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PROTEOLITIC ACTIVITY OF MARINE STRAIN *BACILLUS* SP. 051

The main interest in the study of marine microorganisms is due to their ability to produce a wide range of unique enzymes, including peptidases with different specificities. In recent years, interest has increased in peptidases that are able to cleave elastin as a specific substrate. Streptomyces fradiae and Bacillus thermoproteolyticus elastases are among the most potent elastolytic proteinases discovered to date because they are 4-8-fold more effective than pancreatic elastases. The disadvantages of these producers include the fact that most of them are pathogenic for humans, and the elastase enzyme secreted from them is directly involved in the initiation of the pathogenetic process. All this significantly limits the scope of their practical application. Therefore, the search for new, more effective, safe for humans' producers continues to be an urgent question, taking into account the fact that there are no highly active elastase producers in Ukraine. Previously we found elastase activity in only 4 of the 10 studied isolates of bacteria from the Black Sea. Since among them, the elastase activity of the Bacillus sp. 051 was the highest, the ***aim*** *of this work was to study the physicochemical properties and substrate specificity of the enzyme.* ***Methods****. We used methods of determining proteolytic (caseinolytic, elastolytic, fibrinolytic, fibrinogenolytic) activity. Protein concentration was determined by the Lowry method. The study of the effect of temperature on the enzymatic activity was carried out in the range from 4 to 70 °C and pH values from 2.0 to 12.0, created using 0.01 M phosphate-citrate buffer.* ***Results****. It has been shown that the growing temperature of 12°C is the most optimal for biosynthesis of enzyme by the culture of Bacillus sp. 051. The complex enzyme preparation capable of hydrolyzing elastin, casein and fibrinogen. The enzyme showed maximum activity in relation to elastin (3.65 U/mg). The optimum pH of the enzyme action is 8.0, the thermal optimum is 40°C. The rate of casein hydrolysis compared to elastin was 2.7 times lower and amounted to 1.35 U/mg. The complex enzyme preparation also hydrolyzed fibrinogen (1.16 U/mg).* ***Conclusions****. According to its physicochemical and catalytic properties, the representative of the Black Sea, Bacillus sp. 051 is promising for further research as an enzyme producer with elastolytic activity.*

Keywords: *Bacillus sp. 051, elastolytic activity, pH optimum, thermal optimum, substrate specificity.*

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Marine environment, which encompasses about 71% of the Earth's surface, is not only rich in biodiversity but also a vast resource for potential microorganisms of useful applications. Microbes inhabit various habitats of marine environment including neuston, plankton, nekton, seston, and epibiotic, endobiotic, pelagic, and benthic environments. These habitats harbor a diverse range of microbes including archaebacteria, cyanobacteria, eubacteria, actinomycetes, yeasts, filamentous fungi, microalgae, algae, and protozoa [1]. The main interest in the study of marine microorganisms is due to their ability to produce a wide range of unique enzymes [2—4], including peptidases with different specificities [5—8]. In recent years, interest has increased in peptidases that are able to cleave elastin as a specific substrate. Elastin is the main matrix component responsible for tissue elasticity. It is found in the skin, arteries, lungs, and other tissues. Depending on the type of tissue, elastin is present in varying amounts, forming fibers from a highly cross-linked protein. It is insoluble and has a high degree of hydrophobicity. These characteristics are relatively stable and durable in tissues if proteinases do not destroy elastin fibers [1, 8]. The majority of microbial elastases are produced by *Pseudomonas aeruginosa* [9], *Staphylococcus epidermidis* [10], *Bacillus licheniformis* [11], *Bacillus subtilis* [12], and *Chryseobacterium indologenes* [13]. *Streptomyces fradiae* and *Bacillus thermoproteolyticus* elastases are among the most potent elastolytic proteinases discovered to date as they are 4-8-fold more effective than pancreatic elastases [14]. The disadvantages of these producers include the fact that most of them are pathogenic for humans, and the elastase enzyme secreted from them is directly involved in the initiation of the pathogenetic process. All this significantly limits the scope of their practical application. Therefore, the search for new, more effective, safe-for-humans producers continues to be an urgent question, taking into account that there are no highly active elastase producers in Ukraine. Previously [7], we have found an elastase activity in only 4 of 10 studied isolates of bacteria from the Black Sea. Among them, the elastase activity of the *Bacillus* sp. 051 was the highest (20.83 U/mL). Therefore, the aim of this work was to study the physicochemical properties and substrate specificity of this enzyme.

Materials and Methods. The object was *Bacillus* sp. 051 isolated from the Black Sea at a depth of 2080 m.

For submerged fermentation, the strain was cultivated in Erlenmeyer flasks containing 100 mL of medium with the following composition (g/L): KH_2PO_4 — 1.0; $MgSO_4 \cdot 7H_2O$ — 0.75; $ZnSO_4 \cdot H_2O$ — 0.25; $(NH_4)_2SO_4$— 0.5; maltose — 1.0; gelatin — 10.0; yeast autolysate — 0.15; pH 7.0. The culture was grown at 12, 28, and 37 °C with a rotation speed of 210 rpm for 17 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.

Caseinolytic (total proteolytic) activity was determined by the Anson method [15]. Elastase activity was determined colorimetrically by the color intensity of the solution during the enzymatic hydrolysis of elastin stained with Congo-rot using the method of Trombridg et al. [16] in Bondarchuk's modification [17]. 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, 10 min. The color intensity was measured on an SF-26 spectrophotometer at 515 nm. The activity was calculated from the standard curve obtained by measuring the color of the 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.

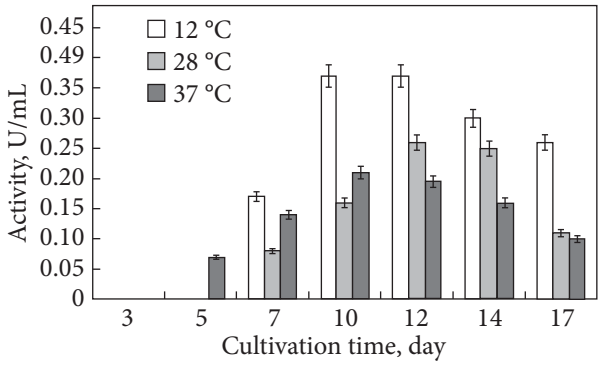

Fig. 1. Dynamics of the synthesis of *Bacillus* sp. 051 caseinolytic (total proteolytic) activity at different temperatures

Fibrinolytic and fibrinogenolytic activities were determined by the recommended methods [5] with fibrin and fibrinogen as substrates.

Protein concentration was determined by the Lowry method [18]. A standard curve of bovine serum albumin (BSA) (1 mg mL-1) was constructed.

To obtain a partially purified enzyme preparation, dry ammonium sulphate was added to the culture liquid to a concentration of 90% saturation under pH control (~6.0). The mixture was kept for 10—12 h at 4 °C, centrifuged at 5000 g for 30 min. The precipitate obtained from the fractionation with ammonium sulfate was dialyzed. The effect of temperature on the enzymatic activity was studied in the range from 4 to 70 °C and pH 2.0 to 12.0, the latter was created using 0.01 M phosphate-citrate buffer (PCB).

All experiments were performed in no fewer than in 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 [19].

Results. The study of the total proteolytic (caseinolytic) activity in dynamics showed (Fig. 1) that only on the fifth day of cultivation in the supernatant of the culture liquid of *Bacillus* sp. 051, there is observed low activity at 37 °C. Starting from the seventh day of cultivation, caseinolytic activity was noted regardless of the cultivation temperature (12, 28, and 37 °C) or the duration of cultivation (up to 17 days). However, the level of activity depended on the cultivation temperature. So, on the 7th day, the maximum activity was at 12 °C (0.17 U/mL), somewhat lower at 37 °C (0.14 U/mL), and at 28 °C it was only 0.08 U/mL. On the 10th day of cultivation, the maximum activity was also noted at 12 °C (0.37 U/mL), slightly lower at 37 °C (0.21 U/mL), and at 28 °C it was 0.16 U/mL. An increase in the cultivation time contributed to an increase in activity when grown at 28 °C (0.26 U/mL), while at 37 °C, the activity decreased (0.195 U/mL), and at 12 °C it remained at the same level (0.37 U/mL). On the 14th—17th days of cultivation, a decrease in activity was noted at all studied temperatures. So, when grown at 12 °C, it was 0.30 U/mL on the 14th day of cultivation, and on the 17th day it was 0.26 U/mL. At 28 °C, the activity decreased more significantly: from 0.25 U/mL on the 14th day to 0.11 U/mL on the 17th day. At 37 °C, the same picture was noted. Thus, the total proteolytic activity was 0.16 U/mL on the 14th day of cultivation and 0.10 U/mL on the 17th day of cultivation.

As seen, the cultivation temperature of 12 °C is the most optimal for the total proteolytic (caseinolytic) activity. An increase in the growing temperature (28 °C and 37 °C) contributed to a decrease in the biosynthesis of the studied enzyme. Thus, during cultivation at 28 °C, the activity was lower, more than by 2 times, compared to cultivation at 12 °C.

The study of elastase activity in dynamics (Fig. 2) showed that only on the 3rd day of cultivation, in the supernatant of the culture liquid of *Bacillus* sp. 051, a slight activity appears at different temperatures. The highest activity (3.0 U/mL) was noted at 12 °C. During cultivation, elastase activity increased to 4.5 U/mL (4th day), and on the 5th day of cultivation, the activity increased more than by 3 times and amounted to 15 U/mL. The maximum activity of the enzyme was on the 6th day of cultivation of the producer

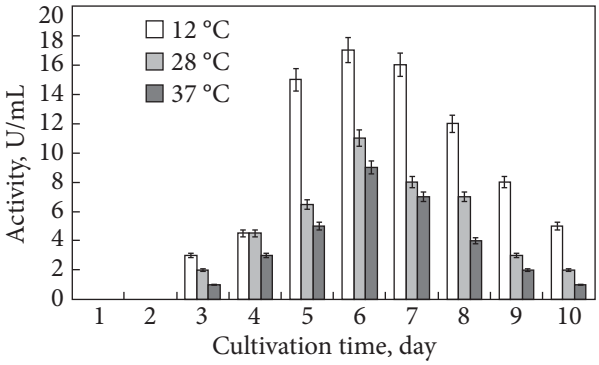

Fig. 2. Dynamics of the synthesis of *Bacillus* sp. 051 elastase activity at different temperatures

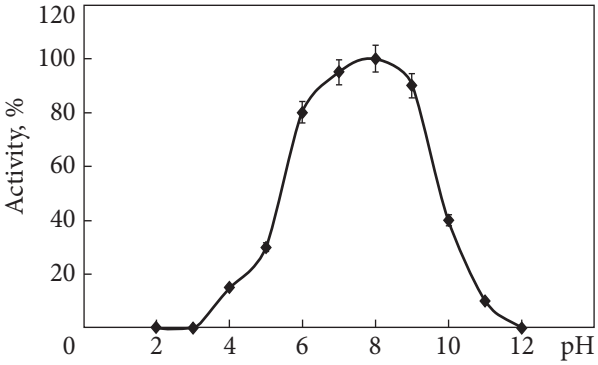

Fig. 3. Effect of pH on the elastase activity of *Bacillus* sp. 051 (40 °C)

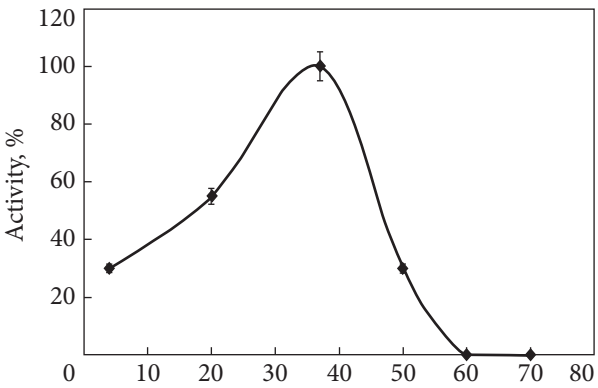

Fig. 4. Effect of temperature on the *Bacillus* sp. 051 elastase activity (pH 8.0)

and amounted to 17 U/mL. With a further increase in the cultivation time, a decrease in enzymatic activity was observed: on the 7th day of cultivation, it was 16 U/mL, on the 8th day of cultivation — 12 U/mL, on the 9th day — 8 U/mL, and on the 10th day — 5 U/mL. A similar picture was noted when grown at 28 °C and 37 °C, but the activity was significantly lower. The maximum activity was also noted on the 6th day of cultivation. As the cultivation time increased, the activity decreased.

Further studies of properties were carried out on a complex enzyme preparation. When studying the effect of pH on the rate of elastin hydrolysis, the optimal activity of the *Bacillus* sp. 051 was found at pH 8.0 (Fig. 3). At pH 7.0, the activity was 95%, at pH 9.0 — 90%, at pH 6.0 — 80%, at pH 10.0 — 40%, at pH 5.0 — 30%, at pH 4.0 — 15%, and at pH 11.0 — only 10%. At pH 2.0, 3.0, and 12.0, no elastase activity was noted.

Study of the elastase activity of *Bacillus* sp. 051 in the range from 4 to 70 °C showed that 40 °C was the optimal temperature at which the activity was maximum (Fig. 4). The increase in temperature contributed to a significant decrease in the activity. Thus, at 50 °C, elastase activity decreased by 70%. Complete inactivation of the enzyme was observed at 60 °C. The complex enzyme preparation was active at sub-optimal temperatures. Thus, at 20 °C, 55% of the activity was retained, and at 4 °C — 30%.

A study of the substrate specificity of the complex enzyme preparation showed (Fig. 5) that, in addition to elastin, it was able to hydrolyze other protein substrates, in particular casein and fibrinogen but not fibrin. The highest activity was noted on elastin (3.65 U/mg). The rate of casein hydrolysis compared to elastin was 2.7 times lower and amounted to 1.35 U/mg.

The complex enzyme preparation also hydrolyzed fibrinogen (1.16 U/mg). When studying the synthesis of fibrinogenolytic activity by the tested culture, a regularity was observed noted in the study of elastase activity (Fig. 6). Fibrinogenolytic activity was also noted on the

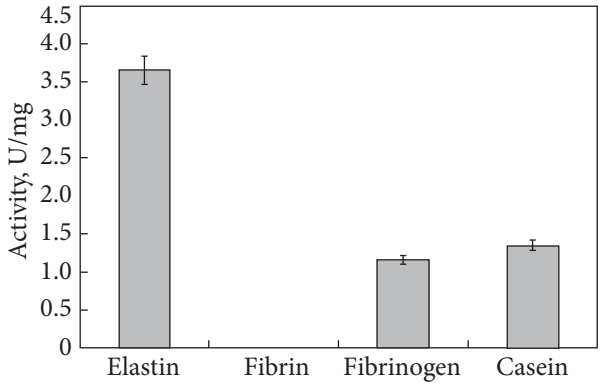

Fig. 5. Substrate specificity of *Bacillus* sp. 051 enzyme preparation

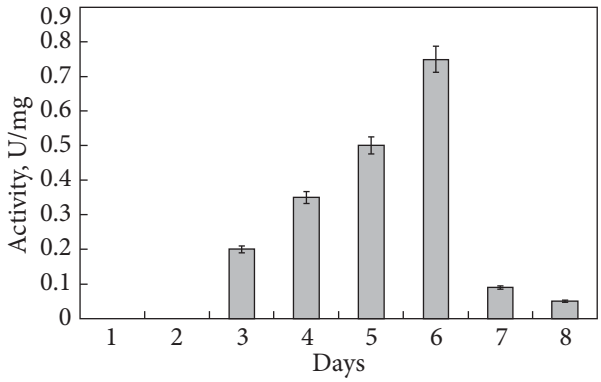

Fig. 6. Fibrinogenolytic activity of *Bacillus* sp. 051

3rd day of cultivation of the producer and was 0.2 U/mL, on the 4th day — 0.35 U/mL, on the 5th day — 0.5 U/mL, and on the 6th day — 0.75 U/mL. It was shown that up to the 6th day, the activity increased, while on the 7th—8th days, it significantly decreased (0.09 and 0.05 U/mL, respectively).

As shown, the growing temperature of 12 °C is the most optimal for biosynthesis by the culture of *Bacillus* sp. 051 a proteolytic enzyme capable of hydrolyzing elastin, casein, and fibrinogen. The enzyme showed maximum activity in relation to elastin. The optimum pH of the enzyme action is 8.0, and the thermal optimum is 40 °C.

Discussion. Microbial enzymes have several advantages over the enzymes derived from plant or animal sources by virtue of their great variety of catalytic activities, cheaper in cost, regular abundant supplies at even quantity and relatively more stability. To date, a large number of hydrolases of terrestrial bacteria have been studied. However, data on the structure and function of enzymes from marine microorganisms that have to survive in habitats characterized by extreme salinity, temperature or pressure conditions are extremely limited. However, the available data indicate such advantages of enzymes of marine origin as high activity, low temperature optimum, resistance to chemical modifications, and long-term storage stability [5, 6, 17]. One of the largest closed and isolated inland water bodies is the Black Sea, connected to the Mediterranean Sea by narrow straits. A large influx of fresh water from rivers creates a low-density surface layer, while the outflow of salty Mediterranean waters through the straits forms a high-density bottom layer [20]. Therefore, it can be assumed that microorganisms isolated from different depths will differ in their activity.

Previously [2], out of 64 bacterial strains, both typical and isolated from water and vertebrates of the Black Sea, we have selected a number of strains with fibrinolytic, collagenase, and keratinase activities. However, none of them showed elastase activity. Therefore, our following studies [7] on the discovery of elastase activity in representatives of the genus *Bacillus* isolated from the deep-water soil of the Black Sea, which manifested itself in all strains on the second day of cultivation, are very important, but differed greatly among the studied strains. The highest activity was identified in *Bacillus* sp. O8, 249, 98, and 1 and amounted to 15.0, 9.8, 8.5, and 6.1 U/mg protein, respectively. Since the activity was not high, we carried out further studies of elastase activity in other representatives of the Black Sea [7]. So, 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), showed elastase activity (20.83 U/mL, 19.96 U/mL, 15.62 U/mL, and 12.15 U/mL, respectively). Cultures isolated from the upper layers of the Black Sea (1499 and 1537 m) either did not show elastase activity at all, or it was low. *Bacillus* sp. 051, which showed the highest activity, was the subject of this study. Of the three studied temperatures for growing the optimal protease production, a temperature of 12 °C was revealed. The optimal activity of the complex preparation with elastase activity was observed at pH 8.0 and a temperature of 40 °C. In addition to elastin, the complex enzyme preparation also hydrolyzed casein and fibrinogen, but no ability to hydrolyze fibrin was not revealed, although many producers are capable to hydrolyze both fibrin and fibrinogen simultaneously.

The study of the fibrinogenolytic activity in the dynamics of the producer growth showed that, like the elastase activity, it was maximal on the 6th day of the cultivation of the producer, but its values were insignificant — only 0.75 U/mL, as well as for the caseinolytic activity, which only within 10—12 days reached the highest activity — 0.37 U/mL.

Thus, from the representative of the Black Sea *Bacillus* sp. 051, a partially purified enzyme preparation with high elastase and low fibrinogenolytic and caseinolytic activities was obtained. The need for unique environmentally friendly bacterial elastase, which can be used in various industrial and pharmaceutical applications, has increased in recent years.

Microbial elastolytic enzymes have great potential for use in industry to hydrolyze raw materials containing elastin fibers. In the meat processing industry, elastases are used in the process of ripening meat and increasing the yield of high-quality meat by 40—43%. In the fishing industry, the use of such enzymes accelerates the processes of salting and ripening of herring by 6—6.5 times and contributes to a more even distribution of salt throughout the carcass. Elastolytic enzymes are also used to disintegrate connective tissue and obtain cell suspensions or cell neoplasms, since selective destruction of the extracellular matrix without damage to the surface of living cells is necessary to maintain cell viability. Elastolytic enzymes of microorganisms can also be used in medicine for the treatment of certain liver diseases, spinal disc herniation, burns, and frostbite and to accelerate the rejection of dead tissues, trophic ulcers, and the cleaning of purulent-necrotic plaques [1, 5, 6, 8, 21—23], as well as in the cosmetic and food industries.

Thus, it is shown that the representative of the Black Sea *Bacillus* sp. 051 is promising for further research as an enzyme producer with elastolytic activity.

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ПРОТЕОЛІТИЧНА АКТИВНІСТЬ МОРСЬКОГО ШТАМУ *BACILLUS* SP. 051

Основний інтерес у вивченні морських мікроорганізмів викликаний їхньою здатністю виробляти широкий спектр унікальних ферментів, а саме пептидаз із різною специфічністю. В останні роки зріс інтерес до пептидаз, які здатні розщеплювати еластин як специфічний субстрат. Еластази *Streptomyces fradiae* і *Bacillus thermoproteolyticus* є одними з найпотужніших еластолітичних протеїназ, виявлених на сьогоднішній день, оскільки вони в 4—8 разів ефективніші, ніж еластази підшлункової залози. До недоліків цих продуцентів можна віднести те, що вони них є патогенними для людини, а виділений з них фермент еластаза бере безпосередню участь в ініціації патогенетичного процесу. Все це істотно обмежує сферу їх практичного застосування. Тому пошук нових, ефективніших та безпечних для людини продуцентів залишається актуальним, зважаючи на те, що в Україні немає високоактивних продуцентів еластази. Раніше ми виявили активність еластази лише в 4 із 10 досліджених нами ізолятів бактерій з Чорного моря. Оскільки серед досліджених ізолятів еластазна активність *Bacillus* sp. 051 була найвищою, **метою** даної роботи було вивчення фізико-хімічних властивостей та субстратної специфічності цього ферменту. **Методи**. Використовували методи визначення протеолітичної (казеїнолітичної, еластолітичної, фібринолітичної, фібриногенолітичної) активності. Концентрацію білка визначали методом Лоурі. Дослідження впливу температури на ферментативну активність проводили в діапазоні від 4 до 70 °С і значеннях pH від 2,0 до 12,0, які досягали за допомогою 0,01 М фосфатно-цитратного буфера. **Результати**. Показано, що для біосинтезу культурою *Bacillus* sp. 51 оптимальною є температура вирощування 12°С. Комплексний ферментний препарат здатен гідролізувати еластин, казеїн і фібриноген з максимальною активністю щодо еластину (3.65 од/мг). Оптимум pH дії ферменту — 8, термооптимум — 40 °С. Швидкість гідролізу казеїну порівняно з еластином була в 2.7 рази нижчою і становила 1.35 од/мг. Комплексний ферментний препарат також гідролізував фібриноген (1.16 од/мг). За своїми фізико-хімічними та каталітичними властивостями представник Чорного моря *Bacillus* sp. 051 є перспективним для подальших досліджень як продуцент ферменту з еластолітичною активністю.

***Ключові слова*:** *Bacillus sp. 051, еластолітична активність, pH-оптимум, термооптимум, субстратна специфічність.*