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Removal of anthracene from model wastewater by immobilized peroxidase from *Momordica charantia* in batch process as well as in a continuous spiral-bed reactor

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ABSTRACT

The aim of this study was to develop a system based on the use of immobilized peroxidase from bitter gourd (*Momordica charantia*) for the removal of polycyclic aromatic hydrocarbon, anthracene. In vitro removal of anthracene by soluble and calcium alginate–starch beads surface immobilized peroxidase was investigated in the presence of a redox mediator, guaiacol. The maximum oxidation of anthracene was observed in the presence of 0.70 mM H_2O_2 and 0.4 U mL^{-1} of the enzyme in sodium acetate buffer, pH 4.0 at 40 °C in 2.5 h. The oxidative degradation and polymerization of anthracene was increased more than two-fold in the presence of 0.1 mM guaiacol. The oxidation of anthracene was quite effective in batch process, 99% anthracene was removed in 7 h by immobilized enzyme. The spiral-bed reactor filled with immobilized enzyme retained more than 40% anthracene removal efficiency even after 1 month of its continuous operation. The absorption spectra of anthracene after treatment exhibited a marked difference in absorbance at respective wavelengths as compared to untreated compound. FT-IR analysis has shown a derivative of anthraquinone as a product of peroxidase catalyzed anthracene conversion. *Allium cepa* toxicity assay showed that the peroxidase catalyzed product of anthracene was less toxic as compared to parent compound.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are pollutants produced via natural and anthropogenic sources, generated during the incomplete combustion of solid and liquid fuels or derived from industrial activities. These compounds are hydrophobic with low water solubility, thus they are easily adsorbed onto organic matter as soils and sediments. The environmental impact associated may cause a potential health risk due their mutagenic and carcinogenic potential. Important drawbacks greatly hamper the natural biological degradation of PAHs [1,2].

There were many enzymes used for the oxidative removal of aromatic pollutants from environment. White rot fungi, which are known to degrade a great variety of complex compounds including PAHs [3]. Lignin peroxidase (LiP) and manganese peroxidase (MnP) from white rot fungi have been used for the removal of variety of PAHs [4]. However, a wider application of these enzymes is hindered by the fact that enzymes work properly in aqueous media. Many nonpolar and polyaromatics compounds showed very less water solubility. Due to hydrophobic nature of PAHs such compounds cannot be treated by enzymes in aqueous environment. The addition of organic solvents in the reaction mixture increased solubility of polyaromatics in aqueous media and thus it increased degradation of these compounds by enzymes [5–7]. A good effort has been made to enhance PAHs solubility to several folds by adding water-miscible-solvents or surfactants [8–10]. Although enzymatic catalysis in organic solvents is considered a promising approach to solve environmental problems but most of the soluble enzymes get denatured in such medium [11]. Immobilization of enzymes provided higher stability, reusability and capability to work in aqueous as well as in organic solvents due to protection of enzymes against denaturants, proteolysis and reduced susceptibility to microbial contamination. It was due to their enhanced resistance to unfolding provided by multipoint covalent and non-covalent attachment of enzymes with the matrix [11–14].

Recently bioaffinity based procedure has attracted the attention of the enzymologists due to their direct immobilization from partially purified preparation or even from crude homogenates

Abbreviations: PAHs, polycyclic aromatic hydrocarbons; BGP, bitter gourd peroxidase; LiP, lignin peroxidase; MnP, manganese peroxidase; Con A, concanavalin A; S-BGP, soluble bitter gourd peroxidase; I-BGP, immobilized bitter gourd peroxidase; DMF, N-N dimethylformamide; DMSO, dimethylsulfoxide HOBT1-hydroxybenzotrizole.

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[15–17]. Surface immobilized enzymes are more superior as compared to entrapped enzymes, as in the later case the diffusion of large molecular size products from inside the gel beads was difficult [18,19]. In order to prevent the possibility of accumulation of products inside the gel, immobilization of enzymes on the surface of a support would be a preferred choice.

Bitter gourd (*Momordica charantia*) peroxidase immobilized on the surface of concanavalin A (Con A) layered calcium alginate–starch hybrid beads has been employed for the removal of anthracene from the model wastewater. Immobilized bitter gourd peroxidase (I-BGP) was successfully used for the removal of anthracene, a PAH model compound, from the polluted water containing water-miscible organic solvents. The oxidation and removal of anthracene was optimized under various experimental conditions. A large scale treatment of anthracene by immobilized enzyme has been investigated in a batch process as well as in a continuous spiral-bed reactor.

2. Materials and methods

2.1. Materials

Bovine serum albumin, violuric acid and *o*-dianisidine HCl were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Ammonium sulphate, azinobis (3-ethyl benzothiazoline-6 sulfonic acid) diammonium salt, jack bean meal, N-N dimethylformamide (DMF), dimethylsulphoxide (DMSO), 1-hydroxybenzotrizole (HOBT), anthracene and guaiacol were purchased from SRL Chemicals Pvt. Ltd. (Mumbai, India). Veratryl alcohol and syringaldehyde were the products of Hi-Media Pvt. Ltd. (Mumbai, India). Bitter gourd (*M. charantia*) was obtained from local vegetable market. The other chemicals and reagents were of analytical grade and were used without any further purification.

2.2. Ammonium sulphate fractionation of M. charantia protein

50 g of bitter gourd (*M. charantia*) collected from local vegetable market was homogenized in 100 mL of 100 mM sodium acetate buffer, pH 4.0. Homogenate was filtered through four layers of cheesecloth. The filtrate was then centrifuged at 10,000 × *g* on a Remi C-24 Cooling Centrifuge. The clear supernatant was subjected to salt fractionation by adding 20–80% (w/v) $(NH_4)_2SO_4$. This solution was stirred overnight at 4 °C to obtain maximum precipitation. The precipitate was collected by centrifugation at 10,000 × *g* on a Remi C-24 Cooling Centrifuge. The obtained precipitate was redissolved in an appropriate volume of 100 mM sodium acetate buffer, pH 4.0 and dialyzed against the assay buffer [20].

2.3. Immobilization of BGP on the surface of Con A layered calcium alginate–starch beads

Jack bean (*Canavalia ensiformis*) 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 3000 × *g* for 30 min. After centrifugation the clear supernatant was collected and this collected supernatant was used as a source of Con A.

Immobilization of BGP on the surface of Con A layered calcium alginate–starch beads was done according to the procedure described earlier [19].

2.4. Anthracene solubility

The solubility of anthracene (5.0 mM, stock solution) was examined by taking concentrations of the mixed solvents in (v/v), i.e. (10–50%) acetone, DMF, DMSO, methanol, propanol and various mixtures of these solvents; (5–25%) acetone + (5–25%) propanol, (5–25%) acetone + (5–25%) DMF, (5–25%) methanol + (5–25%) DMSO, (5–25%) acetone + (5–25%) methanol, (5–25%) acetone + (5–25%) DMSO, (5–25%) methanol + (5–25%) propanol, (5–25%) methanol + (5–25%) DMF, (5–25%) methanol + (5–25%) DMSO, (5–25%) propanol + (5–25%) DMF, (5–25%) propanol + (5–25%) DMSO, (5–25%) DMF + (5–25%) DMSO, prepared in 100 mM sodium acetate buffer, pH 4.0.

2.5. Effect of redox mediators on anthracene removal

The oxidation of anthracene (0.5 mM, 5.0 mL) prepared in 100 mM sodium acetate buffer, pH 4.0 containing a mixture of 17.5% acetone + 17.5% DMF was investigated in the presence of seven different redox mediators; HOBT, violuric acid, veratryl alcohol, phenol, syringaldehyde, guaiacol and azinobis (3-ethyl benzothiazoline-6 sulfonic acid) diammonium salt. The molarity of each redox mediator was 0.1 mM and 0.2 mM. The oxidative removal of anthracene was catalyzed by BGP (0.4 U mL^{-1}) in 100 mM sodium acetate buffer, pH 4.0 in the presence of 0.70 mM H_2O_2 at 40 °C for 2.5 h.

2.6. Effect of water-miscible organic solvent mixtures on removal of anthracene by BGP

Four mixtures of organic solvents; (i) 20% acetone + 20% propanol, (ii) 17.5% acetone + 17.5% DMF, (iii) 20% acetone + 20% DMSO and (iv) 20% propanol + 20% DMSO prepared in 100 mM sodium acetate buffer, pH 4.0 showed maximum solubility of anthracene were selected for the oxidation of this compound by BGP. The solutions of anthracene (0.5 mM, 5.0 mL), prepared in all these mixtures were treated independently by soluble and immobilized enzyme (0.4 U mL^{-1}) in the presence of 0.1 mM guaiacol. The reaction was initiated by adding 0.70 mM H_2O_2. The reaction mixtures were incubated at 40 °C for 2.5 h. The reaction was stopped by heating in a boiling water bath for 5 min. Insoluble product was removed by centrifugation at 3000 × *g* for 15 min. The decrease in absorbance at specific $\lambda_{max(254)}$ was monitored. The percent removal was calculated by taking untreated anthracene solution in each mixture of organic solvents as control (100%).

2.7. Effect of enzyme concentration

Anthracene solution (0.5 mM, 5.0 mL) prepared in a solvent mixture of 17.5% acetone + 17.5% DMF in sodium acetate buffer, pH 4.0 was incubated with soluble enzyme (0.1–0.6 U mL^{-1}) in the presence of 0.1 mM guaiacol and 0.70 mM H_2O_2 at 40 °C for 2.5 h. The percent removal of anthracene was calculated as described above.

2.8. Effect of time on the degradation of anthracene by BGP

Anthracene (0.5 mM, 5.0 mL) polluted water prepared in 100 mM sodium acetate buffer, pH 4.0 containing a solvent mixture of 17.5% acetone + 17.5% DMF was treated by the soluble enzyme (0.4 U mL^{-1}) at 40 °C for 0.5–3.0 h in the presence of 0.70 mM H_2O_2 and 0.1 mM guaiacol. The percent removal of anthracene was calculated as described in Section 2.6.

2.9. Procedure for anthracene degradation by BGP

Anthracene polluted water (0.5 mM, 5.0 mL) prepared in 100 mM sodium acetate buffer, pH 4.0 containing a solvent mixture of 17.5% acetone + 17.5% DMF was treated by the soluble enzyme (0.4 U mL^{-1}) in the presence of 0.1 mM guaiacol and 0.70 mM H_2O_2 at 40 °C for 2.5 h,

The percent removal of anthracene was calculated at its $\lambda_{max(254)}$.

2.10. Effect of pH and temperature

Anthracene (0.5 mM, 5.0 mL) polluted water prepared in the buffers of different pH (2.0–10.0) containing a solvent mixture of 17.5% acetone + 17.5% DMF was independently treated by soluble and immobilized enzyme (0.4 U mL^{-1}) in the presence of 0.70 mM H_2O_2 and 0.1 mM guaiacol for 2.5 h at 40 °C. The buffers used were glycine–HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0 and 5.0), sodium phosphate (pH 6.0–8.0), and Tris–HCl (pH 9.0 and 10.0). The molarity of each buffer was 100 mM.

Anthracene polluted water (0.5 mM, 5.0 mL) prepared in 100 mM sodium acetate buffer, pH 4.0 containing a solvent mixture of 17.5% acetone + 17.5% DMF was independently treated by soluble and immobilized enzyme (0.4 U mL^{-1}) in the presence of 0.70 mM H_2O_2 and 0.1 mM guaiacol at various temperatures (20–80 °C) for 2.5 h. The insoluble product formed after reaction was removed by centrifugation at 3000 × *g* for 15 min.

2.11. Removal of anthracene in a batch process

Anthracene polluted water (0.5 mM, 500 mL) prepared in 100 mM sodium acetate buffer, pH 4.0 containing a solvent mixture of 17.5% acetone + 17.5% DMF was treated independently by soluble and immobilized enzyme (20 U) in batch process for varying times at 40 °C in the presence of 0.70 mM H_2O_2 and 0.1 mM guaiacol. The aliquots of 5.0 mL were taken from the reaction mixture at the gap of 30 min. Percent removal was calculated as described in Section 2.6.

2.12. Removal of anthracene in spiral-bed reactor

Spiral-bed reactor was developed for the continuous removal of anthracene. Reactor (70 cm × 1.5 cm) containing immobilized enzyme (4524 U) was used for the continuous degradation and removal of anthracene. Anthracene polluted water (0.5 mM) prepared in 100 mM sodium acetate buffer, pH 4.0 containing a solvent mixture of 17.5% acetone + 17.5% DMF, 0.70 mM H_2O_2 and 0.1 mM guaiacol was continuously passed through the spiral-bed reactor at room temperature (30 ± 2 °C). Flow rate of the column was maintained at 20 mL h^{-1}. Samples were collected after every 5 days and after centrifugation were analyzed for the remaining anthracene spectrophotometrically.

2.13. Spectra of anthracene

The absorption spectra of treated and untreated anthracene solutions were recorded on Cintra 10*e* UV–visible spectrophotometer.

2.14. FT-IR analysis

The FT-IR spectral studies were performed using KBr pelleting technique with INTERSPEC 2020 model FT-IR instrument, USA. The calibration was done by polystyrene film. The sample was injected by Hamiet 100 μL syringe in ATR box. The syringe was first washed by acetone followed by washing with distilled water. FT-IR analysis was performed to monitor the functional groups present in the native compound and on the product.

2.15. Allium cepa test for toxicity measurement

The *A. cepa* bioassay for parent compound and immobilized enzyme catalyzed products was carried out according to the method of Fiskesjo [21]. For this test small onions of equal size were taken and yellowish brown outer scales and brownish bottom plates were removed using a sharp knife. Care was taken to maintain the ring primordial intact. Boiling tubes filled with control and samples treated by BGP (0.4 U mL^{-1}) in the presence of 0.70 mM H_2O_2 and 0.1 mM guaiacol in 100 mM sodium acetate buffer, pH 4.0 and were kept in dark. Distilled water was used as control in all experiments. One onion was placed at the top of each tube with root primordial downward touching the liquid. After a gap of 12 h the same samples were added in the respective tubes to fill up to the top and care was taken to prevent gap between onion bulb and sample present in the tube. The treatment was continued for 15 days. After completion of the time of treatment, onions were taken out and root length of each bulb was measured. Inhibition in the growth of *A. cepa* roots was considered as an index for the degree of toxicity [21].

2.16. Measurement of peroxidase activity

Peroxidase activity was measured from the change in the optical density ($A_{460\ nm}$) in 100 mM sodium acetate buffer, pH 4.0 at 40 °C by measuring the initial rate of oxidation of 6.0 mM *o*-dianisidine HCl in the presence of 18 mM H_2O_2. Immobilized enzyme preparation was continuously agitated for entire duration of assay. The assay was highly reproducible with immobilized enzyme preparation [22].

One unit (1.0 U) of peroxidase activity was defined as the amount of enzyme protein that catalyzes the oxidation of 1.0 μmol of *o*-dianisidine HCl per min at 40 °C into colored product (ε_m = 30,000 M^{-1} cm^{-1}).

2.17. Estimation of protein

Protein concentration was determined using procedure described by Lowry et al. [23]. Bovine serum albumin was used as a standard protein.

2.18. 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.

3. Results

3.1. Solubility of anthracene in water-miscible organic solvent mixtures

The maximum solubilization of anthracene was obtained in a mixture of 17.5% acetone + 17.5% DMF prepared in 100 mM sodium acetate buffer, pH 4.0. Other water-miscible organic solvent mixtures showed lower solubility of anthracene.

3.2. Removal of anthracene in water-miscible organic solvent mixtures

The effect of water-miscible organic solvent mixtures on the activity and oxidation of anthracene by the soluble enzyme was evaluated. Anthracene was maximally polymerized by enzyme to 40% in a mixture of 17.5% acetone + 17.5% DMF, whereas other mixtures; 20% acetone + 20% propanol, 20% acetone + 20% DMSO and 20% propanol + 20% DMSO oxidized anthracene to 35%, 25% and 20%, respectively.

Table 1
Effect of redox mediators on the degradation of anthracene.

Redox mediator	Conc. of redox mediator (mM)	Anthracene removal (%)
Control (without redox mediator)	0.0	40
HOBT	0.1	64
	0.2	70
Violuric acid	0.1	54
	0.2	60
Veratryl alcohol	0.1	20
	0.2	15
Syringaldehyde	0.1	45
	0.2	51
Phenol	0.1	56
	0.2	59
Guaiacol	0.1	83
	0.2	66
Azinobis (3-ethyl benzothiazoline-6 sulfonic acid) diammonium salt	0.1	51
	0.2	53

Removal of anthracene in the presence of different redox mediators was done as given in Section 2.5. The percent removal was calculated by taking untreated anthracene solution in sodium acetate buffer, pH 4.0 with each redox mediator as control (100%).

Table 2
Guaiacol mediated oxidative degradation of anthracene by BGP.

Composition of mixtures	Anthracene removal (%)	
	S-BGP	I-BGP
Acetone (20%) + propanol (20%)	69	81
Acetone (17.5%) + DMF (17.5%)	83	95
Acetone (20%) + DMSO (20%)	64	78
Propanol (20%) + DMSO (20%)	71	80

Treatment of anthracene in four different solvents mixtures in the presence of guaiacol was done as given in Section 2.6. The percent removal was calculated by taking untreated anthracene solution in sodium acetate buffer, pH 4.0 with of each mixture as control (100%).

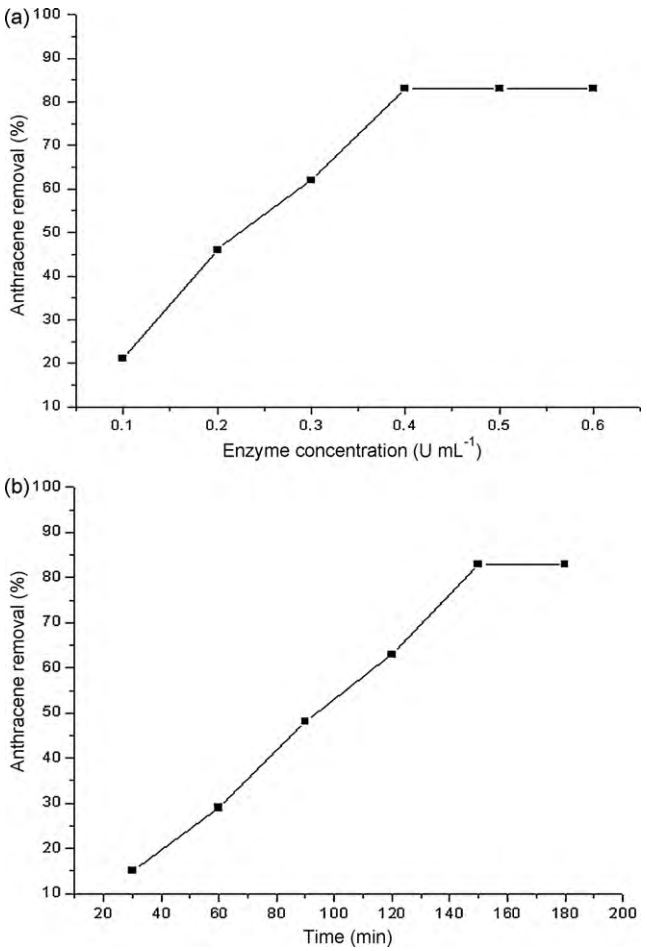

Fig. 1. Effect of enzyme concentration (a) and time (b) on the treatment of anthracene by soluble BGP. Water containing anthracene (0.5 mM, 5 mL) was treated by the soluble enzyme as described in Sections 2.7 and 2.8. The percent removal was calculated by taking untreated anthracene solution as control (100%).

3.3. Removal of anthracene in the presence of different redox mediators

Table 1 demonstrates the oxidative polymerization and removal of anthracene by the soluble enzyme in the presence of seven different redox mediators; HOBT, violuric acid, veratryl alcohol, phenol, syringaldehyde, guaiacol and azinobis (3-ethyl benzothiazoline-6 sulfonic acid) diammonium salt, in the mixture of 17.5% acetone + 17.5% DMF. The maximum removal of anthracene by soluble enzyme was 83% in the presence of 0.1 mM guaiacol followed by other redox mediators (Table 1).

3.4. Guaiacol mediated oxidation of anthracene by soluble and immobilized BGP

The effect of water-miscible organic solvents and their mixtures on the activity of soluble and immobilized enzyme was evaluated in the presence of 0.1 mM guaiacol. The removal of anthracene by soluble and immobilized enzyme in a mixture of 17.5% acetone + 17.5% DMF was 83% and 95%, respectively. However, the removal of anthracene in other mixtures of organic solvents was relatively low. The oxidative polymerization and removal of anthracene by immobilized enzyme was significantly higher as compared to soluble enzyme (Table 2).

3.5. Effect of peroxidase concentrations on the removal of anthracene

The oxidative polymerization and removal of anthracene was increased with increasing concentrations of the enzyme and it reached highest to 83% in the presence of 0.4 U mL^{-1} of the enzyme and 0.1 mM guaiacol in the sodium acetate buffer, pH 4.0 at 40 °C (Fig. 1a).

3.6. Effect of time on the oxidative polymerization of anthracene

The effect of time on the anthracene removal by peroxidase-catalyzed reaction was determined. The oxidation of anthracene was continuously increased with time. The maximum removal of anthracene was observed after 2.5 h of incubation (Fig. 1b). Further increase in the time of incubation had no significant effect on the oxidative removal of anthracene.

3.7. Effect of pH and temperature

The role of pH on the removal of anthracene by soluble and immobilized enzyme has been demonstrated in Fig. 2a. Anthracene was maximally oxidized in the buffer of pH 4.0 by S-BGP while its removal by I-BGP was maximum at pH 5.0.

The removal of anthracene was maximum at 40 °C and 50 °C by S-BGP and I-BGP, respectively. However, further increase in tem-

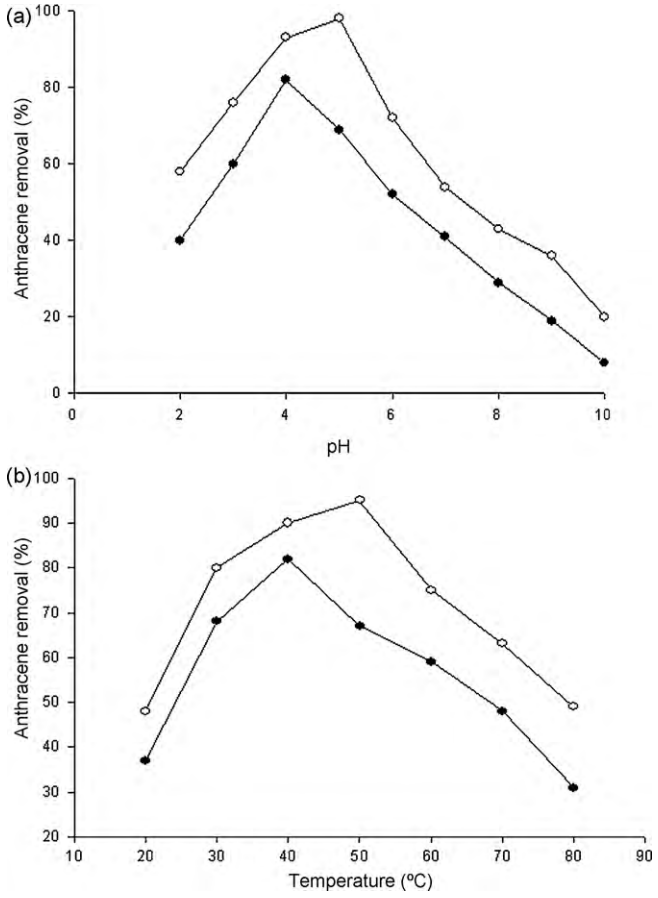

Fig. 2. Effect of pH (a) and temperature (b) on the treatment of anthracene by BGP. Water containing anthracene (0.5 mM, 5 mL) was treated by the enzyme as described in Section 2.10. The percent removal was calculated by taking untreated anthracene solution in the buffer of each pH as control (100%). Symbols indicate treatment of anthracene by soluble (●) and immobilized (○) enzyme.

Table 3
Anthracene removal in batch processes.

Times (h)	Anthracene removal (%)	
	S-BGP	I-BGP
1	5	15
2	12	24
3	23	37
4	31	54
5	48	67
6	57	72
7	66	99
8	71	99
9	71	99
10	71	99

Anthracene removal was done by soluble and immobilized enzyme as described in Section 2.11. The percent removal was calculated by taking untreated anthracene solution as control (100%).

perature resulted in declining the oxidative removal of anthracene (Fig. 2b).

3.8. Oxidation of anthracene in batch process by BGP

Table 3 depicts removal of anthracene by soluble and immobilized enzyme in a batch process. It was observed that I-BGP could oxidize 99% anthracene within 7 h of incubation, whereas S-BGP catalyzed oxidative polymerization and removal of this compound to 71% within 8 h. Increasing time of incubation had no marked effect on the oxidative polymerization of anthracene. However, I-BGP showed higher oxidative polymerization and removal of anthracene as compared to S-BGP with respective time.

3.9. Analysis of oxidized anthracene in spiral-bed reactor

The diagram of the spiral-bed reactor in terms of anthracene oxidative polymerization has been shown in Fig. 3. The oxidative polymerization and removal of anthracene was 100% for first 5 days. There was an inverse relationship in anthracene polymerization and time of operation of reactor. As the time of operation of reac-

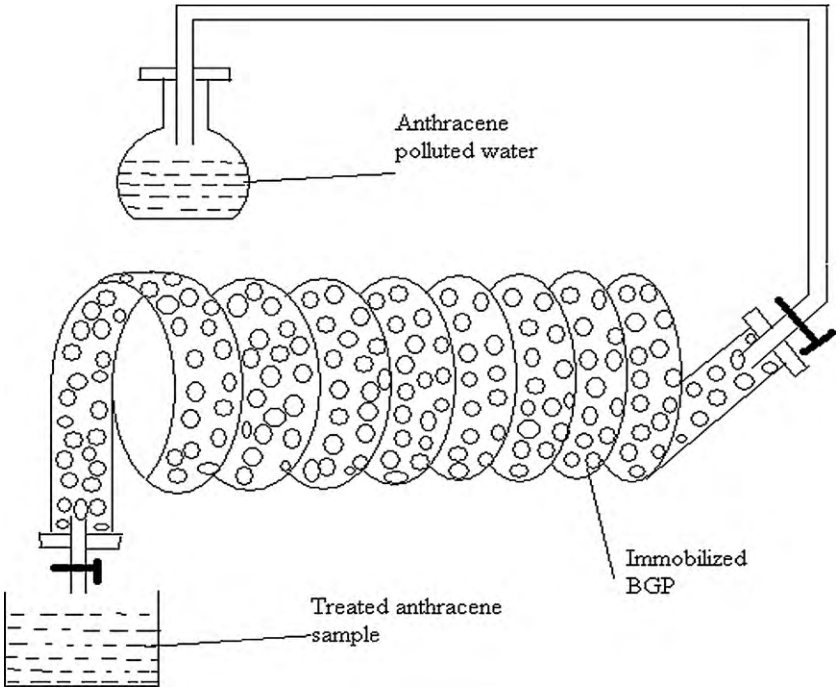

Fig. 3. Diagrammatic representation of spiral-bed reactor.

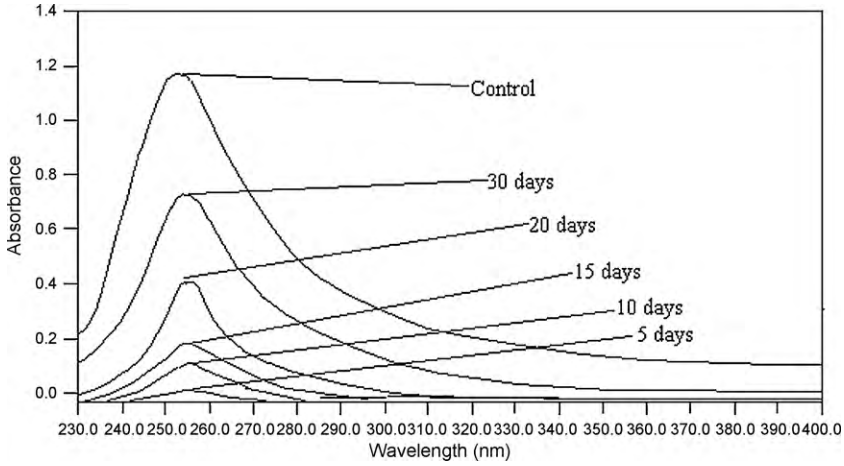

Fig. 4. UV absorbance spectra of anthracene before and after treatment. UV absorbance spectra were recorded before and after the treatment of anthracene by UV–visible Cintra 10*e* spectrophotometer. For treated and untreated anthracene the spectra are labeled in the figure.

tor increased, the oxidative removal of anthracene was decreased. However, only 41% anthracene removal was seen when spiral-bed reactor filled with immobilized enzymes was operated continuously for 30 days.

In order to confirm the conversion of anthracene by I-BGP, some spectral analyses were performed. Fig. 4 demonstrates the absorption spectra of treated and untreated anthracene polluted water with respect to number of days of operation of the spiral-bed reactor. The decrease in absorbance peaks in UV region of the anthracene was a clear evidence regarding the removal of this pollutant from the treated water.

3.10. FT-IR analysis of anthracene and peroxidase catalyzed product

The FT-IR spectrum of anthracene and its catalyzed product were shown in Fig. 5b and a, respectively. The middle of the IR spectrum of anthracene provided a characteristic peak due to aromatic C–H stretch at 3047 cm^{-1} which is very sharp. The sharpness of the peak shows that the hydrogen atoms in the anthracene ring was not exerting any bonding interaction with molecules. The skeletal vibrations (carbon carbon double bond) of the ring could be assigned to peaks at 1614, 1523 and 1447 cm^{-1}. The presence of two sets of four adjacent hydrogen atoms was evidenced by the peak at 718 cm^{-1}, due to C–H out of plane bending vibration. The peak at 885 cm^{-1} was due to C–H out of plane bending vibration which corresponded to two isolated hydrogen atoms. Over tones or combination bands were found at 1923–1700 cm^{-1}. Out of plane (carbon carbon double bond) could be assigned to 596.9–470 cm^{-1}. In second spectrum (Fig. 5b) the IR peaks 819 and 615 cm^{-1} belongs to phenyl rings (C–H), another peaks 1740 and 1653 cm^{-1} was due to C=O (s) and next two peaks 1552 and 1516 cm^{-1} (w) because of aromatic rings. On the basis of this analysis it was clear that the compound was a derivative of anthraquinone.

3.11. Determination of phytotoxicity of untreated and treated samples of anthracene

In order to examine the toxicity caused by BGP treated product of anthracene, the phytotoxicity experiment was performed using *A. cepa* test with untreated and treated anthracene solutions. Table 4 shows the growth of *A. cepa* roots in terms of length in

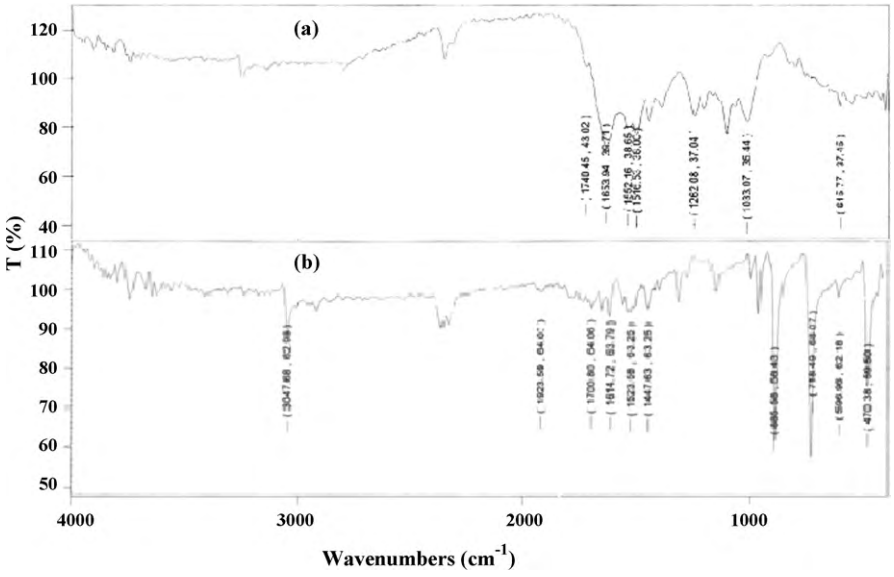

Fig. 5. FT-IR analysis. The FT-IR spectral studies were performed with INTERSPEC 2020 model FT-IR instrument, USA. The calibration was done by polystyrene film. Peaks in (a) and (b) related to the enzyme catalyzed product, one anthraquinone derivatives and anthracene respectively.

Table 4
Allium cepa test of anthracene and enzyme treated anthracene.

Test solution	Root length (cm)	Inhibition (%)
Control	5.0	–
Untreated	0.20	96
Treated	1.1	78

Onion bulbs were placed at the top of each tube containing control and treated samples with root primordial downwards touching the liquid. Distilled water was used as control for all the samples. In order to prevent the gap between onion bulbs and the liquid, respective samples were added to each tube after a gap of 12 h. The experiments were carried out for 15 days in dark. Inhibition in the growth of *Allium cepa* roots with respect to control was considered as an index for the degree of toxicity.

centimeter and percent inhibition brought about by treated and untreated solutions. *A. cepa* incubated with untreated anthracene solution for 15 days and it showed 96% inhibition in root length. The average root length was recorded to be 0.20 cm compared to 5.0 cm in control while BGP treated anthracene solution exhibited an inhibition of 78%.

4. Discussion

The development of an efficient oxidative polymerization and removal system for polyaromatics based on the use of peroxidases *in vitro* requires their increased bioavailability using organic solvents. The use of water-miscible organic solvents is preferred to solubilize hydrophobic substrates [24,25]. The choice of an organic solvent for a given reaction should be based on three factors; (i) ecological toxicity of the solvent; (ii) effects of solvent on the reaction (including solubility of the substrate); and (iii) effect of solvent on the biocatalyst stability. Since the solvent can affect the hydration shell of the enzyme molecule, it is necessary to maintain the native conformation [26–28]. Acetone at a specific concentration had no significant deactivation effect on peroxidases. In monophasic systems, the enzymatic activity loss has been mainly attributed to the fact that water molecules in the enzyme were stripped away or replaced by solvent molecules causing deformation and denaturation of the enzymes [28,29].

The effect of various redox mediators on the oxidative polymerization of anthracene was demonstrated in Table 1. Johannes et al. [30] have already reported the oxidation of anthracene by laccase from *Trametes versicolor* and they found that after 72 h incubation about 35% of the anthracene was transformed to 9,10-anthraquinone. Transformation of anthracene increased rapidly in the presence of different mediators but these mediators were required at very high concentrations (2.0 mM azinobis (3-ethyl benzothiazoline-6 sulfonic acid) diammonium salt and 1.0 mM HOBT). Here, we reported the involvement of a very low concentration of guaiacol (0.1 mM) which was sufficient to convert anthracene into polymerized products.

The maximum 83% anthracene transformation was obtained by 0.40 U mL^{-1} BGP (Fig. 1a). Eibes et al. [31] have used 60–550, 210–1310 and 170–1340 U L^{-1} of MnP for the degradation of anthracene, pyrene and dibenzothiophene respectively. However, in this study we reported a requirement of very low concentration of BGP for the maximum anthracene removal.

The maximum oxidative polymerization time for anthracene by soluble BGP was 2.5 h (Fig. 1b). Several earlier workers have shown the removal of anthracene, dibenzothiophene and pyrene was different and it might depend on the structure of PAH compounds [31].

Anthrecene was maximally oxidized in the buffer of pH 4.0 at 40 °C (Fig. 2a and b). However, the oxidation of anthracene was specifically pH and temperature dependent. A broad range of pH and temperature-optimum for the removal of PAHs has already been reported in an earlier study [31].

Here for the first time an effort has been made to treat water polluted with anthracene using immobilized peroxidase. Immobilized BGP was used for the treatment of anthracene in a batch process. I-BGP was efficient enough to remove 99% anthracene while the S-BGP could remove only 71% anthracene under similar experimental condition (Table 3). It showed the immobilized enzyme was more effective in the oxidative polymerization of industrial pollutants as compared to its soluble form because they are protected against the inhibition caused by product of the reaction [32]. The size of beads of this immobilized preparation was approximates 3.0 μm, immobilization of enzymes on such support not only immobilized large amount of enzyme but also have the capability to degrade large concentration of toxic pollutants to its nontoxic product due to large surface area of enzyme and substrate interaction [33].

Spiral-bed reactor containing immobilized BGP was operated for the continuous oxidative degradation and removal of anthracene. This reactor was operated without any operational problem and had high anthracene removal efficiency. In order to confirm the oxidative degradation and removal of such aromatic compounds from wastewater through a spiral-bed reactor filled with I-BGP, some spectral analysis became an important aspect to show a loss of these compounds after treatment. The decrease in absorbance peaks in UV region provided a strong evidence for the removal of anthracene from polluted water (Fig. 4). The disappearance of absorption peaks in UV region was due to formation of insoluble compounds. Peroxidases have been reported to catalyze free-radical formation followed by spontaneous polymerization of a variety of aromatic compounds including phenols [20], chlorophenols [34] and dyes [35,36].

FT-IR spectra for anthracene and its enzymatically oxidized products were recorded in the range of 1000–4000 nm. FT-IR spectrum peaks of anthracene crystal grown in CS_2 and CCl_4 showed the skeletal vibrations (carbon carbon double bond) of the ring could be assigned to peaks 1619, 1532 and 1447 cm^{-1}. The presence of two sets of four adjacent hydrogen atoms was evidenced by the peak at 725 cm^{-1}. The peak at 883 cm^{-1} was due to C–H out of plane bending vibration which corresponded to two isolated hydrogen atoms. Over tones or combination bands were found at 1926.7–1719.4 cm^{-1}. Out of plane could be assigned to 469.3–438 cm^{-1} [37]. In another study anthracene grown by double run selective self-seeding vertical Bridgmann technique was subjected to FT-IR spectral studies. The characteristic peaks were found at 3047, 1615, 1500, 1445, 719, 469 cm^{-1}. Thus, the spectral data showed the purity of grown crystals. It also exhibited that there was no solvent inclusion in the grown crystals [38]. Anthracene catalyzed by soybean peroxidase (SBP) in the presence of water-miscible organic co-solvents, the product yielded exclusively anthraquinone, thereby demonstrating that SBP catalyzed a formal six electron oxidation of the un-activated aromatic substrate to the quinine [39]. In another published work three PAH_S compounds were treated by manganese peroxidase. The intermediate compounds were determined using gas chromatography–mass spectrometry. Anthracene was degraded to a intermediate, 9,10-anthraquinone and finally to phthalic acid [31]. Organic synthesis of a new compound octaiodo 9,10-anthraquinone was reported by Jiang and Jin [40]. In this study the spectra of many anthraquinone derivatives have been shown. FT-IR spectra of octaiodo 9,10-anthraquinone had peaks at 1624.8, 1336.0, 1182.1, 998.9 and 482.7 cm^{-1} and these peaks were same as given by us for the BGP catalyzed anthracene product, except the peak of iodo.

The proposed degradation pathway of anthracene by BGP is given in Fig. 6. BGP catalyzed conversion of guaiacol to phenoxy guaiacol. This phenoxy radical attack on anthracene and transformed it into anthracene free radical. This free rad-

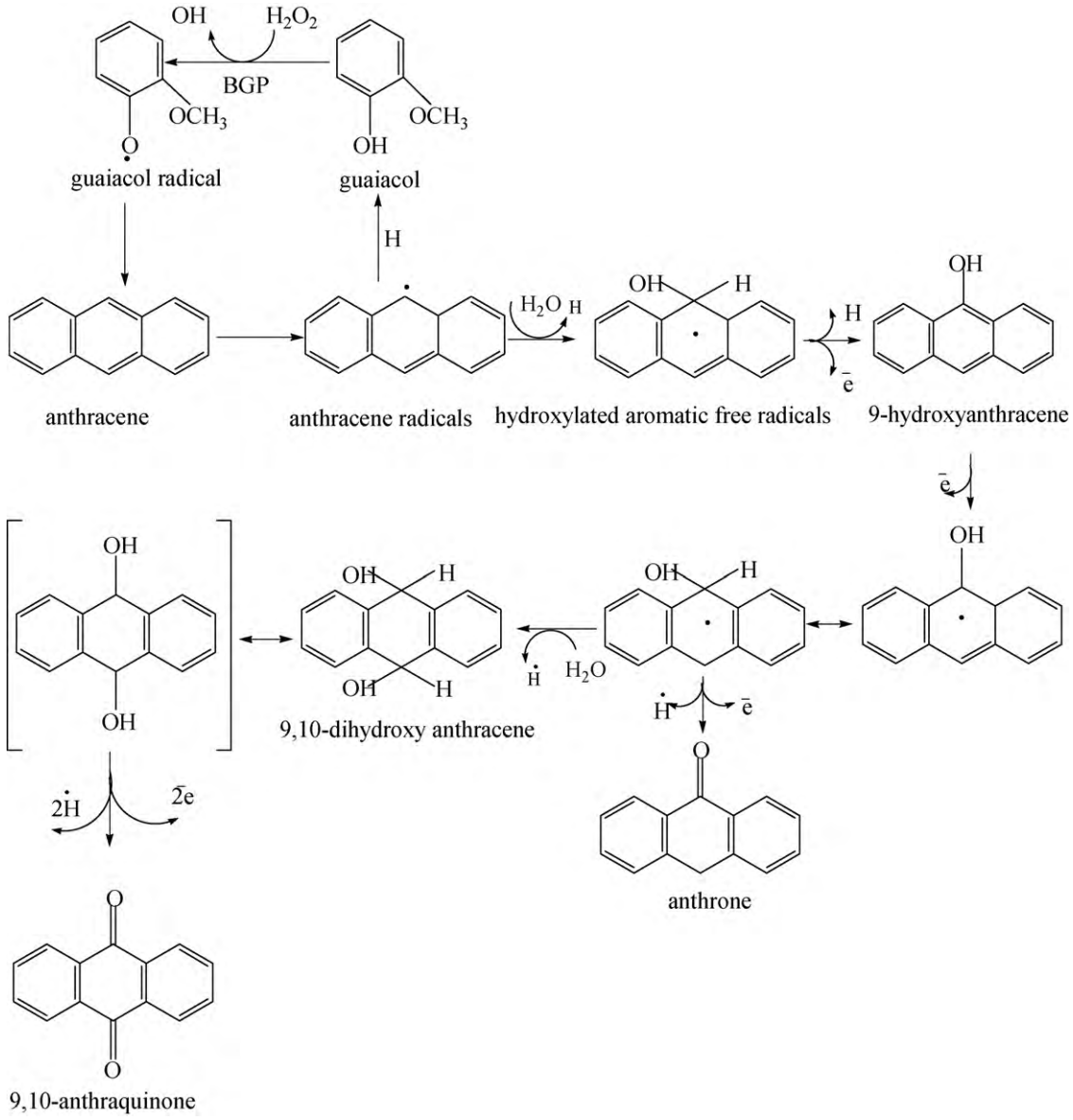

Fig. 6. Proposed degradation pathway of anthracene by BGP.

ical species accept $OH^{\cdot}$ radical from H_2O and changed into 9-hydroxyanthracene intermediate. 9-Hydroxyanthracene further undergoes one-electron oxidation to give a radical form, after an electron oxidation of this radical, anthrone was detected. 9-Hydroxyanthracene radical can then accept a water molecule to give 9,10-dihydrodiol anthracene. Finally, two-electron oxidation of the 9,10-dihydroxy anthracene to 9,10-anthraquinone would also be catalyzed by BGP. Potthast et al. [41] provide evidence that co-oxidant, which transfers an electron to the enzyme, initiating the ability of the enzyme to accomplish electron transfer. Previously it has been shown that the purified extracellular laccases from *P. ostreatus* catalyzed single electron transfer reactions when the lignin-related compound, 3,5-dimethoxy-5-hydroxyacetophenone was used as substrate [42]. In another study degradation of anthracene by MnP, the formation of anthrone was detected, which was an expected intermediate and it was followed by the appearance of 9,10-anthraquinone [1,31]. This compound was produced at high molar yield. Some earlier reports demonstrated the formation of anthraquinone by peroxidases *in vitro* oxidation of anthracene [43,44]. Although LiP, MnP and laccase are likely to oxidized anthracene to anthraquinone by similar mechanism involving single electron transfer reaction [45].

5. Conclusions

BGP can be efficiently used for the oxidative polymerization and removal of anthracene in the presence of various redox mediators. The treatment of anthracene by the enzyme in the presence of redox mediator produced insoluble aggregates which could be easily removed simply by centrifugation. Thus, peroxidase from bitter gourd would be highly useful in the removal of anthracene from polluted water as well as many other hazardous PAHs. These findings suggested that the use of BGP could be extended to the large scale treatment of anthracene and other related PAHs by employing more effective and cheaper redox mediators. This as well as the scale up of enzymatic processes will be the subject of further investigation. The results of this work suggested that the peroxidase and guaiacol system was an effective biocatalyst for the treatment of wide spectrum hazardous PAHs compounds present in wastewaters or effluents. The novel design of spiral-bed reactor not only

minimizes the size of reactor but also it requires minimum space. Due to its smaller size and greater efficiency for the degradation, it can be transported anywhere and can be used directly in the outlet of industries from where toxic substances/pollutants coming out.

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