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α-Galactosidase from *Bacillus megaterium* VHM1 and Its Application in Removal of Flatulence-Causing Factors from Soymilk

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A bacterial strain capable of producing extracellular α-galactosidase was isolated from a sample of sugarcane industrial waste. Microbiological, physiological, and biochemical studies revealed that the isolate belonged to *Bacillus* sp. Furthermore, based on a 16S rDNA sequence analysis, the new isolate was identified as *Bacillus megaterium* VHM1. The production of α-galactosidase was optimized based on various physical culture conditions. Guar gum and yeast extract acted as the best carbon and nitrogen sources, respectively. The optimum pH was 7.5 and the enzyme remained stable over a pH range of 5–9. The enzyme was optimally active at 55°C and thermostable with a half-life of 120 min, yet lost 90% of its residual activity within 120 min at 60°C. One mM concentrations of Ag^2, Cu^2, and Hg^{2+} strongly inhibited the α-galactosidase, whereas the metal ions Fe^2, Mn^{2+}, and Mg^{2+} had no effect on the α-galactosidase activity, and Zn^{2+}, Ni^{2+}, and Ca^{2+} reduced the enzyme activity slightly. When treated with the *B. megaterium* VHM1 enzyme, the flatulence-causing sugars in soymilk were completely hydrolyzed within 1.5 h.

Keywords: *Bacillus megaterium*, α-galactosidase, 16S rDNA analysis, raffinose family oligosaccharides (RFOs), soymilk

α-Galactosidase (α-D-galactoside galactohydrolase; E.C. 3.2.1.22) catalyzes the hydrolysis of α-1,6-linked galactoside residues from simple oligosaccharides, such as melibiose, raffinose, and stachyose and the polysaccharides of galactomannans, locust bean gum, and guar gum. It also acts on glycoconjugates, glycoprotein, and glycosphingolipids and is known to catalyze transglycosylation reactions, especially with higher substrate concentrations [31]. Thus, interest in this enzyme stems from its potential biotechnological and medicinal applications. Currently, the most important industrial applications of α-galactosidase are related to the beet sugar industry, pulp and paper industry, soya food processing, and animal feed processing [3, 25, 27, 34]. Moreover, it is also used in blood group transformation, the treatment of Fabray's disease, and xenotransplantation [8, 23].

Among protein sources, soy products have an excellent nutritional status based on their high protein content, plus soy proteins contain most of the essential amino acids needed to meet biological requirements. Soybeans typically contain 9–12% total sugar, including 4–5% sucrose, 1–2% raffinose, and 3.5–4.5% stachyose [12]. Thus, soymilk is considered as an economical substitute for cows' milk, especially in developing countries, and an ideal nutritional supplement for lactose-intolerant people, as soybeans do not contain lactose [4, 33]. Soya-based foods are also promising supplements to overcome existing protein calorie malnutrition problems [12]. Nonetheless, the widespread use of soya has been limited owing to certain antinutritional compounds that diminish its nutritional value; for example, the production of flatulence in the human small intestine due to a lack of α-galactosidase in the digestive juices. This necessitates the hydrolysis of raffinose and stachyose, which contain α-galactosidic linkages. Raffinose and stachyose, otherwise known as the raffinose family of oligosaccharides (RFOs), are relatively large molecules and not absorbed by the intestinal wall. Therefore, these oligosaccharides enter the large intestine intact, where they are metabolized by the microflora into carbon dioxide, hydrogen, and methane, leading to flatulence [4, 34]. Thus, the removal of oligosaccharides from soya-based foods would significantly improve their nutritive value.

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A literature survey only revealed a few reports on the use of α-galactosidase for reducing oligosaccharides in soymilk [10, 17]. Accordingly, this study isolated a novel bacterial strain, *B megaterium* VHM1, optimized the culture conditions for the production of α-galactosidase, and used the α-galactosidase for treating soymilk. To the best of our knowledge, this is the first report on the production and application of α-galactosidase from *B. megaterium*.

MATERIALS AND METHODS

Organism

An alkalophilic bacterium, *Bacillus megaterium* VHM1, producing extracellular α-galactosidase was isolated from sugar cane industrial waste samples taken near Bijapur, Karnataka, India. The nutrient broth used to isolate the bacterium was supplemented with a defatted soya flour extract at 50°C and the pH adjusted to 7.5. The isolate was maintained on nutrient agar slants containing the following (g/l): peptone, 10; yeast extract, 10; and guar gum, 5. The isolate was preliminarily identified on the basis of morphological, physiological, and biochemical tests as described in B*ergey's Manual of Systematic Bacteriology* [5].

Phylogenetic Analysis of the Isolated Strain

The partial 16S rDNA nucleotide sequences were determined as previously described by Kim *et al*. [14]. The 16S rDNA was amplified by a PCR (94°C for 5 min; 30 cycles consisting of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s; plus a final incubation at 72°C for 10 min) using universal 16S rDNA F and R primers from isolated strains. The PCR-amplified product was purified and the nucleotide sequences were determined by SolGent Co. Ltd (Taejeon, Korea) using an automated sequencing apparatus (ABI PRISM 3777 DNA Sequencer; PE Applied Biosystems, Missisauga, Ontario, Canada) with the 16S rDNA F/R primers. The resulting sequences were then deposited in the NCBI nucleotide sequence database under Accession No. FJ 613521. The 16S rDNA sequences of the isolated strains were compared with DNA sequences already deposited in the NCBI and identified based on their sequence homology, plus phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (http://www.megasoftware.net/)[36].

Enzyme Assay

The α-galactosidase activity was measured according to the method of Dey and Pridham [7]. One ml of the reaction mixture contained 100 µl of the suitably diluted enzyme, 50 µl of a 10 mM chromogenic substrate (*p*-nitrophenyl α-D-galactopyranoside, PNPG), and 850 µl of a 0.2 mM phosphate buffer, pH 7.5, at 50°C for 10 min. The reaction was terminated by adding 2 ml of a 0.2 M sodium carbonate solution. The amount of *p*-nitrophenol released was estimated from the absorbance at 405 nm. The enzyme activity was expressed as the amount of enzyme required to liberate 1 µmole of product per minute under the assay conditions.

The invertase activity in the culture supernatant was assayed at 50°C using 0.5 M sucrose in a 0.2 mM phosphate buffer, pH 7.5, and the amount of reducing sugar released was estimated using the method of Somogyi and Nelson [22]. One unit was defined as the amount of enzyme that catalyzed the hydrolysis of 1 µmole of sucrose per minute under the above conditions.

The protease activity in the culture supernatant was checked using the method described by Kunitz [16].

Protein Estimation

The protein concentration in the culture filtrate was determined using the method of Lowry *et al*. [20] with bovine serum albumin as the standard.

Medium and Culture Conditions

The medium used to optimize the culture conditions contained the following (g/l): peptone, 5; $(NH_4)_2SO_4$, 1.5; urea, 0.3; $MgSO_4$, 0.5; KH_2PO_4, 6; and guar gum, 5. The pH of the medium was 7.5 and was not further adjusted. Fifty ml of the above medium was dispensed in 250-ml Erlenmeyer flasks and sterilized at 121°C for 20 min. The sterilized flasks were then inoculated with 5.0% (v/v) of a culture grown overnight in a nutrient broth at 50°C in an orbital shaker (Remi, India) at 180 rpm. The submerged fermentation was continued for 28 h and the fermented broth checked for bacterial growth and α-galactosidase activity.

Optimization of Culture Conditions for Production of α-Galactosidase

The effect of temperature on the production of α-galactosidase was investigated by growing *Bacillus megaterium* VHM1 at different temperatures (30°C to 55°C) and then assaying the α-galactosidase activity and growth (660 nm) after 28 h of fermentation. The effect of the initial medium pH on growth and the production of α-galactosidase was studied by adjusting the media (with 1 M NaOH or HCl) to different pH levels, ranging from 4 to 9. The effect of the agitation rate on growth and the production of α-galactosidase was studied by cultivating the bacterium under different agitation speeds (0 to 250 rpm). The effect of the bacterial inoculum size (A_{600}=0.5) on growth and the production of α-galactosidase was investigated using different inoculum sizes, ranging from 1% to 10% (v/v). All the experiments were studied at 28 h of fermentation.

Characterization of α-Galactosidase

The 28-h fermented broth was centrifuged at 6,800 ×*g* for 10 min, and the supernatant obtained after centrifugation was subjected to 80% ammonium sulfate precipitation. The precipitated protein was then resuspended in a 0.1 M phosphate buffer (pH 7.5), dialyzed against a 0.01 M solution of the same buffer for 24 h at 4°C with 6 changes of buffer, and the activity assayed. This dialyzed enzyme was then used for further characterization.

The effect of pH on the enzyme activity was determined in a 0.2 M acetate buffer (pH 3 to 6), phosphate buffer (pH 6 to 7), barbitone buffer (pH 7.5 to 8.5), and glycine–NaOH buffer (pH 9 to 10). The pH stability was also determined by incubating the suitably diluted enzyme in the above buffers at 50°C for 16 h and measuring residual enzyme activity under optimal conditions. The optimum temperature was determined over a temperature range of 35–65°C, and the thermal stability was determined at a temperature range of 50–65°C over a period of 120 min. The effect of various metal ions on the α-galactosidase activity was determined by preincubating the diluted enzyme with 1 mM of metal salts ($FeSO_4$, $CuSO_4$, $MgSO_4$, and $MnSO_4$) in a 0.2 M phosphate buffer at pH 7.5 for 10 min at

25°C, and then determining residual enzyme activity under optimal conditions.

Removal of RFOs Present in Soymilk Using Enzymatic Method

The soybean seeds were purchased from a local market in Gulbarga, India and soymilk was prepared using the method of Mulimani and Ramalingam [21]. Fifty-ml aliquots of soymilk and 50 U of α-galactosidase from *Bacillus megaterium* VHM1 were incubated at 55°C for 1.5 h. At every 30 min, samples were withdrawn and the enzyme activity in the samples was terminated by keeping the reaction mixture in a 100°C water bath for 10 min. The proteins were precipitated by the addition of 0.02 ml of 0.03 M barium hydroxide and 0.02 ml of 0.18 M zinc sulfate. The precipitated proteins were then removed by centrifugation (Remi, India), and the reducing sugar liberated in the reaction was estimated using the method of Nelson and Somogyi [22].

Separation of Oligosaccharides Using TLC and HPLC

Fifteen ml of the enzyme-treated/untreated soymilk was poured into 35 ml of absolute ethanol and the precipitated proteins were removed by centrifugation at 3,200 ×*g* and 50°C for 15 min. The resulting supernatant was then concentrated to a syrup by evaporation and dissolved in distilled water when necessary. The amount of sucrose, raffinose, and stachyose in the syrup was estimated using the thiobarbituric acid method [37].

The thin-layer chromatography (TLC) was performed on precoated silica gel plates. The plates were developed at room temperature in a saturated chamber containing n-propanol:ethyl acetate:water [6:1:3 (v/v/v)] as the solvent system. Following the final development, the TLC plates were sprayed with α-naphthol in absolute ethanol containing 10% *ortho*-phosphoric acid and then kept in an oven at 100°C for 10–15 min [37]. The HPLC analysis was performed using a Shimadzu apparatus (Shimadzu Corporation, Japan) equipped with an Lc 10 ATVP 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 bond clone 10 µ CHO column. The mobile phase consisted of acetonitrile:water [70:30 (v/v)] for the 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 completed by comparing the retention times with those of standards.

Results and Discussion

Isolation and Identification of Strain

A bacterial strain capable of producing extracellular α-galactosidase was isolated from a sample of sugarcane industrial waste. The isolated bacterial strain was Gram-positive (central ellipsoidal spores), rod-shaped, and grew optimally at 50°C and pH 7.5. Microbiological, physiological, and biochemical studies (Table 1) revealed that the isolate belonged to a *Bacillus* sp. The 16S rDNA of VHM1 was sequenced and analyzed as described in Materials and Methods. A phylogram was constructed using the neighbor-joining method with bootstrap (500 replicates) values as shown in Fig. 1. The 16S rDNA sequence of VHM1 showed a 98% homology with type strain *Bacillus megaterium* (EU333886.1), and hence the strain was named *Bacillus megaterium* VHM1.

Table 1. Microbiological and biochemical tests.

Microbiological tests

Test	Results
Colony morphology	
Configuration	Round
Size	Large
Margin	Elevated
Pigments	No
Grams staining	Gram +ve
Spore staining	Spore production at center (endospores)
Capsular staining.	Present

Biochemical tests

Tests	Results
Starch hydrolysis	+ve
Gelatin hydrolysis	-ve
Urease	-ve
H_2S production	-ve
Casein hydrolysis	-ve
Catalase	-ve
MR	-ve
VP	+ve
Indole production	+ve
Citrate utilization	-ve
Carbohydrate fermentation	
Glucose	-ve gas +ve acid production
Lactose	-ve gas +ve acid production
Maltose	-ve gas -ve acid production

Preliminary experiments revealed that *Bacillus megaterium* VHM1 grew well in a mineral salt medium containing 0.5% guar gum as the carbon source and produced α-galactosidase. There was no increase in α-galactosidase activity when using metal ions in the medium tested. The strain also showed 1.2 U/ml of invertase activity, and no protease activity was detected in the culture supernatant.

Time Course of α-Galactosidase Production

The growth profile of *Bacillus megaterium* VHM1 is shown in Fig. 2. The organism grew very rapidly during the initial hours of cultivation, reaching a maximum biomass after 12–16 h of fermentation, after which the cell mass declined slowly. Meanwhile, α-galactosidase activity appeared in the culture broth after 8 h of fermentation and increased rapidly, reaching a maximum (1.6 U/ml) after 20 h. Thus, the maximum growth and α-galactosidase activity of *Bacillus megaterium* VHM1 were both higher than those previously reported for *Bacillus stearothermophilus* after 10–12 h [10] and *Bacillus* sp. JF2 after 24 h of

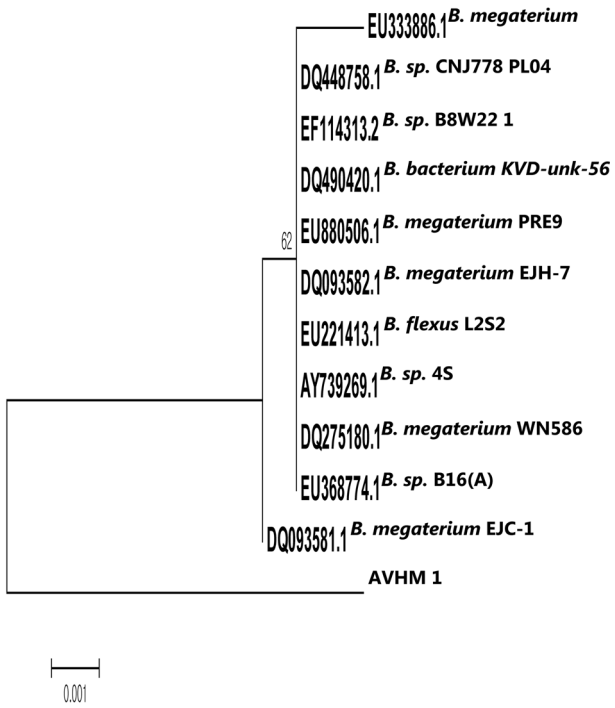

Fig. 1. Phylogram showing taxonomic position of strain VHM1 among reference species for *Bacillus*.
Strain VHM1 showed a 98% homology with the type strain *Bacillus megaterium* (EU333886.1).

fermentation [18]. Although most bacterial α-galactosidases are intracellular when grown in a submerged culture [40], a few extracellular bacterial α-galactosidases have been reported, but their activity is very low [6, 35]. Moreover, although most fungal α-galactosidases are extracellular in nature [25], they require a longer fermentation time.

Effect of Initial pH on Production of α-Galactosidase

Among the various cultural parameters influencing product formation by microorganisms, the hydrogen ion concentration

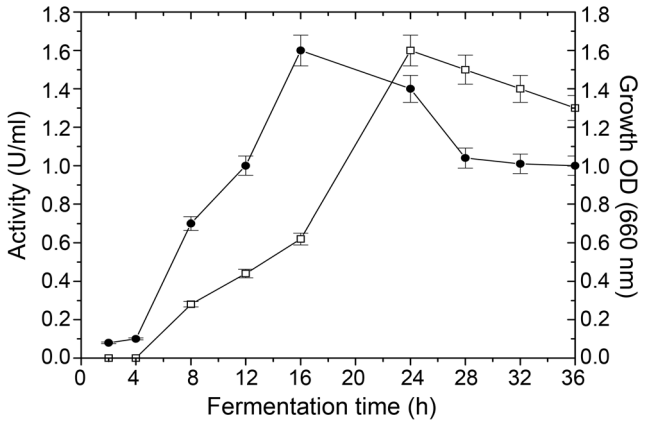

Fig. 2. Time course of the cell growth (●) and production of α-galactosidase (□) by *Bacillus megaterium* VHM 1.

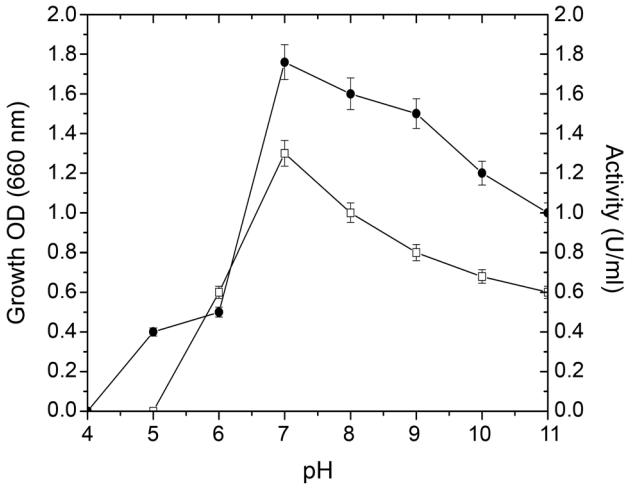

Fig. 3. Effect of pH on the growth (●) and α-galactosidase production (□) by *Bacillus megaterium* VHM1.

in the culture medium is considered extremely important. The effect of the initial pH on the enzyme production and growth of *Bacillus megaterium* VHM1 is depicted in Fig. 3. Growth and α-galactosidase activity were both observed at pH 4–9; however, the optimum growth and enzyme secretion were observed in a pH range of 7–7.5. No growth or enzyme secretion was detected in an acidic range (pH 5). These findings were similar to the observations of Gote *et al.* [10], Tablot and Sygusch [35], and Li *et al.* [18] that reported the maximum growth of *Bacillus* sp. JF2 occurred at pH 5.8, whereas its optimal enzyme production was at pH 7.5.

Effect of Temperature on Production of α-Galactosidase

The effect of temperature on the growth of *Bacillus megaterium* VHM1 and its production of α-galactosidase is depicted in Fig. 4. When grown at different temperatures, ranging from 30°C–60°C, the maximum growth and α-galactosidase production were observed at 50°C, and then decreased at temperatures above 50°C, which agreed with previously reported results on the optimum temperature for α-galactosidase production by *Bacillus* sp. [13]. Whereas *Bacillus sterothermophilus* α-galactosidase production is at 60°C, above this temperature, growth and α-galactosidase activity concomitantly decreases [10]. Other reports also confirmed an optimal temperature of 60°C for growth and α-galactosidase production by *Bacillus sterothermophilus* [6, 35].

Effect of Agitation Rate and Inoculum Size on Production of α-Galactosidase

The effect of the agitation rate on the production of α-galactosidase and bacterial growth was also investigated (Fig. 5). The highest α-galactosidase production and bacterial growth were obtained when the agitation speed

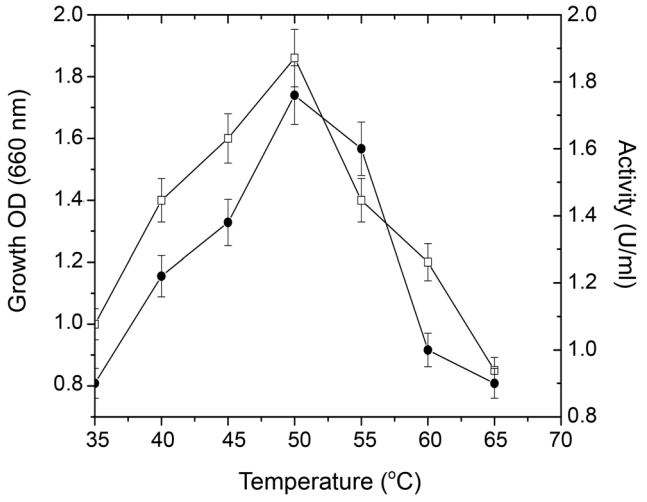

Fig. 4. Effect of temperature on the growth (□) and α-galactosidase (●) production by *Bacillus megaterium* VHM1.

was 200 rpm, which produced a sufficient supply of dissolved oxygen in the medium [15]. However, when the agitation speed was increased to 250 rpm, the production of α-galactosidase was found to decrease, whereas static conditions inhibited enzyme production. Thus, despite the increased oxygen from increasing the agitation speed beyond 200 rpm, the enzyme production did not increase, probably because the higher agitation rate altered the structure of the enzyme [29]. Meanwhile, lowering the aeration rate can also cause a drastic reduction in the enzyme yield [39]. The effect of the inoculum size was studied by varying the inoculum size from 1% to 10%, and the maximum enzyme production was observed with a 5% (v/v) inoculum size. When the inoculum size is too small, the insufficient number of bacteria leads to a reduced amount of secreted α-galactosidase. Conversely, a high inoculum size can lead to a lack of oxygen and depletion of nutrients in the culture media.

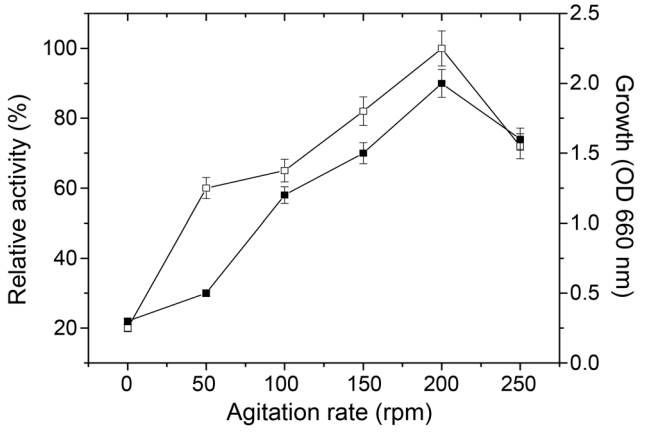

Fig. 5. Effect of agitation rate on α-galactosidase production, ■ growth, and □ relative activity.

Effects of Carbon Sources on α-Galactosidase Production

Carbon occupies a unique position among the essential elements required by microorganisms. When it is consumed, it undergoes three metabolic changes; namely conversion into cell substances, carbon dioxide, and accumulation in several metabolic products, such as enzymes. Based on the type and nature of the carbon source in a medium, an organism will grow and produce various enzymes. Thus, the effects of various carbon sources on α-galactosidase production are shown in Table 2. Strain *Bacillus megaterium* VHM1 was able to grow with all the carbon sources tested, yet grew less in the culture medium including glucose, xylose, and sucrose, when compared with the other sugar sources tested. The maximum enzyme production was induced in the presence of α-galactosides [*viz*, raffinose, and melibiose (0.2–0.4%)], as found in other reports [18]. All the other monosaccharides and disaccharides tested had a lesser influence on the enzyme production. However, in contrast to other reports, *Bacillus megaterium* VHM1 exhibited its maximum activity in guar gum and locust bean gum [6, 10]. However, no α-galactosidase was produced when guar gum was used as the carbon source. The supplementation of glucose in the medium inhibited the α-galactosidase production due to catabolite repression [6].

Effects of Nitrogen Sources on α-Galactosidase Production

The effects of various nitrogen sources on the production of α-galactosidase by *Bacillus megaterium* VHM1 are shown in Table 3. Both organic and inorganic nitrogen sources were tested, and better growth was generally observed with the organic nitrogen sources, where yeast extract, beef extract, and peptone produced the highest α-galactosidase activities, whereas ammonium sulfate and ammonium nitrate among the inorganic nitrogen sources showed moderate growth and α-galactosidase activity.

Table 2. Effects of various carbon sources on the production of α-galactosidase by *Bacillus megaterium* VHM1.

Carbon sources[a]	At 660 nm	Activity (U/ml)[b]
Glucose	0.814	0.10
Fructose	0.874	0.20
Maltose	0.964	0.08
Lactose	0.900	0.06
Xylose	0.780	0.08
Galactose	0.800	0.60
Mannose	0.850	0.10
Melibiose	0.867	0.65
Rafinose	0.853	0.80
Guar gum	0.854	1.10
Locust bean gum	0.913	1.06
Glucose+Galactose	0.800	0.30

[a]Guar gum in the medium was replaced with 0.3% carbon source.
[b]0.03% of each carbon source was added to the medium and the activity checked after 28 h of fermentation.

Table 3. Effects of various organic and inorganic nitrogen sources on α-galactosidase production.

N_2 Sources	At 660 nm	α-Galactosidase activity(U/ml)
Control	1.10	0.80
Yeast extract		
0.3%	1.62	2.53
0.5%	1.90	2.43
Peptone	1.70	1.00
Beef extract	1.70	1.13
Tryptone	1.50	1.00
Ammonium sulfate	1.23	0.60
Ammonium nitrate	0.72	0.75
Urea	1.00	0.40
Soybean flour	1.10	0.60
Sodium nitrate	0.80	0.55
Ammonium sulfate +yeast extract	1.90	2.50
Ammonium nitrate +yeast extract	2.0	1.80

The medium with 0.3% (w/v) peptone was replaced with 0.3% (w/v) nitrogen sources.
Control: no N_2 sources added.

Nonetheless, the combination of an inorganic nitrogen source with yeast extract produced the maximum activity. The α-galactosidase activity produced by *Bacillus megaterium* VHM1 (2.5 U/ml) was higher than that previously reported for *Bacillus stearothermophilus* at 0.276 U/ml [6], 2.0 U/ml [10], 0.09 U/ml [24], and 0.02 U/ml [25], but lower than that reported for the thermophilic *Bacillus* sp JF2 strain when grown on soybean meal [18].

Characterization of Partially Purified α-Galactosidase

Effect of pH on stability. The enzyme activity at various pHs and its stability is shown in Fig. 6. The optimum pH for the α-galactosidase was 7.0 to 7.5, and it remained stable in the range of pH 5.0 to 9. The enzyme exhibited 60% residual activity at pH 5.0 and 88% at pH 9.0. In general, the optimum pH for bacterial α-galactosidases is slightly alkaline (pH 6.0–7.5), whereas that for fungal and yeast α-galactosidases is acidic (pH 3–5) [1, 28]. An acidic form of α-galactosidase is not suitable for soymilk hydrolysis, as the pH of soymilk is around 6.2–6.4 [10], and lowering the pH of soymilk causes the precipitation of soy proteins and leaves a sour taste. Thus, since the *Bacillus megaterium* VHM1 α-galactosidase has an alkaline optimum pH (7–7.5), this makes it appropriate for treating the RFOs present in soymilk.

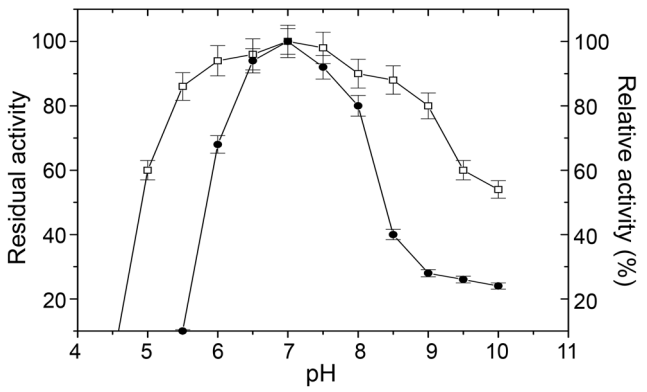

Fig. 6. Effect of pH on α-galactosidase activity (●) and stability (□).

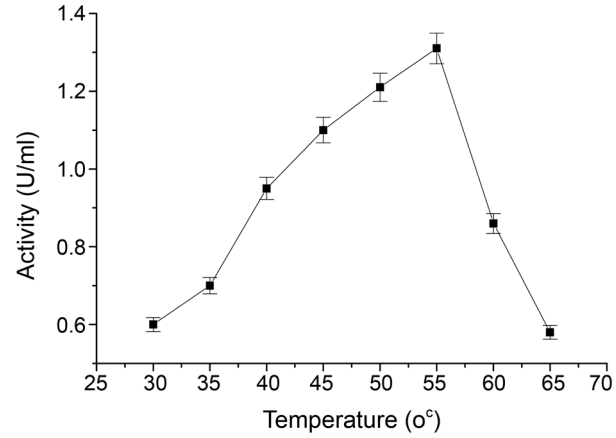

Fig. 7. Effect of temperature on α-galactosidase activity.

Optimum temperature and thermostability. The effect of temperature on the enzyme activity is shown in Fig. 7, where the enzyme activity increased up to 55°C as the optimum temperature and then decreased suddenly. The thermostability of the α-galactosidase is shown in Fig. 8, where the enzyme remained thermostable with a half-life of 120 min at 55°C, but lost 90% of its activity within 120 min at 60°C. The thermostability of α-galactosidase has an added advantage for food processing, as regards minimizing the contamination caused by mesophilic

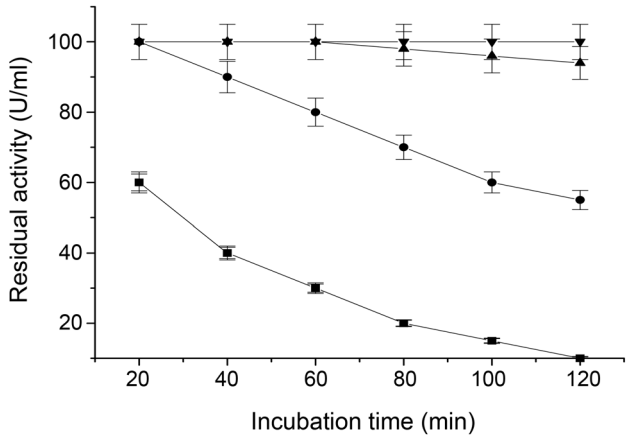

Fig. 8. Effect of temperature on the stability of α-galactosidase (▼40°C, ▲50°C, ●55°C, ■60°C).

Table 4. Effects of various metal ions on α-galactosidase activity.

Metal ions	Relative activity (%)
None	100
Fe^{2+}	100
Cu^{2+}	0
Mg^{2+}	100
Mn^{2+}	100
Ni^{2+}	90
Ag^{2+}	0
Zn^{2+}	92
Ca^{2+}	90
EDTA[a]	100
Hg^{2+}	0

[a]Concentration used was 20 mM.

organisms. A similar result was also reported by Silva *et al*. [31] for the *Lactobacillus fermentum* α-galactosidase, which showed optimal activity at 50°C.

Effects of metal ions on α-galactosidase activity. The effects of various metal ions on the α-galactosidase are shown in Table 4, where 1 mM concentrations of Ag^{2+}, Cu^{2+}, and Hg^{2+} strongly inhibited the α-galactosidase; Fe^{2}, Mn^{2+}, and Mg^{2+} had no effect on the α-galactosidase activity; and Zn^{2}, Ni^{2+}, and Ca^{2+} reduced the enzyme activity slightly (less than 15%). However, the α-galactosidase activity was not inhibited by EDTA, implying that the enzyme is not a metalloenzyme or does not require divalent cations for activity. Similar to the present findings, *Bacillus stearothermophilus* has been reported to be strongly inhibited by Ag^{2+}, Cu^{2+}, Hg^{2+}, and metal salts, and EDTA does not affect its α-galactosidase activity [10]. The same observation has been reported for other α-galactosidases from alkalophilic strains [2], whereas Ag^{2+}, Cu^{2+}, and Hg^{2+} have been reported to inhibit the α-galactosidases from plant and fungal sources [5, 11–32].

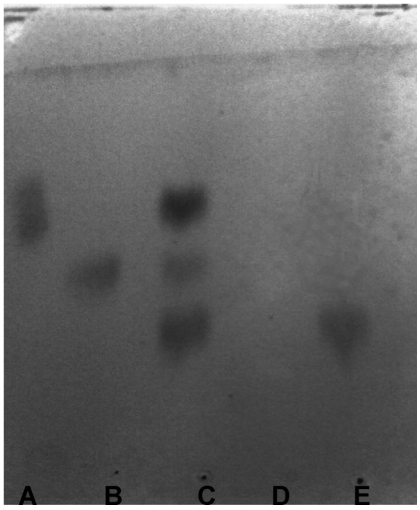

Fig. 9. TLC separation of raffinose family oligosaccharides from soymilk.
(**A**) Standard sucrose, (**B**) standard raffinose, (**C**) untreated soymilk, (**D**) enzyme-treated soymilk, and (**E**) standard stachyose.

Enzymatic hydrolysis of soymilk. Thin-layer chromatography and high-performance liquid chromatography analyses of the enzyme-treated (Fig. 9 and 10) soymilk showed that the α-galactosidase and invertase completely hydrolyzed the raffinose family oligosaccharides, (*i.e*., sucrose, raffinose, and stachyose) after 1.5 h of incubation at pH 7 and 55°C. In previous reports, the α-galactosidase from *L. fermentum* completely degraded raffinose and stachyose in commercial soymilk after 7 h of incubation at pH 5.2 to 6.5 and 37°C [9], α-galactosidase from germinating guar and *A. oryzae* successfully degraded raffinose and stachyose in soymilk [26, 30], and the α-galactosidase from *Bacillus sterothermophilus* [10] successfully hydrolyzed RFOs

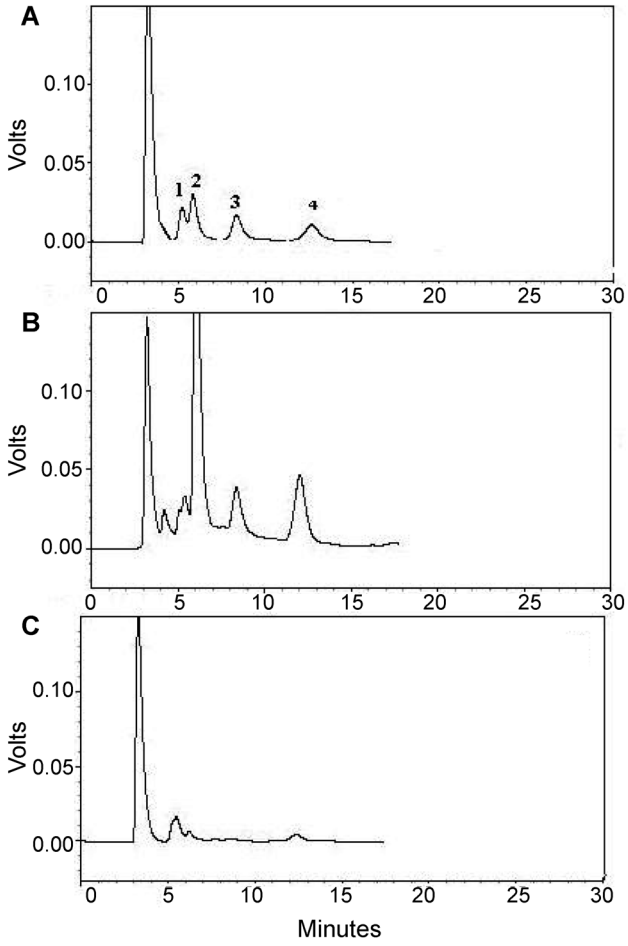

Fig. 10. HPLC separation pattern of raffinose oligosaccharides in soymilk before and after treatment with *B. megaterium* VHM1 α-galactosidase.
A. Standard sugars: 1, galactose; 2, sucrose; 3, raffinose; and 4, stachyose. **B**. Soymilk before enzyme treatment. **C**. Soymilk after treatment with enzyme.

from soymilk after 2 h of incubation. Thus, *Bacillus megaterium* VHM1 took less time (*i.e*., 1.5 h) to hydrolyze the raffinose family oligosaccharides in soymilk.

In conclusion, *Bacillus megaterium* VHM1 is a nonpathogenic organism that utilizes complex polysaccharides and produces an extracellular α-galactosidase that is alkaline and thermostable, without protease activity, allowing it to be utilized in the food and pharmaceutical industries.

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