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KERATINASE, CASEINOLITIC, CELLULASE, AND β-MANANASE ACTIVITIES OF BACTERIA ISOLATED FROM THE BLACK SEA

For a long time, the main interest in the marine environment, considered extreme, was the isolation and identification of natural products with biological properties, and for that, numerous organisms and chemical structures have been studied. Thus, marine bacteria isolated from various substrates, such as sediments, seawater, and mangrove detritus, are producers of enzymes with different activities, i.e., amylase, cellulase, alginate lyase, chitinase, glucosidase, inulinase, keratinase, ligninase, xylanase, and others. Nowadays, researchers are also focusing on the enzymes produced in the marine environment that can present special properties. Therefore, ***the aim*** *of this study was to investigate the ability of marine strains of microorganisms to exhibit cellulase, β-mannanase, keratinase, and caseinolytic activities.* ***Methods.*** *Enzymatic activities were studied in the culture liquid supernatant. To determine β-mannanase and cellulase activities, guar gum galactomannan and Na-carboxymethylcellulose respectively were used as substrates. Casein and crushed defatted feathers served as substrates for the determination of proteolytic activity.* ***Results.*** *Growing 10 cultures of microorganisms on a nutrient medium containing chicken feathers as the sole source of carbon and nitrogen (nutrient medium 1) did not give positive results. When using medium 2, active growth was observed in four of the studied strains (51, 52, 54, 247) in the supernatant of culture liquid (CLS), the activity of which both to keratin (6.0—16.0 U/mL) and casein (0.025—0.33 U/mL) was found. In the CLS of only six of the 10 studied cultures (7, 20, 51, 52, 50, 247), cellulase and β-mannanase activities were observed. The highest cellulase activity was found in culture 20 (1.8 U/mL). The activity of culture 7 was somewhat lower (1.0 U/mL). An insignificant activity was noted in cultures 54 (0.06 U/mL), 56, and 50 (0.05 U/mL). Trace levels of activity were observed in culture 247.* ***Conclusions.*** *Strains 7, 20, 247, and 51, for the first time isolated from the Black Sea, are promising for further studies as producers of cellulase, β-mannanase, keratinase, and caseinolytic enzymes.*

Keywords: *bacteria from the Black Sea; cellulase, β-mannanase, keratinase, and caseinolytic activities.*

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Marine microorganisms have been variously estimated to make up about 70 - 90%, of the biomass in the ocean. Taken together, they form the marine microbiome. Over billions of years, this microbiome has evolved through many lifestyles and adaptations and has come to participate in the global cycling of almost all chemical elements. Microorganisms are crucial to nutrient recycling in ecosystems as they act as decomposers. They are also responsible for nearly all photosynthesis that occurs in the ocean, as well as the cycling of carbon, nitrogen, phosphorus and other nutrients and trace elements. Marine microorganisms sequester large amounts of carbon and produce much of the world's oxygen. For a long time, the main interest in the marine environment, considered extreme, was focused on the isolation and identification of natural products with biological properties, and for that, numerous organisms and chemical structures were studied [1, 2]. Marine bacteria isolated from various substrates, such as sediments, seawater, and mangrove detritus, are producers of enzymes with different activities, including amylase, cellulase, alginate lyase, chitinase, glucosidase, inulinase, keratinase, ligninase, xylanase, and others [3]. Nowadays, researchers are also focusing on the enzymes produced in the marine environment that can present special properties [4].

Therefore, the aim of this study was to investigate the ability of marine strains of microorganisms to exhibit cellulase, β-mannanase, keratinase, and caseinolytic activities.

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, 52, 54	269	2080	N 44° 17.329	E 35° 0.081

Materials and methods. The objects were microorganisms obtained from the collection of cultures of the Department of Microbiology of I.I. Mechnikov Odessa National University. In total, 10 strains of bacteria isolated from the Black Sea were studied (Table 1).

To study the proteolytic activity, microorganism cultures were grown in 50 mL test tubes on a shaker with a rotation speed of 232 rpm at a temperature of 28 °C for 10 days. For this purpose, two basic nutrient media of the following composition (g/L) were used: 1) K_2HPO_4 — 0.3; KH_2PO_4 — 0.4; $MgSO_4 \cdot 7H_2O$ — 0.1; NaCl — 0.5; defatted chicken feathers — 5; H_2O — up to 1 L; pH 6.8—7.0 (specialized medium for the synthesis of keratinases); 2) maltose — 1.0; food gelatin — 10.0; KH_2PO_4— 1.6; $MgSO_4 \cdot 7H_2O$ — 0.75; $ZnSO_4 \cdot 7H_2O$ — 0.25; $(NH_4)_2SO_4$ — 0.5; yeast autolysate — 0.15; distilled water — up to 1 L; pH 7.0—7.2 (medium for the synthesis of the rest investigated enzymes). Chicken feathers were defatted with a 1:1 mixture of chloroform and methanol, washed three times with distilled water, and dried. The culture liquid was centrifuged at 7000 g for 10 min; the resulting supernatant (culture liquid, CLS) was used for further studies.

A daily culture of microorganisms was used as an inoculum.

The caseinolytic activity was determined by the Anson method modified by Petrova [5].

The keratinase activity (KerA) was determined by UV absorption at 280 nm of hydrolysis products of keratin-containing raw materials. The reaction mixture consisting of 10 mg of cut small defatted chicken feathers, 2.5 mL of 0.05 M boron-borate buffer (pH 9.0), and 1 mL of CLS was kept in a thermostat at 37 °C for 3 hr and then filtered. To determine KerA, two controls were used: 10 mg of the feathers, 2.5 mL of 0.05 M boron-borate buffer (pH 9.2), and 1 mL of distilled water (1); 2.5 mL of 0.05 M boron-borate buffer (pH 9.2) and 1 mL of CLS (2). The sum of the two controls was subtracted from the A_{280} values obtained by measuring the optical density

of filtrates. The absorbance increase at 280 nm of the test sample filtrate relative to the controls was taken as the degree of protein release [6]. A unit of keratinase activity (1 U/mL = 0.01) was taken as the amount of enzyme that causes an increase in absorption by 0.01 after 3 hr of incubation.

Protein was determined by the Lowry method [7].

The β-mannanase activity was estimated using a 1% solution of guar gum galactomannan («Sigma-Aldrich») as a substrate in the 0.1 M phosphate citrate buffer, pH 5.0. The reaction mixture contained 0.25 mL of supernatant and 0.25 mL of substrate solution. It was incubated at 40 °C for 30 min. 2.5 mL of a dinitrosalicylic acid reagent solution was added to the reaction mixture after the incubation. then the reaction mixtures were boiled at 100 °C for 5 min. Absorbance was measured at 540 nm [8]. The carboxymethylcellulase (CM-cellulase) activity of the CLS cultures was determined as described above, but a 1% Na-carboxymethylcellulose 25—75 mPas («Millipore») solution was used as a substrate, and glucose was used as a standard [8]. The unit (U) of the CM-cellulase or β-mannanase activity is the amount of enzyme required to liberate, under the assay conditions, 1 μmol/min reducing sugar expressed as a glucose (mannose) equivalent.

All experiments were replicated 3—5 times. Statistical analysis of the results of the experimental series was carried out by standard methods using Student's t-criterion. The results presented graphically were obtained using Microsoft Office Excel 2007.

Results. Growing 10 cultures of microorganisms on a nutrient medium containing chicken feathers as the sole source of carbon and nitrogen (nutrient medium 1) did not give positive results: no keratinase activity was detected in the CLS of any of the strains studied (Fig. 1).

When using medium 2, active growth was observed in four of the studied cultures (51, 52, 54, 247), in the culture liquid of which activity both to keratin (12.0—16.0 U/mL) and casein (0.025—0.33 U/mL) was found (Fig. 1, 2). The

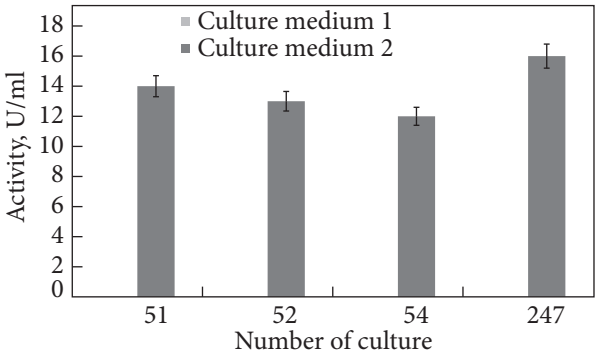

Fig. 1. Keratinase activity of the studied cultures on two types of nutrient medium

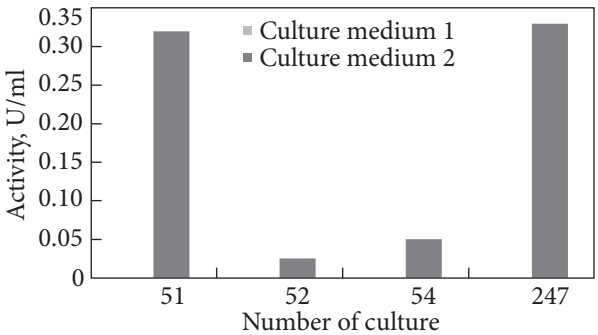

Fig. 2. Caseinolytic activity of the studied cultures

highest keratinase and caseinolytic activities have been shown for culture 247.

In the CLS of only six of the 10 studied cultures (7, 20, 51, 52, 50, 247), the cellulase and β-mannanase activities were observed (Figs. 3, 4). The highest cellulase activity was found in culture 20 (1.8 U/mL) (Fig. 3). The activity of culture 7 was somewhat lower (1.0 U/mL). Insignificant activity was noted in 54 (0.06 U/mL), 56, and 50 (0.05 U/mL) cultures. Trace levels of activity were observed in culture 247 (Fig. 3).

All cultures that exhibited the cellulase activity were also characterized by the β-mannanase activity. The highest β-mannanase activity was detected for culture 20 (3.6 U/mL). The activity of culture 7 was two times lower (1.8 U/mL). Much lower activity was detected in cultures 56, 50 (0.75 U/mL) and insignificant one - in culture

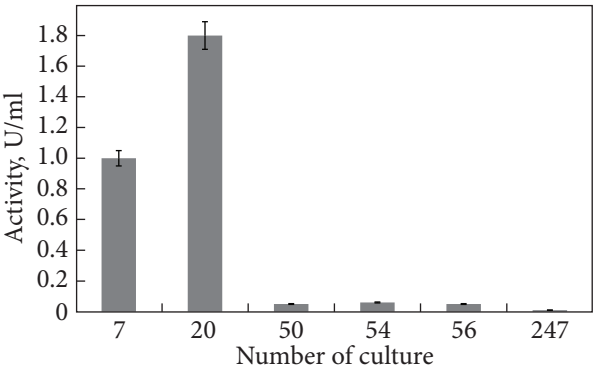

Fig. 3. CM-cellulase activity of marine cultures

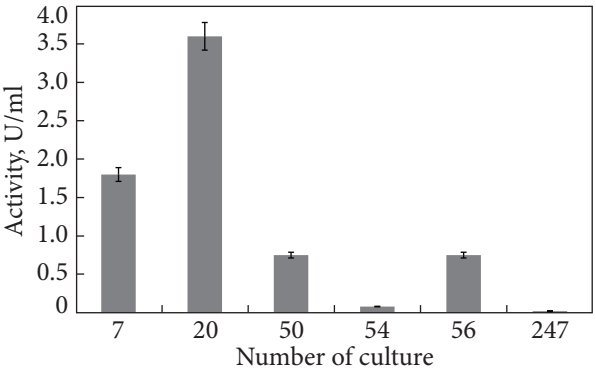

Fig. 4. β-Mannanase activity of marine bacteria

54 (0.08 U/mL). There was disclosed practically no activity in culture 247 (Fig. 4).

Thus, we revealed that cultures 7 and 20 showed the highest cellulase and β-mannanase activity among the studied strains. It should be noted that both cultures were isolated from the water of the Black Sea at a depth of 5—10 m. The highest levels of both keratinase and caseinolytic activities were shown in cultures 247 and 51.

Discussion. The marine environment represents a great opportunity for the exploration of new enzymes and molecules. However, these ecosystems are already threatened by the pollution that might cause the extinction of many species of this poorly studied universe [9]. Marine enzymes are capable of being active in high-salt concentrations, large ranges of temperature, pH, organic solvents, surfactants, metal ions, and high incidence of light and pressure. Additionally, enzymes obtained from cold places such as the Antarctic pole can also present activity at extreme low temperatures [10]. Therefore, these enzymes might remain active under varied operational conditions, providing competitiveness and efficiency to different industrial processes [11].

Very few studies have characterized the enzyme systems of marine bacteria degrading substrates that are relevant in marine systems. In this regard, our studies of the proteolytic and glycolytic activity of bacterial cultures isolated from different depths of the Black Sea water are relevant. In particular, we established that two cultures 20 and 247 show the highest β-mannanase and keratinase activity respectively. Both cultures were isolated from samples taken at the same station 258 and isolated from the same depth 1888 m, with the same latitude and longitude parameters. These results may be promising for further studies to increase the activity of the tested cultures. There are no effective producers with β-mannanase and keratinase activity in Ukraine. However, these enzymes are of great practical importance. So, β-mannanase (EC 3.2.1.78) is an enzyme that catalyze the hydrolysis of the β-mannosidic bond in the main chain of hemicellulose, as well as gluco- and galactomannans with the formation of mannooligosaccharides, mannose, glucose, and galactose. Cellulose and hemicellulose, due to their chemical properties, are substrates of great biotechnological importance. On the one hand, wastes from the woodworking, paper, and agricultural industries can be environmental pollution factors, and, on the other hand, they have a great technological potential as a source of poly- and oligosaccharides. Due to the ability to hydrolyze hemicelluloses, β-mannanase has found application in various industries: pharmaceutical, pulp and paper, in the production of gas, biofuels and cheap energy, food and feed, as well as prebiotic mannooligosaccharides [12]. Therefore, the search for producers of enzymes of the mannan-degrading

complex of a certain specificity remains today an urgent problem with broad prospects.

As for microbial keratinases, interest in the study is due to the fact that they are able to cleave insoluble keratin-containing substrates, as well as to hydrolyze a number of other protein substrates such as collagen, elastin, and fibrin. The possibility of using keratinases in dehairing processes in the leather industry and in the processing of feather keratin, which is the most significant waste in poultry farms, is being studied [13, 14]. Now there is only one way to dispose of feathers - thermal sintering them and grinding into flour. Moreover, most of this product is peptides and ash. The use of enzymes for such processing will allow the use of low temperatures and obtain a better finished product, with a high content of amino acids [12—14]. Today, interest in microbial keratinases has grown due to the accidental discovery of the hydrolyzing effect of keratinase on prion proteins. Thus, the variety of properties of microbial keratinases, in particularisolated from marine bacteria, and the possibilities of using them have not been studied properly. At the same time, this group of proteolytic enzymes has so far been found only in a small number of representatives of the microbial world.

Therefore, strains 7, 20, 247, and 51, for the first time isolated from the Black Sea, are of interest to researchers for further studies as producers of cellulase, β-mannanase, keratinase, and caseinolytic enzymes respectively. Their taxonomic position will be established, as it was determined by authors [15] who identified the facultative anaerobic spore-forming bacteria (*Bacillus, Paenibacillus, Lysinibacillus, Brevibacillus)* in the Black Sea at depths of 888—2080 m.

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КЕРАТИНАЗНА, КАЗЕЇНОЛІТИЧНА, ЦЕЛЮЛАЗНА
ТА β -МАНАНАЗНА АКТИВНОСТІ БАКТЕРІЙ, ВИДІЛЕНИХ ІЗ ЧОРНОГО МОРЯ

Довгий час основним джерелом інтересу до морського середовища, яке вважалося екстремальним, було виділення та ідентифікація природних продуктів з біологічними властивостями, для чого вже вивчено безліч організмів і хімічних структур. Таким чином, морські бактерії, виділені з різних субстратів, таких як відкладення, морська вода, мангровий детрит є продуцентами ферментів різної активності, а саме амілази, целюлази, альгінатліази, хітинази, глюкозидази, інулінази, кератинази, лігнінази, ксиланази та ін. Нині дослідження також зосереджені на ферментах, що виробляються в морському середовищі і можуть проявляти особливі властивості. Тому **метою даної роботи** було дослідити здатність морських штамів мікроорганізмів проявляти целюлазну, β-мананазу, кератиназну та казеїнолітичну активності. **Методи.** Ензиматичні активності досліджували у супернатанті культуральної рідини. Для визначення активності використовували для β-мананазної — галактоманан гуару, для целюлазної — Na-карбоксиметилцелюлозу. Для визначення протеолітичних активностей як субстрат використовували казеїн та обезжирене куряче перо. **Результати.** Вирощування 10 культур мікроорганізмів на живильному середовищі, що містить куряче пір'я як єдине джерело вуглецю та азоту (середовище 1) не дало позитивних результатів. При використанні середовища 2 активний ріст спостерігався у чотирьох досліджених штамів (51, 52, 54, 247), в культуральній рідині (КЛС) активність як до кератину (12,0—16,0 Од/мл), так і до казеїну (0,025—0,33 Од/мл). У КЛС лише у шести з 10 досліджуваних культур (7, 20, 51, 52, 50, 247) спостерігали целюлазну та β-мананазну активності. Найвищу целюлазну активність виявлено в культурі 20 (1,8 Од/мл). Активність культури 7 була дещо нижчою (1,0 Од/мл). Незначна активність відзначена в культурах 54 (0,06 ОД/мл), 56, 50 (0,05 Од/мл). Слідові рівні активності спостерігали в культурі 247. **Висновки.** Штами 7, 20, 247 і 51, вперше виділені з Чорного моря, перспективні для подальшого вивчення як продуценти целюлаз, β-мананаз, кератиназ і казеїнолітичних ферментів.

Ключові слова: *бактерії з води Чорного моря; целюлазна, β-мананазна, кератиназна та казеїнолітична активності.*