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ORIGINAL PAPER

Calcium alginate entrapped preparation of α-galactosidase: its stability and application in hydrolysis of soymilk galactooligosaccharides

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Abstract Thermostable α-galactosidase from *Aspergillus terreus*$_{GR}$ was insolubilized using concanavalin A obtained from jack bean extract and in order to maintain the integrity of complex in the presence of its substrate or products, this complex was crosslinked with glutaraldehyde. Soluble α-galactosidase entrapped in calcium alginate retained 82% of enzyme activity whereas, Con A-α-galactosidase complex entrapped in calcium alginate and crosslinked Con A-α-galactosidase complex entrapped calcium alginate retained 74 and 61% activity, respectively. A fluidized bed reactor was constructed for continuous hydrolysis of galactooligosaccharides in soymilk using crosslinked Con A-α-galactosidase complex entrapped calcium alginate. Optimum conditions such as pH (5.0) and temperature (65°C) were the same for all immobilized enzyme preparations and soluble enzyme. Crosslinked Con A-α-galactosidase entrapped complex exhibited enhanced thermostability and showed 62% of activity (38%) after 360 min at 65°C. Entrapped crosslinked Con A-α-galactosidase complex preparation was superior in the continuous hydrolysis of oligosaccharides in soymilk by batch processes compared to the other entrapped preparations. The entrapped crosslinked concanavalin A-α-galactosidase complex retained 95% activity after eight cycles of use.

Keywords Affinity immobilization · Con A · *Aspergillus terreus*$_{GR}$ · Thermostable α-galactosidase

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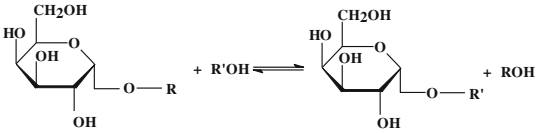
Introduction

Immobilization of enzymes to the adsorbents by covalent bond formation is often leads to loss of biological activity. This can often be overcome by non-covalent immobilization [19, 23]. Bioaffinity-based immobilization is one such technique that offers the possibility of enzyme immobilization directly from partially pure enzyme preparations or even cell lysates [30]. During the last decade, several investigators studied the usefulness of concanavalin-A (lection from *Canavalia ensiformis*) for immobilization of glycoenzymes [31]. From recombinant DNA technology, it is possible to produce most enzymes for a commercially acceptable price. However, industrial application is often hampered by the lack of long-term operational stability and difficulty in recovery and re-use of the enzyme. An immobilized form of enzyme is more convenient to handle and it also provides facile separation from the product, thereby minimizing or eliminating protein contamination of the product [29].

Legumes play an important role in the traditional diet of many regions throughout the world. Among legumes, human consumption of soy products is increasing not only because of their high nutritional value but also in view of their health-promoting effects, such as reduction in cardiovascular disease, osteoporosis, and cancer risks [7]. Soybean protein contains enough of all the essential amino acids to meet biological requirements when consumed at the recommended level [5, 22]. Human consumption of soy products has been limited due to the presence of nondigestible raffinose family of sugars (RFOs: mainly raffinose and stachyose), which are not eliminated by usual soy processing [21, 24].

α-D-Galactose-galactohydrolase (E.C.3.2.1.22) commonly referred to as α-galactosidase, catalyzes the hydrolysis of α-galactosidic linkages in oligosaccharides such as

raffinose, melibiose, stachyose and verbascose, polysaccharides like galactomannans, and glycoconjugates such as glycoproteins and glycolipids. Ceramide trihexosides, with its higher homologues and derivatives, are also cleaved by the action of α-galactosidase. Bau [3] and Fischer and Lindner [9] isolated α-galactosidase from bottom yeast for the first time. Because of its action on melibiose, it was named melibiase, catalyzing the following reaction.



The hydroxylic acceptor molecule R′OH is commonly water, although R and R′ can be aliphatic or aromatic groups.

Monogastric animals lack the ability to synthesize sufficient α-galactosidase in their intestinal system to hydrolyze α-galactosides present in soybeans and other legumes. Thus, these undigested sugars pass into the large intestine, where the intestinal microflora acts on them, causing flatulence and gastrointestinal disturbance. These disturbances reduce both feed efficiency in monogastric animals and general consumer acceptance of soy foods [9, 27]. In an earlier study, we demonstrated that α-galactosidase from *Aspergillus terreus*$_{GR}$ is thermostable [32]. High temperature used during the sugar manufacturing process, the pulping process, and a sterilization step following the soybean processing, lead to denaturation of thermolabile α-galactosidases. Thus, in all cases, thermostable enzymes offer a considerable advantage over their thermolabile counterparts [10]. The present study deals with the application of the hydrolytic properties of immobilized, thermostable α-galactosidase from *Aspergillus terreus*$_{GR}$ in soymilk processing.

Materials and methods

Microorganism

Aspergillus terreus$_{GR}$ capable of producing thermostable extracellular α-galactosidase was isolated from a soil sample in our laboratory. It was maintained on PDA (potato dextrose agar) slants and stored at 4°C. The fungus was subcultured periodically. Submerged fermentation was carried out for α-galactosidase production by *Aspergillus terreus*$_{GR}$ in a chemically defined medium as described previously [32].

Assay of α-galactosidase

α-Galactosidase activity was quantitatively assayed according to the method described by Dey and Pridham [6] using a final concentration of 1 mM (*p*-nitrophenyl-α-D-galactopyranoside) PNPG as substrate. Aliquots of 0.1 ml of PNPG (1 mM) and 0.8 ml of acetate buffer (0.1 M, pH 5) were pre-incubated at 65°C for 2 min, prior to adding 0.1 ml of suitably diluted enzyme to initiate the reaction. This was terminated after 15 min by the addition of 3 ml of 0.1 M Na_2CO_3 and the released *p*-nitrophenol was determined spectrophotometrically at 405 nm (Elico Ltd. India). The activity of immobilized enzyme was estimated by adding 0.1 ml of 1.0 mM PNPG to 1 g of immobilized support and using the same method as for the free enzyme. One unit of α-galactosidase activity was defined as the amount of enzyme liberating 1 μ mol *p*-nitrophenol in 1 min under the assay conditions.

Preparation of jack bean extract

Ten grams of jack bean seeds soaked overnight were homogenized in 100 ml of 0.1 M Tris–HCl buffer, pH 6.1 containing 0.1 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM $CaCl_2$. The mixture was kept on a magnetic stirrer for 2 h at room temperature. Insoluble residue was removed by centrifugation at 12,300*g* at 4°C for 20 min until a clear supernatant was obtained. The collected supernatant was used for the insolubilization of α-galactosidase.

Preparation of Con A-α-galactosidase complex

The 10% jack bean seed extract was added to 0.5 ml diluted α-galactosidase (12 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 [15]. The precipitate was collected by centrifugation at 3,000 × *g* in a cooling centrifuge for 30 min. The precipitate was washed with acetate buffer pH 5 (0.1 M) and the amount of bound enzyme was calculated by the difference of initial activity before the addition of jack bean extract and the activity of the supernatant and washings.

Preparation of crosslinked Con A-α-galactosidase complex by glutaraldehyde

Con A-α-galactosidase complex (22 U) was crosslinked by adding increasing concentration of glutaraldehyde (0.1–0.5%, w/v) for 2 h at 4°C [15]. 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 3,000 × *g* for 30 min at 4°C on a cooling tabletop centrifuge. The precipitate was suspended in sodium acetate buffer pH 5. The activity was determined both in supernatant and pellet.

Entrapment of soluble, Con A-α-galactosidase and crosslinked Con A-α-galactosidase complex into sodium alginate

The soluble, Con A-α-galactosidase and crosslinked Con A-α-galactosidase complex were mixed separately with 2.0% sodium alginate solution and added drop-wise to a stirred solution of 0.2 M $CaCl_2$ prepared in distilled water [26]. A 5.0-ml syringe with attached needle number 20 was used for the preparation of calcium alginate beads. The beads were stirred in $CaCl_2$ solution for 2 h on a magnetic stirrer to make them hard and then suspended in 0.1 M sodium acetate buffer pH 5. The obtained beads were stored for later use.

Effect of pH

The free and immobilized enzyme were incubated with buffers of different pH values at 65°C for 15 min using 2.0 mM PNPG as substrate with respective controls (0.1 M, acetate buffer pH 5). The α-galactosidase activity was assayed as described above.

Effect of temperature

The effect of temperature was studied by incubating both soluble and immobilized enzymes in 0.1 M, pH 5 acetate buffer at different temperatures (ranging from 30 to 70°C) with respective controls for 15 min using 2.0 mM PNPG as substrate.

Thermostability

For determination of thermal stability, the enzyme was pre-incubated with the substrate without any stabilizers at 65°C. Residual activity in each sample was calculated by doing assay against enzyme control sample at pH 5.0 and 65°C.

Preparation of soymilk

Soymilk was prepared according to the method of Mulimani and Ramalingam [25]. Soybean seeds were ground to flours. The soybean flour was defatted with hexane (1:1, w/v). The fat free soybean flour was suspended in distilled water (1:10 w/v) and heated on boiling water bath. Undissolved residue was separated from soymilk by centrifugation for 5 min at 12,300 × *g*. The supernatant containing soymilk was removed and stored at 4°C until further use.

Estimation of oligosaccharides in soymilk

The amount of sucrose, raffinose, and stachyose in raw and enzyme-treated soymilk were estimated by thin layer chromatography (TLC) according to the method of Tanaka et al. [34].

Soybean oligosaccharide hydrolysis by batch reaction

Batch reactions were performed for both free and immobilized enzymes for different incubation periods. For the reaction involving free enzyme, around 10 ml enzyme (5.8 U) was added to 60 ml of soymilk in a 250-ml Erlenmeyer flask. For immobilized enzyme, 300 mg of immobilized granules (0.6 U) were added to 60 ml of soymilk. The hydrolysis reaction was carried out at 65°C in an incubator shaker (200 rpm) for different incubation periods of 2, 4, 8, and 12 h. After the incubation period, the samples of the reaction mixture were taken out and kept in a boiling water bath for 10 min to arrest the enzyme reaction. Afterwards, the sample was analyzed for degradation of oligosaccharides. The control experiments were performed in the same manner with 0.2 M acetate buffer (pH 5) replacing the enzyme.

Repeated batch experiments

For establishing the stability in the oligosaccharide degradation by immobilized enzyme, repeated batch experiments were carried out. The Erlenmeyer flasks containing 60 ml of soymilk and 300 mg of immobilized enzyme granules were kept in an incubator shaker (200 rpm) maintained at 65°C. After every 2 h of incubation, the soymilk was taken out and oligosaccharide concentration was determined. The granules were separated by filtration, washed with sterile water, and transferred into another batch of soymilk (60 ml) for 2-h incubation. The reaction was carried out under identical conditions.

Fluidized bed reactor for the continuous degradation of RFO in soymilk

Fluidized bed reactor studies were carried out in jacketed glass column of 75 cm in length and 1.5 cm in diameter, with bed volume of 150 ml. The jacket temperature was maintained at 65°C using water bath (Julabo, Germany). The soymilk feed solution preheated at 65°C in a water bath was introduced from the bottom of the column through a peristaltic pump (Amersham Pharmacia Biotech, Sweden) and the product was withdrawn from the top. The entrapped preparations of α-galactosidase in calcium alginate were packed in the column. The upward substrate stream fluidized the granules filled up in the column. Different flow rates of 50, 75, 100, and 125 ml/h were used for the degradation of oligosaccharides in soymilk. The outlet stream was continuously collected from the frontal end of

the column in a container. The effluents were analyzed for the degradation of raffinose and stachyose by TLC.

All the experiments were performed independently in triplicate and the results given here are the mean of three values. The standard deviation for all the values were $<\pm 5\%$.

Results and discussion

Immobilization of α-galactosidase and its properties

The α-galactosidase from *Aspergillus terreus*$_{GR}$ is a glycoprotein. The oligosaccharide moieties take part in the interaction with the Con A. Glutaraldehyde was used to maintain further integrity of enzyme in calcium alginate. Yield of enzyme activity is decreased after every step of the immobilization process. This is mainly due to the loss of enzyme during the process of immobilization. Similar results were obtained when *β*-galactosidase from *Aspergillus oryzae* was immobilized into calcium alginate after conjugation with Con A extract [15].

Sodium-alginate is a potential matrix for immobilization of enzymes and cells. However, this technique has disadvantages like leaching of enzymes from the matrix due to high porosity of the polymer. Affinity precipitation is a technique used for immobilization of enzymes, which is likely to enhance the performance of any given enzyme [30]. In the present study, Con A extracted from jack bean seeds was conjugated with *Aspergillus terreus*$_{GR}$ α-galactosidase. Table 1 summarizes immobilization of α-galactosidase into calcium alginate, entrapment of Con A-α-galactosidase complex into calcium alginate, and crosslinked Con A-α-galactosidase entrapment into calcium alginate. It is evident that 82% of immobilized yield was obtained when soluble enzyme was immobilized into calcium alginate. Con A-α-galactosidase and crosslinked Con A-α-galactosidase complex entrapped in calcium alginate resulted in decrease of 8 and 21% of activity, respectively, when compared to that of soluble enzyme (Table 1). Sodium alginate concentration of 2% was found to be optimum for entrapment of α-galactosidase. Further, increase in the concentration of sodium alginate resulted in decrease of enzyme activity yield (data not shown). This could be due to overcrowding of support matrix that competes for enzyme molecule to bind and that result in decreased enzyme activity yield [16]. Figure 1 shows the effect of pH on soluble, entrapped and crosslinked Con A-α-galactosidase entrapped complex. There is no significant change in the optimum activity of all forms of enzyme. Optimum activity for free as well as immobilized enzyme was at pH 5.0. The immobilized preparation of crosslinked Con A-α-galactosidase entrapped complex is optimally active between pH range 3.8–6.0. Therefore, immobilized (crosslinked Con A-α-galactosidase entrapped complex) preparation is suitable for hydrolysis of RFOs of soymilk (pH of commercial soymilk is 6.1). Similar to our results, Haider and Hussain [15] have reported that *β*-galactosidase from *Aspergillus oryzae* immobilized using Con A and calcium alginate and crosslinked Con A-α-galactosidase entrapped complex was active between the pH range of 3.0–5.2. Figure 2 shows the effect of temperature on free and immobilized preparations of *Aspergillus terreus*$_{GR}$ α-galactosidase. The free as well as immobilized forms of α-galactosidase showed optimum temperature at 65°C. However, the crosslinked Con A-α-galactosidase entrapped complex retained significantly high activity at temperatures lower and higher than the temperature optima compared to the soluble and entrapped Con A-α-galactosidase complex. Crosslinked Con A-α-galactosidase entrapped complex retained 64% at 80°C while the free enzyme retained only 21% of initial activity at the same temperature. There was no

Table 1 Relative activities of α-galactosidase immobilization by using Con A from jack bean and calcium alginate

Methods	Relative activity yield (%)
1. Soluble α-galactosidase entrapped in calcium alginate	82
2. Con A- α- galactosidase complex entrapped in calcium alginate	74
3. Crosslinked Con A-α-galactosidase complex entrapped in calcium alginate	61

Relative activity yield is the value-obtained enzyme activity of immobilized enzyme/enzyme activity of soluble enzyme. All the experiments were performed independently in triplicate and the results given here are the mean of three values. The standard deviation for all the values was $<\pm 5\%$

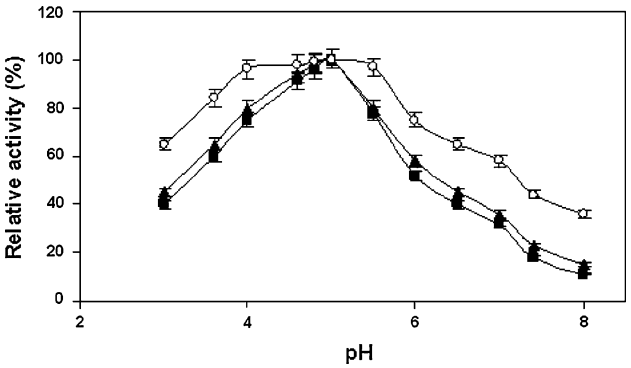


Fig. 1 Effect of pH on enzyme activity. Enzyme activity of soluble (*filled square*), entrapped (*filled triangle*), and crosslinked Con A-α-galactosidase complex entrapped in calcium alginate (*open circle*). All the experiments were performed independently in triplicate and the results given here are the mean of three values. The standard deviation for all the values was $<\pm 5\%$

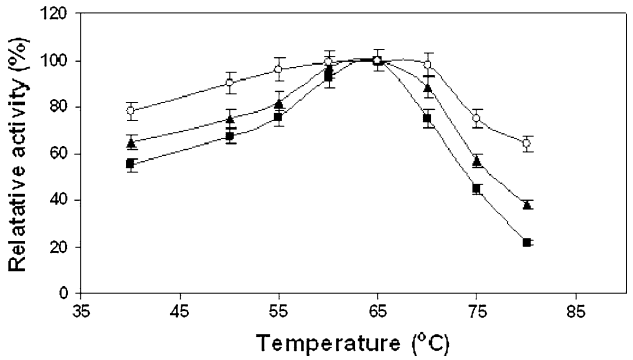


Fig. 2 Effect of temperature on enzyme activity. Enzyme activity of soluble (*filled square*), entrapped (*filled triangle*), and crosslinked Con A-α-galactosidase complex entrapped in calcium alginate (*open circle*). All the experiments were performed independently in triplicate and the results given here are the mean of three values. The standard deviation for all the values was <±5%

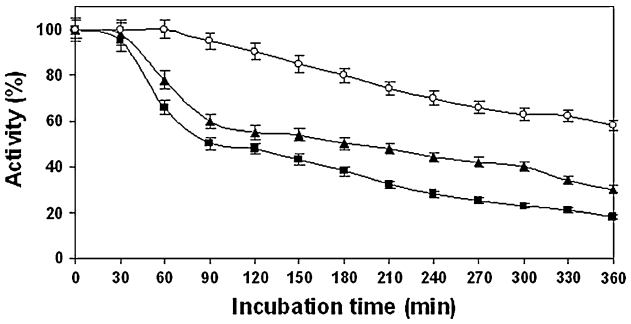


Fig. 3 Thermal stability of α-galactosidase. The enzyme activity of soluble (*filled square*), entrapped (*filled triangle*), and crosslinked con A-α-galactosidase complex entrapped in calcium alginate (*open circle*). All the experiments were performed independently in triplicate and the results given here are the mean of three values. The standard deviation for all the values was <±5%

alteration in temperature and pH optima of the various immobilized enzyme preparations, although there was a broadening in temperature activity profiles for immobilized enzyme preparations. Arica et al. [2] speculated that hydrophobic interaction and other secondary interactions of the immobilized enzyme might impair conformational flexibility necessitating higher temperature for its proper functioning. Similar results were also obtained by Khare and Gupta [20], when β-galactosidase from *E. coli* was conjugated with Con A. Tanriseven and Dogan [35] made an observation that β-galactosidase from *A. oryzae* had an optimal activity at 50°C and upon immobilization in alginate–gelatin fibers its optimum temperature was not affected.

Thermal stability

Figure 3 shows thermal stability of free as well as immobilized forms of α-galactosidase. All forms of α-galactosidase were stable at 65°C for 30 min. After 1 h, a decrease in enzyme activity of 24 and 36% of soluble entrapped and free α-galactosidase was observed, respectively (Fig. 3). Whereas, crosslinked Con A-α-galactosidase entrapped complex exhibited gradual loss of activity (38% after 360 min). Enhanced enzyme stability is one of the important features of the crosslinked Con A-α-galactosidase entrapped complex. It is mainly due to stabilization of glycoenzyme as a result of binding to Con A. It binds specifically to certain structures found in various sugars, glycoproteins, and glycolipids, mainly internal and nonreducing terminal alpha-mannosyl groups [20, 30]. The preparation obtained by the immobilization of *Aspergillus niger* glucose oxidase on the Con-A matrix was more stable than the soluble enzyme and the enzyme coupled via amino groups to the cellulose against urea, guanidine hydrochloride, heat, and pH-induced inactivation [31]. Crosslinking of the preparation resulted in a further increase in stability, although at the expense of some catalytic activity [17]. *Aspergillus oryzae* α-galactosidase was immobilized on the surface of novel bioaffinity support concanavalin A-layered calcium alginate-starch beads. The maximum activity of this immobilized α-galactosidase was obtained at 60°C, approximately 10°C higher than that of the free enzyme [13]. β-Galactosidase from *E. coli* showed a marginal increase in thermal stability compared to the native enzyme. However, the Sephadex-bound galactosidase conjugate showed considerable enhancement in the thermal stability [20]. Immobilized α-galactosidase of *P. griseoroseum* maintained 100% activity after 24 h of incubation at 40°C, while free enzyme showed only 32% activity under the same incubation conditions [8]. Therefore, the higher stability of crosslinked Con A-α-galactosidase entrapped complex would be exploited in hydrolysis of soymilk where the chances of contamination is very much reduced.

Effect of galactose

Galactose is one of the products of α-galactosidase-catalyzed hydrolysis of the raffinose family of sugars. It is a well known fact that galactose inhibits α-galactosidase [1, 11]. Therefore, the effects of various concentrations of galactose (1.5–10%) on activity of soluble, calcium alginate entrapped and cross-linked Con A-α-galactosidase entrapped in calcium alginate preparation was studied (data not shown). Incubation of α-galactosidase in 5% galactose for 1 h at 65°C resulted in a loss of nearly 60% of all initial activity while cross-linked Con A-α-galactosidase complex entrapped in calcium-alginate retained over 80% of the initial activity. Generally, galactose is a competitive inhibitor for many α-galactosidases [11]. However, inhibition studies on α-galactosidase of *M. vinacea* by D-galactose was found to

Table 2 Km and Vmax of free and immobilized (crosslinked con A-α-galactosidase complex entrapped in calcium alginate) α-galactosidase from *Aspergillus terreus*$_{GR}$

	PNPG*		Raffinose	
	Km (mM)	Vmax (Uml^{-1})	Km (mM)	Vmax (Uml^{-1})
Soluble	0.11	7.2	0.42	16.33
Immobilized	0.84	9.40	1.40	20.16

All the experiments were performed independently in triplicate and the results given here are the mean of three values. The standard deviation for all the values was <±5%

* PNPG (*p*-nitrophenyl α-D-galactopyranoside)

be of mixed type, suggesting both competitive and non-competitive binding of D-galactose to the enzyme [33]. D-galactose, D-glucose, melibiose, and raffinose act as competitive inhibitors of α-galactosidase from *A. niger* [28].

Kinetic parameters

Table 2 shows the Km and Vmax values of soluble and crosslinked Con A-α-galactosidase entrapped in ca-alginate. A Lineweaver–Burk plot revealed that soluble enzyme had 0.11 mM and 7.2 Uml^{-1} of Km and Vmax, respectively, for PNPG. Immobilized preparation showed higher Km (0.84 mM) for PNPG as well as for raffinose, which may be because that after immobilization, the substrate is not easily accessible to the enzyme. Bodalo et al. [4] have similarly reported the increase in Km values from 0.28 to 0.44 mM and decrease in Vmax from 295 to 18.58 U/mg protein upon immobilization of α-galactosidase.

Hydrolysis of soymilk by repeated batch

Thin layer chromatography (data not shown) reveals the complete hydrolysis of the raffinose family of oligosaccharides in soymilk after 12 h of incubation. Reduced hydrolysis in soluble entrapped α-galactosidase may be attributed to leakage of the enzyme due to high porosity of alginate beads. It is known that glucose oxidase retained very high enzyme activity when insolubilized by using Con A and polyclonal antibodies [18]. Haider and Husain [14] found that entrapped crosslinked Con A–α-galactosidase was more efficient in the hydrolysis of lactose present in milk (77%) and whey (86%) in batch processes compared to the entrapped soluble α-galactosidase. These reports suggested that the insoluble Con A are quite porous and active sites of the enzyme were easily accessible to the substrate. Hydrolysis of soymilk oligosaccharides by soluble enzyme was 55% and by immobilized Con-A-α-galactosidase entrapped preparation in ca-alginate 48% after 1 h of incubation.

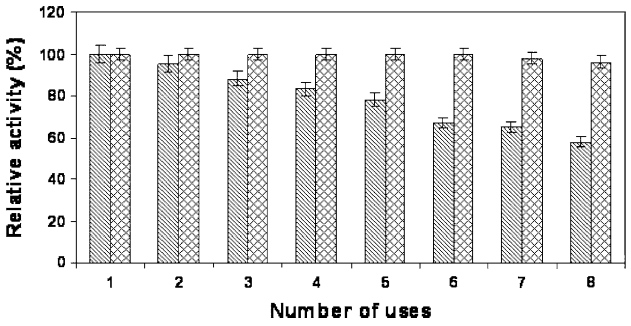


Fig. 4 Reusability of calcium alginate-entrapped α-galactosidase. Enzyme activity of entrapped (▨) and crosslinked Con A-α-galactosidase entrapped in calcium alginate (▩). All the experiments were performed independently in triplicate and the results given here are the mean of three values. The standard deviation for all the values was <±5%

Fluidized bed reaction for the continuous degradation of RFO in soymilk

To explore the feasibility of using the immobilized enzyme in continuous mode, a fluidized bed reactor was constructed and soymilk hydrolysis was studied at different flow rates at 65°C. The raffinose family of oligosaccharides was determined by TLC after passing through the reactor at different flow rates. The oligosaccharide reduction in soymilk was 85, 72, 68, 49, and 32% for 40, 80, 120, 160, and 200 ml/h flow rates. The results from continuous reactor suggest that a low flow rate (40 ml/h) i.e., higher retention time gave a higher percent degradation. However, with an increase in the flow rate percent, degradation was reduced proportionally. Hydrolysis to the extent of 97% was noted at a flow rate of 151 per day, whereas 75–80% conversion was obtained [12]. Therefore, immobilization of α-galactosidase with Con A is proved to be suitable for the hydrolysis of soymilk oligosaccharide.

Repeated batch experiments

Figure 4 shows repeated use of entrapped and crosslinked Con A-α-galactosidase entrapped in Ca-alginate complex. After eight cycles, crosslinked Con A-α-galactosidase-entrapped complex retained 95% of its activity, whereas soluble entrapped α-galactosidase retained only 51% of its hydrolytic activity.

Conclusions

Con A-conjugated alpha-galactosidase from *Aspergillus terreus*$_{GR}$ showed increased thermostability. Immobilized enzyme complex can be repeatedly used for eight numbers of cycles without any leakage of the enzyme. This enzyme complex can be used in the food industry for hydrolysis of

oligosaccharides in soymilk and the matrix used is also non-toxic. This immobilized enzyme preparation was found to be superior in terms of immobilization yield and retention of enzyme activity both in continuous and batch processes. The results have shown that entrapped cross-linked Con A-α-galactosidase preparation could successfully be employed in a reactor for the continuous hydrolysis of RFO present in soymilk.

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