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BACTERIA OF DEEP-SEA SEDIMENTS OF THE BLACK SEA BREAKING DOWN KERATIN

Down and feather raw materials generated at food processing plants are among the main environmental pollutants. Most enterprises use burial and burning methods to deal with this waste, which negatively affects the environmental situation. The use of enzymatic hydrolysis by decomposer microorganisms is a promising and safe method for processing keratin waste. Earlier, it was shown that bacteria isolated from the bottom sediments of the Black Sea are active producers of elastases, fibrinogenases, and fibrinases. Therefore, the ***aim*** *of this work was to evaluate the ability of bacteria isolated from the deep-sea sediments of the Black Sea to exhibit other types of proteolytic activity, in particular, to decompose hard-to-reach protein keratin.* **Methods.** *The objects of the study were 20 cultures of bacteria isolated from deep-sea sediments of the Black Sea represented by the genera Bacillus, Metabacillus, Priestia, and Robertmurraya. The cultures were grown under conditions of submerged cultivation at 28 °C, with a nutrient medium stirring rate of 232 rpm for 4 days. For growth, a basic nutrient medium containing 0.5% defatted chicken feathers as sole sources of carbon and nitrogen was used. The keratinase activity was assessed by UV absorption at 280 nm of hydrolysis products of keratin-containing materials. Protein was determined by the Lowry method, and caseinolytic (total proteolytic) activity was determined by the Anson method. Disulfide reductase activity was measured spectrophotometrically at 412 nm by evaluating the yellow sulfide formed during the reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB).* ***Results.*** *It was shown that the cultures of Metabacillus idriensis 2 and Robertmurraya siralis 57, out of twenty studied, did not grow on a nutrient medium with chicken feathers as the only source of carbon and nitrogen. The remaining 18 cultures exhibited varying degrees of keratinase activity (from 3 to 32 U/mL). The highest level of activity is characteristic of the culture Priestia megaterium 035 (32 U/mL). A study of the ability to break down casein showed that the level of total proteolytic (caseinolytic) activity of most cultures ranged from 0.015 U/mL to 0.14 U/mL. The highest total proteolytic activity was demonstrated by Bacillus pumilus A (0.3 U/mL) and Priestia megaterium 55 (0.24 U/mL) cultures, which also demonstrated high keratinase activity. The highest level of disulfide reductase activity was observed in Bacillus*

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pumilus A (63.3 μmol/min), Bacillus subtilis 248 (62.0 μmol/min), and Priestia megaterium 035 (61.3 μmol/min), and the lowest in Bacillus licheniformis 249. Thus, from the deep-sea sediments of the Black Sea, we have isolated a number of active producers of keratinases, representatives of the two genera Bacillus and Priestia, which, after studying their physicochemical and catalytic properties, may turn out to be promising for practical application, in particular in the development of new technologies for the utilization of down and feather poultry farm waste.

***Keywords**: bacteria of deep-sea sediments of the Black Sea, keratinase activity, total proteolytic (caseinolytic) activity, disulfide reductase activity.*

In recent years, the poultry farming industry associated with the growing and processing of broiler chickens for meat has gained intensive development in Ukraine. As a result, large industrial-scale poultry farms generate a huge amount of non-food waste, in particular down and feathers, which are rarely used by enterprises for feed purposes. Most often they just burn out. At the same time, down and feather waste, due to the high content of keratin protein in its composition, can be used for the production of animal feed. As a result, there will be no environmental pollution (Gladiy et al., 2018; Tesfaye et al., 2017).

Keratin is a fibrillar protein that is the main component of the horny derivatives of the epidermis of the skin: feathers, hair, nails, wool, horns, hooves, etc. This protein is characterized by a high content of cysteine, and, accordingly, disulfide bonds and hydrophobic residues, which ensures mechanical strength and insolubility of keratin in water, weak solutions of acids and alkalis, and resistance to most proteolytic enzymes. Therefore, the proteins of feather keratin raw materials, only after hydrolysis due to the rupture of disulfide bonds, become water-soluble, well-digested, and absorbed in the body of animals (Li, 2021; Vidmar & Vodovnik, 2018).

The main methods used for processing keratin raw materials are alkaline, acid, hydrothermal, and enzymatic hydrolysis (Anbesaw, 2022). Each of these methods has its advantages and disadvantages. In alkaline hydrolysis, which ensures the production of hydrolysates from any type of keratin raw material, a solution of sodium or potassium hydroxide is used, followed by neutralization of the resulting hydrolyzate with hydrochloric or phosphoric acids. However, the salts formed when using phosphoric acid give the product of a bitter taste, and during storage, they can precipitate. All this affects the quality of the resulting product. In addition, this type of hydrolysis destroys amino acids such as cysteine and methionine, the breakdown of arginine into ornithine and ammonia, as well as the deamination of some amino acids (serine, threonine, cystine, cysteine, methionine).

Acid hydrolysis ensures a high degree of breakdown of keratin raw materials and prevents the breakdown of arginine and deamination of some amino acids, although, at the same time, it leads to the loss of tryptophan and tyrosine, as well as the conversion of some amino acids from the L-form to the D-form, which is not absorbed by animals. However, both acid and alkaline hydrolyses are quite lengthy processes, which limits their use for processing keratin raw materials (Dąbrowska et al., 2022).

The hydrothermal treatment method is a fairly simple, short-term, and frequently used method. It is a heat treatment in an aqueous environment under pressure. The resulting product is dried, crushed, sifted, and subsequently used as a feed additive. The most promising, safe, environmentally friendly, and less energy-consuming method is enzymatic hydrolysis, which is carried out at lower temperatures and a neutral pH value. It makes it possible to obtain a protein hydrolyzate with a natural amino acid composition in the L-form and high physiological availability (Anbesaw, 2022; Goda et al., 2022). Enzymatic degradation of keratin is a multistage process that requires the following steps: (i) adsorption of keratinases on the macromolecule surface through electro-

static and hydrophobic interactions, followed by (ii) sulfitolysis (reduction of disulfide bonds) and proteolysis. Sulfitolysis can only occur in the presence of reducing compounds such as sodium sulfide, dithiothreitol (DTT), mercaptoethanol, glutathione, cysteine, thioglycolic acid, or disulfide reductases, which act with keratinases to degrade keratin molecules (Qiu et al., 2020).

Therefore, the search for producers of keratinases that ensure the breakdown of down and feather raw materials is an urgent area of research. Previously, our department has conducted a number of studies to search for microorganisms that produce proteolytic and hydrolytic enzymes. It was shown that bacteria isolated from the bottom sediments of the Black Sea are active producers of elastases, fibrinogenases, and fibrinases (Gudzenko et al., 2022). In connection with the prospect of biomodification of keratin-containing raw materials, research related to the discovery of new producers of keratinases and the development of effective methods for their isolation, purification, and study of properties are becoming very important. Therefore, the aim of this work was to evaluate the ability of bacteria isolated from deep-sea sediments of the Black Sea to exhibit other types of proteolytic activity, in particular, to decompose hard-to-reach protein keratin.

Table 1. **Studied strains**

Strain number (number in Fig. 1)	Station number, depth (m), horizon (cm)	Strain
1 (1)	242, 1499, 5—10	*Bacillus subtilis*
2 (2)	258, 888, 0—5	*Metabacillus idriensis*
08 (3)	242, 1499, 10—15	*Bacillus atrophaeus*
013 (4)	242, 1499, 10—15	*Bacillus subtilis*
033 (5)	242, 1499, 25—30	*Bacillus subtilis*
035 (6)	258, 888, 30—35	*Priestia megaterium*
043 (7)	233, 1537, 15—20	*Bacillus licheniformis*
055 (8)	233, 1537, 0—5	*Bacillus licheniformis*
55 (9)	233, 1537, 0—5	*Priestia megaterium*
57 (10)	242, 1499, 0—5	*Robertmurraya siralis*
116 (11)	269, 2080, 0—5	Not identified
212 (12)	233, 1537, 5—10	*Bacillus subtilis*
231 (13)	258, 888, 5—10	*Bacillus subtilis*
232 (14)	258, 888, 5—10	*Bacillus subtilis*
245 (15)	242, 1499, 25—30	*Bacillus licheniformis*
Myco (16)	258, 888, 0—5	Not identified
A (17)	258, 888, 0—5	*Bacillus pumilus*
021 (18)	258, 888, 10—15	*Bacillus subtilis*
249 (19)	242, 1499, 15—20	*Bacillus licheniformis*
248 (20)	242, 1499, 15—20	*Bacillus subtilis*

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 the ONU for microbiological research by Yu. P. Zaitsev and B. G. Alexandrov (Institute of Marine Biology, NASU). Selected strains were identified previously (Ivanytsia, Shtenikov, & Ostapchuk, 2017) and are listed in Table 1.

For the cultivation of cultures, the basic nutrient medium of the following composition (g/L) was used: K_2HPO_4 — 1.4; KH_2PO_4 — 0.7; $MgSO_4 \cdot 7H_2O$ — 0.1; NaCl — 0.5; defatted chicken feathers — 5; H_2O — up to 1.0 L; pH 7.0—7.2. White chicken feathers were washed with a detergent solution, followed by washing with distilled water about 10 times, drying, and defatting with a mixture of chloroform and methanol in a ratio of 1:1. Daily cultures were used as an inoculum. Bacteria were grown by the submerged cultivation method in 0.75 L Erlenmeyer flasks with 100 mL of the above liquid nutrient medium (pH 7.2) at a temperature of 28 °C, stirring speed 232 rpm for 4 days. To obtain a supernatant (culture liquid supernatant, SCL) for further studies, the culture liquid was centrifuged at 7000 g for 10 min.

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 crushed defatted feathers, 2.5 mL of 0.05 M boron-borate buffer

(pH 9.0), and 1 mL of the SCL was kept in a thermostat at 37 °C for 3 hours, after which it was filtered. Two controls were used to determine KerA: (1) 10 mg of crushed defatted feathers, 2.5 mL of 0.05 M boron-borate buffer (pH 9.0), and 1 mL of distilled water; (2) 2.5 mL of 0.05 M boron-borate buffer (pH 9.0) and 1 mL of the SCL. The sum of the values of the two controls was subtracted from the values obtained by measuring the optical density of the filtrates at A_{280}. The increase in absorption at 280 nm of the test sample filtrate relative to the controls was taken as the degree of protein release. One unit of keratinase activity (1 U/mL = 0.01) was defined as the amount of enzyme that causes an increase in absorption by 0.01 for 3 h of incubation (Nickerson et al., 1963).

The total proteolytic (caseinolytic) activity was determined by the Anson method based on the quantitative determination of tyrosine formed during the enzymatic hydrolysis of casein under the influence of enzymes of the studied supernatants. 0.5 mL of SCL and 0.5 mL of 1% casein were added to the test tube. The control tube contained 0.5 mL of SCL and 2 mL of 4% trichloroacetic acid (TCA). Incubation was carried out in a water bath at 37 °C for 30 min, after which 2 mL of 4% TCA was added to the test tube, kept for 20 min at room temperature, and centrifuged at 10 000 g for 5 min. 2.5 mL of 0.5 M Na_2CO_3 and 0.5 mL of diluted Folin`s reagent (1:3) were added to 0.5 mL of SCL and kept for 20 min at room temperature. Cleavage products were determined on an SF-26 spectrophotometer at a wavelength of 670 nm (cuvette 10 mm). The activity was expressed in units corresponding to the number of micromoles of tyrosine released from casein during enzymatic hydrolysis within 1 min under the experimental conditions (Varbanets & Matseliukh, 2014).

The disulfide reductase activity was determined according to Gupta with minor modifications (Gupta et al., 2015). The reaction mixture contained 2.5 mL of 0.1 M sodium phosphate and 50 μL of DTNB (5,5'-dithiobis-(2-nitrobenzoic acid). Then, 250 μL of the SCL was added to this mixture. Upon reduction of DTNB, a yellow sulfide was formed, which was measured spectrometrically at 412 nm. The amount of the enzyme catalyzing the formation of 1 μmol of sulfide per 1 min was taken as a unit of disulfide reductase activity.

Protein concentration was determined by the Lowry method (Lowry et al., 1951). The standard curve of bovine serum albumin (BSA) (1 mg/mL) was constructed.

All experiments were performed in at least 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.

Results. The studied cultures were grown for 4 days by taking samples of the culture liquid every 24 h. The total proteolytic (caseinolytic) and keratinase activity was determined in the supernatants of culture liquids. It was shown that cultures 2 and 10, out of twenty studied, did not grow on a nutrient medium containing chicken feathers as the only source of carbon and nitrogen. Therefore, neither general proteolytic nor keratinase activity was detected in them. It should be noted that most of the studied cultures did not show high activity toward casein. The activity level for many microorganisms ranged from 0.015 U/mL to 0.14 U/mL (Fig. 1). The highest total proteolytic (caseinolytic) activity was exhibited by cultures 17 (0.3 U/mL) and 9 (0.24 U/mL).

The remaining 18 cultures exhibited varying degrees of keratinase activity (from 3 to 32 U/mL) (Fig. 2). The highest level of activity is characteristic of culture 6 (32 U/mL), slightly lower for cultures 9 (15 U/mL), 13, 17 (14 U/mL), 5, 11, and 20 cultures (12 U/mL). Interestingly, 11 of the 20 studied microorganisms began to synthesize keratinase on the first day of cultivation, two cultures on the second day, and four cultures on the third day. The highest proteolytic (caseinolytic) activity was exhibited by cultures 17 (0.3 U/mL) and 9 (0.24 U/mL), which also demonstrated high keratinase activity. At the same time, culture 6, the level of keratinase activity of which was the highest,

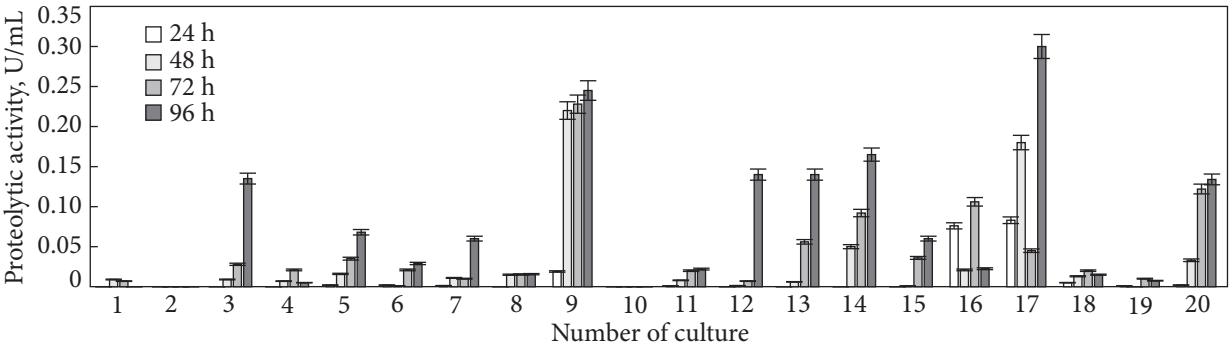

Fig. 1. Total proteolytic (caseinolytic) activity of culture liquid supernatant of bacterial strains isolated from the deep-water bottom sediments of the Black Sea

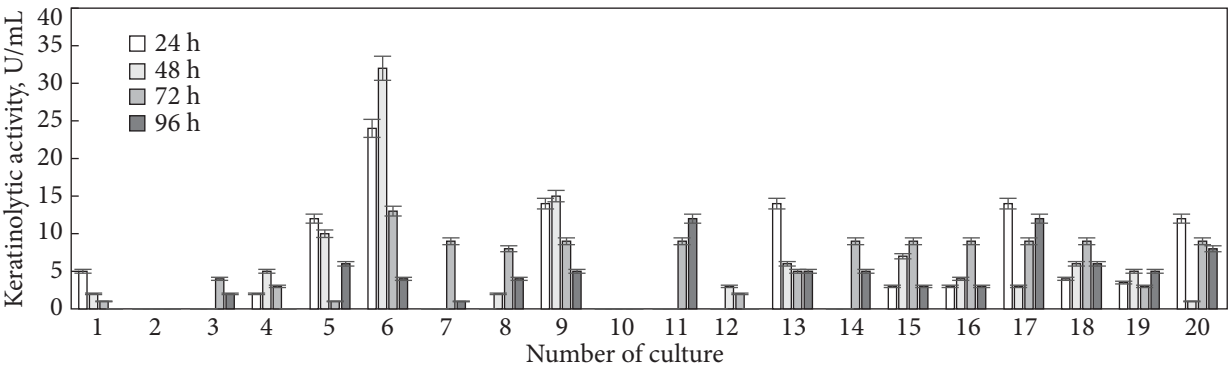

Fig. 2. Keratinase activity of culture liquid supernatant of bacterial strains isolated from the deep-water bottom sediments of the Black Sea

exhibited low caseinolytic activity (0.029 U/mL). That is, there is no direct correlation between the level of keratinase and total proteolytic activity.

During the splitting of feathers, the products of their hydrolysis (peptides, individual amino acids) accumulate in the culture liquid. Therefore, an important indicator of successful substrate degradation is the protein content in the supernatant of the microorganism's culture liquid. It was shown that the majority (11 cultures) of the studied bacteria demonstrated the maximum level of protein in the culture liquid on the third day and only 5 cultures (3, 6, 8, 11, and 12) on the fourth day. The highest protein level was shown by culture 6 (5.85 mg/mL), which was characterized by the highest keratinase activity (Fig. 3).

It is known from the literature that not only keratinases are involved in the process of feather hydrolysis, but also disulfide reductases (or other enzymes or reducing agents), which reduce the number of disulfide bonds in the protein molecule, making it accessible to keratinases (Gupta, 2015). A study of the presence of disulfide reductase activity in the studied cultures showed that 18 out of 20 bacteria (except cultures 2 and 10) synthesized this enzyme (Fig. 4). The highest level of the activity was observed in cultures 17 (63.3 μmol/min), 20 (62.0 μmol/min), and 6 (61.3 μmol/min) and the lowest — in culture 19. The maximum level of disulfide reductase activity, characteristic of each culture, was observed on days 2—3, and only in cultures 16 and 20 the activity increase from the first to the fourth day. Previously (Gudzenko et al., 2023) when studying the keratinase activity of *Bacillus* sp KG7 and KG8 isolated from soil of rice agrocenosis, it was shown that they exhibited low-

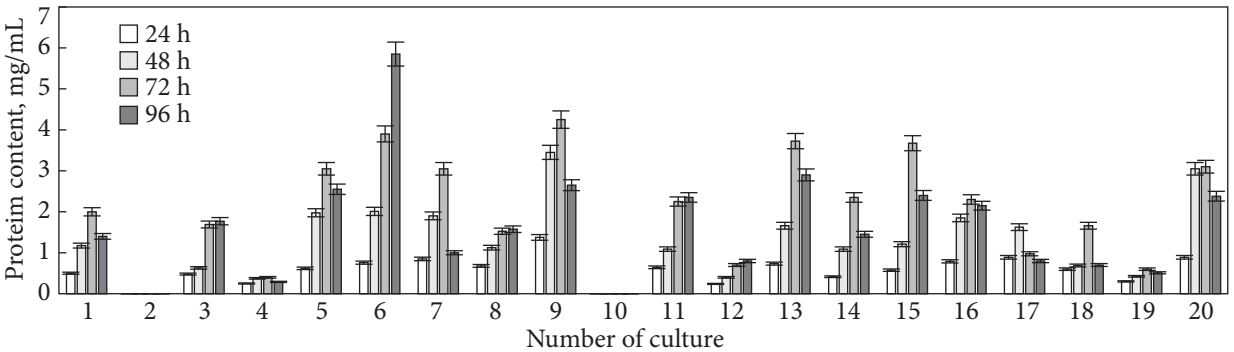

Fig. 3. Protein level of culture liquid supernatant of bacterial strains isolated from the deep-water bottom sediments of the Black Sea

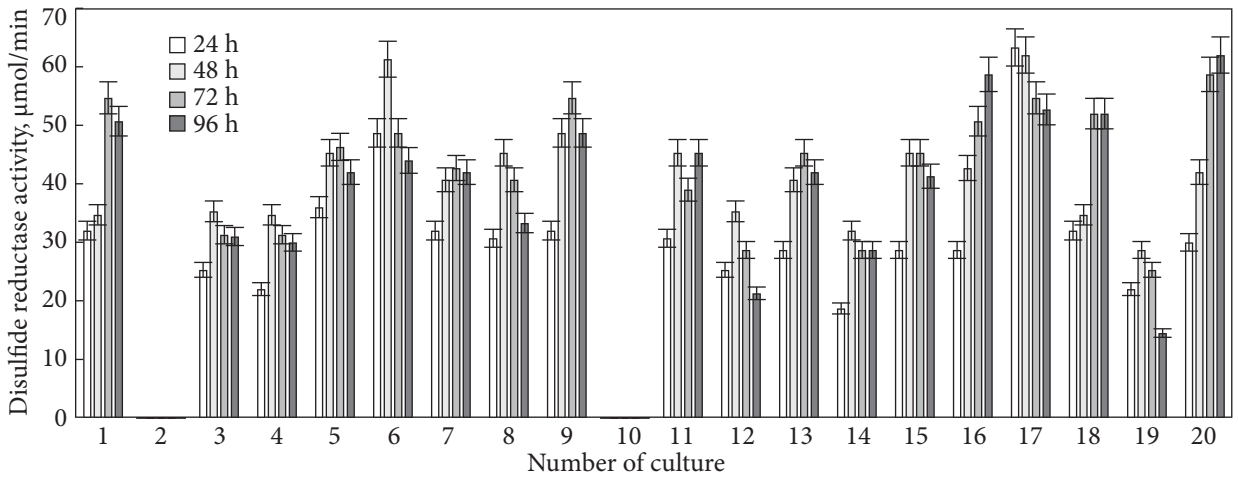

Fig. 4. Disulfide reductase activity of culture liquid supernatant of bacterial strains isolated from the deep-water bottom sediments of the Black Sea

er disulfide reductase activity: 33 μmol/min and 31 μmol/min, respectively.

Discussion. In recent years, a sufficient number of reports have appeared in the literature about microorganisms that synthesize keratinase. It is known that most microbial keratinases are inducible enzymes secreted into the extracellular matrix in the presence of keratin or keratin-containing substrates. Some microorganisms are capable of simultaneously producing extracellular and intracellular keratinases. It is also known about the existence of cell-associated enzymes, which are of great interest for industrial applications since,due to the immobilization on the cell surface, they can be easily used in the processing of keratin waste (Li, 2021).

Sources for the release of keratinase producers can be soil, keratin waste, and water (fresh or sea) (Anbesaw, 2022; Gudzenko et al., 2022). According to many scientists, the marine environment is a reserve of new enzymes and is of great interest as an inexhaustible source of microorganisms (Herzog et al., 2016). Oceans, as aquatic ecosystems covering 71% of the Earth's surface, contain multiple sources of enzymes from the classes of plants, animals, bacteria, and fungi (Hamiche et al., 2019). Marine microorganisms, which are an important part of marine ecosystems, play a significant role in the processes of transformation and mineralization of organic material in the marine environment. They differ from microorganisms

from other habitats in their physiological and biochemical properties, which are determined by the influence of such specific environmental factors as high concentration of salts, hydrostatic pressure, low temperatures, uneven supply of nutrients, as well as in the ability to synthesize unusual substances that exhibit a variety of biological activities (Duran & Cravo-Laureau, 2016).

Marine microorganisms are valuable and promising sources of new secondary metabolites. Thus, 50 unique bacterial isolates capable of breaking down keratin (feather meal) were isolated from feathers collected on the sea coast in the intertidal zone. The isolated microorganisms were identified using 16S rRNA sequencing. The majority of the identified isolates belonged to the genera *Bacillus* (42%) and *Stenotrophomonas* (40%). The remaining 18% were represented by the genera *Alcaligenes, Chryseobacterium, Salinivibrio, Delftia, Stappia,* and *Microbacterium,* which were not previously known to produce keratinase (Herzog et al., 2016). Representatives of the genus *Bacillus* are well-known producers of keratinases (for example, *B. licheniformis*, *B. subtilis, B. amyloliquefaciens*) (Herzog et al., 2016; Hamiche et al., 2019; Vidmar & Vodovnik, 2018). Strain *B. amyloliquefaciens* S13 was isolated from the brown alga *Zonaria tournefortii*, which synthesizes two types of extracellular keratinolytic enzymes with moderate elastolytic activity. These enzymes were effective on feather keratin and dehairing in the leather industry (Hamiche et al., 2019). A new bacterium, *Bacillus tropicus*, capable of destroying feathers, was isolated and identified in a soil sample from a sea duck farm in Beibu Creek in Guangxa (China) (Shen et al., 2022). Of 56 actinobacteria isolated from the southern coastal region of India, only three showed the ability to degrade feathers (Selvam et al., 2013).

Our studies of deep-sea sediments of the Black Sea have shown that they are a suitable source for isolating keratinolytic microorganisms. Interestingly, the most effective producers of keratinase cultures such as *P. megaterium* 035 (6), *B. subtilis* 231 (13), and *B. pumilus* A (17) from the twenty cultures studied were isolated from sediment samples taken at the same depth of 888 m, although *P. megaterium* 55 (9) and *B. subtilis* 248 (20) also exhibited high levels of keratinase activity and were isolated from sediments collected at greater depths of 1537 m and 1499 m, respectively. The presence of keratinase activity in the studied strains practically does not correlate with their manifestation of other proteolytic activities, in particular elastase, fibrinolytic, and fibrinogenolytic. Thus, previously (Gudzenko et al., 2024) the authors have shown that the cultures of *B. licheniformis* 249 (19), *B. subtilis* 248 (20), *B. subtilis* 1 (1), and *B. atrophaeus* 08 (3) are the most active producers of the above enzymes, while a marked keratinase activity was detected only in *B. subtilis* strain 248 (20).

Despite the fact that, in addition to keratinases, disulfide reductases also take part in the hydrolysis of feathers, we did not find a direct correlation in their activity. Thus, the highest level of disulfide reductase activity was observed in cultures *B. pumilus* A (17) (63.3 μmol/min), *B. subtilis* 248 (20) (62.0 μmol/min), and *P. megaterium* 035 (6) (61.3 μmol/min) and the lowest — in culture *B. licheniformis* 249 (19).

Thus, from the deep-sea sediments of the Black Sea, we have isolated a number of active producers of keratinases, representatives of the two genera *Bacillus* and *Priestia*, which, after studying their physicochemical and catalytic properties, may turn out to be promising for practical application, in particular in the development of new technologies for the utilization of down and feather poultry farm waste. This can be significant for the development of livestock feed resources and environmental protection. In connection with the prospect of biomodification of keratin-containing raw materials, research related to the discovery of new producers, the development of effective methods for isolating, purifying, and studying their properties is becoming very important.

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БАКТЕРІЇ ІЗ ГЛИБОКОВОДНИХ ВІДКЛАДЕНЬ ЧОРНОГО МОРЯ, ЯКІ ЗДАТНІ РОЗЩЕПЛЮВАТИ КЕРАТИН

Пухо-перова сировина, що утворюється на переробних підприємствах харчової промисловості, є однією із основних забруднювачів навколишнього середовища. Більшість підприємств для боротьби з цими відходами використовують методи захоронення та спалювання, що негативно впливає на екологічну ситуацію. Використання ферментативного гідролізу за участю мікроорганізмів-деструкторів є перспективним та безпечним методом переробки кератинових відходів. Раніше було показано, що бактерії, виділені з донних відкладень Чорного моря, є активними продуцентами еластаз, фібриногеназ та фібриназ. Тому **метою** даної роботи було оцінити здатність бактерій, виділених із глибоководних відкладень Чорного моря, проявляти інші види протеолітичної активності, зокрема розщеплювати важкодоступний білок кератин. **Методи**. Об'єктами дослідження були 20 культур бактерій, виділених із глибоководних відкладень Чорного моря та представлених родами *Bacillus*, *Metabacillus*, *Priestia* та *Robertmurraya*. Культури вирощували в умовах глибинного культивування при 28 °С зі швидкістю перемішування живильного середовища 232 об/хв протягом 4 діб. Для росту використовували базове живильне середовище, що містило 0,5% знежиреного курячого пір'я як єдине джерело вуглецю та азоту. Активність кератинази оцінювали за УФ-поглинанням при 280 нм продуктів гідролізу кератинвмісних матеріалів. Білок визначали за методом Лоурі, казеїнолітичну (загальну протеолітичну) активність — за методом Ансона. Активність дисульфідредуктази вимірювали спектрофотометрично при 412 нм шляхом оцінки жовтого сульфіду, утвореного під час відновлення 5,5'-дитіобіс-(2-нітробензойної кислоти) (DTNB). **Результати.** Показано, що культури *Metabacillus idriensis* 2 і *Robertmurraya siralis* 57, із двадцяти досліджених, не росли на живильному середовищі з курячим пір'ям як єдиним джерелом вуглецю та азоту. Інші 18 культур проявляли різний ступінь кератиназної активності (від 3 до 32 од/мл). Найвищий рівень активності характерний для культури *Priestia megaterium* 035 (32 од/мл). Вивчення здатності розщеплювати казеїн показало, що рівень протеолітичної активності більшості культур знаходився в межах від 0,015 од/мл до 0,14 од/мл. Найвищу протеолітичну активність виявляли культури *Bacillus pumilus* A (0,3 од/мл) і *Priestia megaterium* 55 (0,24 од/мл), які також демонстрували високий рівень кератиназної активності. Найвищий рівень дисульфідредуктазної активності спостерігався у *Bacillus pumilus* A (63,3 мкмоль/хв), *Bacillus subtilis* 248 (62,0 мкмоль/хв) і *Priestia megaterium* 035 (61,3 мкмоль/хв), а найнижчий — у *Bacillus licheniformis* 249.

Keywords: *бактерії з глибоководних відкладень Чорного моря, кератиназна активність, загальна протеолітична (казеїнолітична) активність, дисульфідредуктазна активність.*