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Microorganisms Resistant to White Phosphorus

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Abstract

We present preliminary results on the successful culturing of different microbial taxonomic groups on media containing white phosphorus (P4) as the sole source of phosphorus. The increase in culture resistance resulting from targeted selection was demonstrated. The highest concentration of P4 used in the study exceeds the threshold limit concentration of P4 in wastewater mud by 5000 times. Putative metabolites of P4 were also investigated.

Keywords: biodegradation; white phosphorus; *Aspergillus niger; Streptomyces* sp. A8.

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White phosphorus P_4 is one of the most dangerous environmental pollutants [1]. On the other hand, the phosphorus element has a unique quality - being the strongest poison in the form of a simple substance, in the oxidized state it is absolutely necessary for all life forms. Thus, it seems appropriate that detoxifying P_4 completely be based on this property. Biodegradation is becoming one of the most popular methods for the neutralization of industrial waste [2].

The study is aimed at biodegradation of white phosphorus (one of the most dangerous substances used in large-scale chemical production) using microorganisms. In the literature adequate information on proven examples of the biological degradation of white phosphorus appears to be lacking. Previous works of our team [3, 4] have shed light on the practically unexplored question of the toxicity of white phosphorus for prokaryotes.

Cultures were obtained on a modified Pridham-Gottlieb medium. The classical Pridham-Gottlieb medium does not contain carbon sources: oil products act as such. Our modification includes glucose, but does not contain sources of phosphorus (white phosphorus acts as such). Inoculation of *Aspergillus niger*, the spores of which were introduced together with white phosphorus, was carried out in a medium containing white phosphorus at a concentration of 0.01 and 0.05% by weight. The grown *A. niger*

was likewise cultures in control media K (+) and K (-), in which phosphate was added to the positive control media K (+), while the negative control media K (-) lacked any source of phosphorus. A reseeding of *A. niger* was performed in media of a similar composition. However, the third reinoculation was cariied out in media with an increased concentration of white phosphorus: 0.05, 0.1, and 0.2% by weight. A strain of *Streptomyces sp*. A8 was cultured under similar conditions. The fourth culturing was carried out in media with a white phosphorus concentration of 0.1, 0.5, and 1% by weight. In this, in addition to *Aspergillus* and *Streptomycete*, the *Trichoderma asperellum* F-1087 fungus, provided by the Department of Biochemistry of the Kazan Federal University, was cultured.

On day 5 following inoculation, the *A. niger* culture which had grown at 0.05% white phosphorus, was reseeded into the control media K (+) and K (-). Six days after inoculation, the following pattern was observed (see Figure 1); a significant number of relatively small colonies grew in K (+) medium with phosphate: this means that most spores have sprouted, which is natural under favorable conditions. In K (-) medium, without phosphorus sources, small colonies grew, occupying a relatively large area, but very weak _ almost transparent, with undeveloped mycelium and distinctive conidiophores, in the appearance of a scattering of black dots, instead of a solid black field. Apparently, this was due to a lack of phosphorus. Even though the agar used to prepare the medium contains an admixture of phosphate, it was insufficient for the full growth of mushrooms. In a medium with 0.05% white phosphorus, the colonies grew less than in K (+), however, they give the impression of completely normal, not experiencing a deficiency of nutrients. It could thus be concluded that not all fungal spores survive in the presence of white phosphorus. However, survivors have the ability to use either white phosphorus itself or the products of its chemical transformations as a source of phosphorus.

The next reseeding was carried out on day 84 after the first in media with a higher concentration of white phosphorus, in order to adapt the fungus to it. P_4 concentrations of 0.05, 0.1, and 0.2% were adopted. The latter, according to [5], corresponds to a thousand-fold excess of the threshold limit concentration (TLC) of white phosphorus in wastewater. Nevertheless, even with such a high content of white phosphorus in the medium, intensive growth of fungal colonies was observed. On the fourth day after inoculation, at all three concentrations of white phosphorus, the beginning of sporulation was observed, but at 0.1 and 0.2% of P_4 fungi lagged in growth as compared to 0.05%. It is possible that the used concentrations of the studied toxicant negatively affect the potency and viability of the fungi. Notwithstanding, these concentrations do not completely suppress them. Nevertheless, the results of sowing suggest that black

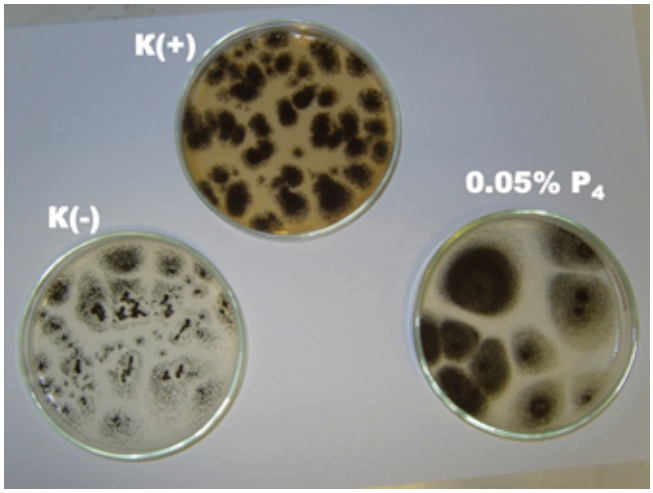

Figure 1: Samples of the first reseeding of resistant fungi A. niger. after 6 days: growth of 49 spore-forming colonies was observed in medium with phosphate (K (+)); growth of 33 weakened colonies was noticed in medium without phosphorus source (K (-)), while growth of 11 large spore-forming colonies was seen in medium with 0.05% white phosphorus

aspergillus easily adapts to the presence of white phosphorus in the medium even at a concentration of 0.2%.

The fourth and second reseeding of aspergillus and streptomycetes, respectively were conducted 112 days after the first inoculation. The concentration of white phosphorus in the medium was again increased to 0.5 and 1% by weight. When such a large amount of P_4 was added, a thick black precipitate in the media was observed instantly. The media emitted a strong specific odor of white phosphorus, even several days after sowing. After a day, the growth of seeded microorganisms was not detected. After four days in a medium with 0.5% white phosphorus, growth of small aspergillus colonies having a whitish appearance was observed (i.e., growth was greatly slowed down). In media with 1% of white phosphorus, no growth was observed on this day. Apparently, the observed black precipitate of phosphides transformed the trace elements present in the medium and necessary for the growth of microorganisms into the insoluble forms. It should be noted that a concentration of white phosphorus of 0.5% corresponds to 2500 times the TLC [5]. In addition, the *Trichoderma asperellum* F-1087 fungus was seeded at concentrations of 0.1, 0.5, and 1%. After four days, in the medium with a lower concentration, one large colony of trichoderma grew, i.e. this fungus likewise posseses the potential to absorb white phosphorus. However, the fungi developed very slowly. Apparently, these concentrations of white phosphorus are close to limiting, at which fungal growth is still possible. For instance, growth of streptomycetes at 0.5% was not even observed 19 days after inoculation. On the eighth day, a scattering of spores formed on the surface of the aspergillus colonies, i.e. the fungus retained the ability to reproduce. Also on the eighth day, a growth of trichoderma colony on white phosphorus

at a concentration of 0.5% was visible. In media containing 1% P_4, trichoderma growth was detected only after 11 days of culturing. In the case of trichoderma, there is a clear dependence: the higher the concentration of white phosphorus in the substrate, the slower the fungus grows. On the 12th day after sowing at 0.1% white phosphorus, the fungus formed an aereal mycelium with a pink color, at 0.5% the colony remained colorless with a shape close to a regular circle, but at 1% the colony consisted of a substrate mycelium.

T. asperellum F-1087 showed greater resistance to white phosphorus than *A. niger* and especially streptomycetes. On the eighteenth day after sowing, it acquired color and began to sporulate at 0.5% white phosphorus. It should be emphasized that trichoderma adapted to such high concentrations of white phosphorus immediately, without preliminary cultivation with a series of reseeding. Previously, this strain of the fungus was never grown in the presence of white phosphorus. Moreover, the concentration of white phosphorus in 1% exceeds the TLC in wastewater by 5000 times!

Third reseeding of *Streptomyces sp*. for the first time demonstrated an increase in the resistance of microorganisms to white phosphorus during selection. On day 22 after seeding, streptomycete growth was observed in the medium containing 0.5% white phosphorus. In previous cultures *Streptomyces. sp*. grew at concentrations notexceeding 0.2%, and clearly after a long delay. Even on the 20th day after sowing, signs of growth were not visible. On day 22, streptomycete appeared to have a substrate mycelium.

On the 27th day after the sixth culturing of *A. niger*, the onset of fungal growth was observed in the medium with 1% white phosphorus. In previous cultures, the maximum concentration of white phosphorus at which aspergillus grew was 0.5%. That is, *A. niger*, like streptomycetes, after several reseeding developed significantly greater stability compared to the original. Hence, streptomycetes showed the best adaptability to white phosphorus (see Figure 2). After five successive cultivation, their resistance increased fivefold. Fungi were observed continually to grow and adapt more slowly (in aspergillus, a doubled resistance was observed after eight successive cultures), but their resistance was initially higher than that of the actinomycetes, especially in trichoderma [6].

For the genetic identification of micromycetes, which stably metabolize white phosphorus and are morphologically classified as *A. niger*, the nucleotide sequence of its ITS1 and ITS2 regions was determined. Comparison with the sequences of the GenBank database using the BLAST system [7] revealed 99% homology with the ITS1 and ITS2 regions of the described strains of *Aspergillus niger*, which allows us to identify this microorganism as a new strain of *Aspergillus niger*. We assigned to it the number

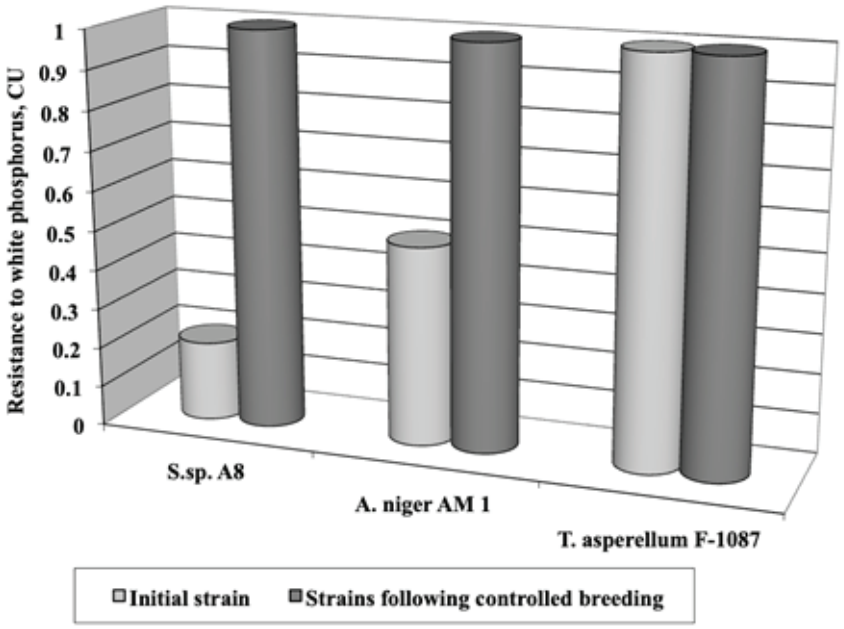

Figure 2: Adaptation and growth of resistance of microorganisms to white phosphorus after controlled breeding

A. niger AM1 [8]. The nucleotide sequence of the strain is published in the GenBank database under the accesion number KT805426.

In the experimental ^{31}P Nuclear Magnetic Resonance spectrum taken from the aqueous phase, signals appeared in the region of 0.3, 3.7, and 6.2 ppm, corresponding to phosphite and hypophosphite. Thus, it corresponds to compounds that are supposedly metabolites of white phosphorus, ie, it confirms our proposed metabolic pathway [9]. Below is a suggested pattern of white phosphorus metabolism (Figure 3).

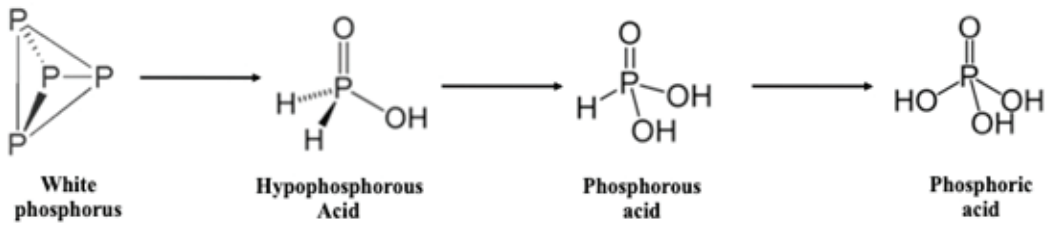

Figure 3: The proposed metabolic pathway of white phosphorus

Since there is no information in the literature about microorganisms resistant to P_4, the presented work has an undeniable novelty.

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