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PROTEOLYTIC ACTIVITY OF *BACILLUS* STRAINS ISOLATED FROM SOIL OF RICE AGROCENOSIS

Microorganisms are the most common sources of commercial enzymes due to their physiological and biochemical properties, facile culture conditions, and ease of cell manipulation. Among microbial enzymes, proteases are ubiquitous in nature and have been found in all living forms encompassing the eukaryotes like plants, animals, fungi, and protists as well as the prokaryotic domains of bacteria and archaea. Proteases are the most important for the industry and constitute approximately 60% of the total industrial enzyme market. Among the bacteria, the genus Bacillus has a very prominent place in terms of the commercial production of proteases. Earlier from the water and bottom sediments of the Black Sea, we have isolated a number of producers of proteolytic enzymes from Bacillus species. The ***aim*** *of this work was to investigate the ability of representatives of a number of soil bacilli species to synthesize enzymes that hydrolyze such protein substrates as elastin, fibrin, fibrinogen, and keratin.* ***Methods****. The objects of the study were 8 cultures (KS 1 — KS 8) isolated from the soil of the rice agrocenosis. Cultures were grown under conditions of deep cultivation at 28 °C, with a mixing speed of for the nutrient medium of 230 rpm for 4 days. Methods of determining proteolytic (caseinolytic, elastolytic, fibrinolytic, fibrinogenolytic, and keratinase) activity in the culture liquid supernatant were used. 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.*** *The study of the spectrum of proteolytic activities of 8 freshly isolated strains showed that only KS 6 under experimental conditions did not show the ability to hydrolyze any of the studied substrates (casein, elastin, fibrin, fibrinogen, and keratin). Strains KS 1, KS 2, KS 7, and KS 8 showed higher levels of activity compared to other strains studied. The most interesting for further research are: I) strain KS 1, which showed the highest fibrinolytic activity, II) strain KS 2 as the most effective producer with elastase and fibrinogenolytic activity, III) KS 7 and KS 8, which simultaneously showed the highest rates as keratinase (7 U/mL and 9 U/mL) and sulfate reductase (33 μmol/min and 31 μmol/min) activity, respectively.* ***Conclusions.*** *According to the catalytic properties, a number of representatives of Bacillus, isolated from the soil of the rice agrocenosis may be promising for further research as an enzyme producer with proteolytic activity.*

Keywords: *Bacillus sp., proteolytic, disulfide reductase activity.*

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Microorganisms are the most common sources of commercial enzymes due to their physiological and biochemical properties, facile culture conditions, and ease of cell manipulation. Among microbial enzymes, proteases are ubiquitous in nature and have been found in all living forms encompassing the eukaryotes like plants, animals, fungi, and protists as well as the prokaryotic domains of bacteria and archaea. Many different proteases have been identified and characterized in viruses; however, from the commercial point of view, these have not gained much importance and mainly serve as targets of various antiviral drugs [1]. Proteases are the most important for industry and constitute approximately 60% of the total industrial enzyme market. These enzymes are used for food processing, pharmaceuticals, leather processing, silver retrieval in the x-ray film industry, industrial waste treatment, and as detergent additives. In bacteria, proteolytic systems are well known to play key roles in various functions, such as nutrition, virulence, protein turnover, regulatory events, protein maturation and export, and so on. Among the bacteria, the genus *Bacillus* has a very prominent place in terms of the commercial production of proteases. Indeed, among the *Bacillus* species isolated from the water and bottom sediments of the Black Sea, earlier [2, 3] we have identified a number of producers of proteolytic enzymes. The **aim** of this work was to investigate the ability of representatives of a number of soil bacilli species to synthesize enzymes that hydrolyze such protein substrates as elastin, fibrin, fibrinogen, and keratin.

Materials and methods. The objects of research were *Bacillus* sp. KS1, KS2, KS3, KS4, KS5, KS6, KS7, and KS8, isolated from the soil of the rice agrocenosis (Odesa region, Kiliya district, Kiliya).

For submerged fermentation, strains were cultivated in Erlenmeyer flasks containing 100 mL of medium of the following compositions: KH_2PO_4 — 1.0; $MgSO_4 \times 7H_2O$ — 0.75; $ZnSO_4 \times H_2O$ — 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 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.

Caseinolytic (total proteolytic) activity was determined by the Anson method modified by Petrova [4]. Elastase activity was determined colorimetrically by the color intensity of the solution during the enzymatic hydrolysis of elastin stained with Congo-rot using the Trombridge method [5] in Bondarchuk's modification [6]. 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 h 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 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.

Fibrinolytic and fibrinogenolytic activities were determined by the recommended methods [7] with fibrin and fibrinogen as substrates.

To determine the keratinase activity (KerA), microorganisms were grown in large 50 ml test tubes on a nutrient medium of the following composition: K_2HPO_4 — 1.4; KH_2PO_4 — 0.7; $MgSO_4 7 \times H_2O$ — 0.1; NaCl — 0.5; defatted chicken feathers — 5; H_2O — up to 1 L; pH 6.8—7.0. Chicken feathers were defatted with a mixture of chloroform and methanol in a 1:1 ratio.

The keratinase activity was determined by the UV absorption at 280 nm of the 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 9.2), and 1 mL of culture liquid, was kept in a thermostat at 37 °C for 3 h, after which it was filtered. To determine KerA, two controls were used: (1) 10 mg of crushed defatted feathers, 2.5 mL of 0.05 M boron-borate buffer (pH 9.2), and 1 mL of distilled water; (2) 2.5 mL of 0.05 M boron-borate buffer (pH 9.2) and 1 mL of culture liquid. 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 filtrate of the test sample relative to the controls was taken as the degree of protein release [8]. 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.

The disulfide reductase activity was determined according to Gupta [9] with minor modifications. 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 enzyme was added to this mixture. Upon reduction of DTNB, a yellow sulfide is 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 method of Lowry [10]. The standard curve of bovine serum albumin (BSA) (1 mg/mL^{-1}) 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 study of the total proteolytic (caseinolytic) activity showed (Fig. 1) that on the fourth day of cultivation, activity was detected in the culture liquid supernatants of 7 *Bacillus* sp. strains. The highest activity was noted in strain KS 1 (2 U/mL). The activity was somewhat lower

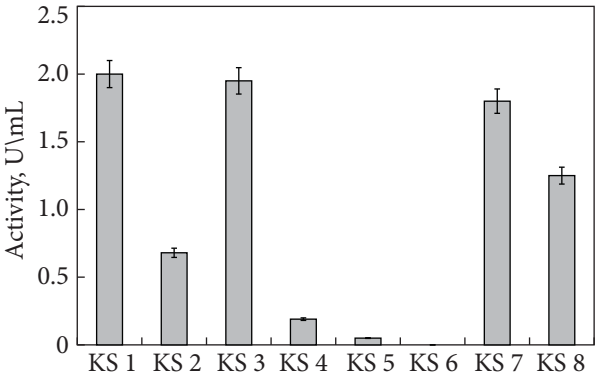

Fig. 1. Caseinolytic (total proteolytic) activity of *Bacillus* sp. KS 1-KS 8

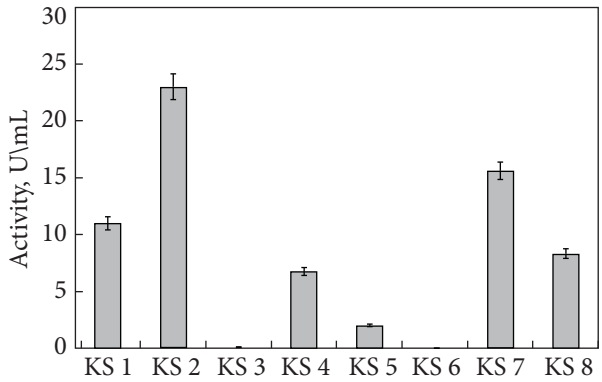

Fig. 2. Elastase activity of *Bacillus* sp. KS 1—KS 8

in strains KS 3 (1.95 U/mL), KS 7 (1.8 U/mL), and KS 8 (1.25 U/mL). Insignificant levels of activity were noted in strains KS 2 (0.68 U/mL), KS 4 (0.19 U/mL), and KS 5 (0.05 U/mL). Only one strain KS 6 was found to have no overall proteolytic (caseinolytic) activity.

Among the 8 studied strains, the ability to hydrolyze elastin was found in 7 studied microorganisms (Fig. 2). The highest activity was noted in strain KS 2 (23 U/mL). The elastase activity of strain KS 7 was slightly lower and amounted to 15.6 U/mL. The activity of strain KS 1 (11 U/mL) was more than twice lower than that of strain KS 2. Strains KS 8 and KS 4 showed lower levels of elastase activity, which were 8.3 and 6.77 U/mL, respectively. Trace levels of activity were noted in strain KS 3 (0.078 U/mL), and

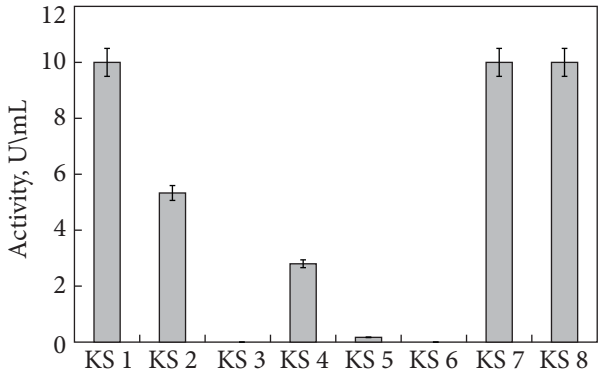

Fig. 3. Fibrinolytic activity of *Bacillus* sp. KS 1—KS 8

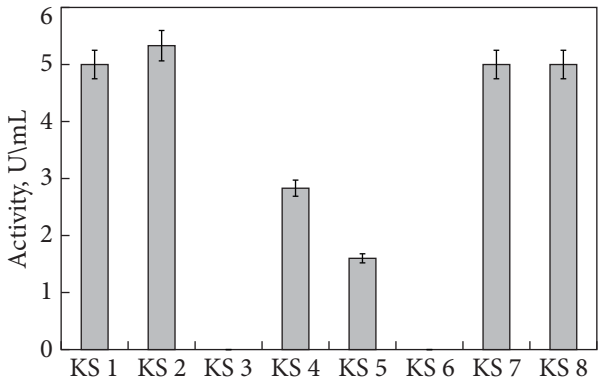

Fig. 4. Fibrinogenolytic activity of *Bacillus* sp. KS 1—KS 8

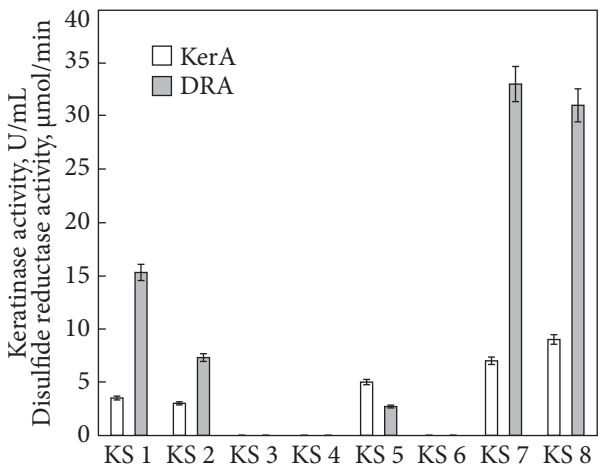

Fig. 5. Keratinase and disulfide reductase proteolytic activities of *Bacillus* sp. KS 1—KS 8

only one of the 8 studied strains (KS 6) did not show any ability to hydrolyze elastin under experimental conditions.

The study of fibrinolytic activity showed that 6 strains were able to hydrolyze fibrin under experimental conditions (Fig. 3). The highest activity was noted in strains KS 1, KS 7, and KS 8, which was 10 U/mL (Fig. 3). Cultures KS 2 and KS 4 showed lower activity of 5.33 and 2.8 U/mL, respectively. The lowest activity was noted in strain KS 5 (0.17 U/mL). Strains KS 3 and KS 6 did not show any ability to hydrolyze fibrin under experimental conditions.

The study of fibrinogenolytic activity showed that 6 cultures displayed different levels of the activity (Fig. 4). The highest activity was noted for strain KS 2 (5.33 U/mL). In strains KS 1, KS 7, and KS 8, it was at the same level and amounted to 5 U/mL. The activity of strains KS 4 and KS 5, which amounted to 2.83 and 1.6 U/mL, respectively, was slightly lower compared to strains KS 3 and KS 6 and did not show the ability to hydrolyze fibrinogen under experimental conditions.

It was found that 5 of 8 cultures showed keratinase activity. The level of keratinase activity varied from 3 to 9 U/mL (Fig. 5). An important role is played not only by the presence of keratinase activity but also by the degree of cleavage of the substrate. Of the 5 cultures, only two, KS 7 and KS 8, completely split the feathers, leaving only feather cores. KS 1, KS 2, and KS 5 cultures synthesized keratinase with an activity of 3 to 5 U/mL, but the degree of feather splitting was insignificant. The ability of the producer to synthesize sulfate reductase also plays a significant role in the process of feather splitting. Insignificant sulfate reductase activity was observed in cultures KS 2 and KS 5 and amounted to 7.3 and 2.7 μmol/min, respectively. The highest rates were typical for cultures KS 7 (33 μmol/min) and KS 8 (31 μmol/min). The KS 1 culture showed an activity of 15.3 μmol/min (Fig. 5). As a result of the research, the cultures KS 7 and KS 8 turned out to be the most promising.

Discussion. Bacteria of the genus *Bacillus* have versatile polyenzymatic activity. Their enzyme systems include a set of enzymes of various classes, which allows them to exist on a variety of substrates. The representatives of *Bacillus* have a particularly developed system of hydrolases. They synthesize proteases, chitinases, α-amylases, lactamases, cellulases, and a number of other enzymes [11]. Of particular interest are the extracellular proteolytic enzymes of *Bacillus,* which have different specificities with respect to the type of hydrolyzable bond. Therefore, the search for new highly efficient producers of proteolytic enzymes capable of splitting various types of proteins, such as keratin, elastin, fibrin, fibrinogen, and casein remains relevant at the present time. Proteases that cleave the above protein substrates are of wide practical use. Thus, keratinases are of great interest from the point of view of solving the global problem of recent decades – the conversion of the main protein of keratin-containing waste (for example, feather waste is 90% protein) into an accessible and easily digestible form (feather hydrolyzates). The latter, in turn, can be used as a source of feed protein and fertilizer for plants [8, 9]. Also, keratinases are successfully used in various industries, such as agriculture, in the production of washing powders, cosmetics, pharmaceuticals, and the leather and textile industries.

Fibrinogenolytic enzymes can be considered as a basis for the creation of drugs aimed at reducing the threat of intravascular thrombus formation by limiting proteolysis of fibrinogen circulating in the patient's bloodstream. Proteases characterized by fibrinogenolytic activity are part of drugs that prevent blood clotting and lower blood pressure. The anticoagulation effect of such proteinases is enhanced by the fact that a significant part of these enzymes also affects the platelet link of the hemostasis system, inhibiting platelet aggregation [12, 13].

Microbial elastolytic enzymes have a huge potential for use in industry for the hydrolysis of raw materials containing elastin fibers, in particular in the meat processing and fishing industries. Elastases and their complex enzyme preparations are widely used in various fields of medicine to treat burn wounds, inflammatory processes, edema, hematomas, and frostbite, to accelerate the rejection of dead tissues, trophic ulcers, and the cleaning of purulent-necrotic plaques [12].

Fibrinolytic enzymes have enormous potential for use as food additives, as well as for the treatment of thrombosis and associated diseases.

Hence, the spectrum of the use of proteolytic enzymes in various branches of industry and medicine is extremely wide, therefore, the identification of new producers of proteolytic enzymes is an important direction of modern research.

The study of the spectrum of proteolytic activities of 8 freshly isolated strains showed that only KS 6 under experimental conditions did not show the ability to hydrolyze any of the studied substrates (casein, elastin, fibrin, fibrinogen, and keratin). Strains KS 1, KS 2, KS 7, and KS 8 showed higher levels of activity compared to other strains studied. The most interesting for further research are I)strain KS 1, which showed the highest fibrinolytic activity, II) strain KS 2 as the most effective producer with elastase and fibrinogenolytic activity, III) KS 7 and KS 8, which simultaneously showed the highest rates in keratinase (7 U/mL and 9 U/mL) and sulphate reductase (33 μmol/min and 31 μmol/min) activities, respectively. Why are the two activities important for keratin breakdown? not only keratinases are involved in the process of keratin destruction but also other enzymes such as peptidases, disulfide reductase or reducing agents such as sulfite, bisulfite, and disulfide. Breaking the disulfide bonds required for keratin folding is an essential step to facilitate keratinase access to the substrate. The synergy of sulfitolysis and proteolysis contributes to the complete degradation of the structure of the keratin complex, resulting in the formation of hydrolyzate, which consists

mainly of soluble oligopeptides and amino acids [14, 15]. Since keratinase synthesis is mainly an inducible process [14], we used chicken feathers during screening, which are among the most commonly used substrates since feather splitting can be easily monitored visually [14, 16].

Conclusions. The data we obtained on the proteolytic activity of *Bacillus* strains with respect to proteins of various nature can indicate to a certain extent the multicomponent nature of the proteases produced by bacilli, reflect the complexity and integration of the life processes occurring in these microorganisms, as well as their ability to adapt to various living conditions. The strains studied by us showed a wide substrate specificity, which makes them promising for further practical application.

REFERENCES

1. Razzaq A, Shamsi S, Ali A, Ali Q, Sajjad M, Malik A and Ashraf M. Microbial Proteases Applications. Front Bioeng Biotechnol. 2019; 7:110.
2. Gudzenko OV, Ivanytsia VO, Varbanets LD. Bacteria of the Black Sea are Producers of Proteolytic Enzymes. Mikrobiol Z. 2022; 3:3—8.
3. Varbanets LD, Avdeeva LV, Borzova NV, Matselukh OV, Gudzenko OV, Kiprianova OH, et al. [The black sea bacteria — producers of hydrolytic enzymes]. Mikrobiol Z. 2011; 73(5):9—20. Ukrainian.
4. Petrova IS, Vintsyunayte MN. [Determination proteolytic activity enzyme preparations of microbial origin]. Prikl Biokhim Mikrobiol. 1966; 2(1):322—327. Russian.
5. Trombridge GO, Moon HD. Purification of human elastase. Proc Soc Exp Biol Med.1972; 141(3):928—931.
6. Nidialkova NA, Matseliukh OV, Varbanets LD. [Physico-chemical properties of *Bacillus thuringiensis* IMV B-7324 fibrinolytic peptidase]. Mikrobiol Z. 2013; 75(4):3—7. Ukrainian.
7. Varbanets LD, Matseliukh EV. Peptidases of microorganisms and methods of their investigations. Kyiv, Naukova Dumka. 2014; 323 p.
8. Nickerson WJ, Noval JJ, Robison RS. Keratinase. I. Properties of the enzyme conjugate elaborated by *Streptomyces fradiae*. Biochim Biophys Acta. 1963; 77(1):73—86.
9. Gupta S, Singh SP, Singh R. Synergistic Effect of Reductase and Keratinase for Facile Synthesis of Protein-Coated Gold Nanoparticles. J Microbiol Biotechnol.2015; 25(5):612—619.
10. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with folinphenol reagent. J Biol Chem. 1951; 193(2):265—275.
11. AlShaikh-Mubarak GA, Kotb E, Alabdalall AH, Aldayel MF. A survey of elastase-producing bacteria and characteristics of the most potent producer, *Priestia megaterium* gasm32. PLoS One. 2023; 18(3):e0282963.
12. Lei Y, Zhao P, Li C, Zhao H, Shan Z, Wu Q. Isolation, identification and characterization of a novel elastase from *Chryseobacterium indologenes*. Appl Biol Chem. 2018; 61(3):365—72.
13. Craik CS, Page MJ, Madison EL. Proteases as therapeutics. Biochem J. 2011; 435(1):1—16.
14. Li Q. Structure, Application, and Biochemistry of Microbial Keratinases. Front Microbiol. 2021; 12:674345.
15. de Menezes CLA, Santos RC, Santos MV, Boscolo M, da Silva R, Gomes E, da Silva RR. Industrial sustainability of microbial keratinases: production and potential applications. World J Microbiol Biotechnol. 2021; 37(5):86—102.
16. Sharma R, Devi S. Versatility and commercial status of microbial keratinases: a review. Rev Environ Sci Biotechnol. 2018; 17(2):19—45.

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ПРОТЕОЛІТИЧНА АКТИВНІСТЬ ШТАМІВ *BACILLUS*,
ІЗОЛЬОВАНИХ З ҐРУНТУ АГРОЦЕНОЗУ РИСУ

Мікроорганізми є найпоширенішими джерелами комерційних ферментів завдяки їхнім фізіологічним і біохімічним властивостям, легким умовам культивування та легкості маніпулювання клітинами. Серед мікробних ферментів протеази є широко розповсюдженими у природі та присутні у всіх живих формах, що охоплюють як еукаріоти – рослини, тварини, гриби, протисти, так і прокаріотичні домени бактерій та архей. Протеази є найважливішими для промисловості та складають близько 60 % загального ринку промислових ферментів. Серед бактерій рід *Bacillus* займає дуже помітне місце з точки зору комерційного виробництва протеаз. Раніше з води та донних відкладень Чорного моря нами було виділено ряд продуцентів протеолітичних ферментів з видів *Bacillus*. **Метою** роботи було дослідити здатність представників видів ґрунтових бацил синтезувати ферменти, що гідролізують такі білкові субстрати, як еластин, фібрин, фібриноген, кератин. **Методи.** Об'єктами дослідження були 8 культур (KS 1—8), виділених з ґрунту агроценозу рису. Культури вирощували в умовах глибинного культивування при 28 °С зі швидкістю перемішування поживного середовища 230 об/хв протягом 4 діб. Використовували методи визначення протеолітичної (казеїнолітичної, еластолітичної, фібринолітичної, фібриногенолітичної та кератиназної) активності в супернатанті культуральної рідини. Дисульфідредуктазну активність вимірювали спектрофотометрично при 412 нм шляхом оцінки сульфіду жовтого кольору, що утворюється при відновленні 5,5'-дітіобіс-(2-нітробензойної кислоти) (ДТНБ). **Результати.** Вивчення спектру протеолітичної активності 8 свіжовиділених штамів показало, що лише KS 6 в експериментальних умовах не виявив здатності гідролізувати жоден із досліджуваних субстратів (казеїн, еластин, фібрин, фібриноген і кератин). Штами KS 1, KS 2, KS 7 і KS 8 показали вищі рівні активності порівняно з іншими дослідженими штамами. Найбільш цікавими для подальших досліджень є: I) штам KS 1, який виявив найвищу фібринолітичну активність, II) штам KS 2 як найефективніший продуцент з еластазною та фібриногенолітичною активністю**,** III) KS 7 та KS 8, які одночасно виявили найвищі показники кератиназної активності (7 Од/мл і 9 Од/мл) і сульфатредуктази (33 мкмоль/хв і 31 мкмоль/хв) відповідно. **Висновки.** Ряд представників *Bacillus,* виділених із ризосфери ґрунту агроценозу рису, за своїми каталітичними властивостями можуть бути перспективними для подальших досліджень як продуценти ферментів з протеолітичною активністю.

Ключові слова: *Bacillus sp., протеолітична активність, дисульфідредуктазна активність.*