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Research article

Horseradish peroxidase-assisted approach to decolorize and detoxify dye pollutants in a packed bed bioreactor

Muhammad Bilal [a], Hafiz M.N. Iqbal [b, *], Syed Zakir Hussain Shah [c], Hongbo Hu [a], Wei Wang [a], Xuehong Zhang [a]

[a] State Key Laboratory of Microbial Metabolism, and School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China
[b] School of Engineering and Science, Tecnologico de Monterrey, Campus Monterrey, Ave. Eugenio Garza Sada 2501, Monterrey, N. L. CP 64849, Mexico
[c] Fish Nutrition Laboratory, Department of Zoology, Wildlife & Fisheries, University of Agriculture, Faisalabad, Pakistan

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ABSTRACT

In this study, horseradish peroxidase (HRP) was covalently immobilized on the calcium-alginate support using glutaraldehyde (GA) as a cross-linking reagent for detoxification and degradation of synthetic dyes. Immobilization procedure furnished significant immobilization efficiency (86.27 ± 3.43%) along with apparent and relative activity of 24.39 ± 1.03 U/g and 84.97 ± 3.54%, respectively, for immobilized-HRP. In comparison to free-state, immobilized-HRP catalyzed the substrate oxidation reaction in a slightly acidic and wider temperature range, with an optimum at 60 °C. After 10 and 60 min of incubation at 60 °C, the immobilized-HRP displayed 99.0% and 89.0% of residual activities, whereas the free counterpart retained only 34.0% and 18.0% of residual activities, respectively. Moreover, the immobilized-HRP showed potential efficiency for the decolorization of dyes in sequential dye-decolorizing batch reactions. Cytotoxicity analysis using a plant bioassay and acute test demonstrated that the Ca-alginate immobilized-HRP may effectively be used for detoxification of dyes and has a great potential for large-scale environmental remediation.

1. Introduction

Remediation of dye-based pollutants has been attempted by many researchers around the globe, though using different procedures (Husain, 2006; Asgher et al., 2013a; Iqbal and Asgher, 2013; Jaiswal et al., 2016). However, none of them has largely been implemented due to high treatment costs, extreme operational conditions, toxic by-products generation, and less adaptability to wide-ranging structurally different dyes (Srinivasan and Viraraghavan, 2010; Hayat et al., 2015). Undesirable effects of discharging dye-comprising waste effluents to the living ecosystem, such as toxicity, mutagenesis, carcinogenesis and teratogenesis (Gao et al., 2015; Dai et al., 2016), all have realized the scientific community to find out best available bioremediation technology (Matto and Husain, 2009). Recently, a significant number of biotechnological approaches have attracted a particular interest in combating environmental pollution in an eco-efficient manner (Bilal and Asgher, 2015). In this context, a bio-based treatment offers a cost-effective and eco-friendlier alternative to remediate a wider spectrum of original dyes, dye-based waste effluent, and other toxins, *etc.* (Asgher et al., 2013b; Bilal et al., 2016a).

Peroxidases (E.C. 1.11.1.7) are widely distributed heme-containing proteins that reduce hydrogen peroxide (H_2O_2) while catalyzing the oxidation of some other compounds (Lavery et al., 2010; Bilal et al., 2016a). HRP is the most studied enzyme because of its availability, relatively easy extraction and increasing the number of potential applications (Monier et al., 2010), particularly from laboratory scale to an industrial level. Most recently reported and in practice applications of HRPs includes, phenolic compounds containing wastewater treatment, organic syntheses, environmental remediation, elimination of toxic compounds from drinking water, dyes and industrial effluents detoxification, analytical purposes and cancer gene therapy (Tonegawa et al., 2003; Lai and Lin, 2005; Kim et al., 2006; Vojinović et al., 2007; Monier et al., 2010).

Enzymes immobilization on different carriers has expanded their potential applications. However, alginate-based supporting matrix has fascinated researcher's attention for the immobilization of the whole cell and/or proteins due to its easy obtainability at low-cost,

* Corresponding author.
E-mail addresses: hafiz.iqbal@my.westminster.ac.uk, hafiz.iqbal@itesm.mx (H.M.N. Iqbal).

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better mechanical strength, physiological inertness, resistance to microbial attack, and biodegradability (Bilal and Asgher, 2015; Bilal et al., 2016b). Plant-based toxicity tests have been documented as efficient bioassays to assess environmental toxicity since these assays are less expensive, pollutants sensitive and exhibit broader prospects (Leme and Marin-Morales, 2009; Rizzo, 2011). Similarly, shrimp *Artemia salina* and *Daphnia magna* have also been reported to serve as suitable bioassays for detection of toxicity of environmental chemicals and pollutants (Zhu et al., 2010; Bilal et al., 2016b). Despite the proven industrial potential, there is a paucity of reports concerning the HRP-catalyzed detoxification and degradation of industrial dyes and effluents. Therefore, an effort has been made, in the present work to immobilize HRP in Ca-alginate beads and used for the treatment of different synthetic dyes. The immobilized-HRP was characterized and compared with the free counterpart regarding pH, temperature, and thermal inactivation behavior. Finally, several successive decolorization batches were run in packed bed bioreactor to evaluate the suitability of immobilized-HRP for industrial exploitability.

2. Materials and methods

2.1. Chemicals/reagents

Sodium-alginate (viscosity 20,000–40,000 cps), calcium chloride dihydrate, disodium hydrogen phosphate dodecahydrate, acetic acid, pyrogallol, and potassium dichromate were obtained from Sigma-Aldrich, USA. Locally procured horseradish roots were used for HRP enzyme isolation. Other chemicals, including The "BCA Protein Assay Kit" was obtained from Beyotime Institute of Biotechnology, China and used for protein determination. The synthetic dyes including Reactive Red 120 (RR 120), Reactive Blue 4 (RB 4) and Reactive Orange 16 (RO 16) were obtained by Sigma-Aldrich, USA. All other chemicals used, in this study, were of analytical laboratory grade and used as received as per supplier's instruction.

2.2. HRP isolation and immobilization

HRP was isolated from fresh horseradish roots. Briefly, the roots were crushed in a wet grinder, filtered and the filtrate was subjected to centrifugation at 5000 × *g* for 10 min (at 4 °C). The clear supernatant, as a crude extract, was precipitated by ammonium sulfate up to 75% saturation at 4 °C and dialyzed extensively against phosphate buffer (pH 7.0) to get it desalted. Following that, the desalted HRP was covalently immobilized. Briefly, the HRP enzyme was added to the Na-alginate solution [4.0% (*w*/*v*)] and mixed carefully. One hundred μL GA solution (0.02%, *v*/*v*) was added with a gentle mixing and extruded dropwise into 200 mM solution of $CaCl_2$ using a syringe needle under continuous stirring (at 4 °C). The HRP containing Ca-alginate beads were filtered and immersed in GA solution for further hardening at 4 °C. Finally, the alginate beads were washed extensively until no protein detected in the washing solutions at 280 nm. The same procedure was adopted, in parallel, to prepare control Ca-alginate beads, without HRP addition.

2.3. Bio-catalytic performance evaluation of immobilized-HRP

The biocatalytic performance was evaluated regarding the percentage of immobilization efficiency and relative activity. Equation (1) was used to calculate the % immobilization efficiency (IE).

$$IE(\%) = \frac{\text{Enzyme immobilized}}{\text{Enzyme loaded}} \times 100 \quad (1)$$

Relative activity (RA) is another parameter determining the efficiency of immobilization technique. The RA is defined as "the ratio of specific activities of the immobilized and free enzyme" (Buthe et al., 2005). Equation (2) was used to calculate the % relative activity (RA).

$$RA(\%) = \frac{\text{Specific activity of immobilized HRP } \left(\frac{U}{mg}\text{protein}\right)}{\text{Specific activity of free HRP } \left(\frac{U}{mg}\text{protein}\right)} \times 100 \quad (2)$$

2.4. Characterization of free and immobilized HRPs

The influence of pH on the catalytic activities of free and immobilized-HRPs was investigated by carrying out using various pH (4.0–9.0) buffers. Effect of varying temperatures was examined by keeping the enzymes in the temperature ranging from 20 °C to 80 °C. The thermal inactivation study was carried out following exposure of enzymes to varying temperatures (60 °C, 65 °C, 70 °C, 75 °C, and 80 °C) for 1 h.

2.5. Dye decolorization studies

Decolorization of different dyes was carried out in packed bed reactor containing immobilized HRP as previously developed (Iqbal and Asgher, 2013), with minor modifications (Bilal et al., 2016c). The schematic illustration of the PBRS, used in this study, is displayed in Fig. 1. Briefly, the operational conditions of a continuous cycle were: 5.0 g of immobilized-HRP as a biocatalyst was loaded into a glass column, and each type of dye solution was placed in an effluent vessel, independently. The dyes solution was passed through the column using a peristaltic pump at a flow rate of

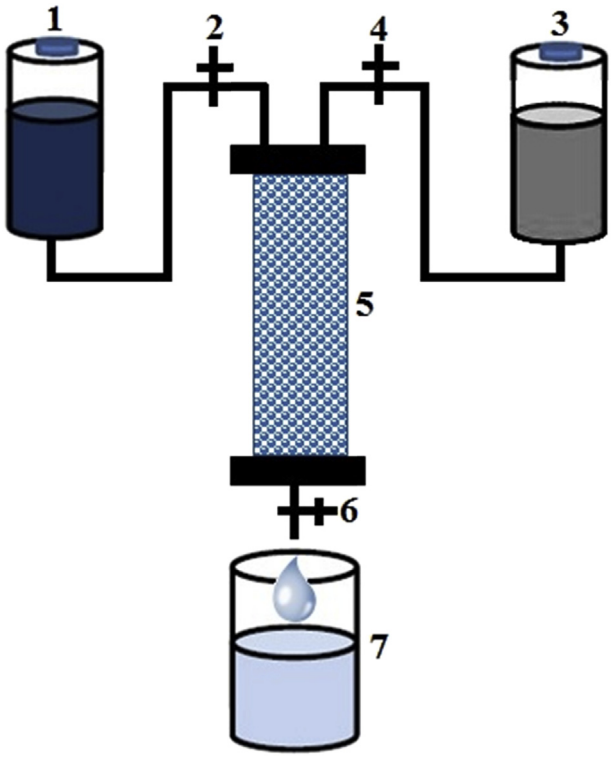

Fig. 1. Packed Bed Reactor System; 1 = Dye solution vessel, 2 = Dye flow control valve, 3 = Substrate vessel, 4 = Substrate flow control valve, 5 = Ca-alginate-immobilized-HRP based column, 6 = mL/min flow control valve, 6 = Decolorized product.