

Original Research

ENZYMATIC DEGRADATION OF PHENOL VIA HRP PERIODATELY
IMMOBILIZED ONTO GMA-CO-EGDMA MACROPOROUS CARRIERS

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Abstract

Wastewater contamination remains a critical environmental challenge, prompting extensive research into the application of immobilized enzymes for remediation. Horseradish peroxidase (HRP), a widely utilized enzyme, exhibits enhanced stability and reusability when tethered to a solid support. In this study, macroporous poly(GMA-co-EGDMA) copolymers were synthesized via dispersion polymerization and functionalized with ethylenediamine. HRP was then immobilized through the periodate method and tested for the removal of phenol in a batch reactor. The resulting biocatalyst has maintained relatively high activity during four cycles of repeated use.

Keywords: wastewater treatment, phenol, HRP, periodate, macroporous beads.

INTRODUCTION

As urban and industrial growth continues to strain the environment, water pollution has emerged as a critical global challenge. Daily, industrial effluents, agricultural runoff, and poor waste management discharge heavy metals, toxic compounds, and organic pollutants into water bodies. Among these contaminants, phenols and phenolic compounds are of particular interest due to their toxicity, persistence and resistance to natural degradation [1]. Therefore, the development of efficient methods for their removal from wastewater has become a critical area of environmental research.

A wide range of enzymes immobilized on different carriers is frequently used for these purposes. By attaching the enzyme to a solid support, enhanced stability and improved reusability can be observed [2]. Among numerous plant-derived enzymes, horseradish

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peroxidase (HRP) is particularly notable as one of the most frequently used peroxidases in these applications [3].

Numerous methods have been developed for the immobilization of HRP, which can be broadly classified into four major groups: adsorption, covalent binding, entrapment and cross-linking. By applying covalent binding, strong linkage between the enzyme and carrier is achieved, thus preventing the leakage and increasing stability and stereospecificity [4].

Carriers, including biopolymers, synthetic polymers and inorganic materials are used for the purpose of enzyme immobilization. There is a growing preference for the usage of synthetic polymers due to their well-defined chemical composition and superior purity. By selecting appropriate monomer units, synthetic polymers can be engineered to exhibit specific mechanical properties, stability and porosity required for a given application. Another advantage of these materials is that they contain different functional groups on the surface (carboxyl, hydroxyl, epoxy, amine etc.), which can be further subjected to the surface modification [5].

MATERIALS AND METHODS

Materials

For the copolymer synthesis, glycidyl methacrylate (GMA), ethylene glycol dimethacrylate (EGDMA), 1-dodecanol and cyclohexanol were purchased from Sigma Aldrich (St. Louise, Mo, USA). Horseradish peroxidase (150–250 U/mg), sodium periodate and pyrogallol were also obtained from Sigma Aldrich and used for enzyme immobilization. Ethylenediamine, used for the amination of copolymer samples, was sourced from Merck (Kenilworth, New Jersey, USA), while hydrogen peroxide was procured from AppliChem GmbH (Darmstadt, Germany).

Copolymer Preparation and Amination

To synthesize the copolymer, a continuous phase of 2.78 wt% PVP ($M_w = 24\,000$ g/mol) in ethanol was heated to 70 °C. A mixture containing GMA/EGDMA monomers (5.0 g, 90/10 ratio), AIBN initiator (0.05 g) and inert porogens (2.25 g of 1-dodecanol and 2.25 g of cyclohexanol) was added and stirred for 6 hours. The resulting copolymer was rinsed five times with ethanol and air-dried. Copolymer was resuspended in toluene and further subjected to amination with ethylenediamine. The samples were washed with ethanol and water until reaching pH 6 and then oven-dried at 50 °C. Amino group concentrations were subsequently quantified via acid-base titration.

Copolymer Characterization

The pore size distribution of the poly(GMA-co-EGDMA) copolymers was determined using mercury porosimetry (Carlo Erba 2000, software Milestone 200). Surface morphology was further characterized via scanning electron microscope (Tescan FE-SEM Mira 3 XMU).

Horseradish Peroxidase Immobilization by Glutaraldehyde Method

To immobilize the enzyme, aminated copolymers were deaerated for 10 minutes, washed twice with 0.1 mol/L sodium phosphate buffer pH 7.0, and incubated in glutaraldehyde for 2 hours. Horseradish peroxidase was then added at concentration of 25 mg/g. Following

incubation, the samples were washed twice, resuspended in the same phosphate buffer, and stored at 4 °C.

Horseradish Peroxidase Immobilization by Periodate Method

To immobilize the enzyme via periodate oxidation, HRP was first oxidized with 50 mmol/L sodium periodate in sodium acetate buffer (pH 5.0) and dialyzed overnight. Aminated copolymer particles were deaerated, rinsed with 0.1 mol/L sodium phosphate buffer pH 7.0, and incubated with appropriate concentration of oxidized HRP (25 mg/g) for 48 hours. Following incubation, the samples were washed and stored in phosphate buffer at 4 °C.

Enzyme Activity Studies

Peroxidase activity was measured spectrophotometrically at 420 nm (Shimadzu Corporation UV-2501PC, Japan). For the soluble enzyme assay, 10 μ L of sample and 10 μ L of H_2O_2 were added to 1 mL of pyrogallol (13 mmol/L). The immobilized enzyme assay utilized 9 mg of copolymer-bound HRP, 30 μ L of H_2O_2 and 3 mL of pyrogallol. Filtered aliquots were analyzed at 60-second intervals to determine activity.

Phenol removal

Phenol removal with immobilized HRP was conducted in a batch reactor by introducing appropriate amount of immobilizate into phenol solution (2 mmol/L in 50 mmol/L Tris-HCl buffer pH 7). For in situ delivery of hydrogen peroxide, glucose oxidase (0.187 U/mL) and glucose (3 mmol/L) were used. Aliquots were collected every 60 seconds, mixed with potassium hexacyanoferrate (III) and 4-aminoantipyrene. Following 10 minutes of color development, absorbance was measured at 510 nm. Phenol concentration was then quantified using a standard calibration curve.

RESULTS AND DISCUSSION

The diblock copolymers of glycidyl methacrylate and ethylene glycol dimethacrylate were synthesized by dispersion polymerization, which leads to the formation of spherical particles with pore size diameter of around 90 nm. SEM image of macroporous beads and chemical structure of copolymer are presented in Figure 1.

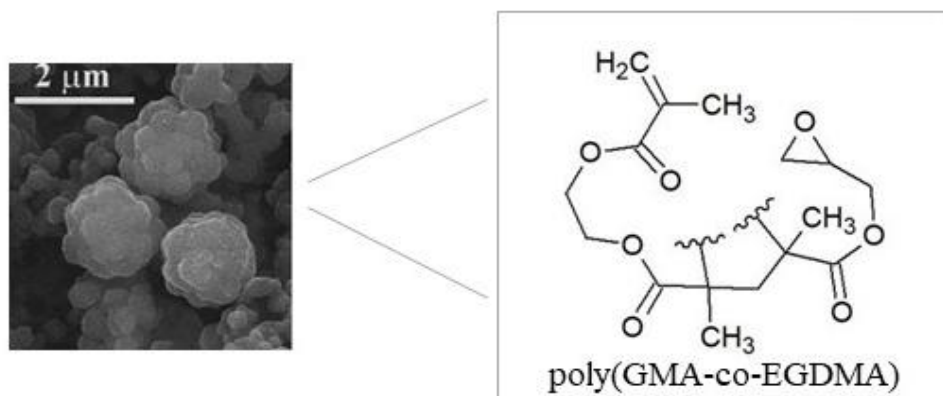


Figure 1 SEM image and chemical structure of macroporous copolymers

This study compares two methods for the immobilization of horseradish peroxidase (HRP) onto macroporous copolymers: the periodate method, which covalently bonds the enzyme's carbohydrate moiety to a copolymer surface, and the glutaraldehyde method, which attaches the enzyme to an activated carrier via its surface amino groups. An appropriate amount of HRP was added per gram of the copolymer (25 mg/g) and specific activity of immobilized enzyme was calculated (Table 1).

Table 1 Specific activities of HRP immobilized by glutaraldehyde and periodate method

	Glutaraldehyde method	Periodate method
Enzyme activity (U)	0.027	0.072
Specific activity (U/g)	2.96	7.36
Specific activity (50 mg of polymer)	148.05	367.87

Results presented in Table 1 show that higher specific activity was achieved by using periodate method (7.36 U/g) compared to the glutaraldehyde method (2.96 U/g). This biocatalyst was used for the removal of phenol in a batch reactor. As previously described in our research [6], higher phenol removal efficiency can be observed when using system composed of glucose oxidase (GOx) and glucose for the delivery of hydrogen peroxide into the reaction mixture for phenol removal. Oxidation reaction in the presence of glucose and GOx is presented in Figure 2. Hydrogen peroxide is essential for peroxidase-catalyzed reactions because it oxidizes the native enzyme. This activation enables the enzyme to accept and oxidize phenolic compounds at its active state.

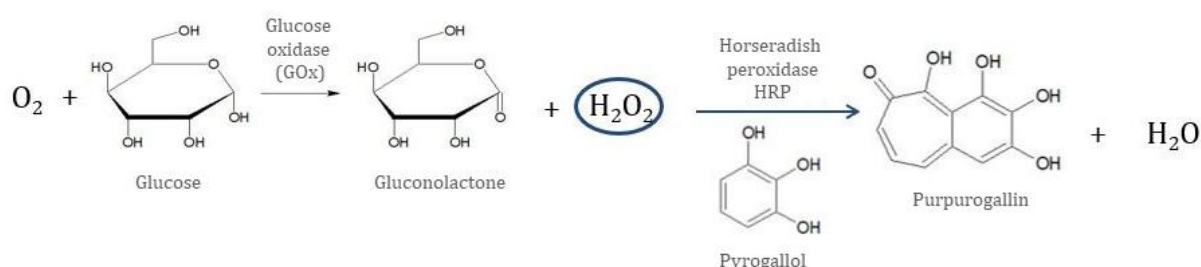


Figure 2 Hydrogen peroxide oxidation in the presence of glucose and glucose oxidase

Within five hours of repeated use of 25 mg/g HRP immobilized on macroporous beads in a batch reactor with the hydrogen peroxide delivery system composed of 0.187 U/mL of glucose oxidase and 3 mmol/L of glucose, efficiency of phenol removed in the solution was 35% (Figure 3).

To evaluate operational stability, immobilized HRP (25 mg/g) was tested over multiple phenol removal cycles. Results presented in Figure 4 indicate that enzyme could be reused for up to four cycles, with efficiency and activity monitored after each use. After four consecutive cycles, the immobilized enzyme retained 45% of its initial activity.

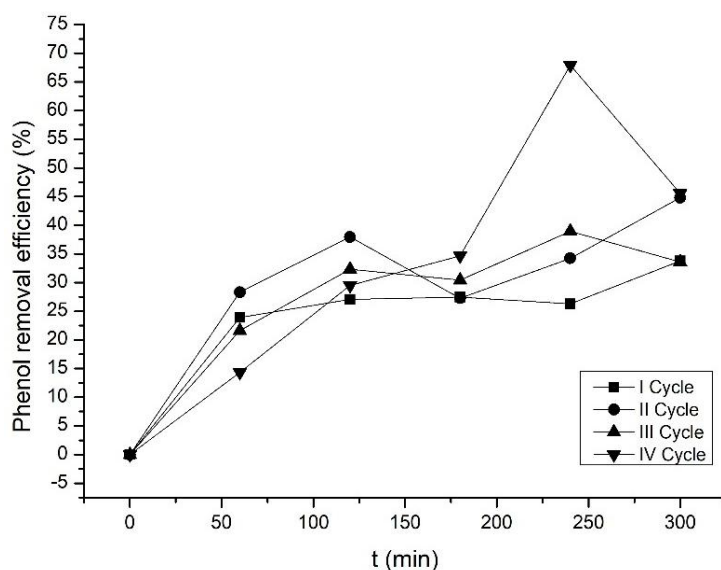


Figure 3 Phenol removal in a batch reactor by periodate-immobilized HRP

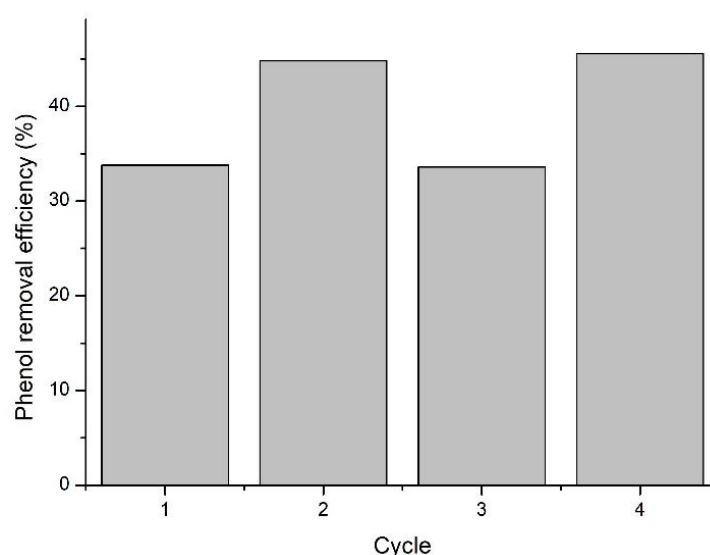


Figure 4 Operational stability of immobilized HRP onto microporous beads

CONCLUSION

Macroporous copolymers of glycidyl methacrylate and ethylene glycol dimethacrylate were synthesized by dispersion polymerization, resulting in 90 nm spherical particles. In comparing immobilization strategies for horseradish peroxidase, the periodate method yielded higher specific activity than the glutaraldehyde method. Periodate-immobilized HRP (25 mg/g) was tested for the removal of phenol in a batch reactor. Using glucose/glucose oxidase system for controlled hydrogen peroxide delivery, the residual phenol concentration reached 75.55 mmol/L after five hours of repeated use. The biocatalyst maintained 45% of its initial activity after four consecutive cycles.

ACKNOWLEDGEMENT

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REFERENCES

- [1] Bayramoglu, G., Arica, M.Y. J. Hazard. Mater. 2008, 156, 148–155.
- [2] Magnin, D., Dumitriu, S., Chornet, E. J. Bioact. Compat. Polym. 2003, 18, 355–373.
- [3] Alemzadeh, I., Nejati, S. J. Hazard. Mater. 2009, 166, 1082–1086.
- [4] Fernández-Lorente, G., Terreni, M., Mateo, C., *et al.* Enzyme Microb. Technol. 2001, 28 (4–5), 389–396.
- [5] Prodanović, R.M., Milosavić, N.B., Jovanović, S.M., *et al.* J. Serb. Chem. Soc. 2006, 71 (4), 339–347.
- [6] Pantić, N., Spasojević, M., Stojanović, Ž., *et al.* J. Polym. Environ. 2022, 30 (7), 1–16.