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PURIFICATION AND CHARACTERIZATION OF A THERMOSTABLE α-GALACTOSIDASE FROM *THIELAVIA TERRESTRIS* NRRL 8126 IN SOLID STATE FERMENTATION

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Several seeds and husks of some plants belonging to leguminosae, Graminae, Compositae and Palmae were evaluated as carbon substrates to produce α-galactosidase (α-Gal) by the thermophilic fungus, *Thielavia terrestris* NRRL 8126 in solid substrate fermentation. The results showed that *Cicer arietinum* (chick pea seed) was the best substrate for α-Gal production. The crude enzyme was precipitated by ammonium sulphate (60%) and purified by gel filtration on sephadex G_{-100} followed by ion exchange chromatography on DEAE-Cellulose. The final purification fold of the enzyme was 30.42. The temperature and pH optima of purified α-Gal from *Thielavia terrestris* were 70 °C and 6.5, respectively. The enzyme showed high thermal stability at 70 °C and 75 °C and the half-life of the α-Gal at 90 °C was 45 min. K_m of the purified enzyme was 1.31 mM. The purified enzyme was inhibited by Ag^{2+}, Hg^{2+}, Zn^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+} and Fe^{2+} at 5 mM and 10 mM. Also, EDTA, sodium arsenate, L-cysteine and iodoacetate inhibited the enzyme activity. On the other hand, Ca^{2+}, Cu^{2+}, K^+ and Na^+ slightly enhanced the enzyme activity at 5 mM while at 10 mM they caused inhibition. The molecular weight of the α-Gal was estimated to be 82 kDa by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. This enzyme displays a number of biochemical properties that make it a potentially strong candidate for biotechnological and medicinal applications.

Keywords: Hemicelluloses – α-galactosidase – thermostability – *Thielavia terrestris*

INTRODUCTION

The α-galactosidases (α-D-galactoside galactohydrolase, EC 3.2.1.22) are carbohydrases which catalyse the hydrolysis of α-1,6-linked α-galactoside residual from simple oligosaccharides such as melibiose, raffinose, stachyose and from polymeric galactomannans [1, 3, 6, 10, 11]. These enzymes are often described as hydrolytic, but some of them also show transglycosylation activity.

α-Galactosidases are widely distributed in microorganisms, plants and animals. Microorganisms have the advantage of being highly active producers, and among them the α-galactosidases from filamentous fungi are the most suitable for techno-

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logical applications because of their extracellular localization, acidic pH optimum and broad stability profiles [3, 11, 23, 34]. To our knowledge, this is the first report on production and purification of α-Gal from a thermophilic fungus, *"Thielavia terrestris"*. Thermophilic fungi can be grown in minimal media with metabolic rates and growth yields comparable to those of mesophilic fungi. They have a powerful ability to degrade polysaccharide constituents of biomass. Their extracellular enzymes are more heat stable than those of the mesophilic fungi. The use of thermostable enzymes to carry out hydrolysis at higher temperatures is generally advantageous because it increases the speed of reaction and avoids microbial contamination contributing to increase technical and economical viability of the process [2, 3, 6, 18, 27, 30–32, 35].

Solid-state fermentation (SSF) is an attractive process to produce enzymes economically due to enhanced enzyme yield, enzyme titre, higher product stability, lower catabolite repression and lower capital, operating and recovery costs [4, 11, 14, 17, 21, 33, 36]. For the production of α-Gal enzyme, some natural agro-industrial wastes or seeds and husks of certain leguminous plants were used so as to decrease the production costs of this enzyme [3, 6, 11].

α-Galactosidases have many applications in everyday life. They are used in the hydrolysis of raffinose and stachyose present in leguminous food that causes intestinal discomfort, flatulence and low feed utilization in monogastrites [24]. This enzyme is also used to improve the gelling properties of galactomannans to be used as food thickeners [8]. In the pulp and paper industry, α-Gal could enhance the bleaching effect of β-mannanases on softwood pulp [16]. In human medicine, this enzyme can be used for the treatment of Fabry's disease [5] or for the blood type conversion [22]. Due to the wide industrial application of α-Gal, their production process has had a great development and capital investment. For that reason, it is important to search for new substrates and microorganisms able to produce α-Gal in great proportions on an economic scale.

The aim of the present paper is to describe the production, purification and some characteristics of α-Gal from the thermophilic fungus, *Thielavia terrestris* after investigating the capacity of this fungus to produce a high yield of extracellular α-Gal in solid-state fermentation using seeds and husks of some plants as carbon sources.

MATERIALS AND METHODS

Materials

P-Nitrophenyl-α-D-galactopiranoside (PNPG) was purchased from Sigma Chemical Co., USA. Sephadex $G\text{-}_{100}$ and DEAE cellulose were purchased from Sigma Pharmaceutical Industries, Nasr City, Cairo, Egypt. All other substrates used (seeds and husks of plants which are presented in Table 1) were obtained from the Agricultural Research Center, Dokky, Cairo, Egypt. These substrates were oven dried at 60 °C, grounded and sieved through 40-mesh screen.

Fungal strain

The strain *Thielavia terrestris* (Apinis) Malloch & Cain NRRL 8126 used in this work was obtained from NRRL (Agricultural Research Service Culture Collection). It was provided by United States Department of Agriculture (USDA), New Orleans, Louisiana 70179. The strain was grown on malt extract agar (MEA) slants at 50 °C for 4 days.

Screening medium (solid state culture) and enzyme production

Cultures were carried out in 250 ml Erlenmeyer conical flasks containing 8 ml of culture medium (g L^{-1}) K_2HPO_4, 1.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4$, 0.5 and 20 g of each substrate which was used as carbon source (listed in Table 1). After sterilization at 121 °C for 20 min, each flask was inoculated with 2 ml of spore suspension (10^6 spores ml^{-1}). The flasks were then incubated at 40 °C for 4 days [33]. The content of each flask was gathered up and thoroughly mixed with 15 ml cooled sterilized distilled water, then the enzyme production was determined. Assays were carried out to investigate the substrate that showed the greatest enzyme production. Each trial was made in triplicate.

Determination of hemicellulose of substrates

Acid (HCl 5%) hydrolyzed seeds and husks of plants (listed in Table 1) were analyzed in terms of hemicelulose contents as described by Jermyn [15].

α-Gal assay

α-Gal activity was assayed by the modified method of Dey [9] using *p*-nitrophenyl-α-D-galactopyranoside (PNPG) as the substrate. Reaction mixture contained supernatant (0.2 ml), acetate buffer 0.2 M (0.3 ml, pH 5.0) and PNPG 3 mM (0.5 ml). The reaction was carried out at 37 °C for 15 min and was stopped by adding 3 ml of 0.1 M sodium carbonate. *P*-nitrophenol (PNP) was quantified spectrophotometrically at λ 405 nm. One unit (U) of α-Gal activity is defined as amount of enzyme liberating 1 μmol of PNP in 1 min under assay conditions.

Protein assay

The protein concentration was determined by Bradford method [7] using bovine serum albumin as a standard.

Purification of α-Gal

The enzyme was purified from the culture supernatant. All operations were done at 0 to 4 °C. The cooled culture supernatant (200 ml) was dialysate in Sigma dialyzing bags against distilled water for 24 h. The obtained cell free dialyzed was precipitated by 60% $(NH_4)_2SO_4$ and then centrifuged, dissolved in 3 ml of 50 mM citrate phosphate buffer (pH 6.0). The enzymatic protein was fractionated through Sephadex $G\text{-}_{100}$ column (18 × 2 cm) eluted with the same buffer (180 ml) at 20 ml h^{-1}. Five ml fractions were collected and assayed for protein and α-Gal activity [29]. The active fractions (Fractions 11–17) with the highest specific activity of enzyme were pooled and dialyzed. The pooled fractions of enzyme were further fractionated through DEAE-Cellulose column (diethylaminoethyl-cellulose) eluted with a gradient of 0–0.8 M NaCl in citrate phosphate buffer (150 ml) at a flow rate of 10 ml h^{-1}. Five ml fractions were collected and assayed for protein and enzyme activity. The most active fractions (Fractions 11–15) were collected and dialyzed once again to remove Na^+ and Cl^- [26, 28]. The prepared enzyme was freeze dried and stored at 0 °C for further investigations.

Characterization of the purified α-Gal

pH optimum and stability

The effect of pH on α-Gal was measured over a pH range from 3.5 to 9.0 by using either 0.05 M citrate-phosphate or 0.05 M Tris-HCl buffer. To determine pH stability, enzyme was incubated in buffers within the above cited range for 12 h. The residual activity for enzyme was assayed.

Temperature optimum and thermal stability

The effect of temperature on enzyme activity was assessed by incubating the enzyme with PNPG at various temperatures ranging from 30 to 90 °C. Enzyme activity was measured to determine the optimum temperature for activity. However, thermal stability was determined after incubating the enzyme for variable durations (15 to 90 min) at fixed temperatures (70 to 90 °C). The remaining activity was determined under standard assay condition.

Determination of Michaelis constant

The effect of PNPG concentration, ranging from 0.2 to 1.2 mg ml^{-1} on α-Gal activity was evaluated under optimal assay conditions. The Michaelis–Menten constant (K_m) was estimated by linear regression from double-reciprocal plots according to Lineweaver and Burk [20].

Effect of different salts and some enzyme inhibitors

The effects of salts and chemical reagents on the enzyme were examined by incubating 0.2 ml of enzyme solutions with each of the salts and chemical reagents at a concentration of 5 or 10 mM in 50 mM citrate phosphate buffer (pH 6.0). The remaining activity of the enzyme was measured under the standard assay conditions.

Molecular mass determination

The molecular mass of the purified inulinases was estimated in sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) using standard protein markers: -phosphorylase-b, 97.0 kDa; bovine serum albumin, 67.0 kDa; egg albumin, 45.0 kDa; glyceraldehyde-3-phosphate, 36.0 kDa; beta-lactoglobulin, 18.4 kDa [19].

Statistical validation of treatment effects

The mean, standard deviation, T-score and probability "P" values of 3 replicates of the investigated factors and the control were computed according to the mathematical principles described by Glantz [13]. Results were considered highly significant, significant or non-significant where $P < 0.01$, > 0.01 and < 0.05, > 0.05, respectively.

RESULTS AND DISCUSSION

Several natural plant products [Leguminosae (seeds of *Cicer arietinum, Lens esculenta, Lupinus termis* and *Pisum sativum;* husk of *Arachis hypogaea*); Graminae (bran of *Hordeum vulgare, Triticum aestivum;* seeds of *Panicum miliaceum, Phalaris canariensis;* straw of *Oryza sativa*); Compositae (seed husks of *Helianthus annuus*) and Palmae (fruits of *Hyphaene thebaica*)] were evaluated as carbon sources to produce α-Gal by *Thielavia terrestris* in solid state fermentation (Table 1). The highest enzyme production was obtained with *Cicer arietinum* seeds (5.86 U/g) followed by *Lupinus termis* (5.49 U/g); *Lens esculenta* (4.74 U/g); *Pisum sativum* (4.19 U/g) and the husk of *Arachis hypogaea* (3.72 U/g), while the other substrates belonging to Graminae, Compositae and Palmae produced lower amounts of the enzyme. Similar results were stated by Gaillard [12], he stated that the Leguminosae plants material were favorable substrates for the enhancement of α-Gal production, because they contained relatively high amounts of hemicelluloses, particularly uronic acid, in addition to galactose and arabinose. α-Gal aid in the digestion of legumes, they play a crucial role in improving the nutritional value of legume-based food [4, 11]. Chemical composition of hemicelluloses of the investigated substrates after acid treatment was demonstrated in (Table 1). The analytical data represent the percentage of hemicelluloses as referred to the original weight. The results show that the high amount of

hemicelluloses was found in *Cicer arietinum* (43.0%) followed by the other legume plants. These results were in conformity with the data in Table 1, concerning the high production of the hemicellulase enzyme (α-Gal) from *Thielavia terrestris* when grown on *Cicer arietinum* seeds.

In order to obtain an enzyme purified to homogeneity, we chose only the most active fractions from individual purification steps. Thereby taking into account rather low levels of recovery of activity. The enzyme was purified by precipitation with $(NH_4)_2SO_4$ (60%), with 67.0% yield and 3.25 purification fold followed by gel filtration using sephadex $G\text{-}_{100}$. In this step, α-Gal was purified 14.4 with yield of about 49.1 and specific activity 40.3 U/mg protein (Table 2). The activity was located in

Table 1

Evaluation of some natural substances for α-Gal production by *Thielavia terrestris* in solid state culture with percentage weight of hemicelluloses of substances after acid treatment

Substrate	α-Gal yield (unit)	Hemicelluloses, %
Arachis hypogaea (Peanut husk)	3.72±0.21 (H.S)	24.6±1.7 (H.S)
Cicer arietinum (Chick pea seed)	5.86±0.02 (H.S)	43.0±3.6 (H.S)
Helianthus annuus (Sunflowers husk)	3.07±0.01 (H.S)	19.8±2.1 (H.S)
Hordeum vulgare (Barley bran)	2.88±0.02 (H.S)	12.5±1.5 (H.S)
Hyphaene thebaica (Doum Palm fruit)	1.77±0.03 (S)	5.4±0.6 ●
Lens esculenta (Lentils seed)	4.74±0.05 (H.S)	37.8±2.21 (H.S)
Lupinus termis (Lupins seed)	5.49±0.03 (H.S)	39.6±2.34 (H.S)
Oryza sativa (Rice straw)	1.12±0.01 ●	11.4±0.72 (H.S)
Panicum miliaceum (Panicum seed)	3.44±0.01 (H.S)	12.7±0.8 (H.S)
Phalaris canariensis (Canary seed)	3.63±0.01 (H.S)	14.3±1.74 (H.S)
Pisum sativum L. (Garden pea seed)	4.19±0.02 (H.S)	28.4±2.11 (H.S)
Triticum aestivum (wheat bran)	3.44±0.01 (H.S)	12.6±0.73 (H.S)
L.S.D 1%	0.68	4.76
L.S.D 5%	0.47	3.31

Table 2

A summary of treatments used for the purification of α-Gal from *Thielavia terrestris*

Treatment	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1} protein)	Recovery (%)	Purification (folds)
*C.F.F. (200 ml)	425	1200	2.82	100	1.0
**C.F.D.	425	1157	2.72	92.96	0.96
***C.F.P. $(NH_4)_2SO_4$	87.5	804	9.19	67.00	3.25
Gel filtration (Sephadex G-100)	14.6	589	40.34	49.08	14.30
Ion-exchange chromatography DEAE-Cellulose	3.8	326	85.78	27.17	30.42

* Cell-free filtrate. ** Cell-free dialysate. *** Cell-free precipitate.

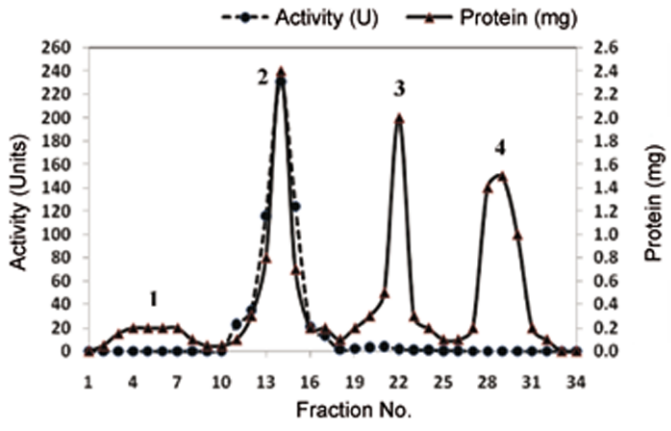

Fig. 1. Purification of α-Gal from *Thielavia terrestris* using gel filtration on Sephadex G-100 (1–4 are the protein peaks; the α-Gal activity is located in peak 2)

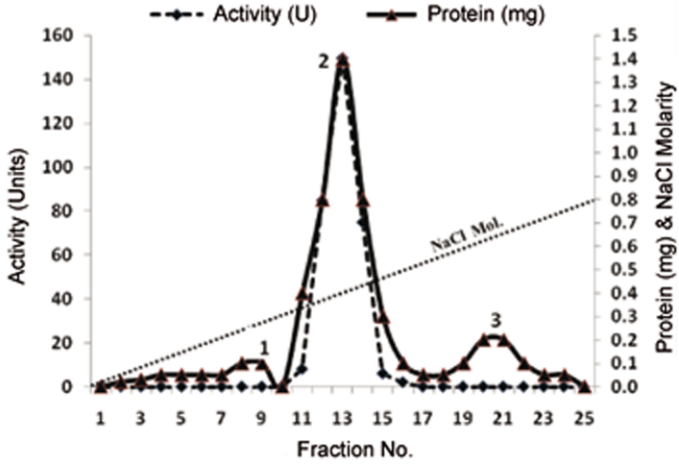

Fig. 2. Purification of α-Gal from *Thielavia terrestris* using ion exchange on DEAE-Cellulose (1–3 are the protein peaks; the α-Gal activity is located in peak 2)

peak 2 (Fractions 11–17, the elution profile was represented in Fig. 1). The purification procedure was completed by anion exchange chromatography on DEAE-cellulose using a linear sodium chloride gradient. The final enzyme preparation displayed only 30.42-fold increase in specific activity (85.78 U mg^{-1} protein) compared with the culture filtrate, with a level of recovery of the original activity of 27.17% (Table 2). The highest activity was detected in peak 2 (Fractions 11–15, the elution profile was represented in Fig. 2). These fractions were eluted at 0.38–0.5 M of NaCl. 3.8 mg of homogeneously purified α-Gal was obtained from 200 ml of crude filtrate.

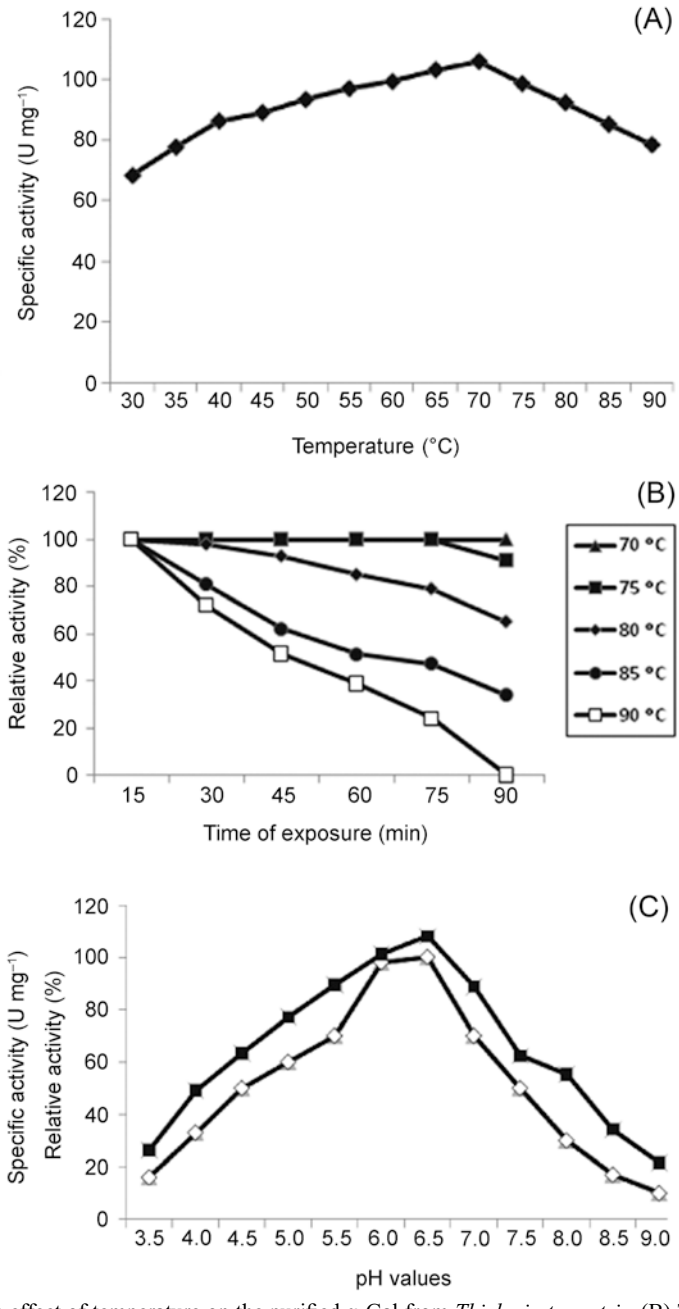

Fig. 3. (A) The effect of temperature on the purified α-Gal from *Thielavia terrestris.* (B) Thermal stability of the purified α-Gal from *Thielavia terrestris.* (C) pH profile (-■-) and pH stability (-□-) of purified α-Gal from *Thielavia terrestris*

α-Gal has been previously purified from culture filtrate using $(NH_4)_2SO_4$ precipitation, gel filtration and ion-exchange chromatography [6, 25].

The optimum temperature of the purified enzyme was recorded at 70 °C (Fig. 3A) compared to the 55 °C observed with both *Aspergillus awamori* and *Aspergillus carbonarius* [11]. The optimum temperature of the purified α-Gal was similar to that of α-Gal from *Trichoderma reesei* RUT C-30 [37]. These enzymes are usually more thermostable than those of mesophilic fungi [16]. Thermal stability of α-Gal was investigated in the temperature range of 70–90 °C. The enzyme retained 100% of its activity after incubation at 70 °C for 90 min. and 75 °C for 75 min. Also, it retained 50% of its activity after 1 h incubation at 85 °C (Fig. 3B). Thus, the thermal stability of this enzyme was higher than that of the *Humicola* sp. and *Thermomyces lanuginosus* α-Gals in previous studies [18, 30, 32].

Most of the so far reported α-Gals had an optimum pH between 5.0 and 7.0 [6, 25]. The purified α-Gal from *Thielavia terrestris* had an optimal pH 6.5 (Fig. 3C). Interestingly, according to the pH stability data, the enzyme retained complete activity after being incubated for 1 h at this degree of pH value (6.5). These data are in line with the previously reported α-Gal isolated from some thermophilic fungi [2, 18, 32]. The residual activity was almost 25% at pH 3.5 and pH 9. This can be attributed to the decreasing affinity of the enzyme to its substrate and/or due to an irreversible

Table 3
Effect of different salts and some enzyme inhibitors on the purified α-Gal

Salts or inhibitors	Relative α-galactosidase activity (%)	
	5 mM	10 mM
Non	100 ●	100 ●
$AgCl_2$	37±1.43 (–H.S)	11±1.0 (–H.S)
$BaCl_2$	87±4.88 (–H.S)	61±2.3 (–H.S)
$CaSO_4$	109±3.3 (+H.S)	91±3.0 (–H.S)
$CuSO_4$	111±3.7 (+H.S)	94±2.5 (–H.S)
$FeSO_4$	77±5.0 (–H.S)	52±1.0 (–H.S)
$HgCl_2$	24±0.9 (–H.S)	7.6±0.2 (–H.S)
KCl	139±5.5 (+H.S)	99±3.3 (N.S)
$MgSO_4$	90±3.3 (–H.S)	70±3.1 (–H.S)
$MnSO_4$	95±4.5 (–H.S)	66±2.2 (–H.S)
Na_2CO_3	112±6.1 (+H.S)	89±3.2 (–H.S)
$ZnSO_4$	42±1.6 (–H.S)	36±1.0 (–H.S)
EDTA	88±2.1 (–H.S)	75±2.5 (–H.S)
Sodium arsenate	72±1.7 (–H.S)	67±0.5 (–H.S)
L-Cysteine	90±2.5 (–H.S)	78±2.0 (–H.S)
Iodoacetate	43±0.5 (–H.S)	39±1.1(–H.S)
L.S.D 1%	4.80	3.32
L.S.D 5%	2.21	1.81

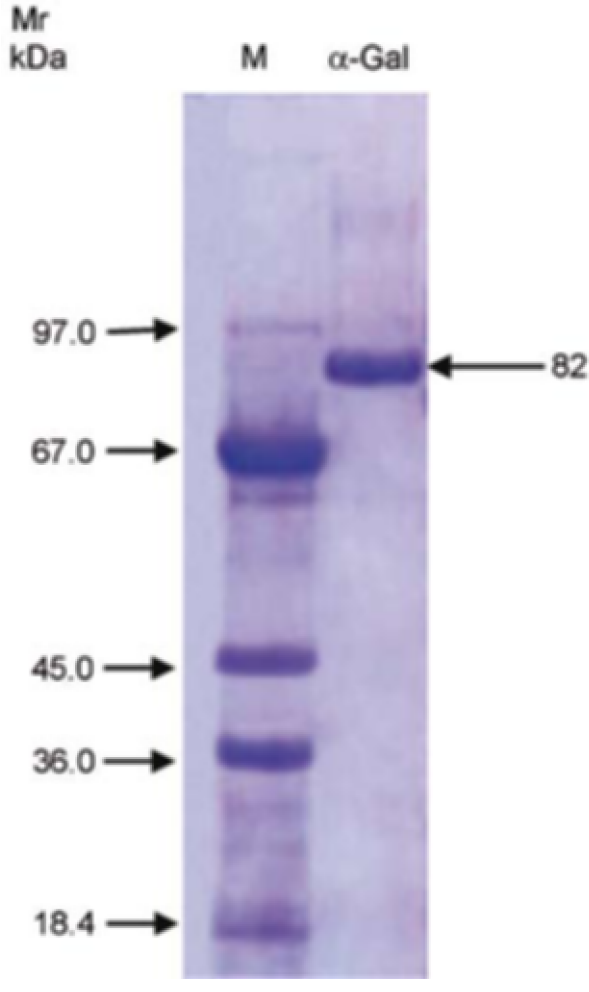

Fig. 4. SDS-PAGE of the purified α-Gal eluted from DEAE-cellulose column (standard protein markers: -phosphorylase-b, 97.0 kDa; bovine serum albumin, 67.0 kDa; egg albumin, 45.0 kDa; glyceraldehyde-3-phosphate, 36.0 kDa; beta-lactoglobulin, 18.4 kDa). The molecular mass of α-Gal was estimated to be 82 kDa

destruction of the enzyme protein. Our enzyme, therefore, exhibits a number of highly appealing and promising features that make it a strong candidate for future industrial applications.

Effect of PNPG concentration on the enzyme activity was studied. The optimum concentration was 0.9 mg/ml. The Lineweaver–Burk plot of the reciprocals of initial velocities and PNPG concentration was studied and the apparent K_m value of α-Gal for PNPG was calculated to be 1.31 mM. K_m value of α-galactosidase from *T. reesei* was recorded at 1.2 mM [37].

The effect of some salts or potential inhibitors on the purified α-Gal is shown in (Table 3). α-Gal activity of *Thielavia terrestris* was strongly affected by $HgCl_2$, $AgCl_2$, $ZnSO_4$ and Iodoacetate at the two investigated levels (5 and 10 mM) which are strong inhibitor of most α-Gal including those from *Aspergillus awamori* and *Aspergillus carbonarius* [11]. The ions Ca^{2+}, Cu^{2+}, K^{+} and Na^{2+} stimulated α-Gal activity at a concentration of 5 mM (Table 3), while at a final conc. of 10 mM, the enzyme was slightly inhibited by these ions. Moreover, $BaCl_2$, $FeSO_4$, $MgSO_4$, $MnSO_4$ had an inhibitory effect on the enzyme activity at the two investigated levels. Also, ethylenediaminetetraacetic acid (EDTA), a chelating reagent, sodium arsenate and L-cysteine were found to inhibit the enzyme activity. The inhibition of α-Gal by heavy metals and some inhibitors has been previously reported [18, 32]. The molecular mass of α-Gal was estimated to be 82 kDa by using SDS-PAGE (Fig. 4). The lower molecular mass of this enzyme than the another α-Gals from *Aspergillus niger,* 99 kDa [6] and *Humicola* sp., 87.1 kDa [18] often evaluate the relative affinities of this enzyme.

CONCLUSIONS

The production of α-galactosidase by *Thielavia terrestris* using seeds and husks of some plants as substrate in solid state culture showed to be a feasible process for obtaining the enzyme. For the different substrates tested, the *Cicer arietinum* seeds presented the highest enzyme activity. Using these substrates is considered as an advantage because it has economical benefits, not much energy and economical investments are required to process this kind of raw materials. Furthermore, the purification and characterization of α-Gal from *Thielavia terrestris* indicate that this enzyme displays a number of biochemical properties that make it a potentially strong candidate for commercial application in pulp, paper and pharmaceutical industry. It is also an enzyme which has a thermal stability and a low molecular weight when compared to the currently used commercial fungal α-Gals. This thermostable enzyme is used in high-temperature processing. These features actually provide a strong stimulation for further research on the structure of the purified α-galactosidase.

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