

Hydrolysis of galacto-oligosaccharides in soymilk by immobilized α -galactosidase from *Chaetomium madarasense* AUMC 9376

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Abstract

*α -galactosidase 3.2.1.22 (α -Gal) enzyme was produced from *Chaetomium madarasense* AUMC 9376 under solid state fermentation using banana peels as substrate. Optimal α -Gal production was obtained at initial pH 5 and, 40°C then the enzyme was completely purified resulting in 25.32 folds purification (1561.67 U/mg). The enzyme was immobilized in technical grade sodium alginate and gelatin. The characteristics of the free and immobilized enzyme were also investigated. The optimum temperature for both enzymes was 50°C and 60°C, respectively, while, the optimum pH for both was 5. Besides, the pH stability and thermal stability of the free and immobilized enzyme were also studied. Immobilized α -Gal was more stable at high pH and temperatures than the soluble one. The results showed that the catalytic properties of the immobilized enzyme were improved. Moreover, HPLC chromatograms revealed that the immobilized enzyme has the ability to reduce raffinose and stachyose oligosaccharides in soymilk by 69.2 and 64.3%, respectively.*

Keywords: *Chaetomium madarasense*, α -galactosidase, immobilization, soymilk, oligosaccharides

Introduction

The α -galactosidase (α -D-galactoside galactohydrolase) are carbohydrases that catalyse hydrolysis of α -1,6-linked α -galactoside residual from simple oligosaccharides such as melibiose, raffinose, stachyose [1]. It plays an essential role in improving the nutritional value of legume based food and reduction of raffinose sugars that causes flatulence and also have various interesting applications in the pulp and paper industries, probiotic formulations, etc. [2].

Therefore various strategies have been employed to increase enzyme stability including genetic engineering, immobilization and operating in non-aqueous media [3]. The main aim of enzyme immobilization is the industrial re-use for many reaction cycles [4]. The immobilized enzymes is characterized over soluble enzymes arise from their enhanced stability and ease of separation from the reaction media which lead to significant savings in enzyme consumption [5, 6]. If the enzyme molecules are bonded to the supports by multipoint covalent attachment, these immobilized preparations are more resistant to any kind of distortion by different agents such as organic solvents or heating [5]. The composition, morphology, and surface characteristics of support material also play crucial roles in regulating the activity of immobilized enzyme; moreover, the supporting material should prevent enzyme aggregation or denaturation and maintain the native conformation of the

enzymes [7]. Affinity matrix [8], glass [9], magnetic beads [7], nano-materials [7, 10], Amberlite and Chitosan [11] are examples of support materials which have been used for enzyme attachment.

Raffinose and stachyose are α -galactosides of sucrose including three and four monomeric units respectively and are non-digestible in the gut due to the absence of α -galactosidase in the human intestinal mucosa, consequently, intact oligosaccharides pass directly into the lower intestine and are metabolized by bacteria that possess this enzyme, resulting in the production of gases [12]. Enzymatic hydrolysis of galacto-oligosaccharides by α -galactosidases may be an alternative way to improve the nutritional quality of soy products for animal and human consumption [13].

The aim of this study is to immobilize the purified microbial α -galactosidase produced under solid state fermentation and to evaluate the catalytic properties of the immobilized enzyme and the free one. Also, to eliminate the soymilk oligosaccharides by enzymatic treatment.

Materials and methods

Chemicals

P-Nitrophenyl- α -D-galactopyranoside (PNPG) was purchased from Sigma Chemical co., USA while, sephadex G-100 and DEAE sepharose were purchased from sigma Pharmaceutical Industries, Nasr City, Cairo, Egypt.

Microorganisms

Ten microbial isolates were screened for α -galactosidase production. The microbial isolates screened, *Aspergillus niger*, *A. terreus*, *A. ustus*, *Penicillium olsonii*, *P. citrinum*, *P. crustosum*, *Ulocladium sp.*, *Chaetomium sp.*, *Trichoderma viride*, and *T. harzianum*. These fungal species were isolated from soil in Cairo governorate and identified in Taxonomy Department, Ain-Shams University, Cairo city, Egypt. The identification of the most potent isolate was confirmed in Assiut University Mycological Centre and was identified as *Chaetomium madarasense* Natarajan AUMC 9376.

Solid state fermentation

The SSF was carried out using *Musa sapientum* (banana) peels. Ten gram of dry finely ground solid substrate was taken in 250 ml Erlenmeyer flasks and was moistened with 5 ml culture medium as follows (g l^{-1}) K_2HPO_4 , 1.0; $(\text{NH}_4)_2\text{SO}_4$, 1.0; MgSO_4 , 0.5 (1:2 w/v) and pH was adjusted to 5.5, then the flasks were autoclaved at 121°C for 15 minute. Each flask was inoculated with 2 ml of spore suspension (10^6 spores ml^{-1}) of each of the tested isolate and incubated in bench-top shaker at 30 °C for 6 days. The content of each flask was gathered up and thoroughly mixed with 10 ml cooled sterilized distilled water, then the mixture was filtered through a double-layered cotton wool and was centrifuged at 10,000 rpm for 10 min. The enzyme activity was determined in the supernatant.

Enzyme assay

Enzyme activity was assayed according to the method of [14]. One ml of reaction mixture containing 0.1 ml of supernatant, 0.8 ml of 0.2 M acetate buffer (pH 4.8) and 0.1 ml of 2mM of *p*-nitrophenyl- α -D-galactopyranoside (PNPG), and incubated at 40°C for 15 min. The reaction was terminated by adding 3 ml of 0.1 M sodium carbonate. *P*-nitrophenol (PNP) was quantified spectrophotometrically at $\lambda 405$ nm. One unit (U) of α -Gal activity is defined as amount of enzyme liberating 1 μmol of PNP in 1 min under assay conditions.

Protein assay

Total protein content in the supernatant was determined by the method described by [15] using bovine serum albumin as standard.

Optimization conditions

Optimum initial pH for the enzyme production was determined by adjusting the pH of the previous medium to varying ranges (3.0, 4.0, 5.0, 6.0, 7.0, and 8.0). The effect of incubation temperature on α -galactosidase biosynthesis was also investigated by incubating the inoculated standard fermented medium at different temperatures including 20°C, 30°C, 40°C, 50°C and 60°C. The optimal initial moisture content for α -galactosidase production was investigated by adjusting the initial moisture content of the fermentation substrates to varying levels (60, 70, 80, 90 and 100%). Medium enrichment with some natural additives, i.e., yeast, malt and beef extracts, peptone and corn-steep liquor 0.05% (w/v) was also tested, and the levels of enzyme production in the enriched media were statistically compared to that of the banana peels medium.

α – galactosidase complete purification

The enzyme purification process was started from the culture supernatant. 200 ml of cooled culture supernatant was dialysate in Sigma dialyzing bags against distilled water for 24 h. The obtained cell free dialyzed was precipitated overnight with 60% isopropanol, then centrifuged and dissolved in 5 ml acetate buffer (0.2 M, pH 6.0), dialyzed overnight against the same buffer. The resulting precipitate was fractionated on a DEAE-Sepharose ion-exchange column previously equilibrated with the same buffer. The flow rate was adjusted to 3.0 ml/3min, and 3.0 ml fractions were eluted with the equilibrium buffer at 4 °C. The material retained by the column was eluted with a linear NaCl gradient (0 - 1 M). α -galactosidase activity was determined in each fraction, and the fractions that showed the highest activities were dialyzed against deionized distilled water for 24 h [16]. The previous fractions were separately concentrated and applied on a Sephadex G-100 affinity column equilibrated with diluted 1:4 (v/v) in 1 M citrate-phosphate buffer (pH 4.5) at 37°C. The flow rate was adjusted to 3.0 ml/3 min, and 3.0 ml fractions were eluted with the equilibrium buffer. The material retained by the column was eluted with the same buffer containing 100 mM lactose and 0.5M NaCl. The column elute was monitored at 280nm for protein and assayed for α -galactosidase activity.

Molecular mass determination

The molecular mass of the purified α -galactosidase was estimated in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by [17]. After electrophoresis, the gel was stained for 1 h with Coomassie Blue R250 dye in a methanol-acetic acid-water solution (4: 1: 5, by volume), then destained in the same solution without the dye.

Immobilization on alginate beads by Entrapment-cross linking technique

The enzyme was immobilized in technical grade sodium alginate which was purchased from Sigma and used as organic carrier (natural polysaccharide polymer) to immobilize purified α -galactosidase via entrapment by cross-linking and gelatin as described by [18]. Encapsulation: 2% sodium alginate solution was used for the preparation of the cationic solution; xanthan was dissolved in a 4% calcium chloride solution to give a final xanthan concentration of 0.4%. About 2.2 ml of the cationic solution was then added drop

wise to 50 ml of the sodium alginate solution. The capsules were rinsed with 100 mM acetate buffer pH 4.8 and hardened in 2% calcium chloride for 2 h, at 4 °C. The immobilization beads were rinsed again with acetate buffer and incubated in 1.25% glutaraldehyde for 30 min at 4 °C to activate the alginate beads. Beads were rinsed again with acetate buffer and stored in glycerol overnight. Prior to use for bioconversion trials, the immobilized enzyme was thoroughly rinsed with acetate buffer. The glutaraldehyde-activated alginate beads were placed into contact with α -galactosidase solution (1ml, 9.35 unit/g carrier) and the residual inulinase activity in the solution was assayed. Immobilized enzymes were kept under refrigeration. Then, they were sequentially with distilled water and acetate buffer. After these washes, the enzymes were immersed in 0.05 mol/L calcium chloride solution. Samples were taken periodically for protein content assay. The immobilization yield was calculated by the following equation:

$$\% \text{ imm yield} = \text{immobilized enzyme} / \text{total enzyme added}$$

Kinetics of the free and immobilized enzyme

The effect of pH values (3.0- 8.0) on the free and immobilized α -galactosidase activity was investigated using ONPG as substrate. The pH stability in the range 3.0-10.0 was determined after 30min of exposure at 55°C. The influence of the temperature on the activity of two enzymes was also determined using ONPG as substrate of the range from 25°C to 65°C. For the determination of thermal stability, the two enzymes were incubated for variable durations (15 to 90min) at fixed temperatures (50 to 65°C).

Storage stability of free and immobilized α -galactosidase under refrigeration

The immobilized α -galactosidase enzyme was stored in Tris buffer (pH 7.0) at 4°C. The storage stability of the immobilized enzyme was determined by measuring the residual activity of the enzyme at predetermined time interval of 5 days and consequently up to 32 days.

Operational stability of immobilized α -galactosidase

The operational stability of the immobilized α -galactosidase enzyme was determined by soaking 0.05g of the immobilized enzyme in 1.8ml phosphate buffer overnight. After the mixture was incubated at 55°C for 15min, the reaction was started by adding 0.2ml (1.5 mgml⁻¹) ONPG and then the reaction mixture was analyzed as above. The above experiment was repeated twelve times under the same conditions.

Treatment of soymilk by immobilized α -galactosidase and HPLC analysis

The enzymatic treatment of soymilk was performed by taking two ml of soymilk (pH 6) and 0.2ml of immobilized α -galactosidase (20 Uml⁻¹) and incubated for 2 hours. After the incubation, the reaction was arrested by adding 0.1ml of 0.3M barium hydroxide and 0.2ml 0.18M zinc sulfate. The resulted precipitation of protein was removed by centrifugation and the supernatant obtained was subjected to HPLC analysis. Sugar solutions extracted and purified (10 μ l) were analyzed by HPLC, Shimadzu Class-VPV 5.03 (Kyoto, Japan) equipped with refractive index RID-10A Shimadzu detector, LC-16ADVP binary pump, DCou-14 A degasser and Shodex PL Hi-Plex Pb column (Sc 1011 No. H706081), Guard column Sc-Lc Shodex, and heater set at 80°C. The mobile phase consisted of a gradient with 20% water at the beginning and 40 % water at 16 min with a flow rate of 1.0ml min⁻¹. The peak identification of the chromatographs was done by comparing the retention times with the standards.

Data analysis

The similarity matrices were done using Gel works ID advanced software UVP-England Program.

Statistical validation of treatment effects

The mean, standard deviation, T-3 replicates of the investigated factors and the control were computed according to the mathematical principles described by [19]. Results were considered highly significant, significant or non-significant where $P < 0.01$, > 0.01 and < 0.05 , > 0.05 , respectively.

Results*Compositional analysis of Musa sapientum peels*

The chemical composition of *Musa sapientum* peels is shown in Table 1. The percentage of protein, crude lipid, carbohydrate, crude fiber were 0.90, 1.70, 59.00 and 31.70, respectively.

Table 1. Composition analysis of *Musa sapientum* peels

Constituent	Dry weight (%)
Protein	0.90
Crude lipid	1.70
Carbohydrate	59.00
Crude fiber	31.70

Screening of isolates for α -galactosidase production

Table 2 displayed that different fungal species were tested for their capability to grow and produce α -galactosidase on banana peels as a rich organic source under solid state fermentation (SSF). The superiority of *Chaetomium madarasense* in α -galactosidase production (1561.67U/mg protein) compared to the other tested fungi is clear from Table 1. Thus, *Chaetomium madarasense* was chosen for further investigation.

Table 2. Screening of isolates for α -galactosidase activity

Isolate	Specific activity U/mg ml ⁻¹ protein
<i>Aspergillus niger</i>	43.6±0.2
<i>A. Terreus</i>	23.2±0.4
<i>A. Ustus</i>	34.5±0.12
<i>Penicillium olsonii</i>	55.1±0.23 ^b
<i>P. citrinum</i>	27.4±0.15
<i>P. crustosum</i>	32.6±0.21 ^b
<i>Ulocladium</i> sp.	38.5±0.22 ^b
<i>Chaetomium madarasense</i>	61.68±0.17 ^a
<i>Trichoderma viride</i>	22.7±0.18 ^a
<i>T. harzianum</i>	23.5±0.22 ^b

The same letters are nonsignificant ($P < 0.05$). Values are expressed as mean ± SD.

Optimizing of α -galactosidase production

It is clear from Table (3) that the highest enzyme production was at pH 5 and decreased at the two sides of optimum value. The effect of initial incubation temperature was also studied. The study revealed that the optimum temperature for α -Gal production is 40°C and the enzyme production was declined as the incubation temperature increased. The moisture level of the medium is a fundamental parameter for microbial growth and metabolite formation through solid state fermentation. The experimental results showed that 80% moisture content was sufficient for α -galactosidase production. The effect of various natural additives on α -galactosidase production is depicted in Table 3. The results revealed that the maximum enzyme production was obtained in the presence of peptone followed by yeast extract.

Table 3. Optimization conditions for α -galactosidase production

Parameter	α -Gal specific activity Umg ⁻¹ protein
Optimum pH value	5
Optimum temperature (°C)	40
Optimum moisture content (%)	80
Optimum natural additive	Peptone

α -galactosidase

purification

The different purification steps and recovery are shown in Table 4. The results indicate that after dialysis, a 5.03 purification fold with yields of 70.64 of the original activities obtained. Most of the α -galactosidase activities were applied onto DEAE-Sephrose and recovered in 80 fractions representing 47.19% of the original activity with a purification fold of 21.61. These fractions were pooled, lyophilized and subjected to further fractionation onto Sephadex G-100. This resulted in 25.32 purification fold with yields of 23.69% of the initial α -galactosidase.

Table 4. Purification scheme for α -galactosidase

Purification step	Total activity Uml ⁻¹	Total protein (mg ml ⁻¹)	Specific activity Umg ⁻¹ protein	Yield %	Purification fold
Crude enzyme	39.54	0.641	61.68	100.00	1.00
Dialysis	27.93	0.090	310.33	70.64	5.03
Cell free precipitate	22.34	0.032	698.12	56.50	11.32
DEAE-Sephrose	18.66	0.014	1332.86	47.19	21.61
Sephadex G-100	9.37	0.006	1561.67	23.69	25.32

Molecular mass determination

A protein band with a molecular mass of 86 kDa was observed on SDS-page electrophoresis (Fig.1).

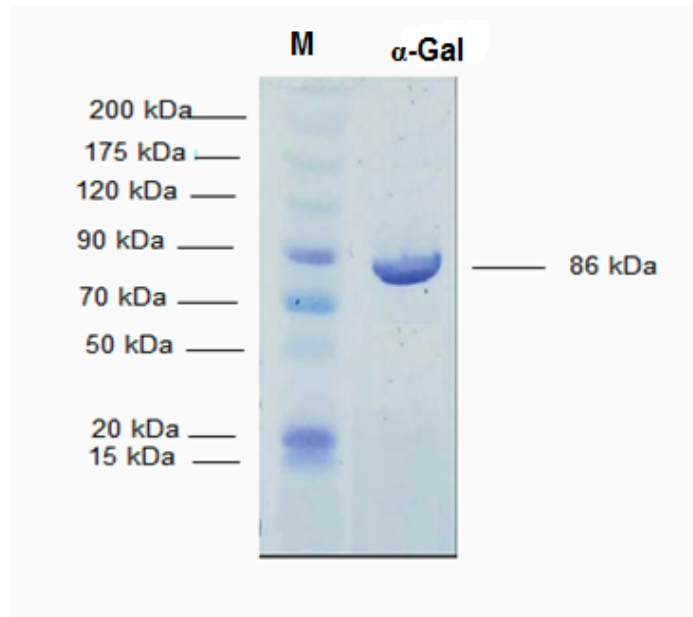


Fig 1. SDS-PAGE of the purified α -Gal with molecular mass of 86 kDa

The overall performance of the free and immobilized α -galactosidase by entrapment-cross linking technique

The results in Table 5 showed that the activity of the immobilized enzyme on the alginate beads reached maximum of 1846.66U/mg protein. The obtained enzyme activity was approximately 2.5 times values of the free one explained that the immobilization technique

Carrier	Form of enzyme	Protein mg ml ⁻¹	Activity Uml ⁻¹	Specific activity Umg ⁻¹ protein	Retained activity (%)	Amount of glucose mg ml ⁻¹
Alginate beads	Free α -galactosidase	0.006	9.35	1558.33	23.69	0.83
	Immobilized α -galactosidase	0.003/g carrier	5.54	1846.66	59.25	1.59

would favor higher adsorption capacity for the enzyme.

Table 5. The overall performance of the free and immobilized α - galactosidase by entrapment-cross linking technique

Characterization of free and immobilized α -galactosidase

Testing the effect of pH-dependence on the free and immobilized α - galactosidase, Fig. 2 revealed that the optimum pH for free and immobilized enzyme activity was 5. Moreover, the free enzyme retained 12% of its initial activity at pH 9 for 30 min, while, the immobilized enzyme retained 35% of its initial activity at the same interval (Fig. 3). With regard to the effect of incubation temperature, Fig. 4 showed that the optimum temperature for both enzymes activity is 50 and 60, respectively. It was clarified from Fig. 5 that

immobilized α -galactosidase was more stable at high temperatures, where the free enzyme retained 45 % of its activity at 50°C after 90 min and retained 30% of its activity at 65°C after the same time. While the immobilized one retained 70 % of its activity at 65°C after 90min of exposure.

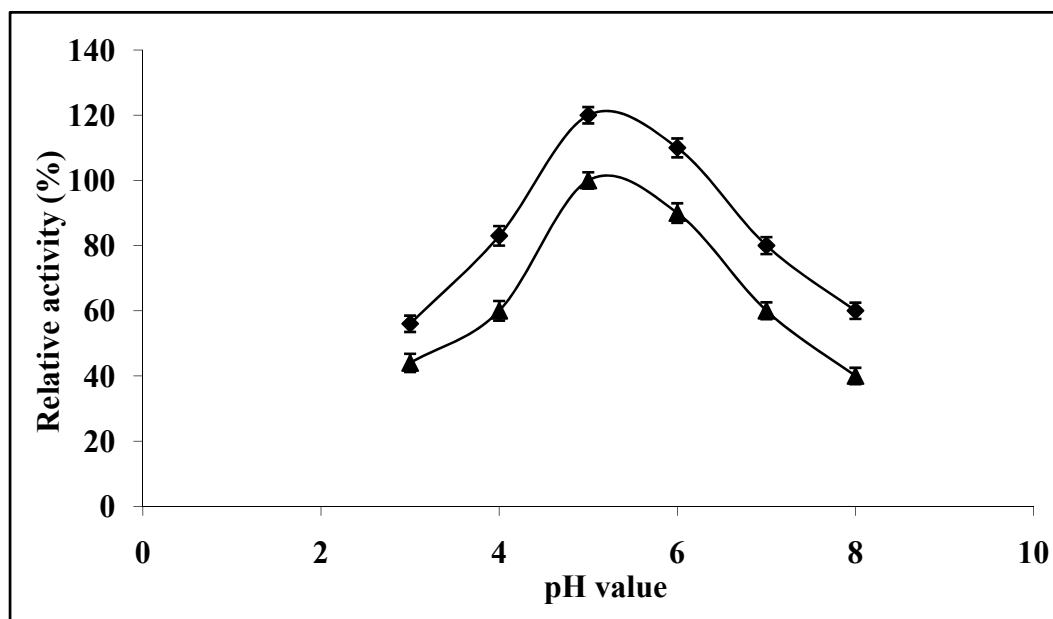


Fig 2. Effect of pH values on the free (-▲-) and immobilized (-◆-) α -Gal from *Chaetomium madarasense*

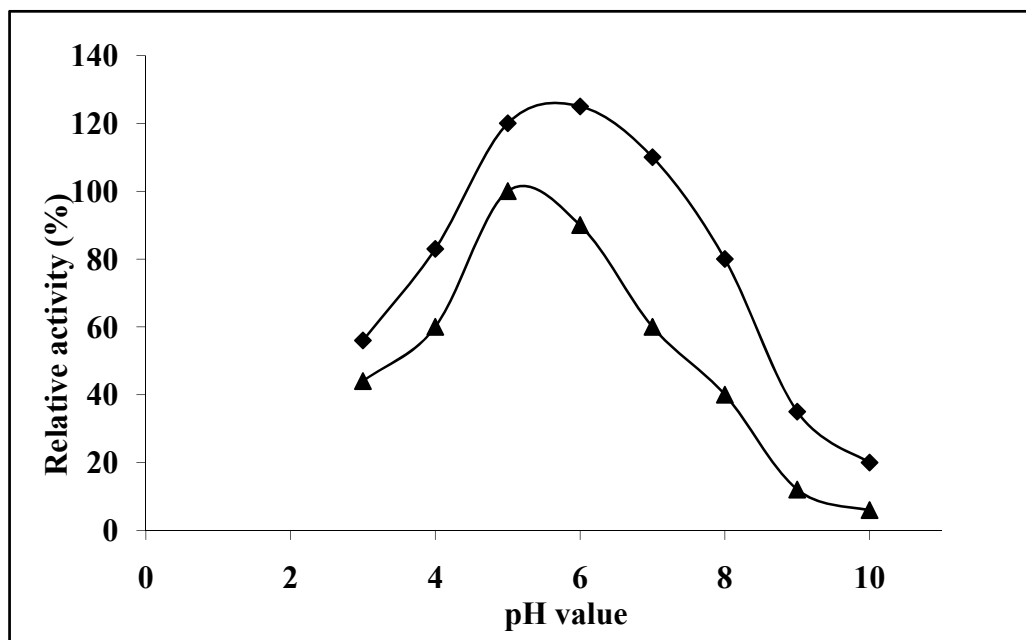


Fig 3. pH stability of the free (-▲-) and immobilized (-◆-) α -Gal from *Chaetomium madarasense*

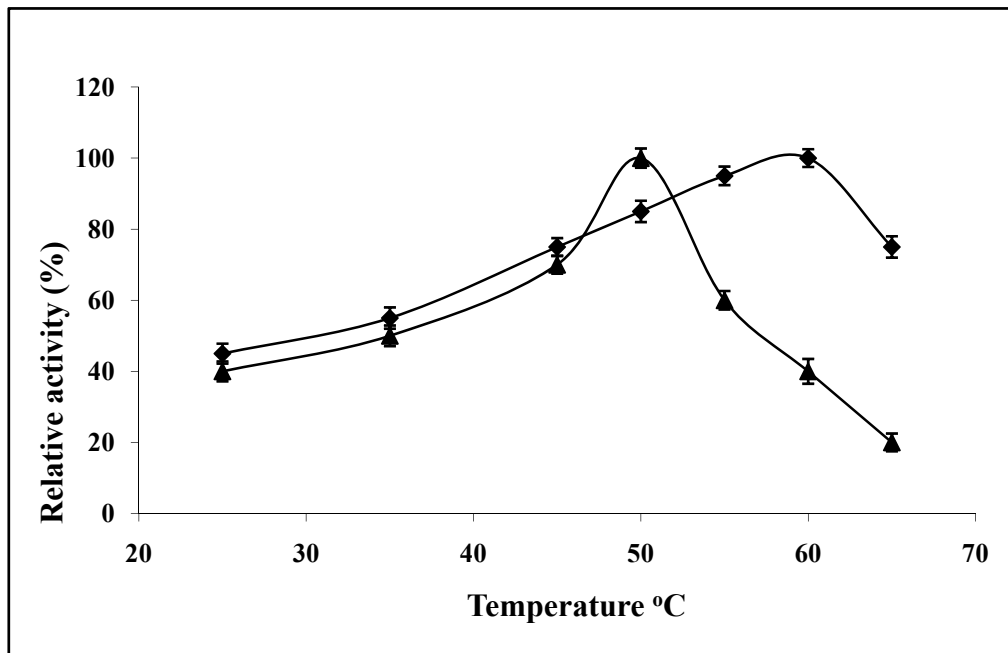


Fig 4. Effect of temperature on the free (-▲-) and immobilized (-◆-) α -Gal from *Chaetomium madarasense*

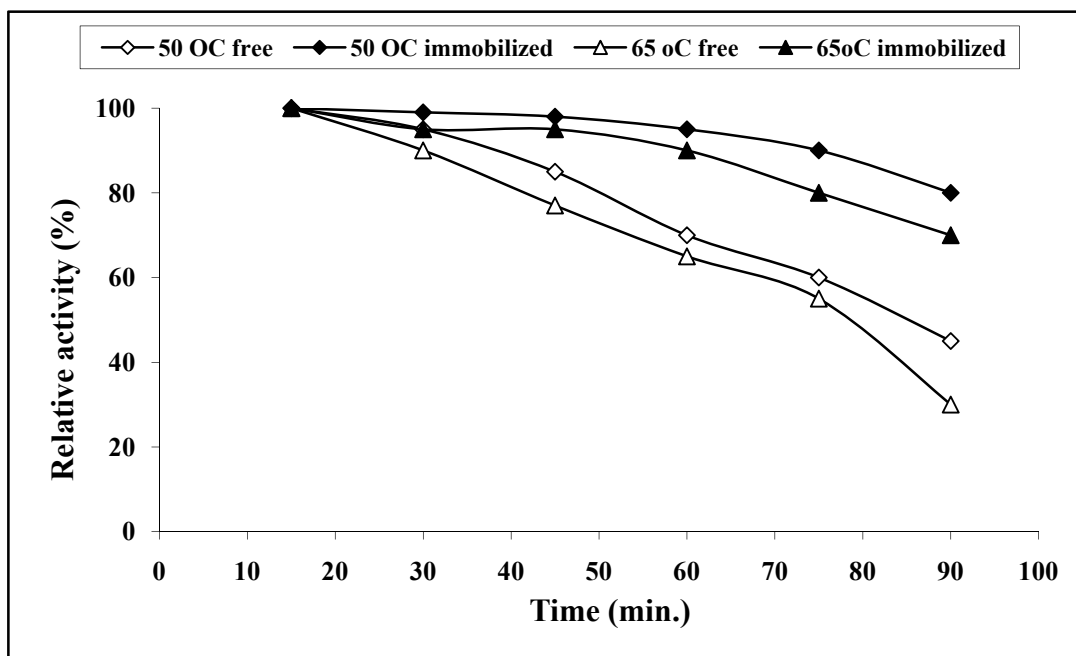


Fig 5. Thermal stability of the free and immobilized α -Gal from *Chaetomium madarasense*

Storage stability of α -galactosidase under refrigeration (4°C)

After 32 days of storage under refrigeration at 4 °C, the activity of the immobilized α -galactosidase decreased slightly from 97.7 to 88.0% (Fig. 6). While, there was a dramatic decrease in the activity of the free α -galactosidase from 90.2 to 58% units after 32 days. This result indicated that there are no ideal or easy storage conditions for free α -galactosidase.

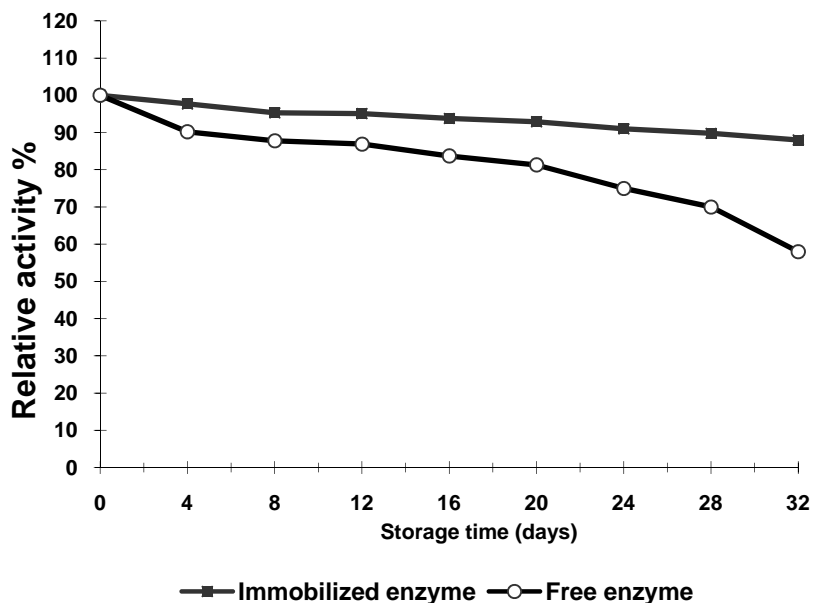


Fig 6. Storage stability of α -galactosidase under refrigeration (4°C)

Operational stability of immobilized α -galactosidase

The operational stability of the immobilized α -galactosidase was evaluated in a repeated batch process (Fig. 7). The experiment was repeated 12 times by using the procedures mentioned above with the same immobilized enzyme at the same initial concentration of ONPG. The results clarified that the immobilized α -galactosidase was used for 6 times without significant loss in activity and the hydrolysis efficiency declined distinctly and in the 12th cycle it achieved a value of about 14.6%. These results indicated that the bound enzyme could be used repeatedly for 8 cycles with slight appreciable loss in hydrolysis efficiency which reached average value of 62.3 %.

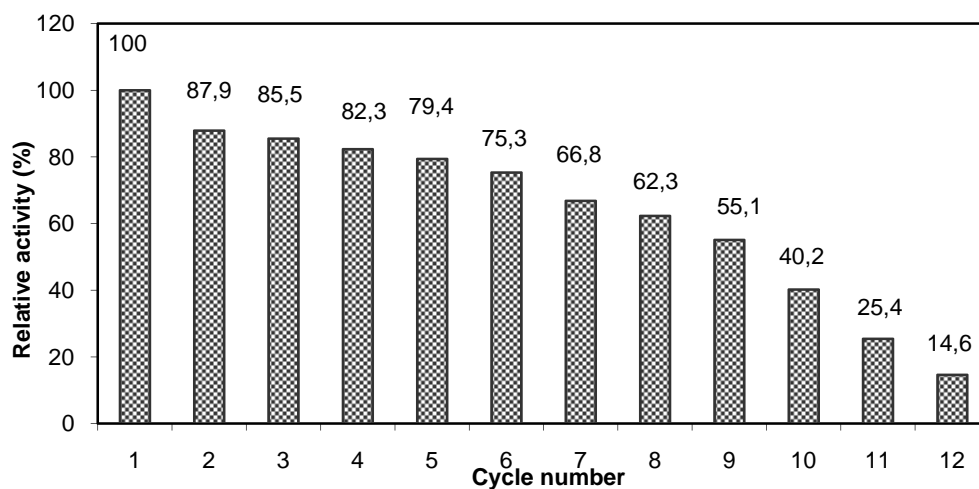
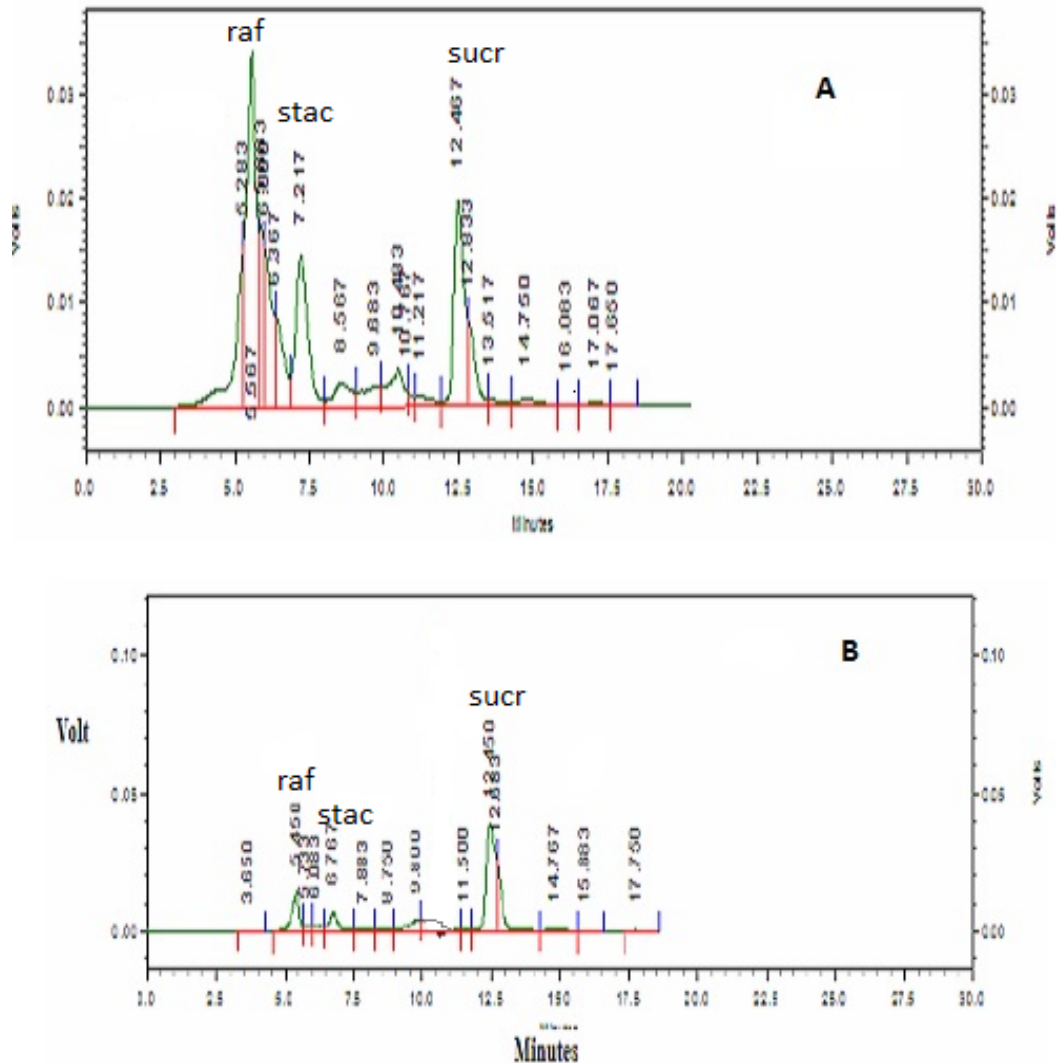


Fig 7. Operational stability of immobilized α - galactosidase*Sugar hydrolysis in soymilk*

It was clarified from Fig. 8 that soymilk oligosaccharides were reduced after treatment with immobilized α -galactosidase, where, raffinose and stachyose were reduced from 3.9 to 1.2 and from 6.17 to 2.2 mg/100 ml resulting in 69.2 and 64.3%, respectively.

**Fig 8.** Chromatograms profiles of oligosaccharides detected using HPLC method from soymilk

- A. Levels of oligosaccharides before enzyme treatment
B. Levels of oligosaccharides after enzyme treatment

Discussion

The previous results showed the superiority of *Chaetomium madarasense* AUMC 9376 in α -galactosidase productivity. In this manner, [20] stated that *Aspergillus parasiticus* is a new source for α -galactosidase production. Moreover, [21] found that a thermostable α -galactosidase was produced from *Thielavia terrestris*.

The temperature control is one of the factors that affect the fermentation process. In this respect, [22] found that the optimum temperature for β -galactosidase production by *A. oryzae* is 30°C. While, α -galactosidase produced from *A. terreus* was optimum at 60°C [23]. In respect of the effect of pH on α -galactosidase production, it is worthy to note that the optimum pH for α -galactosidase production was 5. This result is in accordance with [23] who found that the optimal α -galactosidase production by *A. terreus* was achieved at pH 5. While, [24] reported that the highest β -galactosidase produced by *Cluveromyces marxianus* was at pH 5.5. The initial moisture content is a crucial factor affecting the formation of metabolites through solid state fermentation. In this respect, [25] found that 80% moisture content was appropriate for solid state fermentation. Also, [22] reported that 90% moisture content was sufficient for β -galactosidase production by *A. oryzae*. The effect of some natural additives was also studied, in this context; [26] stated that peptone was found to be the best protein source for promoting the α -galactosidase enzyme production, while, ammonium nitrate was found to be a better for α -galactosidase production from *A. niger* [27]. With referring to enzyme purification, [21] found that The final purification fold of the α -galactosidase enzyme from *Thielavia terrestris* was 30.42 with recovery of the original activity of 27.17%. The molecular weight of the free purified enzyme obtained (86 kDa) was similar to that of *Humicola* sp. α -galactosidase (87.1 kDa) [28], while, the molecular weight of *Pleurotus florida* enzyme was 99 kDa (29).

The free and immobilized enzyme characters were also studied. Regarding to the effect of pH and pH stability, [30] found that The optima pH for both soluble and immobilized enzyme activities were found to be 6.5 and they added that the immobilized enzyme derivative was slightly more stable at higher pH so, the soluble and the immobilized enzyme retained 15% and 25%, respectively, of its initial activity at pH 8.0. In respect of incubation temperature effect, [29] reported that alpha-galactosidase was optimally active at 55°C; moreover, [30] reported that the soluble β -galactosidase lost all activity after 10 h of incubation at 35°C whereas the immobilized one retained 47% of its initial activity at the end of one day of incubation. The increased stability of the immobilized enzyme should be attributed to a reduction in the protein structure mobility, due to anchorage to the support promoted by the covalent bonds and subsequent translation of the rigidity at each anchorage point to the whole enzyme structure, thus protecting it from damaging effects of the environment [31].

With referring to the operational stability of the immobilized enzyme, [30] stated that, the immobilized β -galactosidase from *Kluyveromyces lactis* was successively reutilized for 20 cycles at 25°C and retained approximately half of its initial activity at the end the enzymatic reaction. This decrease may be caused by the mass loss of the enzymatic derivative during the washing procedure, while, the immobilized β -galactosidase from *A. oryzae* were used for 6 times without significant loss in activity [32].

With respect to reducing the flatulence factors in soymilk by enzymatic treatment, there are several reports on the use of partially purified α -galactosidase from *A. awamori* [33, 34], *A. oryzae* [35] for hydrolysis of oligosaccharides of soymilk.

Conclusion

This study presenting data supporting the improvement of the catalytic properties of the immobilized α -galactosidase and treatment of soymilk by the immobilized enzyme to decrease some oligosaccharides which lead to strong fermentation flavor.

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