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BACTERIA OF THE BLACK SEA ARE PRODUCERS OF PROTEOLYTIC ENZYMES

Despite the fact that in recent years there has been a certain enhancing interest in the study of marine microorganisms, nevertheless, marine bacteria as producers of biologically active substances, in particular enzymes, are still poorly. studied. The marine biota is significantly different from the terrestrial one; therefore, there is a high probability of detecting in the marine environment different from terrestrial bacteria producers of enzymes with unique specificity and activity, for the needs of modern biotechnology. Proteolytic enzymes play an important role in these studies. Since the majority of microbial producers are characterized by a number of serious deficiencies, in particular, most of the elastase producers described in the literature are pathogenic for humans, the search for new, effective producers continues to be an urgent problem, given that highly active producers of proteolytic enzymes, in particular elastase, are generally absent in Ukraine. In this regard, ***the purpose*** *of this work was to screen microorganisms isolated from the Black Sea for the presence of effective producers of proteolytic enzymes.* ***Methods.*** *We used methods of determining proteolytic (caseinilytic, elastolytic, fibrinolytic, fibrinogenolytic) activity.* ***Results.*** *The study of the enzymatic activity of the isolates showed that on the 10th day of cultivation in the supernatant of the culture liquid, caseinolytic activity was not detected only in one isolate 56, whereas very insignificant activity was observed in isolates 7, 20, and 50. The maximum activity was detected in isolate 247 (0.2 units/mL), and lower one - in isolates 46 (0.16 U/mL), 52 (0.15 U/mL), 51 (0.135 U/mL), 54 (0.08 U/mL), and 44 (0.05 U/mL). Of the 10 studied isolates, elastase activity was found only in four of them. The highest activity was found in isolates 51 and 54 (20.83 and 19.96 U/mL, respectively). Lower levels of activity (15.62 U/mL and 12.15 U/mL, respectively) were shown by isolates 52 and 247. The studied isolates also differed in their ability to hydrolyze fibrin and fibrinogen. The highest fibrinolytic activity (2.33 U/mL) was found in isolates 46 and 54, significantly lower in isolate 20 (0.5 U/mL) and isolate 44 (0.33 U/mL). The rest isolates did not show fibrinolytic activity. As for fibrinogenolytic activity, it was noted in 6 studied cultures. The highest levels of activity*

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were observed in isolate 51 (1.16 U/mL). Lower activity was found in isolates 54 (0.66 U/mL), 7 (0.5 U/mL), and 247 (0.33 U/mL). In isolate 50, it was minimal (0.083 U/mL). ***Conclusions.*** *No correlation was found between elastase, fibrinolytic and fibrinogenic activity in the studied isolates. Thus, isolates 51, 54 and, to a lesser extent, 52 and 247 synthesize elastase activity. The highest fibrinolytic activity was in isolates 46 and 54, and fibrinogenolytic activity was in isolate 51. It was shown that the Black Sea is rich in marine bacterial species, which can be effective producers of a number of practically important enzymes, in particular, proteolytic ones with specificity to elastin, fibrin, and fibrinogen, which can be promising for implementation in biotechnological processes.*

Keywords: *actinobacteria, the Black Sea, proteolytic activity.*

Despite the fact that in recent years there has been a certain enhancing interest in the study of marine microorganisms, nevertheless, marine bacteria as producers of biologically active substances, in particular, enzymes, are still poorly understood. The marine biota is significantly different from the terrestrial one; therefore, there is a high probability of detecting in the marine environment different from terrestrial bacteria producers of enzymes with unique specificity and activity, for the needs of modern biotechnology. Proteolytic enzymes play an important role in these studies. The number of proteolytic enzymes produced by industry in the world exceeds the output of all major enzymes that have applications in biotechnological processes. They are used in medicine, cosmetology, detergent industry, food and leather industries, as well as for the enzymatic synthesis of peptides. However, despite the wide variety of natural enzymes, their properties are often not optimal for technological processes [1—9]. Two approaches can be used to solve this problem. The first is the search for natural enzymes with suitable characteristics, and the second is the targeted modification of already-known and well-characterized proteins. Although the second approach seems to be more attractive, since it allows designing enzymes that are optimal for a given biotechnological process, the first approach makes it possible to find enzymes that are unique in their biological properties and specificity. It is impossible not to take into account the ecological significance of such works. The study of links in the fundamental chain of biodiversity-biotechnology is also of great importance.

Since the majority of microbial producers are characterized by a number of serious deficiencies, in particular, most of the elastase producers described in the literature are pathogenic for humans, the search for new effective producers continues to be an urgent problem, given that highly active producers of proteolytic enzymes, in particular elastase, are generally absent in Ukraine.

In this regard, the **purpose** of this work was to screen microorganisms isolated from the Black Sea for the presence of effective producers of proteolytic enzymes and to show that marine actinobacteria may be promising enzyme producers.

Materials and methods. The objects were microorganisms obtained from the collection of cultures of the Department of Microbiology of I.I. Mechnikov Odesa National University. In total, 10 strains of bacteria isolated from the Black Sea were studied.

For submerged fermentation, strains were cultivated in Erlenmeyer flasks containing 100 mL of a medium of the following composition (g/L): KH_2PO_4 — 1.0; $MgSO_4 \cdot 7H_2O$ — 0.75; $ZnSO_4 \cdot 7H_2O$ — 0.25; $(NH_4)_2SO_4$ — 0.5; maltose — 1.0; gelatin — 10.0; yeast autolysate — 0.15, pH 7.0. Cultures were grown at a temperature of 27 °C, under the conditions of rocking chairs (210 rpm) for 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.

The caseinolytic (total proteolytic) activity was determined by the Anson method modified by Petrova [10]. Elastase activity was determined colorimetrically by the color intensity of

the solution during the enzymatic hydrolysis of elastin stained with Congo red using the Trombridg G.O. et al. method [11] in Bondarchuk's modification [12]. The incubation mixture contained 5 mg of elastin, 2.0 mL of 0.01 M Tris-HCl buffer (pH 7.5) supplemented with 0.005 M $CaCl_2$, and 1 mL of the test drug solution. The mixture was incubated for 5 hr at 37 °C. Non-hydrolyzed elastin was separated by centrifugation at 8000g for 10 min. The color intensity was measured on an SF-26 spectrophotometer at 515 nm. The activity was calculated from a standard curve, which was obtained by measuring the color of the supernatant from complete enzymatic hydrolysis of known amounts of elastin stained with Congo red. An activity unit was taken as the amount of enzyme that catalyzes the hydrolysis of 1 mg of the substrate for 1 min under standard conditions.

Fibrinolytic and fibrinogenolytic activities were determined by the recommended methods [13] with fibrin and fibrinogen as substrates.

Protein concentration was determined by the Lowry method [14]. The standard curve of bovine serum albumin (BSA) (1 mg/mL) was constructed.

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 the 5% significance level [15].

Results. The study of the enzymatic activity of the isolates showed that on the 10th day of cultivation in the supernatant of the culture liquid, caseinolytic activity (Fig. 1) was not detected only in one isolate 56, very insignificant activity was observed in isolates 7, 20, and 50. The maximum activity was detected in isolate 247 (0.2 U /mL) and lower one — in isolates 46 (0.16 U/mL), 52 (0.15 U/mL), 51 (0.135 U/mL), 54 (0.08 U/mL), and 44 (0.05 U/mL).

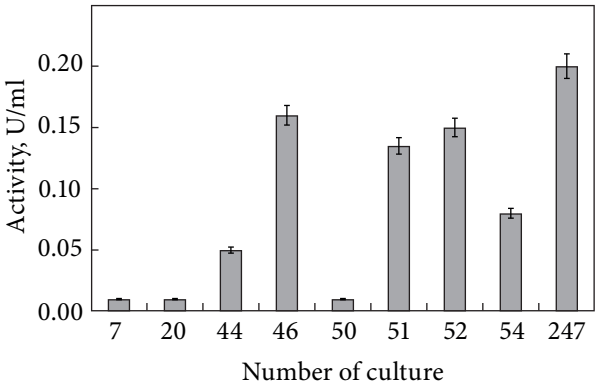

Fig. 1. Caseinolytic (total proteolytic) activity of bacteria isolated

Of the 10 studied isolates, elastase activity was found only in four of them (Fig. 2). The highest activity was found in isolates 51 and 54 (20.83 and 19.96 U/mL, respectively). Lower levels of activity (15.62 U/mL and 12.15 U/mL, respectively) were shown by isolates 52 and 247.

The studied isolates also differed in their ability to hydrolyze fibrin and fibrinogen (Fig. 3). The highest fibrinolytic activity (2.33 U/mL) was found in isolates 46 and 54, significantly lower in isolate 20 (0.5 U/mL) and isolate 44 (0.33 U/mL). The rest isolates did not show fibrinolytic activity. As for fibrinogenolytic activity, it was noted

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

Strain	Station	Depth (m)	Coordinates	
			Latitude	Longitude
7	242	1499	N 41° 31.138	E 37° 37.347
44, 50, 56	233	1537	N 41° 32.670	E 37° 37.460
20, 46, 247	258	1888	N 44° 37. 243	E 35° 42.286
51, 052, 54	269	2080	N 44° 17.329	E 35° 0.081

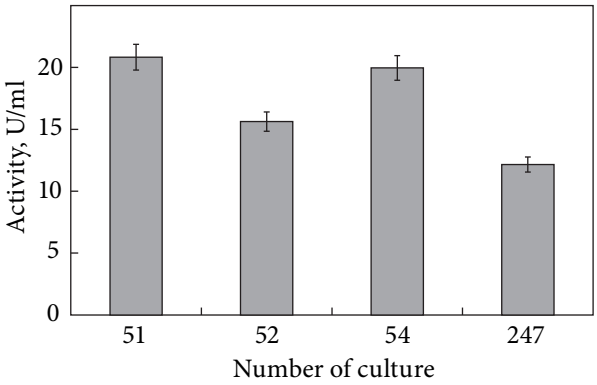

Fig. 2. Elastase activity of bacteria isolated

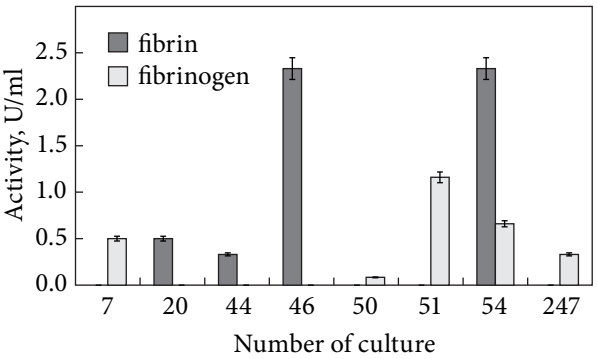

Fig. 3. Fibrinolytic and fibrinogenolytic activity of bacteria isolated

in 6 studied cultures. The highest levels of activity were observed in isolate 51 (1.16 U/mL). Lower activity was found in isolates 54 (0.66 U/mL), 7 (0.5 U/mL), and 247 (0.33 U/mL). In isolate 50, it was minimal (0.083 U/mL). There was no correlation between the levels of fibrinolytic and fibrinogenolytic activities.

No correlation was found between elastase, fibrinolytic, and fibrinogenolytic activities in the studied isolates. Thus, isolates 51, 54, and, to a lesser extent, 52 and 247 synthesize elastase activity. The highest fibrinolytic activity was in isolates 46 and 54, and fibrinogenolytic activity was in isolate 51.

Discussion. The task set in this work to screen among microorganisms isolated from the Black Sea for the presence of effective producers of proteolytic enzymes is relevant both from fundamental and practical aspects. Since the microorganisms of the marine environment and bottom sediments are poorly understood, the study of their ability to synthesize physiologically active substances can provide new knowledge about the role of microflora in the life of organisms inhabiting the Ocean and, ultimately, the functioning of marine ecosystems. Moreover, the identification of the features of the distribution of bacterial protease producers can contribute to a more successful search for these enzymes, which in the future can serve as the basis for the biotechnological production of enzymes with given substrate specificity.

Earlier [16] in our study of the proteolytic activity of 64 bacterial strains, both typical and isolated from the water and invertebrates of the Black Sea, it was shown that out of 9 studied strains that showed significant general (caseinolytic) activity, 8 showed fibrinolytic activity from 0.15 to 2.175 U/mL. The highest fibrinolytic activity was found in *Pseudoalteromonas flavipulchra* 11129 (2.175 U/mL) and *Alteromonas macleodii* 11107 (2.1 U/mL). That is, we can say that in terms of the fibrinolytic activity, the studied isolates do not differ from the activity that we previously established for *Pseudoalteromonas flavipulchra* 11129 (2.175 U/mL) and *Alteromonas macleodii* 11107 (2.1 U/mL). However, if none of the previously studied strains had elastase activity, then out of 10 isolates studied in this work, elastase activity was found in four of them. The absence of elastase activity in the strains we studied earlier was somewhat unexpected since elastase hydrolyzes the insoluble fibrillar protein elastin, which is found in the tissues of most vertebrates, including those living in marine environments, which could be due to the absence of any enzyme inducer in the growth medium. This assumption is supported by the data of the authors [9] that *Bacillus pumilis* KMM 521 isolated from the water of the Pacific Ocean synthesizes secreted elastase with high specific activity and, unlike other elastases,

the enzyme is not completely (but only by 25%) inactivated by NaCl solution.

Equally important are our data on the presence of producers with fibrinogenolytic and fibrinolytic activity. Proteolytic enzymes with fibrinogenolytic activity can be considered as the basis for the creation of drugs aimed at reducing the threat of intravascular thrombosis by limited proteolysis of fibrinogen, which circulates in the patient's bloodstream. In the case of targeted delivery, fibrinogenolytic enzymes can be used to break down intravascular thrombi, which prevent blood supply to organs in such pathologies as myocardial infarction, ischemic stroke, pulmonary embolism, etc. [1—9]. The unique specificity of fibrinogenolytic enzymes makes it possible to obtain, with their help, non-physiological fragments of the fibrinogen molecule, which, due to their power, are classified as physiological products of plasmin hydrolysis. The active functioning of the individual fragments of the molecule can generate buds along with the significance of marine isolates cells in protein-protein and protein-cell interactions [3, 4].

Thus, as a result of the work carried out, it was shown that the Black Sea, like the Pacific Ocean, is rich in marine bacterial species, which can be effective producers of a number of practically important enzymes, in particular, proteolytic ones with specificity to elastin, fibrin, and fibrinogen. This can be promising for implementation in biotechnological processes.

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БАКТЕРІЇ ЧОРНОГО МОРЯ — ПРОДУЦЕНТИ ПРОТЕОЛІТИЧНИХ ФЕРМЕНТІВ

Незважаючи на те що в останні роки спостерігається певне посилення інтересу до вивчення морських мікроорганізмів, тим не менш, морські бактерії як продуценти біологічно активних речовин, зокрема ферментів, досі мало вивчені. Морська біота істотно відрізняється від наземної, тому існує велика ймовірність виявлення в морському середовищі відмінних від наземних бактерій продуцентів ферментів з унікальною специфічністю та активністю, для потреб сучасних біотехнологій. Важливу роль у цих дослідженнях відіграють протеолітичні ферменти. Оскільки більшість мікробних продуцентів характеризуються рядом серйозних недоліків, зокрема більшість описаних у літературі продуцентів еластази є патогенними для людини, пошук нових ефективних продуцентів продовжує залишатися актуальною проблемою, враховуючи високу активність продуцентів протеолітичних ферментів, зокрема еластази, які в Україні взагалі відсутні. У зв'язку з цим метою даної роботи був скринінг виділених із Чорного моря мікроорганізмів на наявність ефективних продуцентів протеолітичних ферментів. **Методи.** Використовували методи визначення протеолітичної (казеїнолітичної, еластолітичної, фібринолітичної, та фібриногенолітичної) активності. **Результати.** Дослідження ферментативної активності ізолятів показало, що на 10-ту добу культивування в супернатанті культуральної рідини казеїнолітична активність не виявлена лише в одному ізоляті 56, і дуже незначна активність спостерігалася в ізолятах 7, 20 і 50. Максимальна активність виявлена в ізоляті 247 (0,2 од/мл), нижча — в ізолятах 46 (0,16 од/мл), 52 (0,15 од/мл), 51 (0,135 од/мл), 54 (0,08 од/мл) і 44 (0,05 од /мл). З 10 досліджених ізолятів активність еластази виявлена лише у чотирьох з них. Найвищу активність виявили в ізолятах 51 та 54 (відповідно 20,83 та 19,96 од /мл). Нижчі рівні активності (15,62 од /мл і 12,15 од /мл відповідно) показали ізоляти 52 та 247. Досліджені ізоляти також відрізнялися за здатністю гідролізувати фібрин та фібриноген. Найвищу фібринолітичну активність (2,33 од /мл) виявили в ізолятах 46 та 54, значно нижчу — в ізолятах 20 (0,5 од /мл) та 44 (0,33 од /мл). Решта ізолятів не виявляли фібринолітичної активності. Що стосується фібриногенолітичної активності, то вона відзначена у 6 досліджуваних культурах. Найвищі рівні активності спостерігалися в ізоляті 51 (1,16 од/мл). Нижчу активність виявили в ізолятах 54 (0,66 од /мл), 7 (0,5 од/мл) та 247 (0,33 од/мл). В ізоляті 50 вона була мінімальною (0,083 од/мл). Не виявлено кореляції між рівнями фібринолітичної та фібриногенолітичної активності. **Висновки.** Не виявлено кореляції між еластазною, фібринолітичною та фібриногенолітичною активністями в досліджуваних ізолятах. Так, ізоляти 51, 54 і, меншою мірою, 52 і 247 синтезують еластазну активність. Найвища фібринолітична активність була в ізолятах 46 і 54, а фібриногенолітична — в ізоляті 51. Показано, що Чорне море багате на види морських бактерій, які можуть бути ефективними продуцентами ряду практично важливих ферментів, зокрема протеолітичних, специфічних до еластину, фібрину та фібриногену, які можуть бути перспективними для впровадження в біотехнологічні процеси.

Ключові слова: *актинобактерії, Чорне море, протеолітичні ферменти.*