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ISOLATION OF *BACILLUS* STRAINS WITH KERATINOLITIC ACTIVITY

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ABSTRACT

Environmental safety and economic feasibility determine the search for new ways of processing waste in poultry farms. Most of this waste is down and feathers, which are 90% β-keratin. Feathers can be a valuable source of amino acids and peptones when properly processed. The most effective is enzymatic treatment of feather keratin. The search for new strains producing keratinolytic enzymes seems to be a promising direction. On the territory of the poultry farm, 4 strains were isolated from the places of accumulation of feathers. They are able to use chicken feathers as their sole source of organic matter. Based on morphological, genomic, and proteomic analyzes, the isolated strains were identified as *Bacillus* sp. It was found that the strains secrete proteolytic enzymes that hydrolyze collagen, casein, β-keratin and do not hydrolyze bovine serum albumin. Feather hydrolysis experiments showed that the *Bacillus* sp. A5.3 possesses maximum keratinolytic activity, and on the second day, the destruction of the second order barbs is observed. The keratinase activity of the strain on azokeratin after an hour of incubation on feather medium was 27.4 U/ml. The optimal conditions for the complex of secreted proteolytic enzymes are pH 7.0-8.0 and temperature 35-40 °C. The isolated *Bacillus* sp. A5.3 strain is a promising source of proteases and keratinases.

Keywords: feather, keratin, protease, keratinase, *Bacillus* sp.

INTRODUCTION

Environmental safety and economic feasibility determines the search for new ways of processing waste in the poultry farms. Global consumption of poultry meat is growing every year and the amount of poultry waste generated annually is increasing. Most of this waste is down and feathers, which are composed of about 90% of β-keratin [1] and which can used as source of amino acids and peptones [2,3]. B-keratin is rich in such essential amino acids as leucine, valine, arginine, isoleucine, phenylalanine and threonine [4]. B-keratins are zigzag-like polypeptide chains stabilized by disulfide bonds, hydrophobic interactions and hydrogen bonds, which leads to their greater hardness in comparison with α-keratins. Keratins contain a high percentage of hydrophobic amino acid residues, are insoluble in water and are highly resistant to proteases [5]. For enzymatic hydrolysis of keratin keratinases capable of cleaving disulfide bonds are used. Keratinases (EC 3.4.99.11) are serine- or metalloproteases [6] and a large number of microorganisms have been reported to produce keratinases [7], including fungi [8,9] and actinomycetes [10]. At the same time, it is known that bacteria of the genus Bacillus have proteolytic activity against various protein substrates [11], including α- and β-keratins [12,13]. The aim of the study was to isolate bacteria of the genus *Bacillus* with keratinolytic activity with the prospect to obtain the keratinase-producing strain.

Materials and methods

Bacteria isolation and strain identification. The soil with rotten feathers were collected from a local poultry plant. 1 g of the sample with 9 mL 0.9% NaCl were shaken for 30 min, and 100 µl of the suspension was plated on skim-milk agar plates (2% skim-milk powder, 0.1% NaCl, 1% tryptone, 1% agar), followed by cultivation at 37°C for 48 h. Well-grown single colonies were isolated and tested on feather agar plate (0.07% KH_2PO_4, 0.14% Na_2HPO_4, 0.5% feather powder, 1.5% agar). Grown colonies were isolated and identified.

Three methods were used for identification: morphological studies of colonies, analysis of ribosomal protein profile on MALDI-TOF Biotyper, sequence of fragment 16S rRNA.

Collagenase assay. Collagenase activity was tested on gelatin agar using trichloroacetic acid (TCA). Gelatin agar plates (3% gelatin, 1% peptone, 1% NaCl, 1.5% agar) identify hydrolysis by change in the opacity around the colony. Isolates were plated on gelatin agar and incubated for 24 h. After incubation TCA was added to the plate and observations were made immediately for a period of at least 4 min

Feather degradation. Single colony was grown on Luria-Bertani medium (1% peptone, 0.3% yeast extract, 0.5% NaCl, pH 7.2) for 16 h at 37°C and 150 rpm. The feathers were washed by detergents and dried. The minimal salt medium (0.7 g/L KH_2PO_4, 1.4 g/L Na_2HPO_4, pH 11.0) with feather powder (5 g/L) was prepared and the feather with the medium together were autoclaved. The grown bacterial culture was inoculate to medium with feather and incubated for 168 h at 37°C and 150 rpm. The feather medium without adding of bacterial culture was used as the negative control.

Assay of keratinase activity. The azokeratin was prepared accordingly the protocol for azoalbumin preparation [14]. The feather powder was treatment with 10% NaHCO3, sulfanilic acid, 0.2 N NaOH, NaNO3 and 5 M HCl. After neutralization with 5 N NaOH solution incubated at 50°C and filtered. The pellet was washed with 50 mM potassium phosphate buffer (pH 7.5) and lyophilized at -90°C. The strain cells were inoculated into feather medium and grow for 168 h at 37°C. The supernatant was clarified by centrifugation (10,000×g, 4°C, 10 min) and filtered throw 0.45 µm and used as the sample for keratinase assay and protein hydrolysis. For keratinase assay 5 µg in 800µL 50 mM potassium phosphate buffer (pH 7.5) and 200µL of sample were mixed and incubated at 50°C for 1 h. The reaction was stopped by adding 10% TCA. Absorption was measured at 450 nm. TCA was added to the control sample prior to incubation.

Protein hydrolysis. The bovine serum albumin (BSA) and casein sodium salt (CSS) were dissolved in 50 mM potassium phosphate buffer (pH 7.5) and incubated at 37°C with the sample for 2 h. The reaction was stopped by adding 10% TCA. TCA was added to the control sample prior to incubation. The hydrolysis products were separated in SDS-PAGE by Laemmli method [15].

RESULTS AND DISCUSSION

Four bacterial strains were isolated from the places of rotten feathers accumulation on the poultry farm territory (30 km from the city of Nur-Sultan): A5.3; A5.5; A7.1; A11.2. The strains have capable of growing on salt medium with feather as the only organic substances source. On nutrient agar, the colonies were 0.5 mm in diameter, beige, and with wave edges after 24 h. Cells are gram-positive, oval, mobile, form spores. MALDI-TOF MS-based identification suggests that they belong to genus *Bacillus.* By sequencing and comparing the nucleotide sequence of 16S rRNA gene with GenBank data, the strains also were identified as bacteria of the genus *Bacillus*.

The strains grow on gelatin plate that evidence about its collagenase activity (Figure 1).

A5.3 A5.5 A7.1 A11.2

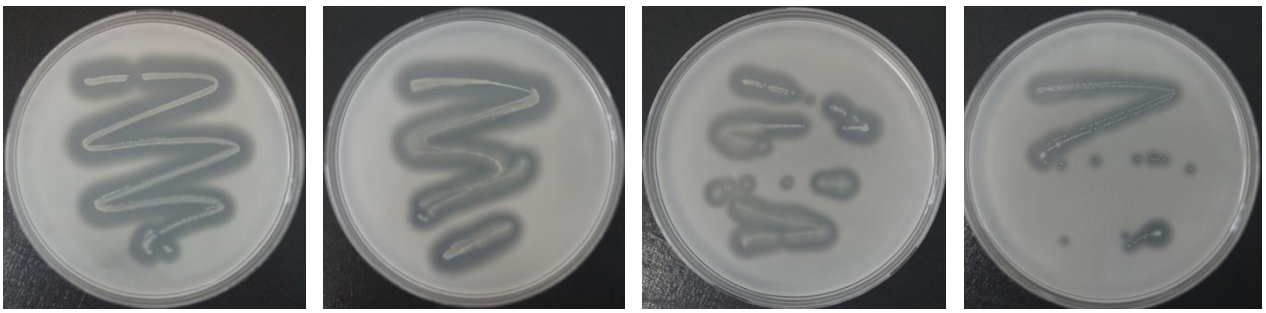

Fig. 1. *Bacillus* sp. A5.3 A5.5; A7.1; A11.2 on gelatin agar

Incubation of feathers with a culture of four strains showed that the A5.3 strain has a higher keratinolytic activity than other strains (Fig. 2). Degradation of feather barbules was apparent from 48 h of incubation onwards and the degradation of barbs was observed after the third day. The degradation of barbs was continued during all incubation and resulted to the complete exposure of the shaft. The medium becomes milky.

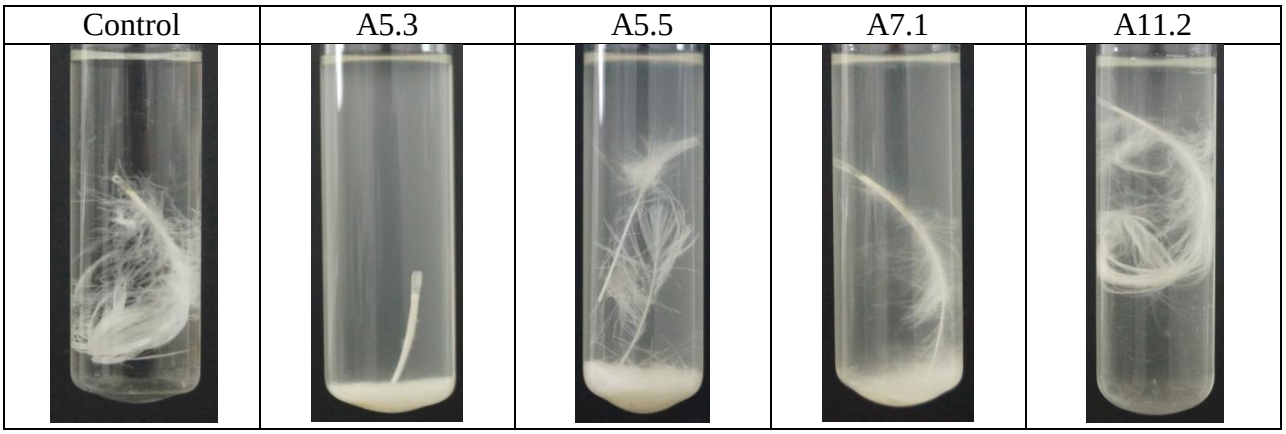

Fig.2. Feather degradation by *Bacillus* sp. A5.3 A5.5; A7.1; A11.2 after 168 h.

Keratinase activity was measured on azokeratin. *Bacillus* sp. A5.3 supernatant presented the highest keratinase activity with 27.4 U/mL. Optimal condition was pH 7.0-8.0 and temperature 30-40°C. Incubation of the supernatant of *Bacillus* sp. A5.3 with casein sodium salt and bovine serum albumin showed that the supernatant enzymes hydrolyze CSS within 30 min and do not degrade BSA.

CONCLUSION

Four bacterial strains were isolated from soil samples collected from the poultry farm. All of them could grow in a feather medium and exhibit protease and keratinase activity. The isolates were identified as bacteria of the genus *Bacillus* by genomic and proteomic analysis. All strains showed good activity in feather hydrolysis. The highest activity was noted in the *Bacillus* sp. A5.3, which indicates its high producing ability of keratinolytic enzymes. After 7 days of growth on a minimal salt medium, the keratinase activity of the *Bacillus* sp. A5.3 was 27.4 U/mL. Thus, the resulting strain is promising for use in biotechnology as a keratinase producer for processing feather waste.

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КЕРАТИНОЛИТИКАЛЫҚ БЕЛСЕНДІЛІККЕ ИЕ БАЦИЛЛАЛАР ШТАММДАРЫН БӨЛІП АЛУ

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ТҮЙІН

Экологиялық қауіпсіздік және экономикалық мақсаттылық құс фабрикаларында қалдықтарды қайта өңдеудің жаңа тәсілдерін іздестіруді талап етеді. Бұл қалдықтардың көп бөлігі –мамықтар мен қауырсындар, олар 90% β-кератинді құрайды. Қауырсынды кератин дұрыс өңделгенде, ол аминқышқылдары мен пептондардың құнды көзі бола алады. Ең тиімдісі - қауырсын кератинін ферментативті өңдеу. кератинолитикалық ферменттер шығаратын жаңа штаммдарды іздестіру перспективалы бағыт болып көрінеді. Құс фабрикасы аумағында қауырсындар жиналатын жерлерден тауық қауырсындарын органикалық заттардың жалғыз көзі ретінде қолдана алатын 4 штамм оқшауланды. Морфологиялық, геномдық және протеомдық талдаулар негізінде оқшауланған штамдар *Bacillus* sp. ретінде анықталды. Штамдар коллагенді, казеинді, β-кератинді гидролиздейтін протеолитикалық ферменттер бөлетіні және бұқаның сарысу альбуминін гидролиздей алатындығы анықталды. қауырсын гидролизі бойынша тәжірибелер көрсеткендей, *Bacillus* sp. a5.3 максималды кератинолитикалық белсенділікке ие, ал екінші күні құрылысы бұзылуы байқалады. Азокератин штаммының кератиназа белсенділігі қауырсынды ортада бір сағат инкубациялаудан кейін 27,4 бірлік/мл құрады. Бөлінетін протеолитикалық ферменттер кешенінің оңтайлы шарттары – pH 7.0-8.0 және температура 35-40 °C. *Bacillus* sp.a5.3 оқшауланған штамы - протеазалар мен кератиназалардың перспективалы көзі.

Негізгі сөздер: қауырсын, кератин, протеаза, кератиназа, *Bacillus* sp.

ВЫДЕЛЕНИЕ ШТАММОВ *BACILLUS*, ОБЛАДАЮЩИХ КЕРАТИНОЛИТИЧЕСКОЙ АКТИВНОСТЬЮ

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АБСТРАКТ

Экологическая безопасность и экономическая целесообразность определяют поиск новых способов переработки отходов в птицефабриках. Большая часть этих отходов - это пух и перья, которые на 90% состоят из β-кератина. При правильной

обработке перьевой кератин может быть ценным источником аминокислот и пептонов. Наиболее эффективным является ферментативная обработка перьевого кератина. Поиск новых штаммов, продуцирующих кератинолитические ферменты представляется перспектиным направлением. Из мест скопления перьев на территории птицефабрики было выделено 4 штамма, способных использовать куриные перья в качестве единственного источника органических веществ. На основании морфологического, геномного и протеомного анализов выделенные штаммы были идентифицированы как *Bacillus* sp. Установлено, что штаммы секретируют протеолитические ферменты, гидролизующие коллаген, казеин, β-кератин и не гидролизующие бычий сывороточный альбумин. Эксперименты по гидролизу перьев показали, что штамм *Bacillus* sp. A5.3 обладает максимальной кератинолитической активностью и уже на вторые сутки наблюдается разрушение бородок второго порядка. Кератиназная активность штамма на азокератине после часовой инкубации на перьевой среде составила 27,4 ед/мл. Оптимальными условиями для комплекса секретируемых протеолитических ферментов составляет pH 7,0-8,0 и температура 35-40°C. Выделенный штамм *Bacillus* sp.A5.3 является перспективным источником протеаз и кератиназ.

Ключевые слова: перо, кератин, протеаза, кератиназа, *Bacillus* sp.