https://doi.org/10.15407/microbiolj85.05.003

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SUBSTRATE SPECIFICITY OF *BACILLUS MEGATERIUM* UCM B-5710 KERATINASE

The specifics of the processing of livestock and poultry products is that in the process of obtaining the main marketable products, about half the feedstock at various stages of the technological process turns into waste that pollutes the environment. These by-products contain large amounts of the hard-to-digest keratin protein. The use of specific enzymes capable of degrading this protein helps not only to reduce the negative anthropogenic impact on nature but also to obtain valuable hydrolysates that can be used as a fertilizer for plants or a feed additive. The ***aim*** *of this work was to study the ability of Bacillus megaterium UCM B-5710 to split various keratin-containing substrates: black and white chicken feathers, white turkey feathers, parrot feathers of various colors, sheep wool, pig bristles, and baby hair and nails.* ***Methods.*** *The culture was grown under conditions of submerged cultivation at 40 °C, with a nutrient medium stirring rate of 201 rpm for 6 days. For growth, a basic nutrient medium containing 0.5% defatted chicken feathers or other keratin-containing substrates as sole sources of carbon and nitrogen were used. Keratinase activity was assessed by UV absorption at 280 nm of hydrolysis products of keratin-containing raw materials. Protein was determined by the Lowry method, caseinolytic (total proteolytic) activity was determined by the Anson method modified by Petrova, and amino acid content was determined by the ninhydrin method. The degree of hydrolysis of the substrates was evaluated by the ratio of the initial and final weight of the substrate.* ***Results.*** *It was shown that the synthesis of keratinase by the culture of B. megaterium UCM B-5710 begins from the 6th hour of cultivation. The level of protein and proteolytic activity and the content of amino acids increased throughout the entire period of culture growth. The supernatant of the culture liquid of B. megaterium UCM B-5710 was most effective in splitting white chicken's and turkey's feathers, a little slower — feathers of black chicken and blue parrots, as well as wool of white sheep. According to the degree of splitting, the substrates used can be arranged in the following order: white turkey feathers > white chicken feathers > black chicken feathers > blue parrot feathers > white sheep wool > baby nails > pig bristle > baby hair. The study of the effect of feather color on the resistance to decomposition showed that black, blue, and red feathers are more resistant, which coincides with the literature data.* ***Conclusions.*** *B. megaterium UCM B-5710 produces keratinase capable of splitting both α- and β-keratins, however, with different efficiencies and rates.*

Keywords: *Bacillus megaterium UCM B-5710, keratinase, substrate specificity, keratin-containing substrates, α-keratin, β-keratin.*

Citation: Avdiyuk K.V., Varbanets L.D. Substrate Specificity of *Bacillus megaterium* UCM B-5710 Keratinase. *Microbiological journal.* 2023 (5). P. 3—11. https://doi.org/10.15407/microbiolj85.05.003

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Processing of livestock products, poultry farming, and wool-processing industries are associated with the formation of a large number of protein-containing by-products, such as feathers, hooves, horns, wool, etc. [1]. So, for example, about 400 tons of soft waste (intestines, skin, paws, heads) and 180 tons of feathers are generated per day at the Vinnytsia poultry farm. For most enterprises, the disposal of the resulting by-products is one of the main problems [2]. At the same time, it is known that protein-containing wastes make up about 85—90% keratin [1, 3]. Keratin is a structural fibrillar protein, which is the third most abundant in nature after cellulose and chitin [4]. Natural keratins are rich in acidic and basic amino acids but have a fairly low level of histidine, lysine, and methionine along with an unusually high content of cysteine residues. The latter, in turn, largely determines the special physicochemical properties of keratins: they are insoluble in water, solutions of neutral salts, dilute acids, and alkalis and resistant to conventional proteases (pepsin, trypsin, and papain). The presence of a large number of thiol groups in keratin determines the significant role of disulfide covalent bonds in the formation and maintenance of the native structure of polypeptide [1, 5]. Therefore, the disposal of keratin-containing waste is a complex technological process associated with the use of high temperatures and pressures. To date, the most common methods are hydrothermal and chemical treatment (acid and alkaline hydrolysis), however, they are energy-consuming, characterized by low digestibility and lead to a product with a deficiency of some amino acids (methionine, lysine, tryptophan) [6, 7]. It is not uncommon for waste to be taken directly to the fields, or to be buried, which can adversely affect the environmental situation [4]. However, the most promising, environmentally friendly, and safe approach is a biotechnological method for processing keratin-containing raw materials, based on the ability of microorganisms to convert the initial substances of the substrate into new useful metabolites due to the synthesis of specific keratinase enzymes. Keratinases are extracellular proteolytic enzymes that catalyze the hydrolysis of the insoluble protein keratin. Keratinase producers have been found among bacteria (genus *Bacillus*, *Enterococcus*, *Micrococcus*, *Staphylococcus*, *Streptomyces*, *Pseudomonas*) and micromycetes (genus *Aspergillus*, *Alternaria*, *Epidermophiton*, *Fusarium*, *Keratinomyces*, *Microsporum*, *Penicillium*, *Trichophyton*) [1, 4]. These enzymes have a wide range of applications: in the leather industry, primarily at the dehairing stage, in the food industry for tenderizing meat and fish, in the production of food and feed protein, fertilizers for plants, in the production of glue, in the creation of cosmetic products (nourishing creams, lotions). The use of keratinases in medicine is very effective for the preparation of diagnostic media, therapeutic sera and vaccines, as well as in the production of cleaning agents and wastewater treatment [1—4, 7].

In connection with the foregoing, studies aimed at finding highly active producers of keratinases and studying their ability to split various keratin-containing substrates are relevant. Previously [8], we have shown that the culture of *Bacillus megaterium* UCM B-5710 is an active producer of keratinase. The aim of this work was to study the substrate specificity of *B. megaterium* UCM B-5710 keratinase.

Materials and Methods. In this work, we used a strain of the bacterium *B. megaterium*, deposited under the number UCM B-5710 in the collection of microorganisms of the Zabolotny Institute of Microbiology and Virology, NAS of Ukraine.

For the cultivation of *B. megaterium* UCM B-5710, the basic nutrient medium of the following composition (g/L) was used: K_2HPO4 — 1.4; KH_2PO4 — 0.7; $MgSO_4x7H_2O$ — 0.1; NaCl — 0.5; defatted chicken feathers (or other keratin-containing substrates) — 5; H_2O — up

to 1 l; pH 6.8—7.0. For the experiment, we used white chicken feathers, white turkey feathers, black chicken feathers, blue and yellow parrot feathers, red Ara parrot feathers, baby hair and nails, white sheep wool, pig bristles. Keratin-containing substrates 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.

A daily culture of *B. megaterium* UCM B-5710 was used as an inoculum. *B. megaterium* UCM B-5710 strain was grown by the submerged cultivation method in 0.75 L Erlenmeyer flasks with 100 mL of the above liquid nutrient medium (pH 8.9) and in large 50 mL test tubes (experiment with different keratin-containing substrates) with 10 mL of the medium at a temperature of 40 °C, stirring speed 201 rpm for 6 days. To obtain a supernatant (culture liquid supernatant, SCL), which was used 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 culture liquid 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 supernatant of culture liquid. From the values obtained by measuring the optical density of the filtrates at A_{280}, the sum of the values of the two controls was subtracted. The increase in absorbance at 280 nm of the studied filtrate relative to controls was taken as the degree of protein release [6]. The amount of enzyme that causes an increase in absorption by 0.01 for 3 hours of incubation (1 U/mL = 0.01) was taken as a unit of keratinase activity [9]. The total proteolytic (caseinolytic) activity was determined by the Anson method modified by Petrova [10], which is based on the quantitative determination of tyrosine formed during the enzymatic hydrolysis of casein under the influence of the studied enzymes.

The protein content was determined by the Lowry method [11], the total amount of free amino acids was determined by the ninhydrin method [10]. The degree of hydrolysis of keratin-containing substrates D (%) was determined by the formula: D = (Gi — Gf) / Gi × 100%, where Gi is the initial weight of the intact substrate before the fermentation process, Gf is the final weight of the residual substrate after the fermentation process [12].

All experiments were carried out in 3—5 repetitions. The analysis of the obtained results was carried out by their statistical processing using Student's t-test. The results presented graphically were drawn up using Microsoft Excel 2016.

Results. The study of the dynamics of culture growth on a medium containing white chicken feathers as a substrate showed that a slight synthesis of keratinase was observed already after 6 hours, and the maximum keratinase activity (10 U/mL) was observed after 48 hours from the start of cultivation, then the synthesis of the enzyme sharply decreased. The level of protein, proteolytic activity, and amino acid content in the culture liquid supernatant increased throughout the entire cultivation period (Fig. 1). In addition, during the growth of the *B. megaterium* UCM B-5710 culture, a slight increase in pH from 8.9 to 9.34 was observed. The authors for the *B. velezensis* NCIM 5802 culture [12] obtained similar results.

To study the ability of the SCL of *B. megaterium* UCM B-5710 to split various keratin-containing substrates, white and black chicken feathers, white turkey feathers, blue parrot feathers, white sheep wool, pig bristles, baby nails and hair were taken as the only source of carbon and nitrogen. As known from the literature [1], these

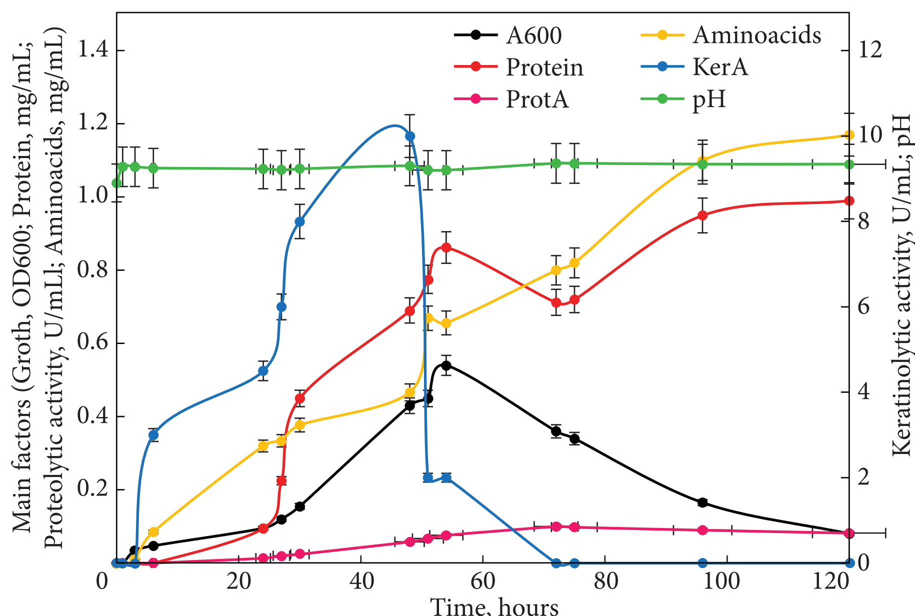

Fig. 1. Dynamics of changes in activity, amino acid content, and pH during cultivation of *B. megaterium* UCM B-5710 on white chicken feathers

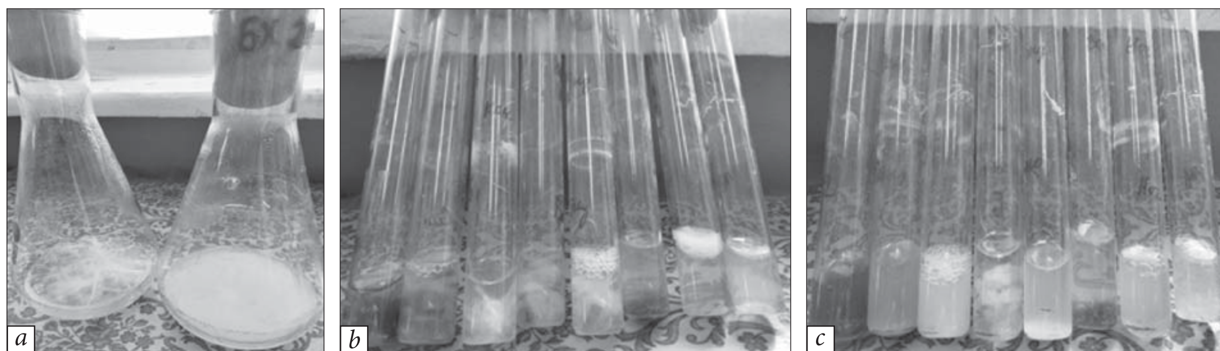

Fig. 2. Degradation of different keratin-containing substrates by *B. megaterium* UCM B-5710 (72 hours): *a* — the control flask on the left and the experimental flask with degraded feathers on the right; *b* — the control tubes with different substrates: black chicken feathers, blue parrot feathers, white chicken feathers, white sheep wool, white turkey feathers, baby hair, pig bristles, and baby nails (from left to right); *c* — test tubes with different substrates: black chicken feathers, blue parrot feathers, white chicken feathers, white sheep wool, white turkey feathers, baby hair, pig bristles, and baby nails (from left to right)

substrates differ in amino acid composition and the type of keratin from which they are composed. To compare the efficiency of cleavage of different keratin-containing products, we used the method of measuring soluble protein in the SCL of *B. megaterium* UCM B-5710 at A_{280} and studied the degree of substrate hydrolysis (Table 1). The *B. megaterium* UCM B-5710 SCL was most effective in splitting white chicken and turkey feathers, and a little slower — black chicken feathers and blue parrot feathers, as well as white sheep wool. According to the degree of splitting, the used substrates can be arranged in the following order (Table 1): white turkey feathers > white chicken feathers > black chicken feathers > blue parrot feathers > white sheep wool > baby nails >

pig bristles > baby hair. The results obtained were confirmed by visual observations (Fig. 1), which demonstrate the above regularity in the splitting of various keratin-containing substrates.

A number of authors have shown [13, 14] that the pigments that make up the feather are able to partially protect it from degradation. To establish whether the color of feathers affects the rate of their splitting, we took for comparison feathers containing different pigments such as feathers of white chicken and turkey, black chicken, yellow and blue feathers of a parrot, and red Ara parrot feathers. It is shown (Table 2) that after 72 hours of incubation with culture liquid supernatant, *B. megaterium*'s efficiency as depending on the feather color can be arranged in the following order: white chicken feathers > red parrot feathers > yellow parrot feathers > black chicken feathers > blue parrot feathers. Thus, the culture of B. megaterium UCM B-5710 has a high potential for splitting different types of feathers.

Discussion. Keratinases are unique enzymes capable of breaking down the hard-to-split protein keratin. The studied culture of *B. megaterium* UCM B-5710, as we showed earlier [8], is an effective producer of keratinase. The study of the growth curve of this culture, the level of keratinase and proteolytic activity, protein, the content of free amino acids in the SCL in dynamics showed that keratinase synthesis began 6 hours after the start of cultivation, and the levels of the above indicators increased with increasing culture cultivation time. Keratinase activity reached its maximum (10 U/mL) 48 hours after the start of cultivation, then the level of enzyme activity decreased. A similar decrease in the level of activity was observed in the case of *Bacillus putida* keratinase [15]. An increase in the content of protein, amino acids, and pH in the nutrient medium during cultivation is characteristic of many cultures capable of degrading keratin-containing substrates [12].

An increase in the level of proteolytic activity and free amino acids during the entire process of *B. megaterium* UCM B-5710 cultivating may indicate that, in addition to keratinase, *B. megaterium* synthesizes some other proteolytic enzymes capable of cleaving peptides or splitting off free amino acids. An important indicator of the action of keratinase is not only the level of keratinase activity of the culture but also the ability to split different types of keratin-containing substrates. The latter, in turn, differ in the structure and content of different types of keratin. It is known that on the basis of the secondary structure, keratin is divided into two

Table 1. **Degradation efficiency of various keratin-containing substrates by *B. megaterium* UCM** B-5710 **(M ± m)**

Keratin-containing substrates	A_{280}	Degree of substrate hydrolysis,% (72 hours)
White chicken feathers	0.710 ± 0.035	66
Black chicken feathers	0.430 ± 0.021	43
White turkey feathers	0.610 ± 0.030	60
Blue parrot feathers	0.440 ± 0.022	39
White sheep wool	0.500 ± 0.025	28
Baby hair	0.012 ± 0.001	7
Baby nails	0.280 ± 0.014	20
Pig bristles	0.090 ± 0.004	12
Control	—	0

Table 2. **Degradation efficiency of colored feathers by *B. megaterium* UCM** B-5710 **(M ± m)**

Keratin-containing substrates	A_{280}	Degree of substrate hydrolysis,% (72 hours)
White chicken feathers	0.710 ± 0.035	66
Black chicken feathers	0.430 ± 0.021	43
Yellow parrot feathers	0.640 ± 0.032	54
Blue parrot feathers	0.440 ± 0.022	39
Red parrot feathers	0.405 ± 0.020	59
Control	—	0

types: α- and β-keratin. α-keratin consists of α-helices, which form fibrils due to interchain interactions, while β-keratin is mainly built up of β-sheets [3, 4]. α-keratin is most often found in the epidermal structures of mammals, such as hair, wool, horns, and in small amounts in the epidermis between the scales of reptiles and in bird feathers, providing the mechanical strength of epithelial cells, their adhesion, and change in shape when stretched. At the same time, β-keratin is the main component of the tissues (feathers) of birds and reptiles (scales). An exception is the integument of the pangolin (a unique scaly anteater), whose keratin scales contain both types of keratin. β-keratin, which is part of the scales of reptiles, has limited extensibility, exhibits microbiological stability and hydrophobicity, providing a protective function. Some authors [1] believe that these two types of keratin are not evolutionarily related to each other.

The keratinase of *B. megaterium* UCM B-5710 studied by us most effectively and quickly split white chicken (66%) and turkey feathers (60%), which consist mainly of β-keratin and a small amount of α-keratin. Similar properties were exhibited by *B. pacificus* RSA27 keratinase [16], which completely hydrolyzed feathers (94.5%) in a fermenter with a volume of up to 5 l for 24 hours with high activity (789 U/mL) and a total amino acid content of 153.97 μmol/mL. Cultures of *Bacillus cereus* S15, S26, and S1 showed the highest keratinase activity when using white chicken feather as a substrate (6.3 U/mL, 5.3 U/mL, and 4.3 U/mL, respectively), followed by white sheep wool (3.8 U/mL for S26), black chicken feather (3.0 U/mL for S15), black sheep wool (2.9 U/mL for S15), and baby hair (2.8 U/mL for S1) [17]. *Pseudomonas aeruginosa* (E2) and *Pseudomonas putida* (B2) also showed the highest keratinase activity on the nutrient medium with feathers, and the lowest activity on the medium with nails [15]. Similar results were obtained for *B. licheniformis* dcs1 keratinase, whose ability to hydrolyze various keratin-containing substrates can be ranked as follows: feathers (48.5 U/mL) < bovine hair (35 U/mL) < baby nails (12 U/mL) < baby hair (10 U/mL) [18].

B. cereus 35 keratinase is able to split chicken feathers, hooves, hair, and nails with practically the same efficiency [19]. Since feathers are mostly composed of β-keratin, this indicates a high ability and specificity of the culture to degrade this particular type of keratin. *B. subtilis* KD-N2 keratinase had a wide range of substrate specificity and could degrade substrates containing both α- and β-keratins. It was active against such insoluble substrates as feather meal, feather keratin, human hair, and sheep wool, but was unable to split calf hair and silk [20]. Comparison of the ability of *B. megaterium* UCM B-5710 culture to degrade various substrates consisting of α-keratin (wool, nails, hair, pig bristles) showed that they were partially degraded, with different rates and efficiency. Among the above substrates, *B. megaterium* UCM B-5710 hydrolyzed white sheep wool best of all (the degree of its hydrolysis was 28%), whereas baby nails (20%), pig bristles (12%), and baby hair (7%) were split worse. Differences in the ability of culture to decompose substrates consisting of α-keratin are associated with differences in their structure. Thus, hair and nails have a similar structure, although the nail is more uniform than hair. Micro- and macrofibrils of the nail are organized like a hair cortex, their layers contain a large number of disulfide bridges along the periphery of each nail cell. A distinctive feature of wool keratin is a significantly higher (from 3 to 5%) sulfur content in it than in other proteins.

Interesting are the studies related to the investigation of the ability of various microorganisms to split bird feathers of different colors. The black and brown color of feathers is due to melanin pigments included in the feather structure the during its development. Melanins strengthen feathers, reduce their wear, especial-

ly in those birds that fly long distances or live in a marine environment filled with abrasive sand, salt, and intense sunlight [13]. The brightest colors of bird plumage are provided by carotenoid-based pigments, which come in red, yellow, and orange. However, birds do not synthesize their own carotenoids: these pigments are borrowed from their diet and «introduced» into the growing feathers. Parrots are considered unique among birds: the bright red, yellow, or orange color of their feathers is acquired not due to food carotenoids, but through the synthesis of their own pigments, called «psittacofulvins» [14]. Interestingly, these fat-soluble pigments are found nowhere else in the animal kingdom. The data obtained in [13] showed that some pigments in bird feathers not only act as color generators, but also maintain the plumage integrity, increasing the resistance of feathers to bacterial decomposition. There is an assumption that pigments can reduce the degree of microbial damage to feathers. White feathers degrade more rapidly than black, blue, green, and red feathers, which are almost at the same rate of degradation. This was also confirmed by our research. Thus, white and yellow feathers were decomposed by *B. megaterium* UCM B-5710 culture after 1—1.5 days, while black, blue, and red feathers took twice longer time. It is known from the literature [13] that yellow feathers containing yellow psittacofulvins are destroyed almost as quickly as white feathers, free of pigments. And the combination of pigments (yellow psittacofulvin and melanin), which are part of green feathers, does not slow down the rate of decomposition of feathers by bacteria compared to feathers that contain only melanin or only red psittacofulvin. The reason for this lies in the biochemical structure of the molecules of yellow and red psittacofulvins. Yellow pigment molecules are formed by small carbon chains with a small number of double bonds, while red pigment molecules — by longer carbon chains with a large number of double bonds, so they are more difficult to cleave [13]. The scientists [21] showed that non-melanized feathers degraded earlier, supported more bacterial growth, and lost more mass than melanized feathers. These results confirm that melanized feathers are more resistant to bacterial degradation than non-melanized feathers.

Studies on the ability of *B. megaterium* UCM B-5710 to decompose various types of keratin-containing substrates showed that this culture decomposes β-keratin more preferentially, which is indicated by the ability of the culture to decompose various types of feathers well. At the same time, *B. megaterium* UCM B-5710 is also able to act on α-keratin but with much less efficiency. Thus, the bioutilization of difficult feather waste through the production of the most important proteolytic enzymes (keratinases) is very attractive from both an environmental and a biotechnological points of view.

Thus, *B. megaterium* UCM B-5710 has demonstrated a high potential in terms of the ability to process keratin-containing substrates, especially white and colored chicken feathers and sheep wool, and can be used as an effective decomposer of these substrates. It has been shown that the color of feathers plays a significant role in their rate of decomposition.

Acknowledgement. The authors express their gratitude to A.A. Roy, PhD, Senior Researcher at the Department of Microbiological Processes on Solid Surfaces, who kindly provided the culture of *B. megaterium* UCM B-5710 for the study.

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Received 1.06.2023

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СУБСТРАТНА СПЕЦИФІЧНІСТЬ КЕРАТИНАЗИ *BACILLUS MEGATERIUM* УКМ В-5710

Специфіка переробки продуктів тваринництва та птахівництва полягає у тому, що в процесі отримання основної товарної продукції близько половини вихідної сировини на різних стадіях технологічного процесу перетворюється на відходи, які забруднюють довкілля. Ці побічні продукти містять велику кількість важкодоступного кератинового білка. Застосування специфічних ферментів, здатних розщеплювати даний білок, допомагає як знизити негативний антропогенний вплив на природу, так і отримати цінні гідролізати, які можна використовувати як добриво для рослин чи кормову кормову добавку для тварин. **Метою** даної роботи було дослідити здатність *Bacillus megaterium* УКМ В-5710 розщеплювати різні кератинвмісні субстрати: чорне і біле куряче пір'я, біле індиче пір'я, пір'я папуг різних кольорів, овечу вовну, свинячу щетину, дитяче волосся. **Методи.** Культуру вирощували в умовах глибинного культивування при 40 °С зі швидкістю перемішування живильного середовища 201 об/хв протягом 6 діб. Для росту використали базове живильне середовище, що містить як єдине джерело вуглецю і азоту 0,5% знежирене куряче пір'я або інші кератинвмісні субстрати. Кератиназну активність оцінювали за поглинанням в УФ при 280 нм продуктів гідролізу кератинвмісної сировини. Білок визначали методом Лоурі, казеїнолітичну (загальну протеолітичну) активність — методом Ансона у модифікації Петрової, вміст амінокислот — нінгідриновим методом. Ступінь гідролізу субстратів оцінювали за співвідношенням початкової та кінцевої ваги субстрату. **Результати.** Показано, що синтез кератинази культурою *Bacillus megaterium* УКМ В-5710 починається з 6-ої години культивування. Рівень білка та протеолітичної активності, вміст амінокислот підвищувалися протягом усього періоду росту культури. Найбільш ефективно супернатант культуральної рідини *B. megaterium* УКМ В-5710 розщеплював біле куряче та індиче пір'я, трохи повільніше — чорне куряче та синє папуже пір'я, а також білу овечу вовну. За ступенем розщеплення використані субстрати можна розташувати в такому порядку: біле індиче пір'я > біле куряче пір'я > чорне куряче пір'я > блакитне папуже пір'я > біла овеча вовна > дитячі нігті > свиняча щетина > дитяче волосся. Вивчення впливу кольору пір'я на його стійкість до розкладання показало, що більш стійким є чорне, синє та червоне пір'я, що збігається з літературними даними. **Висновки.** Культура *Bacillus megaterium* УКМ В-5710 є продуцентом кератинази, здатної розщеплювати як α-, так і β-кератини, але з різною ефективністю та швидкістю.

Ключові слова: *Bacillus megaterium УКМ В-5710, кератиназа, субстратна специфічність, кератинвмісні субстрати, α-кератин, β-кератин.*