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BACTERIA OF THE BLACK SEA ARE PRODUCERS OF α-L-RHAMNOSIDASES

*The search for new producers of α-L-rhamnosidases and the study of their properties are constantly carried out, which makes it possible to identify enzymes with unique properties. Thus, the α-L-rhamnosidases producers of marine species of microorganisms can radically differ in habitat conditions from terrestrial ones. Previously, we have isolated the producers of α-L-rhamnosidases from a number of representatives of the Black Sea microbiota. However, the results of these studies did not allow us to isolate a strain promising for further study of the α-L-rhamnosidase synthesized by it. Therefore, **the purpose** of this work was to further search for effective producers of α-L-rhamnosidases among the microbiota of the Black Sea obtained from its different depths. **Methods**. Glycosidase activities were determined by the Romero and Davis methods. **Results.** The study of α-L-rhamnosidase activity in the dynamics of growth of 10 cultures isolated from the Black Sea showed that the only glycosidase activity, which was found on the third day of cultivation in six (07, 044, 050, 052, 054, 247) of ten cultures studied, **was toward*** α-L-rhamnosidase. *However, on the 5th day of cultivation, an increase (and in some strains, the appearance) of activity was noted in all tested cultures (from 0.01 to 0.12 U/mL). On the 7th day of cultivation, α-L-rhamnosidase activity in the supernatant of the culture liquid ranged from 0.02 to 0.2 U/mL. The highest activity (0.2 U/mL) was found in strain 052. On the 10th day of cultivation, the maximum activity (0.55 U/mL) was noted in culture 052 and slightly lower (0.35 U/mL, 0.28 U/mL, and 0.23 U/mL) in cultures 044, 050, and 054 respectively. Cultures 051, 020, and 247, which showed the same activity (0.1 U/mL), as well as 056 (0.09 U/mL) were an order of magnitude less active. The minimum activity was noted in culture 046 (0.03 U/mL). In the supernatant of the culture liquid of strain 07 on the 10th day of cultivation, α-L-rhamnosidase was absent at all. Since the highest α-L-rhamnosidase activity was found in the supernatants of culture liquids of 5 strains (044, 052, 054, 056, and 247), partially purified complex preparations of those* α-L-*rhamnosidases were obtained for further research. The study of the substrate specificity of complex enzyme preparations of α-L-rhamnosidases of strains 044, 051, 052, 056, and 247 on natural flavonoids, such as naringin, neohesperidin, and rutin, indicated that α-L-rhamnosidase obtained from strain 052 showed the highest activity on three investigated substrates: rutin, naringin (0.55 U/mL), and neohesperidin (0.52 U/mL). In addition to natural substrates, complex preparations of α-L-rhamnosidases of strains 052, 054, 056, and 247 also hydrolyzed synthetic derivatives of monosaccharides,*

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such as p-nitrophenyl-α-L-rhamnopyranoside and p-nitrophenyl-β-D-glucopyranoside. The maximum activity (0.15 U/mL) was noted in strain 052, whereas strain 044 was unable to hydrolyze synthetic substrates. ***Conclusions.*** *Complex enzyme preparations of α-L-rhamnosidase obtained from strain 052 are promising for further investigations. They showed the highest activity both on three natural substrates, such as rutin, naringin, and neohesperidin, as well as on such synthetic derivatives of monosaccharides as p-nitrophenyl-α-L-rhamnopyranoside and p-nitrophenyl-β-D-glucopyranoside.*

Keywords: *bacteria from the Black Sea, α-L-rhamnosidase activity, substrate specificity.*

Modern industrial biotechnology companies pay considerable attention to the development of new enzymes for the use in various fields of production. Among such enzymes, an important place is occupied by α-L-rhamnosidase (EC 3.2.1.40), which is characterized by specificity towards terminal rhamnose residues that are present in natural glycoconjugates and synthetic glycosides. However, despite the wide variety of natural enzymes, their properties are often not optimal for technological processes [1—3]. Therefore, the search for new producers of α-L-rhamnosidases and the study of their properties are constantly carried out, which makes it possible to identify enzymes with unique properties. These may be α-L-rhamnosidases, the producers of which are marine species of microorganisms, radically different in habitat conditions from the terrestrial ones. Previously [4, 5], we have isolated the producers of α-L-rhamnosidases from a number of representatives of the Black Sea microbiota. However, the results of these studies did not allow us to isolate a strain promising for further study of α-L-rhamnosidase synthesized by it.

This determined **the purpose of this work** to further search for effective producers of α-L-rhamnosidases among the microbiota of the Black Sea obtained from its different depths.

Materials and methods. Microorganisms from the culture collection of the Department of Microbiology, Virology and Biotechnology of the Mechnikov Odesa National University were used in the work. In total, 10 strains of bacteria isolated from the Black Sea water were studied: 07, 020, 044, 046, 050, 051, 052, 054, 056, and 247 (Table 1).

To identify glycosidase activities, the bacteria were cultivated under submerged conditions in flasks containing 100 mL of a nutrient medium of the following composition, g/L: rhamnose — 4; $NaNO_3$ — 2.0; KH_2PO_4 — 1.0; $MgSO_4 \cdot 7H_2O$ — 0.5; KCl — 0.5; $FeSO_4 \cdot 7H2O$ — 0.015 at pH 6.0, temperature 27 °C, rotation speed of 220 rpm, and cultivation time of 10 days. At the end of fermentation, the biomass was separated by centrifugation at 5000 g for 30 min.

Enzymatic activities were determined in the culture liquid supernatant (CLS) and complex enzyme preparation.

To isolate the complex enzyme preparation, dry ammonium sulfate was added to the CLS to obtain 30% saturation under pH control (~ 6.0). The mixture was kept for 10—12 h at 4 °C and centrifuged at 5000 g for 30 min. The obtained precipitate was removed, and ammonium sulfate was added to the supernatant to obtain 90% saturation. The mixture was kept for 6 h at 4 °C and centrifuged under the same conditions. The precipitate was dialyzed and used for further research.

In determining the activity of glycosidases, the following nitrophenyl carbohydrate derivatives (Sigma-Aldrich, USA) were used as substrates: *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-glucuronide, *p*-nitrophenyl-α-D-mannopyranoside, *p*-nitrophenyl-α-D-xylopyranoside, *p*-nitrophenyl-α-D-fucopyranoside, *p*-nitrophenyl-β-D-xylopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-N-acetyl-β-D-glucosaminide, *p*-nitrophenyl-N-acetyl-α-D-glucosaminide, *p*-nitrophenyl-N-acetyl-β-D-galactosaminides, *p*-nitrophenyl-α-D-glucopyranoside, and *p*-nitrophenyl-α-L-rhamnopyranoside. To determine the activity of glycosidases, 0.1 mL of 0.1 M phosphate-citrate buffer (PhCB) pH 5.2 and 0.1 mL of a 0.01 M solu-

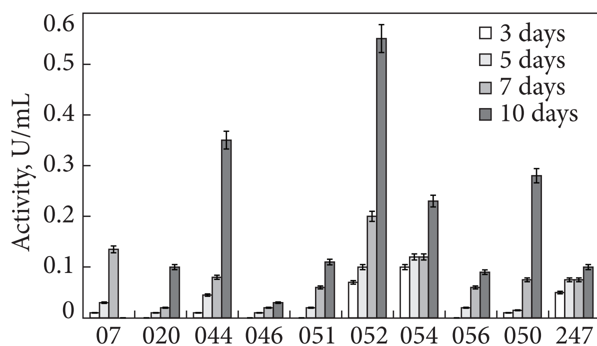

Fig. 1. α-L-Rhamnosidase activity of the bacteria from the Black Sea

tion of the corresponding substrate in PhCB were added to 0.1 mL of CLS.The reaction mixture was incubated for 10 min at 37 °C and stopped by adding 2 mL of 1 M sodium bicarbonate solution. The same components were added to the control sample, however, in the reverse order. The amount of *p*-nitrophenol formed as a result of hydrolysis was determined by the absorption colorimetric method at 400 nm [6]. One unit of activity of the studied glycosidase (U) was taken as its amount that hydrolyzes 1 μmol of the corresponding substrate (Sigma-Aldrich, USA) for 1 min under experimental conditions [6]. The Davis method was used to determine the α-L-rhamnosidase activity using natural substrates of naringin, neohesperidin, and rutin [7]. To determine the activity, 1 mL of 0.05% natural substrate in 0.1 PhCB, pH 5.2 was added to 1 mL of enzyme preparation solution. The reaction mixture was incubated for 60 min at 37 °C. Then 0.2 mL aliquots were taken, and 10 mL of diethylene glycol and 0.2 mL of 4M NaOH were added. The mixture was kept at room temperature for 10 min, and the staining intensity was measured on a spectrophotometer at 420 nm.

Table 1. **Depth coordinates of sampling points**

Strain	Station	Depth (m)	Coordinates	
			Latitude	Longitude
07	242	1499	N 41° 31.138	E 37° 37.347
044, 050, 056	233	1537	N 41° 32.670	E 37° 37.460
020, 046, 247	258	1888	N 44° 37.243	E 35° 42.286
051, 052, 054	269	2080	N 44° 17.329	E 35° 0.081

All experiments were performed in no less than 3—5 replications. Statistical processing of the results of was carried out by standard methods using Student's t-test at the 5% significance level [8].

Results. The study of α-L-rhamnosidase activity in the dynamics of growth of 10 cultures isolated from the Black Sea showed that, regardless of the cultivation time, CLS of all the studied strains did not show β-D-galactosidase, β-D-glucuronidase, α-D-mannosidase, α-D-xylosidase, α-D-fucosidase, β-D-xylosidase, N-acetyl-β-D-glucosaminidase, N-acetyl-α-D-glucosaminidase, N-acetyl-β-D-galactosaminidase, and α-D-glucosidase activity. The only activity, which was found on the third day of cultivation only in six (07, 044, 050, 052, 054, 247) of ten cultures studied was related to α-L-rhamnosidase (Fig. 1). However, on the 5th day of cultivation, an increase (and in some strains, the appearance) of activity was noted, which ranged from 0.01 to 0.12 U/mL.

With an increase in cultivation time, an increase in α-L-rhamnosidase activity in the CLS of all studied cultures was noted. So, on the 7th day of cultivation, α-L-rhamnosidase activity in the CLS ranged from 0.02 to 0.2 U/mL (Fig. 1). The highest activity (0.2 U/mL) was found in strain 052. Somewhat lower (0.135 U/mL, 0.12 U/mL, 0.08 U/mL, 0.075 U/mL, 0.075 U/mL, and 0.06 U/mL) was activity in cultures 051, 052, 044, 050, 247, and 056, respectively. The minimum activity in CLS was found in strains 020 and 046 (0.02 U/mL). On the 10th day of cultivation, the maximum activity (0.55 U/mL) was noted in culture 052, slightly lower (0.35 U/mL, 0.28 U/mL, and 0.23 U/mL) in cultures 044, 050, and 054, respectively. Cultures 051 (0, 20, and 247 (the strains showed the same activity, which amounted to 0.1 U/mL), as well as 056 (0.09 U/mL) were an order of magnitude less active. The minimum activity was noted in culture 046 (0.03 U/mL). In the CLS of strain 07 on the 10th day of cultivation, α-L-rhamnosidase was

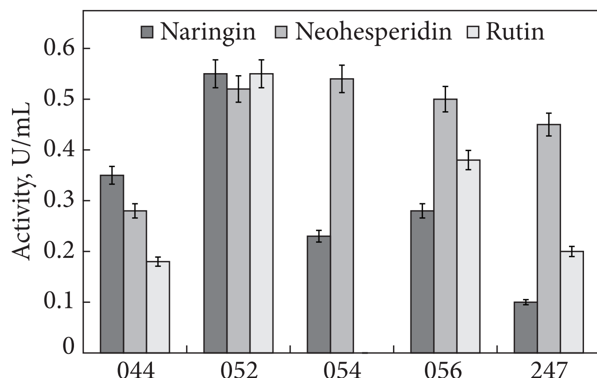

Fig. 2. Substrate specificity of α-L-rhamnosidase of bacteria from the Black Sea

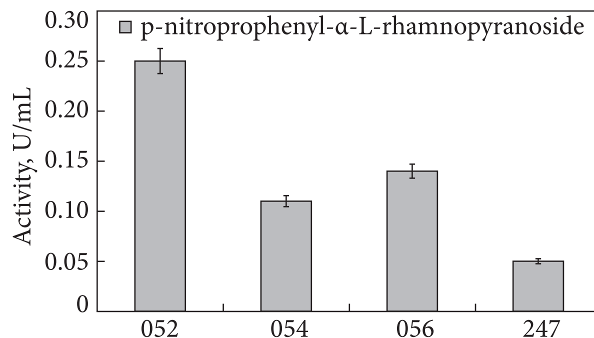

Fig. 3. α-L-Rhamnonosidase activity of bacteria from the Black Sea

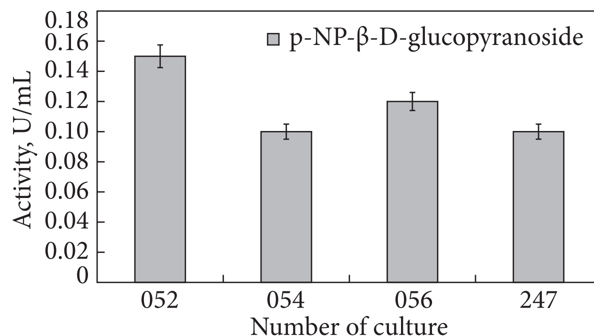

Fig. 4. β-D-Glucosidase activity of bacteria from the Black Sea

absent at all. Since the highest α-L-rhamnosidase activity was found in the CLS of 5 strains (044, 052, 054, 056, and 247), partially purified complex preparations of α-L-rhamnosidases were obtained for further studies. The substrate specificity of complex enzyme preparations of α-L-rhamnosidases of strains 044, 051, 052, 056, and 247 was studied both on natural flavonoids, such as naringin, neohesperidin, and rutin, and on synthetic *p*-nitrophenyl derivatives of monosaccharides. α-L Rhamnosidase obtained from strain 052 showed the highest activity on three natural substrates: rutin, naringin (0.55 U/mL), and neohesperidin (0.52 U/mL).

The complex preparation of α-L-rhamnosidase of strain 044 showed the highest activity on naringin (0.35 U/mL) and neohesperidin (0.28 U/mL) and lower — on rutin (0.18 U/mL), while strain 056 hydrolyzed neohesperidin (0.5 U/mL) and rutin (0.38 U/mL) more efficiently than naringin (0.28 U/mL). Similar results were noted for the complex preparation of α-L-rhamnosidase strain 054, which showed higher activity on neohesperidin (0.54 U/mL) than on naringin (0.28 U/mL), but was unable to hydrolyze rutin. The complex preparation of L-rhamnosidases of strain 247 hydrolyzed neohesperidin (0.45 U/mL) and rutin (0.2 U/mL) more efficiently than naringin (0.1 U/mL). In addition to natural, complex preparations of α-L-rhamnosidases of strains 052, 054, 056, and 247 also hydrolyzed synthetic derivatives of monosaccharides such as *p*-nitrophenyl-α-L-rhamnopyranoside and *p*-nitrophenyl-β-D-glucopyranoside. Strain 044 was unable to hydrolyze synthetic substrates.

Since the preparations of α-L-rhamnosidases from a number of producers are also capable of exhibiting β-D-glucosidase activity, complex enzyme preparations of four strains were tested for their ability to hydrolyze *p*-nitrophenyl-β-D-glucopyranoside (Fig. 4). The maximum activity was noted for strain 052 (0.15 U/mL). Slightly lower results were obtained for strain 056 (0.12 U/mL) as well as for strains 054 and 247 (0.1 U/mL).

Discussion. Industrial enzymology is a fundamental direction of current biotechnology. It contributes to the intensification of many branches of

industry and medicine. As known, in recent years, researchers have focused on enzyme preparations of microbial origin, since biotechnological processes with their participation have a number of significant advantages over the use of plant or animal raw materials. This stimulated the efforts of researchers to search for highly productive strains of microorganisms to solve technological issues in obtaining enzyme preparations with various action spectra. Among them, an important place is occupied by α-L-rhamnosidase, which is widely used in the food, pharmaceutical, and chemical industries. Scientific interest in α-L-rhamnosidase is going on expanding, which is associated with the development of a new era of drugs, where rhamnose-free bioflavonoids are used.

The high biotechnological potential of marine microorganisms has recently stimulated the active screening of glycosidases among marine bacteria [9—13]. Previously, we have shown [5] that α-L-rhamnosidase of a representative of actinobacteria isolated from the Black Sea, namely strain Acty 5, is more active toward such natural substrates as rutin, naringin, and neohesperidin than toward synthetic substrates. Similar specificity was also found in other bacterial α-L-rhamnosidases [1—3]. With regard to synthetic derivatives of monosaccharides, α-L-rhamnosidase Acty 5 hydrolyzed only *p*-nitrophenyl-α-L-rhamnopyranoside. A similar result was obtained by us in the study of the complex preparation of α-L-rhamnosidase of strain 044, while the enzyme preparations of the four other studied strains 052, 054, 056, and 247, except for *p*-nitrophenyl-α-L-rhamnopyranoside, hydrolyzed *p*-nitrophenyl-β-D-glucopyranoside as well. The presence of α-L-rhamnosidase and β-D-glucosidase activity in the strains we studied were appropriate, since it is more technologically advanced to transform natural substrates by using a producer capable of simultaneously synthesizing both enzymes and thereby acting on the substrate in a complex. Our results on the ability of complex preparations of α-L-rhamnosidases from a number of studied strains to hydrolyze such bioflavonoids as naringin, neohesperidin, and rutin are also of great importance. Their modification by splitting off the terminal residue of L-rhamnose leads to the improvement in the properties of such bioflavonoids, which makes it possible to create drugs based on them for the treatment of cardiovascular diseases, as well as drugs with antiviral and immunotropic effects, and to use them in various industries, in particular, in the food industry, in the production of wine and citrus juices.

Conclusions. α-L-Rhamnosidases obtained from strain 052 are promising for further investigations. They have shown the highest activity both on three natural substrates, namely rutin, naringin, and neohesperidin, and synthetic derivatives of monosaccharides, such as *p*-nitrophenyl-α-L-rhamnopyranoside and *p*-nitrophenyl-β-D-glucopyranoside.

REFERENCES

1. Borzova N., Gudzenko O., Varbanets L. Purification and characterization of a naringinase from Cryptococcus albidus // Appl Biochem Biotechnol V. 184, No 3, 2018, P. 953–969. doi: 10.1007/s12010-017-2593-2
2. Puri M. Updates on naringinase: structural and biotechnological aspects. Appl Microbiol Biotechnol. 2012; 93(1):49—60. DOI: 10.1007/s00253-011-3679-3
3. Borzova N, Gudzenko O, Varbanets L. α-L-Rhamnosidase from *Penicillium tardum* and its application for biotransformation of citrus rhamnosides. Appl Biochem Biotechnol. 2022. https://doi.org/10.1007/s12010-022-04008-1.
4. Varbanets LD, Avdeeva LV, Borzova NV, Matseliukh EV, Gudzenko AV, Kiprianova EA.[The Black Sea bacteria-producers of hydrolytic enzymes]. Mikrobiol Z. 2011; 73(5):9—15. Ukrainian.
5. Varbanets LD, Gudzenko OV, Ivanytsia VA. Marine Actinobacteria — Producers of Enzymes with α-L-Rhamnosidase**.** Mikrobiol Z. 2020; 82(5):3—10. doi: https://doi.org/10.15407/microbiolj82.05.003
6. Chaplin ME, Kennedy JE (Eds.). Carbohydrate analysis: a practical approach. Washington: Oxford IRL Press. 1986.
7. Davis BJ. Assay of naringinase. Anal Biochem. 1985; 149(2):566—571.

8. Lakyn HF. [Byometryia]. Moscow: Vysshaya Shkola; 1990. Russian.
9. Gudzenko O.V., Borzova N.V., Varbanets L.D. α-l-Rhamnosidase activity of antarctic strain of *Pseudomonas mandelii* U1 Microbiol. Z. 2021; 83(5):3-10
10. Gudzenko O.V., Ivanytsia V.O., Varbanets L.D. Bacteria of the Black Sea are Producers of Proteolytic Enzymes. Microbiological journal. 2022 (3). P. 3—8. https://doi.org/10.15407/microbiolj84.03.003
11. Maqtari QA, Mahdi AA. Cold-active enzymes and their applications in industrial fields — A review. International Journal of Research in Agricultural Sciences 2019; 6(4):2348—3997.
12. Varbanets LD, Matselyukh OV, Gudzenko OV, Borzova NV, Seifullina II, Khitrich CN. [Coordinative compounds of zinc with n-substituted thiocarbamoil-N'-pentamethylensulfenamides — activity modifiers of enzymes of proteolytic and glycolytic action[Ukr.Biochem.J. 2011; 83(3):25-36. Ukrainian, а також додати посилання
13. Varbanets LD, Avdeeva LV, Borzova NV, Matselukh OV, Gudzenko OV, Kiprianova O.H. et al. [The black sea bacteria — producers of hydrolytic enzymes] Mikrobiol Z. 2011; 73(5):9–20. Ukrainian.

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α-L-РАМНОЗИДАЗНА АКТИВНІСТЬ БАКТЕРІЙ, ВИДІЛЕНИХ ІЗ ЧОРНОГО МОРЯ

Дослідниками постійно ведеться пошук нових продуцентів α-L-рамнозидаз та вивчаються їхні властивості, що дає змогу ідентифікувати ферменти з унікальними характеристиками. Тому продуценти α-L-рамнозидаз морських видів мікроорганізмів можуть кардинально відрізнятися за умовами проживання від наземних. Раніше ми виділили продуценти α-L-рамнозидаз із ряду представників мікробіоти Чорного моря. Проте результати цих досліджень не дозволили виділити штам, перспективний для подальшого вивчення синтезованої ним α-L-рамнозидази. Тому **метою даної роботи** був подальший пошук ефективних продуцентів α-L-рамнозидаз серед мікробіоти Чорного моря, отриманої з різних його глибин. **Методи.** Активність глікозидаз визначали методами Ромеро і Девиса. **Результати.** Дослідження активності α-L-рамнозидази в динаміці росту 10 культур, виділених з Чорного моря, показало, що єдина активність глікозидази, яка була виявлена вже на третій день культивування у шести (07, 044, 050, 052, 054, 247) з десяти досліджених культур, була α-L-рамнозидазна активність. Проте вже на 5-й день культивування у всіх досліджуваних культурах відмічено підвищення (а у деяких штамів і поява) активності (від 0,01 до 0,12 од/мл). На 7-й день культивування активність α-L-рамнозидази в супернатанті культуральної рідини становила від 0,02 до 0,2 од/мл. Найвищу активність (0,2 од/мл) виявлено у штаму 052. На 10-ту добу культивування максимальна активність відзначена також у культурі 052 (0,55 од/мл), дещо нижча (0,35 од/мл, 0,28 од/мл та 0,23 од/мл) - у культурах 044, 050 та 054 відповідно. На порядок менш активними були культури штамів 051, 020 та 247 (штами виявили однакову активність, яка становила 0,1 од/мл), а також 056 (0,09 од/мл). Мінімальна активність відзначена в культурі 046 (0,03 од/мл). У супернатанті культуральної рідини штаму 07 на 10-ту добу культивування α-L-рамнозидаза була відсутня. Оскільки найвищу активність α-L-рамнозидази виявлено в супернатантах культуральних рідин пяти штамів (044, 052, 054, 056, 247), для подальших досліджень з них отримано частково очищені комплексні препарати α-L-рамнозидаз. Вивчення субстратної специфічності комплексних ферментних препаратів α-L-рамнозидаз штамів 044, 051, 052, 056 і 247 на природних флавоноїдах, таких як нарингін, неогесперидин та рутин, показало, що α-L-рамнозидаза, отримана зі штаму 052, виявила найвищу активність на трьох досліджуваних субстратах: рутині, нарингіні (0,55 од/мл) та неогесперидині (0,52 од/мл). Крім природних, комплексні препарати α-L-рамнозидаз штамів 052, 054, 056, 247 також гідролізували синтетичні похідні моносахаридів, а саме *п*-нітрофеніл-α-L-рамнопіранозид і *п*-нітрофеніл-β-D-глюкопіранозид. Максимальна активність відзначена у штаму 052 (0,15 од/мл). Штам 044 не зміг гідролізувати синтетичні субстрати. **Висновки.** Комплексний ензимний препарат α-L-рамнозидази, отриманий зі штаму 052, є перспективним для подальших досліджень. Найвищу активність він проявив як на трьох природних субстратах, а саме рутині, нарингіні і неогесперидині, так і на синтетичних похідних моносахаридів, а саме *п*-нітрофеніл-α-L-рамнопіранозид і *п*-нітрофеніл-β-D-глюкопіранозиді.

Ключові слова: *бактерії з Чорного моря, α-L-рамнозидазна активність, субстратна специфічність.*