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Immobilization of *Aspergillus oryzae* β galactosidase on concanavalin A-layered calcium alginate-cellulose beads and its application in lactose hydrolysis in continuous spiral bed reactors

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In this study, *Aspergillus oryzae* β galactosidase was immobilized on concanavalin A layered calcium alginate-cellulose beads as a bioaffinity support. Immobilized enzyme showed a remarkable broadening in temperature-activity profiles as compared to the native enzyme and exhibited 65% activity in the presence of 5% galactose. Michaelis constant (K_m) was 2.57 mM and 5.38 mM for the free and the immobilized β galactosidase, respectively. Crosslinked β galactosidase showed greater catalytic activity in the presence of Mg^{2+} and was more stable during storage at 4°C for 6 weeks. Immobilized enzyme hydrolyzed 67% lactose in milk in 8 h and 85% lactose in whey in 9 h in the stirred batch process at 50°C. The continuous hydrolysis of lactose by crosslinked β galactosidase in spiral bed reactor exhibited 93% and 88% hydrolysis of lactose at flow rate of 20 ml/h and 30 ml/h, after 1 month operation, respectively.

Keywords: β galactosidase. Immobilization. Cellulose-alginate. Concanavalin A. Spiral bed reactor. Lactose hydrolysis.

INTRODUCTION

β Galactosidase (3.2.1.23) is a hydrolytic enzyme that catalyzes the breakdown of lactose into glucose and galactose. It is found in plants, animals and microorganisms. This enzyme has been employed in producing lactose free whey, milk and milk products[1]. Hydrolysis of lactose improves the product's sweetness and makes milk consumption much easier for the people suffering from lactose intolerance and increases the quality of the product in dairy industry[2,3].

The widespread industrial application of enzymes is often hampered by their lack of long term operational stability and shelf-storage life, their cumbersome recovery and re-use. These drawbacks can generally be overcome by immobilization of enzymes, and a major challenge in industrial biocatalysis is the development of stable, robust and preferably insoluble biocatalysts[4,5]. Immobilized biocatalyst can be reused several times and provides several benefits, especially in the food technologies, including easy separation from reaction mixture, no contamination of the product by the enzyme, operational and long term stability, continuous processing and multienzyme reaction systems[6]. Several methods utilize the affinity of biomolecules and ligands for immobilization of enzymes which are widely used in constructing enzyme based analytical devices and other applications[7–9]. The suitability of support and the method of immobilization varies from enzyme to enzyme and their intended use, and therefore a separate optimization of each system is essential[10,11].

In the present study, an attempt was made to immobilize *Aspergillus oryzae* β galactosidase on a bioaffinity support, Concanavalin A (Con A) layered calcium alginate-cellulose beads. The stability of immobilized β galactosidase (IβG) has been studied against various physical and chemical denaturants, and compared with its soluble counterpart. Immobilized β galactosidase was used in a stirred batch process for the hydrolysis of lactose from milk and whey at various temperatures and in a spiral bed reactor for continuous hydrolysis of lactose in solution.

MATERIALS AND METHODS

Materials

Aspergillus oryzae β galactosidase (3.2.1.23), α-methyl β-D-glucopyranoside, glucose oxidase, o-dianisidine HCl and glucose were obtained from Sigma Chem. Co. (St. Louis, MO, USA). Cellulose was purchased from Titan Biotech (Rajasthan, India), Peroxidase, glutaraldehyde 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). Jack bean meal was procured from Loba Chemical Co. (Mumbai, India). All other chemicals and reagents used were of analytical grade and used without purification.

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 a 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 4.6 at 4°C, until further use.

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. Calcium alginate-cellulose beads (500 beads) were incubated overnight with jack bean extract (25 ml) containing Con A at room temperature with slow stirring on a magnetic stirrer. Con A bound calcium alginate-cellulose beads were washed thrice with 0.1 M sodium acetate buffer, pH 4.6.

Adsorption of β galactosidase on Con A layered calcium alginate-cellulose beads

Con A layered calcium alginate-cellulose beads were incubated with β galactosidase (2250 U) overnight at 32°C, with slow stirring. The unbound enzyme was removed by repeated washing with 0.1 M sodium acetate buffer, pH 4.6.

Crosslinking of immobilized β galactosidase

β Galactosidase immobilized on the surface of Con A layered calcium alginate-cellulose beads was suspended in 0.1 M sodium acetate buffer, pH 4.6 for 2 h at 4°C. Adsorbed enzyme preparation was crosslinked by 0.5% (v/v) glutaraldehyde for 2 h at 4°C. Finally the immobilized enzyme was incubated with 0.01% (v/v) ethanolamine for 90 min at 30°C to stop crosslinking. The integrity of the crosslinked immobilized enzyme was examined by incubating the beads in 1.0 M α-methyl-β-D-glucopyranoside for 2 h at room temperature.

Effect of temperature

The activity of the soluble and immobilized β galactosidase (20 λ) was assayed in 0.1 M sodium acetate buffer, pH 4.6 at various temperatures (20–80°C) for 15 min. The activity obtained at 50°C was considered as control (100%) for the calculation of remaining percent activity at different temperatures.

Soluble and immobilized β galactosidase preparations were incubated at 60°C in 0.1 M sodium acetate buffer, pH 4.6 for varying times. Aliquots of each preparation (20 λ) were taken at indicated time intervals and chilled quickly in crushed ice for 5 min. The enzyme was brought at room temperature. The enzyme activity without incubation at 60°C was taken as control (100%) for the calculation of the remaining percent activity.

Effect of galactose and $MgCl_2$

The activity of soluble and immobilized β galactosidase (20 λ) was independently measured in the presence of increasing concentrations of galactose/$MgCl_2$ (1.0–5.0%, w/v) in 0.1 M sodium acetate buffer, pH 4.6 for 1 h at 37°C. The activity of the enzyme without added galactose/$MgCl_2$ was considered as control (100%) for the calculation of the remaining percent activity.

Storage stability

Soluble and the immobilized β galactosidase preparations were stored at 4°C in 0.1 M sodium acetate buffer, pH 4.6 for over 6 weeks. The aliquots from each preparation (20 λ) were taken in triplicates at the gap of 10 days and were then analyzed for the remaining enzyme activity. The activity determined on the first day was taken as control (100%) for the calculation of remaining percent activity.

Hydrolysis of milk lactose in batch process

The cold milk was skimmed by centrifugation at 8000×g for 20 min. The fat layer was removed from the milk and stored at 4°C for further use. Skimmed milk (500 ml) was independently treated with soluble and immobilized β galactosidase (250 U) in a stirred batch process at 50°C and 60°C. The aliquots of 250 μl were taken at indicated time intervals for 10 h. The hydrolysis of lactose was estimated by glucose oxidase-peroxidase assay procedure as described by Ansari and Husain (2010)[3].

Hydrolysis of whey lactose in batch process

Skimmed milk was acidified by HCl until the pH reached to 4.8 and casein was removed by centrifugation. Whey (500 ml) was separately treated with soluble and immobilized β galactosidase (125 U) in a stirred batch process at 50°C and 60°C. The aliquots of 250 μl were taken out at indicated times and hydrolysis of lactose was estimated using a procedure described by Ansari and Husain (2010)[3].

Hydrolysis of lactose in spiral bed reactors

A spiral column (70 x 1.5 cm) filled with immobilized β galactosidase (2520 U) was used for the continuous hydrolysis of lactose in solution for 6 weeks. 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 independent columns with flow rates of 20 ml/h and 30 ml/h at room temperature (32°C). After a gap of 5 days, the samples were collected and analyzed for the hydrolysis of lactose according to the procedure described by Ansari and Husain (2010)[3].

Glucose estimation

Glucose released from lactose hydrolysis was monitored using a glucose oxidase-peroxidase coupled assay procedure according to the procedure described by Ansari and Husain (2010)[3].

Assay of β galactosidase

The hydrolytic activity of β galactosidase was determined by measuring the release of *o*-nitrophenol from ONPG at 405 nm. The reaction was performed by continuous shaking in an assay volume of 2.0 mL containing 1.7 ml of 0.1 M sodium acetate buffer, pH 4.6, 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 product (*o*-nitrophenol) formation was measured spectrophotometrically at 405 nm.

One unit (1.0 U) of β galactosidase activity is 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 according to the procedure described by Ansari and Husain (2010)[3].

Estimation of protein

Protein concentration was determined by the dye binding method[12].

Statistical analysis

Each value represents the mean for three independent experiments performed in triplicates, with average standard deviation <5%. The data expressed in various studies were plotted using Sigma Plot-9 and expressed with standard deviation of error (±). A student's t-test was used to examine statistically significant differences. The analysis of variance was performed using ANOVA, P values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Cellulose and alginate have been independently used by several workers to immobilize enzymes. Cellulose is an inexpensive and easily available matrix. It is widely used as an enzyme immobilization material due to its unique hydrophilic characteristics, relatively high chemical stability and remarkable environmental protecting capability[13]. The use of sodium alginate has also been reported for immobilizing and microencapsulating enzymes, cell organelles, plants and animal cells[14]. However, these supports have some limitations like compact packing of cellulose in the reactor affects the flow rate of the column or sometimes block the reactor completely. Although calcium alginate beads have been used for the entrapment of enzymes, but they are not normally used as their large pore size results in enzyme leakage[15]. To overcome this problem, we developed a hybrid gel of calcium alginate-cellulose beads which were layered with Con A. β galactosidase from *Aspergillus oryzae* was then immobilized on Con A layered support.

Immobilization of β galactosidase

In order to prevent the desorption of the enzyme from the surface of beads, adsorbed enzyme was crosslinked by glutaraldehyde. Crosslinking decreased the enzyme activity from 78% to 70% (Table 1). It has earlier been reported that while crosslinking of enzyme by glutaraldehyde prevented desorption of the enzyme, the activity of the enzyme was decreased slightly[16]. It is a well-known fact that the crosslinking of enzymes by bi-functional or multi-functional agents enhanced thermal stability due to the formation of several linkages between enzyme and support[17].

Effect of temperature

The temperature-optima for both the soluble and immobilized β galactosidase preparations were same at 50°C. However, the immobilized β galactosidase showed greater enzyme activity at higher and lower temperatures as compared to its soluble counterpart (Fig 1a). Such enhanced stability was brought about by the immobilization process, which resulted in the formation of molecular cage around the protein molecules (enzyme) thereby enhancing the thermal stability of the immobilized enzyme[18]. Fig 1b illustrates the thermal denaturation of soluble and immobilized β galactosidase. The immobilized enzyme retained 93% of the initial activity after 30 min exposure at 60°C while the soluble enzyme lost 40% activity under similar incubation conditions. The temperature tolerance of the immobilized enzyme increased due to diffusional affects where the reaction velocity is more likely to be diffusion limited, so that improvements in thermal diffusion would correspondingly result in proportionately higher reaction rates. Bayramoglu et al. (2007) has also shown that β galactosidase from *Escherichia coli* immobilized on magnetic poly beads led to an increase in its stability when incubated at high temperatures[19]. The immobilized enzyme could work in harsh environmental conditions with less activity loss than the soluble β galactosidase.

Effect of magnesium chloride and galactose

Several workers have investigated that galactose is one of the end product of β galactosidase catalyzed hydrolysis of lactose and it competitively inhibited the activity of the enzyme[20,21]. Incubation of the soluble β galactosidase with 5.0% galactose for 1 h at 37°C resulted in a significant loss of 71% activity while the immobilized β galactosidase retained over 65% of the original enzyme activity during such exposure. The immobilized β galactosidase was significantly more resistant to inhibition mediated by galactose as compared to the free enzyme. Nevertheless, it had been reported earlier that Mg^{+2} ions are important for the activity of β galactosidase. Thus, the effect of $MgCl_2$ was monitored on the activity of soluble and the immobilized enzyme (Fig 1c). It was observed that at 5% $MgCl_2$, the activity of the soluble β galactosidase decreased while the immobilized enzyme exhibited an enhancement in the activity upto 135%. Sutendra et al. (2007) had observed that *E. coli* β galactosidase has several metal binding sites particularly Mg^{+2} binding site which bring about an increase in enzyme activity[22].

Storage stability

The storage stability of the soluble and immobilized β galactosidase has been shown in Fig 1d. The immobilized enzyme retained 80% of the initial enzyme activity after 6 weeks of storage at 4°C while the soluble β galactosidase exhibited only 56% of the original activity after similar storage. The generally observed higher stability of β galactosidase may be related to the specific and strong binding of enzyme with bioaffinity support which prevented the unfolding/denaturation of enzyme upon long storage.

Kinetic properties

Table 2 shows that the immobilization of the enzyme resulted in an increase in "Km" and a decrease in "Vmax" value. Mass transfer resistance, electrostatic and steric effects may have caused the increase in "Km" value after immobilization. Mass transfer resistance appears to be significant for macromolecular substrates

Table 2. Influence of immobilization on the kinetic constants

Derivative	K_m (mM)	V_{max} (mM/min)
Soluble β galactosidase	2.57±0.48[+]	0.48±0.75[/]
Immobilized β galactosidase	5.38±0.31*	0.38±0.42[//]

*P-values <0.03 with respect to the soluble β galactosidase[+]
[//]P-values <0.03 with respect to the soluble β galactosidase[/]

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 x100
			Theoretical (X-Y)=A	Actual=B	
Enzyme adsorbed on the surface of beads	2250	831	1419	1106	78±0.93*
Enzyme adsorbed on the surface of beads and crosslinked	1106	0	1106	774	70±0.55[//]

[//]P-values <0.03 with respect to crosslinked β galactosidase*

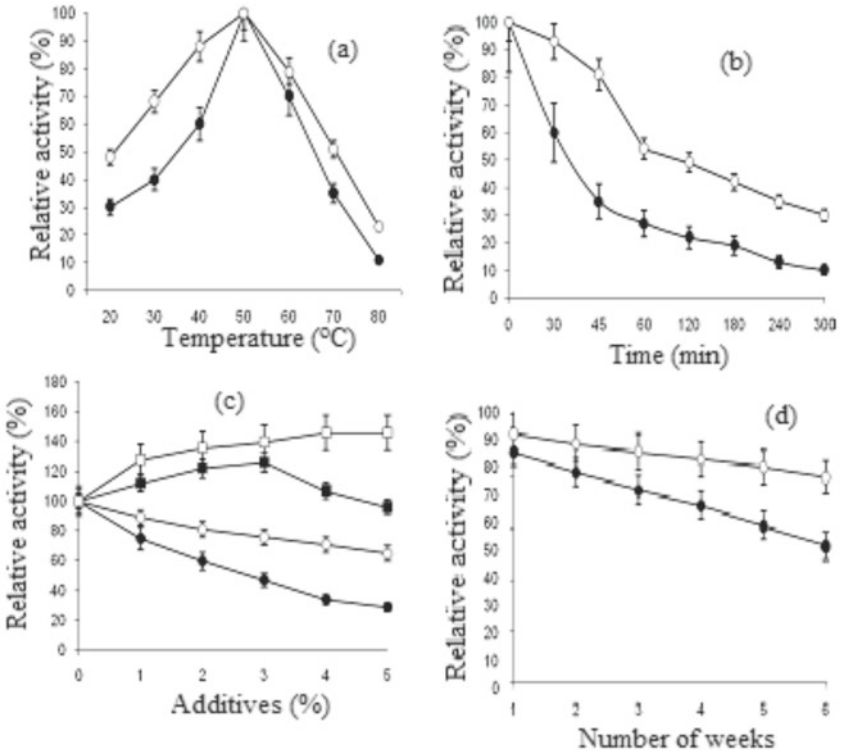

Figure 1. (a) Temperature activity profiles of the soluble (●) and the immobilized (○) β galactosidase
(b) Thermal denaturation plot of the soluble (●) and the immobilized (○) β galactosidase
(c) Effect of galactose on the soluble (●) and the immobilized (○) β galactosidase, and $MgCl_2$ on soluble (■) and immobilized (□) β galactosidase
(d) Storage stability of the soluble (●) and the immobilized (○) β galactosidase

such as ONPG because the substrate must contact the enzyme adsorbed on the surface of cellulose-alginate beads efficiently. Greater "Km" value may have been attributable to enzyme active sites being less accessible to the substrate than in free solution[23]. Nevertheless, immobilization of β galactosidase may also have reduced its ability to undergo conformational changes that are intrinsic to enzyme-substrate interaction, thereby resulting in a noticeable decrease in "Vmax" value for the immobilized enzyme. The increase in "Km" values and decrease in "Vmax" values for the immobilized enzyme seen here are in agreement with the observations of other researchers who conducted work on the immobilized β galactosidase[24,25].

Lactose hydrolysis from milk and whey

Table 3 describes the hydrolysis of lactose from milk and whey by soluble and immobilized β galactosidase for various times. It was observed that immobilized enzyme hydrolyzed 67% and 85% lactose from milk and whey, respectively after 10 h at 50°C (Table 3). The efficiency of lactose hydrolysis in milk and whey by the immobilized enzyme was found to be several folds higher than that mentioned in the earlier work[26,27]. The greater efficiency of the immobilized β galactosidase in hydrolyzing lactose from whey compared to milk lactose has been explained by a difference in pH between the tests, that is, pH 4.5–5.0 for whey and pH 6.5–6.8 for milk. *Aspergillus*

Table 3. Hydrolysis of lactose in milk/whey by the soluble and the immobilized β galactosidase in a batch process at 50°C/60°C

Time (h)	Lactose hydrolysis (%)							
	50 °C				60 °C			
	Milk		Whey		Milk		Whey	
	SβG	IβG	SβG	IβG	SβG	IβG	IβG	SβG
1	10±0.25^{+}	8±0.30*	16±0.39^{++}	12±0.18**	8±0.39$^{/}$	5±0.40$^{\#}$	13±0.48$^{//}$	9±0.22$^{\#\#}$
2	17±0.35^{+}	20±0.49*	25±0.31^{++}	31±0.52**	12±0.54$^{/}$	19±0.48$^{\#}$	21±0.69$^{//}$	25±0.40$^{\#\#}$
3	25±0.11^{+}	36±0.59*	31±0.79^{++}	48±0.47**	17±0.39$^{/}$	27±0.51$^{\#}$	27±0.10$^{//}$	41±0.51$^{\#\#}$
4	37±0.65^{+}	51±0.57*	42±0.51^{++}	59±0.39**	26±0.62$^{/}$	38±0.38$^{\#}$	34±0.29$^{//}$	49±0.29$^{\#\#}$
5	44±0.49^{+}	55±0.37*	49±0.49^{++}	65±0.49**	35±0.79$^{/}$	47±0.62$^{\#}$	40±0.82$^{//}$	56±0.48$^{\#\#}$
6	49±0.73^{+}	61±0.21*	57±0.56^{++}	70±0.19**	40±0.39$^{/}$	54±0.82$^{\#}$	46±0.90$^{//}$	63±0.56$^{\#\#}$
7	52±0.56^{+}	65±0.29*	61±0.71^{++}	75±0.73**	42±0.49$^{/}$	49±0.92$^{\#}$	51±0.56$^{//}$	70±0.49$^{\#\#}$
8	53±0.25^{+}	67±0.47*	69±0.91^{++}	77±0.72**	48±0.29$^{/}$	59±0.33$^{\#}$	57±0.73$^{//}$	77±0.61$^{\#\#}$
9	53±0.37^{+}	67±0.28*	69±0.77^{++}	85±0.19**	48±0.67$^{/}$	59±0.82$^{\#}$	57±0.40$^{//}$	77±0.48$^{\#\#}$
10	53±0.49^{+}	67±0.76*	69±0.38^{++}	85±0.38**	48±0.94$^{/}$	58±0.38$^{\#}$	57±0.72$^{//}$	77±0.38$^{\#\#}$

*P-values <0.03 with respect to the soluble β galactosidase at 50 °C $^{+}$
**P-values <0.05 with respect to the soluble β galactosidase at 50 °C $^{++}$
$^{\#}$P-values <0.03 with respect to the soluble β galactosidase at 60 °C $^{/}$
$^{\#\#}$P-values <0.05 with respect to the soluble β galactosidase at 60 °C $^{//}$

Milk and whey (500 ml, each) were treated independently with the soluble and the immobilized β galactosidase in a stirred batch process at 50 °C/60 °C. The aliquots of 250 µl were taken out at indicated time intervals for 10 h. The hydrolysis of lactose was estimated by using assay procedure described in the text (Ansari and Husain 2010).

oryzae β galactosidase showed 100% activity at pH 4.6 but its activity considerably decreased above pH 6.0 [28]. Despite the reduced hydrolytic activity shown by the immobilized β galactosidase above pH 6.0, the rate of hydrolysis of milk lactose (67%) can be considered quite satisfactory. Roy and Gupta (2003) had investigated that lactose hydrolysis by lactozym immobilized on cellulose beads took 48 h in a continuous batch mode[29]. According to recent reviews, some traditional enzymatic processes for milk lactose removal available in the market present a final hydrolytic value of 50–70% at reaction times larger than 18 h. Thus, Con A layered calcium alginate-cellulose bead can be exploited commercially for lactose hydrolysis in milk and whey. Moreover, the enzyme activity of β galactosidase decreased substantially at temperature higher than 50°C [30], thus a decreased lactose hydrolyzing activity was noticed at 60°C both in milk and whey in a stirred batch process (Table 3).

Hydrolysis of lactose in continuous spiral-bed reactors

Table 4 summarizes the influence of varied flow rates on the hydrolytic rate of lactose in a spiral bed reactor by taking same enzyme activity of the immobilized enzyme. It was observed that 95% and 90% of lactose was hydrolyzed by the immobilized β galactosidase after 20 d and 40 d, respectively when the flow rate was 20 ml/h. At a flow rate of 30 ml/h, the immobilized enzyme could attain 92% and 83% lactose hydrolysis after 20 d and 40 d of continuous operation of the reactor (Table 4). Mammarella and Rubiolo (2006) have earlier reported that higher lactose hydrolysis took place at lower flow rates[30]. The results indicated that the extent of lactose hydrolysis might be simply controlled in a way of adjusting the flow rate of the continuous operation mode. Novalin et al. (2005) had reported 78% conversion of lactose in skimmed milk at flow rate of 9.9 L/h at an enzyme activity of 120 U/ml and at a temperature of 23±2°C in a hollow fibre reactor[26].

Since the immobilization procedure discussed in this manuscript is cheap and eliminates any complicated step while leading to high enzyme activity, it finds promising application in the continuous production of novel products in food and dairy industry.

Table 4. Lactose hydrolysis in continuous spiral bed reactors

Number of days	Lactose hydrolysis at different flow rates	
	20 ml/h	30 ml/h
Control	100^{+}	100^{++}
5	100±0.91*	100±0.73**
10	100±1.33*	95±0.95**
15	95±0.67*	92±0.96**
20	95±0.88*	92±1.03**
25	95±1.07*	92±1.38**
30	93±1.23*	88±1.46**
35	90±1.54*	86±1.39**
40	90±1.38*	83±1.54**

*P < 0.05 with respect to control+
**P < 0.05 with respect to control++
Hydrolysis of lactose in continuous reactors containing immobilized β galactosidase (2520 U) was performed as described in the text (Ansari and Husain 2010).

CONCLUSION

Immobilized β galactosidase was found to be remarkably stable against various physical and chemical denaturants and was successfully employed for the hydrolysis of lactose from milk and whey in a batch process. 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.

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