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ИЗВЕСТИЯ

НАЦИОНАЛЬНОЙ АКАДЕМИИ НАУК
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каб. 310, тел. 291-62-80, факс 291-57-22, e-mail:orgcat@nursat.kz
Адрес типографии: ИП «Аруна», г. Алматы, ул. Муратбаева, 75

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A. Z. Mindubaev[1], E. V. Babynin[2], A. D. Voloshina[1], K.A. Saparmyradov[2], Y. A. Akosah[2], E. K. Badeeva[1], S. T. Minzanova[1], L. G. Mironova[1]

[1]State Budgetary-Funded Institution of Science A.E. Arbuzov Institute
of Organic and Physical Chemistry of Kazan Scientific Center of Russian Academy of Sciences;
[2]Kazan (Volga region) Federal University, Kazan

THE POSSIBILITY OF NEUTRALIZING WHITE PHOSPHORUS USING MICROBIAL CULTURES

Abstract. The growth of microorganisms of various taxonomic groups (fungi, streptomyces and bacteria) was observed for the first time in culture media containing white phosphorus P_4 as the source of phosphorus. This is the first known example of the incorporation of white phosphorus into the biospheric circulation of the phosphorus element. The highest concentration applied in this study exceeds the Threshold Limit Value (TLV) of white phosphorus in wastewater by 5000 times, and in drinking water by up to 100,000,000 times! The growth selection of cultures resistant to P_4 was carried out for the first time. We identified the microorganisms, growing on white phosphorus, as new strains of *Aspergillus niger* and *Streptomyces sampsonii.* The number *A. niger* AM1 and *S. sampsonii* A8 were assigned to them, respectively. Strains of the *A. niger* isolates were shown to better adapt to P_4 than the bacteria.

Keywords: biodegradation, white phosphorus, environmental protection, chemical pollution.

Introduction

White phosphorus P4 is widely used in industry and is a key compound in the production of drugs, phosphate fertilizers, polymers and a number of other important substances and materials. According to the review article [1], Russia's portion amidst global consumption of white phosphorus in 2004 was estimated to be 5.7%, Kazakhstan - 8.1%, China - 71.1%, USA - 8.6%, Western Europe - 5.8% and India - 0.7%. Therefore, the penetration of white phosphorus into the environment is undoubtedly possible. Meanwhile, white phosphorus is one of the most dangerous environmental pollutants [2]. Protocol III of the 1980 Convention on Certain Conventional Weapons officially prohibits the use of P_4 for military purposes. However, the ban is constantly violated, which leads to human casualties and severe environmental pollution. Effective methods of purifying the envirinment from this pollutant have not yet been developed [2]. The only approach to P_4 detoxification currently involves its oxidation into phosphoric acid with the aid of copper sulfate solution; however, the implementation of this method is limited due to its high cost and toxicity. Biodegradation is considered to be one of the most practically significant and frequently used methods of industrial waste disposal [3, 4].

At the same time, phosphorus posseses a unique quality - being in the form of a simple substance, it remains a very strong poison, while in its oxidized state, it acts as a biogenic element that is absolutely necessary for all living organisms (Fig. 1, above). Thus, the primary task lies in finding an efficient and environmentally friendly approach to oxidize white phosphorus into phosphate. We propose to use microorganisms for this purpose.

The lower picture of Figure 1, illustrates the coassimilation of several toxic substances in a single metabolic pathway, demonstrating the perfect nature of the biochemistry of microorganisms, depicted on the basis of literature sources [6-8]. The inclusion of two toxic xenobiotics (formaldehyde and hydrocyanic acid) into the composition of sugars and amino acids is perhaps the most significant example of biodegradation. This is a substantial fundamental argument in favor of the possibility of biodegradation of even such dangerous xenobiotics as white phosphorus.

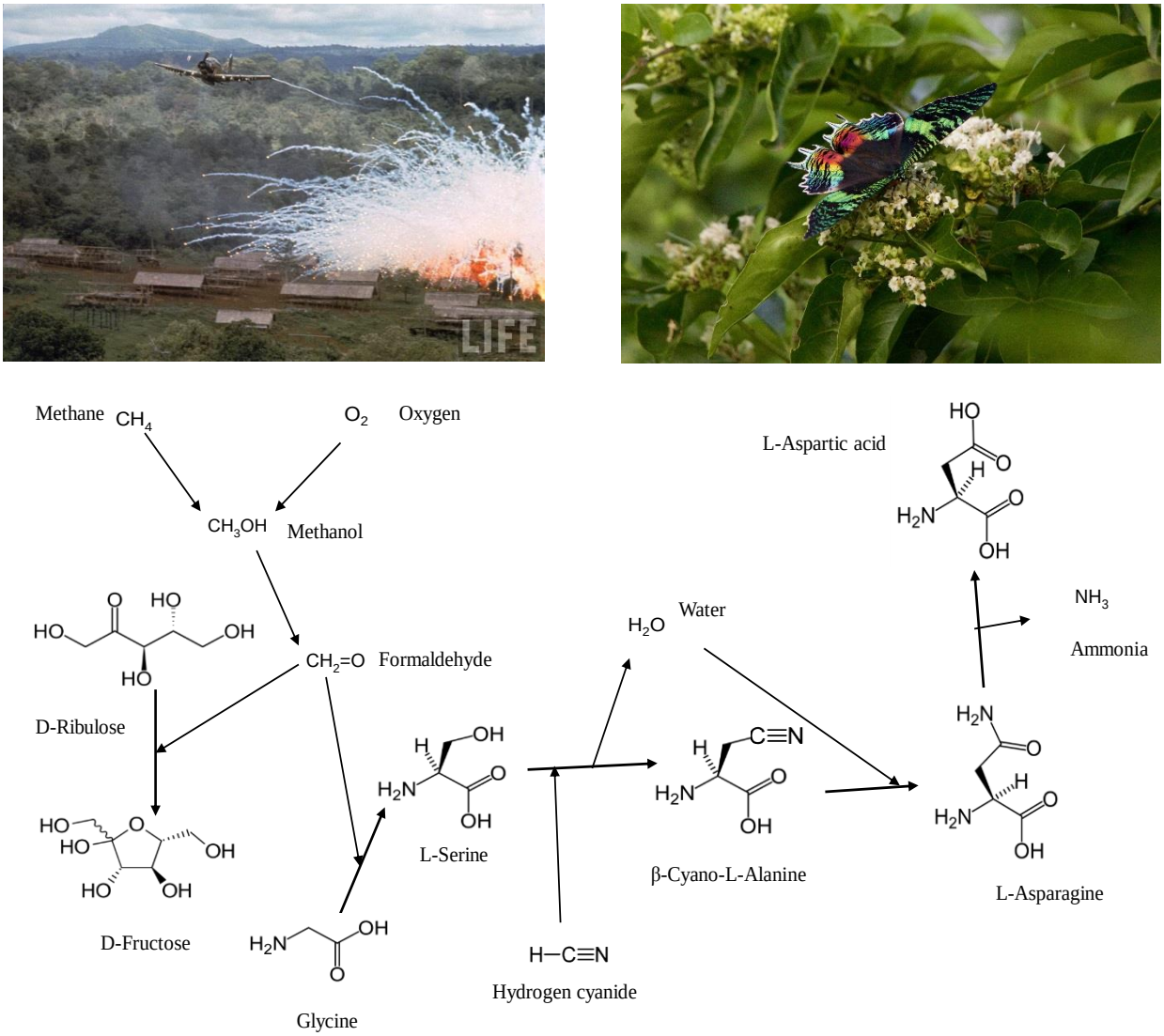

Figure 1 - Phosphorus in its diverse forms. Top left: Explosion of a phosphorus bomb during the Vietnam War (image from https://www.pinterest.ru). Top right: the final oxidized form of phosphorus, phosphate is a food source for plants and plays a crucial role in the existence of all forms of life. Image from https://elementy.ru. Below. The inclusion of formaldehyde and cyanide into the structure of sugars and amino acids is a strong example of biodegradation. Synthesis of methanol from methane (which in itself is a product of microbial metabolism [5]), is carried out by methanotrophic bacteria; serine and fructose from methanol - by some methylotrophic bacteria and yeast; β-cyano-L-alanine from hydrocyanic acid and serine - by the bacteria *Chromobacterium violaceum*; asparagine from cyanoalanine - by a number of plants. A figure of A.Z. Mindubaev

This publication is a continuation of the series of work done by of our team [9, 10], which showed that microorganisms survive upon being in contact with white phosphorus, adapt to its presence in the environment and process it into less dangerous compounds. The purpose of this work is to compare the resistance and ability of microorganisms of different strains and taxonomic groups to the biodegradation of white phosphorus. And also to identify the minimum inhibitory concentration (MIC) of this substance in each case.

Experimental part

For the first time, we have successfully observed growth of a stable microbiota, cultured in an artificial nutrient medium containing white phosphorus as the sole source of phosphorus in concentrations ranging from 0.01 to 1. Culturing was carried out in a modified Pridhem-Gottlieb medium. The classical Pridham-Gottlieb medium does not contain monosaccharides as carbon sources, and petroleum products act as such. Our modification includes glucose as a carbon source. 1 L of the modified medium contained:

glucose – 5 g, $(NH_4)_2SO_4$ – 2,64 g, $MgSO_4$ – 0,49 g, $CuSO_4 \cdot 5H_2O$ – 0,1 g, $FeSO_4 \cdot 7H_2O$ – 0,02 g, $MnCl_2 \cdot 4H_2O$ – 0,15 g, $ZnSO_4 \cdot 7H_2O$ – 0,27 g, and white phosphorus in different concentrations. 7.4 g of $K_2HPO_4 \cdot 3H_2O$ and 2.38 g of KH_2PO_4 were added to the control medium as a source of phosphorus. Semi-fluid modification of the medium was achieved after adding 4.8 g of agar. White phosphorus was emulsified in sterile distilled water. The concentration of white phosphorus prior to inoculation was calculated as follows. An emulsion was prepared from 1 g of white phosphorus in 50 ml of water to achieve a 2% solution. After cultivation, the concentrations of P_4 in the media were not measured. However, the growth of the microorganism itself indicates its decline, since phosphate is essential for growth, and in this case, it could only be formed from white phosphorus.

Aspergillus niger AM1, the spores of which were introduced together with white phosphorus, was initially cultured in a medium with white phosphorus concentrations (w/v) of 0.01 and 0.05%. Phosphate was added to the positive control. No sources of phosphorus were added to the negative control. After 60 days, the fungi were reinoculated into media with concentrations of white phosphorus 0.05; 0.1 and 0.2%. Following another 60 days, the strains were subcultured to higher concentrations of P_4 0.5; and 1%. The cultivation of *Streptomyces sp*. A8 isolated from sewage sludge containing white phosphorus was carried out in a similar manner. The concentration (w/v) of white phosphorus 0.05; 0.1 and 0.2% was used for the culture of fungi and streptomycetes, but not for bacteria, since the latter do not tolerate high concentrations of this substance.

Inoculation of *A. niger* AM1 and *S. sp*. A8 was carried out using spores, while bacteria were cultivated in the form of vegetative cells. Spore suspension contained 10^8 fungal bodies per ml; 0.2 ml was added per 20 ml of medium. Cultures were grown in flasks with 20 ml of culture medium without aeration, as well as Petri dishes. Incubation was carried out in a thermostat, at 25°C.

For genetic analysis, DNA samples from the culture of the fungus A. niger AM1 and streptomycete S. sp. A8 were isolated according to the method described in [11]. Next, the polymerase chain reaction (PCR) of the obtained DNA fragments was carried out using primers to the conservative sites of the 5.8S rDNA gene ITS1 (AAATTTAGGGGAATT) and ITS2 (GGGTTGGTTGGCCCGT).

To compare the resistance of black Aspergilus strains to white phosphorus, our strain Aspergillus niger AM1 was used, as well as three strains from the All-Russian collection of microorganisms in the Skryabin Institute of Biochemistry and Physiology of Microorganisms (IBPM RAS) (Table 1).

Table 1 - *Aspergillus niger* strains from the All-Russian collection of microorganisms (ARCM), with which the study was conducted

Species	Strain	Substrate of isolation	Place of isolation
Aspergillus niger	BKM FW-650	Permafrost deposits, 170 years old, Depth: 20.50-20.55 m deep	Taglu, Canada
Aspergillus niger	BKM FW-2664	Frozen volcanic ash, Depth:1.8-1.85 m	Kamchatka Peninsula, Russia
Aspergillus niger	BKM FW-2731	Permafrost, volcanic ash, Depth: 14.5 m	Kamchatka Peninsula, Russia

The cultures were grown in 96-well Corning microplates, the growth rate was estimated by measuring the optical density (OD) at λ 550 nm using a microplate reader Infinite F200 Pro, Tecan (Austria). This was done to conduct parallel cultivation of different strains, while comparing their growth rate in media with different concentrations of white phosphorus. In our experiments, the maximum concentration of white phosphorus in the wells of the plates reached 1%.

For comparison, cultures of bacteria *Achromobacter xylosoxidans*, *Pseudomonas aeruginosa*, *Bacillus firmus* and *Salmonella typhimurium* were sown. The purpose of these studies was to detect the minimum inhibitory concentration (MIC) of white phosphorus for these microorganisms.

To establish the nature of Aspergillus' resistance to P_4, reseeding was carried out in a medium with phosphate as a source of phosphorus, without P_4. The culture grown in this medium was re-inoculated into a medium with 0.2% white phosphorus. As a control, A. niger AM1, which had previously grown in an environment with white phosphorus, was also cultured.

An Avance 400 (Bruker) high resolution NMR spectrometer was used to monitor the P_4 processing. Sampling was done using insulin syringes. The test medium was purified from fungal hyphae and mycelia using a Millex®-HV filter (Syringe-driven Filter Unit) with pore diameter is 0.45 microns. Parameters for spectrum analysis: Bruker Avance III 400 MHz 31P {1H} - (161.9 MHz, 25 ºC).

Since white phosphorus actively reacts with ions of divalent copper at room temperature, until recently its biodegradation was not confirmed: the transformations could be explained by a chemical reaction. For the first time, we carried out a further modification of the Pridhem-Gottlieb medium, excluding from its composition copper sulfate ($CuSO_4$) and phosphate. The growth of the microbes under similar conditions, but in the absence of $CuSO_4$ and phosphates would provide stronger foundation for the biodegradation of P_4. Statistical data processing was performed using Microsoft Excel 2013.

Results and discussion

In the negative control without sources of phosphorus, a few (11) colonies of Aspergillus niger AM1 were observed. They occupied a relatively large area and grew very slowly (with undeveloped mycelium and weak sporulation). Without doubt, this was due to the lack of phosphorus. Interestingly, in the experimental medium with 0.05% white phosphorus, there were fewer colonies (33 colonies) than in the positive control (49 colonies), but their growth and sporulation was normal in comparison with the phosphorus deficient medium.

Subsequently, it can be concluded that not all fungal spores survive in a medium with white phosphorus. However, the survivors have the ability to utilize either P_4 itself or the products of its chemical transformations as a source of phosphorus. Reseeding was carried out in media with the following concentrations of white phosphorus, 0.05; 0.1; 0.2; 0.5 and 1% P4, in order to facilitate their adaptation to the toxicant. The results suggest that black Aspergillus tolerates the presence of white phosphorus in the medium, even at a concentration of 1%. The highest concentration of white phosphorus studied by us, being 1% P4 corresponds to about 5000 times the Threshold Limit Value (TLV) of white phosphorus in wastewater. Moreover, the TLV of elemental phosphorus in water bodies for household drinking and use is only 0.0001 mg / l, which is lower than 1% by one hundred million ($1 \cdot 10^8$) times [12]. In environments with a lower content of P_4, the growth of fungi is more intense - this was determined visually. After the fourth reseeding, streptomycete also began to grow at a P_4 concentration of 1%, i.e., it developed resistance even faster and more efficiently than the fungus.

For the genetic identification of a fungus, the nucleotide sequence of its regions ITS1 and ITS2 was determined according to morphological characters attributed to the species A. niger. Comparison of the obtained sequence with the sequences of the GenBank database (NCBI) using the BLAST system allowed us to identify this microorganism as a new strain of Aspergillus niger and thus, the number *A. niger* AM1 was assigned to it [9].

For strain A8, the similarity of the 16S rDNA nucleotide sequences (amplification by primers fD1 and rP2) of the compared streptomycete fragments selected from the NCBI database and the isolates tested was 94–99.7%. In particular, the greatest similarity - 99.74% - was found between isolate A8 and *Streptomyces sampsonii*. It is proposed to attribute our strain to this species.

Intriguingly, in the medium with white phosphorus, a spontaneous appearance of an *Aspergillus niger* AM1 culture with altered morphology and color, which grows faster in the medium with the xenobiotic under study was observed. In one of the culture replicates, the colony began to develop faster than in others, although the conditions were completely identical. 55 days after inoculation, the leading culture began to produce pigment and acquired a more saturated yellow color, which stained both the colonies and the medium and thus, hinting on the fact that this pigment is water soluble. Judging by the fact that this fungus efficiently gathered biomass in the medium with white phosphorus, we conclude that it adapts faster in this medium in comparison to the ancestral culture. We labelled this strain *A. niger* AM2.

The NMR analysis revealed the resistance of the AM1 culture to products of partial oxidation of P4. The very fact of the emergence of resistance to this group of substances (phosphites and hypophosphites, which are antimicrobial agents [13]) is very interesting, however, the expected result — complete metabolic conversion of white phosphorus to phosphate is yet to be confirmed.

It turned out that all four *A. niger* FW-650, FW-2664 and FW-2731 and AM1 strains show resistance to white phosphorus at a concentration of 1% and the MIC was not found for them with respect to the

chosen range of P_4 concentrations. Since all the studied strains were randomly sampled strains of *A. niger*, it can be assumed that high resistance to white phosphorus is a sign that characterizes all black aspergillus, or at least most of them. In addition, at concentrations of 0.5 and 0.25%, the AM1 strain grew faster, i.e. proved to be more adaptable (Fig. 2). In the case of bacteria, the MIC was identified to be 0.125% for *A. xylosoxidans*, 0.25% for B. firmus, and 0.5% for both *P. aeruginosa* and *S. typhimurium*. From this it follows that black Aspergillus is more resistant to white phosphorus than bacteria.

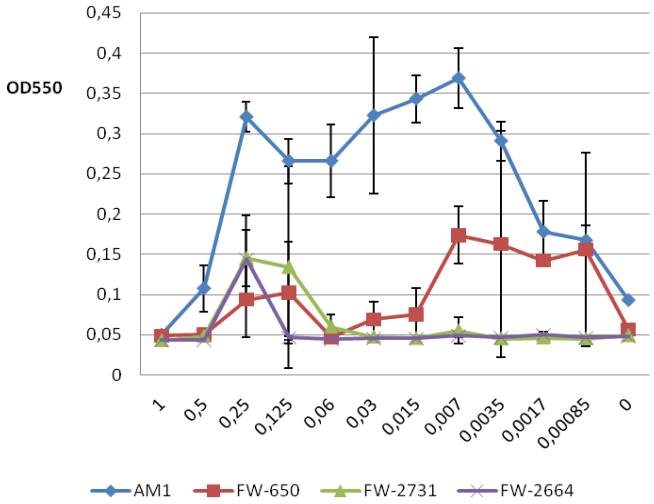

Figure 2 - The growth of *A. niger* strains in a phosphate deficient medium containing white phosphorus, on the third day of cultivation. X- axis: concentration (%) of white phosphorus. Y-axis: optical density at λ=550 nm. The growth rate of strain AM1 is emphasized

It was expected that following growth under favorable conditions, reculturing A. niger AM1 in a medium with P4 could lead to loss in resistance to white phosphorus. In fact, the fungus continued to grow normally in both P4 after being subjected to a medium with phosphate [14]. In line with this, it can be concluded that the resistance to white phosphorus in the studied *A. niger* strain is fixed in the genome, and is an inherited trait transmitted in a number of generations even in the absence of P_4. The vital role of salts of transition metals in the growth and development of microbes is well known [15, 16]. However, excluding copper sulfate from the composition of the nutrient medium revealed that there was no statistical diference between the fungal growth in the presence and absence of $CuSO_4$ (Fig. 3).

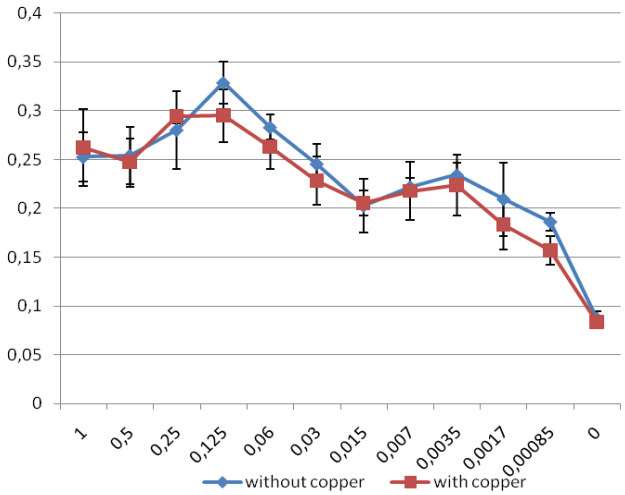

Figure 3 - The growth of *A. niger* AM1 on the fifth day after inoculation. It can be seen that there is no significant differencee between culture growth in the medium variant with copper and without copper. The abscissa axis is the concentration of white phosphorus,%. Y-axis - Optical Density (OD) at λ = 550 nm

It should be noted that when emulsion of white phosphorus was introduced into the copper--dificient medium, no black precipitate (something we noted in earlier studies) was observed. This means that P_4 does not enter into a chemical reaction and remains in the medium for a longer time. This fact is an additional argument in favor of the fact that the biodegradation of white phosphorus takes place, and not the chemical neutralization of copper ions.

Conclusion

The work presented has demonstrated for the first time the inclusion of white phosphorus in the natural cycle of the phosphorus element. The uniqueness of phosphorus is in the fact that this element is absolutely necessary for the vital activity of all life forms. Nonetheless, as a simple substance, white phosphorus is a poison of first class danger, which with great difficulty is subjected to destruction in the environment. Prior to the beginning of our work, the biodegradation of white phosphorus (like its other allotropic modifications) had not been described in the literature.
The significance of the presented work precisely involves the fact that for the first time, it showed the possibility of the growth of microorganisms from different and distant taxonomic groups in culture media containing white phosphorus as the sole source of phosphorus. Thus, oxidation of white phosphorus to phosphate, the harmless component of all living cells has been shown and its further incorporation into the microbial biomass.

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А.З. Миндубаев[1], Э.В. Бабынин[2], А.Д. Волошина[1], К.А. Сапармырадов[2], Й.А. Акосах[2], Е.К. Бадеева[1], С.Т. Минзанова[1], Л.Г. Миронова[1]

[1]Институт органической и физической химии им. А.Е. Арбузова ФИЦ КазНЦ РАН, Россия, 420088, г. Казань, ул. Арбузова, 8;
[2]Казанский (Приволжский) федеральный университет, Россия, 420008, г. Казань, ул. Кремлёвская, 18

ВОЗМОЖНОСТЬ ОБЕЗВРЕЖИВАНИЯ БЕЛОГО ФОСФОРА МИКРОБНЫМИ КУЛЬТУРАМИ

Аннотация. Впервые наблюдался рост микроорганизмов различных таксономических групп (грибов, стрептомицетов и бактерий) в культуральных средах, содержащих в качестве источника фосфора белый фосфор P_4. Это первый известный пример включения белого фосфора в биосферный круговорот элемента фосфора. Самая высокая концентрация, применённая в данном исследовании, соответствует превышению ПДК белого фосфора в сточных водах в 5000 раз, а в питьевой воде – в 100000000 раз! Впервые проведена селекция на рост устойчивости культур к P_4. Мы идентифицировали микроорганизмы, растущие на белом фосфоре, как новые штаммы *Aspergillus niger* и *Streptomyces sampsonii*, которым были присвоены номера *A. niger* AM1 и *S. sampsonii* A8. Показано, что штаммы грибов *Aspergillus niger* адаптируются к P_4 лучше, чем бактерии.

Ключевые слова: биодеградация, белый фосфор, защита окружающей среды, химическое загрязнение.

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