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Production, purification and characterization of a constitutive intracellular α-galactosidase from the thermophilic fungus *Humicola* sp

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The thermophilic fungus *Humicola* sp constitutively produces intracellular α-galactosidase (1.33 U mg^{-1} protein) within 48 h at 45°C in shaken flasks, when grown in a medium containing 7% wheat bran extract as a carbon source and 0.5% yeast extract as a nitrogen source. The enzyme has been purified to homogeneity by ultrafiltration, ethanol precipitation, DEAE cellulose and Sephacryl S-300 chromatography with a 124-fold increase in specific activity and 29.5% recovery. The molecular weight of the enzyme is 371.5 kDa by gel filtration on Sephacryl S-300 and 87.1 kDa by SDS-polyacrylamide gel electrophoresis. The enzyme has an optimum temperature of 65°C and an optimum pH of 5.0. *Humicola* α-galactosidase is a glycoprotein with 8.3% carbohydrate content and is acidic in nature with a pI of 4.0. The K_mS for *p*-nitrophenyl-α-D-galactopyranoside, *o*-nitrophenyl-α-D-galactopyranoside, raffinose and stachyose are 0.279, 0.40, 1.45 and 1.42 mM respectively. The enzyme activity was strongly inhibited by Ag^{+} and Hg^{2+}. D-Galactose inhibited α-galactosidase competitively and the inhibition constant (K_i) for galactose was 11 mM.

Keywords: α-galactosidase; intracellular; thermophilic fungus; *Humicola* sp; purification

Introduction

There is considerable interest in enzymes that catalyze hydrolysis of glycoside bonds due to their extensive industrial, therapeutic and biochemical applications [29]. α-Galactosidase (E.C.3.2.1.22, α-d-galactoside galactohydrolase) catalyzes hydrolysis of simple and complex oligo- and polysaccharides containing α-1,6 linked terminal galactosyl groups, which are widely distributed among plants [5,9], humans [3,25] and microorganisms [4,21,29]. It plays a crucial role in enzymatic hydrolysis of raffinose which inhibits crystallization of sucrose from sugar beet molasses [19]. It is also used in the hydrolysis of raffinose and stachyose present in soybean milk, as these sugars cause intestinal discomfort and flatulence [9]. Studies on α-galactosidase are also of interest in medicine as its deficiency leads to Fabry's disease [14]. It is also involved in blood group specificity [23] and is required for ingestion of seed proteins by monogastric animals [28].

α-Galactosidases have been purified from such mesophilic fungi as *Aspergillus ficcum* [30], *A. niger* [21], *Mortierella vinacea* [27], *Penicillium purpurogenum* [26] and *Trichoderma reesei* [31]. Although extracellular α-galactosidases have been reported from fungi, the constitutive intracellular α-galactosidase has not been reported from the same thermophilic fungi. We have reported the extracellular α-galactosidase from *Humicola* sp [15–17] and in this paper we report the production, purification and characterization of a constitutive intracellular α-galactosidase from the thermophilic fungus *Humicola* sp, and its comparison with the extracellular enzyme.

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Materials and methods

Organism

The strain used throughout the present work was *Humicola* sp NCIM 1252. It was maintained on YpSs (yeast extract-peptone-starch agar) and PDA (potato dextrose agar) slants. YpSs medium contains (per litre distilled water): Difco yeast extract, 4 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; soluble starch (British Drug House), 15 g; and agar 20 g. Potato dextrose agar (PDA) contains (per litre distilled water): extract from 200 g potatoes; glucose, 20 g; Difco yeast extract, 1 g; and agar 20 g.

Medium and culture conditions

The basal medium contained (per liter of distilled water): KH_2PO_4, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; yeast extract, 5.0 g; and wheat bran extract, 20.0 g. Wheat bran extract was prepared according to Kotwal *et al* [15]. The pH of the basal medium was 6.0 and was not further adjusted. It was sterilized at 121°C for 40 min. The medium was inoculated with a 1×1 cm piece of 7-day-old sporulated culture from a PDA slant. The cultivation was carried out in 250-ml Erlenmeyer flasks each containing 50 ml of medium. The flasks were incubated at 45°C on a rotary shaker at 200 rpm. The fungal biomass was removed from the culture broth by filtration through muslin cloth and Whatman No. 1 filter paper.

Preparation of cell free extracts

Two methods were used for enzyme extraction from the biomass.

(1) Solvent extraction. One gram (wet weight) of mycelial cake was suspended in 10 ml of potassium phosphate buffer (100 mM, pH 7.0) containing 2% isopropanol.

The suspension was held at 45°C for 2 h on a rotary shaker at 200 rpm. Mycelium was separated by filtration through muslin cloth, followed by centifugation ($7000 \times g$, 20 min) and the clear supernatant was used as the source of the crude enzyme.

(2) Sonication. One gram of biomass (wet weight) was suspended in 40 ml of potassium phosphate buffer (100 mM, pH 7.0) in a beaker. The beaker was kept in an ice bath and sonicated using a Ralsonics ultrasonic processor at 10 kilocycles s^{-1} for 5 min. After sonication the biomass was removed by filtration followed by centrifugation as above, and the clear supernatant was used as the source of the crude enzyme.

Effect of carbon, nitrogen, initial pH, temperature and metal ions on enzyme production

Humicola sp was grown in media containing different carbon and nitrogen sources to determine a medium for optimum production of the enzyme. The fungus was also grown at different initial pHs (4–9) at 45°C and enzyme production was monitored for 60 h. To check the effect of temperature on production of enzyme, the fermentation was carried out at 37, 45, 50 and 55°C. Samples were removed after 48 h and assayed for α-galactosidase activity. The effect of metal ions on enzyme production was determined by adding 1 mM salt to the fermentation medium and intracellular enzyme activity was checked after incubating the culture at 45°C for 48 h.

Enzyme assay

α-Galactosidase activity was assayed in a water bath at 50°C for 10 min. The assay mixture contained 100 μl of suitably diluted enzyme, 50 μl of 666 μM pNPG and 850 μl of 100 mM citrate-phosphate buffer (pH 5.0). The reaction was terminated by adding 2 ml of 1 M sodium carbonate and the *p*-nitrophenol released was determined spectrophotometrically at 405 nm. When raffinose or stachyose was used as a substrate, the reducing sugars produced were estimated by the method of Nelson [22]. One unit (U) of enzyme activity was the amount of enzyme that produced 1 μmol of *p*-nitrophenol or reducing sugar per min.

Protein determination

Protein was determined according to Lowry *et al* with BSA as standard [20].

Purification of the intracellular α-galactosidase

Unless otherwise mentioned all the purification steps were carried out at 4°C. The clear supernatant of mycelia containing 900 mg protein (1200 units of α-galactosidase) was concentrated by ultrafiltration using an Amicon membrane YM-10 and dialyzed for 24 h against 20 mM potassium phosphate buffer pH 7.0. The dialyzate was further precipitated with three volumes of chilled distilled ethanol. The mixture was allowed to stand overnight and the resulting precipitate was collected by centrifugation ($7000 \times g$, 20 min) and dissolved in the same buffer. The solution was dialyzed for 24 h against 20 mM phosphate buffer, pH 7.0, and applied on DEAE cellulose equilibrated with 20 mM phosphate buffer, pH 7.0. The enzyme was eluted by the stepwise addition of 0.1, 0.2, 0.3, 0.4 and 0.5 M NaCl and fractions containing the highest activity were pooled, dialyzed, concentrated by ultra filtration and loaded on a Sephacryl S-300 (1×120 cm) column, equilibrated with 20 mM phosphate buffer pH 7.0 containing 0.15 M NaCl, at a flow rate 10 ml h^{-1}. Fractions containing α-galactosidase were pooled, concentrated by ultrafiltration, dialyzed against deionised water and used for further studies.

Electrophoresis

Under non denaturing conditions polyacrylamide gel electrophoresis was carried out on 7.5% gel at pH 8.3 using bromophenol blue as marker. SDS-PAGE (10% w/v) was carried out according to Laemmli [18]. Protein bands were stained either by Coomassie Blue R-250 or by silver staining.

Molecular weight determination

Gel filtration: The molecular weight of the native enzyme was estimated by gel filtration on a Sephacryl S-300 (1×120 cm) column equilibrated with 20 mM phosphate buffer, pH 7.0 containing 0.15 M NaCl using thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa) and bovine serum albumin (66 kDa) as standard proteins by the method of Andrews [1].

SDS-PAGE: The subunit molecular weight of α-galactosidase was determined by SDS-PAGE according to Laemmli [18]. The sigma molecular weight standard mixture of individual proteins: phosphorylase *b*, bovine plasma albumin, an egg albumin and carbonic anhydrase were used to calibrate the gel.

Isoelectric focusing

Isoelectric focusing was performed by using ampholytes (BioRad, New Delhi, India) of pH range 3–11, according to Chinnatambi *et al* [6].

Glycoprotein nature

Gels were stained for the detection of glycoprotein by the method of Gander [12]. The total neutral sugar content of the enzyme was determined by the phenol-sulfuric acid method of Dubois *et al* [11].

Kinetic studies

These were carried out using 666 μM pNPG in 100 mM citrate phosphate buffer (pH 5.0) as a substrate solution. The effect of pH on enzyme activity was studied for the pH range 3.0–8.0 using citrate phosphate, potassium phosphate and Tris-HCl buffer. The pH stability was studied by pre-incubating enzyme at room temperature for 24 h in the same pH range. The optimum temperature was determined over the range 40–70°C and the thermal stability was investigated by incubating the enzyme at the same temperature range for 1 h and the residual activity was then determined under standard assay conditions. The effect of substrate concentration on the α-galactosidase activity (K_m and V_{max} was determined using the Lineweaver–Burk plot.

Inhibition studies

Effect of salts and reagents: The inhibitory effect of salts on enzyme activity was studied by incubating 100 μl of the enzyme solution with 50 μl metallic ions and reagents in 100 mM citrate phosphate buffer pH 5.0 for 10 min at room temperature. The residual activity was determined under standard assay conditions.

Effect of sugars and sugar alcohols: One hundred microlitres of the enzyme were incubated with different sugars and sugar alcohols (10 mM effective concentration) with pNPG as substrate and the residual activity was determined under standard assay conditions. The inhibition constant for galactose was determined using pNPG as a substrate according to Dixon [10].

Results

Optimum conditions for mycelial growth and enzyme production

Among the carbon and nitrogen sources [yeast extract, malt extract, tryptone, peptone, casamino acid, beef extract and constip liquor (0.5%)] tested for enzyme production, maximum activity was obtained when 7% wheat bran extract was used as carbon source (Table 1) and 0.5% yeast extract as nitrogen source (data not shown). However, α-galactosidase activity was also detected when such inducers as raffinose, melibiose and galactose were used (Table 1). The maximum activity (1.33 U mg^{-1} of protein) was obtained within 48 h of fermentation and enzyme production was associated with biomass production (data not shown). Effect of initial pH (4–8) of the medium on α-galactosidase production indicated maximum production was obtained in a medium with initial pH 6.0. Maximum secretion of α-galactosidase occurred at 45°C and the fungus did not grow at 60°C. Hg^{2+} inhibited enzyme production strongly while Cu^{2+} had comparatively less inhibitory effect on the production of α-galactosidase (Table 2).

Table 1 Effect of various carbon sources on production of α-galactosidase

Carbon source	Final pH	Specific activity (U mg^{-1} protein)
Galactose 2.0%	5.9	0.18
Glucose 2.0%	6.0	0.06
Xylose 2.0%	6.0	0.88
Sucrose 2.0%	6.3	0.23
Raffinose 2.0%	6.2	0.60
Melibiose 2.0%	6.4	0.72
Starch 2.0%	6.2	0.64
Soyaflour 2.0%	7.1	1.22
Wheat bran extract		
2.0%	6.0	0.75
5.0%	6.0	0.95
7.0%	6.7	1.33
10%	6.9	1.05

Humicola sp was grown in shake culture at 45°C as described in Materials and Methods. Wheat bran extract in the medium was replaced by other carbon sources as listed.

Purification of intracellular α-galactosidase

The purification scheme for intracellular α-galactosidase from *Humicola* sp is summarized in Table 3. The yield of the enzyme after the final step was 2.14 mg of protein (29.5%) with a specific activity of 165 U mg^{-1} and a 124.8-fold purification. PAGE of the purified α-galactosidase under denaturing conditions (Figure 1) showed a single protein band. The purified enzyme had a molecular weight of 87 100 Da by SDS-PAGE and 371 500 Da by gel filtration on Sephacryl S-300. Carbohydrate content estimated was 8.3% using mannose as standard. The enzyme stained red with a yellow background indicating the presence of a carbohydrate moiety in the protein. The enzyme was an acidic protein with pI of 4.0 (Table 4).

Effect of pH and temperature on the activity of the purified α-galactosidase

The optimum pH for activity of the α-galactosidase was 5.0 and the optimum temperature was 65°C. The enzyme was more than 80% stable in the pH range 4.0–6.5 (Table 4). It retained complete activity at 55°C, half of its original activity at 60°C for 60 min and was completely inactivated at 65°C in 60 min (Figure 2).

Effect of various metal ions and reagents on the activity of purified α-galactosidase

Ag^{+}, Hg^{2+} and Ni^{2+} inhibited enzyme activity and there was no activation by any of the metal ions or reagents tested (Table 5). *p*-Chloromercuribenzoate (PCMB) and *p*-hydroxymercuribenzoate (PHMB) strongly inhibited the enzyme.

Effect of sugars and sugar alcohols on purified α-galactosidase activity

The effects of sugars are shown in Table 6. In the presence of 10 mM sugars only galactose was inhibitory. It inhibited the enzyme competitively and the inhibition constant (K_i) was 11 mM (Figure 3).

Substrate specificity

Mycelial α-galactosidase of *Humicola* sp hydrolyzed various glycosides and oligosaccharides containing α-galacto-

Table 2 Effect of metal ions on the production of α-galactosidase

Metal ion	Relative activity (%)
None	100
Zn^{2+}	96
Mg^{2+}	100
Ca^{2+}	102
Co^{2+}	100
Cu^{2+}	53
Hg^{2+}	18
Mn^{2+}	103

Humicola sp was grown with the above salts (1 mM effective concentration) in fermentation medium at 45°C under shaking conditions as described in Materials and Methods.

Table 3 Summary of purification procedure of intracellular α-galactosidase from *Humicola* sp

Purification steps	Activity (Units)	Protein (mg)	Sp. activity (U mg^{-1})	Purification (fold)	Yield (%)
Mycelial extract	1200	900	1.33	–	100.00
Amicon concentrate	1000	330	3.03	2.3	83.33
Alcohol precipitation	928	101	9.18	6.9	77.33
DEAE-cellulose	724	25	30.16	22.7	62.83
Sephacryl S-300	354	2.14	165.0	124.8	29.50

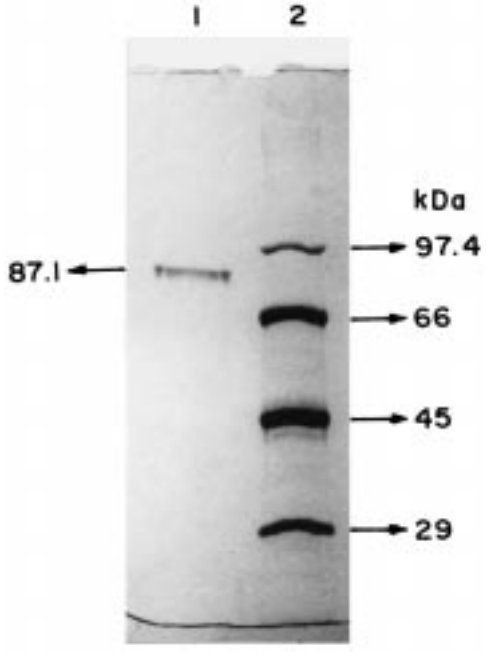

Figure 1 Electrophoresis of intracellular α-galactosidase under denaturing conditions (SDS-PAGE). Purified enzyme was electrophoresed on 10% gel and stained with Coomassie blue R-250. Lane 1: 5 μg denatured α-galactosidase; lane 2: sigma molecular weight standard mixture of individual proteins: phosphorylase-*b*, bovine plasma albumin, egg albumin and carbonic anhydrase.

Table 4 Properties of purified mycelial α-galactosidase from *Humicola* sp

Property	Value
Molecular mass (M_r) by:	
Sephacryl S-300	371.5 kDa
SDS-PAGE	87.1 kDa
pI	4.0
Optimum pH	5.0
pH stability	6.5–8.5 for 24 h at RT
Optimum temperature	65°C
Temperature stability	55°C for 60 min 60°C for 30 min 65°C for 10 min

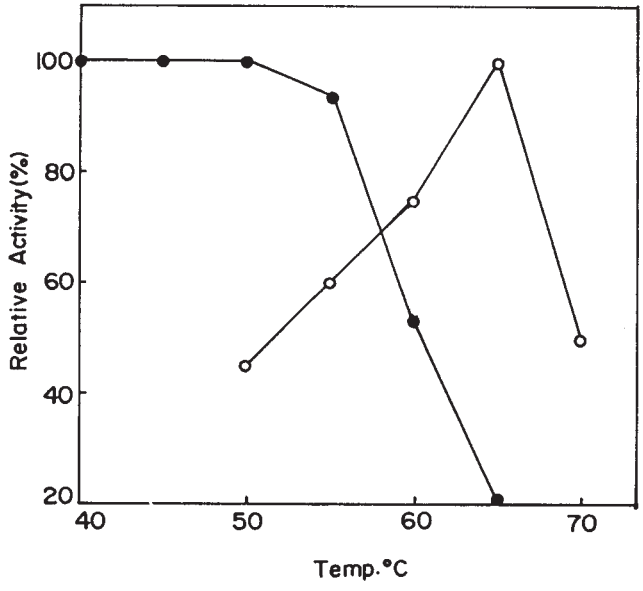

Figure 2 Effect of temperature on the activity (○--○) and stability (●--●) of *Humicola* α-galactosidase. Samples were incubated at temperatures ranging from 40–70°C for 1 h and the residual enzyme activity determined as described in Materials and Methods.

Table 5 Effect of metal ions and reagents on α-galactosidase activity

Metal ions	Residual activity (%)	Reagents	Residual activity (%)
None	100	KCl (1 mM)	95
Ag^{+}	0	EDTA (10 mM)	97
Ca^{2+}	97	NaF (10 mM)	100
Co^{2+}	93	NaN_3 (10 mM)	100
Cu^{2+}	93	Tris (10 mM)	81
Hg^{2+}	0	Urea (10 mM)	100
Mg^{2+}	100	Idoacetate (1 mM)	100
Mn^{2+}	103	2-Mercaptoethanol (1 mM)	100
Ni^{2+}	87	PCMB (1 mM)	0
Zn^{2+}	105	PHMB (1 mM)	0
–	–	N-Ethylmalieamide (1 mM)	100

100 μl of the enzyme was incubated with different metal ions (5 mM) concentrations) and reagents (as indicated) in 100 mM citrate phosphate buffer, pH 5.0 as described in Materials and Methods, and the residual activity was calculated.

Table 6 Effect of sugars and sugar alcohols on the activity of α-galactosidase

Sugars and sugar alcohols	Residual activity (%)
None	100
Arabinose	98
Galactose	63
Glucose	97
Glycerol	96
Fructose	95
Lactose	91
Maltose	88
Mannose	90
Mannitol	98
Sucrose	100
Sorbitol	93
Xylose	100

The enzyme (100 μl) was incubated with sugars and sugar alcohols (10 mM final concentrations) and the residual activity was calculated as described in Materials and Methods.

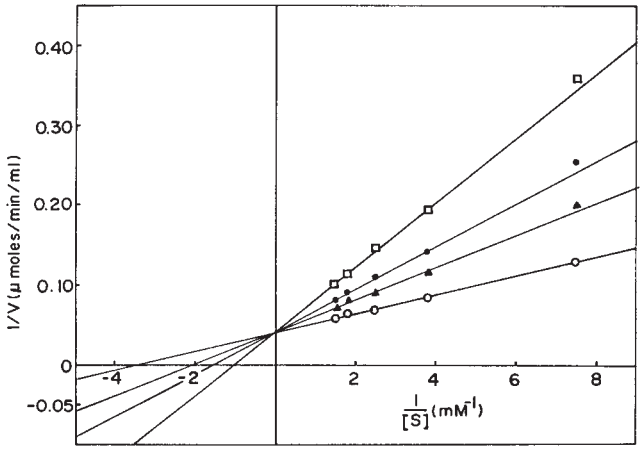

Figure 3 Inhibition of α-galactosidase by galactose at pH 5.0. Galactose concentrations: ○, 0 mM; ▲, 5 mM; ●, 10 mM; ◇, 15 mM.

sidic linkages at their non reducing ends, *o*- and *p*-nitrophenyl-α-d-galactopyranoside (oNPG, pNPG), melibiose, raffinose, stachyose, guar gum and locust bean gum (Table 7). It did not hydrolyze β-galactoside, α- and β-glucoside, mannoside and N-acetyl-α-d-galactopyranoside. α-Galactosidase from *Humicola* sp showed greater affinity towards *o*- and *p*-nitrophenyl-α-d-galactopyranoside as compared to raffinose and stachyose which are natural substrates of the enzyme. The values for apparent Michaelis constant (K_m) and maximum velocity (V_{max}) for several substrates are shown in Table 7.

Discussion

α-Galactosidases from bacteria, yeast and fungi are well documented. However, among thermophilic fungi there is a single report on inducible and partially purified intracellular α-galactosidase from *Penicillium dupontii* [2]. The α-galactosidase from the thermophilic fungus *Humicola* sp is the first report of production of constitutive and invertase-free intracellular α-galactosidase. The maximum enzyme activity (1.33 U mg^{-1}) found when the fungus is grown on 7% wheat bran extract is more than any extracellular α-galactosidase produced by other mesophilic fungi eg *Aspergillus nidulans* (sp. activity 0.1) and *Trichoderma reesei* (sp. activity 0.9) [24,31].

The pH and temperature optima of mycelial α-galactosidase were 5.0 and 65°C respectively and were similar to α-galactosidases from bacteria, yeast and other fungi [13,29,31], although higher temperature optima (75°C) have been reported for the enzyme from yeast, *Candida javanica* [4].

The mycelial α-galactosidase from *Humicola* was strongly inhibited by Ag^+ and Hg^{2+} similar to the enzyme from *Aspergillus tamarii* [7,8] and *Mortierella vinacea* [27]. Inhibition of the α-galactosidase activity by thiol modifiers such as PCMB and PHMB indicates the involvement of a thiol group in enzyme activity. However, this was not true with the enzyme from *M. vinacea* indicating that not all α-galactosidases require a SH group for activity [27]. d-Galactose at 10 mM inhibited the enzyme competi-

Table 7 Kinetic parameters of α-galactosidase from *Humicola* sp[b]

Substrates[a]	V_{max} (μmol min^{-1} mg^{-1})	Action	K_m (mM)
*p*NP-α-d-galactopyranoside	188.58	Hydrolysed	0.279
*o*NP-α-d-galactopyranoside	88.33	Hydrolysed	0.4
*m*NP-α-d-galactopyranoside	ND	Hydrolysed	ND
Melibiose	ND	Hydrolysed	ND
Raffinose	1.95	Hydrolysed	1.45
Stachyose	7.57	Hydrolysed	1.42
Guar gum	ND	Hydrolysed	ND
Locust bean gum	ND	Hydrolysed	ND

[a]*p*NP-N-acetyl-α-d-galactopyranoside, *p*NP-β-d-galactopyranoside, *p*NP-α-d-glucopyranoside, *p*NP-N-acetyl-α-d-glucopyranoside and *p*NP-α-d-mannopyranoside were not hydrolyzed.
[b]Substrates were prepared in 100 mM phosphate buffer pH 5.0. A suitable quantity of enzyme was added to produce a linear increase of *p*-nitrophenol, *o*-nitrophenol, glucose and galactose during the first 10 min of the reaction. The kinetic parameters were determined under standard assay conditions using the concentrations in the range of 0.05–1 mg for synthetic substrates and 0.5–10.0 mM for natural substrates. The values were determined by fitting the data to linear regression using Lineweaver–Burk or Eadie–Hofstee plots.
ND, Not determined.

tively and the inhibitor constant (K_i) was 11 mM (Figure 3). Competitive inhibition by d-galactose has been reported for α-galactosidase from *Trichoderma reesei* [31]. However, in the case of *C. javanica* α-galactosidase, 10 mM galactose did not have any effect [4].

The molecular weight of α-galactosidase by gel filtration on Sephacryl S-300 was 371.5 kDa and its subunit molecular weight by SDS-PAGE was 87.1 kDa indicating that the enzyme is a tetramer (Figure 1). Molecular weights of α-galactosidases from various microorganisms range from 45 kDa to 330 kDa and these are composed of several identical subunits [7,24,29,30]. The isoelectric point of this enzyme is 4.0 and other α-galactosidases have an isoelectic point in the range of 4.4–6.3 [13,26,30].

Mycelial α-galactosidase from *Humicola* hydrolyzed pNPG, oNPG, mNPG, melibiose, raffinose, stachyose, guar gum and locust bean gum (Table 7). The enzyme showed more affinity towards pNPG (K_m 0.279 mM) and oNPG (K_m 0.4) than raffinose (K_m 1.45 mM) and stachyose (K_m 1.42). The enzyme did not hydrolyze other substrates indicating that the configuration of the substrate is important for the enzyme to act catalytically. K_m and V_{max} values for these substrates are comparable to those previously reported from other organisms [4,13,24]. The K_m values of mycelial α-galactosidase for pNPG from various microorganisms range from 0.16–0.60 mM while for oNPG it ranges from 0.30–11.0 mM. Only in case of α-galactosidase for *M. vinacea*, is K_m for oNPG (0.36 mM) lower than for pNPG (0.43 mM) [27]. However, the enzyme from *T. reesei* does not show significant differences in affinity for various α-d-galactosides having different oligosaccharide chain lengths [31]. The mycelial α-galactosidase of *Humicola* is a glycoprotein similar to other fungal α-galactosidases [7,8,27,30,31] and it contains 8.3% neutral sugar.

The intracellular and extracellular α-galactosidases from *Humicola* have different properties. The molecular weight of intracellular α-galactosidase was higher (371 500) than the extracellular α-galactosidase (354 800). The optimum temperature of the intracellular enzyme was 65°C and that for extracellular enzyme was 60°C. The isoelectric points for intracellular and extracellular enzyme were 4.0 and 4.90 respectively. Intracellular enzyme showed more affinity towards pNPG (K_m 0.279), oNPG (K_m 0.4), raffinose (K_m 1.45) and stachyose (K_m 1.42) compared to extracellular enzyme in which K_m for pNPG, oNPG, raffinose and stachyose were 0.31, 0.54, 3.3 and 7.6 mM respectively. d-Galactose inhibited the intracellular α-galactosidase competitively and the extracellular α-galactosidase noncompetitively. There are reports of both intra- and extracellular α-galactosidase production and characterization of mesophilic fungi *viz Aspergillus nidulans* [24] and *A. tamarii* [7,8], showing similar and different properties of intracellular and extracellular α-galactosidases.

The high yield of α-galactosidase by *Humicola* sp using wheat bran, its acidic pH optima and heat stability may prove useful in industrial applications for degradation of raffinose from beet molasses as well as for hydrolysis of oligosaccharides having α-galactoside linkages.

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