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Research article

Development of horseradish peroxidase-based cross-linked enzyme aggregates and their environmental exploitation for bioremediation purposes

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ABSTRACT

In the present study, horseradish peroxidase (HRP), in-house isolated crude cocktail enzyme, from *Armoracia rusticana* was cross-linked using a new type of cross-linking agent, i.e., ethylene glycol-bis [succinic acid N-hydroxysuccinimide, (EG-NHS)], which is mild in nature as compared to the glutaraldehyde (GA). The HRP-immobilized cross-linked enzyme aggregates (HRP-CLEAs) were developed using a wider range of EG-NHS and notably no adverse effect was observed. In a comparative evaluation, in the case of EG-NHS, a high-level stability in the residual activity was recorded, whereas a sharp decrease was observed in the case of glutaraldehyde. Following initial cross-linker evaluation, the HRP-CLEAs were tested to investigate their bio-catalytic efficacy for bioremediation purposes using a newly developed packed bed reactor system (PBRS). A maximal of 94.26% degradation of textile-based methyl orange dye was recorded within the shortest time frame, following 91.73% degradation of basic red 9, 84.35% degradation of indigo, 81.47% degradation of Rhodamin B, and 73.6% degradation of Rhodamine 6G, respectively, under the same working environment. Notably, the HRP-CLEAs retained almost 60% of its original activity after methyl orange dye degradation in seven consecutive cycles using PBRS. Furthermore, after HRP-CLEAs-mediated treatment in the PBRS, a significant toxicity reduction in the dye samples was recorded as compared to their pristine counterparts. In conclusion, the results suggest that the newly developed HRP-CLEAs have a great potential for industrial exploitation, to tackle numerous industrial dye-based emergent pollutants.

1. Introduction

From the past several years, the engineering of novel enzyme-based cross-linked aggregates as versatile and efficient bio-catalysts have substantially increased both at laboratory scale and industrial level (Straathof et al., 2002). Consequently, a highly selective and eco-friendlier HRP has appeared as a robust bio-based green catalyst for the degradation and detoxification of various emerging pollutants including industrial-based synthetic dye (Šekuljica et al., 2015). Moreover, the original dye-based formulations sometimes ended up with the generation of intermediate metabolites of these dyes which are even more toxic than their pristine counterparts. Therefore, a proper remediation treatment is inevitable, before discharge of this dye-based wastewater into receiving water bodies. Peroxidases and particularly HRP has gained significant concern in new research arena around the globe. Such scientific interests are because of its potential ability to transform harmful compounds into innocuous and environmentally safe products in the presence of hydrogen peroxide under mild reaction conditions (Shakeri and Shoda, 2008; Celebi et al., 2013; Jiang et al., 2014; Šekuljica et al., 2015).

Despite the functional versatility of peroxidases, the utilization of such industrially relevant enzymes in native-state is often hampered, in the industry, due to lack of stability under aggressive processing conditions and impossibility of repeated uses (Khanahmadi et al., 2015; Bilal and Asgher, 2015: Bilal et al., 2016a). The enzyme immobilization can fulfill these inadequacies, thereby rendering it more cheap, stable and recoverable having prolonged lifespan (Sheldon, 2007). Moreover, immobilized enzyme systems

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have been justified, both from economic and eco-friendlier viewpoint, owing to their great potential for reutilization several times, overall cost-effective ratio, elimination of harsh chemicals/reagents, mild reaction procedures, and protection and de-protection steps, thus significantly, trimming down the overall process costs (Šulek et al., 2011).

Based on the data reported in the literature, previously, authors have immobilized various enzymes using various techniques through different types of supporting carriers. Among them magnetic beads, alginate/acrylamide gel, sol-gel, modified chitosan beads, cyclodextrin-chitosan complex, activated wool, electrospun microfibrous membranes, kaolin, phospholipid-templated titania particles and Fe_3O_4/Graphene oxide nanocomposite have been investigated so far (Monier et al., 2010; Asgher et al., 2013; Iqbal and Asgher, 2013; Mohamed et al., 2013; Xu et al., 2015; Jiang et al., 2014; Kalaiarasan and Palvannan, 2015; Šekuljica et al., 2015; Bilal et al., 2016a,b). In this background, in an earlier study from our group, an immobilize HRP in Ca-alginate beads was developed for the treatment of different synthetic dyes using GA as a cross-linking agent (Bilal et al., 2016b). However, it was observed that the carrier bound enzyme showed instability towards leaching in aqueous media (Khanahmadi et al., 2015). Therefore, in recent years, the research scientists, around the globe, have diverted their research interests in carrier free immobilization approaches. Furthermore, the critical issues of the above-mentioned carrier-based immobilization methodologies could be mitigated by developing a novel form of CLEAs (Aytar and Bakir, 2008). In CLEAs preparation, the enzyme is precipitated first by adding precipitants followed by cross-linking of the resulting physical aggregates with glutaraldehyde or other milder cross-linkers. The relative simplicity and robustness of the method ideally lend itself to be of great value given economic and environmental applications. CLEAs present many advantages, like enhanced shelf life and functional stability toward heat denaturation, organic solvents, and autoproteolysis (Šekuljica et al., 2015).

The present work dealt with HRP-CLEAs synthesis using a new type of cross-linking agent, i.e., EG-NHS, which is mild in nature as compared to the glutaraldehyde. The newly developed HRP-CLEAs were tested for the oxidation and decolorization of different synthetic dyes. Towards the end of this study, the toxicity characteristics were also evaluated. The hemolytic and *Artemia salina* (brine shrimp) assays were adopted for toxicity evaluation studies.

2. Materials and methods

2.1. Chemicals

Standard laboratory grade chemicals including sodium alginate, calcium chloride dihydrate, acetic acid, sodium malonate dibasic monohydrate, acetone, pyrogallol, GA, chitosan (MW 20,000, degree of deacylation = 89.2%), and EG-NHS were mainly bought from Sigma-Aldrich, USA and Merck, Germany. Whereas, "BCA Protein Assay Kit" used for protein determination was delivered by Thermo Scientific, Rockford, IL, USA. The synthetic textile dyes, used in this work, namely Indigo (indigo blue), Methyl Orange, and Pararosaniline (Basic Red 9) were obtained from Sigma-Aldrich, USA, while, Rhodamine B and Rhodamine 6G were procured from Santa Cruz Biotechnology (Shanghai) Co., Ltd. The physiochemical characteristics of the dyes used in this study are summarized in Table 1.

2.2. The extraction protocol for HRP

HRP was isolated from the fresh horseradish roots, purchased from local vegetable market (Shanghai) as per the procedure described by Bhunia et al., 2001. Briefly, the roots were crushed in a wet grinder, filtered and the filtrate was subjected to the centrifugation (5000 × *g*) for 10 min at 4 °C. After the stipulated time, the collected residual-free supernatant (crude enzyme extract) was precipitated by ammonium sulfate up to 75% saturation at 4 °C and dialyzed for 24 h against phosphate buffer (pH 7.0) to remove the salts.

2.3. Development of HRP-CLEAs

Cross-linked aggregates of HRP were synthesized as reported earlier (Rehman et al., 2016). Up to a three (03) volume of ice-cold acetone as a precipitant was mixed with the enzyme solution. After 45 min of constant agitation (200 rpm) at 4 °C, the glutaraldehyde (GLA) and EG-NHS using various concentrations i.e. 50 mM, 100 mM, 150 mM, 200 mM, 250 mM, and 300 mM were slowly added to the resulting mixture. The above-mentioned each concentration was then allowed to react for various reaction periods ranging from 50 min to 300 min with 50 min interval at room temperature (25 °C) to obtain CLEAs. The CLEAs were recovered by centrifugation at 5000 × *g* for 10 min at 4 °C. A 20 mM phosphate buffer with 7.0 pH value was used to wash the collected CLEAs. Each experimental trial was washed up to three to four times until no traces of protein detected in the resultant supernatant. Following that the percent activity recovery was determined using Equation (1), as mentioned below.

$$\text{Activity recovery } (\%) = \frac{\text{Total activity of HRP} - \text{CLEAs}}{\text{Total activity of free HRP used for CLEAs synthesis}} \times 100 \quad (1)$$

2.4. HRP-CLEAs-mediated dyes degradation

Uninterrupted degradation of different textile-based synthetic dyes was carried out in a newly developed HRP-CLEAs based PBRS. The schematic representation of the PBRS, employed in this study, is shown in Fig. 1. To avoid leaching issues, a cotton wool was positioned at the bottom of the column. A 5.0 g sample was loaded into a glass column as a biocatalyst, and each type of dye sample was passed through the column using a peristaltic pump (Thermo Fisher Scientific), at a flow rate of 2.0 mL/min. The treated samples were collected at the outlet stream, filtered (Whatman No.1 filter paper), centrifuged (5000 × *g*, 15 min) and dye degradation was recorded Spectrophotometrically at the maximum wavelength of respective dye using the formula given in Equation (2). After each cycle, the reactor was rinsed with a continuous flow of phosphate buffer (pH 7.0) for 30 min, and the entire process was repeated for several cycles under the same working conditions.

$$\text{Degradation } (\%) = \left(\frac{(\text{Ai}) - (\text{At})}{(\text{Ai})}\right) \times 100 \quad (2)$$

Where, A*i* = initial absorbance of dye before enzymatic treatment