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Immobilization of lignin peroxidase from *Alcaligenes aquatilis* and its application in dye decolorization

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ABSTRACT

Purefied lignin peroxidase produced from *Alcaligenes aquatilis* DB8 was immobilized by entrapment on Ca-alginate and chitosan by adsorption and cross linker. Immobilization yields of entrapment, adsorption and cross linking methods were 79.6%, 31.4% and 91.2%, respectively. The thermostability of immobilized LiP by entrapment, adsorption and crosslinking retained residual activity of 68%, 63% and 85%, respectively after 180 min at 50 °C. While the residual activity of free enzyme was decreased to almost half after 180 min. Lignin peroxidase immobilized by crosslinking showed high thermal stability, more than 70% of the enzymatic activity remained after 240 min. Also, immobilized lignin peroxidase by crosslinking showed better storage stability about 55% at 30 days. The application of immobilized lignin peroxidase in a packed bead bioreactor enhanced decolorization of malachite green at 20 mg/l, and the percent of decolorization was 97% at 4 h.

Keywords: *Immobilization; decolorization; Wastewater; bioreactor; lignin peroxidase; Alcaligenes aquatilis.*

1. INTRODUCTION

Wastewater discharge from paper, textile, leather, plastic, food and cosmetic industries polluted the environment seriously [1,2]. Colored water from dyeing process in the textile industries is a crucial environmental problem. Dyes varied chemically and can be divided into triphenylmethane, azo, anthraquinone, nitro, nitroso and indigoid dyes [3]. Dye containing wastewater disposal into aquatic ecosystems reduces light penetration into deeper layers and consequently impedes the photosynthetic activities causes dissolved oxygen (DO) depletion and biodiversity loss [4,5]. Before discharging into water bodies, biological, chemical and physical treatments have been used for treating textile dye effluents [6].

Biological methods in liquid state fermentation are less effective in removing dyes from continuous effluents [7]. Recently, treatment of textile dyes through enzymatic process has attracted much more attention owing to they are rapid ecofriendly decolorization process [8].

Lignin peroxidase (LiP; EC 1.11.1.14) is a heme-cotaining enzyme belonging to the family of oxidoreductases [9]. LiP has the ability to oxidize xenobiotic compounds that include polychlorinated phenols, polycyclic aromatic hydrocarbons, azo and nitroaromatics dyes [10].

LiP has a great potential for industrial processes application due to its high redox potential and wide range of substrates [11]. Recovery of enzymes from the reaction solutions as well as the separation of enzymes from substrates is very difficult. Immobilization of the enzyme on various matrices has various advantages including unreacted substrate contamination, great operational control, enhanced stability, easy separation from catalyst and flexibility of the reactor design [12,13].

Enzyme immobilization can be achieved by several methods such as adsorption, entrapment, covalent binding and cross-linking [14].

In the present study, LiP isolated from *Alcaligenes aquatilis* DB8 has been immobilized on various beads to improve its thermostability. Also, studying storage stability, reusability and capability of immobilized LiP for malachite green (MG) dye decolorization.

2. MATERIALS AND METHODS

2.1. Microorganism and culture condition.

Alcaligenes aquatilis DB8 used in this study was previously isolated from the water sample for LiP-catalyzed dye decolorization. The LiP production was performed by response surface methodology with optimized concentration at: soluble starch, 10.2 (g/l); ammonium nitrate, 5.0 (g/l); KH_2PO_4, 2.0 (g/l); inoculums size, 10%; and time course, 36.6 (h).

2.2. Enzyme immobilization.

LiP was purified to 30.4 folds through ion exchange chromatography using DEAE sephadex A-50 followed by gel filtration chromatography using sephadex G-100 (data not shown) and used for immobilization studies.

2.3. Immobilization of Lignin peroxidase on Alginate bead.

The pure LiP was mixed with 40 ml sodium alginate (3%). The alginate beads were then prepared by dropping gently the alginate mixture into a beaker containing 200 ml 3% $CaCl_2$ solution at a rate of approximately 30 drops/min., stirring gently for 3h to allow the bead to harden. the bead was filtered out of the $CaCl_2$ solution and thoroughly washed [15]. The effect of different

concentrations of sodium alginate (1-5%) on immobilization of LiP was studied to obtain stable beads.

2.4. Immobilization of Lignin peroxidase on Chitosan bead.

2.4.1. Preparation of Chitosan beads.

Chitosan powder (3 g) was suspended in distilled water (99 ml) followed by 20 min stirring then glacial acetic acid (1 ml) was added with stirring for 4h at room temperature. The solution was filtered then dried. Drops of 2% (w/v) NaOH were added for neutralizing the acetic acid in the chitosan beads. The pretreated chitosan beads were washed twice with distilled water and finally dried again [16].

2.4.2. Immobilization of LiP by direct adsorption on pretreated chitosan beads.

Pretreated chitosan beads were added into 1 ml LiP solution (2 mg/ml). The solution was stirred at 25 ˚C, 150 rpm for 5h then the chitosan beads with the adsorbed enzymes were separated and washed three times with 100 mM potassium phosphate buffer, pH 6.0; to remove unbound enzymes [17].

2.4.3. Activation of prepared Chitosan beads.

Prepared chitosan beads were activated using glutaraldehyde ranging from 0.5 to 3% (v/v) and allowed to stand at room temperature for 3h. Activated chitosan beads were washed thoroughly with 100 mM potassium phosphate buffer, pH 6.0; two or three times to remove any free glutaraldehyde. Activated chitosan beads were stored at their optimal pH, 50mM Tris buffer at 4 ˚C, until used.

2.4.4. Immobilization of LiP on chitosan beads.

Activated chitosan beads were mixed with 2 mg/ml LiP solution in 100 mM potassium phosphate buffer, pH 6.0; for 3 h with slight stirring and washed at 4 ˚C. Then, the beads dried at room temperature and stored at 4 ˚C. The activity of the immobilized enzyme was estimated by subtracting the activity recorded in the supernatant after immobilization from the activity added to chitosan bead.

2.4.5. Determination of Immobilization Yield for LiP.

The immobilization yield of the enzyme was defined as the yield of the enzyme immobilized in chitosan and expressed as:

$$Immobilization\ yield\ (\%) = \frac{Activity\ of\ immobilized\ enzyme\ \times 100}{Activity\ of\ free\ enzyme\ used}$$

2.5. Enzyme activity and protein estimation.

LiP activity was determined by assaying the formation of purpurogallin at 420 nm in a reaction mixture containing 2.4 ml of 100 mM potassium phosphate buffer, pH 6.0; 0.3 ml of 5.33% pyrogallol, 100 µ of enzyme and 200 µ of 10 mM H_2O_2 [18]. One unit of LiP activity (U/ml) was defined as the amount of enzyme required to oxidize 1 µmol pyrogallol to purpurogallin per min under standard assay conditions.

$$Lip\ activity\ (U/mL) = \frac{\Delta A\ (420)\ x\ Vt}{€\ x\ min\ x\ Vs}$$

Where; Vt is the final volume of reaction mixture, Vs is the sample volume of enzyme, $€_{(420)}$ is the extinction coefficient of pyrogallol = 12,000 $M^{-1}cm^{-1}$

Protein was determined by Folin-Lowry method using bovine serum albumin as a standard [19].

2.6. Thermal stabilitiy of immobilized LiP.

Thermal satability was assayed by incubating the free and immobilized LiP at 50 ˚C at different time intervals 30 - 240 min, then The reaction mixture was assayed by standard LiP assay protocols.

2.7. Storage stability of immobilized LiP.

The storage stability of immobilized LiP was investigated at 4 ˚C for thirty days. The residual activity was checked from time to time with pyrogaloll as a substrate according to the standard assay protocol that previously described. In each set, the highest value of enzyme activity was assigned as the value of 100 % activity.

2.8. Reusability of immobilized LiP.

To study the reusability of the immobilized LiP, the used beads were recovered at the end of each cycle and washed three times with 100 mM potassium phosphate buffer, pH 6.0 to be used in the next fresh dye decolorization [20]. The residual activity of LiP was calculated by taking the enzyme activity in the first cycle as 100%, and the recycling process was repeated several times and calculated as:

$$Residual\ activity\ (\%) = \frac{Enzyme\ activity\ in\ n^{nt}\ cycle\ x\ 100}{enzyme\ activity\ in\ 1^{st}\ cycle}$$

2.9. Decolorization of Malachite Green in packed bed column.

Twenty five ml of MG solution in 50 mM Tris-HCl buffer, pH 8.0 was used with a concentration of (5-30 mg/l) circulated through a column (1.5 × 40 cm) packed with immobilized Lip (5.0 g) at a flow rate of 3 ml/min. Dye solution put on a hot plate at 35 ˚C was continuously loaded to the column reactor by using a peristaltic pump [21]. The absorbance of the dye solution was monitored for 0, 30, 60, 90, 120, and 150 min. of reaction. The decolorization was determined spectrophotometrically using a UV-Vis spectrophotometer (Jenway - 6800) at 620 nm by measuring the decrease in the absorbance. The decolorization percentage was calculated according to the following equation:

$$Decolorization\ (\%) = \frac{Initial\ O\ D - Final\ O\ D\ x\ 100}{Initial\ O\ D}$$

3. RESULTS

3.1. Immobilization of LiP from *Alcaligenes aquatilis* DB8.

One of the main objectives of enzyme immobilization is to enhance the stability and efficiency of dye degrading enzyme to be stable under various conditions. The LiP was immobilized by entrapment on Ca-alginate and chitosan by cross linker and on chitosan by adsorption (Fig. 1). The immobilization yields were 79.6%, 91.2% and 31.4%, respectively. Lip was immobilized on various supports including immobilization on nanoporous gold [22] and immobilized on carbon nanotubes [23].

The results obtained were so similar to other previous result [24]. Enzyme immobilization causes an increase in enzyme rigidity, which is commonly reflected by an increase in stability toward denaturation by raising the temperature. The biocompatibility of the support to the enzyme molecule plays an important role in stabilization of enzyme conformation.

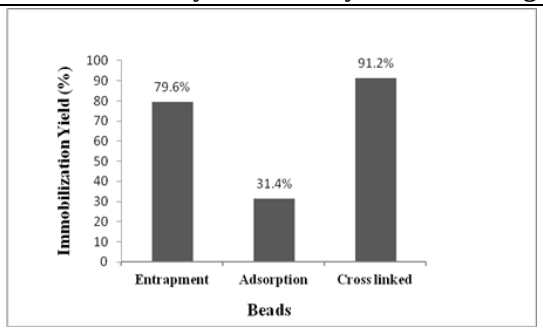

Figure 1. Immobilization of LiP on different beads.

Enzyme immobilization has been a popular strategy for most large-scale applications owing to the ease in continuous operation, storage, recycling and product purification [25].

3.2. Effect of sodium alginate concentration on LiP immobilization.

Enzymes entrapment in beads relies on sodium alginate concentration followed by $CaCl_2$ addition (Fig. 2). The immobilization yield of LiP was 79.6% at 3% sodium alginate concentration which may be due to the strong cross linked gel formation as discussed by [26]. However, at higher concentration decrease of immobilization yield noticed which may be due to decreasing the porosity of the gel beads [27].

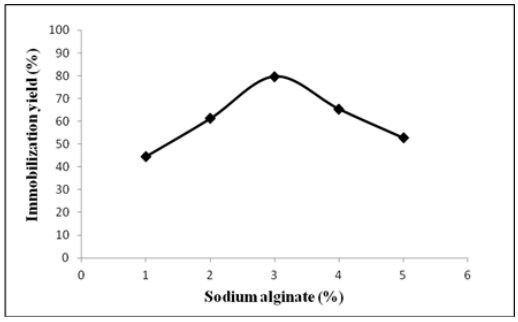

Figure 2. Effect of sodium alginate concentration on immobilization yield (%).

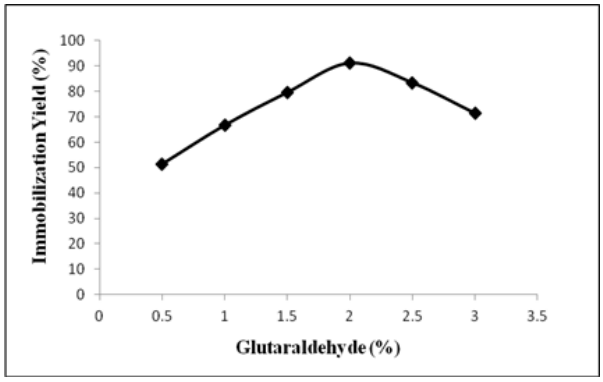

Figure 3. Effect of glutaraldehyde concentration on immobilization yield (%)

3.3. Effect of glutaraldehyde concentration on LiP immobilization.

Chitosan beads were activated using glutaraldehyde ranging from 0.5 to 3% (v/v), Figure 3 showed the activated beads with 2% glutaraldehyde give maximum immobilization yield (91.2%). However, the lower concentrations cause lower immobilization efficiency. The chitosan beads activated with maximum efficiency at 1.5% glutaraldehyde [28]. But, the lower concentrations of glutaraldehyde cause lower immobilization efficiency [29]. Moreover, excessive glutaraldehyde concentrations might affect the tridimensional structure of the enzyme [30].

3.4. Thermostability of Immobilized LiP.

The thermostability of the free and immobilized LiP was studied at 50 ˚C (Fig. 4). All immobilization treatments retained more than 90% of enzyme activity after 90 min when tested at 50 ˚C. The immobilized LiP by entrapment, adsorption and crosslinking retained residual activity 68%, 63% and 85%, respectively after 180 min. However, the free enzyme residual activity was decreased to 50% after 180 min. In addition, LiP immobilized by crosslinking showed high thermal stability with remaining enzyme activity more than 70% after 240 min. These results were in agreement with earlier studies [17, 31].

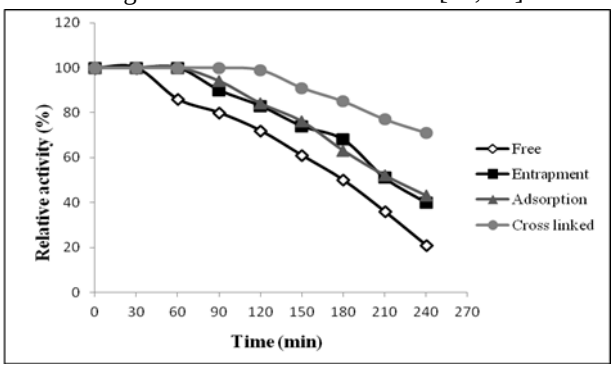

Figure 4. Thermal stability of free and immobilized LiP at 50 ˚C.

Immobilization of LiP on various beads improved enzyme stability at 50 ˚C compared to the free enzyme. The immobilization support generally has a protective effect at high temperature at which deactivation takes place. The enzyme conformational flexibility is affected by immobilization [32]. At higher temperature, the loss of activity of immobilized enzyme was less than the free enzyme.

Calcium alginate might have a protecting effect at higher temperatures at which enzymes deactivation occurs. Immobilization of peroxidase on these beads can cause an increase in the enzyme rigidity which is commonly reflected by increase in stability towards denaturation by raising the temperature [33].

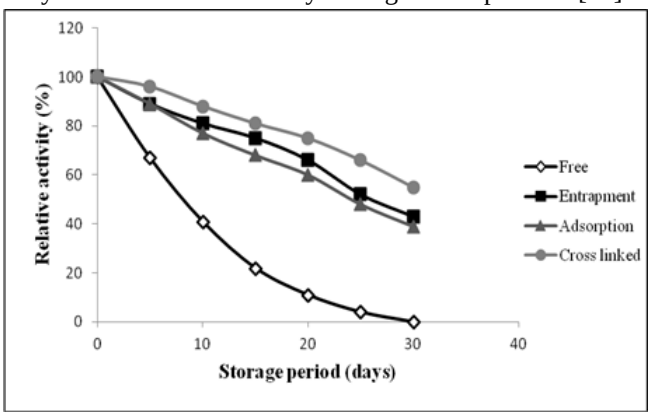

Figure 5. Storage stability of free and immobilized LiP at 4 ˚C.

The major factor accountable for this thermostability improvement might be due to the interactions between the carrier and LiP, such as hydrogen bonds and electrostatic interactions, which can promote further rigidity of the enzyme structure and greater resistance against thermal denaturation [34]. The process of immobilization can recover the enzyme feasibly from the reaction mixtures and therefore can be used to catalyze more reactions. However, the soluble enzyme cannot make it feasibly [35].

3.5. Storage stability of immobilized LiP.

The immobilized LiP by entrapment, adsorption and crosslinking was retained residual activity of 43%, 39% and 55%, respectively after 30 days when preserved in buffer at 4 ˚C. However, the free enzyme retained activity less than 41% after ten days (Fig. 5). The stability of storage enzyme may be due to the contacts of charged residues by interaction between the matrix and enzyme [36]. Enhanced storage stability of enzymes via immobilization has also been studied previously [37,38].

3.6. Reusability of immobilized LiP.

Reusability of immobilized enzymes is an important aspect of biotechnological applications. Immobilized enzymes decrease the production cost owing to their continuous, repeated or batch uses. Reusability of the immobilized LiP for MG decolorization was assayed at 35 ˚C for seven cycles using the same LiP beads.

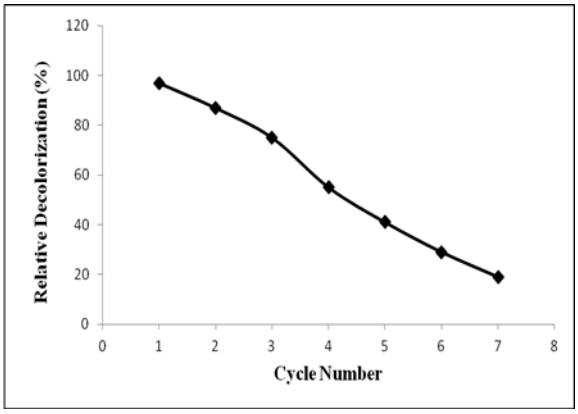

Figure 6. Reusability of immobilized LiP in MG decolorization.

In the 4th cycle, the activity of immobilized crosslinked chitosan coupled LiP was reduced to 55% (Fig. 6). At the 6th cycle, the decolorization potential of immobilized LiP decreased to 29%. The immobilized enzyme activity diminishes gradually with increasing cycles of reuse. This probably due to decreasing the binding strength between the enzyme and support beads by repeated wash and uses [39]. The decreased activity may also be due to the possibility of enzyme denaturation during the operation process [40].

This observation could be explained by the denaturation of enzyme on the surfaces of carriers, as well as enzyme leakage from carriers during the reactions [41]. Similar results were obtained previously with the chitosan for enzyme immobilization [28,37].

3.7. Malachite Green Decolorization in packed bed column.

The decolorization of MG by immobilized LiP from *Alcaligenes aquatilis* DB8 in the bioreactor at various concentrations (5-25 mg/L) was investigated in Table 1. Crosslinked chitosan was the best bead as the immobilized enzyme showed appreciable thermostability at 50 ˚C. At 5 mg/l, 98% MG decolorization was obtained after 1h. However, at 20 mg/l, the decolorization was 97% after 4 h. At MG of 30 mg/l, the decolorization was decreased to 67% after 7 h. This may be due to the decrease in enzyme activity and this was similar to other results [42].

The immobilized LiP on crosslinked chitosan beads showed great potential for MG decolorization with higher catalytic efficiency, stability and capability to be reused in the dye decolorization process. Furthermore, the immobilized LiP on different supports also showed a higher capability of decolorizing dyes [43,23].

Table 1. Decolorization of MG at different concentration and time consumed

MG mg/l	Decolorization (%)	Time (h)
5	98	1
10	97	2
15	97	3
20	97	4
25	83	6
30	67	7

4. CONCLUSIONS

Results obtained from this study explain that extralcellular LiP from *Alcaligenes aquatilis* DB8 can be successfully immobilized with different support. Crosslinked chitosan was the best bead among those others tested. The immobilized enzyme showed appreciable thermostability at 50 ˚C particularly on crosslinked chitosan, storage stability and reusability. Thus, the immobilized LiP could be used in dye decolorization and various biotechnological applications.

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