Results of primary screening of proteolytically active microorganisms that are promising for the transformation of organic pollution

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Abstract. In order to optimize the methods of remediation of contaminated areas, strains of microorganisms were selected that are promising for the transformation of excess amounts and the accumulation of organic matter in surface water bodies. Research has been carried out on the isolation and selection of proteolytically active strains of microorganisms from the microflora of water. Among the isolated isolates, representatives of 3 genera dominated: Pseudomonas sp., Micrococcus sp., Bacillus sp., Which differed in the rate of biomass accumulation / optical density (OD), the number of viable cells (CFU/ml) and the concentration of residual protein (mg/ml). The highest proteolytic activity was found in representatives of the genus Bacillus sp. For bacilli, within 5 days of the experiment, a 7-fold increase in biomass was observed, and the number of viable cells increased 8-fold. Due to the proteolytic activity of Bacillus sp. the concentration of the protein in the test solution decreased from 0.2412 to 0.1123 mg/ml. The research carried out opens up prospects for the creation of biopreparations of targeted action based on mono- and polycultures of proteolitics for the purposes of ecobiotechnology.

1. Introduction
One of the tasks of ecobiotechnology is the development of alternative methods for remediation of contaminated areas through microbiological transformation of various groups of xenobiotics [1, 2]. A variety of the latter are high- and low-molecular nitrogen-containing organic compounds in the form of proteids, polypeptides, amino acids, amides and urea (carbamide), which are often toxic and can disrupt the stable state of the ecosystem. The main sources of excess nitrogen-containing organic matter include untreated or insufficiently treated industrial, agricultural and municipal wastewater, including waste from livestock complexes, with which up to 4000 mg/l of nitrogen-containing organic matter can enter the environment annually [3, 4]. Under the action of specific groups of microorganisms with biochemical activity, complete or partial mineralization of excess organic matter (deamination or decarboxylation) becomes possible, while metabolites of microorganisms accumulate in the medium, and the synthesis of substances de novo is not observed [5, 6]. It is experimentally proved that the most active producers of proteases are representatives of the genus Bacillus and Pseudomonas [7], as well as microscopic fungi [8]. Such microorganisms are part of a large group of biological products, consisting of monocultures or associations of microorganisms that can accelerate the decomposition of organic waste and neutralize toxic compounds [9, 10]. For example, the biopreparations «Komponaza» and «Biocomplex-BTU» (manufactured by BTU-Center) are intended for composting organic waste of industrial, agricultural, household origin and obtaining a humified substrate - biofertilizer; granular bacterial concentrate «Bacti-Bio 9800» (manufactured by A&V Envirotec) is proposed to be used for...
the complete and intensive decomposition of organic matter and sediments due to the metabolic activity of anaerobic and aerobic strains of microorganisms.

To optimize the composition of drugs, the action of which is aimed at the destruction of pollutants due to proteolytic groups of microorganisms, the constant search for new biochemically active strains, promising for the purposes of ecobiotechnology, is considered relevant.

2. Material and research methods
The isolation of bacteria capable of synthesizing proteolytic enzymes into a pure culture was performed from the microflora of the water of the Kondopoga Bay of Lake Onega. Samples were taken from 5 stations, differing in the degree of anthropogenic pollution, distance from the coastal zone, depth, soil quality, and the degree of fouling of the coastline. The layout of the sampling stations for water microflora is shown in Figure 1.

![Figure 1. Location of water microflora sampling stations in the water area of the Kondopoga Bay of Lake Onega (scale 1: 1,000,000).](image)

Under laboratory conditions, 0.1 ml of water was inoculated on a nutrient medium of the following composition (g/l): KH₂PO₄ - 0.01; MgSO₄×7H₂O - 0.2; CaCl₂×2H₂O - 0.1; NaCl -1.0; FeCl₃×6H₂O - 0.075; amino acid or peptone hydrolyzate - 0.5; agar - 15. Isolated colonies were used to obtain pure cultures, which were kept on a nutrient agar slant at 4 °C for further study of morphophysiological and biochemical properties for identification by phenotypic criteria regulated in the Bergey Bacteria Guide [11]. To accumulate the biomass of microbial cells, a peptone-containing medium was used: peptone (10 g/l), agar (20 g/l) and milk agar: agar (25 g/l), 0.5% milk in a 1:2 ratio. Bacteria were cultivated in a TSO-200 SPU thermostat at a temperature of 37 ± 0.2 °C.

The diversity of the isolated strains was assessed by a complex of cultural, tinctorial, physiological and biochemical characteristics. The morphological, tinctorial and cultural characteristics of the strains were studied using immersion (biological microscope Motic DM-BA-30) and inverted (Thermo Fisher, Evos M5000) microscopy techniques. The cultural traits of the isolated microorganisms were: colony diameter, edge shape, pigment color, consistency, position on a nutrient medium.
2.1. Study of the proteolytic activity of isolated microorganisms

To detect proteolytic activity, the studied bacterial culture was inoculated under aseptic conditions in sterile nutrient broth (pH 7.2–7.4) containing coagulated egg white. Inoculated culture plates were placed in a thermostat at a temperature of 37 ± 0.5 °C and periodically examined for 10 months. The result was taken into account according to the biochemical activity of proteolytically active cultures of microorganisms. At the same time, the protein fragments contained in the medium decreased in size, turned into a crumb-like mass, or completely dissolved. When analyzing the result, attention was drawn to the fact that the proteolytic activity of different types of microorganisms is manifested differently, which is due to the specificity of the enzymatic activity [12]. The end result was evaluated by the change in the protein concentration in the reaction mixture.

All experiments to study the proteolytic activity of isolated microorganisms were performed in triplicate. To ensure stationary conditions of the experiment, the following were controlled: inoculum inoculum dose, temperature and cultivation period, and the rate of stirring of the reaction mixture. To select the most active proteolytics, a complete loop of the studied strain was introduced into 50 ml of sterile nutrient broth and incubated using a Biosan OS-20 orbital shaker with a microprocessor for controlling time and revolutions for 24 h at a temperature of 24 ± 3 °C and stirring at 150 rpm/min. After cultivation, microbial cells were concentrated by centrifugation at 4000 rpm for 10 min and suspended twice in 10 ml of physiological solution to obtain an optical density of a bacterial suspension of 0.5 u.p., which was measured on a LEKI spectrophotometer (MEDIORA OY, Finland) at a wavelength of 640 nm and a green filter [13].

The selection of the most active proteolytics was carried out under experimental conditions on media enriched with peptone. Peptone was used as the only nitrogen source needed for the growth and reproduction of bacteria. For this, 5 ml of the bacterial suspension was transferred into 100 ml conical flasks containing 50 ml of mineral saline solution (MSP) and 0.5 ml of peptone as the sole nitrogen source. A sterile solution consisting of 50 ml of MSP and 0.5 ml of peptone served as a control. Experimental and control flasks were placed on a shaker at a temperature of 24 ± 3 °C with a stirring speed of 150 rpm for 5 days.

Peptone degradation was assessed spectrophotometrically by the optical density of the bacterial suspension and by the number of viable cells growing on BEA (beef extract agar) medium. To determine the number of viable cells, 1 ml of broth culture of the investigated strain was used, which was mixed with 9 ml of sterile saline solution and a series of dilutions from 10⁻¹ to 10⁻¹⁰ was performed. To obtain statistically significant results from the last three dilutions, 0.1 ml of the culture solution was dispersed in Petri dishes containing BEA. The inoculations were incubated at 38 °C for 48 hours. At the end of incubation, the colonies of the studied strain were counted and the result was expressed in CFU/ml.

2.2. Determination of the concentration of residual protein by the Lowry method

The proteolytic activity of the isolated bacteria was assessed by the Lowry method [14] by determining the concentration of residual protein. For the study, the following were preliminarily prepared: 2% Na₂CO₃ solution prepared in 0.1 M NaOH solution (reagent 1); 0.5% CuSO₄ solution prepared in 1% Na₃C₆H₅O₇ solution (reagent 2); reagent 3 (1 ml of reagent 1 was mixed with 50 ml of reagent 2). The Folin-Chocalteu reagent was prepared immediately before the study. Next, a test sample with a volume of 0.4 ml (400 μl) was mixed with 2 ml of reagent 3 and left at room temperature for 10 minutes. Added 0.2 ml (200 μl) of the Folin-Chocalteu reagent, stirred and after 30–40 minutes measured the optical density at 750 nm on a spectrophotometer. The result obtained was expressed in units of u.a./mg of protein (author certificate No. 1811854 dated April 30, 1993) and was calculated using the formula:

\[ c.u.a. = \frac{u.a.}{[P]}, \]

where:
- c.u.a. – conditional units of activity
- u.a. – units of activity
- [P] – protein concentration in the test sample (mg/ml)
3. Research results

3.1. Results of isolating bacteria from water microflora
A variety of heterotrophic bacteria capable of assimilating organic forms of nitrogen in the composition of products of incomplete hydrolysis of protein from peptone, which is used as the main source of nitrogen, have been isolated from the microflora of the water of the Kondopoga Bay of Lake Onega. The cultural diversity of bacteria isolated from the microflora of water sampled at stations 1–5 is shown in photographs (Figure 2).

![Photographs of bacterial cultures](image)

**Figure 2.** Cultural diversity of bacteria isolated from the microflora of water at 1 sampling station (a), at 2 (b), at 3 (c), at 4 (d) and 5 (e).

By phenotypic traits, 10 proteolytic strains were identified, belonging to 4 morphotypes: coccoid, rod-shaped, bacillary, and sarcino-like cells (Figure 3).
Figure 3. Variety of morphotypes of proteolytic bacteria: coccoid (a), rod-shaped (b), bacillary (c) and sarcino-like bacteria (d); immersion microscopy × 1000; coloring with gentian violet and basic fuchsine.

The isolated bacteria were assigned to 2 phyla according to tinctorial characteristics: Firmicutes (gram-positive bacteria - strains No. 2, 4, 5, 6, and 8) and Gracilicutes (gram-negative bacteria - strains No. 1, 3, 7, 9 and 10). Among the described morphotypes, globular cells dominated; polymorphic rods were found singly. According to cultural characteristics, cultures dominated, giving small (1–3 mm), medium (5–7 mm) and large colonies (32–45 mm) when growing on agar media. The pigment color corresponded to white or cream shades; pigment was absent in 2 isolates. For 8 cultures, the mucous surface of the colonies was described and for 2 - rough.

The biochemical activity of the isolated strains is shown in the diagram (Figure 4). According to the ability to synthesize hemin catalase, 85% of the isolated bacteria are attributed to aerobes. All cultures were actively grown in the presence of amino acid hydrolysates or peptone as the only source of carbon, nitrogen, and energy. Growth activity in the presence of proteins (the product of incomplete protein hydrolysis - peptone and albumin) was established for 96% of the isolated strains, respectively. 80% of the isolated cultures fermented glucose, but only 40% of the isolates fermented sugar to acid and gas; 70% of the isolated cultures grew in the presence of mannitol and actively assimilated it.
According to the efficiency of albumin decomposition in the composition of coagulated chicken protein, for further study of the proteolytic activity of the isolated cultures, 3 strains were selected, identified to the genus: *Pseudomonas sp.*, *Micrococcus sp.* and *Bacillus sp.*

### 3.2. Results of a study of the proteolytic activity of isolated bacteria

The activity of the isolated proteolytics was assessed according to three parameters: 1. the rate of accumulation of proteolytic biomass/optical density (o.d.u.); 2. the number of viable cells (CFU/ml); 3. the concentration of residual protein (mg/ml). A sterile culture medium enriched with peptone was used as a control. Observations of the proteolytic activity of microorganisms were performed for 5 days; all experiments were performed in triplicate. Tables 1–3 show the results obtained.

#### Table 1. Proteolytic activity of bacteria of the genus *Pseudomonas* (n = 3).

| Day | Optical density, o.d.u. | Number of cells, CFU/ml | Amount of residual protein, mg/ml |
|-----|------------------------|--------------------------|----------------------------------|
| 1   | 0.309                  | 2.5 × 10³                | 0.2561                           |
| 2   | 0.580                  | 2.6 × 10³                | 0.1109                           |
| 3   | 1.207                  | 5.1 × 10³                | 0.1004                           |
| 4   | 1.609                  | 6.9 × 10³                | 0.1093                           |
| 5   | 1.780                  | 1.1 × 10⁴                | 0.1061                           |

* o.d.u. – optical density units

When studying the proteolytic activity of bacteria of the genus *Pseudomonas sp.* it was found that during the five days of the experiment the optical density increased from 0.309 o.d.u. to 1,780 o.d.u., which is 5,58 times more than the initial indicators. At the same time, the number of viable cells changed from 2.5 × 10³ CFU/ml to 1.1 × 10⁴ CFU/ml. The number of viable cells increased 4.5 times. The protein concentration in the culture mixture decreased by 2 times and by the end of the 5th day of the experiment was 0.1061 mg / ml.

The results of proteolytic activity of *Micrococcus sp.* are presented in Table 2.

#### Table 2. Proteolytic activity of bacteria of the genus *Micrococcus* (n = 3).

| Day | Optical density, o.d.u. | Number of cells, CFU/ml | Amount of residual protein, mg/ml |
|-----|------------------------|--------------------------|----------------------------------|
| 1   | 0.092                  | 94                       | 0.2813                           |
| 2   | 0.076                  | 1.0 × 10²                | 0.2672                           |
| 3   | 0.109                  | 3.1 × 10²                | 0.2413                           |
| 4   | 0.308                  | 3.2 × 10²                | 0.2217                           |
| 5   | 0.206                  | 2.7 × 10²                | 0.2310                           |
For 5 days of the experiment, the optical density of bacteria increased from 0.092 o.d.u. up to 0.206 o.d.u., which is 2.2 times more than the initial indicators. At the same time, the number of viable cells increased 3 times - from 94 to $2.7 \times 10^2$ CFU/ml, and the concentration of protein assimilated by micrococci changed insignificantly from 0.2813 to 0.2310 mg/ml.

When studying the activity of proteolytics of the genus Bacillus sp. (Table 3) it was found that for five days of the experiment the optical density increased from 0.123 o.d.u. up to 1.408 o.d.u., which is 11.4 times more than the initial values. At the same time, the number of viable cells increased from $1.2 \times 10^3$ CFU / ml to $9.6 \times 10^6$ CFU / ml. Due to the active proteolytic activity of bacilli, the protein concentration in the culture mixture decreased by 2 times and by the end of the 5th day of the experiment was 0.1123 mg/ml.

Table 3. Proteolytic activity of bacteria of the genus Bacillus ($n = 3$).

| Day | Optical density, o.d.u. | Number of cells, CFU/ml | Amount of residual protein, mg/ml |
|-----|------------------------|-------------------------|----------------------------------|
| 1   | 0.123                  | $1.2 \times 10^3$       | 0.2412                           |
| 2   | 0.268                  | $4.1 \times 10^3$       | 0.2309                           |
| 3   | 0.541                  | $4.8 \times 10^3$       | 0.1578                           |
| 4   | 1.203                  | $5.9 \times 10^3$       | 0.1124                           |
| 5   | 1.408                  | $9.6 \times 10^6$       | 0.1123                           |

4. Conclusion

According to the results of the study, it was proved that microorganisms in the isolated bacterial complex have pronounced proteolytic activity. However, in order to optimize the biodegradation of organic pollutants, it is necessary to take into account their concentration in the ecosystem, the applied dose of inoculum, and temperature conditions [15, 16]. Microorganisms with proteolytic activity are widespread in nature and are ubiquitous in the utilization of nitrogen-containing organic pollutants. Within the framework of the study, primary data were obtained indicating that the bacteria of the genus Bacillus sp. Have the highest proteolytic activity among bacteria isolated from the microflora of water. For bacilli, within 5 days of the experiment, an increase in biomass was observed 7 times from the initial indicators. At the same time, the number of viable cells increased by 8 times, and the concentration of the protein in the test solution decreased by 2 times. The data obtained by Khaziev A.F. [17], Contesini F. J., Melo R. R., Sato H. H. [18] on the example of B. subtilis also indicate a high proteolytic activity of Bacilli. No data has been found that studies of the proteolytic ability of bacteria isolated from the microflora of water in the Kondopoga Bay of Lake Onega have already been carried out. In this regard, the results obtained in the course of the study can be considered unique and, in the future, can be used for the purposes of ecobiotechnology.

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