Bulgarian Chemical Communications, Special Issue E, (pp. 101 – 106) 2017

Alpha-galactosidase and invertase from *Penicillium chrysogenum* sp.23: purification, characteristics and hydrolysis of raffinose

B.K. Yakimova, B.P. Tchorbanov, I.B. Stoineva*

Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Acad.G. Bonchev str. bl.9, 1113 Sofia, Bulgaria

Received September 30, 2016; Revised February 3, 2017

The shaking fermentation of fungal strain *Penicilium chrysogenum* sp.23 on soy meal resulting in a high α-galactosidase yield of 4200 U/l and was accompanied by the excretion of invertase activity. An ultrafiltration method was applied to obtain enzyme product and further purification procedure was developed to obtain pure α-galactosidase. The optimal parameters pH 4.5-5.0 and temperature 50°C of α-galactosidase fraction were determined by using p-nitrophenyl-α-D-galactopyranoside as substrate. The hydrolysis of raffinose catalyzed by α-galactosidase in the presence of side excreted invertase was followed by HPLC analysis. The results were compared with data from raffinose hydrolysis of α-galactosidase without any traces of invertase using fungal strain *Humicola lutea* 120-5. It is shown clearly that the presence of invertase in this case provoked a significant transformation of the raffinose to melibiose and fructose mainly.

Key words: α-galactosidase, *Penicillium chrysogenum* sp.23, invertase, hydrolysis of raffinose

INTRODUCTION

Human consumption of soy products is increasing due to their high nutritional value, acceptable price as well as their health effects, such as reduction the cardiovascular diseases, osteoporosis and cancer risks [1]. In spite of these advantages, they also contain a certain amount of oligosaccharides of raffinose type, which are not assimilated by humans and non-ruminants animals causing flatulence and discomfort. They pass on intact into the large intestine, where anaerobic microorganisms ferment them and cause gastrointestinal disturbances [2]. Degradation of these oligosaccharides from soybeans and legumes foods to low molecular weight compounds such as glucose and fructose is necessary to reduce substantially or completely prevent of the flatus formation (flatulence). Many researchers have noted the degradation of these oligosaccharides using a mixed crude enzyme system consisting of alpha-galactosidase and invertase [3]. The main problem is that the hydrolysis of these oligosaccharides as raffinose does not run completely to the formation of galactose, glucose and fructose, and in the hydrolyzate were found significant amounts of melibiose.

α-Galactosidase (EC 3.2.1.22) and invertase (EC 3.2.1.26) with official name β-fructosidase are enzymes belonging to the class of hydrolases, subclass glycosidases (3.2.1) i.e enzymes hydrolyzing glycosyl oxygen compounds. α-Galactosidase is an enzyme which is a glycoprotein with a carbohydrate and a protein part in a ratio of 1:6. It catalyzes the hydrolysis of non-reducing α-1, 6 linked galactose residues of different substrates, including linear and branched oligosaccharides, polysaccharides, and synthetic substrates such as p-nitrophenyl-α-D-galactopyranoside [4]. This enzyme is widely distributed in microorganisms, plants and animals [4]. Among all the sources of α-galactosidases, the fungal α-galactosidases were most suitably exploited for their biotechnological applications mainly due to their extracellular localization, acidic pH optima, and broad stability profiles.

At present, the industrial applications of α-galactosidase are related to the beet sugar industry, pulp and paper industry, soy food processing, and animal feed processing [5, 6]. Another important application of α-galactosidase is its use in blood group transformation, the treatment of Fabry's disease and xenotransplantation [7,8].

The invertase is an enzyme that has the ability to hydrolyze α-1,2 glycosidic bonds, and thus degrades sucrose to glucose and fructose in a ratio of 1:1 [9]. There are several isoforms of invertase, differing in pH optimum of activity, which may be neutral, acid or alkaline [10]. The enzymatic activity of invertase has been characterized mainly in plants [11] and microorganisms [12,13].

In the present study we describe the production, purification, pH and termal stability of an α-

* To whom all correspondence should be sent:
E-mail: istoineva@yahoo.com

galactosidase from *Penicillium chrysogenum* sp. 23. The special attention was focused on to the raffinose hydrolysis in the presence of the secondary enzyme invertase.

EXPERIMENTAL

Medium and culture conditions

In this research we used mesophilic filamentous strain *Penicillium chrysogenum* sp.23, cultivated as a source of extracellular α–galactosidase The fungal culture was maintained on beer agar slants. Five ml of spore suspension (approximately $2x10^9$ – 10^{10} spores/ml) were added to flasks (500 ml capacity) containing 50 ml of soy meal extract (SME) which is an extract of waste processing of soy protein isolates with 5% dry content. The cultivation was carried out stationary on rotary shaker at 30˚C. In a laboratory bioreactor (working volume 3 l) the cultivation conditions were 30˚C, agitation 600 rpm and aeration 1.0 l/min^{-1}. Samples were taken in different hours of cultivation and at the end of the fermentation process fungal biomass was separated from the culture fluid by filtration through paper filter. The filtrate was used for assaying α-galactosidase and invertase activity. After the fermentation, the culture supernatant was subjected to ultrafiltration for concentration and desalination and after that it was lyophilized, as in that form the enzyme retains activity for a long time.

Enzyme assay

α–Galactosidase activity was assayed by the modified method of *Dey et al*. [14], which is very accurate and sensitive, using 0,003 M p-nitrophenyl–α-D-galactopyranoside (pNPG) as substrate at pH 5.5 supporting by 0.1 M 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 is defined as the amount of enzyme liberating 1 μmol of p-nitrophenol per min under the described conditions and corresponds to 16.7 nkat.

Invertase activity was determined using sucrose as a substrate [15] and the reducing sugars produced was then determined by the dinitro-salicylic acid method [16] using glucose as standard. One unit of the enzyme was defined as the amount of protein necessary to produce 1 μmol glucose equivalent in 1 ml of solution per minute at pH 5 and 37˚C. The absorbance was measured at 530 nm.

The data presented are mean values of triplicate assays. Standart deviations values were always smaller than 5 % of the mean value.

Determination of protein content by Lowry method

To determine the protein content was used the method of Lowry [17]. The method is based on the colorimetric measurement of the blue coloration which is obtained as a result of the reaction of the peptide bonds in proteins with copper ions under alkaline conditions and reduction of phosphotungstic acid and phosphomolybdic acid from Folin–Ciocalteu reagent. The amount of protein is determined from a standard curve using bovine serum albumin with different concentrations (0-100 μg/ml). The absorbance was measured at λ =750 nm. All measurements were performed in triplicate.

α-Galactosidase purification

For separation and purification of enzyme sample were used gel-filtration and ion-exchange chromatography. The lyophilised enzyme sample (700 mg) was dissolved in 5 ml 0.02 M sodium-acetate buffer pH 5.5 and applied on Sephadex G_{100} column (80 x 2.5 cm) equilibrated with 0.02 M sodium acetate buffer with pH 5.5. The absorbance was monitored at λ=280 nm. The proteins were eluted at a flow rate of 18 ml/h and 3.8 ml fractions were collected. Fractions of the active peak after gel-filtration chromatography containing α–galactosidase and invertase activity were pooled and were applied on DEAE-cellulose column (16 cm × 1.7 cm), equilibrated with 0.02 M sodium acetate buffer with pH 5.5. Proteins were eluted at a flow rate of 30 ml/h, with a linear gradient of NaCl (0.1-0.5 M).Fractions containing α–galactosidase and invertase activity were pooled, lyophilized and used in further studies.

HPLC analysis of saccharides hydrolysis

HPLC analysis was performed with a Agilent 1100 chromatograph equipped with Evaporative Light-scatering detector. An analytical column LiChrosorb NH_2 (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, raffinose and melibiose were purchased from Sigma. Stock solutions of mono-,

di- and trisaccharides were prepared with equal concentrations - 10 mg/ml, 0.1 M citrate-phosphate buffer with pH 5.5, which was used for the enzyme hydrolysis.

After determination the retention times of the standart sugars it was conducted the hydrolysis of raffinose catalyzed by the enzyme sample from *Penicillium chrysogenum* sp.23 with the activity - 2U. The reaction mixtures contained 100 µl 0.05 mmol raffinose in 800 µl 0.1 M citrate-phosphate buffer with pH 5.5, 2 units of the enzyme and 100 µl buffer. The reaction mixtures were incubated at 40 C for 5 min, cooled and applied to HPLC.

To prove the effect of invertase accompanying α-galactosidase produced by strain *Penicillium chrysogenum* sp.23, were conducted comparative kinetic studies using pure α-galactosidase produced by strain *Humicola lutea* 120-5 with the activity - 2U and without invertase activity.

Determination of pH optimum and pH stability

The effect of pH on the enzyme activity was established by 0.1 M citrate - phosphate buffer with different pH from 2.6 to 7.0. The enzyme activity was measured by the method described above using the synthetic substrate p-nitrophenyl–α-D-galactopyranoside. The amount of p-nitrophenol, which releases the enzyme was determined spectrophotometrically at SPECORD UV VIS at λ= 405 nm.

The pH stability was determined by incubating the suitably diluted enzyme in the above buffers incubated at room temperature for 2 h and for 24 h at 4°C and measuring residual enzyme activity at pH 4.5 , 50°C and reaction time 15 min by the modified method of *Dey et al.*

Thermal stability experiments of the enzyme

The thermal stability of α-galactosidase was investigated by measuring the residual activity of the enzyme after incubation at different temperatures in the range from 25 ° to 70° C. The diluted with distilled water enzyme solutions were incubated for 2 h at a thermostat and the activity of the samples was measured under standard conditions described above.

RESULTS AND DISCUSSION

The fungal strain *Penicillium chrysogenum* sp.23 produces high levels extracellular α-galactosidase in combination with invertase, like other eukaryotic microorganisms. After the fermentation, the cultural supernatant was subjected to ultrafiltration for concentration and desalination. The cultural supernatant was lyophilized because in that form the enzyme retains activity for a long period of time. After purification of the α-galactosidase by gel filtration on Sephadex G-100 column, we registered two closed protein fractions, which have a common enzymatic activity of 65.6 U (fig.1)

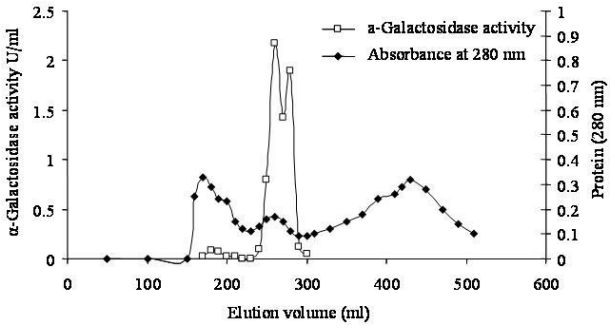

Fig 1. Elution profile of α-galactosidase from *Penicillium chrysogenum* sp.23 on Sephadex G-100 column

The fractions of the active peaks after gel filtration on Sephadex G-100 column were subjected to ion exchange chromatography on DEAE-cellulose column (16 cm × 1.7 cm), equilibrated with 0.02 M sodium acetate buffer with pH 5.5. Proteins were eluted at a flow rate of 30 ml / h, with a linear gradient of NaCl (0.1-0.5M). There is one major peak, having the α-galactosidase activity of 27 U, which was eluted with a linear gradient at a concentration of 0.3 M NaCl (fig,2).

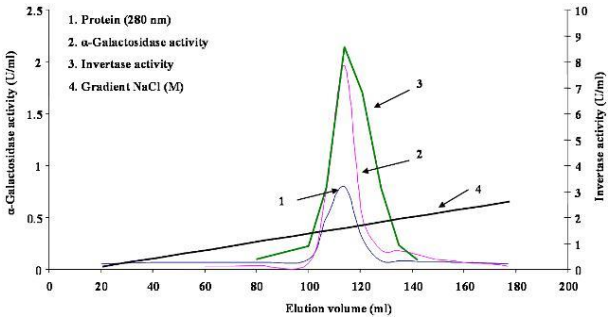

Fig.2. Elution profile of the α-galactosidase from *Penicillium chrysogenum* sp.23 on DEAE-cellulose column

It was established that the active fraction exhibiting α-galactosidase activity coincides with that of invertase activity. After DEAE ion-exchange chromatography the two enzymes are still not separated. This procedure resulted in a partially purified α-galactosidase fraction, which has and invertase activity with specific enzyme activity of 30 U/mg, purification factor 65.2 and recovery level about 28 %.

The results of the purification of the enzyme are summarized in the Table 1.

Table 1. Summary of protein content, enzymatic activity and yield at different purifaction steps of extracellular α-galactosidase produced by *Penicillium chrysogenum* sp.23

Purification	Total protein [mg]	Total activity [U]	Specific activity [U/mg]	Purification factor	Yeild [%]
Lyophilized extract	210	96.6	0.46	1	100
Gel-filtartion	9	65.6	7.28	15.8	67.9
Ion-exchange chromatography	0.9	27	30	65.2	28

Kinetic studies were performed by using HPLC analysis for tracking the hydrolysis of raffinose using two different enzymes from fungal strains- *Penicillium chrysogenum* sp.23 and *Humicola lutea* 120-5.

The HPLC analysis of the kinetic study with enzyme from fungal strain *Humicola lutea* 120-5 clearly shows that the enzyme does not possess invertase activity (fig.3, fig.4)

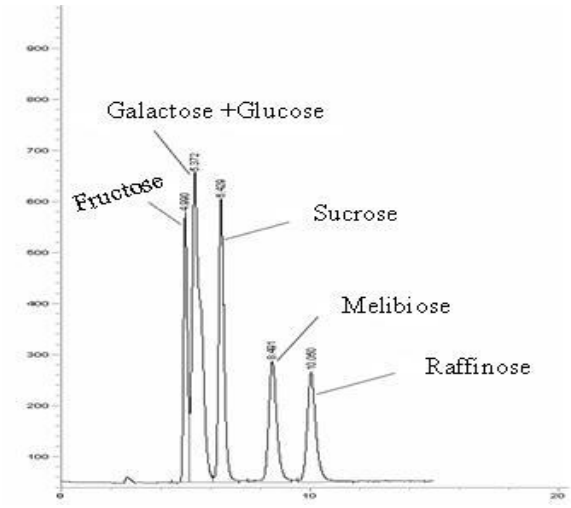

Fig 3. HPLC chromatogram of a standard mixture of sugars (mobile phase -acetonitrile: water (70:30 v/v, flow rate -1 ml/min)

Kinetic studies using HPLC analysis of the hydrolysis of raffinose catalyzed by *Penicillium chrysogenum* sp.23 showed the presence of fructose and depletion of raffinose. The appearance of a new peak for a disaccharide, probably is due to the α-glucosidase activity produced by *Penicillium chrysogenum* sp.23 invertase. The presence of invertase activity in the α-galactosidase preparations could contribute to the complete hydrolysis of the raffinose oligosaccharides, because they are substrates for both enzymes (fig.5).

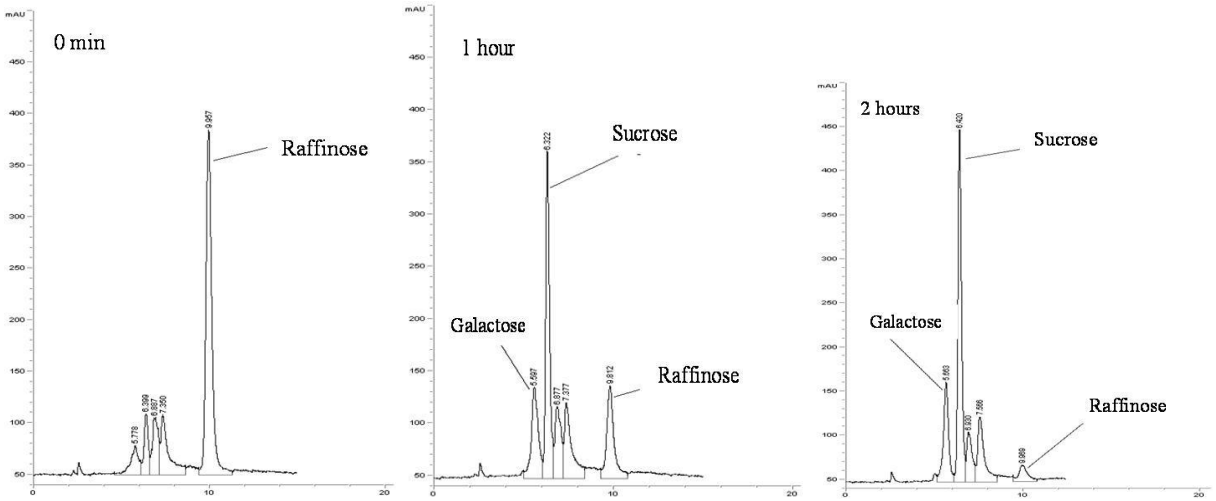

Fig 4. HPLC chromatograms of the hydrolysis of raffinose catalyzed by the enzyme sample from *Humicola lutea*. 120-5 with α- galactosidase activity - 2U.

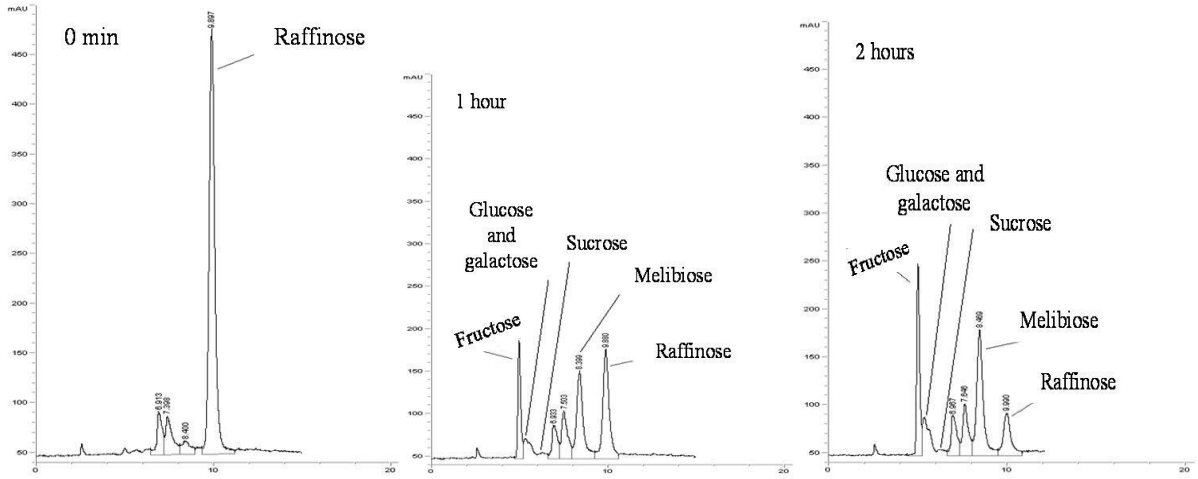

Fig. 5. HPLC chromatograms of the hydrolysis of raffinose catalyzed by an enzyme preparation of *Penicillium chrysogenum* sp.23 with α- galactosidase activity - 2U.

The obtained results show that the partially purified enzyme sample from *Penicillium chrysogenum* sp.23 should be particularly beneficial in the processing of soy crops for food purposes because of action of the two enzymes. This makes α-galactosidase of *Penicillium chrysogenum* sp.23 very promising for application in the food industry, since this enzyme has a much higher activity compared to the α-galactosidases produced by other strains.

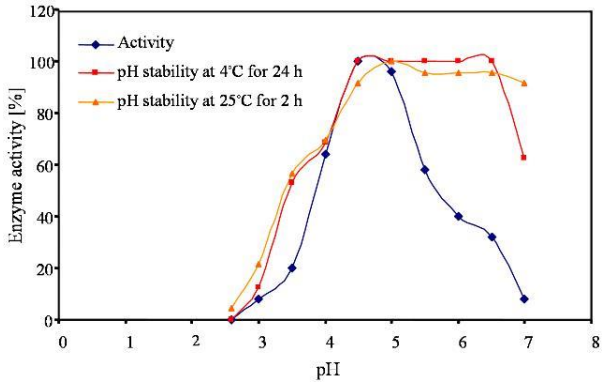

Fig. 6. Effect of pH on α-galactosidase activity and stability.

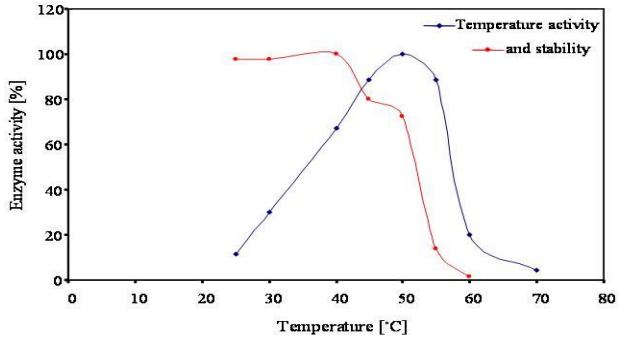

Fig. 7. Effect of the temperature on α-galactosidase activity and stability

It was established that the enzyme has a pH optimum 4.5-5.0, and pH stability extends over a wide range of 4.5-6 (fig.6).

The temperature optimum of α-galactosidase was 50°C, and the enzyme is stable from 25 to 40°C, at 45°C the activity falls to 80%, at 50°C falls to 72% and at 60°C the activity is completely lost (fig.7).

Most α-galactosidases are stable over a broad range of activity. For example the α-galactosidase from *Penicillium* sp. F63 CGMCC1669 has an optimum pH of 5.0 and an optimum temperature of 45 degrees C. The enzyme is stable between pH 5.0 and 6.0 below 40°C [14].

CONCLUSION

In this paper was proven that the fungus strain *Penicillium chrysogenum* sp.23 produces both enzymes α-galactosidase and invertase. By gel-filtation chromatography was partially purified a crude extract of the enzyme sample obtained from *Penicillium chrysogenum* sp.23. The purification was achieved of α-galactosidase from accompanying substances by ion exchange chromatography, but does not achieve separation of the invertase and α-galactosidase from each other. This requires the research of more efficient methods for the separation of the two enzymes.

The ability of fungus strain of *Penicillium chrysogenum* sp.23 to produce both α-galactosidase and invertase makes it especially attractive for industrial applications. Due to the synergism of action of the two enzymes it is expected fast hydrolysis of oligosaccharides in soy foods and legumes releasing digestible monosaccharides and removing discomfort of flatulence.

REFERENCES

1. H. Jooyandeh., *Middle–East Journal of Scientific Research* **7**(1), 71 (2011).
2. D. Tsangalis, J. Ashton, A. McGill, N. Shah, *J.Food Sci*, **67**, 3104 (2012).
3. K.S Dhananjay., V.H. Mulimani, *Biotechnol. Lett*., **30**, 1565 (2008).
4. P. M.. Dey and J.B. Pridham ' *Adv. Enzimol*., **36**, 911, (1972).
5. G. P. Aravind Goud and V. H. Mulimani *Biotechnol. Bioprocess Eng*. **13**, 354 (2008)
6. S. J. Prashanth, V. H. Mulimani., *Process Biochem*. **40,** 1199 (2005).
7. M. M. Fuller, Lovejoy, D. A. Brooks, M. L. Harkin, J. J. Hopwood and P. J. Mickle.. *Clin. Chem.*, **50**, 1979 (2004).
8. M. L.Olsson, C. A. Hill, H. Dela Vega, Q. P. Liu, M. R. Stroud, and J. Valdinocei.. *Transfus. Clin. Biol*. **11**, 33 (2004).
9. C. Goosen, Xiao-Lian Yuan, M. Jolanda van Munster, F. Arthur, J.Ram. *EUKARYOTIC CELL*, 674 (2007).
10. H. Winter, SC. Huber .*Crit Rev Plant Sci*,, **19**, 31 (2000).
11. A. Tauzin and T. Giardina *Frontiers in Plant Science*, **5**, 293 (2014).
12. M. Giraldo, T. da Silva, F. Salvato, H. Terenzi, J. Jorge, L. Guimaraesh *World J.of Microb. and Biotechnol*., **28**, 463 (2012).
13. G. Arumugam, A. Sadiq , M. Nagalingam., and A. Panneerselvam, *European Journal of Experimental Biology*, **4**, 29 (2014).
14. P.M. Dey, S. Patel, M.D.Brownleader *Biotechnol. Appl. Biochem*., **17**, 361 (1993)
15. S. T. de Rezende,. C.R. Felix *Biotechnol. Lett*., **19**, 217 (1997).
16. G. L. Miller, *Anal. Chem.*, **31**, 426 (1959).
17. O. Lowry, N. Resenbrough, A. Farr, K. Rawdall *J. Biol. Chem*., **193**, 265 (1951).

АЛФА-ГАЛАКТОЗИДАЗА ОТ *PENICILLIUM CHRYSOGENUM* SP.23: ПРЕЧИСТВАНЕ, ХАРАКТЕРИСТИКИ И ХИДРОЛИЗА НА РАФИНОЗА В ПРИСЪСТВИЕТО НА ИНВЕРТАЗА

Б. К. Якимова, Б. П. Чорбанов, И. Б. Стойнева

Лаб.Химия и биофизика на белтъци и ензими, Институт по Органична Химия с Център по Фитохимия, Българска Академия на Науките, ул. Акад.Г. Бончев, бл.9, 1113, София, България

Постъпила на 30 септември, 2016 г.; Коригирана на 3 февруари, 2017 г.

(Резюме)

В това изследване установихме, че щамът *Penicillium Chrysogenum* sp.23 може да продуцира едновременно α-галактозидаза и инвертаза с максимална ензимна активност (4000 $U.l^{-1}$) когато се използва екстракт от соево брашно, съдържащ 5% сухо вещество като среда. Определени са оптималните параметри pH 4,5-5,0 и температура 50°C на α-галактозидазата фракция чрез използване на p-нитрофенил-α-D-галактопиранозид като субстрат. Хидролизата на рафиноза катализирана от α-галактозидаза, съпътствана от инвертаза е регистрирана с високоефективна течна хроматография.

Установено е, че в присъствие на инвертаза се наблюдава преобразуване на рафинозата главно до мелибиоза и фруктоза. Ензимната хидролиза на рафинозните олигозахариди, която се осъществява от α-галактозидаза и/или инвертаза в соевите семена използвани в храната на човека и животните води до подобряване на нейните хранителни свойства и значително намалява или отстранява стомашния дискомфорт. Получените резултати показват, че частично пречистен ензимен препарат от *Penicillium chrysogenum* sp.23 би бил много полезен при преработката на соеви култури за хранителни цели, тъй като се реализира синергизъм в действието на двата ензима.