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Degradation of Raffinose Oligosaccharides in Soymilk by Immobilized α-Galactosidase of *Aspergillus oryzae*

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Abstract α-Galactosidase was immobilized in a mixture of *k*-carrageenan and locust bean gum. The properties of the free and immobilized enzyme were then determined. The optimum pH for both the soluble and immobilized enzyme was 4.8. The optimum temperature for the soluble enzymes was 50°C, whereas that for the immobilized enzyme was 55°C. The immobilized enzyme was used in batch, repeated batch, and continuous modes to degrade the raffinose-family sugars present in soymilk. Two hours of incubation with the free and immobilized α-galactosidases resulted in an 80% and 68% reduction in the raffinose oligosaccharides in the soymilk, respectively. In the repeated batch, a 73% reduction was obtained in the fourth cycle. A fluidized bed reactor was also designed to treat soymilk continuously and the performance of the immobilized α-galactosidase tested at different flow rates, resulting in a 90% reduction of raffinose-family oligosaccharides in the soymilk at a flow rate 40 ml/h. Therefore, the present study demonstrated that immobilized α-galactosidase in a continuous mode is efficient for reducing the oligosaccharides present in soymilk, which may be of considerable interest for industrial application.

Keywords: *Aspergillus oryzae*, *k*-carrageenan, locust bean gum, α-galactosidase, soymilk

Soybeans are a legume crop and excellent food and feed source all over the world. Soybean protein also has a well-balanced amino acid pattern [25], and since soybeans do not include lactose, soymilk is a low-cost dairy-milk substitute for the lactose-intolerant population [8]. Soymilk is a raw material, which industrially serves as a base material for a variety of beverages and the production of proteinacious foods [25]. Yet, soy usage has been limited, as its antinutritional compounds diminish its nutritive value and wider acceptance. Flatulence-causing oligosaccharides are one such antinutritional factor [26], resulting from the lack of α-galactosidase in the human intestinal tract, which is required for the hydrolysis of these oligosaccharides. This is of particular concern in the development of soymilk for infant feeding [8].

α-Galactosidase (E.C.3.2.1.22) is widely distributed in microorganisms, plants, and animals, and hydrolyzes α-D-galactose from melibiose, raffinose, stachyose, verbascose, galactomannan, glycoprotein, ceramide trihexoside, and the higher homologs, as well as derivatives [14]. α-Galactosidase is used to degrade raffinose-family oligosaccharides in food and feed materials, such as soya meal or soymilk, and there have already been several reports on the use of α-galactosidases from plants [24], bacteria [12], and fungal sources [19, 20, 27] for the removal of raffinose-family sugars from soymilk. α-Galactosidase-treated soymilk is also widely accepted in diets for undernourished children, as well as lactose-intolerant individuals [8].

Aspergillus oryzae is a known source of α-galactosidase [11], and Cruz and Park [11] successfully demonstrated significant oligosaccharide removal from soymilk using a crude extract of *A.oryzae* α-galactosidase. Their results also suggest the possibility of using α-galactosidase in an immobilized form, which could lead to a process with the advantages of reusability and cost-effectiveness.

Polysaccharides (carrageenans, agarose, or alginate) form hydrogels under mild conditions and can be employed for enzyme entrapment for application in food product treatment. Several reports have already been published on the immobilization of α-galactosidase in calcium alginate [21], *k*-carrageenan [17], and a polyacrylamide gel [30, 31], although a polyacrylamide support is not useful in food processing because of its toxicity.

One of the most widely used immobilization materials is *k*-carrageenan, which is a linear and sulfated polysaccharide extracted from marine red algae with a primary structure made up of alternating α-(1,3)-D-galactose-4-sulfate and

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β-(1,4)-3,6-anhydro-D-galactose residues. *k*-Carrageenan forms a gel through a transition from a coil (disordered sol state) to a helix (ordered state), which is triggered by a reduction in temperature and/or ionic interactions [13].

The enrichment of *k*-carrageenan with locust bean gum (ratio of 2:1) provides a noticeable reinforcement of the gel strength, elastic texture, and prevention of syneresis [22, 28]. Locust bean gum is a galactomannan that includes the substitution of one mannose to four units of galactose. However, since this substitution is irregular, some locust bean gum regions are unsubstituted, and these mannose-free regions are then able to associate with the repeating helical structure of carrageenan dimers to form gels.

Accordingly, the present report investigates the immobilization of *A.oryzae* α-galactosidase in a gel matrix of *k*-carrageenan and locust bean gum for the treatment of soymilk, along with the extent of oligosaccharide removal using batch, repeated batch, and continuous modes of enzyme reaction.

MATERIALS AND METHODS

Microorganism and Cultivation

Aspergillus oryzae capable of producing extracellular α-galactosidase was isolated in the authors' laboratory. The active culture was produced through submerged cultivation in a chemically defined medium, as described by Prashanth and Mulimani [21]. The pH of the medium was adjusted to 5.5, and after autoclaving, the flask was inoculated with spores (2×10^6) of *A. oryzae*. The submerged fermentation was carried out in a 250-ml Erlenmeyer flask containing 50 ml of the culture medium that was incubated at 37°C for 5 days on an orbital shaker at 120 rpm. The mycelia were then separated from the culture broth by filtration through Whatman No. 1 filter paper and the filtrate used as the crude α-galactosidase solution. Acetone (1:1 v/v) was added to the crude enzyme solution and the mixture allowed to stand for 2 h at 0°C. The precipitated material that contained α-galactosidase was then recovered by centrifugation at 15,000 rpm for 20 min at 4°C. Thereafter, the precipitate was dissolved in a minimal amount of a 0.1 M acetate buffer (pH 4.8), and then dialyzed against water for 12 h at 4°C.

Enzyme Assay

The α-galactosidase was assayed using the method of Dey and Pridham [14]. One ml of the reaction mixture contained 0.1 ml of the suitably diluted enzyme, 0.8 ml of a 0.2 M acetate buffer (pH 4.8), and 0.1 ml of 2.0 mM *p*-nitrophenyl-α-D-galactopyranoside (PNPG). It was incubated at 37°C for 15 min. The reaction was arrested by adding 3 ml of a 0.2 M Na_2CO_3 solution and the absorbance read at 405 nm using a spectrophotometer (Elico Ltd, India). One unit of enzyme activity was defined as the amount of enzyme preparation required to liberate 1 μM of *p*-nitrophenol from PNPG per minute under the standard assay conditions. The activity yield represented the percent of the ratio of the enzyme activity of the immobilized enzyme to that of the soluble enzyme used for immobilization. In addition, the activity yield of the α-galactosidase immobilized in the gel matrix was also calculated.

Preparation of Soymilk

The soymilk was prepared according to the method of Mulimani and Ramalingam [20]. The soybeans were ground to flour and defatted with hexane (1:1 w/v). The fat-free soybean flour was then suspended in 10 volumes of distilled water and heated to boiling. The undissolved residue was separated from the soymilk by centrifugation for 5 min at 5,000 rpm. The supernatant, which contained the soymilk, was then stored at 4°C for a short period until further use.

Estimation of Oligosaccharides in Soymilk

Fifteen ml of soymilk was poured into 35 ml of absolute ethyl alcohol and centrifuged at 6,000 rpm for 15 min at 37°C. The centrifugate was then evaporated and redissolved in 15 ml of distilled water. The amount of sucrose, raffinose, and stachyose was estimated using the method of Tanaka *et al.* [29].

Separation of Oligosaccharides by HPLC

The HPLC analysis was performed using a Shimadzu (Shimadzu Corporation, Japan) equipped with an LC 10ATVP pump and refractive index detector. The sample injection was *via* a Rheodyne injector equipped with a 20 μl sample loop. The carbohydrates were separated on a Phenomenex Bondclone 10 μ CHO column (column size 300×3.9 mm). The mobile phase consisted of acetonitrile: water (70:30 v/v) for separation, and the flow rate was fixed at 1 ml/min. The chromatographic data were collected and plotted using Class-VP 6.1 software, and the peak identification of the chromatographs was performed by comparing the retention times with those of standards. The fructose, sucrose, raffinose, and stachyose were all purchased from Sigma Chemicals (St. Louis MO, U.S.A.).

Immobilization of α-Galactosidase

The ammonium sulfate-precipitated α-galactosidase was entrapped in a gel matrix of *k*-carrageenan and locust bean gum, and the entrapment carried out using the modified method of Audet *et al.* [5]. Ten ml (6.2 U) of the enzyme and 3 g of the gel matrix were dissolved in 60 ml at 4°C. This solution was then dropped at a constant speed into a solution containing cold 0.3 M KCl. The resulting beads were treated for 3 min with 1% glutaraldehyde. The beads were then filtered off, washed with sterile water, and stored at 4°C.

General Properties of Free and Immobilized α-Galactosidase

Optimum pH and Temperature. To determine the optimum pH, 100 µl of the suitably diluted (in a 0.2 M acetate buffer, pH 4.8) ammonium sulfate-precipitated enzyme was incubated with 100 µl of 2.0 mM PNPG in different buffers (800 ml) with a pH ranging from 3.6 to 6.2 for 15 min at 37°C. The following buffers were used: pH 3.6–6.2. Ten mg of beads containing the immobilized enzyme was then used instead of the free enzyme. The optimum temperature was determined by incubating a mixture of 100 µl of the diluted enzyme (10 mg of the immobilized beads), 800 µl of a buffer (0.2 M, pH 4.8), and 100 µl of PNPG (2 mM) for 15 min at different temperatures, ranging from 5° to 65°C.

Thermostability. To determine the thermal stability, the enzyme (10 mg of the immobilized beads) was incubated with the substrate without any stabilizer at 50°C for different incubation periods. The residual activity in each sample was assayed at pH 4.8 and 50°C.

Treatment of Soymilk with Enzyme

Batch Mode. Batch reactions were performed using both the free and immobilized enzyme for different incubation periods. Approximately 10 ml of the free enzyme (4.53 U/ml) was added to 60 ml of soymilk in a 250-ml Erlenmeyer flask. For the immobilized enzyme, approximately 220 mg (0.21 U/mg) of immobilized beads was added to 60 ml of soymilk. The hydrolysis reaction was carried out at 50°C in an incubator shaker (200 rpm) for different incubation periods of 2, 4, 8, and 12 h, and then samples of the reaction mixture were extracted and kept in a boiling water bath for 10 min to arrest the enzyme reaction. Finally, each sample was analyzed for degradation of the oligosaccharides. A control experiment was also performed in the same manner using a 0.2 M acetate buffer (pH 4.8).

Repeated Batch Mode

To establish the stability of the oligosaccharide degradation by the immobilized enzyme, repeated batch experiments were also carried out. An Erlenmeyer flask containing 60 ml of soymilk and 220 mg of the immobilized enzyme beads (0.21 U/mg) was kept in an incubator shaker (200 rpm) at 50°C. After every 4 h of incubation, an aliquot of the soymilk was taken out and the oligosaccharide concentration determined. The beads were then separated by filtration, washed with sterile water, and transferred into another new batch. The reaction was then carried out under identical conditions.

Continuous Mode with Fluidized Reactor

A continuous reaction with a fluidized bed reactor was carried out in a jacketed glass column (75 cm length and 1.5 cm diameter) with a bed volume of 150 ml. The jacket temperature was maintained at 50°C (water bath from Julabo, Germany). The soymilk feed solution preheated to 50°C in a water bath was introduced from the bottom of the column through a peristaltic pump (Amersham Pharmacia Biotech, Sweden) and the product withdrawn from the top. The gel matrix beads containing *A. oryzae* α-galactosidase were packed in the column. The upward substrate stream then fluidized the beads that filled the column. Different flow rates of 25, 50, 75, and 100 ml/h were used to degrade oligosaccharides in the soymilk. The outlet stream was continuously collected from the front end of the column in a container, and the effluent analyzed for the degradation of raffinose and stachyose.

RESULTS AND DISCUSSION

An inherent problem in enzyme immobilization is enzyme leakage from the beads, even at higher concentrations, resulting in a gradual loss of enzyme activity. Thus, to avoid such leakage, a 1% glutaraldehyde solution was used as a hardener, as Ates and Mehmetoglu [4] previously reported that treatment with a 1% glutaraldehyde solution stabilized the activity of β-galactosidase in alginate beads.

Effect of pH and Temperature on Free and Immobilized α-Galactosidases

The effect of pH on the soluble and immobilized enzyme is depicted in Fig. 1, where 4.8 was the optimum pH value for both. Mansour and Dawoud [18] also reported that an invertase from *Saccharomyces cerevisiae* showed optimum activity at pH 4.6, with no shift in the optimum pH for the celite and polyacrylamide immobilized enzyme. Godbole *et al.* [17] and Arruda and Vitolo [3] reported that invertase activity was dependent on the pH, and the optimum pH for immobilized invertase activity was 4.6 with no shift from the optimum pH for the soluble enzyme.

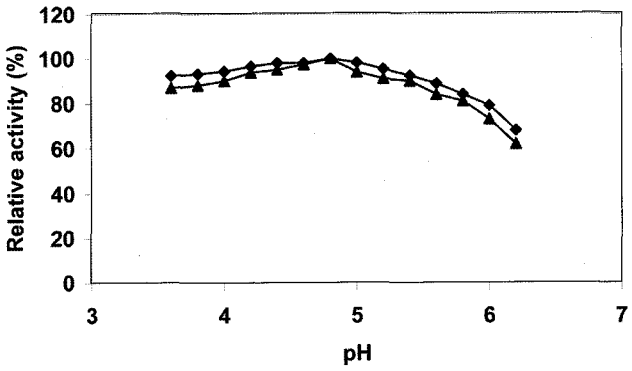

Fig. 1. Effect of pH on free (▲) and immobilized (◆) α-galactosidases.

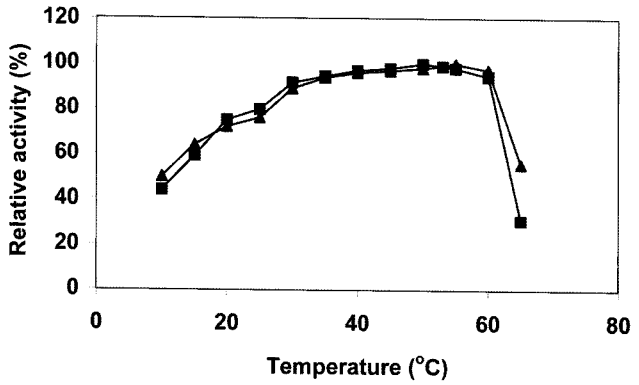

Fig. 2. Effect of temperature on free (■) and immobilized (▲) α-galactosidases.

In contrast, the optimum temperature for the activity of the immobilized enzyme shifted to a higher value than that for the soluble enzyme (Fig. 2). The maximum activity of the entrapped enzyme was obtained at 55°C as compared with 50°C for the soluble enzyme. Arica *et al.* [2] speculated that hydrophobic and other secondary interactions of immobilized enzymes may impair conformational flexibility, necessitating a higher temperature for the enzyme molecules to reorganize and attain a proper conformation as regards functioning and binding to a substrate. Thus, the current immobilization system of *k*-carrageenan and locust bean gum seemed to improve the thermal stability of the enzyme, which maintained its activity even at 55°C, which is appropriate, since a high temperature diminishes microbial contamination during the treatment of soymilk.

Fig. 3 depicts the effect of immobilization on the α-galactosidase stability at 50°C, where after 24 h, the immobilized enzyme retained 76% of its activity. Moreover, the half-life of both the soluble and immobilized enzymes at different temperatures is shown in Table 1, where the immobilized enzyme exhibited a higher thermal stability than the free enzyme, due to the reduced conformational

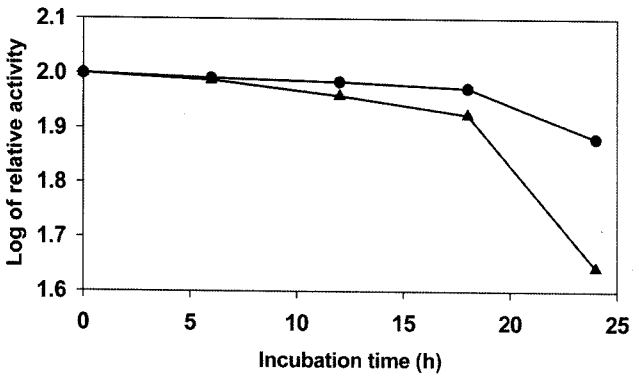

Fig. 3. Thermostability of free (▲) and immobilized (●) α-galactosidases.

Table 1. Half-life of free and immobilized α-galactosidases at different temperatures.

Storage temperature (°C)	Free enzyme ($t_{1/2}$)	Immobilized enzyme ($t_{1/2}$)
4	15 days	45 days
37	5 days	20 days
50	23 h	42 h
65	0.5 h	8 h

The calculated value of $t_{1/2}$, defined as the temperature at which 50% of the initial activity was retained at pH 4.8.

flexibility of the immobilized enzyme [10]. The greater stability of the immobilized enzyme may also have been due to the stabilizing effects of the immobilization [23].

Treatment of Soymilk

Batch Reaction. The hydrolysis of raffinose-family sugars by the soluble and immobilized α-galactosidase in the batch experiment is presented in Fig. 4. In the batch experiments, the soluble and immobilized enzymes were incubated with soymilk for different incubation periods of up to 4 h based on 30-min increments. An HPLC analysis of the soymilk treated with the enzyme for 4 h exhibited hydrolysis of the raffinose sugar components (Fig. 5), while 30 min and 1 h incubations resulted in 67% and 73% hydrolysis with the soluble enzyme and 55% and 61% hydrolysis with the immobilized enzyme, respectively. After 2 and 4 h incubations, the soluble α-galactosidase led to 80% and 88% degradation of the raffinose oligosaccharides in soymilk, whereas the immobilized α-galactosidase resulted in 68% and 77% reductions, respectively. Thus, the soluble enzyme showed a better percent of degradation than the immobilized enzyme, possibly due to a diffusional limitation with the immobilized enzyme (*i.e.*, resistance of the substrate to diffuse into the immobilization matrix and resistance of the products to diffuse out) [1]. Ninety-two % hydrolysis

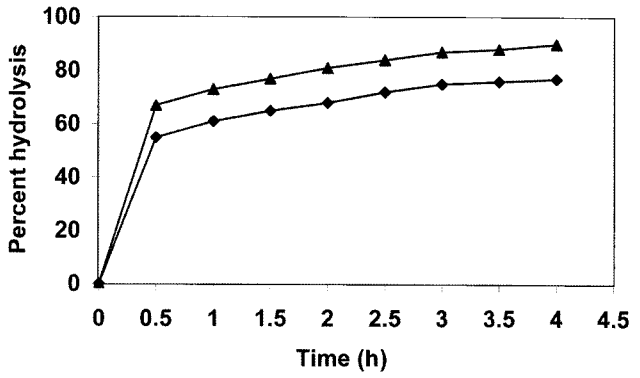

Fig. 4. Percent hydrolysis of raffinose-family sugars after treatment with free (▲) and immobilized (◆) α-galactosidases in soymilk for different incubation periods.

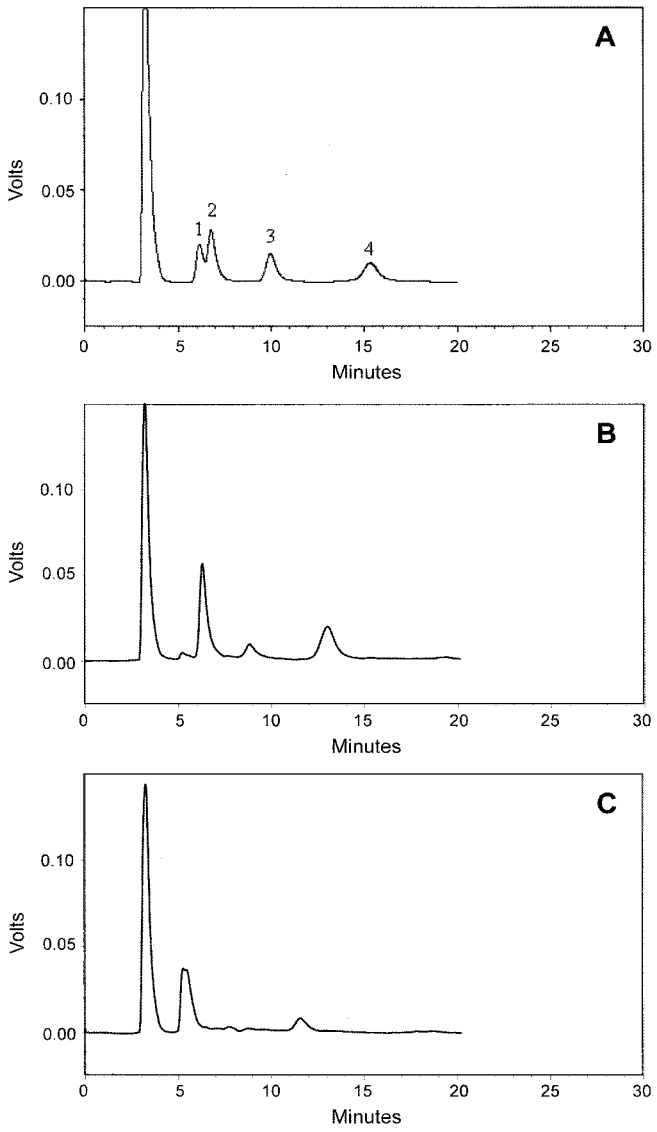

Fig. 5. HPLC separation of *A. oryzae* α-galactosidase-treated soymilk.
A. Authentic sugars. 1, Galactose; 2, Sucrose; 3, Raffinose; 4, Stachyose. **B.** Soymilk before α-galactosidase treatment. **C.** Soymilk after α-galactosidase treatment.

of the raffinose sugars in soymilk by immobilized α-galactosidase after 12 h is the highest reduction so far among previous reports [15, 17]. Yet, interestingly, 80% of the oligosaccharide hydrolysis occurred within 2 h of incubation with the soluble enzyme, indicating that prolonged incubation did not significantly increase the hydrolysis. The hydrolysis after 2 h was very slow, probably due to substrate depletion or product inhibition [27]. Thananunkul *et al.* [30] previously reported 50% hydrolysis, whereas Thippeswamy and Mulimani [31] reported 71% hydrolysis after incubating soymilk for 12 h with α-galactosidase immobilized in polyacrylamide.

Repeated Batch Reaction

The operational stability of the immobilized α-galactosidase was also evaluated in a repeated batch process, and the results indicated that the catalytic activity of the immobilized enzyme remained resilient after repeated use. As such, the immobilized enzyme was able to maintain 52% hydrolysis even after 5 cycles, with 76% hydrolysis after a 4-h incubation for the first cycle, followed by 74%, 71%, 67%, and 64% hydrolysis for the second, third, fourth, and fifth cycles, respectively. Even after 5 cycles, there was no drastic decrease in the hydrolysis percent, possibly due to the prevention of enzyme leakage with the glutaraldehyde-treated beads. Ates and Mehmetoglu [4] also found that treatment with glutaraldehyde allowed a Cu-alginate-immobilized enzyme to be used 8 times with high activity, whereas β-galactosidase immobilized in PVA was used 30 times for the hydrolysis of lactose in an acetate buffer and 8 times in whey [7].

Continuous Reaction

The use of an immobilized enzyme makes it economically feasible to operate an enzyme process in a continuous mode. Thus, a fluidized bed reactor was constructed to explore the practicability of using the current immobilized enzyme in a continuous system. Bodalo *et al.* [9] previously suggested that the final choice of a derivative for use in fluidized bed reactors should be based not only on the enzyme activity but also on the hydrodynamic behavior of the support. Moreover, for the operation of a fluidized reactor, small-sized beads help to minimize the mass transfer resistance. When the continuous degradation of oligosaccharides in soymilk was carried out in a fluidized bed reactor at 50°C, the oligosaccharide reduction in the soymilk was 90%, 77%, 73%, 51%, and 37% with a flow

Table 2. Summary of α-galactosidase immobilized on various matrices for soymilk raffinose oligosaccharide hydrolysis.

Method	Immobilized matrix	Flow rate (ml/h)	Percent hydrolysis	References
Entrapment	Polyacralamide	30	60	[30]
	Polyacralamide	25	84	[31]
	Calcium alginate	40	90	[21]
	k-Carrageenan	30	92	[17]
	k-Carrageenan and locust bean gum	40	90	This study
Cross-linking	Chitosan	60	92	[15]

rate of 40, 80, 120, 160, and 200 ml/h, respectively, suggesting that a low flow rate (40 ml/h), *i.e.*, higher retention time, led to a higher percent of degradation (90%). The optimal flow rate is an important parameter for the effective operation of a fluidized column reactor. A comparison of the raffinose oligosaccharide hydrolysis of soymilk by α-galactosidase immobilized on various matrices was also conducted (Table 2).

A. oryzae is considered as an excellent host for the safe production of harmless products [6]. In addition, the immobilization of the enzyme in *k*-carrageenan and locust bean gum is simple, its subsequent use is comparatively safe and cheap, and the enzyme activity is robust. The immobilized α-galactosidase in the present study showed a high optimum temperature, diminishing microbial contamination during the treatment of soymilk, and also demonstrated a better operational stability, remaining stable even after 5 cycles, when compared with the soluble enzyme. This high stability of the preparation under common conditions is especially advantageous.

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