SCREENING OF ENZYME PRODUCERS WITH KERATINASE ACTIVITY AMONG MARINE ACTINOBACTERIA

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About 2 million tons of feathers are produced annually around the world as a by-product of poultry farming. Due to the lack of funds and the complexity of processing, they have become one of the main environmental pollutants. The biodegradation of feathers by keratinolytic microorganisms has proven to be an effective, environmentally friendly and cost-effective method of bioconverting feather waste into a nutritious, balanced and easily digestible product that contains free amino acids, peptides and ammonium ions. **Aim.** To investigate the ability of marine actinobacteria to synthesize enzymes with keratinolytic activity and to study some of the physicochemical properties of the most active enzyme preparation. **The object** of the study was 10 strains of actinobacteria isolated from bottom sediments in the area of the Pradneprovsky trench of the Black Sea shelf. **Methods.** Caseinolytic (general proteolytic) activity was determined by the Anson method modified by Petrova, based on the quantitative determination of tyrosine, which is formed during the enzymatic hydrolysis of casein. Keratinase activity was determined by UV absorption at 280 nm of the hydrolysis products of keratin-containing raw materials. The cultivation of actinobacteria was carried out in a liquid nutrient medium with the addition of defatted chicken feathers as the main source of carbon and nitrogen. **Results.** The ability to hydrolyze keratin was found in five cultures. Moreover, all the strains studied were practically unable to break down casein. The Acty 9 strain (12 U/ml) showed the highest keratinase activity. Additional introduction of NaCl to the nutrient medium did not have a positive effect on the enzymes synthesis. The study of the physicochemical properties of the enzyme preparation Acty 9 showed that the pH and thermooptimum were 9.0 and 60 °C, respectively. It retained 100 % of the initial activity in the range of pH 7.0–10.0 after 3 h and 95 % activity at pH 8.0 after 24 h of incubation. The studied enzyme preparation was thermostable, since it remained active for 3 h at 50 °C and 1 h at 60 °C. **Conclusions.** The extracellular keratinase synthesized by actinobacterium Acty 9 is promising for further research, since the enzyme is pH and thermostable and is not inferior in its physicochemical properties to those previously described in the literature.

Keywords: actinobacteria, keratinolytic enzymes, producers, caseinolytic activity.

Keratin is a fibrillar protein that is mainly found in higher spinal, such as mammals, birds, reptiles, and is one of the main components of human epithelial tissues. It is found in hair, wool, nails, feathers, horns, and hooves, which accumulate in large quantities as a by-product of the meat, wool and poultry industries [1, 2]. Although keratin waste is 90 % protein, keratin is usually insensitive to the action of common proteases. Therefore, the traditional methods that are used for the neutralization and disposal of these wastes are expensive and energy-intensive [3].

In nature, keratin waste does not accumulate, which indicates the existence of microorganisms that are capable of destroying this substrate. To

date, many articles are devoted to the study of keratinolytic enzymes [1, 2, 4]. However, the majority of keratinase producers were found among bacteria and fungi, and a significantly smaller number among actinobacteria. It is known that soil samples taken near poultry farms contain a large number of actinobacteria. Some representatives of the genus *Streptomyces*, capable of splitting feathers and hairs, were isolated from soil, feather waste, hair and garbage [5]. At the same time, there is a little information on keratinases, which are synthesized by marine actinobacteria [3, 6]. Marine microorganisms have always been and will be the objects of fundamental research in various fields of science. Despite the fact that the Ocean

is still poorly understood, it is obvious that it is the marine microbiota that plays a key role in the classical circuit of the cycle of energy and matter, turning them into sources of essential elements at higher trophic levels.

In this regard, the **aim** of the work was to screen among the strains of actinobacteria, isolated from the Black Sea, for their ability to degrade native feathers, followed by the study of the physicochemical properties of the keratinolytic enzyme of the most promising producer.

Materials and methods. The objects of the study were 10 strains of actinobacteria isolated from bottom sediments in the area of the Pradneprovsky trench of the Black Sea shelf: Acty 1, Acty 2, Acty 3, Acty 3-1, Acty 4, Acty 5, Acty 7, Acty 8, Acty 9 and Acty 10. These cultures were isolated by the staff of the Department of Microbiology, Virology and Biotechnology of Odessa I.I. Mechnikov National University in 2019.

To study the keratinase and caseinolytic activity, actinobacteria cultures were grown in test tubes ($V = 50$ ml) at a temperature of 19 °C and 28 °C, with 232 rpm shaking, for 14 days. The basal medium used in the study consisted of (g/l): maltose – 40.0, chicken feathers – 20.0, KH_2PO_4 – 0.5, NaCl – 0.5, $\text{ZnSO}_4 \times 7\text{H}_2\text{O}$ – 0.02, CaCl_2 – 0.01, $\text{MnCl}_2 \times 4\text{H}_2\text{O}$ – 0.01, distilled water – up to 1 liter, pH 7.0–7.2. Since the studied cultures are marine bacteria, in parallel we added 5 g/l NaCl to some of the test tubes in the basal nutrient medium. The culture liquid was centrifuged at 7000 g for 10 min, the resulting supernatant (supernatant of culture liquid, SCL) was used for further studies.

Chicken feathers used in research were defatted as follows: they were washed extensively with tap water, anionic detergent, delipidated with chloroform: methanol (1:1, v/v), washed finally with distilled water and dried again at 60 °C [7].

Caseinolytic (general proteolytic) activity was determined by the Anson method modified by Petrova [8], which is based on the quantitative determination of tyrosine formed during enzymatic hydrolysis of casein under the action of the studied enzymes.

Keratinase activity (KerA) was determined by UV absorption at 280 nm of the products of hydrolysis of keratin-containing materials. The reaction mixture, consisting of 10 mg of chicken defatted feathers, 2.5 ml of 0.05 M borate buffer (pH 9.2) and 1 ml of culture liquid supernatant, was incubated at 37 °C for 3 hours. At the end of

the period, the reaction mixture was filtered. To determine KerA, two controls were used: 10 mg of chicken defatted feathers, 2.5 ml of 0.05 M borate buffer (pH 9.2) and 1 ml of distilled water (1); 2.5 ml of 0.05 M borate buffer (pH 9.2) and 1 ml of culture liquid supernatant (2). The sum of the two controls was subtracted from the A_{280} values obtained by measuring the optical density of the filtrates (samples). The increase in the absorbance at 280 nm of the filtrate of the test sample relative to the controls was taken as the degree of protein release [9]. One unit of keratinase activity (1 U) was defined as the amount of the enzyme that causes an increase in the absorbance value by 0.01 for 3 hours of incubation.

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

Results. The study of the keratinase activity level dynamics of 10 actinobacteria strains (Acty 1, Acty 2, Acty 3, Acty 3-1, Acty 4, Acty 5, Acty 7, Acty 8, Acty 9 and Acty 10) for 14 days showed that the highest results were obtained on the 4th day. Keratinase synthesis was observed already on the 2nd day of cultivation, reached a maximum on the 4th day, after which it subsided. At a temperature of 19 °C on the 4th day of cultivation, the Acty 1, Acty 3-1, Acty 5, Acty 9 and Acty 10 cultures showed a maximum keratinase activity: 4 U/ml, 7 U/ml, 8 U/ml, 12 U/ml and 9 U/ml, respectively.

At the same time, the cultures were practically unable to break down casein (data not presented, since the level of caseinolytic activity was minimal). The study of keratinase activity showed that out of 10 cultures studied, the ability to synthesize keratinolytic enzymes was found in half of the strains (Acty 1, Acty 3-1, Acty 5, Acty 9 and Acty 10). Cultivation of actinobacteria at a temperature of 19 °C promoted a higher level of keratinase activity (12 U/ml) in the SCL than at a temperature of 28 °C (10 U/ml) (Fig. 1).

Additional introduction of NaCl to the nutrient medium did not have a positive effect on the synthesis of enzymes (Fig. 2). The activity level was either the same (4 U/ml and 6 U/ml for Acty 3-1 and Acty 5 at 28 °C) as without the addition of NaCl, or lower (Acty 3-1 (6 U/ml), Acty 5 (3 U/ml) at 19 °C and Acty 9 (6 U/ml and 8 U/ml), Acty 10 (5 U/ml and 6 U/ml) at both temperatures).

Fig. 1. The level of actinobacteria keratinolytic activity at different temperatures (19 °C and 28 °C, 4th day of cultivation. There was no keratinase activity in the control tube, which contained the basal medium with feathers without the addition of actinobacteria).

Fig. 2. The level of actinobacteria keratinolytic activity in the presence of NaCl at different temperatures (19 °C and 28 °C, 4th day of cultivation. There was no keratinase activity in the control tube, which contained the basal medium with feathers without the addition of actinobacteria).

Thus, among 10 cultures of actinobacteria taken for research, based on the level of keratinase activity, one Acty 9 strain was selected for further investigations. The study of the physicochemical properties of the enzyme synthesized by this culture was carried out on a concentrated enzyme preparation obtained as a result of precipitation of the SCL with ammonium sulfate 90 % saturation.

It is known that keratinases are characterized by a wide variety of biochemical and biophysical properties. Many of them are most active in the neutral and alkaline pH range and at 40–60 °C [4, 10, 11]. Although there are thermo- and alkalophilic keratinolytic enzymes [1, 12–14].

The study of the effect of pH on keratinase activity showed that the enzyme preparation Acty

9 was active in the pH range from 7.0 to 10.0 and showed maximum activity at pH 9.0 (Fig. 3). In addition, the study of the pH-stability of the tested enzyme showed that it retained 100 % of the initial activity in the range of pH 7.0–10.0 after 3 hours of incubation and 95 % of the activity at pH 8.0 after 24 hours of incubation (Fig. 4).

The effect of temperature on the activity of the enzyme preparation Acty 9 showed that the optimal temperature of this enzyme is 60 °C (Fig. 5), although the optimal temperature for the action of many keratinases is in the lower temperature range of 35–40 °C [11].

With regard to thermal stability, the investigated enzyme preparation was shown to remain active for 3 hours at 50 °C and 1 hour at 60 °C (Fig. 6).

F i g. 3. The influence of pH on Acty 9 keratinolytic activity

F i g. 4. pH-stability of Acty 9 keratinolytic enzyme

F i g. 5. The influence of temperature on Acty 9 keratinolytic activity

Fig. 6. Thermostability of Acty 9 keratinolytic enzyme

This indicates its competitiveness with previously described keratinases [15, 16].

Discussion. Marine microbiology and the chemistry of marine natural compounds are now developing rapidly, providing the basis for marine biotechnology. A characteristic feature of microorganisms living in the sea, in addition to psychro-, halo-, barophilicity and tolerance, is also the presence of a complex of hydrolytic enzymes in them, which are involved in the processes of microbial degradation of various molecules, such as agar, chitin, carrageenan, starch and others. At the same time there are few data in the literature about keratinase producers among marine actinobacteria. Therefore, we were interested in this topic. It was shown that among the studied actinobacteria, the Acty 9 culture was the most active, which on the 4th day cultivation at 19° C showed a maximum keratinase activity at a level of 12 U/ml. These data are in agreement with the results of others authors [10, 17], which showed that the maximum level of actinobacteria keratinase activity observed between 4–5 days. At the same time a commercial preparation of keratinase was obtained from the culture liquid of the actinomycete *Streptomyces fradiae* IM No. 3739, which cultivation carried out in a nutrient medium with a keratin substrate for 7 days [18]. The cultivation of the producer for 4 days at a temperature of 19 °C will make it possible to quickly obtain the required product without significant energy costs to maintain a high cultivation temperature. Since the addition of 5 g/l NaCl to the nutrient medium had a positive effect on the level of rhamnosidase activity of marine actinobacteria [19], we suppose that

the addition of NaCl to cultivation medium of Acty 9 may also cause the increase of its keratinase activity. However this effect has not been obtained in our experiments.

Earlier [20], when studying bacteria isolated from water and invertebrates of the Black Sea, they found insignificant (0.17–0.30 U/ml) keratinase activity in *Alteromonas macleodii* 11130. Studies of actinobacteria isolated from bottom sediments in the area of the Pradneprovsky trench of the Black Sea shelf allowed select from 10 strains one of the most active – Acty 9, with keratinase activity 12 U/ml.

It is known from the literature that keratinases are optimally active at neutral or alkaline pH values, which coincides with our data: Acty 9 keratinase exhibits maximum activity at pH 9.0. Similar results are characteristic of the *Streptomyces sclerotialis* and *Nocardioopsis* sp. SD 5 keratinases with pH optimum 9.0 [21, 10] and also of *Streptomyces* sp. MAB18 and *Micrococcus luteus* keratinases, which optimal pH is in the range of pH 8.0–10.0 [15] and 9.4, respectively [17].

The enzyme preparation obtained by us retained 100 % activity at pH 7.0–10.0 for 3 hours, while the keratinase of *Thermoactinomyces* sp. RM4 was completely stable in the pH range 6.0–10.0 for 2 hours with a noticeable loss of enzyme activity at higher pH values [12]. Keratinase of *S. sclerotialis* after 60 min of incubation at the optimum temperature (55 °C) and pH optimum 9.0 retained 64 % of the initial activity [21].

The study of the optimum temperature for the action of Acty 9 keratinase showed that the enzyme exhibited maximum activity at 60 °C. Similar data were obtained for the keratinases of *M. luteus*

[17], *S. albidoflavus* [16], *S. sclerotialis* [21], *Streptomyces* sp. MAB18 [15], *S. thermoviolaceus* [22], the optimum temperature for the action of which was 55–60 °C. Retention of 100 % activity by Acty 9 keratinase for 3 hours at 50 °C and 1 hour at 60 °C indicates its thermal stability. *Streptomyces* sp. MAB18 keratinase also retained 100 % activity at 60 °C after 1 hour of incubation [15]. At the same time, *S. albidoflavus* keratinase with the optimum temperature 60 °C retained only 80 % of the initial activity after 1 hour of incubation at 50 °C [16].

Thus, since the enzyme synthesized by actinobacterium Acty 9 is pH- and thermostable and is not inferior in its physicochemical properties to the enzymes previously described in the literature, it can be assumed that it is promising as a producer of extracellular keratinase.

СКРИНІНГ ПРОДУЦЕНТІВ ФЕРМЕНТІВ З КЕРАТИНАЗНОЮ АКТИВНІСТЮ СЕРЕД МОРСЬКИХ АКТИНОБАКТЕРІЙ

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Резюме

Щорічно в усьому світі як побічний продукт птахівництва виробляється близько 2 млн тонн пір'я. Через нерентабельність і складнощі в переробці вони стали одними з основних забруднювачів навколишнього середовища. Біодеградація пір'я кератинолітичними мікроорганізмами є ефективним, екологічно чистим і рентабельним методом біоконверсії відходів пір'я в живильний, збалансований і легко засвоюваний продукт, що містить вільні амінокислоти, пептиди та іони

амонію. **Мета.** Дослідити здатність морських актинобактерій синтезувати ферменти з кератинолітичною активністю і вивчити деякі фізико-хімічні властивості отриманого ферментного препарату. Об'єктами дослідження були 10 штамів актинобактерій, виділених з донних відкладень в районі Придніпровського жолобу шельфу Чорного моря. **Методи.** Казеїнолітичну (загальну протеолітичну) активність визначали методом Ансона в модифікації Петрової, що базується на кількісному визначенні тирозину, який утворюється при ферментативному гідролізі казеїну. Кератиназну активність визначали за поглинанням в УФ при 280 нм продуктів гідролізу кератинвмісної сировини. Культивування актинобактерій проводили в рідкому поживному середовищі з додаванням знежиреного курячого пір'я як основного джерела вуглецю і азоту. **Результати.** Здатність гідролізувати кератин була виявлена у п'яти культур. При цьому всі досліджені штами майже не здатні були розщеплювати казеїн. Найвищу кератиназну активність виявив штам Acty 9 (12 Од/мл). Додаткове внесення NaCl до складу поживного середовища не викликало позитивного впливу на синтез ферментів. Вивчення фізико-хімічних властивостей ферментного препарату Acty 9 показало, що рН і термооптимум становили 9.0 і 60 °C відповідно. Він зберігав 100 % вихідної активності в області рН 7.0–10.0 через 3 год та 95 % активності при рН 8.0 через 24 год інкубування. Досліджений ферментний препарат виявився термостабільним, оскільки залишався активним протягом 3 год при 50 °C і 1 год при 60 °C. **Висновки.** Штам актинобактерій Acty 9 є перспективним продуцентом позаклітинної кератинази, оскільки синтезований ним фермент є рН- і термостабільним та не поступається за фізико-хімічними властивостями раніше описаним у літературі ензимам.

Ключові слова: актинобактерії, кератинолітичні ферменти, продуценти, казеїнолітична активність.

1. Qingxin Li. Progress in Microbial Degradation of Feather Waste. *Front Microbiol.* 2019; 10:2717. doi:10.3389/fmicb.2019.02717
2. Hassan MA, Abol-Fotouh D, Omer AM, Tamer TM, Abbas E. Comprehensive insights into microbial keratinases and their implication in various biotechnological and industrial sectors: A review.

International Journal of Biological Macromolecules. 2020; 154:567–583. doi: 10.1016/j.ijbiomac.2020.03.116

3. Selvam K, Vishnupriya B, Yamuna M. Isolation and description of keratinase producing marine actinobacteria from South Indian Coastal Region *Afr. J Biotechnol.* 2013; 12(1):19–26. doi:

- 10.5897/AJB12.2428
4. Brandelli A, Daroit DJ, Riffel A. Biochemical features of microbial keratinases and their production and applications. *Appl Microbiol Biotechnol.* 2010; 85(6):1735–1750. doi: 10.1007/s00253-009-2398-5
5. Allure N, Madhusudhan DN, Agsar D. Detection of keratinolytic actinobacteria and evaluation of bioprocess for production of alkaline keratinase. *Int J Curr Microbiol Appl Sci.* 2015; 4(7):907–918.
6. Gushterova A, Tonkova EV, Dimova E, Nedkov P, Haertle T. Keratinase production by newly isolated Antarctic actinobacteria strains. *World J Microbiol Biotechnol.* 2005; 21:831–834.
7. Mazotto AM, Coelho RR, Cedrola SM, de Lima MF, Couri S, Paraguai de Souza E, et al. Keratinase Production by Three *Bacillus* spp. Using Feather Meal and Whole Feather as Substrate in a Submerged Fermentation. *Enzyme Res.* 2011; 2011:523780. doi:10.4061/2011/523780
8. Varbanets LD, Matseliukh EV. [Peptidases of microorganisms and methods of their investigations]. Kyiv: Naukova Dumka; 2014. Russian.
9. 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.
10. Saha S, Dhanasekaran D, Shanmugapriya S, Latha S. *Nocardiopsis* sp. SD 5: A potent feather degrading rare actinobacterium isolated from feather waste in Tamil Nadu, India. *J Basic Microbiol.* 2013; 53(7):608–616. doi:10.1002/jobm.201100105
11. Jadhav RS, Karad DD, Kulakrni SW. Isolation, Identification and Characterization of Keratinolytic *Streptomyces coelicoflavus*. *Int J Curr Microbiol App Sci.* 2016; 5(7):153–163. doi:10.20546/IJCMAS.2016.507.015
12. Verma A, Singh H, Anwar MS, Kumar S, Ansari MW, Agrawal S. Production of Thermostable Organic Solvent Tolerant Keratinolytic Protease from *Thermoactinomyces* sp. RM4: IAA Production and Plant Growth Promotion. *Front Microbiol.* 2016; 7:1189. doi:10.3389/fmicb.2016.01189
13. Gong JS, Wang Y, Zhang DD, Zhang RX, Su C, Li H, et al. Biochemical characterization of an extreme alkaline and surfactant-stable keratinase derived from a newly isolated actinomycete *Streptomyces aureofaciens* K13. *RSC Adv.* 2015; 5:24691–24699. doi: 10.1039/C4RA16423G
14. Habbeche A, Saoudi B, Jaouadi B, Haberra S, Kerouaz B, et al. Purification and biochemical characterization of a detergent-stable keratinase from a newly thermophilic actinomycete *Actinomadura keratinilytica* strain Cpt29 isolated from poultry compost. *J Biosci Bioeng.* 2014; 117(4):413–421. doi: 10.1016/j.jbiosc.2013.09.006
15. Manivasagan P, Venkatesan J, Sivakumar K, Kim SK. Production, characterization and antioxidant potential of protease from *Streptomyces* sp. MAB18 using poultry wastes. *Biomed Res Int.* 2013; 2013:496586. doi: 10.1155/2013/496586
16. Bressollier P, Letourneau F, Urdaci M, Verneuil B. Purification and Characterization of a Keratinolytic Serine Proteinase from *Streptomyces albidoflavus*. *Appl Environ Microbiol.* 1999; 65(6):2570–2576. doi: 10.1128/AEM.65.6.2570-2576.1999
17. Laba W, Choinska A, Rodziejewicz A, Piegza M. Keratinolytic abilities of *Micrococcus luteus* from poultry waste. *Braz J Microbiol.* 2015; 46(3):691–700. doi:10.1590/S1517-838246320140098
18. Nickerson WJ, Princeton, Novel JJ. Process of treating keratinaceous material and a keratinase produced thereby. US 2988487 13.06.1961
19. Varbanets LD, Gudzenko OV, Ivanytsia VA. Marine Actinobacteria – Producers of Enzymes with α -L-Rhamnosidase. *Mikrobiol Z.* 2020; 82(5): 3–10. doi: https://doi.org/10.15407/mikrobiolj82.05.003
20. Varbanets LD, Avdeeva LV, Borzova NV, Matseilyukh OV, Gudzenko OV, Kiprianova OA, Yaroshenko LV. [The Black sea bacteria – producers of hydrolytic enzymes]. *Mikrobiol Z.* 2011; 73(5):9–15. Russian.

21. Yadav AK, Vardhan S, Yandigeri MS, Srivastava AK, Arora DK. Optimization of keratin degrading enzyme from thermophilic strain of *Streptomyces sclerotialis*. Res J Microbiol. 2011; 6(9):693–705. doi:10.3923/jm.2011.693.705
22. Chitte RR, Nalawade VK, Dey S. Keratinolytic activity from the broth of a feather-degrading thermophilic *Streptomyces thermoviolaceus* strain SD8. Lett Appl Microbiol. 1999; 28:131–136. doi:10.1046/j.1365-2672.1999.00484.

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