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Analysis of Petroleum Biodegradation by a Bacterial Consortium of *Bacillus amyloliquefaciens* ssp. *Plantarum* and *Bacillus subtilis*

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ABSTRACT

Features of change of phytotoxic influence of the soil polluted with oil at the use of a complex biological product based on strains of microorganisms *Bacillus amyloliquefaciens* subsp. *plantarum* NSh-2 and *Bacillus subtilis* NSh-4 in the laboratory were investigated. The level of destruction of petroleum hydrocarbons at different combinations of pollutant and biological product concentrations was determined, as well as in the absence of oil pollution to ensure the assessment of the biological product's impact on the environment. Soil phytotoxicity was assessed by the method of biotesting using radish seeds of the Sora variety by the ratio of seedling height and the obtained mass of organic matter.

Keywords: *Bacillus amyloliquefacieni subsp. plantarum, Bacillus subtilis*, biotesting, phytotoxicity, biosurfactant, oil-contaminated soil

INTRODUCTION

The study subject was the phytotoxic properties of artificially contaminated soil by oil, exposed to oil-destroying microorganisms. Soil is one of the main ecological objects, the central link between the biotic and anabiotic components of the biosphere. Assessing the quality of soils and soil cover requires understanding and applying a range of analytical and theoretical methods available in the arsenal of soil science [Hrodzyns′kyy et al. 2006]. Currently, there are various methods of controlling environmental pollution. One of the promising methods of environmental research is biotesting, which allows establishing environmental toxicity, i.e. studies the response of living organisms to pollutants. The essence of this method is to determine the effect of toxicants on specially selected organisms under standard conditions, with registration for behavioural, physiological or biochemical parameters. Biotesting is aimed at assessing the overall toxic effects of the whole complex of pollutants using the studied objects [Dzhura 2011].

The impact of petroleum pollution on bioindicator plants occurs in two ways: – directly through the receipt of petroleum components by the root and foliar route with then inclusion in the metabolism; – indirectly due to changes in the physical and chemical composition of the soil and violation of its biological characteristics. The components of the liquid part of petroleum products enter the plant's body through the root system, which can cause mutagenic reactions or deviations from normal development in morphological parameters [Shestopalov et al. 2015; Filonov 2016].

It is known that the seeds can absorb oil, which, in turn, leads to changes in metabolic reactions, resulting in reduced germination or, potentially, seeds do not germinate at all.

The biotesting method has unique and powerful tools for sensitive and specific quantitative assessment of the environment toxic effects in the

case of different types of pollution [Vasilyev et al. 2012; Shevchyk et al. 2016].

When petroleum hydrocarbons do not exceed the maximum allowable ecological load, which is determined by the maximum volume and protective capacity of the ecosystem to this pollutant, the system is capable of self-cleaning and self-recovery. The mechanism of this process is based on the involvement of petroleum hydrocarbons in the metabolic pathways of substances (representatives of the micro- and phytocenosis) oxidation due to changes in enzymatic activity [Moebius et al. 2007]. Among the measures taken to protect the environment from oil pollution, one of the most promising and environmentally friendly is the method of bioremediation of soils and waters [Liu et al. 2016]. It is based on the ability of some microorganisms to remove petroleum products from the environment mainly through biotransformation [Rusin et al. 2015; Poi et al. 2017]. The use of biosurfactant to remove pollution from the environment is not new, but it is still an insufficiently studied area of research. The search for new types of oil hydrocarbon destructors and identifying optimal conditions for their effective use is currently underway. With the degradation of oil component in the soil occur the process of decreasing in residual content and also a change in fractional compositions, due to the processes of redistribution of oil in the soil profile [Sharma 2012]. Under conditions of natural microbiocenosis, various groups of microorganisms assimilate different degrees of oil fractions.

It is known that strains of bacterias such as *Acinetobacter, Pseudomonas, Bacillus, Micrococcus* et al. can be used to clean oil-contaminated soils by the bioremediation method [Salvatore et al. 2008]. Biological Solutions based on strains of *Bacillus amyloliquefaciens* can utilize petroleum hydrocarbons in the pH range of 5–9, as well as degrade its comportments in concentrations up to 20%. The advantage of the proposed proposed biological product is the high rate of oxidation of oil and petroleum products, which allows its effective use in the biological treatment of soils and waters contaminated with petroleum products [Sakthipriya et al. 2015; Datta et al. 2018].

An easily soluble bacterial protein is formed in the environment as a result of treatment of oil pollution with biological oil-destroyers. Along with it, nontoxic oil decomposition products are formed, and both do not require any further utilization. The products of bacterial activity are low molecular weight compounds, and the bacteria themselves decompose, giving the basis for the formation of humus (when using biosurfactant for soil purification) or forming a bottom sludge (when using for water purification) [Filonov 2016; Purnomo 2020].

Numerous studies have examined the features of purification processes using biosurfactants based on the strains of *Bacillus* microorganisms. For example, using the halotolerant marine bacterium *Bacillus licheniformis* LRK1 to test its biosurfactant production potential showed a 70% oil emulsification effect. The purified biosurfactant was analyzed by Thin Layer Chromatography (TLC) and Fourier-transform Infrared Spectroscopy (FTIR), which confirmed the nature of the obtained biosurfactant as lipopeptide. The gravimetric method showed 24.23% decomposition of engine oil by the biosurfactants after 21 days of incubation [Rahman et al. 2003; Ossai et al. 2019; Nayak et al. 2020].

Previous studies of the *Bacillus subtilis* strain have proved its resistance to changes in pH, temperature, salinity and showed its effective emulsifying properties when interacting with petroleum hydrocarbons. Even in the semi-purified form, the biosurfactant based on *Bacillus subtilis* microorganisms was not toxic to *Lactuca sativa* (lettuce) or *Artemia salina* bioindicators [Varjani et al. 2017; Zhang et al. 2020].

The analysis of the previously obtained results showed that the use of biosurfactants based on *Bacillus bacteria* has significant potential in bioremediation and protection of the environment from petroleum pollutants.

Therefore, given the above, the study aimed to determine the phytotoxicity of soil samples artificially contaminated with petroleum, which was affected by oil-destroying microorganisms. The core of the experiment is based on biotesting and analyzing the morphological parameters of the indicator plant (*Raphanus sativus var. sativus*).

MATERIALS AND METHODS

During the experiment as oil destroyers used biosurfactants based on the strain *Bacillus amyloliquefaciens ssp. plantarum* were isolated from drilling sludge from Semirenkivsky GKR of Myrhorod district of Poltava region in Ukraine.

Determination of the bisurfactant effectiveness using the method of "Growth test" was carried out. The essence of the growth test is to analyze changes in the germination of indicator culture grown on the studied soil samples. To determine the toxicity of the soil, radish seeds of the Sora variety were used, which is due to the high sensitivity of the seeds to toxic substances. Phytotesting is based on the sensitivity of plants to exogenous exposure to chemicals, which is reflected in the growth and morphological characteristics of plants. The method's main advantages are clarity, convenience and simplicity of experiments, repeatability and reliability, economy and objectivity of results. Phytotests can be used to detect the phytotoxicity of soil and the aquatic environment because bioindcator plants can respond to contamination, which are related to the parameters of seed germination and the growth rate of roots and shoots and act as indicators of soil toxicity [Ziółkowska et al. 2010].

The experiment was performed using identical beakers with a capacity of 180 ml. In equal amounts samples of oil-contaminated soil (the appropriate amount of oil was added to the soil up to the required concentration) were placed in beakers, then moistened with the same (5 ml) volume of water. The seeds were germinated at a temperature of 20–23°C for 26 days. Plant germination, seedling height and dry weight of the obtained organic matter were chosen as the basis for soil toxicity assessment. Mineral fertilizers were not used during the experiment.

Phytotoxicity effect (PE) was determined as a percentage of plant height by the formula:

$$\mathrm{PE} = \frac{M_0 - M_x}{M_0} \cdot 100, \% \qquad (1)$$

where: M_o – the height of sprouts in a beaker with a control group;
M_x – the value of a similar bioparameter in a beaker with the tested groups.

Soil phytotoxicity is a general indicator that can be used to characterize the effect of soil on plants. If there are toxic substances in the soil, they can affect seed germination or plant growth. One of the main parameters for soil toxicity is the number of germinated seeds over time and their growth dynamics [Shestopalov et al. 2015].

RESULTS AND DISCUSSION

Biotesting was performed in the laboratory using the standard test system *"Raphanus sativus var. sativus"*, which made it possible to identify the environmental toxicity of contaminated soil samples that were affected by tested oil destructors in controlled and reproducible conditions.

The dynamics of bioindicators' growth in different conditions of soil pollution and oil destructors concentrations represents the quality of biosurfactant effect (Figure 1, Figure 2).

Germination of the first sprouts was recorded in the control groups on days 10–11 of the experiment. In groups with a high concentration of oil sprouting occurred later, on 12–13 days of the experiment, shortly after the 4th watering with

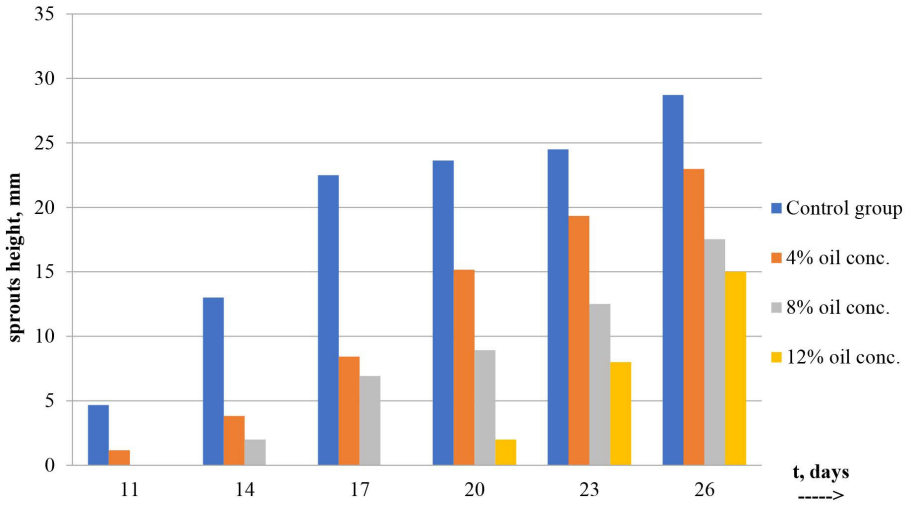

Figure 1. Dynamics of bioindicator growth in oil-contaminated soil using 2% concentrate of oil-destroying microorganisms

a solution based on the "Bacillus amyloliquefaciens ssp. plantarum" bacterial consortium.

There was no significant difference in growth processes between the control groups with and without using biosurfactant in high concentrations on the non-polluted soil. The germination of sprouts under the action of a 4% solution concentrate does not differ significantly from the control.

At the same time, in oil-contaminated soil without using oil destructors, withering processes were taking place stably, sprouts above 3–4 mm do not germinate. There was inhibition of growth processes in all control groups from 4 to 16% of the pollutant concentration.

According to the data obtained, contamination of the soil with oil without using biosurfactant had a more negative effect on the growth of indicator plants than contamination with the use of *"Bacillus amyloliquefaciens"* microorganisms in the same doses of oil pollution.

Withering processes took their place in terms of oil pollution even with the use of tested oil destructors, but the number of recorded cases, compared to the control samples, was considerably lower.

The statistical difference between the average values of the bioparameter in the control group and study variants indicated significant changes in the growth processes of bioindicators.

The allotted area of the plot in the beaker for each test was the same 0.02123 dm^2. Given the amount of organic matter obtained, there was a tendency of stable increase in yield production with increasing biosurfactant concentration from 2% to 4% in all tested variants.

According to the results, the soil in the studied groups using oil destructors in beakers without pollution was almost the same quality as in the control experiment. The impact of biosurfactant does not increase toxicity due to the absence of difference in the sprout height or amount of organic material obtained.

There was a significant phytotoxic effect in control beakers without the use of biosurfactant with an almost complete absence of organic (Table 2). At the same time, parallel testing using the solution showed a significant reduction of adverse effects from petroleum pollution. The primary indicator was the data obtained from measurements of the dry mass of organic matter, as it is the most informative indicator in determining the degree of soil toxicity. The obtained results showed that soil samples were only moderately toxic to indicator plants even with petroleum pollution of 4 and 8% and the use of the oil-destroying microorganisms at the concentration of 4%.

A significant decrease in the germination of sprouts was observed in all samples without the use of solution concentrate. The phytotoxic effect was 96.04%, 96.38%, and 97.06% in groups with 4, 8, 12% of petroleum. It should be noted that sprouting did not occur at all in the beakers, with pollution close to 16%.

The results of comprehensive biotesting and analysis of organic mass (Fig. 3) showed that the doses petroleum concentration of 4 and 8% did not have an acute toxic effect on bioindicators with the using biosurfactant of 4%. The significant level of suppression was only 21.86% and 27.91%, respectively.

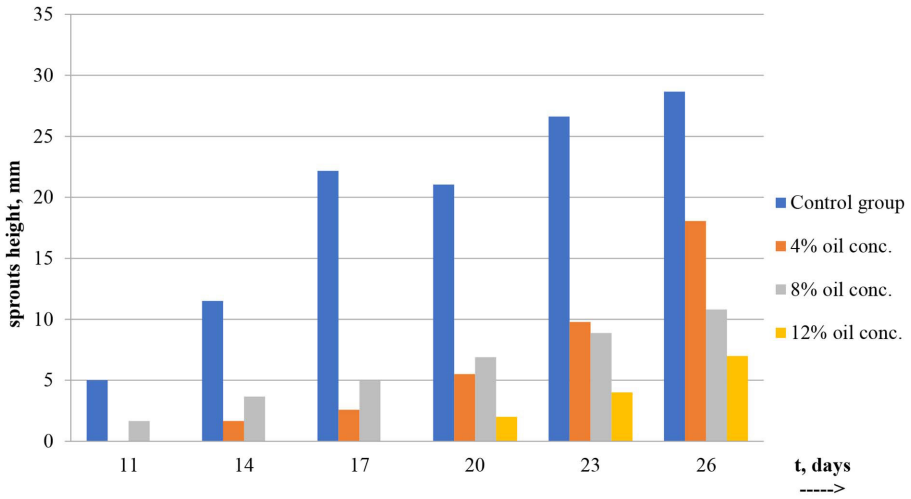

Figure 2. Dynamics of bioindicator growth in oil-contaminated soil using 4% concentrate of oil-destroying microorganisms

Table 1. The average height of plants at the end of the experiment (by groups), mm

Biosurfactant concentration, %	Petroleum concentration, %				
	0	4	8	12	16
0	28,71 ± 3,97	-	-	-	-
2	-	18,06 ± 3,57	14,80 ± 1,93	15	-
4	28,66 ± 4,45	22,99 ± 3,74	17,54 ± 2,28	7,00 ± 0,72	-

Table 2. Phytotoxicity effect statistic of the oil-contaminated soil using oil destructors, %

Biosurfactant concentration, %	Petroleum concentration, %			
	4	8	12	16
0	96,04	96,38	97,06	100
2	44,68	63,57	85,52	100
4	21,86	27,91	61,40	100

The greatest effect was found when exposed to contaminated (4% oil) soil with a solution in concentrations of 2% and 4%, the phytotoxic effect in these cases decreased from 96.04% to 44.68% and 21.86%, respectively.

A significant reduction in oil concentration is obtained due to the multifunctionality of the tested oil destructors. Expressed emulsifying, antifungal and growth-stimulating properties along with, the ability to oxidize hydrocarbons reduce the phytotoxicity of the soil to a level acceptable for plant growth. Emulsification (solubilization) of hydrocarbons with the help of substances secreted by microorganisms improves the flow of contaminants from the soil into microbial cells and promotes their degradation. The antifungal and growth-stimulating effect of biosurfactant works as antagonistic action against pathogenic microorganisms, ensure prevention and treatment of mycotoxicosis with further protection of plants from phytopathogenic fungi. Enzymes of microorganisms that degrade hydrocarbons belong to the class of mixed functions of oxidoreductases (oxygenases) and are related to the membrane structures of bacteria cells. Oxygenases catalyze the inclusion of one oxygen atom from its molecular form in the terminal methyl group of a hydrocarbon, further degrading its phytotoxic properties.

Microorganisms secrete various biologically active substances into the environment, including cytokinins. The biomass of these microorganisms can play the role of an effective biological fertilizer that stimulates the development of the entire microbiota in the oil-contaminated soil.

Comparison of the dry organic mass obtained was grown in the field of oil-contaminated soils with biotesting data of the experiment leads to the absence of fundamental contradictions between them. That means that the yield of terrestrial biomass in the field experience confirms the results of a laboratory study of the polluted soil productivity by biotesting.

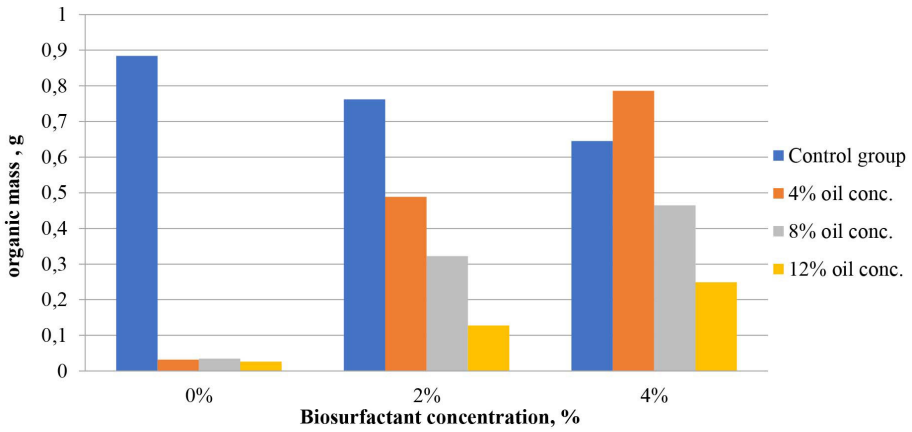

Figure 3. Weight of dry organic mass/yield per 0.02123 dm^2

CONCLUSIONS

1. The study showed that using biosurfactant based on the strain *"Bacillus amyloliquefaciens ssp. plantarum"* can be appropriate. The main role of the presented strain of oil destructors is enzymatic oxidation of petroleum products, with the further reduction of phytotoxicity of contaminated soil, even at high (12%) oil concentrations.
2. Thus, the use of biosurfactants based on the suspension of microorganisms-oil destructors is a rational approach in bioremediation methods. The use of the strain *"Bacillus amyloliquefaciens ssp. plantarum"* in the studied areas of oil-contaminated soils provided a reduction of phytotoxic effect at the level of 51.36–74.18%. It should be noted that the study was conducted for 26 days, which is quite a short time in the process of cleaning the environment from oil pollution.
3. A well-defined dependence was detected between suppressing morphometric parameters of the studied bioindicators *"Raphanus sativus var. sativus"* at oil concentrations in the soil in the range of 4–16% when using 2–4% solution of the biosurfactant. The tested biosurfactant can create acceptable conditions for plant germination in the contaminated soil at pollution concentrations from 4 to 12%. The presence of 16% of oil in the soil within tested biosurfactant concentrations (2–4%) was not enough to ensure plant germination.
4. The plans of future part of experiments include a chromatographic analysis of oil destruction degree in soil samples due to the impact of destructive microorganisms to develop a method of bioremediation of contaminated soil. Also, based on the positive results of previous studies in the use of biosurfactants based on strains of *Bacillus* microorganism in the purification of aquatic ecosystems contaminated with oil and petroleum products [25–28], it is planned to conduct a series of experiments to establish the effectiveness of tested biosurfactant in the purification of oil-contaminated water.

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