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An accomplished procedure of horseradish peroxidase immobilization for removal of acid yellow 11 in aqueous solutions

Melda Altikatoglu Yapaoz and Azade Attar

ABSTRACT

Horseradish peroxidase (HRP) characteristics were improved by two techniques, Na-alginate entrapment and glutaraldehyde crosslinking prior to alginate entrapment, in order to enhance the stability, functionality and removal of dyes in waste water. Free, entrapped and crosslinked-entrapped enzymes were compared by activity assays, which indicated the optimum temperature is 25 °C and pH 4.0–5.0. Kinetics results showed that alginate entrapment and crosslinking prior to entrapment increased V_{max} and did not cause any significant decrease in K_m. The thermal resistance of the free enzyme was short-term, zero residual activity after 250 min, while the immobilized enzymes preserved more than 50% of their activity for 5 h at 60 °C. Immobilized HRP was resistant to methanol, ethanol, DMSO and THF. The storage stability of free HRP ended in 35 days whereas entrapped and crosslinked-entrapped HRPs had 87 and 92% residual activity at the 60th day, respectively. HRP was used in the decolorization of azo dye Acid yellow 11 and total decolorization (>99%) was obtained using crosslinked-entrapped HRP. Reusability studies presented the improvement that crosslinked-entrapped HRP reached 74% decolorization after 10 batches. The results demonstrated that the novel immobilized HRP can be used as an effective catalyst for dye degradation of industrial waste effluents.

Key words | alginate entrapment, azo dye removal in aquatic solutions, decolorization, enzyme immobilization, glutaraldehyde crosslinking, horseradish peroxidase

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HIGHLIGHTS

- First study on the removal of acid yellow 11 in aqueous solutions using horseradish peroxidase.
- HRP immobilization methods are compared and glutaraldehyde crosslinking prior to alginate entrapment is suggested.
- Suggested method is practical and the enzyme exhibited enhanced characteristics.
- The immobilized HRP is a successful catalyst for decolorization of industrial waste water.

INTRODUCTION

Immobilization of enzymes on natural biopolymers is increasingly becoming important as compared to free enzymes (Monier *et al.* 2010; Amin *et al.* 2017; Zdarta *et al.* 2018). Immobilized enzymes exhibit many advantages such as activity, stability, reusability, selectivity, improved product yield and ease of enzyme recovery from a reaction mixture (Bilal *et al.* 2019). Polymers constitute a cross-linked hydrophilic form and therefore provide an available environment. Horseradish peroxidase (HRP, EC1.11.17) is a heme-containing enzyme that catalyzes oxidative reactions using hydrogen peroxide as a co-substrate and different aromatic structures as substrates. It is a

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glycoprotein having approximately 18% N-linked oligosaccharides in its composition (Altikatoglu *et al.* 2009; Janović *et al.* 2017) and has been used for practical analytical applications and in decolorization studies (Regalado *et al.* 2004).

Azo dyes have been widely used as colorants in a variety of products such as textiles, paper, food and pharmaceuticals (Rajmohan *et al.* 2019). Azo dyes are resistant to biodegradation due to their complex structures and xenobiotic nature (Selvankumar *et al.* 2019). In addition, they may cause acute toxicity or even mutagenic effects on exposed aquatic organisms (Ayed *et al.* 2019). Therefore, removal of azo reactive dyes is of great importance and has gained much attention in recent years. Biological process, including plants, fungi or bacteria, is considered an environmentally friendly and effective tool in the decolorization of azo dyes (Waghmode *et al.* 2019). This decolorization is carried out by enzymes activated in the biological process and HRP is one of the leading enzymes used in phenol removal (Janović *et al.* 2017). There is a considerable number of reports dealing with the degradation of industrial dyes by free or immobilized HRP (Onder *et al.* 2011; Husain 2019; Wasak *et al.* 2019).

HRP has been immobilized through adsorption and covalent bonding on many polymeric supports, such as alginate, chitin, and chitosan, which have the advantages of being nontoxic, biocompatible, and biodegradable (Sun *et al.* 2018). Among the reported immobilization techniques, enzyme entrapment in alginate beads is an eco-friendly, cost effective and simple immobilization technique. However, this technique is restricted by the porosity of the matrix, which may introduce enzyme leakage (Brandi *et al.* 2006; Lassouane *et al.* 2019). In this study, a recent approach consisting in crosslinking HRP enzyme prior to entrapment into sodium alginate beads was investigated. Further, the decolorization of azo dye acid yellow 11 by free and immobilized HRP was evaluated for the first time in literature. The effect of pH, temperature, and time on the decolorization of acid yellow 11 and the reusability of the immobilized HRP were also examined.

MATERIALS AND METHODS

Chemicals

Horseradish peroxidase (E.C. 1.11.1.7) (Mw ~ 40.000 Da) and Acid yellow 11 (Mw: 380.35 Da) were purchased from Sigma Aldrich. All chemicals used in the experiments were of analytical grade and utilized as received without further purification.

HRP immobilization

HRP was immobilized by entrapment and crosslinking prior to entrapment into alginate beads. Therefore, two methods were used in the immobilization of horseradish peroxidase (HRP): sodium alginate was applied in the entrapment of the enzyme and glutaraldehyde was utilized in the crosslinking. In the first method, various concentrations of sodium alginate (1.0–3.0%) and calcium chloride (0.5–2.5%) were used for immobilization of HRP. Sodium alginate (2.5%, w/v) solution in deionized water was mixed well by shaking with HRP (1%, w/v) solution prepared in 20 mL of assay buffer (50 mM acetate buffer, pH 5.0). The mixture was dropped by means of a burette into 2% (w/v) calcium chloride solution and the obtained beads were left to harden for 2 h under gentle shaking. Then, the beads were assembled out of the solution and washed twice with water and stored in the assay buffer at 4 °C for its further use. In the second method, glutaraldehyde in deionized water was added slowly into the HRP solution in order to crosslink the enzyme with glutaraldehyde (1.0%, v/v). The mixture was stored at room temperature under stirring for 2 h and the steps of the first method were performed.

The immobilization yield (IY) was calculated as the ratio of the immobilized HRP activity to the activity of the free enzyme and expressed as a percentage. The 1:1 ratio between the enzyme and alginate was preserved in all experiments. The entrapped and crosslinked-entrapped HRP beads were placed in a definite volume of acetate buffer (pH 5.0) for 24 h in order to determine the enzyme leakage. Afterwards, the beads were collected and HRP activity released from the beads in the buffer solution was measured and compared to the immobilized HRP activity at time zero. The leakage was expressed in terms of percentage.

Activity determination

HRP activity was spectrophotometrically measured (UV-Visible spectrophotometer, Shimadzu) at 460 nm by the method of Altikatoglu *et al.* (2009) with o-dianisidine as substrate (Altikatoglu *et al.* 2009). One unit of activity was defined as the amount of enzyme that oxidized 1 μmol of o-dianisidine per minute. Ten beads were used in the activity assay of the immobilized HRP and the samples were incubated under agitation for 5 min.

Enzyme kinetics

The kinetic constants (K_m and V_{max}) of free and immobilized HRP were analyzed for various concentrations of

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o-dianisidine (0.0125–0.2 mM) using the standard assay procedure under optimum conditions (pH 5.0, 30 °C). The experimental data were analyzed according to the Michaelis-Menten model by Lineweaver-Burk plots and by non-linear regression analysis.

Stability studies of the free, entrapped and crosslinked-entrapped HRP

Optimal temperature and pH

The optimal pH of the free, entrapped and crosslinked-entrapped HRP activities was determined at a pH range from 2.0 to 9.0 at 30 °C. The buffers used were acetate buffer (pH 3.0–5.0), phosphate buffer (pH 6.0–8.0) and Tris-HCl buffer (pH 9.0). The effect of temperature on the biocatalyst activity was assayed at different temperatures between 25 and 80 °C at optimum pH. Relative activities were measured regarding the highest activity as 100%.

Thermal inactivation

Free and immobilized enzymes were incubated at 60 °C in 50 mM acetate buffer at pH 5.0. Aliquots were collected at scheduled times, cooled rapidly and assayed for residual enzymatic activity.

Effect of organic solvent

The effects of organic solvents were determined in the presence of 10% (v/v) methanol, ethanol, tetrahydrofuran (THF), and dimethyl sulfoxide (DMSO) at pH 5.0, 30 °C. The samples were pre-incubated with the organic solvent for 10 min and relative activities were measured using o-dianisidine as substrate.

Storage stability

Free, entrapped and crosslinked-entrapped HRP were stored in acetate buffer (pH 5.0) at 4 °C for 60 days. The samples were taken at certain time intervals for residual activity determination of HRP.

Decolorization studies and reusability assay

The decolorization was carried out directly in the spectrophotometer cuvette. The reaction was started by adding buffer solution (50 mM acetate buffer, pH 5.0), 12 µl Acid yellow 11 (0.1 mM), HRP solution (0.0033 mg ml^{-1}) and finally 10 µl H_2O_2 (0.3 mM) as the initiator into the cuvette respectively, in a total volume of 3 ml. The percentage decolorization was determined by a reduction in absorbance measured by UV-Vis spectrophotometer (Model UV-1700 Pharmaspec Shimadzu) at the specific λ_{max} of dyes for 60 minutes, using the following equation;

$$D = \frac{Ai - Af}{Ai} \times 100$$

where, D is dye decolorization (%), A_i is the initial absorbance of the reaction mixture and A_f is the final absorbance of the reaction mixture.

Reusability experiments were performed in order to determine the reusability of the immobilized HRP. The immobilized HRP was removed and washed with 50 mM acetate buffer (pH 5.0) after each cycle and the solution replaced with fresh dye. The procedure was repeated for 10 cycles. The activity of freshly prepared enzyme in the first cycle was defined as 100%.

Statistical analysis

All assays were performed in triplicate and each value represents the mean for three-independent experiments. Standard deviation was calculated by the Microsoft Excel software.

RESULTS AND DISCUSSION

Various concentrations of sodium alginate (1.0–3.0%, w/v) and $CaCl_2$ (0.5–2.5%, w/v) were utilized in the process of HRP immobilization; consequently, HRP leakage and immobilization yield were taken into account in order to determine the suitable concentration of the two components. Immobilized HRP activity increased as the increase in the sodium alginate concentration till 2.5% and $CaCl_2$ 2.0% (w/v). This is most probably due to the unstable, soft and weak formation of the beads under this concentration. Low crosslinking rate below the optimum constituent concentration is another handicap in immobilization. Besides, the decrease in immobilization yield at higher concentrations implies the inadequate substrate diffusion into the HRP immobilized beads. Therefore, sodium alginate 2.5% (w/v) and $CaCl_2$ 2.0% (w/v) were selected as the optimum according to the results by decrease in HRP leakage and increase in immobilization yield. HRP was immobilized by two methods in order to compare the

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crosslinking efficiency: entrapment and crosslinking prior to entrapment into alginate beads. Crosslinking prior to entrapment showed higher immobilization yield (83%) than HRP immobilized only by entrapment (67%). Also, HRP leakage significantly decreased (about 5-fold) by alginate entrapment after glutaraldehyde crosslinking (data not shown). This result evaluates the goal of including glutaraldehyde and it is parallel with the literature (Bhushan *et al.* 2015).

Kinetic parameters of free and immobilized HRP

Kinetic parameters of the free, entrapped and crosslinked-entrapped HRP were determined with respect to o-dianisidine by Lineweaver-Burk plot. As illustrated in Table 1, the Michaelis Menten kinetics constant (K_m) values of the immobilized enzymes are not significantly different (entrapped HRP 33.5 ± 1.85 mg/mL and crosslinked-entrapped HRP 33.6 ± 2.58 mg/mL) than the K_m for the free enzyme (35.2 ± 2.31 mg/mL) when the standard deviations are taken into account. On the other side, the maximal velocity (V_{max}) value of the HRP increased from 112 U/mL to about 125 U/mL upon immobilization. The K_m and V_{max} for immobilized enzymes were almost in the same range as of the free enzyme. Increase in V_{max} value means variation in the 3-D structure of the enzyme or depends on the characteristics of the support material (Akpınar *et al.* 2020). In this case, depending on the kinetic parameters, it can be said that immobilization of HRP in calcium alginate beads did not cause steric hindrance or substrate diffusion resistance. Some of the binding sites of the enzyme are usually captured after immobilization, which causes reduction of the surface area for substrate interaction; therefore, decrease in V_{max} and increase in K_m were reported in some studies (Almulaiky *et al.* 2019; Yu *et al.* 2019). However, the results of immobilized HRP exhibited good kinetic characteristics with constant K_m and slight increase in V_{max}, which means that the suggested immobilization method did not affect the substrate affinity and catalytic activity.

Table 1 | Kinetic parameters of free and immobilized HRP

		Immobilized HRP	
Kinetic parameters	**Free HRP**	**Entrapment**	**Crosslinked-entrapment**
K_m (mg/mL)	35.2 ± 2.31	33.5 ± 1.85	33.6 ± 2.58
V_{max} (U/mL)	112 ± 2.95	124 ± 2.86	125 ± 3.05

Optimal temperature and pH

The effect of pH and temperature on relative activity by free, entrapped and crosslinked-entrapped HRP at room temperature were studied. The optimum pH of the free and immobilized HRP were found to be pH 4.0 and 5.0 (Figure 1(a)). Previous studies pointed out the optimal working pH of HRP as between 4.0 and 5.0 (Altikatoglu *et al.* 2009; Celebi *et al.* 2013). Immobilized enzyme indicated higher activity compared to the free enzyme between pH 6.0 and 9.0 and crosslinked-entrapped HRP was more active than the entrapped one. Free HRP lost its total activity at pH 9.0; however, entrapped HRP and crosslinked-entrapped HRP showed 30 and 37% of relative activity, respectively. Figure 1(b) displays the rapid activity decline of the free enzyme with the increase in temperature, whilst immobilized enzymes had higher resistance against temperature. The optimum temperature of free and immobilized enzyme was found to be 25 °C. The immobilized enzymes indicated higher activity than the free enzyme over 30 °C. It was observed that the immobilized enzymes had lower activity than the free enzyme at 25 and 30 °C, but their values were not changed significantly before 60 °C. The entrapped and crosslinked-entrapped HRP presented relative activity of 35 and 40%, respectively; however, free HRP maintained at most 20% of its initial activity at 70 °C. This situation is related with alginate entrapment that covers the HRP enzyme, resisting the temperature change in the bulk solution (Yamak *et al.* 2009; Lassouane *et al.* 2019).

Thermal inactivation

Figure 2 reports the kinetics of thermal inactivation of the enzyme forms exposed to 60 °C. The free HRP progressively lost activity with time according to inactivation kinetics, but the immobilized enzymes were more resistant to thermal treatment than the native counterpart. It can be monitored that both kinds of immobilization exhibited a similar trend. The residual activities declined less and slowly along with the prolonged reaction time. Free HRP lost its activity after 240 min, while immobilized enzymes did not lose their activity until the end of the reaction time, which was 300 min. Both kinds of immobilized HRP maintained more than 58 and 61% of their activity after a 5 h period, so were inactivated at a much slower rate than that of the native HRP. On the basis of this observation, the results indicate that the thermostability of immobilized HPR elevated remarkably. Parallel results were obtained and reported as

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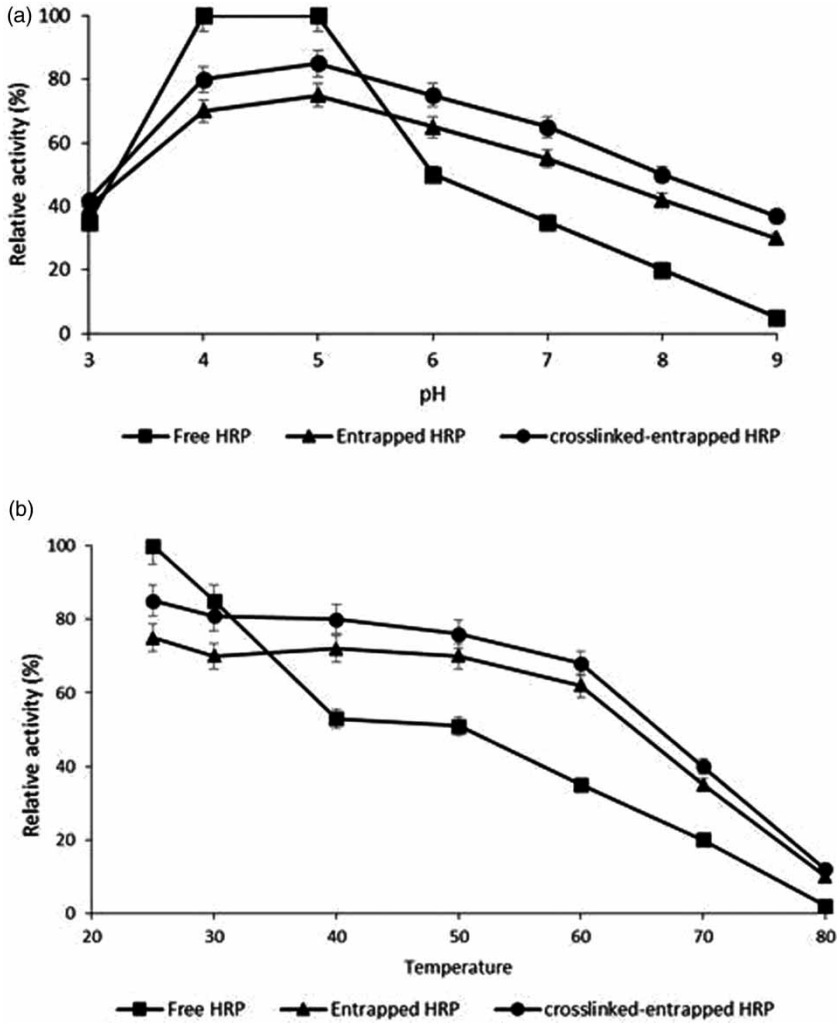


Figure 1 | Optimal pH profile at room temperature of the free, entrapped and crosslinked-entrapped HRP (a) and optimal temperature profile at pH 5.0 of the free, entrapped and crosslinked-entrapped HRP (b).

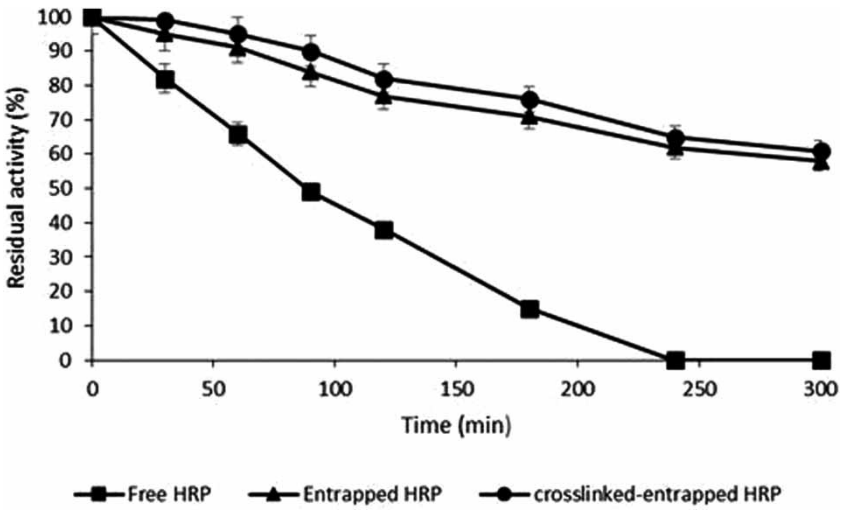


Figure 2 | Thermal inactivation of free, entrapped and crosslinked-entrapped HRP incubated at 60 °C pH 5.0 for 5 h.

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immobilization significantly increased the thermal stability of HRP (Akpınar *et al.* 2020).

Effect of organic solvent

It was known that organic solvents are inhibitory on the enzyme activity, therefore some commonly used solvents such as methanol, ethanol, THF and DMSO were applied in enzyme activity assay in order to measure the resistance. Free, entrapped and crosslinked-entrapped HRP solutions were incubated with the solvents at pH 5.0, 30 °C for 10 min. Figure 3 demonstrates the effect of organic solvents on the activity of free and immobilized enzymes. Free HRP was deactivated in the solvent media, but the entrapped and crosslinked-entrapped HRP showed higher residual activity compared to the free enzyme. There are some reports on activity of non-modified HRP that was not lost by addition of low concentration of organic solvents (Santucci *et al.* 2002; Hassani & Nourozi 2014); however, it was also reported that some organic solvents inhibit enzyme activity by promoting protein unfolding (Altikatoglu & Celebi 2011), which is in agreement with our result. It can be concluded that inhibition depends on the type and origin of the HRP. Also, THF and DMSO have higher inhibitory effect on enzyme activity because of their organic nature when compared to alcohols such as methanol and ethanol.

Storage stability

Storage stability of the free and immobilized HRP was studied for 4 weeks. The solutions were stored in acetate buffer (0.05 M, pH 5.0) at 4 °C. Free enzyme lost its activity after 30 days at +4 °C whereas immobilized enzymes were stable, showing good activity values for 60 days (Figure 4). A little difference was observed between the entrapped and crosslinked-entrapped HRP, whose retained activities were 87 and 92%, respectively, after 35 days, evaluating that the crosslinking renders further resistance to peroxidase enzyme, which extends shelf life. The immobilized enzyme had a longer storage lifetime compared to the free enzyme, which is considered as a good feature for usage in practice. The high storage stability of the immobilized HRP can be referred to the protective micro environment offered by the alginate matrix (Chaudhari *et al.* 2015). Alginate support should bring stabilization by minimizing deformity possibilities imposed from aqueous medium on the active site of the HRP enzyme; therefore, it is suggested that the immobilization method supplies a higher shelf life compared to that of its free complement (Bayramoğlu & Arıca 2008).

Studies on the decolorization of acid yellow 11

Azo dyes are used in dyeing in many industries, especially in textiles. The removal of these dyes from wastewater is an important process for the environment. Therefore, in this study, Acid yellow 11 (AY11) was studied in order to determine the use of immobilized HRP enzyme in decolorization of azo dyes. The decolorization effect of HRP enzyme on many azo dyes is available in the literature. However, no study using HRP enzyme has been found to remove AY11 dye. Key parameters in enzymatic decolorization, including pH, temperature and reaction time, were investigated in

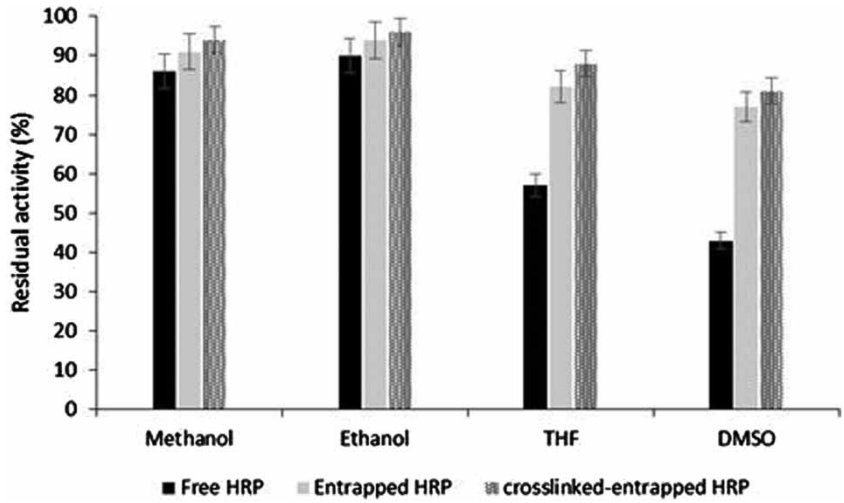


Figure 3 | Effect of organic solvents on the activity of the free, entrapped and crosslinked-entrapped HRP.

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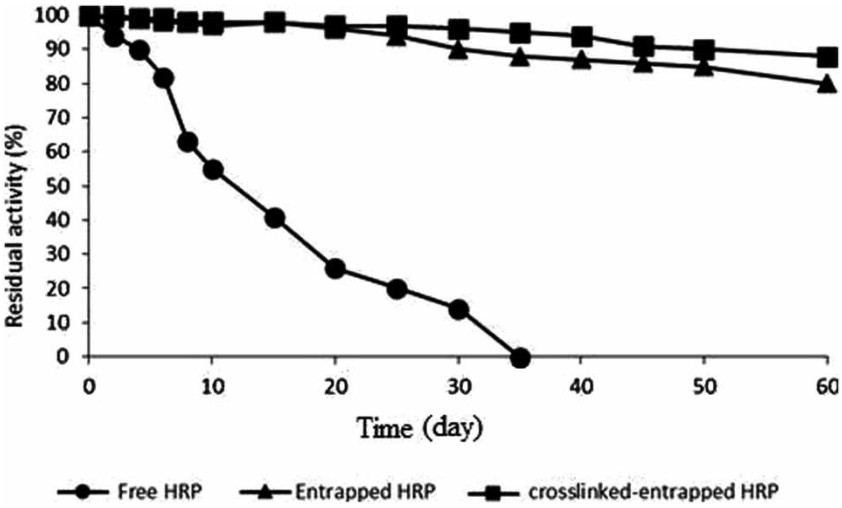


Figure 4 | Storage stability at 4 °C for the free, entrapped and crosslinked-entrapped HRP.

order to characterize *in vitro* degradation of AY11 by free and immobilized enzymes. Optimum pH was tested and pH 5.0 was found to be the most suitable for all variations of HRP in decolorization studies (Figure 5(a)). Free HRP, entrapped HRP and crosslinked HRP have 77, 88 and 93% removal, respectively at pH 5.0. The highest decolorization was obtained by free HRP at 25 °C as 85% where the entrapped HRP and crosslinked counterpart were at 30 °C as 85 and 90% (Figure 5(b)). The dye degradation rates were much slower for the temperatures above 35 °C. The results of decolorization versus time indicated that decolorization percentage increases with time. Total

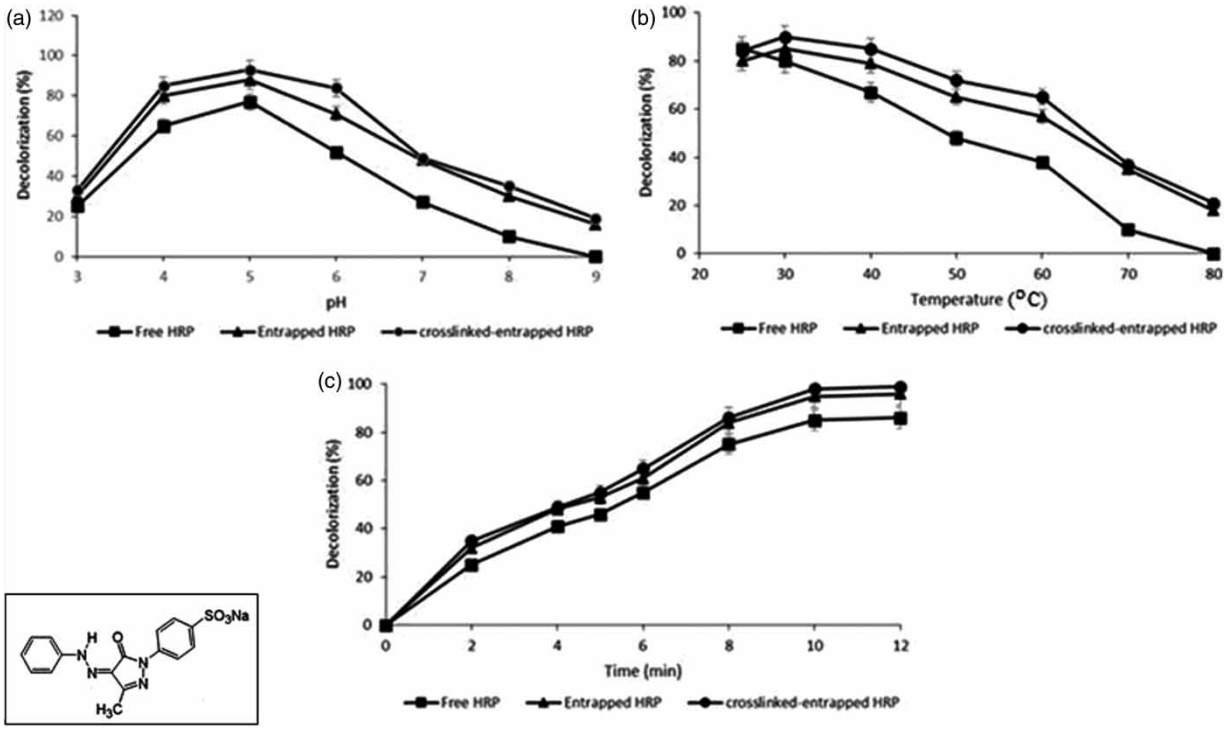


Figure 5 | Effect of pH, temperature and reaction time on dye removal by free, entrapped and crosslinked-entrapped HRP. (a) Decolorization of dye in the buffer of varying pH at 30 °C, (b) decolorization of dye at different temperatures at pH 5.0, and (c) effect of reaction time on dye decolorization for 30 °C at pH 5.0. Conditions: dye concentration: 0.1 mM; H_2O_2 concentration: 0.3 mM; enzyme concentration: 0.0033 mg/ml, incubation time: 60 min. Structure of acid yellow 11 (left bottom).

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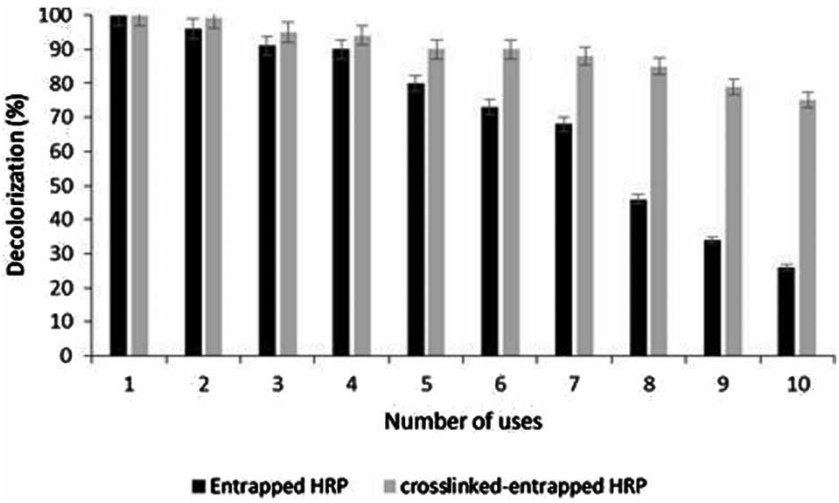


Figure 6 | Reusability of the entrapped and crosslinked-entrapped HRP in stirred batch process.

dye removal was obtained by cross-linked HRP after 12 min (Figure 5(c)). It was evaluated by the decolorization experiments that the best results were obtained using cross-linked HRP, and almost complete decolorization (>99%) of AY11 was attained at a pH of 5.0, temperature of 30 °C, H_2O_2 concentration of 0.3 mM, and enzyme dose of 0.0033 mg/mL of immobilized HRP. There are reports on dye removal of immobilized HRP between 86 and 99.9% (Bayramoğlu & Arıca 2008; Wang *et al.* 2016). However, a parallel study using calcium alginate gel in order to decolorize acid orange 7 and acid blue 25 reported 75 and 84%, respectively (Gholami-Borujeni *et al.* 2011). Our results showed that crosslinked HRP is very successful in dye removal, which concluded by total removal of AY11 in all cases. Also, the possible effect of the adsorption of AY11 on the alginate beads was considered in order to correctly evaluate the role of HRP action. AY11 adsorption of alginate spheres was tested by experimenting with empty spheres. The experimental results have shown that the dye adsorption of alginate beads is negligible.

Reusability of immobilized HRP

The reusability of the immobilized HRP was assessed by decolorization of AY11 (0.1 mM) and was quantified during 10 cycles of assay. The decolorization of azo dye declined gradually throughout the successive batches for both biocatalysts (Figure 6). The HRP immobilized by crosslinking prior to entrapment maintained its activity throughout the 10 batches, with an AY11 degradation of 75% at the end of the last batch. These superior results pointed out the advanced usage of the immobilized HRP by the suggested method when compared with the previous reports (Alemzadeh & Nejati 2009; Monier *et al.* 2010; Gholami-Borujeni *et al.* 2011). The probable reason for the reduction in phenol removal efficiency might be blockages on the alginate membrane and radical augmentation that cause inactivation of the enzyme.

CONCLUSION

Two immobilization methods were applied on horseradish peroxidase using sodium alginate. The preparation and application of alginate entrapped and glutaraldehyde crosslinked prior to alginate entrapped HRP was analyzed by measuring residual activity, and decolorization of azo dye acid yellow 11 was investigated. The immobilization technique of crosslinking prior to entrapment gave the best results. The optimum temperature of free and immobilized enzyme was found to be 25 °C. Thermal inactivity studies revealed the success of the immobilization technique. Also, the crosslinked HRP was more stable during storage and more effective in dye degradation compared with the free enzyme. Therefore, crosslinking prior to alginate entrapment is suggested as a suitable, practical and cost effective immobilization method in order to obtain highly active, thermostable and reusable HRP. Consequently, the suggested immobilization method brings stability and improved characteristics without decreasing the substrate binding affinity of the HRP. Total decolorization was obtained using crosslinked-entrapped HRP, indicating considerable potential for large scale enzymatic dye degradation.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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