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SELECTION OF POPS DEGRADING MICROORGANISMS AND THEIR MOLECULAR GENETIC IDENTIFICATION

Currently, one of the environmentally friendly problems is the contamination of the natural environment, which is resistant to low-pollutants, possesses a high toxicity. Pesticides, including chlorine-based coefficients, are particularly suitable for environmental and human use. Toxic substances from repositories for pesticides can cause a serious threat to all living organisms. Most of the studies conducted are devoted to studying the effect of pesticides on microbial populations in the soils of agrocenoses, while the study of soil microbial complexes in pesticide burial areas has not been adequately addressed. At the same time, microorganisms isolated from ecosystems exposed to prolonged exposure to pesticides have the potential to decompose these compounds more quickly, which makes it necessary to study the microbial communities of soils contaminated with pesticides, both for assessing biological risk and for selecting promising destructor microorganisms for bioremediation technologies of natural objects.

In connection with the above purpose of this study, a screening of prospective microorganisms – POP destructors and molecular genetic identification of the selected strains was performed.

Soil samples were taken from 4 points (v. Kyzylkayrat, v. Amangeldy №1, v. Amangeldy №2, v. Brigada-2 – Almaty Plemzavod, v. Bashy (control) of the Talgar territory the area of Almaty region adjacent to the pesticide burial sites. As a result of studies, strains of microorganismsdestructors with destructive activity against persistent organic pollutants were selected. These strains can be used to create a biological product, to clean up soil contaminated with chlorine pesticides.

Key words: organochlorine pesticides, microbial diversity, screening, destructive microorganisms, identification, chemical pollutants.

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Тұрақты органикалық қосылыстарды ыдыратушы микроорганизм – деструкторларды іріктеу және молекулалық-генетикалық идентификациясы

Қазіргі таңда өзекті экологиялық проблемалардың бірі табиғи объектілердің токсинділігі жоғары тұрақты органикалық қосылыстармен ластануы болып табылады. Пестицидтердің ішінде, хлорорганикалық қосылыстар қоршаған орта мен адам үшін аса қауіптілігімен ерекшеленеді. Пестицидтерге арналған қоймалардың токсинді заттары барлық тірі организмдер үшін үлкен қауіп төндіруі мүмкін. Жүргізілген зерттеу жұмыстарының көп бөлігі агроценоз топырағындағы пестицидтердің микробтық алуантүрлілігін зерттеуге арналған, ал пестицидтер көмілген жерлерде топырақ микробиоценозын зерттеу тиісті деңгейде қарастырылмаған. Сонымен қатар ұзақ уақыт пестицидтер әсеріне ұшыраған экожүйелерден оқшауланған микроорганизмдер пестицидтерді ыдырату қабілетіне ие болғандықтан, пестицидтермен ластанған топырақтың микробтық алуантүрлілігін зерттеуді қажет етеді, биологиялық қауіпті бағалау және перспективті деструктор – микроорганизмдерді таңдау табиғи объектілерді биоремедиациялау технологияларын ұйымдастыруға мүмкіндік береді.

Жоғарыда айтылған мәселені шешуге байланысты зерттеудің мақсаты іріктеліп алынған перспективті деструктор-микроорганизмдерінің скринингін жүргізу және молекулалық-генетикалық идентификациясы.

Топырақ үлгілерінің сынамалары 4 аймақтан (Қызылқайрат, Амангелді №1, Амангелді №2, Бригада-2 – «Алматы» Племзавод, Басшы аумақтарынан (бақылау) алынды. Зерттеулер нәтижелеріне сәйкес тұрақты органикалық ластағыштарға төзімді деструктивті белсенділігі жоғары, деструктор-микроорганизм штаммдары іріктеліп алынды. Бұл штамдар хлорорганикалық пестицидтермен ластанған топырақты тазалау процесінде биопрепарат жасауда қолданылуы мүмкін.

Түйін сөздер: хлорорганикалық пестицидтер, микробтық алуантүрлілік, скрининг, деструктор- микроорганизмдер, идентификация, химиялық ластағыштар.

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Отбор микроорганизмов-деструкторов стойких органических загрязнителей и их молекулярно-генетическая идентификация

В настоящее время одной из экологических проблем является загрязнение природных экосистем стойкими органическими загрязнителями, обладающими высокой токсичностью. Пестициды, в том числе хлоорганические соединения, представляют особую опасность для окружающей среды и человека. Токсичные вещества из хранилищ для пестицидов могут вызвать серьезную угрозу для всех живых организмов. Большинство проводимых исследований посвящено изучению влияния пестицидов на популяции микроорганизмов в почвах агроценозов, тогда как вопросы изучения почвенных микробных комплексов в районах захоронения пестицидов освещены недостаточно. В то же время микроорганизмы, выделенные из экосистем, подвергающихся длительному воздействию пестицидов, обладают потенциалом к более быстрому разложению данных соединений, что делает необходимым изучение микробных сообществ почв, загрязненных пестицидами, как для оценки биологического риска, так и для отбора перспективных деструкторов – микроорганизмов для технологии биоремедиации природных объектов.

В связи с вышесказанным целью данного исследования был скрининг перспективных микроорганизмов – деструкторов СОЗ и проведение молекулярно-генетической идентификации отобранных штаммов.

Пробы почвенных образцов отбирали из 4 точек (п. Кызылкайрат, п. Амангельды №1, п. Амангельды №2, п. Бригада-2 – АО Племзавод «Алматы», п. Басшы (контроль) территории Талгарского района Алматинской области, прилегающей к местам захоронения пестицидов. В результате исследований были отобраны штаммы микроорганизмы-деструкторы, обладающие деструктивной активностью в отношении стойких органических загрязнителей. Эти штаммы могут быть использованы для создания биопрепарата для очистки почвы, загрязненной хлорорганическими пестицидами.

Ключевые слова: хлорорганические пестициды, микробное разнообразие, скрининг, микроорганизмы-деструкторы, идентификация, химические загрязнители.

Introduction

Currently, the Republic of Kazakhstan is facing an acute environmental problem related to the consequences of the long-term use of pesticides in agriculture – chemicals for controlling pests of agricultural plants.

As you know, at present their use is practically prohibited, but due to their high toxicity with respect to biological objects of soils and water bodies, another problem arose, the leveling of which is associated with the need to create new environmentally hazardous facilities – storages (warehouses) for huge quantities of unused pesticides – substances highly dangerous to all living organisms [1]. The construction of such storage facilities was dictated by the fact that in recent years the use of these drugs, due to a noticeable decrease in demand for them, has decreased markedly. The reason for this strategy was the following factors: low efficiency and negative impact on environmental objects [2].

However, given the possibility of the successful development of new tools to mitigate the negative effects of pesticides on organisms that inhabit objects of the environment – soil, water and air, their intended use in the future is not excluded, but, on the contrary, may become necessary [3].

Based on the foregoing, in some regions special storage facilities have been built for pesticides available in warehouses. In particular,

they are available in the Talgar district of the Almaty region.

Although, as is known, all the studies carried out earlier in this direction were devoted to studying the target activity of pesticides in the process of their application in agriculture, at present, the problems associated with the influence of pesticide storages on the ecology. The environment on the physiological activity of microorganisms also require attention and other living objects of water and soil, which in such studies can serve as markers of changes, which are based on the presence of pesticides and their decomposition products in environmental objects yes [4].

It is important that their metabolic activity can assess the intensity and nature of the effects of toxic substances, for example pesticides, on metabolic processes in the cells of soil microorganisms directly, i.e. metabolic potential of soil microbial communities in pesticide burial areas [5].

Such studies, on the one hand, will provide reliable information not only about the impact of pesticide storages on the environmental situation of the studied region, but also have prognostic value, because allow us to judge the possible changes in the microbial potential in environmental objects in the presence of significant amounts of pesticides in storage [6]. It is equally important that the study of microbial communities of soils contaminated with pesticides is necessary both for assessing biological risk and for selecting promising agents for remediation activities in contaminated areas [7].

Materials and research methods

The study of the study of microbial specimens of soil samples of the Almaty region, which is in contact with places of contamination of pesticides is carried out. Soil samples were taken from 4 points (v. Kyzylkayrat, v. Amangeldy №1, v. Amangeldy №2, v. Brigada-2 – Almaty Plemzavod, v. Bashy (control) of the Talgar territory the area of Almaty region adjacent to the pesticide burial sites.

Methods of screening POPs degrading microorganisms of chemical contaminants and methods for the determination of destructive activity of selected positive stamps.

To search for destructors, we used strains from dominant populations of bacteria. For this, all isolated strains were seeded on Petri dishes with M9 agar medium supplemented with a pesticide as a carbon source of 0.01%, 2,3,5 – triphenyl tetrazolium chloride (TTC) as an indicator of bacterial dehydrogenase activity [8]. The ability of microorganisms to disinfect pesticides shows the color of them from the medium in red, which indicates the appearance of triphenyl form. For these features of microorganisms, strain-destructors were selected for further research [9].

Molecular – genetic identification of distinguished POPs degrading microorganisms.

Strain identification was performed by determining the nucleotide sequence of the 16s rRNA fragment of the gene, followed by determining the nucleotide identity with sequences deposited in the international Gene Bank database. Phylogenetic trees with nucleotide sequences of reference strains were constructed [10].

Results and Discussion

Screening of POPs degrading microorganisms and their degradation products

One of the urgent tasks of modern biotechnology is the creation of biological products based on strains of destructors isolated from indigenous microflora to solve a complex of tasks related to the rehabilitation of soils contaminated with xenobiotics [11]. Soils are exposed to especially severe destructive effects due to the intensive use of pesticides in violation of the norms and rules of their use, which leads to their significant accumulation in soils [12]. Of particular danger are landfills for unused or prohibited chemicals. Natural processes of soil self-purification are not able to cope with such volumes of pollution [13,14].

It is known that soil fertility and self-cleaning directly depend on the activity of microbiological processes, however, as a result of high soil intoxication, autochthonous microflora is inhibited [15]. Therefore, the development of integrated technologies aimed at restoring the basic functions of soils and increasing their fertility is of considerable scientific interest, both for theoretical and applied microbiology. Currently, methods of biological remediation are considered as priorities for solving the problems of cleaning contaminated soils [16, 17].

Biodegradation is considered the most promising area in the technologies for the rehabilitation of soil systems infected with organic pollutants, including pesticides.

In connection with the above, the goal of further research was to screen effective destructive microorganisms, study the destructive potential of cultures and select the most promising strains. The search for destructors was carried out among cultures from the dominant populations of microorganisms

[18]. Destruction strains in the total soil microbiota were indicated on solid M9 medium with the addition of DDT pesticide as a carbon source of 0.01%, 2,3,5 – triphenyl tetrazolium chloride (TTC) as an indicator of bacterial dehydrogenase activity [19]. Destructive activity of cultures was evaluated by the activity of growth and preservation of cell viability in the presence of organochlorine compounds. Screening of active microorganisms-destructors of POPs and their decay products was carried out in all 40 strains of pure cultures isolated from soil and water adjacent to the burial sites of pesticides. According to screening studies, 10 strains did not show growth in a medium supplemented with DDT as the sole carbon source. The lack of growth of cultures indicates that the strains do not have destructive activity. 20 strains showed the least activity against pesticide. The strains *K2, K3, AK3, AK4, AK 5, AC1, BR1, BR3, BR7* isolated from the burial site of pesticides, have a high destructive activity against DDT.

There is data in the literature that shows that the use of a wide range of pesticides suggests different mechanisms of action of these substances on prokaryotic and eukaryotic cells of microorganisms, on heterotrophic microorganisms, and the spectrum of these mechanisms is very wide [20]. Derivatives of carbamates are known to affect cell division; organic copper compounds and dithiocarbamates – for membrane permeability and oxidative phosphorylation, electron transfer in the respiratory chain; organic mercury compounds react with cellular components, reacting with carboxylic, sulfhydryl, amino groups, metal ions [21]. Therefore, strains of isolated pure cultures showed different growth activities in the medium with the organochlorine preparation DDT.

As a result of screening among 20 destructively active strains, we selected 4 promising cultures of microorganisms that can actively grow on a medium with the organochlorine compound DDT for further work. The results are presented in figures 1, 2.

Figure 1 shows the growth dynamics of cultures of promising strains in the medium with the addition of DDT as the sole carbon source.

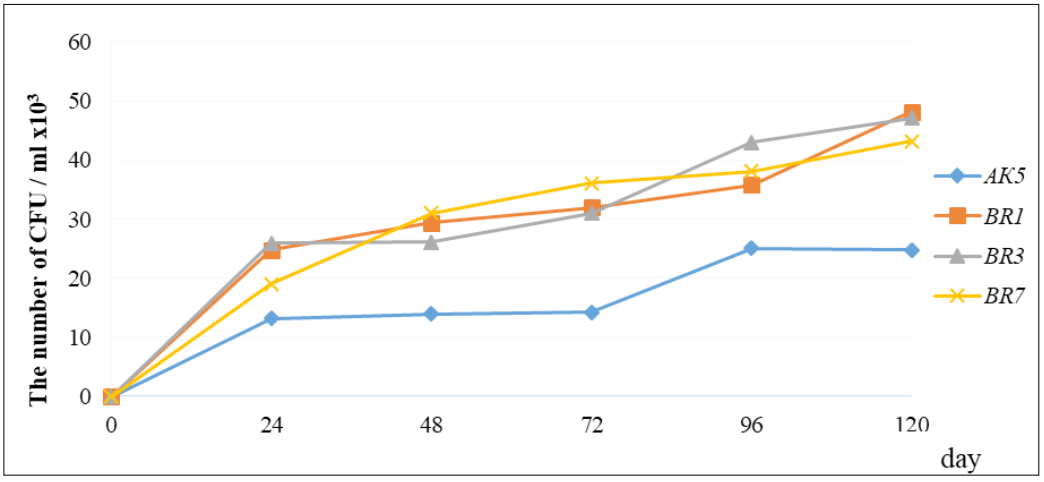

Figure 1 – The growth dynamics of crops in the environment with the addition of DDT as the sole carbon source

Among the studied strains, the highest growth activity was shown by strains *AK5, BR1, BR3, BR7.* The growth of strain *BR7* on the first day of cultivation showed active growth, the number of cells in the medium was $2.9x10^4$ CFU / g. The abundance of all cultures at the end of the experiment was within 2.4×10^4–4.8×10^4 CFU / g (Fig. 1). Active growth of cultures in a medium with the addition of DDT show that the strains use organochlorine compounds as the sole carbon source.

Figure 2 shows the growth of cultures of microorganisms – destructors on the environment with the addition of DDT.

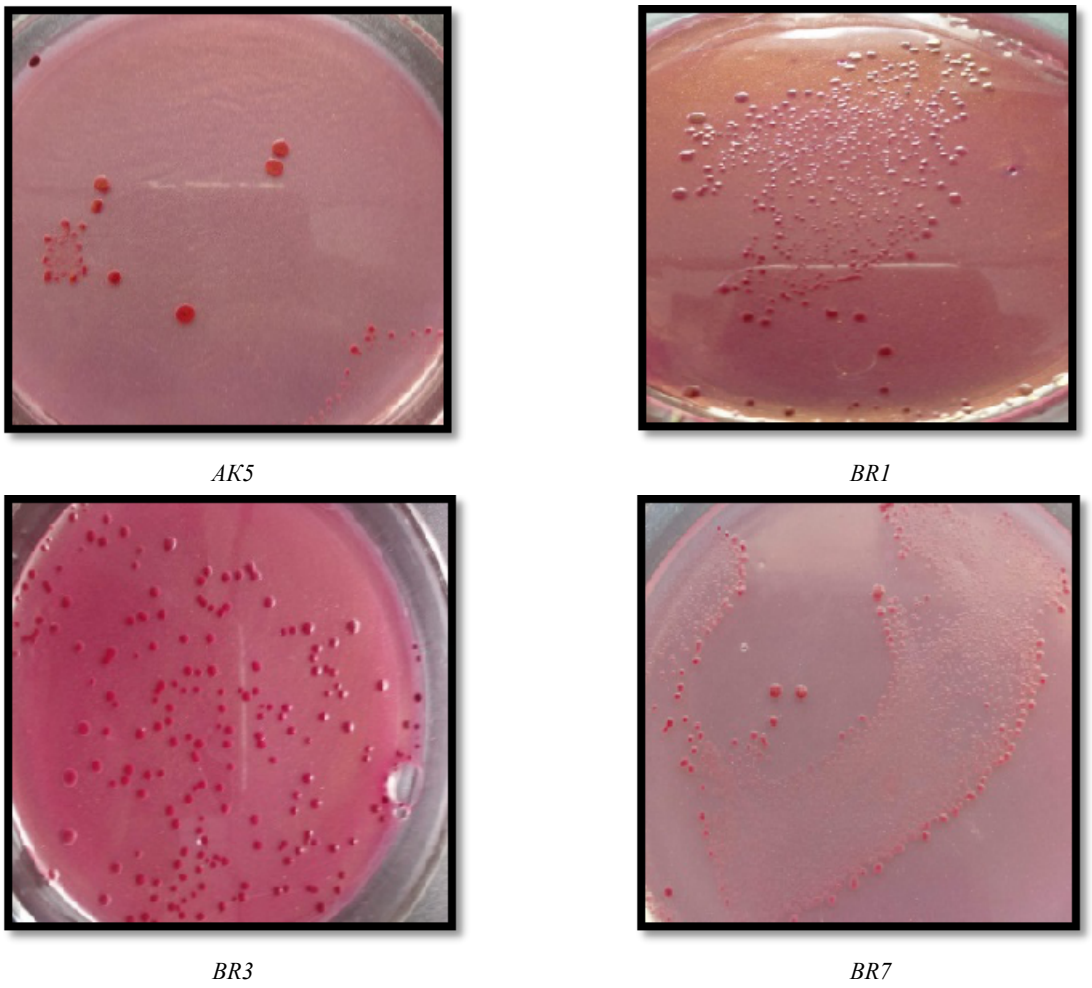

Figure 2 – Macromorphology of cultures of microorganisms – destructors on the environment with the addition of DDT

I would like to note that on the dense M9 medium all the colonies were only smooth, shiny S – red. Cultures on M9 medium in addition to DDT, as the only carbon source, change the initial white – matte color to red, this is due to the formation of reduced triphenylformazan (TPP) in the medium, which indicates the dehydrogenase activity of the microorganism. The scientific literature provides numerous examples of the transformation of various pesticides under the influence of microorganisms in certain conditions and certain soils [22]. So, for example, organochlorine preparations (DDT) under the influence of microflora undergo deep decomposition with the splitting of aromatic rings.

As a result of studying the destructive activity of promising strains of chemical pollutants, it was found that strains *AK5, BR1, BR3, BR7* are capable of destroying pesticides, this was indicated by the staining of the colonies and the environment around them in red, indicating the formation of reduced triphenylformazan (TFP). Since, under aerobic conditions, the first stage of xenobiotic biodegradation is oxidative metabolism reactions catalyzed by various oxidoreductases, the main of which are dehydrogenases, the identification of these enzymes in microorganisms indicates the destructive potential of the culture [23].

In future work, it was planned to conduct an analysis on the molecular genetic identification of selected strains of destructors *AK5, BR1, BR3, BR7.*

Molecular genetic identification of isolated strains of POPs degrading microorganisms.

AK5, BR1, BR3, BR7 strains can be recommended for the creation of a comprehensive product designed to clean land contaminated with organochlorine pesticides.

Selected cultures were identified to species. Molecular genetic identification of microorganisms was carried out by Sanger sequencing [26]. A fragment of the 16S rRNA gene, about 700 bp in size, was amplified by PCR. The nucleotide sequences and identification results are presented in table 1.

Table 1 – Results of identification by analysis of the nucleotide sequence of the 16S rRNA gene

	The name of the strain	The sequence of the 16S r RNA gene fragment	Identification of nucleotide sequences in the international database (http://www.ncbi.nlm.nih.gov/) BLAST algorithm		
			GenBank Inventory Number (Accession number)	The name of the strain	% coincide
№	1	2	3	4	5
1	AK5	GTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACG CATTTCACCGCTACACGTGGAATTCCACTCTCCTCTT CTGCACTCAAGTTCCCCAGTTTCCAATGACCCTCCCC GGTTGAGCCGGGGGCTTTCACATCAGACTTAAGGAA CCGCCTGCGAGCCCTTTACGCCCAATAATTCCGGAC AACGCTTGCCACCTACGTATTACCGCGGCTGCTGGC ACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTC AAGGTACCGCCCTATTCGAACGGTACTTGTTCTTCCC TAACAACAGAGCTTTACGATCCGAAAACCTTCATCA CTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTG CGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCT GGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCT CTCAGGTCGGCTACGCATCGTTGCCTTGGTGAGCCG TTACCTCACCAACTAGCTAATGCGCCGCGGGTCCAT CTGTAAGTGGTAGCCGAAGCCACCTTTTATGTTTGA ACCATGCGGTTCAAACAAGCATCCGGTATTAGCCCC GGTTTCCCGGAGTTATCCCAGTCTTACAGGCAGGTT ACCCACGTGTTACTCACCCGTCCGCCGCTAACATCAG GGAGCAAGCTCCCATCTGTCCGCTCGACTTGCATGT ATTAGGCACGCCGCC	*BGSC 3A28*	*Bacillus subtillis*	*99%*
2	BR1	AGCTTGCTCTCTGGGTGACGAGCGGCGGACGGGTGA GTAATGTCTGGGAAACTGCCTGATGGAGGGGGATA ACTACTGGAAACGGTAGCTAATACCGCATAACGTCT TCGGACCAAAGTGGGGGACCTTCGGGCCTCACGCCA TCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGG GTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTC TGAGAGGATGACCAGCCACACTGGAACTGAGACACG GTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT TGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGC GTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTT CAGCGAGGAGGAAGGGTAGTGTGTTAATAGCACATT GCATTGACGTTACTCGCAGAAGAAGCACCGGCTAAC TCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAA GCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCA GGCGGTTTGTTAAGTCAGATGTGATATCCCCGCGCT TAACGTGGGAACTGCATTTGATACTGGCAAGCTAGA GTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCG GTGAAATGCGTAGAGATCTGGAGGAATACCGGTGG CGAAGGCGGCTCCCCTG	*NR 114575.1*	*Serratia quinivorans*	*99%*

Continuation of table 1

	The name of the strain	The sequence of the 16S r RNA gene fragment	Identification of nucleotide sequences in the international database (http://www.ncbi.nlm.nih.gov/) BLAST algorithm		
			GenBank Inventory Number (Accession number)	The name of the strain	% coincide
№	1	2	3	4	5
3	BR3	GGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTC TACGCATTTCACCGCTACACCTGGAATTCTACCCCCC TCTACAAGACTCTAGCCTGCCAGTTTCGAATGCAGTT CCCAGGTTGAGCCCGGGGATTTCACATCCGACTTGAC AGACCGCCTGCGTGCGCTTTACGCCCAGTAATTCCGA TTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGG CACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTC AATTGCTGCGGTTATTAACCACAACACCTTCCTCCCC GCTGAAAGTACTTTACAACCCGAAGGCCTTCTTCATA CACGCGGCATGGCTGCATCAGGCTTGCGCCCATTGTG CAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGA CCGTGTCTCAGTTCCAGTGTGGCTGGTCATCCTCTCA GACCAGCTAGGGATCGTCGCCTAGGTGAGCCGTTAC CCCACCTACTAGCTAATCCCATCTGGGCACATCTGAT GGCAAGAGGCCCGAAGGTCCCCCTCTTTGGTCTTGC GACGTTATGCGGTATTAGCTACCGTTTCCAGTAGTTA TCCCCCTCCATCAGGCAGTTTCCCAGACATTACTCACC CGTCCGCCACTCGTCACCCGAGAGCAAGCTCTCTGTG CTACCGTTCGACTTGCATGTGTTAGGCCTGCCGCC	*NR 118011.1*	*Enterobacter cloacae subsp. dissolvens*	*100%*
4	BR7	TCTACGCATTTCACCGCTACACCTGGAATTCCACCAT CCTCTACCGCACTCTAGCTTGCCAGTATCGAATGCA ATTCCCAGGTTGAGCCCGGGGATTTCACATCTGACTT AACAAGCCGCCTACGCGCGCTTTACGCCCAGTAAAT CCGATTAACGCTTGCACCCTCTGTATTACCGCGGCTG CTGGCACAGAGTTAGCCGGTGCTTATTCTGCCAGTA ACGTC	*NR 025254.1*	*Alkanindiges illinoisensis*	*95%*

Taking into account the literature data [24], indicating the presence of nucleotide sequences in international banks GeneBank (http://www.ncbi.nlm.nih.gov/), Ribosomal Database Project (RDP-II) (http: // rdp. cme.msu.edu/html/), errors, in addition, phylogenetic trees with 16S rRNA nucleotide sequences of the gene of reference strains of these species were constructed (http://www.bacterio.net).

The analysis included nucleotide sequences of the 16S rRNA gene, the most phylogenetically related microorganisms [25].

To construct a phylogenetic tree for strain *AK5*, the nucleotide sequences of 16S rRNA reference strains included in the *Bacillus subtillis* group were used [27].

Figure 3 shows the phylogenetic analysis of genetically related species of *Bacillus mojavensis* and *Bacillus subtillis.*

As can be seen from figure 4, strain *BR1* is located on the same branch as *Serratia plymuthica* and *Serratia quinivorans*, given the high identity of 16S rRNA in these species.

As can be seen from figure 5, strain *BR3* is located on the same branch as *Enterobacter ludwigi* and *Enterobacter cloacae subsp*. dissolvens given the high identity of 16S rRNA in these species.

Figure 6 shows the phylogenetic analysis of genetically related species *Alkanindiges hongkongens* and *Alkanindiges illinoisensis.*

As a result of molecular genetic identification of the selected cultures of microorganisms, strain *AK5* was assigned to the species *Bacillus subtillis*, *BR1 – Serratia quinivorans, BR3 – Enterobacter cloacae subsp. dissolvens, BR7 – Alkanindiges illinoisensis.*

Thus, the studies indicate the need for continuous monitoring of the state of microbial diversity in the area adjacent to the burial sites of pesticides, as well as studying the possibility of using microorganisms as indicators of environmental pollution. The search and identification of promising cultures of microorganisms among bacteria, micromycetes, capable of actively degrading persistent organic pollutants, is the basis in the development of bioremediation measures to clean the soil of residual pesticides.

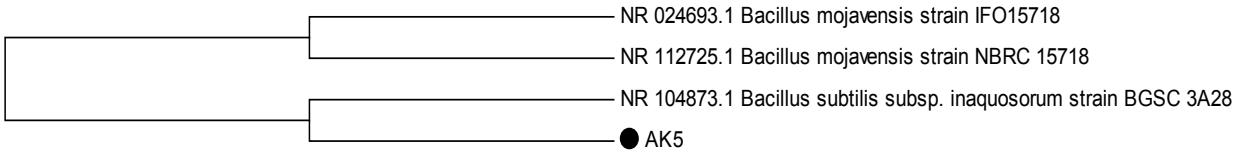

Figure 3 – Phylogenetic tree, built on the basis of analysis of the 16S rRNA gene fragment of *Bacillus subtillis*

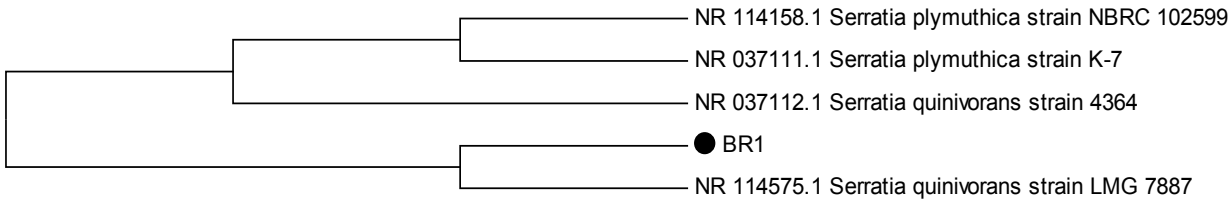

Figure 4 – Phylogenetic tree, built on the basis of analysis of the 16S rRNA gene fragment of *Serratia quinivorans*

Figure 5 – Phylogenetic tree, built on the basis of analysis of the 16S rRNA gene fragment of *Enterobacter cloacae subsp. dissolvens*

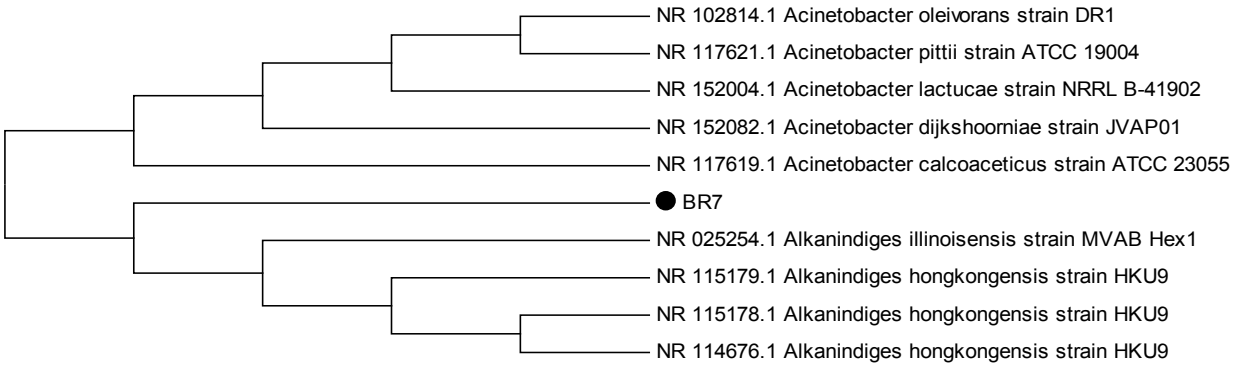

Figure 6 – Phylogenetic tree, built on the basis of analysis of the 16S rRNA gene fragment of *Alkanindiges illinoisensis*

Conclusion

As a result of screening, 4 promising strains of microorganism cultures were selected that can actively grow on a medium with the organochlorine compound DDT. The number of cells of the studied strains in a day amounted to $1.7x10^3$ -$8.6x10^3$ CFU / g, on the fifth day it was in the range of $2.1x10^4$-$1.2 * 10^4$ CFU / g.

Among the studied strains, the highest growth activity was shown by strains AK5, BR1, BR3, BR7. The growth of strain BR7 on the first day of cultivation showed active growth, the number of cells in the medium was $2.9x10^4$ CFU / g. The number of all cultures at the end of the experiment was in the range of 2.4×10^4–4.8×10^4 CFU / g. Active growth of cultures with the environment with the addition of DDT show that the strains use organochlorine compounds as the sole carbon source.

As a result of molecular genetic identification of the selected cultures of microorganisms, strain *AK5* was assigned to the species *Bacillus subtillis*, *BR1 – Serratia quinivorans, BR3 – Enterobacter cloacae subsp. dissolvens, BR7 – Alkanindiges illinoisensis.*

The search and identification of promising cultures of microorganisms among bacteria, micromycetes, capable of actively degrading persistent organic pollutants, is the basis in the development of bioremediation measures to clean the soil of residual pesticides.

Conflict of interest. All authors have read and are familiar with the content of the article and do not have a conflict of interest.

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