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Original Article

# USE OF *BACILLUS* STRAINS WITH PROTEASE, KERATINASE AND COLLAGENASE ACTIVITY IN THE PROCESSING OF AGRICULTURAL PRODUCTS

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## ABSTRACT

The significant accumulation of protein-rich agro-industrial waste requires the urgent development of biotechnological methods for its processing and usage. Strains of *B. subtilis* and *B. paralicheniformis* were isolated and identified from soil samples obtained from a chicken farm in the Akmola region and the city of Taraz. These strains produce proteases with peak activity at 70 °C and pH 10.5 for *B. subtilis*, and at 60 °C and pH 9.0 for *B. paralicheniformis*. The protease, keratinase, and collagenase activity of *B. subtilis* enzymes are 158.8 ± 2.5 U/mL, 109.3 U/mL, and 10.4 U/mL, respectively. The enzyme values for *B. paralicheniformis* were 323 ± 4.8 U/mL, 122.6 U/mL, and 23.6 U/mL, respectively. Enzymatic hydrolysis of poultry and livestock waste, including chicken feathers, hooves, and cattle hides, was conducted using the *B. paralicheniformis* strain. Following 96 hours of treatment, the hydrolysis rates of feathers, hooves, and hides were 100%, 29.6%, and 97.2%, respectively. Examination of the hydrolyzed feathers using scanning electron microscopy revealed the degradation of essential structural components of the feathers and the adherence of bacterial cells to the surface of the feather keratin. The acquired data suggest the potential of the *B. paralicheniformis* strain as a producer of alkaline proteolytic enzymes and its use in technologies for processing protein-rich agro-industrial waste.

Key words: *Bacillus,* proteases, bioconversion, agrobiotechnology, feather, hoof, hide

## INTRODUCTION

Modern agriculture is characterized not only by growth in production volumes but also by an increase in the number of by-products generated at all stages of the agro-industrial cycle [1]. Such materials include feather and wool waste from poultry and sheep farming, leather and connective tissue residues from meat processing, and plant substrates rich in structural proteins [2]. The accumulation of these difficult-to-degrade substrates leads to environmental risks, requires additional disposal costs, and reduces the overall efficiency of the industry. At the same time, there is a growing need for cost-effective and environmentally safe technologies for the deep processing of biological waste, allowing the production of value-added products and their integration into circular production schemes [3].

Microbiological methods of waste processing have gained wide recognition due to the high efficiency of enzymatic catabolism, the selectivity of biocatalysts, and the possibility of cultivating microorganisms on inexpensive substrates [4]. Among the available biocatalytic platforms, strains of the genus *Bacillus* occupy a special place. Representatives of this genus are distinguished by their physiological plasticity, resistance to pH and temperature fluctuations, ability to form spores, and high potential for extracellular protein secretion [5]. Many of them have GRAS or QPS status, which greatly facilitates their introduction into the food, agricultural, and biotechnology industries [6]. Thanks to these properties, *Bacillus* has become one of the key microbial producers of enzymes of industrial importance [6-9].

Of particular interest are bacillary proteolytic complexes, which include a wide range of serine proteases and metalloproteases [7]. These enzymes demonstrate high activity against difficult-to-break-down structural proteins such as keratin and collagen [10]. Keratins, which have a dense structure stabilized by numerous disulfide bridges, form the basis of feathers, wool, and horny structures, making them one of the most stable biopolymers in nature [11, 12]. Collagen, which predominates in animal connective tissue, forms an extracellular matrix with high mechanical strength [13]. The hydrolysis of both types of proteins is a complex task, but bacillary keratinases and collagenases are capable of effectively destroying these substrates, converting them into soluble oligopeptides and amino acids.

The use of *Bacillus* strains with protease, keratinase, and collagenase activities opens up opportunities for the development of new methods for the bioconversion of agro-industrial waste [14]. The products obtained in this process – protein hydrolysates, amino acid mixtures, plant growth biostimulants, feed, or functional ingredients – make it possible to expand the resource base of production and reduce the burden on the environment [15]. In addition, biological processing is characterized by mild conditions, lower energy consumption, and the absence of toxic by-products, making it an attractive alternative to chemical disposal methods.

The aim of this work is to isolate new strains with protease, keratinase, and collagenase activity and to apply them in the enzymatic hydrolysis of protein, keratin, and collagen substrates.

## MATERIALS AND METHODS

### Chemicals and media

Chemical reagents from Sigma-Aldrich (St. Louis, MO, USA) and AppliChem (Darmstadt, Germany) were used in this study. The following media were used for culturing *Bacillus subtilis* and *Bacillus paralicheniformis*: Lysogeny broth (0.5% yeast extract (Condalab, Madrid, Spain), 1% tryptone plus (Sigma-Aldrich), 0.5% NaCl (Sigma-Aldrich), pH 7.0), Nutrient broth (Himedia, Mumbai, India), Feather medium

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with yeast extract (0.03% $NaH_2PO_4$, 0.035% $Na_2HPO_4$, 0.2% yeast extract, 0.75% feather powder, pH 7.0).

**Isolation and identification of strains**

Strains was isolated from soil sample collected in Kazakhstan at **a local poultry plant** close to Akmol village with coordinates (51°04′23″ N 70°58′45″ E) and at Taraz city territory with coordinates 42°54′00″N, 71°22′00″E. One gram of a sample with 9 mL of 0.9% (w/v) NaCl was shaken for 30 min, and 100 µL of the suspension was seeded in Nutrient agar plates, followed by cultivation at 37 °C for 48 h.

**Strain identification**

Identification of the strains was performed by morphological analysis of colonies and cells and sequencing of the 16S rRNA fragment. Identification by morphological characteristics was carried out in accordance with Bergey's identification guide [16]. For sequencing, the genomic DNA of the strain was isolated using a Genomic Wizard Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. The 16S rRNA gene fragment was amplified by PCR using universal primers 27F and 1492R. The amplified DNA fragment was sequenced by the Sanger method [17] using BigDye™ Terminator v3.1 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. DNA fragments were separated using an ABI 3730xl automated sequencer (Applied Biosystems, Foster City, CA, USA). Chromatograms were analyzed and compared with the reference sequence using Vector NTI software version 11 (Thermo Fisher Scientific) and the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

**Screening for protease, keratinase and collagenase activities**

Well-grown stand-alone colonies were isolated and tested for a proteolytic activity on skim-milk agar plate. Plates were grown for 8 h at 37 °C and followed by 16 h incubation at RT (23–24 °C) for clearing zones appear. Activity-positive colonies were again seeded feather agar plates and gelatin agar plates.

**Enzyme extract preparation**

*B. subtilis* and *B. paralicheniformis* cultures were grown independently in 1-liter flasks in a KS4000i control shaker incubator (IKA, Germany). One colony of each strain was inoculated into 5 mL of nutrient broth and cultured at 37 °C in a shaker incubator at 180 rpm for 18 hours. The grown culture was transferred to 200 mL of Feather medium with the addition of 0.2% yeast extract and grown under the same conditions for 48 hours. The cultures were freed from cells and substrate residues by centrifugation at 11,000 × g and sterilized by microfiltration using membrane filters with a cut-off threshold of 0.22 µm. The resulting enzyme extracts were stored at 4 °C and used in experiments.

**Enzyme assay**

Proteolytic activity was determined according to [18] using azocasein (Sigma-Aldrich, St. Louis, MO, USA) as a substrate. The reaction mixture consisted of 0.5 mL of azocasein substrate and 0.5 mL of enzyme extract. The mixture was incubated for 1 hour, after which the reaction was stopped by the addition of 0.5 mL of 20% TCA. A sample to which TCA had been added prior to incubation served as a blank. After stopping the reaction, the mixture was cooled on ice for 15 min and then centrifuged at 4000 × g for 10 min. 0.5 mL of 0.5 N NaOH was added to 0.5 mL of the supernatant and the absorbance of azocasein was measured at 415 nm. One unit of protease activity was defined as the amount of enzyme extract required to cause a change in optical density of 0.01 per minute under the experimental conditions.

Keratinolytic activity was measured according to [19] using azokeratin as the substrate. To summarize, 0.8 mL of 50 mM sodium phosphate buffer (pH 7.5) was used to resuspend 5 mg of azokeratin. Subsequently, 0.2 mL of the enzymatic extract was introduced to the azo-keratin suspension. The mixture underwent incubation at 50 °C for 1 hour, adhering to standard assay conditions. The reaction was terminated using 0.2 mL of 10% (w/v) trichloroacetic acid (TCA). The resulting mix underwent centrifugation for 10 minutes at 10,000 × g. The absorbance of the supernatant was quantified at 450 nm. A suitable blank was used, with 10% TCA added to the mixture prior to incubation. An increase in absorbance of 0.01 units at 450 nm was accepted as 1 unit of keratinolytic activity.

Collagenase activity was determined according to [20] with using azocoll (Sigma-Aldrich, St. Louis, MO, USA) as substrate. Azocoll (75 mg) was washed twice in 50 mL of buffer solution (50 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.8) to remove degraded peptides. The washed azocoll was then suspended in 50 mL of the same buffer. For analysis, 100 µl of the sample was mixed with 400 µl of the substrate suspension. The reaction mixture was then incubated for two hours at 37 °C with constant stirring at 300 rpm. After incubation, the mixture was centrifuged at 10,000 × g for 10 min. The optical density of the supernatant was then measured at 540 nm. A change in optical density of 0.01 under these conditions was defined as one unit of activity.

**Determination of temperature and pH optimum**

To find the optimum, the effect of temperature on enzymatic activity was determined by conducting proteolysis reactions in the temperature range of 30–80 °C (at 10 °C intervals). Maximum enzymatic activity was taken as 100%. Enzymatic activity was measured in the pH range from 3.0 to 11.0. The following buffer systems were used: citrate buffer (pH 3.0–6.0), sodium phosphate buffer (pH 6.0–7.5), Tris-HCl (pH 7.5–9.0), and glycine-NaOH (pH 9.0–11.0). The values were converted to relative units with a maximum value of 100%. The conditions under which protease activity was maximal were taken as optimal.

**Treatment of feather, hoof, and hide**

The ability of the *Bacillus* strains to degrade animal waste (chicken feathers, hooves, hides) was investigated. *Bacillus* were cultured in 15 mL of nutrient broth in a shaker incubator at 37 °C and 170 rpm for 18 h. Thereafter, 1 mL inoculum was added to glass tubes containing hoof (1.405 g), leather (1.055 g), and feather (0.25 g) fragments in 10 mL of sodium phosphate buffer (pH 7.0). Next, the samples were degraded in a shaker incubator at 37 °C and 250 rpm for 7 days. The degree of degradation of the samples was determined by measuring the dry weights of unhydrolyzed samples. For this purpose, the culture was passed through a pre-weighed filter paper, and the remaining residue was washed twice with distilled water and dried at 60 °C to a constant weight. The results are ex-

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pressed as a percentage of the initial weight of the samples, which was taken as 100%.

**Scanning electron microscopy**

Feather samples were inoculated with 1 mL of sterile enzymatic extract in 10 mL of 0.1 M Tris-HCl buffer (pH 8.5) supplemented with 0.02% sodium azide. Incubation was conducted for 2–7 d at 37 °C. To evaluate the effect of *B. paralicheniformis* T7 on the degradation process, hydrolysis was performed using an unfiltered enzymatic extract without sodium azide under the same conditions. Samples prepared under the same conditions but without enzymatic extracts were used as controls. After incubation, the samples were analyzed using scanning electron microscopy (SEM) to evaluate the degree of degradation. The samples were placed on stubs and sputtered with gold (10 nm). Images were captured using an Auriga CrossBeam 540 Scanning Electron Microscope (Carl Zeiss, Jena, Germany) at 3 kV.

## RESULTS AND DISCUSSION

*Isolation and identification of strains of Bacillus subtilis and Bacillus paralicheniformis*

From soil samples collected at a feather waste accumulation site on the territory of the Kurochka-Ryaba poultry farm in the village of Akmol, Akmola region, and the territory of the city of Taraz, cultures were sown on nutrient agar, and primary colonies were obtained. To test protease, keratinase, and collagenase activity, the colonies were sequentially sown on appropriate selective media: milk, feather, and gelatin agar (Figure 1). Milk agar served as an indicator of the strains' ability to break down casein, feather agar showed the strains' ability to hydrolyze keratin, and gelatin agar allowed the hydrolysis of denatured collagen to be assessed. The formation of clear zones around the colonies demonstrates the corresponding type of hydrolase activity. As a result, two strains were selected for further study, as they showed the highest proteolytic activity among all the isolates tested.

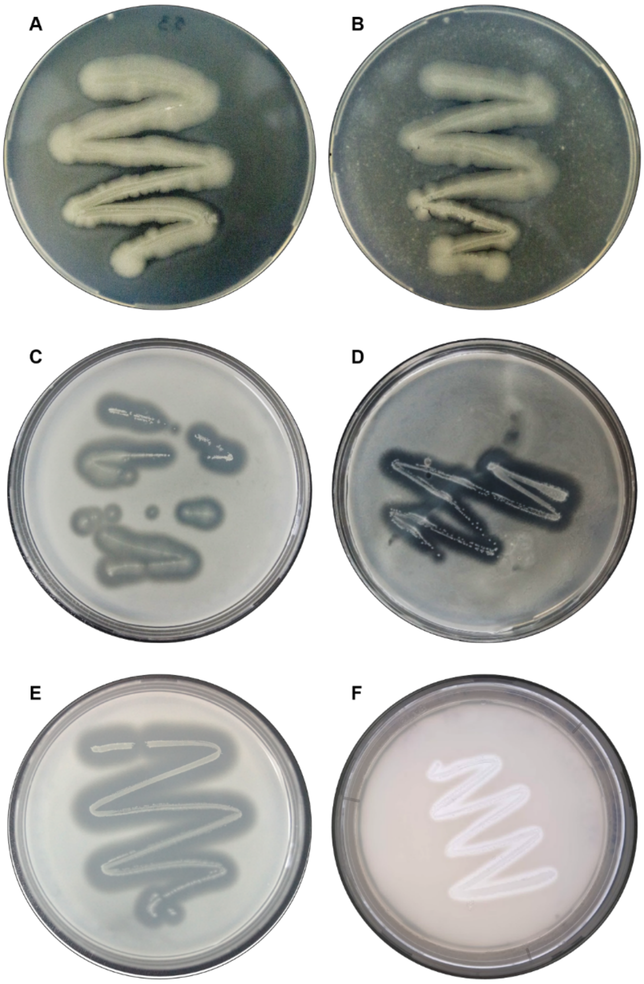

Figure 1 – Screening on selective media. **A** – feather agar, isolate from a poultry farm; **B** – feather agar, isolate from Taraz; **C** – gelatin agar, isolate from a poultry farm; **D** – gelatin agar, isolate from Taraz; **E** – milk agar, isolate from a poultry farm; **F** – milk agar, isolate from Taraz

After 16 hours of incubation on nutrient agar, both isolates formed beige opaque colonies 2-4 mm in diameter with scalloped edges and a convex or slightly curved profile. The surface of the colonies was smooth or slightly folded, matte, with a viscous and fibrous consistency. Microscopy of Gram-stained smears showed that the cells of the strains are Gram-positive rods, occurring singly or in chains, which corresponds to the morphology of bacteria of the genus *Bacillus* (Figure 2). Sheffer-Fulton staining revealed the presence of oval subterminal endospores, forming one per cell in both isolates, which is also characteristic of bacillary strains capable of forming endospores.

Sequencing of the 16S rRNA fragment and comparison of the obtained sequence with NCBI data using BLAST showed that the isolates obtained from the poultry farm and Taraz city, with homology above 99%, correspond to the strains *Bacillus subtilis* and *Bacillus paralicheniformis*, respectively.

*Determination of enzymatic activity*

Sterile enzyme extracts of the studied strains were used to perform biochemical tests. In the first stage, keratinase and collagenase activities were evaluated. The keratinase activity of the *B. subtilis* extract was 109.3 U/mL, and the activity of the *B. paralicheniformis* extract was 122.6 U/mL. Collagenase activity values reached 10.4 U/mL for *B. subtilis* and 23.6 U/mL for *B. paralicheniformis*. The presence of keratinase and collagenase activity indicates that these strains possess a complex set of hydrolytic enzymes capable of breaking down both soluble proteins and structurally stable protein substrates such as keratin and collagen. The presence of several types of proteolytic enzymes indicates a wide range of hydrolytic potential and suggests the promise of these strains as producers of keratinases and collagenases.

After a general assessment of the enzyme profile, the dependence of the proteolytic activity of enzyme extracts from *Bacillus subtilis* and *Bacillus paralicheniformis* on tempera-

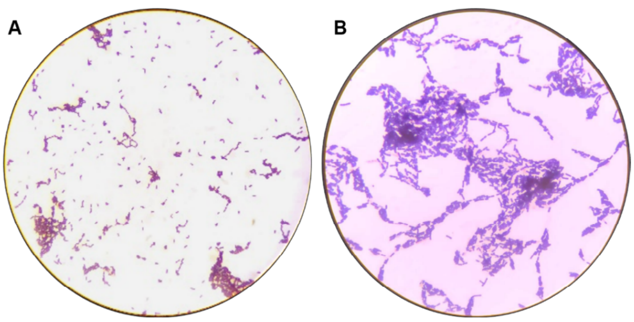

Figure 2 – Gram staining: A – isolate from a poultry farm, B – isolate from Taraz

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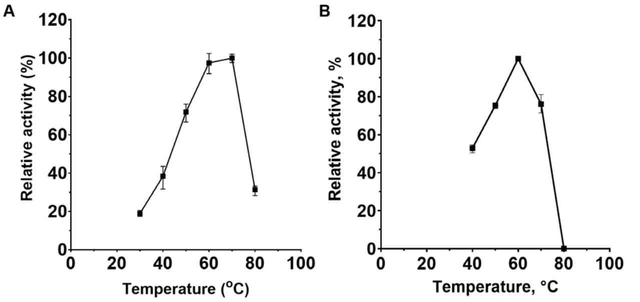


Figure 3 – Dependence of proteolytic activity of enzymatic extracts of *B. subtilis* (**A**) and *B. paralicheniformis* (**B**) strains on temperature

ture in the range of 30–80 °C was studied. The study found that the enzyme extract from *B. subtilis* has maximum activity at 70 °C (Figure 3A). The maximum activity of the *B. paralicheniformis* extract is reached at 60 °C (Figure 3B).

The dependence of proteolytic activity of enzyme extracts from strains *B. subtilis* and *B. paralicheniformis* on pH was studied in buffers: citrate buffer (pH 4.0-6.0), phosphate buffer (pH 6.0-7.5), Tris-HCl buffer (pH 7.5-9.0), and phosphate buffer (pH 9.0-11.0). The results showed that proteolytic enzymes are active in a wide pH range. *B. subtilis* enzymes retain more than 60% of their activity at pH values of 7.5-11.0, and *B. paralicheniformis* enzymes at pH values of 6.0-10.0. The optimal pH value for the proteolytic activity of *B. subtilis* enzymes is glycine buffer with a pH of 10.5, and for *B. paralicheniformis*, Tris-HCl with a pH of 9.0 (Figure 4).

The biochemical characteristics of proteases produced by B. subtilis and B. paralicheniformis allow them to be compared with a wide range of bacillary enzyme systems described in the literature (Table 1). Proteases from *B. paralicheniformis* demonstrate optimums at 60 °C and pH 9.0, which is characteristic of alkaline enzymes from moderately

Table 1 – Temperature and pH optima of various bacillary proteases

| Strain | Optimal temperature (°C) | pH optimum | Reference |
|---|---|---|---|
| *B. paralicheniformis* | 60 | 9.0 | This work |
| *B. subtilis* | 70 | 10.5 | This work |
| *Bacillus* sp. CL18 | 55 | 8.0 | [37] |
| *Bacillus* sp. RAM | 50 | 9.0 | [34] |
| *Bacillus* sp. DPUA 1728 | 50 | 9.0 | [36] |
| *B. velezensis* Y1 | 50 | 6.0 | [21] |
| *B. safensis* RH12 | 60 | 9.0 | [25] |
| *B. amyloliquefaciens* D1 | 50 | 6.0 | [39] |
| *Bacillus* sp. ZG20 | 45 | 7.0 | [22] |
| *B. licheniformis* K7A | 70 | 10.0 | [35] |
| *B. clausii* GMBE 22 | 60 | 12.0 | [38] |
| *Bacillus sp.* P45 | 55 | 7.5 | [40] |

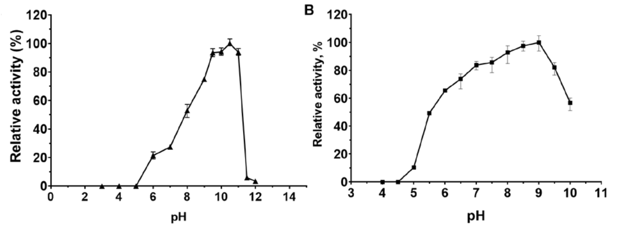


Figure 4 – Dependence of proteolytic activity of enzymatic extracts of *B. subtilis* (**A**) and *B. paralicheniformis* (**B**) strains on pH

thermotolerant strains. The closest parameters are observed in *Bacillus sp.* RAM, *Bacillus sp.* DPUA 1728, and *B. safensis* RH12 (pH 9.0; 50–60 °C), as well as in *Bacillus sp.* CL18 (55 °C, pH 8.0). These values fall within the range typical for bacterial proteases used in the hydrolysis of animal protein substrates.

*B. subtilis* proteases have a different profile, with higher optimum values of 70 °C and pH 10.5. Similar characteristics are found in a limited number of strains, in particular *B. licheniformis* K7A (70 °C, pH 10.0) and *B. clausii* GMBE 22 (60 °C, pH 12.0). Thus, *B. subtilis* belongs to a group of producers of highly alkaline proteases with elevated temperature optima, which are in demand in processes operating at extreme temperatures and pH values.

The other strains in the table have a more moderate profile: in *B. amyloliquefaciens* D1 and *B. velezensis* Y1, the optima are shifted to a slightly acidic range (pH 6.0 at 50 °C), while *Bacillus sp.* ZG20 and *Bacillus sp.* P45 show activity at neutral pH values (7.0–7.5) and temperatures of 45–55 °C. These data highlight the high variability of enzymatic systems within the genus Bacillus and reflect the ecological adaptation of strains to different substrates and environmental conditions.

Against this background, *B. paralicheniformis* is a strain with moderately thermophilic and alkaline properties typical of bacilli, while *B. subtilis* has the highest temperature and pH optimum values among the compared isolates. The data show that the former corresponds to the most common range of operating conditions, while the latter demonstrates parameters characteristic of industrially significant, highly thermostable, and highly alkaline enzyme systems.

The measured activity of the enzymatic extract from *B. paralicheniformis* under optimal conditions was 323 ± 4.8 U/mL, while that of *Bacillus subtilis* enzymes was 158.8 ± 2.5

Table 2 – Proteolytic activity (U/mL)

| Strain | Proteolytic activity (U/mL) | Reference |
|---|---|---|
| *B. paralicheniformis* | 323 ± 4.8 | This work |
| *B. subtilis* | 158.8 ± 2.5 | This work |
| *B. subtilis* RD7 | 176.00 | [41] |
| *B. subtilis* NRD9 | 163.76 | [41] |
| *B. cereus* FT1 | 187.00 | [42] |
| *B. subtilis* VBC7 | 246.0 | [43] |
| *B. haynesii* BK1H | 157.630 ± 16.240 | [44] |
| *Bacillus* sp. DPUA 1728 | 86.3 | [36] |
| *B. velezensis* Y1 | 301 | [46] |

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U/mL. A comparison of the proteolytic activity of the two strains studied, *B. paralicheniformis* and *B. subtilis*, with the data presented for other bacillary producers in Table 2 demonstrates marked differences in the level of enzyme secretion.

The high protease activity of *B. paralicheniformis* makes this strain one of the most productive natural strains used without extensive optimization interventions. This indicator exceeds the values recorded for a number of strains described in the literature, such as *B. subtilis* NRD [41], *B. subtilis* RD7 [41], *Bacillus cereus* FT1 [42], and *Bacillus haynesii* BK1H [44]. It is particularly noteworthy that the activity of *B. paralicheniformis* is on par with the upper range of values described in recent studies for cultures without optimization of growth conditions, where activities typically range from 160 to 250 U/mL. Higher values of up to 295 U/mL, mentioned for thermostable bacillary isolates, only partially approach the activity demonstrated by *B. paralicheniformis*. This emphasizes that the studied strain has a significant natural potential for protease secretion, exceeding most comparable strains obtained from various ecological niches.

The second strain, *B. subtilis*, exhibited an activity of 158.8 ± 2.5 U/mL, which corresponds to the middle range typical of many bacillary protease producers. Similar values are observed in *B. haynesii* BK1H [44] and *B. subtilis* NRD9 [41]. Such activity is considered typical for strains that have not undergone targeted optimization of the medium composition and cultivation conditions. Against this background, the example of *B. subtilis* VBC7 is particularly striking: the baseline activity of this isolate is 246 U/mL, but after optimization it reaches 712 U/mL, which demonstrates the ability of proteolytic systems of the genus *Bacillus* to significantly increase productivity with minimal changes in growth conditions [43]. Nevertheless, even against the backdrop of such examples, *B. subtilis* from this study remains a representative strain with average activity, and its potential for enhancing protease production when fermentation conditions are adjusted remains highly probable.

Based on these data, the strain *B. paralicheniformis* was selected for further experiments on the hydrolysis of protein-containing substrates (feathers, hides, and hooves) as the most promising object for subsequent study of the effectiveness of enzymatic treatment.

Enzymatic hydrolysis of hoof, hide, and chicken feather samples

Treatment of hoof, hide, and chicken feather samples with

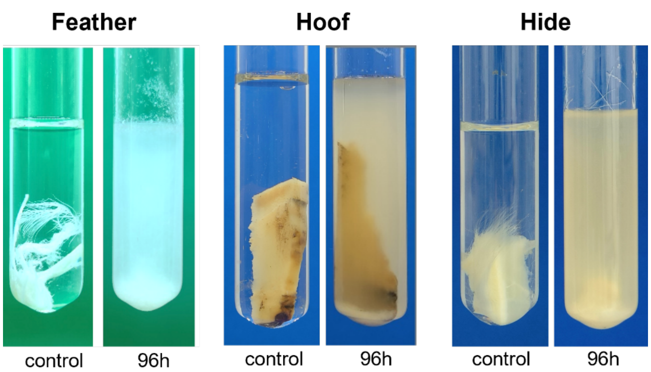


Figure 5 – Hydrolysis of protein substrates by enzymes of the *B. paralicheniformis* strain

Table 3 – Hydrolysis of horn, hoof, hide, cattle wool, and chicken feathers after 4 days

| Substrate | Degree of hydrolysis (%) | Proteolytic activity (U/ml) |
|---|---|---|
| Hoof | 29,6 | 115 ± 2,12 |
| Hide | 97,2 | 53 ± 2,11 |
| Chicken feather | 100 | 134 ± 0,20 |

*B. paralicheniformis* culture showed that this strain exhibits degrading properties with respect to all samples. Figure 5 shows the dynamics of degradation of hoof, hide, and feather samples.

As can be seen in Figure 6, after 4 days of treatment, the samples of feathers, hooves, and hide produce different results. Visual analysis indicates that all three samples are susceptible to proteases, keratinases, and collagenases of the *B. paralicheniformis* strain. Table 3 presents data on the degree of hydrolysis in the samples and protease activity after 4 days of treatment.

The quantitative data in Table 3 confirm the results of visual analysis: the enzymes of the strain hydrolyze the studied substrates in different ways. The activity is also influenced by the type of protein substrate used. Treatment of hoof horn, skin, and chicken feather samples with *B. paralicheniformis* culture showed pronounced substrate specificity of the enzyme complex: after 4 days, the degree of hydrolysis was 29.6% for hoof, 97.2% for skin, and 100% for feathers, with significant differences in proteolytic and keratinolytic activity levels. Complete hydrolysis of feathers is consistent with data in the literature, where bacillary strains demonstrate the ability to almost completely destroy feather keratin at moderate levels of keratinase and protease activities [47, 48], confirming the effectiveness of *B. paralicheniformis* enzymes against β-keratins. The high degree of skin hydrolysis at a relatively low total proteolytic activity indicates the significant role of collagenolytic enzymes; similar conclusions about the specificity of bacterial collagenases are presented in the work of Hoppe et al. [49], where it was established that active bacillary collagenases are capable of effectively destroying structural proteins of the dermis even at moderate enzyme titers. In contrast, the low level of hoof hydrolysis is explained by the high stability of rigid keratins rich in disulfide bonds; the need to use either more specialized keratinases or pre-treatment of such substrates is emphasized in reviews by Brandelli et al. [50] and Vidmar & Vodovnik [51]. Thus, the results obtained confirm that *B. paralicheniformis* is effective on feather and skin substrates, but for the decomposition of dense keratin structures, such as hoof horn, either a different enzyme combination or modification of the treatment conditions is required.

Treatment of chicken feathers with *B. paralicheniformis* culture showed that this strain effectively degrades feathers. Twenty-four hours after the start of incubation, the culture fluid was clouding. On the second day, degradation of the barbs was observed. On the third day, destruction of the shafts was noted. The culture acquired a milky color and lost its transparency. After incubation for 96 hours, the feather vane was completely destroyed, and the shafts were completely degraded or lost their mechanical strength, indicating the high keratinolytic potential of the bacterium *B. paralicheniformis*.

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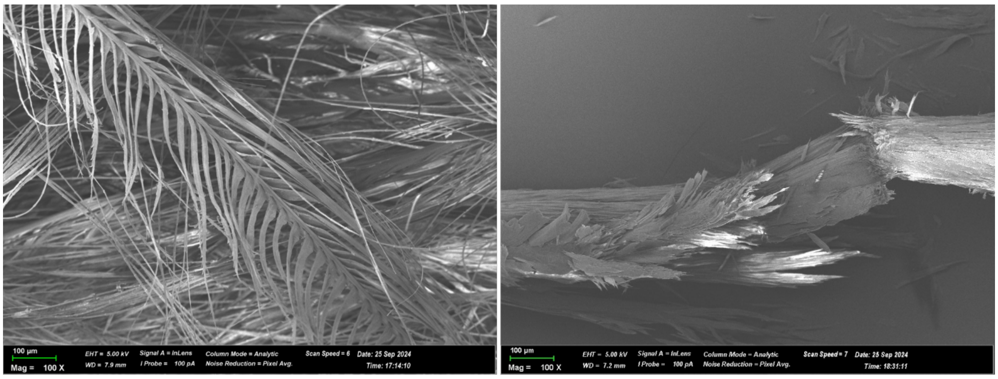


A B

Figure 6 – Scanning electron microscopy of a chicken feather after hydrolysis by the bacterial culture *B. paralicheniformis*: A – feather before treatment, B – feather after treatment, 96 hours.

Changes in the microstructure of the feather were observed using a scanning electron microscope during the destruction by *B. paralicheniformis* enzymes. Figure 6 shows photographs of a feather that underwent hydrolysis. Feather shafts with barbs and barbs with hooks were clearly visible in the SEM images of untreated feathers. The hooks and barbs had almost completely decomposed by 96 hours (Figure 6).

In addition to the action of enzymes in the destruction of feathers, the adhesion of live *B. paralicheniformis* bacteria to the feather substrate is of great importance. The use of scanning electron microscopy made it possible to visualize bacterial cells and confirm the direct involvement of *B. paralicheniformis* bacteria in the destruction of feathers (Figure 7).

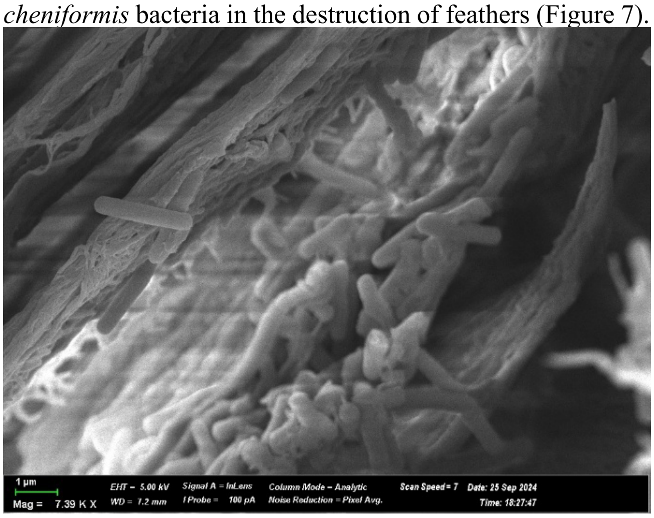


Figure 7 – Scanning electron microscopy of *B. paralicheniformis* cell adhesion to the surface of feathers, 96 hours

The significance of live bacteria in feather hydrolysis was confirmed in experiments on feather destruction involving live bacteria, enzymatic extract obtained by filtering the culture through 0.22 µm, and the addition of antibiotics. It was found that feather destruction in a sample with live bacteria proceeds faster than without microorganisms. This fact is consistent with literature sources [52, 53]. Thus, the extracellular keratinolytic activity of *B. licheniformis* RG1 without live bacterial cells does not lead to feather degradation [52]. In this study, structural analysis indicated that these bacteria adhere closely to the barbs and produce keratinase, which diffuses laterally, destroying the barbs and barbs with hooks.

Thus, the strain *B. paralicheniformis*, isolated from the soil of the city of Taraz, showed a high level of proteolytic enzyme secretion, possesses protease, keratinase, and collagenase activity, and demonstrated the ability to hydrolyze feathers, hooves, and hides. Based on the results obtained, it can be concluded that the use of the strain *B. paralicheniformis* opens up prospects for the creation of innovative methods of bioconversion of agro-industrial waste with the formation of valuable protein hydrolysate products, which allows expanding the raw material base of production and simultaneously reducing the environmental impact of poultry and livestock farming.

## CONCLUSION

Strains of *B. subtilis* and *B. paralicheniformis* with protease, keratinase, and collagenase activity were isolated and identified from soil samples taken in Central and Southern Kazakhstan. A study of their biochemical properties showed that the secreted proteases are alkaline enzymes, with maximum activity at 70 °C and pH 10.5 for B. subtilis, and at 60 °C and pH 9.0 for *B. paralicheniformis*. The levels of protease, keratinase, and collagenase activity of *B. subtilis* were 158.8 ± 2.5 U/mL, 109.3 U/mL, and 10.4 U/mL, respectively. In *B. paralicheniformis*, these values were 323 ± 4.8 U/mL, 122.6 U/mL, and 23.6 U/mL. The *B. paralicheniformis* strain was used for enzymatic treatment of feathers, hooves, and hides. As a result of 96 hours of treatment, the degree of hydrolysis was 29.6–100% for different substrates. Microstructural analysis confirmed the destruction of key structural elements of the feather, reflecting the high specificity and efficiency of the secreted enzymes. The data obtained convincingly demonstrate the applicability of the *B. paralicheniformis* strain in technologies for processing protein-rich agro-industrial waste. Their ability to act on stable biopolymers such as keratin and collagen makes these strains a promising basis for the development of environmentally friendly and economically viable biotechnological processes for the utilization and bioconversion of livestock products.

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**CONFLICTS OF INTEREST**

The authors declared no conflicts of interest

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*ӘОЖ* **579.222; 579.6; 579.62**

## АУЫЛ ШАРУАШЫЛЫҚ ӨНІМДЕРІН ӨҢДЕУДЕ ПРОТЕАЗАЛЫҚ, КЕРАТИНАЗАЛЫҚ ЖӘНЕ КОЛЛАГЕНАЗАЛЫҚ БЕЛСЕНДІЛІККЕ ИЕ *BACILLUS* ШТАМДАРЫН ҚОЛДАНУ

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**ТҮЙІН**

Ақуыз негізді агроөнеркәсіптік қалдықтардың айтарлықтай мөлшері жиналатындықтан, оларды өңдеу мен кәдеге жаратудың биотехнологиялық тәсілдерін әзірлеу өзекті мәселе болып табылады. Тараз қаласындағы және Ақмола облысындағы құс фабрикасынан жиналған топырақтан *B. subtilis* және *B. paralicheniformis* штамдары бөлініп алынып, сәйкестендірілді. Берілген *B. subtilis* және *B. paralicheniformis* штамдар сәйкесінше 70°C және pH 10,5 пен 60°C және 9,0 жағдайларында максималды белсенділікке ие протеазаларды бөліп шығарады. *B. subtilis* ферменттерінің протеазалық, кератиназалық және коллагеназалық белсенділігі сәйкесінше 158,8 ± 2,5 U/мл, 109,3 U/мл және 10,4 U/мл тең. *B. paralicheniformis* ферменттері үшін бұл мәндер 323 ± 4,8 U/мл, 122,6 U/мл және 23,6 U/мл жетті. *B. paralicheniformis* штаммын қолдана отырып, тауық қауырсындарын, ірі қара малдың тұяқтары мен терісін қоса сияқты құс және мал шаруашылығы қалдықтарының ферментативті гидролизі жүргізілді. 96 сағаттық өңдеуден кейін қауырсындардың, тұяқтардың және терінің гидролиз деңгейі сәйкесінше 100%, 29,6% және 97,2% тең болды. Гидролизденген қауырсындарды сканерлеуші электронды микроскопиялық талдау барысында, қауырсынның негізгі құрылымдық элементтерінің ыдырауын және қауырсын кератинінің беткейінде бациллярлық жасушалардың адгезиясы анықтады. Алынған деректер бойынша *B. paralicheniformis* штамының сілтілі протеолитикалық ферменттерді өндіруші ретінде және оның ақуыз негізді агроөнеркәсіптік қалдықтарды өңдеу технологияларында қолданылуында мүмкіндігі зор екендігін көрсетеді.

**Негізгі сөздер:** *Bacillus, протеазалар, биоконверсия, агробиотехнология, қауырсын, тұяқб тері*

*УДК* **579.222; 579.6; 579.62**

## ПРИМЕНЕНИЕ ШТАММОВ *BACILLUS* С ПРОТЕАЗНОЙ, КЕРАТИНАЗНОЙ И КОЛЛАГЕНАЗНОЙ АКТИВНОСТЬЮ В ПЕРЕРЕРАБОТКЕ СЕЛЬСКОХОЗЯЙСТВЕННОЙ ПРОДУКЦИИ

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**АБСТРАКТ**

В условиях накопления значительных объёмов белковых агропромышленных отходов актуальной задачей является разработка биотехнологических подходов к их переработке и утилизации. Из почвы, собранных с территории птицефабрики Акмолинской области и городской черты г. Тараз выделены и идентифицированы штаммы *B. subtilis* и *B. paralicheniformis*. Данные штаммы секретируют протеазы с максимальной активностью при 70°C и pH 10,5 и 60°C 9,0 для *B. subtilis* и *B. paralicheniformis*, соответственно. Протеазная, кератиназная и коллагеназная активность ферментов *B. subtilis* составляет 158,8 ± 2,5 U/mL, 109,3 U/mL и 10.4 U/mL, соответственно. Для ферментов *B. paralicheniformis* эти значения составили 323 ± 4,8 U/mL, 122,6 U/mL и 23,6 U/mL. С помощью штамма *B. paralicheniformis* проведен ферментативный гидролиз отходов птицеводства и животноводства: куриных перьев, копыта и шкуры КРС. В результате 96 часовой обработки степень гидролиза пера, копыта и шкуры составила 100%, 29,6% и 97,2%, соответственно. Анализ гидролизованного пера, проведенный с помощью сканирующей электронной микроскопии, показал разрушение ключевых структурных элементов пера и адгезию бациллярных клеток на поверхности перьевого кератина. Полученные данные свидетельствуют о перспективности штамма *B. paralicheniformis* в качестве продуцента щелочных протеолитических ферментов и его применимости в технологиях переработки белковых агропромышленных отходов.

**Ключевые слова:** *Bacillus, протеазы, биоконверсия*, *агробиотехнология*, перья, копыта, шкура