Bioaffinity based immobilization of almond (*Amygdalus communis*) β-galactosidase on Con A-layered calcium alginate-cellulose beads: Its application in lactose hydrolysis in batch and continuous mode

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Abstract

In this study, immobilization of partially purified almond (*Amygdalus communis)* β-galactosidase on Con A layered calcium alginate-cellulose beads was investigated. Immobilized β-galactosidase retained 72% of the initial activity after crosslinking by glutaraldehyde. Both soluble and immobilized enzyme exhibited the same pH and temperature optima at pH 5.5 and 50ºC, respectively. However, the immobilized enzyme showed a remarkable broadening in pH and temperature-activity profiles as compared to the native enzyme. Immobilized enzyme was significantly more stable against thermal denaturation at 60ºC. Immobilized β-galactosidase exhibited 67% residual activity in the presence of 5% D-galactose while its soluble counterpart retained only 35% activity under identical conditions. Soluble enzyme showed 69% residual activity after exposure to pepsin (0.15 mg/ml) for 1 h whereas the immobilized β-galactosidase was more stable and retained nearly 84% activity under identical experimental conditions. The activity of immobilized enzyme was enhanced to 156% whereas soluble β-galactosidase showed an enhancement upto 134% when exposed to trypsin (0.1 mg/ml) for 1 h. Moreover, immobilized β-galactosidase exhibited greater enhancement in enzyme activity against exposure to various ions present in milk such as Na^+, K^+, Ca^{+2}, Mg^{+2} and citrate ions. The higher concentration of lactose was hydrolyzed from whey as compared to the hydrolysis from milk by immobilized enzyme at 50ºC and 60ºC in batch processes. Lactose was hydrolyzed to 86% and 78% after 20 days continuous operation of reactors at the flow rates of 20 ml/h and 30 ml/h, respectively. In view of its stability and utility in batch and continuous processes, such preparation could be exploited for the hydrolysis of lactose from milk and whey in a more convenient and cheaper way in dairy industries.

Keywords: β-galactosidase; almond; lactose hydrolysis; cellulose-alginate beads; batch process

INTRODUCTION

β-Galactosidase (3.2.1.23) is a hydrolytic enzyme that catalyzes breakdown of lactose into glucose and galactose. It is widely present in microorganisms, plants and animals (Haider and Husain, 2009 a). The enzymatic hydrolysis of lactose is a popular technology which is used to produce lactose-reduced milk and its derived dairy products for consumption by lactose-intolerant persons whose metabolism exhibited a decline in the level of β-galactosidase (Haider and Husain, 2008 b; Heyman, 2006). In addition, it catalyzes the formation of galacto-oligosaccharides, which are prebiotic additives for the so-called "healthy foods" (Grosova *et al.*, 2008).

Immobilized enzymes are more favorable than free enzymes since they offer the possibility of continuous flow processing so that easy regeneration of the enzyme and low cost operation can be achieved (Zhou and Chen, 2001). Moreover, the soluble enzymes are normally used for batch processes while the immobilized enzyme lends itself to continuous operation (Haider and Husain, 2009 b; Bodalo *et al.*, 2001). Thus, immobilization of enzymes has been a widely employed technique in industrial applications due to enhanced stability, imparted reusability, inhibition by reaction products, selectivity towards non-natural substrates thereby making immobilization process cost

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effective and viable (Iyer and Ananthanarayan, 2008; Mateo *et al.*, 2007).

Several techniques that have been used earlier for enzyme immobilization included entrapment (Betancor *et al.*, 2008; Haider and Husain, 2007 b), crosslinking (Wang *et al.*, 2008), adsorption (Salah *et al.*, 2008) or a combination of these methods (Husain, 2010). The suitability of support and method of immobilization varies from enzyme to enzyme and their intended use and needs to be optimized for each system (Plessas *et al.*, 2005; Burton *et al.*, 2002). Several immobilized enzyme derivatives have been reported for β-galactosidase from *Kluveromyces fragilis* (Roy and Gupta, 2003), *Kluveromyces lactis* (Zhou and Chen, 2003), *Thermus* sp. (Pessela *et al.*, 2007; Ladero *et al.*, 2006), *Escherichia coli* (Bayramoglu *et al.*, 2007) and *Aspergillus oryzae* (Ansari and Husain, 2010; Haider and Husain, 2008 a; Gaur *et al.*, 2006). Reactors such as fluidized fixed bed, hollow fibre, plug flow, capillary bed and rolled membrane - all containing immobilized β-galactosidase have been developed for lactose hydrolysis (Jurado *et al.*, 2005). The choice of lactose hydrolysis in batch and continuous mode depends primarily on the enzymatic characteristics and the economy encompassing the production, storage and reusability (Haider and Husain, 2007 b). Moreover, the use of packed bed reactors in biological processes would allow the application of new methodologies to transform environmental problems such as to permeate whey elimination of dairy industries for commercial purposes (Mammarella and Rubiolo, 2006).

The present study deals with the immobilization of almond β-galactosidase on the surface of a bioaffinity support, concanavalin A (Con A) layered calcium alginate-cellulose beads. The activity of soluble and immobilized β-galactosidase was compared against various physical and chemical parameters such as pH, temperature, proteolytic enzymes (pepsin and trypsin) and numerous ions present in milk and whey such as Na^+, K^+, Ca^{+2}, Mg^{+2} and citrate ions. The effect of product inhibition on the activity of soluble and immobilized β-galactosidase has also been investigated. The hydrolysis of lactose from milk and whey was also examined in a stirred batch process at different temperatures and in continuous reactors at different flow rates.

MATERIALS AND METHODS

Materials: Galactose and glucose were purchased from Sigma Chem. Co. (St. Louis, MO, USA). Cellulose, peroxidase, ethanolamine and *o*-nitrophenyl β-D-galactopyranoside (ONPG) were obtained from SRL Chemicals (Mumbai, India). Sodium alginate was the product of Koch-Light Lab. (Colnbrook, UK). Glutaraldehyde was the product of Thomas Baker Chemical Co. (Mumbai, India). Jack bean meal was procured from Loba Chemical Co. (Mumbai, India). Almonds were purchased from local market. All other chemicals and reagents were employed of analytical grade and were used without further purification.

Preparation of partially purified almond β-galactosidase: Twenty five grams of almond were homogenized in 250 ml of 0.1 M sodium phosphate buffer, pH 5.5. The homogenate was kept at 4ºC and passed through four layers of cheese cloth. The filtrate was then centrifuged at 6000 rpm on a Remi R-24 cooling centrifuge for 10 min at 4ºC. The clear supernatant was subjected to salt fractionation by adding $(NH_4)_2SO_4$ (0-80%, w/v). The solution was stirred overnight at 4ºC and the precipitate obtained was collected by centrifugation at 6000 rpm for 10 min at 4ºC. The precipitate collected was re-dissolved in 0.1 M sodium phosphate buffer, pH 5.5 and dialyzed against the assay buffer (Haider and Husain, 2007 a).

Assay of β-galactosidase: The hydrolytic activity of β-galactosidase was determined by the release of *o*-nitrophenol from ONPG at 405 nm. The reaction was carried out with continuous agitation in an assay volume of 2.0 ml containing 1.7 ml of 0.1 M sodium acetate buffer, pH 5.5, 2.0 U β galactosidase and 0.2 ml of 20 mM ONPG. The reaction was stopped by adding 2.0 ml of 2.0 N sodium carbonate solution and the product (*o*-nitrophenol) formation was measured spectrophotometrically at 405 nm.

One unit (1.0 U) of β-galactosidase activity was defined as the amount of enzyme that liberates 1.0 μmole of *o*-nitrophenol (ε_m = 4500 l/mol/cm) per min under standard assay conditions (Haider and Husain, 2008 b).

Estimation of protein: Protein concentration was determined according to the procedure described by Lowry *et al.* (1951). Bovine serum albumin was used as standard.

Preparation of calcium alginate-cellulose beads: An aqueous mixture of sodium alginate (2.5%, w/v) and cellulose (2.5%, w/v) was prepared and the resulting mixture was slowly extruded as droplets through a 5.0

ml syringe with attached needle no. 20 into 0.2 M calcium chloride solution. The formation of calcium alginate-cellulose beads was instantaneous and the solution was gently stirred for 2 h. The beads were then washed and stored in 0.1 M sodium acetate buffer, pH 5.5 at 4ºC until further use (Haider and Husain, 2008 a).

Binding of Con A on calcium alginate-cellulose beads: 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 6.2 (Haider and Husain, 2007 b). Calcium alginate-cellulose beads (500 beads) were incubated overnight with jack bean extract (25 ml) containing Con A at 32ºC with slow stirring. Con A bound calcium alginate-cellulose beads were washed 3-4 times with 0.1 M sodium acetate buffer, pH 5.5.

Adsorption of β-galactosidase on Con A layered calcium alginate-cellulose beads: Con A layered calcium alginate-cellulose beads were incubated with β-galactosidase (1850 U) overnight at 32ºC with slow stirring. The unbound enzyme was removed by repeated washing with 0.1 M sodium acetate buffer, pH 5.5.

Crosslinking of immobilized β-galactosidase by glutaraldehyde: β-galactosidase immobilized on the surface of Con A layered calcium alginate-cellulose beads was suspended in 0.1 M sodium acetate buffer, pH 5.5 for 2 h at 4ºC. This preparation was crosslinked by 0.5% (v/v) glutaraldehyde for 2 h at 4ºC. Finally the beads were incubated with 0.01% (v/v) ethanolamine for 90 min at 30ºC to stop crosslinking. The integrity of crosslinked immobilized enzyme was examined by incubating beads in 1.0 M α-methyl-β-D-glucopyranoside for 2 h (Haider and Husain, 2008 a).

Effect of pH on soluble and immobilized β-galactosidase: The activity of soluble and immobilized β-galactosidase (2.0 U) was measured in the buffers of various pH (2.0-9.0). The buffers used were glycine-HCl (pH 2.0-3.0), sodium acetate (pH 3.5-6.0), sodium phosphate (6.5-7.0) and Tris-HCl (pH 7.5-9.0). The molarity of each buffer was 0.1 M. The activity at pH 5.5 was taken as control (100%) for the calculation of remaining percent activity.

Effect of temperature on soluble and immobilized β-galactosidase: The activity of soluble and immobilized β-galactosidase (2.0 U) was assayed in 0.1 M sodium acetate buffer, pH 5.5 at various temperatures (20-80ºC) for 15 min. The activity obtained at 50ºC was considered as control (100%) for the calculation of remaining percent activity at different temperatures.

Soluble and immobilized β-galactosidase preparations were incubated at 60ºC in 0.1 M sodium acetate buffer, pH 5.5 for varying times. Aliquots of each preparation (2.0 U) were taken out at indicated time intervals and chilled quickly in crushed ice for 5 min. The enzyme was brought at room temperature and its activity without incubation at 60ºC was taken as control (100%) for the calculation of remaining percent activity.

Effect of D-galactose: Activity of soluble and immobilized β-galactosidase (2.0 U) was measured in the presence of increasing concentrations of galactose (1.0-5.0%, w/v) in 0.1 M sodium acetate buffer, pH 5.5 for 1 h at 37ºC. The activity of enzyme without added galactose was considered as control (100%) for the calculation of remaining percent activity.

Effect of KCl and trisodium citrate: Soluble and immobilized β-galactosidase (2.0 U) was independently incubated with increasing concentrations of KCl and trisodium citrate (0.5-5.0%, w/v) in 0.1 M sodium acetate buffer, pH 5.5 for 1 h at 37ºC. The activity of enzyme without incubation with ions was considered as control (100%) for the calculation of remaining percent activity.

Combined effect of $CaCl_2$ and $MgCl_2$: Soluble and immobilized β-galactosidase (2.0 U) was incubated at 37ºC with $CaCl_2$ (123 mg/100 ml) and $MgCl_2$ (12 mg/100 ml) for different times. The activity of enzyme without incubation with ions was considered as control (100%) for the calculation of remaining percent activity.

Combined effect of NaCl and KCl: Soluble and immobilized β-galactosidase (2.0 U) was incubated at 37ºC with KCl (141 mg/100 ml) and NaCl (58 mg/100 ml) for different times. The activity of enzyme without incubation with ions was considered as control (100%) for the calculation of remaining percent activity.

Combined effect of $CaCl_2$, $MgCl_2$, KCl, NaCl and trisodium citrate ions: Soluble and immobilized β-galactosidase (2.0 U) was incubated with KCl (141 mg/100 ml), $CaCl_2$ (123 mg/100 ml), $MgCl_2$ (12 mg/100 ml), NaCl (58 mg/100 ml) and trisodium citrate (160 mg/100 ml) at 37ºC for different times. The

activity of enzyme without incubation with ions was considered as control (100%) for the calculation of remaining percent activity.

Effect of trypsin/pepsin on soluble and immobilized β-galactosidase: Soluble and immobilized β-galactosidase (2.0 U) was independently incubated with increasing concentrations of trypsin/pepsin (0.025-0.150 mg/ml) at 37ºC for 1 h. After incubation period, β-galactosidase activity was determined according to the procedure described in an earlier section.

Hydrolysis of milk lactose by β-galactosidase in batch processes: The milk was skimmed by centrifuging cold milk at 4500 rpm for 20 min. The fatty layer was removed from milk and stored at 4ºC for further use. Skimmed milk (500 ml) was treated by soluble and immobilized β-galactosidase (125 U) independently in batch processes at 50ºC and 60ºC for 10 h. The aliquots of 250 µl were taken out at indicated time intervals and the hydrolysis of lactose was estimated by the assay procedure described by Haider and Husain (2009 a).

Hydrolysis of whey lactose by β-galactosidase in batch processes: Skimmed milk was acidified by HCl until the pH reached to 4.8. The casein was removed by centrifugation. The obtained whey was stored at 4ºC for further use. Whey (500 ml) was treated by soluble and immobilized β-galactosidase (125 U) independently in batch processes at 50ºC and 60ºC for 10 h. The aliquots of 250 µl were taken out at indicated time intervals and the hydrolysis of lactose was estimated by assay procedure described by Haider and Husain (2009 a).

Continuous hydrolysis of lactose through the packed-bed reactors containing immobilized enzyme: Immobilized β-galactosidase (2000 U) was packed in two columns of similar dimensions (2.0 ×10.0 cm). The packed volume of each column was 8.0 ml. Lactose (0.1 M) dissolved in 0.1 M sodium acetate buffer, pH 5.5 containing 0.001 M sodium azide was passed through both the columns at different flow rates of 20 ml/h and 30 ml/h at room temperature (30-32ºC).

Glucose estimation by glucose oxidase-peroxidase coupled assay procedure: Lactose hydrolysis was monitored for the formation of glucose by using glu-cose oxidase-peroxidase coupled assay procedure. An appropriate amount of lactose hydrolyzed by β-galactosidase, suitably diluted with 0.5 M phosphate buffer, pH 7.0 was taken. The hydrolysis of lactose was estimated by using solution C. Solution C was prepared by taking 5.0 mg glucose oxidase, 15.0 mg *o*-dianisidine HCl prepared in 2.5 ml distilled water, 40.0 ml glycerol (20%, v/v) and 1.0 mg of peroxidase dissolved in 5.0 ml 0.1 M potassium phosphate buffer, pH 7.0. To each tube, 1.5 ml of solution C was added. The test tubes were again incubated at 37ºC for 15 min and the reaction was stopped by adding 1.0 ml of 6.0 N HCl and the developed color was measured at 540 nm (Haider and Husain, 2009 a).

Acid mediated hydrolysis of lactose: Lactose (5.0 ml, 2.0 M) was mixed with 1.0 ml of 20% H_2SO_4 and was heated for 10 min in a boiling water bath. This acid treated lactose solution was further neutralized with 10% NaOH at room temperature. Hydrolyzed lactose was taken in increasing concentrations for different time intervals till 1.0 ml and the products were estimated by using glucose oxidase-peroxidase coupled assay procedure (Haider and Husain, 2007 a).

Statistical analysis: Each value represents the mean for three independent experiments performed in duplicates, with average standard deviation <5%. The data expressed in various studies was plotted using Sigma Plot-9 and expressed with standard deviation of error (±). Data was analyzed by one-way ANOVA. P-values <0.05 were considered statistically significant.

RESULTS

Immobilization of β-galactosidase on bioaffinity support: Table 1 demonstrates the immobilization of β-galactosidase on Con A layered calcium alginate-cellulose beads. The bioaffinity bound â galactosidase retained nearly 79% of the original activity. However, crosslinking with glutaraldehyde resulted in a marginal loss of 7% enzyme activity.

Effect of pH: Figure 1 demonstrates pH-activity profiles of soluble and immobilized β-galactosidase. The pH-optimum for both soluble and immobilized enzyme was same at 5.5. However, immobilized β-galactosidase exhibited a remarkable broadening in pH-activity profiles as compared to the native enzyme.
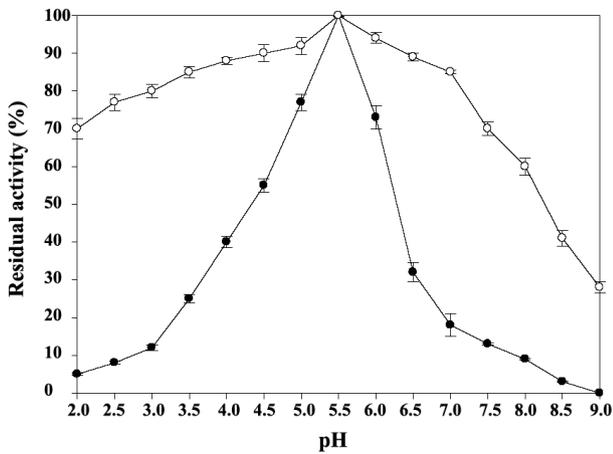
Figure 1. pH activity profiles for soluble and immobilized β-galactosidase. The activity of soluble and immobilized β-galactosidase (2.0 U) was measured in the buffers of various pH. The buffers used were glycine-HCl (pH 2.0-3.0), sodium acetate (pH 3.5-6.0), sodium phosphate (6.5-7.0) and Tris-HCl (pH 7.5-9.0). The molarity of each buffer was 0.1 M. The activity at pH 5.5 was taken as control (100%) for the calculation of remaining percent activity. The symbols show soluble (●) and immobilized (o) β-galactosidase.
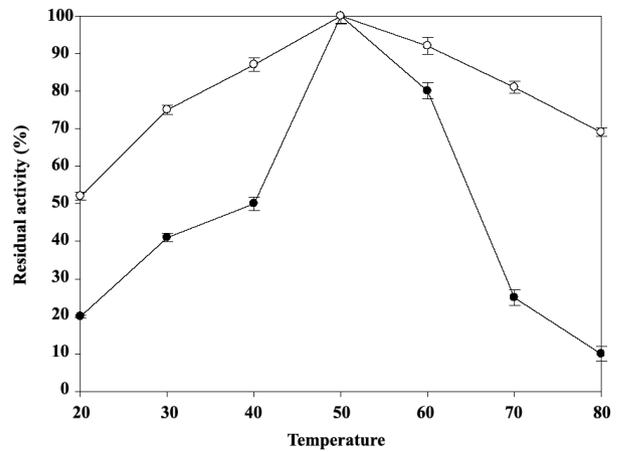
Figure 2. Temperature activity profiles for soluble and immobilized β-galactosidase The activity of soluble and immobilized β-galactosidase (2.0 U) was assayed in 0.1 Msodium acetate buffer, pH 5.5 at various temperatures (20-80ºC) for 15 min. The activity obtained at 50ºC was considered as control (100%) for the calculation of remaining percent activity. For symbols, refer to Figure 1 legend.

The soluble enzyme retained 40% activity at pH 4.0 while the immobilized β-galactosidase exhibited 88% enzymatic activity at the same pH.

Effect of temperature: The temperature-activity profiles of soluble and immobilized β-galactosidase have been shown in Figure 2. The temperature-optimum for both soluble and immobilized enzyme was same, i.e. 50ºC. Immobilized β-galactosidase exhibited greater fractions of enzyme activity at lower and higher temperatures as compared to its soluble counterpart. The soluble enzyme showed 41% activity at 30ºC whereas the immobilized β-galactosidase retained 75% activity at the same temperature.

Soluble β-galactosidase exhibited 32% enzyme

activity after 2 h exposure at 60ºC while the immobilized enzyme retained 65% of the original activity under similar incubation conditions (Fig. 3). Moreover, the immobilized β-galactosidase retained 28% residual activity even after 5 h incubation at 60ºC while the soluble enzyme lost its entire activity.

Effect of D-galactose: Fig. 4 shows the inhibitory effect of galactose on the activity of soluble and immobilized β-galactosidase. The soluble enzyme retained 35% activity in the presence of 5.0% galactose while the immobilized β-galactosidase exhibited much higher enzyme activity, 67% under identical experimental conditions.

**Effect of KCl and trisodium citrate:** Potassium and

Table 1. β-Galactosidase immobilized on Con A layered calcium alginate-cellulose beads.

| Enzyme preparation	Enzyme activity loaded X (U)	Enzyme activity in washes Y (U)	Activity bound/g cellulose-alginate beads		Activity yield (%) B/A x 100
			Theoretical (X-Y)=A	Actual=B	
Enzyme adsorbed on the surface of beads	1850	204	1646	1300	79
Enzyme adsorbed on the surface of beads and crosslinked	1300	0	1300	1185	72

Each value represents the mean for three independent experiments performed in duplicates, with average standard deviations, < 5%.
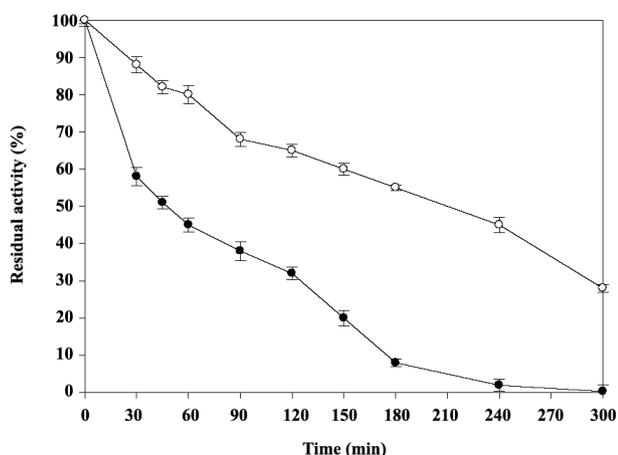
Figure 3. Thermal denaturation for soluble and immobilized β-galactosidase. Soluble and immobilized β-galactosidase was incubated at 60ºC in 0.1 M sodium acetate buffer, pH 5.5 for varying times. Aliquots of each preparation (2.0 U) were taken out at indicated time intervals and chilled quickly in crushed ice for 5 min. The enzyme was brought at room temperature. The activity of enzyme without incubation at 60ºC was taken as control (100%) for the calculation of remaining percent activity. For symbols, refer to Figure 1 legend.
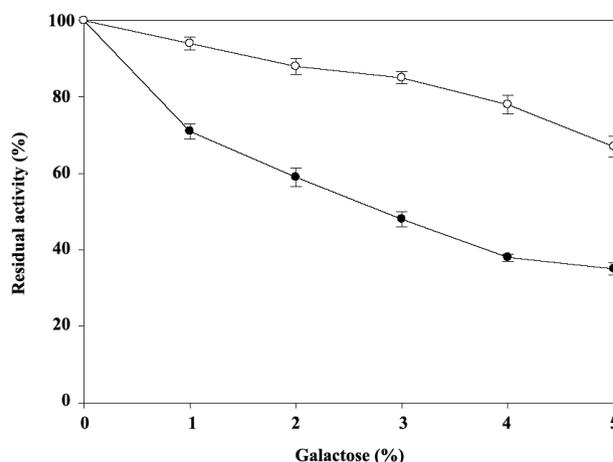
Figure 4. Effect of D-galactose on soluble and immobilized β-galactosidase. Effect of galactose on soluble and immobilized β-galactosidase (2.0 U) was measured in the presence of increasing concentrations of galactose (1-5%, w/v) in 0.1 M sodium acetate buffer, pH 5.5 for 1 h at 37ºC. The activity of enzyme obtained without added galactose was taken as control (100%) for the calculation of remaining percent activity. For symbols, refer to Figure 1 legend.
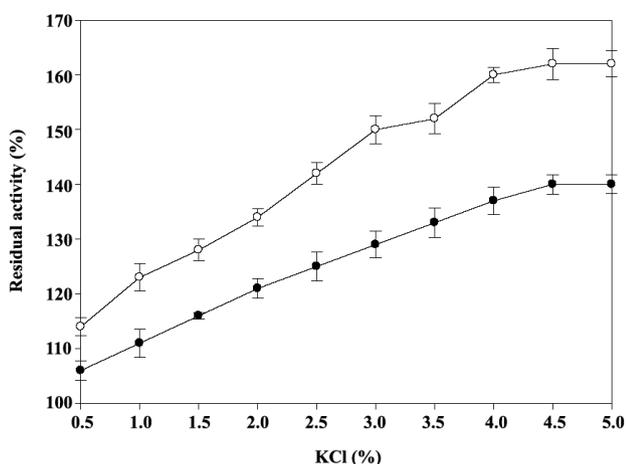
Figure 5. Effect of KCl on soluble and immobilized b-galactosidase. Soluble and immobilized b-galactosidase (2.0 U) was incubated with increasing concentration of KCl (0.5-5.0%) in 0.1 M sodium acetate buffer, pH 5.5 for 1 h at 37ºC. The activity of enzyme without incubation with KCl was considered as control (100%) for the calculation of remaining percent activity. For symbols, refer to Figure 1 legend.
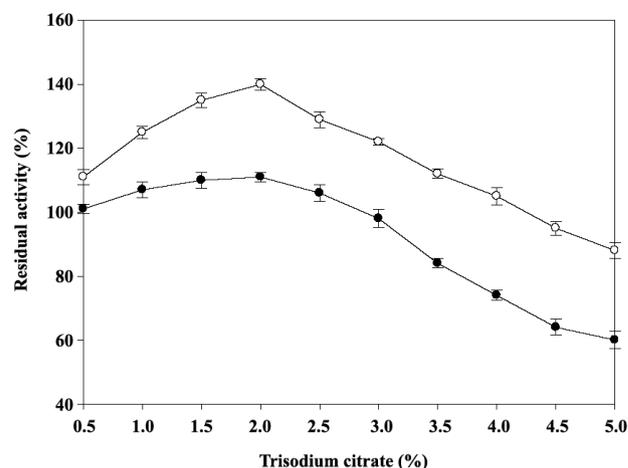

Figure 6. Effect of trisodium citrate on soluble and immobilized β-galactosidase. Soluble and immobilized β-galactosidase (2.0 U) was incubated with increasing concentration of trisodium citrate (0.5-5.0%) in 0.1 M sodium acetate buffer, pH 5.5 for 1 h at 37ºC. The activity of enzyme without added trisodium citrate was considered as control (100%) for the calculation of remaining percent activity. For symbols, refer to Figure 1 legend.

citrate ions are present in milk. Therefore, it is important to evaluate the stability of soluble and immobilized β-galactosidase in the presence of different concentrations of K+ (KCl) and citrate (trisodium citrate) ions. The incubation of soluble and immobilized β-galactosidase with increasing concentrations of KCl for 1 h at 37ºC showed an enhancement in the activity of enzyme. The activity of soluble enzyme was increased to 140% in the presence of 4.0% KCl while the immobilized β-galactosidase showed an enhancement in activity upto 160% (Fig. 5).

Soluble and immobilized β-galactosidase was exposed to different concentrations of citrate (trisodium citrate) for 1 h at 37ºC (Fig. 6). The soluble enzyme exhibited maximum activity 111% at 2.0% trisodium citrate exposure while the immobilized β-
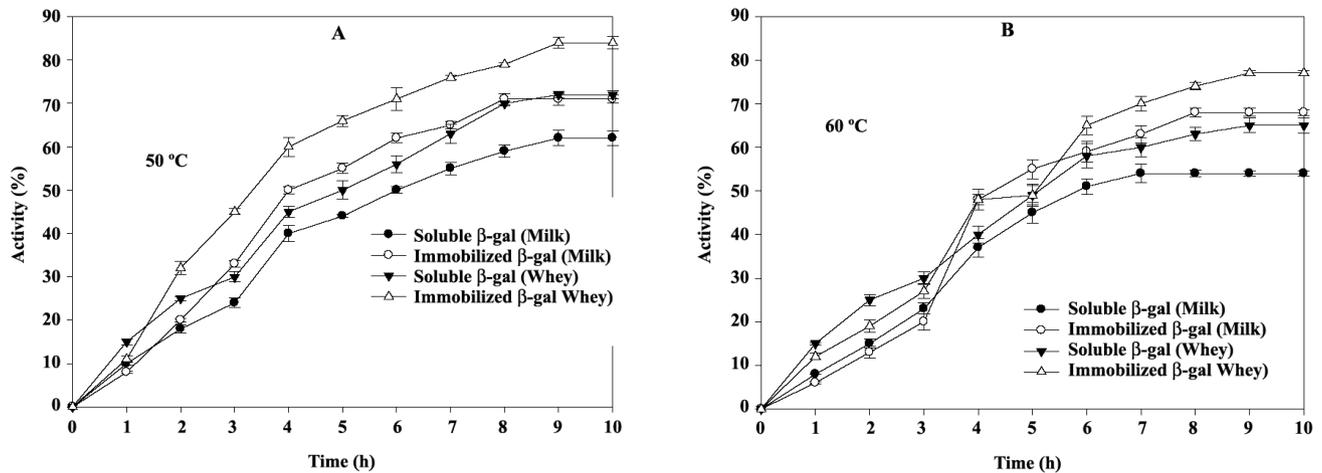
Figure 7. Hydrolysis of lactose in milk/whey by soluble and immobilized β-galactosidase in batch processes. Milk and whey (500 ml, each) was treated by soluble and immobilized β-galactosidase (125 U) independently in stirred batch processes at 50ºC and 60ºC for 10 h. The aliquots of 250 μl were taken out at indicated time intervals. The hydrolysis of lactose was estimated by using assay procedure as described in text. The symbols show hydrolysis of milk by soluble (•) and immobilized (o) β-galactosidase and hydrolysis of whey by soluble (▼) and immobilized (Δ) β-galactosidase.

galactosidase showed much enhanced activity upto 140% under similar experimental conditions.

Combined effect of CaCl₂ and MgCl₂: The combined effect of Ca^{+2} and Mg^{+2} ions on the activity of soluble and immobilized β-galactosidase is illustrated in Table 2. The activity of immobilized enzyme was enhanced to 168% after 1 h incubation with combined ions while the enhancement was 133% in case of soluble β-galactosidase. An enhancement in immobilized enzyme activity was 217% upon combined exposure with these ions for 6 h whereas the activation in case of soluble β-galactosidase

was only 161% under similar incubation conditions.

Combined effect of NaCl and KCl: Table 3 demonstrates the combined effect of Na^+ and K^+ ions on the activity of soluble and immobilized β-galactosidase. The activity of soluble β-galactosidase was increased to 120% at 1.5 h incubation at 37ºC while the immobilized enzyme showed significantly higher enhancement of 156% activity under identical conditions.

Combined effect of CaCl₂, MgCl₂, KCl, NaCl and trisodium citrate: Ca^{+2}, Mg^{+2}, K^+, Na^+ and citrate

Table 2. Combined effect of CaCl₂ and MgCl₂ on soluble and immobilized β-galactosidase.

Time (min)	Relative activity (%)	
	SβG	IβG
15	103±1.80	124±1.47
30	116±0.68	131±0.83
45	130±0.47	144±2.49
60	133±1.36	168±1.61
90	142±1.49	177±1.94
120	150±0.69	200±0.73
180	156±1.57	205±0.19
240	161±1.52	217±2.02
300	161±0.94	217±1.63
360	161±2.46	217±1.59

Soluble and immobilized b-galactosidase (2.0 U) was incubated for different time intervals at 37ºC with calcium chloride (123 mg/100 ml) and magnesium chloride (12 mg/100 ml) in 0.1 M sodium acetate buffer, pH 5.5. The activity of the enzyme without these ions was considered as control (100%) for the calculation of remaining percent activity.

Table 3. Combined effect of KCl and NaCl on soluble and immobilized β-galactosidase.

Time (min)	Relative activity (%)	
	SβG	IβG
15	97±1.86	109±1.56
30	102±2.17	124±1.83
45	107±0.94	137±1.38
60	114±1.63	148±0.52
90	120±1.55	156±0.79
120	123±2.09	167±2.19
180	124±2.63	175±1.60
240	128±1.48	175±1.99
300	128±0.72	175±0.62
360	128±0.39	175±1.52

Soluble and immobilized β-galactosidase (2.0 U) was incubated for different times at 37ºC with potassium chloride (141 mg/100 ml) and sodium chloride (58 mg/100 ml) in 0.1 M sodium acetate buffer, pH 5.5. The activity of the enzyme without these ions was considered as control (100%) for the calculation of remaining percent activity.

ions are important ions of milk. Thus, in the present study, we investigated the synergistic effect of these five ions on the activity of soluble and immobilized β-galactosidase. Both the enzyme preparations were incubated together with all these ions for different times with the same concentration as they are present in milk. The activity of both soluble and immobilized β-galactosidase was enhanced till 5 h (Table 4). Soluble β-galactosidase showed an increase in enzyme activity upto 130% upon 3 h incubation while the activity of immobilized enzyme was enhanced to 175% under identical exposure.

Effect of Pepsin and Trypsin: The effect of pepsin and trypsin on the activity of soluble and immobilized enzyme is shown in Table 5. The soluble enzyme retained 69% residual activity after treating with 0.15 mg/ml pepsin whereas the immobilized enzyme retained 84% activity under identical pepsin treatment. Moreover, the maximum activity of 156% was achieved by the immobilized β-galactosidase in the presence of trypsin at a concentration of 0.10 mg/ml while the activity retained by the soluble enzyme under similar conditions was 134%.

Lactose hydrolysis from milk and whey in stirred batch processes at 50ºC and 60ºC: Figure 7 illustrates the hydrolysis of lactose from milk and whey at regular time intervals for 10 h at 50ºC and 60ºC. It was observed that hydrolysis of lactose in milk by soluble enzyme was 59% after 8 h at 50ºC whereas immobilized β-galactosidase exhibited 71% hydrolysis under similar experimental conditions. Moreover, the soluble β-galactosidase hydrolyzed 72% lactose from whey after 9 h while immobilized enzyme showed greater amount of lactose hydrolysis 84% under identical conditions.

Table 4. Combined effect of $CaCl_2$, $MgCl_2$, KCl, NaCl and trisodium citrate ions on soluble and immobilized β-galactosidase.

Time (min)	Relative activity (%)	
	SβG	IβG
15	62±1.01	90±0.93
30	100±1.30	100±1.51
45	103±1.19	109±1.83
60	111± 0.72	123±0.82
90	120±2.18	143±1.97
120	123±1.72	152±2.15
180	130±2.82	175±1.83
240	140±1.70	183±0.92
300	143±1.92	187±2.11
360	143±0.83	187±1.71

Soluble and immobilized β-galactosidase (2.0 U) was incubated for different times at 37ºC with calcium chloride (123 mg/100 ml), magnesium chloride (12 mg/100 ml), potassium chloride (141 mg/100 ml), sodium chloride (58 mg/100 ml) and trisodium citrate (160 mg/100 ml) in 0.1 M sodium acetate buffer, pH 5.5. The activity of the enzyme without these ions was considered as control (100%) for the calculation of remaining percent activity after exposure to these components.

Similarly, the hydrolysis of milk lactose at 60ºC after 4 h was 37% and 48% by free and immobilized enzyme, respectively. However, lactose hydrolysis achieved in whey after 7 h was 63% and 70% by soluble and immobilized β-galactosidase, respectively (Fig. 7).

Continuous hydrolysis of lactose through the packed-bed reactors filled with immobilized β-galactosidase: The rate of lactose hydrolysis was monitored at different flow rates in reactors containing immobilized enzyme. The greater extent of lactose was hydrolyzed inside the column containing immobilized β-galactosidase when the flow rate was 20 ml/h

Table 5. Effect of pepsin/trypsin on soluble and immobilized β-galactosidase.

Concentration (mg/ml)	Relative activity (%)			
	Pepsin		Trypsin	
	SβG	IβG	SβG	IβG
0.025	88±1.24	96±1.79	111±0.92	126±1.01
0.050	84±0.83	93±1.01	123±2.82	142±3.11
0.075	81±1.82	90±0.29	129±2.19	151±1.84
0.100	77±1.92	88±2.08	134±1.84	156±0.99
0.125	73±2.06	85±1.89	127±2.65	150±2.98
0.150	69±2.47	84±1.70	122±1.61	145±3.06

Soluble and immobilized β-galactosidase (2.0 U) was independently incubated with increasing concentrations of pepsin/trypsin (0.025-0.150 mg/ml) in 0.1 M sodium acetate buffer, pH 5.5 for 1 h at 37ºC. The activity of the enzyme without pepsin/trypsin treatment was considered as control (100%) for the calculation of remaining percent activity.

Table 6. Continuous lactose hydrolysis through the packed-bed reactors containing immobilized β-galactosidase.

Number of days	Lactose hydrolysis at different flow rates	
	20 ml/h	30 ml/h
Control	100	100
5	92±2.16	87±2.82
10	90±2.98	84±3.06
15	86±1.82	84±0.93
20	86±2.60	78±2.94
25	79±3.72	74±3.10
30	74±3.19	70±2.84

Hydrolysis of lactose in a continuous reactor containing immobilized β-galactosidase (2000 U) was performed as described in text. Each value represents the mean for three independent experiments performed in duplicates, with average standard deviations, < 5%.

while the hydrolytic rates slightly decreased as the flow rate was increased to 30 ml/h. It was seen that 90% lactose was hydrolyzed by the immobilized preparation after 10 d of the operation of reactor when the flow rate was 20 ml/h. However, immobilized β galactosidase exhibited 78% and 70% conversion of lactose after 20 d and 30 d of continuous operation of the reactor at a flow rate of 30 ml/h (Table 6).

DISCUSSION

In the recent past, cellulose and alginate supports have been exploited independently by several researchers for the purpose of enzyme immobilization. Cellulose is used for enzyme immobilization due to its unique hydrophilic characteristics, relatively high chemical stability and remarkable environmental protecting capability (Zhang *et al.*, 2009; Lee *et al.*, 2008). Several studies also reported the use of sodium alginate for immobilizing and microencapsulating enzymes, cell organelles, plants and animal cells (Haider and Husain, 2007 b; Prashanth and Mulimani, 2005). The use of calcium alginate beads have been used by Smidsrod and Skjak-Braek (1990) for entrapment of enzymes but due to their large pore size, leakage of enzyme occurs through them. Thus, the present study aimed to work out a novel and inexpensive approach of preparing hybrid gel from calcium alginate-cellulose beads. These gel beads were layered with Con A obtained directly from jack bean extract. Partially purified almond β-galactosidase was then immobilized on this Con A layered support. In order to prevent the leaching of enzyme from the surface of

beads, this immobilized enzyme preparation was crosslinked by glutaraldehyde (Table 1). Crosslinking resulted in a loss of 7% enzyme activity. It has earlier been reported that crosslinking leads to genuine enhancement of conformational stability of an enzyme (Haider and Husain, 2008 b; Betancor *et al.*, 2006; Alonso *et al.*, 2005).

The catalytic activity of the enzyme depends on the presence of a given conformational structure in the folded polypeptide chain, even minor alterations in the tertiary structure resulted in a loss of biocatalytic activity (Neri *et al.*, 2008). Immobilized β-galactosidase showed significant stability at lower and higher pH values as compared to its soluble counterpart. It could be due to greater alteration produced in the tertiary structure of free enzyme in highly acidic and basic solutions as compared to the immobilized enzyme. Moreover, the loss in activity of soluble enzyme at higher temperatures can be attributed to the denaturation of enzyme molecules resulting in the rupturing of native conformation of proteins. Thus, the immobilized enzyme showed significant improvement in thermal and chemical stability (Figs. 1-3).

Several workers have investigated that galactose is one of the end products of β-galactosidase catalyzed hydrolysis of lactose and it competitively inhibited the enzyme (Park and Oh, 2010; Zhou *et al.*, 2003). Our results (Fig. 4) were in accordance with the previously published observations indicating that immobilized β-galactosidase was significantly more resistant to inhibition mediated by galactose as compared to the free enzyme (Haider and Husain, 2009 b).

The activity of immobilized β-galactosidase was better protected in the presence of KCl and trisodium citrate (Figs. 5, 6). Weixia *et al.* (1999) have reported a decrease in activity of *Cicer arietinum* β-galactosidase in the presence of citric acid and enhancement in the activity of enzyme in the presence of KCl. Their studies suggested 98.8% enzyme activity in the presence of 40 mM KCl in case of free enzyme whereas enzyme immobilized on carrier 1 and carrier 2 exhibited 121% and 147% of enzyme activity, respectively. Inhibitory effect of citric acid (0.1 M) on the activity of *Cicer arietinum* β-galactosidase showed 82% and 89% residual activity for the enzyme immobilized on carrier 1 and carrier 2, respectively.

The results for the continuous hydrolysis of lactose in batch process exhibited a higher rate of hydrolysis in case of soluble β-galactosidase for the first few hours as compared to the immobilized enzyme (Fig. 7). This might be due to the easy accessibility of solu-

ble enzyme for first few hours but after prolonged incubation, the rate of lactose hydrolysis decreased. Mammarella and Rubiolo (2006) suggested product inhibition as a probable reason for the decrease in lactose hydrolysis by the soluble enzyme. Our observations showed that immobilized β-galactosidase was more suitable for lactose hydrolysis on prolonged incubation. Haider and Husain (2007 b) have described that calcium alginate entrapped β-galactosidase was quite effective in the hydrolysis of lactose in batch process at 37ºC. The rate of hydrolysis of lactose in whey and milk is dependent on the activity of β-galactosidase which in turn depends on the reaction conditions such as pH, temperature, concentration of enzyme and processing time (Sener *et al.*, 2006). Since pH of whey ranges from 4.5 to 5.0 whereas the pH of milk ranges from 6.5 to 6.8, almond β-galactosidase having an optimum pH of 5.5 showed 78% activity at pH 5.0 (Fig. 1). Thus, whey was hydrolyzed to a greater extent as compared to skimmed milk in continuous stirred batch process at 50ºC and 60ºC.

Minerals contribute to the buffering capacity of milk by maintaining its pH, ionic strength and osmotic pressure. Thus, we have studied the synergistic effect of Ca^{+2} and Mg^{+2} ions (Table 2), Na^+ and K^+ ions (Table 3) and the combined effect of Na^+(NaCl), K^+(KCl), Ca^{+2}(CaCl$_2$), Mg^{+2}(MgCl$_2$) and citrate ions (trisodium citrate) on the activity of soluble and immobilized enzyme (Table 4). Activity enhancement of 123% was observed for soluble enzyme in presence of all these ions while the immobilized β-galactosidase showed a significant enhancement in the activity of enzyme, 152% under similar conditions. These results showed that a synergistic effect of CaCl$_2$, MgCl$_2$, NaCl and KCl has a stimulating effect on the activity of both soluble and immobilized enzyme. However, this effect was more pronounced in case of immobilized enzyme. The observation of the effects of various cations are in agreement with the reports on the requirements for mono and divalent metal ions for optimal activity and stability for a number of different β-galactosidases (Nguyen *et al.*, 2007; Nguyen *et al.*, 2006; Rajakala and Karthigai, 2006; Nakayama and Amachi, 1999). Ca^{+2} is a known inhibitor of some β-galactosidases (Haider and Husain, 2009 b; Fernandes *et al.*, 2002; Lee *et al.*, 2002; Garman *et al.*, 1996; Smart *et al.*, 1985) while the stimulation of almond β-galactosidase by Mg^{+2} ion has already been demonstrated (Haider and Husain, 2007 a). Huang *et al.* (1996) reported the combined effect of Ca^{+2} and Mg^{+2} ions on both soluble and immobilized *Streptavidin* β-galactosidase.

Immobilized β-galactosidase was significantly stabilized against the action of proteolytic enzymes; pepsin and trypsin (Table 5). Moreover, the activity of immobilized enzyme was enhanced drastically in the presence of trypsin as compared to its soluble counterpart. Our results are in agreement with the previously published observation indicating that immobilized β-galactosidase showed significant enhancement in enzyme activity in the presence of trypsin as compared to pepsin (Haider and Husain, 2008 a).

Table 6 describes the hydrolysis of lactose by immobilized β-galactosidase in continuous packed bed reactors at different flow rates. It was observed that the maximum hydrolysis occurred at a flow of 20 ml/h while the hydrolytic rate decreased at 30 ml/h. It was due to residence time of lactose inside the column containing immobilized β-galactosidase (Zhou and Chen, 2001). Mammarella and Rubiolo (2006) have earlier reported higher lactose hydrolysis at low flow rates. In view of the stability offered by the immobilized β-galactosidase together with its high porosity and excellent flow properties, it appeared to be a promising system for the continuous production of lactose-free milk and whey in dairy industries.

The enzyme bound to Con A layered calcium alginate-cellulose beads has large surface area which provided easy contact with more substrate molecules and caused no problem of substrate and product diffusion as it has been reported earlier in case of microencapsulated and entrapped enzyme (Nogales and Lopez, 2006). Since immobilization yield was high (72%) and the immobilized enzyme remained active for longer durations without a reasonable decrease in activity, this method could be employed for the hydrolysis of lactose from whey and milk in batch as well as in continuous reactors.

CONCLUSIONS

Con A layered calcium alginate-cellulose beads have large surface area that has been exploited for the immobilization of β-galactosidase. The enzyme present on its surface makes easy contact with more substrate and has no problem of substrate and product diffusion as it has been reported in case of microencapsulated and entrapped enzyme. Immobilized enzyme exhibited significantly higher activity and stability at different pH and temperatures. This immobilized enzyme preparation was found to be remarkably stable

against the inactivation mediated by pepsin and trypsin and also retained very high activity in presence of various cations. On the basis of these findings, we suggest that biological and/or technical relevance of this study is that such immobilized enzyme preparations could be employed for the hydrolysis of lactose for lactose intolerant patients. In view of its stability and utility in batch process, such preparation could be exploited for the continuous conversion of lactose from milk and whey for longer durations in a reactor in a more convenient and cheaper way.

Acknowledgments

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