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Production of thermostable α-galactosidase from thermophilic fungus *Humicola* sp

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The thermophilic fungus, *Humicola* sp isolated from soil, secreted extracellular α-galactosidase in a medium containing wheat bran extract and yeast extract. Maximum enzyme production was found in a medium containing 5% wheat bran extract as a carbon source and 0.5% beef extract as a carbon and nitrogen source. Enzyme secretion was strongly inhibited by the presence of Cu^{2+}, Ni^{2+} and Hg^{2+} (1 mM) in the fermentation medium. Production of enzyme under stationary conditions resulted in 10-fold higher activity than under shaking conditions. The temperature range for production of the enzyme was 37° C to 55° C, with maximum activity (5.54 U ml^{-1}) at 45° C. Optimum pH and temperature for enzyme activity were 5.0 and 60° C, respectively. One hundred per cent of the original activity was retained after heating the enzyme at 60° C for 1 h. At 5 mM Hg^{2+} strongly inhibited enzyme activity. The K_m and V_{max} for *p*-nitrophenyl-α-D-galactopyranoside were 60 μM and 33.6 μmol min^{-1} mg^{-1}, respectively, while for raffinose those values were 10.52 mM and 1.8 μmol min^{-1} mg^{-1}, respectively.

Keywords: α-galactosidase; thermophilic fungus; *Humicola*

Introduction

α-Galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) is widely distributed in nature among microorganisms, plants and animals [3]. The enzyme catalyses the hydrolysis of α-1,6-linked galactose moieties present in galacto-, oligo- and polysaccharides, especially those which occur among legume seeds [5,13]. The enzyme hydrolyses galactosaccharides and liberates free sugars which may serve as a ready energy source [4]. The enzyme is very important in the processing of beet sugar, in which it is used to remove raffinose which inhibits normal crystallization of beet sugar [9]. It is also used in the hydrolysis of raffinose and stachyose present in soybean milk, as these sugars cause intestinal discomfort and flatulence [2,16]. There are several reports on the use of mycelia containing α-galactosidase for commercial hydrolysis of raffinose from sugar beet molasses [10]. Several patents have been awarded for production of α-galactosidase from mesophiles, eg *Pseudomonas fluorescens* H-601 α-galactosidase showing pH and temperature optima of 6–7 and 45° C, respectively [8], thermostable (100% active at 60° C after 1 h) α-galactosidase from *Candida guillermondii* having an activity of 0.8 units ml^{-1} [7] and from *Paecilomyces varioti* HS-1001 [6].

Although there are several reports of α-galactosidase from mesophilic fungi [17,19–21], an extracellular α-galactosidase has not yet been reported from thermophilic fungi. Among other thermophiles *B. stearothermophilus* [18] produces extracellular α-galactosidase. In the present paper we report the production of extracellular α-galactosidase from a newly isolated thermophilic fungus, *Humicola* sp.

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Materials and methods

Culture

The thermophilic fungus *Humicola* sp was isolated from soil samples collected around decaying plant material. It was maintained on YpSs and PDA slants. YpSs medium contains (per liter distilled water): Difco yeast extract, 4 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; soluble starch (BDH), 15 g and agar 20 g. Potato dextrose agar (PDA) contains (per liter of distilled water): extract from 200 g potatoes; glucose, 20 g; Difco yeast extract, 1 g and agar 20 g. The *Humicola* sp is deposited with the National Collection of Industrial Micro-organisms (NCIM), Division of Biochemical Sciences, National Chemical Laboratory, Pune 411 008, India, with accession number 1252.

Medium and culture conditions

The basal medium contained (per liter of distilled water): K_2HPO_4, 3 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; yeast extract, 5 g and wheat bran extract 20 g. Wheat bran extract was prepared by steaming 100 g wheat bran in 1 liter distilled water for 30 min. The supernatant fluid was decanted and its volume was adjusted to 2 liters. The pH of the basal medium was 6 and was not further controlled. The medium was inoculated with a 1 × 1-cm piece of 7-day-old sporulated culture from a PDA slant. Cultivation was carried out in 250-ml Erlenmeyer flasks containing 50 ml of medium. The cultures were incubated at 45° C on a rotary shaker at 200 rpm or kept under stationary conditions. The mycelium was removed from the culture broth by filtration through muslin cloth followed by Whatman no 1 and the clear supernatant phase was used for determining α-galactosidase activity. Enzyme activity was precipitated with ammonium sulfate at 90% saturation and the precipitate was dialysed against 20 mM phosphate buffer, pH 7.0. The dialysate was used to study enzyme properties.

Effect of pH, temperature and metal ions on enzyme production

The fungus was grown at different initial pH's (4–9) at 45° C and enzyme production was monitored for 12 days. To check the effect of temperature on production of enzyme, fermentation was carried out at 37, 45, 50 and 55° C. Samples were removed on the 10th day and assayed for α-galactosidase activity. The effect of metal ions on enzyme production was monitored by adding 1 mM salt to the fermentation medium and extracellular enzyme activity was checked after incubating the culture at 45° C for 10 days without shaking.

Enzyme assay

α-Galactosidase was assayed by incubating 100 μl of suitably diluted enzyme with 50 μl of 666 μM substrate (*p*-nitrophenyl-α-D-galactopyranoside) and 850 μl of 100 mM citrate-phosphate buffer (pH 5.0) at 50° C for 10 min. The reaction was terminated by adding 2 ml of 1 M sodium carbonate and the *p*-nitrophenol released was determined from absorbance at 405 nm. When raffinose was used as substrate, the reducing sugar produced was determined by the method of Somogyi [15] and Nelson [12]. One unit (U) of α-galactosidase activity was expressed as the amount of enzyme that liberates 1 μmol of product (*p*-nitrophenol or reducing sugar) per minute under the assay conditions. Protein was measured by the method of Lowry *et al* [11] with bovine serum albumin as standard.

All experiments were carried out in quadruplicate and values reported are mean values of four such experiments in which 3–5% variability was observed.

Results

Isolation of thermophilic fungi

Fifteen different fungi were isolated from decaying plant material containing soil samples which were collected randomly. Single spores were inoculated on YpSs agar plates which were incubated at 45° C. Isolates were screened for the production of α-galactosidase in wheat bran extract liquid medium. One of the fungi, *Humicola* sp NCIM 1252 producing α-galactosidase was selected for further work.

Effect of carbon sources on production of α-galactosidase

The effect of different carbon sources on the production of α-galactosidase in stationary culture is summarised in Table 1. The enzyme secretion was constitutive and maximum activity (3.47 U ml^{-1}) was found in a medium supplemented with raffinose as a carbon source. Since 5% wheat bran extract also gave fairly high activity (2.59 U ml^{-1}) and is an economic carbon source, it was used for further fermentation studies.

Among the different organic nitrogen sources tested for enzyme production, beef extract (5.54 U ml^{-1}) was the best nitrogen source (Table 2).

Time course of α-galactosidase production

The time course of α-galactosidase production in medium containing 5% wheat bran extract and 0.5% beef extract in stationary and in shaken cultures is shown in Figure 1a and b, respectively. Under both conditions enzyme secretion was growth-associated. In stationary cultures maximum enzyme activity 5.3 U ml^{-1} (2.34 U mg^{-1} protein) was obtained on the 10th day of fermentation while in shaken cultures maximum enzyme activity was 0.53 U ml^{-1} (0.84 U mg^{-1} protein) after 48 h. Increase in biomass was rapid in shake culture compared to stationary cultures. This may be due to increased oxygen availability to the fungus in shake cultures.

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

Carbon source	Final pH	Enzyme activity (U ml^{-1})
Glucose 2.0%	6.8	0.78
Sucrose 2.0%	7.1	1.46
Xylose 2.0%	6.7	0.64
Starch 2.0%	6.8	0.85
Lactose 2.0	6.7	0.51
Galactose 2.0%	6.8	0.93
Fructose 2.0%	6.7	0.88
Maltose 2.0%	6.8	1.03
Cellobiose 2.0%	6.6	0.68
Raffinose 2.0%	7.8	3.47
Melibiose 2.0%	6.8	0.75
Wheat bran extract		
2.0%	7.0	0.94
5.0%	7.4	2.59
7.0%	7.4	2.50

The fungus was grown without shaking at 45° C as described in Materials and Methods. Wheat bran extract in the medium was replaced by other carbon sources as listed

Table 2 Effect of nitrogen sources on production of α-galactosidase

Nitrogen source (0.5%)	Final pH	α-galactosidase activity (U ml^{-1})
Yeast extract	7.8	3.93
Malt extract	7.6	2.80
Corn steep liquor	7.7	3.44
Peptone	7.1	2.33
Liver extract	7.8	4.57
Beef extract	7.8	5.54
Tryptone	7.2	2.61
Casamino acid	7.0	2.07

The fungus was grown in stationary culture at 45° C as described in Materials and Methods. Yeast extract in the medium was replaced by other nitrogen sources as listed

Effect of temperature on production of α-galactosidase

The effect of different temperatures (37–55° C) on production of α-galactosidase is shown in Figure 2. Enzyme secretion was maximum at 45° C with or without shaking. Less enzyme activity was detected above 50° C in shaken cultures. At 60° C the fungus did not grow.

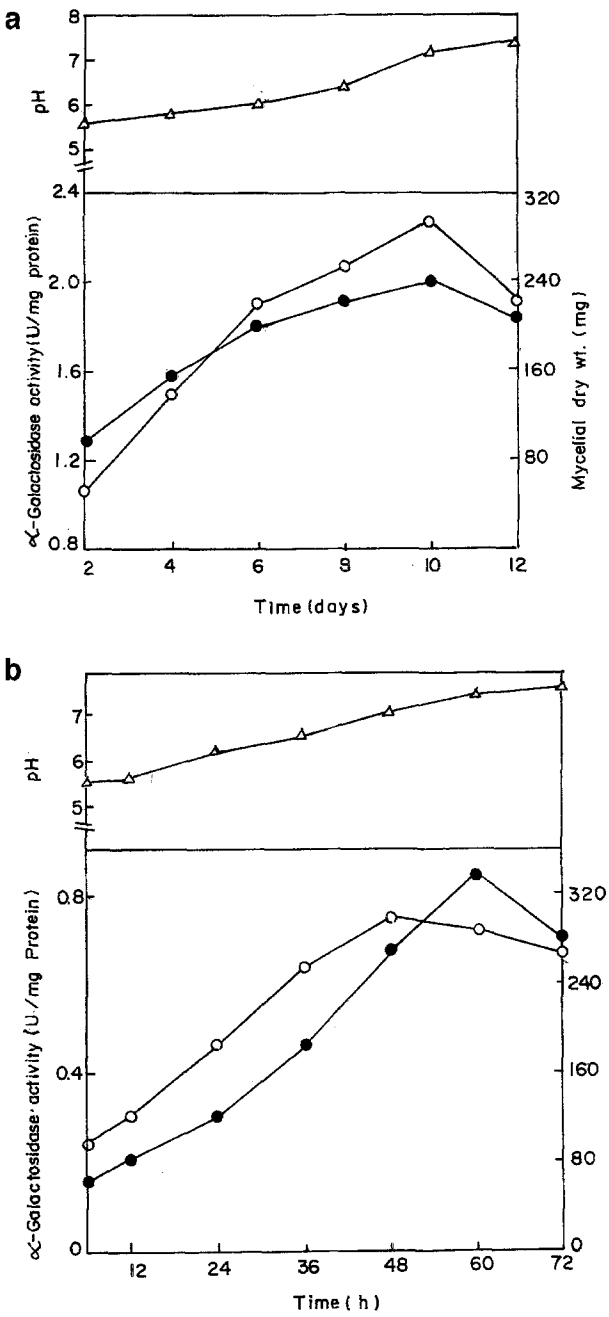

Figure 1 Time course of α-galactosidase production by *Humicola* sp using 5% wheat bran extract medium. (a) Stationary cultures: specific activity (○), pH (△) and biomass (●); (b) Shaken cultures: specific activity (○), pH (△) and biomass (●)

Effect of initial pH on enzyme production

The effect of initial pH (4–9) of the medium on α-galactosidase production in stationary cultures is shown in Figure 3. Even though the fungus grew over a wide pH range, maximum enzyme secretion occurred in a medium having an initial pH of 6.0.

Effect of metal ions on enzyme production

The effect of different metal ions (1 mM) on production of α-galactosidase was examined (Table 3). Cu^{2+}, Co^{2+}, Ni^{2+} and Hg^{2+} had a strong inhibitory effect on enzyme production, Ca^{2+} and Mn^{2+} had a lesser inhibition, and Zn^{2+} and Mg^{2+} had a stimulatory effect.

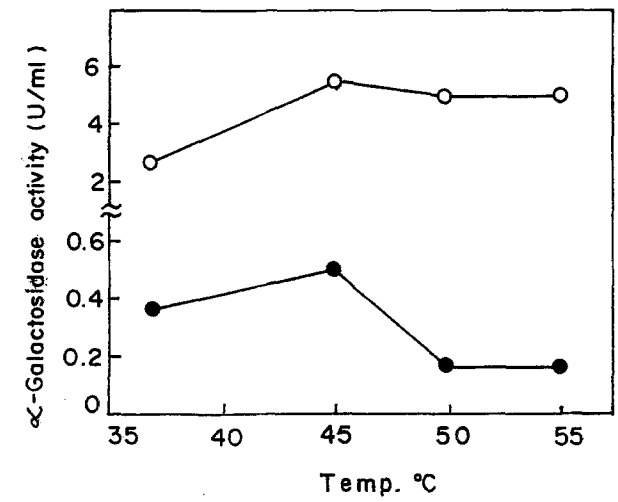

Figure 2 Effect of temperature on the production of α-galactosidase in stationary (○) and shaken (●) cultures

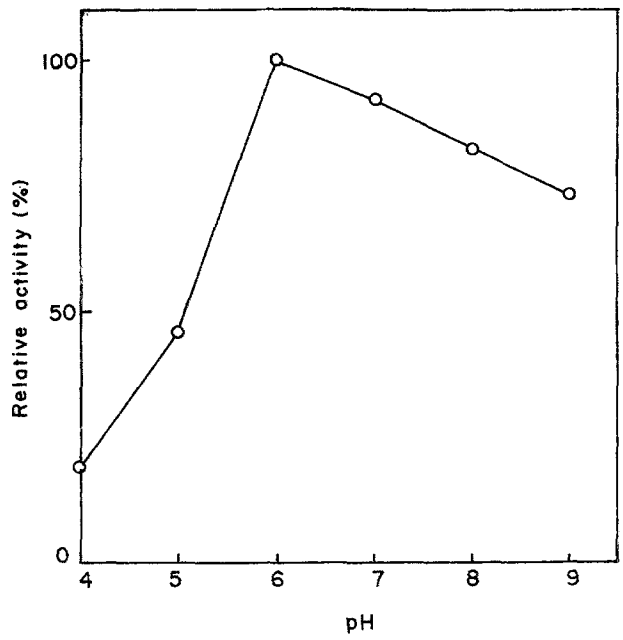

Figure 3 α-Galactosidase production by *Humicola* sp at different initial pHs. pH was adjusted with 0.1 N NaOH or 0.1 M HCl

Effect of pH on enzyme activity and stability

Enzyme was most active at pH 5 and was 100% stable in the pH range 4–5 (Figure 4). Only 12% inactivation was found at pH 3 and 7 when enzyme was incubated at 4° C for 16 h at the respective pH and residual activity was determined under standard assay conditions.

Effect of temperature on enzyme activity and stability

The effect of temperature on enzyme activity and stability is shown in Figure 5. The optimum temperature for enzyme activity was 60° C. It was stable at 60° C for 1 h. The enzyme was rapidly inactivated above 60° C.

Table 3 Effect of metal ions on production of α-galactosidase

Metal ion (1 mM)	Relative activity (%)
None	85
Cu^{2+}	6
Ca^{2+}	20
Zn^{2+}	118
Ni^{2+}	4
Mg^{2+}	100
Hg^{2+}	3
Co^{2+}	10
Mn^{2+}	26

Humicola sp was grown with the above salts (1 mM) in fermentation medium at 45° C in stationary cultures as described in Materials and Methods. Mg^{2+} in the medium was replaced by the metal ion as listed

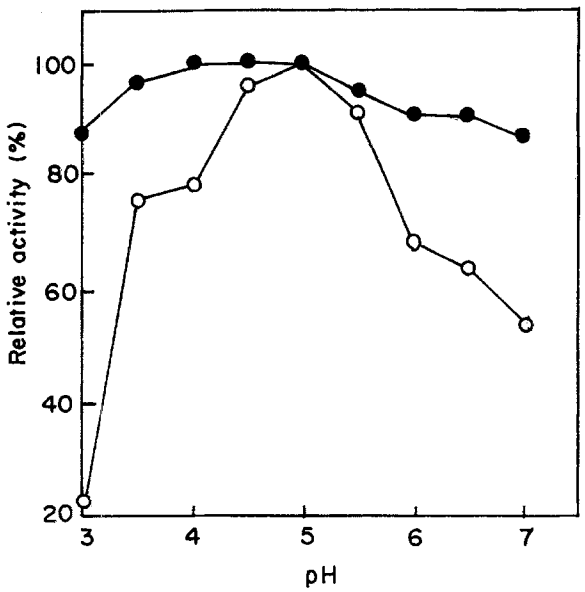

Figure 4 Effect of pH on α-galactosidase activity (○) and stability (●). For optimum pH, α-galactosidase activity was measured using desired buffers (100 mM citrate-phosphate and sodium phosphate) at 50° C for 10 min. For pH stability the enzyme solutions were kept at the desired pH at 4° C for 16 h and then used for activity measurement

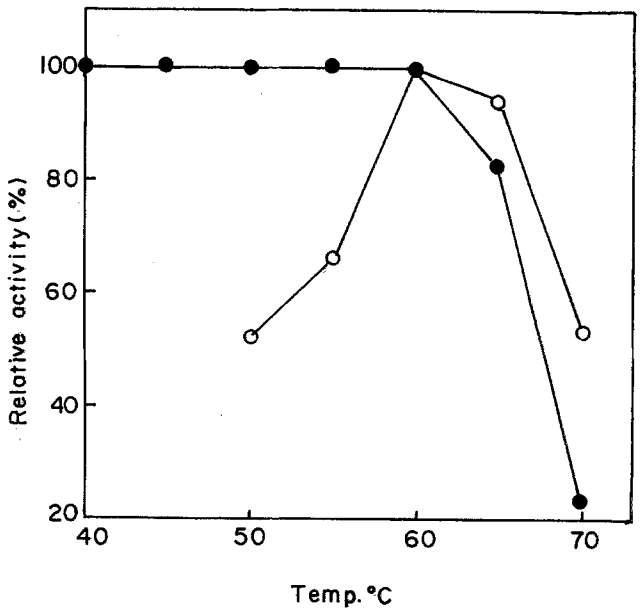

Figure 5 Effect of temperature on α-galactosidase activity (○) and stability (●). For thermal stability the enzyme solution was held at the temperature indicated for 10 min then cooled immediately in ice and the residual activity was measured

Table 4 Effect of metal ions on α-galactosidase activity

Metal ion (5 mM)	Relative activity (%)
None	100
Hg^{2+}	9
Mn^{2+}	21
Ni^{2+}	82
Cu^{2+}	50
Ca^{2+}	84
Zn^{2+}	90
Co^{2+}	72
Mg^{2+}	100

α-Galactosidase was obtained by the procedure described in Materials and Methods. The metal ions to be tested were added to the assay system and the results expressed as a percentage of the control

Effect of metal ions on enzyme activity

The effect of metal ions on the activity of α-galactosidase was examined (Table 4). Mg^{2+} had no apparent effect on activity. The enzyme was strongly inhibited by 5 mM Hg^{2+}, and it was inhibited to lesser degrees by the other metals tested.

The K_m and V_{max} for *p*-nitrophenyl-α-D-galactopyranoside were 60 μM and 33.6 μmol min^{-1} mg^{-1}, respectively and for raffinose 10.52 mM and 1.823 μmol min^{-1} mg^{-1}, respectively.

Discussion

α-Galactosidase from mesophilic bacteria, yeast and fungi is well documented. Among thermophilic fungi there is only one report of intracellular α-galactosidase, from *Penicillium dupontii* [1]. There is no report of extracellular α-galactosidase from thermophilic fungi. Results in the present paper indicate that the newly isolated thermophilic fungus, identified as *Humicola* sp NCIM 1252 produces higher levels of α-galactosidase (5.54 U ml^{-1} when 33.3 μM *p*-nitrophenyl-α-D-galactopyranoside was used as substrate or 0.389 U ml^{-1} when 25 mM raffinose was used as a substrate) than other mesophilic fungi, eg *Aspergillus nidulans* (specific activity 0.1), *Trichoderma reesei* (specific activity 0.9) [14,21] and yeast *Candida guillermondii* (0.8 U ml^{-1}) [7]. Moreover, extract from a cheap agricultural residue like wheat bran can be used instead of raffinose, melibiose and stachyose as a carbon source. The enzyme from *Humicola* sp had a pH optimum of 5 but

retained 50% activity at pH 7. Similar observations were reported for the enzyme from *A. nidulans* [14]. However the enzyme from *Humicola* is highly thermostable compared to the enzyme from *A. nidulans* and *Trichoderma reesei* RUT C-30 which were inactivated above 40° C [14,21]. α-Galactosidase from *Humicola* sp had a temperature optimum at 60° C.

Similar results were reported for the enzyme from *Aspergillus ficcum* NRRL 3135 and *T. reesei* RUT C-30 [19,21]. Further work on purification and characterization of the enzyme is in progress.

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