CHAPTER V

Immobilization of *Aspergillus oryzae* β galactosidase on immunoaffinity support
5.1. INTRODUCTION

β-Galactosidases from different sources are currently being used in the production of lactose-free milk products. Hydrolysis of lactose improves product sweetness, makes milk consumption easier by people who suffer from lactose intolerance, increases product quality and process efficiency in the dairy industry. Lactose is the main carbohydrate present in milk (4-5%) and dairy products. Its concentration in food products is indicative of the amount of lactose present in these products (Hatzinikolaou et al., 2005). Lactose is determined in dairy products in the milk industry in order to provide process efficiency and product quality control (Ozdural et al., 2003). There are several methods to detect lactose concentration such as spectrophotometry, polarimetry, titrimetry and chromatography. However, these methods are tedious and time-consuming due to long sample preparation. These disadvantages stimulated the development of a bioaffinity-based method for the detection of lactose (Merino et al., 2005).

Bioaffinity-based methods have several advantages over other known methods used for the immobilization of enzymes (Kulshrestha and Husain, 2006). It provides oriented immobilization of enzymes that facilitates good expression of activity and possibility of direct enzyme immobilization (Mislovicova et al., 2000; Jan et al., 2001). Among the bioaffinity pairs, the antigen and antibody pair is highly specific and this pair could be exploited for the immobilization of all kinds of enzymes. Immobilized antibodies have become powerful tools in biosensor technology, diagnostics and therapeutics (Karyakin et al., 2000; Nisnevitch and Firer, 2001). In the last few years, purification of antibodies has grown to become the largest class of proteins in clinical-phase development intended for therapeutic and diagnostic applications (Guerrier et al., 2001; Tanaka et al., 2003; Zhu et al., 2006). Antibodies are routinely recognized as analytical reagents in clinical and research laboratories. Two of their most common applications are in immunoassays and immunoaffinity separation but interest is also increasing in immunosensors (Haupt and Mosbach, 1998).

Here an effort has been made to immobilize β-galactosidase from Aspergillus oryzae on an immunoaffinity support, IgG-cellulose. The stability of immunoaffinity...
immobilized β galactosidase has been investigated against denaturants such as pH, heat, urea, proteolytic enzymes (pepsin, trypsin), CaCl₂ and galactose. Immunoaffinity support immobilized β galactosidase was also investigated for its storage stability and reusability.

5.2. MATERIALS & METHODS

5.2.1. Materials

Aspergillus oryzae [G-7138, Lot121H0055] β galactosidase (3.2.1.23), cyanogen bromide (CNBr), galactose and glucose were obtained from Sigma Chem. Co. (St. Louis, MO) USA. Cellulose, DEAE-cellulose, α-nitrophenyl β-D-galactopyranoside (ONPG) and all chemicals used in electrophoresis and immunodiffusion were obtained from SRL Chem. Mumbai, India. All the other chemicals and reagents used were of analytical grade.

5.2.2. Immunization

Commercially available purified β galactosidase was injected into healthy male albino rabbits weighing 2-3 kg for the production of anti-β galactosidase polyclonal antibodies. The animals received subcutaneously 300 µg of β galactosidase (0.5 mL) mixed and emulsified with equal volume of Freund’s complete adjuvant as first dose. Booster doses of 150 µg of β galactosidase mixed and emulsified with Freund’s incomplete adjuvant were administered weekly after resting the animal for 15 d. After each booster dose blood was collected from the ear vein of the animal and allowed to clot at room temperature for 3 h. Serum was collected by centrifugation at 1600 xg for 20 min at 4 °C and later it was decomplimented by incubating at 56 °C for 30 min. After adding sodium azide (0.2%) serum was stored at -20 °C (Fatima and Husain, 2007).

5.2.3. Purification and characterization of polyclonal antibodies

The antiserum was fractionated with 20-40% ammonium sulphate. The sample was kept overnight with constant stirring at 4 °C to precipitate out proteins. The
precipitated proteins were collected by centrifugation at 1600 xg for 20 min at 4 °C. The pellet obtained was re-dissolved in a minimum volume of 0.02 M phosphate buffer, pH 7.2 and was subjected to extensive dialysis against the same buffer to remove the traces of ammonium sulphate.

Further antibodies against β galactosidase were purified by ion exchange chromatography. The dialyzed protein sample from ammonium sulphate precipitated antiserum was passed through DEAE-cellulose column (1.20 cm X 10.0 cm) and the fractions containing purified anti-β galactosidase antibodies were pooled for further use (Khan et al., 2005).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% gel under denaturing conditions and native PAGE on 7.5% gel were run to separate proteins present in DEAE-cellulose purified anti-β galactosidase antibodies (Laemmli, 1970). The staining and de-staining of the gel was also performed by the same procedure. Molecular weight marker proteins (myosin, 205 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; soybean trypsin inhibitor, 20 kDa and lysozyme, 14.3 kDa) were also run in one lane adjacent to the purified IgG.

5.2.4. Immunodiffusion

Ouchterlony double immunodiffusion was used to prove the presence of antibodies against β galactosidase. Immunodiffusion was performed in 1.0% (w/v) agarose prepared in normal saline (Jan et al., 2001). The purified anti-β galactosidase antibodies were employed for preparing immunoaffinity support.

5.2.5. Direct binding ELISA

Polystyrene (96 well) microtitre plate was coated with 100 µL of antigen (β galactosidase) at a concentration of 10 µg/mL prepared in antigen coating buffer (bicarbonate buffer, 0.05 M, pH 9.6) and then incubated for 2 h at 37 °C followed by overnight storage at 4 °C. The wells were then washed three-times with TBS-T buffer. The unoccupied sites were blocked with 2% fat milk in TBS (150 µL, each well) followed by incubation for 5-6 h at room temperature. The wells were then washed twice.
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with TBS-T. The test and control wells were then diluted with 100 µL of serially diluted serum. Each dilution was in TBS buffer. Serially diluted blanks corresponding to each dilution were also present. The plate was then incubated for 2 h at room temperature and kept overnight at 4 °C. The plate was washed again with TBS-T buffer (five times). Bound antibodies were assayed with an appropriate conjugate of anti-rabbit IgG alkaline phosphatase, 100 µL of it was coated in each well and kept at room temperature for 2 h. Washing of the plate with TBS-T (five times) and with distilled water (two times) was followed by addition of p-nitrophenyl phosphate (50 µg/100 µL) in each well and incubation at 37 °C for 30-45 min. The absorbance of each well was monitored at 405 nm on a Lab system ELISA Reader (Fatima and Husain, 2007).

5.2.6. Preparation of immunoaffinity support

Cellulose powder (5.0 g) was activated by cyanogen bromide as described by Porath et al. (1967). Cellulose was washed thoroughly with distilled water in a sintered glass funnel. The gel was sucked dry and suspended in 10.0 mL of 1.0 M Na₂CO₃ and stirred slowly by placing on a magnetic stirrer at 4 °C for 30 min. CNBr (1.0 g) dissolved in 1.0 mL of acetonitrile was added to the beaker containing cellulose and was again stirred for 10 min in cold. The whole mass was transferred immediately to a sintered funnel and washed thoroughly with sufficient volume of 0.1 M bicarbonate buffer, pH 8.5, distilled water, and again with same buffer. Washed activated cellulose was dried and re-suspended in 5.0 mL of 0.1 M bicarbonate buffer, pH 8.5. Purified antibodies (60 mg) were mixed with activated cellulose (5.0 g) and stirred overnight in cold. Cellulose unbound antibodies was removed by centrifugation at 1600 xg for 20 min at 4 °C. Antibody bound matrix was extensively washed with 0.1 M bicarbonate buffer, pH 8.5 containing 1.0 M NaCl. The washed suspension was treated with 7.0 mL of 0.1 M glycine for 2 h at 4 °C. Antibody bound cellulose was successively washed with 0.1 M sodium bicarbonate buffer, pH 8.5 containing 1.0 M NaCl, distilled water and finally with 0.05 M sodium phosphate buffer, pH 7.0. The quantity of bound antibody was calculated by subtracting the unbound protein in the washings from that of total added protein.
5.2.7. Immobilization of \( \beta \) galactosidase on IgG-cellulose

\( \beta \) Galactosidase (5200 U) was mixed with 5.0 g of IgG-cellulose. The mixture was stirred overnight at 4 °C. The gel was then thoroughly washed with 0.05 M sodium phosphate buffer, pH 7.0 to remove unbound enzyme (Jan and Husain, 2004).

5.2.8. Effectiveness factor (\( \eta \))

The effectiveness factor (\( \eta \)) represents the ratio of actual and theoretical activity of the immobilized enzyme (Matto and Husain, 2006).

5.2.9. Measurement of \( \beta \) galactosidase activity

The \( \beta \) galactosidase activity was determined as described in the text (Chapter II, Section 2.2.6).

5.2.10. Protein estimation

Protein was estimated by the method of Lowry et al. (1951). A suitable aliquot of the protein sample was diluted to 1.0 mL with distilled water. To this, 5.0 mL of freshly prepared alkaline copper reagent was added. The alkaline copper reagent was prepared by mixing copper sulphate (1%, w/v), sodium potassium tartarate (2%, w/v) and sodium carbonate (2.0 g) in 0.1 N NaOH in the ratio of 1:1:100. After 10 min incubation at room temperature, 0.5 mL of 1.0 N Folin's reagent was added. The tubes were instantly vortexed. The color developed was read at 660 nm after 30 min against a reagent blank. A standard curve was prepared using bovine serum albumin.

5.2.11. Effect of pH on soluble and immobilized \( \beta \) galactosidase

The activity of \( \beta \) galactosidase (2.0 U) was measured in the buffers of various pH. The buffers used were glycine-HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0-6.0) and Tris-HCl (pH 7.0-10.0). The molarity of each buffer was 0.1 M. The activity at pH 4.6 was taken as control (100%) for the calculation of remaining percent activity.
5.2.12. Effect of temperature on soluble and immobilized β galactosidase

The activity of soluble and immobilized enzyme was assayed at various temperatures (25°C-80°C) for determining temperature-activity profile.

Further soluble and immobilized β galactosidase (2.0 U) was incubated at 60 °C for varying time intervals in 0.1 M sodium acetate buffer, pH 4.6. After each incubation period the enzyme was taken out and quickly chilled in crushed ice for 5 min. The enzyme was brought to room temperature and then β galactosidase activity was determined as described in the text (Chapter II, Section 2.2.6).

5.2.13. Effect of urea on soluble and immobilized β galactosidase

Soluble and immobilized β galactosidase was incubated in 4.0 M urea dissolved in sodium acetate buffer, pH 4.6. Aliquots from each preparation (2.0 U) were removed at varying times and the activity was determined as described in the text (Chapter II, Section 2.2.6). The activity obtained without incubation with urea was taken as control (100%) for the calculation of remaining percent activity.

5.2.14. Effect of calcium ions/galactose on soluble and immobilized β galactosidase

The effect of various concentrations of calcium chloride/galactose (1.0-5.0%, w/v) on the activity of soluble and immobilized β galactosidase was measured in 0.1 M sodium acetate buffer, pH 4.6. The activity obtained without incubation with calcium chloride/galactose was taken as control (100%) for the calculation of remaining percent activity.

5.2.15. Effect of trypsin/pepsin on soluble and immobilized β galactosidase

Soluble and immobilized β galactosidase (2.0 U) were incubated with increasing concentrations of trypsin/pepsin (0.25-2.5 mg mL⁻¹) in 0.1 M sodium acetate buffer, pH 4.6 at 37 °C for 1 h. For the calculation of the remaining percent activity, trypsin/pepsin untreated samples were considered as control (100%).
5.2.16. Storage stability of soluble and immobilized β galactosidase

Soluble and immobilized β galactosidase was stored at 4 °C in 0.1 M sodium acetate buffer, pH 4.6 for over 2 months in duplicates. The aliquots from each preparation were taken in triplicates at a gap of 10 d and then were analyzed for the remaining enzyme activity. The enzyme activity measured on first day was considered as control (100%) for the calculation of further storage activity.

5.2.17. Reusability of immunoaffinity bound immobilized β galactosidase

The reusability of immunoaffinity support immobilized β galactosidase was monitored at the gap of 12 h. The immobilized preparation was taken in triplicates and was assayed for the remaining activity. After each assay immobilized enzyme was centrifuged at 1600 ×g and was stored in 0.1 M sodium acetate buffer, pH 4.6 at 4 °C. The activity was assayed for 10 successive uses.

5.2.18. Statistical analysis

The statistical analysis was done as described in Chapter II, Section 2.2.15.

5.3. RESULTS

5.3.1. Production and purification of anti-β galactosidase polyclonal antibodies

β Galactosidase purified to homogeneity was highly immunogenic in rabbits. Purified antibodies raised against β galactosidase showed a clear single precipitin line (Figure 22). It suggested that β galactosidase was immunogenic in rabbits. DEAE-cellulose purified IgG loaded on SDS-PAGE clearly showed two bands, which corresponded to heavy and light chains of the antibody (Figure 23a). However, the separation of antibodies on native PAGE showed a single band (Figure 23b) and this result supported the purity of the purified antibodies. The titer obtained through direct binding ELISA was greater or equal to 25600 (Figure 24). Overnight incubation of fixed
amount of β galactosidase with increasing concentrations of purified IgG exhibited no loss in enzyme activity. These observations further supported that the antiserum raised against the purified β galactosidase contained only non-inhibitory antibodies.

5.3.2. Immobilization of β galactosidase on anti-β galactosidase IgG-cellulose

IgG produced against Aspergillus oryzae β galactosidase were used for the construction of cellulose-anti-β galactosidase immunoaffinity support. Anti-β galactosidase polyclonal antibody bound cellulose specifically retained β galactosidase nearly 911 U/g of the IgG-cellulose support. The obtained immobilized β galactosidase preparation was quite active and exhibited very high effectiveness factor (η) 0.97 (Table 15).

5.3.3. Stability properties of soluble and immobilized β galactosidase

The stability of immobilized β galactosidase against various types of denaturants is of significant importance in various applications.

There was no difference in pH-optima of immobilized β galactosidase between pH 4.6-5.5, unlike the soluble β galactosidase that exhibited activity peak at pH 4.6 (Figure 25). Immunoaffinity bound β galactosidase exhibited no difference in activity between 50-60 °C whereas free β galactosidase had a temperature-optima at 50 °C (Figure 26). Immobilized β galactosidase retained 87% of the original activity after 2 h incubation at 60 °C while the soluble enzyme lost 85% activity under similar experimental conditions (Figure 27).

The effect of 4.0 M urea for different times on the activity of soluble and immobilized β galactosidase has been demonstrated in Figure 28. The soluble enzyme lost nearly its complete activity after 2 h exposure to 4.0 M urea at 37 °C while the immobilized β galactosidase retained 64% activity under identical experimental conditions.
Figure 22: Ouchterlony double immunodiffusion of β galactosidase against purified anti-β galactosidase antibody

Anti-β galactosidase polyclonal antibodies were produced in male albino rabbits. Immunodiffusion was performed in 1.0% (w/v) agarose gel prepared in normal saline. The central well contains antigen while the outer wells A, B and C contain equal volume of antiserum.
Figure 23 (a): SDS-PAGE for anti-β galactosidase polyclonal antibodies and marker proteins

Lane A: Antiserum proteins (30 µg)
Lane B: Ammonium sulphate fractionated proteins (30 µg)
Lane C: Purified anti-β galactosidase polyclonal antibodies (30 µg)
Lane D: Marker proteins (30 µg)
Figure 23 (b): Native PAGE for purified anti-β galactosidase polyclonal antibodies

Lane A: Antiserum proteins (30 µg)
Figure 24: Direct Binding ELISA

Serially diluted antiserum and pre-immune serum were incubated in a polystyrene microtitre plate with the antigen (β galactosidase). Serially diluted blanks corresponding to each dilution were also present. The absorbance of each well (after addition of substrate, p-nitrophenyl phosphate) was monitored at 405 nm on Lab systems ELISA Reader.
Table 15: Immobilization of β galactosidase on IgG-cellulose support

<table>
<thead>
<tr>
<th>Amount of enzyme loaded (X) (U)</th>
<th>Amount of enzyme activity in washes (U)</th>
<th>Activity bound /g of IgG-cellulose (U)</th>
<th>Activity yield (%) (B/Ax100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1040</td>
<td>96</td>
<td>944</td>
<td>97.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>911</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Each value represents the mean for three-independent experiments performed in duplicates, with average standard deviation, <5%.
Figure 25: pH-activity profiles of soluble and IgG-cellulose immobilized β galactosidase

An appropriate and equal amount of soluble and IgG-cellulose immobilized β galactosidase was taken for assaying the activity in the buffers of various pH. The buffers used were glycine-HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0-6.0), and Tris-HCl (pH 7.0-10.0). The molarity of each buffer was 0.1 M.
**Figure 26: Temperature-activity profiles of soluble and IgG-cellulose immobilized \( \beta \) galactosidase**

The activity of soluble and IgG-cellulose immobilized \( \beta \) galactosidase was measured in 0.1 M sodium acetate buffer, pH 4.6 at various temperatures.
Figure 27: Thermal denaturation of soluble and IgG-cellulose immobilized β galactosidase

Soluble and immobilized β galactosidase was incubated at 60 °C for various time intervals in 0.1 M sodium acetate buffer, pH 4.6. Aliquots from each preparation (2.0 U) were removed at different time intervals and the activity was determined according to the procedure described in the text (Chapter II, Section 2.2.6).
Figure 28: Effect of urea on soluble and IgG-cellulose immobilized β-galactosidase

Soluble and IgG-cellulose immobilized β-galactosidase preparations were incubated in 4.0 M urea dissolved in 0.1 M sodium acetate buffer, pH 4.6 at 37°C. Aliquots from each preparation (2.0 U) were removed at indicated time intervals and activity was determined. Each enzyme preparation without urea exposure was considered as control (100%) for the calculation of remaining percent activity.
The effect of various concentrations of calcium chloride/galactose (1.0-5.0%, w/v) on the activity of β galactosidase has been illustrated in Figure 29. The exposure of soluble β galactosidase to 5.0% calcium chloride for 1 h at 37 °C resulted in a loss of 58% of its original activity, whereas the immobilized β galactosidase retained 71% activity under similar treatment. Due to product inhibition soluble β galactosidase resulted in a loss of 72% activity after exposure to 5.0% of galactose for 1 h at 37 °C while the immobilized enzyme retained 65% of the original activity under similar treatment (Figure 29).

Soluble and immobilized β galactosidase was treated with increasing concentrations of trypsin/pepsin (0.25-2.50 mg mL⁻¹) for 1 h at 37 °C (Figure 30). Immobilized β galactosidase showed 85% and 79% activity when exposed to 1.0 mg mL⁻¹ trypsin and same concentration of pepsin for 1 h, respectively. However, soluble enzyme exhibited a loss of 29% and 50% of the initial activity under similar incubation conditions. Immobilized β galactosidase retained 60% and 50% activity after exposure to 2.5 mg mL⁻¹ trypsin and same concentration of pepsin whereas the soluble counterpart lost 73% and 80% activity, respectively under identical experimental conditions. It showed that immobilized β galactosidase exhibited significantly very high stabilization to proteolytic digestion.

The reusability of immunoaffinity bound β galactosidase has been shown in Figure 31. After tenth repeated use the immobilized enzyme retained nearly 46% of its initial activity. Immobilized β galactosidase retained significantly very high activity on storage for 2 months at 4 °C (Table 16).

5.4. DISCUSSION

The enzyme electrodes based on immobilized β galactosidases have been successfully employed for measuring lactose in milk and milk products (Amarita et al., 1997; Merino et al., 2005). Selective and sensitive devices for lactose hydrolysis have proved to be very useful in the determination of lactose in whey, milk and milk products. Lukacheva et al. (2007) developed lactose biosensor based on Berlin blue which acts as a signal transducer.
Figure 29: Effect of calcium chloride/galactose on soluble and IgG-cellulose immobilized β galactosidase

Soluble and IgG-cellulose immobilized β galactosidase were incubated with increasing concentrations of calcium chloride/galactose (1.0-5.0%, w/v) in 0.1 M sodium acetate buffer, pH 4.6 for 1 h at 37 °C.
Figure 30: Effect of trypsin/pepsin on soluble and IgG-cellulose immobilized β galactosidase

Soluble and IgG-cellulose immobilized β galactosidase were incubated with increasing concentrations of trypsin/pepsin (0.25-2.5 mg mL⁻¹) in 0.1 M sodium acetate buffer, pH 4.6 at 37 °C for 1 h. For calculating the remaining percent activity untreated samples were considered as control (100%).
Figure 31: Reusability of immunoaffinity bound IgG-cellulose immobilized β-galactosidase

The reusability of immunoaffinity bound immobilized β galactosidase was monitored at the gap of 12 h. The samples of each preparation were taken in triplicates and were assayed for the remaining enzyme activity.
Table 16: Storage stability of soluble and IgG-cellulose immobilized β galactosidase

<table>
<thead>
<tr>
<th>Number of days</th>
<th>Remaining activity (%)</th>
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<th></th>
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<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>Soluble β galactosidase</td>
<td>Immobilized β galactosidase</td>
</tr>
<tr>
<td>10</td>
<td>87 ±2.85</td>
<td>99 ±0.14</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>75 ±1.96</td>
<td>96 ±1.69</td>
<td></td>
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<td>30</td>
<td>69 ±1.65</td>
<td>94.3 ±1.56</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>55 ±3.4</td>
<td>89.6 ±1.09</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>44 ±1.69</td>
<td>85 ±2.58</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>35 ±2.1</td>
<td>80 ±2.16</td>
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</table>

Soluble and IgG-cellulose immobilized β galactosidase was stored at 4 °C in 0.1 M sodium acetate buffer, pH 4.6 for over 2 months. The aliquots of each enzyme preparation (2.0 U) were taken in duplicates at a gap of 10 d and were then analyzed for the remaining enzyme activity. The enzyme activity measured on the first day was considered as control (100%) for calculating remaining storage activity. Each value represents the mean for three-independent experiments performed in duplicates, with average standard deviation, <5%.
For the effective application of the immunoaffinity based devices for the analysis of lactose, the immunoaffinity layer must have a high surface area and a large number of binding sites for the determination of lactose in milk and whey (Pyun et al., 2005). We have developed a high yield procedure for the immobilization of β galactosidase on an immunoaffinity support and this preparation could be exploited for the determination of lactose in milk/whey.

Immunoaffinity bound β galactosidase retained significantly very high activity (Table 15). Effectiveness factor of an immobilized enzyme is a measure of internal diffusion and reflects the efficiency of the immobilization procedure. A large number of earlier studies have described that the enzymes immobilized on the antibody support showed very high effectiveness factor (Jan et al., 2001; Jan and Husain, 2004). The strength of association of an enzyme with its support depends upon its affinity for the antibody. The multiplicity of antigen-antibody interactions may add remarkably to the strength of association with support and in turn to the operational life of the device (Rao et al., 1998; Khan et al., 2005).

IgG-cellulose immobilized β galactosidase higher activity at pH other than pH-optima as compared to soluble β galactosidase (Figure 25). Such changes are generally analyzed as a result of immobilization which greatly helps in the stabilization of enzyme at a wider pH range (Zhou and Chen, 2001a). An amperometric lactose biosensor was developed by immobilizing lactase and galactose oxidase in Langmuir-Blodgett (LB) films of poly (3-hexyl thiophene) (P3HT)/stearic acid (SA) for estimation of lactose in milk and its products in order to confirm the purity of tested sample. The enzyme immobilized on LB film was used as working electrode and platinum as reference electrode. It shows the maximum activity at pH range 7.0-7.2 (Sharma et al., 2004).

Immobilized β galactosidase was significantly more stable at higher temperatures as compared to its free form. The immobilized enzyme was very active at temperature above 60 °C which was due to the increased stability of the IgG-cellulose bound β galactosidase (Figure 26 and 27). Goktug et al. (2005) showed that the temperature-optimum of glucose oxidase-β galactosidase hybrid biosensor was at 40 °C, but this preparation lost nearly 53% of its initial activity when incubated for a period of 6 h at the same temperature. It is well established that thermal inactivation starts with the unfolding
of the protein molecule 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 state (Ladero et al., 2006).

Milk is a common health drink consumed by people of all age groups. A large population in our country depends on milk from local suppliers. Due to the increasing demand, adulteration of milk by urea has been very common (Renny et al., 2005). Therefore, it is necessary to evaluate the stability of immobilized preparation against denaturation caused by urea. IgG-cellulose immobilized β-galactosidase retained 64% activity after 2 h incubation with 4.0 M urea whereas the soluble β-galactosidase showed only 4% activity (Figure 28). Urea is a strong denaturant of proteins. Some earlier studies have described that protein can be unfolded by the direct interaction of urea with a peptide backbone via hydrogen bonding/hydrophobic interaction, which contributes to the maintenance of protein conformation (Khan et al., 2005). Glucose oxidase bound to Co$^{3+}$-IDA Sepharose via antienzyme antibodies or F(ab)$^*$ fragment retained very high enzyme activity when exposed to 4.0 M urea for 2 h at 37 °C, whereas the soluble enzyme rapidly lost complete activity under similar incubation conditions (Jan et al., 2001). However, IgG-cellulose immobilized β-galactosidase is quite stable against denaturation caused by urea.

Dairy products are the main source of calcium which cannot be taken by lactose intolerant children (Reily et al., 2006). Here we have investigated the stability of IgG-cellulose immobilized β-galactosidase in the presence of calcium chloride (Figure 29). Immobilized preparation, however, showed greater stability when treated in the presence of calcium. Calcium has an inhibitory effect on soluble β-galactosidase which has already been reported (Li et al., 2001). IgG-cellulose immobilized β-galactosidase showed greater resistance against product inhibition (Figure 29). It is generally agreed that the hydrolysis of lactose by β-galactosidase is inhibited as a result of competitive inhibition by galactose both for soluble and immobilized enzyme preparation (Ozdural et al., 2003; Jurado et al., 2006).

IgG-cellulose bound β-galactosidase was more stable than soluble β-galactosidase against inactivation caused by proteolytic enzymes; trypsin and pepsin (Figure 30). In a recent study, Fatima and Husain (2007) have demonstrated that immunoaffinity bound
immobilized bitter gourd peroxidase was highly stable against the exposure caused by proteolytic enzymes.

High reusability and prolonged storage stability of β galactosidase have further supported that IgG-cellulose immobilized β galactosidase could be exploited for the conversion of lactose from milk and whey (Figure 31 and Table 16). The reusability of glucose oxidase-β galactosidase hybrid biosensor based on glassy carbon electrode and modified with mercury for lactose determination decreased, due to the insufficient stability of mercury thin films (Goktug et al., 2005). Adanyi et al. (1999) also showed the long-term storage stability of lactose biosensors for the determination of lactose in milk and dairy products.

The generally observed higher stability of the immunoaffinity bound β galactosidase against various forms of inactivation may be related to the specific and strong binding of enzyme with the antibody support which prevents the unfolding/denaturation of the enzyme. Thus, the aim of this study was to find an inexpensive, stable and high yield procedure for the purification and immobilization of β galactosidase on an immunoaffinity support. This could be of great interest for the analysis of lactose present in whey, milk and different milk products. Immunoaffinity bound procedure is reversible and the matrix can be regenerated for binding to the fresh batch of enzyme.
Summary
Enzymes are biological catalysts that serve different functions in the body and have attracted a wide range of interest from fundamental academic research to many different industrial applications. The interest of the dairy industry in lactose hydrolysis has been driven mainly by the fact that more than 70% of the world’s population suffers from the inability to digest lactose or lactose containing products due to lactose intolerance symptoms caused by the lack of β galactosidase activity. In this work various immobilized preparations of *Aspergillus oryzae* β galactosidase have made and these preparations have been compared with its free form. Immobilized β galactosidase preparations have also been employed for the hydrolysis of lactose from whey and milk in batch process as well in continuous reactors.

Here, a simple, stable and high yield procedure for the immobilization of glycosylated β galactosidase has been developed. In order to minimize the cost of processes, we have taken an inexpensive support, calcium alginate. Concanavalin A is finding increasing applications as a useful ligand in glycoenzyme immobilization. Concanavalin A-β galactosidase complex was obtained by adding increasing concentration of jack bean extract 10% (w/v) to soluble β galactosidase. Insoluble concanavalin A-β galactosidase preparation was crosslinked by 0.5% (v/v) glutaraldehyde for 2 h at 4 °C. Concanavalin A-β galactosidase complex retained 92% enzyme activity. Crosslinking of concanavalin A-β galactosidase complex resulted in a marginal loss of 6% enzyme activity. The soluble, concanavalin A complex and crosslinked concanavalin A complex of β galactosidase were entrapped into calcium alginate beads. Stability of soluble and entrapped β galactosidase preparations was compared against various denaturing agents such as heat, pH, urea, calcium ions and galactose. Immobilized β galactosidase preparations showed no change in their pH-optima and temperature-optima, however, there was a remarkable broadening in pH-activity and temperature-activity profiles as compared to native enzyme. The soluble enzyme lost its complete activity after 2 h exposure to 4.0 M urea at 37 °C whereas the entrapped crosslinked concanavalin A-β galactosidase complex retained more than 30% of its original activity under identical exposure. Entrapped crosslinked concanavalin A-β galactosidase complex retained more than 80% of the original activity after incubation with 5% CaCl₂ at 37 °C for 1 h while soluble β galactosidase lost nearly half of the
original activity. The incubation of soluble β galactosidase with 5% galactose for 1 h at 37 °C resulted in a significant loss of 70% activity while the entrapped crosslinked concanavalin A-β galactosidase complex retained over 60% of the original activity under similar exposure. Ki_{app} values have been calculated which demonstrated that the concentration of galactose was inversely proportional to Ki_{app} value. Entrapped crosslinked concanavalin A-β galactosidase complex exhibited significantly very high Ki_{app} value at 1% galactose as compared to other β galactosidase preparations. It indicated that entrapped crosslinked concanavalin A-β galactosidase complex was more stable and less affected by galactose inhibition.

The Michaelis constant (K_m) and maximum velocity (V_{max}) were calculated from Lineweaver Burk plots. The K_m for soluble β galactosidase and entrapped crosslinked concanavalin A-β galactosidase was 2.51 mM and 5.18 mM, respectively, whereas V_{max} for soluble β galactosidase and entrapped crosslinked concanavalin A-β galactosidase was 4.8 X 10^{-4} mol/min and 4.2 X 10^{-4} mol/min, respectively.

The reusability experiment further supported that entrapped crosslinked concanavalin A-β galactosidase complex did not leach out of the gel beads therefore such preparation could be exploited for the continuous conversion of lactose from milk or whey for longer durations in reactor. The entrapped crosslinked concanavalin A-β galactosidase complex retained 93% activity after 2 months storage at 4 °C, whereas the soluble β galactosidase exhibited only 40% activity under identical conditions. Thus, entrapped crosslinked concanavalin A-β galactosidase complex was highly stable at 4 °C.

Entrapped crosslinked concanavalin A-β galactosidase complex was more efficient in hydrolyzing greater fraction of lactose as compared to entrapped soluble β galactosidase. It was also demonstrated that entrapped crosslinked concanavalin A-β galactosidase complex was more efficient in hydrolyzing lactose from whey and milk. Nearly 86% and 77% lactose from whey and milk was hydrolyzed after 3 h of incubation by entrapped crosslinked concanavalin A-β galactosidase, while 69% and 54% lactose was hydrolyzed from whey and milk by entrapped soluble β galactosidase under similar experimental conditions. The rate of lactose hydrolysis was seen at different flow rates in a packed bed reactor by taking entrapped preparations of soluble β galactosidase and crosslinked concanavalin A-β galactosidase. It was seen that the maximum hydrolysis
Summary

occurred at the flow rate of 10 mL h⁻¹ while the hydrolytic rate decreased at 20 mL h⁻¹ and 30 mL h⁻¹. It was due to the residence time of lactose inside the column containing entrapped preparations of β galactosidase.

Alginate as entrapment media has some limitations for the immobilization of enzymes such as the problem of substrate and product diffusion in and out of the alginate beads. Therefore, an attempt has been made to synthesize a hybrid gel of alginate and starch for the purpose of bioaffinity based immobilization of enzymes on the large surface area of beads via concanavalin A. The presence of starch in hybrid gel would also help in minimizing the cost of the process. Concanavalin A was adsorbed on the surface of calcium alginate-starch beads by incubating with jack bean extract. The immobilized β galactosidase beads were further crosslinked by glutaraldehyde in order to maintain its integrity. Calcium alginate-starch beads retained nearly 76% of the original activity. However, crosslinking with glutaraldehyde resulted in a marginal loss of enzyme activity and it showed nearly 71% of the initial activity. The temperature-optimum of the immobilized β galactosidase shifted from 50 °C to 60 °C. Immobilized β galactosidase retained nearly 65% activity after 5 h incubation at 60 °C while the soluble enzyme showed an insignificant activity of only 2% under identical conditions. Immobilized β galactosidase exhibited increased activity on exposure to 5% of MgCl₂. However, the exposure of soluble β galactosidase to 5% calcium chloride for 1 h at 37 °C resulted in a loss of 52% its original activity whereas the immobilized β galactosidase retained 61% activity under similar treatment. Immobilized β galactosidase exhibited greater stabilization against urea induced denaturation and product inhibition due to galactose. β Galactosidase immobilized on the surface of concanavalin A layered calcium alginate-starch beads would be more accessible to its substrate and thus the problem of end product inhibition could be minimized when such preparation would be used in reactors for the continuous hydrolysis of lactose. Calcium alginate-starch beads surface immobilized β galactosidase was more superior in stability on prolonged storage at 4 °C.

It was noticed that 70% and 89% of lactose was hydrolyzed from whey and 61% and 79% of lactose was hydrolyzed from milk by soluble and immobilized β galactosidase in 3 h and 4 h, respectively in batch process at 32 °C. Thus the calcium alginate-starch beads surface immobilized β galactosidase has been successfully
employed for the hydrolysis of lactose from whey and milk in reactors without any appreciable loss in its activity.

The suitability of β galactosidase immobilized on the surface of concanavalin A layered calcium alginate-starch beads, as an oral therapeutic agent for the treatment of patients suffering from lactose intolerance has also been investigated. The stability of immobilized β galactosidase against the conditions of alimentary canal/digestive system, such as varying pH, trypsin, pepsin and salivary α amylase were also monitored. Immobilized β galactosidase retained 84% and 95% of original activity while soluble enzyme showed 52% and 78% of the original activity at pH 3.0 and 5.0.

The reusability of immobilized enzyme in the buffers of varying pH and in the assay buffer also showed that the immobilized preparation was remarkably stable at extreme conditions of acidic and alkaline media and digestion by proteases. The effect of α amylase activity demonstrated that immobilized β galactosidase had no loss in its activity over prolonged incubation with high concentrations of salivary amylase. Thus, we have tried to focus on the aspect that if the enzyme immobilized on the surface of beads were taken orally as a drug it would greatly help in reducing the problem of lactose intolerance. The size of these beads was spherical in shape, thus the area calculated was found to be 341.94 X 10^-3 cm^2 and the volume of the spherical beads was 18.80 X 10^-3 cm^3, which would remarkably help in the hydrolysis of lactose as they can easily reach near to the lumen of the small intestine and would help in the hydrolysis of lactose.

Purified β galactosidase from Aspergillus oryzae was injected into healthy male albino rabbits for the production of anti-β galactosidase polyclonal antibodies. Antibodies raised against β galactosidase were further purified by ion exchange chromatography. The dialyzed protein sample from ammonium sulphate fractionated antiserum was passed through DEAE-cellulose column. The fractions contained purified anti-β galactosidase antibodies. DEAE-cellulose purified IgG loaded on SDS-PAGE clearly showed two bands corresponding to the heavy and light chains of the antibody. The native PAGE showed a single band and this result further supported the purity of the purified antibodies. Purified anti-β galactosidase antibodies gave a clear precipitin line with the purified β galactosidase from Aspergillus oryzae when Ouchterlony double immunodiffusion was performed. The IgG isolated were used for the construction of
cellulose-anti-β galactosidase immunoaffinity support. Immunoaffinity immobilized β galactosidase exhibited high yield of immobilization. The immunoaffinity immobilized β galactosidase preparation exhibited a very high effectiveness factor (η), 0.97. There was no difference in pH-optima of immobilized β galactosidase between pH 4.6-5.5, unlike the soluble β galactosidase that exhibited activity peak at pH 4.6. Immunoaffinity bound β galactosidase exhibited no difference in its activity between 50-60 °C whereas free β galactosidase had a temperature-optima at 50 °C. Immobilized β galactosidase exhibited significantly very high stabilization to urea and proteolytic digestion, i.e. against pepsin and trypsin. The immobilized β galactosidase retained 71% activity when treated with 5% calcium chloride for 1 h at 37 °C. Due to product inhibition, soluble β galactosidase resulted in a loss of 72% activity after exposure to 5% of galactose for 1 h at 37 °C while the immunoaffinity immobilized β galactosidase retained 65% of the original activity under similar treatment. After tenth repeated use the IgG-cellulose immobilized enzyme preparation retained nearly 46% of its initial activity. Immunoaffinity immobilized β galactosidase also retained 80% activity on storage for over 2 months at 4 °C. Thus, immunoaffinity bound procedure is reversible and the matrix can be regenerated for binding to the fresh batch of enzyme. It could be of great interest in the area of clinical analysis of lactose.