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EFFECT OF DIFFERENT CARBON AND NITROGEN SOURCES ON α-GALACTOSIDASE ACTIVITY ORIGINATED FROM *THERMOMYCES LANUGINOSUS* CBS 395.62/B

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α-Galactosidase activity from *Thermomyces lanuginosus* strain of CBS 395.62/b was investigated in cultivation media with various compositions. Among the seven nitrogen sources only three L-asparagine, yeast extract and ammonium acetate supported the α-galactosidase synthesis. Ammonium acetate proved to be the best candidate as nitrogen source. When glucose or galactose was used as main carbon source, very low level, constitutive α-galactosidase activity was observed. In presence of raffinose, considerable α-galactosidase activity was detected. Raffinose can be replaced by sucrose in the cultivation medium, because the productivity reached by it was superior to that of raffinose. α-Galactosidase activity was improved by the optimisation of the concentrations of sucrose and ammonium acetate in the medium. Applying medium composition with 3% (w/v) sucrose and 0.6% (w/v) ammonium acetate led to at least 5 times higher activity which was observed in the reference medium containing 1.5% (w/v) raffinose and 0.45% (w/v) L-asparagine.

Keywords: α-galactosidase, *Thermomyces lanuginosus,* composition of medium, thermophilic fungus

α-Galactosidases catalyse the hydrolysis of α-1,6 linked α-galactose residues from oligosaccharides such as melibiose, raffinose, stachyose and verbascose, from polymeric galactomannans and from galactolipids, as well (DEY & PRIDHAM, 1972). The occurrence of these enzymes is widespread in nature. α-Galactosidase activity has been reported from animals, plants and microbes. Several prokaryotic and eukaryotic micro-organisms synthetize α-galactosidase enzymes. Purification and properties of bacterial α-galactosidase from *Lactobacillus fermentum* (GARRO et al., 1996), *Bifido-bacterium adolescentis* (LEDER et al., 1999), *Bacillus stearothermophilus* (TALBOT & SYGUSCH, 1990) and a hyperthermophilic eubacterium *Thermotoga neopolitana* strains (DUFFAUD et al., 1997) were reported. HASHIMOTO and co-workers (1993) isolated a yeast strain – *Candida guilliermondii* H-404 – from soil, which produced α-galactosidase. As a result of the purification procedure two isoenzymes were gained. These two enzymes had the same molecular mass, but they had different isoelectric points, and differed from each other in pH and temperature stability.

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Several reports deal with the synthesis of α-galactosidase enzymes originated from filamentous fungi. A simple method was developed for the production of α-galactosidase in crystalline form from the mycelia of *Mortierella vinacea*. The crystalline α-galactosidase was free from protease and other glycosidases (SUZUKI et al., 1970). To analyse the primary structure of α-galactosidase from *Mortierella vinacea*, a cDNA library was constructed from mRNA of *M. vinacea* in λgt10 vector. A clone, which has an insert size of about 1.4 kb by pairs, was found to contain the coding region of the mature enzyme. The mature enzyme consisted of 397 amino acid residues with molecular mass of 44.350 Da (SHIBUYA et al., 1995b). FODA and co-workers (1995) observed intracellular α-galactosidase synthesis by *Penicillium janthinellum*, while SHIBUYA and co-workers (1995a) reported that they found a *Penicillium purpurogenum* strain, which secreted α-galactosidase enzyme into the culture filtrate. *Aspergillus niger* produces several α-galactosidase enzymes and this phenomenon indicates that these enzymes are active on different substrates (DE VRIES et al., 1999). Extracellular α-galactosidase enzyme was produced in solid state fermentation by a thermophilic *Humicola* sp. applying soy flour as carbon source (KOTWAL et al., 1998).

The industrial potential of the application of α-galactosidase enzyme is increasing, especially in food industry. It is applicable to reduce or eliminate the antinutritive galacto-oligosaccharide content from legume-based food (HOSCHKE et al., 1999). By the enzymatic removal of so-called raffinose family sugars from legume-based food products, their nutritional value can be enhanced and the digestion discomfort avoided.

In our previous study seventeen strains of the thermophilic fungus *Thermomyces lanuginosus* were screened for production of extracellular α-galactosidase enzyme. Strain CBS 395.62/b showed the best productivity. The optimum temperature and pH for enzyme activity were determined as 58 °C and pH 4.2. In the range from 5.0 to 7.8 of pH values the α-galactosidase from *Thermomyces lanuginosus* is fairly stable at 47 °C for a week. More than 80% of the enzyme activity was recovered after 10 days of incubation in water bath at 55 °C. The half-life times of the enzyme activity were 15 h, 1.2 h and 3.5 min in the case of temperatures 60 °C, 65 °C and 70 °C, respectively (REZESSY-SZABÓ et al., 2000). In this study we are presenting the results of the optimisation of media composition to maximise the α-galactosidase productivity of the strain *Thermomyces lanuginosus* CBS 395.62b.

1. Materials and methods

All chemicals used were of analytical grade and purchased either from Merck, Sigma, Reanal or from other companies.

1.1. Micro-organism

Thermomyces lanuginosus CBS 395.62/b strain was kindly provided by Dr. M.K. Bhat (Institute of Food Research, UK). The strain was maintained on slants, containing Potato-Dextrose-Agar (PDA) and stored at 4 °C until use.

1.2. Medium and culture conditions

Five ml of conidia suspension prepared by using 0.1% Triton X-100, was added to 100 ml of glucose-asparagine medium (glucose 2 g, L-asparagine 0.4 g, KH_2PO_4 0.3 g, K_2HPO_4 0.2 g, $MgSO_4.7H_2O$ 0.05 g and 0.1 ml of Vogel's trace elements solution (VOGEL, 1956) in 100 ml distilled water, pH 6.0) to initiate the cultivation. The inoculum was prepared in orbital shaker at 47 °C and 220 r.p.m. for 1 or 2 days. The enzyme production was carried out in 500-ml Erlenmeyer flasks containing 150 ml of fermentation medium using 5 ml of inoculum. The basal medium consisted of 3 g of KH_2PO_4, 2 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$ and 1 ml of Vogel's trace elements solution in 1000 ml distilled water. The enzyme production was investigated in basal medium complemented with various nitrogen and carbon sources of different concentration. The initial pH of the medium was about 6.4 and was not further controlled. Samples were taken regularly, the fungus was harvested by filtration and the α-galactosidase activity was assayed in the culture filtrates.

1.3. α-Galactosidase 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 of suitably diluted enzyme solution (ferment 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 international unit (IU) of α-galactosidase activity is defined as the amount of enzyme that liberates 1 μmol p-nitrophenol in 1 min under the assay conditions.

1.4. Optimisation of the medium composition

Enhancement of the productivity is achieved by the optimisation of the composition of the medium. Two different strategies were followed: classical one factor trials and factorial experiment design. In the later one more factors are changed at the same time and the statistical evaluation makes it possible to estimate the effects of the individual components on the formation of α-galactosidase activity. The tested components are presented in the "Results and discussion" part.

2. Results and discussion

The strain CBS 395.62/b of *Thermomyces lanuginosus* gives 16 IU ml^{-1} α-galactosidase activity in a medium containing 1% (w/v) raffinose as carbon source and 0.4% (w/v) L-asparagine as nitrogen source (REZESSY-SZABÓ et al., 2000).

2.1. Two-level, two-factorial design I

The effects of raffinose as a quantitative factor, L-asparagine and yeast extract as qualitative factor on the α-galactosidase productivity of the relevant strain were investigated. The experimental design is presented in Table 1.

Table 1. Experimental design I

Trials	Raffinose % (v/w)	Nitrogen source
1	1.5	0.4% (w/v) L-asparagine
2	1.5	0.8% (w/v) yeast extract
3	2.5	0.4% (w/v) L-asparagine
4	2.5	0.8% (w/v) yeast extract

Nitrogen sources were adjusted to the same nitrogen content. Results are shown in Fig. 1. The α-galactosidase activity is plotted against the time course.

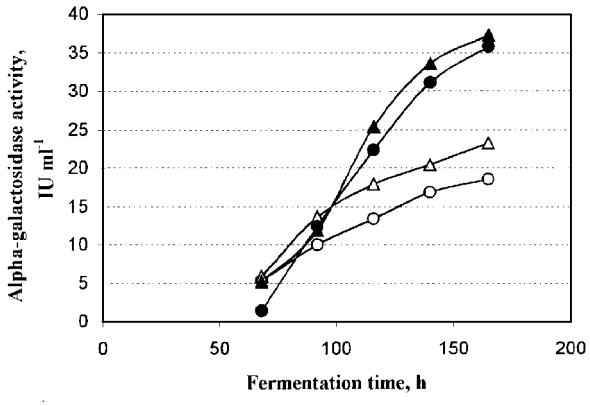

Fig. 1. α-Galactosidase activities in the course of fermentation experimental design I.
–O–: Trial 1; –Δ–: Trial 2; –●–: Trial 3; –▲–: Trial 4

On the basis of the results it can be concluded, that the tested nitrogen sources caused no significant difference in α-galactosidase activities in the course of fermentation. Enhancement of the raffinose concentration increased the α-galactosidase activity.

2.2. Effect of various nitrogen sources on the α-galactosidase activity

The effects of the various nitrogen sources were tested in media containing raffinose of 2.5% (w/v). The concentrations of nitrogen sources were defined on the base of same

nitrogen contents. Seven different compounds were tested, namely yeast extract, L-asparagine, ammonium acetate, ammonium nitrate, sodium nitrate, ammonium sulphate and ammonium dihydrogen-phosphate. The fungus grew very well on all tested nitrogen sources. However, only the organic compounds supported the synthesis of α-galactosidase enzyme (Fig. 2).

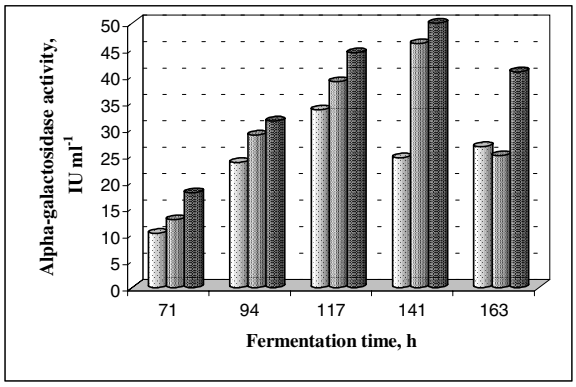

Fig. 2. Effect of various nitrogen sources on the α-galactosidase activity.
▨: L-asparagine; ▩: yeast extract; ■: ammonium acetate

The best results were gained by using ammonium acetate. By the sixth day, the enzyme activity reached the value of 50 IU ml^{-1} and on the seventh day it was still higher than 40 IU ml^{-1}.

2.3. Effect of various carbon sources on the α-galactosidase activity

The α-galactosidase synthesis of *Thermomyces lanuginosus* CBS 395.62/b strain was investigated by applying different chemically defined compounds, which did not contain α-galactosidic linkages, such as glucose, galactose, and sucrose, and besides them raffinose was used as carbon source in control trial. The growth media contained 0.45% (w/v) ammonium acetate as nitrogen source and 1% (w/v) of each carbon source, respectively. The α-galactosidase activity during the fermentation is shown in the Fig. 3.

In the presence of glucose and galactose the fungus showed very low levels of α-galactosidase activity. It was about 1 IU ml^{-1} in the case of glucose and about 2.5 IU ml^{-1} when galactose was used as carbon source. The maximum activity was achieved in all cases at the 127th hour of the fermentation. The α-galactosidase activity gained in medium containing sucrose as carbon source, was higher than in the medium containing raffinose. The maximum activity was 49 IU ml^{-1} in sucrose containing medium.

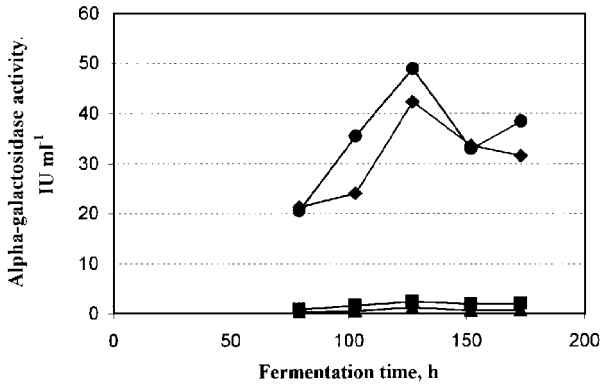

Fig. 3. Effect of various carbon sources on the α-galactosidase activity.
–▲–: glucose; –■–: galactose; –●–: sucrose; –◆–: raffinose

2.4. Two-level, two-factorial design II

Ammonium acetate was selected as nitrogen source and sucrose was chosen as main carbon source for the production of α-galactosidase enzyme by the strain CBS 395.62/b of *Thermomyces lanuginosus*. Therefore, a two-level, two-factorial experimental design was created and performed, where the effect of the concentration of ammonium acetate and sucrose as quantitative factors was investigated in respect of the production of α-galactosidase enzyme. The individual trials were carried out in two replicates. The experimental design and the results are shown in Table 2.

Table 2. Two-level, two-factorial experimental design II and the gained results

Trials	Sucrose	NH_4-acetate	α-Galactosidase activity (IU ml^{-1})				
			72 h				168 h
	% (w/v)	% (w/v)		96 h	120 h	144 h	
1	3	0.60	10.5	17.25	31.7	39.0	90.4
2	3	0.30	7.3	8.2	12.8	27.2	47.7
3	1	0.60	11.65	13.9	14.8	18.3	32.3
4	1	0.30	15.75	14.7	22.2	34.2	16.3

Data of α-galactosidase activity are expressed as average of two replicate experiments

As Table 2 reveals, a full factorial design was accomplished. The upper levels of the concentration of sucrose and ammonium acetate were 3.0% (w/v) and 0.6% (w/v) respectively, while the lower levels of the concentration of sucrose and ammonium acetate were 1% (w/v) and 0.3% (w/v). Transformations of the independent variables make the evaluation easier. After standardisation their values will be equal to 1 at the upper level and (–1) at the lower level. α-Galactosidase activity, as the function of the time, is given in the Fig. 4, where the results of the individual experimental runs are shown in separate graphs.

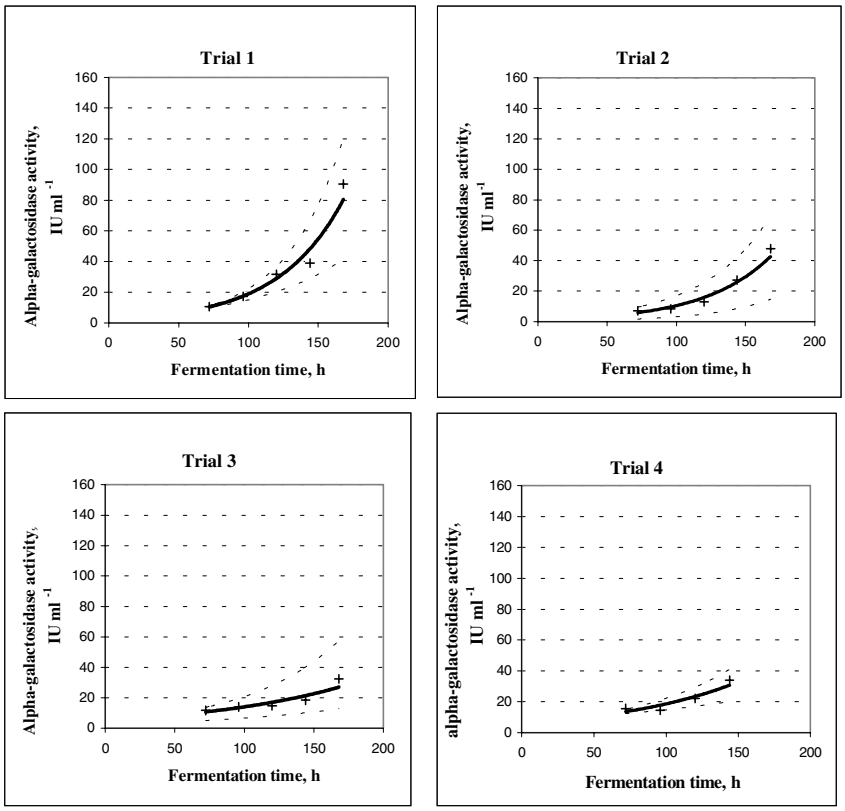

Fig. 4. Formation of α-galactosidase activity in the experimental design I. Trial 1: $y = 2.2405e^{0.0213x}$ ($R^2 = 0.9748$); Trial 2: $y = 1.3159e^{0.0207x}$ ($R^2 = 0.9491$); Trial 3: $y = 5.3620e^{0.0096x}$ ($R^2 = 0.8610$); Trial 4: $y = 5.9957e^{0.0114x}$ ($R^2 = 0.8338$)

The graphs of Fig. 4 contain the averages of α-galactosidase activities measured at different times. Exponential curves were fitted to the measurement results in the ranges, where the activities were increasing constantly as a function of time. All trials showed the highest values at the 168th hour except trial 4, where the concentrations of both nutrients were at the lower level. Therefore, in the case of trial 4 the evaluation of data was made in the range from 72 to 144 h. The dashed lines indicate the confidence intervals at 5% significance level. The confidence interval was broadened with the increasing fermentation time. The highest α-galactosidase activity was reached in the case of trial 1, where both tested nutrients were applied in higher concentration, namely the growth medium contained 3% (w/v) sucrose and 0.6% (w/v) ammonium acetate.

Table 3. Estimation of the coefficients of a linear model (SPSS 9.0 for Windows software)

	Unstandardized coefficients B	Std. error	Standardized coefficients Beta	t	Sig
Constant	46.650	6.984		6.680	0.001
Sucrose	22.375	6.984	0.722	3.204	0.024
Ammonium acetate	14.675	6.984	0.474	2.101	0.090

Dependent variable: α-galactosidase activity (AGAL)

To predict the effect of the tested components on the activity of α-galactosidase, the results obtained at different trials were statistically evaluated by using SPSS 9.0 for Windows program package. The experimental data were evaluated at the 168th hour of the fermentation. A linear model was fitted to the experimental results. For the calculation the transformed factors were applied. The statistical results are summarised in Table 3.

The increase of the concentration of both sucrose and ammonium acetate exerts positive effects on the α-galactosidase activity. The enhancement of the concentration of sucrose succeeds at α=0.05 significance level, whereas the increase of the concentration of ammonium acetate gets on at α=0.1 significance level.

The proposed linear model for the description of the experimental system is the following:

$$\alpha\text{-galactosidase activity} = 46.65+22.37x_1+14.67\ x_2$$

where x_1 is the amount of sucrose (transformed variable)
x_2 is the amount of ammonium acetate (transformed variable).
The predicted model was created with SPSS 9.0 for Windows software package.

Based on the calculations the highest α-galactosidase activity can be gained in the medium of trial 1. The predicted value of α-galactosidase activity should be located in the range between 50 and 118 with mean of 84 IU ml^{-1} at α=0.05 significance level.

The results were confirmed in two separate experimental series. In the first series four replicate fermentations were made, whereas in the second one five replicates were accomplished. Fermentation time was elongated to determine the time of maximum α-galactosidase activity. In case of the first fermentation the maximum α-galactosidase activity was reached by the 168th hour, while in the second fermentation it was detected at the 192nd hour. Data are shown in Fig. 5.

The maximum α-galactosidase activities gained in the relevant medium were in the range from 52 to 99 IU ml^{-1}. They were in the range of the predicted values. Further studies are needed to standardise the fermentation and fix the time of maximum α-galactosidase activity.

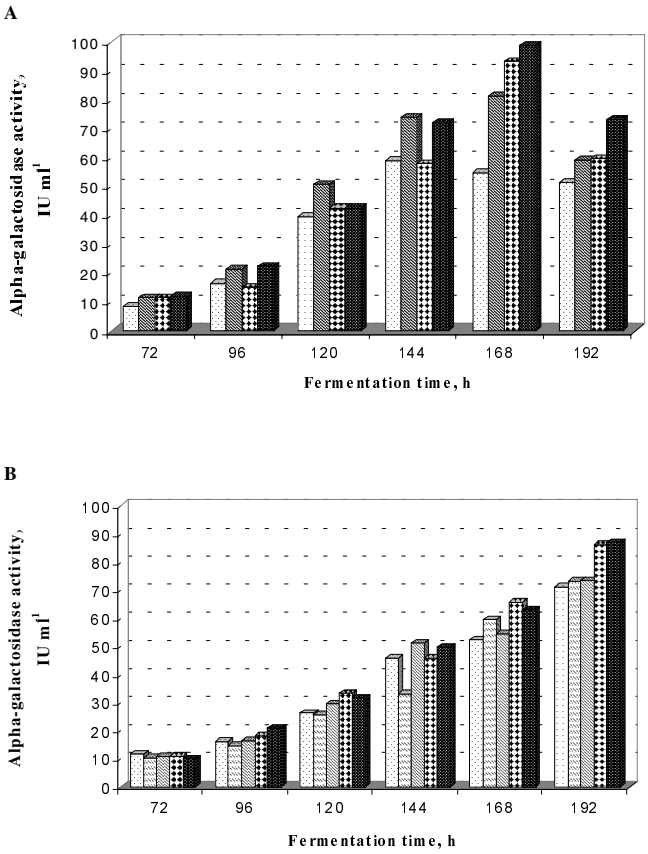

Fig. 5. Formation of α-galactosidase activity in medium containing sucrose (3 % w/v) and ammonium acetate (0.6 % w/v). **A:** experimental series with 4 parallels. : replicate 1; : replicate 2; : replicate 3; : replicate 4. **B:** experimental series with 5 parallels. replicate 1; : replicate 2; : replicate 3; : replicate 4; : replicate 5.

3. Conclusions

There was no extracellular α-galactosidase activity from *Thermomyces lanuginosus* CBS 395.62/b detected when it was grown on ammonium nitrate, sodium nitrate, ammonium-sulphate or ammonium-di-hydrogen-phosphate as a sole nitrogen source.

Very low level of α-galactosidase activity was observed in the fermentation broth, when glucose or galactose was present in the cultivation media. It is a remarkable result that raffinose can be replaced by sucrose in the medium, because sucrose is a readily available fermentation carbon source.

α-Galactosidase activity of *Thermomyces lanuginosus* CBS 395.62/b strain was enhanced by the modification of the composition of the medium. In respect of the α-galactosidase synthesis and secretion the most promising carbon and nitrogen sources are sucrose and ammonium acetate. Applying medium, which contains sucrose of 3% (w/v) and ammonium acetate of 0.6% (w/v) the activity of the relevant strain could be improved about five times.

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