doi: https://doi.org/10.15407/microbiolj82.05.003

MARINE ACTINOBACTERIA – PRODUCERS OF ENZYMES WITH A-L-RHAMNOSIDASE ACTIVITY

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In recent years researchers have attracted their attention to such glycosidases as α-L-rhamnosidase (α-L-rhamnoside-rhamnohydrolase – EC 3.2.1.40). The substrates of their action are widespread in the plant world glycosides such as naringin, quercetrin, hesperidin, neohesperidin, and rutin, from which α-L-rhamnosidases cleave the terminal unreduced L-rhamnose residues. This specificity of α-L-rhamnosidases can be used in various industries: food – to improve the quality of drinks (reducing bitterness in citrus juices, enhancing the aroma of wines), as well as production of food additives; in the pharmaceutical industry – to improve the biological properties of bioflavonoids, in particular anti-inflammatory. A number of them are characterized by cardio- and radioprotective effects, have antioxidant, cytotoxic, antibacterial, antisclerotic properties, and are used in the complex treatment of coronary heart disease, including angina pectoris. The use of α-L-rhamnosidases in the chemical industry is associated with a reduction in the cost of rhamnose production as well as various plant glycosides and rutinosides. In the literature available to us, no data were found on the producers of α-L-rhamnosidases among the representatives of actinobacteria, which are known to synthesize a wide range of biologically active compounds, including antibiotics and enzymes. ***Purpose.*** *To study the ability of actinobacteria isolated from water and bottom sediments of the Black Sea, to produce a-L-rhamnosidase, and also to study the properties of the most active producer.* ***Methods.*** *Glycosidase activity was determined by the Romero and Davis methods, protein – by the Lowry method.* ***Results.*** *The study of 12 glycosidase activities in 10 strains of actinobacteria isolated from bottom sediments of the Black Sea indicated that 6 investigated strains showed the ability to synthesize an enzyme with a-L-rhamnosidase and b-D-glucosidase activity. Studies have shown that the highest α-L-rhamnosidase activity (0.14 U/mg protein) was manifested by Acty 5 isolate with an optimum pH of 7.0 and a temperature optimum of 38° C. The enzyme preparation showed substrate specificity both for natural (rutin, naringin, neohesperidin) and synthetic (p-nitrophenyl derivatives of L-rhamnose and D-glucose) substrates.* ***Conclusions.****., Promising Acty 5 isolate with high a-L-rhamnosidase and low b-D-glucosidase activity was found among marine actinobacteria. Bacteria with two enzymes activity expand the possibilities of their practical use.*

Keywords: actinobacteria, Black Sea, a-L-rhamnosidase.

One of the most actively developing areas of modern biotechnology is the use of a wide range of enzymes in technological processes. Among these enzymes, glycosidases play an important role, because are used in medical and technological processes, in food industry, biotechnologies for the purification and processing of raw materials, as well as in many other areas of human activity. In recent years, researchers have attracted their attention to such representatives of glycosidases as α-L-rhamnosidase (α-L-rhamnoside-rhamnohydrolase – EC 3.2.1.40) [1–5]. The substrates of their action are glycosides widespread in the plant world, such as naringin, quercetrin, hesperidin, neohesperidin, rutin, from which α-L-rhamnosidases cleave the terminal unreduced L-rhamnose residues. This specificity of α-L-rhamnosidases can be used in various industries: food – to improve the quality of drinks (reduces bitterness in citrus juices, enhance the aroma of wines), and also in the production of food additives [1, 3, 4]. α-L-rhamnosidases are used in the pharmaceutical industry to improve the biological properties of bioflavonoids, in particular anti-inflammatory. A number of them are characterized by cardio- and radioprotective effects, have antioxidant, cytotoxic, antibacterial, antisclerotic properties, and are used in the complex treatment of coronary heart

disease, including angina pectoris [1, 2]. The use of α-L-rhamnosidases in the chemical industry is associated with a reduction in the cost of rhamnose production, as well as various plant glycosides and rutinosides.

Earlier studies, both made by us [6, 7] and other authors [3, 8], showed the ability of representatives of various taxonomic groups of microorganisms to produce α-L-rhamnosidase. However, despite the wide variety of natural enzymes, their properties are often not optimal for technological processes. Currently, only two preparations of α-L-rhamnosidases, hesperidinase and naringinase, obtained from representatives of the *Aspergillus* and *Penicillium* genera, respectively, are produced by Sigma (USA). Well-known commercial preparations of α-L-rhamnosidase (hesperidinase and naringinase, Sigma, USA), in addition to α-L-rhamnosidase, also contain β-D-glucosidase, which expands the possibilities of their practical use. In Ukraine, the production of α-L-rhamnosidase preparations is completely absent. In this regard, the search for new effective producers of highly specific α-L-rhamnosidases with suitable characteristics continues to be relevant, both for scientific, theoretical and practical purposes. No data were found in the available literature on producers of α-L-rhamnosidases among representatives of actinobacteria, which, as it is known [2, 4, 5], synthesize a wide range of biologically active compounds, including antibiotics and enzymes.

Therefore the **aim** of this work was to evaluate the ability of a number of actinobacteria isolated from the Black Sea to produce α-L-rhamnosidase. Since enzymes with both narrow and broad substrate specificity are used in biotechnological processes, depending on the tasks, we investigated not only the α-L-rhamnosidase activity of a number of isolates, but also evaluated the potential of their other glycosidase activities.

Materials and methods. Microorganisms from the culture collection of the Department of Microbiology, Virology and Biotechnology of Odessa National University were used in the work. The object of the study were 10 actinobacteria isolates: Acty 1, Acty 2, Acty 3, Acty 3-1, Acty 4, Acty 5, Acty 7, Acty 8, Acty 9, Acty 10, isolated from samples of bottom sediments of the Black Sea.

Microorganisms were grown (5–7 days) on Nutrient Agar medium of the following composition, g/l: NaCl – 5.0; agar-agar – 5.0; peptone – 5.0; yeast extract – 1.5; meat extract – 1.5.

To identify glycosidase activities, actinobacteria were cultured on two media under deep conditions in test tubes containing 10 ml of culture medium at a temperature of 27° C, with a rotation speed of 220 rpm for 5–10 days. The medium No. 1 contained, g/l: NaCl – 5.0; peptone – 5.0; rhamnose – 5.0; yeast extract – 1.5; meat extract – 1.5. The medium No. 2 was similar to medium No. 1, but did not contain NaCl. The most active Acty 5 isolate was grown at different temperatures: 4, 16, and 28° C in flasks on medium No. 1. The cultivation at 16 and 28° C was carried out both at orbital shaker at 220 rpm and without stirring. The cultivation of this culture at 4° C was carried out without stirring in the refrigerator.

After fermentation, the biomass was separated by centrifugation at 5000 g, 30 min. Enzymatic activities were determined in the supernatant of the culture liquid.

When determining the activity of glycosidases, the following nitrophenyl carbohydrate derivatives (Sigma-Aldrich, USA) were used as substrates: *p*-nitrophenyl-b-D-galactopyranoside, *p*-nitrophenyl-b-D-glucuronide, *p*-nitrophenyl-a-D-mannopyranoside, *p*-nitrophenyl-a-D-xylopyranoside, *p*-nitrophenyl-a-D-fucopyranoside, *p*-nitrophenyl-b-D-xylopyranoside, *p*-nitrophenyl-b-D-glucopyranoside, *p*-nitrophenyl-N-acetyl- b-D-glucosaminide, *p*-nitrophenyl-N-acetyl-α-D-glucosaminide, *p*-nitrophenyl-N-acetyl-b-D-galactosaminides, *p*-nitrophenyl-α-D-glucopyranoside, *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 solution of the corresponding substrate in PhCB were added to 0.1 ml of the culture liquid supernatant. The reaction mixture was incubated for 10 min at a temperature of 37° C. The reaction was 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, which is formed as a result of hydrolysis, was determined by the absorption colorimetric method at 400 nm [9]. The unit of activity of the studied glycosidases (U) was taken to be such an amount that hydrolyzes 1 μmol of the corresponding substrate (Sigma-Aldrich, USA) in 1 min under experimental conditions [9].

The Davis method was used to determine the a-L-rhamnosidase activity using natural substrates of naringin, neohesperidin, and rutin [10]. 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. 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, the staining intensity was measured on a spectrophotometer at 420 nm. The unit of enzyme activity (U) was taken to be such as amount that hydrolyzes 1 μmol of substrate in 1 min under experimental conditions. Specific activity was calculated as the number of units of activity per 1 mg of protein.

Protein concentration in culture liquid supernatant was determined by the Lowry method [11]. A calibration curve was constructed using bovine serum albumin as a standard.

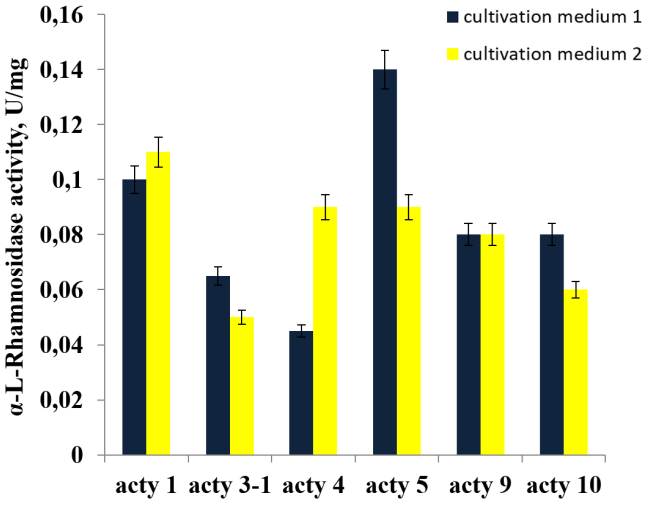
To isolate the enzyme preparation, dry ammonium sulfate was added to the supernatant of the culture liquid to obtain concentration of 30 % saturation under pH control (~ 6.0). The mixture was kept for 10–12 h at 4° C, centrifuged at 5000 g, 30 min. The precipitate was discarded; ammonium sulfate was added to the supernatant to obtain 90 % saturation. The mixture was kept for 6 h at 4° C, centrifuged under the same conditions. The precipitate obtained by fractionation with ammonium sulfate was dialyzed. The study of temperature effect on the enzymatic activity was carried out in the range from 4 to 60° C and pH values from 2.0 to 6.0, the latter was created using 0.01 M PhCB.

All experiments were performed in no less than 3–5 replications. Statistical processing of the results of the experimental series was carried out by standard methods using Student's t-test at 5 % significance level [12].

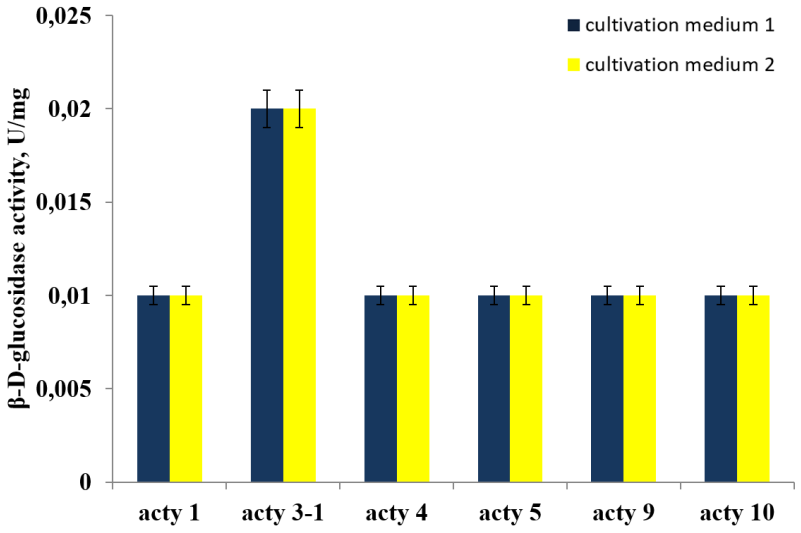
Results. The studies of 12 glycosidase activities in 10 tested isolates indicated that 6 of them showed a-L-rhamnosidase activity (Fig. 1). In the supernatant of the culture liquid, it ranged from 0.045 to 0.14 U/mg of protein. The highest activity was found in Acty 5 isolate (0.14 U/mg of protein in nutrient medium No. 1). The activity of Acty 1 was slightly lower (0.10 and 0.11 U/mg of protein in nutrient media No. 1 and No. 2 respectively); Acty 9 (0.08 U/mg of protein); Acty 10 (0.08 and 0.06 U/mg of protein in nutrient media No. 1 and No. 2 respectively), Acty 3–1 (0.065 and 0.05 U/mg of protein in nutrient media No. 1 and No. 2 respectively) and Acty 4 (0.045 and 0.09 U/mg of protein in nutrient media No. 1 and No. 2 respectively). Thus, it was shown that for some cultures (Acty 9 and Acty 1), the a-L-rhamnosidase activity was almost the same when grown in both media, while the activity of Acty 4 culture in medium 2 was twice higher. The presence of NaCl in the culture medium stimulated the enzymatic activity of Acty 3-1, Acty 5, and Acty 9 cultures.

In the culture liquid of the six studied isolates, which showed a-L-rhamnosidase activity, insignificant β-D-glucosidase activity was also noted (Fig. 2). The highest activity was revealed in the Acty 3-1 strain (0.02 U/mg of protein). Unlike of a-L-rhamnosidase activity, which depended on the composition of the nutrient medium, β-D-glucosidase activity was the same when grown both in medium No. 1 and No. 2.

All studied isolates lacked b-D-galactosidase, b-D-glucuronidase, a-D-mannosidase, a-D-xylosidase, a-D-fucosidase, b-D-xylosidase, N-acetyl-b-D-glucosaminidase, N-acetyl-α-D-glucosaminidase, N-acetyl-b-D-galactosaminidase, α-D-glucosidase activity.



F i g. 1. α-L-rhamnosidase activity of the studied actinobacteria depending on the culture medium

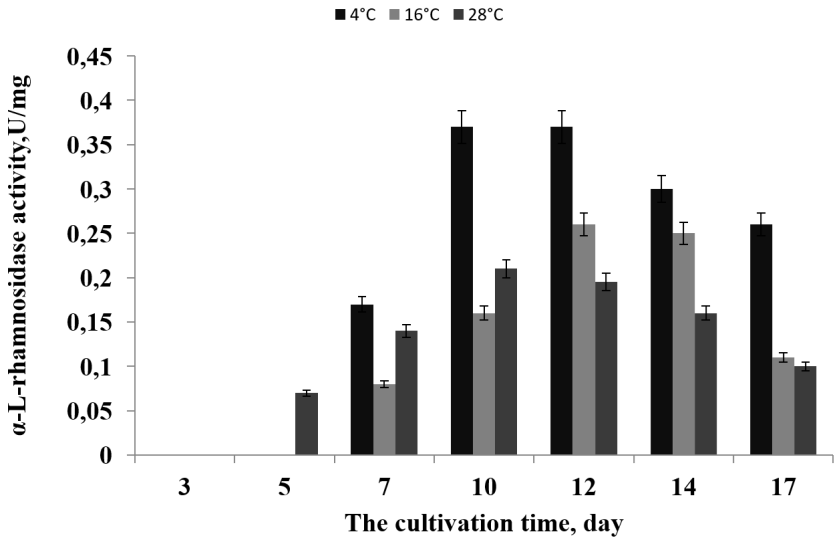


F i g. 2. β-D-glucosidase activity of tested actinobacteria

Since Acty 5 isolate showed the highest α-L-rhamnosidase activity, subsequent studies were performed with this microorganism.

A significant influence on the synthesis of enzyme is exerted by the temperature of producer growth [3–5]. So, it was shown (Fig. 3) that the cultivation at temperature of 4° C is optimal for the synthesis of α-L-rhamnosidase by Acty 5. The increase of temperature negatively affects biosynthesis.

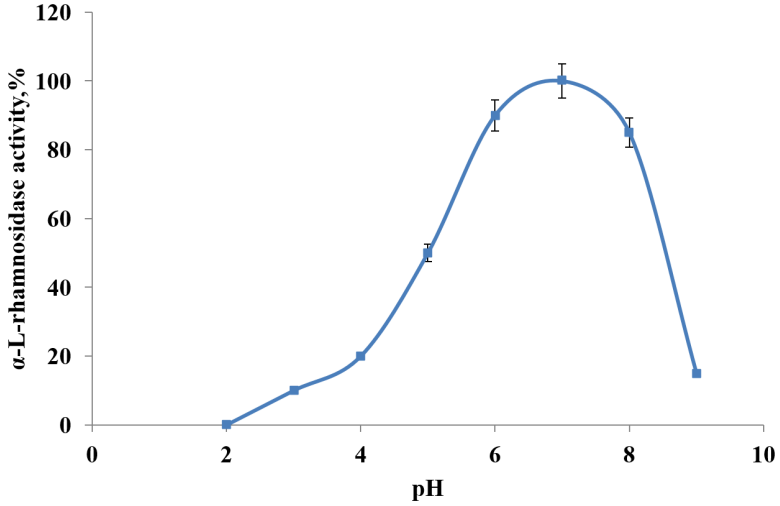
Figure 3 shows the data where the culture was grown at 16 and 28° C on an orbital shaker with stirring (since there was no parallel activity in flasks without stirring) and at 4° C without stirring. A study of the dynamics of a-L-rhamnosidase synthesis by Acty 5 culture showed (Fig. 3) that the maximum enzymatic activity was observed on the 10–12th day of cultivation at 4° C.



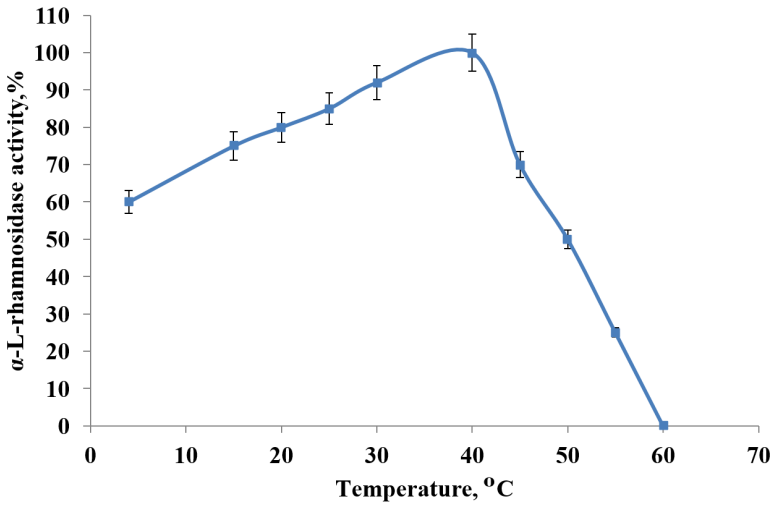
F i g. 3. The dynamics of a-L-rhamnosidase synthesis by Acty 5 isolate at different temperatures

It was established that the pH optimum for α-L-rhamnosidase synthesis by Acty 5 was 7.0, and up to 80–90 % of the maximum activity remained in the pH range from 6.0 to 8.0 (Fig. 4).

Typically, the reaction rate catalyzed by the enzymes increases to the optimum value with increasing temperature to the point at which the enzyme is inactivated. It was determined that the thermal optimum of Acty 5 α-L-rhamnosidase activity is 38° C (Fig. 5). It is known [1–5] that the temperature optimum of the action of most bacterial α-L-rhamnosidases is 40–45° C.



F i g. 4. Effects of pH on Acty 5 α-L-rhamnosidase activity (37° C)

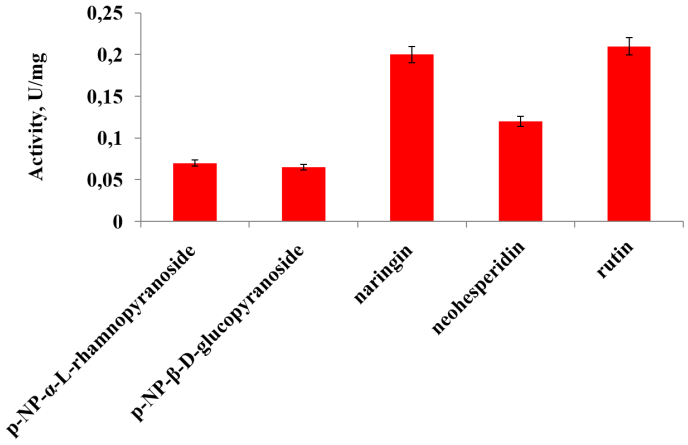


F i g. 5. Effects of temperature on the Acty 5 α-L-rhamnosidase activity (pH 7.0)

The substrate specificity of Acty 5 α-L-rhamnosidase was studied both on synthetic *p*-nitrophenyl derivatives of monosaccharides and on natural flavonoids such as naringin, neohesperidin and rutin. Acty 5 α-L-rhamnosidase showed higher activity to rutin (0.21 U/mg of protein), naringin (0.2 U/mg of protein) and neohesperidin (0.12 U/mg of protein) than to synthetic substrates. Similar specificity is found in other bacterial α-L-rhamnosidases [1–5]. As for synthetic derivatives of monosaccharides, it should be noted the narrow specificity for glycon: so, it is shown the ability of Acty 5 α-L-rhamnosidase to hydrolyze only *p*-nitrophenyl-α-L-rhamnopyranoside (0.07 U/mg of protein) and *p*-nitrophenyl-β-D-glucopyranoside (0.065 U/mg of protein) (Fig. 6).

The detected ability of the Acty 5 enzyme to hydrolyze the synthetic substrates of *p*-nitrophenyl-α-L-rhamnopyranoside and *p*-nitrophenyl-β-D-glucopyranoside, as well as natural substrates – naringin, neohesperidin and rutin, suggest the possibility of its use in food technology and obtaining biologically active flavonoids.

Discussion. The level of enzyme biosynthesis by microorganisms depends not only on the genetic characteristics of the producers, but to a large extent on the conditions in which they are cultivated, primarily the composition of the nutrient medium. Culture media should ensure the viability, growth and development of the respective producers, as well as the synthesis of the target product with maximum efficiency. Since the studied actinobacteria were isolated from samples of bottom sediments of the Black Sea, they were grown on media containing or not containing NaCl.



F i g. 6. Substrate specificity of Acty 5 α-L-rhamnosidase

Screening for glycosidase activities indicates that the biosynthesis of α-L-rhamnosidase in most cultures did not depend on the presence of NaCl in the culture medium. The exceptions were Acty 4 and Acty 5 cultures. For Acty 4 isolate, higher activity was observed on NaCl-free medium, while Acty 5 culture was most active on NaCl-containing medium. Since this isolate turned out to be more active (0.14 U/mg of protein) compared with other studied cultures, it was taken for further studies.

The most important characteristics of enzyme preparations include the optimal pH and temperature at which they exhibit enzymatic activity, as well as their substrate specificity. α-L-rhamnosidases are known to have pH optimum from 2.0 to 11.0. The main differences between fungal and bacterial enzymes are a significant difference in the pH action: for fungal enzymes, the optimum pH is in the acidic region from 4.0 to 6.0, while for bacterial enzymes it is close to neutral or alkaline. These properties determine the use of fungal and bacterial α-L-rhamnosidases in different areas. So, fungal α-L-rhamnosidases are more used in such processes as production of wines [1, 2] and juices [3, 8], while bacterial enzymes are mainly used in the production of L-rhamnose by hydrolysis of hesperidin and various flavonoid glycosides [4, 5].

The results obtained by studying the optimal pH values on a partially purified enzyme preparation obtained by 90 % saturation with ammonium sulfate of the supernatant of the culture liquid of Acty 5 isolate indicate that its optimum pH is 7.0, as well as other enzymes of bacterial origin. As for the temperature optimum, its value for Acty 5 α-L-rhamnosidase preparation differs from other α-L-rhamnosidase preparations studied and is 38° C. Temperature optimum action of most α-L-rhamnosidases are 40–80° C. An exception is the enzyme isolated from *Pseudoalteromonas* sp., which was active at 4° C [2] and *Aspergillus kawachii* α-L-rhamnosidase, which at 60° C retained up to 80 % of its maximum activity for 1 h [8].

Since rhamnose in both natural glycoconjugants and synthetic glycosides is linked by different types of bonds (α-1,2, α-1,4, α-1,6), an important characteristic of α-L-rhamnosidases is their substrate specificity. The specificity of enzymes is explained primarily coincidence of the spatial configuration of their substrate center with the substrate. Only if this coincidence is sufficiently complete and an enzyme – substrate complex can form, the process of enzymatic catalysis begins. Studying this question can give an answer whether the enzyme has absolute specificity (that is, it stimulate the rate of a single reaction) or catalyzes reactions, the main feature of which is the type of bond. They are characterized by group specificity or differ in stereochemical specificity, that is, they act on one of the spatial isomers.

In the study of substrate specificity, both synthetic (*p*-nitrophenyl derivatives of α-L-rhamnose and β-D-glucose) and natural derivatives of flavonoids (naringin, neohesperidin and rutin) were taken. The latter are plant flavonoids in which the L-rhamnose residue is attached to the β-D-glucoside α-1,6 (in rutin and hesperidin) or α-1,2 bond (in naringin). It was established that the enzyme preparation exhibits a greater affinity for natural substrates (rutin> naringin>neohesperidin) than for synthetic derivatives, as was noted by other researchers [1–5].

Since the Acty 5 enzyme preparation exhibits both α-L-rhamnosidase and β-D-glucosidase activity, as well as cleaves naringin well, it is

likely that naringinase is present. Naringin is a natural substrate containing L-rhamnose as well as D-glucose. α-L-Rhamnosidase breaks down naringin to L-rhamnose and prunin, and β-D-glucosidase, which often, as in our producer, is found together with α-L-rhamnosidase, breaks down prunin to naringenin and rhamnose. Therefore producers exhibiting the activity of two enzymes expand the possibilities of their practical use.

МОРСЬКІ АКТИНОБАКТЕРІЇ – ПРОДУЦЕНТИ ФЕРМЕНТІВ З A-L-РАМНОЗИДАЗНОЮ АКТИВНІСТЮ

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Резюме

Останніми роками увагу дослідників привертають такі представники глікозидаз, як α-L-рамнозидази (α-L-рамнозид-рамногідролаза – К.Ф. 3.2.1.40). Субстратами їх дії є широко поширені в рослинному світі глікозиди: нарингин, кверцитрин, гесперидин, неогесперидин, рутин, від яких α-L-рамнозидази відщеплюють термінальні залишки L-рамнози, які не відновлюються. Така специфічність α-L-рамнозидаз може бути використана в різних галузях промисловості: харчовій – для поліпшення якості напоїв (зменшення гіркоти в цитрусових соках, посилення аромату вин), а також у виробництві харчових добавок; у фармацевтичній промисловості – для поліпшення біологічних властивостей біофлавоноїдів, зокрема з протизапальною дією. Деякі з них характеризуються кардіо- та радіопротекторною дією, мають антиоксидантні, цитотоксичні, антибактеріальні, антисклеротичні властивості, використовуються в комплексній терапії ішемічної хвороби серця, в тому числі стенокардії. Використання α-L-рамнозидаз у хімічній промисловості пов'язано зі здешевленням виробництва рамнози, а також різних глікозидів і рутинозидів рослинного походження. У доступній нам літературі не виявлено даних щодо продуцентів α-L-рамнозидаз у представників актинобактерій, які, як відомо, синтезують широкий спектр біологічно активних сполук, включаючи антибіотики, ферменти. Мета. Вивчити здатність актинобактерій, виділених з води і донних осадів Чорного моря продукувати a-L-рамнозидази, а також дослідити властивості найбільш активного продуцента. Методи. Глікозидазні активності визначали методами Romero і Davis, білок – методом Lowry. Результати. Вивчення 12 глікозидазних активностей у 10 штамів актинобактерій, виділених з донних осадів Чорного моря показало, що 6 досліджених штамів проявили здатність синтезувати фермент з a-L-рамнозидазною і b-D-гюкозидазною активністю. Дослідження показали, що найвищу α-L-рамнозидазну активність (0.14 од/мг білка) виявив ізолят Acty 5 з оптимумом pH 7.0 і температурним оптимумом 38° C. Ферментний препарат виявляв субстратну специфічність як до природних (рутин, нарингин, неогесперидин), так і синтетичних (*p*-нітрофенільних похідних L-рамнози і D-глюкози) субстратів. Висновки. Серед морських актинобактерій виявлений перспективний ізолят Acty 5 з високою a-L-рамнозидазною і низькою b-D-гюкозидазною активністю. Бактерії, що мають активність двох ферментів розширюють можливості їх практичного використання.

Ключові слова: актинобактерії, Чорне море, a-L-рамнозидаза.

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Received 10.06.2020