CHAPTER II

Calcium alginate entrapped Aspergillus oryzae β galactosidase its stability and applications in the hydrolysis of lactose from milk/whey
2.1. INTRODUCTION

Extensive research efforts have been made to investigate the possibilities offered by β galactosidase in dairy technology (Roy and Gupta, 2003; Al-Muftah and Abu-Reesh, 2005). Some peculiar properties of the enzymes, such as their non-reusability, high sensitivity to several denaturing agents and presence of adverse sensory or toxicological effects may hamper the effective use of soluble enzymes. Many of these undesirable limitations can be overcome by the use of immobilized enzymes (Nijpels, 1981; Gekas and Lopez-Leiva, 1985).

Enzyme immobilization offers a number of advantages over the soluble enzymes. Immobilization permits the reuse of the enzymes and may provide a better environment for catalytic activity. It also reduces the cost of downstream processing in addition to good product quality. The use of immobilized lactase in the production of lactose free milk has been described by a number of workers (Bakken et al., 1989; Lartillot, 1993; Roy and Gupta, 2003).

The immobilization of enzymes, through their amino acid side chain groups sometimes resulted in the loss of enzyme activity (Ladero et al., 2005). However, an alternative strategy has been suggested for the immobilization of glycoenzymes via their glycosyl moiety (Bakken and Hill, 1992; Jan and Husain, 2004). The carbohydrate part of the enzymes do not participate in catalysis, therefore the immobilization of such enzymes via glycosyl moieties is quite safe. Lectins are carbohydrate-binding proteins and they interact specifically with glycoproteins/glycoenzymes and form insoluble complexes (Jan et al., 2006).

Various types of supports and techniques have been used for β galactosidase immobilizations and their applications (Gekas and Lopez-Levia, 1985). Membrane reactors often lower enzymatic activity in comparison to the use of soluble enzyme in batch reactors (Jurado et al., 2005). The use of PBRs in biological processes would allow the application of new methodologies to transform an environmental problem, such as permeate whey elimination of dairy industries in a commercial affair. The choice of lactose hydrolysis in batch and continuous mode depends primarily on the enzymatic
characteristics and the economics encompassing the production, storage and reusability (Mammarella and Rubiolo, 2006).

The objective of the present work is to develop a simple and high yield procedure for the immobilization of glycosylated β galactosidase from Aspergillus oryzae. The activity of soluble and immobilized β galactosidase was compared against various chemical and physical denaturants, such as heat, pH, urea and calcium chloride. The effect of product inhibition on the activity of soluble and immobilized preparations of β galactosidase has been investigated. Reactor efficiency and operational stability both in terms of continuous and batch processes have also been studied.

2.2. MATERIALS & METHODS

2.2.1. Materials

Aspergillus oryzae [G-7138, Lot121H0055] β galactosidase (3.2.1.23) and galactose were obtained from Sigma Chem. Co. (St. Louis, MO) USA. o-nitrophenyl β-D-galactopyranoside (ONPG), glutaraldehyde, ethanolamine are obtained from SRL, Chem. Mumbai, India. All other chemicals and reagents used were of analytical grade. Milk was purchased from local dairy in Aligarh, India.

2.2.2. Preparation of jack bean extract

Jack bean extract (10%, w/v) was prepared by adding 5.0 g of jack bean meal to 50 mL of 0.1 M Tris-HCl buffer, pH 6.1 containing 0.1 M NaCl, 0.001 M MgCl₂, 0.001 M MnCl₂, and 0.001 M CaCl₂. The mixture was kept on a magnetic stirrer for 2 h at room temperature. Insoluble residue was removed by centrifugation at 12,000 xg on a Remi Cooling Centrifuge R-24 for 20 min, until a clear supernatant is obtained. The collected supernatant was used for the insolubilization of β galactosidase.
2.2.3. Preparation of Con A-β galactosidase complex

The increasing concentration of jack bean extract (10%, w/v) was added to 0.5 mL diluted β galactosidase (200 U) making the volume up to 2.0 mL by 0.1 M Tris-HCl buffer, pH 6.1. The reaction mixture was incubated overnight at 37 °C. The precipitate was collected by centrifugation at 3000 xg on a Remi Cooling Centrifuge R-24 for 30 min. The activity was determined both in supernatant and pellet (Jan et al., 2006).

2.2.4. Crosslinking of Con A-β galactosidase complex

Con A-β galactosidase complex (220 U) was crosslinked by adding increasing concentrations of glutaraldehyde (0.1-0.5%, v/v) for 2 h at 4 °C. Ethanolamine was added to a final concentration of 0.01% (v/v) to stop crosslinking. The solution was allowed to stand for 90 min at room temperature and the pellet was collected by centrifugation at 3000 xg for 30 min on a cooling tabletop centrifuge. The precipitate was suspended in 1.0 M methyl α-D-glucopyranoside and allowed to stand for 1 h at room temperature. Centrifuged again at 3000 xg for 30 min, the precipitate obtained was suspended in 0.1 M sodium acetate buffer, pH 4.6. The activity was determined both in supernatant and pellet. The activity of Con A-β galactosidase complex without methyl α-D-glucopyranoside treatment was considered as control (Jan et al., 2006).

2.2.5. Entrapment of soluble, Con A complex and crosslinked Con A complex of β galactosidase into calcium alginate

The soluble, Con A-β galactosidase complex and crosslinked Con A-β galactosidase complex were mixed independently with 5.0% aqueous sodium alginate solution and added drop wise to a stirred solution of 0.2 M CaCl₂ prepared in distilled water. A 5.0 mL syringe with attached needle number 20 was used for the preparation of calcium alginate beads. The beads were stirred in CaCl₂ solution for 2 h on a magnetic stirrer to make them hard and then suspended in 0.1 M sodium acetate buffer, pH 4.6. The obtained beads were stored and further used (Musthapa et al., 2004).
2.2.6. Assay of β-galactosidase

The enzyme activity of β-galactosidase was determined by measuring the release of o-nitrophenol from ONPG (o-nitrophenyl β-D-galactopyranoside) at 405 nm (Batra et al., 2002). Enzymatic reaction was carried out in a total volume of 2.0 mL containing 1.7 mL of 0.1 M sodium acetate buffer, pH 4.6, 0.1 mL suitably diluted enzyme and 0.2 mL of 20 mM ONPG at 37°C for 15 min. The reaction was stopped by adding 2.0 mL 2.0 N sodium carbonate solution. The immobilized preparations were continuously agitated for entire duration of assay. The assay was highly reproducible with immobilized preparation.

One unit of β-galactosidase activity (U) is defined as the amount of enzyme that liberates 1.0 μmole of o-nitrophenol (ε₄₅₀ = 4500 L Mol⁻¹ Cm⁻¹) per min under standard assay conditions.

2.2.7. Protein estimation

Protein concentration was determined by using Bradford dye binding method (Bradford, 1976). Bradford dye was prepared by dissolving 30 mg of commassie brilliant blue G 250 in 15 mL ethanol and 30 mL o-phosphoric acid. The contents were properly dissolved in a brown bottle and the final volume was then made up to 300 mL with distilled water. Prepared dye solution was filtered through a whatman filter paper. Aliquots of protein were taken in a set of tubes and final volume was made up to 1 mL with distilled water. Bradford dye solution (5.0 mL) was then added to each tube. The color developed was read at 595 nm after 5 min incubation at room temperature against a reagent blank. Bovine serum albumin was used as a standard protein.

2.2.8. Glucose estimation by glucose oxidase/peroxidase coupled assay procedure

The lactose hydrolysis was monitored for the formation of glucose by using glucose oxidase/peroxidase coupled assay procedure. An appropriate amount of β-galactosidase treated lactose, suitably diluted with 0.5 M phosphate buffer at pH 7.0 was
taken. The hydrolysis of lactose was estimated by using solution C. Solution C was prepared by taking 5 mg of glucose oxidase, 1 mg of peroxidase dissolved in 5.0 mL of 0.1 M potassium phosphate buffer, pH 6.1, 15 mg o-dianisidine HCl prepared in 2.5 mL of distilled water and 40 ml. glycerol (20%). Solution C, 1.5 mL was added in all assay tubes. The test tubes were again incubated at 37 °C for 15 min and the reaction was stopped by adding 1.0 mL of 6.0 N HCl and developed color was measured at 540 nm (Hatton and Regoezci, 1976).

2.2.9. Calculation of Michaelis constant ($K_m$) and maximum reaction velocity ($V_{max}$)

The Michaelis constant ($K_m$) and maximum reaction velocity ($V_{max}$) for soluble β galactosidase and entrapped crosslinked Con A-β galactosidase was calculated from Lineweaver Burk plots of $1/v$ vs. $1/s$ where $v$ = velocity of reaction and $s$ = substrate concentration. The reaction velocity ($V_{max}$) was measured at different ONPG concentrations, keeping the amount of free and immobilized β galactosidase constant (Rejikumar and Devi, 2001).

2.2.10. Calculation of $K_{i_{app}}$ ($K_{i_{app}}$)

The $K_{i_{app}}$ values were calculated to explain galactose inhibition in terms of Henderson equation, which indicates competitive inhibition mechanism.

$$I_o = \frac{1 - v_i}{v_o} = K_i \left(1 + [S]_o / K_m \right)$$

$$K_{i_{app}} = K_i \left(1 + [S]_o / K_{m} \right)$$

where $[I]_o$, $[E_o]$ and $[S]_o$ are the initial concentrations of inhibitor, enzyme and substrate respectively, $v_o$ is the velocity without inhibitor and $v_i$ is the velocity in the presence of inhibitor. $K_i$ is inhibition constant, $K_{i_{app}}$ is apparent inhibition constant and $K_{m}$ is Michaelis-Menton constant (Henderson 1972; Pagano et al., 1984).
Chapter II

2.2.11. Reusability of alginate entrapped β galactosidase

Alginate entrapped preparations of β galactosidase; soluble, Con A complex, cross linked Con A complex were taken in triplicates for assaying the activity of enzyme. After each assay immobilized enzyme were taken out from assay tubes and were washed and stored in 0.1 M sodium acetate buffer, pH 4.6 overnight at 4 °C. The activity was assayed for seven successive days.

2.2.12. Storage stability of soluble and entrapped β galactosidase preparations

Soluble and all the immobilized preparations of β galactosidase were stored at 4 °C in 0.1 M sodium acetate buffer, pH 4.6 for over 2 months. The aliquots from each preparation (2.0 U) were taken in duplicates at the 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 further storage activity.

2.2.13. Hydrolysis of lactose from milk/whey by β galactosidase in batch process

The milk was skimmed by centrifugation of cold milk at 8000 xg for 20 min in a Remi Cooling Centrifuge. The fat layer was removed from milk and stored at 4 °C for further use. Skimmed milk (500 mL) was treated with β galactosidase (1000 U) in batch process at 32 °C. The aliquots of 250 μL were taken out at indicated time intervals for 12 h.

Whey was prepared from skimmed milk by acidifying with HCl until the pH reached 4.8. The casein was removed by centrifugation. Prepared whey was stored at 4 °C for further use (Roy and Gupta, 2003). Whey (50 μL) was treated with β galactosidase (400 U) in batch process at 32 °C. The aliquots of 250 μL were taken out at indicated time intervals for 12 h. The hydrolysis of lactose was estimated by glucose oxidase/peroxidase assay procedure (Hatton and Regoezci, 1976).
2.2.14. Hydrolysis of lactose using packed bed columns

Calcium alginate entrapped crosslinked Con A-β galactosidase (1525 U) was packed in a (2.0 x 10 cm) column. A column of similar dimensions containing alginate entrapped soluble β galactosidase (1500 U) was also prepared. The packed volume of the column was 8 mL. Lactose (0.1 M) dissolved in 0.1 M sodium acetate buffer; pH 4.6 containing 0.001 M sodium azide was passed through both the columns at different flow rates at room temperature (32 °C).

2.2.15. Statistical analysis

The data expressed in various studies was plotted using Sigma Plot-10 and Origin-6.1 expressed as mean with standard deviation of error (±). Each value represents the mean for three-independent experiments performed in duplicates with average standard deviation, <5%.

2.3. RESULTS

2.3.1. Formation of Con A-β galactosidase complex

The addition of increasing concentrations of jack bean extract to the fixed concentration of β galactosidase resulted in increased precipitation of enzyme activity. The maximum precipitation exhibited 92% of the initial enzyme activity (Figure 4).

2.3.2. Crosslinking of Con A-β galactosidase by glutaraldehyde

In order to maintain the integrity of Con A-β galactosidase complex in the presence of substrate and product, the complex was crosslinked by increasing concentrations of glutaraldehyde. The maximum crosslinking was obtained by using 0.5% (v/v) glutaraldehyde. Crosslinking further resulted in a marginal loss of 6% of the initial activity (Figure 5).
2.3.3. Entrapment of β galactosidase into calcium alginate beads

Entrapment of soluble β galactosidase, Con A-β galactosidase complex and crosslinked Con A-β galactosidase complex into calcium alginate beads further resulted in the loss of enzyme activity and these entrapped β galactosidase preparations retained 72%, 63% and 57% of the original enzyme activity, respectively (Table 2).

2.3.4. Determination of kinetic constants

The Michaelis constant \( (K_m) \) and maximum velocity \( (V_{max}) \) were calculated from Lineweaver Burk plots. It was found that \( V_{max} \) was affected less than \( K_m \) upon immobilization (Table 3).

2.3.5. Effect of pH

Figure 6 demonstrates the pH-activity profiles of soluble and immobilized β galactosidase. All immobilized β galactosidase preparations showed no change in pH-optima but had a remarkable broadening in pH-activity profiles as compared to the native enzyme. However, the crosslinked Con A-β galactosidase complex and entrapped crosslinked Con A-β galactosidase complex retained significantly very high activity at acidic and alkaline side of the pH-optima as compared to the soluble and other immobilized β galactosidase preparations. Entrapped crosslinked Con A-β galactosidase complex retained 81% and 21% activity at pH 3.0 and pH 8.5, respectively whereas the soluble enzyme exhibited 49% and 7% of the initial activity, respectively.

2.3.6. Effect of temperature

There was no alteration in temperature-optima of the immobilized β galactosidase preparations although there was a significant broadening in temperature-activity profiles for immobilized enzyme preparations (Figure 7).
Figure 4: Precipitation of β galactosidase by using jack bean extract

β Galactosidase (200 U) was incubated with increasing concentrations (0.1-1.0 mL) of jack bean extract (10%, w/v) in a total volume of 2 mL of 0.1 M Tris-HCl buffer, pH 6.1 for 12 h at 37 °C. Precipitate of each preparation was separated by centrifugation at 3000 xg for 15 min. Each precipitate was further washed with assay buffer and activity was determined in each precipitate as well as in supernatant as described in text (Section 2.2.6).
Figure 5: Crosslinking of Con A-β galactosidase complex by glutaraldehyde

Con A-β galactosidase complex (220 U) was incubated with increasing concentrations of glutaraldehyde (0.1-0.5%, v/v) for 2 h at 4 °C. After glutaraldehyde treatment in each tube 0.01% ethanolamine was added and further incubated for 30 min at room temperature. Crosslinked complex was collected by centrifugation at 3000 xg for 15 min. The complex was washed with assay buffer. Crosslinked complex was incubated with 1.0 M methyl α-D glucopyranoside for over 1 h at room temperature. The activity of enzyme was determined in each supernatant and precipitate.
Table 2: Immobilization of β galactosidase by using jack bean extract and calcium alginate matrix

<table>
<thead>
<tr>
<th>Methods</th>
<th>Activity expressed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A-β galactosidase</td>
<td>92 ±2.3</td>
</tr>
<tr>
<td>Crosslinked Con A-β galactosidase</td>
<td>88 ±1.8</td>
</tr>
<tr>
<td>Calcium alginate entrapped soluble β galactosidase</td>
<td>72 ±2.95</td>
</tr>
<tr>
<td>Calcium alginate entrapped Con A-β galactosidase</td>
<td>63 ±1.65</td>
</tr>
<tr>
<td>Calcium alginate entrapped crosslinked Con A-β galactosidase</td>
<td>57 ±2.89</td>
</tr>
</tbody>
</table>

Each expressed activity is the percentage of the initial soluble enzyme activity. Each value represents the mean for three-independent experiments performed in duplicates, with average standard deviation, < 5%.
Table 3: Influence of immobilization process on kinetic constants

<table>
<thead>
<tr>
<th>Derivative</th>
<th>$K_m$ (mM)</th>
<th>$V_m$ (mol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble $\beta$ galactosidase</td>
<td>2.51</td>
<td>$4.8 \times 10^{-4}$</td>
</tr>
<tr>
<td>Entrapped crosslinked Con A-$\beta$ galactosidase</td>
<td>5.18</td>
<td>$4.2 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

The Michaelis constant ($K_m$) and maximum velocity ($V_{max}$) were calculated from Lineweaver Burk plots of $1/v$ vs. $1/s$ where $v$ = velocity of reaction and $s$ = substrate concentration. Each value represents the mean for three-independent experiments performed in duplicates, with average standard deviation, < 5%.
Figure 6: pH-activity profiles of soluble and entrapped β galactosidase preparations

The enzyme activity of soluble and entrapped β galactosidase preparations (2.0 U) was measured in the buffers of various pH. The molarity of each buffer was 0.1 M. The activity at pH 4.6 for all the preparations was taken as control (100%) for the calculation of remaining percent activity as described in the text (Section 2.2.6).
However, the crosslinked Con A-β galactosidase complex and entrapped crosslinked Con A-β galactosidase complex retained significantly very high activity at temperatures lower and higher than the temperature-optima as compared to the soluble and other immobilized β galactosidase preparations. Entrapped crosslinked Con A-β galactosidase preparation retained significantly higher fraction of catalytic activity, 63% at 80 °C while the free enzyme exhibited only 2% of the initial enzyme activity at the same temperature.

Figure 8 illustrates the thermal denaturation plot of soluble and immobilized preparations of β galactosidase. All the immobilized β galactosidase preparations retained significantly high catalytic activity even after 6 h incubation at 60 °C. Crosslinked Con A-β galactosidase complex showed nearly 41% of the initial activity after 6 h exposure. Entrapped crosslinked Con A-β galactosidase complex exhibited nearly 53% activity after 6 h whereas the native enzyme showed only marginal activity of 3% after 5 h exposure under similar incubation conditions.

2.3.7. Effect of 4.0 M urea

The urea-induced denaturation of β galactosidase preparations is shown (Figure 9). The soluble enzyme almost lost its complete activity after 2 h exposure with 4.0 M urea at 37 °C while the immobilization provides more stability against the denaturation induced by urea. Increased stability of various immobilized preparations of β galactosidase was in order of entrapped soluble β galactosidase < Con A-β galactosidase complex < entrapped Con A-β galactosidase complex < crosslinked Con A-β galactosidase complex < entrapped crosslinked Con A-β galactosidase complex. However, entrapped crosslinked Con A-β galactosidase complex retained more than 50% of its original activity after 1 h exposure with 4.0 M urea at 37 °C whereas the soluble enzyme had a marginal activity of 14% under identical incubation conditions.
Figure 7: Temperature-activity profiles of soluble and entrapped β galactosidase preparations

The enzyme activity of soluble and entrapped β galactosidase preparations (2.0 U) was measured in 0.1 M sodium acetate buffer, pH 4.6 at various temperatures. The activity obtained at 50 °C was taken as control (100%) for the calculation of remaining percent activity as described in the text (Section 2.2.6).
Chapter II

Soluble β galactosidase

Entrapped soluble β galactosidase

Con A-β galactosidase complex

Alginate entrapped Con A-β galactosidase complex

Crosslinked Con A-β galactosidase complex

Alginate entrapped crosslinked Con A-β galactosidase complex

Figure 8: Thermal denaturation of soluble and entrapped β galactosidase preparations

Soluble and entrapped β galactosidase preparations were incubated at 60 °C in 0.1 M sodium acetate buffer, pH 4.6 for various time intervals. The aliquots of each enzyme preparation (2.0 U) were collected at various times and activity of the enzyme was determined as described in the text (Section 2.2.6). The enzyme unexposed at 60 °C was considered as control (100%).
Soluble and entrapped β galactosidase preparations were incubated in 4.0 M urea dissolved in 0.1 M sodium acetate buffer, pH 4.6 at 37 °C of the urea exposed enzyme preparations. Aliquots of each preparation (2.0 U) were removed at various times and the remaining activity was determined as described in the text (Section 2.2.6).
2.3.8. Effect of calcium chloride

Calcium is one of the important components of milk. Therefore, it is necessary to evaluate the stability of various preparations of β galactosidase in the presence of different concentrations of calcium chloride. The effect of various concentrations of calcium chloride (1.0-5.0%, w/v) on the activity of β galactosidase is demonstrated in Figure 10. The exposure of soluble β galactosidase by 5.0% calcium chloride for 1 h at 37°C resulted in a loss of nearly half of the initial activity. Entrapped crosslinked Con A-β galactosidase complex was remarkably more stable as compared to the soluble and other immobilized β galactosidase preparations. This preparation retained more than 80% of the original activity after incubation with 5% CaCl₂ at 37°C for 1 h.

2.3.9. Effect of galactose

Galactose is one of the products of β galactosidase catalyzed hydrolysis of lactose. It has been reported that galactose can also inhibit reaction catalyzed by β galactosidase; therefore an effort has been made to investigate the effect of various concentrations of galactose on the activity of soluble and immobilized preparations of β galactosidase. The effect of increasing concentrations of galactose (1.0-5.0%, w/v) has been examined on soluble and immobilized β galactosidase (Figure 11). The preincubation of soluble β galactosidase with 5.0% galactose for 1 h at 37°C resulted in a significant loss of 70% activity while the entrapped crosslinked Con A-β galactosidase complex retained over 60% of the original activity under similar exposure.

$K_{iapp}$ values has been calculated which demonstrated that the concentration of galactose was inversely proportional to $K_{iapp}$ value. Entrapped crosslinked Con A-β galactosidase complex exhibited significantly very high $K_{iapp}$ value at 1% of galactose as compared to other β galactosidase preparations. It indicated that this preparation was more stable and less affected by galactose inhibition (Table 4).
Figure 10: Effect of calcium chloride on soluble and entrapped β-galactosidase preparations

Soluble and entrapped β-galactosidase preparations (2.0 U) were incubated with increasing concentration of calcium chloride (1.0-5.0 %, w/v) in 0.1 M sodium acetate buffer, pH 4.6 for 1 h at 37 °C. The remaining activity of the enzyme was determined as described in the text (Section 2.2.6).
Figure 11: Effect of galactose on soluble and entrapped β galactosidase preparations

Soluble and entrapped β galactosidase preparations (2.0 U) were incubated with increasing concentrations of galactose (1.0-5.0 %, w/v) in 0.1 M sodium acetate buffer, pH 4.6 for 1 h at 37 °C. The remaining activity of the enzyme was determined as described in the text (Section 2.2.6).
Table 4: $K_{iapp}$ of soluble and entrapped $\beta$ galactosidase preparations in the presence of galactose

<table>
<thead>
<tr>
<th>Galactose conc. (%)</th>
<th>Soluble $\beta$ galactosidase</th>
<th>Entrapped soluble $\beta$ galactosidase</th>
<th>Con A-$\beta$ galactosidase</th>
<th>Entrapped Con A-$\beta$ galactosidase</th>
<th>Crosslinked Con A-$\beta$ galactosidase</th>
<th>Entrapped crosslinked Con A-$\beta$ galactosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>220.17 ±2.8</td>
<td>282.21 ±2.6</td>
<td>316.2 ±1.6</td>
<td>350 ±3.1</td>
<td>418.05 ±1.9</td>
<td>574 ±0.9</td>
</tr>
<tr>
<td>2</td>
<td>173.61 ±3.2</td>
<td>186.62 ±2.2</td>
<td>209 ±1.32</td>
<td>249 ±2.9</td>
<td>302.08 ±1.5</td>
<td>392.16 ±2.4</td>
</tr>
<tr>
<td>3</td>
<td>151 ±2.9</td>
<td>156.28 ±3.5</td>
<td>174 ±1.8</td>
<td>214.2 ±3.4</td>
<td>249 ±2.4</td>
<td>332 ±2.5</td>
</tr>
<tr>
<td>4</td>
<td>148 ±2.1</td>
<td>148.15 ±1.9</td>
<td>155 ±3.2</td>
<td>190.4 ±2.8</td>
<td>204 ±2.2</td>
<td>227 ±2.8</td>
</tr>
<tr>
<td>5</td>
<td>146 ±1.6</td>
<td>147.2 ±1.2</td>
<td>153.3 ±2.5</td>
<td>168.31 ±2.1</td>
<td>174 ±2.3</td>
<td>180 ±1.74</td>
</tr>
</tbody>
</table>

The $K_{iapp}$ values were calculated to explain galactose inhibition in terms of competitive inhibition mechanism:

$$I_0 + \frac{v_i}{v_0} = K_i \left(1 + \frac{[S]_o}{K_m}\right) \frac{v_i}{v_0} + [E_0]; \quad K_{iapp} = K_i \left(1 + \frac{[S]_o}{K_m}\right)$$

Where $[I]_o$, $[E_o]$ and $[S]_o$ are the initial concentrations of inhibitor, enzyme and substrate respectively, $v_0$ is the velocity without inhibitor and $v_i$ is the velocity in the presence of inhibitor. $K_i$ is inhibition constant, $K_{iapp}$ is apparent inhibition constant and $K_m$ is Michaelis-Menten constant. Each value represents the mean for three-independent experiments performed in duplicates, with average standard deviation, < 5%.
However as the concentrations of galactose was increased, more inhibition was noticed which suggested that at high concentration of galactose, a significant loss of enzyme activity occurred in soluble β galactosidase whereas the entrapped crosslinked Con A-β galactosidase complex retained more enzymatic activity under similar conditions.

2.3.10. Reusability of beads containing β galactosidase

Figure 12 demonstrates the reusability of three different calcium alginate entrapped preparations of β galactosidase. After seventh repeated use the entrapped soluble enzyme showed a marginal activity of 21% whereas the entrapped Con A-β galactosidase complex and entrapped crosslinked Con A-β galactosidase complex retained 85% and 95% of its activity, respectively.

2.3.11. Storage activity of soluble and entrapped β galactosidase preparations

Storage stability of soluble and immobilized preparations of β galactosidase at 4 °C was monitored at the gap of 10 d for over 2 months (Table 5). The entrapped crosslinked Con A-β galactosidase complex retained almost 93% of the original activity after a period of 2 months storage at 4 °C, whereas the soluble β galactosidase exhibited only 40% activity under identical storage conditions. Thus, entrapped crosslinked Con A-β galactosidase is highly stable at 4 °C.

2.3.12. Hydrolysis of lactose from milk/whey by entrapped β galactosidase

The hydrolysis of lactose from milk and whey was monitored by sampling at regular time intervals for 12 h at 32 °C in stirred batch process (Table 6). It was noted that after 1 h, the rate of hydrolysis by entrapped soluble β galactosidase was 54% in whey and 45% in milk while entrapped crosslinked Con A-β galactosidase showed slightly less hydrolysis of lactose in whey and milk, 43% and 36%, respectively.
The reusability of entrapped soluble \( \beta \) galactosidase, entrapped Con A-\( \beta \) galactosidase complex and entrapped crosslinked Con A-\( \beta \) galactosidase was monitored at the gap of 6 h. The samples of each preparation were taken in triplicates and were assayed for the remaining enzyme activity as described in the text (Section 2.2.6).
Table 5: Storage activity of soluble and entrapped β galactosidase preparations

<table>
<thead>
<tr>
<th>Number of days</th>
<th>Soluble β galactosidase</th>
<th>Entrapped soluble β galactosidase</th>
<th>Con A-β galactosidase</th>
<th>Entrapped Con A-β galactosidase</th>
<th>Crosslinked Con A-β galactosidase</th>
<th>Entrapped crosslinked Con A-β galactosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>91 ± 1.23</td>
<td>94 ± 2.7</td>
<td>96 ± 0.98</td>
<td>98 ± 1.23</td>
<td>99 ± 0.76</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>73 ± 3.22</td>
<td>84 ± 2.67</td>
<td>89 ± 1.67</td>
<td>93 ± 1.32</td>
<td>97 ± 1.11</td>
<td>100</td>
</tr>
<tr>
<td>30</td>
<td>61 ± 2.56</td>
<td>70 ± 2.98</td>
<td>79 ± 3.41</td>
<td>87 ± 1.8</td>
<td>94 ± 1.56</td>
<td>98 ± 1.34</td>
</tr>
<tr>
<td>40</td>
<td>57 ± 1.43</td>
<td>67 ± 2.45</td>
<td>71 ± 2.5</td>
<td>80 ± 2.8</td>
<td>93 ± 1.67</td>
<td>97 ± 1.85</td>
</tr>
<tr>
<td>50</td>
<td>51 ± 3.2</td>
<td>60 ± 1.54</td>
<td>69 ± 2.33</td>
<td>79 ± 3.34</td>
<td>89 ± 2.76</td>
<td>95 ± 1.86</td>
</tr>
<tr>
<td>60</td>
<td>40 ± 2.5</td>
<td>51 ± 1.78</td>
<td>60 ± 2.97</td>
<td>70 ± 2.99</td>
<td>84 ± 3.2</td>
<td>93 ± 1.2</td>
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</tbody>
</table>

Soluble and all the entrapped β galactosidase preparations were stored at 4 °C in 0.1 M sodium acetate buffer, pH 4.6 for over 2 months. The aliquots from each preparation (2.0 U) were taken in duplicates at the 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 the calculation of further storage activity. Each value represents the mean for three-independent experiments performed in duplicates, with average standard deviation, < 5%.
Table 6: Hydrolysis of lactose from milk/whey by entrapped β galactosidase

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Lactose hydrolysis (•/')</th>
<th>Milk</th>
<th>Whey</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Entrapped soluble β galactosidase</td>
<td>Entrapped crosslinked Con A-β galactosidase</td>
</tr>
<tr>
<td>1</td>
<td>45 ±1.32</td>
<td>36 ±1.56</td>
<td>54 ±1.23</td>
</tr>
<tr>
<td>2</td>
<td>50 ±1.22</td>
<td>63 ±1.45</td>
<td>61 ±1.45</td>
</tr>
<tr>
<td>3</td>
<td>54 ±1.11</td>
<td>77 ±1.99</td>
<td>69 ±1.23</td>
</tr>
<tr>
<td>4</td>
<td>54 ±1.09</td>
<td>77 ±1.78</td>
<td>69 ±1.65</td>
</tr>
<tr>
<td>5</td>
<td>54 ±1.67</td>
<td>77 ±1.32</td>
<td>69 ±1.22</td>
</tr>
<tr>
<td>6</td>
<td>54 ±1.56</td>
<td>77 ±0.99</td>
<td>69 ±1.21</td>
</tr>
<tr>
<td>7</td>
<td>54 ±1.32</td>
<td>77 ±1.34</td>
<td>69 ±1.45</td>
</tr>
<tr>
<td>8</td>
<td>54 ±1.54</td>
<td>77 ±1.32</td>
<td>69 ±1.27</td>
</tr>
<tr>
<td>9</td>
<td>54 ±1.32</td>
<td>77 ±1.22</td>
<td>69 ±1.32</td>
</tr>
<tr>
<td>10</td>
<td>54 ±1.21</td>
<td>77 ±1.11</td>
<td>69 ±1.54</td>
</tr>
<tr>
<td>11</td>
<td>54 ±1.21</td>
<td>77 ±1.11</td>
<td>69 ±1.23</td>
</tr>
<tr>
<td>12</td>
<td>54 ±1.60</td>
<td>77 ±1.62</td>
<td>69 ±1.09</td>
</tr>
</tbody>
</table>

Skimmed milk and whey (500 mL) were treated with β galactosidase 1000 U and 400 U respectively, in batch process at 32 °C, under stirred conditions. The aliquots from each preparation in batch process (250 μL) were taken out at indicated time intervals for 12 h. The hydrolysis of lactose was estimated by glucose oxidase/peroxidase assay procedure as described in the Section 2.2.8. Each value represents the mean for three-independent experiments performed in duplicates, with average standard deviation, < 5%. 

59
However, 86% and 77% lactose was hydrolyzed from whey and milk after 3 h by entrapped crosslinked Con A-β galactosidase, respectively while the 69% and 54% lactose was hydrolyzed in whey and milk by entrapped soluble β galactosidase, respectively. There was no further increase in the hydrolysis of lactose up to 12 h (Table 6).

2.3.13. Lactose hydrolysis in packed bed column

The rate of lactose hydrolysis was seen at different flow rates by taking entrapped preparations of soluble β galactosidase and crosslinked Con A-β galactosidase. It was seen that the maximum hydrolysis occurred at the flow of 10 mL h⁻¹ while the hydrolytic rate decreased at 20 and 30 mL h⁻¹ (Table 7). It was due to the residence time of lactose inside the column containing entrapped preparations of β galactosidase. At 10 mL h⁻¹ greater percent of lactose was hydrolyzed inside the column whereas, the hydrolytic rate reduced considerably when the flow rate was increased.

It was seen that 96% of lactose was hydrolyzed by soluble entrapped β galactosidase and 91% of lactose was hydrolyzed by entrapped crosslinked Con A-β galactosidase. This was due to more accessibility of substrate in the entrapped preparation of soluble β galactosidase but it was seen that after 1 month the lactose hydrolysis decreased to 87% and after 2 months the hydrolysis of lactose reduced to only 30%. However entrapped crosslinked Con A-β galactosidase showed 86% lactose hydrolyzing activity after 2 months of continuous lactose hydrolysis. Similarly at 20 and 30 mL h⁻¹ entrapped soluble β galactosidase exhibited nearly 29% and 25% hydrolytic activity, respectively after 2 months whereas the entrapped crosslinked Con A-β galactosidase could hydrolyze 80% and 77% of lactose under similar experimental conditions.
Table 7: Lactose hydrolysis in continuous process in a packed bed column

<table>
<thead>
<tr>
<th>Number of days</th>
<th>10 mL h⁻¹</th>
<th>20 mL h⁻¹</th>
<th>30 mL h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Entrapped soluble β galactosidase</td>
<td>100</td>
<td>96.0 ±1.21</td>
</tr>
<tr>
<td></td>
<td>Entrapped crosslinked Con A-β galactosidase</td>
<td>100</td>
<td>96.0 ±1.43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>96.0 ±1.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>94.2 ±1.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>90.0 ±1.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>87.0 ±1.87</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>80.2 ±2.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>73.1 ±3.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>61.34 ±1.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>50.0 ±2.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>39.2 ±2.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>30.1 ±1.45</td>
</tr>
</tbody>
</table>

Lactose (0.1 M) dissolved in 0.1 M sodium acetate buffer, pH 4.6 containing 0.001 M sodium azide was passed through two columns: Alginate entrapped crosslinked Con A-β galactosidase (1525 U) column and entrapped soluble β galactosidase (1500 U) column, at different flow rates at 32 °C. Each value represents the mean for three-independent experiments performed in duplicates with average standard deviation, < 5%.
2.4. DISCUSSION

Sodium alginate has proved to be an efficient substance for the immobilization of enzymes, cell organelles, microorganisms, plant and animal cells (Smidsrod and Gudmund, 1990; Kierstan and Bucke, 2000). But this property has turned out to be a draw back as far as immobilization of enzymes is concerned. Due to the high porosity of alginate beads, the entrapped enzymes were leached out of the polymer matrix (Blandino et al., 2000; Musthapa et al., 2004). In order to circumvent this leaching problem of the enzymes from the polymeric matrix several efforts has already been made but the cost of the process always restricted their applications (Betancor et al., 2005). In order to prevent the leaching of the enzymes from the calcium alginate beads, an insoluble Con A-β galactosidase complex has been prepared by using a simple extract of jack bean meal. It was seen that a very insignificant quantity of jack bean extract was required to form the insoluble complex of β galactosidase (Figure 4).

Con A-β galactosidase complex retained very high enzyme activity. It has already been reported that glucose oxidase exhibited remarkably very high enzyme activity when complexed with Con A or polyclonal antibodies (Jan et al., 2006). These reports suggested that the insoluble Con A-enzyme complexes are quite porous and the active sites of the enzymes were easily accessible to the substrates. The enzyme was precipitated at a low Con A/enzyme ratio, thereby reducing the non-enzymatic content of the complex. This would be advantageous in minimizing the valuable reactor space occupied by non-enzymatic material.

This is well known fact that Con A complexes of glycoenzymes could be dissociated in the presence of glucose, mannose and N-acetyl glucosamine (Akhtar et al., 2005; Jan et al., 2006). In order to maintain the integrity of Con A-β galactosidase complex in the presence of its substrates and products, such complex was crosslinked by glutaraldehyde. Con A-β galactosidase complex showed a marginal loss of 6% enzyme activity upon crosslinking (Figure 5). Crosslinking of Con A-β galactosidase complex prior to entrapment in alginate, for its use in an enzyme reactor, was essential since the enzymes acted on carbohydrate substrate that disaggregates the complex. Crosslinking of Con A-β galactosidase complex with glutaraldehyde resulted in a small loss of enzyme activity.
activity (Table 2). Glutaraldehyde based chemistry is an effective method for enzyme immobilization and stabilization. Glutaraldehyde stabilizes the alginate gel, helping in the prevention of the leakage of enzymes (Tanriseven and Dogan, 2002). However, there was further decrease in enzyme activity as a result of entrapment, the fractions of entrapped activity that was expressed in case of soluble β galactosidase and Con A-β galactosidase complex were certainly higher as compared to crosslinked Con A-β galactosidase complex.

Due to entrapment of crosslinked Con A-β galactosidase $K_m$ value has been increased as compared to soluble β galactosidase (Table 3). However, $V_{max}$ values for the free and immobilized enzyme showed that there was no conformational change in the enzyme during immobilization. The change in the affinity of the enzyme for its substrate is caused by lower affinity of the substrate to the active site of the immobilized enzyme (Arica et al., 1998; Hernaiz and Crout, 2000).

Enhancement in stability appeared to be another attractive feature of the crosslinked Con A-β galactosidase and entrapped crosslinked Con A-β galactosidase. The marked stability exhibited by these immobilized preparations (Figures 6-11) is not unanticipated, in view of the earlier reports on the stabilization of glycoenzymes as a result of binding to Con A (Akhtar et al., 2005; Jan et al., 2006).

Immobilized β galactosidase preparations exhibited no change in pH and temperature-optima as compared to native enzyme (Figures 6 and 7). Similar results were obtained when β galactosidase from Aspergillus oryzae immobilized in fibers comprised of alginate and gelatin and hardened by glutaraldehyde (Tanriseven and Dogan, 2002). However, the entrapped crosslinked Con A-β galactosidase was far superior in thermal stability (Figure 8) as compared to other methods used for the immobilization of Aspergillus oryzae β galactosidase. Crosslinked Con A-β galactosidase and entrapped crosslinked Con A-β galactosidase preparations were markedly more stable against the denaturation induced by urea (Figure 9). These high stability immobilized preparations of β galactosidase could be successfully exploited for the hydrolysis of lactose even in the presence of such type of denaturants. Although the action mechanism of urea on the protein structures has not yet been completely understood, several earlier studies have proposed that protein is unfolded by the direct interaction of urea molecule with a peptide.
backbone via hydrogen bonding/hydrophobic interaction, which contributes to the maintenance of protein conformation (Makhatadze and Privalov, 1992; Khan et al., 2005).

Here we have noticed that *Aspergillus oryzae* β galactosidase activity was also significantly decreased after incubation with 5.0% (w/v) calcium chloride (Figure 10). However, the entrapped crosslinked Con A-β galactosidase was remarkably more stable against the denaturation mediated by calcium chloride exposure. Some investigators have demonstrated that the activity of peach β galactosidase inhibited by Ca$^{2+}$ ions and other bivalent cations (Lee et al., 2003). Demirhan et al. (2008) reported that β galactosidase from *Kluyveromyces marxianus* lactis in a batch reactor system also showed a decrease in activity on the addition of Ca$^{2+}$ ions.

In order to maintain the catalytic efficiency of reactor containing immobilized β galactosidase, the activity of the enzyme should not be affected by presence of its products. However, there are several reports which indicated that galactose was one of the products of β galactosidase catalyzed hydrolysis of lactose and it has competitively and non-competitively inhibited the activity of the enzyme (Shukla and Chaplin, 1993; Portaccio et al., 1998). Our findings indicated that the crosslinked Con A-β galactosidase and entrapped crosslinked Con A-β galactosidase preparations were significantly more resistant to the inhibition mediated by galactose (Figure 11). Here some preliminary investigations have been done to find out the $K_{i_{app}}$ values so as to explain galactose inhibition in terms of Henderson equation, which indicates competitive inhibition mechanism and this may be explained as follows:

$$I_o = \frac{V_i - V_o}{V_o} = K_i \left(1 + \frac{[S]_o}{K_m}\right) \frac{V_i}{V_o} + [E_o]$$

$$K_{i_{app}} = K_i \left(1 + \frac{[S]_o}{K_m}\right),$$

where $I_o$, $[E_o]$ and $[S]_o$ are the initial concentrations of inhibitor, enzyme and substrate respectively, $V_o$ is the velocity without inhibitor and $V_i$ is the velocity in the presence of inhibitor (Henderson, 1972; Pagano et al., 1984). As reported by Portaccio et al. (1998) that a lower value for $K_i$ denotes stronger inhibition as in case of β galactosidase/immunodyne system whereas β galactosidase/chitosan system has higher $K_i$ value. Hence the latter system seemed to be more appropriate to perform lactose
hydrolysis. Entrapped crosslinked Con A-β galactosidase complex has higher $K_{i_{app}}$ value as compared to soluble β galactosidase, therefore entrapped crosslinked Con A-β galactosidase retained more enzyme activity in the presence of high concentration of galactose (Table 4).

One of the limitations associated with the industrial application of enzymes is their high cost and instability under operational conditions. The process becomes cost effective if the preparation shows higher catalytic efficiency and reusability. Entrapped crosslinked Con A-β galactosidase and entrapped Con A-β galactosidase complex retained significantly very high activity on their repeated uses whereas the entrapped soluble enzyme lost its activity rapidly on similar reuses (Figure 12). Thus, the reusability of calcium alginate entrapped preparations of β galactosidase showed that entrapped crosslinked Con A-β galactosidase complex could be exploited for the continuous conversion of lactose from milk or whey for a longer duration in a reactor. In view of the earlier reports on leakage of peroxidase (Matto and Husain, 2006), polyphenol oxidases (Davis and Burns, 1990) and glucose oxidase (Blandino et al., 2000) from alginate gels, activity inhibition by galactose may be responsible for this behavior. However, some investigators have showed that chitosan-immobilized β galactosidase from Aspergillus oryzae could be reused for four cycles of lactose hydrolysis without significant loss in activity (Gaur et al., 2006).

The entrapped crosslinked Con A-β galactosidase retained almost 93% of the original activity after a period of 2 months storage at 4 °C whereas the soluble β galactosidase exhibited only 40% activity under identical storage conditions. Thus, entrapped crosslinked Con A-β galactosidase was highly stable at 4 °C (Table 5).

The hydrolysis of lactose from milk and whey was investigated for 12 h by entrapped preparations of β galactosidase at room temperature. Entrapped crosslinked Con A-β galactosidase was more efficient in hydrolyzing lactose in whey than in milk. Similarly, entrapped soluble β galactosidase showed a higher hydrolytic rate in whey than in milk (Table 6). It has been earlier reported that lactose hydrolysis by lactozym™ immobilized on cellulose beads took 48 h in continuous batch mode at 30 °C (Roy and Gupta, 2003). Higher percent of lactose hydrolysis was seen in whey as compared to
Chapter II

skimmed milk. As the pH of whey ranges from 4.5 to 5.0 whereas the pH of milk ranges from 6.5 to 6.8, β galactosidase showed 100% activity at pH 4.6 but its activity considerably decreased at higher pH. Due to this reason 1000 U of β galactosidase is required for the hydrolysis of lactose in skimmed milk whereas only 400 U are needed in case of whey.

Entrapment of crosslinked Con A-β galactosidase in calcium alginate beads thus appeared to be a useful method for continuous conversion of lactose into glucose and galactose (Table 7). Crosslinked Con A-β galactosidase complex is insoluble yet it is rather unsuitable for direct use in reactor due to its fineness and tendency to pack compactly.

In view of the high stability offered by the crosslinked Con A-β galactosidase and entrapped crosslinked Con A-β galactosidase against heat, pH, urea, calcium chloride and galactose, it suggested that entrapped crosslinked Con A-β galactosidase preparation could successfully be employed in a reactor for the continuous conversion of lactose. This immobilized enzyme preparation was found to be more superior in terms of immobilization yield and retention of enzyme activity both in continuous and batch process. The reusability and storage experiments further supported that crosslinked Con A-β galactosidase complex did not leach out of the gel beads, therefore such preparations could be exploited for the continuous conversion of lactose from milk or whey for a longer duration in a reactor.