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# *Thermomyces lanuginosus* CBS 395.62/b Strain as Rich Source of α-Galactosidase Enzyme

*Judit M. Rezessy-Szabó*, Quang D. Nguyen, Erika Bujna, Krisztina Takács, Magdolna Kovács and Ágoston Hoschke*

Szent István University, Department of Brewing & Distilling, Ménesi út 45, H-1118 Budapest, Hungary

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**Summary**

Seventeen *Thermomyces lanuginosus* strains, cultivated on raffinose and sucrose, were ranked on the basis of α-galactosidase activities. *T. lanuginosus* CBS 395.62/b strain showed the highest α-galactosidase activity on both investigated carbohydrates. Several carbon sources were tested as potential inducers for the α-galactosidase synthesis. On melibiose substrate α-galactosidase activity was higher in the intracellular fraction than in the filtrate of the fermentation broth, although both values were very low and did not reach the value of 1 U/mL. Raffinose, sucrose and Lactosucrose® proved to be inducers for α-galactosidase production. The highest titer (about 30 U/mL) was achieved on 1 % sucrose and 0.45 % ammonium acetate. The optimum sucrose and ammonium acetate concentrations, at which about 90 U/mL α-galactosidase activity was reached during an 8-day fermentation, were 3 and 0.9 %, respectively.

*Key words*: α-galactosidase, fermentation, enzyme production, *Thermomyces lanuginosus*

## Introduction

The management of environment focuses attention on biotechnologies. These technologies, among other, apply microorganisms or constituents of microbes such as enzymes. Enzymes can be used in bioconversion for production of desired products and to eliminate environmental pollutions. One of the promising enzymes is α-galactosidase, widespread in nature, and it is synthesised by both prokaryotes and eukaryotes *(1)*. α-Galactosidases generally catalyse the hydrolysis of α-1,6-linked α-galactose residues from oligosaccharides such as melibiose, raffinose, stachyose and galactomannans as well as galactolipids *(2)*. Transgalactosidase activity was also demonstrated in case of some α-galactosidases *(3)*. Nowadays, α-galactosidases have an increasingly practical potential in biotechnology. In order to improve the nutritional value of legume-based food, they can be applied for reduction or elimination of antinutritive galacto-oligosaccharides (called raffinose family sugars) that cause flatulence *(4,5)*. Galacto-oligosaccharides produced by transfer reaction of α-galactosidase can be used as a prebiotic in functional food *(6)*. In beet industry they are used to remove raffinose from molasses in order to facilitate the crystallisation and improve the yield of sucrose *(7,8)*. Furthermore, in the pulp and paper industry the use of hemicellulases, including α-galactosidase, has gained interest. Moreover, the potential of α-galactosidase can be exploited for medical purposes and as a biochemical tool in structure analysis.

* Corresponding author; Phone: ++36 (0)1 372 6396; Fax: ++36 (0)1 372 6214; E-mail: jrezessy@omega.kee.hu

To find the most suitable enzyme source for a given purpose, production and characterisation of the enzymes are necessary. To achieve this goal the first step is the establishment of the enzyme production technology. Our previous results *(9,10)* have demonstrated that *Thermomyces lanuginosus* CBS 395.62/b strain shows outstanding extracellular α-galactosidase activity when cultivated on the medium containing raffinose and asparagine. Moreover, it was proved that raffinose and asparagine can be successfully replaced by sucrose and ammonium acetate in the growth medium. *T. lanuginosus* is a real thermophilic fungus, which presumably synthesises enzymes of higher working temperatures due to its higher optimum temperature and better thermal stability than its mesophilic counterparts. Therefore, it seems to be an ideal source for thermostable enzyme production. In the present report *T. lanuginosus* strain selections are shown in the presence of 0.45 % ammonium acetate and raffinose or sucrose as carbon sources. The strain, which possesses the best α-galactosidase activity, was cultivated on various compounds for assessment of α-galactosidase activity. The main nutrient concentrations were optimised to develop fermentation technology for the production of α-galactosidase enzyme.

## Materials and Methods

### *Microorganisms*

The seventeen *Thermomyces lanuginosus* strains, originating from various culture collections, were kindly donated by Dr. Bhat (Institute of Food Research, Norwich, UK). The matured cultures were stored on Potato Dextrose Agar (PDA) medium at 4 °C until use.

### *Chemicals*

*p*-Nitrophenyl-α-D-galactopyranoside was purchased from Sigma Company (USA). Lactosucrose® and Oligotime® are prebiotics, which were a gift from Ensuiko Sugar Refining Company (Japan) and Showa Sangyo Company (Japan), respectively. Raffinose, sucrose, asparagine and other chemicals were from Reanal (Hungary).

### *Culture conditions*

Shaken flask technique was used applying Erlenmeyer flasks (500 mL) containing 100 or 150 mL of medium. Composition of the inoculum medium was the following: glucose 2 g, L-asparagine 0.4 g, $KH_2PO_4$ 0.3 g, $K_2HPO_4$ 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.05 g and 0.1 mL of Vogel's trace elements solution *(11)* in 100 mL distilled water, pH=6.0. The inoculum culture was cultivated in 100 mL medium at 47 °C and 220 rpm in orbital incubator for 2 days and initiated by 5 mL of conidia suspension prepared with 0.1 % Triton X-100 solution. Five mL of inoculum culture was transferred into 150 mL production medium. The constant components of the medium were 3 g $KH_2PO_4$, 2 g $K_2HPO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$ and 1 mL of Vogel's trace elements solution in 1000 mL medium. The applied carbohydrates and their concentrations as well as the concentration of ammonium acetate are given in Results and Discussion part. After the completion of fermentation (168-192 h), the fungus was harvested by filtration and α-galactosidase activity was assayed in the culture filtrates.

### *Preparation of cell-free extracts*

Cells from 20 mL of fermentation broth were harvested by filtration and washed three times by distilled water, then resuspended in the appropriate amount of buffer and disintegrated by grinding with quartz sand. The cell debris was removed by centrifugation at 13000 rpm at 4 °C for 15 min. The supernatant was used as cell-free extract for the determination of intracellular α-galactosidase activity.

### *Enzyme activity assay*

A reaction mixture containing 0.5 mL of 15 mM *p*-nitrophenyl-α-D-galactopyranoside and 0.3 mL of 100 mM McIlvaine buffer (pH=4.2) was preincubated at 58 °C for 10 min before adding 0.2 mL suitably diluted enzyme solution (filtrate of fermentation broth). After 5 min, the reaction was terminated by adding 5 mL of 0.1 M $Na_2CO_3$ and the released *p*-nitrophenol was determined by measuring the absorbance at 405 nm. One unit of α-galactosidase activity was defined as the amount of enzyme that liberates 1 μmol *p*-nitrophenol in one minute under the assay conditions.

### *Determination of protein concentration*

Protein concentration of the filtrate of the fermentation broth was determined by the Biuret method. Protein concentration was calculated from the calibration curve created by bovine serum albumin. The method is sensitive in the concentration range between 0.5–5.0 mg/mL.

## Results and Discussion

### *Strain selection procedure for extracellular α-galactosidase activities*

A comprehensive *Thermomyces lanuginosus* strain selection was carried out in media containing 0.45 % ammonium acetate, and in the presence of either 1 % raffinose or 1 % sucrose. For the final evaluation, beside the α-galactosidase activities, the protein concentrations and pH values were determined in the culture filtrate (Table 1).

Maximum activities were reached between the fifth and the seventh day of fermentation. pH values were found in alkaline range between 7.5 and 8.2. The extracellular protein concentration showed divergence, it was characteristically the function of the strain applied and did not depend on the carbohydrates tested. The protein concentration ranged from 2.94 to 11 mg/mL. From the seventeen *T. lanuginosus* strains, only two strains (ATCC 16455 and CBS 218.34) gave higher activities on raffinose than on sucrose. α-Galactosidase activities of two strains (ATCC 28083 and ATCC 34626) did not reach the value of 1 U/mL. *T. lanuginosus* CBS 395.62/b strain showed outstanding activities on both carbon sources. Further studies focused on this strain to reveal the correspondence between the α-galactosidase activities and carbon sources used.

Table 1. Maximum α-galactosidase activities of the tested *T. lanuginosus* strains, relevant protein concentrations and pH values

| Substrate | Raffinose | | | | Sucrose | | | |
|---|---|---|---|---|---|---|---|---|
| Strain | Activity | Protein | Spec. activity | pH | Activity | Protein | Spec. activity | pH |
| | U/mL | mg/mL | U/mg | | U/mL | mg/mL | U/mg | |
| ATCC 16455 | 22.27 | 8.17 | 2.73 | 7.8 | 9.88 | 7.68 | 1.29 | 7.8 |
| ATCC 28083 | 0.33 | 6.00 | 0.06 | 7.5 | 0.06 | 7.10 | 0.01 | 7.6 |
| ATCC 34626 | 0.36 | 5.60 | 0.06 | 7.7 | 0.87 | 7.02 | 0.12 | 7.6 |
| ATCC 36350 | 4.42 | 4.63 | 0.95 | 7.7 | 5.44 | 3.83 | 1.42 | 7.9 |
| ATCC 44008 | 11.97 | 7.59 | 1.58 | 7.7 | 12.69 | 6.81 | 1.86 | 7.8 |
| ATCC 46882 | 2.60 | 2.94 | 0.88 | 7.7 | 3.11 | 3.28 | 0.95 | 7.7 |
| ATCC 84400 | 5.54 | 7.30 | 0.76 | 7.9 | 10.33 | 8.98 | 1.15 | 7.7 |
| CBS 218.34 | 14.16 | 3.69 | 3.84 | 7.9 | 9.95 | 4.13 | 2.41 | 7.8 |
| CBS 224.63 | 6.84 | 5.74 | 1.19 | 8.1 | 6.81 | 5.14 | 1.32 | 8.1 |
| CBS 288.54 | 1.29 | 3.77 | 0.34 | 8.1 | 1.86 | 4.26 | 0.44 | 8.1 |
| CBS 395.62/a | 17.24 | 9.54 | 1.81 | 7.9 | 29.62 | 10.86 | 2.73 | 7.8 |
| CBS 395.62/b | 24.28 | 9.26 | 2.62 | 7.8 | 29.76 | 9.74 | 3.06 | 8.0 |
| DSM 5826 | 6.62 | 8.10 | 0.82 | 8.0 | 7.03 | 8.13 | 0.86 | 7.9 |
| IMI 096218 | 3.45 | 4.54 | 0.76 | 7.9 | 2.63 | 4.91 | 0.54 | 8.0 |
| IMI 110803 | 2.74 | 9.03 | 0.30 | 8.1 | 2.30 | 9.11 | 0.25 | 7.8 |
| IMI 131010 | 6.94 | 10.54 | 0.66 | 8.2 | 6.72 | 8.93 | 0.75 | 8.1 |
| IMI 140524 | 4.36 | 7.42 | 0.59 | 7.9 | 6.70 | 7.70 | 0.87 | 7.9 |

ATCC American Type Culture Collection
CBS Centraalbureau voor Schimmelculture
DSM Deutsche Sammlung von Microorganismen und Zellkulturen
IMI International Mycological Institute

### *α-Galactosidase activity of Thermomyces lanuginosus CBS 395.62/b grown on various carbon sources*

Several carbohydrates and their derivatives were tested as growth substrates in 1 % concentration and α-galactosidase activities were monitored during the fermentation. The fungus grew on all tested carbohydrates. In the presence of most of the tested compounds the extracellular α-galactosidase activity showed a basic level value, which was lower than 1 U/mL. These compounds were the following: ribose, glucose, fructose, galactose, mannitol, melibiose, lactose, maltose, inulin, meso-inosit and Oligotime®. Among the above mentioned carbohydrates only melibiose possesses α-galactosidic linkage and it is a substrate for α-galactosidase. To reveal the correlation between the utilisation of melibiose and α-galactosidase activities, both extracellular and intracellular activities were investigated. To get more detailed information about the effect of the concentration of melibiose on formation of α-galactosidase activity, the α-galactosidase activity was investigated in media with 1, 2 and 3 % melibiose. The results are summarised in Fig. 1. The maximum intracellular activities exceeded the extracellular ones by at least one order of magnitude. Maximum intracellular activities were in the range from 0.23 to 0.27 U/mL. These values were determined on the first day of the fermentation, but they declined rapidly during the cultivation.

In presence of 1 % L-arabinose slight induction of extracellular α-galactosidase activity was observed, but

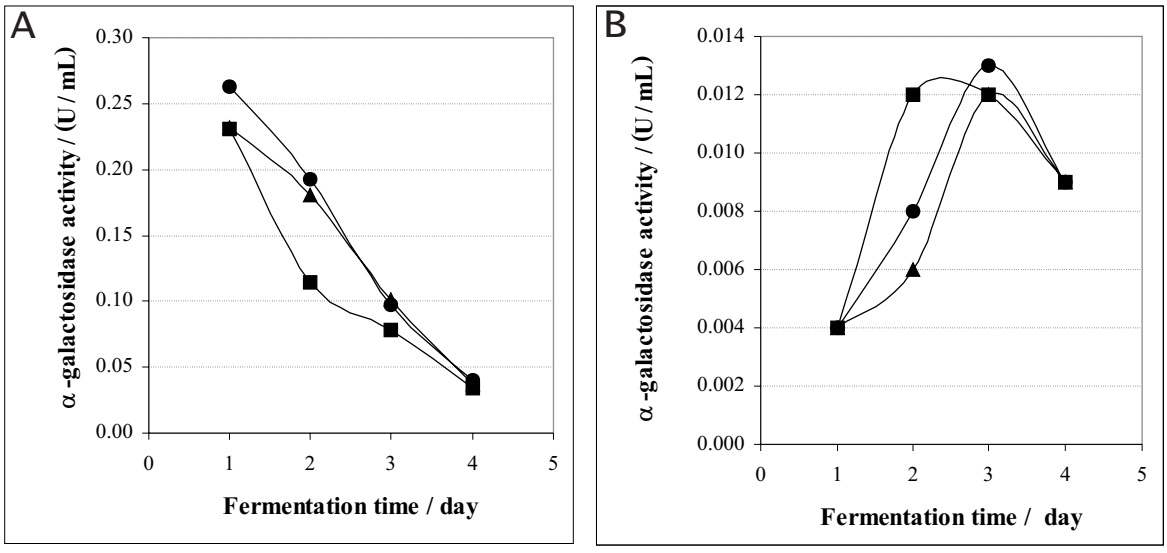


**Fig. 1. α-Galactosidase activities (A intracellular and B extracellular) of *T. lanuginosus* grown on melibiose** ■ 1 %, ● 2 %, ▲ 3 %

its value was lower than 2 U/mL on the sixth day of fermentation. The carbohydrates containing α-galactosidic linkages such as Lactosucrose® and raffinose induced the production of extracellular α-galactosidase enzyme. The induction of α-galactosidase was most promising on sucrose. The synthesis of α-galactosidase on Lactosucrose®, raffinose and sucrose during a six-day cultivation is shown in Fig. 2. The graphs show monotone increase, but they clearly demonstrate the ability of tested carbohydrates to induce α-galactosidase enzyme synthesis. Applying Lactosucrose® as growth substrate about 9 Um/L of α-galactosidase activity was achieved. The comparison reveals that the α-galactosidase activities on raffinose and sucrose were 2.5 and 3.0 times higher than on Lactosucrose®, respectively. The α-galactosidase activity of 28 U/mL achieved on sucrose substrate is promising for development of an enzyme fermentation technology.

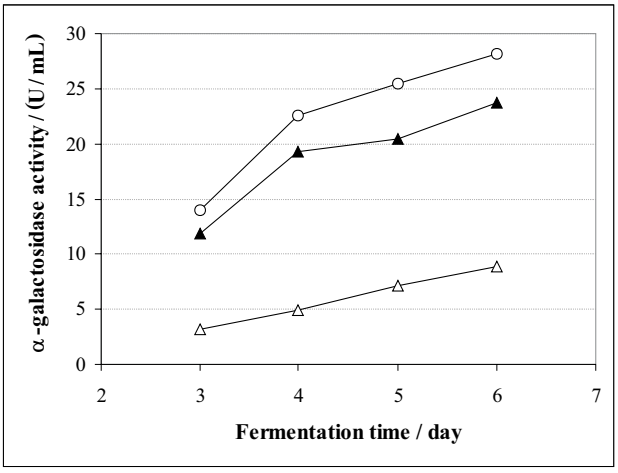


**Fig. 2. Development of extracellular α-galactosidase activities on media containing sucrose, raffinose and Lactosucrose®** ❍ sucrose, ▲ raffinose, Δ Lactosucrose®

To create a medium suitable for the production of α-galactosidase correlation between the enzyme activity and sucrose concentration was studied on media containing 0.45 % ammonium acetate. The tested sucrose concentrations were in the range of 2-4 % and the results are summarised in Fig. 3. Analysing the results it may be concluded that in case of 2 % sucrose, α-galactosidase activity reached lower value (35 U/mL) than in the case of higher concentrations. α-Galactosidase activities increased when the concentration of sucrose was in the range from 2.5 to 3.0 %. At higher concentrations the development of α-galactosidase activities was delayed. As a compromise 3 % sucrose is suggested to apply in α-galactosidase fermentation.

Applying 3 % sucrose solution, the optimum ratio of the amounts of sucrose and ammonium acetate was determined. The tested concentrations of ammonium acetate were between 0.6 and 1.2 %. The development of the α-galactosidase activities in the course of fermentation is shown in Fig. 4. As the graphs reveal, 0.6 and 0.75 % ammonium acetate were insufficient to achieve maximum α-galactosidase activity. After the seventh day of fermentation the activities decreased. At higher concentrations the activities increased proportionally with time. In the case of 1.05 and 1.2 % ammonium acetate the increase of α-galactosidase activity was slower than at lower concentration. On the eighth day of the cultivation the highest activity (90 U/mL) was observed in medium containing 0.9 % ammonium acetate, and it exceeded the value of 100 U/mL by the ninth day. These results suggest that 3 % sucrose and 0.9 % ammonium acetate can be proposed for the production of α-galactosidase by *T. lanuginosus* CBS 395.62/b strain.

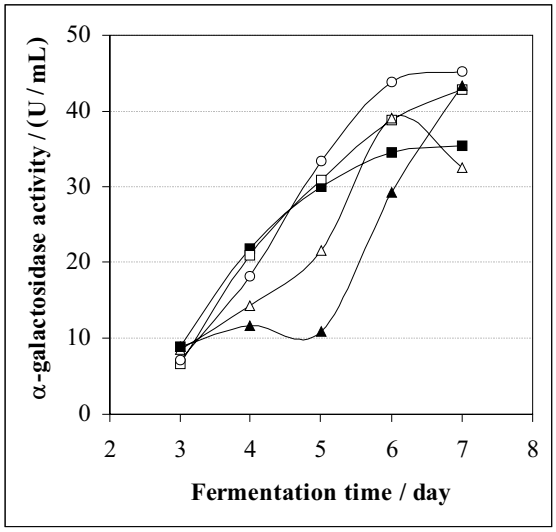


**Fig. 3. Formation of extracellular α-galactosidase activities of *T. lanuginosus* CBS 395.62/b strain in presence of various sucrose concentrations at 0.45 % ammonium acetate** ■ 2 %, ❐ 2.5 %, ❍ 3 %, Δ 3.5 %, ▲ 4 %

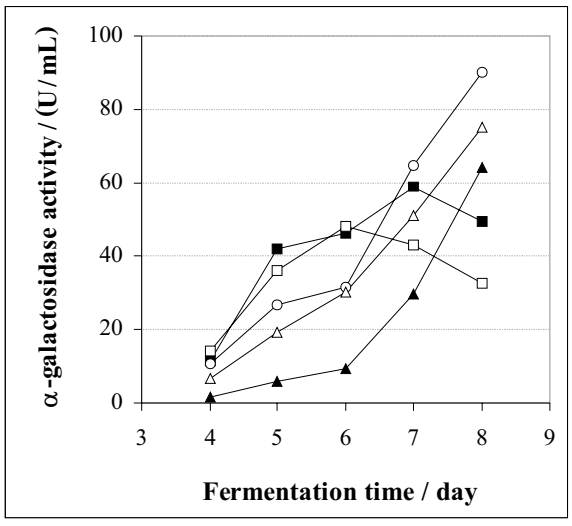


**Fig. 4. Formation of extracellular α-galactosidase activities of *T. lanuginosus* CBS 395.62/b strain in presence of various ammonium acetate concentrations at 3 % sucrose** ■ 0.6 %, ❐ 0.75 %, ❍ 0.9 %, Δ 1.05 %, ▲ 1.2 %

## Conclusion

In respect to the enzyme production there are considerable differences among the various microbial genera and species. In case of inducible enzymes it is very important to determine the compounds which can act as inducers. The cell-associated α-galactosidase enzyme of *Bacillus* sp. $JF_2$ was induced by melibiose and raffinose, but completely repressed by sucrose. The α-galactosidase induction by raffinose was enhanced when glucose was added to medium (*12*). In the case of *Aspergillus ficuum* NRRL 3135 strain the extracellular α-galactosidase production was induced by galactose (*13*). The synthesis and secretion of α-galactosidase by *Aspergillus nidulans* can be induced by both lactose and galactose, but not by melibiose (*14*), and it is in contradiction with extracellular α-galactosidase of *Cephalosporium acremonium* 237, which was induced neither by galactose nor lactose (*15*). α-Galactosidase activity of *T. lanuginosus* CBS 395.62/b strain can be induced by raffinose, Lactosucrose® and sucrose. These facts confirm that different species and strains may have different enzyme induction pattern. Moreover, enzyme activities vary from species to species or even from strain to strain. In our experiments α-galactosidase activities of the seventeen *Thermomyces lanuginosus* varied in the range of activity from 0.1 to 30 U/mL under the same conditions. Puchart *et al.* reported the secretion of α-galactosidase enzyme by *T. lanuginosus* IMI 158749 grown on galactomannan (*16*). Their aim was not the bulk enzyme production, but the enzyme production for purpose of purification and characterisation. Medium composition has a great influence on production and secretion of enzymes. α-Galactosidase productivity of *T. lanuginosus* CBS 395.65/b was enhanced three times by optimisation of the medium composition. Comparing the reached α-galactosidase activity of 90-100 U/mL with the values found in the literature, *T. lanuginosus* CBS 395.62/b strain may be regarded as potential α-galactosidase producing strain for industrial purposes.

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# Soj *Thermomyces lanuginosus* CBS 395.62/b kao bogati izvor enzima α-galaktozidaze

**Sažetak**

Sedamnaest sojeva *Thermomyces lanuginosus*, uzgojenih na rafinozi i saharozi, bilo je razvrstano prema aktivnostima α-galaktozidaze. *Thermomyces lanuginosus* CBS 395.62/b pokazao je najveću α-galaktozidoznu aktivnost uzgojem na obama ispitivanim ugljikohidratima. Nekoliko izvora ugljika ispitano je kao potencijalnih induktora za sintezu α-galaktozidaze. Na melibioznom supstratu aktivnost je α-galaktozidaze bila veća u intracelularnoj frakciji nego u filtratu podloge, iako su obje vrijednosti bile vrlo niske i nisu dostigle vrijednost od 1 U/mL. Dokazano je da su rafinoza, saharoza i Lactosucrose® induktori proizvodnje α-galaktozidaze. Najviši titar (oko 30 U/mL) postignut je uzgojem na 1 %-tnoj saharozi i 0,45 %-tnom amonijevu acetatu. Optimalna koncentracija saharoze iznosila je 3 %, a amonijeva acetata 0,9 %. S takvom podlogom postiže se α-galaktozidazna aktivnost otprilike 90 U/mL tijekom osmodnevne fermentacije.