# SOY MEAL WASTE EXTRACT AS CULTIVATION MEDIUM FOR PRODUCTION OF EXTRACELLULAR α-GALACTOSIDASE FROM THE FUNGUS *HUMICOLA LUTEA* 120-5

L. Nacheva[1], P. Aleksieva[1], E. Bratovanova[2], I. Stoineva[2], B. Yakimova[2], B. Tchorbanov[2]
Bulgarian Academy of Sciences, Institute of Microbiology, Sofia, Bulgaria[1]
Bulgarian Academy of Sciences, Institute of Organic Chemistry with Centre of Phytochemistry, Sofia, Bulgaria[2]
Correspondence to: Lilyana Nacheva
E-mail: lin1@abv.bg

## ABSTRACT

*Soy meal extract presenting a waste product from soy protein concentrate manufacture was applied as cultivation medium for the production of extracellular α-galactosidase in submerged fermentation of the filamentous fungus Humicola lutea 120-5. Maximum enzyme activity (2000 U.l$^{-1}$) was produced when the soy meal extract containing 5% dry substances (~1% raffinose oligosaccharides) was used at 120th h cultivation and 35°C. Chromatography on Sephadex G-100 and DEAE-cellulose resulted in a purified α-galactosidase fraction with specific enzyme activity of 37 U.mg$^{-1}$. The optimum conditions for the enzyme reaction were pH 4.0 to 4.5 at 50°C. The partially purified enzyme was stable up to 40°C at pH 2.0 to 7.5. The crude enzyme preparation readily hydrolyzes raffinose according HPLC analysis, but no activity against disaccharides as sucrose and melibiose was observed. The soy meal waste extract can be recommended as a suitable medium for the industrial cultivation of strains producing α-galactosidase for food and fodder purposes.*

**Keywords:** Soy meal extract, α-galactosidase, *Humicola lutea*

## Introduction

α-D-galactoside galactohydrolase, [EC 3.2.1.22], (α-galactosidases) catalyzed the hydrolysys of α-1,6-linked galactosyl residues in oligosaccharides and galactomannans. The raffinose oligosaccharides (RO) are considered the major factors responsible for flatulence after ingestion of soybean or other legumes. α-Galactosidases have an wide application in biotechnology for the nutritional improvement of legumes based foods and fodders (13). Several fungal genera have been studied preferably for production of extracellular α-galactosidases: *Aspergillus* (1, 9, 13), *Penicillium* (8), *Thermomyces* (11, 12) and *Humicola* (6, 7). The fermentations have been carried out on media containing mono- and oligo-saccharadides (13); galactomannan (1); wheat arabinoxylan (9) as well as waste cakes from the oats, coconut, wheat and soy processing, locust bean gum, out-husk meal (7, 8).

The aim of the present work is to study the production of extracellular α-galactosidase for food and fodder purposes by submerged cultivation of the filamentous fungus *Humicola lutea* 120-5. In this paper we are focused on the usage of a waste product from the soy protein concentrate manufacture as effective fermentation medium alone.

## Materials and Methods

### Microorganism and nutrient media

The fungal strain *Humicola lutea* 120-5, registered in National bank for industrial microorganisms and cell cultures: No 391, Sofia, Bulgaria (5) was used in this study. Waste extract from the production of soya protein isolates was used as fermentation medium only. For the experiments the soya meal extract was prepared following the next procedure: **i**) 150 g defatted soy meal was dispersed in 1 l water and pH was adjusted at pH 8.0 by ammonia solution, the mixture was heated to 80-85°C and the solution was removed from the insoluble part by centrifugation; **ii**) the solution obtained was cooled to room temperature and the protein fraction was precipitated by adjustment to pH 4.5 by ortho-phosphoric acid and further separated by centrifugation; **iii**) the residual waste extract with a dry content of 5.0-5.5% was used further as production medium. For comparable experiments the medium thus obtained was twice diluted or 150% concentrated. The medium was autoclaved at 115°C for 15 min and pH after the sterilization was 5.8-6.0. Comparable fermentations using different mono- and oligosaccharides were performed as control processes.

The cultivation was carried out in 500 ml Erlenmeyer flasks each containing 50 ml of medium, inoculated with spore suspension (density $10^6$–$10^7$ spores/ml) from one test tube. The flasks were incubated on a rotary shaker at 220 rpm during 168h at different temperatures (30°C, 35°C and 40°C). In order to select the carbon source, spores were transferred to 50 ml of control media consisting of g.L$^{-1}$: $KH_2PO_4$ 1.0, $MgSO_4$ 0.5, KCl 0.5, $NaNO_3$ 4.0, yeast extract 0.6 and 1% (w/v) of one of the follows: galactose, lactose, melibiose, raffinose, glucose, or sucrose. At the end of the fermentation process the fungal biomass was removed from the culture broth by filtration through filter paper. The culture supernatants were subjected for analysis of α-galactosidase activity. The variation of the enzyme yield during the 3 parallel fermentations was within 4-5 %.

### Preparation of cell free extract

After a grinding of biomass by quartz sand suspended in citrate-phosphate buffer pH 5.5 and consequent centrifugation at 10 000 g for 20 min the clear supernatant was checked for intracellular enzyme activity.

### Enzyme assay

α-Galactosidase activity was assayed by the method of Dey et al. (4) using 0,003 M p-nitrophenyl-α-D-galactopyranoside as substrate at pH 5.5 supporting by citrate-phosphate buffer. The reaction mixture was incubated at 50°C for 15 min. The reaction was stopped by the addition of 0.1 M sodium carbonate. The amount of p-nitrophenol released was measured from absorbance at 405 nm. One unit (U) of α-galactosidase activity was defined as the amount of enzyme which liberates 1 μmol of p-nitrophenol per min under the described conditions.

### α-Galactosidase purification

The lyophilized enzymatic sample resuspended in 20 mM sodium acetate buffer, pH 5.5 was subjected to gel filtration chromatography in a Sephadex G-100 column (80 cm x 2.5 cm) equilibrated with 20 mM sodium acetate buffer. The proteins were eluted at a flow rate of 18 $ml.h^{-1}$ and 3.8 ml fractions were collected. Fractions containing α-galactosidase activity were pooled and subjected to ion exchange chromatography in a DEAE-cellulose (16cm x 1.7 cm) equilibrated with 20 mM sodium acetate buffer, pH 5.5. The proteins were eluted at a flow rate of 30 $ml.h^{-1}$, with a linear gradient of NaCl (0-0.6 M) in 20 mM sodium acetate buffer. Fractions containing α-galactosidase activity were pooled, lyophilized and used further studies.

### Enzyme characterization

The effect of pH and temperature on enzyme activity was studied using citrate-phosphate buffer at pH 2.0 to 8.0 (the enzyme was kept for 24h at 4°C) or various incubation temperatures (20-70°C) under the standard conditions (pH 5.5 for 15 min).

### HPLC analysis of saccharides hydrolysis

The hydrolysis of di- and oligosaccharides catalyzed by aliqouts of crude cultural filtrates (demonstrating 100 mU α-galactosidase activity) were carried out using 500 μl 1% substrate solution in 50 mM sodium acetate buffer, pH 4.5 at 40°C. HPLC analysis was performed with a Agilent 11100 chromatograph equipped with Evaporative Light-scatering Detector. An analytical column LiChrosorb NH2 (250-4 mm, 5 μm) was applied for carbohydrates separation. Sample injection was *via* a Rheodyne injector equipped with a 10 μl sample loop. The mobile phase consisted of acetonitrile: water (70:30 v/v) for separation and flow rate was fixed at 1 ml/min. Peak identification of the chromatographs was done by comparing the retention time with the standards. Galactose, glucose, sucrose, melibiose and raffinose were purchased from Sigma.

## Results and Discussion

### Effect of carbon sources

The fungal strains are suitable sources of α-galactosidase because of extracellular enzyme localization and broad profiles of pH and temperature stability. The strain *H. lutea* 120-5 was found to utilize soy meal waste extract (SME) containing about 1% RO and different mono- and oligosaccharides as carbon sources (**Table 1**). Lactose and galactose were poor carbon sources. Glucose was converted into biomass visibly but did not induce α-galactosidase biosynthesis. Surprisingly, although the raffinose and mellibiose sustained a good growth (2.6 -2.8 $g.l^{-1}$) these galactose containing oligosaccharides were low effective inducers. Similar results have been obtained by de Rezende et al. (13) investigating α-galactosidase excreted by the fungus *A. fumigatus*. According to the authors, the raffinose supported substantial growth but was a poor inducer as lactose, probably due to the presence of the invertases, which in combination with background α-galactosidase leading hydrolysis to simple sugars. Galactose, glucose, and fructose could be used for the production of the biomass, but were unable for further inducing α-galactosidase biosynthesis. The highest α-galactosidase activity (1100 $U.l^{-1}$) was detected in cultural supernatant obtained after the fermentation using SME (5% dry content). Abundant galacto-oligosaccharides in soybean meal induced α-galactosidase biosynthesis described previously by Cruz and Park (3). A high enzyme yield (44.6 $U.g^{-1}$) observed after solid state cultivation of *Humicola sp.* on soya flour (7) has been explained with the presence of intermediates in the metabolism of oligosaccharides, but the mechanism is still to be probed.

### Extracellular and intracellular α-galactosidase activity

The study on the α-galactosidase activity distribution between culture liquid (extracellular) and mycelial extract (intracellular) shows that after 48h of cultivation, 55% of the total enzyme activity was in culture filtrate. At the transition of the culture from the exponential to the stationary phase (120h), the enzyme activity in culture liquid increased to 98% (**Fig. 1**).

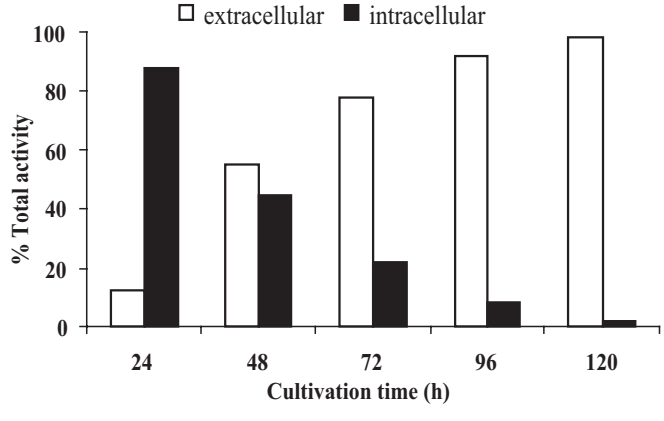



**Fig. 1.** Distribution of *H. lutea* α-galactosidase activity in culture liquid and mycelial extract

**TABLE 1**

α-Galactosidase activity in the culture medium and mycelial mass produced by the fungus *H. lutea* 120-5 grown on different carbon sources at 30°C for several times. $U.L^{-1}$ = units per L of culture supernatant; $g.L^{-1}$ = grams of mycelium dry weight per L of culture medium

| Carbon source | Growth time (h) | | | | | |
|---|---|---|---|---|---|---|
| | 96 | | 120 | | 144 | |
| | $U.L^{-1}$ | $g.L^{-1}$ | $U.L^{-1}$ | $g.L^{-1}$ | $U.L^{-1}$ | $g.L^{-1}$ |
| galactose | 4 | 1.0 | 10 | 1.3 | 11 | 1.2 |
| melibiose | 5 | 3.0 | 110 | 2.8 | 105 | 2.6 |
| raffinose | 40 | 2.3 | 90 | 2.6 | 80 | 2.5 |
| lactose | 3 | 0.1 | 5 | 0.1 | 4 | 0.1 |
| sucrose | 34 | 1.5 | 74 | 2.4 | 70 | 2.3 |
| glucose | 8 | 2.4 | 14 | 2.2 | 15 | 2.1 |
| Soy meal waste extract | 900 | 9.0 | 1100 | 8.5 | 1000 | 8.0 |

**TABLE 2**

Summary of *H. lutea* 120-5 extracellular α-galactosidase purification

| Purification step | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Purification factor | Recovery (%) |
|---|---|---|---|---|---|
| Lyophilized extract | 632 | 62 | 0.098 | 1 | 100 |
| Sephadex G-100 | 4.8 | 35 | 7.3 | 74 | 56.4 |
| DEAE-Cellulose | 0.6 | 22.2 | 37 | 377 | 35.8 |

Consequently: (**i**) the majority of the activity was found in the culture broth, e.g. *H. lutea* 120-5 α-galactosidase is a typical extracellular enzyme. (**ii**) SME is the best carbon source up to now for fungus cultivation releasing α-galactosidase due to its composition as well as to the availability as waste product during soy protein isolates production providing a basis for scale-up purposes of the process on α-galactosidase production.

**Cultivation parameters**

SME with 5% dry content (about 1% galactosides) was optimal and corresponded to the technological parameters for protein concentrate production from soy meal (**Fig. 2**). The cell growth increased visibly during the first stage of the fermentation reaching a maximum of 11 g per l at the 96-th h. The fungus produced the enzyme mainly during the exponential and post-exponential phase and α-galactosidase activity reached up to 1100 units per l at the stationary phase (120th h).

Using twice diluted SME as fermentation medium 50% less of the enzyme yield was registered, while the application of 150% concentrated SME did not influence the maximal result (**Fig. 2**). Consequently, SME (5% dry content) gave the best results and for this reason our next experiments were carried out with this extract.

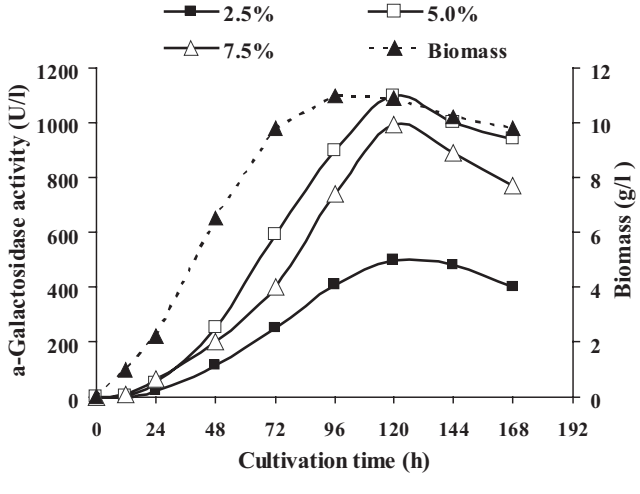


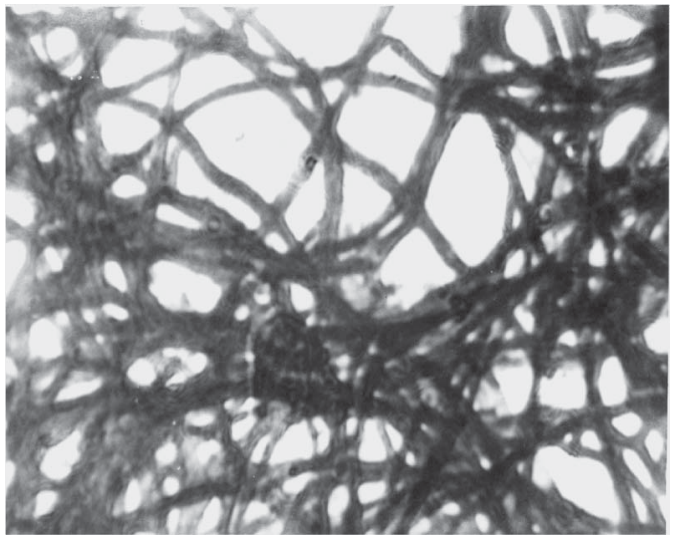

**Fig. 2.** Influence of SME concentration on the yield of α-galactosidase during submerged cultivation of the fungus *H. lutea* 120-5

During the cultivation in shake flasks, the fungal strain *H. lutea* 120-5 grew as small pellets. Light microscopy picture of 96th h culture (**Fig. 3A**) shows mycelial network composed of many densely entangled and intensively stained hyphae, which preserved their basophilic character after 120th h of the cultivation. Partly autolysed mycelia (**Fig. 3B**) and lateral egg-shaped spore formation was observed at the end of the fermentation (168th h).

Even though the strain *Humicola lutea 120-5* was considered as mesophilic fungus (2), we carried out cultivations at 35°C and 40°C (**Fig. 4**). The sharp increasing of the enzyme yield – up to 1900 $U.l^{-1}$ after the cultivation at 35°C demonstrated the importance of all fermentation factors for industrial development of α-galactosidase production under low cost.

**Enzymatic hydrolysis of oligosaccharides**

The potential of *H. lutea* α-galactosidase (crude preparation) to hydrolyze the oligosaccharides melibiose and raffinose was demonstrated using HPLC analysis (**Fig. 5**). As can be seen the enzyme readily hydrolyses raffinose (**Fig. 5B**) but no activity against disaccharides as sucrose and melibiose (**Fig. 5A**) was observed. Our results indicate that *H. lutea* α-galactosidase showed no invertase activity, as it was previously discussed (15) for extracellular α-galactosidase from *Debaryomyces hansenii*.



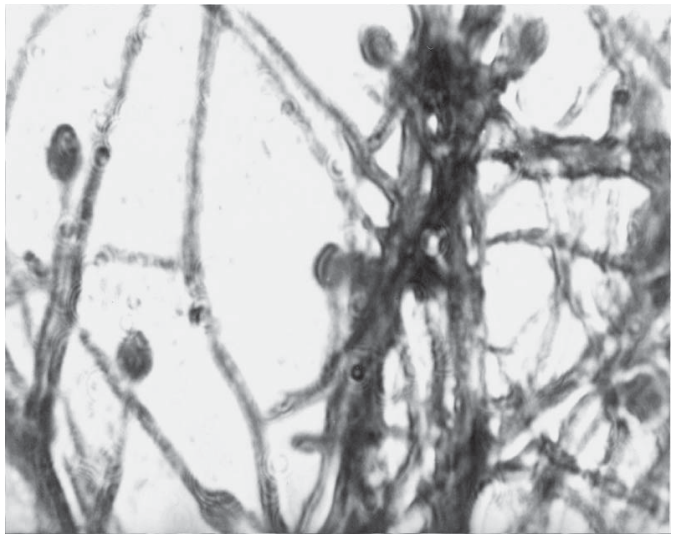


**Fig. 3.** Light microscopy of *H. lutea* mycelia after 96 h (A) and 168 h (B) submerged shake flask cultivation on SME (5% dry content). Magnification: x 2500

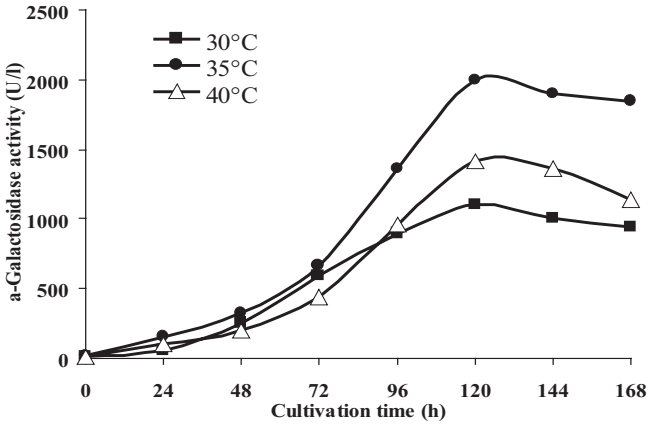
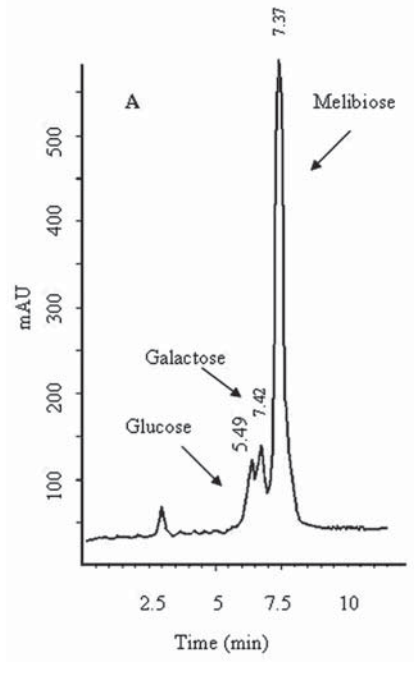



**Fig. 4.** Influence of incubation temperatures on the a-galactosidase production. The fungus was grown on SME (5% dry content)



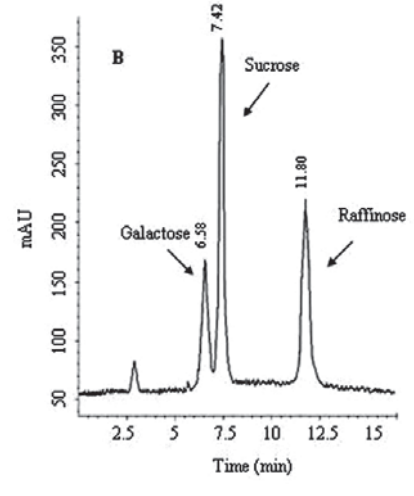




**Fig. 5.** HPLC analysis of hydrolysis product of melibiose (A) and raffinose (B) by extracellular α-galactosidase after 16 h incubation

**Purification and characterization**

SME-grown *H. lutea* 120-5 produced high levels of extracellular α-galactosidase similar to other eukaryotic microorganisms (11, 15). Results of the enzyme purification are summarized in **Table 2**. The lyophilized concentrate from the culture supernatant was subjected to gel filtration chromatography resulting in the separation of two protein fractions with α-galactosidase activity (**Fig. 6A**). The molecular mass of α-galactosidase from *H. lutea* 120-5 was evaluated to about 110-120 kDa and it is possibly due to the dimmer formation. This is expected because some small peaks with MW of about 50 kDa were detected from the elution profile. The active peak was with specific activity of 7.3 U.mg$^{-1}$. The rechromatography of the first active fraction on an ion exchange DEAE-Cellulose also resulted in an α-galactosidase activity peak, which was eluted with NaCl, at a concentration of about 0.3 M (**Fig. 6B**).

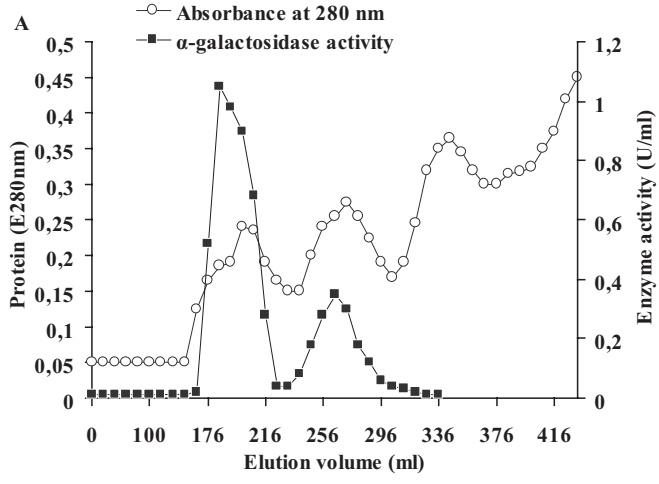



**Fig. 6.** Elution profile of the α-galactosidase from *H. lutea* 120-5 on (A) Sephadex G-100 column and (B) DEAE –Cellulose column

This procedure resulted in a purified α-galactosidase fraction with specific enzyme activity of 37 U.mg$^{-1}$, purification factor 377 and recovery level of about 36%. For comparison, the α-galactosidase secreted by *A. fumigatus* was purified 133.8-fold with specific activity 6.7 U.mg$^{-1}$ and a recovery of 52 % (13).

The pH-activity profile (**Fig 7**) indicated that the optimum pH of *H. lutea* 120-5 α-galactosidase was 4.0 to 4.5; the enzyme was stable between pH 2.0 and 7.5. Generally, the pH optimum of fungal α-galactosidases by *A. niger* (1), *A. fumigatus* (13), *A. flavipes* (10), *P. simplicissimum* (8) was about pH 3.5 to 5.0. Most α-galactosidases are stable over a broad range of activity.

The optimum temperature for PNPG hydrolysis by the of *H. lutea* 120-5 α-galactosidase at pH 5.5 was 55°C (**Fig. 8**) which is within the range of values reported for *A. niger* (14), *P. ochrochloron* (4) α-galactosidases. *H. lutea* 120-5 α-galactosidase was stable up to 40°C for 2h, whereas at 50°C the enzyme retained 70% of its original activity (**Fig. 8**).

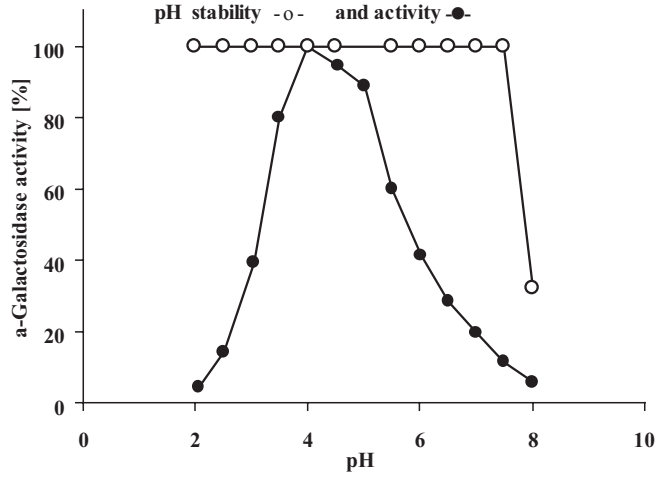



**Fig. 7.** Effect of pH on α-galactosidase activity and stability

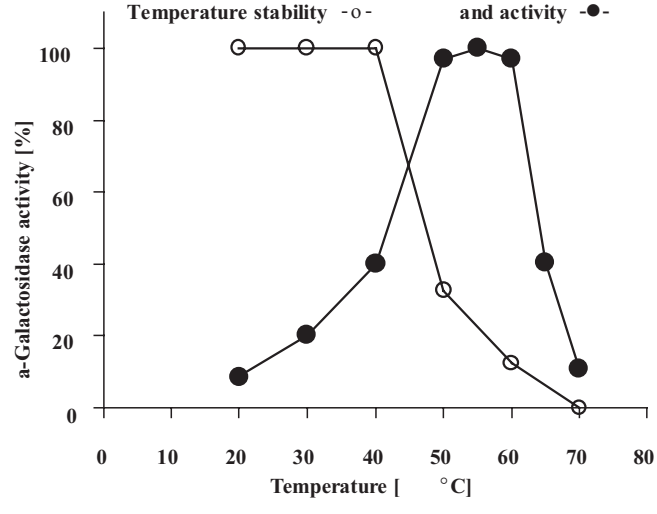



**Fig. 8.** Effect of the temperature on α-galactosidase activity and stability

## Conclusions

The waste soy meal extract can be recommended as a suitable medium for cultivation of fungal strain *H. lutea* 120-5

producing α-galactosidase. The obtaining of cultural filtrates with α-galactosidase activity can be included in the technology for legumes seeds treatment for food and fodder purposes.

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