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# Purification and Characterization of an α-Galactosidase from *Aspergillus fumigatus*

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## ABSTRACT

*Aspergillus fumigatus secreted invertase (β-fructofuranosidase) and α-galactosidase enzymatic activities able to hydrolyzing raffinose oligosaccharides (RO). α-Galactosidase was induced by galactose, melibiose and raffinose, but galactose was the most efficient inducer. It was purified by gel filtration and two ion exchange chromatographies 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 $K_M$ for pNPGal was 0.3 mM. Melibiose was hydrolyzed by the enzyme but raffinose was very poor substrate.*

**Key words:** *Aspergillus fumigatus,* α-galactosidase, raffinose oligosaccharides

## INTRODUCTION

The α-galactosidases (α-D-galactoside galactohydrolase, EC 3.2.1.22) are a group of exotype carbohydrases which catalyze the cleavage of terminal α-1,6-linked galactosyl residues from a wide range of substrates, including linear and branched oligosaccharides, polysaccharides and synthetic substrates such as ρ-nitrophenyl-α-D-galactopyranoside (Dey and Pridman, 1972). α-Galactosidases have a number of biotechnological applications: in beet sugar industry, these enzymes are used to remove raffinose from beet molasses and to increase the yield of sucrose (Shibuya et al., 1995); they are also used to improve the gelling properties of galactomannans to be used as food thickeners (Bulpin et al., 1990) and to degrade the raffinose family sugars (raffinose, stachyose and verbascose) in food and feed materials such as soya meal or soya milk (Guimarães et al., 2001).

The raffinose oligosaccharides (RO), specially raffinose and stachyose, are considered the major factors responsible for flatulence after ingestion of soybean or other legumes. The enzymic hydrolysis of the RO, therefore, may be of biotechnological interest. The hydrolysis of the RO may be accomplished by the α-galactosidase, invertase or both. The α-galactosidase cleaves the α-1,6 linkage, joining the galactosyl residue to sucrose, yielding galactose and sucrose, while the invertase hydrolyses the α-1,2 linkage, producing melibiose

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and fructose. α-Galactosidases are produced either by microorganisms, plants or animals, often in multiple forms (Dey and Pridman, 1972). The fungus *Aspergillus fumigatus* is a good producer of several proteolytic enzymes (Moser et al., 1994). In our laboratory, this mold was found to utilize native keratin (Santos et al., 1996) and several sugars including galactose, raffinose and melibiose (de Rezende and Felix, 1997, 1999). Here, we report the isolation, purification and characterization of an α-galactosidase produced by a strain *A. fumigatus.*

## MATERIALS AND METHODS

### *Organism growth and α-galactosidase production*

*Aspergillus fumigatus* was isolated from a hot water fountain in Brazil (Caldas Novas - GO) and was maintained on potato-dextrose-agar medium. In order to select the carbon source, spores ($10^7$.$mL^{-1}$) were transferred to 25 mL of liquid medium consisting of g.$L^{-1}$ $KH_2PO_4$ 7.0, $K_2HPO_4$ 2.0, $MgSO_4.7H_2O$ 0.1, $(NH_4)_2SO_4$ 1.0, 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. After growth for 36 h at 42 °C, the culture was filtered and the supernatants containing α-galactosidase activity were concentrated approximately 10-fold by freeze-drying.

### *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.

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 (Miller, 1956). The activity against melibiose was assayed using the same reaction system and determining the glucose formed by the glucose-oxidase method (Bergmeyer and Bernt, 1974).

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.

### *α-Galactosidase purification*

A portion of the concentrated crude enzyme was applied to a BioGel P-100 (Pharmacia) 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 $mL.h^{-1}$ at 4 °C. 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 $mL.h^{-1}$. 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% (Laemmli, 1970) and was stained with silver reagent (Blum et al.,1987). The protein was quantified by the Coomassie Brilliant Blue binding method (Bradford, 1976) using bovine serum albumin as standard.

### *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 ($K_M$) 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.

## RESULTS AND DISCUSSION

The fungus *A. fumigatus* grew efficiently on a medium containing galactose, melibiose, or raffinose as carbon sources. Lactose was a poor carbon source (Table 1). α-Galactosidase activity in the culture supernatant varied with the carbon source and the growth time (Table 2).

**Table 1 -** 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 | 36.30 | 18.80 | 25.80 | 44.90 |
| 24 | 69.50 | 23.10 | 62.70 | 65.50 |
| 36 | 84.20 | 19.50 | 85.20 | 79.00 |
| 48 | 96.70 | 14.30 | 72.50 | 67.40 |

**Table 2 -** 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 | |
| | $U.mL^{-1}$ | $U.mg^{-1}$ | $U.mL^{-1}$ | $U.mg^{-1}$ | $U.mL^{-1}$ | $U.mg^{-1}$ | $U.mL^{-1}$ | $U.mg^{-1}$ |
| Galactose | 0.56 | 0.14 | 5.78 | 0.82 | 35.68 | 3.96 | 24.09 | 4.01 |
| Lactose | 1.23 | 0.62 | 1.69 | 0.85 | 2.37 | 0.79 | 3.11 | 0.78 |
| Melibiose | 3.54 | 0.71 | 7.76 | 0.86 | 24.83 | 1.13 | 29.15 | 1.94 |
| Raffinose | 1.81 | 0.96 | 4.79 | 0.50 | 6.54 | 0.74 | 9.61 | 0.71 |

1U = 1μmol of ρNP produced per minute.

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* (de Rezende and Felix, 1997, 1999), *Trichoderma reesei* (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 activity 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 (C1 and C2), showing both α-galactosidase activities (Fig 1). While the C1 fraction was eluted in the column void volume, the C2 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 its 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 C1 fraction all contained several protein forms, while the CM-Sepharose C2 fraction contained a single protein with *Mw* of 54.7 kDa (Fig 2).

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 *Aspergillus 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 Lineweaver-Burk reciprocal plot for hydrolysis of ρNPGal were 0.38 mM and 0.16 μmol.min$^{-1}$, respectively (Fig 5).

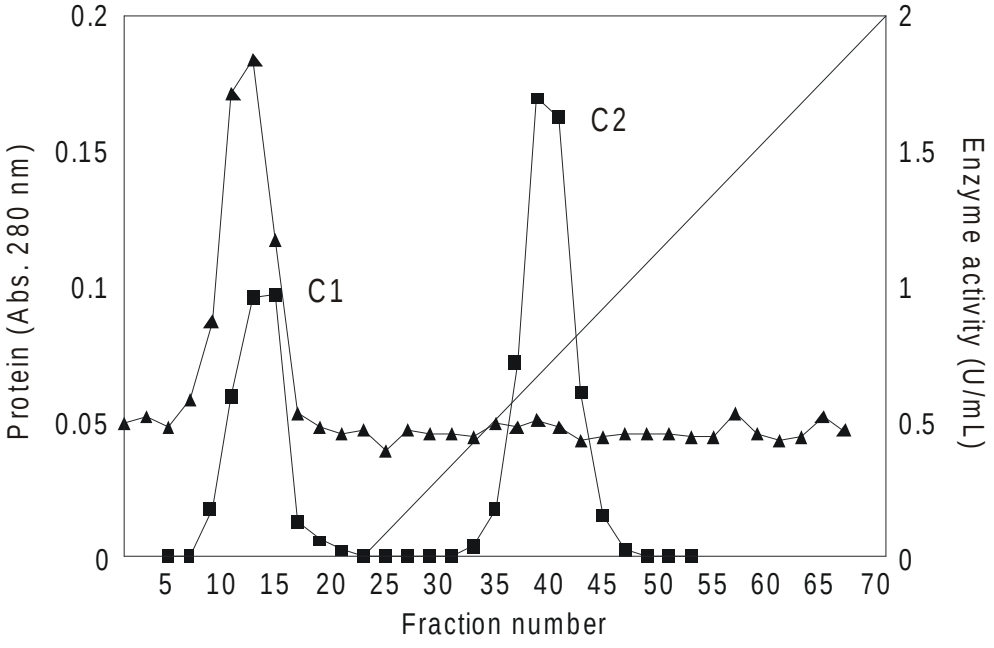


**Figure 1** - Elution profile of the α-galactosidase from *Aspergillus fumigatus* chromatographed on the CM-Sepharose column. Absorbance at 280 nm (π); α-galactosidase activity (■); linear gradient of NaCl (0 - 0.8 M) (—)

**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 (%) |
|---|---|---|---|---|---|
| Crude extract | 1230.50 | 69.94 | 0.05 | 1 | 100 |
| BioGel P100 | 16.66 | 54.20 | 3.25 | 65.0 | 77.49 |
| Q-Sepharose | 5.10 | 35.34 | 6.69 | 133.8 | 50.52 |
| CM-Sepharose (P2) | 0.24 | 2.13 | 8.85 | 177.0 | 3.04 |

$U.mg^{-1}$ = units of enzyme per mg of protein.

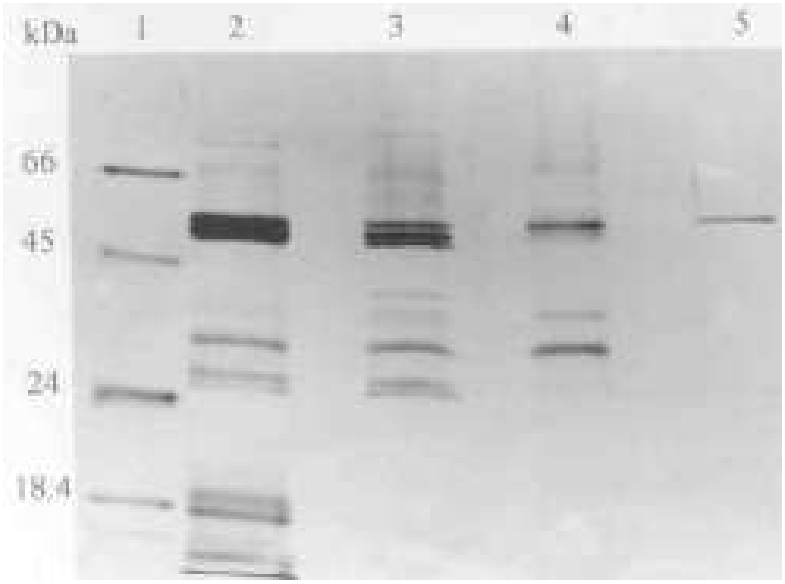


**Figure 2 -** SDS-PAGE (12%) of *Aspergillus fumigatus* α-galactosidase samples; 1- molecular mass standards; 2- concentrated culture medium; 3- fraction P1 from the BioGel P-100 column; 4- fraction Q1 from the Q-Sepharose column; 5- fraction C2 from the CM-Sepharose column

The $K_M$ 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). Although the purified α-galactosidase didnot hydrolyze raffinose and stachyose, the culture hydrolyzed the RO (de Rezende and Felix, 1997, 1999), indicating the ability of *A. fumigatus* to produce other α-galactosidase forms. These reported results indicated the potential of *A. fumigatus* for decreasing RO in soymilk.

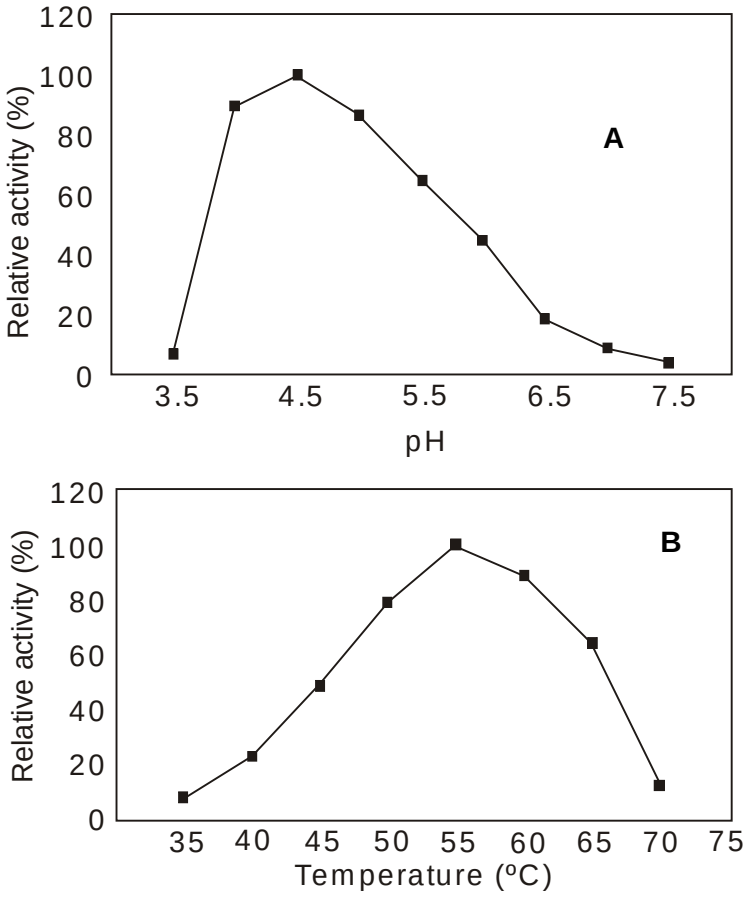


**Figure 3 -** Effects of pH (A) and temperature (B) on the activity of the *Aspergillus fumigatus* purified α-galactosidase

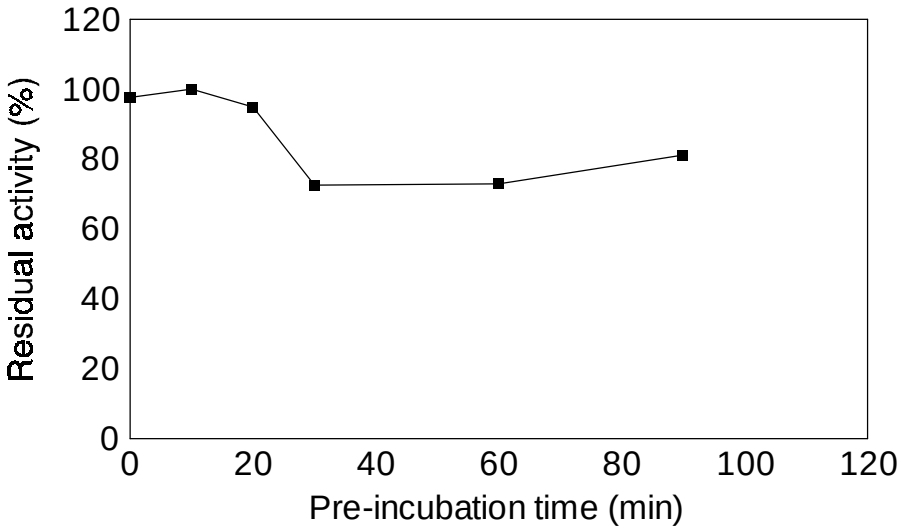


**Figure 4 -** Thermal stability of the *Aspergillus fumigatus* purified α-galactosidase. The enzyme samples were pre-incubated for several times at 50 ºC, and then assayed as described in the text

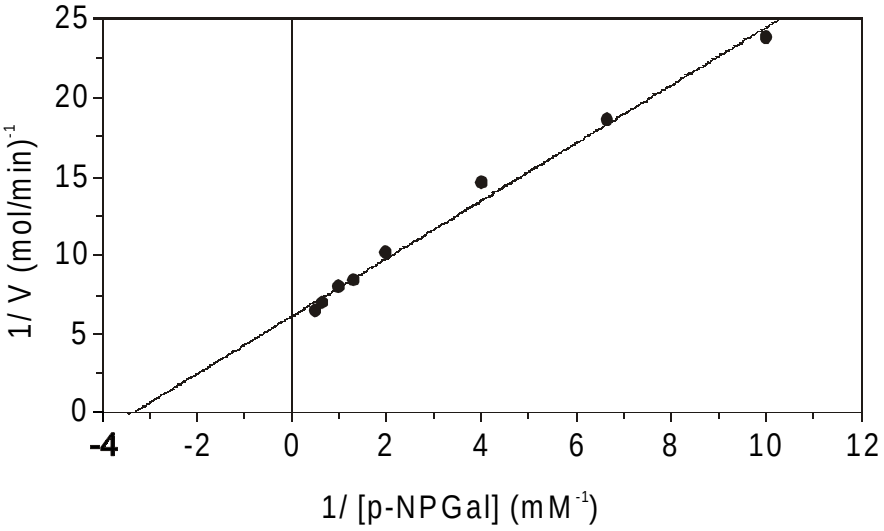


**Figure 5 -** Lineweaver-Burk plot for the *Aspergillus fumigatus* purified α-galactosidase.

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## RESUMO

O fungo termofílico *Aspergillus fumigatus* secreta as enzimas invertase (β-frutofuranosidase) e α-galactosidase (α-D-galactosídeo galactohidrolase) que estão envolvidas na hidrólise completa dos oligossacarídeos de rafinose. A enzima α-galactosidase foi produzida em meio de cultura do fungo *Aspergillus fumigatus* crescido por 36 h a 42 °C em meio mineral mínimo contendo os açúcares galactose, ou melibiose, ou rafinose como fontes de carbono. A enzima foi purificada por filtração em gel, seguida por duas cromatografias de troca iônica. A massa molecular da α-galactosidase determinada por SDS-PAGE foi de 54,7 kDa. A atividade máxima da enzima purificada, utilizando o substrato ρ-nitrofenil-α-D-galactopiranosídeo (ρNPGal) foi na faixa de pH entre 4,5 e 5,5 e a 55 °C. A enzima manteve aproximadamente 80% de sua atividade original mesmo após pré-incubação por 90 minutos a 50 °C. O valor de $K_M$ para o substrato ρNPGal foi 0,3 mM. A enzima foi capaz de hidrolisar melibiose, mas sua atividade foi muito reduzida na presença do substrato rafinose.

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