Journal of Applied Biology & Biotechnology Vol. 9(05), pp. 13-19, September, 2021
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2021.9502

Purification, characterization of α-galactosidase from a novel *Bacillus megaterium* VHM1, and its applications in the food industry

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ARTICLE INFO

Article history:
Received on: December 22, 2020
Accepted on: February 05, 2021
Available online: September 01, 2021

Key words:
Bacillus megaterium VHM1, α-Galactosidase, purification, food industry

ABSTRACT

An extracellular thermostable α-galactosidase from *Bacillus megaterium* VHM1 was purified 94.26-fold by precipitation with ethanol, followed by sequential column chromatography with DEAE-Sephacel and G_{75} column. The purified enzyme was homogeneous on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). It was found to be a monomeric protein with a molecular weight of about 66 kDa. The purified enzyme showed optimum activity in p-nitrophenyl α-D-galactopyranoside (PNPG) at pH 7.0 and a temperature of 60°C. The enzyme was thermostable, showing complete activity even after heating at 55°C for 60 minutes. The substrate specificity of α-galactosidase on PNPG, ortho-Nitrophenyl-β-galactoside, and raffinose was investigated and K_m was found to be 0.508, 0.529, and 5.0 mM and V_{max} was 3.492, 4.287, and 14.20 μ mol/ml/minute, respectively. Among the metal ions and reagents tested, Hg^{2+}, Cu^{2+}, and Ag^{2+} strongly inhibited the α-galactosidase activity, and ethylenediaminetetraacetic acid showed no effect on enzymes. The present study supports the application of α-galactosidase from *B. megaterium* VHM1 as a potent enzyme in the food processing industry.

1. INTRODUCTION

α-Galactosidase (EC.3.2.1.22) is an exogalactosidase that catalyzes the hydrolysis of terminal α-1-6-linked-D-galactose residues from simple galactose containing oligosaccharides such as melibiose, raffinose, stachyose, and verbascose, as well as more complex polysaccharides including galactomannans [1]. α-Galactosidases are of particular interest in view of their many potential biotechnological and medicinal applications. The most important industrial applications being in beet sugar industry [2], soy food processing [3], animal feed processing [4], and in pulp and paper industries [5]. α-Galactosidase is also used for the removal of a qualitative proportion of galactose moieties from guar gum in order to improve the gelling prosperities of the polysaccharides and to make them comparable to those of locust been gum [6]. Furthermore, galacto-oligosaccharides produced by the transfer reaction of α-galactosidase can be used as a prebiotic in functional food [7]. An increased interest in α-galactosidase can be seen in human medicine as it is used in blood group transformation [8], treatment of Fabry's disease, and in xenotransplantation [9]. Some of the α-galactosidases are also known to have trans-glycosidase activity [10,11].

α-Galactosidases have been isolated from various sources. Most of the α-galactosidases have been extensively isolated and purified from fungal sources [12,13]. *Bacillus megaterium* is Gram-positive, mainly aerobic spore-forming bacterium found in various habitats, from soil to sea water. The organism is industrially employed as it possesses some very useful and unusual enzymes and has a high capacity for the production of exoenzymes. The seven plasmids of *B. megaterium* strain QM B1552 contain several unusual metabolic genes that may be useful in bioremediation. Altogether, a "toolbox" of hundreds of genetically characterized strains, genetic methods, vectors, hosts, and genomic sequence makes *B. megaterium* an ideal organism for industrial and environmental applications [14]. Sindhu *et al.* [15] reported on the production of cellulose-free xylanase from *B. megaterium* by solid-state fermentation for bio-bleaching of pulp. *Bacillus. megaterium* also produced β-amylase by solid state fermentation using starch waste [16]. *Bacillus* sp. JF2 strain produced extracellular thermostable α-amylase (EC 3.2.1.1)

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and intracellular α-galactosidase (EC 3.2.1.22) [17]. Intracellular α-galactosidase production was expressed by the growth-associated model and relationship equation between the substrate and cell growth, two enzyme productions were also defined, and all constants were defined.

Thermostable enzymes are gaining wide and excellent applications in industrial sectors that take advantage of their function of high temperature. *Bacillus. megaterium* VHM1 has been found to secrete extracellular thermostable α-galactosidase. Here we report on the purification and characterization of α-galactosidase from a novel strain *B. megaterium* VHM1.

2. MATERIALS AND METHODS

2.1. Isolation and Characterization of the Bacteria

A strain of alkalophilic bacterium *B. megaterium* VHM1, producing extracellular α-galactosidase, was isolated from sugar cane industrial waste samples near Bijapur (Karnataka, India). The nutrient broth used for isolation of bacterium was supplemented with defatted soya flour extract at 50°C and pH was adjusted to 7.5. The isolate was maintained on nutrient agar slants containing the following (per liter of distilled water): peptone, 10 g; yeast extract, 10 g; and guar gum, 5 g. The isolate was identified on the basis of morphological, physiological, and biochemical tests as described in Bergey's Manual of Systematic Bacteriology [18]. The partial 16S rDNA nucleotide sequences were determined as previously described Kim *et al.* [19]. The 16S rDNA was amplified by polymerase chain reaction (PCR) (94°C for 5 minute, 30 cycles consisting of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 90 seconds), followed by a terminal incubation at 72°C for 10 minutes) with universal 16S rDNA F and R primers from isolated strains. The PCR amplified product was purified and nucleotide sequences were determined at SolGent Co. Ltd (Taejeon, Korea) with an automated sequencing apparatus (ABI PRISM 377, PE Biosystems Inc.) using 16S rDNA F/R primers. The sequences thus generated were deposited in National Center for Biotechnology Information (NCBI) nucleotide sequence database under accession no. FJ 613521. The 16S rDNA sequences of isolated strains were compared with the DNA sequences deposited in NCBI and identified based on sequence homology. Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 4 [20].

2.2. Enzyme Production

The medium used for production of α-galactosidase was mineral salt guar gum medium (0.5% guar gum and 0.5% peptone containing mineral salt medium). The sterilized Erlenmeyer flasks (250 ml capacity containing 50 ml of medium) were inoculated with 24-hour grown culture of *B. megaterium* VHM1. Fermentation was carried out on rotary orbital shaker at 120 rpm at 40°C for 26 hours. After 26 hours of submerged fermentation, the broth was centrifuged at 10,000 rpm for 10 minutes. The supernatant is taken as crude enzyme source and was kept at 4°C until further studies.

2.3. Enzyme Assay

α-Galactosidase activity was carried out according to the method of Dey and Pridham [1]. 1 ml of reaction mixture contains 100 µl of suitably diluted enzyme, 50 µl of 10 mM chromogenic substrate (p-nitrophenyl α-D-galactopyranoside, PNPG), and 850 µl of 0.2 mM phosphate buffer (pH 7.5) at 50°C for 10 minutes. The reaction was terminated by adding 2 ml of 0. 2 M sodium carbonate solution. The amount of p-nitrophenol released was estimated from absorbance at 405 nm. Enzyme activity was expressed as the amount of enzyme required to liberate 1 µmol of product per minute under the assay conditions.

2.4. Protein Assay

Protein concentration was determined by the method of Lowry *et al.* [21]. Fractions obtained during the purification procedure were screened for protein content by UV absorbance spectrophotometer (Elico India Ltd) at 280 nm.

2.5. Purification of α-Galactosidase

The precooled ethanol was added slowly to the crude enzyme extract with constant stirring to give a concentration of 1:1.5 (*v/v*) and this solution was kept at 4°C for 6 hours, centrifuged at 12,000 × g for 20 minutes and the supernatant were discarded. The precipitate was dissolved in Tris-Hcl buffer (0.05 M pH 7.5) and dialyzed against the same buffer at 10°C for 12 hours, and then used for further purification. The dialyzed enzyme was loaded onto a DEAE-Sephacel column (2.3 × 10 cm) equilibrated with phosphate buffer (0.05 M; pH 7.5) and the column was washed with the same buffer. The enzyme bound to DEAE-Sephacel was eluted with linear gradient of 0.5 M NaCl in an equilibration buffer (50 mM; pH 7.5); fractions were collected at the flow rate of 40 ml/hour using a peristaltic pump p-1 (Pharmacia); and fractions (2.5 ml) were collected using the fractional collector. All the fractions were checked for protein (A_{280}) and α-galactosidase activity (A_{450}). The fractions having maximum enzyme activity were pooled, concentrated, and stored at 4°C for further purification. G_{75} gel-filtration matrix in a column (1.8 × 50 cm) was equilibrated with phosphate buffer (50 mM; pH 7.5). The concentrated enzyme obtained after ion exchange chromatography was loaded on the column and allowed to enter the gel. The protein was then eluted with equilibration buffer. The flow rate was adjusted to 32 ml/hour using peristaltic pump p-1(Pharmacia). Fractions (2.5 ml) were collected using fractional collector. The entire fractions were checked for protein and enzyme activities. The fractions showing α-galactosidase activity were pooled, concentrated, and dialyzed. This dialyzed enzyme solution was then used as for purified enzyme preparation.

2.6. Electrophoresis and Zymogram Analysis

The purity of the enzyme was checked using 10% denaturating sulfateSDS-PAGE under reducing conditions. The polyacrylamide gel was prepared by the method of Laemmli [22]. The Mr of the purified α-galactosidase was calculated based on the relative mobility of the molecular mass markers from GENE, Bangalore (phosphorylase B = 97.4 kDa, ovalbumin = 43 kDa, carbonic anhydrase = 29 kDa, soybean trypsin inhibitors = 20.05 kDa, and lysozyme = 14.3 kDa). For zymogram analysis, the SDS-PAGE gel was treated with Triton X for 10 minutes to remove SDS; the triton X treated gel was

incubated with 2% $Pb(NO_3)_2$ + 1 mM $MgCl_2$ + p-nitrophenyl α-D-galactopyransoside + acetate buffer pH 6.0 for 10 minutes. After 10 minutes, 2% glacial acetic acid was added to terminate the reaction for 10 minutes and washed with distilled water. After that, diluted ammonium sulfide was added and kept for 10 minutes and then washed with distilled water. Black color band corresponds to α-galactosidase activity. Here, the phosphate ions liberated from PNPG will react with ammonium sulfide and $MgCl_2$ to produce colored bands.

2.7. Optimum pH and Stability

The optimum pH of α-galactosidase was determined by assaying α-galactosidase activity using a standard assay method over a pH range of 4.0–9.0 using either 50 mM acetate buffer (pH 4.0–6.0), 50 mM phosphate buffer (pH 6.0–7.5), or barbitone buffer (pH 8.0–9.0). Enzymatic activities at different pH were expressed as relative values (%) with reference to 100% activity at the optimal pH. The pH stability was determined by pre-incubating the enzyme in 50 mM acetate buffer (pH 4.0–6.0), 50 mM phosphate buffer (pH 6.0–7.5), or barbitone buffer (pH 8.0–9.0).for a period of 2 hours at 55°C. Small aliquots were withdrawn from all the samples and residual enzyme activity was determined by standard assay methods.

2.8. Optimum Temperature and Stability

The optimum temperature of α-galactosidase was determined by carrying out assays at temperatures ranging from 30°C to 75°C. Thermal stability of the enzyme was determined by incubating the enzyme at temperatures ranging from 65°C to75°C for several time periods. The residual activity of the enzyme was estimated under standard assay conditions.

2.9. Determination of Kinetic Parameters (K_m and V_{max})

The kinetic parameters (K_m and V_{max}) were determined with the use of varying concentrations of artificial substrates PNPG and natural substrates raffinose in a phosphate buffer of pH 7.0 and incubated at 60°C for 10 minutes. When PNPG was used, the reaction (assay) was arrested by adding 3 ml of 0.2 M sodium carbonate solution and the absorbance was measured at 405 nm. In case of raffinose, the reaction was arrested by adding 1 ml of alkaline copper reagent. Test tubes were kept in a boiling water bath for 20 minutes. 1 ml of arsenomolybdate reagent was added and the absorbance was read at 540 nm [23]. The kinetic parameters were determined by the plot method Lineweaver and Burk [24].

2.10. Effect of Inhibitors and Metal Ions on α-Galactosidase

The sensitivity of the enzyme to metals was examined by running the standard α-galactosidase assay at optimum temperature and pH. Metal ions ($AgNO_3$, $CaCl_2$, Cu SO_4, $HgCl_2$, $MgCl_2$, MnSO4, $NiCl_2$, and $ZnSO_4$) were dissolved to a final concentration of 10 mM in 50 mM phosphate buffer of pH 7.0. To determine the effect of inhibitors on α-galactosidase, the enzyme was pre-incubated with final concentrations of 10 mM 1, 10-Phenthraline; N-bromosuccinamide, PMSF, Mercaptoethonol, ethylenediaminetetraacetic acid (EDTA), and urea. After 30 minutes of pre-incubation, the reaction was initiated by addition of 1 mM PNPG.

3. RESULTS AND DISCUSSION

3.1. Purification of α-Galactosidase

The enzyme purification protocol is summarized in Table 1. α-Galactosidase was purified 94.26-fold with 42.20% yield from the crude enzyme extract. First, the proteins in the crude extract were precipitated with chilled ethanol at 1:1.5 (*v/v*). This step achieved a 4.11-fold enzyme purification, and 90.05% of the enzyme could be recovered. Then, the concentrated enzyme preparation was fractionated by anionic exchange chromatography using a DEAE-Sephacel column, in which the enzyme was eluted with linear gradient of 0.5 M NaCl in equilibration buffer (50 mM; pH 7.5). The elution profile is shown in Figure 1. The remaining protein contaminants were removed by gel filtration using G_{75} (Fig. 2). The fractions with maximal enzyme activity were collected and further examined for purity by gel electrophoresis.

Earlier, purification of fungal α-galactosidase was achieved by multistep purification procedures, which were tedious and time-consuming [25–27]. However, the purification scheme employed here is relatively simple and highly reproducible. Garro *et al.* [28] purified α-galactosidase from *Lactobacillus ferementum* up to homogeneity using three steps, ammonium sulfate precipitation, gel filtration, and ion exchange chromatography, to the yield of 5.50% and purification fold of 21.03%. Gote *et al.* [29] purified α-galactosidase from *Bacillus stearothermophilus* using three steps amicon concentration, followed by ultrafiltration, alcohol precipitation, and hydrophobic interaction chromatography to the yield of 44.6% and purification fold of 369%.

3.2. Electrophoretic Analysis of α-Galactosidase on SDS-PAGE

After simple three purification steps, SDS-PAGE of the final enzyme preparation showed a single band. The molecular weight

Table 1: Summary of the purification of α-galactosidase from *B. megaterium* VHM1.

Purification steps	Total volume (ml)	Total enzymeactivity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	500	545	446	1.22	100	0
Ethanol precipitation	15	402	80	5.22	90.05	4.11
DEAE-Sephacel chromatography	3	298	10	29.8	54.67	17.21
Sephedex G_{75}	1.5	230	2.0	115.0	42.20	94.26

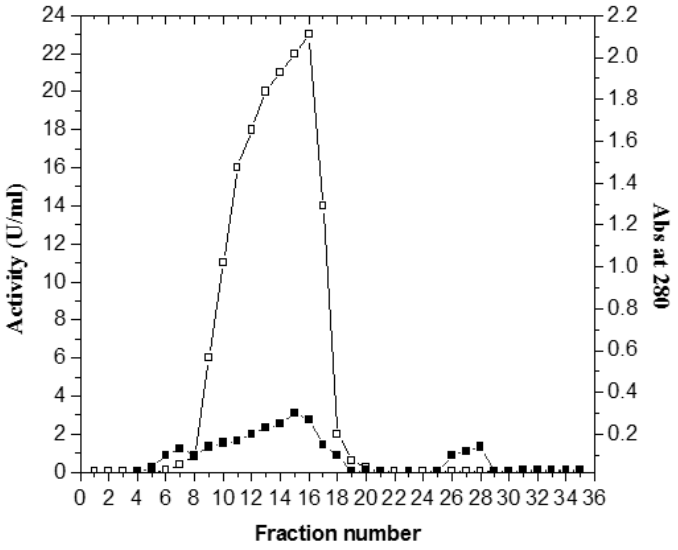

Figure 1. Elution profile DEAE-Sephacel column chromatography of α-galactosidase *B. megaterium* VHM1 -□- α-Galactosidase activity; -■-Absorbance at 280 nm.

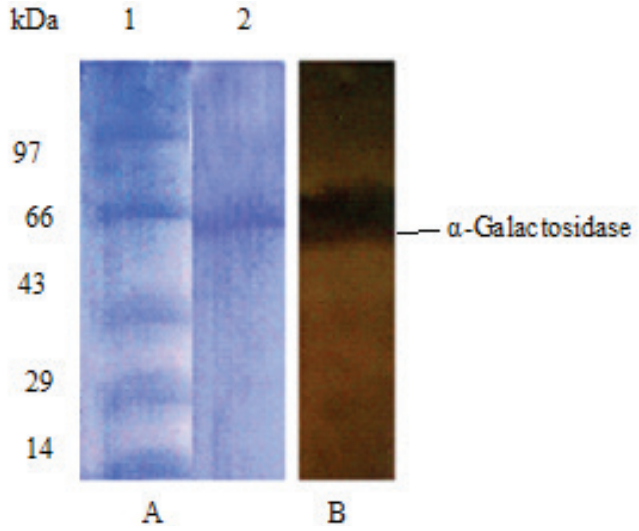

Figure 2: SDS-PAGE of purified α-galactosidase. (A) Lane 1: molecular weight markers and Lane 2: purified α-galactosidase. (B) Activity staining of α-galactosidase.

of the purified enzyme by SDS-PAGE was between 60 and 64 KDa (Fig. 3). Similar to our results, the molecular weights of α-galactosidase *B. stearothermophilus* and *Debromyces hansenii* were in range from 57 to 80 kDa [29,30].

3.3. Effect of pH on α-Galactosidase Activity and Stability

Figure 4 shows that purified α-galactosidase from *B. megaterium* VHM1 was considerably active from pH 6.0 to 9.0. Optimum pH of α-galactosidase is 7.0. The purified α-galactosidase showed broader stability over a pH range of 4.5–8.5 (Fig. 5). The enzyme exhibited 65% residual activity at pH 4.5 and 75% at pH 8.0. From the literature, it is evident that α-galactosidase from *B. stearothermophilus* has an optimum activity at neutral to basic

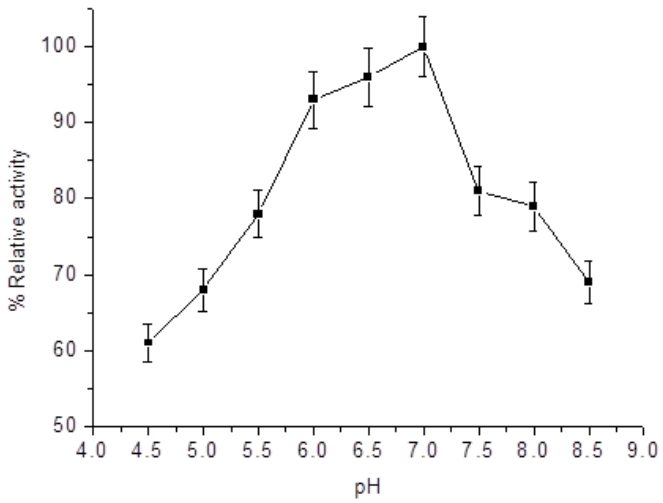

Figure 3. Effect of pH on the activity of α-galactosidase from *B. megaterium* VHM1.

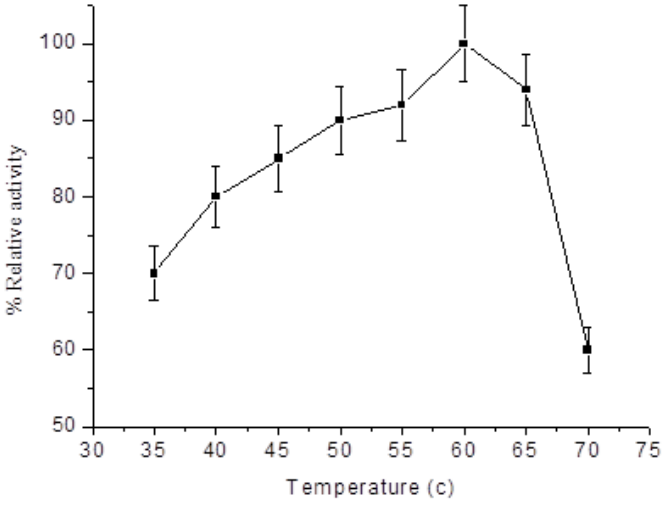

Figure 4. Effect of temperature on activity of α-galactosidase activity from *B. megaterium* VHM1.

side. α-Galactosidase from *B. stearothermophilus* was optimally active between pH 7 and 7.5, and stable in pH 4.8 [31–33]. Li *et al.* [34] reported *Bacillus* spp. JF2 α-galactosidase optimal activity at pH 7.2, whereas in case of fungal α-galactosidase it showed optimal activity toward acidic pH (4.0–6.0) [30,35].

3.4. Effect of Temperature on Activity and Stability of α-Galactosidase

Figure 5 shows the temperature optima for *B. megaterium* VHM1 α-galactosidase. The enzyme maximally hydrolyzed PNPG at 60°C (pH 7.0). Figure 4 shows a thermostability profile of α-galactosidase. It is clear from Figure 4 that the enzyme is 100% stable up to 60 minutes at 55°C. More than 65% activity is retained up to 40 minutes at 65°C. The enzyme was thermostable with a half-life of 50 minutes at 65°C. After 50 minutes incubation at 65°C, enzyme activity decreased rapidly. Li *et al.* [34] reported

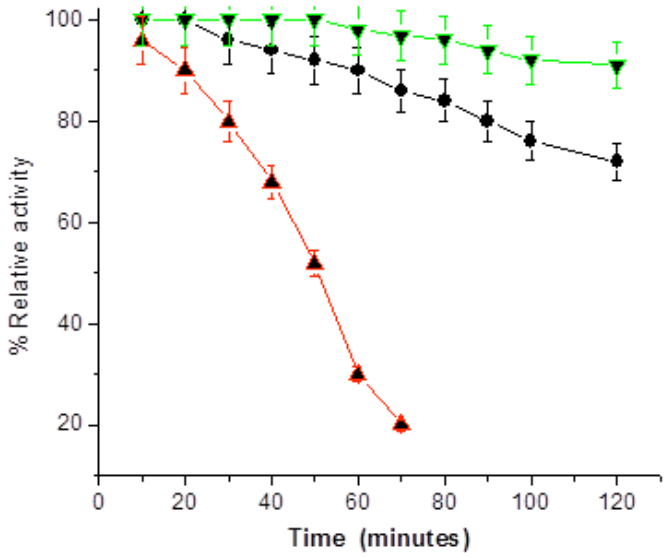

Figure 5. Effect of temperature stability on α-galactosidase from B. megaterium VHM1, ▲ 65°C, ● 60°C, ▼ 55°C

Table 2: K_m and V_{max} values of *B. megaterium* VHM1 α-galactosidase.

Substrates	Relative activity (%)	$K_{m\ (mM)}$	$V_{max(\mu\ mol/ml/minute)}$
PNPG	100	0.508	3.492
ONPG	96	0.529	4.287
Raffinose	80	5.0	14.20
Guar gum	76	–	–
Locust bean gum	60	–	–

α-galactosidase from *Bacillus* JF2 spp. optimal activity at 55°C and thermostability at 60°C for 40 minutes. Garro *et al.* [28] reported α-galactosidase from *L. ferementum* optimal temperature was 45°C and half-life of the enzyme was 30 minutes at 60°C. Gote *et al.* [32] reported *B. stearothermophilus* optimal α-galactosidase activity at 65°C and its half-life inactivation (*t* ½) at 70°C for 30 minutes. Fridjonsson *et al.* [36] reported α-galactosidase from *B. stearothermophilus* optimal temperature at 70°C and *t* ½ of the enzyme was 19 hours at 70°C. Other reports from *B. stearothermophilus* showed optimal temperature at 70°C [29,31].

3.5. Substrate Specificity and Determination of Kinetic Parameters

Kinetic parameters K_m and V_{max} of *B. megaterium* VHM1 had higher affinity toward PNPG (K_m 0.508 mM) than that of ortho-Nitrophenyl-β-galactoside (ONPG) (0.529 mM). Other natural substrates also had a higher K_m value. The relative substrate specificity toward synthetic and natural galactosidase is in the order PNPG>ONPG>Raffinose>Guargum (Table 2). The kinetic study revealed that enzyme prefers synthetic as well as natural polysaccharides. Therefore, enzyme is applicable in the food processing industry for elimination of flatulence causing factors in legume-based foods. In the literature, α-galactosidase from different organisms has different K_m and V_{max} values. King *et al.* [37] reported

Table 3: Effect of inhibitors and metal ions on α-galactosidase from *B. megaterium* VHM1.

Inhibitors/metal ions	Residual activity (%)
None	100
N-bromosuccinamide	000
PMSF	100
EDTA	100
1–10-Phenanthroline	100
Mercaptoethanol	100
Mg^{2+}	105
Hg^{2+}	0
Zn^{2+}	69
Cu^{2+}	0
Ag^{2+}	0
Ni^{2+}	89
Co^{2+}	94
Mn^{2+}	100
Ca^{2+}	82

Thermoanaerobacterium *Polysacchrolyticum* α-galactosidase K_m value for PNPG was 0.38 mM and raffinose was 16.4 mM.

3.6. Effect of Inhibitors and Metal Ions on α-Galactosidase Activity

The effect of different inhibitors and metal ions on α-galactosidase activity is depicted in Table 3. Among the inhibitors tested, EDTA and 1, 10, phenantherene did not inhibit the α-galactosidase activity and this indicates that α-galactosidase is not a metalloenzyme. Inhibition of α-galactosidase from *B. megaterium* VHM1 by N-bromosuccinamide indicates that role of tryptophan at or near the active site. Among the metal ions tested on α-galactosidase activity, Hg^{2+}, Cu^{2+}, and Ag^{2+} were strongly inhibited. Inhibition of Hg^{2+} ions suggested that the enzyme contains sulfahydryl group. Similar to our report, Garro *et al.* [28] reported complete inhibition of α-galactosidase in the presence of Hg^{+} and the enzyme was not inhibited by mercaptoethanol and EDTA. Fridjonsson *et al.* [34] reported that *B. stearothermophilus* from α-galactosidase is inhibited by Hg^{2+} and Cu^{2+} metal ions. King *et al.* [37] reported that *T. polysacchrolyticum* α-galactosidase is completely inhibited by Hg^{2+} ions, whereas fungal α-galactosidase was strongly inhibited by Ag^{2+}, Hg^{2+}, and metal ions [33,38].

4. CONCLUSION

The present study reveals that the application of thermostable novel α-galactosidase from *B. megaterium* VHM1 as a potent enzyme in the degradation of raffinose family oligosaccharides and food processing industry.

5. ACKNOWLEDGMENTS

Author Aravind Goud G Patil is thankful to the Council of Scientific and Industrial Research (CSIR), New Delhi, for

providing financial support in the form of Senior Research Fellowship (SRF).

6. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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How to cite this article:
Patil AGG, Kote NV, Manjula AC, Vishwanatha T. Purification, characterization of α-galactosidase from a novel *Bacillus megaterium* VHM1 and its applications in food industry. J Appl Biol Biotech 2021; 9(05):13–19.