Chapter VI

Removal of anthracene from polluted water by immobilized *Momordica charantia* peroxidase in batch process as well as in a continuous reactor
6.1. INTRODUCTION

PAHs are pollutants produced via natural and anthropogenic sources, generated during 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 to their mutagenic and carcinogenic potential (Cerniglia, 1992; Shuttleworth and Cerniglia, 1995).

LiP and MnP from WRF have been used for the removal of variety of PAHs (Bumpus and Steven, 1987; Field et al., 1992). However, a wider application of these enzymes is hindered by the fact that enzymes work properly in aqueous media. Many nonpolar and polyaromatics compounds such as PAHs showed very less water solubility. Due to hydrophobic nature of PAHs such compounds cannot be removed by enzymes in aqueous solution. The addition of organic solvents in the reaction mixture increased solubility of PAHs in aqueous media and thus it increased enzymatic degradation of these compounds (Cerniglia and Heitkamp, 1984; Bumpus, 1989; Kilbane, 1997). A good effort has been made to enhance PAHs solubility to several folds by adding water-miscible co-solvents or surfactants (Field et al., 1995b; Lee et al., 2001; Zheng and Obbard, 2002) 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 (Husain and Husain, 2008). Immobilization of enzymes provided higher stability, reusability and capability to work 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/non-covalent attachment to the matrix (Zaks and Klibanov, 1998; Klibanov, 2001; Husain and Husain, 2008; Husain and Ulber, 2010).

Recently bioaffinity based procedure has attracted attention of the enzymologists due to their direct immobilization from partially purified preparation or
even from crude homogenates (Akhtar et al., 2005c; Kulshrestha and Husain, 2006b; Khan and Husain, 2007a; 2007b). Surface immobilized enzymes are more superior as compared to entrapped enzymes as in the later case the diffusion of large molecular size substrate/products from in and out the gel beads was difficult (Le-Tien et al., 2004; Matto and Husain, 2009c). In order to prevent the possibility of accumulation of products inside the gel beads, the immobilization of enzymes on the surface of a support would be a preferred choice.

BGP immobilized on the surface of Con A layered calcium alginate-starch hybrid beads (SI-BGP) has been employed for the removal of anthracene in the presence of water miscible organic solvents from polluted water. The oxidation and removal of anthracene by immobilized BGP has been 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.

6.2. MATERIALS AND METHOD

6.2.1. Materials

BSA, VLA and o-dianisidine HCl were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Ammonium sulphate, DMF, DMSO, HOBT, anthracene and guaiacol were purchased from SRL Chemicals Pvt. Ltd. (Mumbai, India). VA and syringaldehyde were the products of Hi-Media Pvt. Ltd (Mumbai, India). Bitter gourd was obtained from local vegetable market. The other chemicals and reagents of analytical grade and were used without any further purification.

6.2.2. Ammonium sulphate fractionation of bitter gourd protein

Ammonium sulphate fractionation of bitter gourd peroxidase in sodium acetate buffer, pH 4.0 was done as described in Section 2.2.2, Chapter 2.
6.2.3. 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 6 h. Insoluble residue was removed by centrifugation at 3,000xg for 30 min. After centrifugation clear supernatant was collected and the collected supernatant was used as a source of Con A.

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 4.0 in three batches and beads were prepared by dropping this mixture into 0.2 M CaCl₂ solution. Beads of each batch were incubated with jack bean extract (10.0 mL) overnight with slow stirring at room temperature. Next day, Con A bound calcium alginate-starch beads were collected and washed with assay buffer. Con A layered calcium alginate-starch beads from all the batches were pooled and incubated with BGP overnight at room temperature with slow stirring (Matto and Husain, 2009c). The unbound enzyme was removed by repeated washing with 100 mM sodium acetate buffer, pH 4.0.

BGP immobilized on the surface of Con A layered calcium alginate-starch beads was crosslinked by 0.5% (v/v) glutaraldehyde for 2 h at 4 °C with 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 4.0 (Matto and Husain, 2009c).

6.2.4. Anthracene solubility

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

### 6.2.5. 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% (v/v) acetone + 17.5% (v/v) DMF was investigated in the presence of seven different redox mediators; HOBT, VLA, VA, phenol, syringaldehyde, guaiacol and ABTS. 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 H₂O₂ at 40 °C for 2.5 h.

### 6.2.6. Effect of water miscible organic-solvent mixtures on anthracene oxidation

Four mixtures of organic solvents; (i) 20% (v/v) acetone + 20% (v/v) propanol, (ii) 17.5% (v/v) acetone + 17.5% (v/v) DMF, (iii) 20% (v/v) acetone + 20% (v/v) DMSO and (iv) 20% (v/v) propanol + 20% (v/v) 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 H₂O₂. 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 3000xg for 15 min. The decrease in absorbance at specific λ_max(254) was monitored. The percent removal was calculated by taking untreated anthracene solution in each mixture of organic solvents as control (100%).

### 6.2.7. Effect of enzyme concentration

Anthracene solution (0.5 mM, 5.0 mL) prepared in a solvent mixture of 17.5% (v/v) acetone and 17.5% (v/v) 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 H₂O₂ at 40 °C for 2.5 h. The percent removal of anthracene was calculated as described in Section 6.2.6.

6.2.8. 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% (v/v) acetone and 17.5% (v/v) 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 H₂O₂ and 0.1 mM guaiacol. The percent removal of anthracene was calculated as described in Section 6.2.6.

6.2.9. 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% (v/v) acetone and 17.5% (v/v) DMF was independently treated by soluble and surface immobilized enzyme (0.4 U mL⁻¹) in the presence of 0.70 mM H₂O₂ 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% (v/v) acetone and 17.5% (v/v) DMF was independently treated by soluble and surface immobilized enzyme (0.4 U mL⁻¹) in the presence of 0.70 mM H₂O₂ 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 3,000xg for 15 min.

6.2.11. 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% (v/v) acetone and 17.5% (v/v) DMF was treated independently by soluble and surface immobilized enzyme (20 U) in batch process for varying times at 40 °C in the presence of 0.70 mM H₂O₂ 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 6.2.6.
6.2.12. Removal of anthracene in spiral-bed reactor

Spiral-bed reactor was developed for the continuous removal of anthracene. Reactor (70x1.5 cm) containing surface 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% (v/v) acetone and 17.5% (v/v) DMF, 0.70 mM H₂O₂ 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 d and after centrifugation were analyzed for the remaining anthracene spectrophotometrically.

6.2.13. Spectra of anthracene

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

6.2.14. FT-IR analysis

The FT-IR spectral studies were performed using KBr pelleting technique with INTERSPEC 2020 model FT-IR instrument, USA as described in Section 2.2.11, Chapter 2.

6.2.15. Allium cepa test for toxicity measurement

The *Allium cepa* bioassay for the parent compound and immobilized enzyme catalyzed products was performed according to the procedure described by Fiskesjö (1985). For this test small onions of equal size were taken and yellowish brown outer scales and brownish bottom plates were removed by 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 H₂O₂ 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 d. After completion of the time of treatment, onions were taken out and root length of each bulb was measured. Inhibition in the growth of *Allium cepa* roots was considered as an index for the degree of toxicity.

**6.2.16. Measurement of peroxidase activity**

Peroxidase activity was estimated as described in Section 2.2.12, Chapter 2.

**6.2.17. Estimation of protein**

The protein concentration was determined as described in Section 2.2.13, Chapter 2.

**6.2.18. Statistical Analysis**

Statistical Analysis was performed as described in Section 2.2.14, Chapter 2.

**6.3. RESULTS**

**6.3.1. Solubility and removal of anthracene in water-miscible organic solvent mixtures**

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

The effect of water-miscible organic co-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% (v/v) acetone + 17.5% (v/v) DMF, whereas other mixtures; 20% (v/v) acetone + 20% (v/v) propanol, 20% (v/v) acetone + 20% (v/v) DMSO and 20% (v/v) propanol + 20% (v/v) DMSO oxidized anthracene to 35%, 25% and 20%, respectively.
6.3.2. Removal of anthracene in the presence of different redox mediators

Table 15 demonstrates the oxidative polymerization and removal of anthracene by the soluble enzyme in the presence of seven different redox-mediators; HOBT, VLA, VA, phenol, syringaldehyde, guaiacol and ABTS in the mixture of 17.5% (v/v) acetone + 17.5% (v/v) 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 15).

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

The effect of water-miscible organic co-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 surface immobilized enzyme in a mixture of 17.5% (v/v) acetone + 17.5% (v/v) 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 16).

6.3.4. Effect of peroxidase concentrations on the removal of anthracene

The oxidative polymerization and removal of anthracene was increased with increasing concentrations of enzyme and it reached highest 83% in the presence of 0.4 U mL⁻¹ enzyme and 0.1 mM guaiacol in sodium acetate buffer, pH 4.0 at 40 °C (Fig. 20).

6.3.5. Effect of time on the oxidative polymerization of anthracene

Fig. 21 demonstrates the effect of time on the peroxidase catalyzed anthracene removal. The oxidation of anthracene was continuously increased with time. The maximum removal of anthracene was observed after 2.5 h of incubation. Further increase in the time of incubation had no significant effect on the oxidative removal of anthracene.
Table 15: Effect of redox mediators on the degradation of anthracene

<table>
<thead>
<tr>
<th>Redox-mediators</th>
<th>Redox-mediator (mM)</th>
<th>Anthracene removal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (without redox mediator)</td>
<td>0.0</td>
<td>40</td>
</tr>
<tr>
<td>HOBT</td>
<td>0.1</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>70</td>
</tr>
<tr>
<td>VLA</td>
<td>0.1</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>60</td>
</tr>
<tr>
<td>VA</td>
<td>0.1</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>15</td>
</tr>
<tr>
<td>Syringaldehyde</td>
<td>0.1</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>51</td>
</tr>
<tr>
<td>Phenol</td>
<td>0.1</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>59</td>
</tr>
<tr>
<td>Guaiacol</td>
<td>0.1</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>66</td>
</tr>
<tr>
<td>ABTS</td>
<td>0.1</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>53</td>
</tr>
</tbody>
</table>

Removal of anthracene in the presence of different redox mediators was done as given in section 6.2.5. 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 16: Guaiacol mediated oxidative degradation of anthracene by BGP

<table>
<thead>
<tr>
<th>Composition of mixtures</th>
<th>Anthracene removal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S-BGP</td>
</tr>
<tr>
<td>Acetone (20%, v/v) + propanol (20%, v/v)</td>
<td>69</td>
</tr>
<tr>
<td>Acetone (17.5%, v/v) + DMF (17.5%, v/v)</td>
<td>83</td>
</tr>
<tr>
<td>Acetone (20%, v/v) + DMSO (20%, v/v)</td>
<td>64</td>
</tr>
<tr>
<td>Propanol (20%, v/v) + DMSO (20%, v/v)</td>
<td>71</td>
</tr>
</tbody>
</table>

Treatment of anthracene in four different solvents mixtures in the presence of guaiacol was done as given in Section 6.2.6. The percent removal was calculated by taking untreated anthracene solution in sodium acetate buffer, pH 4.0 with each mixture as control (100%).
Fig. 20: Effect of enzyme concentration on the treatment of anthracene by soluble BGP

Water containing anthracene (0.5 mM, 5 mL) was treated by soluble enzyme as described in Section 6.2.7. The percent removal was calculated by taking untreated anthracene solution as control (100%).
Fig. 21: Effect of time on the treatment of anthracene by soluble BGP

Water containing anthracene (0.5 mM, 5 mL) was treated by soluble enzyme as described in Section 6.2.8. The percent removal was calculated by taking untreated anthracene solution as control (100%).
6.3.6. Effect of pH and temperature

The role of pH on the removal of anthracene by soluble and surface immobilized BGP has been demonstrated in Fig. 22. Anthracene was maximally oxidized in the buffer of pH 4.0 by S-BGP while its removal by SI-BGP was maximum at pH 5.0.

The removal of anthracene was maximum at 40 °C and 50 °C by S-BGP and SI-BGP, respectively. However, further increase in temperature resulted in declining the oxidative removal of anthracene (Fig. 23).

6.3.7. Oxidation of anthracene in batch process by BGP

Table 17 depicts removal of anthracene by soluble and immobilized enzyme in batch processes. It was observed that SI-BGP could oxidize 99% anthracene within 7 h of incubation, whereas S-BGP oxidative polymerization and removal of this compound was 71% after 8 h. Increasing time of incubation had no marked effect on the oxidative polymerization of anthracene. However, SI-BGP showed higher oxidative polymerization and removal of this compound as compared to S-BGP with respective time.

6.3.8. 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. 24. The oxidative polymerization and removal of anthracene was 100% for first 5 d. There was an inverse relationship in the anthracene polymerization and time of operation of reactor. As the time of operation of reactor 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 d.

In order to confirm the conversion of anthracene by SI-BGP, some spectral analyses were performed. Fig. 25 demonstrates the absorption spectra of treated and untreated anthracene polluted water with respect to number of days of operation of the
Fig. 22: Effect of pH on the treatment of anthracene by BGP

Water containing anthracene (0.5 mM, 5 mL) was treated by the enzyme as described in Section 6.2.10. 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 (○) BGP.
Fig. 23: Effect of temperature on the treatment of anthracene by BGP

Water containing anthracene (0.5 mM, 5 mL) was treated by the enzyme as described in Section 6.2.10. 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 (○) BGP.
Table 17: Anthracene removal in batch processes

<table>
<thead>
<tr>
<th>Times (h)</th>
<th>Anthracene removal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S-BGP</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>23</td>
</tr>
<tr>
<td>4</td>
<td>31</td>
</tr>
<tr>
<td>5</td>
<td>48</td>
</tr>
<tr>
<td>6</td>
<td>57</td>
</tr>
<tr>
<td>7</td>
<td>66</td>
</tr>
<tr>
<td>8</td>
<td>71</td>
</tr>
<tr>
<td>9</td>
<td>71</td>
</tr>
<tr>
<td>10</td>
<td>71</td>
</tr>
</tbody>
</table>

Anthracene removal was done by soluble and immobilized enzyme as described in Section 6.2.11. The percent removal was calculated by taking untreated anthracene solution as control (100%).
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

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

The FT-IR spectrum of anthracene and its catalyzed product were shown in Fig. 26b and 26a 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 to 1700 cm\(^{-1}\). Out of plane (carbon carbon double bond) could be assigned 596.9 to 470 cm\(^{-1}\). In second spectrum (Fig. 24b) 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.

6.3.10. 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 *Allium cepa* test with untreated and treated anthracene solutions. Table 18 shows the growth of *A. cepa* roots in terms of length in centimeter and percent inhibition brought about by treated and untreated solutions. *A. cepa* incubated with untreated anthracene solution for 15 d 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%.
Fig. 24: Diagrammatic representation of spiral-bed reactor
Fig. 25: UV absorbance spectra of anthracene before and after treatment

UV absorbance spectra were recorded before and after treatment of anthracene by UV-visible Cintra 10e spectrophotometer. For treated and untreated anthracene the spectra are labeled in the Fig.
Fig. 26: FT-IR analysis of anthracene and 4-IPP

The FT-IR spectral studies were performed with INTERSPEC 2020 model FT-IR instrument, USA. The calibration was done by polystyrene film. Peaks in Figs. (a) and (b) related to the enzyme catalyzed product, one anthraquinone derivatives and anthracene, respectively.
Table 18: *Allium cepa* test for BGP treated anthracene solution

<table>
<thead>
<tr>
<th>Test solution</th>
<th>Root length (cm)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.0</td>
<td>_</td>
</tr>
<tr>
<td>Untreated</td>
<td>0.20</td>
<td>96</td>
</tr>
<tr>
<td>Treated</td>
<td>1.1</td>
<td>78</td>
</tr>
</tbody>
</table>

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 d in dark. Inhibition in the growth of *Allium cepa* roots with respect to control was considered as an index for the degree of toxicity.
6.4. 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 by using organic solvents. The use of water-miscible organic solvents is preferred to solubilize hydrophobic substrate (Hansen et al., 2000; Ogino and Ishikawa, 2001). 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 (Gorjup et al., 1999; Jouyban et al., 2002; Yoshida et al., 2009). 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 (Jouyban et al., 2002; Schlosser and Hofer, 2002).

The effect of various redox mediators on oxidative polymerization of anthracene was demonstrated in Table 15. Johannes et al. (1996) have already reported 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 ABTS 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. 20). Eibes et al. (2006) have used 550 U L$^{-1}$ of MnP for degradation of anthracene, pyrene and dibenzothiophene. 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. 21). Several earlier workers have described removal of anthracene, dibenzothiophene and pyrene was different and it might depend on the structure of PAH compounds (Eibes et al., 2006). Anthracene was maximally oxidized in the buffer of pH 4.0 at 40 °C (Figs. 22 and 23). 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 (Eibes et al., 2006).

Here for the first time an effort has been made to treat water polluted with anthracene by using immobilized peroxidase. Immobilized BGP was used for the treatment of anthracene in a batch process. SI-BGP was efficient enough to remove 99% anthracene while the S-BGP could remove only 71% anthracene under similar experimental condition (Table 17). It showed that the immobilized enzyme was more effective in the oxidative polymerization of industrial pollutants as compared to its soluble counter part because they are protected against inhibition caused by product of the reaction (Azani and Katayon, 2003). 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 (Matto et al., 2008).

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 oxidative degradation and removal of such aromatic compounds from wastewater through a spiral-bed reactor filled with SI-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. 25). 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
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 CS₂ and CCl₄ showed 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 to 1719.4 cm⁻¹. Out of plane could be assigned to 469.3-438 cm⁻¹ (Madhurambal and Srinivasan, 2006). 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 (Arul et al., 2002). Anthracene catalyzed by 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 (Kraus et al., 1999). In another published work three PAH₅ compounds were treated by MnP. The intermediate compounds were determined using GC-MS. Anthracene was degraded to a intermediate, 9,10-anthraquinone and finally to phthalic acid (Eibes et al., 2006). Organic synthesis of a new compound octaiodo 9,10-anthraquinone was reported by Jiang and Jin (2007). 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. 27. BGP catalyzed conversion of guaiacol to phenoxy guaiacol. This phenoxy radical attack on anthracene and transformed it into anthracene free radical. This free radical species accept OH radical from H₂O 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. (1995) 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 (Youn et al., 1995). 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 (Cerniglia, 1992; Eibes et al., 2006). This compound was produced at high molar yield. Some earlier reports demonstrated the formation of anthraquinone by peroxidases *in vitro* oxidation of anthracene (Hammel et al., 1986). Although LiP, MnP and laccase are likely to oxidized anthracene to anthraquinone by similar mechanism involving single electron transfer reaction (Cerniglia, 1992)
Fig. 27: Proposed degradation pathway of anthracene by BGP