Application of immobilized horseradish peroxidase for removal and detoxification of azo dye from aqueous solution

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Abstract

The development and application of an immobilized horseradish peroxidase (HRP) on calcium alginate gel beads are reported for the successful and effective decolorization textile industrial effluent. The optimal condition for the immobilization of HRP on calcium alginate gel beads which leads to a specific activity of 15 U/g calcium alginate was identified. Upon immobilization, considering enzyme encapsulation efficiency, retention activity and enzyme leakage of the capsules, the best gelation condition was found to be 2 % w/v of sodium alginate solution and 2 % w/v of calcium chloride hexahydrate. Upon immobilization, pH profile of enzyme activity changes as it shows higher value at basic and acidic solution. The capsules were reusable up to ten cycles but decreased in their efficiency. The ratio of hydrogen peroxide/dye at which highest dye removal occurs is 1.25. Optimum conditions for removal of AO-7 and AB-25 were: 90 min, reaction time; 0.8 units/g, enzyme concentration for 500 ADMI colore, ratio of H_2O_2 /dye, 1.25/1; temperature, 25-50°C that cause 75% and 84% removal in AO-7 and AB-25 solution respectively. There was a significant reduction in toxicity of dye solution after the enzymatic treatment.

Keywords: Azo dye, Decolorization, Enzyme, Horseradish peroxidase, Immobilization.

Introduction

Dye wastewater from textile and dyestuff industries is very difficult to treat. Synthetic dyes, classified by their chromophore as azo, anthraquinone, triphenylmethane, heterocyclic or phthalocyanine dyes, are very stable and resistant to microbial attack and therefore it is difficult to remove them from effluents by conventional biological processes[1]. Decolorization of industrial dyes can be achieved by physico-chemical methods, such as adsorption, precipitation or chemical degradation, but these are very expensive, which limits their application[2]. The decolorization of the dyes through intermediates of different color has been proposed to be a series of multiple reactions. In case of anaerobic decolorization of azo dyes by some bacteria, arising intermediates and final products are carcinogenic and mostly more toxic than the starting dyes. In recent years, the utilization of biodegradative abilities of some white rot fungi seems to be promising[1, 3]. Owing to their extracellular nonspecific free radical-based enzymatic system, they can completely eliminate a variety of xenobiotics, including synthetic dyes, giving rise to non-toxic compounds[4, 5].

Enzymes can act on specific recalcitrant pollutants to remove them by precipitation or transformation to other (innocuous) products and also can change the characteristics of a given waste to render it more amenable for treatment[6]. 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[7]. They can catalyze reactions at relatively low temperature and in the entire aqueous phase pH range. Though much attention has been paid in the utilization of biocatalysts in several fields, their involvement has been felt very recently in solving the environmental problems[8, 9]. Horseradish peroxidase (HRP) is a promising candidate for industrial application due to its low cost, obtaining convenience, stability, broad substrate specificity and tolerance to wide ranges of pH and temperature[10]. Horseradish peroxidase has been isolated from many species of plants, animals, and microorganisms[11].

These enzymes are able to act on a variety of aromatic compounds in the presence of hydrogen peroxide. Several limitations prevent the use of free enzymes as the stability and catalytic ability of free enzymes decrease with the complexity of the effluents[12]. Some of these limitations are overcome by the use of enzymes in immobilized form which can be used as catalysts with long lifetime[12, 13]. Immobilization with different polymeric materials is studied for enzyme encapsulation along with their application in treatment of various pollutants[12]. However, appropriate selection of encapsulation material specific to the enzyme and optimization of process conditions is still under investigation.

This communication reports results pertaining to systematic evaluation of hydrogen peroxidase oxidoreductase extracted from horseradish (EC 1.11.1.7)

also called as horseradish peroxidase in the process of acid azo dye (Acid Orange 7 and Acid Blue 25) removal. Effect of parameters such as aqueous phase pH, H_2O_2 and HRP concentration, contact time, repeated application of immobilized HRP and dye concentration has been investigated to optimize the system conditions. Also, evaluation of immobilized HRP (in calcium alginate) performance in the process of dye removal was evaluated in order to study its reusability.

Material and Methods

Material: Aqueous solution of hydrogen peroxide (30% w/v, specific gravity=1.12), 4-aminoantipyrine, HRP from Sigma-Aldrich. AO-7 and AB-25 were purchased from ALVAN SABET (color manufacture in Iran). All other chemicals used were of analytical grade.

Enzyme immobilization

Sodium alginate was dissolved in reagent water. For dissolving sodium alginate in water a backer equipped with a magnetic stirrer was used. Dissolving process was so slow that it took up to 5 hours for preparing a 1-4 % w/v of alginate gel. For expelling air bubble, occasional mixing was performed with a glass rod. After dissolving sodium alginate, the gel was placed in the room temperature followed by continuous stirring to obtain a homogenous gel. The gel was being stored in 4 °C for further usage. Calcium alginate capsules were prepared by extrusion using a simple one- step process similar to that described by Nigma et al.[14] Pre-determined enzyme was dissolved in 10 mL calcium chloride solution and was dropped through a silicon tube, using a peristaltic pump, into 100 mL of alginate solution. The sodium alginate solution was maintained under constant stirring (200 rev/min) using a magnetic stirrer situated at the bottom of the backer, in order to avoid the droplets sticking together and minimize the external mass transfer resistance. 10 cm dropping height was chosen to obtain spherical capsule. After 20 min gelation time, the capsule was removed by dilution of alginate solution to 5 times with distilled water followed by filtration of capsules. The average capsule's diameter was 2-3 mm.

Protein determination: The amount of protein initially offered, in the wash liquid after immobilization and also the protein content in capsule after leakage test were obtained by Lowry's procedure as modified by Peterson[15].

Enzyme encapsulation efficiency: To assess the enzyme encapsulation efficiency, it was necessary to measure HRP concentration both in calcium chloride solution and capsule. To measure the encapsulated enzyme concentration, capsules were cut in half and put in 5 mL phosphate buffer (pH=7.4) solution. The concentration of protein in buffer was measured according to the Lowry's assay after 2 hours in order to obtain encapsulated protein[15]. The percentage of encapsulated enzyme was obtained from the difference between initial protein introduced to the calcium chloride hexahydate solution and encapsulated protein measured as mentioned above.

Enzyme leakage: Enzyme leakage measurement was carried out by placing capsules in a test tube filled with tris buffer (pH=8.0) for 18 hours. Then the capsules were removed, cut in half and put in phosphate buffer (pH=7.4) solution. The protein concentration was measured according to the Lowry's assay and the leakage percentage was calculated from the differences between encapsulated protein at the beginning of time interval and the value found according to the above procedure [16].

Peroxidase Activity assay: HRP enzyme activity was measured using phenol, 4-aminoantipyrine (4-AAP) and hydrogen peroxide as substrates. The approach was to provide all components except enzyme near saturation concentration so that the initial rate of reaction became directly proportional to the amount of enzyme present. The assay mixture contained 2.5 ml 9.6 mM of 4-AAP, 1 ml 100 mM of phenol, 1 ml 2 mM of hydrogen peroxide, 4.5–5.0 ml 100 mM of phosphate buffer pH=7.4 and 0.5–2g enzyme. The rate of reaction was measured by monitoring the rate of formation of the products which absorbed light at a peak wavelength of 510 nm upon addition of the enzyme; thus, one unit of activity (U) used in this study is defined as the number of mM peroxide converted per min at pH=7.4 and 25˚C [11, 16- 18].

Experimental procedure: Experiments were conducted in a stirred batch reactor of 50 ml total volume at room temperature. Substrates, azo dye and hydrogen peroxide, were added to the reactor as aqueous solutions, and immobilized derivative as a suspension in phosphate buffer 0.1 M pH 7.4. The same buffer was used to complete the reactor volume, giving a final buffer concentration of 70 mM. First, azo dye and the buffer solutions were placed in the reactor. When room temperature was reached (some solutions were stored in a refrigerator), the enzyme suspension and finally, hydrogen peroxide solution was added. Experiments were carried out at different azo dye, hydrogen peroxide and immobilized enzyme concentrations. The reaction course was followed by taking 5 ml samples. Aliquots were removed every 5 min, being centrifuged at 6000 rpm and submitted to analytical control. The reaction was being allowed to progress until this value remained constant.

Analytical process: AO-7 and AB-25 that are used for decolorization experiments were determined by American Dye Manufactures Institute (ADMI) method (according to the APHA). The ADMI color value for each sample was measured via the following procedure: sample pH adjustment; centrifuge (6000 rpm); spectrophotometer; and ADMI measurement. For the pH adjustment step, two different pH values were chosen: original (ambient) pH and pH=7.6[19]. For the spectrophotometer step, Hach DR 5000 was used. For the ADMI measurement step, 31 wavelength

ADMI color values were measured according to the APHA method. There is linear correlation between ADMI and concentration of AO-7 and AB-25 by the following equations:

$$ADMI = 42200X + 22 \text{ and}$$
$$ADMI = 47530X + 4.5$$

respectively. In this equation X is concentration of dye as g/l.

Acute toxicity test with *Daphnia Magna:* The acute toxicity tests with *Daphnia Magna* were carried out according to the ABNT norms. The sensitivity tests were carried out with young organisms (6–24 h of life), which were not fed during the test period. For each concentration 10 organisms were used in a 25 ml beaker, in duplicate for each concentration, along with the controls with the dilution water (basic medium). The acute toxicity tests with the effluent samples had duration of 24-96 h, and after this time of exposure the number of immobile organisms was observed and noted. The organisms were considered immobile, if they did not show any mobility during 20 s of observation [20-22]. In order to calculation of LC_{50}, probit analysis with SPSS (ver 11.5) was applied.

Results and Discussion

Different concentrations of sodium alginate and sodium chloride (1-4% w/v) respectively were used to obtain the optimal condition for immobilization of HRP. In order to find these concentrations two factors were taken into consideration: enzyme leakage and encapsulation efficiency. The results are shown in figs 1 and 2. According to results the best biocatalytic properties including lower enzyme leakage and higher enzyme encapsulation achieved when the calcium chloride and sodium alginate solution were 2 % w/v. By selecting these concentrations, encapsulation efficiency increases to 90% and leakage decreases to lower than 8 %.The enzyme concentration of these immobilized HRP was measured according to previous described method was15 units/g.

Effect of pH: The variations of residual activity of free and immobilized HRP with pH are shown in fig. 3. The immobilized HRP was stable in the pH 7.4 with over 90% residual activity after incubating for 90 min at 25 °C, also the free HRP was stable in the pH with over 85%. This indicated that the immobilization appreciably improved the stability of HRP.

Thermal stability of HRP: For survey of thermal stability of free and immobilized HRP, these enzyme were incubated at 20-80°C for 90 min; the thermal stability of the free and immobilized HRP in terms of relative activities are compare in Fig. 4. Results show that relative activity of immobilized HRP at 40-70°C is more than free enzyme. Relative activities of free and immobilized HRP were decreased after incubation at above of 30 and 50 °C respectively and at 80°C relative activity of to source enzymes were decreased more than 85%. These results indicated that immobilization led to a significant effect on thermal stability of enzyme.

Reusability: The immobilized enzyme could be easily removed and assessed for its remained catalytic activity. To demonstrate the reusability of encapsulated enzyme, capsules were separated after 90 min of reaction time and then rinsed thoroughly with distilled water. The capsules were used for subsequent batches. After 4 times of the repeated test, the AO-7 and AB-25 removal efficiency was reduced to higher than 82 % of its initial value. These results are shown in Fig. 5.

Effect of reaction time: Experiments were performed in order to assess the optimum reaction time required for the dye removal by application of immobilized HRP. Fig. 6 shows the effect of reaction time on dye removal by immobilized HRP. It is evident from the figure that 90 min of the reaction time is sufficient for Acid Orange 7 (AO-7) and Acid Blue 25 (AB-25) removal efficiency of 80 and 70% respectively. However, Mohan et al[8] reported that 45 min of reaction time was required for HRP catalyzed Acid Black 10 BX removal. After 90 min reaction time, the removal reaction followed by a very slow removal process. This slowdown can be attributed to the simultaneous decrease in concentration of reactants. Subsequent experiments were conducted for 90 min of reaction time. The price of HRP was very high. The cost of enzyme had always been the bottleneck of application of enzymatic process on the treatment of wastewater. Thus, one could increase the reaction time to offset the reduction in decolorization efficiency at low enzyme concentration.

Effect of enzyme concentration: Normally removal of dye is dependent on the amount of catalyst added. To study the effect of enzyme concentration on dye removal, five different enzyme concentrations were used to compare the efficiency of encapsulated enzyme. The dyes and hydrogen peroxide concentrations along with the physical condition of reaction remained unchanged (dye concentration, 500 ADMI; H_2O_2, 0.8 mmol/l, pH 7.4; enzyme concentration, 0-2 units/g alginate). Fig. 7 depicts the effect of enzyme dose on initial dye concentration and differs from case to case. It is found that for a 500 ADMI of each colore solution, increasing enzyme concentration from 0 to 0.8 units/g results in gradual increase in dye removal and after that nearly remained constant. Therefore, the remained dye concentration, which is the difference between initial dye and removal dye, decreases. Further increases in enzyme concentration have no significant effect on dye removal. The enzyme concentration of 0.8 units/g was found to be the optimal dose for the experiment condition.

Effect of hydrogen peroxide concentration: In order to determine the effect of H_2O_2 concentration on AO-7 and AB-25 removal, 90 min contact time and 0.8U/g of

immobilized HRP and H_2O_2/dye was varied between 0.5 and 2.25 at room temperature (25°C). Some authors introduced an optimal molar ratio of hydrogen peroxide to dye resulting in higher removal efficiency. The optimum peroxide concentration is totally depends on initial dye concentration and differ from case to case. The behavior of the dye removal efficiency was similar in too dye types. First, the amount of dye removed was sharply increased with an increase in hydrogen peroxide up to an optimal point. It shows that hydrogen peroxide is a limiting factor in this range. Second, after dye conversion reached its optimum point adding hydrogen peroxide significantly reduced the conversion. A reason for this phenomenon would be that an excess amount of hydrogen peroxide results in higher concentrations of intermediate products which inhibit the activity of enzyme and/or that enzyme is inactivated by an excess of hydrogen peroxide. The deviation of the aforementioned ration might be the result of polymer produced in the catalytic process larger than dimmer.

Acute toxicity test: The toxicity study of the raw dye solution and the effluent after the enzymatic treatment was carried out with the purpose of evaluating whether the reaction products would be less toxic than the raw dye solution.

In the present study, toxicity was evaluated at concentrations of 10-30 mg/l for AO-7 and AB-25 to determine LC_{50} ranges. Also, control solutions (dye concentration of 0.0 mg/l) were conducted to confirm the accuracy of the test and after treatment at optimum condition, toxicity of the effluent solution was evaluated.

The results indicate that the LC_{50} of AO-7 and AB-25 for *Daphnia magna* are about 5.22 and 6.15 mg/l at 24h, 3.73 and 4.87 mg/l at 48h and for 96 h tests were 2.94 and 3.85 mg/l respectively. But after treatment of AO-7 and AB-25 by immobilized HRP this process can significantly decrease toxicity of AO-7 and AB-25, LC_{50} of AO-7 and AB-25 after treatment for *Daphnia magna* are about 11.32 and 12.3 mg/l at 24h, 9.12 and 11.25 mg/l at 48h and for 96 h tests were 26.71 and 11.35 mg/l respectively. This means, application of this process to detoxification of AO-7 and AB-25, significantly decreased the toxicity of solution. LC_{50} of the AO-7 and AB-25 before and after degradation with immobilized HRP are presented in figure 9. The enzyme HRP, in the immobilized form, was shown to be effective for the decolorization of textile dyes and effluents, as well as for achieving a reduction in the toxicity of the effluent after the enzyme treatment.

Conclusion

In this study, HRP was immobilized on alginate beads in order to study the use of this enzyme for removal of dye compounds from aqueous solutions. In immobilization process, highest activity of HRP was taken when the calcium chloride and sodium alginate solution was 2 % w/v. By selecting these concentrations, encapsulation efficiency increases to 90% and leakage decreases to lower than 8 %.The enzyme concentration of these immobilized HRP was 15 units/g. The immobilized HRP was stable in the pH 7.4 with over 90% residual activity after incubating for 90 min at 25 °C. This indicated that the immobilization appreciably improved the stability of HRP. Activity of immobilized HRP at 40-70°C is more than free enzyme which means immobilization led to a significant effect on thermal stability of enzyme. Optimum conditions for removal of AO-7 and AB-25 were: 90 min, reaction time; 0.8 units/g, enzyme concentration for 500 ADMI colore, ratio of H_2o_2 /dye, 1.25/1; temperature, 25-50°C. Application of this process to removal of AO-7 and AB-25 significantly decreased the toxicity of solution. The enzyme horseradish peroxidase showed a good decolorization of textile dyes.

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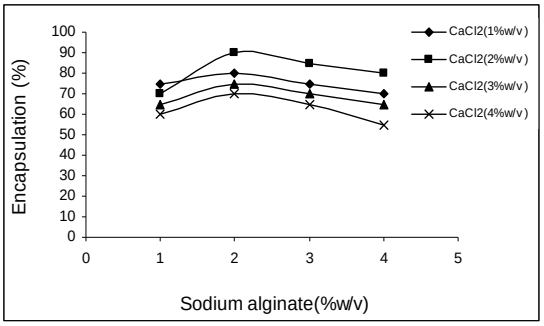

Fig. 1: Encapsulation efficiency, under different gelation condition, T= 25°C

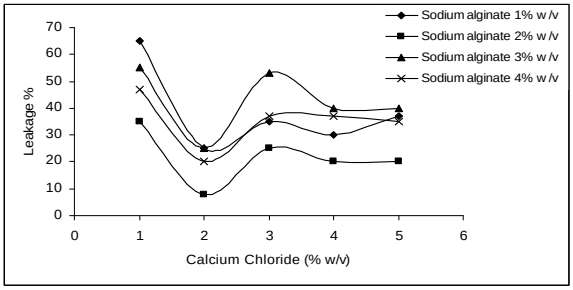

Fig. 2: Leakage percentage under different sodium alginate and calcium chloride condition

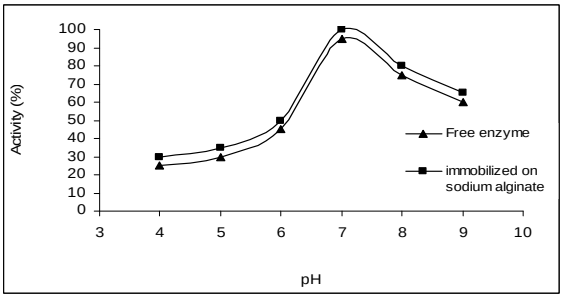

Fig.3: Effect of pH on the Activity of free and immobilized HRP

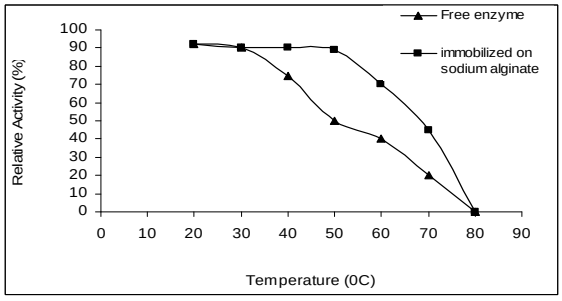

Fig. 4: The effect of temperature on relative activity free and immobilized Alginate. Conditions: enzyme concentration, 1 units/g alginate; 0.8 mmol/l, H_2O_2; buffer phosphate, (pH=7.4); reaction time, 90 min; 20-80 °C.

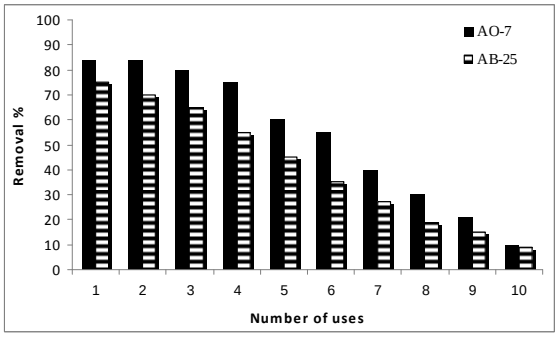

Fig.5: Reusability of capsules (cycles), Conditions: AO-7 and AB-25 concentrations, 500 ADMI; enzyme concentration, 1 units/g alginate; 0.8 mmol/l, H_2O_2; buffer phosphate, (pH=7.4); reaction time, 90 min at 25 °C.

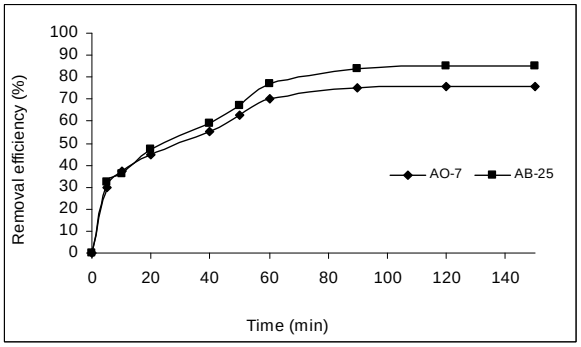

Fig.6: Effect of reaction time on dye removal by immobilized HRP. Conditions: dye concentration, 500 ADMI; enzyme concentration, 1 units/g alginate; 0.8 mmol/l, H_2O_2; buffer phosphate, (pH=7.4); reaction time, 90 min at 25 °C.

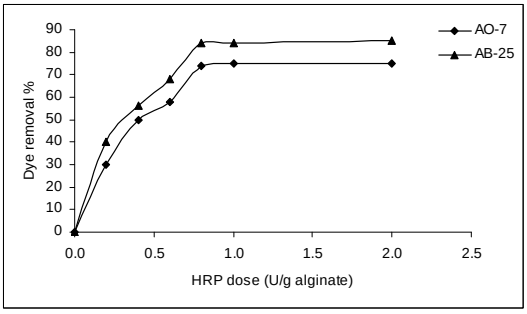

Fig. 7: Effect of immobilized HRP dose on dye removal. Conditions: dye concentration, 500 ADMI; enzyme concentrations, 0-2 units/g alginate; H_2O_2 , 0.8 mmol/l; buffer phosphate, (pH=7.4); reaction time, 90 min at 25°C.

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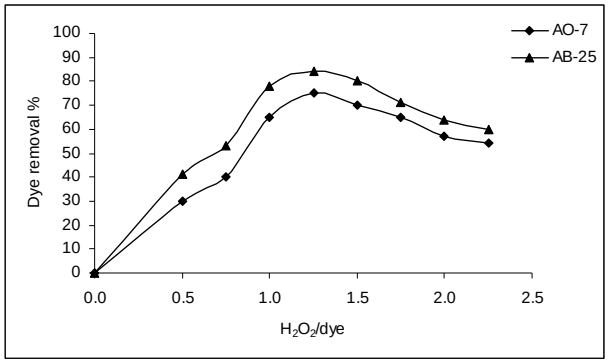
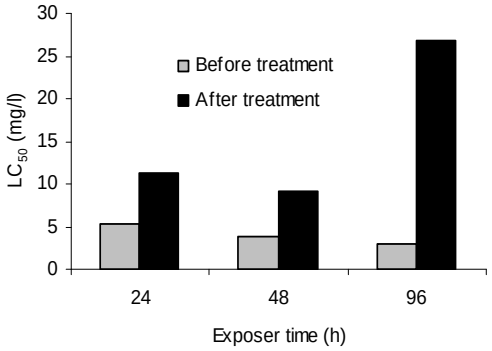
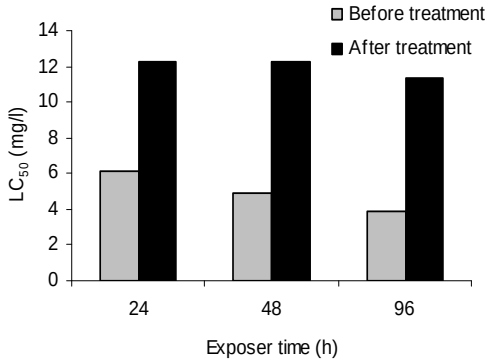


Fig. 8: Effect of hydrogen peroxide concentration on maximum conversion reached at three different dye concentrations (500-1500 ADMI).



(a)



(b)

Fig.9: Results for *Daphnia magna* exposed to the raw and after enzymatic treatment of AO-7 (A) and AB-25 (B).

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