Chapter III

Calcium alginate-starch hybrid support for both surface immobilization and entrapment of bitter gourd peroxidase
3.1. INTRODUCTION

Among the various techniques employed for the immobilization of enzymes, entrapment may be a good choice owing to an inert aqueous environment within the matrix and causing relatively little damage to the structure of the native enzyme (Musthapa et al., 2004). Alginate appears to be one of the most suitable polymers for the entrapment of enzymes, cell organelles and even cells because of the following advantages: hydrophilic nature, presence of carboxylic groups, natural origin, mechanical stability and stability over extreme experimental conditions (Arica et al., 2001; Zhu et al., 2005; Lu et al., 2007).

However, it has been reported that because of the porous nature of sodium alginate, most of the entrapped material is released from the gel beads during its application. In order to optimize the encapsulation efficiency and controlled release of enzyme from the gel matrix, entrapment of crosslinked or pre-immobilized enzymes has been done (Lee et al., 1993; Musthapa et al., 2004). However, the major limitation of physical entrapment is that large molecular size substrates/products cannot easily be diffuse in and out of the gel (Liang et al., 2000).

In this work an effort has been made to prepare a hybrid gel of alginate and starch which could be exploited for the entrapment of enzymes as well as bioaffinity attachment of glycosylated enzymes on the surface of gel beads. These calcium alginate-starch beads were layered with Con A. Con A layered calcium alginate-starch beads were used for the surface immobilization of glycosylated peroxidases from bitter gourd. Con A-BGP complex was also entrapped inside the calcium alginate-starch beads. A comparative stability study of entrapped and surface immobilized peroxidase has been carried out against various physical and chemical denaturants. Immobilized BGP preparations have also been studied for their reusability.

3.2. MATERIALS AND METHODS

3.2.1. Materials

Jack bean meal was procured from DIFCO, Detroit, USA. o-dianisidine HCl was obtained from the Centre for Biochemical Technology, CSIR, India. Cadmium
chloride (CdCl₂), dioxane, n-propanol, mercuric chloride (HgCl₂), starch and Tween 20 were obtained from the SRL Chemicals Pvt. Ltd. Mumbai, India. Bovine serum albumin, glutaraldehyde and ethanolamine were procured from Sigma Chemical Co. (St. Louis, MO) USA. Ethylenediamine tetracetic acid (EDTA) and sodium azide were purchased from the Merck Chemicals Pvt. Ltd. Worli, 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.

3.2.2. Ammonium sulphate fractionation of bitter gourd proteins

Bitter gourd (100 g) was homogenized in 200 mL of 100 mM sodium acetate buffer, pH 5.0. Homogenate was filtered through four layers of cheese-cloth. The filtrate was then centrifuged at 10,000 g on a Remi R-24 Cooling Centrifuge for 20 min at 4 °C. The clear supernatant was subjected to salt fractionation by adding 20-80% (w/v) ammonium sulphate. The solution was stirred overnight at 4 °C and the obtained precipitate was collected by centrifugation at 10,000 g on a Remi R-24 Cooling Centrifuge for 20 min at 4 °C. The collected precipitate was redissolved in 100 mM sodium acetate buffer, pH 5.0 and dialyzed against the assay buffer (Akhtar et al., 2005a).

3.2.3. Preparation of Con A-BGP complex

Jack bean extract (10%, w/v) was prepared according to the method described in Chapter II, Section 2.2.3. The collected supernatant was used as source of Con A.

BGP (1200 U) was incubated with increasing concentrations of jack bean extract (0.1-1.0 mL) containing Con A and the final volume was adjusted to 4.0 mL with 100 mM sodium phosphate buffer, pH 6.2. The mixtures were incubated at 37 °C for 12 h. The insoluble complex was collected by centrifugation at 3000 g for 15 min at room temperature and the precipitates were washed thrice with sodium phosphate buffer, pH 6.2 to remove unbound protein. Finally each precipitate was suspended in assay buffer and peroxidase activity was determined.

The Con A-BGP complex (840 U) was crosslinked prior to entrapment in calcium alginate-starch beads with 0.5% glutaraldehyde for 2 h at 4 °C with constant shaking. Ethanolamine was added to a final concentration of 0.1% (v/v) to stop
crosslinking. The solution was allowed to stand for 90 min at room temperature and the complex was collected by centrifugation at 3000 g for 15 min at room temperature. The precipitate was washed thrice with assay buffer and then suspended in the same buffer (Jan et al., 2006).

3.2.4. Entrapment of crosslinked Con A-BGP into calcium alginate-starch beads

The crosslinked Con A-BGP complex (792 U) was mixed with sodium alginate (2.5%, w/v) and starch (2.5%, w/v) prepared in 10.0 mL of assay buffer. The resulting mixture was slowly extruded as droplets through a 5.0 mL syringe with attached needle No. 20 into 200 mM calcium chloride solution and further gently stirred for 2 h. The obtained calcium alginate-starch entrapped crosslinked Con A-BGP (E-BGP) was washed with 100 mM sodium acetate buffer, pH 5.0 and stored in the assay buffer at 4 °C for its further use.

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

Sodium alginate (2.5%, w/v) and starch (2.5%, w/v) beads were prepared without enzyme according to the procedure described in the above section and these beads were incubated in 10.0 mL jack bean extract, a source of Con A for 12 h at room temperature with slow stirring. After incubation period, Con A bound calcium alginate-starch beads were collected and washed with assay buffer. Con A layered calcium alginate-starch beads were then incubated with BGP (1200 U) overnight at room temperature with slow stirring on a magnetic stirrer. 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 (Jan et al., 2006). Surface immobilized and glutaraldehyde crosslinked BGP (SI-BGP) was stored at 4 °C for its further use.

3.2.6. Measurement of peroxidase activity

Peroxidase activity was estimated in 100 mM sodium acetate buffer, pH 5.0 at
3.2.7. Determination of protein concentration

The concentration of protein was determined as described in Chapter II, Section 2.2.7. Bovine serum albumin was used as a standard.

3.2.8. Effect of pH

Appropriate and equal amounts of S-BGP, SI-BGP and E-BGP were taken to determine the activity of peroxidase in the buffers of different pH. The buffers used were glycine-HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0 and 5.0) and Tris HCl (pH 6.0-10.0). The activity at pH-optimum was considered as control (100%) for the calculation of percent activity at other pH.

3.2.9. Effect of temperature

The activity of soluble and immobilized BGP (1.3 U) was determined at various temperatures (30-80 °C) in 100 mM sodium acetate buffer, pH 5.0. The activity at temperature-optimum was considered as control (100%) for the calculation of percent activity at other temperatures.

In another set of experiment, all three BGP preparations were incubated at 60 °C for varying time intervals in 100 mM sodium acetate buffer, pH 5.0. After each incubation period the enzyme was quickly chilled in crushed ice for 5 min. The enzyme was brought to room temperature and the peroxidase activity was measured. The activity without incubation at 60 °C was taken as control (100%) for the calculation of remaining percent activity.

3.2.10. Effect of urea

Soluble and immobilized BGP (1.3 U) were incubated with 4.0 M urea for varying time intervals in 100 mM sodium acetate buffer, pH 5.0 at 37 °C. Peroxidase activity was determined at the indicated time intervals. The activity of enzyme
without incubation with urea was taken as control (100%) for the calculation of remaining percent activity.

3.2.11. Effect of water-miscible organic solvents

Soluble and immobilized BGP (1.3 U) were incubated independently with varying concentrations of water-miscible organic solvents; dioxane and n-propanol (10-60%, v/v) in 100 mM sodium acetate buffer, pH 5.0 at 37 °C for 1 h. Activity of enzyme without organic solvent was taken as control (100%) for the calculation of remaining percent activity.

3.2.12. Effect of detergents

Soluble and immobilized BGP (1.3 U) were incubated with Tween 20 (0.5-5.0%, v/v) in 100 mM sodium acetate buffer, pH 5.0 at 37 °C for 1 h. The activity of enzyme without Tween 20 was taken as control (100%) for the calculation of remaining percent activity.

3.2.13. Effect of sodium azide and EDTA

The inhibitory effect of sodium azide/EDTA (0.01-0.1 mM) was examined on BGP preparations (1.3 U). Soluble and immobilized BGP were pre-incubated with inhibitors in 100 mM sodium acetate buffer, pH 5.0 at 37 °C for 1 h. The activity of enzyme without exposure to sodium azide/EDTA was considered as control (100%) for the calculation of remaining percent activity.

3.2.14. Effect of HgCl₂/CdCl₂

Soluble and immobilized BGP (1.3 U) were incubated independently with HgCl₂/CdCl₂ (0.01-0.1 mM) in 100 mM sodium acetate buffer, pH 5.0 at 37 °C for 1 h. The activity of enzyme without exposure to heavy metal was taken as control (100%) for the calculation of remaining percent activity.
3.2.15. Effect of NaCl

Soluble and immobilized BGP preparations (1.3 U) were incubated with chloride (0.1-1.0 M) in 100 mM sodium acetate buffer, pH 5.0 at 37 °C for 1 h. Activity of enzyme without sodium chloride was taken as control (100%) for the calculation of remaining percent activity.

3.2.16. Reusability of immobilized BGP

E-BGP and SI-BGP were taken in triplicates for assaying the peroxidase activity. After each assay the immobilized enzyme preparations were taken out, washed and stored in 100 mM sodium acetate buffer, pH 5.0 overnight at 4 °C. The activity was assayed for seven successive days. The activity determined for the first time was considered as control (100%) for the calculation of remaining percent activity after each use.

3.3. RESULTS

3.3.1. Entrapment and surface immobilization of BGP

The entrapment and surface immobilization of BGP into/on calcium alginate-starch beads is demonstrated in Fig 7. Insoluble Con A-BGP complex retained 70% of the initial activity. In order to prevent the dissociation of Con A-BGP complex, this complex was crosslinked by 0.5% glutaraldehyde. The activity of enzyme was decreased after crosslinking and crosslinked preparation retained 66% of the activity. However, the entrapment of crosslinked Con A-BGP complex into calcium alginate-starch beads further resulted in a loss of 14% activity (Table 7).

BGP immobilized on the surface of Con A layered calcium alginate-starch beads exhibited 69% of the original activity. Moreover, the surface immobilized enzyme was also crosslinked by 0.5% glutaraldehyde in order to maintain its integrity. The crosslinking of surface immobilized BGP also resulted in a loss of 6% of its initial activity (Table 7).
Fig. 7: Schematic diagram of (a) E-BGP and (b) SI-BGP
Table 7: Immobilization of BGP into and on calcium alginate-starch beads

<table>
<thead>
<tr>
<th>Enzyme immobilized preparations</th>
<th>Activity expressed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A-BGP complex</td>
<td>70</td>
</tr>
<tr>
<td>Crosslinked Con A-BGP complex</td>
<td>66</td>
</tr>
<tr>
<td>E-BGP</td>
<td>52</td>
</tr>
<tr>
<td>Peroxidase immobilized on Con A layered calcium alginate-starch beads</td>
<td>69</td>
</tr>
<tr>
<td>SI-BGP</td>
<td>63</td>
</tr>
</tbody>
</table>

Each value shows the mean for three-independent experiments performed in duplicates, with average standard deviation, < 5%.
3.3.2. Effect of pH

Fig. 8 demonstrates the effect of pH on the activity of soluble and immobilized BGP. Both immobilized BGP preparations showed no change in pH-optima, pH 5.0 but had remarkable broadening in pH-activity profiles as compared to S-BGP. However, E-BGP retained significantly very high enzyme activity at acidic and alkaline side of pH-optima as compared to SI-BGP and S-BGP. The E-BGP retained 57% and 60% of the maximum activity at pH 3.0 and pH 8.0, respectively whereas soluble enzyme exhibited only 36% and 41% activity under similar incubation conditions.

3.3.3. Effect of temperature

Both immobilized BGP preparations exhibited same temperature-optima as its soluble counterpart at 40 °C (Fig. 9). E-BGP retained remarkably higher fraction of catalytic activity at temperatures below and above the temperature-optima as compared to SI-BGP and S-BGP. E-BGP exhibited 50% activity at 80 °C while SI-BGP and S-BGP retained 40% and 30% activity at this temperature, respectively.

Soluble and immobilized BGP were incubated at 60 °C for various time intervals. Incubation of S-BGP at 60 °C for 2 h resulted in a loss of 53% of initial activity. However, E-BGP and SI-BGP retained 73% and 60% of the original activity under similar incubation conditions, respectively (Fig. 10).

3.3.4. Effect of urea

Fig. 11 demonstrates the effect of 4.0 M urea on the activity of BGP. E-BGP and SI-BGP retained 70% and 50% of their activity after 2 h incubation, whereas soluble enzyme lost nearly 68% of the initial activity under identical urea exposure.

3.3.5. Effect of organic solvents

The effect of increasing concentrations of water-miscible organic solvents; dioxane and n-propanol (10-60%, v/v), on the activity of soluble and immobilized BGP is shown in Table 8. E-BGP showed more than 55% of the initial activity when
Soluble and immobilized BGP (1.3 U) were incubated in the buffers of varying pH. The morality of each buffer was 100 mM. The activity at pH 5.0 for all the preparations was taken as control (100%) for the calculation of remaining percent activity. The symbols show S-BGP (●), SI-BGP (○) and E-BGP (▼).
Fig. 9: Temperature-activity profiles for soluble and immobilized BGP

The activity of soluble and immobilized BGP (1.3 U) was measured in 100 mM sodium acetate buffer, pH 5.0 at various temperatures (30-80 °C). The activity obtained at 40 °C was taken as control (100%) for the calculation of remaining percent activity. The symbols show S-BGP (●), SI-BGP (○) and E-BGP (▼).
Fig. 10: Thermal denaturation of soluble and immobilized BGP

Soluble and immobilized BGP (1.3 U) were incubated at 60 °C for various times in 100 mM sodium acetate buffer, pH 5.0. Aliquots of each preparation were taken out at indicated time intervals and chilled quickly in crushed ice for 5 min. Enzyme activity was determined as described in text. Activity obtained without incubation at 60 °C was taken as control (100%) for the calculation of remaining percent activity. The symbols show S-BGP (●), SI-BGP (○) and E-BGP (▼).
Fig. 11: Effect of 4.0 M urea on soluble and immobilized BGP

Soluble and immobilized BGP (1.3 U) were incubated with 4.0 M urea in 100 mM sodium acetate buffer, pH 5.0 for various times. Activity obtained without urea exposure was taken as control (100%) for the calculation of remaining percent activity. The symbols show S-BGP (●), SI-BGP (○) and E-BGP (▼).
Table 8: Effect of organic solvents on soluble and immobilized BGP

<table>
<thead>
<tr>
<th>Organic solvent (v/v, %)</th>
<th>Remaining activity (%)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dioxane</td>
<td>n-propanol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S-BGP</td>
<td>SI-BGP</td>
<td>E-BGP</td>
<td>S-BGP</td>
<td>SI-BGP</td>
<td>E-BGP</td>
</tr>
<tr>
<td>10</td>
<td>76</td>
<td>85</td>
<td>93</td>
<td>51</td>
<td>68</td>
<td>89</td>
</tr>
<tr>
<td>20</td>
<td>64</td>
<td>72</td>
<td>85</td>
<td>38</td>
<td>50</td>
<td>81</td>
</tr>
<tr>
<td>30</td>
<td>41</td>
<td>58</td>
<td>67</td>
<td>29</td>
<td>40</td>
<td>77</td>
</tr>
<tr>
<td>40</td>
<td>34</td>
<td>40</td>
<td>58</td>
<td>23</td>
<td>35</td>
<td>56</td>
</tr>
<tr>
<td>50</td>
<td>26</td>
<td>31</td>
<td>38</td>
<td>18</td>
<td>30</td>
<td>43</td>
</tr>
<tr>
<td>60</td>
<td>19</td>
<td>24</td>
<td>29</td>
<td>14</td>
<td>27</td>
<td>35</td>
</tr>
</tbody>
</table>

Soluble and immobilized BGP (1.3 U) were incubated independently with dioxane/n-propanol (10-60%, v/v) in 100 mM sodium acetate buffer, pH 5.0 at 37 °C for 1 h. The activity of BGP without exposure to organic solvent was taken as control (100%) for the calculation of remaining percent activity. Each value represents the mean for three independent experiments performed in duplicates, with average standard deviation, < 5%.
exposed to 40% (v/v) dioxane/n-propanol for 1 h at 37 °C. However, S-BGP exhibited only 34% and 23% of activity after exposure to 40% (v/v) dioxane and n-propanol, respectively.

### 3.3.6. Effect of detergent (Tween 20)

The effect of Tween 20 on the activity of soluble and immobilized BGP is shown in Fig. 12. E-BGP and SI-BGP retained 57% and 40% of the original activity when exposed to 5.0% (v/v) Tween 20 for 1 h at 37 °C. However, soluble enzyme was sensitive to Tween 20 and it lost nearly 70% of its activity under similar exposure.

### 3.3.7. Effect of sodium azide/EDTA

Table 9 demonstrates the effect of sodium azide/EDTA on the activity of soluble and immobilized BGP. E-BGP and SI-BGP retained 43% and 40% activity after 1 h exposure to 0.05 mM sodium azide. Moreover, SI-BGP and E-BGP retained 68% and 80% activity after 1 h exposure to 0.05 mM EDTA, respectively while the soluble BGP lost nearly 50% of its activity under identical exposure (Table 9).

### 3.3.8. Effect of HgCl₂/CdCl₂

The chemical contamination of water from a wide range of toxic derivatives, in particular heavy metals is a serious environmental problem owing to their potential human toxicity. In view of their presence in wastewater, it becomes important to examine the effect of some heavy metals on the activity of BGP. E-BGP and SI-BGP retained 71% and 58% activity in the presence of 0.1 mM HgCl₂, respectively whereas S-BGP exhibited only 53% activity under similar treatment conditions (Table 10).

Immobilized BGP preparations were more resistant to inactivation induced by CdCl₂. E-BGP and SI-BGP retained 69% and 59% activity after 1 h exposure to 0.1 mM CdCl₂, respectively. However, S-BGP lost 48% of its original activity when it was pre-incubated to 0.1 mM CdCl₂ (Table 10).
Fig. 12: Effect of Tween 20 on soluble and immobilized BGP

Soluble and immobilized BGP (1.3 U) were incubated with Tween 20 (0.5-5.0%, v/v) in 100 mM sodium acetate buffer, pH 5.0. The activity of BGP without Tween 20 was taken as control (100%) for calculation of remaining percent activity. The symbols show S-BGP (●), SI-BGP (○) and E-BGP (▼).
Table 9: Effect of sodium azide/EDTA on soluble and immobilized BGP

<table>
<thead>
<tr>
<th>Inhibitor (mM)</th>
<th>Remaining activity (%)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sodium azide</td>
<td>S-BGP</td>
<td>SI-BGP</td>
<td>E-BGP</td>
<td>S-BGP</td>
<td>SI-BGP</td>
<td>E-BGP</td>
</tr>
<tr>
<td>0.01</td>
<td>70</td>
<td>82</td>
<td>89</td>
<td>82</td>
<td>87</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>0.02</td>
<td>63</td>
<td>71</td>
<td>75</td>
<td>76</td>
<td>85</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>0.03</td>
<td>55</td>
<td>65</td>
<td>69</td>
<td>71</td>
<td>78</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>0.04</td>
<td>43</td>
<td>52</td>
<td>59</td>
<td>60</td>
<td>72</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>32</td>
<td>40</td>
<td>43</td>
<td>52</td>
<td>68</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>0.06</td>
<td>28</td>
<td>35</td>
<td>39</td>
<td>49</td>
<td>64</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>0.07</td>
<td>24</td>
<td>30</td>
<td>36</td>
<td>46</td>
<td>61</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>0.08</td>
<td>21</td>
<td>27</td>
<td>31</td>
<td>41</td>
<td>57</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>0.09</td>
<td>16</td>
<td>24</td>
<td>27</td>
<td>34</td>
<td>54</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>0.10</td>
<td>12</td>
<td>20</td>
<td>24</td>
<td>30</td>
<td>51</td>
<td>61</td>
<td></td>
</tr>
</tbody>
</table>

Soluble and immobilized BGP (1.3 U) were incubated independently with (0.01-1.0 mM) of sodium azide and EDTA in 100 mM sodium acetate buffer, pH 5.0 at 37 °C for 1 h. The activity of soluble and immobilized BGP without exposure to inhibitor was taken as control (100%) for the calculation of remaining percent activity. Each value represents the mean for three independent experiments performed in duplicates, with average standard deviation, < 5%.
Table 10: Effect of HgCl₂/CdCl₂ on soluble and immobilized BGP

<table>
<thead>
<tr>
<th>HgCl₂/CdCl₂ (mM)</th>
<th>Remaining activity (%)</th>
<th>HgCl₂</th>
<th>CdCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S-BGP</td>
<td>SI-BGP</td>
<td>E-BGP</td>
</tr>
<tr>
<td>0.01</td>
<td>72</td>
<td>80</td>
<td>90</td>
</tr>
<tr>
<td>0.02</td>
<td>69</td>
<td>72</td>
<td>86</td>
</tr>
<tr>
<td>0.04</td>
<td>64</td>
<td>70</td>
<td>82</td>
</tr>
<tr>
<td>0.06</td>
<td>60</td>
<td>63</td>
<td>76</td>
</tr>
<tr>
<td>0.08</td>
<td>56</td>
<td>61</td>
<td>74</td>
</tr>
<tr>
<td>0.10</td>
<td>53</td>
<td>58</td>
<td>71</td>
</tr>
</tbody>
</table>

Soluble and immobilized BGP (1.3 U) were incubated with HgCl₂/CdCl₂ (0.01-1.0 mM) in 100 mM sodium acetate buffer, pH 5.0 at 37 °C for 1 h. The activity of BGP without exposure to HgCl₂/CdCl₂ was taken as control (100%) for the calculation of remaining percent activity. Each value represents the mean for three independent experiments performed in duplicates, with average standard deviation, < 5%.
3.3.9. **Effect of sodium chloride**

The effect of NaCl on the activity of BGP has been illustrated in Fig. 13. The activity of immobilized BGP preparations was slightly enhanced in the presence of 0.2 M NaCl. E-BGP and SI-BGP showed 104% and 114% activity after 1 h of incubation with 0.2 M NaCl. However, S-BGP exhibited a marginal loss of 6% of the enzyme activity under similar incubation conditions.

3.3.10. **Reusability of immobilized BGP**

Reusability of two immobilized preparations of BGP has been shown in Fig. 14. After 7th repeated use the E-BGP retained 75% of the original activity, whereas SI-BGP showed 69% activity.

3.4. **DISCUSSION**

Sodium alginate has been considered since a long time for the entrapment of enzymes, cell organelles, microorganisms due to its biocompatibility and processibility (Zhu et al., 2005; Kasipathy et al., 2006). Calcium alginate entrapped enzyme preparations have one inherent limitation that the large Mr substrates or products cannot easily diffuse in and out of gel beads (Norouzian, 2003). In order to circumvent this problem, the immobilization of enzymes on the surface of such beads would be a preferred choice. In this work an effort has been made to prepare a hybrid calcium alginate-starch gel bead which has successfully been employed both for entrapment of BGP and its immobilization on the surface of such beads layered with Con A.

The immobilization of BGP on the surface of Con A layered calcium alginate-starch beads has presented a strategy to overcome the problem of diffusion limitation of the substrate/product and to increase the surface area of contact between enzyme and substrate so that such preparation could be exploited for the treatment of large volume of industrial waste. The enzyme activity expressed by SI-BGP was more than E-BGP due to the compact structure of E-BGP, which decreased the flexibility of enzyme as well as considerably limited diffusion of the substrate (Fig. 7, Table 7). It
Soluble and immobilized BGP (1.3 U) were incubated with NaCl (0.1-1.0 M) in 100 mM sodium acetate buffer, pH 5.0 at 37 °C for 1 h. The activity of BGP without incubation with NaCl was taken as control (100%) for the calculation of remaining percent activity. The symbols show S-BGP (●), SI-BGP (○) and E-BGP (▼).
E-BGP and SI-BGP (1.3 U) were independently assayed in 100 mM sodium acetate buffer, pH 5.0 as described in the text. After each assay immobilized enzyme preparations were taken out and stored in assay buffer overnight for next use. This procedure was repeated for seven consecutive days.

Fig. 14: Reusability of immobilized preparations of BGP
has already been reported that the enzyme immobilized on the surface of gel beads has minimum mass transfer constrain (Le-Tien et al., 2004; Gomez et al., 2006).

Immobilization of an enzyme to a support often limits its freedom to undergo drastic conformational changes and thus resulted in increased stability towards denaturants such as organic solvents, heat, inhibitors and urea (Fig. 9-13, Table 8-10). However, some earlier workers have demonstrated that the stability of surface immobilized enzymes was significantly higher against pH, heat and proteolysis than the free enzyme (Le-Tien et al., 2004). Thus the enhanced resistance to the stability of immobilized BGP against denaturants offered a potential advantage for the application of such type of enzyme preparation in treatment of wastewater.

The pH-activity profiles of E-BGP and SI-BGP have the same pH-optima as that of S-BGP (Fig. 8). However, entrapped and surface immobilized BGP preparations showed significant broadening in pH activity profiles indicating a marked increased stability in the buffers of varying pH. This predicts that the entrapment of enzyme in the gel beads provides a microenvironment for the enzyme, which might play an important role in the state of protonation of the protein molecules. However, the surface immobilized BGP provided stability due to multiple point attachment between enzyme and Con A. The crosslinking of BGP present on the Con A layered calcium alginate-starch beads by glutaraldehyde further increased stability.

Furthermore, immobilized peroxidase preparations retained their structure and remarkably high activity at elevated temperatures as compared to soluble peroxidase (Fig. 9, 10). These observations were in agreement with the findings of some earlier investigators (Al-Adhami et al., 2002; Dodor et al., 2004). It is well established that thermal exposure initiate unfolding of protein molecules which is followed by irreversible changes due to aggregation and formation of scrambled structures which takes place more in soluble form as compared to the immobilized enzyme (Querol et al., 1996).

SI-BGP and E-BGP were markedly more stable to the denaturation induced by 4.0 M urea (Fig. 11). Although the action mechanism of urea on the protein structure has not yet been completely understood, some earlier workers have proposed that the presence of carbohydrate moieties in enzymes increased their resistance to inactivation caused by urea (Kwon and Yu, 1997).
The E-BGP followed by SI-BGP was remarkably more resistant to the inactivation mediated by water-miscible organic solvents; dioxane and n-propanol (Table 8). It has already been reported that the stabilization of insoluble complexes against various organic solvents which could possibly be due to low water requirement or enhanced rigidity of the enzyme structure (Xin et al., 2005).

Immobilized BGP was significantly more resistant to denaturation induced by Tween 20 as compared to its soluble counterpart (Fig. 12). These observations suggested that the presence of lower concentrations of detergents was not harmful to the enzyme's native conformation. Flock et al. (1999) reported an increase in the activity of soybean peroxidase when the enzyme was treated with low concentrations of SDS and Tween 20. The enhancement of enzyme activity by 0.5% (v/v) Tween 20/Triton X 100 and stabilization of BGP immobilized on a bioaffinity support (Con A-Sephadex) against high concentrations of such type of detergents has also been reported by some earlier workers (Akhtar et al., 2005c).

The immobilized peroxidase preparations showed more than 80% of their original enzyme activity in the presence of 0.1 mM inhibitory compounds; sodium azide and EDTA (Table 9). A number of studies have already been performed on the inhibitory effect of such compounds on HRP (Ortiz de Montellano et al., 1988). Sodium azide has been shown to be a potent inhibitor of many hemeprotein-catalyzed reactions (Kvaratskhelia et al., 1997). Peroxidase in the presence of sodium azide and H$_2$O$_2$ mediates one electron oxidation of azide ions and forming azidyl free radicals, which bind covalently to the heme moiety thus inhibiting the enzyme activity (Tatarko and Bumpus, 1997).

Metals induce conformational changes in enzymes; however peroxidases remains active even in the presence of a number of metal ions, as a part of their detoxifying role. BGP has exhibited more resistant to its stability against heavy metals induced inhibition. Some recent reports have also indicated that HRP was remarkably inhibited by heavy metal ions (Keyhani et al., 2003; Einollahi et al., 2006). However, in this study the strength of inhibition of immobilized BGP by heavy metal ions was quite low as compared to soluble enzyme (Table 10). The stability of immobilized BGP against several metal compounds showed that such preparations could be exploited to treat aromatic pollutants even in the presence of heavy metals.

Enzyme reuse provides a number of cost effective advantages that are often an essential prerequisite for establishing an economically viable enzyme catalyzed
process (Norouzian, 2003). However, E-BGP and SI-BGP retained more than 60% of their original activity even after its seven successive uses (Fig. 14). The activity loss during repeated use might be due to the inhibition of enzyme by product or by leaching of enzyme from the gel bead or damage to the beads (Gaserød et al., 1999; Musthapa et al., 2004).

On the basis of results obtained in the present work, it can be concluded that the stability offered by E-BGP followed by SI-BGP against various denaturants suggested that these preparations could successfully be employed in reactors for the treatment of effluents containing phenolic and other aromatic pollutants.