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Turkish Green Clay–Based Immobilized Polyphenol Oxidase as a Sustainable Biocatalyst for Phenol Removal from Water

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Abstract Enzymatic treatment systems play a significant role in the removal of phenolic pollutants from water, and naturally occurring clay minerals such as Turkish Green Clay (TGC) offer effective and environmentally friendly support materials for enzyme immobilization. In this study, polyphenol oxidase (PPO) was partially purified 10.70-fold with a yield of 15.21% from the zeferan peach (*Prunus persica* L.) cultivar using the cold acetone precipitation method. Enzyme was immobilized onto TGC, and immobilization was confirmed by scanning electron microscopy (SEM). The immobilization yield was calculated as 37.26% based on activity measurements before and after immobilization. Both free and immobilized PPO exhibited an optimum pH of 7.0 and an optimum temperature of 30 °C. Kinetic analysis revealed K_m and V_{max} values of 2.13 mM and 1250.00 $EU.mL^{-1}.min^{-1}$. for the free enzyme and 1.0 mM and 1111.11 $EU.mL^{-1}.min^{-1}$. for the immobilized enzyme, indicating an increased affinity toward 4-methylcatechol upon immobilization. The immobilized enzyme exhibited superior thermal and pH stability compared to the free enzyme. Storage stability was evaluated at 4 °C for 15 days by measuring residual activity at 24-h intervals. After 15 days, the immobilized enzyme retained 69.75% of its initial activity, whereas the free enzyme maintained only 23.41% of its original activity. To assess the potential of the immobilized polyphenol oxidase for water treatment applications, phenol solutions at concentrations of 4, 8, 15, and 20 mg/mL were prepared and subjected to removal studies. The immobilized PPO eliminated phenol by 95.14%, 47.95%, 18.01%, and 11.7%, respectively. These results demonstrate that the immobilized enzyme is highly effective at removing phenol, particularly at low concentrations, and highlight its promise as an environmentally friendly biocatalyst for the treatment of phenol-contaminated water.

Keywords Polyphenol oxidase · Turkish Green Clay · Immobilization · Phenol removal

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1 Introduction

Phenols and phenolic compounds rank among the most prevalent pollutants in industrial waste waters

generated from coal processing, petrochemical operations, pulp and paper manufacturing, and oil refining. Because of their high toxicity and poor biodegradability, phenols can pose serious environmental risks even at trace concentrations (Saputera et al., 2021). Phenol is included in the U.S. Environmental Protection Agency's (EPA) priority pollutant list and is known to exert toxic, carcinogenic, and mutagenic effects on humans, animals, and aquatic organisms (Eryılmaz & Genç, 2021). The proper treatment of these compounds before discharge is of critical importance to protect the environment.

Traditional treatment techniques, including distillation, absorption, extraction, and both chemical and electrochemical oxidation, are generally effective in eliminating a wide range of phenolic pollutants. Advanced processes such as fenton oxidation, ozonation, wet air oxidation, and photochemical methods use fewer chemicals but require high energy. Biological treatments are eco-friendly and use less energy, but they work less effectively when pollutant concentrations are high. Enzymatic processes are considered an effective and eco-friendly alternative for the removal of phenolic compounds, especially at low to moderate pollutant concentrations (Villegas et al., 2016). Oxidoreductase enzymes, including peroxidases (Sellami et al., 2022), polyphenol oxidases (Zhou et al., 2025), and laccases (Younus et al., 2025), have demonstrated significant potential in the oxidative removal of pollutants such as phenols and aromatic amines from contaminated environments. These enzymes show broad substrate specificity and remain effective even at low substrate concentrations (Huseyin & Jan, 2000).

Polyphenol oxidases (PPOs) can oxidize a wide variety of phenolic compounds into quinones, which subsequently form high-molecular-weight and insoluble polymers that can be easily removed from aqueous environments. This property makes PPOs valuable tools for the removal of phenolic pollutants from wastewater and industrial effluents (Mukherjee et al., 2013). However, the limitations of free enzymes, such as low stability, limited reusability, and sensitivity to pH and temperature, have been shown to be overcome through immobilization of the enzymes onto insoluble matrices (Almulaiky & Almaghrabi, 2022).

Polyphenol oxidases enzymes (PPO's, tyrosinase, catechol oxidase) is an oxidoreductase with a binuclear copper center coordinated by histidine residues in the active site. This enzyme is widely distributed among microorganisms, plants, and animals (Martinez & Whitaker, 1995; Murniati et al., 2018). In plants, polyphenol oxidase is commonly localized in cellular organelles and plays important roles in photosynthesis, defense against pests and diseases, growth and development, and flower color formation (Zhou et al., 2025). In the presence of molecular oxygen, two sequential types of reactions are observed to occur: (i) the *o*-hydroxylation of monophenols, known as cresolase or monophenolase activity (EC 1.14.18.1), and (ii) the oxidation of *o*-diphenols to *o*-quinones, known as catecholase or diphenolase activity (EC 1.10.3.1) (Yoruk & Marshall, 2003). Through these catalytic activities, polyphenol oxidases (PPOs) can oxidize a broad spectrum of phenolic compounds, including L-tyrosine, catechol, dopamine, phenol, and their derivatives. Due to their broad substrate specificity, PPOs have been widely investigated as effective biocatalysts for the remediation of phenolic pollutants in wastewater (Mukherjee et al., 2013). PPOs have been applied in phenol removal from industrial wastewater, food quality preservation, and biosensor development (Agunbiade et al., 2025).

Despite its valuable biocatalytic properties, the industrial applications of PPO are currently hindered by its poor long-term stability, high cost and inability to be reused. To address these limitations, immobilization techniques have been widely employed, providing enhanced stability and practical applicability in phenol removal (Wang et al., 2020). The primary objective of immobilization is to improve the economic viability of biocatalytic processes by enabling the reuse of enzymes, facilitating the separation of catalysts from products and enhancing enzyme properties such as stability in organic solvents, tolerance to different pH levels, thermal stability and durability (Guzik et al., 2014). Immobilization enhances the operational stability and reusability of enzymes, which increases their practicality in environmental and industrial applications (Ersöz & Bilgin, 2012; Munjal & Sawhney, 2002).

Immobilized polyphenol oxidases have been reported to be effective in the removal of phenols and their derivatives from wastewater (Bayramoğlu et al., 2013; Dinçer et al., 2012; Li et al., 2021; Zhong et al., 2021), in the enzymatic decolorization of textile dyes (Arabaci & Usluoglu, 2014), in amperometric biosensors for cyanide detection (Shan et al., 2003), and in

biosensors for the detection of pharmaceuticals such as paracetamol, acetaminophen, acetylsalicylic acid, methyldopa, and ascorbic acid (Antunes et al., 2018).

Various inorganic and organic supports have been utilized as carriers for immobilization (Brena et al., 2013). Among them, clays stand out as promising biocomposite materials for biomolecules such as enzymes due to their low toxicity, biocompatibility, and unique physicochemical properties (Olivera et al., 2018). Clay minerals are widely applied in industry owing to their physical and chemical stability, high surface area, structural diversity, and abundance. Their availability from local sources and low cost make them attractive alternatives to other raw materials (Bergaya & Lagaly, 2006). Clay minerals have been used in pharmaceuticals, soaps, paper, detergents, ceramics, and casting industries, as well as for bleaching beer, wine, vegetable oils, and fruit juices, in rubber and plastic production, in hazardous waste removal, animal feed production, and cosmetics (Bergaya & Lagaly, 2006; Olivera et al., 2018).

The green clay is a mineral material composed of illite, iron oxides, and decomposed plant matter. Due to its antibacterial properties, it has been reported to be effective in the treatment of skin infections (Williams et al., 2008). The green clay employed in this research was obtained from mineral deposits in Gürpınar, Van Province, Turkiye, and is herein referred to as "Turkish green clay" (Demirbaş & Nas, 2016). In this study, TGC is examined as a natural, low-cost support material for PPO immobilization, and its potential applicability for phenol removal in water treatment is assessed. To the best of our knowledge, there is no published study investigating the immobilization of polyphenol oxidase onto Turkish green clay or evaluating the phenol removal performance of such a biocatalytic system.

2 Material and Method

2.1 Material

2.1.1 Peach Material

In August 2019, Zeferan peaches (*Prunus persica* L.) were obtained in the Iğdır province of Türkiye and subsequently preserved at –20 °C until analysis.

2.1.2 Clay Material

The clay mineral used in this study was Turkish green clay, supplied by Mehmet Salih Nas (Gürpınar district of Van, Turkey). Its physicochemical properties have been previously reported (Demirbaş & Nas, 2016). To prepare the green clay for immobilization experiments, a 1% clay suspension was prepared to remove impurities. The suspension was mechanically stirred at room temperature for 24 h and allowed to settle briefly, after which the intermediate phase was decanted to separate the clay from sedimented impurities. The purified clay was then filtered using white tape filter paper with a water trom, dried in an oven at 110 °C for 24 h, and sieved through a 300–400 mesh screen to obtain a uniform particle size suitable for immobilization procedures (Nas, 2016).

2.1.3 Chemicals

The 4-methylcatechol (4-MC), polyethylene glycol, buffers, and solvents (ethanol, acetone, and methanol) were of analytical grade and purchased from Sigma-Aldrich, Merck, and Carlo Erba.

2.2 Exraction and Partial Purification Method of Polyphenol Oxidase

Polyphenol oxidase (PPO) was extracted from the peeled edible flesh (mesocarp) of Zefaran peach (Prunus persica L.). The peach mesocarp tissue (150 g) was homogenized using a mortar and the freeze–thaw method. The sample was homogenized in 600 mL of 0.1 M phosphate buffer (pH 7.0) containing 1% polyethylene glycol (PEG). After filtration, the homogenate was centrifuged at 10.000 rpm for 25 min to obtain the crude enzyme extract, and the supernatant was collected. Cold acetone (–20 °C) was then slowly added to the supernatant in a 1:1 ratio with stirring in an ice bath, and the proteins were precipitated by incubating the mixture overnight at 4 °C. The resulting precipitate was collected by centrifugation at 10.000 rpm for 30 min at 4 °C, dissolved in 50 mM phosphate buffer (pH 7.0), and used as the enzyme extract for kinetic studies (Kolcuoğlu, 2012; Özen et al., 2004).

2.3 Immobilization of Polyphenol Oxidase on Turkish Green Clay (TGC)

For enzyme immobilization, 0.4 g of Turkish green clay (TGC) was added to 50 mL of PPO enzyme solution prepared in 50 mM phosphate buffer (pH 7.0). No chemical or physical activation was applied to the clay prior to immobilization, and TGC was used in its native form. The mixture was continuously stirred for 3 h at room temperature (25 °C) and subsequently incubated at 4 °C overnight to allow complete enzyme adsorption. The immobilization process was based on physical adsorption method (Sayahi, et al., 2025). After immobilization, the samples were washed three times with phosphate buffer to remove unbound proteins. The immobilized PPO was then stored at 4 °C and used throughout the study (Türkhan et al., 2018a, 2018b). The immobilization efficiency was determined by comparing the activity of the enzyme in the filtrate before and after immobilization. Immobilization yield was calculated using the following equation (Eq. 1).

$$\text{Immobilization yield } (\%) = \frac{x - y}{x} * 100 \quad (1)$$

In equation x, is the initial activity of PPO enzyme before immobilization, y is the total activity of the PPO remaining in the filtrate after the immobilization process (Hou et al., 2016).

Protein loading efficiency (%) was calculated based on the difference between the initial protein content in the enzyme solution and the residual protein content in the filtrate after immobilization, according to Eq. (2). The initial protein concentration (C_i) and final protein concentration in the filtrate (C_f) were determined spectrophotometrically using the Bradford method (Bradford, 1976), and the corresponding volumes (V_i and V_f) were used for the calculation (Dinçer et al., 2012).

$$\text{Loading efficiency } (\%) = \frac{(Ci.Vi - Cf.Vf)}{Ci.Vi} * 100 \quad (2)$$

Protein loading capacity was defined as the amount of protein bound per gram of clay and calculated as follows (Eq: 3):

$$\text{Protein loading capacit } (\text{mg/gclay}) = \frac{\text{Ci.Vi} - \text{Cf.Vf}}{\text{W support}} \quad (3)$$

where C_i and C_f are the initial and final protein concentrations (mg mL^{-1}), V_i and V_f are the corresponding volumes (mL), and $W_{support}$ is the weight of the clay support (g)(Cheng et al., 2023).

2.4 Characterization of PPO Immobilization on Turkish Green Clay (TGC)

To examine the surface characteristics of TGC prior to and following enzyme immobilization, scanning electron microscopy (SEM; ZEISS EVO LS10).

2.5 Activity Assay

Enzyme activity was assayed spectrophotometrically by monitoring the increase in absorbance at 496 nm using 4-methylcatechol (4-MC) as the substrate in the presence of 3-methyl-2-benzothioazolinone (MBTH) as a chromogenic coupling agent. The reaction mixture (1 mL) consisted of 10 mM 4-methyl catechol, 10 mM 3-methyl-2-benzothioazolinone (MBTH), 2% dimethylformamide (DMF, used as a co-solvent to improve the solubility and stability of the reaction products), and 50 mM phosphate buffer (pH 7.0) (Espin et al., 1995). The reaction was initiated by adding 100 µL of enzyme solution, and the change in absorbance over 1 min was recorded spectrophotometrically (Galeazzi & Sgarbieri, 1981).

One unit of PPO enzyme was determined as the amount of PPO that caused an increase of 0.001 in absorbance in 1 mL of reaction mixture in per min at optimum conditions (Özen et al., 2004). One unit (EU) of immobilized PPO enzyme activity was calculated as enzyme unit per mg clay ($EU.min^{-1}.mg\ clay^{-1}$).

2.6 Protein Determination

Qualitative protein determination was carried out according to the Bradford method (Bradford, 1976). 1 mg/ml Bovine serum albumin (BSA) was used as protein standard.

2.7 Effect of Temperature Free and Immobilized PPO

The optimum temperature of free and immobilized PPO was determined by measuring enzyme activity in the range of 10–70 °C at 10 °C intervals. The reaction mixtures were incubated in a water bath at the specified temperatures for 10 min. Enzyme activity

was measured at each temperature, and a graph of relative activity (%) versus temperature was plotted for both free and immobilized PPO. The point of highest activity was defined as the optimum temperature (Kolcuoğlu, 2012). The thermal stability test was performed by incubating free and immobilized PPO at 4 °C, 30 °C, and 60 °C for 90 min. Residual activities, measured after cooling the samples to room temperature, were expressed as percentages relative to the activity of the untreated enzyme (Colak et al., 2007).

2.8 Effect of pH Free and Immobilized PPO

The effect of pH was carried out by measuring the activity of PPO utilizing sodium acetate (pH 4.0–5.0), phosphate (6.0–7.0) and Tris (pH 8.0–9.0) buffer systems. Relative activity (%) versus pH graphs were plotted for both free and immobilized enzymes. The pH value showing the highest activity was defined as the optimum pH. The pH stability of free and immobilized PPO was tested by mixing the enzyme solutions (1:1) with the buffer systems described above and incubating them at 4 °C for 72 h. Enzyme activity was measured at 24-h intervals, and residual activity (%) versus time graphs were plotted (Türkhan et al., 2020).

2.9 Determination of $K_m$and V_{max}

Activity determinations of free and immobilized PPO were performed under optimum conditions at different 4-MC concentrations. Substrate saturation curve was plotted. The Linewear-Burk curve was plotted using 1/V and 1/S values. K_m and V_{max} values were calculated from the equation of the curve (Lineweaver and Burk, 1934).

2.10 Storage Stability

To assess storage stability, enzyme activities of free and immobilized PPO were recorded at 48 h intervals over 15 days. Initial activity was defined as 100%, and residual activities were expressed as a percentage of this value and graphed over time (Arıca et al., 2004).

2.11 Phenol Removal

For the phenol removal study, a calibration curve was first prepared using phenol solutions at concentrations between 1.25 and 20 mg/L, based on absorbance measurements at 270 nm (Özçelik, 2005).

Phenol removal using immobilized polyphenol oxidase was carried out with a modification of the method described by Özçelik (2005) (Özçelik, 2005). Phenol solutions (1 mL) with final concentrations of 4, 8, 15, and 20 mg/L were prepared in 50 mM phosphate buffer (pH 7.0) and mixed with 8 mg of immobilized enzyme. To check the potential phenol removal capacity of the clay sample itself, clay samples without the same amount of enzyme were added to phenol solutions at corresponding concentrations. The mixtures were incubated in a shaker at 100 rpm for 3 h at room temperature. Afterwards, they were centrifuged at 10000 rpm for 20 min to separate the clay, and the supernatants were filtered. The absorbance of the filtrates was measured at 270 nm to determine the residual phenol concentration, which was calculated from the calibration curve. All experiments with the immobilized enzyme were repeated using the same amount of clay without enzyme, and the percentage of phenol removal was calculated. A buffer solution containing clay at the same concentration as the immobilized enzyme was used as a blank (Bayramoğlu et al., 2013; Özçelik, 2005).

3 Results and Discussion

3.1 Extraction and Partial Purification of PPO

In this study, PPO enzyme was partially purified 10.70-fold with 15.21% yield from Zeferan peach (*Prunus persica* L.) using acetone precipitation method (Table 1). In similar studies, polyphenol oxidase was partially purified 3.58 times with a yield of 9.5% from hawthorn using the cold acetone precipitation method (Türkhan et al., 2018a, 2018b). The specific activity increased from 8697.94 EU/mg in the crude extract to 93,109.24 EU/mg after cold acetone precipitation. This increase reflects protein enrichment resulting from the removal of non-enzymatic proteins during the precipitation step. In similar studies, polyphenol oxidase was partially purified from bitter leaf (*Vernonia amygdalina Delile*) using an aqueous two-phase system, where the specific activity was reported as 1686.4 U/mg protein in the crude extract and 62,600 U/mg protein in the lower phase (Agunbiade et al., 2025).

Table 1 Purification steps of polyphenol oxidase from peach (*Prunus persica* L.)

Purification steps	Volume (mL)	Activity (EU. mL^{-1} min^{-1})	Total Activity	Protein (mg/mL)	Total Protein (mg)	Spesific Activity (EU/mg)	Yield (%)	Purification Fold
Crude extract	600	1821	1092600	0.20936	125.616	8697.94	100	1
Cold Acetone precipitation	150	1108	166200	0.0119	1.785	93109.24	15.21	10.70

3.2 Immobilization of Polyphenol Oxidase Enzyme on Turkish Green Clay (TGC)

The surface morphology of green clay before and after the immobilization of polyphenol oxidase was displayed using scanning electron microscopy (SEM) images. In the SEM images, the clay samples prior to immobilization (A) exhibited particles that were largely dispersed, irregularly distributed, and characterized by a porous surface structure. This morphology indicates that the clay is a suitable support for enzyme immobilization due to its high surface area and accessible binding sites. In the samples after immobilization (B), the surface appeared more compact and homogeneous, with increased particle aggregation and partial coverage of the clay surface. These structural changes support the successful binding of the enzyme to the clay surface and confirm that the immobilization process occurred. (Fig. 1 A, B).

3.3 Immobilization Efficiency

The immobilization efficiency of PPO on Turkish green clay (TGC), calculated based on enzyme activity measurements, was found to be 37.26%, considering the initial activity of PPO and the remaining activity after immobilization. In addition, based on protein loading calculations, the protein loading capacity was determined as 1.041 mg protein per gram of clay, corresponding to an immobilization efficiency of 60.07% based on protein content.

Tyrosinase immobilization on chitosan–clay composite beads showed 67% efficiency, calculated from the enzyme activity before and after immobilization (Dinçer et al., 2012). Polyphenol oxidase immobilized on carboxymethyl cellulose hydrogel beads exhibited an immobilization efficiency of 44%, calculated from enzyme activity before and after immobilization (Arıca, 2000).

3.4 Optimum Temperature and Thermal Stability

To assess the impact of temperature on both free and immobilized PPO, enzyme activity was measured at temperatures ranging from 10 °C to 70 °C, with increments of 10 °C. Within the temperature range of 40–70 °C, the immobilized enzyme exhibited greater stability in relative activity compared to the free enzyme (Fig. 2).

Previous reports have shown that tyrosinase purified from the shells of *Musa cavendish* was immobilized on Ca–alginate after cross-linking with glutaraldehyde, with the optimum temperature shifting from 60 °C for the free enzyme to 70 °C for the immobilized form (Adeyanju & Ademakinwa, 2024). In another study, tyrosinase immobilized on SnO_2 thin films (ATO–TF) exhibited an optimum temperature of 30 °C (Türkhan et al., 2020). Similarly, PPO obtained from *Pleurotus* sp. mushrooms was immobilized on different matrices including sodium alginate (SA), sodium alginate–polyvinyl alcohol (SA–PVA), and SA–PVA–silver nanoparticle composites (SA–PVA–AgNPs), where the immobilized enzyme demonstrated improved activity within the 30–50 °C range. Evaluation of p-cresol degradation with polyphenol oxidase (PPO) immobilized in various matrices. *3 Biotech*, *6*(2), 229.) (Edalli et al., 2016). Furthermore, immobilization of tyrosinase onto pre-activated biosilica via covalent attachment using 3-aminopropyl triethoxysilane (APTES) and glutaraldehyde resulted in an optimum temperature that was approximately 10 °C higher than the free enzyme, along with a broader thermal activity profile (Bayramoğlu et al., 2013).

The thermal stability of free and immobilised PPO was investigated by incubation in a water bath at 4 ℃, 30 ℃ and 60 ℃ for 90 min at 15 min intervals. After bringing the free and immobilized PPO to room temperature, we performed activity

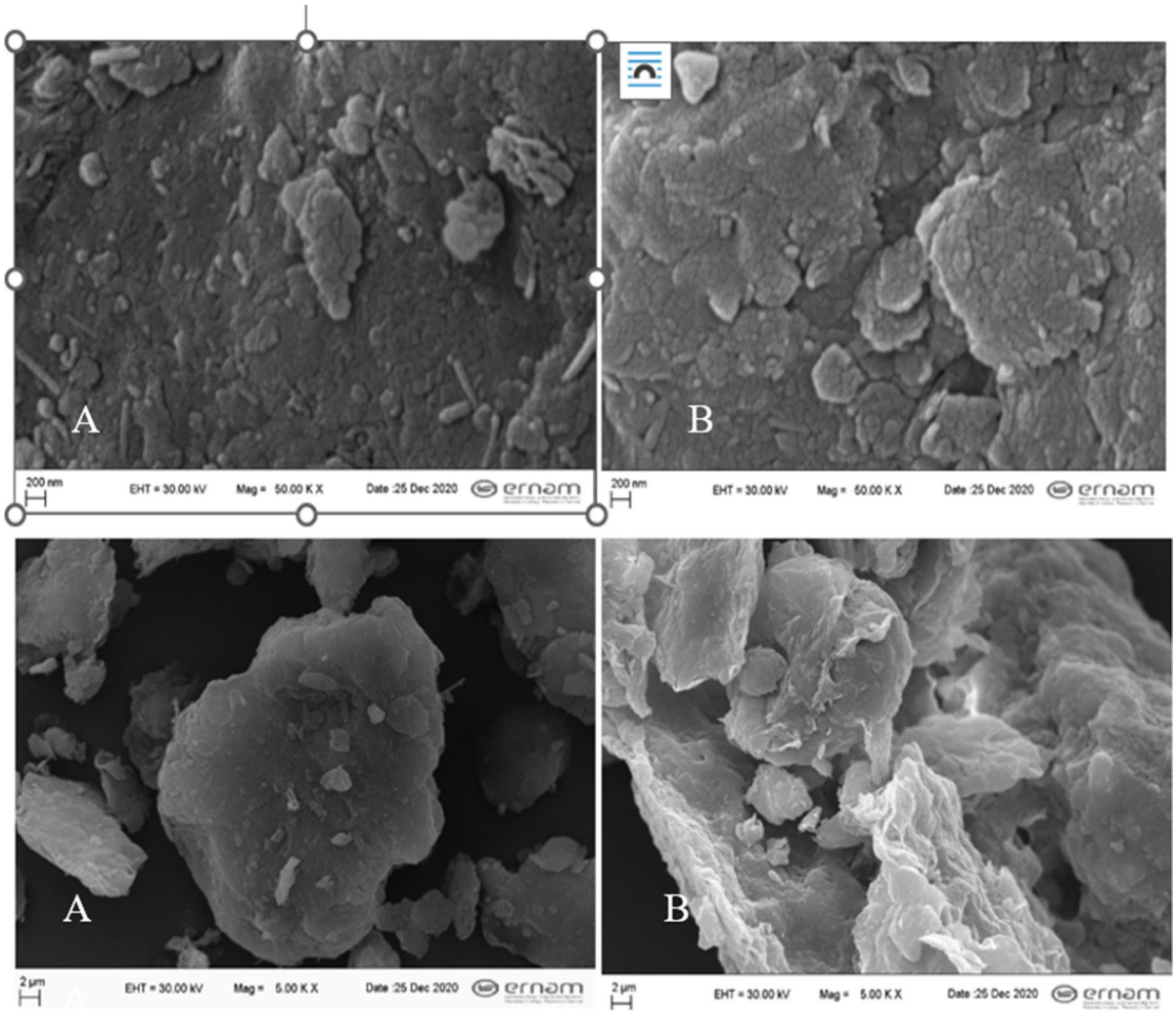


Fig. 1 SEM images of Turkish green clay (TGC) before and after PPO immobilization. **A**: Turkish green clay, **B**: clay surface after immobilization

determinations and plotted % remaining activity versus time for each temperature range (Fig. 3).

The results indicated that immobilization enhanced PPO stability, as the immobilized enzyme outperformed the free form across all temperatures during the 90 min incubation. The higher thermal stability of the immobilized enzyme can be attributed to the stabilization of the enzyme by preventing conformational denaturation at high temperatures. This result is important for the use of the enzyme in industry (Türkhan et al., 2018a, 2018b).

3.5 Optimum pH and pH Stability

pH is a crucial factor that influences both the formation of the enzyme's 3D structure and the ionization of the substrate (Zhong et al., 2021). The optimum pH of free and immobilized PPO was assessed across a range of 4.0–9.0 using Na-AC (pH 4.0–5.0), phosphate (pH 6.0–7.0), and Tris (pH 8.0–9.0) buffer solutions. The data obtained are presented in a % relative activity vs. pH graph (Fig. 4). Both free and immobilized PPO reached maximum activity at pH 7.0.

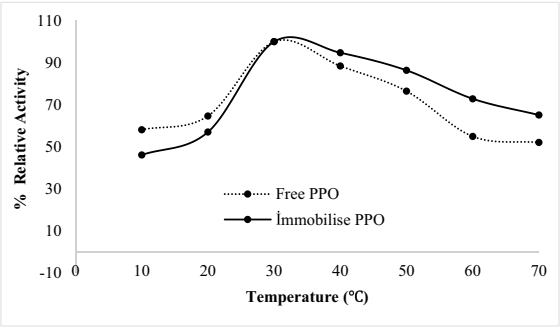


Fig. 2 Effect of temperature on the relative activity of free and immobilized polyphenol oxidase. Enzyme activity was measured using 4-methylcatechol as substrate in 50 mM phosphate buffer (pH 7.0)

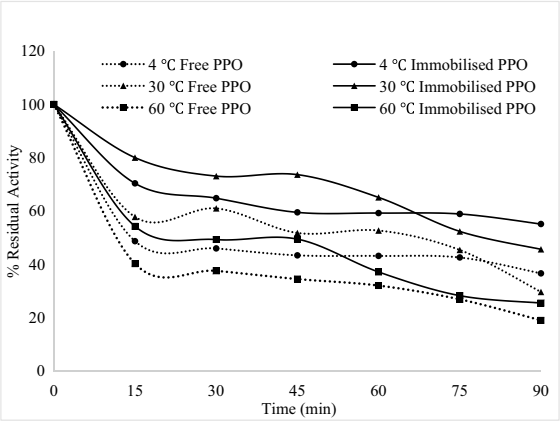


Fig. 3 Thermal stability of free and immobilized polyphenol oxidase at 4, 30, and 60 °C. Residual activities were determined using 4-methylcatechol as substrate in 50 mM phosphate buffer (pH 7.0)

Compared with the free enzyme, the immobilized form demonstrated greater resistance to pH fluctuations, thereby maintaining higher activity.

Polyphenol oxidase extracted from quince leaves (*Cydonia oblonga*) was immobilized on calcium alginate beads, and the optimum pH of both free and immobilized PPO was determined to be 7.5 (Arabaci & Usluoglu, 2014). Polyphenol oxidase was immobilized on carboxymethyl cellulose beads via covalent binding, and the optimum pH values for the free and immobilized forms were reported as 6.5 and 7.0, respectively. The immobilized PPO exhibited a broader pH activity profile than the free enzyme, with a shift towards the neutral region.

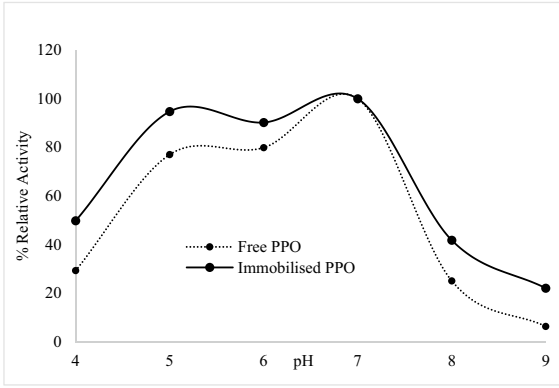


Fig. 4 Effect of pH on the Relative activity of free and immobilized PPO determined using 4-methylcatechol as substrate at 30 °C

This phenomenon was attributed to the formation of ionic and polar interactions between the enzyme and the polymeric matrix (Arıca, 2000). Tyrosinase purified from different yam species was immobilized on Ca-alginate, polyacrylamide gel, and cross-linked enzyme aggregates (CLEA), with optimum pH values of 6.5 for polyacrylamide gel and CLEA, and 7.0 for Ca-alginate (Ilesanmi & Adewale, 2020).

To examine pH stability, activities of free and immobilized PPO were determined at 24-h intervals for 72 h using buffers covering pH 4.0–9.0. The immobilized enzyme exhibited a more stable profile than the free enzyme at all examined pH values. Specifically, at pH 7.0, the remaining activity of immobilized PFO after 72 h was determined to be 81.27%, while for the free enzyme it was 61.74% (Fig. 5).

Tyrosinase was immobilized on chitosan–clay beads cross-linked with glutaraldehyde, and the pH stability of free and immobilized forms was tested following 1 h of incubation (pH 4.5–8.0). At pH 7.0 both forms retained their initial activities, whereas activity losses of 11% (free enzyme) and 15% (immobilized enzyme) were observed at pH 8.0 (Dinçer et al., 2012). The pH stability of PPO immobilized on chitosan/montmorillonite (IPPO) and chitosan–gold nanoparticles/montmorillonite (IPPO-Au) composites was examined at pH 3.0, 7.0, and 11.0 for 8 h. Both free and immobilized enzymes were stable at pH 7.0; however, the free enzyme lost all activity at pH 3.0 and 11.0, whereas

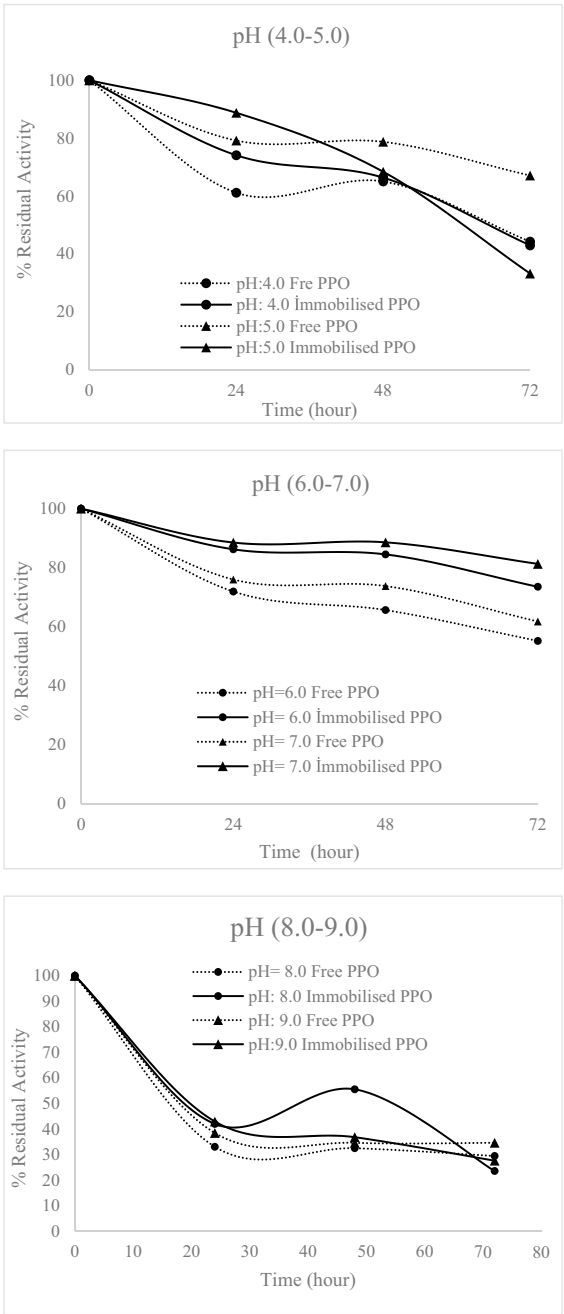


Fig. 5 Comparative pH stability of free and immobilized PPO across different buffer systems using 4-methylcatechol as substrate

IPPO and IPPO-Au retained 42.5% and 75.4% activity, respectively. These results indicate a considerable enhancement in pH stability after immobilization (Wang et al., 2020).

3.6 K_m and V_{max}

The maximum velocity (V_{max}) of free and immobilized PPO was determined to be 1250 EU.mL^{-1}.min^{-1} and 1111.11 EU.mL^{-1}.min^{-1}, respectively, while the Michaelis–Menten constant (K_m) was found to be 2.13 mM and 1.00 mM, respectively (Table 2, Fig. 6).

The K_m value of an immobilized enzyme differs from that of the free enzyme because immobilization introduces diffusion limitations, steric hindrance, and changes in the local ionic environment. These factors can restrict substrate access to the active site and alter

Table 2 Kinetic parameters (K_m and V_{max}) of free and immobilized polyphenol oxidase determined from Lineweaver–Burk plots

Enzyme	K_m (mM)	V_{max} (EU. mL^{-1} min^{-1})
Free PPO	2.13	1250.00
Immobilized PPO	1.00	1111.11

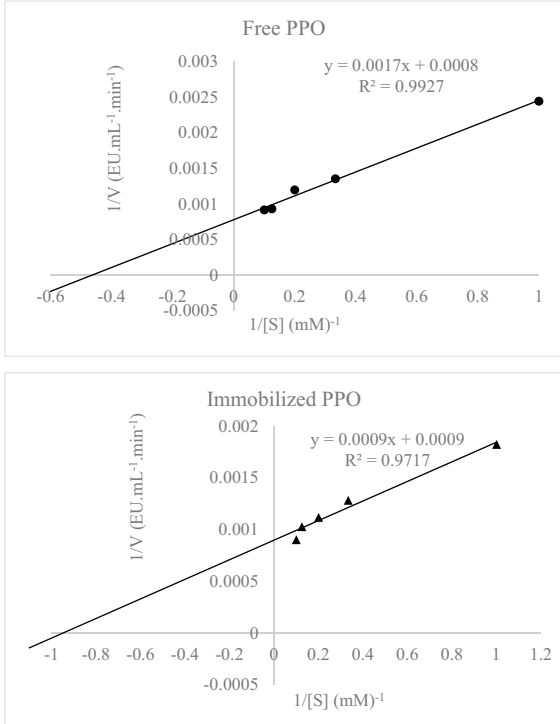


Fig. 6 Lineweaver–Burk plots of free and immobilized polyphenol oxidase determined using 4-methylcatechol as substrate in 50 mM phosphate buffer (pH 7.0) at 30 °C

enzyme–substrate interactions, leading to differences in the apparent substrate affinity. Limitations in substrate partitioning between the solution and the support, together with diffusion constraints, may contribute to a lower apparent V_{max} for the immobilized enzyme compared to the free enzyme. (Dinçer et al., 2012). In this study, the decrease in K_m observed after immobilization indicates an enhanced apparent substrate affinity, likely resulting from favorable microenvironmental conditions provided by the support material and partial conformational stabilization of the enzyme.

In similar study the K_m values were evaluated for polyphenol oxidase purified from *Coleus forskohlii* after immobilization on calcium alginate combined with zinc oxide nanoparticles (Ca-ALG-ZnO NPs) were determined to be 4.99 mM and 3.12 mM, respectively, while the V_{max} values were 255.75 unit.mL^{-1} and 251.89 unit.mL^{-1} when using catechol as the substrate (Almulaiky & Almaghrabi, 2022). After immobilization, the K_m value decreased, similar the findings in our study.

3.7 Storage Stability

The storage stability of immobilized PPO was evaluated at 4 °C in phosphate buffer (pH 7.0) over a total period of 15 days, with measurements recorded at 48-h intervals. After 15 days, PPO immobilized on green clay preserved 69.75% of its original activity, while the free enzyme maintained only 23.41%. Immobilization markedly enhanced storage stability by limiting enzyme inactivation (Fig. 7).

In similar, PPO enzyme immobilized on alginate/ZnO nanoparticle beads was stored at 4 °C in phosphate buffer (pH 6.8, 10 mM) for 8 weeks and was reported to retain 68% of its initial activity (Almulaiky & Almaghrabi, 2022). Polyphenol oxidase (PPO) partially purified from potatoes was immobilized on an alginate–SiO_2 (ALG–SiO_2) organic/inorganic hybrid gel. Storage of free and immobilized PPO was carried out in buffer at 4 °C over 30 days. During this period, the free enzyme retained only 47.1% of its initial activity, whereas the ALG–SiO_2/PPO preparation preserved about 93.3% (Shao et al., 2009).

3.8 Phenol Removal of Immobilized Polyphenol Oxidase

The use of immobilized polyphenol oxidases is known to be a potentially powerful tool for removing

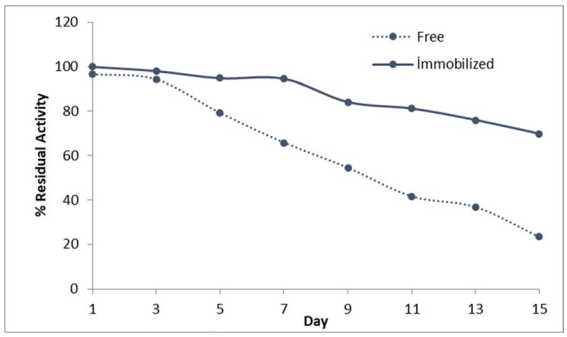


Fig. 7 Storage stability of free and immobilized polyphenol oxidase stored at 4 °C in 50 mM phosphate buffer (pH 7.0)

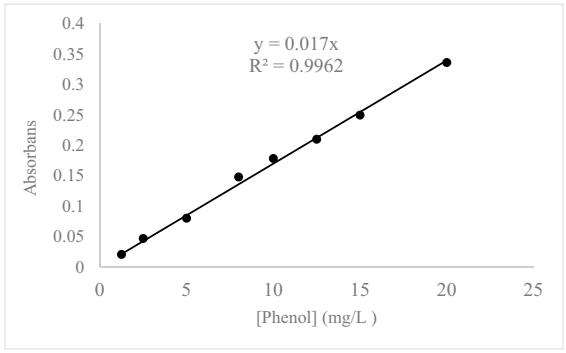


Fig. 8 Standard curve of phenol in phosphate buffer (pH 7.0)

phenolic compounds from industrial wastewater (Shao et al., 2009). For the standard phenol graph, phenol solutions were prepared in the concentration range of 1.25 mg/L to 20 mg/L. The absorbances of these solutions were measured at 270 nm, starting from the lowest concentration, and a calibration curve was plotted (Fig. 8).

The polyphenol oxidase enzyme immobilized on Turkish Green Clay was incubated with synthetic phenol solutions prepared in phosphate buffer (pH 7.0) at concentrations of 4 mg/L, 8 mg/L, 15 mg/L, and 20 mg/L for 3 h. After incubation, the amount of phenol removal by immobilized PPO was calculated using the standard phenol graph, and the results were expressed as % removal (Table 3).

In Table 3, phenol removal was first evaluated using Turkish green clay alone to assess its adsorption contribution and subsequently investigated using PPO immobilized on the clay. While phenol removal remained low when only clay was used, the

Table 3 Phenol removal efficiency of clay (control) and immobilized PPO–clay at different phenol concentrations

Phenol Concentration (mg/L)	Clay (Control) % Phenol Removal	Immobilized PPO (Clay + PPO) % Phenol Removal
4 mg/L	23.15	95.14
8 mg/L	15.25	47.95
15 mg/L	4.25	18.01
20 mg/L	3.3	11.70

immobilized PPO system exhibited high removal efficiency. These results indicate that phenol removal is primarily driven by enzymatic oxidation. As phenol concentration increased, the removal efficiency of immobilized PPO decreased, suggesting a substrate-dependent limitation of the enzymatic system. This decrease can be attributed to enzymatic saturation and the accumulation of reactive oxidation intermediates such as o-quinones, which inhibit polyphenol oxidase activity at high substrate levels (Xu & Yang, 2013).

In the present study, polyphenol oxidase immobilized on TGC exhibited a high phenol removal efficiency, achieving 95.14% removal at an initial phenol concentration of 4 mg/L, while the clay support alone removed only 23.15%, clearly demonstrating the catalytic contribution of the immobilized enzyme. Although the removal efficiency decreased with increasing phenol concentration, the immobilized PPO system consistently outperformed the control, confirming the suitability of green clay as an effective support material for enzymatic phenol degradation.

In similar studies, the PPO enzyme isolated from potato was immobilized on a chitosan-SiO_2 hybrid substrate, and its usability in phenol removal from water was investigated. It was observed that 1 g of immobilized polyphenol oxidase removed 86% of phenol in an analysis conducted with 10 mg/L phenol in artificial phenolic wastewater at 25 °C after 20 h. In the study repeated five times, it was determined that the immobilized enzyme removed phenol with an average efficiency of 60% (Shao et al., 2007). Adeyanju and Ademakinwa (2024) investigated the phenol removal potential of tyrosinase purified from Musa cavendish peels and immobilized within a calcium alginate matrix. Phenol removal experiments were conducted using a 650 µM (0.06 mg/mL) phenol solution prepared in 10 mM phosphate buffer (pH 6.5). The reaction system consisted of 5.0 mL of phenol solution and 100 mg of immobilized tyrosinase, and the experiments were carried out at 60 °C. Under these conditions, the immobilized tyrosinase removed approximately 70% of the initial phenol concentration after 2 h (Adeyanju & Ademakinwa, 2024). Lončar et al. (2011) immobilized potato PPO on synthetic and mineral carriers such as Eupergit C250L, Celite, and CelluloseM, with the highest phenol removal obtained using the Eupergit C250L support, achieving approximately 20% removal at 2.5 mM after 5 h. These findings highlight that while artificial supports can enhance enzyme stability and reusability, these findings highlight that enzyme immobilization is an effective strategy for phenol removal. In the present study, the Turkish green clay (TGC) demonstrated high phenol removal efficiency, particularly at environmentally relevant low concentrations, offering a sustainable and low-cost alternative for wastewater treatment applications.

4 Conclusion

In this study, polyphenol oxidase (PPO), enzymes were partially purified from Zeferan Peach (*Prunus persica L.*), using the cold acetone precipitation method. PPO was immobilized onto TGC, a natural, cost-effective, and durable support material with practical potential. Owing to their high surface area, natural abundance, and physicochemical stability, clay minerals represent a suitable support for enzyme immobilization. The immobilization of PPO was further confirmed and characterized through SEM analyses. Prior to and following immobilization, the enzyme was evaluated in terms of optimum pH, optimum temperature, pH stability, and thermal stability. Immobilization significantly improved the activity, thermal stability, and pH stability of PPO compared with the free enzyme. Evaluation of the kinetic parameters (K_m and V_{max}) of the free and immobilized enzymes revealed that immobilization increased the enzyme's affinity for the substrate (lower K_m), while no significant change was observed in the maximum reaction rate (V_{max}). Immobilization positively influenced the stability of storage of the enzyme, indicating that the immobilized form is more suitable for potential industrial applications. The potential application of immobilized polyphenol oxidase in phenol

removal was investigated. Phenol solutions at concentrations of 4, 8, 15, and 20 mg/L were treated with immobilized PPO–clay, resulting in removal efficiencies of 95.14%, 47.95%, 18.01%, and 11.7%, respectively. These results highlight the potential of immobilized PPO as a promising tool for the elimination of phenol, a common contaminant in drinking water.

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Authors' Contributions Elif Duygu KAYA(A): Conceptualization, Investigation, Writing Original Draft, Visualization. Methodology, Formal analysis. Ayşe TÜRKHAN (B): Conceptualization, Investigation, Writing Original Draft, Visualization. Methodology, Formal analysis. Mehmet Salih NAS (C): Resources (Clay), Formal analysis. A and B wrote the main manuscript text; A and B prepared the figures; A, B and C reviewed the manuscript.

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Data Availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics Approval Not applicable.

Consent for Publication Not applicable.

Competing interests The authors declare no competing interests.

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