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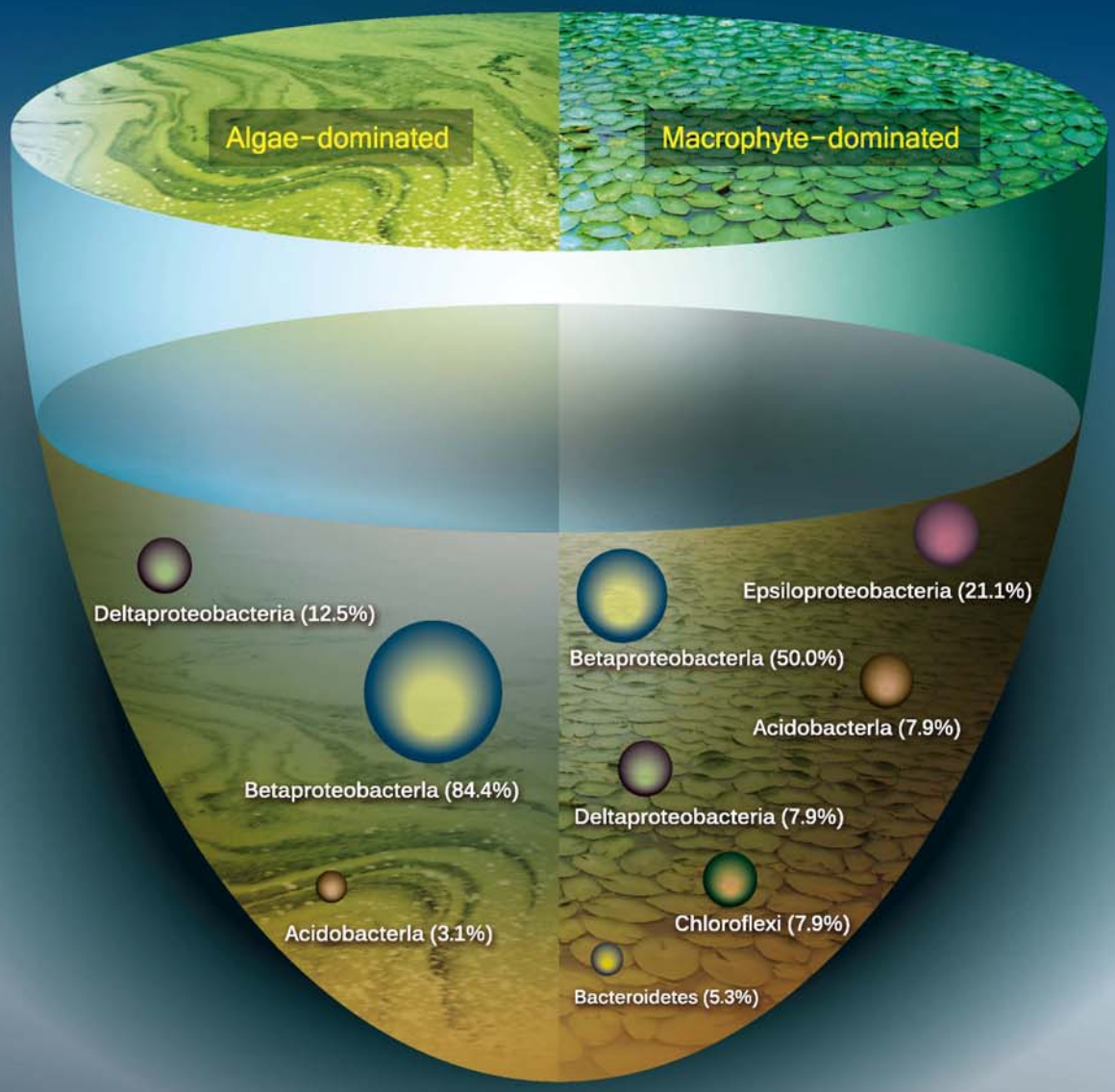

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Degradation of direct azo dye by *Cucurbita pepo* free and immobilized peroxidase

Nabila Boucherit[1,2], Mahmoud Abouseoud[2,3,*], Lydia Adour[1,4]

1. Université Mouloud Mammeri, Tizi-Ouzou (15000), Algerie
2. Laboratoire de Biomatériaux et de Phénomènes de Transports, Université Dr. Yahia Fares, Médéa (26000), Algerie
3. Laboratoire de Génie de la Réaction, Faculté de Génie Mécanique et Génie des Procédés, Université Houari Boumediene, Bab Ezzouar, Alger (16111), Algerie
4. Laboratoire des Biotechnologies Environnementales et Génie des Procédés, BIOGEP, ENP, El-Harrach (16000), Algerie

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Abstract

Enzymatic decolourization of the azo dye, Direct Yellow (DY106) by *Cucurbita pepo* (courgette) peroxidase (CP) is a complex process, which is greatly affected by pH, temperature, enzyme activity and the concentrations of H_2O_2 and dye. Courgette peroxidase was extracted and its performance was evaluated by using the free-CP (FCP) and immobilized-CP (ICP) forms in the decolourization of DY106. Immobilization of peroxidase in calcium alginate beads was performed according to a strategy aiming to minimize enzyme leakage and keep its activity at a maximum value by optimizing sodium alginate content, enzyme loading and calcium chloride concentration. The initial conditions at which the highest DY106 decolourization yield was obtained were found at pH 2, temperature 20°C, H_2O_2 dose 1 mmol/L (FCP) and 100 mmol/L (ICP). The highest decolourization rates were obtained for dye concentrations 50 mg/L (FCP) and 80 mg/L (ICP). Under optimal conditions, the FCP was able to decolorize more than 87% of the dye within 2 min. While with ICP, the decolourization yield was 75% within 15 min. The decolourization and removal of DY106 was proved by UV-Vis analysis. Fourier transform infrared (FT-IR) spectroscopy analysis was also performed on DY106 and enzymatic treatment precipitated byproduct.

Key words: *Cucurbita pepo*; peroxidase; immobilization; decolourization; azo dye

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Introduction

Wastewater effluents from different industries including dyeing, textile, dye manufacturing, leather, cosmetics, food processing and paper are considered as main sources of dye pollution (Bhatnagar and Jian, 2005). There are more than 10^5 kinds of commercially available dyes with over 8×10^5 metric tons of dye stuffs is lost and released to industrial effluents (Palmieri et al., 2005). Nearly a half of all known dyestuffs are azoic type, which makes them the most abundant group of synthetic dyes (Selvam et al., 2003). These dyes can be hardly degraded in the environment because of their resistance to the oxidizing agents, light and water due to their chemical structure (O'Neill et al., 1999). The decolourization of azo dyes by microorganisms usually starts by reductive cleavage of azo bond under anaerobics conditions leads to the formation of aromatic amines which may are toxics on microorganisms (Gottlieb et al., 2003). Even though, the physical and chemical methods have been applied in most studies (Robinson et al., 2001; Lin and Chen, 1997; Zhang et al., 2009), they present some drawbacks of being economically unfeasible, and being unable to completely remove the recalcitrant azo dyes and/or their organic metabolites, generating an important amount of sludge that may cause secondary pollution problems (Forgacs et al., 2004; Zhang et al., 2004). One approach that is more advantageous is the use of enzyme-based methods, which generate compounds with lower toxicity and a minimal impact on ecosystems. They also present some other interesting properties as low energy requirements, easy process control and operation over a wide range of pH, temperature and ionic strength. Furthermore, enzymes are active in the presence of high concentrations of organic solvents in which hydrophobic molecules are soluble. In addition, enzyme-based treatments used alone could be sufficient when the enzymes transform toxic compounds to less harmful products. In this case, complete degradation of the contaminants is

* Corresponding author. E-mail: aseoud2002@yahoo.fr

therefore not necessary (De Souza et al., 2007; Khouni et al., 2011; Husain, 2006; Shaffiqu, 2002). Peroxidases are among the oxidative useful enzymes in wastetreatment. Among the heme-containing peroxidases, those from plants, fungi, and bacteria share similar amino acid sequences and similar catalytic activities, but are slightly different in terms of substrate specificities and subsequent reaction products (Welinder, 1992). Removal of complex azo dyes by peroxidases from plants such as horse radish horse radish, turnip and bitter gourd, was thoroughly studied (Bhunia et al., 2001; Shaffiqu et al., 2002). Peroxidase can act on the soluble azo dye substrate in the presence of hydrogen peroxide, leading to a non soluble product which can be easily removed by precipitation or filtration. The high commercializing costs for applying horse radish led to the search for alternative cheaper sources of plant PODs to substitute HRP in various applications. Crude or purified extracts of several vegetables such as peach (*Prunus persica*), yam (*Alocasia macrorhiza*), manioc (*Manihot utilissima*), artichoke (*Cynara scolymus* L.), sweet potato (*Ipomoea batatas* (L.) Lam.), turnip (*Brassica campestre* ssp. rapifera), and zucchini (*Cucurbita pepo*) were also investigated as the source of peroxidase. Among those, zucchini (*Cucurbita pepo*) peroxidase represents one of the highest activities, thermal and chemical stabilities (Ghaemmaghami et al., 2010; Neves et al., 2012).

Enzyme recovery and substrate inhibition which are the major constraints in enzymatic process development could be easily overcome by immobilization on various supports (Akhtar et al., 2005b; Mohan et al., 2005; Shakeri and Shoda, 2010). Immobilization into calcium alginate beads represents however several advantages such as high support porosity and chemical stability, with a mild, fast, simple and low cost immobilization method (Alemzadeh and Nejati, 2009; Taqieddin and Amiji, 2004; Mohan et al., 2005).

The purpose of the present study, was firstly, to extract a peroxidase from fruit organs of *Cucurbita pepo*, then optimize its immobilization in beads of calcium alginate. Secondly, we attempted to apply the free and immobilized form of peroxidase on a Direct Yellow (DY106) dye solution from the class of azo dye. Effects of parameters such as: aqueous phase pH, temperature, substrates and enzyme concentrations, contact time, have been investigated to optimize the reaction conditions. The reusability of immobilized enzyme in repeated batch processes was also tested. Products of enzymatic transformation were analyzed by Fourier transform infrared (FT-IR) and compared with DY106 dye.

1 Materials and methods

1.1 Chemicals

Direct azo dye: C.I. Direct Yellow 106 (DY106), from stilbene azo dye, was provided by SOITEX (textile manufacturing unit located to Tlemcen, in Algeria and which was purchased from Ciba Colors Ltd. The DY106 was chosen since it was widely used to color cotton and silk.

Acetone, hydrogen peroxide, calcium chloride were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All others chemicals were of analytical grade and were used without further purification.

C-Peroxidase was extracted from fresh vegetal courgette which was collected from local market.

1.2 Methods

1.2.1 Acetone precipitation of courgette peroxidase

Courgette (100 g) was homogenized in a blender with 200 mL of 50 mmol/L of cold phosphate buffer pH 7. The extract was filtered through multi-layers of cheese cloth. The obtained solution is considered as a crude courgette peroxidase (C-peroxidase). The filtrate thus obtained was subject to acetone precipitation by drop wise addition under permanent agitation at 0°C. The precipitate was collected by centrifugation at 4000 r/min for 30 min at 4°C. The obtained precipitate was redissolved in 100 mL of 50 mmol/L of phosphate buffer pH 7 (Şimşek and Yemenicioğlu, 2005). This solution was considered as a partially purified C-peroxidase.

1.2.2 Entrapment of C-peroxidase in alginate gel

Peroxidase immobilization was realized by entrapment in calcium alginate (Ca-alginate) beads according to the method described by Nigma et al. (1988). The optimization strategy was similar to that described by other authors (Won et al., 2005; Cheirsilp et al., 2009; Ozyilmaz and Gezer, 2009). The aim was to retain maximum enzyme at the highest activity with minimum leakage. Sodium alginate was dissolved in 10 mL of enzymatic solution containing (2.53 ± 0.08) UI/mL, and kept under magnetic stirring. The solubilization process lasted 2 hours to prepare different alginate/enzyme solutions (1%, 2%, 3% and 4% (W/V)). Finally, the gel mixture was dropped through a syringe into 50 mL of calcium chloride ($CaCl_2$) solution (100, 200 and 300 mmol/L). The mixture was kept under permanent magnetic agitation 250 r/min for 2 hr (Alemzadeh and Nejati, 2009), forming beads of 1.7–2.5 mm diameter. The beads were separated from $CaCl_2$ solution by vacuum filtration, washed twice with distilled water and kept in phosphate buffer solution pH 7 at 4°C. The immobilization efficiency and the retained activity were calculated from Eqs. (1) and (2). Enzyme leakage estimation was carried out by placing beads in a test tube with phosphate buffer pH 7 during 24 hr. Beads were then removed and released enzyme activity in the buffer solution was measured by phenol-4 aminoantipyrine method (Nicell and Wright, 1997). Immobilization efficiency (IE), retained activity (RA) and enzyme leakage EL

were calculated by the following equations:

$$\text{IE}\ (\%) = \left(\frac{A_0 \times V_0 - A_F \times V_F}{A_0 \times V_0}\right) \times 100 \quad (1)$$

$$\text{RA}\ (\%) = \left(\frac{A_{imm} \times V_{imm}}{A_0 \times V_0 - A_F \times V_F}\right) \times 100 \quad (2)$$

$$\text{EL}\ (\%) = \left(\frac{A_{buffer}^{24\ hr} - A_{buffer}^{t=0}}{A_{buffer}^{t=0}}\right) \times 100 \quad (3)$$

where, A_0, A_{imm} and A_F are activities of C-peroxidase solution, immobilized C-peroxidase and filtrate respectively, V_0, V_{imm} and V_F are the volumes of C-peroxidase solution, immobilized C-peroxidase and filtrate respectively, $A_{buffer}^{t=0}$ and $A_{buffer}^{24\ hr}$ are activities of C-peroxidase in buffer solution at initial time and after 24 hr respectively.

The stability of free and immobilized enzyme was studied by incubating separately 15 g of immobilized C-peroxidase immobilized C-peroxidase (ICP) in 50 mL of 50 mmol/L of phosphate buffer pH 7 and 150 mL of free C-peroxidase (FCP) at 4°C. The activity was assessed daily for both FCP and ICP in the buffer solution during three months.

1.2.3 C-peroxidase activity assay

Activities of crude, partially purified free and immobilized C-peroxidase were assessed by employing 4-aminoantipyrene method involving colorimetric estimation using phenol and hydrogen peroxide as substrates and 4-aminoantipyrene as chromogen (Nicell and Wright, 1997). Peroxidase activity was measured from the change in the optical density ($A_{517\ nm}$) at 25°C by adding phosphate buffer pH 7.4 containing reagents with time. One unit of peroxidase activity was defined as 1 μmol/(min·mL) (for enzyme solution) or 1 μmol/(min·g) (for enzyme beads) of hydrogen peroxide transformed at 25°C and pH 7.4 respectively. For ICP, enzyme activity (EA, IU/g support) was reported to beads mass ($m_{support}$) as shown in Eq. (4) (Yücel et al., 2011):

$$\text{EA} = \frac{A_{imm}}{m_{support}} \quad (4)$$

Protein concentration in FCP was determined according to the Bradford method (Bradford, 1976). Bovine serum albumin was used as standard for making a calibration curve.

Specific activity (SA) giving the degree of purification was calculated by the following equations:

$$\text{SA}_{PPE} = \frac{A_{PPE}}{C_{protein}} \quad (5)$$

$$\text{Purification fold}\ (\%) = \frac{\text{SA}_{PPE}}{\text{SA}_{CE}} \quad (6)$$

where, A_{PPE} (UI/mL): activity of partially purified C-peroxidase; SA_{PPE} (IU/mg): specific activity of partially purified C-peroxidase; $C_{protein}$ (mg/mL): protein concentration; SA_{CE} (UI/mg): specific activity of crude C-peroxidase.

1.2.4 Dye enzymatic degradation

Experiments were conducted to compare the capacities of the free and immobilized forms of C-peroxidase in the decolourization reaction of DY 106. Batch experiments were conducted in glass beakers containing 100 mL of the reaction synthetic mixture under permanent magnetic agitation at 300 r/min for a fixed duration. The reaction was then started by adding 0.48 UI/mL of FCP and 0.32 UI/g of ICP.

These experiments were carried out to study effect of parameters, such as initial conditions of DY106 concentration (10–160 mg/L), H_2O_2 dose (0.1–100 mmol/L), temperature (20–80°C) and initial pH fixed in range of 2 to 10 on decolourization efficiency and initial decolourization rate.

The effect of pH was investigated by choosing different types of buffer solutions including: 50 mmol/L of potassium chloride/HCl buffer (pH 2), 50 mmol/L of hydrochloric acid/phthalate buffer (pH 3 and pH 4), 50 mmol/L of phosphate buffer (pH 6, 7 and 8), 50 mmol/L of borax/NaOH buffer (pH 9 and 10). After centrifugation at 4000 r/min for 10 min, the residual dye concentration after treatment with free and immobilized C-peroxidase was carried out by UV-Vis spectrophotometer (Perkin-Elmer 550A) at the maximum wavelengths 396 nm. The initial decolourization rate (IDR) on treated dye by FCP and ICP was calculated from the slope of the dye concentration versus time, at the beginning of decolourization. The decolourization efficiency (DE) with both FCP and ICP was calculated from Eq. (7):

$$\text{DE}\ (\%) = \left(\frac{A_0 - A_t}{A_0}\right) \times 100 \quad (7)$$

where, A_0 and A_t were the absorbance at 396 nm of dye before and after treatment.

1.2.5 Dye decolourization reusability of immobilized C-peroxidase

Experiments were performed repeatedly using the same sample of ICP (0.32 UI/g) in five consecutive agitated batches during 30 min in the presence of 100 mmol/L H_2O_2 in pH 2 buffer solution. Once reaction was completed, beads containing ICP were separated from the mixture, washed with distilled water, and used again in a fresh decolourization medium. Dye decolourization was monitored by UV-Vis at λ_{max} (396 nm) at the end of each batch.

2 Results and discussion

2.1 Purification efficiency by acetone precipitation method

Partially purified peroxidase extracted from *Cuccurbita pepo* was obtained by a simple, economic and less denaturing method. Enzymatic and specific activities of crude and partially purified C-peroxidase are shown in **Table 1**. It shows that the C-peroxidase acetone powder specific activity increased when compared to crude enzyme extract by 1.34 fold. Besides, partially purified peroxidase showed greater storage stability.

2.2 Optimization of C-peroxidase immobilization conditions

Spherical particles having a mean diameter of 2 ± 0.2 mm were obtained by a simple inclusion method with no risk of denaturation or inactivation.

Effects of alginate and $CaCl_2$ concentrations on immobilized C-peroxidase activity, retained activity and enzyme leakage were investigated by the one factor at a time method (Won et al., 2005; Ozyilmaz and Gezer, 2010). Results are illustrated in **Table 2**.

For a fixed FCP activity of (2.53 ± 0.08) IU/mL, different concentrations of sodium alginate and calcium chloride solution were used to obtain the optimal condition for producing immobilized biocatalysts which are effective in dye removal from aqueous phase. From **Table 2**, immobilization efficiencies were not significantly affected by alginate concentration. The values were in the range of 86.70%–92.48%. Nevertheless, the immobilized C-peroxidase activity decreased. This might be due to a limitation of substrate transfer from the bulk phase into the alginate bead to access the enzyme (Cheirsilp et al., 2009). A significant decrease in enzyme leakage (more than 47%), was detected when alginate concentration increased to 2% (*W*/*V*), at constant calcium chloride concentration.

No significant change was detected in enzyme leakage when varying $CaCl_2$ concentration. The maximal retained activity of ICP was reached at 200 mmol/L and 2% (*W*/*V*) of calcium chloride and sodium alginate concentrations respectively. The effect of enzyme concentration on C-peroxidase immobilization was determined by varying enzyme concentrations while keeping the alginate and $CaCl_2$ concentrations at the optimal level. With increasing enzyme concentrations, the ICP activity and retained activity also increased but they were stabilized at FCP activity of 1.4 IU/mL. This could be due to diffusion limitations of the substrate in alginate gel. In conclusion, the best biocatalyst performance including higher IE, RA and lower EL were achieved when calcium chloride and sodium alginate concentration were 200 mmol/L and 2% (*W*/*V*) respectively and activity of FCP was 1.4 IU/mL. The prepared ICP under optimal conditions, could be stored at 4°C in pH 7 buffer solution for three months with no significant changes in either enzyme leakage (< 5%) or loss of activity (< 10%).

Table 1 Results of *Cucurbita pepo* peroxidase purification

Sample	Activity (IU/mL)	Protein (mg/mL)	Specific activity (IU/mg)	Purification fold
Crude extract of C-peroxidase	2.19	2.63	0.83	1
Partially purified C-peroxidase	1.39	1.24	1.12	1.34

2.3 DY106 degradation by free and immobilized C-peroxidase

2.3.1 Effect of pH

The effect of pH on DY106 degradation was investigated by incubating separately 0.28 IU of FCP or 0.16 IU of ICP at 20°C in a reaction mixture containing 50 mg/L of DY106 and 5 mmol/L of H_2O_2. The pH of solutions was kept constant between 2 and 10. The mixture was kept under constant agitation 100 r/min for one hour. The effect of pH on decolourization efficiency is depicted in **Fig. 1**.

For FCP, 73.71% of dye decolourization was obtained with an acidic medium (buffer pH = 2). Dye removal efficiency decreased rapidly from pH 2 to 5, then less dramatically between pH 5 and 8, followed by a decrease down to less than 20% from pH 8 to 10. In a similar study with the enzyme HRP, Bhunia et al. (2001) found that the maximum degradation yield of Remazol blue by free HRP was obtained at pH 2.5. Similar results were obtained by treating other azo dyes (Turquoise Blue G133, Lanaset Blue 2R and Direct Yellow 12) with HRP as a catalyst (De Souza et al., 2007; Maddhinni et al., 2006)

On the other hand, with ICP (**Fig. 1**), about 51% of the dye was removed at pH 2. Increasing pH above 2 conducted to a slight decrease down to 10%–20% in decolourization efficiency (from pH 2 to 6, removal decrease was about 15%). Thus, alginate entrapped enzyme presented a certain stability against changing acidity. The

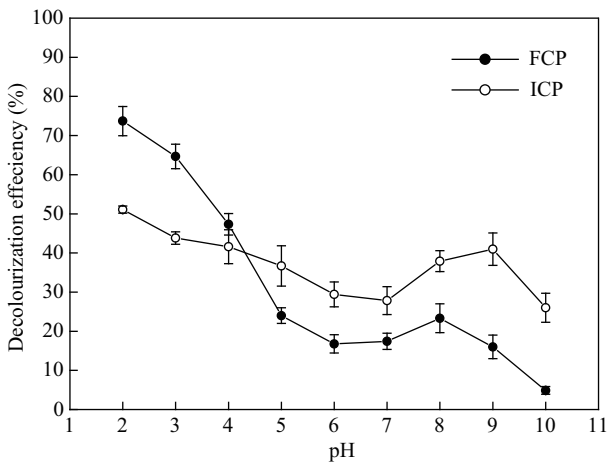

Fig. 1 Effect of pH on dye decolourization by free C-peroxidase (FCP) and immobilized C-peroxidase (ICP).

Table 2 Immobilization efficiency (IE), immobilized C-peroxidase and retained activities (RA) and enzyme leakage (EL) of alginate beads obtained under different gelation conditions

Test 1	Sodium alginate concentration at calcium chloride 100 mmol/L, FCP = 2.53 IU/mL			
	1%, *w/V*	2%, *w/V*	3%, *w/V*	4%, *w/V*
IE (%)	86.70 ± 4.94	90.43 ± 4.01	89.48 ± 3.06	92.48 ± 5.36
IEA (IU/g)	0.47 ± 0.04	0.37 ± 0.08	0.13 ± 0.02	0.02 ± 0.006
RA (%)	19.17 ± 1.69	16.16 ± 1.52	7.77 ± 1.05	5.99 ± 0.10
EL (%)	9.52 ± 0.05	14.97 ± 0.05	3.97 ± 0.01	1.25 ± 0.02
Test 2	Calcium chloride at sodium alginate concentration = 2% (*w/V*), FCP = 2.53 IU/mL			
		100 mmol/L	200 mmol/L	300 mmol/L
IE (%)		90.34 ± 4.01	82.42 ± 3.39	88.90 ± 4.55
IEA (IU/g)		0.37± 0.08	0.41 ± 0.03	0.16 ± 0.01
RA(%)		16.16 ± 1.52	13.64	13.76 ± 2.04
EL (%)		14.97 ± 0.05	4.19 ±0.38	4.95 ± 0.11
Test 3	FCP at sodium alginate concentration 2% (*w/V*), calcium chloride 200 mmol/L			
	0.45 IU/mL	0.98 IU/mL	1.4 IU/mL	2.53 IU/mL
IE (%)	84.33 ± 4.95	90.69 ± 2.03	89.14 ± 3.59	82.42 ± 3.39
IEA (IU/g)	0.02 ± 0.001	0.22 ± 0.01	0.38 ± 0.02	0.41 ± 0.03
RA (%)	4.25 ± 1.65	22.77 ± 1.76	29.25 ± 2.41	13.64 ± 2.05
EL (%)	2.43 ± 0.06	2.19 ± 0.55	3.79 ± 0.13	4.19 ± 0.38

reincreasing yield between pH 8 and 9 could be due to isoenzymes. Mohan et al. (2005) obtained the maximum of decolourization of Acid Black 10BX, by free and immobilized HRP in alginate at pH 2, with decreasing in decolourization efficiency on immobilized compared to free enzyme.

2.3.2 Effect of contact time

Series of mixtures containing different initial concentration of dye (10–160 mg/L), H_2O_2 5 mmol/L, FCP (0.28 IU) or ICP (0, 16 IU), were prepared. Temperature was maintained at 20°C under constant magnetic agitation (200 r/min) at pH 2 during one hour. At 5 min time intervals, the reaction was stopped and solution was analyzed for the residual dye concentration. **Figure 2a** shows a rapid dye decolourization was recorded during the first 5 min, for all dye concentrations. With an initial dye concentration of 25 mg/L, more than 80% of color was removed within 5 min. After this period, a negligible dye removal was noticed up. Decolourization yield was significantly related to initial dye concentration. Tests with ICP showed that the decolourization process could take more time to achieve the highest yield (**Fig. 2b**). Equilibrium time was 30 min. and the highest yield was obtained at 25 mg/L initial concentration. This could be due to diffusional limitations in alginate beads. Similar results were noted, where the reaction profile of Acid Black BX 10 decolourization with alginate immobilized HRP reached an optimal decolourization yield of 54% after 45 min against 67% removed by the free form (Mohan et al., 2005).

2.3.3 Effect of temperature

Effect of temperature was investigated for a reaction mixture containing 50 mg/L DY106, H_2O_2 (5 mmol/L), FCP (0.28 UI),or ICP (0.16 UI). Contact time was fixed at 5 min for FCP and 30 min for ICP. **Figure 3** shows the

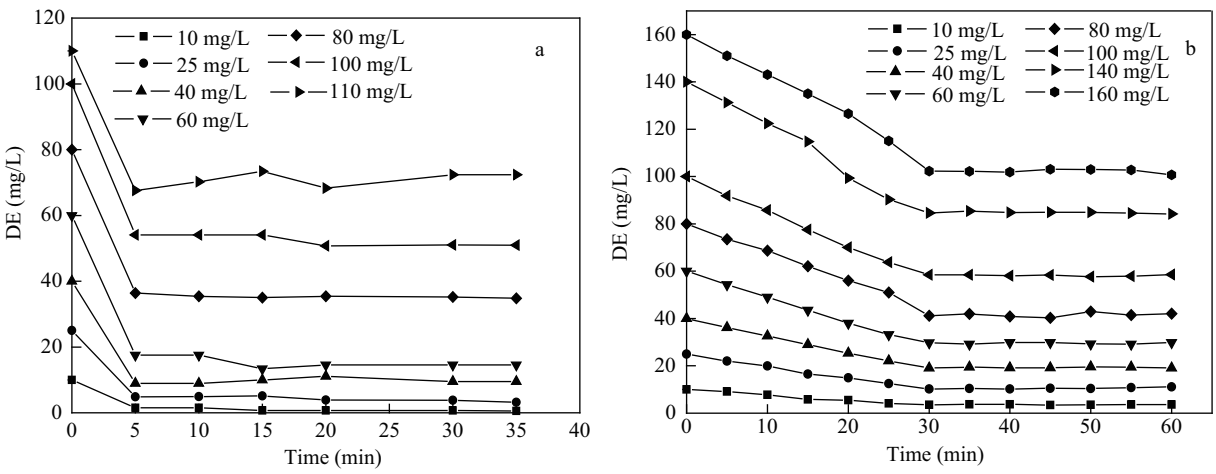

Fig. 2 DY106 decolourization efficiency (DE) kinetics by FCP (a) and ICP (b) at different dye concentrations.

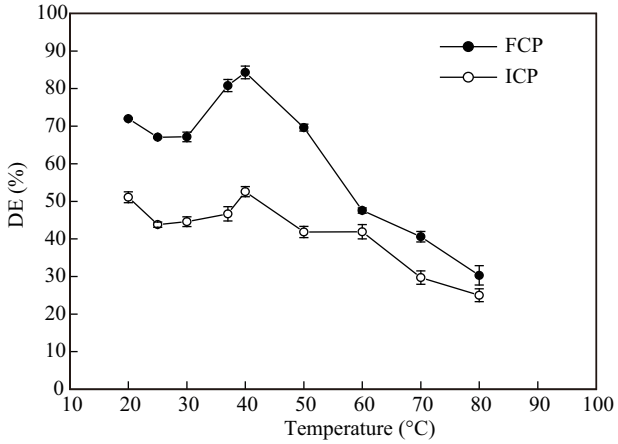

Fig. 3 Effect of temperature on decolourization efficiency (DE) of DY106 by FCP and ICP.

variation of decolourization efficiency (DE) at different temperatures for DY106 treatment by FCP and ICP. The optimal temperature was 40°C, corresponding to more than 84% and 52% of dye removal by FCP and ICP respectively.

Above 40°C, the DE with FCP decreased dramatically (up to 40% loss) due to enzyme partial denaturation. This decrease was less pronounced with ICP (less than 10%) when temperature was increased from 40°C to 60°C. The enzyme is more protected in alginate gel against temperature increase. The immobilization in alginate beads significantly improves the thermal stability of the peroxidase. The greater stability of the entrapped enzyme may be ascribed to the stabilizing effects of immobilization (Cao, 2005).

2.3.4 Effects of dye, H_2O_2 and enzyme concentrations

The optimization of dye decolourization was carried out for the same conditions as mentioned earlier with fixed duration 5 and 30 min by FCP and ICP, respectively. High decolourization by C-peroxidase was achieved at low concentration of DY106. DE dropped at higher dye concentrations (**Fig. 4**). However, initial decolourization rate increased with increasing concentration as also observed in decolourization by other peroxidases (Alam et al., 2009; Yu et al., 2006; Yousefi and Kariminia, 2010). Optimum dye concentration was selected for maximum initial decolourization rate (IDR) and DE, it has a value of 50 and 80 mg/L for FCP and ICP respectively (**Fig. 4**). For free enzyme, the plot of initial rate vs. dye concentration follows a hyperbolic pattern as expected for Michelis-Menten kinetics. The lineweaver-Burke plot of inverse of initial rate vs inverse of concentration giving the values of the apparent Michaels constant $(K_M)_{app}$ at 0.109 mmole/L and the apparent maximum velocity $(V_{max})_{app}$ at 0.01 mmole/(L·min). This value is close to that found by Bhunia et al. (2001) who studied the degradation of remazol blue by HRP. Values of kinetic parameters were estimated as: $(K_M)_{app}$ 0.04 mmole/L and $(V_{max})_{app}$ 0.015 mmole/(L·min).

In order to find out optimum H_2O_2 concentration for maximal degradation, experiments were carried out by varying concentration of H_2O_2 from 0.1 to 7.5 mmol/L for FCP (**Fig. 5a**) and from 1 to 200 mmol/L in the case of ICP (**Fig. 5b**). All other parameters were kept constants (pH 2, dye concentration: 50 mg/L for FCP and 80 mg/L for ICP). Reaction time and enzyme activity were fixed respectively to 5 min and 0.28 IU with FCP and 30 min and 0.16 IU with ICP. Optimal concentration is obtained from the intersection of IDR and DE curves.

Initial decolourization rate and decolourization efficiency for the treatment by FCP increased when H_2O_2 concentration increase from 0.1 to 1 mmol/L, but decreased at higher concentrations. Maximum decolourization (76.92%) was observed in the presence of 1 mmol/L H_2O_2 that was near to those reported by using HRP (Klibanov et al., 1983) and BGP (Akhtar and Husain, 2006) for phenol removal. Also, the inhibition effect at high concentrations of H_2O_2 was observed by Yousefi and Kariminia (2010) for decolourization of Acid Orange 7 by *Coprinus cinereus* peroxidase. The high concentration of H_2O_2 acted as an inhibitor of peroxidase activity by irreversibly oxidizing the enzyme ferric heme group essential for peroxidase activity (Duarte-Vázquez, 2001). Thus, immobilization has the advantage to protect enzyme against

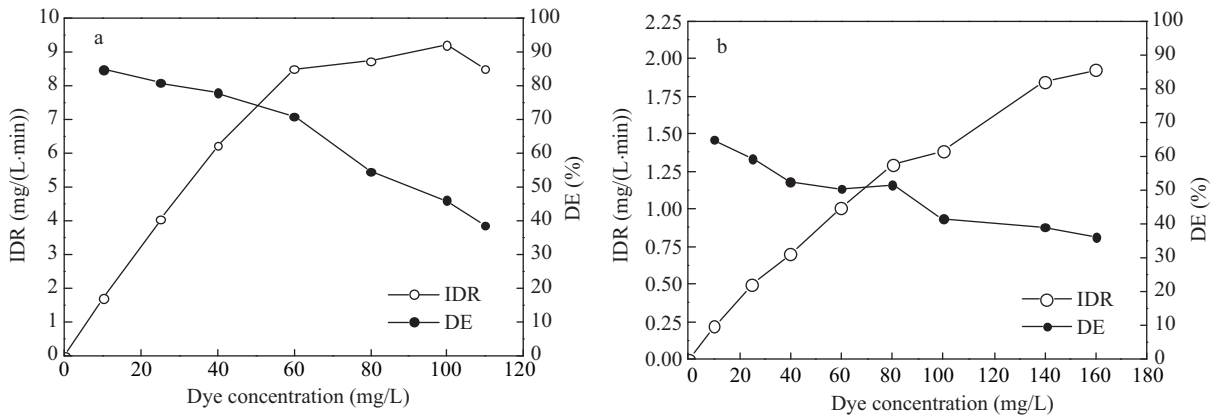

Fig. 4 Effect of initial DY106 concentration on initial decolourization rate (IDR) and DE by free (a) and immobilized (b) C-peroxidase.

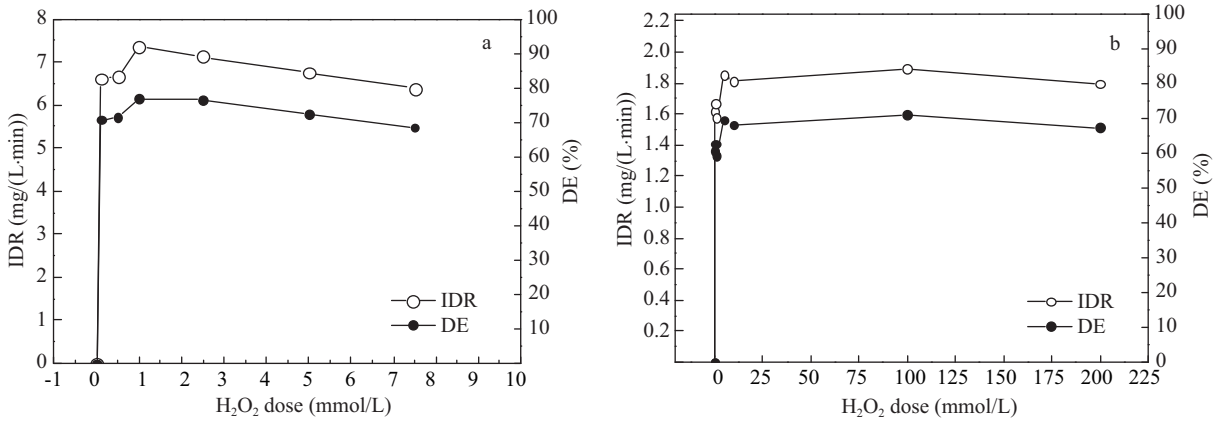

Fig. 5 Effect of hydrogene peroxide concentration on IDR and DE by FCP (a) and ICP (b).

high peroxide concentrations. The optimum H_2O_2 dose was 100 mmol/L on ICP corresponding to the maximum dye removal (70.92%).

To study the effect of C-peroxidase dose on DY106 removal, eight different enzyme concentrations were used. All other parameters were kept constants at their optimum values: DY106 50 and 80 mg/L, H_2O_2 1 and 100 mmol/L and pH 2. **Figure 6a** shows that when enzyme concentration was 0.16 IU/mL, the decolourization yield was only 29%. However, when enzyme activity was increased to 0.66 IU, the decolourization increased to 47%. The highest decolourization efficiency (90%) was noticed at 2.25 IU/mL enzyme concentration. Further increase in enzyme activity did not improve the yield. The enzyme activity of 2.25 IU/mL was found to be the optimal dose for experiment conditions. Similar results were found by Mohan et al. (2005). An enzyme activity of 2.2 IU/mL was sufficient to remove more than 84% of acid black BX10. It could be noticed in **Fig. 6b** that IDR and DE of treated dye solutions by ICP were lower than those obtained by FCP. Increasing the activity from 0.025 to 0.5 IU/g resulted in increasing dye decolourization, after which it remained constant (76%).

Under optimum DY106 degradation conditions with FCP and ICP, the residual dye concentration and DE are reported in **Fig. 7**.

It was observed that contact time is reduced from 5 to 2 min and from 30 to 15 min for treatment by 2.25 IU/mL and 0.5 IU/g of FCP and ICP respectively with increasing decolourization from 73.71% to 89.45% and from 51% to 75.66% for both cases. Different results were found if other dyes/peroxidase/support systems were used (Mohan et al., 2005).

2.3.5 UV-Vis and FT-IR spectra analysis

Decolourization tests were carried out under optimum conditions in order to perform spectral analysis of products before and after enzymatic treatments. When the reaction was achieved, the solution was centrifuged at 4000 r/min during 10 min, the supernatant was analyzed by UV-Vis spectrophotometer and the precipitate was dried and analyzed by FT-IR. In **Fig. 8**, the scanning spectra of dye solution before and after enzymatic treatment show that the DY106 spectrum in visible region exhibits a main peak with a maximum absorbance at 396 nm, the other peaks indicates the presence of auxiliary chemical substance initially in the dye sample. Their decrease indicating that they were removed after enzymatic treatment. The decrease in peak intensity at λ max was the result of the cleavage of azo bonds. It could be speculated that azo bonds (–N=N–)

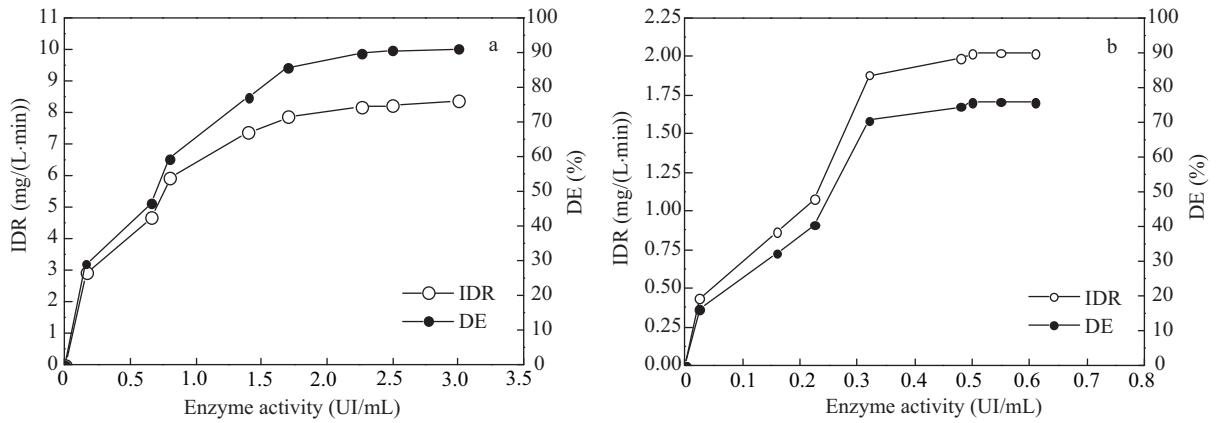

Fig. 6 Effect of FCP (a) and ICP (b) activity on initial rate and efficiency of decolourization.

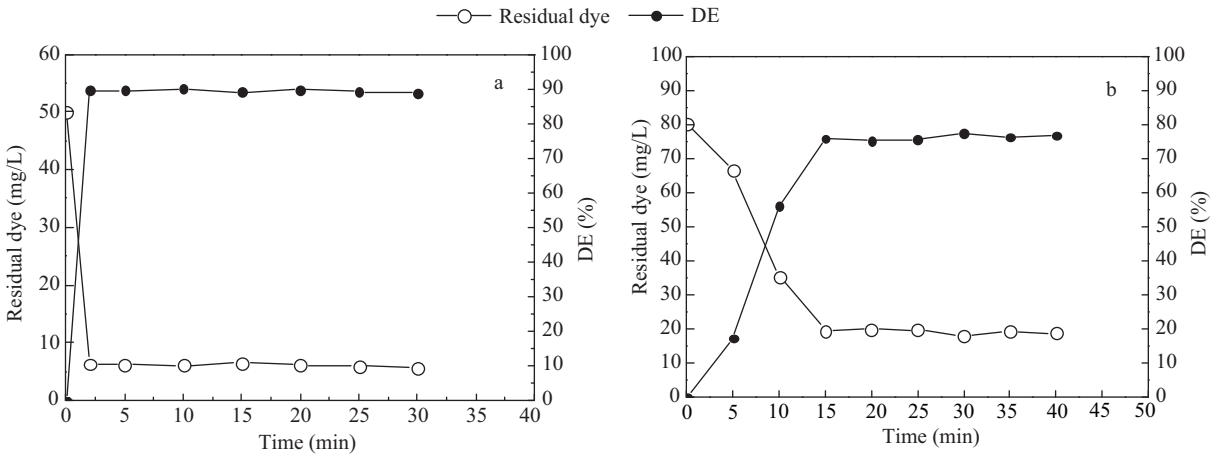

Fig. 7 Effect of contact time on residual dye and DE after enzymatic treatment by FCP (a) and ICP (b) under optimum conditions.

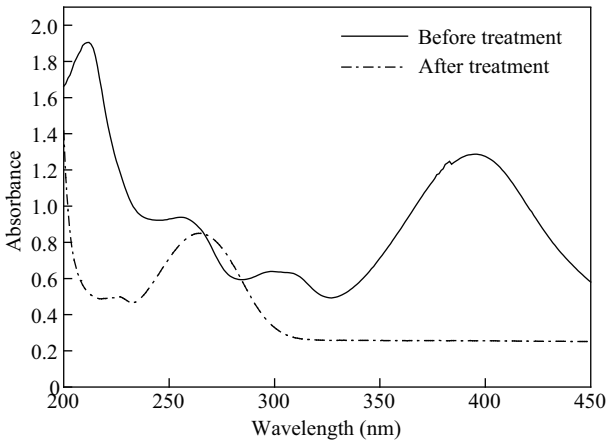

Fig. 8 UV-Vis spectrum of DY106 dye aqueous solutions before and after enzymatic treatment by CP under optimum conditions.

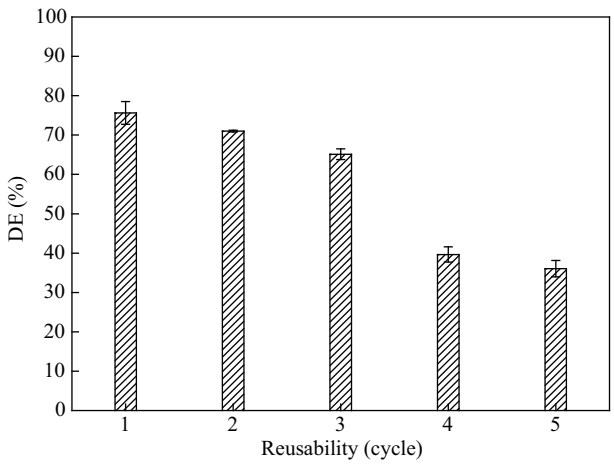

Fig. 9 Reusability of ICP for the treatment of DY106 under optimum conditions.

of characteristic conjugated chromophores in azo dyes molecule were broken (Wang et al., 2009). Also, according to **Fig. 8**, an extra absorbance peaks appeared in the treated solution at 265 nm, probably resulting from the absorbance of metabolites or degraded fragments of the dye molecule (Wang et al., 2009).

The comparison of FT-IR spectrum (data not shown) between the dye and its precipitate clearly indicated the transformation of the parent dye compounds by peroxidase. The absorption bands in the dye spectrum represented the stretching vibrations of –C–S– at 613.2 cm^{-1} and –S=O– at 1199.6 cm^{-1} as well as a stretching vibration at 1045.3 cm^{-1} for –C–N. The stretching vibration of C–H was reported at 2923.9 cm^{-1}, whereas the band at 1431.1 cm^{-1} represented –N=N– stretching of azo group. The FT-IR spectrum of the precipitate showed a significant change in the band positions compared to dye spectra. The bands corresponding to azo group disappeared and a new band appeared at 1649 cm^{-1} which may be due to the formation of aromatic compounds like aromatic amines.

2.3.6 Reusability of immobilized C-peroxidase

Experiments were carried out at optimum conditions fixed for dye treated by ICP. The assay consisted in employing ICP in repeated batch mode. Beads were separated from the reaction mixture after 15 min, rinsed with distilled water, then put in a fresh reaction mixture respecting the same procedure described previously. **Figure 9** shows the performance stability of ICP in repeated batch treatments of DY106. After three repeated uses, the ICP retained 87% dye decolourization of its initial capacity. It decreased to a 36% removal capacity after five uses. This may be due to inactivation of peroxidase induced by the reaction metabolites or it could be attributed to the blockage of pores making the enzyme inaccessible to reaction. Other investigators for immobilized bitter gourd peroxidase were found that 59% of textile effluent was removed after eight repeated uses (Matto and Husain, 2009a, 2009b).

3 Conclusions

The preparation and optimization of immobilized C-peroxidase in Ca-alginate beads and application on treatment of direct azo dye from aqueous solutions was investigated. The immobilized C-peroxidase and retained activities and enzyme leakage are influenced by the gel preparation conditions. The results obtained in the present study revealed the effectiveness of the free and immobilized C-peroxidase in DY 106 decolourization. The performance of DY106 removed with the use of free and immobilized C-peroxidase was found to be highly dependent on dye and enzyme concentrations, aqueous phase pH and H_2O_2. The results of spectral analysis indicated that enzymatic process can cause the disappearance of azo bond in both the supernatant and the precipitate of treated dye. The catalytic capacity of peroxidase for dye treatment with ICP remained relatively constant after three repeated uses.

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