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Microorganisms Cultures Screening with High Proteolytic Properties and Capable Fix Atmospheric Nitrogen to Speed up the Poultry Manure Biodegradation

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Abstract

In this work, we studied screening strains of microorganisms that exhibit high proteolytic properties and can fix atmospheric nitrogen to speed up the process of biodegradation of bird droppings. There had been established that the Pseudomonasputida 90 biovar A (171) strain exhibits the highest enzymatic ability. Its addition to chicken manure contributed to an increase in the microflora titer from 104 CFU/ml to 1011 CFU/ml after 15-20days. The study of strains capable of utilizing atmospheric nitrogen showed the best property of the culture Azotobacterchroococcum 31/8R. Treatment of bird droppings with the studied strain reduced the level of ammonia in the environment from 93mg/m^3 to 14mg/m^3. In the structure of the total DNA of this strain, it identified genes encoding all the enzymes responsible for the process of nitrogen fixation. The combined use of selected cultures of microorganisms can be the basis for the development of a biological product that speeds up the process of natural decomposition of bird droppings.

Disciplinary: Veterinary, Sustainability.

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1 Introduction

Industrial poultry farming is the most important branch of agriculture, both in the Russian Federation and in the world. In recent years, this industry has been actively developing and innovative technologies are being introduced into its structure that increases the profitability of production (Neverova et al., 2021; Rozhkova and Stepanova, 2021). This is all because of the high level of consumption of poultry products, which shows the prospects of this industry in the coming years (Micciche et al., 2019; Mir et al., 2017; Shepon et al., 2016).

Today, in the poultry industry, there are problems associated with the processing and use of poultry manure because of the long period of natural biodegradation. The problem of the disposal of bird droppings is relevant since it used a significant amount of arable land for its storage. These storages are a source of a fetid odor that spreads over extensive areas. In 2002, the Ministry of Natural Resources of the Russian Federation approved the "Federal Classification Catalog of Waste", which included chicken manure with its assignment to the third and fourth hazard classes. It introduced penalties for the disposal of third-class waste (in particular, bird droppings), which led to significant financial costs (Bamire and Amujoyegbe, 2021; Logan et al., 2021; Muhammad et al., 2020; Subedi and Dhakal, 2018; Tian et al., 2021).

A promising direction for the use of bird droppings is its processing for further use as an organic fertilizer (Ulimbashev et al., 2019; Luneva et al., 2022).

Thus, the purpose of the research is the selection of strains of microorganisms for effective biodegradation of chicken manure.

2 Materials And Methods

The research was at the Scientific and Testing Center for Toxic and Pharmacological Research and Development of Veterinary Medicines, Feed Additives and Disinfectants (Research Center for Veterinary Farmbiocenter). Part of the work was in the laboratories of the Kuban State Agrarian University named after I.T. Trubilin.

For microorganisms screening, there had studied strains with the ability to fix atmospheric nitrogen (representatives of the genus Azotobacter) and strains synthesizing proteolytic enzymes (producing complex proteases - three strains, alkaline proteases - two strains, neutral proteases - three strains). There had also studied two strains of aromatic compounds destructor (Anokhina et al., 2019). (Table 1).

There had selected cultures based on previous experience, their thermotolerance and purpose. Each of the involved groups of microorganisms solves a specific problem:

- the destruction of complex substances to easily accessible substrates;

- decomposition of organic matter with the release of ammonium nitrogen for its subsequent conversion into a form available to plants;

- suppression of the development of pathogenic microflora, conservation of nutrients in the resulting compost, etc.

Table 1: Natural and collection strains of microorganisms used in research work

Scope of the strain/synthesized product	Strain Source		
	Collection KubanSAU	All-Russian Collection of Industrial Microorganisms (RCIM)	Natural
Atmospheric Nitrogen Fixer	*Azotobacterchroococcum* B 35	*Azotobacterchroococcum* 31/8 R	*Azotobacter* sp._21
		Azotobacterchroococcum B-8739	*Azotobacter* sp._25
		Azotobacter vinelandii B-932	*Azotobacter* sp._26
			Azotobacter sp._28
			Azotobacter sp._G1
			Azotobacter sp._G2
Aromatic Destructor	*Pseudomonasputida* ATCC 12633	*Pseudomonasputida* 90 biovar A (171)	–
Protease neutral	–	*Bacillusmegaterium* BM-11	–
		Bacillussubtilis 203	
		Bacillussubtilis 103	
Protease alkaline	–	*Bacillus subtilis* 310	–
		Bacillussubtilis «Research Institute of Genetics 90»	
Proteolytic Enzymes	–	*Bacilluslicheniformis* L-34	–
		Bacillusmesentericus B-2466	
		Bacillussubtilis «Research Institute of Genetics-45»	

When selecting a strain with the best litter biodegradation properties, the study of proteolytic activity was according to GOST 20264.2-88. In the experiments, the total microbial number was determined and analyzed by seeding on meat-peptone agar (CFU/ml) and the content of ammonium nitrogen.

For choosing the best microbial nitrogen fixer from the genus Azotobacter, there had studied the content of ammonia nitrogen in the atmospheric air over experimental batches of litter, using a universal gas analyzer UG-2.

In studies of bird droppings, the number of viable fly larvae was determined according to Guidelines 3.5.2.1759-03 and helminthological contamination of bird droppings according to Guidelines 4.2.796-99. The results of sanitary and biological studies of the litter were according to GOST 31461-2012. The calculation of the hazard class of by-products of poultry farming was according to Sanitary rules 2.1.7.1386-03 and «Criteria for attributing waste to I-V hazard classes according to the negative impact on the environment».

Confirmation of the species belonging to the selected microbial cultures was carried out by conventional molecular genetic methods (method of 16S rRNA sequence sequencing).

3 Result and Discussion

Selection of cultures with enzymatic activity

The first stage of research was assessing the proteolytic activity of the selected strains of microorganisms. The research results are in Table 2.

The results of the study of enzymatic activity showed that all strains have proteolytic properties since they produced proteases to one degree or another. Of the strains that demonstrated high proteolytic activity, *Pseudomonasputida* 90 biovar A (171), *Pseudomonasputida* ATCC 12633, *Bacilluslicheniformis* L-34 and *Bacillusmesentericus* B-2466 should be distinguished.

However, the *Pseudomonasputida* 90 biovar A (171) strain demonstrated the highest proteolytic activity, which amounted to 74.6 U/g.

Table 2: Proteolytic activity of experimental strains

Producer strain	Proteolytic activity, units/g
Pseudomonasputida ATCC 12633	58.2±2.2
Pseudomonasputida 90 biovar A (171)	74.6±3.1
Bacillusmegaterium BM-11	20.2±0.8
Bacillussubtilis 203	39.4±1.7
Bacillussubtilis 103	45.7±1.9
Bacillus subtilis 310	49.3±1.9
Bacillussubtilis «Research Institute of Genetics 90»	40.4±1.6
Bacilluslicheniformis L-34	61.7±2.8
Bacillusmesentericus B-2466	55.2±2.3
Bacillussubtilis «Research Institute of Genetics -45»	43.3±2.1

The study of the molecular genetic properties of the strains was also carried out by the polymerase chain reaction method for the presence of genes encoding proteases of various groups. Express analysis determined the presence of genes similar to the sequences of seven important groups of proteolytic enzymes (serine, aspartate, threonine, glutamine, cysteine, metalloproteases and asparagine) using specific primers.

The conducted studies revealed positive signals for all analyzed cultures of microorganisms, but to varying degrees:

- *Pseudomonasputida* ATCC 12633 – serine-, aspartate-, glutamine-, cysteine- and metalloprotease-like genes;

- *Pseudomonasputida* 90 biovar A (171) – serine-, aspartate-, threonine-, glutamine-, cysteine-, metalloprotease- and asparagine-like genes;

- *Bacillusmegaterium* BM-11 – threonine- and cysteine-like genes;

- *Bacillussubtilis* 203 – serine-, aspartate-, and glutamine-like genes;

- *Bacillussubtilis* 103 – serine-, aspartate-, and glutamine-like genes;

- *Bacillussubtilis* 310 – serine-, aspartate-, glutamine- and cysteine-like genes;

- *Bacillussubtilis* «Research Institute of Genetics 90» – serine-, glutamine- and cysteine-like genes;

- *Bacilluslicheniformis* L-34 – serine-, aspartate-, threonine-, glutamine-, cysteine-, and asparagine-like genes;

- *Bacillusmesentericus* B-2466 – serine-, aspartate-, threonine-, glutamine- and cysteine-like genes;

- *Bacillussubtilis* «Research Institute of Genetics-45» – serine-, aspartate-, glutamine- and cysteine-like genes.

These results show a greater preference for using *Pseudomonasputida* 90 biovar A (171) as a decomposer strain. Because there had been discovered seven different genes encoding proteases in the structure of its total DNA.

The obtained results indicate a greater preference for using *Pseudomonasputida* 90 biovar A (171) as a decomposer strain since the genes encoding proteases of seven different groups were identified in the structure of its total DNA.

The effect of cultures of the studied microorganisms on the biodegradation of chicken manure without litter had been studied for 30 days. Every five days, as analyzed indicators, had been recording the total microbial number (TMC) and the content of ammonium nitrogen. There had used an active microbial culture of the studied strains with a cell titer of at least 10^9CFU/ml for this research. The dose of introducing strains-producers for screening was 10.0% by weight of the by-product. It presented the dependence of the bioconversion of chicken manure on the processing time and the culture of the microorganism used in Table 3.

Table 3: Dependence of chicken manure bioconversion on treatment time and microorganism culture

Producer strain	Research time, days													
	0		5		10		15		20		25		30	
	Analyzed indicator													
	TMC, cells/g	Ammon. nitrogen, mg/l	TMC, cells/g	Ammon. nitrogen, mg/l	TMC, cells/g	Ammon. nitrogen, mg/l	TMC, cells/g	Ammon. nitrogen, mg/l	TMC, cells/g	Ammon. nitrogen, mg/l	TMC, cells/g	Ammon. nitrogen, mg/l	TMC, cells/g	Ammon. nitrogen, mg/l
Pseudomonas putida ATCC 12633	10^4	340	10^5	302	10^6	264	10^9	174	10^9	169	10^9	170	10^8	172
Pseudomonas putida 90 biovar A (171)	10^4	340	10^6	281	10^8	228	10^{11}	132	10^{11}	130	10^{11}	134	10^{10}	129
Bacillus megaterium BM-11	10^4	340	10^4	334	10^4	323	10^5	307	10^5	303	10^5	304	10^5	301
Bacillus subtilis 203	10^4	340	10^4	321	10^4	304	10^5	304	10^5	296	10^5	289	10^5	294
Bacillussubtilis 103	10^4	340	10^4	326	10^4	305	10^5	305	10^5	304	10^5	298	10^5	302
Bacillus subtilis 310	10^4	340	10^4	317	10^5	297	10^6	288	10^6	283	10^6	290	10^6	289
Bacillussubtilis Research Institute of Genetics 90	10^4	340	10^4	330	10^4	320	10^5	320	10^5	314	10^5	310	10^5	312
Bacillus licheniformis L-34	10^4	340	10^5	311	10^6	295	10^6	295	10^7	273	10^6	261	10^5	254
Bacillus mesentericus B-2466	10^4	340	10^4	319	10^5	298	10^6	298	10^7	276	10^6	271	10^6	268
Bacillussubtilis Research Institute of Genetics-45	10^4	340	10^4	326	10^5	312	10^5	312	10^5	305	10^5	301	10^5	297
Litter without processing	10^4	340	10^4	341	10^4	339	10^4	338	10^4	341	10^4	338	10^4	335

It achieved the highest number of microbial cells in bird droppings using the microbial culture *Pseudomonasputida* 90 biovar A (171). At the beginning of the study, the microbial count was 10^4 CFU/ml. By the 15th day, the microbial count was 10^{11} CFU/ml, and then the microflora titer

stopped increasing in all cases. Most likely, this is because of the termination of the action of the enzyme complex of proteolytic microorganisms.

Analyzing the content of ammonium nitrogen in chicken manure, the maximum decrease in the studied indicator was on 15-20th day from the start of treatment. This data correlates with the dynamics of an increase in the total number of microorganisms. The lowest level of ammonium nitrogen had discovered by treatment of its microbial culture *Pseudomonasputida* 90 biovar A (171). This indicator decreased from 340 mg/l from the beginning of treatment to 132 and 130 mg/l at the end of the experiment.

The analyzed indicators of chicken manure untreated with microbial culture did not change significantly during the experiment.

3.1 Selection of Crops with Nitrogen-fixing Ability

Microorganisms of the genus *Azotobacter* are often used to increase soil fertility, biological remediation of soils, and enrich it with nitrogen compounds. However, in the presence of ammonia, the process of nitrogen fixation stops and the microbial culture of the genus *Azotobacter* uses free ammonia, which gives a high deodorizing effect (Aasfar et al., 2021; Din et al., 2019; Hindersah et al., 2018, 2021; Nosrati et al., 2014; Sumbul et al., 2020).

At the next stage of research, it screened bacteria of the genus Azotobacter of collection and natural strains. There was analysis of the content of ammonia nitrogen in the environment over experimental batches of chicken manure treated with experimental cultures (Figure 1).

Figure 1: Introduction of microbial culture of the genus Azotobacter into chicken manure and analysis of ammonia nitrogen using a gas analyzer UG-2

For analyzing ammonia nitrogen in the environment, had used a universal gas analyzer UG-2. Gas analyzer UG-2 measures mass concentrations of harmful substances in the air, including industrial emissions. The device comprises an air intake device and a set of indicator tubes. The principle of operation of the gas analyzer UG-2 is based on the change in color of the indicator powder layer in the indicator tube after passing the test air through it. The length of the colored column of indicator powder in the tube is proportional to it measured by the mass concentration of the harmful substance in the air and on a graduated scale in mg/m^3. The research results are in Table 4.

Table 4: Dependence of the content of ammonia nitrogen in the environment at the time of treatment for chicken manure and the microbial culture.

Microbial culture	Research time, days						
	0	5	10	15	20	25	30
	Analyzed indicator						
	Ammonia nitrogen, mg/m^3						
Azotobacter chroococcum B 35	93	74	65	57	55	52	50
Azotobacter chroococcum B-8739	93	80	71	66	62	59	60
Azotobacter chroococcum 31/8 R	93	63	38	14	12	12	11
Azotobacter vinelandii B-932	93	82	75	68	65	66	63
Azotobacter sp._21	93	90	91	90	87	83	84
Azotobacter sp._25	93	92	90	87	85	82	82
Azotobacter sp._26	93	86	89	88	85	84	83
Azotobacter sp._28	93	90	93	91	87	86	85
Azotobacter sp._G1	93	86	85	87	90	83	83
Azotobacter sp._G2	93	88	87	86	86	85	85
Litter without processing	93	93	93	92	91	92	90

On the first day of the experiment, the gas content over fresh chicken manure was 93mg/m^3, which is above the limit of permissible concentration (LPC 20mg/m^3). During the experiments, it had revealed that the isolated natural strains of the genus *Azotobacter* had an insignificant effect on the content of ammonia nitrogen in the environment over the experimental batches of chicken manure. Only one strain, *Azotobacterchroococcum* 31/8 R, demonstrated the best fixing ability of atmospheric nitrogen. There had found that on the 15th day of the experiment, the ammonia level over chicken manure treated with *Azotobacterchroococcum* 31/8 R decreased to 14mg/m^3, which is below the LPC level. On the 20th, 25th and 30th day of the study, the content of ammonia nitrogen in this group changed slightly. There were no changes in the rest of the remaining study options. However, in none of the experimental batches, the content of ammonia nitrogen was lower than the LPC value. For selecting the most suitable strain, it studied genes encoding a complex of enzymes involved in the process of nitrogen fixation. Using express analysis detected DNA of genes similar to the sequences of important enzymes - ferredoxins, hydrogenases and the nitrogenase complex (Mo-Fe-nitrogenase, V-nitrogenase and Fe-nitrogenase).

The conducted studies revealed positive signals for all analyzed cultures of microorganisms, but to varying degrees:

- *Azotobacterchroococcum* B 35 - ferredoxin-, hydrogenase-, and Mo-Fe-nitrogenase-like genes;

- *Azotobacterchroococcum* B-8739 and *Azotobactervinelandii* B-932 - ferredoxin-, hydrogenase- and Fe-nitrogenase-like genes;

- *Azotobacterchroococcum* 31/8 R - ferredoxin-, hydrogenase-, Mo-Fe-nitrogenase-, V-nitrogenase- and Fe-nitrogenase-like genes;

- *Azotobacter* sp._21, *Azotobacter* sp._25, *Azotobacter* sp._26, *Azotobacter* sp._28, *Azotobacter* sp._G1 and *Azotobacter* sp._G2 -Fe- nitrogenase-like genes.

These results showed that the most promising strains are the nitrogen-fixing culture *Azotobacterchroococcum* 31/8R. In the structure of total DNA, it identified genes encoding all the studied enzymes responsible for the process of nitrogen fixation.

Molecular genetic identification of selected microbial cultures

For confirmation of the species affiliation of the selected microbial cultures (*Pseudomonasputida*, *Azotobacterchroococcum*), conventional molecular genetic methods identified them. The DNA of bacterial strains was isolated by freezing.

PCR was performed using the standard primers 27f and 1495r for identifying the 16S rRNA gene (Table 5).

Table 5: Primers used in the work

Name	Subsequence
27f	5´-GAGAGTTTGATCCTGGCTCAG-3´
1495r	5´-CTACGGCTACCTTGTTACGA-3´

DNA amplification was performed using a Bio-RadT100 apparatus (Bio-Rad, USA).

After PCR, there had checked TAE-agarose the amplification results of the fragments. Further, these fragments had isolated from the gel and purified using the ColGen kit from Synthol.

The sequencing of the isolated PCR fragments, according to Sanger, was on an ABI Prism 3130 sequencer. The volume of the mixture was 6ml with a concentration of 50ng/ml. Here, sequencing was using forward and reverse primers to bring the two sequences together later.

The resulting DNA sequences were compared using the NCBIBLAST service, after reflecting the reverse sequences using the web version of the Reverse-Complement tool. Both sequences after that were connected. Next, the online program aligned with each other the samples Clustal Omega (Figure 2).

Figure 2 shows the sequenced of the 16S rRNA gene of the *Azotobacterchroococcum* strain and *Pseudomonasputida* strain. As expected, there had revealed differences in the structure of these sequences between the species.

After comparison with each other, the DNA sequences of the presented strains were deposited with the US National Center for Biotechnology Information (NCBI) under the numbers

Azotobacterchroococcum KA (MZ411408.1) and *Pseudomonasputida* AA (MZ411409.1).

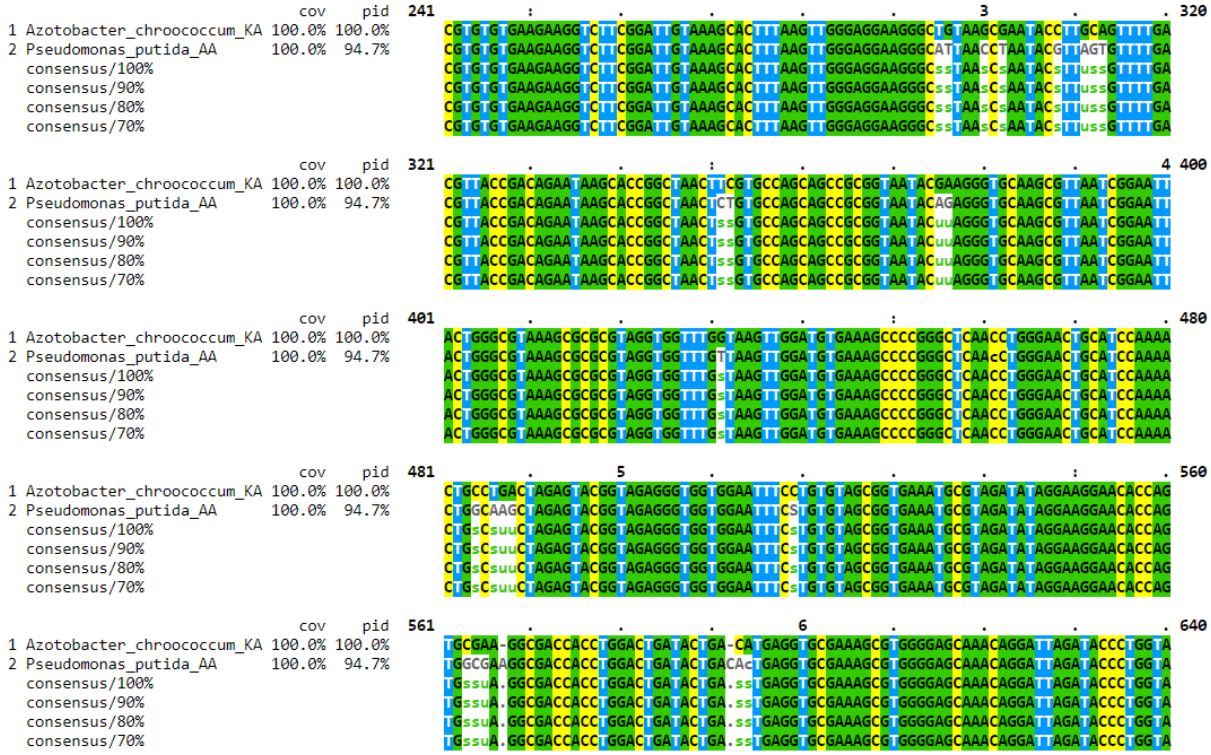

Figure 2: Alignment of DNA sequences of 16S rRNA genes of the studied strains

Thus, the conducted modern molecular genetic analysis confirmed the species affiliation of the selected strains.

4 Conclusion

The conducted studies have shown that the culture of *Pseudomonasputida* 90 biovar A (171) has a high proteolytic ability. Introducing this strain into chicken manure leads to its biodegradation. This is indicated by a decrease in ammonium nitrogen and an increase in the total microbial number, which speed up the decomposition process of poultry waste. It was also found that the best ability to fix atmospheric nitrogen is the *Azotobacterchroococcum* 31/8 R strain since its use helped to reduce the level of ammonia produced by chicken manure in the environment. Thus, the combined use of selected cultures of microorganisms can be the basis for the development of a biological product that speeds up the process of natural decomposition of bird droppings.

5 Availability of Data and Material

Data can be made available by contacting the corresponding author.

6 Acknowledgement

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