Հայաստանի Գիտությունների Ազգային Ակադեմիա
Национальная Академия Наук Армении
National Academy of Sciences of Armenia

Հայաստանի Կենսաբանական Հանդես
Биологический Журнал Армении
Biological Journal of Armenia

• Փորձարարական և տեսական հոդվածներ • Экспериментальные и теоретические статьи •
• Experimental and theoretical articles •

Biolog. Journal of Armenia, 2 (73), 2021

PECULIARITY OF EXOINULINASE OF A NOVEL PRODUCER OF CITRIC ACID FROM INULIN

I.E. MELKUMYAN[1], R.S. HOVHANNISYAN[1], N.S. KHACHATURYAN[2], L.S. MARKOSYAN[1,*]

[1]Institute of Microbiology of the Scientific and Production Center "Armbiotechnology" of NAS of Armenia
[2]Microbial Depository Center of the SPC "Armbiotechnology" of NAS of Armenia
[*]lmark@sci.am

This paper presents the results of research of biochemical and physicochemical properties of extracellular inulinase synthesized by the new citric acid producer *Aspergillus niger-1* (12000). A method for the isolation of extracellular homogeneous inulinase from the culture broth has been developed. It was revealed that this exoinulinase, possessing at the same time invertase activity (I/S=1.14), showed inulolytic activity and stability in a wide range of pH (3.0-7.0) and temperature (20-50 ^{o}C). The metal ions $MnCl_2$, NaCl, $MgSO_4$, $CoCl_2$ partially increase, and $FeCl_3$, $ZnSO_4$, $AgNO_3$ suppress or completely inhibit the catalytic activity of exoinulinase. The molecular weight is 63 kDa which is typical for inulinases produced by the strains belonging to the genus *Asprgillus.* Fructose and glucose, which are the end products of enzymatic hydrolysis of inulin, indicate the exo-type action of the studied exoinulinase, as well as the possibility of its application in biotechnology for the production of various products from inulin. The revealed properties of this exoinulinase are also important for the optimization of the processes of citric acid synthesis by *Aspergillus niger-1* from inulin under varying conditions of the fermentation.

Exoinulinase – inulin – invertase – citric acid

Սույն աշխատանքում ներկայացված են կիտրոնաթթվի նոր արտադրիչ *Aspergillus niger*-1-ի արտաբջջային ինուլինազայի կենսաքիմիական և ֆիզիկաքիմիական հատկությունների հետազոտությունների արդյունքները: Մշակվել է միջավայրից արտաբջջային միատար ինուլինազայի մեկուսացման եղանակ: Պարզվել է, որ նշված էկզոինուլինազը, որը միաժամանակ ինվերտազային ակտիվություն ունի (I / S=1.14), ցուցաբերում է ինուլինը տարրալուծող ակտիվություն ու կայունություն pH 3.0-7.0 և ջերմաստիճանի 20-50^{0}C պայմաններում: Նշված ցուցանիշների բարձրացումը կամ նվազեցումը ճնշում է ֆերմենտի գործունեությունը: Ցույց է տրվել, որ $MnCL_2$, NaCl, $MgSO_4$, $CoCL_2$ մետաղների իոնները մասամբ խթանում են, իսկ $FeCL_3$, $ZnSO_4$, $AgNO_3$-ը ճնշում կամ ամբողջովին արգելակում են ֆերմենտի կատալիտիկ ակտիվությունը: Մոլեկուլային զանգվածը՝ 63КДа, համահունչ է *Asprgillus* տեսակին պատկանող շտամների ինուլինազների տվյալներին: Ֆրուկտոզը և գլյուկոզը՝ որպես ինուլինի ֆերմենտային տարրալուծման վերջնական արգասիքներ, վկայում են նրա ազդեցության էկզոտեսակի, ինչպես նաև ինուլինից տարբեր պրոդուկտների ստացման կենսատեխնոլոգիաներում կիրառման հնարավորությունների մասին: Նշված էկզոինուլինազի բացահայտված հատկությունները կարևոր են նաև *Aspergillus niger-1* կիրառմամբ ինուլինից կիտրոնաթթվի սինթեզի գործընթացների օպտիմալացման համար ֆերմենտացիայի միջավայրի փոփոխվող պայմաններում:

Էկզոինուլինազ – ինուլին – ինվերտազ – կիտրոնաթթու

В настоящей работе представлены результаты исследований биохимических и физико-химических свойств внеклеточной инулиназы, синтезируемой новым продуцентом лимонной кислоты *Aspergillus niger-1*. Разработан способ выделения внеклеточной гомогенной инулиназы из среды культивирования гриба. Выявлено, что указанная экзоинулиназа, обладающая одновременно инвертазной активностью (I/S=1.14), проявляет инулолитическую активность и устойчивость в широком диапозоне pH 3.0-7.0 и при температуре 20-50^{0}C. Повышение или понижение указанных параметров подавляют активность фермента. Показано, что ионы металлов $MnCL_2$, NaCL, $MgSO_4$, $CoCL_2$ частично повышают, а $FeCL_3$, ZnSO4, $AgNO_3$ подавляют или полностью ингибируют каталитическую активность. Молекулярная масса 63КДа характерна для инулиназ продуцируемыми штаммамы рода *Asprgillus.* Фруктоза и глюкоза, являющиеся конечными продуктами ферментативного гидролиза инулина, свидетельствуют об экзо- типе действия изучаемой экзоинулиназы, а также о возможности ее применения в биотехнологии для получения различных продуктов из инулина. Выявленные свойства указанной экзоинулиназы важны также для оптимизации процессов синтеза лимонной кислоты штаммом *Aspergillus niger-1* при меняющихся условиях среды ферментации.

Экзоинулиназа – инулин – инвертаз – лимонная кислота

Inulinases are biocatalysts of great practical importance and are classified according to the type of their action on inulin: endo-inulinases (β-D-fructan-fructanohydrolase, EC3.2.1.7) and exo-inulinases (β-D-fructopyranoside fructose hydrolase, EC3.2.1.8) - splitting inulin with the formation of inulo-oligosaccharides and fructose, glucose, respectively. Inulinase producers are mainly fungi, yeast and bacteria. Comprehensive research of inulinases produced by microorganisms belonging to various systematic groups of microorganisms was carried out. It was found that there were significant differences in the physicochemical and biochemical properties of inulinases synthesized by various strains of microorganisms, including the strains belonging to the same genus [1-14]. The production of biofuels, fructose-glucose syrup, fructose, inulooligofructans, cycloinulofructans and other products from inulin (polyfructan), widely distributed in plants belonging to the families *liliacea, Compositae, Amaryllidace, Asteraceae,* was carried out by microorganisms having inulinase activity. [1-2, 13-18]. However, currently there is no microbiological technology for the direct bioconversion of inulin by the same strain into citric acid, which is widely used in the food, chemical and pharmaceutical industries. It should be noted that the main raw material of industrial production of citric acid is molasses.

Previously, we have isolated a new original strain *Aspergillus niger-1* (#12000) performing one-step bioconversion of inulin into citric acid [19, 20]. To optimize the processes and develop an efficient biotechnology for the bioconversion of inulin into citric acid, it is necessary to reveal the biochemical, physicochemical features of *Aspergillus niger-1* exoinulinase, which is the main goal of this research.

Materials and methods. Microorganism: The producer of exoinulinase and citric acid *Aspergillus niger-1* (# 12000) was isolated by the screening of the strains of genus *Aspergillius* isolated from the rhizosphere of *Jerusalem artichoke* and stored in the collection of microorganisms in the Microbial Depository Center of the SPC "Armbiotechnology" of NAS of Armenia [19, 20].

Enzyme Assay

Isolation of exoinulinase: To isolate extracellular inulinase, the strain of *Aspergillus niger-1* was cultivated in a 3-liter bioreactor in Czapek's medium: (g/L) $NaNO_3$-2.0, KH_2PO_4-1.0, $MgSO_4.7H_2O$-0.5, $FeSO_4.7H_2O$-0.001, KCl - 0.5 and inulin - 15 as the only source of carbon, pH 6.0 at a temperature of 30 ^{o}C, aeration 0.8 l/min. for 4-5 days. The enzyme was isolated from the culture broth by the method developed by us (research results).

The homogeneity of the enzyme was determined by disk electrophoresis in polyacrylamide gel [21].

Molecular weight of extracellular inulinase was determined by gel filtration on a Sephadex G200 column (1.5x55 cm).

Determination of inulolytic and invertase activity of extracellular inulinase: Inulolytic activity was measured by the amount of fructose formed as a result of inulin hydrolysis by exoinulinase. The incubation medium contained 1 ml of an inulinase solution, 2 ml of a 3% inulin solution in 0.1 M acetate buffer, pH was 5.5. The mixture was incubated at 50°C for 30 min. The reaction was stopped by heating the incubation medium at 100°C for 5 min. The amount of resulted fructose was determined by the Somogy method [22]. One unit of enzyme activity was defined as the amount of enzyme which produced 1 μmol of fructose for 1 min.

The invertase activity was determined under the indicated conditions using 2 ml of a 3% sucrose solution as a substrate.

Determination of the optimal temperature of enzyme activity: The incubation medium containing 1 ml of exoinulinase solution and 2 ml of 3% inulin in 0.1 M acetate buffer, pH - 5.0 was incubated at different temperatures (20-70 ^{o}C) for 30 minutes. Then after heating the mixture at 100°C for 5 min. the enzyme activity was measured by the amount of reducing sugars in the inulin hydrolyzate [21].

Determination of thermostability of exoinulinase: One ml of enzyme solution was preincubated at different temperatures (20-70°C) for 30 min. Then 2 ml of a 3% inulin solution was added to the cooled solutions. After incubation at 50°C for 30 min. and heating at 100°C for 5 minutes, the residual enzyme activity was determined.

Determination of the effect of pH on exoinulinase activity: Enzyme solutions containing 3% inulin were incubated at different pH values (3.0-7.0.0) for 30 min at 50°C. The reaction was stopped by heating the tubes at 100°C for 5 min, and the enzyme activity was measured.

Determination of pH stability of exoinulinase: Enzyme solutions in acetate buffer at different pH values (3.0–7.0) were preincubated for 30 min. at 50°C. Then the exoinulinase activity was measured after adding inulin (3%) and incubating at 50°C for 30 min.

Determination of the products of enzymatic hydrolysis of inulin: The hydrolyzate content was analyzed by high performance liquid chromatography (HPLC) on a Shimadzu 2010 C analyzer, Ultkon PS-80-H column, 2x250 mm, mobile phase 0.1 mM acetate buffer/acetonitrile 1:5, pH - 5.8, rate flow - 1ml/min. The measurements were carried out according to the refractometric index (RI).

Determination of the effect of metal ions on the activity of enzyme: Enzyme solutions in 0.1M acetate buffer, pH - 5.5 with various metal ions (10^{-3}M) were preincubated at 30 ^{o}C for 60 min. Then after adding inulin (3%) to the solutions and incubating the mixtures at 50°C for 30 min. the activity of inulinase was measured.

Results and Discussion.

Properties of A. niger-1 Exoinulinase

The research carried out has shown that *A. niger-1* during its growth in Czapek's medium, containing inulin as the only carbon source, bioconverts inulin into citric acid due to extracellular inulinase hydrolyzing inulin to simple sugars: fructose and glucose [19, 20]. To optimize the processes of hydrolysis of inulin and the formation of citric acid during the growth *of A.niger -1*, the studies of the biochemical and physicochemical properties of extracellular inulinase were carried out. The enzyme was isolated through the method developed by us. The culture was grown in a bioreactor in Czapek's medium containing inulin at 30 ^{o}C and aeration (0.8 L / min.) for 4 days. The biomass of the fungi was separated by the culture broth filtration with paper pulp. Then the filtrate was centrifuged (10000g/10min.) and decolorized with activated carbon. To concentrate proteins, including inulinase, the solution was passed through a column of DEAE-cellulose-52 (4x6cm), equilibrated with 0.1 M acetate buffer, at pH 5.5, followed re-adsorption by the 1M NaCl solution. The eluate was desalted by dialysis against distilled water. Further, partially purified exoinulinase was obtained by chromatography

on a DEAE-ToyoPerl 650 M (2x20 cm) column equilibrated with 0.1 M acetate buffer, pH - 5.5 (fig. 1).

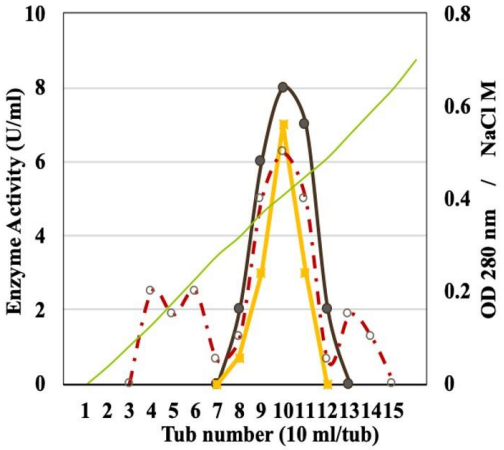

Fig. 1. Isolation of inulinase by chromatography on the DEAE-ToyoPerl 650 M column (3x8cm) equilibrated with 0.1 M acetate buffer, pH - 5.5 The linear gradient elution implemented with 0.7 NaCl solution, flow rate - 0.7ml/min. -•- enzyme activity, -•- protein, - NaCl, -■- Invertase activity

Fractions with inulinase activity (7-13) were combined, desalted by dialysis against distilled water and concentrated under the vacuum (at 30^0C up to 15-20 ml). Then to obtain homogeneous exoinulinase, the gel chromatography on a Sephadex-G100 column (2x50 cm), equilibrated with 0.1 M acetate buffer, pH - 5.5 was carried out (fig. 2).

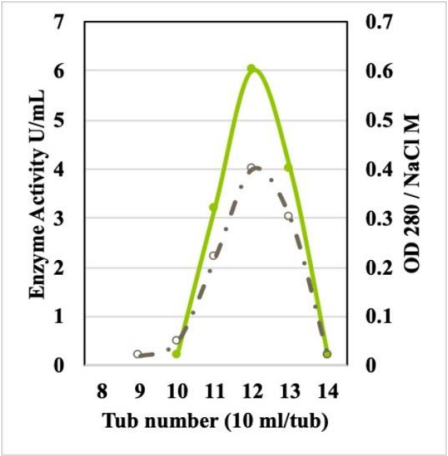

Fig. 2. Gel filtration on Sephadex-G100 column (2x50cm) equilibrated with 0.1 M acetate buffer, pH - 5.5, flow rate - 0.5 ml/min. -•- enzyme activity, -•- protein

Fractions with inulinase activity (10-14) were pooled and by means of polyacrylamide gel electrophoresis the homogeneity of the isolated exoinulinase was shown, the enzyme migrated as a single protein band (fig. 3).

Fig. 3. Disc electrophoresis of extracellular inuliase in polyacrylamide gel

It is well-known that the molecular weight of inulinases of microorganisms varies within a fairly wide range: 50-300 kDa [1-2, 10-13]. Our studies have shown that the molecular weight of the isolated exoinulinase is 63 kDa fig. 4).

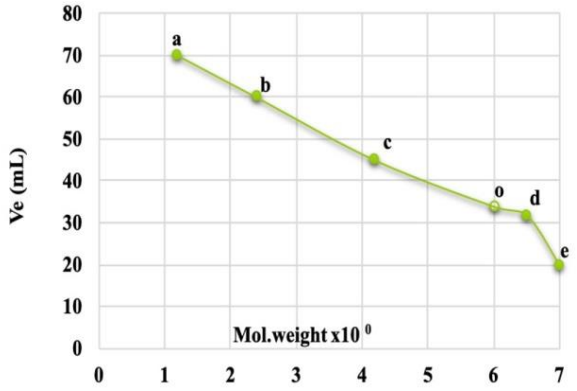

Fig. 4. Estimation of molecular weight of extracellular inulinase by gel filtration on Sephadex-G200 column (1.5x55cm). Protein markers: a-cytochrom C (12 kDa), b-trypsin C (24 kDa), c-ovalbumin C (42 kDa), d-bovine ovalbumin (66 kDa), e-gamma globulin (140 kDa), o-inulinase. The void volume of the column was determined with blue dextran

Previous studies have shown that exoinulinases synthesized by microorganisms: fungi, yeast, bacteria, overwhelmingly have also invertase activity [1-2,5,9,10] due to what the exoinulinases provide complete hydrolysis of inulin with the formation of fructose and glucose, which are very important in the production of various products from inulin: ethanol, fructose – glucose syrup, fructose, citric acid and other products. Studies have revealed that the studying extracellular inulinase also possesses invertase activity (fig. 1) and as additional evidence only fructose and glucose have been identified by HPLC in the enzymatic hydrolysate of inulin (fig. 5). The ratio of the catalytic activity of the enzyme to inulin (I) and sucrose (S) is I/S=1.14 (fig.1).

The presence of inulinase and invertase activity caused by the subcenter structure of the active center of exoinulinases, which provides splitting of both β-(2,1) bond between fructose monomers and α-(1,2) connection, like sucrose [23]. The obtained results also indicate that extracellular inulinase of *A. niger-1* is related to exoinulinases.

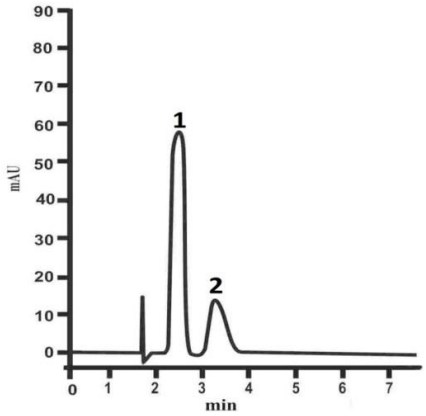

Fig. 5. Chromatography of the enzymatic hydrolysate of inulin (1-fructose, 2-glucose) analyzed by HPLC

The presence of inulinase and invertase activity caused by the subcenter structure of the active center of exoinulinases, which provides splitting of both β-(2,1) bond between fructose monomers and α-(1,2) connection, like sucrose [23]. The obtained results also indicate that extracellular inulinase of *A. niger-1* is related to exoinulinases.

Research has shown that isolated exoinulinase exhibits a sufficiently high catalytic activity already at a temperature of 30-35^{0}C reaching a maximum activity at 45-50^{0}C and at higher temperatures the enzyme loses activity completely being inactivated at 70 ^{o}C (fig. 6).

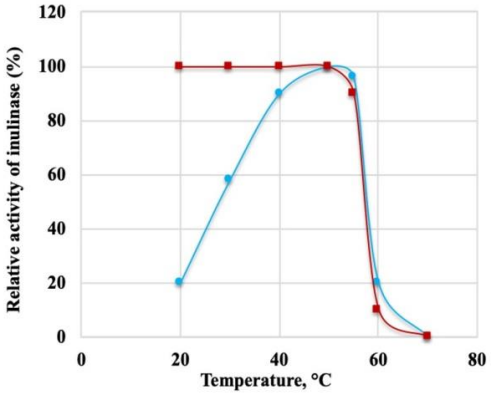

Fig. 6. Influence of temperature on exoinulinase activity (-•-) and thermostability (-■-)

It should be noted that for inulinases produced by different strains of *A. niger*, these parameters range from 35-60 ^{o}C [2, 10, 11, 13].

Studies of the influence of pH on the inulolytic activity of exoinulinase revealed that already at pH 3.5, the enzyme showed sufficient activity reaching a maximum at pH 5.5-6.0, and at higher pH 7.5 significant drop of activity was observed. The studied exoinulinase has also been shown to be resistant to the influence of pH of the incubation medium up to pH 6.0. A further increase in pH lowers the enzyme stability (fig. 7).

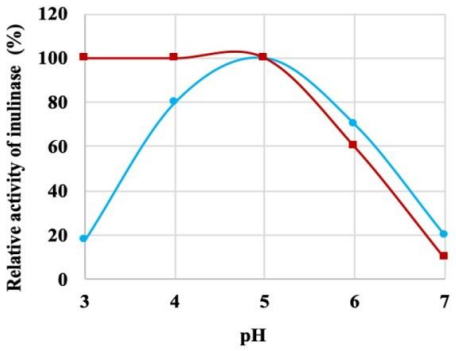

Fig. 7. Influence of pH on exoinulinase activity (-•-) and pH stability (-■-)

It is well-known that inulinases of different origin, including producers from the same genus of microorganisms, exhibit different sensitivity to different metal ions [1, 2, 10, 11, 13]. The research has shown that the ions of various divalent metals affect the catalytic activity of the studied exoinulinase (tab. 1).

Table 1. Effect of metal ions on exoinulinase and invertase activity of *A. niger-1*

Metal ions*	Relative activity (%)	
	inulinase	invertase
Control	100	100
NaCl	110	105
$CaCl_2$	103	90
KCl	100	100
$CuSO_4$	90	95
$FeCl_3$	57	45
$ZnSO_4$	80	89
$MnCl_2$	120	106
$MgSO_4$	105	100
$CoCl_2$	110	105
$AgNO_3$	0	0

Ions of NaCl, $MnCl_2$, $CoCl_2$ significantly stimulate and $FeCl_2$, $ZnSO_4$ inhibit the activity of the enzyme. Complete inhibition of activity with $AgNO_3$ was as in the case of other enzymes by the blocking of sulfhydryl groups in the active site of the enzymes.

It should be noted that the study of the effect of various ions on the activity of cxoinulinase is important not only for the general characteristics of the enzyme, but also for practical significance in the production of citric acid from inulin-containing raw materials, since possible presence of metal ions in the extract from *Jerusalem artichoke* tubers can affect the efficiency of hydrolysis of inulin and consequently the level of bioconversion of inulin into citric acid.

The research of the biochemical, physicochemical properties of exoinulinase produced by *A. niger-1* isolated by the method developed by us, has revealed that the enzyme has a molecular weight of 63 kDa, shows sufficient high inulolitic activity at a temperature range of around 25-50^{o}C and pH range 3.0-6.0. It has also invertase activity

and is reasonably stable in the presence of divalent cations. Fructose and glucose, which are the end products of enzymatic hydrolysis of inulin, indicate not only the exo-nature of the enzyme action, but also its possible application in biotechnology for the production of various products from inulin, as well as for the optimization of one-stage inulin bioconversion into citric acid under varying conditions of inulin fermentation by *A.niger-1*, and promote the rational use of the substrate in the fermentation process. The obtained result complements our knowledge of the distribution and properties of inulinases expressed by microorganisms.

REFERENCES

1. *Vandamme E.J., Derycke D.C.* Microbial inulinases: fermentation process, properties and applications, Adv. in App. Microbiol. *29*, 139-176, 1983. http://DOI:10.1016/s0065-2164(08)703356-3
2. *Pandey A., Soccol C.R., Selvacumar P., Soccol V.T., Krieger N., Fontana J.D.N.* Recent developments in microbial inulinasees: Its Production, properties and industrial application, App. Biochem. and Biotechnol. *81*, 35-52, 1999. http://DOI:10.1385/abab:81:1:35
3. *Nascimento D.S., Valasqes Junior G., Fernandes P., Riberio G.C., Lime D.M., Goes-Neto A., Oliveria R.Q., de Cassia R., Ribeiro F., de Assis S.A.* Production, characterization and application of inulinase from fungal endophite CCMB 328. Annals of the Braziklian Academy of Sciences, *84*, 2-9, 2012.
4. *Xia A., Zhong Guo L., Lu W.D.* Alkaline inulinase production by a newly isolated bacterium Marinimicrobium sp. LS-A18 and inulin hydrolysis by the enzyme, World J. of Microbial Biotechnol. *28*, 81-89, 2012. http://DOI:10.1007/s11274-011-0794-3
5. *Laowklom N., Chantanaphan R., Pinphanichakara P.* Production and characterization of inulinase from a newly isolated *Sterptomyces sp.* CP01, Natural Researches, 3, 137-144, 2012. http://DOI:10.4236/nr.2012.33018
6. *Kumar U., Karan R., Kapoor S., Singh S.P., Khare S.K.* Screening and isolation of halophilic bacteria producing industrially important enzymes, Brazilian J. of Microbiol. *43*, 1595-1603, 2012.
7. *Kang S., Chang Y., Oh S., Kim S.* Purification and properties of an endo-inulinase from an Arthrobacter sp., Biotechnol. Letters, *20*, 983-986, 1998. http://doi.org/10.1023/A:1005440910352
8. Singh P., Kaur P., Production of Inulinases: Recent advances, Food Technol. and Biotechnol. 44 (2006)151-162.
9. Zhi C., Chi Z., Zhang T., Liu G., Yue L., Inulinase-expressing microorganisms and applications of inulinases, Appl. Microbiol. Biotechnol. *82*, 211-220, 2009. http://DOI:10.1007/s00253-008-1827-1
10. *Skowronek M., Fiedurek J.* Purification and properties od extracellular endoinulinase from Asperigillus niger 20 OSM. Food Technol. And Biotechnol. *44*, 53-58, 2006.
11. *Parivuguna P., Kumari P.S., Sanmughaqm S.* Characterisation of inulinase from Aspergillus niger AM270052.1 and scale up of conditions for mass production. Inter. J. of Pharmacy and Biological Science, *9*, 395-404, 2019.
12. *Vijayaraghavan K., Yamini D., Ambika V., Sowgamini S.* Trends inulinase production. A review. Critical Reviews in Biotechnol. *29*, 67-77, 2009.
13. *Kago N., Jain S.C.* Production and Properties of Microbial Inulinase, Resent Advances, Food Biotechnol., *25*, 165-212, 2011.
14. *Nascimento D.S., Valasques Junior G., Fernandes P., Ribeiro G.C.A., Lima D.M., Góes-Neto A., Oliveira R.Q., de Cassia R., Figueiredo-Ribeiro L., de Assis S.A.* Production, characterization and application of inulinase from *fungal endophyte* CCMB 328. Annals of the Brazilian Academy of Sciences, *84*, 443-453, 2012.

16. *Chi Z.M., Zang T., Cao T.S., Liu X.Y., Cui W., Zhao C.H.* Biotechnological potential of inulin for bioprocesses, Bioresour Technol., *102*, 4295-4303, 2011. http://DOI:10.1016/j.biortech.2010.12.086
17. *Neagu C., Bahrim G.* Inulinase-A versatile tool for biotechnology. Innov. Romanian Food Biotechnol., *9*, 1-11, 2011.
18. *Maragrube M.B., Upite D., Kaminskaq E., Danilevich A., Viesturs U.* Inulin syrop from dries Jerusalem arishoke, LLU Raksti 21(315), 116-121, 2008.
19. *Wang D., Li F.L., Wang S.A.* A one- step bioprocess for production of high-content fructo-oligosaccharides from inulun by yeast, Carbohydr. Polim., *151*, 1220-1226, 2016. https://DOI: 10.1016/j.carbpol.2016.06.059
20. *Markosyan L.S., Hovhannisyan R.S., Melkumyan I.E., Vardanyan A.K.* Production of citric acid from Heliantus tuberozus by Aspergillus niger sp. 4th congress of European Microbiologist FEMS 2011, Geneva, June 26-30. Abstracts, pdg/1225.
21. *Melqumyan L.E., Hovhannisyan R.S., Marksoyan L.S.* Topinambur (Heliantus tuberosus) as a new row material for citric acid production. Materials of the International Youth Conference "Innovation Approaches in Science, Yerevan, Armenia, 113-118, 2014.
22. *Devis B., Ornstain L.* Disc electrophoresis. I. background and theory, Ann. N. Y. Acad. Sci., *121*, 321-349, 1964. http://DOI:10.1111/j.1749-6632.1964.tb14213.x
23. *Somogyi M.* Determination of Redusing Sugar.Methods in Enzimology. 1966, 8, p.326.
24. Holyavka M., Artyukhov V., Kovaleva T., Structural and functional properties of inulinases: A review. Biocatalysis and biotransformaqtion *34*, 1-17, 2016. https://doi.org/10.1080/10242422.2016.1196486

Received on 28.04.2021