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RESEARCH ARTICLE

Aspergillus fumigates α-Galactosidase: Purification and Characterization

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Abstract

α-Galactosidase (α-D-galactoside galactohydrolase) is an exotype glycoside hydrolase that catalyzes the hydrolysis of α-1,6-galactosidic linkages in galactose-containing oligosaccharides such as melibiose (galactose- α-1,6-glucose), raffinose (galactose- α-1,6-sucrose), and stachyose (galactose- α-1,6-raffinose) and in galactomannan. An α-galactosidase (a-D-galactosidegalactohydrolase was purified to homogeneity from the culture filtrate of Aspergillus fumigatus. α-Galactosidase was induced by galactose, melibiose and raffinose, but galactose was the most efficient inducer. α-galactosidase was purified by gel filtration and two ion exchange chromatography's and showed Mw of 54.7 kDa. The purified enzyme showed maximal activity against p-nitrophenyl-α-D-galactopyranoside (pNPGal) at pH 4.5-5.5 and 55 °C, and retained about 80% of the original activity after incubation for 90 minutes at 50ºC. The KM for pNPGal was 0.3 mM. Melibiose was hydrolyzed by the enzyme but raffinose was very poor substrate.

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INTRODUCTION

α-galactosidases are the group of glycoside hydrolases (glycosidases or carbohydrases); the enzymes that catalyze hydrolytic cleavage of O-glycoside bond and belong to the enzymes of carbohydrate catabolism (Ademark et al., 2000). *α*-galactosidases (*α*-D-galactoside galactohydrolase) hydrolyze the terminal *α*-1,6-linked nonreducing *α*-D-galactose residues from linear and branched oligosaccharides and polysaccharides like melibiose, raffinose, stachyose, shortfragments of galacto(gluco)mannans, and galactolipid (Guimarães et al 2001). According to their substrate specificities, *α*-galactosidases can be divided in to two groups .The first group contains *α*-galactosidases active only on oligosaccharides with low degree of polymerization, for example melibiose, raffinose, stachyose, and short fragments of galacto(gluco)mannans. These enzymes are usually very active on artificial substrates like p-nitrophenyl- *α*-D-galactopyranosides (Jiao et al., 2000). The second group of *α*-galactosidases is active on polymeric substrates. However, similar to the enzymes of the first group, they attack short oligosaccharides, mainly fragments of degraded polymers, as well asartificial *α*-galactosides (Rezende et al., 2005).
α-Galactosidase (α-galactoside galactohydrolase) is an exoglycosidase that catalytically removes α-linked terminal non-reducing galactose residues from small oligosaccharides likes melibiose and raffinose and larger galactopolysaccharides and galactolipids (Manzanares et al., 1998) . It is widely distributed in biological systems and the genes encoding α-galactosidases have been isolated from microorganisms, plants and animals and classified into three well-conserved families based on sequence similarity (Marraccini et al., 2005; Viana et al., 2006).

Several species of filamentous fungi produce extracellular α-galactosidases (Cao et al., 2007). They have been identified and purified from many Aspergillus species. The most of these enzymes are glycoproteins (Cao et al.,

2009 and 2010) . The corresponding encoding genes have also been identified in A. fumigates, can probably produce at least two different polypeptides (encoded by separate genes). These are probably induced by, and subsequently degrade different, galactose-containing substrates within the extracellular environment (Rezende el al., 2005).

The aim of the present research paper is to isolate potential fungal strains for α-galactosidase production, identify their specific strains, and to optimize basal liquid culture media conditions in order to induce maximum growth and enzyme production by selecting different carbon sources in standardized media. Total protein secreted by different fungal strains in the culture broth was also monitored along with evaluation of The stability of enzyme activity in culture filtrates.

1. Material and Methods:

Aspergillus fumigatus strain DD41 dissociated from outdoor air in India, which were preserved and were used to accomplish this research. In this study, isolates were grown in saboraud glucose agar in sterile conditions, and placed in incubator at 27°C for 48-72 hours, and examined after appropriate growth of fungi in terms of morphological colony and Microscopic, Separating Fungus Colonies: Fungus colonies were separated from medium by using wattman paper number 1 and Bookhner funnel in condition completely sterile and were washed with sterile PBS, three stages (Murayama et al., 1998).

2.1 Mass Cultivation:

A. fumigatus was grown in the carbon source, spores (107.mL-1) were transferred to 50 mL of liquid medium consisting of gram/liter Ammonium sulphate 2.0, potassium dihydrogen phosphate 14.0, Di-potassium hydrogen phosphate 84.0, magnesium sulphate 0.2, ferric citrate 0.2, sodium citrate 0.25, dextrose, glycerol were dissolved in 1.0 liter distilled water and pH was adjusted to yeast extract 0.6, and 1% (w/v) of galactose, or lactose, or melibiose, or raffinose. After incubation at 42°C and 120 rpm for 12, 24, 36 and 48 h, the culture supernatants were collected by filtration through filter paper. The enzyme sample used for the purification process was produced in 4.75 L of the medium containing the galactose (Singh et al., 2009).

2.2 Cell Fractionation

Cells Disruptions Were Performed Using Two Methods: (i) Freeze and Thaw was used to break up the fungus piles, (ii) Glass beads: disruption was performed using glass beads (diameter, 1mm) on a vortex mixture for 1 min until about 80-90% cells were disrupted. The membrane of Aspergillus fumigatus was more solid and rigid than that of others, so liquid nitrogen was used to break it .

2.3 Preparing Crude Extracts and Measuring Protein Value

After cell disruption, the crude extracts were separated from intact cells and cell walls remaining by centrifugation at 25000 rpm for 30 min through three stages. The clear supernatants obtained. The protein content of these solutions was determined according to the method of Bradford. The supernatants were kept in micro tubes at-20°C until used (Steven et al., 2011).

2.4 Estimation of protein concentration in samples

The concentration of the proteins in samples was estimated using BCA assay. A volume of 10.0 µl of each sample was pipette in to microtiter wells in duplicates. The volume was made up to 90.0 µl using distilled water. Then added 100 µl of standard working reagent to the wells containing samples. The wells containing reagent and distilled water, with the sample in duplicates served as blank. The sample in plates was incubated at 37 °C for 30 min. The absorbance was read at 562 nm by spectrophotometer against the blank after incubation period. The concentration of protein in samples was determined by using the standard Curve (Stoscheck, 1990).

2.5 Enzyme assay

α-Galactosidase was assayed using ρ-nitrophenyl-α-D-galactopyranoside (ρNPGal) as substrate. The assay system contained 200 µL of 100 mM sodium acetate buffer pH 5.0, 250 µL of 2 mM ρNPGal solution and 50 µL of enzyme preparation. The reaction was carried out for 15 min at 50°C and was stopped by the addition of 1 ml of 0.5 M sodium carbonate. The amount of ρ-nitrophenol (ρNP) released was determined at 410 nm. A unit (U) of enzyme was defined as the amount of α-galactosidase which liberates 1 µmol of ρNP per min under the given assay conditions (Schnell et al., 2006). The activities against raffinose and sucrose were assayed for 60 min at 50 °C using a reaction mixture containing 400 µL of 100 mM sodium acetate buffer, pH 5.0, 350 µL of enzyme extract and 250 µL of 4% (w/v) substrate solutions. The amount of the reducing sugar produced was determined by the dinitrosalicylate method (Todd and Gomez 2001). The activity against melibiose was assayed using the same reaction system and determining the glucose formed by the glucose-oxidase method. Except for the chromatographic experiment, the enzyme activity values presented are mean values of triplicate assays. Standard deviations values were always smaller than 10% of the mean value (Zhao et al., 2012).

2.6 α-Galactosidase purification

A portion of the concentrated crude enzyme was applied to a BioGel P-100 column (80 x 1.5 cm), packed and equilibrated with 50 mM sodium phosphate buffer, pH 7.0. The column was washed with the same buffer and the samples were collected at a flow rate of 18 mL/h at 4 ℃. The active protein fractions were pooled, concentrated by ultrafiltration on an Amicon membrane (PM 10), and chromatographed on a Q-Sepharose column (15 x 1.5 cm), equilibrated and eluted with 100 mM sodium phosphate buffer pH 7.0, followed by a linear gradient formed with 210 mL of phosphate buffer and 210 mL of the same buffer containing 0.8 M NaCl. Samples of 4 mL were collected at a flow rate of 45 mL/h . The active protein fractions were concentrated by ultrafiltration as above, and loaded on a CM-Sepharose (15 x 2.0 cm), equilibrated and eluted with 100 mM sodium acetate buffer pH 5.0, followed by a linear gradient formed with 80 mL of 100 mM of acetate buffer and 80 mL of the same buffer containing 0.8 M NaCl. The enzyme preparation was analyzed by SDS-PAGE 12% and was stained with silver reagent (Naoko et al., 2010). The protein was quantified by the Coomassie Brilliant Blue binding method using bovine serum albumin as standard (Rath et al., 2009).

2.7 Enzyme characterization

The influence of the pH and temperature on the α-galactosidase activity was studied using the assay system described above, but different buffer solutions (pH 3.0-7.5) or different incubation temperatures (30-70 °C). For determination of thermal stability, the enzyme fractions were pre-incubated with buffer solution at 50 °C for several time periods at the optimum pH. The residual activity was determined using the standard assay. Kinetics experiments were performed at 50 °C and pH 5.0. The Michaelis-Menten constant (KM) and maximal reaction rates (V max) were calculated by Michaelis-Menten and Lineweaver-Burk plots, using the computer software Curve Expert version 1.3 for Windows (Zhao et al., 2012).

2.8 Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis

The extracts of fungi were analyzed by making use of SDS-PAGE method with 11% separating gel and 4% stacking gel in a discontinuous buffer system according to the method of Laemmli (Rath et al., 2009)..The extracts were boiled for 5 min with a reducing sample buffer (containing 2-mercaptoethanol) and 35 microlitre of each sample was loaded on a gel. Along with the samples, standard marker (Fermentase) was also electrophoresis, which is a mixture of fourteen pieces of pure neoformed proteins in different sizes, thus, these protein pieces are distinguished as fourteen bands from 10 to 200KD, which respectively are 200, 150, 120, 110, 85, 70, 60, 50, 40, 30, 25, 15 and 10KD. Staining was done by using coomassi brilliant blue G250 (sigma) (Rath et al., 2009).

2. RESULTS AND DISCUSSION

The isolated fungus A. fumigatus grown efficiently on a medium containing different carbon sources. α-Galactosidase activity in the culture supernatant different with the carbon source and the growth time (Table 1). Mycelial mass (mg/dry wt) produced on the culture fluid by the Aspergillus fumigatus growth at 28 °C for several times (table 2).

The highest activity (35.68 U.mL-1) was induced by galactose which was followed by melibiose. This was in agreement with the results previously reported for the production of α-galactosidase by A. fumigatus (Rezende et al., 2005), Trichoderma (Zeilinger et al., 1993) and in Penicillium simplicissimum (Luonteri et al., 1998). Surprisingly, although the raffinose sustained substantial growth, this substrate was almost as poor inducer as lactose. This could be due to the presence of the invertases, which in combination with background α-galactosidase, hydrolyse the raffinose, producing simple sugars. These sugars could then be used for the production of the micelial mass, but were unable for further inducing α-galactosidase production.

The gel filtration chromatography of the culture fluid of the A. fumigatus grown for 36 h at 42 ºC in galactose resulted in a single α-galactosidase activity peak (not shown). The rechromatography of this active fraction on an ion exchange Q-Sepharose column also resulted in an α-galactosidase ctivity peak, which was eluted with NaCl, at a concentration of about 0.5 M (not shown). Further chromatography of this fraction on a CM-Sepharose column resulted in two main protein peaks (F1 and F2), showing both α-galactosidase activities (Fig 2). While the F1 fraction was eluted in the column void volume, the F2 fraction was eluted with a NaCl concentration of about 0.5 M. As for the elution of the first peak of α-galactosidase in the void volume of the column it's unlikely that we overcharged the system as this also happened with smaller volumes of enzyme. It was more likely that it represented a distinct isoform of the enzyme. However, we did not determine the pI values for these potential isoforms. The purification results are summarized in Table 3. α-Galactosidase secreted by A. fumigatus was purified 177-fold with a recovery of 3.04 %. The

SDS-PAGE analysis revealed that the Biogel fraction, the Q-Sepharose fraction and the CM-Sepharose F1 fraction all contained several protein forms, while the CM-Sepharose F2 fraction contained a single protein with Mw of 54.7 kDa (Fig 1).

It was previously reported that α-galactosidases from A. niger, T. reesei, Mortierella vinacea and Thermotoga neapolitana 5068 had Mw of 45 kDa (Adya and Elbein, 1977), 50 kDa (Zeilinger et al., 1993), 53 kDa (Shibuya et al., 1995) and 61 kDa (Duffaud et al., 1997), respectively.

The purified α-galactosidase could not hydrolyze sucrose and raffinose was a very poor substrate, but the enzyme was able to hydrolyze melibiose. However, the affinity of the enzyme for melibiose was much lower than its affinity for ρNPGal. It is known (Varbanetes et al., 2001) that most microbial α-galactosidases hydrolyze the synthetic substrate ρNPGal more efficiently than the natural α-galactosides. Moreover, α-galactosidase from A. niger was reported (Kaneko et al., 1991) to hydrolyze exclusively the synthetic substrate and failed to split off the terminal α-1,6-bound galactose in linear structures like the melibiose, raffinose and stachyose. Nevertheless, previous findings indicated that α-galactosidases from T. reesei (Zeilinger et al., 1993) and Bifidobacterium breve (Xiao et al., 2000) were able to hydrolyze raffinose.

A substantial activity against ρNPGal was determined for the A. fumigatus purified α-galactosidase at the temperature range of 45-65 ºC and at the pH range of 4.0-5.0 (Figs. 3 A and B). The maximal substrate hydrolysis was achieved at 55 °C and pH 4.5. These optimum pH and temperature values were close to those determined for hydrolysis of ρNPGal by α-galactosidase from P. purpurogenum (Shibuya et al., 1995) by the fungal raffinose hydrolysing enzymes (de Rezende and Felix, 1999) and by α-galactosidase from germinating soybean seed (Guimarães et al., 2001). The enzyme was thermostable and retained about 80% of its original activity after pre-incubation for 90 min at 50 °C (Fig 4). The K M and V max values calculated by the Line weaver-Burk reciprocal plot for hydrolysis of ρNPGal were 0.38 mM and 0.16 µmol.min -1, respectively (Fig 5).

The KM value was close to that determined for the α-galactosidases from coffee bean (Zhu et al., 1996) and from A. niger (Ademark et al., 2001), but lower than those reported for hydrolysis of ρNPGal by α-galactosidases from T reesei (Zeilinger et al., 1993) and Penicillium sp. 23 (Varbanetes et al., 2001).

Table 1 - α-Galactosidase activity in the culture medium, concentrated of Aspergillus fumigatus grown on several carbon sources at 42 ºC. U.mL-1 = units per ml of culture, U.mg-1 = units per mg of protein.

Carbon source	Growth time (h)			
	12	24	36	48
Galactose	0.58	0.21	5.78	0.98
Lactose	1. 46	0.89	1.69	0.99
Melibiose	4.02	0.98	7.76	0.96
Raffinose	2.01	100.2	4.79	0.56

Table 2 - Mycelial mass (mg/dry wt) produced on the culture fluid by the Aspergillus fumigatus growth at 42 °C for several times.

Growth time (h)	**Carbon source (1 %, w/v)**			
	Galactose	**Lactose**	**Melibiose**	**Raffinose**
12	**37.2**	**17.98**	**26.23**	**45.78**
24	**71.5**	**24.20**	**63.98**	**67.58**
36	**85 .75**	**20.10**	**86.23**	**80.28**
48	**98.2**	**15.20**	**73.53**	**67.98**

Table 3 - Summary of the purification steps of the α-galactosidase from Aspergillus fumigatus.

Purification step	**Total protein (mg)**	**Total activity (U)**	**Specific activity (U.mg-1)**	**Purification (fold)**	**Recovery (%)**
BioGel P100	16.66	54.20	3.25	65.0	77.49
Q-Sepharose	5.10	35.34	6.69	133.8	50.52
Crude extract	1230.50	69.94	0.05	1	100
CM-Sepharose (P2)-1	0.24	2.13	8.85	177.0	3.04

U.mg-1 = units of enzyme per mg of protein.

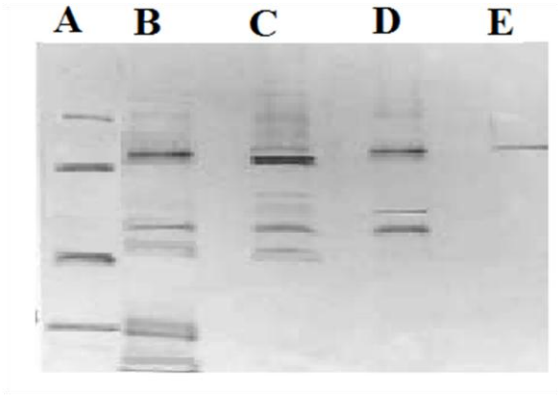

Fig .1 SDS-PAGE (12%) of Aspergillus fumigatus α-galactosidase samples; A- molecular mass standards; B-concentrated culture medium; C- fraction P1 from the BioGel P- 100 column; 4-fraction F1 from the Q-Sepharose column; 5- fraction f2 from the CM-Sepharose column.

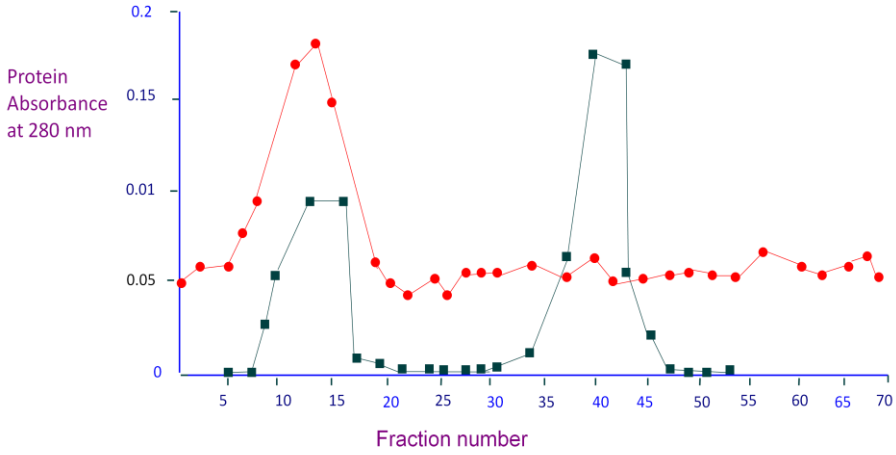

Fig. 2 Elution profile of the α-galactosidase from Aspergillus fumigatus chromatographed on the CM-Sepharose column Absorbance at 280 nm (π); α-galactosidase activity linear gradient of NaCl (0 - 0.8 M)

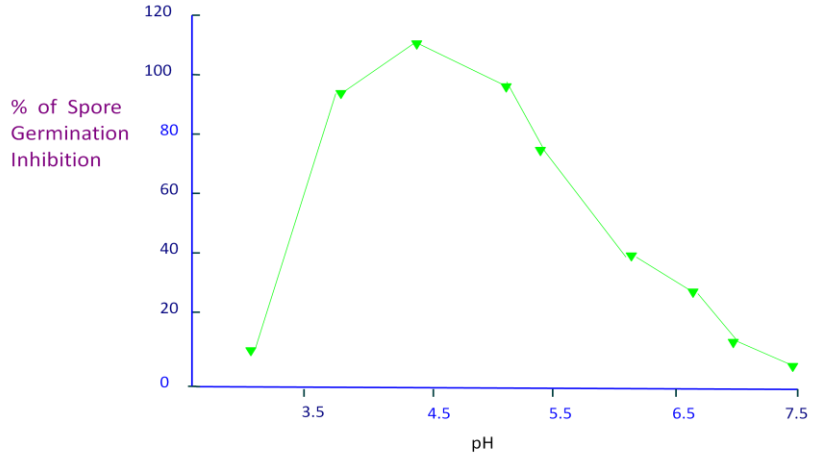

Fig. 3 Effects of pH on the activity of the Aspergillus fumigates purified α-galactosidase.

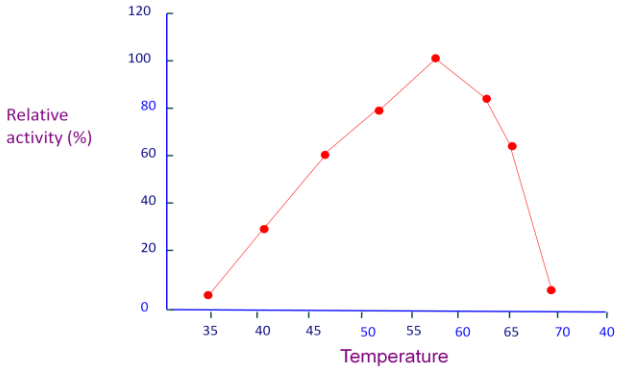

Fig. 4 Effects of temperature on the activity of the Aspergillus fumigates purified α-galactosidase

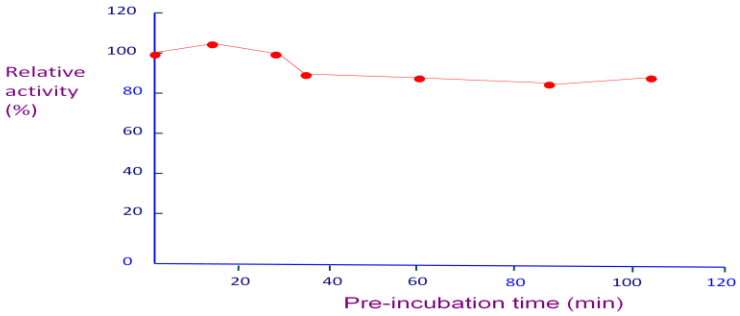

Fig. 5 Thermal stability of the Aspergillus fumigatus purified α-galactosidase. The enzymesamples were pre-incubated for several times at 50 °C, and then assayed as described in the text

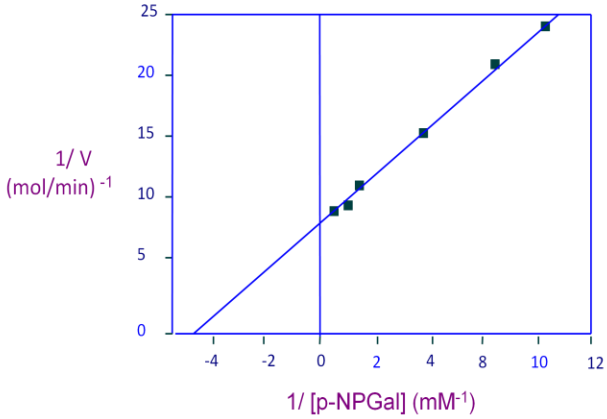

Fig. 6 Lineweaver-Burk plot for the Aspergillus fumigatus purified α-galactosidase.

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