Computational Analysis of Phenolic Compound Biodegradation in Polluted Water

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Keywords: Enzyme Applications, Immobilized Enzymes, Peroxidase, Phenolic Compounds.

Abstract: This study aims to achieve environmental sustainability by using peroxidase extracted from Welsh onion (*Allium fistulosum* L.) and immobilized on bentonite clay for the biodegradation of phenolic compounds in polluted water. Peroxidase was purified using $(NH_4)_2SO_4$, DEAE-Cellulose, and Sephacryl S-200. It was obtained with a specific activity of 1345.3 U mg^{-1}, a 4.8-fold purification, and a 23.37% yield. The efficiency of immobilization on bentonite clay was 83%. The optimum pH for the activity of the immobilized enzyme was 7. It was stable at the pH range 5-8 for 30 min with a residual activity of 86% and 82%, respectively. The optimum temperature for the activity and stability was 50°C, with residual activity of 53% at 70°C. The immobilized enzyme retained 100% and 84% of activity after 21 and 30 d, respectively, during storage at 4°C. The activity of the enzyme was stable up to 19 reuses, while it retained 75% after 30 reuses. The treatment of polluted water with immobilized peroxidase 20 U mg^{-1} led to the removal of 95% of 4-chlorophenol and 100% of phenol at 30 and 25 min, respectively.

1 INTRODUCTION

Economic growth in recent years has led to a rise in pollution to high levels worldwide. Phenolic compounds are a leading cause of water pollution, primarily due to their widespread use in various industries such as dyes, textiles, pharmaceuticals, pesticides, printing, and others [1]. The hazard of these compounds, especially phenol, lies in their low Biodegradability and high stability, which is attributed to their chemical structure [2]. Phenol is a toxic pollutant for the health and safety of life [3]. Biological treatment of wastewater and pollutant water solutions with phenolic compounds is catching increasing interest due to their environmental friendliness [1]. Enzymatic methods have gained popularity due to their high efficiency, low cost, specificity, and environmental safety, which surpass those of other chemical methods [4]. Peroxidase POD EC: 1.11.1.7 belongs to the oxidoreductases group, considered one of the common enzymes used in the environmental treatment of phenolic compounds in wastewater and pollutant aqueous solutions [5]. It was purified from different plant sources, including citron (*Citrus medica*) leaves [6]; white cabbage (*Brassica oleracea var. capitata f. alba*) [7]; chestnut kernel (*Castanea mollissima*) [8]; roots of horseradish (*Armoracia rusticana*) [9]; soybean or soya bean (*Glycine max*) [10]; and prickly lettuce (*Lactuca serriola* L.) leaves [11]. Immobilization technology has been used as a promising method in this field by different support materials, such as Alginate [12], microporous starch [13], nanocomposite [14], chitosan [10], and nanocrystals [15]. This technology is an environmentally friendly approach that enhances the catalytic properties of the enzyme, including stability, storage, and reuse [16]. Thus, this study aims to use peroxidase extracted from Welsh onion (*Allium fistulosum* L.) and immobilized on bentonite clay for the biodegradation of phenolic compounds in polluted water.

2 MATERIALS AND METHODS

2.1 POD Extraction

After washing and drying, 100 g of Welsh onion (*Allium fistulosum* L.) was cut into small pieces of approximately 1 cm^2 in size, then extracted using an

electric mixer for 5 min at 4°C. The extraction solution consisted of 500 mL of 0.1 M sodium phosphate buffer at pH 6, containing 1 mM L-cysteine, and 1 g of Polyclar AT was added to inhibit the phenols released during extraction. Then, centrifuge the mixture at 10,000 rpm for 20 min at 4°C. The supernatant was used as a crude extract for the enzyme purification [6].

2.2 POD Activity

The activity was estimated using Whitaker and Bernhard [17] method, using guaiacol as a substrate for the enzyme, as in

$$POD\ activity \frac{U}{mL} = \frac{\Delta A\ 420\ nm}{t \times v \times 0.001}. \quad (1)$$

Where: ΔA420 nm: Absorbance nm at final reaction time-Absorbance nm at initial reaction time; 0.001: unit of enzymatic activity; v: amount of free and immobilized peroxidase (IPOD) that was used, 0.1 mL of free or 0.1 g of immobilized enzyme; t: reaction time min.

The reaction began at 25°C by adding 0.1 mL of the enzyme to the 3 mL of reaction solution, which contained 0.05 M guaiacol and 0.02 M hydrogen peroxide 30% in phosphate buffer 0.05 M, pH 7.0. The reaction was continued for 3 min, and then ΔA420 nm was used for the calculation of activity. The unit of enzymatic activity is defined as the amount of POD that causes a change in absorbance at 420 nm of 0.001 units per minute at reaction [17].

2.3 Protein Estimation

Protein concentration was estimated using the Bradford method [18].

2.4 POD Purification

The method of Aziz [11] is used to purify POD through precipitation using 40-80% of $(NH_4)_2SO_4$. After dialyzing against deionized distilled water for 24 h at a refrigerator, centrifuging at 10,000 rpm for 30 min, and drying by freeze-drying. Ten mL of 10 mg mL^{-1} of it was applied to a DEAE-Cellulose column 1.5 × 30 cm that was equilibrated with a 0.1 M sodium phosphate buffer, pH 7. The washing step was performed twice with the column volume filled with dialysis buffer to remove all unbound proteins. Then, an elution step was done by a gradient of NaCl 0-1 M. The fractions were collected in a 4.5 mL tube at 90 mL h^{-1}, and their absorbency was measured at 280 nm, with enzyme activity U mL^{-1} estimated for all separated fractions. The fractions that showed the POD activity were collected and dialyzed as previously described steps, then 10 mL, 10 mg mL^{-1} of it was added to a Sephacryl S-200 column 1.5 × 60 cm that was equilibrated and washed with 0.1 M Tris-HCl buffer solution, pH 7, in a 3 mL tube, 18 mL h^{-1}. The fractions were collected for analysis of POD activity U mL^{-1} and protein mg mL^{-1}. Then dialyzed against dialysis buffer for 24 h at 4°C and dried by freeze-drier until use.

2.5 POD Immobilization

Bentonite was activated by stirring with a 10% solution of 3-Aminopropyl triethoxysilane (APTES) in acetone v/v for one h at 25°C, followed by filtration and washing with acetone. Bentonite was dried at 80°C and then mixed with a 10% aqueous glutaraldehyde v/v for one h. Next, the mixture was filtered and washed with 0.05 M potassium phosphate buffer, pH 7.0, and then the activated clay was stored in a refrigerator. The immobilization was done by mixed-activated clay with POD 10 mg mL^{-1} in 0.05 M potassium-phosphate at pH 7, 1:1 v/v, then the mixture was stirred for one h at 4°C and centrifuged at 10,000 rpm for 10 min. The precipitate (IPOD) was washed many times by the same buffer and stored in the refrigerator until use [19].

2.6 Immobilization POD Efficiency

The immobilization efficiency (IE) was estimated using Mohamed *et al.* [12], as in

$$IE\ \% = \frac{Activity\ of\ IPOD}{Intial\ activity\ of\ soluble\ POD} \times 100. \quad (2)$$

2.7 IPOD Characterization

The optimum pH for the activity of 1 g of IPOD was estimated using a 0.05 M buffer solution at pH from 3 to 5.5 with citrate-phosphate, 6 to 7.5 with sodium-phosphate, and 8 to 9 with Tris-HCl. The optimum temperature for the activity of 1 g of IPOD was estimated between 30 and 70°C. The stability was estimated by incubating the IPOD for 30 min [5].

2.8 Storage Period and Reuse

IPOD storage period for 30 d and 30 reuse was estimated at 4°C [9].

2.9 Estimation of Phenolic Compounds

The concentration of phenolic compounds mg mL^{-1} was estimated using the Folin-Ciocalteau method using gallic acid 10-100 mg mL^{-1} as a standard curve and absorbency at 765 nm [19].

2.10 Removal of Phenolic Compounds

The ability of removal (%) was estimated by adding 2.5 g of IPOD 10 mg mL^{-1} to 0.5 L of the 10 mM of 4-chlorophenol and phenol, then incubating for 10 h at 50 rpm at 30°C, and estimation of the remaining concentration of these compounds [5].

3 RESULTS AND DISCUSSION

3.1 Purification

POD was purified using 40-80% $(NH_4)_2SO_4$, DEAE-Cellulose (Fig. 1), and Sephacryl S-200 (Fig. 2), giving rise to a specific activity of 1345.3 U mg^{-1}, a 4.8-fold purification, and a 23.37% yield (Table 1).

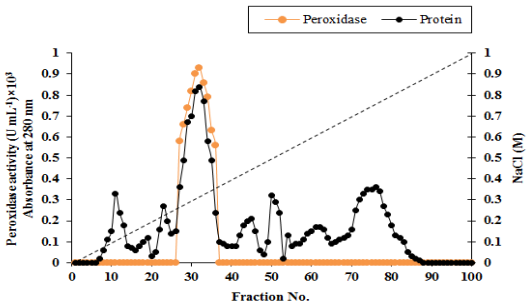

Figure 1: Purification of peroxidase from Welsh onion (*Allium fistulosum* L.) by DEAE-Cellulose.

POD was purified from plants using different methods and techniques. Likewise, gel filtration by Sephadex G-100 with specific activity of 62080 U mg^{-1}, a 15.10-fold purification, and a 28.6% yield from citron leaves leaf [6]; affinity by 4-Amino-Benzohydrazide with specific activity of 964.5 U mg^{-1}, a 24.7-fold purification, and a 4.3% yield from white cabbage [7]; concentration by 30-80% ammonium sulfate and Ion exchange by DEAE-Cellulose 52 with specific activity of 16500 U mg^{-1}, a 9.52-fold purification, and a 4.21% yield from chestnut kernel [8]; Concentration by sucrose and gel filtration by Sephadex G-150 with specific activity of 6103 U mg^{-1}, a 2.09-fold purification, and a 25.55% yield from soybean or soya bean [10]; concentration by 40-80% ammonium sulfate, Ion exchange by DEAE-Cellulose 52, and gel filtration by Sephacryl S-200 with specific activity of 15496 U mg^{-1}, a 5.8-fold purification, and a 23.24% yield from prickly lettuce leaves [11].

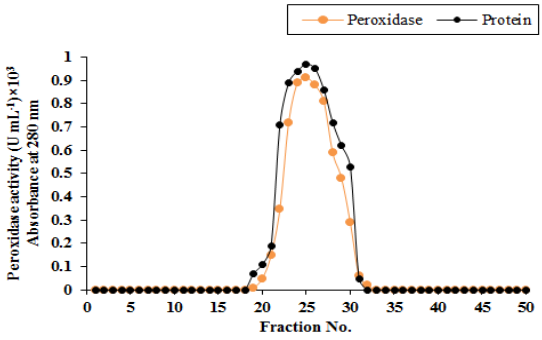

Figure 2: Purification of peroxidase from Welsh onion (*Allium fistulosum* L.) by Sephacryl S-200.

The methods used in enzyme purification vary depending on the properties of the enzymes, such as ion exchange based on charge, gel filtration based on molecular weight, and affinity based on the enzyme's tendency to bind to a specific substance. Therefore, choosing the appropriate purification method based on the available capabilities, the required degree of purification, and the type of application for the purified enzyme is one of the important indicators of the success of the goal of the purification process [6], [7].

Table 1: Purification steps of peroxidase from Welsh onion (*Allium fistulosum* L.).

Step	Volume mL	Protein mg mL^{-1}	Enzyme activity U mL^{-1}	Specific activity U mg^{-1}	Total activity U	Fold purification	Yield %
Crude extract	100	4.52	1536.8	278.41	153680	1	100
Precipitation 40-80% $(NH_4)_2So_4$	10	13.76	10468.68	760.81	104686.8	2.7	68.12
DEAE-Cellulose	45	0.91	873.59	959.99	39311.34	3.45	25.58
Sephacryl S-200	30	0.89	1197.32	1345.3	35919.6	4.8	23.37

3.2 Immobilization POD Efficiency

The efficiency of immobilization POD on bentonite was 83%. Generally, immobilization efficiency depends on the type of immobilization method and the support material used [19]. It was 58.07% on chitosan [10], 86% on microporous starch [13], 75% on Alginate polymer [12]. In the same context, immobilization aims to enhance the activity and stability by using several techniques, such as the use of glutaraldehyde, which leads to increased recovery in activity [20], as in IPOD, using the cross-linked enzyme aggregate technique [21]; nanocomposite [14]; and nanocrystals [15].

3.3 pH and Temperature Value

The optimum pH activity for IPOD was 7. It was stable at the pH range 5-8 for 30 min with residual activity of 86% and 82% of its original activity, respectively (Fig. 3). The optimum temperature for activity was 50°C. It remained stable at 50°C for 30 min, while the residual activity was 53% at 70°C (Fig. 4).

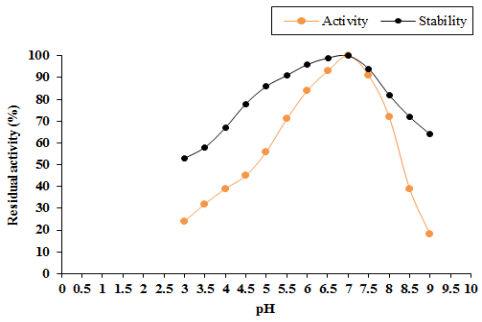

Figure 3: The optimum pH of activity and stability for immobilized peroxidase from Welsh onion (*Allium fistulosum* L.) on bentonite clay.

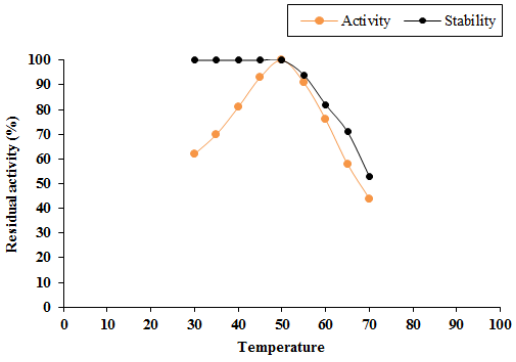

Figure 4: The temperature of activity and stability for immobilized peroxidase from Welsh onion (*Allium fistulosum* L.) on bentonite clay.

Many articles refer to differences in pH and temperature. On this basis, Mohamed *et al.* [12] found that the optimum pH and temperature for IPOD on alginate polymer were 6 and 50°C, respectively. At the same time, El-Naggar *et al.* [13] report on 6 and 40°C, respectively, for IPOD on microporous starch. Additionally, the optimum pH of IPOD on the nanocomposite was 7, and the enzyme retained 90% of its activity after incubation for 90 min at 40°C [14]. In a related context, the optimal pH activity of IPOD on polycarbonate discs was 7, and the enzyme retained 100% and 89% after 60 min of incubation at 50°C and 60°C, respectively [9].

Generally, immobilization improves pH and temperature stability due to changes in charge interactions during the formation of a strong covalent bond between the enzyme and the support material, and it protects the enzyme conformation from damage that may occur in the active site [9], [20].

3.4 Storage and Reuse

The enzyme retained 100% and 84% of its activity after 21 and 30 d of storage at 4°C, respectively. At the same time, the IPOD retained 100% and 75% of its activity after 19 and 30 reuses, respectively (Fig. 5).

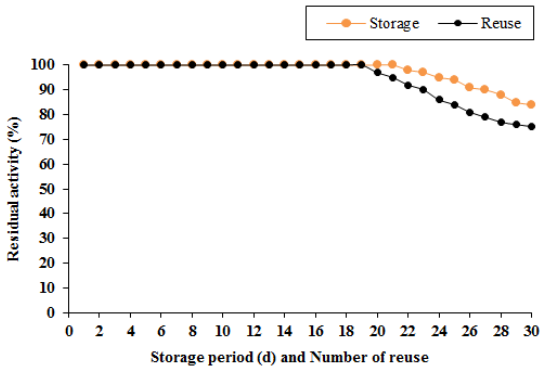

Figure 5: Effect of storage at 4°C and number of reuse on immobilized peroxidase from Welsh onion (*Allium fistulosum* L.) on bentonite clay.

IPOD on alginate polymer retained about 60% after 10 reuses [12]; IPOD on alginate micro-beads preserved 60% after four cycles [22]; IPOD on functionalized reduced graphene oxide retained 70% after 10 cycles and 97% after 35 d [23]. In a related context, IPOD, on microporous starch retained 80 and 60% of original activity after 5 and 10 reuse, respectively [13]; on nanocomposite have 100% of its activity after 35 d of storage at 4°C, while its remaining about 70% of original activity after 10

reuse [14]; on biopolymers from sugarcane bagasse retained 77 after 30 d of storage at 4°C, while its remaining 47% of original activity after eight reuse [24]; on polycarbonate discs could have fully activity up to 14 d of storage at 4°C, while the efficiency of its reduced up to 60% after four reuse [9].

Stability at storage and reuse is considered one of the essential characteristics of the immobilized enzymes [14]. Therefore, this stability is related to many factors, including the type of immobilization method, support material, forming bonds, infiltration, reaction conditions, partial damage of the active site due to product accumulation, inhibition of the enzyme by H_2O_2, and breakage of the support [13].

3.5 Removal of Phenolic Compounds

The treatment by IPOD 20 U mg^{-1} led to the removal of 22, 39, 67, 79, 88, and 95% of 4-chlorophenol and 43, 61, 85, 96, 100, and 100% of phenol at 5, 10, 15, 20, 25, and 30 min, respectively (Fig. 6).

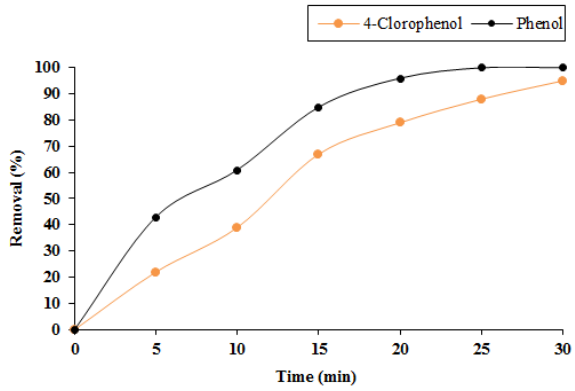

Figure 6: Removal of 4-chlorophenol and phenol (%) from aqueous solutions by immobilized peroxidase from Welsh onion (*Allium fistulosum* L.) on bentonite clay.

IPOD is a vital biocatalyst used for the removal of phenolic compounds from wastewater treatment in a short duration [20]. Therefore, several researchers have reported the use of this application. For instance, the removal efficiency for phenol was 90% using tyramine-alginate [22], 100% using nanocomposite [14], 100% using graphene oxide [23], 79% using biopolymers from sugarcane bagasse [24], 96% using Polycarbonate discs [9]. Also, the removal efficiency for phenol and *p*-chlorophenol was 78 and 58%, respectively, using Alginate polymer [12], phenol and *p*-chlorophenol was 60 and 52%, respectively, using microporous starch [13], phenol and *p*-cresol was 98 and 92%, respectively, using cross-linked enzyme aggregate technique [21], phenol, 2,4-dimethoxyphenol, *p*-chlorophenol, 4-methoxyphenol, and 3-aminophenol were 86.1, 43.6, 25.9, 88.2, and 92.2%, respectively, using ZnO nanocrystals [15].

Phenolic compounds are considered one of the most hazardous pollutants in industrial wastewater [12]. The removal of its by specific enzymes is considered an environmentally friendly treatment classified as part of the green economy [5]. Therefore, these enzymes need to be protected from environmental conditions, such as pH and temperature, by immobilization to improve their stability for large-scale removal of these compounds [21].

4 CONCLUSIONS

This study demonstrated the successful application of immobilized peroxidase extracted from Welsh onion (*Allium fistulosum L.*) for the biodegradation of phenolic compounds in polluted water. The purification process using ammonium sulfate precipitation, DEAE-Cellulose, and Sephacryl S-200 chromatography achieved high enzymatic activity and satisfactory purification yield. In addition, immobilization on bentonite clay provided high immobilization efficiency together with improved operational stability under different pH and temperature conditions.

The immobilized enzyme retained most of its catalytic activity during storage and repeated reuse cycles, indicating its suitability for sustainable environmental applications. Experimental results confirmed the effective removal of phenol and 4-chlorophenol from aqueous solutions within relatively short treatment periods.

From an applied analytical perspective, the study also demonstrates the importance of computational and quantitative evaluation in optimizing biodegradation processes, including activity measurement, stability assessment, and efficiency analysis under varying operational parameters. The obtained results support the potential integration of enzyme-based treatment strategies with intelligent environmental monitoring and data-driven wastewater management systems.

Overall, the proposed approach provides a low-cost, environmentally friendly, and reusable solution for wastewater treatment and highlights the applicability of biochemical process optimization methods in environmental technology research.

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