African Journal of Biotechnology Vol. 11(57), pp. 12053-12062, 17 July, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.4228
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Redox-mediated polymerization and removal of benzidine from model wastewater catalyzed by immobilized peroxidase

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Accepted 17 February, 2012

Peroxidase from *Momordica charantia* was highly effective, active and stable for the oxidation of benzidine from model wastewater. There was no oxidative polymerization of benzidine without any redox mediator. Various experimental parameters were standardized for the maximum oxidation of benzidine by peroxidase. The maximum oxidation of this pollutant was observed in the presence of 0.05 mM phenol, 0.75 mM H_2O_2 and 0.2 U mL^{-1} bitter gourd peroxidase (BGP) in a buffer of pH 5.0 at 40°C. Comparative study was performed by soluble as well as surface immobilized bitter gourd peroxidase on Con A layered calcium alginate-starch beads for the degradation of benzidine from model wastewater. Immobilized bitter gourd peroxidase was used for the successful and effective removal of water polluted with benzidine in batch as well as in continuous reactor. The effect of detergents and some water miscible organic solvent was also reported for the oxidation of benzidine from polluted water. Oxidation of benzidine in batch process by soluble and immobilized peroxidase was highly effective and it could remove 72 and 100% benzidine by soluble and immobilized bitter gourd peroxidase, respectively. The reactor filled with immobilized enzyme retained more than 45% benzidine removal efficiency even after 30 days of its continuous operation. The absorption spectra of the treated benzidine exhibited a marked difference in absorption at its λ_{max} as compared to untreated benzidine polluted water.

Key words: Alginate, bitter gourd peroxidase, concanavalin A, removal, immobilization.

INTRODUCTION

Environmental pollution implies any alternation in the surroundings, but the term is restricted in use especially to mean any deterioration in the physical, chemical, and biological quality of the environment. All types of pollution directly or indirectly affect human health. Benzidine is a moderately persistent pollutant in the environment and the exposure to populations with benzidine's waste-disposal is a matter of great concern with regard to human health (Bi et al., 2003).

Benzidine has been used for over a century as an intermediate in the production of various dyes, color salts and naphthols. In the past, benzidine has also been used in the production of rubber, plastic films and for quantitative determination of nicotine in drugs (Stavric, 2000; Hai et al., 2007).

Benzidine was listed as a carcinogenic agent for the first time in the First Annual Report on Carcinogens (Tannenbaum, 2000). Numerous studies in various locations have reported a strong relation between the exposure to benzidine and bladder cancer. Some workers have also reported a link between benzidine

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Abbreviations: I-BGP, Immobilized bitter gourd peroxidase; **S-BGP,** soluble bitter gourd peroxidase; **ABTS,** 2,2- azinobis (3-ethyl benzothiazoline-6 sulfonic acid) diammonium salt; **HOBT,** hydroxybenzotriazole; **Con A,** concanavalin A; **VA,** veratryl alcohol; **VLA,** violuric acid; **BGP,** bitter gourd peroxidase; **DMF,** dimethylformamide; **DMSO,** dimethyl sulfoxide.

exposure and cancer at other tissue sites, especially liver, kidney, nervous system, and various other organs of human's body (Pinheiro et al., 2002; Won et al., 2004).

Therefore, prior to benzidine's final disposal to the environment, its removal from polluted water is required (Duran and Esposito, 2000; Torres et al., 2003; Christian et al., 2005; Palmieri et al., 2005). Several chemical, physical and biological procedures have been exploited for this purpose. However, these procedures have certain demerits such as hazardous radiations, toxicity of chemicals, high costs of production of microbial culture, limited mobility and survival of cells in the soil, completeness of the indigenous populations and metabolic inhibition (Duran and Esposito, 2000). In view of these limitations, recently enzymatic methods for the treatment of aromatic pollutants which offer several advantages have been suggested. Recent works on the treatment of aromatic pollutants by peroxidases have shown that the oxidative degradation and polymerization of such compounds was enhanced to several folds in the presence of some redox mediators (Xu et al., 2000; Huang et al., 2003; Won et al., 2004; Kulshrestha and Husain, 2007).

Hence, in this study an attempt was made to investigate the oxidative degradation and removal of benzidine from model wastewater by immobilized bitter gourd peroxidase (I-BGP) in the presence of some redox mediators. Various experimental condition for the treatment of benzidine by I-BGP such as the effect of redox mediator, pH, temperature, time and H_2O_2 have been standardized. The effect of some organic solvents and detergents on the oxidation of benzidine was also investigated. A comparative study for the oxidation of benzidine by soluble BGP (S-BGP) and I-BGP was also evaluated in batch as well as in continuous processes. The removal of benzidine after treatment and its progress was followed by ultraviolet (UV)-vis spectroscopy.

MATERIALS AND METHODS

Materials

Bovine serum albumin, *o*-dianisidine HCl and 2,2- azinobis (3-ethyl benzothiazoline-6 sulfonic acid) diammonium salt (ABTS) were obtained from Sigma Chemical Company (St. Louis, MO, USA). Benzidine, starch, guaiacol, 1-hydroxybenzotriazole (HOBT), syingaldehyde, phenol, Concanavalin A (Con A) and veratryl alcohol (VA) were procured from SRL Chemicals Co. (Mumbai, India). Violuric acid (VLA) was purchased from Fluka Chemicals (Austria). Sodium alginate was the product of Koch-Light (Pool, England). Jack bean meal was purchased from Loba Chemical Company (Mumbai, India). Bitter gourd was purchased from the local vegetable market. Other chemicals and reagents employed were of analytical grade and were used without any further purification.

Ammonium sulfate fractionation of bitter gourd protein

Bitter gourd (50 g) was homogenized in 100 mL of 100 mM sodium acetate buffer, pH 5.0. The homogenate was filtered through four layers of cheesecloth. The filtrate was then centrifuged at 10,000 rpm on a Remi C-24 cooling centrifuge. The clear supernatant was subjected to salt fractionation by adding 20 to 80% (w/v) $(NH_4)_2SO_4$. This solution was stirred overnight at 4°C to obtain maximum precipitate. The obtained precipitate was collected at 10,000 rpm on a Remi C-24 cooling centrifuge. The collected precipitate was redissolved in 100 mM sodium acetate buffer, pH 5.0 and dialyzed against the same buffer solution.

Immobilization of BGP on the surface of Con A layered calcium alginate-starch beads

Jack bean extract (10%, w/v) was prepared in 200 mL of 100 mM Tris HCl buffer, pH 6.2. The mixture was stirred at room temperature for 12 h. Insoluble residue was removed by centrifugation at 3,000 rpm for 30 min. The collected supernatant was used as a source of Con A (Matto and Husain, 2009a).

Sodium alginate (2.5%, w/v) and starch (2.5%, w/v) were mixed in a total volume of 20 mL of 100 mM sodium acetate buffer, pH 5.0 in three batches and beads were prepared by dropping this mixture into 0.2 M $CaCl_2$ solution. Beads of each batch were incubated in jack bean extract (10.0 mL) overnight with slow stirring at room temperature. The Con A bound calcium alginate-starch beads were collected the following day and were washed with assayed buffer. Con A layered calcium alginate-starch beads from all the batches were pooled and incubated with BGP (3733 U) overnight at room temperature with slow stirring (Matto and Husain, 2009a). The unbound enzyme was removed by repeated washing with 100 mM sodium acetate buffer, pH 5.0.

BGP immobilized on the surface of Con A layered calcium alginate-starch beads was crosslinked by 0.5% glutaraldehyde for 2 h at 4°C under constant shaking. Ethanolamine was added to a final concentration of 0.01% (v/v) and incubated for 90 min at room temperature. The beads were washed and suspended in 100 mM sodium acetate buffer, pH 5.0 (Matto et al., 2009). I-BGP was stored and used for further studies.

General procedure for treatment of benzidine

Benzidine polluted water (0.5 mM, 5.0 mL) was treated by BGP (0.20 U mL^{-1}) in 100 mM sodium acetate buffer, pH 5.0 at 40°C. The reaction was carried in the presence of 0.75 mM H_2O_2 and 0.05 mM phenol for 1 h at 40°C. The insoluble product was removed by centrifugation at 3,000 rpm for 15 min. Untreated benzidine polluted water (containing all the reagents that are present in treated solution with heated enzyme) was considered as the control (100%) for the calculation of percentage of oxidation. In a blank solution, we take the heated enzyme (denatured enzyme), H_2O_2 and assay buffer having 5 mL of total volume. Such solutions preparations short out the problem of protein absorbance at specific wavelength. The percentage of oxidation and removal of benzidine was defined as:

$$\frac{\text{Absorbance of untreated benzidine} - \text{Absorbance of benzidine after treatment}}{\text{Absorbance of untreated benzidine}} \times 100$$

Effect of redox mediators on the oxidation of benzidine by BGP

The effect of various redox mediators (0.025 and 0.05 mM) on BGP (0.20 U mL^{-1}) which catalyzed benzidine polymerization and the removal efficiency was investigated in 100 mM sodium acetate buffer, pH 5.0 in the presence of 0.75 mM H_2O_2 at 40°C for 1 h. The redox mediators; HOBT, syingaldehyde, phenol, VA, VLA, guaiacol, ABTS, 2,4-dichlorophenol, *p*-chlorophenol, 4-nitrophenol and quinol

were used. The reaction was stopped by boiling the sample in a water bath for 5 min. Oxidative polymerization and removal of benzidine from polluted water was monitored at 285 nm. The percentage of oxidation was calculated by taking untreated benzidine polluted water as the control (100%).

Effect of enzyme concentration

Benzidine (0.5 mM, 5.0 mL) was incubated with increasing concentrations of BGP (0.1- 0.5 U mL^{-1}) in the presence of 0.05 mM phenol and 0.75 mM H_2O_2 in 100 mM sodium acetate buffer, pH 5.0 at 40°C for 1 h. The insoluble product was removed by centrifugation at 3,000 rpm for 15 min. The decrease in the absorbance at λ_{max} (285 nm) was monitored. The percentage of oxidation was calculated by taking benzidine untreated polluted water as the control (100%).

Effect of pH on the oxidation and removal of benzidine by BGP

Benzidine polluted water (0.5 mM, 5.0 mL) was treated with BGP (0.20 U mL^{-1}) in the buffers of different pHs (2.0-10.0) in the presence of 0.75 mM H_2O_2 and 0.05 mM phenol at 40°C for 1 h. The buffers used were glycine-HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0 and 5.0), sodium phosphate (6.0 and 8.0), and Tris-HCl (pH 9.0 and 10.0). The molarity of each buffer was 100 mM. Untreated benzidine polluted water of each buffer was considered as the control (100%) for the calculation of percentage of oxidation at each pH.

Effect of temperature on the oxidation of benzidine by BGP

Benzidine polluted water (0.5 mM, 5.0 mL) was treated by BGP (0.20 U mL^{-1}) in the presence of 0.75 mM H_2O_2 and 0.05 mM phenol in 100 mM sodium acetate buffer, pH 5.0 at various temperatures (20 to 80°C) for 1 h. The insoluble product was removed by centrifugation at 3,000 rpm for 15 min. Untreated benzidine polluted water was considered as the control (100%) for the calculation of percentage of oxidation at each temperature.

Effect of detergents on oxidation of benzidine

The effect of Triton X-100 and Tween-20 (0.5 to 5.0%, v/v) on the BGP catalyzed oxidative degradation and polymerization of benzidine was investigated under the experimental conditions mentioned above. Similarly, the insoluble product was removed by centrifugation at 3,000 rpm for 15 min. The decrease in absorbance of benzidine at the respective λmax was monitored. The percentage of oxidation was calculated by taking untreated benzidine polluted water as the control (100%).

Effect of water-miscible organic solvents on the oxidation of benzidine

The effect of dioxane, DMF, DMSO and *n*-propanol, 10 to 60% (v/v) on BGP catalyzed oxidation that is removal of benzidine was also investigated under the experimental conditions mentioned above. The insoluble product was removed by centrifugation at 3,000 rpm for 15 min. Likewise, the decrease in the absorbance of benzidine at the respective λmax was monitored and the percentage of oxidation (removal) was calculated by taking untreated sample as control (100%).

Oxidation of benzidine in a batch process

Benzidine polluted water (0.5 mM, 500 mL) was treated by soluble and immobilized BGP (15 U) in batch processes under various time at 40°C in the presence of 0.05 mM phenol and 0.75 mM H_2O_2. The aliquots were taken from the reaction mixture at different time intervals. The reaction was stopped by heating the collected sample in a boiling water bath for 5 min. The insoluble product was removed by centrifugation at 3,000 rpm for 15 min. The percentage of oxidation was calculated by taking untreated benzidine polluted water as the control (100%).

Treatment of benzidine polluted water in a continuous reactor

A continuous reactor was developed for the removal of benzidine polluted water. A column (16.0 x 1.5 cm^2) was filled with I-BGP (1462 U). The benzidine model wastewater (0.5 mM) containing 0.05 mM phenol and 0.75 mM H_2O_2 was passed through the reactor at room temperature (30±2°C). The flow rate of the column was maintained at 10 mL h^{-1}. The samples were collected every 5 days and after centrifugation the samples were analyzed spectrophotometrically.

Analysis of oxidative polymerization and removal of benzidine via continuous reactor system

Benzidine polluted wastewater was passed through the reactor containing I-BGP. Samples from the column outlet were collected, centrifuged and then analyzed spectrophotometrically for the remaining benzidine. Spectra of the control and I-BGP treated benzidine solution were taken on Cintra 10*e* UV-visible spectrophotometer.

Assay of peroxidase activity

The activity of peroxidase was estimated from the change in the optical density (A_{460} nm) in the 100 mM sodium acetate buffer, pH 5.0 at 40°C by measuring the initial rate of oxidation of 6.0 mM *o*-dianisidine HCl by 18 mM H_2O_2 . Immobilized enzyme preparation was continuously agitated during entire duration of assay. The assay was highly reproducible with immobilized enzyme preparation.

One unit (1.0 U) of peroxidase activity was defined as the amount of enzyme protein that catalyzed the oxidation of 1.0 µ mole of *o*-dianisidine HCl per min at 40°C into colored product (ε_m =30000 mol^{-1} cm^{1}).

Statistical analysis

Each value represents the mean of three independent experiments performed in duplicates, with average deviations, < 5%. The data expressed in various studies was plotted using sigma plot-10.0 and Microsoft Excel 2003. *P*-values < 0.05 were considered statistically significant.

RESULTS

Role of redox mediators on the oxidation of benzidine by BGP

Table 1 demonstrates the effect of some redox mediators on the oxidative degradation and polymerization of benzidine by BGP. Among the used redox mediators,

Table 1. Effect of redox-mediators on polymerization of benzidine by BGP.

Redox-mediator	Benzidine removal (%)	
	0.05 mM	0.025 mM
HOBT	90	89
VLA	77	40
VA	21	12
Syringaldehyde	75	54
Phenol	99	88
Guaiacol	79	72
ABTS	52	44
2, 4- dichlorophenol	96	92
p-chlorolophenol	93	84
4-nitrophenol	90	81
Quinol	95	93

The effect of various redox mediators on BGP (0.2 U mL^{-1}) catalyzed benzidine oxidative degradation and polymerization was investigated as described in the text. HOBT, hydroxybenzotriazole; VLA, violuric acid; VA, veratryl alcohol; ABTS, 2,2- azinobis (3-ethyl benzothiazoline-6 sulfonic acid) diammonium salt.

Table 2. Effect of enzyme concentration on the polymerization of benzidine.

Enzyme concentration (U mL^{-1})	Benzidine removal (%)
0.1	75
0.2	99
0.3	99
0.4	99
0.5	99

Benzidine polluted water (0.5 mM, 5.0 mL) was treated by BGP (0.1- 0.5 U mL^{-1}) as described in the text.

0.05 mM of phenol showed highest (99%) oxidative degradation and polymerization of benzidine from the polluted model wastewater. Other redox mediators however, showed lower oxidation/removal of benzidine.

Effect of enzyme concentration

To determine the minimum BGP dose required to remove maximum benzidine from polluted model wastewater, the reaction was conducted under standard conditions in the presence of 0.5 mM benzidine, 0.75 mM H_2O_2 and 0.05 mM phenol. The removal efficiencies increased with enzyme doses and reached its maximum to 99% at 0.20 U mL^{-1} of BGP (Table 2).

Effect of pH

The role of pH on the oxidation and removal of benzidine from polluted water by BGP was demonstrated in Figure 1. Benzidine was maximally oxidized in the buffer of pH 5.0. Above and below this pH, the removal of benzidine was remarkably decreased. However, immobilized BGP exhibited more removal of benzidine in the buffer of other pH as compared to soluble enzyme.

Effect of temperature

Figure 2 demonstrates the effect of temperature on the oxidative polymerization and removal of benzidine from polluted water. The removal efficiency of benzidine was enhanced with increasing temperature until 40°C. Further increase in temperature resulted in reduced polymerization of benzidine by BGP. I-BGP exhibited removal of greater fractions of benzidine at higher temperatures as compared to its soluble counterpart, S-BGP.

Effect of detergents

The effect of Triton X-100 and Tween-20 on the polymerization of benzidine from polluted water is shown in Table 3. Benzidine was removed up to 80 and 90% by

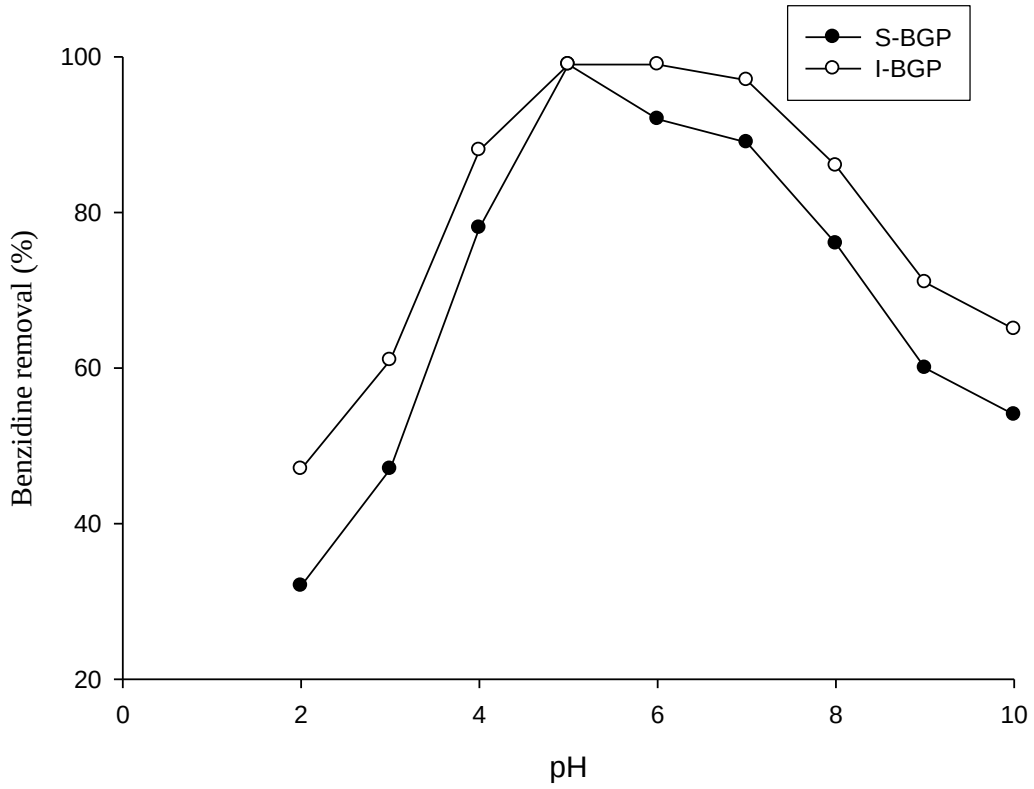

Figure 1. Effect of pH on the oxidation and removal of benzidine.

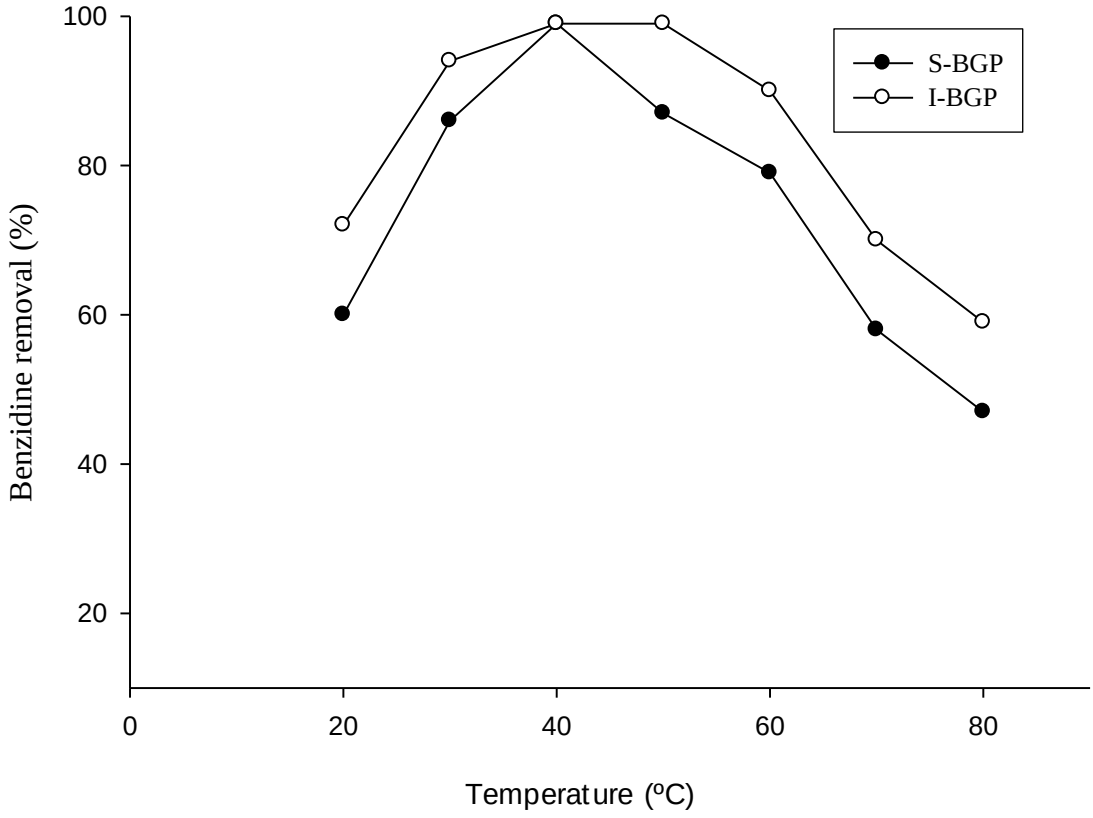

Figure 2. Effect of temperature on the oxidation and removal of benzidine.

Table 3. Effect of the detergents on the oxidative polymerization of benzidine.

Detergent (%, v/v)	Benzidine removal (%)			
	S-BGP		I-BGP	
	Tritron X-100	Tween-20	Tritron X-100	Tween-20
0.5	99	97	99	99
1.0	99	97	99	99
1.5	98	94	99	99
2.0	98	93	99	98
2.5	97	91	99	97
3.0	90	90	99	95
3.5	88	82	99	93
4.0	86	78	97	92
4.5	82	76	96	91
5.0	79	74	94	89

Benzidine polluted model wastewater (0.5 mM, 5.0 mL) incubated with BGP (0.2 U mL^{-1}) catalyzed benzidine oxidative degradation and transformation was investigated as described in the manuscript. The percentage oxidation was calculated by taking untreated benzidine polluted water as the control (100%). S-BGP, soluble bitter gourd peroxidase; I-BGP, immobilized bitter gourd peroxidase.

Table 4. Effect of water-miscible organic solvents on the oxidation of benzidine.

Organic solvents (%,v/v)	Benzidine removal (%)							
	S-BGP				I-BGP			
	DMF	DMSO	Dioxane	*n*-propanol	DMF	DMSO	Dioxane	*n*-propanol
10	94	96	98	99	99	99	99	99
20	87	86	80	97	99	99	98	99
30	78	79	48	92	97	99	95	99
40	68	68	38	87	95	97	91	99
50	53	59	25	81	88	95	86	99
60	35	42	15	76	86	92	79	97

Benzidine polluted water (0.5 mM, 5.0 mL) incubated with BGP (0.2 U mL^{-1}) catalyzed benzidine oxidation was investigated as described in text. DMF, dimethylformaamide; DMSO, dimethyl sulfoxide; S-BGP, soluble bitter gourd peroxidase; I-BGP, immobilized bitter gourd peroxidase.

soluble and immobilized BGP in the presence of 3.5% detergents concentrations, respectively. However, the oxidation of benzidine decreased in the presence of increasing concentrations of detergents. BGP catalyzed the removal of benzidine from polluted model wastewater more effectively in the presence of Triton X-100 as compared to Tween-20.

Effect of water-miscible organic solvents

The effect of dioxane, DMF, DMSO and *n*-propanol on the polymerization of benzidine by BGP are summarized in Table 4. The removal of benzidine decreased in the presence of increasing concentrations of organic solvents. I-BGP removed significantly higher concen-tration of benzidine in the presence of all the concentrations of investigated organic solvents as compared to S-BGP.

Oxidation of benzidine in batch process by BGP

Figure 3 depicts polymerization of benzidine by both soluble and immobilized BGP in batch processes. S-BGP removed 72% benzidine after 150 min while the I-BGP showed 82% removal of the compound. The maximum oxidation of benzidine (72%) was achieved in 150 min by S-BGP while its oxidation by I-BGP reached 99% within 210 min.

Analysis of benzidine oxidized through continuous-reactor

The removal of benzidine was 98% even after operating for 5 days (Figure 4). There was an inverse relationship in the benzidine removal and the time of operation.

Some spectral analyses were also illustrated to confirm

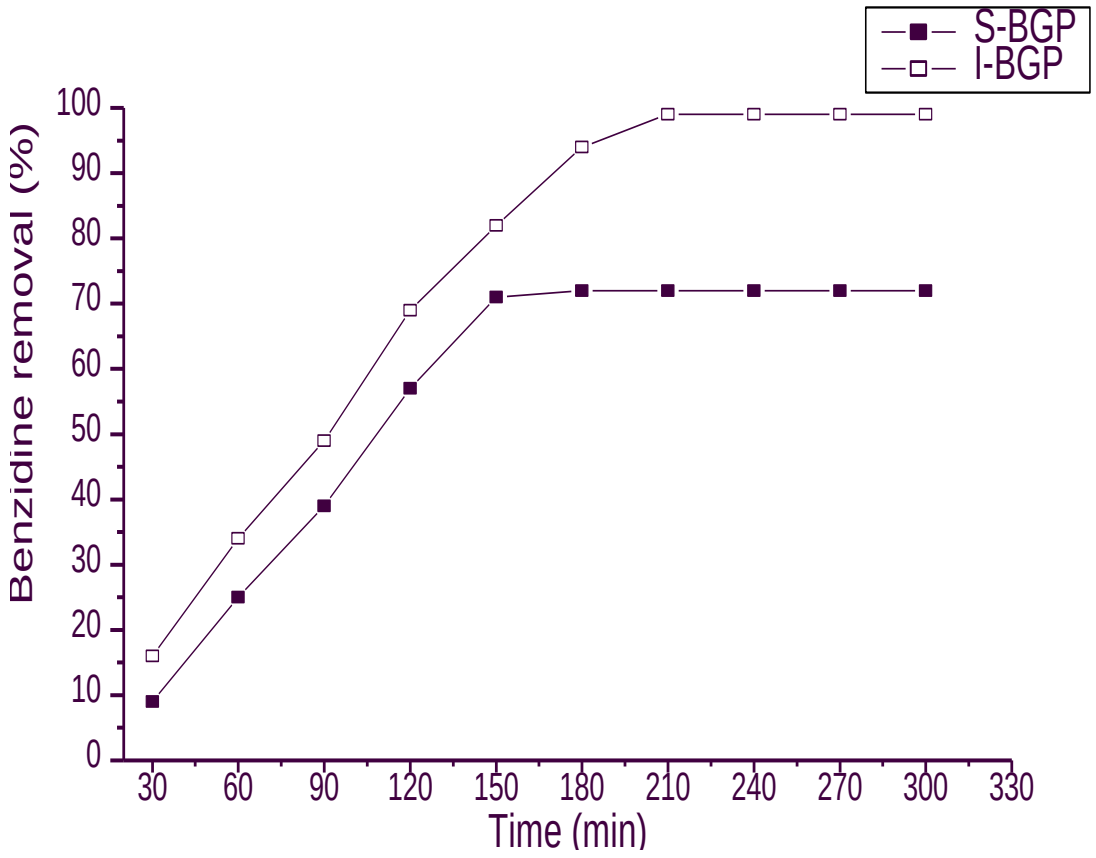

Figure 3. Oxidative polymerization of benzidine in batch processes**.**

the removal of benzidine by BGP. Figure 5 demonstrates the absorption spectra for the treated and untreated benzidine with respect to the time of operation. Diminution in the absorbance peak of the treated sample in the UV region clearly showed the removal of benzidine from the treated wastewater.

DISCUSSION

Plant peroxidases are receiving increasing attention due to their extensive properties for the treatment of aromatic compounds present in wastewater. It has already been described previously that peroxidases can be used in the detoxification of various phenols and aromatic amines. In order to reduce the cost of wastewater treatment, simple ammonium sulphate precipitated proteins from bitter gourd were considered for the treatment of benzidine present in polluted water.

The maximum oxidative degradation and removal of benzidine was observed at 40°C in the buffer solution of pH 5.0 (Figures 2 and 3). However, these results show the oxidative degradation and polymerization of benzidine was specifically pH and temperature dependent. Peroxidases from various plant sources had already been reported to catalyze the removal of different aromatic compounds in a broad range of pH and temperature (Kulshrestha and Husain, 2007; Matto and Husain, 2009a; Matto et al., 2009).

Benzidine was a recalcitrant compound for peroxidase as there was 0% oxidative polymerization of benzidine without any redox mediator. BGP was able to oxidize benzidine in the presence of 11 redox mediators. The maximum oxidative degradation and polymerization of benzidine from polluted water was observed in the presence of 0.05 mM of phenol (Table 1). Several workers have demonstrated that the use of redox mediator would enhance the rate of oxidation of aromatic pollutants present in wastewater to several folds but these mediators were required in very high concentrations, that is, 5.7 mM Violuric acid and 2.0 mM of HOBT (Soares et al., 2002; Claus et al., 2002). Phenol was an effective redox mediator and was used in the oxidative degradation and removal of many aromatic pollutants present in wastewater by peroxidases and many other enzymes (Camarero et al., 2005; Calcaterra et al., 2008). Here, we have shown for the first time, the oxidation of benzidine by BGP in the presence of very low concentration (0.05 mM) phenol. Redox mediators facilitated recalcitrant compounds degrading activities of enzymes and enhance their specificity to a wide range of substrates/recalcitrant compounds. Redox mediators are

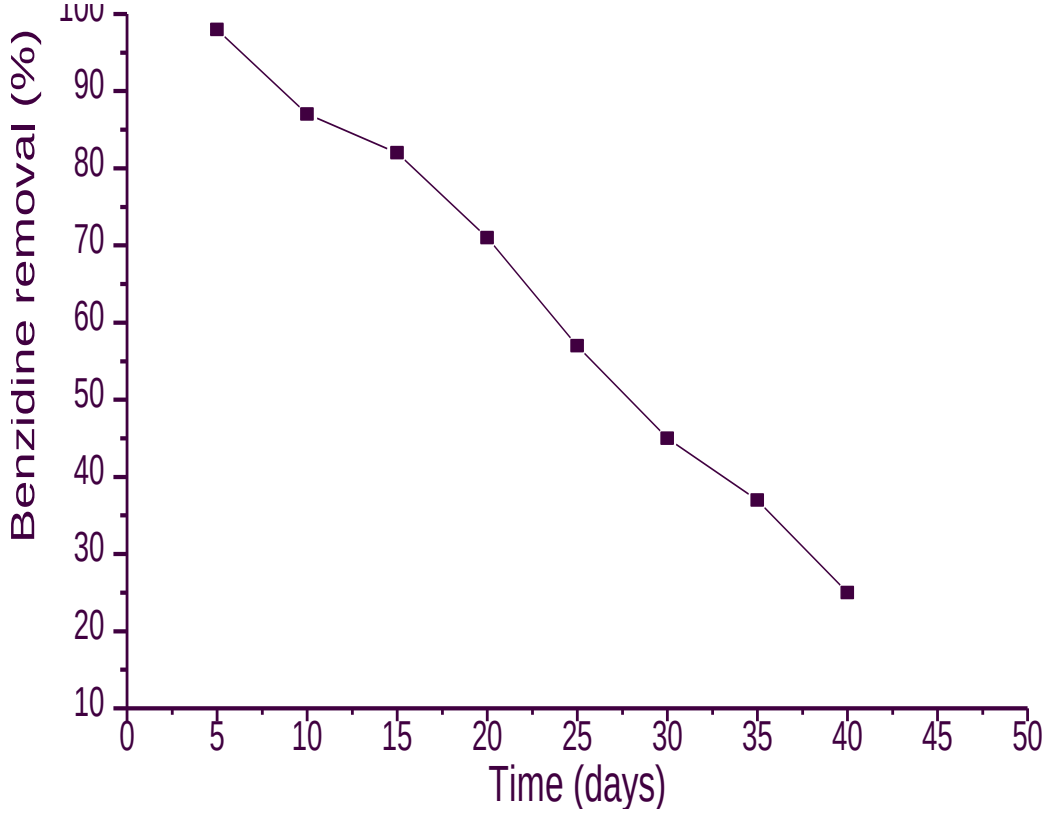

Figure 4. Removal of benzidine from polluted water through a continuous reactor filled with I-BGP.

the compounds that speed up the reaction rate by shuttling electrons from the biological oxidation of primary electron donors or from bulk electron donors to the electron accepting organic compounds. Low molecular weight, diffusible redox mediators provide high redox potentials (>900 mV) to attack on recalcitrant structural analogs and were able to migrate into the aromatic structure of the compound. Among the redox mediators, those presenting the >NOH moiety were highly effective towards benzylic substrates through a radical H-abstraction route of oxidation involving the aminoxyl radical (>N-O$^{\cdot}$) intermediate. These compounds helped BGP to degrade benzidine as (i) it is oxidized by one-electron directly by the action of enzyme to produce free radical, (ii) it produced free radical, stable enough to diffuse and react with the target compound and (iii) it has an appropriate redox potential (Husain, 2006;

Maximum oxidative degradation and removal of benzidine from polluted water was obtained by 0.2 U mL^{-1} of BGP (Table 2). Hisae et al. (2003) used oxidation assay having 66.7 U mL^{-1} of HRP and 3.0 mM H_2O_2 for the treatment of aromatic pollutants, other enzymes used for the oxidation of aromatic compounds were 10 U mL^{-1} of laccase (Kumarasamy et al., 2006) and 50 U mL^{-1} of tyrosinase (Yamada et al., 2006). As compared to all these catalytic oxidative reactions, we have successfully used 0.20 U mL^{-1} of BGP for the treatment of benzidine. Treatment of benzidine with peroxidase or H_2O_2 alone exhibited no appreciable removal of such compounds. Enzymes employed for the treatment of wastewater containing pollutants would be affected by the presence of detergents and organic solvents. Industrial effluents and municipal wastewater also contain detergents and organic solvents together with other aromatic pollutants. Therefore, it is necessary to investigate the role of some detergents and water miscible organic solvents on the activity of immobilized BGP. Our observations suggested that immobilized BGP was remarkably more stable against the exposure caused by very high concentrations of several detergents and organic solvents (Tables 3 and 4). These results showed that the inactivation of enzyme increased with rise in the concentrations of detergents and organic solvents. However, the immobilized BGP retained significantly higher activity against such exposures as compared to soluble enzyme. Recently, some investigators have demonstrated that calcium alginate-starch surface immobilized and entrapped BGP preparations were significantly more stable against denaturation mediated by heat, urea, organic solvents and detergents (Matto and Husain, 2009b). Benzidine was removed to 99% by immobilized BGP in 210 min in a batch process. This shows that the immobilized enzyme was more effective in the oxidation of industrial pollutants because these enzymes were protected against.

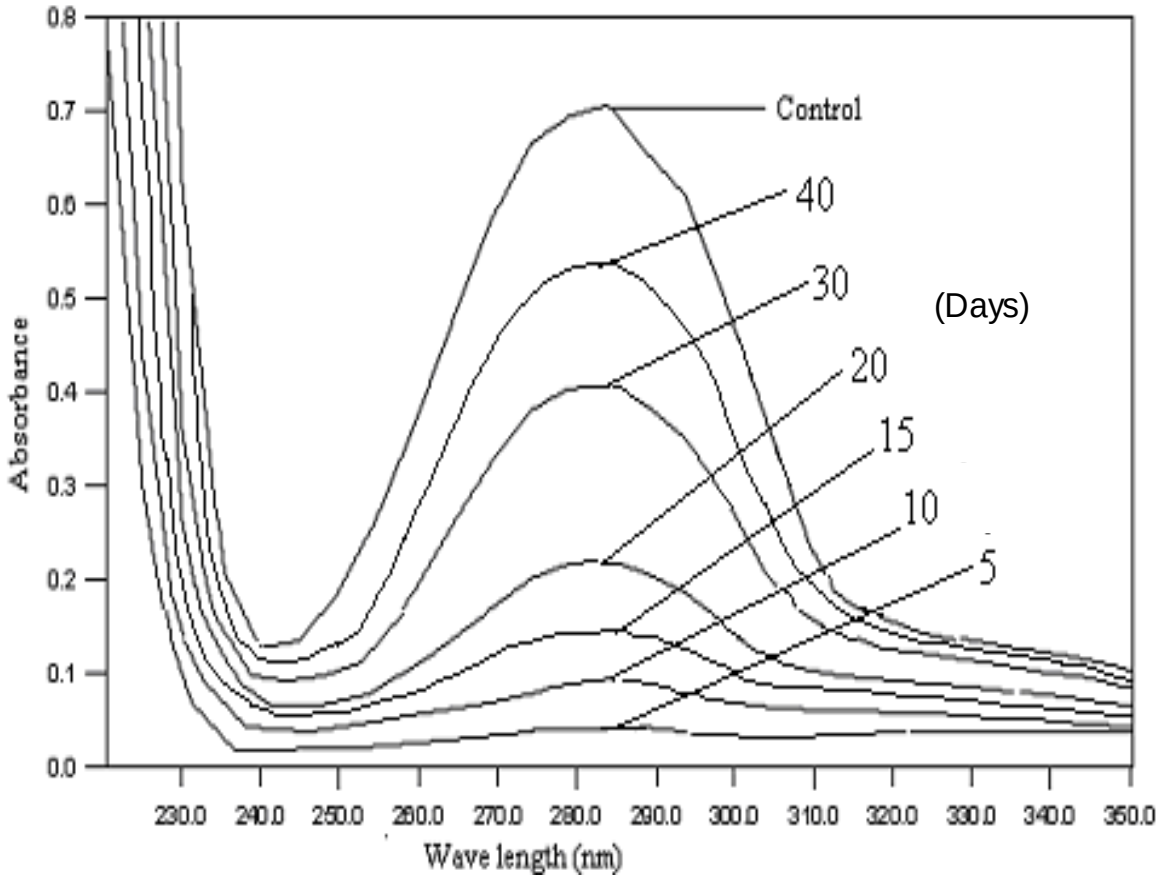

Figure 5. UV spectra of benzidine.

The reactor was ope-rated with very high benzidine removal efficiency without any practical problem.

These results are in agreement with earlier studies where investigators have reported that the treatment of effluent containing aromatic compounds resulted in the formation of free radicals (Matto and Husain, 2006; Husain and Husain, 2008). Removal of reaction products as insoluble complex was an important signal for the detoxification of wastewater contaminated with aromatic compounds. It has already been demonstrated that horseradish peroxidase (HRP) and BGP could catalyze free-radical formation, followed by spontaneous polymerization of a variety of aromatic compounds including phenols (Huang et al., 2003), chlorophenols (Tatasumi e al., 1996), bisphenol A (Karim and Husain, 2009), dyes (Bhunia et al., 2001; Akhtar et al., 2005) and aromatic amines.In order to confirm the oxidation and removal of toxic compounds from wastewater, spectral analysis became an important aspect to show the removal of benzidine after treatment with immobilized BGP present in the reactor. The decrease in absorbance peaks in UV region from 250-310 nm provides a strong evidence for the removal of this aromatic pollutant from polluted model wastewater (Figure 5). The disappearance of absorption peak in the UV region was due to the oxidation of the benzidine from model wastewater.

Conclusions

Here, it is shown that BGP can be efficiently used for the oxidative polymerization and removal of benzidine in the presence of various redox mediators. The treatment of benzidine by BGP in the presence of redox-mediators produced insoluble aggregates which could be easily removed simply by centrifugation or filtration. On the basis of these findings one can suggest that the use of immobilized BGP could be extended to the large-scale treatment of benzidine and other related compounds by employing more effective and cheaper redox-mediators. Our data suggest that the peroxidase/phenol system was an effective biocatalyst for the treatment of benzidine present in polluted water.

ACKNOWLEDGEMENTS

The authors are thankful to Dr. Faisal M. Al-Tobaigy, Dean, Faculty of Applied Medical Sciences, Jazan University, Jazan, Kingdom of Saudi Arabia for constant encouragement and support and Universiti Sains Malaysia for financial support through USM RU grant

(Grant no. 1001/PKIMIA/815160).

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