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REPRESENTATIVES OF *BACILLUS* FROM DEEP-WATER BOTTOM SEDIMENTS OF THE BLACK SEA — PRODUCERS OF ELASTASES, FIBRIN(OGEN)ASES, AND COLLAGENASES

Among microorganisms, bacteria and fungi are reported to be good sources of different types of enzymes, in particular proteases, which have a broad range of applications in industrial processes and products and are representative of most worldwide enzyme sales. The genus Bacillus is probably the most important bacterial source of proteases and is capable of producing high yields of neutral and alkaline proteolytic enzymes with remarkable properties, such as high stability toward extreme temperatures, pH, organic solvents, detergents, and oxidizing compounds. Earlier we have shown the ability of a number of strains of Bacillus sp. isolated from the bottom sediments of the Black Sea: 051, 054, 052 (depth 2080 m), and 247 (depth 1888 m) to display elastase activity (20.83 U/mL, 19.96 U/mL, 15.62 U/mL and 12.15 U/mL, respectively). Since the bacterial population of the deep-sea bottom sediments of the Black Sea has been little studied, the purpose of this work was to search for effective protease producers among the microbiota of the Black Sea water and sediments obtained from its various depths. Methods. The objects of the study were 20 cultures isolated from bottom sediments from 4 points at depths of 888—2080 m in the Black Sea. The cultures were grown under conditions of deep cultivation at 28 °C, with a mixing speed of the nutrient medium of 230 rpm for 2 days. Methods for determining proteolytic (elastolytic, fibrinolytic, fibrinogenolytic, and collagenase) activity in the culture liquid supernatant were used. Results. The research on the ability of the supernatants of the studied cultures to hydrolyze various proteolytic substrates has shown that promising for further investigations can be cultures 248 and 249, isolated under the same conditions (1499 m, 15—20 cm), but being representatives of different species, namely Bacillus subtilis and B. licheniformis, respectively. Supernatants of their culture liquids showed the greatest activity toward fibrin (20.5 U/mL and 19.0 U/mL) and fibrinogen (21.66 U/mL and 20 U/mL, respectively), while cultures of B. licheniformis 249 (1499 m, 15—20 cm), Priestia

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megaterium 55 (1537 m, 0–5 cm), and *B. subtilis* 1 (1499 m, 5–10 cm), which were isolated under different conditions, showed high activity toward elastin (33.3 U/mL, 31.2 U/mL and 29 U/mL, respectively). *B. subtilis* 1 is able to hydrolyze all investigated proteolytic substrates: elastin, fibrin, fibrinogen, and collagen, but the level of these activities was lower than in the above-mentioned strains. **Conclusions.** According to their catalytic properties, a number of representatives of bacteria *Bacillus licheniformis* 249, *Priestia megaterium* 55, and *Bacillus subtilis* 1 isolated from the deep-water bottom sediments of the Black sea may be promising for further research as producers of enzymes with elastolytic, fibrinolytic, and fibrinogenolytic activity.

Keywords: bacteria isolated from the deep-water bottom sediments of the Black sea; elastolytic, fibrinolytic, fibrinogenolytic, collagenase activity.

Marine environments, as a large ecosystem, are among the most significant sources of natural bioactive compounds (Rigogliuso, Campora, & Ghersi, 2023) and are among the richest and most sophisticated ecosystems on earth in terms of biodiversity (Esposito et al., 2022). The harsh conditions of marine ecosystems produce in marine organisms various types of molecules with unique structural and functional characteristics (Zhang & Kim, 2010). Compared to terrestrial species, marine species produce a greater number of bioactive compounds, in particular, enzymes. Researchers have recently become more interested in studying the various applications of marine enzymes due to their high biodiversity and ease of extraction (Dionisi, Lozada & Olivera, 2012). Among microorganisms, bacteria and fungi are reported to be good sources of different types of enzymes, in particular, proteases, which have a broad range of applications in industrial processes and products and are representative of most worldwide enzyme sales. The genus *Bacillus* is probably the most important bacterial source of proteases and is capable of producing high yields of neutral and alkaline proteolytic enzymes with remarkable properties, such as high stability toward extreme temperatures, pH, organic solvents, detergents, and oxidizing compounds.

Earlier (Gudzenko et al., 2022) we have shown the ability of a number of strains of *Bacillus* sp., isolated from the bottom sediments of the Black Sea, namely 051, 054, 052 (depth 2080 m), and 247 (depth 1888 m), display elastase activity (20.83 U/mL, 19.96 U/mL, 15.62 U/mL, and 12.15 U/mL, respectively). Since the bacterial population of the deep-sea bottom sediments of the Black Sea has been little studied, the purpose of this work was to search for

effective protease producers among the microbiota of the Black Sea water and sediments obtained from its various depths.

Materials and Methods. The objects of research were 20 strains that were isolated from bottom sediments from 4 points at depths of 888–2080 m in the Black Sea, from the horizons of cylindrical cores with an interval of 5 cm. The samples from which the strains were identified were taken during the M84/2 expedition of the University of Bremen on the Meteor ship in March 2011 and transferred to Mechnikov Odesa National University for microbiological research by Yu.P. Zaitsev and B.G. Alexandrov (Institute of Marine Biology, NASU). Selected strains were identified previously (Ivanytsia et al., 2017) and are listed in Table 1.

For submerged fermentation, the strains were cultivated in Erlenmeyer flasks containing 100 mL of medium of the following compositions: KH_2PO_4 — 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ — 0.75; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ — 0.25; $(\text{NH}_4)_2\text{SO}_4$ — 0.5; maltose — 1.0; gelatin — 10.0; yeast autolysate — 0.15, pH 7.0. Cultures were grown at a temperature of 28 °C, with a rotation speed of 230 rpm for 4 days. At the end of fermentation, the biomass was separated by centrifugation at 5000 g for 30 min. Enzymatic activity was determined in the culture liquid supernatant.

Elastase activity was determined colorimetrically by the color intensity of the solution during the enzymatic hydrolysis of elastin stained with Congo-rot using the Trowbridge et al. method (Trowbridge & Moon, 1972). 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 test drug solution. The mixture was incubated for 5

hours at 37 °C. Non-hydrolyzed elastin was separated by centrifugation at 8000 g, 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 culture liquid supernatant from complete enzymatic hydrolysis of known amounts of elastin stained with Congo rot. 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.

To determine fibrinolytic activity, fibrinogen was used as a substrate (Nidialkova et al., 2016). 1 mg of fibrinogen, 1.8 mL of Tris-HCl buffer (pH 7.5), and 0.2 mL of the studied preparation were added to the test sample. Incubation lasted 30–45 min at 37 °C. The reaction stopped by adding 2 mL of 10% trichloroacetic acid (TCA). TCA was added to the control sample immediately. Samples were kept at room temperature for 20 min and then centrifuged

at 10 000 g for 10 min to remove precipitated protein. Absorption was measured on an SF-26 spectrophotometer at a wavelength of 275 nm. The amount of enzyme that, under the conditions of the experiment, increased absorption by 0.01 for 1 min was taken as a unit of activity.

Collagenase activity was defined by the content of free amino acids in the reaction mixture in the reaction with ninhydrin (Moore & Stein, 1948). The unit of activity was the number of micromoles of released amino acids according to the standard curve constructed for leucine. Fibrinolytic activity was determined by the Masada method (Nidialkova et al., 2016). The formation of fibrin cleavage products was measured on an SF-26 spectrophotometer at 275 nm. The amount of enzyme that increases the optical density of the reaction mixture by 0.01 for 1 min was taken as a unit of fibrinolytic activity.

All experiments were performed in no less than 3–5 replications. Statistical processing of the results

Table 1. Studied strains

Strain number (number in Fig. 1)	Station number, depth (m), horizon (cm)	Strains
1 (1)	242, 1499, 5–10	<i>Bacillus subtilis</i>
2 (2)	258, 888, 0–5	<i>Metabacillus idriensis</i>
08 (3)	242, 1499, 10–15	<i>Bacillus atrophaeus</i>
013 (4)	242, 1499, 10–15	<i>Bacillus subtilis</i>
033 (5)	242, 1499, 25–30	<i>Bacillus subtilis</i>
035 (6)	258, 888, 30–35	<i>Priestia megaterium</i>
043 (7)	233, 1537, 15–20	<i>Bacillus licheniformis</i>
055 (8)	233, 1537, 0–5	<i>Bacillus licheniformis</i>
55 (9)	233, 1537, 0–5	<i>Priestia megaterium</i>
57 (10)	242, 1499, 0–5	<i>Robertmurraya siralis</i>
116 (11)	269, 2080, 0–5	Unknown
212 (12)	233, 1537, 5–10	<i>Bacillus subtilis</i>
231 (13)	258, 888, 5–10	<i>Bacillus subtilis</i>
232 (14)	258, 888, 5–10	<i>Bacillus subtilis</i>
245 (15)	242, 1499, 25–30	<i>Bacillus licheniformis</i>
Myco (16)	258, 888, 0–5	Unknown (possibly <i>B. mycooides</i> by specific morphology)
A (17)	258, 888, 0–5	<i>Bacillus pumilus</i>
021 (18)	258, 888, 10–15	<i>Bacillus subtilis</i>
249 (19)	242, 1499, 15–20	<i>Bacillus licheniformis</i>
248 (20)	242, 1499, 15–20	<i>Bacillus subtilis</i>

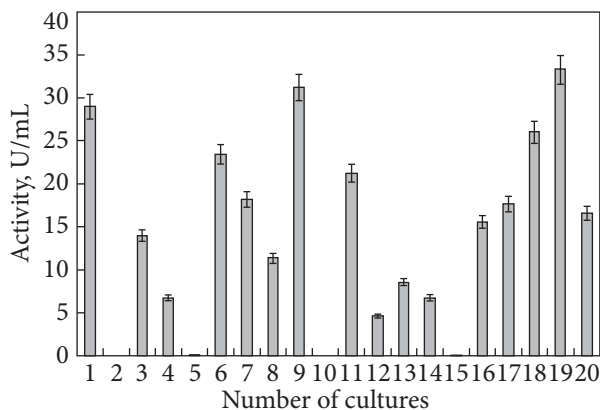


Fig. 1. Elastase activity of culture liquid supernatant of *Bacillus* strains isolated from the deep-water bottom sediments of the Black sea

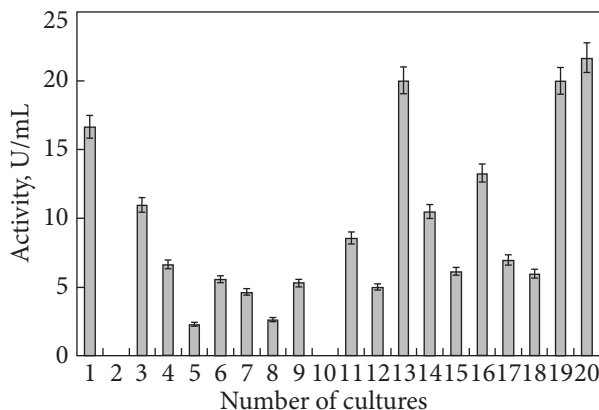


Fig. 2. Fibrinolytic activity of culture liquid supernatant of *Bacillus* strains isolated from the deep-water bottom sediments of the Black sea

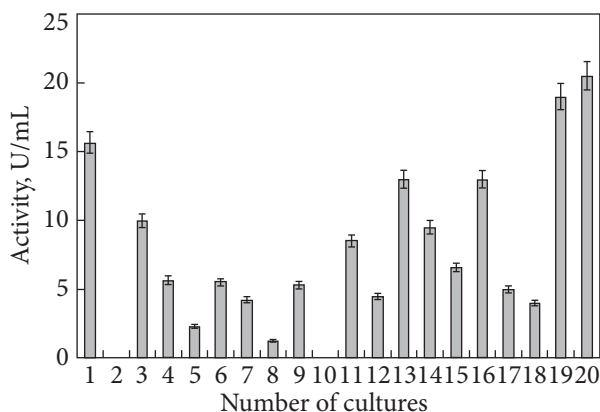


Fig. 3. Fibrinogenolytic activity of culture liquid supernatant of *Bacillus* strains, isolated from the deep-water bottom sediments of the Black sea

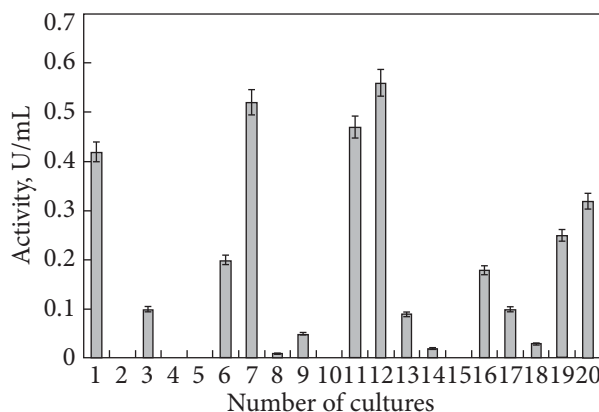


Fig. 4. Collagenase activity of culture liquid supernatant of *Bacillus* strains isolated from the deep-water bottom sediments of the Black sea

of the experimental series was carried out by standard methods using Student's t-test. The value of the null hypothesis $p < 0.05$ was taken as the critical level of reliability.

Results. The study of elastase activity showed (Fig. 1) that on the fourth day of cultivation in the supernatant of the culture liquid, activity was detected in 16 of 20 *Bacillus* strains studied. The highest activity was observed in strain 19 (33.3 U/mL). The activity was slightly lower in strains 9 (31.2 U/mL), 1 (29 U/mL), 18 (26 U/mL), 6 (23.4 U/mL), and 11 (21.3 U/mL). Slightly less activity was observed in strains 7 (18.2 U/mL), 17 (17.7 U/mL), 20 (16.6 U/mL),

16 (15.6 U/mL), 3 (14 U/mL), and 8 (11.4 U/mL). Lower levels of activity were noted for strains 13 (8.59 U/mL), 14 and 4 (6.77 U/mL), and 12 (4.68 U/mL). Traces of activity levels were found in strains 5 and 15 (0.05 and 0.038 U/mL, respectively). Only two of the 20 studied strains (2 and 10) did not show the ability to hydrolyze elastin under experimental conditions.

The study of fibrinolytic activity showed that 18 strains were able to hydrolyze fibrin under experimental conditions (Fig. 2). The highest activity was observed in strains 20 (20.5 U/mL), 19 (19.0 U/mL), 1 (15.66 U/mL), 16 and 13 (13 U/mL), 3 (10 U/mL), and

14 (9.5 U/mL). Strains 11 (8.5 U/mL), 15 (6.6 U/mL), 4 (5.66 U/mL), 5 (2.33 U/mL), 6 (5.5 U/mL), 9 (5.33 U/mL), 17 (5 U/mL), 7 (4.26 U/mL), 18 (4 U/mL), and 8 (1.26 U/mL) showed lower fibrinolytic activity.

Like fibrinolytic, fibrinogenolytic activity was not detected under experimental conditions only in 2 cultures (2, 10) (Fig. 3). The highest activity was observed in strains 20 (21.66 U/mL), 19 and 13 (20 U/mL), 1 (16, 66 U/mL), and 16 (13.3 U/mL). The activity was slightly lower in strains 3 (11 U/mL), 14 (10.5 U/mL), 11 (8.6 U/mL), 17 (7 U/mL), 4 (6.66 U/mL), 15 (6.16 U/mL), 18 (6.0 U/mL), 6 (5.6 U/mL), 9 (5.33 U/mL), 12 (5.0 U/mL), 7 (4.66 U/mL), and 5 (2.33 U/mL). There is a correlation between the presence of fibrin- and fibrinogenolytic activity, but the levels of activity differed among the strains studied.

As for collagenase activity (Fig. 4), it was detected in 15 out of 20 *Bacillus* cultures studied, however, its level was insignificant and ranged from 0.58 to 0.05 U/mL. The highest activity was demonstrated by 5 strains, namely 12 (0.56 U/mL), 7 (0.52 U/mL), 11 (0.47 U/mL), 1 (0.42 U/mL), and 20 (0.32 U/mL). Lower levels of activity were observed in strains 19 (0.25 U/mL), 6 (0.2 U/mL), 16 (0.18 U/mL), 3 and 17 (0.1 U/mL), and 13 (0.09 U/mL). Traces of activity were found in strains 8 (0.01 U/mL), 9 (0.05 U/mL), 14 (0.02 U/mL), and 18 (0.03 U/mL). The supernatants of culture liquids of 5 strains — 2, 4, 5, 10, and 15 — did not hydrolyze collagen.

Discussion. The consumption of various enzymes in industrial applications around the world has increased immensely. Nowadays, industries are more focused on incorporating microbial enzymes in multiple processes to avoid the hazardous effects of chemicals. Among the commercially exploited enzymes, proteases are the most abundantly used enzymes in different industries. Moreover, many commercial proteases have been characterized and purified from different *Bacillus* species.

In biotechnological processes involving proteases, there are important enzymes with different physico-

chemical and catalytic properties and substrate specificity. We investigated 20 strains of microorganisms isolated from different horizons of deep-sea deposits of the hydrogen sulfide zone of the Black Sea by the authors (Ivanytsia et al., 2017) and classified by the morphological, physiological, and biochemical characteristics as a group of facultatively anaerobic spore-forming bacteria. The analysis of the ability of the supernatants of the studied cultures to hydrolyze various proteolytic substrates showed that promising for further research can be cultures 248 and 249 isolated under the same conditions (1499 m, 15–20 cm) but representatives of different species, in particular *B. subtilis* and *B. licheniformis*, respectively. Supernatants of their culture liquids showed the greatest activity toward fibrin (20.5 U/mL and 19.0 U/mL) and fibrinogen (21.66 U/mL and 20 U/mL, respectively), while cultures of *B. licheniformis* 249 (1499 m, 15–20 cm), *Priestia megaterium* 55 (1537 m, 0–5 cm), and *B. subtilis* 1 (1499 m, 5–10 cm) isolated under different conditions, showed high activity toward elastin (33.3 U/mL, 31.2 U/mL, and 29 U/mL, respectively). Interestingly, strain *B. subtilis* 1 is able to hydrolyze all investigated proteolytic substrates: elastin, fibrin, fibrinogen, and collagen, but the level of all activities was lower than in the above-mentioned strains. The analysis of the obtained data does not make it possible to establish a relationship between certain enzyme activity and the conditions under which the cultures are isolated, as well as the species of the strain. Most likely, the different activity of the studied cultures is determined by the structural features of both the enzyme and the strain of the microorganism. The only exceptions are representatives of the rare and little-studied genera *Metabacillus* and *Robertmyrraya*. Taking into account the observation of the limited ability to form endospores in strain 2 even in the presence of Mn^{2+} , we can assume the aboriginality of these strains for the deep-sea bottom sediments of the Black Sea and, as a result, the inability to utilize proteins of animal origin.

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ПРЕДСТАВНИКИ *BACILLUS* ІЗ ГЛИБОКОВОДНИХ ВІДКЛАДЕНЬ ЧОРНОГО МОРЯ — ПРОДУЦЕНТИ ЕЛАСТАЗ, ФІБРИН(ОГЕН)АЗ ТА КОЛАГЕНАЗ

Серед мікроорганізмів бактерії та гриби вважаються хорошими джерелами різних видів ферментів, зокрема протеаз, які мають широкий спектр застосування в промислових процесах і продуктах і є представниками більшості світових продажів ферментів. Рід *Bacillus*, ймовірно, є найважливішим бактеріальним джерелом протеаз і здатний виробляти високі кількості нейтральних і лужних протеолітичних ферментів з такими властивостями, як висока стабільність до екстремальних температур, рН, органічних розчинників, детергентів і окислювачів. Раніше було показано здатність ряду штамів *Bacillus* sp., виділених із донних відкладень Чорного моря: 051, 054, 052 (глибина 2080 м) та 247 (глибина 1888 м) проявляти еластазну активність (20,83 од/мл, 19,96 од/мл, 15,62 од/мл та 12,15 од/мл відповідно). Оскільки бактеріальна популяція глибоководних донних відкладень Чорного моря мало вивчена, метою даної роботи був пошук ефективних продуцентів протеаз серед мікробіоти Чорного моря та осадів, отриманих з різних його глибин. **Методи.** Об'єктами дослідження були 20 культур, виділених із донних відкладень із 4 точок на глибині 888—2080 м у Чорному морі. Культури вирощували в умовах глибокого культивування при 28 °С, зі швидкістю перемішування живильного середовища 230 об/хв протягом 4 діб. Використовували методи визначення протеолітичної (еластолітичної, фібринолітичної, фібриногенолітичної та колагеназної) активності у супернатанті культуральної рідини. **Результати.** Дослідження здатності супернатантів досліджуваних культур гідролізувати різні протеолітичні субстрати показало, що перспективними для подальших досліджень можуть бути культури 248 і 249, виділені в однакових умовах (1499 м, 15—20 см), але представників різних видів, *Bacillus subtilis* і *B. licheniformis* відповідно. Супернатанти їх культуральних рідин виявили найбільшу активність щодо фібрину (20,5 од/мл і 19,0 од/мл) і фібриногену (21,66 од/мл і 20 од/мл відповідно), тоді як культури *B. licheniformis* 249 (1499 мкм, 15—20 см), *Priestia megaterium* 55 (1537 м, 0—5 см) і *B. subtilis* 1 (1499 м, 5—10 см), виділені в різних умовах, виявляють високу активність щодо еластину (33,3 од/мл, 31,2 од/мл і 29 од/мл відповідно). *B. subtilis* 1 здатний гідролізувати всі досліджені протеолітичні субстрати: еластин, фібрин, фібриноген і колаген, але рівень усіх цих активностей був нижчим, ніж у вищезазначених штамів. **Висновки.** Ряд представників бактерій: *Bacillus licheniformis* 249, *Priestia megaterium* 55 та *Bacillus subtilis* 1, виділених із глибоководних донних відкладень Чорного моря, за своїми каталітичними властивостями можуть бути перспективними для подальших досліджень як продуценти ферментів з еластолітичною, фібринолітичною та фібриногенолітичною активністю.

Ключові слова: бактерії, виділені з глибоководних донних відкладень Чорного моря, еластолітична, фібринолітична, фібриногенолітична, колагеназна активність.