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EFFECT OF CULTIVATION TEMPERATURE ON ELASTIC, FIBRINOGENOLITIC, AND COLLAGENASE ACTIVITY OF MARINE BACTERIA *BACILLUS ATROPHAEUS* 08, *BACILLUS LICHENIFORMIS* 043, AND *BACILLUS SUBTILIS* 248

Marine microorganisms are the main suppliers of vital organic compounds in the complex ecosystem of the World Ocean thanks to the expression of a wide range of unique enzymes, including proteases. The activity of proteases depends on both the taxonomic affiliation of the strain and the source and place of isolation of the microorganism. Environmental factors of bacteria, such as water salinity, its temperature, pressure, and illumination, largely determine both the composition and the physicochemical properties and substrate specificity of the enzymes produced by them. Since not only the temperature of the residence of microorganisms but also the temperature of their cultivation can significantly affect the activity, the ***purpose*** *of the work was to determine the optimal cultivation temperatures of producers, necessary to achieve the maximum elastase, fibrinogenolytic, and collagenase activities of the studied strains Bacillus atrophaeus 08, Bacillus licheniformis 043, and Bacillus subtilis 248, which were isolated from different depths of the Black Sea.* ***Methods****. The objects of research were three strains: Bacillus subtilis 08, Bacillus licheniformis 043, and Bacillus subtilis 248, which were isolated from bottom sediments* from *3 points at depths of 888—2080 m in the Black Sea. Cultures were grown at a temperature of 12, 28, and 42 °C with a rotation speed of 210 rpm for 5 days. At the end of fermentation, the biomass was separated by centrifugation at 5000 g for 30 min. Methods of determining proteolytic (elastolytic, fibrinogenolytic, and collagenase) activity in the culture liquid supernatant were used.* ***Results.*** *Although the studied cultures were isolated from almost the same depth: Bacillus atrophaeus 08, Bacillus subtilis 248 (1499 m), and Bacillus licheniformis 043 (1537 m), the supernatants of their culture liquids showed different enzymatic activity depending on temperature and growth dynamics. So, for Bacillus atrophaeus 08, the highest elastase and collagenase activities were detected at 28 °C on the second day of cultivation, while fibrinogenolytic activity was detected at 12 °C on the second day of cultivation. The maximum elastase and collagenase activites of Bacillus licheniformis 043 were manifested at 28 °C on the fourth and second day of cultivation, respectively. The highest fibrinogenolytic*

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activity was at 42 °C on the second day of cultivation. When studying Bacillus subtilis 248, it was shown that the maximum elastase activity is achieved at 12 °C on the second day of cultivation, the highest fibrinogenolytic activity was noted at 28 °C on the fourth day of cultivation, and to achieve maximum collagenase activity, it is necessary to grow at 42 °C for four days. ***Conclusions****. The temperature of cultivation of microorganisms plays a significant role in achieving maximum proteolytic activity. Choosing the optimal growing temperature allows for increasing elastase, fibrinogenase, and collagenase activities by several times. It was established that the dynamics of synthesis in different strains is significantly different.*

Keywords: *bacteria isolated from deep-sea sediments of the Black Sea, cultivation temperature, elastolytic, fibrinogenolytic, and collagenase activities.*

Marine microorganisms are the main suppliers of vital organic compounds in the complex ecosystem of the World Ocean thanks to the expression of a whole range of unique enzymes, including hydrolases (Mou & Zhu, 2022). Microbial hydrolases have long been used in various industries, and the rapid development of biotechnology has created a need for enzymes with new properties. The most well-studied class of hydrolases includes microbial proteases, which occupy a key position in terms of their commercial application. They play an important role in physiological processes due to their ability to hydrolyze various protein substrates. Thus, proteases are involved in such biological processes as blood clotting, control of cell death, and tissue differentiation. They catalyze a number of processes in tumor diseases and during infections caused by microorganisms and viruses. They are one of the three large groups of industrially important enzymes, accounting for about 60% of the worldwide sale of enzymes. So, they are widely used in various industries (meat processing, cheese making, cosmetology, detergents, etc.) and medicine (Song & Wei, 2023). The activity of proteases depends on both the taxonomic affiliation of the strain and the source and place of isolation of the microorganism. Environmental factors of bacteria, such as water salinity, temperature, pressure, and illumination, largely determine both the composition and physicochemical properties and substrate specificity of the enzymes produced by them. Since not only the temperature of the residence of microorganisms but also the temperature of their cultivation can significantly affect the activity, the **purpose** of the work was to determine the optimal cultivation temperatures of producers, necessary to achieve the maximum elastase, fibrinogenolytic, and collagenase activities of the studied strains *Bacillus atrophaeus* 08, *Bacillus licheniformis* 043, and *Bacillus subtilis* 248, which were isolated from different depths of the Black Sea.

Materials and Methods. The objects of research were 3 strains: *Bacillus subtilis* 08, *B. licheniformis* 043, and *B. subtilis* 248, which were isolated from bottom sediments from three points at depths of 888—2080 m in the Black Sea, from the horizons of cylindrical sediment 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 (ONU) for microbiological research by Yu.P. Zaitsev and B.G. Aleksandrov (Institute of Marine Biology, NASU). Selected strains were identified previously (Ivanytsia & Ostapchuk, 2017) and are listed in Table 1.

The strains were grown and maintained on meat peptone agar slants by cultivating for 24 h at 28 °C.

For submerged fermentation, strains were cultivated in Erlenmeyer flasks containing 100 mL of medium of the following composition (g/L): KH_2PO_4 — 1.0; $MgSO_4x7H_2O$ — 0.75; $ZnSO_4xH_2O$ — 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 12, 28, and 42 °C with a rotation speed of 210 rpm for 5 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.

The culture, grown in a medium of the above composition was used as an inoculum. The inoculum with the number of bacteria 10^4—10^5 cells/mL was added to the medium volume in the amount of 10%.

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 enzyme solution. The mixture was incubated for 5 h 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 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 within 1 min under standard conditions.

To determine fibrinogenolytic activity, fibrinogen was used as a substrate (Nidialkova & Chernyshenko, 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 incubated for 30—45 min at 37 °C. The reaction was stopped by adding 2 mL of 10% trichloroacetic acid (TCA) the control sample immediately. The 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 increases absorption by 0.01 within 1 min was taken as a unit of activity.

Collagenase activity was determined 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.

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. The value of the null hypothesis $p < 0.05$ was taken as the critical level of reliability.

Results. The study of the influence of the cultivation temperature on the elastase activity of *Bacillus atrophaeus* 08 showed (Fig. 1 A) that it was noted from the very first day of cultivation at all temperatures studied but the highest activity was observed at 28 °C, regardless of the day of cultivation. The maximum elastase activity was 35.6 U/mL on the 2nd day of cultivation.

Fibrinogenolytic activity was also observed at all investigated temperatures during the entire cultivation period, but the optimal temperature for cultivation of *B. atrophaeus* 08 to achieve maximum fibrinogenolytic activity was 12 °C, after 3 days of cultivation (25.0 U/mL) (Fig. 1B). Moreover, it should be noted that at this cultivation temperature, starting from the 2nd day of cultivation, the fibrinogenolytic activity was higher than during cultivation at temperatures of 28 and 42 °C.

The study of the influence of cultivation temperature on the collagenase activity of *B. atrophaeus* 08 showed (Fig. 1 C) that it is insignificant, its maximum level (0.1 U/mL) is reached on the 2nd day of cultivation at 28 °C. This level of collagenase activity was maintained on the 3rd and 4th days of cultivation. On the 5th day,

Table 1. **Studied strains**

Strain	Station number, depth (m), horizon (cm)
Bacillus atrophaeus 08	242, 1499, 10—15
Bacillus licheniformis 043	233, 1537, 15—20
Bacillus subtilis 248	242, 1499, 15—20

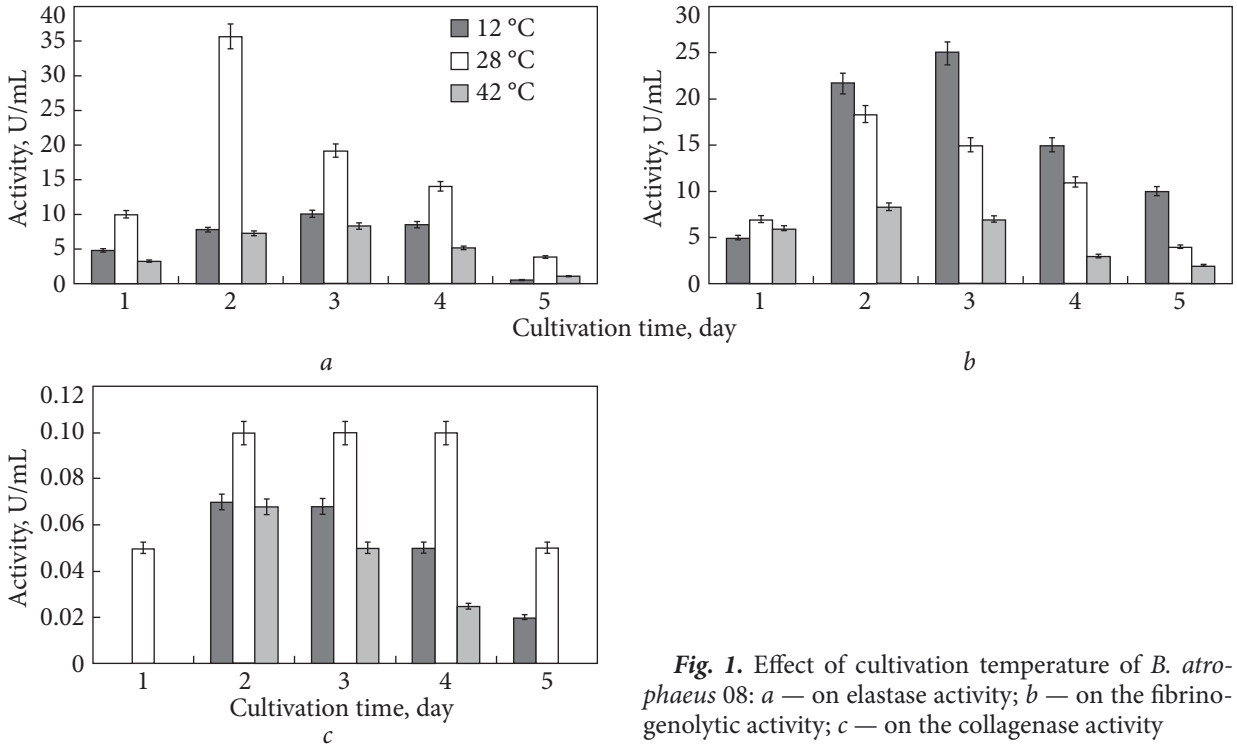

Fig. 1. Effect of cultivation temperature of *B. atrophaeus* 08: *a* — on elastase activity; *b* — on the fibrinogenolytic activity; *c* — on the collagenase activity

the activity decreased more than by 2 times and amounted to 0.05 U/mL.

The study of the influence of the growing temperature of *Bacillus licheniformis* 043 on the dynamics showed (Fig. 2 A) that the maximum elastase activity was observed at a temperature of 28 °C (18.85 U/mL) on the 4th day of cultivation. We consider as an interesting result the absence of elastase activity when cultivating the producer at 42 °C already on the 4th or 5th days. Significantly different results were noted for the fibrinogenolytic activity of *B. licheniformis* 043 (Fig. 2 B).

Thus, the greatest fibrinogenolytic activity was at a temperature of 42 °C throughout the entire cultivation period. Lowering the growing temperature of *B. licheniformis* 043 contributed to a significant decrease in activity. The maximum fibrinogenolytic activity, 41.66 U/mL, was on the 2nd day of cultivation at 42 °C, while at 28 °C it was 6 times lower (7.66 U/mL), and at 12 °C — more than 15 times and was only 2.66 U/mL.

The study of the level of collagenase activity of *B. licheniformis* 043 showed that its highest values were at 28 °C (Fig. 2 C).

On the 1st day of cultivation at this temperature, the activity was 0.25 U/mL. On the 2nd to 4th days of cultivation, it was at the same level — 0.52 U/mL. On the fifth day of cultivation, it slightly decreased and amounted to 0.35 U/mL. A slightly lower activity was observed at a cultivation temperature of 42 °C. At this temperature, the maximum collagenase activity, 0.42 U/mL, was reached on the 2nd day. Starting from the 3rd day of cultivation, it gradually decreased from 0.35 U/mL down to 0.1 U/mL on the 5th day of cultivation.

When cultivating *B. licheniformis* 043 at 12 °C, collagenase activity was not detected at all.

Studying the influence of the cultivation temperature on the elastase activity of *B. subtilis* 248 revealed (Fig. 3 A) that its maximum level, 18.6 U/mL, was reached at 12 °C on the 2nd day of cultivation.

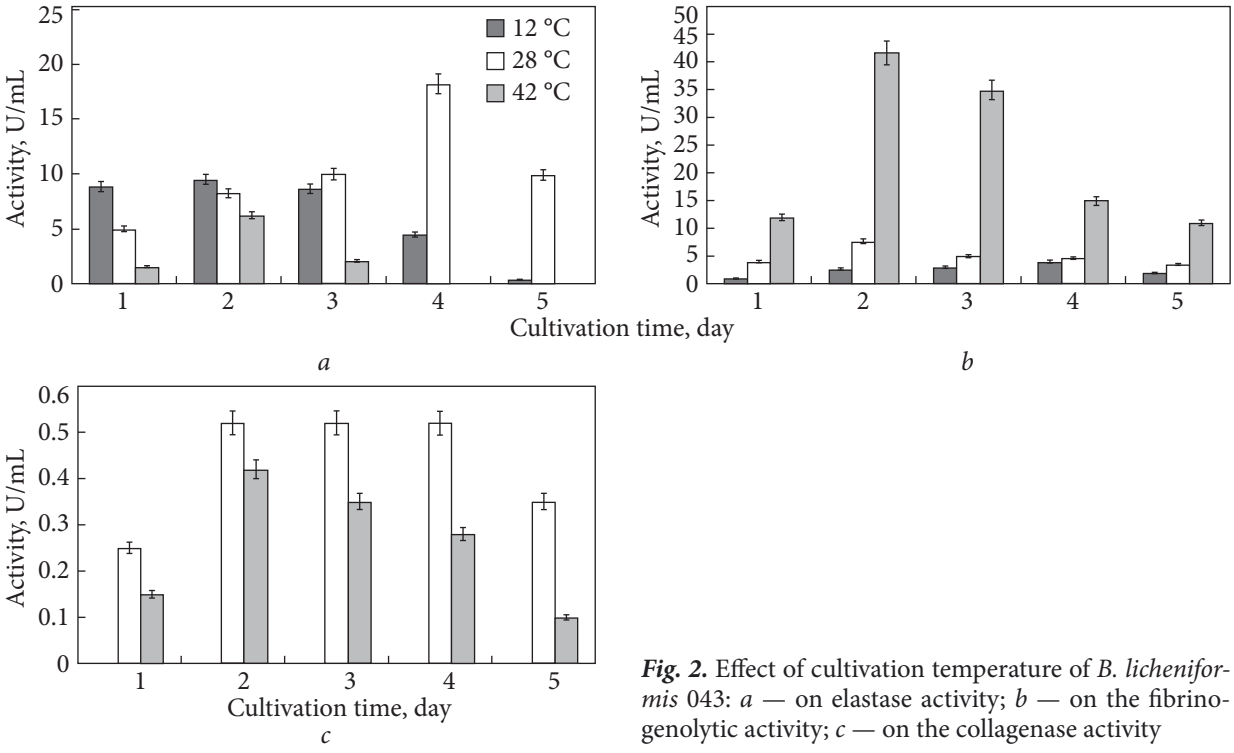

Fig. 2. Effect of cultivation temperature of *B. licheniformis* 043: *a* — on elastase activity; *b* — on the fibrinogenolytic activity; *c* — on the collagenase activity

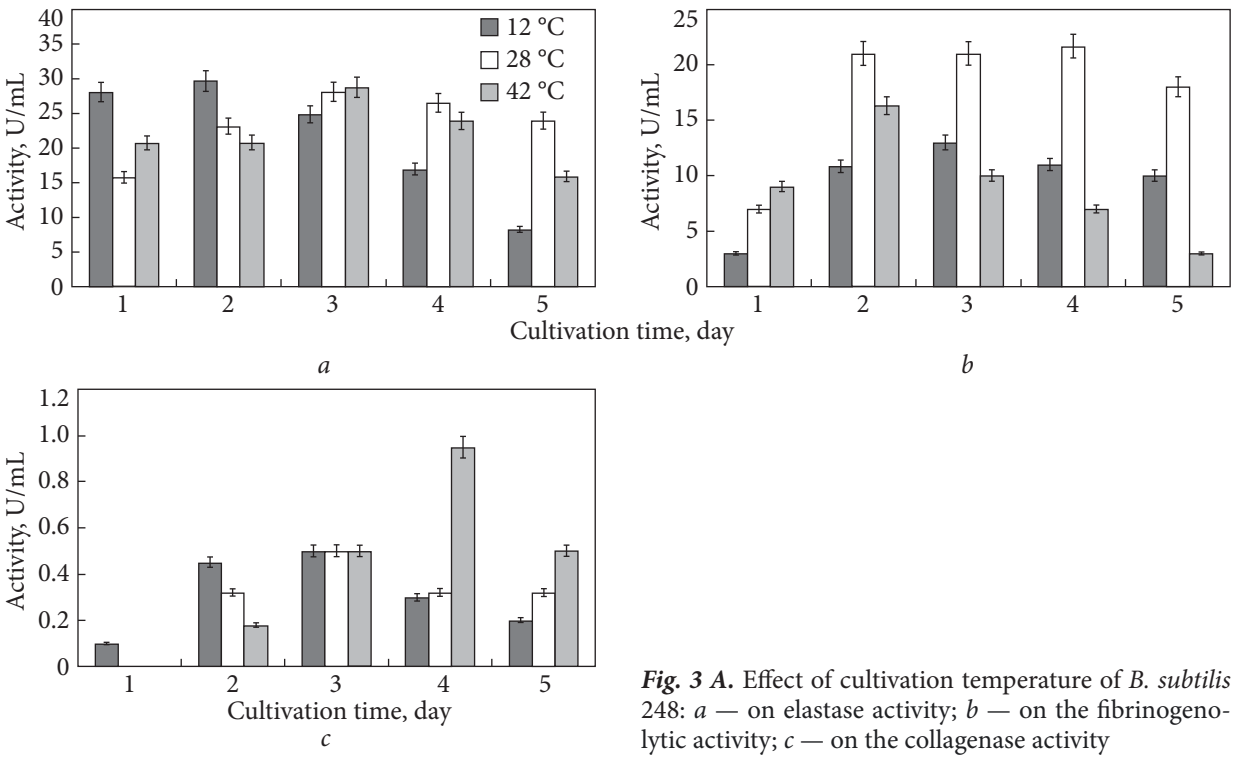

Fig. 3 A. Effect of cultivation temperature of *B. subtilis* 248: *a* — on elastase activity; *b* — on the fibrinogenolytic activity; *c* — on the collagenase activity

Elastase activity was somewhat lower at this temperature for 1 day of cultivation (17.6 U/mL). With an increase in the time of cultivation of *B. subtilis* 248, a decrease in enzymatic activity was noted at 12 °C, with increasing the temperature of cultivation, the activity also increased, and at 42 °C for 3 days it amounted to 17.3 U/mL.

The study of the influence of the cultivation temperature on the fibrinogenolytic activity of *B. subtilis* 248 showed that a maximum activity was achieved at 28 °C (Fig. 3 B).

At this temperature, the highest fibrinogenolytic activity, 21.66 U/mL, was noted on the 4th day of cultivation, whereas on the 2nd and 3rd days of cultivation, it was almost at the same level — 21 U/mL. When growing at 42 °C, the maximum fibrinogenolytic activity, 16.3 U/mL, was already on the 2nd day of cultivation Starting from the 3rd day of cultivation, it decreased at this temperature.

When growing *B. subtilis* 248 at 12 °C, the activity was maximal on the 3rd day of cultivation (13 U/mL). An increase in the cultivation time contributed to a decrease in fibrinogenolytic activity.

To achieve the maximum collagenase activity of the *B. subtilis* 248 strain (Fig. 3 C), it is necessary to grow the strain for four days at 42 °C. Under these conditions, the activity was 0.95 U/mL.

At this cultivation temperature, activity was noted starting from the second day of cultivation. As shown in Fig. 3, on the 2nd day, it was 0.18 U/mL, on the 3rd and fifth days — 0.5 U/mL. It turned out to be interesting that on the first day of cultivation, collagenase activity was detected only at 12 °C.

Thus, the temperature of cultivation of microorganisms plays a significant role in achieving maximum proteolytic activity. Choosing the optimal growing temperature allows for an increase in elastase, fibrinogenase, and collagenase activities by several times. It was established that the dynamics of synthesis in different strains is significantly different.

Discussion. Each microorganism is characterized by an optimal growth temperature, so any temperature above or below this level worsens metabolic processes, as they slow down at such temperatures. In some microorganisms, the production of enzymes can be significantly reduced when grown at higher temperatures, because their denaturation is observed (Engqvist, 2018; Mehta & Sharma, 2016). At the same time, in some microorganisms, when they are grown at lower temperatures (Médigue et al., 2005), an increase in both the synthesis and activity of enzymes can be observed. Since the bacteria we studied were isolated from deep-sea sediments of the Black Sea, where the temperature is +5 — +9°C, we decided to investigate how the cultivation temperature can affect the activity of proteolytic enzymes. Although the studied cultures were isolated from almost the same depth: *Bacillus atrophaeus* 08 and *Bacillus subtilis* 248 at 1499 m, and *Bacillus licheniformis* 043 at 1537 m, the supernatants of their culture liquids showed different enzymatic activity depending on both temperature and growth dynamics. So, for *Bacillus atrophaeus* 08, the highest elastase and collagenase activities were detected at 28 °C on the 2nd day of cultivation, while fibrinogenolytic activity was detected at 12 °C on the 2nd day of cultivation. Elastase and collagenase activities of *Bacillus licheniformis* 043 were maximum at 28 °C on the 4th and 2nd day of cultivation, respectively. The highest fibrinogenolytic activity was at 42 °C on the second day of cultivation. When studying the temperature of cultivation of *Bacillus subtilis* 248, it was shown that the maximum elastase activity is achieved at 12 °C on the 2nd day of cultivation, the highest fibrinogenolytic activity was noted at 28 °C on the 4th day of cultivation, and to achieve maximum collagenase activity, it was necessary to grow at 42 °C for four days.

Thus, based on the conducted research, it is possible to mark the most effective producers of elastase — *Bacillus atrophaeus* 08 and fibrinoge-

nase — *Bacillus licheniformis* 043. If the elastolytic activity of *Bacillus atrophaeus* 08 is 35.6 U/mL, then the elastase activity of the culture liquid supernatant of the previously described (Matselukh, 2010) mutant strain of *Bacillus* sp. 27, obtained with the help of N-methyl-N'-nitro-N-nitrosoguanidine, was 16.0 U/mg of protein. Since 1 mL usually contains about 1 mg of protein, the elastase activity of *Bacillus atrophaeus* 08 is much higher.

The fibrinogenolytic activity of *Bacillus licheniformis* 043 (41.66 U/mL) is almost 10 times higher than the activity of *Bacillus thuringiensis* IMV B-7324 (Nidialkova, 2014), with deep cultivation of which under certain conditions, an enzyme preparation with an activity of 4.17 U/mg of protein can be obtained. Today in Ukraine, there are no highly active producers of proteinases of microbial origin with fibrinogenolytic activity.

Currently, it is known (Suphatharaprateep & Jongjareonrak, 2011; Wanderley et al., 2017) that collagenases are synthesized by various microorganisms, many of which are pathogens for humans, which significantly limits the scope of their practical application. Therefore, non-pathogenic collagenase producers are of great theoretical and practical interest. Unfortunately, the collagenase activity of the *Bacillus subtilis* 248 strain we studied was insignificant (0.95 U/mL). It was several times lower than the activity of the collagenase enzyme preparation of *Streptomyces* sp. (Abdel-Fattah, 2013), as well as complex enzyme preparation *Bacillus thuringiensis* var. i*sraelensis* (Nidialkova & Chernyshenko, 2016), the collagenase activity of which reached 34.5 U/mg of protein, which is almost 13 times higher than the collagenase activity of *Streptomyces* sp. 1349. Therefore, *Bacillus subtilis* 248 as a collagenase producer is not promising for further research.

At the same time, *Bacillus subtilis* 248 and *Bacillus atrophaeus* 08, the elastase and fibrinogenolytic activities of which, respectively, are manifested at 12 °C on the 2nd day of their cultivation, may be promising for further research. Enzymes of psychrophilic microorganisms have attracted increased attention of researchers in recent years. This increased interest is attributed to the attractive properties of such proteins, namely high specific activity and low thermal stability, and thus, such cold-active enzymes represent a huge potential for basic research and biotechnological applications. Structural and functional studies of such enzymes allow for obtaining information on the mechanisms of their adaptation to functioning at low temperatures. Increasing the activity of enzymes obtained from cultures grown at low temperatures can be considered one of the adaptation mechanisms of the organism to environmental conditions. An important concept in adaptation to cold is the flexibility of the protein structure of such enzymes (Yang & Huang, 2023). The study of enzymes of marine microorganisms makes it possible to compare the properties of enzymes isolated from both thermophilic and psychrophilic microorganisms. This will make it possible to understand the molecular basis of cold or heat adaptation of enzymes of microorganisms that live in the appropriate conditions.

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ВПЛИВ ТЕМПЕРАТУРИ КУЛЬТИВУВАННЯ НА ЕЛАСТАЗНУ, ФІБРИНОГЕНОЛІТИЧНУ ТА КОЛАГЕНАЗНУ АКТИВНОСТІ МОРСЬКИХ БАКТЕРІЙ *BACILLUS ATROPHAEUS 08, BACILLUS LICHENIFORMIS* 043 І *BACILLUS SUBTILIS* 248

Морські мікроорганізми є основними постачальниками життєво важливих органічних сполук у складній екосистемі Світового океану завдяки експресії цілого спектра унікальних ферментів, включаючи протеази. Активність протеаз залежить як від таксономічної приналежності штаму, так і від джерела та місця виділення мікроорганізму. Фактори довкілля бактерій, такі як солоність води, її температура, тиск, освітленість значною мірою визначають склад, фізико-хімічні властивості та субстратну специфічність продукованих ними ферментів. Оскільки не лише температура середовища мікроорганізмів, а й температура їх вирощування може істотно впливати на активність, **метою** роботи було визначити оптимальні температури культивування продуцентів, необхідні для досягнення максимальної еластазної, фібриногенолітичної та колагеназної активності досліджуваних штамів *Bacillus atrophaeus* 08, *Bacillus licheniformis* 043, *Bacillus subtilis* 248, які були виділені з різних глибин Чорного моря. **Методи**. Об'єктами дослідження були 3 штами: *Bacillus subtilis* 08, *Bacillus licheniformis* 043, *Bacillus subtilis* 248, виділені з донних відкладень із трьох точок на глибинах 888—2080 м у Чорному морі. Культури вирощували за температур 12, 28 і 42 °С зі швидкістю обертання 210 об/хв протягом 5 діб. Після закінчення ферментації біомасу відокремлювали центрифугуванням при 5000 g протягом 30 хв. Використовували методи визначення протеолітичної (еластолітичної, фібриногенолітичної та колагеназної) активності в супернатанті культуральної рідини. **Результати.** Хоча досліджувані культури були виділені майже з однакової глибини (*Bacillus atrophaeus* 08 і *Bacillus subtilis* 248 — 1499 м, *Bacillus licheniformis* 043 — 1537 м), супернатанти їхніх культуральних рідин проявляли різну ензиматичну активність в залежності як від температури, так і динаміки росту. Так, якщо для *Bacillus atrophaeus* 08 найбільша еластазна і колагеназна активності були виявлені за температури 28 °С на другу добу культивування, то фібриногенолітична – за 12 °С на другу добу вирощування. Максимальна еластазна і колагеназна активності *Bacillus licheniformis* 043 проявлялися за температури 28 °С на четверту і другу добу культивування відповідно**.** Найвища фібриногенолітична активність була за температури 42 °С на другу добу культивування. При вивченні впливу температури культивування *Bacillus subtilis* 248 показано, що максимальна еластазна активність досягається за температури 12 °С на другу добу культивування, найвища фібриногенолітична активність – при 28 °С на четверту добу культивування, а для досягнення максимальної колагеназної активності необхідно вирощувати культуру при 42 °С протягом чотирьох діб. **Висновки.** Температура культивування мікроорганізмів відіграє суттєву роль у досягненні максимальної протеолітичної актвності. Підбір оптимальної температури вирощування дозволяє збільшити в декілька разів еластазну, фібриногеназну і колагеназну активності. Встановлено, що динаміка синтезу у різних штамів суттєво відрізняється.

***Ключові слова**: бактерії, виділені з глибоководних відкладень Чорного моря, температура культивування, еластолітична, фібриногенолітична, колагеназна активності.*