optimum PH was 7.5, the optimum temperature was 25 °C, under dark conditions.

Assay conditions for peroxidase activity were optimized by evaluating pH (4.5–10.5) ((Mohammed and M-Ridha, 2025), temperature (0–80 °C), H₂O₂ concentration (0.05–0.50 mM), and light exposure (Figures 1–4). Enzyme activity increased progressively from acidic conditions to reach a maximum at pH 7.5, followed by a gradual decline at higher pH values, consistent with pH-dependent changes in active-site ionization and enzyme stability (Lemos et al., 2000; Nicolis et al., 2007). Temperature showed the highest activity at 25 °C, with reduced activity at 60 °C and pronounced decrease in activation at 80 °C, indicating thermal elevation at temperatures reduces catalytic efficiency without fully inactivating the enzyme ((Wu et al., 2024), (Neves-Petersen et al.). In addition, storage under ambient light markedly reduced activity (≈1.1 U/mL) compared with dark conditions (≈4.1 U/mL), confirming that light protection is necessary to preserve enzyme performance (Veitch, 2004). Therefore, subsequent assays and kinetic experiments were conducted at pH 7.5, 25 °C, 0.20 mM H₂O₂, with samples protected from light.

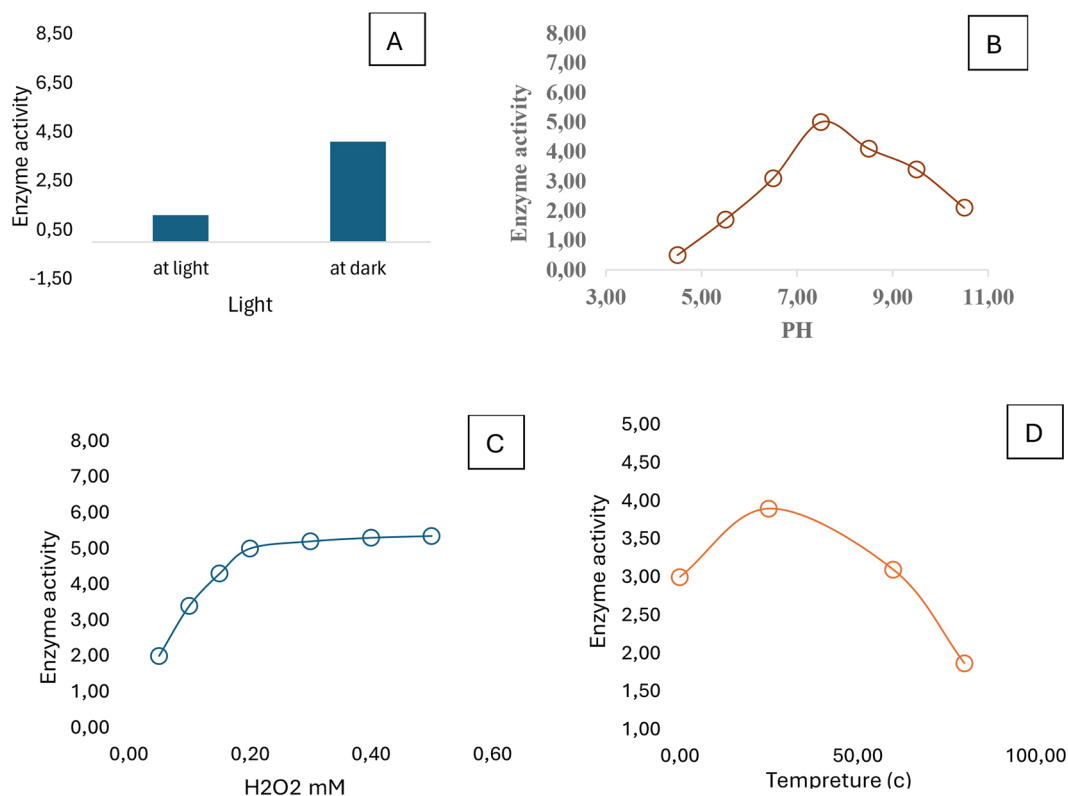


Figure 1. enzyme activity parameters A: Effect of light. Peroxidase activity was measured under light exposure and dark conditions. B: Effect of pH, pH range (4.5–10.5). C: H₂O₂ Concentration, Peroxidase activity was determined at different (0.01, 0.015, 0.1, 0.2, 0.3, 0.4, 0.5) mM. D: Effect of temperature (0, 25, 60 and 80 °C)

Kinetic analysis of the isolated peroxidase of green bean

Peroxidase kinetics were evaluated using the phenol/4-aminoantipyrine (4-AAP) coupling assay by varying H_2O_2 concentration. The enzyme showed Michaelis–Menten behavior, with an apparent K_m of 0.064 mM and a fitted $V_{\max}$ of 4.91 U/mL, indicating high affinity toward H_2O_2 . The reaction followed first-order kinetics at low H_2O_2 concentrations (0.05–0.15 mM), showed a transition region at 0.15–0.30 mM, and approached near-zero-order behavior at ≥ 0.30 mM, reflecting progressive saturation of the enzyme active sites. While activity still increased somewhat at higher substrate concentrations, the rate enhancement became limited – indicative of approaching maximum catalytic capacity (Table 2).

Optimization of anisidine removal from water using GBP

The purified GBP was applied in wastewater treatment. Optimization is essential for achieving efficient enzymatic removal of environmental pollutants. The conditions of the reaction can either enhance or decrease the enzyme's catalytic activity, which affects how quickly contaminants are removed. Determining the optimal conditions is necessary to maximize reaction efficiency and avoid enzyme inhibition caused by unsuitable reactant levels.

pH effect

Enzymatic degradation as a function of pH was evaluated using laboratory-scale controlled assays. As shown in Figure 2, removal efficiency was strongly affected by the acidity of the reaction medium. The degradation of p-anisidine was more effective than o-anisidine at pH 4.5, o-anisidine was susceptible to degradation at pH 5.5, demonstrating a strong sensitivity to an acidic environment. Enzymatic degradation of anisidine isomers was highest under slightly acidic conditions, with optimum pH values of 4.5 for p-anisidine and 5.5 for o-anisidine.

Enzymatic degradation of anisidine isomers was highest under slightly acidic conditions. This behavior is consistent with the strong pH dependence of plant peroxidases, which is governed by both the ionization state of catalytic residues (affecting peroxide activation/electron transfer) and the substrate's protonation state (Mazloun et al., 2016; Wu et al., 2024). The single one-pH-unit difference between isomers suggests structure-specific effects: the ortho-methoxy group appears to impose steric constraints that disfavor productive binding around the heme pocket; thus, a modest elevation of pH enhances binding/electron transfer. Furthermore, the isomers differ in basicity ($\text{pK}_a \approx 5.36$ for p-anisidine vs ≈ 4.53 for o-anisidine), and thus each possesses a unique protonation fraction at any given pH value, which modulates electron donation and susceptibility to one-electron oxidation. Together, the results support selective reactivity of *Phaseolus vulgaris* peroxidase with both ortho and para isomers associated with increased reactant access at more acidic pH and modest elevation in pH, enhancing reaction kinetics for ortho relative to steric/ionization limitation (Krainer & Glieder, 2015).

Optimum H_2O_2 -to-substrate concentration ratio

The effect of H_2O_2 concentration on anisidine degradation was assessed under controlled conditions. As shown in Figure 3, increasing H_2O_2 reduced the remaining percentage of both isomers, with the greatest improvement observed up to about 0.2 mM, after which the response approached a plateau. It was found that p-Anisidine showed a greater decline than o-anisidine. In contrast, peroxide-free controls showed little change, confirming that anisidine removal depends on a peroxide-driven enzymatic pathway, rather than spontaneous loss.

The peroxide dependence shown in Figure 4 reflects the known mechanism of plant heme peroxidases, in which H_2O_2 is required to form the reactive intermediates responsible for anisidine oxidation; without H_2O_2 , conversion remains

Table 2. Kinetic constants (K_m and $V_{\max}$) and reaction-order ranges of isolated green bean peroxidase with H_2O_2

K_m (mM)	$V_{\max}$ (U/mL)	1st order (H_2O_2 , mM)	1st + zero order (H_2O_2 , mM)	Zero order (H_2O_2 , mM)
0.064	4.91	0.05 – 0.15	0.15 – 0.30	≥ 0.30

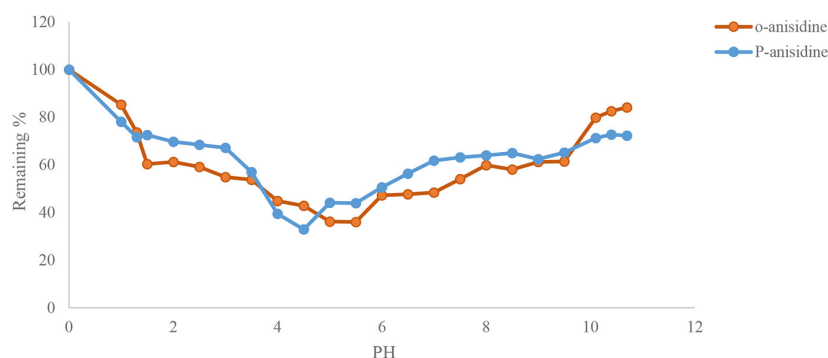


Figure 2. pH optimization for Para-anisidine and o-anisidine. Conditions: The enzyme activity was 3.1 U/mL, the hydrogen peroxide and substrates were equimolar (0.05 mM), and the reaction lasted three hours

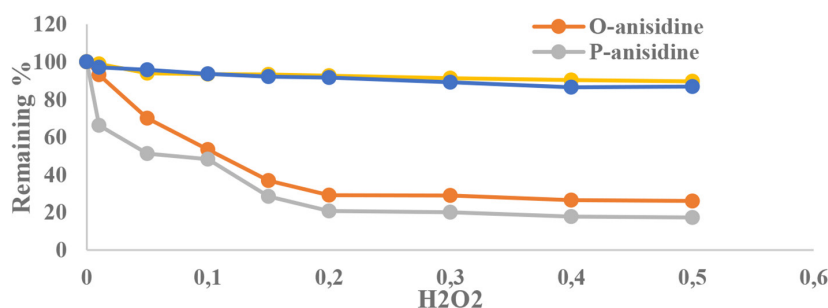


Figure 3. Optimization of H₂O₂ concentration for Ortho-anisidine and Para-anisidine. Conditions: The enzyme activity was 3.1U/mL, the substrates were 0.05 mM, at pH 4.5 for Para-anisidine and pH 5.5 for Ortho-anisidine, and the reaction lasted three hours

minimal until it reaches a rich, limited concentration which any increase in H₂O₂ concentrations do not further increase the reactions; this plateau indicates that when H₂O₂ is no longer limiting, side reactions and partial enzyme inactivation limit the reaction (a feature of peroxide/H₂O systems) (Valderrama et al., 2002).

Enzyme activity

The impact of peroxidase dose on anisidine degradation was assessed by gradually increasing the enzyme loading under otherwise constant operating conditions. This design allowed the role of catalytic capacity to be evaluated across a practical range of enzyme doses.

The dose–response pattern agrees with the known mechanism of heme peroxidases, where higher enzyme levels increase the availability of active sites and enhance substrate oxidation until the enzyme is no longer limiting. Beyond this point, further increases produce only limited improvement because the reaction becomes constrained by other factors, as commonly reported for H₂O₂-dependent peroxidase systems.

Reaction time

Time-course profiles of anisidine isomer degradation were studied in synthetic and natural wastewaters for the best operational conditions. As can be seen in the black and red trends presented in Figures 5 and 6, about 95% removal was achieved in ≤ 3 hours.

Synthetic wastewater

The p-Anisidine and o-anisidine degradation under laboratory conditions exhibited an exponential decrease, which was described satisfactorily by an apparent first-order equation. Both compounds underwent rapid degradation in the first 30 min, with p-anisidine having a slightly higher apparent rate constant and lower half-life (17.6 min) than o-anisidine (19.6 min) at this early stage, suggesting that the initial removal rate favored faster decomposition of p-anisidine. When the fitting period was extended to 180 min, the apparent rate constants decreased. This behavior is consistent with peroxidase-mediated oxidation systems, in which substrate oxidation

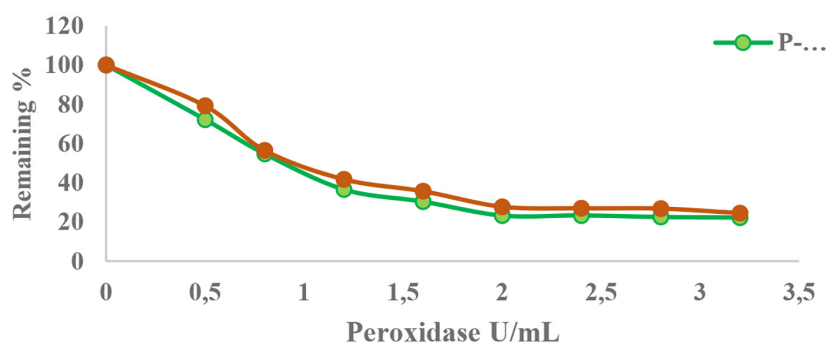


Figure 4. Optimization of crude peroxidase dose for Ortho-anisidine and Para-anisidine
Conditions: The substrates were 0.05 mM, pH 4.5 for Para-anisidine and pH 5.5 for Ortho-anisidine, H_2O_2 concentration was 0.2 mM, and the reaction time was three hours

may slow with time because of reduced catalytic efficiency, peroxide-related enzyme inactivation, and the accumulation of oxidation intermediates or products (de Oliveira et al., 2021), (Shen and Wang, 2024). The slightly faster degradation of p-anisidine may also reflect substrate-structure effects, since the oxidation of aromatic amines by peroxidase is known to depend on ring substituents and their influence on electron-transfer reactivity (Table 3).

Degradation adhered to first-order kinetics ($y = ae^{bx}$). At 30 minutes, the rate constants were 3.93×10^{-2} and $3.54 \times 10^{-2} \text{ min}^{-1}$ for p- and o-anisidine, with half-lives of 17.6 and 19.6 minutes, respectively ($R^2 = 0.971$ and 0.988). Over 180 minutes, rates diminished to 2.64×10^{-2} and $1.91 \times 10^{-2} \text{ min}^{-1}$ ($t_{1/2} = 26.3$ and 36.3 minutes; $R^2 = 0.900$ and 0.863), respectively.

Natural wastewater

Both compounds in natural wastewater were degraded following an apparent first-order kinetic model with rapid removal over 30 min. In the first stage, p-anisidine exhibited a higher rate constant for degradation ($3.28 \times 10^{-2} \text{ min}^{-1}$) and shorter half-life (21.1 min) than o-anisidine ($2.95 \times 10^{-2} \text{ min}^{-1}$ and 23.5 min,

respectively), suggesting that p-anisidine was oxidized at a faster rate than o-anisidine. For a 180 min fitting period, longer half-life and lower rate constant values were obtained for both compounds, with the corresponding derivatives being $2.32 \times 10^{-2} \text{ min}^{-1}$ and 29.9 min for p-anisidine; $1.95 \times 10^{-2} \text{ min}^{-1}$ and 35.6 min for o-anisidine. This trend reflects a progressive decrease in the degradation process over time. In general, both isomers could be degraded well, but with species of p-anisidine, there was the fastest apparent elimination in the above condition. This behavior is typical of peroxidase-mediated oxidation systems, where the reaction may shift from an initially rapid stage to a slower one as substrate concentrations decline and oxidation products accumulate (Altahir et al., 2020). In natural wastewater, this slowing may be further enhanced by matrix effects, such as dissolved organic matter and inorganic constituents, which can compete for oxidizing equivalents, alter effective reactivity, or reduce the extent to which a single exponential term adequately describes the full reaction course (Pignatello et al., 2006). The long-window fits remained moderate ($R^2 \approx 0.92$), suggesting only slight deviation from ideal single-term first-order behavior over the full treatment period (Table 4).

Table 3. Exponential decay fit parameters for the enzymatic degradation of o-anisidine and p-anisidine in synthetic wastewater at 30 and 180 min

Pollutant	Time window	k value (min^{-1})	Half-life (min)	Apparent initial value, a (%)	R^2
p-anisidine	30 min	$(3.93 \pm 0.33) \times 10^{-2}$	17.6	94.8 ± 3.6	0.971
o-anisidine	30 min	$(3.54 \pm 0.19) \times 10^{-2}$	19.6	96.7 ± 2.2	0.988
p-anisidine	180 min	$(2.64 \pm 0.43) \times 10^{-2}$	26.3	85.2 ± 6.3	0.9
o-anisidine	180 min	$(1.91 \pm 0.36) \times 10^{-2}$	36.3	82.7 ± 6.4	0.863

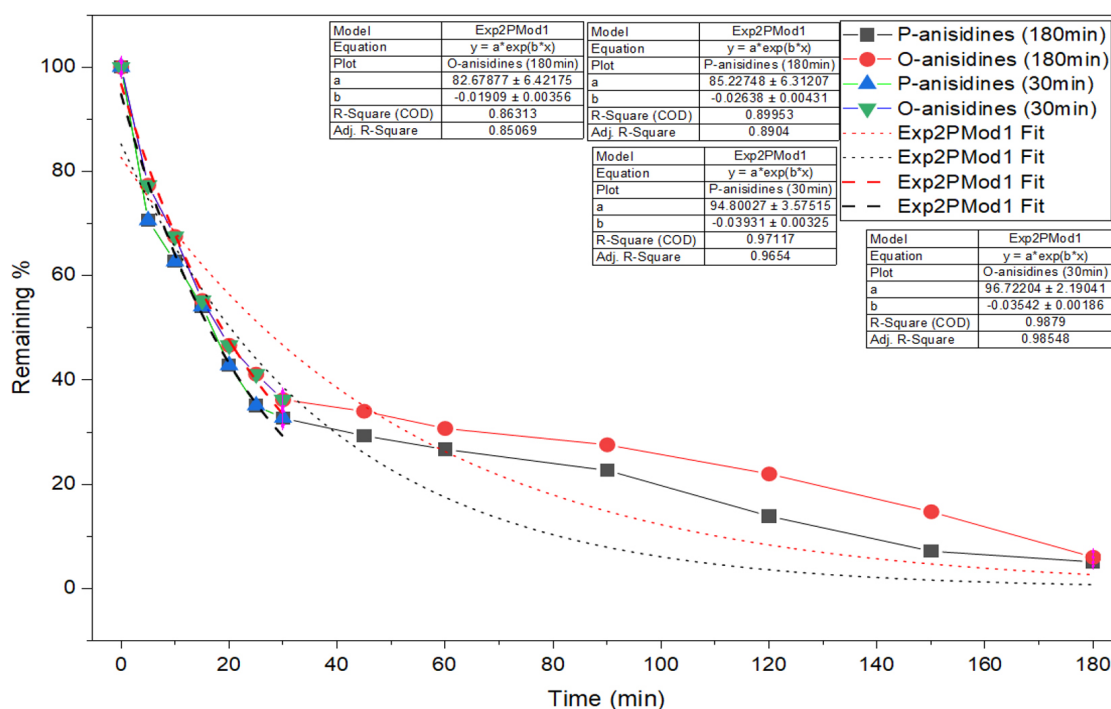


Figure 5. Exponential decay of o-anisidine along with p-anisidine (95 %) in synthetic wastewater for a duration of 0–180 minutes under ideal circumstances (pH 5.5 and 4.5, respectively; 0.2 mM H_2O_2 ; 3.1 U mL^{-1} enzyme)

Degradation followed an apparent first-order kinetic model ($y = ae^{bx}$). During the first 30 min, the apparent rate constants for *p*-anisidine and *o*-anisidine were 3.282×10^{-2} and $2.952 \times 10^{-2} \text{ min}^{-1}$, respectively, corresponding to half-lives of 21.1 and 23.5 min, with R^2 values of 0.981 and 0.962. Over the full 180 min interval, the rate constants decreased to 2.322×10^{-2} and $1.947 \times 10^{-2} \text{ min}^{-1}$, giving half-lives of 29.9 and 35.6 min, respectively, with R^2 values of 0.926 and 0.923. These results indicate a rapid initial oxidation phase followed by a slower degradation stage at longer reaction times.

Environmental greenness assessment using Analytical Eco-Scale

The Analysis Eco-Scale method was employed to investigate the environmental greenness

of this enzymatic process. Calculation of this score involves the allocation of penalty points for not environmentally friendly aspects (e.g., dangerous reagents, solvents, energy requisition, and waste production), and the final mark is obtained as 100 minus the sum of all penalty points (Gałuszka et al., 2012).

As depicted in Figure 7, the overall penalty was 5 points. Thus, the Eco-Scale score was determined as follows: Eco-Scale score = $100 - 5 = 95$. On the Eco-Scale, this rating corresponds to an excellent green/eco-friendly! The high score is primarily due to the fact that a plant-based enzyme recovered from agricultural residues was utilized; lack of heavy-metal catalysts and organic solvents, mild conditions employed, and a low level of waste (Sajid and Płotka-Wasyłka, 2022). Comparison with previous non-enzymatic methods was compared and arranged in Table 5.

Table 4. Fit of progress curves by parameters for the removal of Anisidines from natural wastewater

Pollutant	Time window	k value (min^{-1})	Half-life (min)	Fitted initial percentage, a (%)	R^2
p-anisidine	30 min	$(3.28 \pm 0.22) \times 10^{-2}$	21.1	103.3 ± 2.9	0.9809
o-anisidine	30 min	$(2.95 \pm 0.28) \times 10^{-2}$	23.5	105.0 ± 3.9	0.9622
p-anisidine	180 min	$(2.32 \pm 0.32) \times 10^{-2}$	29.9	94.7 ± 5.7	0.9261
o-anisidine	180 min	$(1.95 \pm 0.27) \times 10^{-2}$	35.6	95.0 ± 5.5	0.9226

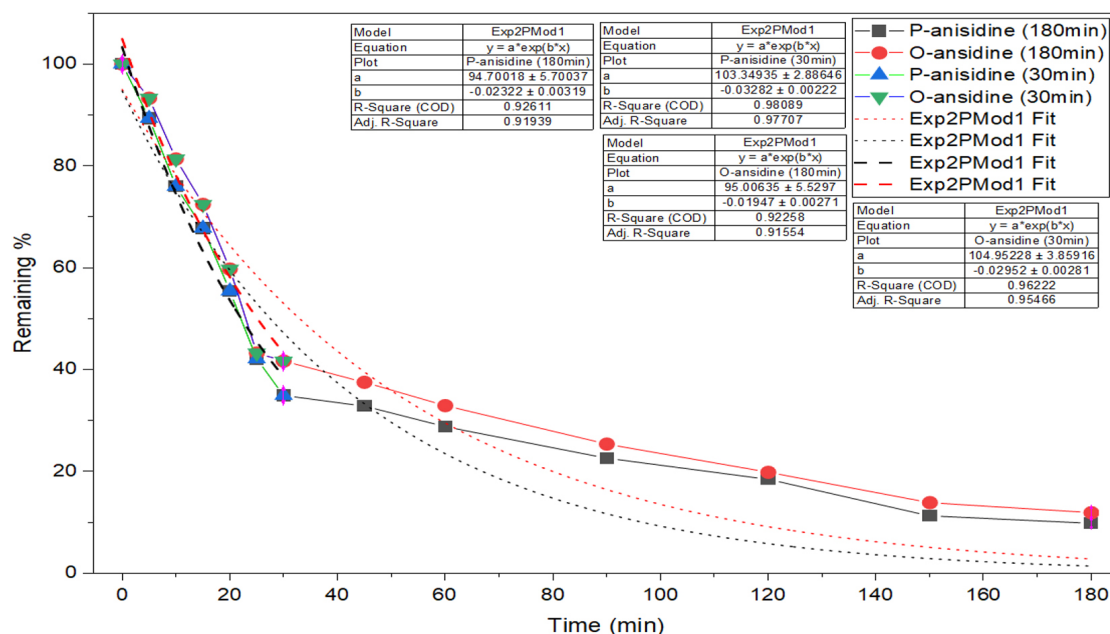


Figure 6. Exponential decay of o-anisidine and p-anisidine remaining (%) in natural wastewater during 0–180 min ($C_0 = 0.05$ mM); conditions: pH 5.5 (o) and pH 4.5 (p), 0.2 mM H_2O_2 , and enzyme activity 2 U mL⁻¹

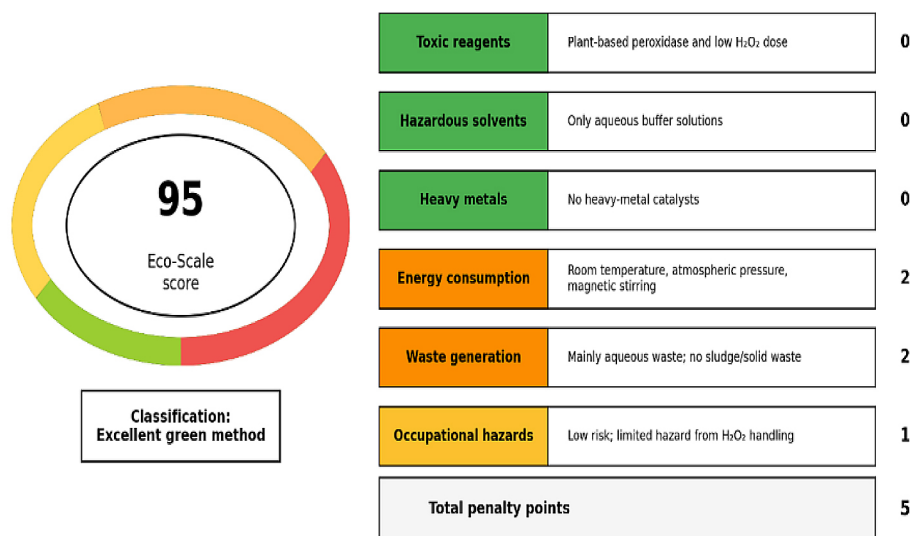


Figure 7. Analytical Eco-Scale representation of the enzymatic treatment for anisidine removal, showing a high score (95) and excellent environmental greenness

Mechanism of action

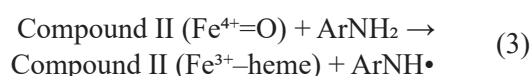
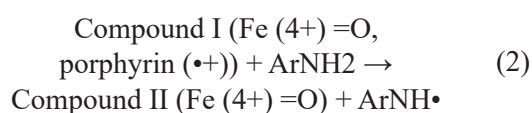
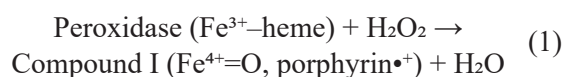
Peroxidase-catalyzed oxidation proceeds through a heme-mediated redox cycle in which the active-site iron alternates between different oxidation states. In its native form, the enzyme contains ferric heme iron (Fe^{3+}). Upon reaction with hydrogen peroxide, the resting enzyme is converted into the highly reactive Compound I,

which contains an oxoferryl iron center ($Fe^{4+}=O$) and a porphyrin radical cation. Compound I then oxidizes anisidine ($ArNH_2$) by one-electron transfer, generating an anisidine radical ($ArNH\bullet$) and reducing the enzyme to Compound II. Following, Compound II oxidizes a second anisidine molecule and returns the enzyme to its resting ferric state. The anisidine radicals formed are highly reactive and subsequently undergo

Table 5. Comparison of treatment methods for aromatic amines reported in the literature and this study.

Method	Pollutant	pH	Oxidant	Additional conditions	Reaction time	Removal efficiency/performance	Reference
Fenton oxidation	p-Anisidine	~2.5	H ₂ O ₂ + Fe ²⁺	Fe ²⁺ ≈ 0.05 mM	24 h	~89% removal	(Chaturvedi and Katoch, 2019)
Electro-Fenton	Aniline	~3.0	In-situ H ₂ O ₂	Pilot flow reactor	5 h	>95% removal	(Brillas and Casado, 2002)
Catalytic ozonation	Aniline	—	O ₃	TiO ₂ -based catalyst + AC	30 min	>95% removal	(Zhang et al., 2021)
Photocatalytic ozonation	Aniline	—	O ₃ / UV	TiO ₂ + MWCNT	3 h	Near complete mineralization	(Orge et al., 2017)
Persulfate activation (ZVI)	Aniline	~7.0	Persulfate	Sulfate radical pathway	5 h	72% removal, TOC 61%	(Gao et al., 2020)
Immobilized laccase–mediator system	Phenol Red dye	—	—	Commercial laccase	120 min	89–100% removal	(Taha et al., 2020)
Membrane distillation + Fenton	Aromatic amines	Variable	H ₂ O ₂ + Fe ²⁺	Pretreatment for ZLD	30 h	~91% removal	(Kyomuhimbo et al., 2023)
Electrocoagulation	Aniline Blue	7	—	Stainless steel mesh electrodes; horizontal arrangement;	15 min	96% removal	(Atiya et al., 2020)
Adsorption (activated carbon)	Aromatic amines	Variable	—	Carbon Black (Vulcan XC-72R)	1 h	≈ 80% removal	(Bezerra et al., 2014)
Green bean peroxidase	o- & p-Anisidine	4.5–5.5	H ₂ O ₂	Mild conditions, no metal catalyst	30–180 min	95% removal	

non-enzymatic reactions such as radical coupling, bond cleavage, further oxidation, and polymerization, which contribute to anisidine degradation. Thus, the catalytic cycle enables effective oxidation of anisidine without consumption of the enzyme (Mazloum et al., 2016). As shown in the equations below:



CONCLUSIONS

In the present work, POD from green bean (*Phaseolus vulgaris* L.) pod waste was used as an efficient biocatalyst for ortho- and para-anisidine removal from industrial wastewater. The enzyme was purified by ammonium sulfate precipitation, ultrafiltration, dialysis, ion-exchange chromatography, and gel filtration, achieving a specific activity of 40.8 U mg^{−1}. Optimal degradation conditions were pH 4.5–5.5, enzyme

activity 3.1 U/mL, H₂O₂ at 0.2 mM, and 3 hours treatment time, removing about 95% of anisidine compounds following pseudo-first-order kinetics, with p-anisidine degrading slightly faster than o-anisidine. Green bean peroxidase proved to be an efficient, selective, and sustainable alternative to traditional methods, saving energy and avoiding secondary pollution. Future research should focus on enzyme immobilization and reactor design for industrial applications.

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