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Advancing livestock waste fermentation via strategic organic substrate selection

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Abstract. Increasing volumes of livestock waste have created a growing demand for efficient biotechnological methods for its management. Properly processed animal waste can not only reduce organic matter accumulation but also serve as an effective substitute for mineral fertilizers, thereby improving soil fertility. This study aimed to investigate the use of various organic substrates in the fermentation of animal waste for the production of biohumus. Thermophilic microorganisms capable of growth at 50°C were isolated from cattle manure samples. All isolates were identified as members of the genus *Bacillus.* Enzymatic activities were evaluated using qualitative assays for cellulase, protease, amylase, and urease, along with biochemical tests for catalase and oxidase. The range of proteolytic index values produced by isolates with protease potential varied from 0.12 to 1.5. Amylase enzyme was detected in isolates I, II, and IV, with the highest activity observed in isolate IV (AI = 1.1). Among them, isolates I and II showed strong enzymatic activity across all investigated substrates, forming distinct hydrolysis zones that indicated efficient degradation of complex organic matter. The most active strains, characterized by high viable cell concentrations, are recommended for incorporation into biopreparations designed to accelerate organic waste fermentation and improve biohumus quality.
Keywords: livestock waste, fermentation, organic substrates, thermophilic microorganisms, microbial activity, biohumus

Introduction

Global agricultural production continues to expand each year in response to population growth, making effective management an increasingly urgent priority [1]. According to the 2023 census, there were more than 8.185 million cattle in Kazakhstan, which is 4.3 percent more than the previous year, according to the National Bureau of Statistics. In January of that

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year, the livestock population increased further, reaching 8.264 million head. The majority of cattle are kept on private subsidiary farms (52.4%), followed by farms (38.2%), and agricultural enterprises (9.4%). Approximately 4.8 million cattle of this, 58.8% are used for dairy farming [2]. Such an increase in the number of farm animals leads to an increase in the volume of organic waste, which in turn creates problems of biological safety.

Animal waste can be broadly divided into two main categories: biodegradable and non-biodegradable. Biodegradable waste consists of solid materials that can be naturally broken down by microorganisms over time, reducing long-term pollution. Examples include animal waste such as paper products, plant residues, carrion, feces, and poultry by-products [3].

Biodegradable waste decomposes relatively quickly, it often produces an unpleasant odor and negatively affects the visual appeal of the environment. In addition, it can contaminate soil and water sources in urban areas, as it is a favorable environment for the growth of pathogenic microorganisms (Figure 1) [4].

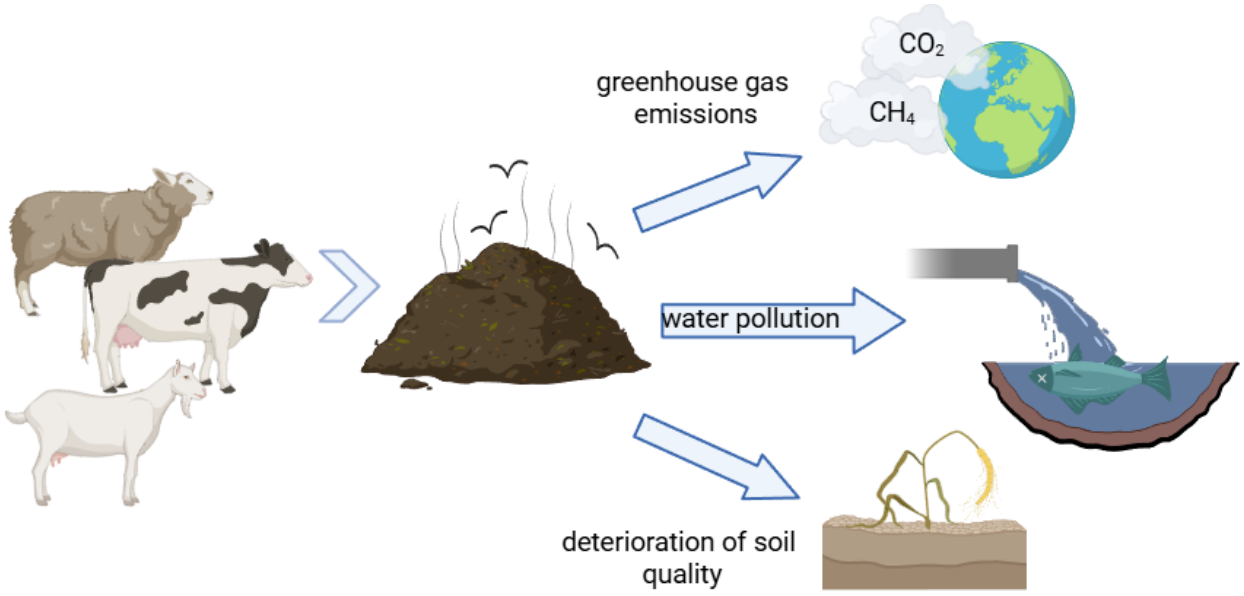

Figure 1. Biosecurity issues arising from the accumulation of animal waste. (This image was created using the BioRender scientific illustration platform)

Furthermore, a substantial amount of ammonia (NH_3), nitrous oxide (N_2O), "knox" (a mixture of NO_x, NO, and NO_2), methane (CH_4), and carbon dioxide (CO_2) are also released into the atmosphere by the agricultural sector. Nonetheless, it is well known that nitrogenous gaseous molecules (NO_x, N_2O, and NH_3) can result in serious biological issues. NO_x helps create ozone in the troposphere, N_2O is a greenhouse gas that contributes to the loss of stratospheric ozone, and ammonia can cause soil acidification and eutrophication of water systems. Methane, with a global warming potential (GWP) of 21, is a potent greenhouse gas that influences the climate directly through interactions with long-wave infrared radiation and indirectly through atmospheric oxidation processes. Methane accounts for around 18% of the greenhouse effect and is the second most important greenhouse gas after CO_2 [5].

However, assuming proper application of animal waste resources can not only decrease the amounts of waste but also effectively replace mineral fertilizers, traditional fodder, and cooking gas [6]. By breaking down organic waste into simpler compounds like carbon dioxide (CO_2), methane (CO_4), hydrogen (H_2), hydrogen sulfide (H_2S), and water (H_2O), microorganisms are

able to receive the energy they require for growth and reproduction. They aid in the breakdown of organic matter, the soil's capacity to clean itself, the development of its fertility, the conversion of humus, and the preservation of the mineral and cellulose balance. Furthermore, it is well known that microorganisms actively participate in the removal of different organic pollutants from the environment [7-8]. Thus, recycling animal waste not only produces energy and organic fertilizers but also reduces the pollution problems associated with such waste. By treating waste as a potential resource rather than a harmful element, air, water, and soil pollution, as well as the spread of toxic substances, can be prevented [9].

In addition, poor soil and erosion can lead to the need to increase its fertility, which in turn opens up the possibility of rational use of livestock waste. The use of agricultural waste based on the bioenzymatic activity of microorganisms that improve soil fertility is an economically rational solution.

The technological parameters of fermentation by microorganisms are aimed at the efficient transformation of the initial mixture into high-quality, environmentally friendly organic fertilizer [10]. Understanding the fermentation processes allows for the controlled fermentation of organic matter, which leads to the production of natural organic fertilizer and the extraction of humic substances from it [11].

The purpose of this study is to investigate the activity of new strains of thermostable microorganisms on various substrates, which can be used to optimize fermentation processes and produce high-quality biohumus.

Materials and research methods

Isolation and selection of thermophilic microorganisms

Isolation of pure bacterial cultures included a number of stages: selection of sources, sampling, inoculation of microorganisms onto solid media by serial dilution, and isolation of pure cultures.

To isolate microorganisms, 1g of cattle manure (Cattle manure samples were collected from farms in the Akmola region, Kazakhstan) was suspended in sterile NaCl (9%) solution, serially diluted, and plated onto meat-peptone agar (MPA) using the 2-4-6 method and incubated at 50°C for 48 hours (Figure 2). After incubation, isolated colonies were selected and isolated onto fresh MPA medium by Gold streaking for further study.

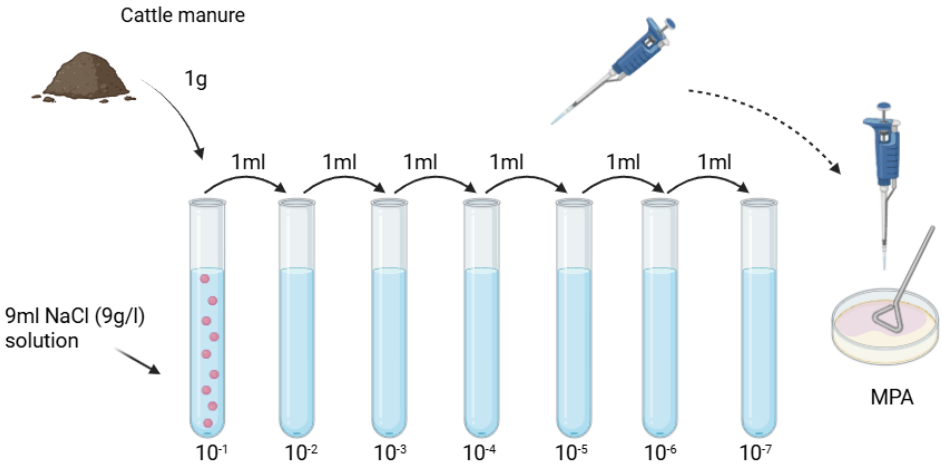

Figure 2. Isolation of microorganisms using the serial dilution method. (This image was created using the BioRender scientific illustration platform)

Cultural and morphological analysis of microbial isolates

For each isolate in Petri dishes, a morphological study was carried out, depending on the shape, color, transparency, and bacterial cell ends. Then, Gram staining was performed for microscopic examination of the morphology of the isolates. First, the suspension of microorganisms was collected from the Petri dish using a bacteriological loop and transferred onto the surface of a glass slide containing a drop of distilled water. The smear was then air-dried or heat-fixed by briefly passing it through a flame. Then the preparation was stained with crystal violet solution for 1 min. Crystal violet was treated with Lugol's solution (1 min), and after stabilization, it was decolorized by dropping ethanol or acetone. The preparation is additionally stained with Safranin solution to give color to Gram-negative bacteria. After each stage, the preparation is rinsed with water.

The stained preparations are examined using immersion microscopy to study the morphology of the bacteria.

Study of the biochemistry of microorganisms

(a) The catalase test was performed using 3% hydrogen peroxide (H_2O_2). A small amount of 24-hour pure culture of bacteria was smeared onto a glass slide using a sterile bacteriological loop. Then, 3% H_2O_2 solution was dripped onto the test (1-2 drops). As a result, the appearance of visual gas bubbles was considered a positive reaction [12]. (b) Commercial oxidase discs (HiMedia, India) were used to conduct the oxidase test. The test was performed according to the manufacturer's instructions: the oxidase disc was treated with the bacterial culture. The results were evaluated by the change in the color of the disc to purple within 30 seconds.

Preparation of various substrates for the evaluation of the enzymatic activity of microorganisms

(a) To test the proteolytic activity of microorganisms, a culture medium based on skim milk was prepared (skim milk agar medium by MicroMaster, prepared according to manufacturer specifications). The culture medium was prepared by dissolving 3.5 g peptone, 3 g yeast extract, and 15 g agar in 700 ml distilled water and sterilized in an autoclave at 1 ATM. 300 ml of skim milk was added to the culture medium after sterilizing in an autoclave at 0.5 ATM and allowed to cool slightly. The culture medium was poured into Petri dishes. Bacterial isolates were inoculated by the spot inoculation method and incubated at 50°C for 48 hours. After incubation, the results were obtained based on the appearance/absence of a clear zone. If the microorganisms are able to degrade casein (a protein in milk), a transparent zone appears around the areas where the bacteria have grown.

(b) The culture medium for determining the amylolytic activity of microorganisms was prepared by dissolving 10 g of peptone, 5 g of KH_2PO_4, 18 g of agar-agar, and 2 g of starch, the main substrate, in 1L of distilled water. The culture medium was sterilized in an autoclave and poured into Petri dishes. A 48-hour culture of bacterial isolates was incubated at 50°C for 48 hours. To visualize the results, the tests were stained with Lugol's solution (2 ml) after incubation [13].

(c) The medium for identification of cellulolytic activity was prepared based on $NaNO_3$-2g, $MgSO_4$-0.5g, KCl-0.5g, K_2HPO_4-1g, peptone-0.2g, agar-agar-17g and the substrate CMC-Na (Sodium Carboxymethyl Cellulose). The medium was sterilized in an autoclave at 1 ATM and poured into Petri dishes. Bacterial isolates were inoculated using the plaque method and incubated at 50°C for 48 hours. To visualize the results, the samples were stained with 5ml of 0.1% Congo red solution for 30 minutes after incubation and washed by treating with 1 M NaCl for 5 minutes [5].

(d) Urea-degrading microorganisms were studied on a medium based on 25 g urea base agar and 20 g urea solution in 1L of distilled water. The medium was sterilized in an autoclave and poured into test tubes in a slanting position. Bacterial isolates were inoculated into test tubes by streaking and incubated at 50°C. Samples were monitored for 6-24 hours, and the results were obtained after 72 hours of incubation. Cultivation of microorganisms in this condition is used to ensure rapid reaction (in the presence of microbial activity, the pH of the medium changes due to the release of nitrogen from urea, turning it pink) and to reduce urea evaporation. The results are visually observed by a change in the intensity of the color of the medium [14].

To quantitatively assess proteolytic, amylolytic, and cellulolytic activity, an additional hydrolysis index (I) was calculated according to the following formula (2) [15]:

$$I = \frac{Dz - Dc}{Dc} \quad (2)$$

Where:
I-activity index
D_z-diameter of the hydrolysis zone
D_c-diameter of the colony

Identification of microorganisms

Pure cultures, which were re-examined microscopically, were grown in the medium for 24 hours at 50°C. After incubation, the samples were sent to a specialized laboratory for identification by MALDI-TOF mass spectrometry, performed according to standard protocols.

Checking the viability of microorganisms

Serial dilutions of the test sample are prepared using the Miles and Misra method. The bacterial culture is serially diluted up to 10^{-10} in physiological saline (0.9% NaCl). Then the agar in a Petri dish is divided into eight equal sectors. Starting from the third dilution (10^{-3}), the resulting test was inoculated in the form of drops on the surface of solid culture medium. The cultures are then incubated to allow the growth of colonies. Colony counting is carried out in the drop zones that show the highest number of discrete colonies that do not coalesce after appropriate incubation. The samples were incubated at 50°C for 24 h. After a specified time, the presence and density of colonies in each sector are assessed, which allows determining the viability of bacteria at different dilution levels.

Statistical analysis

All experiments were performed in quadruplicate, and the results are presented as mean ± standard deviation (SD). Statistical significance of differences among isolates was evaluated using one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) test at a significance level of $P < 0.05$. All analyses were performed using Jump (version 8).

Results

Isolation and selection of thermophilic microorganisms

Microorganisms were isolated by the serial dilution method based on samples taken from cattle feces. As a result of incubation on meat-peptone agar (MPA) at a temperature of 50°C for 48 hours, numerous colonies with different morphological features were formed.

After incubation, individual colonies that differed in macroscopic characteristics were selected from the colonies formed. As a result of pure cultures obtained by re-inoculation by

the Gold method, 15 different thermophilic bacterial isolates were obtained. These isolates had different colony morphology and strains were selected from them for further study.

Among the isolates obtained, five strains demonstrating the highest enzymatic activity were selected for further analysis.

Colony description

The colonies of the selected strains grown on nutrient media had different morphological characteristics (Figure 3):

Colonies of isolate I were shiny, yellowish, with a dry shell, had a complex shape, and were distinguished by a convex structure, teardrop-like elevations, and crater-like formations.

Isolate II formed yellowish colored colonies with a dry surface; their edges were rhizoid, and their structure was flat.

Colonies of isolate III were flat, shiny, and round, with irregular, jagged ends.

Isolate IV produced dark, flat colonies with a yellow-orange hue and a structure resembling concentric rings.

Isolate V developed flat, dark-yellowish colonies with a round shape and clearly irregular ends.

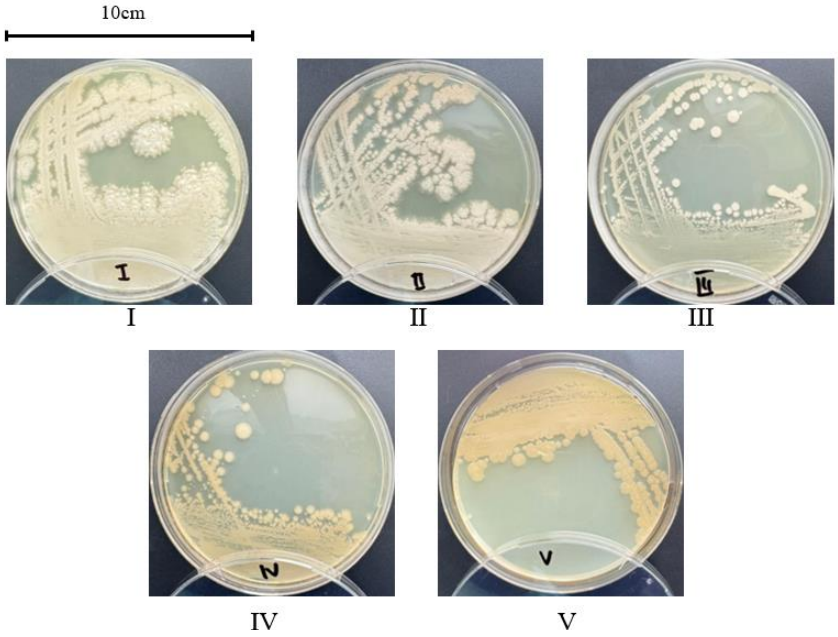

Figure 3. Colonies of microorganisms. I-V-correspond to isolates numbers; Scale bar = 10cm

Microscopic description

When examining the microscopic appearance of microorganisms, it was found that all the isolates studied were gram-positive, round rods with pointed ends (Figure 4). Spores were observed in all isolates. They are often located on the outside, but also inside the bacteria. The morphology of bacteria differs from each other in shape and length of the rods.

Gram +, short rods with rounded ends, spores on the outside and in the middle.

Gram +, short rods with rounded ends, rare spores on the outside and in the middle of the rods

Gram +, short rods with rounded ends, many spores, located on the outside and inside (in the middle)

Gram +, short rods, "microphone"-shaped rods are often found, spores are located separately.
Gram +, short, thin rods with rounded ends, few spores, "microphone"-shaped rods are found.

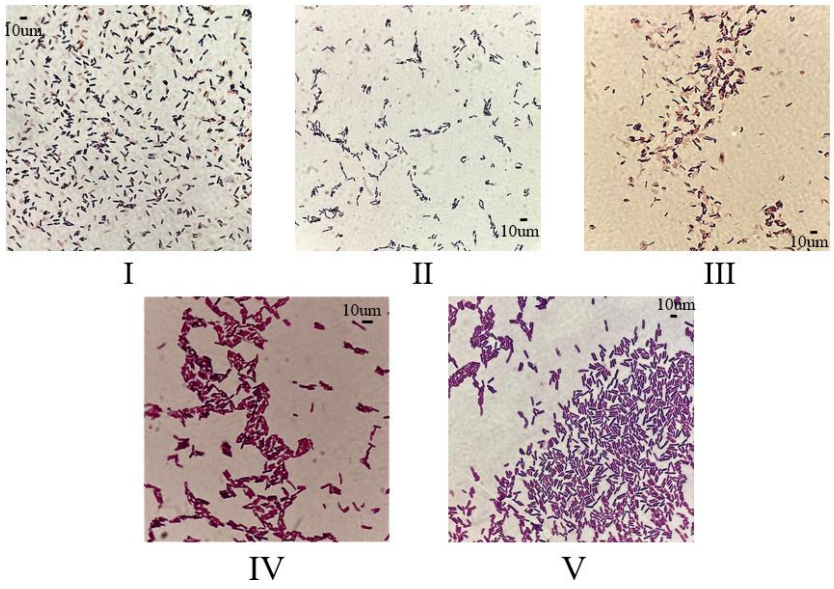

Figure 4. Microscopic morphology of bacterial isolates. **I-V** corresponds to isolates numbers; Scale bar = 10 μm.

Biochemical assay results

Assays performed to characterize the biochemical properties of the bacterial isolates, all tested isolates exhibited positive activity for both enzymes. However, the intensity of the oxidase test showed lower indicators.

As a result of the catalase reaction (1), the decomposition of hydrogen peroxide on the surface of the glass slide by the catalase enzyme led to the formation of O2 gas bubbles (Figure 5a):

$$2H_2O_2 \rightarrow 2H_2O+O_2\uparrow \quad (1)$$

A positive result of the oxidase test confirms that the bacteria possess the *cytochrome C oxidase* enzyme, which uses oxygen as an electron donor in the final stage of the respiratory chain. The result of the reaction can be observed by the color change of the *cytochrome-C* oxidase enzyme when it interacts with a chemical indicator on the oxidase disk (Figure 5b).

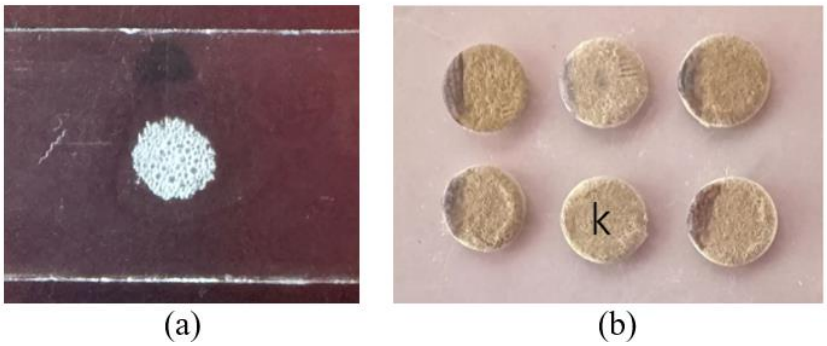

Figure 5. Biochemical **(a)** catalase and **(b)** oxidase tests. k-indicates control

Evaluation of the enzymatic activity of microorganisms based on various substrates

The qualitative assessment of four enzymatic activities: proteolytic, amylolytic, cellulolytic, and urease, was performed on the isolated microorganisms, representing a key component of the experimental work. The results were observed due to the appearance of transparent zones around the colony or a change in the color of the culture medium. The clear zones formed in the activity test qualitatively indicate the ability of bacterial isolates to produce hydrolysis enzymes. It is believed that the larger the formed transparent zone, the more positively it correlates with the ability of these isolates to produce the corresponding enzymes.

Calculating the activity index allowed us to compare the efficiency of enzymatic activity between different isolates, since it took into account not only the presence of the zone, but also the ratio of the hydrolysis zone diameter to the diameter of the bacterial colony itself. A higher ratio indicates a greater enzymatic activity exhibited by the microorganism.

Proteolytic activity was determined on a medium containing casein proteins. In this study, the appearance of transparent zones around the colonies indicates the area of protein degradation. Hydrolysis by the protease enzyme was shown by isolates I, II, IV, and V (Figure 6). The proteolytic activity index values are shown in Table 1 and Figure 7. The range of proteolytic index values produced by isolates with protease potential varied from 0.12 to 1.5. Isolate No. V showed the most pronounced proteolytic activity in terms of the volume of the hydrolysis zone and the calculated index.

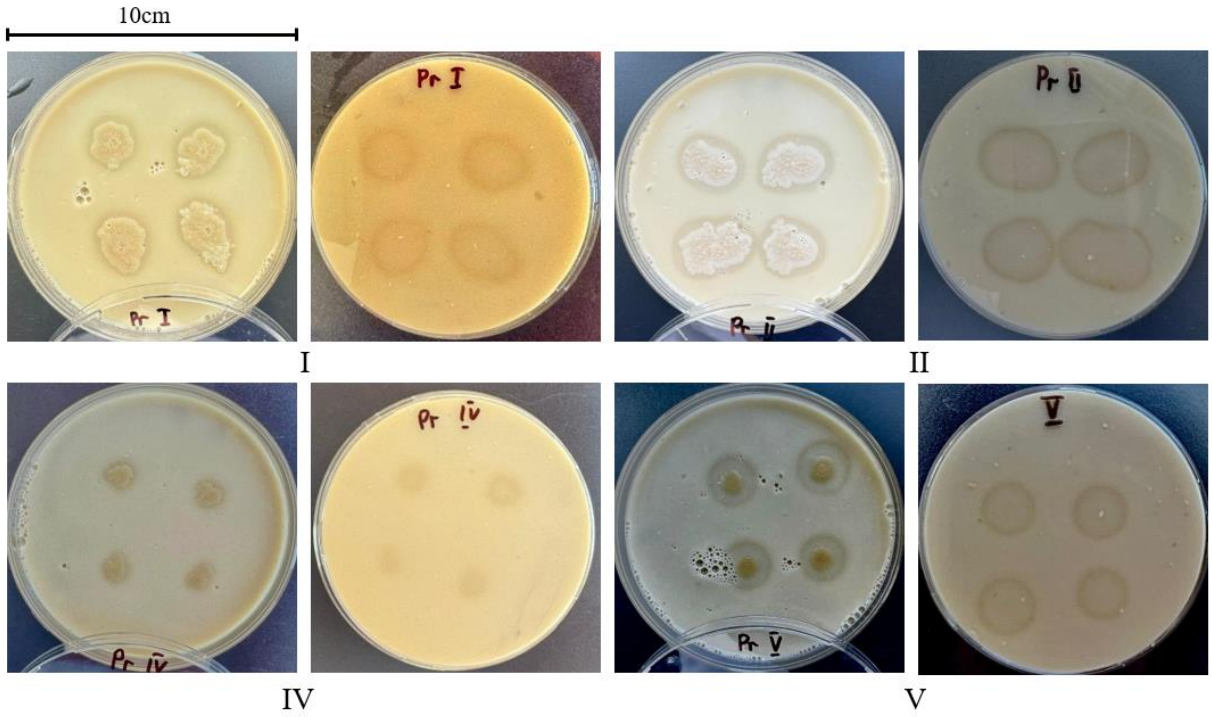

Figure 6. Proteolytic activity. **I, II, IV, V** correspond to isolates numbers; Scale bar = 10cm

Table 1

Average proteolytic index (PI) of thermophilic bacteria isolated from cattle manure (n-4)

№	Isolate	Diameter of the colony (mm)	Diameter of the hydrolysis zone (mm)	Proteolytic Index (PI)
1	I	13	17	0.30

1	I	15 22 17	18 25 20	0.2 0.14 0.18
2	II	17 18 17 24	21 23 19 27	0.23 0.28 0.12 0.125
3	IV	8 7 7 7	10 9 9 8	0.25 0.28 0.28 0.143
4	V	8 6 7 7	16 15 14 14	1 1.5 1 1

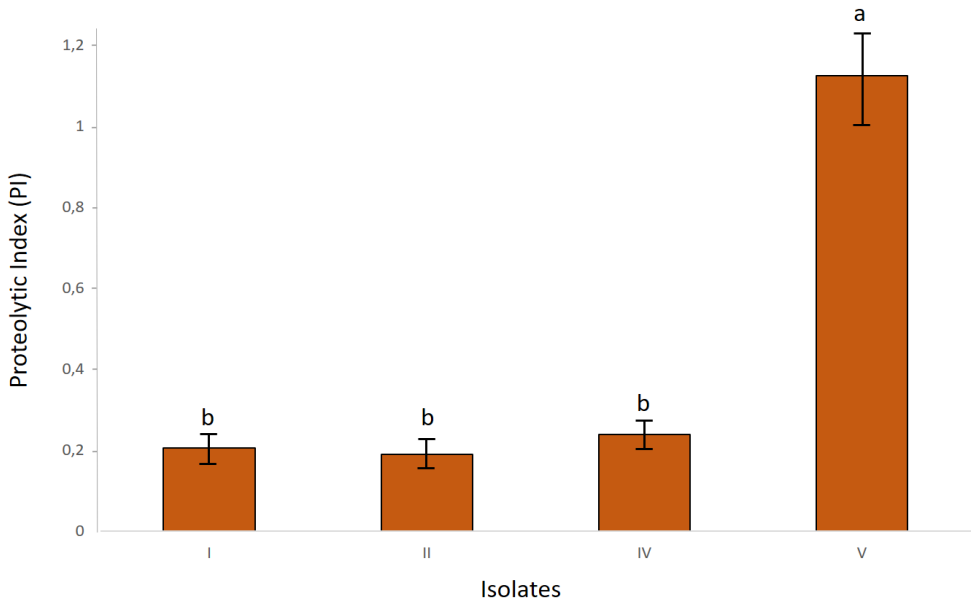

Figure 7. Comparison of proteolytic activity (PI) among thermophilic isolates. Bars represent mean ± SD (n = 4). Different letters indicate significant differences between isolates (Tukey's HSD, $P < 0.05$)

Amylolytic bacteria break down starch and glycogen using amylases, including α- and β-amylases. The resulting hydrolysis products, such as glucose, serve as a source of carbon and energy for microorganisms and a source of nutrients for plants [16].

Therefore, amylolytic activity was assessed on a starch-based medium. The results were visualized, and the area of hydrolysis was stained with iodine solution, an indicator of starch. Clear zones around the colonies indicate starch degradation. Amylase enzyme was detected in isolates I, II, and IV, with very high activity observed in isolate IV (AI=1.1). The results are shown in Figure 8, and the amylolytic index is shown in Table 2 and Figure 9.

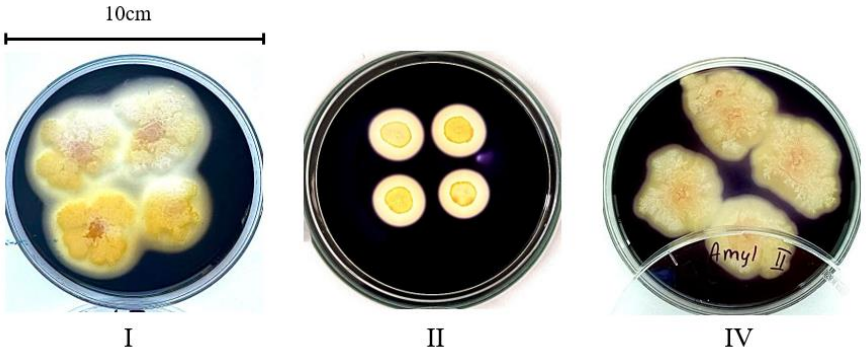

Figure 8. Amylolytic activity. **I, II, IV** correspond to isolates numbers; Scale bar = 10cm

Table 2

Average amylolytic index (AI) of thermophilic bacteria isolated from cattle manure (n-4)

№	Isolate	Diameter of the colony (mm)	Diameter of the hydrolysis zone (mm)	Proteolytic Index (PI)
1	I	24 20 23 27	30 32 28 34	0.25 0.6 0.30 0.26
2	II	27 33 28 28	35 35 30 30	0.29 0.06 0.07 0.07
3	IV	11 10 9 10	21 20 19 20	0.9 1 1.1 1

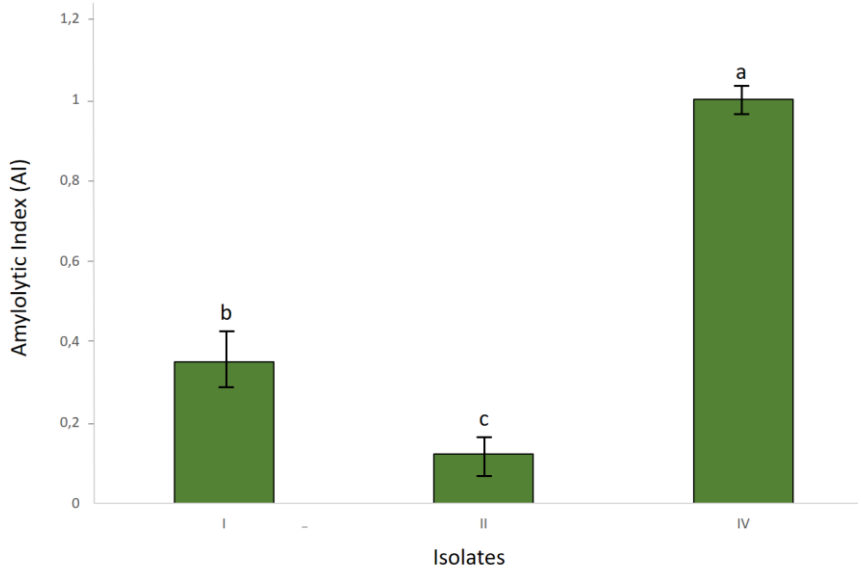

Figure 9. Comparison of amylolytic activity (AI) among thermophilic isolates. Bars represent mean ± SD (n = 4). Different letters indicate significant differences between isolates (Tukey's HSD, $P < 0.05$)

Cellulolytic activity was studied in a medium containing sodium carboxymethylcellulose (CMC-Na). Degradation was visualized as decolorized zones surrounding the colonies following staining of the medium with Congo red solution. Cellulolytic activity was detected in isolates I, II, III, and V (Figure 10). However, the hydrolytic activity exhibited by isolates III and V was markedly low (Table 3, Figure 11).

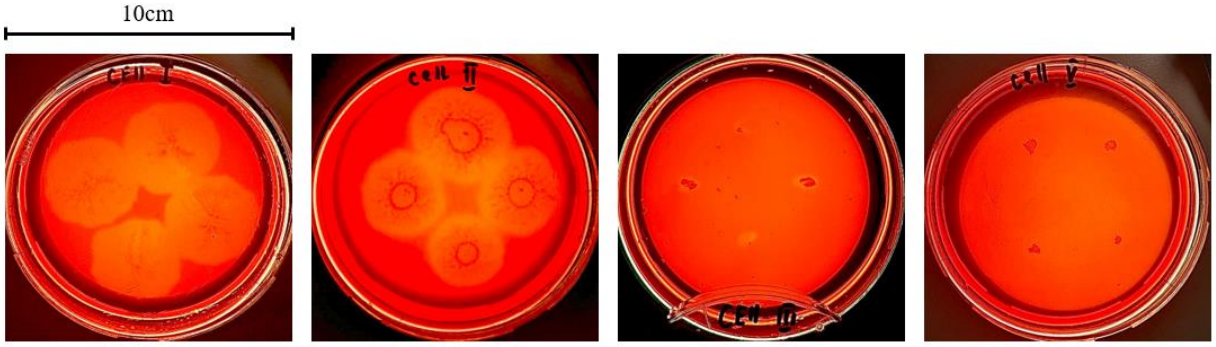

Figure 10. Cellulolytic activity. **I, II, III, V** correspond to isolates numbers; Scale bar = 10cm

Table 3

Average cellulolytic index (CI) of thermophilic bacteria isolated from cattle manure (n-4)

№	Isolate	Diameter of the colony (mm)	Diameter of the hydrolysis zone (mm)	Proteolytic Index (PI)
1	I	18 16 17 17	30 28 29 30	0.66 0.75 0.70 0.76
2	II	18 20 19 20	25 33 27 33	0.39 0.65 0.63 0.65
3	IV	5 5 6 4	7 7 8 5	0.4 0.4 0.33 0.25
4	V	4 4 3 4	5 5 4 5.5	0.25 0.25 0.33 0.375

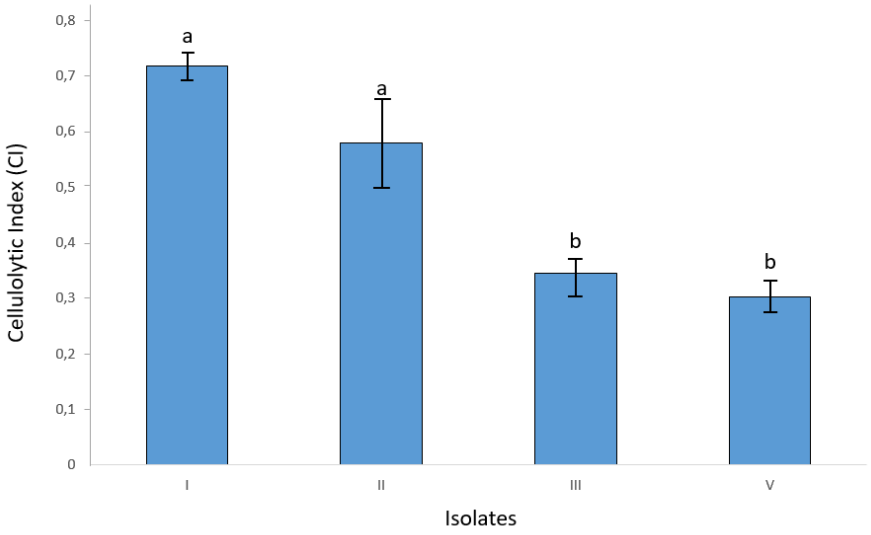

Figure 11. Comparison of cellulolytic activity (CI) among thermophilic isolates. Bars represent mean ± SD (n = 4). Different letters indicate significant differences between isolates (Tukey's HSD, $P < 0.05$)

Urease enzyme activity was assessed by the color change of a medium containing urea and red phenol as a pH indicator. The results were interpreted in terms of the intensity of the pink color change of the medium, which occurred due to the formation of ammonia during the decomposition of urea and the increase in the pH of the medium. Isolates I, II, III, and IV, shown in Figure 12, showed very high urease activity.

A culture of *Klebsiella pneumoniae,* known for its high urease activity, was used as a positive control, which caused the medium to turn a deep pink color. As a negative control, E. Coli, which does not show urease activity, was inoculated, resulting in a yellow coloration. Also, as a control, the pure medium without inoculation retained its original orange color.

Note: Klebsiella-positive control, Empty-clean medium without microorganisms, *E. Coli*-negative control

Figure 12. Urease Activity Assay. **I, II, III, IV** correspond to isolates numbers

Currently, microbial enzymes have almost completely replaced synthetic analogues in a number of industrial sectors [17]. Thus, the obtained results showed that most of the isolates studied had several types of enzymatic activity. Isolates I and II exhibited positive results across

all four tested enzymatic activities, demonstrating notable activity potential. Based on their high enzymatic performance, these isolates were selected for further investigation.

Identification of microorganisms

To determine the taxonomic affiliation of the isolated microorganisms, identification was carried out using MALDI-TOF mass spectrometry. The results of the analysis of protein profiles showed that all the isolates studied belong to the genus Bacillus.

As a result of the identification, it was determined that the isolates studied belong to the species *Bacillus licheniformis, Aeribacillus pallidus, Geobacillus thermodenitrificans,* and *Lysinibacillus sphaericus* (Table 4). All of these species are microorganisms with high enzymatic activity, the ability to decompose complex organic compounds, and have valuable properties from a biotechnological point of view.

Table 4

Taxonomic affiliation of the studied isolates

№	Isolate	Genus	Species
1	I	*Bacillus*	*B. licheniformis*
2	II	*Bacillus*	*B. licheniformis*
3	III	*Aeribacillus*	*A. pallidus*
4	IV	*Geobacillus*	*G. thermodenitrificans*
5	V	*Lysinibacillus*	*L. sphaericus*

Assessment of microbial viability

To assess the viability of microorganisms, a tenfold dilution series of the test samples was performed up to the 10^{-10} level. 10 µl of the sample from each dilution was evenly dropped onto the surface of the solid medium in separate sectors of a Petri dish. After incubation under optimal conditions, the number of colonies grown was counted.

Since the droplet volume used was 10 µl (i.e., 1/100 of 1 ml), the following formula (3) was used to convert the obtained data to a standard unit of measurement (CFU/ml) [18]:

$$CFU/ml = Number\ of\ colonies \times 100 \times Dilution\ level \quad (3)$$

Where:

Number of colonies – the number of colonies grown in 10 µl of suspension,

100 – the conversion factor for 1 ml of solution,

Dilution level – takes into account the dilution of the original sample.

Thus, each cultured colony showed the presence of one viable cell per drop of the test dilution, and the final re-counting allowed us to determine the total number of viable microorganisms in the original sample (Table 5).

Table 5

Determination of the viability of isolates

№	Isolates	Dilution degree	Number of colonies	CFU/ml
1	I	10^{-7}	no separate colony	> 10^{10} CFU/ml

2	II	10^{-6}	2	2×10^{8} CFU/ml
3	III	10^{-6}	20	2×10^{9} CFU/ml
4	IV	10^{-6}	9	9×10^{8} CFU/ml
5	V	10^{-6}	5	5×10^{8} CFU/ml

The obtained data show that the viability of microorganisms was maintained in all studied samples to the order of 10^{-6} and 10^{-7} (Figure 13), and the concentration of viable cells varied from 5×10^{8} to 10_{10} CFU/ml. In the first sample, the concentration of viable cells was so high that it was not possible to separate colonies even at a dilution of 10^{-7}, which indicates that the permissible limit of standard counting was exceeded. The viability of samples II, III, IV, and V ranged from 5×10^{8} to 2×10^{9} CFU/ml. These values confirm the presence of a large number of viable microorganisms in the studied samples.

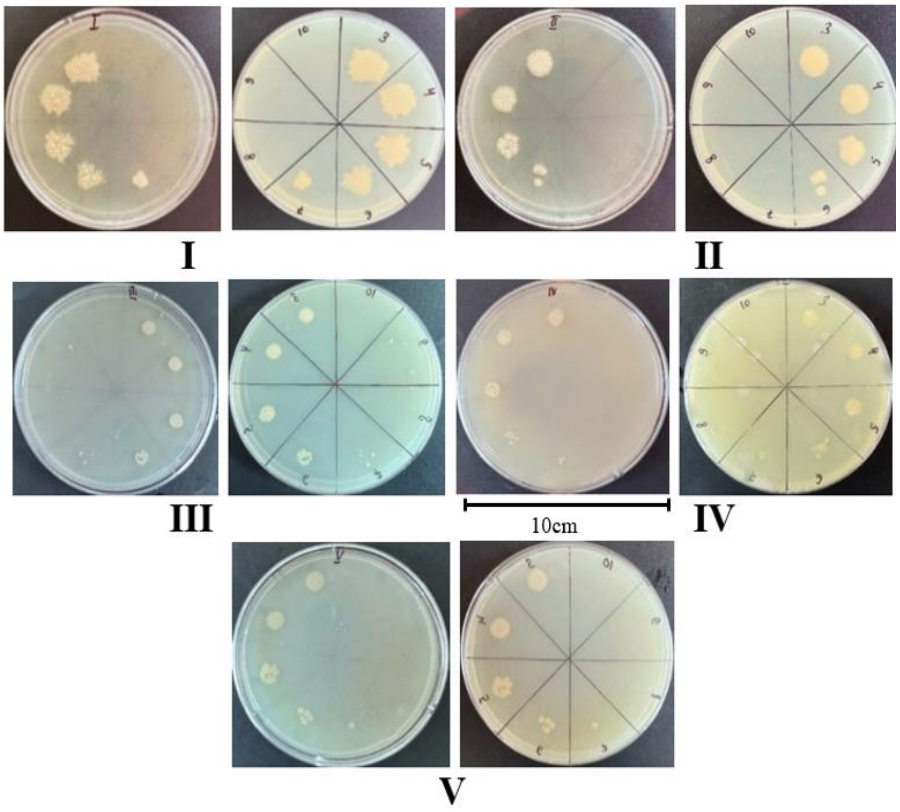

Figure 13. Bacterial viability. **I-V** correspond to isolates numbers; Scale bar = 10cm

Discussion

Cattle feces are a rich source of microbial diversity, as evidenced by the isolation of thermophilic microorganisms from them. The obtained isolates show potential for biotechnological applications, particularly in the context of fermenting organic waste.

These strains were distinguished by their ability to accelerate the decomposition of substrates and were recognized as promising for future use as the main components of a bioconsortium in the production of biopreparations.

Recent research highlighting the significance of thermophilic bacterial strains in high-temperature composting and waste bioconversion is consistent with the isolation and characterization of these organisms from cattle manure. Temperatures between 50 and 80°C promote the growth of thermophilic microorganisms during the thermogenic phase of composting, especially spore-forming Gram-positive bacteria like those in the Bacillus genus, which predominate microbial communities and play a major role in the breakdown of organic matter [19].

The tests showed that the isolates exhibit various enzymatic activities, indicating their role in organic matter decomposition. Studies by T. A. Ogunnusi and O. Olorunfemi have shown that cattle manure is a rich substrate for the growth and activity of proteolytic microorganisms due to the high content of organic compounds, including proteins. The protein components of manure serve as a source of amino acids and peptides formed as a result of enzymatic decomposition [20]. Protein hydrolysis in this substrate is carried out due to the activity of bacteria that synthesize proteases capable of breaking down casein and other complex protein structures.

The observed amylase activity, especially pronounced in isolate IV, indicates that these microorganisms are capable of efficient starch degradation. Such enzymatic potential not only reflects their adaptability but also points to their possible role in enhancing nutrient cycling and soil fertility. Amylolytic activity has also been reported in other Bacillus strains isolated from cattle manure. For example, Bacillus subtilis CM3 produces thermostable α-amylase with an optimum at 50-70°C and pH 5-9, making it highly relevant for composting and feed bioconversion applications [21].

Cellulose can be degraded by cellulases produced by thermophilic bacteria, mainly Bacillus spp., effectively hydrolyzing cellulose even at temperatures up to 70°C and under variable pH conditions [22].

Cellulose is the most abundant renewable organic resource in nature, comprising approximately 45% of the dry weight of wood. The microbial degradation of cellulose has been extensively studied for decades. The function of cellulase is to hydrolyze cellulose by breaking the α-1,4-glycosidic bond in the main structural polysaccharide of plants. The mechanisms of cellulose degradation are constrained by many factors, including the complexity of the substrate and the number of enzymes involved [23].

Recent data show that thermotolerant Bacillus inoculation increased cellulose degradation rates up to 57% in composting systems, supporting the reliability of our observed enzymatic potential [24].

Several isolates demonstrated cellulolytic potential, consistent with previous findings that thermophilic Bacillus strains are key degraders of cellulose in compost environments. Nevertheless, the hydrolytic index of isolates III and V is significantly lower than other indicators. Notably, the third isolate demonstrates weak cellulase activity and have no detectable proteolytic, amylolytic or catalase functions. However, it showed strong urease activity, indicating that, despite its low enzymatic activity, this isolate could still contribute to nitrogen cycling during organic matter decomposition.

The main source of nitrogen in animal waste is urea, which is converted to ammonia by the enzyme urease [25]. Urease is highly specific and catalyzes the breakdown of urea to unstable ammonium carbonate, which then decomposes to form ammonia and carbon dioxide [26]. Supporting our findings on urease activity and nitrogen-cycle involvement of isolates, Cao et al. (2024) reported significant enhancement of nitrification gene abundances and nitrogen retention in manure composts inoculated with Bacillus strains [27].

The results of the oxidase and catalase tests, in addition to serving as indicators of the biochemical characteristics of the studied isolates, provide essential preliminary data for their further identification. These enzymatic assays offer insight into the physiological properties of the bacterial strains and contribute to their classification within specific taxonomic groups.

Catalases are one of the ROS defense enzymes, which can decompose harmful oxygen compounds such as hydrogen peroxide (H_2O_2) [28]. Therefore, this property proves that they are adapted to live in aerobic or facultative anaerobic environments.

Oxidative activity indicates that the bacteria use oxygen for energy metabolism, which confirms that they belong to aerobic or facultative aerobic organisms. However, the weak reaction may indicate low enzymatic activity in the studied bacteria.

The above-mentioned species belonging to Bacillus genus are considered promising agents for the creation of bioconsortiums and their use in various application areas, such as environmental biotechnology, agriculture, and organic waste processing. Choudhary & Johri (2009) and Ubani et al. (2016) found that 87% of randomly selected colonies in the thermophilic phase of compost belonged to the genus Bacillus [29, 30]. Then, Nayara Noreen et al. (2019) reported similar results. The abundance of thermotolerant and thermophilic Bacillus species in compost samples is due to the thick spore walls of these microorganisms, which are characterized by their high adaptability to extreme conditions [31].

These isolates were recovered from cattle manure in the Akmola region, which to our knowledge has not been previously reported for thermophilic Bacillus strains applied in biohumus production, thereby representing a locally-adapted candidate set for industrial waste-fermentation applications. In this context, the genus Bacillus includes a wide range of species with important biotechnological properties, such as enzymatic activity, antagonism to pathogens and the ability to biodegrade complex compounds. Therefore, the combined enzymatic potential (proteolytic, amylolytic, cellulolytic, and ureolytic activity), together with oxidative stress resistance and high adaptability, suggests that Bacillus isolates from cattle manure are excellent candidates for industrial bioprocesses, compost enhancement, and sustainable waste valorization strategies. What makes them useful for the formation of bioconsortiums and further biotechnological applications.

Conclusion

The development of microbial technologies that allow the reuse of organic waste as fertilizer ensures the efficient use of valuable biological resources [32]. Thermophilic bacteria are particularly promising in the management of livestock waste, since their activity at high temperatures allows for significantly reducing the fermentation time and obtaining a product with high agrotechnical properties. Therefore, in the context of the present study, thermophilic bacterial cultures belonging to the Bacillus genus were isolated from cattle manure, which are distinguished by high viability and enzymatic activity. The study of their activity on four different organic substrates showed that a number of isolated microorganisms exhibited stable activity on all tested substrates. This indicates their ability to effectively decompose complex organic compounds into simpler forms, which significantly improves the availability of nutrients to plants. The results obtained confirm the prospects for further study of the isolated strains. Since this study is part of a larger scientific project aimed at creating a biopreparation based on thermophilic bacteria, it is planned to further study the antagonistic activity and biocompatibility of the isolated strains in order to create an effective bioconsortium for obtaining an industrial biopreparation in the future. Thus, the practical significance of the conducted studies, in turn,

opens prospects for environmentally safe processing of livestock waste and contributes to solving the problems of increasing soil fertility in conditions of sustainable agriculture.

Authors contributions

M.E., N.Zh., and **T.A. –** conceptualization; **T.Zh., K.K.** – data curation; **T.A., E.D., A.A.** – formal analysis; **M.E.** and **N.Zh., T.A.** – writing – original draft; **M.E., N. Zh., T.A.,** and **T.Zh.** – writing – review & editing; **N.Zh.,** and **T.A.**– supervision. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest. Compliance with ethical standards. This article does not contain a description of studies performed by the authors involving people or using animals as objects.

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Органикалық субстратты стратегиялық таңдау арқылы мал шаруашылығы қалдықтарының ферментациясын оңтайландыру

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Аңдатпа. Жануарлар қалдықтарының өсіп келе жатқан көлемі оны өңдеудің тиімді биотехнологиялық әдістеріне сұраныстың артуына әкеліп соғады. Мұндай қалдықтар тез ыдырайды, жиі жағымсыз иістерді шығарады және қоршаған ортаның эстетикалық сапасын нашарлатады. Сонымен қатар, топырақ пен судың ластануы патогендік микроорганизмдердің көбеюіне қолайлы жағдай болып табылады. Мал шаруашылығы қалдықтарын дұрыс өңдеу органикалықзаттардыңжинақталуыназайтыпқанақоймай,минералдытыңайтқыштардытиімді алмастырып, топырақ құнарлылығын арттырады. Бұл зерттеудің мақсаты биогумус өндіру үшін жануарлар қалдықтарын ашытуда әртүрлі органикалық субстраттарды пайдалануды зерттеу болып табылады. Ірі қара мал көңі үлгілерінен 50°C температурада өсуге қабілетті термофильді микроорганизмдер бөлініп алынды. Барлық изоляттар *Bacillus* туысының өкілдері ретінде анықталды. Ферменттік белсенділік целлюлаза, протеаза, амилаза және уреаза үшін сапалы талдаулар және каталаза мен оксидаза биохимиялық сынақтары арқылы бағаланды. Өміршең

жасушаларының концентрациясы жоғары, ең белсенді штаммдар органикалық қалдықтардың ферментациясын жеделдетуге және биогумус сапасын арттыруға арналған биопрепараттардың құрамына енгізу үшін ұсынылады.
Түйін сөздер: мал шаруашылығы қалдықтары, ферментация, органикалық субстраттар, термофильді микроорганизмдер, микробтық белсенділік, биогумус

Оптимизация ферментации отходов животноводства посредством стратегического выбора органического субстрата

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Аннотация. Растущие объемы отходов животноводства создали растущий спрос на эффективные биотехнологические методы их переработки. Такие отходы быстро разлагаются, часто выделяя неприятные запахи и ухудшая эстетическое качество окружающей среды. Более того, загрязнение почвы и воды создает благоприятные условия для размножения патогенных микроорганизмов. Правильно переработанные отходы животноводства могут не только снизить накопление органического вещества, но и служить эффективной заменой минеральных удобрений, тем самым повышая плодородие почвы. Целью данного исследования было изучение использования различных органических субстратов при ферментации отходов животноводства для производства биогумуса. Из образцов навоза крупного рогатого скота были выделены термофильные микроорганизмы, способные расти при температуре 50 °C. Все изоляты были идентифицированы как представители рода *Bacillus.* Ферментативная активность оценивалась с помощью качественных анализов на целлюлазу, протеазу, амилазу и уреазу, а также биохимических тестов на каталазу и оксидазу. Наиболее активные штаммы, характеризующиеся высокой концентрацией жизнеспособных клеток, рекомендуются для включения в состав биопрепаратов, предназначенных для ускорения ферментации органических отходов и улучшения качества биогумуса.
Ключевые слова: отходы животноводства, ферментация, органические субстраты, термофильные микроорганизмы, микробная активность, биогумус

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