Degradation of azo dye with horse radish peroxidase (HRP)

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Abstract

Azo dyes are recalcitrant carcinogenic compounds and have dermal and immunological effect on human beings. Conventional methods are not effective in the treatment of azo dyes. The oxidation capability of horse radish peroxidase (HRP) and hydrogen peroxide (H_2O_2) on direct yellow 12 dye was investigated and was found to be very effective treatment methodology. HRP was extracted from horse radish roots, and its performance was evaluated in soluble and immobilized form by conducting batch experiments in the presence of H_2O_2. The oxidation of direct yellow-12 dye was tested as a function of HRP at fixed concentration of H_2O_2, and at constant HRP activity (1.8 units/ml). Parameters such as aqueous-phase pH, H_2O_2 concentration, dye and enzyme concentrations were studied.

Keywords: Direct yellow-12 dye, horse radish peroxidase, hydrogen peroxide, immobilization, oxidation.

1. Introduction

Among the chemical classes of dyes, azo dyes are considered to be recalcitrant, non-biodegradable and persistent. Treatment of dye-based effluents is considered to be one of the challenging tasks in environmental fraternity. Dyes are complex aromatic compounds, which are normally used for coloration of various substrates like leather, textiles, papers, etc. They are sometimes fused with heavy metals on the structural interface and are considered to have relatively bad consequence on the surrounding environment due to their toxic and inhibitory nature [1–3]. Dye-based effluents are normally not amenable for conventional biological wastewater treatment due to their recalcitrant and inhibitory nature [4]. Azo dyes are largely resistant to biodegradation and persist in conventional wastewater treatment processes. Chemical contamination of soil and groundwater is widespread and frequent [5]. The electron-withdrawing nature of the azo linkages obstructs the susceptibility of azo dye molecules to oxidative reactions. Only specialized azo dye-reducing enzymes were found to degrade the dyes [6].

1.1 *Bioremediation is a viable tool for restoration of contaminated subsurface environments*

A combination of enzymatic catalysis and electrochemical generation of hydrogen peroxide (H_2O_2), which can be a potential alternative to traditional processes, was developed. The

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enzyme horseradish peroxidase (HRP) is a plant glycohemeprotein [7] and enzyme activity is a cyclic reduction and oxidation of the iron atom in the hematin group [8] in a suitable medium.

The catalytic action of enzymes is extremely efficient and selective compared to chemical catalysts due to higher reaction rates, milder reaction conditions and greater stereospecificity. They can catalyze reactions at relatively low temperature and in the entire aqueous-phase pH range. Though biocatalysts are widely used in several fields, their role in solving the environmental problems has been felt very recently only [9, 10].

Enzymes from various sources (fungus and plant based) are used for the treatment of dye-based compounds [11]. The source of the selected enzyme and its nature along with system conditions are found to have significant influence on the overall performance for pollutant removal. Fungal-extracted enzymes have been studied quite significantly in the process of dye removal [12–15] but plant-based peroxidases for the removal of pollutants are less documented [16–18].

Several limitations prevent the use of free enzymes as the stability and catalytic ability of free enzymes decrease with the complexity of the effluents [19]. For the treatment of phenolic compounds, enzyme catalysis are more efficient. For the removal of 2-chlorophenol, the combination method was more effective than sonolysis and enzyme treatment individually. A comparison between HRP and soybean peroxidase (SBP), the two most widely used commercial peroxidases for the removal of phenol from wastewater, was made [20–21].

In this study, the reaction mechanism of HRP was applied to the transformation of direct yellow-12 dye, which is commonly found in the contaminated aquifers of textile industries. This study is aimed at documenting the capabilities of HRP and H_2O_2 coupled oxidation of direct yellow-12 dye. The effect of parameters such as aqueous-phase pH, H_2O_2 and HRP concentration, and contact time was investigated.

2. Materials and methods

Direct yellow-12 dye ($C_{28}H_{24}O_8N_4S_2Na_2$) was a generous gift by Morparia Industrials Ltd, Hyderabad. It belongs to a chemical class of di-azo and the structure is shown in Fig. 1. The aqueous solution of the dye was prepared prior to the experiments by dissolving the requisite amount of dye in double distilled water.

2.1. *Extraction of HRP*

HRP was extracted from horse radish roots [18]. Roots (500 g) were cleaned and crushed in a wet grinder with the addition of acetate buffer and the extract was centrifuged at 10,000 rpm for 6 min at 4°C. The precipitate was dialyzed using 0.1 M acetate buffer (pH 4.5) at 4°C, by three buffer changes for 3 h.
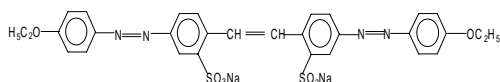

FIG. 1. Chemical structure of direct yellow-12 dye.

2.2. *HRP activity*

HRP activity was assessed by employing 4-amino antipyrene method involving colorimetric estimation using phenol and H_2O_2 as substrates and 4-aminoantipyrene as chromogen [18]. The assay was performed at 25°C by adding phosphate buffer (pH 7.4) containing 1.0×10^2 M phenol, 2.4×0^3 M aminoantipyrene and 2×10^{-4} M H_2O_2. The consumption was estimated by measuring the absorption of the colored product at 510 nm. HRP extracted from horseradish roots was found to contain 1.8 units ml^{-1} of the enzyme after dialysis.

2.2.1. *Quantitative estimation of the dye*

Quantitative estimation of the yellow-12 dye in the aqueous phase was carried out by colorimetry. A solution of 10 mg^{-1} mg/L concentrations was scanned over a range of 190–800 nm by using UV-VIS spectrophotometer and λ_{max} wavelength was determined to be 390 nm. The spectral curve was prepared at maximum wavelength. λ_{max} was used for the estimation of the dye concentration in aqueous phase. After treatment, the sample was centrifuged and the supernatant was assayed for the residual dye concentration.

2.2. *Immobilization of HRP: Calcium alginate gel entrapment and polyacrylamide gel entrapment methods*

Gel entrapment method was used for the immobilization of HRP enzyme. The beads were prepared and cured in 0.2 M $CaCl_2$ for 2 h to permit complete reaction with Ca^{2+} ions. The beads were stored at 4°C in distilled water. Alternatively, polyacrylamide gel entrapment method was applied containing 225 mg of acryl amide monomer and 6.75 mg of bis acryl amide in 0.2 M acetate buffer pH 6.0, to which HRP enzyme was added. To this mixture, 0.25 ml of 5 and 1% ammonium per sulphate solution was added. The mixture was incubated at 30°C for 10 min. The gel was washed thoroughly with cold buffer [22].

2.4. *Removal of yellow-12 dye in aqueous phase by free and immobilized enzyme*

Experiments were conducted at a constant temperature (25°C) by varying process parameters such as pH, dye and HRP concentration, and reaction time. Initially, kinetics were carried out in a series of vials containing 10 mg/L dye, by maintaining aqueous-phase pH at 4.0, HRP concentration at 1.8 units and H_2O_2 dose 1.5 μl/L constant. The reaction mixtures in the vials were kept for agitation on a horizontal shaker at 100 rpm for the requisite concentration in aqueous phase after centrifugation at 5000 rpm for 5 min at 24°C. Each vial was removed at a predetermined time and residual dye concentration in aqueous phase was estimated to determine the optimum contact time. Subsequent series of experiments were performed by varying the aqueous-phase pH between 2 and 10, dye concentration at 5–40 mg/L and H_2O_2 dose between 1 and 3 μl/L to understand the optimum conditions for dye removal by the agitation for optimum contact time. Similarly, kinetic studies were carried out in a series of vials (at 20 mg/L concentration) by keeping aqueous-phase pH at 4.0 and H_2O_2 dose was at 1.5 μl/L using alginate-immobilized HRP enzyme. The reaction mixtures in vials were kept for agitation on a horizontal shaker at 100 rpm for the requisite contact time and the solutions were analyzed for residual dye concentration in aqueous after cen-

trifugation (5000 rpm, 5 min, 24°C). Each vial was removed at a predetermined time and residual dye concentration in aqueous phase was estimated to know the optimum contact time for the alginate-immobilized enzyme. Subsequent series of experiments were performed by varying the pH from 1 to 10, dye concentration 5–40 mg/L and H_2O_2 dose 1–3 μl/L to understand the optimum conditions for dye removal by keeping the agitation for the optimum contact time using the alginate-immobilized HRP and acryl amide-immobilized HRP.

3. Results and discussion

3.1. *Removal of direct yellow-12 dye in aqueous phase by free and immobilized enzyme*

3.1.1. *Optimum contact time*

Initially, experiments were conducted to assess the optimum contact time required for dye removal. To a series of vials containing 5 ml of dye solution (10 mg/L), 1.8 units of enzyme 1.5 μl/L of H_2O_2 were added and the reaction mixture (24°C, pH 4) was agitated for 2 h 15 min. At every 15 min interval, one vial was removed and analyzed for the residual dye concentration. From Fig. 2(a), it is evident that 1 h 45 min of contact time is the optimum duration for maximum dye removal. After this period, negligible dye removal was noticed up to remaining 1 h 35 min. Subsequent experiments were performed for 2 h 15 min.

3.1.2. *Optimum pH*:

The enzymes have an optimum pH range at which their activity is maximum. The studies were carried out on the yellow-12 dye by varying aqueous-phase pH of the reaction mixture between 2 and 10 by keeping the dye concentration at 10 mg/L, enzyme concentration at 1.8 units, H_2O_2 dose at 1.5 μl/L, reaction temperature at 24°C and the contact time (1 h 45 min) constant. Variation of dye removal at various pH values is depicted in Fig. 2(b). About 70% of the dye was removed due to HRP-catalyzed reaction at an aqueous-phase pH of 4. Subsequently, the dye removal dropped significantly from pH 5 to 8 and the same trend continued up to an aqueous phase of pH 10. Aqueous phase of pH 4 resulted in higher HRP activity compared to other pH ranges from 3 to 9.

3.1.3. *Optimization of co-substrate concentration*

Hydrogen peroxide acts as a co-substrate to activate the enzymatic action of peroxide radical. It contributes in the catalytic cycle of peroxidase, to oxidize the native enzyme to form an enzymatic intermediate which accepts the aromatic compound to carry out its oxidation to form a free radical form. Experiments were carried out to find out the optimum H_2O_2 concentration required to bring out the conversion of dye by varying the H_2O_2 dose from 1 to 3 μl/L in the reaction mixture by keeping all the other experimental conditions constant (dye concentration 10 mg/L; temperature 24°C; enzyme concentration 1.8 units; reaction time 1 h 45 min). The studies were conducted in a series of vials. The results obtained are presented in Fig. 2(c) relating dye removal with the function of H_2O_2 concentration. Maximum dye removal was seen at H_2O_2 concentration of 2 μl/L and similar degradation was seen with 2.5 and 3 μl/L; therefore, 2 μl/L was taken as the optimum H_2O_2 dose for dye removal [5].
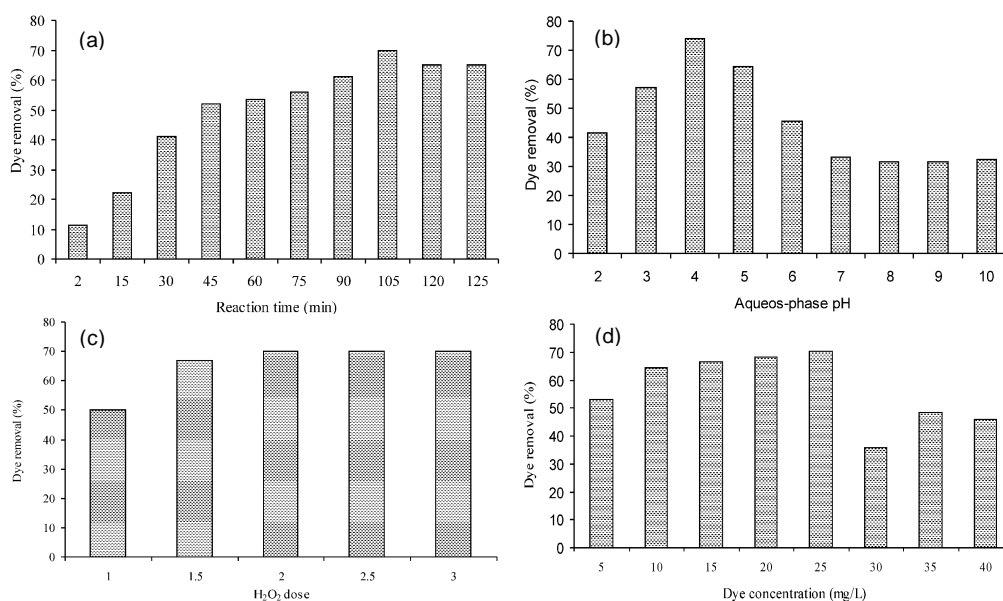
FIG. 2. The impact of (a) reaction time (b) pH (2–10), (c) co-substrate concentration H_2O_2 ($\mu l/1$), and (d) concentration of substrate on the removal of direct yellow-12 dye in the presence of HRP enzyme (1.8 units/ml).

3.1.4. *Optimization of substrate concentration*

The concentration of the substrate present in the aqueous phase has significant influence on any enzyme-mediated reaction. If the amount of enzyme concentration is kept constant and the substrate concentration is gradually increased the reaction will increase until it reaches maximum. After obtaining the equilibrium state any further addition of the substrate will not change the rate of reaction. Studies were carried out at different concentrations of the dye, i.e. 5–40 mg/L, keeping the other parameters constant. The results indicate that any increase in dye concentration above 25 mg/L resulted in relatively low dye removal (Fig. 2(d)). This is acknowledged to be the cut-off concentration of the dye for the optimum removal at the specified experimental conditions (Fig. 3) to retain the activity of HRP enzyme, and to protect the protein from denaturation.

FIG. 3. Impact of HRP on the removal of direct yellow-12 dye. A: Direct yellow-12 dye; B: Reaction mixture; C: End result.
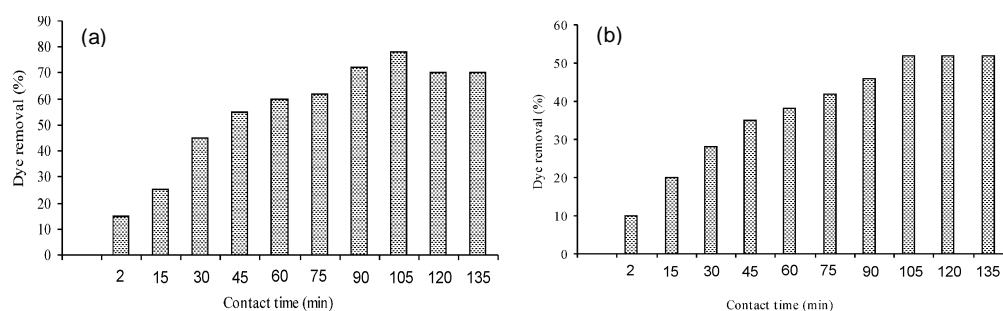
FIG. 4. The impact of contact time for the removal of direct yellow-12 with (a) acryl amide-immobilized HRP, and (b) alginate-immobilized HRP.

3.2. *Effect of immobilization on HRP enzyme activity*

Application of free enzyme in industrial process is not economically viable, while immobilization/entrapment of enzyme results in repeated application. In the present study, two types of polymeric materials—alginate and acryl amide—have been used to study their relative efficiency in dye removal for the entrapment of peroxidase.

Normally, enzyme immobilization is expected to provide stabilization effect [23] restricting the protein unfolding process as a result of the introduction of random intra- and intermolecular crosslinks. Zille *et al.* [19] reported less availability of the enzyme for interaction with anionic dyes due to the immobilization in a particular matrix.

Experiments were carried out separately with both entrapped HRP at a dye concentration of 25 mg/L (H_2O_2 dose 2 μl/L; pH 4; temperature at 24°C, contact time 1 h 45 min). Experimental results are shown in Fig. 4 for alginate- and acryl amide-immobilized HRP enzyme. Acryl amide gel was more efficient in dye removal compared to alginate matrix. About 78% of dye removal was observed with acryl amide gel-immobilized beads, while with alginate matrix it was only 52%. Gel-immobilized HRP was effective in dye removal compared to free HRP (69%), and alginate-immobilized HRP showed inferior performance. The objective of immobilization is the reusability of the matrix in the process.
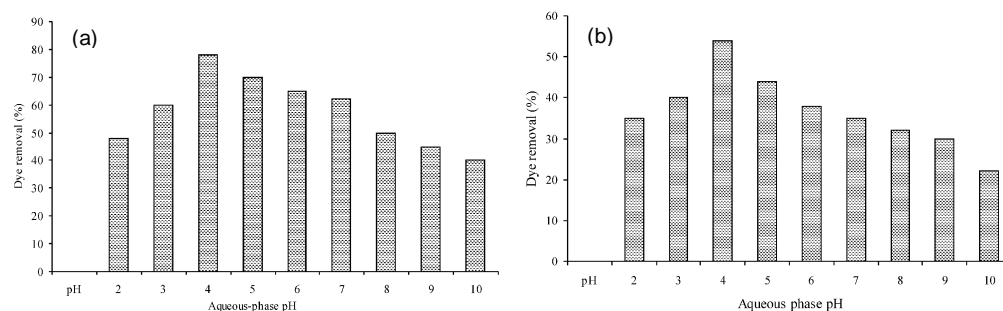

FIG. 5. The impact of pH on the removal of direct yellow-12 with (a) acryl amide-immobilized HRP, and (b) alginate-immobilized HRP.

Effect of aqueous-phase pH on the enzyme-catalyzed degradation with both types of immobilized HRP was studied (pH 2 to 10). The results showed that increase in pH after pH 4 resulted in decrease in the dye removal capacity for both types of the entrapped matrices studied. About 78 and 54% of the dye removal was observed at an aqueous phase of pH 4 for acryl amide and alginate-entrapped beads (Fig. 5). This observation correlates with the performance of free HRP. The relative inferior performance of alginate-immobilized HRP compared to acryl amide may be due to lesser availability of the peroxidase structure to the dye molecule in the alginate mix compared to acryl amide. The effective performance of acryl amide-entrapped beads may be also attributed to the nonionic nature of the beads, which results in minimum modification of the enzyme properties and unaffected nature of the charged substrate as well as product diffusion.

4. Conclusions

The electron-withdrawing nature of the azo linkages obstructs the susceptibility of azo dye molecules to oxidative reactions. Only specialized azo dye-reducing enzymes were found to degrade azo dyes. The results of this study proved that the use of an enzymatic treatment process is a viable approach for the degradation of azo dyes from aqueous solutions. In addition, when direct yellow-12 dye was reduced using the enzymatic method, the oxidation capacity increased with increasing concentrations of HRP and H_2O_2 at pH 4. Gel/alginate-based enzyme immobilization reduced the azo dye. The immobilized enzyme beads were further used two–three times for the removal of the same dye with lower efficiency.

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