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EMULSIFYING ABILITY OF BIOCOMPOSITION BASED ON HUMIC SUBSTANCES AND MICROORGANISMS OF THE GENUS *RHODOCOCCUS* IN THE RELATION TO OIL

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Abstract: The stabilizing ability of humic substances of peats in the relation to oil has been studied. The maximum dispersion of oil drops was observed in the presence of *Rhodococcus erythropolis X5* microorganisms and humic substances of sphagnum high-moor peat, as well as *Rhodococcus erythropolis S67* microorganisms and humus substances of reed fen peat. Conducted assay proved that the reduction of the oil film can be used as a test to prove dispersing ability of microorganisms *Rhodococcus X5* or *S67* and humic substances. It was found that in the presence of humic substances oil emulsions stabilized in the best way, which is explained by the nature of the interaction of humic substances with the alkyl moieties only through hydrophobic interactions. It this study stability of aqueous emulsions of oil in the presence of microorganisms *Rhodococcus erythropolis S67* and humic substances of reed fen peat was proved. The proposed dual systems based on humic substances of peats and microorganisms of the genus *Rhodococcus* were capable of stabilizing emulsions of oil-in-water hydrocarbons by binding them as non-toxic complexes with humic substances.

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Introduction

Bioremediation (the purification of natural environment from pollution with help of biological methods) is currently taking the first place in cleaning up oil-polluted water areas. At the same time, it is necessary to interfere less with the ecosystem that has already been affected by pollution. A biostimulation of native microflora by fertilizing directly into a polluted ecosystem or introducing specialized preparations of microorganisms in order to clean polluted ecosystems.[1]

The use of oil-oxidizing microorganisms for the purification of the aquatic environment is insufficiently studied research area. The search for the new destructors of petroleum hydrocarbons continues and the identification of optimal conditions for the effective use of existing drugs is a developing research area. An attractive field envisage the interaction of several active oil destructors oriented to different fractions of oil and several heterotrophic microorganisms that do not have hydrocarbon-oxidizing ability but are able to assimilate products of intermediate oxidation of hydrocarbons, which are often toxic to hydrocarbon-oxidizing organisms.[2] The problem of creating such consortia is that it is very difficult to assess the potential of hydrocarbon-oxidizing enzyme systems.

In addition, methods are known for detoxifying contaminated natural objects with oil through the use of a complex of microorganisms, oil destructors and environmentally friendly, natural compounds such as humic substances.[3] The use of these methods contributes to the growth of the number and activity of microorganisms due to the presence of humic substances as a nutrient substrate involved in the decomposition of petroleum hydrocarbons, which, after applying them to the surface being cleaned, are attached to the oil film at the oil-natural phase section and are involved in the petroleum biodegradation process. In this regard, the treatment of oil-polluted soils with such microorganisms is considered the most promising method to combat oil pollution. In addition, humic substances due to their accumulative and regulatory functions exhibit a high environmental effect in relation to polluted environments, which consists in the rapid restoration of natural geobotanical processes.[4]

As a result of processing oil pollution with biological products based on humic substances and oil-degrading microorganisms, an easily decomposing bacterial protein-based macromolecules remains in the environment, which does not require subsequent disposal, and non-toxic products of oil decomposition. The waste products of bacteria and the dead bacteria themselves are easily absorbed by the native microflora, giving the basis for the formation of humus (when using the drug to clean the soil) or forming bottom sludge (in the case of use on water). The degree of cleaning depends on the initial value of pollution, the type of oil product, and the mechanical composition of the soil.

The purpose of this work is to study the stabilizing ability of humic substances of peats separately and in the joint presence of microorganisms of the genus *Rhodococcus* in the relation to oil.

Experimental

Materials and bacterial strains

The objects of research were humic substances of peats of the Tula region: red fen peat (RFP), black alder fen peat (BFP), sphagnum high-moor peat (SHP) and sphagnum transition peat (STP),[5] identified by the method described in previously published works[6, 7]. Bacterial strains of *Rhodococcus erythropolis S67*, *Rhodococcus erythropolis X5*, provided by the plasmid laboratory of the Institute of Biochemistry and Physiology of Microorganisms named after GK Scriabin RAS (Pushchino, Russia). The bacteria *Rhodococcus erythropolis S67, Rhodococcus erythropolis X5* are part of the "MicroBac" biological product used for bioremediation of oil-contaminated territories.[8] Oil from the refinery of GAZPROMNEFT-MNPZ JSC was chosen as a model pollutant. All experiments were carried out in fresh water at room temperature.

Humic solution (HS) solutions at a concentration of 50 mg/L were prepared by dissolving precise weights of humic substances in 0.1 M NaOH; neutral pH values were adjusted by adding 0.05 M HNO_3 or 0.05 M NaOH, (monitored with Anion 4154 pH-meter, Anion, Russia) and the solutions were adjusted to the mark with a background electrolyte solution (0.1 M $NaNO_3$).[9]

Microorganism cultivation in the presence of oils/humic substances

Microorganisms were cultivated in a full-fledged Luria-Bertani medium (LB).[10] The prepared media were sterilized by autoclaving for 30 minutes at 120 °C. Bacteria were grown in a liquid nutrient medium LB for 24 h to obtain an inoculum.[11, 12]

To conduct a qualitative experiment aimed at studying the effect of humic substances and microorganisms of the genus *Rhodococcus* on the state of oil drops, individually and jointly, 30 ml of sterile water was poured into control petri dishes (d = 15 cm) and carefully pipetted with 2% vol. oil to the surface. To the test samples, a solution of humic substances (initial concentration 50 mg/L) was added to 30 mL of sterile water and a hydrophobic substance was placed on the surface. In the next version of the experiment the amount of inoculum (105-106 CFU/mL) from *Rhodococcus erythropolis S67* or *Rhodococcus erythropolis X5* microorganisms, was added as a suspension to 30 mL of sterile distilled water, and then the investigated contaminant was added to each Petri dish. In the next version of the experiment, humic substances from peat and a microorganism of the genus *Rhodococcus* were added to Petri dishes. The cups were closed and left on a flat horizontal surface in diffused light at room temperature. Assessment of the state of the drops of hydrophobic compounds was carried out after 2, 24, 48, 72, 168 hours.[10]

To study the stabilization of oil emulsions in the presence of humic substances and microorganisms (genus *Rhodococcus)*, a solution of humic substances with a concentration of 50 mg/L, a suspension of microorganisms (the amount of seed dose 105-106 CFU/ml) and 2% by volume of supposed pollutant were introduced into the flasks. During the experiments, a solution of contaminants suspended in sterile water served as a control.

To study the effect of HS preparations on the aggregate state of petroleum products, the solutions were placed in an ultrasonic bath for 2 minutes and kept for 1 day at room temperature. Samples were taken at a depth of 4 cm; the optical density was measured at 535 nm using an SF-104 spectrophotometer (NV-Lab, Russia).[13] The obtained values of optical densities were used in order to calculate transmittance values (T, %), which served as a criterion for the stability of an oil-in-water emulsion, according to formula (1)[14, 15]:

$$T = 10^{-D} \cdot 100\% \quad (1)$$

where D – optical density.

Results and discussion

The surface tension of a hydrophilic emulsion of oil in water can be reduced by introducing substances that possess both a hydrophobic (hydrocarbonated moieties) and hydrophilic part (polar group). In this study, humic molecules were used to decrease surface tension of these emulsions.

After applying oil (Figure 1) in the control sample, its slow and uniform spreading over almost the entire surface of the water was observed. After 2 hours, the spreading practically ceased and an uniform film formation with smooth edges around the entire circumference was noticed. When humic substances of reed fen peat were introduced, insignificant spreading around the periphery and concentration of a drop of a hydrophobic substance of oil in the center of a cup of humic substances was observed on the periphery (Figure 1d). The condition of the film formed by the oil on the surface of the HS of peats was comparable to the control:

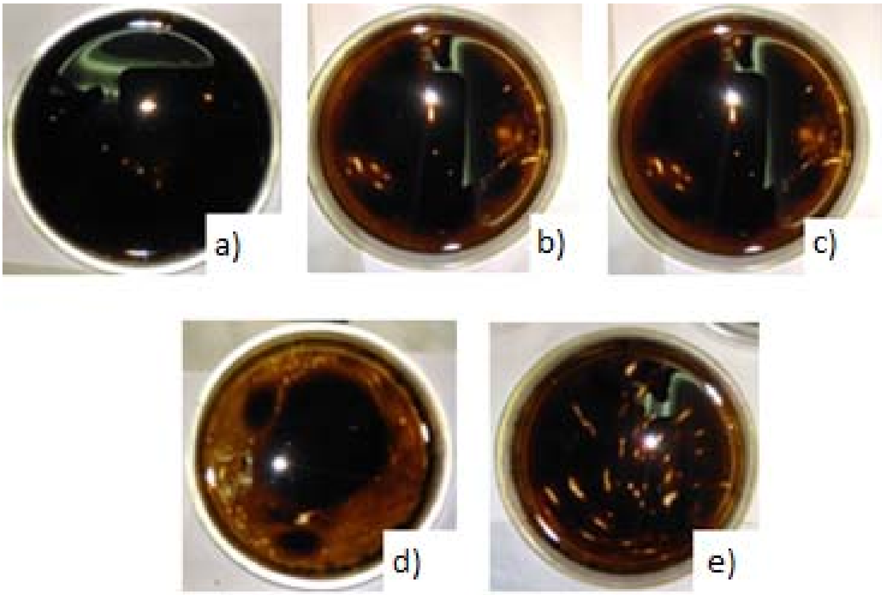

Figure 1. Distribution of oil after 7 days on the surface
a) control b) HS (BFP) c) HS (STP) d) HS (RFP) e) HS (SHP).

In the joint presence in the solution of humic substances and microorganisms *Rh. erythropolis X5* or *Rh. erythropolis S67* was observed non-uniform spreading of oil over the entire surface of the cup for only 3 days (Figure 2). After 5 days of exposure, a drop of oil in variants containing microorganisms of the genus *Rhodococcus* was a thinner, especially in the peripheral part, non-uniform film with long ragged edges. Moreover, after 5 days, in the presence of microorganisms of oil destructors and humic substances of peats, an oil film was clarified. This behavior was not noticed in both control samples and samples containing only humic substances of peat, or only oil-degrading microorganisms of the genus *Rhodococcus*.

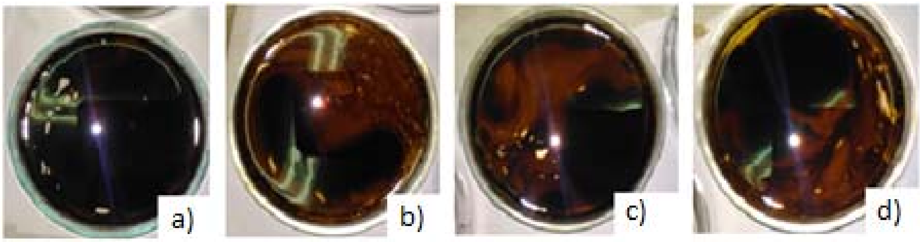

Figure 2. The distribution of oil in the presence of a) microorganisms *Rh. erythropolis X5* and HS (STP); b) *Rh. erythropolis X5* and HS (SHP); c) *Rh. erythropolis S67* and HS (RFP); d) *Rh. erythropolis S67* and HS (SHP) after 3 days on the surface.

After 7 days, there was a depletion of the film surface, division, splitting and tearing of the edges of the drops, as well as the formation of small drops (Figure 3). These experimental data confirm the emulsifying and oil depleting capacity of humic substances of peats and microorganisms *Rh. erythropolis X5* and *Rh. erythropolis S67*, as well as the stimulating effect of humic substances in the relation to these microorganisms.

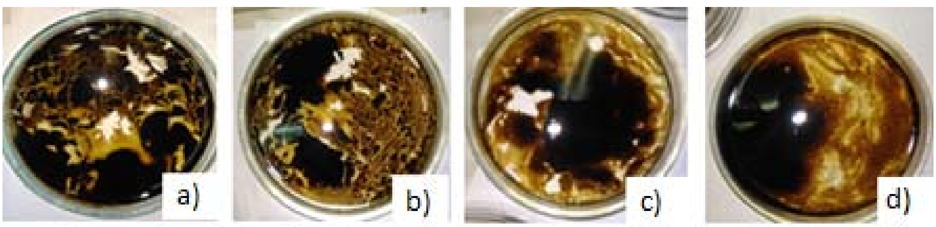

Figure 3. The distribution of oil in the presence of a*) Rh. erythropolis X5* and HS (STP); b) *Rh. erythropolis X5* and HS (SHP); c) *Rh. erythropolis S67* and HS (RFP); d) *Rh. erythropolis S67* and HS (SHP) after 7 days on the surface.

The maximum degree of biodegradation of oil was observed in systems based on humic substances of sphagnum transitional peat (Figure 3a) and high-moor peat (Figure 3b) peat in the presence of *Rh. erythropolis X5*, as well as humic substances from reed fen peat (Figure 3c) and sphagnum transitional peat in the presence of *Rh. erythropolis S67* (Figure 3d).

Polluted oil degradation using a system composed of humic substances and microorganisms can proceed as follows: HS molecules capture the heavy and light fractions of oil with their hydrophobic moieties and sequestered them as non-toxic complexes, with the heavy fractions of oil being bound to the aromatic fragments of the HS, and the light ones to the peripheral parts of the hydrophobic fragments of hydrocarbon radicals, the addition of oil-degrading microorganisms increase the capability of utilizing unreacted oil, and oxidize available organic contaminants on the surface HS.

The characteristic picture of crushing droplets of hydrophobic substances of oil, reducing the film of oil, dividing and crushing drops can be used as an express test for the presence of *Rhodococcus erythropolis X5* or *S67* oil-oxidizing microorganisms activity and humic substances of peats on the ability to disperse and decompose oil films in the hydrosphere.

Further, a quantitative experiment was conducted in order to study the stabilizing ability of humic substances of peats separately and in the joint presence of microorganisms of the genus *Rhodococcus* in the relation to oil.

It is known that the time of localization of oil spills on water should not exceed 4 hours,[16] and the "time windows" of the optimal use of dispersants, depending on the type of oil,[17] are estimated up to 5 hours. In view of this, a comparison of the stabilizing capacity of HS was performed on average four hours after mixing the components. Additional measurements were performed after 2, 4, 6 and 26 hours.

The data on the stabilization of aqueous emulsions of oil by humic substances of peats of various origin and microorganisms of the genus *Rhodococcus* has been obtained.

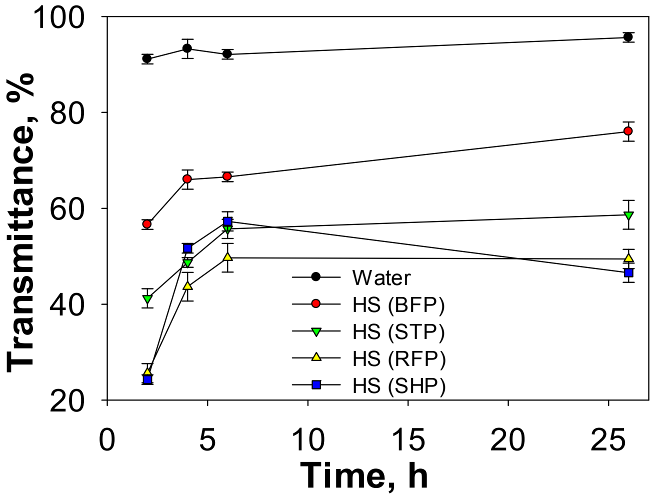

Figure 4. Stabilization of oil emulsion by humic substances.

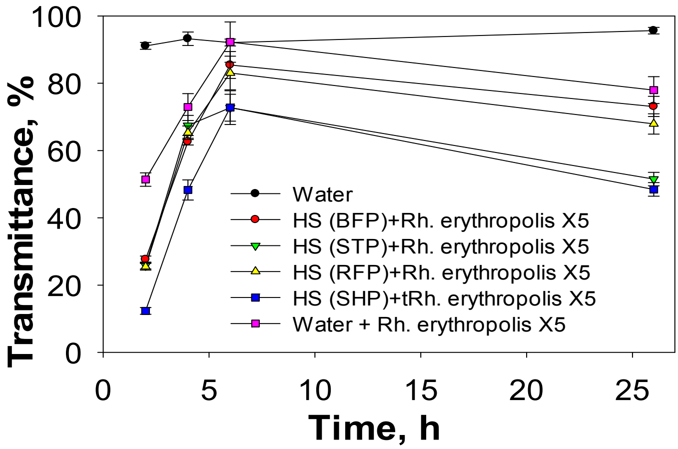

Figure 5. Stabilization of oil emulsions with humic substances and *Rh. Erythropolis X5.*

It has been established that the presence of HS stabilized oil-in-water emulsions. HS (RFP) and HS (SHP) demonstrated the maximum stabilizing ability, *T* values at t = 2 hours are 4 times less, and by the time of the end of the experiment 2 times less than control values (Figure 4). It was found that HS (RFP) most accurately stabilizes the oil emulsion at the initial stage (2 hours after the start of the experiment). The maximum stabilizing effect was observed for HS (SHP) after 26 hours. A decrease of stabilizing ability of HS was in the following order: HS (RFP)> HS (SHP)> HS (STP)> HS (BFP).

The minimum values of the transmittance of oil emulsions were noticed in the joint presence of humic substances and *Rh. erythropolis S67*. In the case of HS (RFP) and *Rh.erythropolis S67*, the action was most effective, the amount of transmission of the emulsion of oil to water changed from 37% to 43% during the whole period of the experiment, which is 2 times less relative to the control and emulsion microorganisms *Rh.erythropolis S67*.

The transmittance calculated for the model pollutant in the system humic substances-*Rhodococcus erythropolis X5* decreased relative to the control and the emulsion containing only HS. Humic substances of sphagnum high-moor and transitional peat have stabilized to a greater extent the oil emulsion (Figure 5).

The presence of microorganisms *Rhodococcus X5* and HS (SHP) or HS (STP) stabilizes the oil in water emulsion 3-8 times better relative to the control solution and 2-4 times relative to the solution containing only microorganisms 2 hours after experiment initiation. The best transmission values after 26 hrs from the start of the experiment are also demonstrated by humic substances of sphagnum high-moor and transitional peat: 12 and 25% at t = 2 and 48 hrs and 52%, respectively, at t = 26 hrs. These data are consistent with previous experimental results, since the humic substances have the maximum stimulating effect on the growth of microorganisms such as *Rhodococcus erythropolis X5*.[18]

The use of humic substances, which are amphoteric amphiphilic ionophore redox compounds,[19,20] for cleaning the hydrosphere from oils is realized due to their action as stabilizers of an oil-in-water emulsion by forming a structural-mechanical barrier (Figure 6) around oil drops, which is a particular advantage of the proposed method, since due to the irreversibility of this process, the possible release of bound oil is prevented. When using humic substances together with oil-degrading microorganisms to detoxify aquatic ecosystems from oil, several mechanisms for stabilizing an oil emulsion are possible: firstly, humic substances are a nutrient substrate for microorganisms involved oil decomposition, which, after applying them to the surface being cleaned, attach to oil film at the section of the oil-natural environment and are included in the process of biodegradation of oil. Secondly, the biosurfactants resulting from the chemical processing of oil by microorganisms contribute even more to the solubilization of oil, thereby ensuring their assimilation by microbial cells. Moreover, sedimentation of the formed mixes of oil emulsions takes place with their subsequent decomposition to carbon dioxide and water (Figure 6).

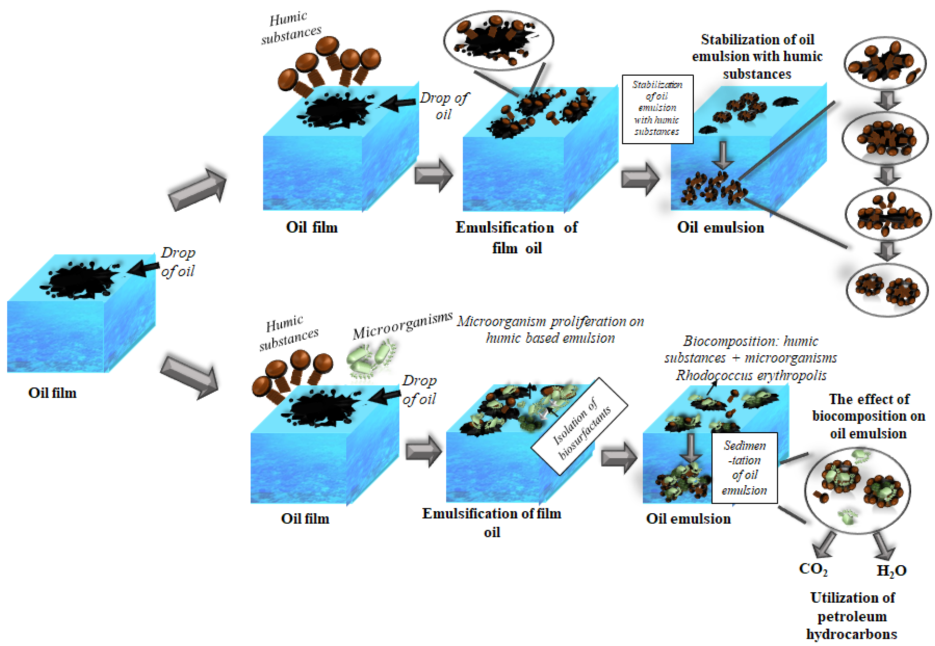

Figure 6. Oil emulsion stabilization by humic substances and microorganisms.

Hydrophilic groups in the molecules of humic substances provide their solubility in water, and rather long hydrocarbon radicals provide solubility in hydrocarbons. The resulting emulsion of oil with humic substances is deposited in aqueous layer, becoming available for biodegradation by native microorganisms due to the destruction of oil micelles. Thus, the cleaning of the water surface from oil pollution is provided.

Conclusions

Maximum emulsification of oil was observed in the presence of *Rhodococcus erythropolis X5* microorganisms and humic substances of sphagnum high-moor peat, as well as *Rhodococcus erythropolis S67* microorganisms and humic substances of reed fen peat. This circumstance is explained by the degree of decomposition and structural characteristics of the original biomaterial from which these humic substances were separated: the greater the degree of decomposition of peat, the more developed the peripheral hydrophobic part of the molecules of humic substances. These molecules act as dispersing agents in particularly for hydrocarbon oil, and therefore they generate conglomerates with drops of oil. The addition of oil-degrading microorganisms is capable of utilizing unreacted oil products together with humic substances, as well as oxidizing the available organic pollutants on the surface of humic substances. Based on our data, the surface reduction of oil films is nicely correlated with the dispersing ability of *Rhodococcus X5* or *S67* oil-degrading microorganisms and enhanced by humic substances.

It was established that HS (SHP) and HS (RFP) mostly stabilized the oil-in-water emulsion and by the time the experiment was completed the value of T decreased by 8 and 10%, respectively. The most stable are aqueous emulsions of oil in the presence of microorganisms *Rhodococcus erythropolis S67* and HS (RFP), the transmittance is minimal and amounts to 37-43%. The obtained biocomposition based on humic substances of peats and microorganisms of the genus *Rhodococcus* is able to stabilize oil emulsions in water by binding them with humic substances of peats as non-toxic complexes. Moreover, the peripheral parts of HS molecules are associated with light fractions of oil, mostly paraffins, due to hydrophobic bonds, the interaction of the aromatic framework with heavy fractions proceeds in the "stacking-interaction" type, which is carried out between parallel arranged aromatic structures due to van der Waals forces combining several types of intermolecular interactions. Persistent, difficult to exfoliate emulsions are formed, since the dispersed phase droplets, protected by a peculiar shell, an adsorption film, cannot merge with each other. Drops of oil surrounded by molecules of humic substances acquire negative buoyancy and pass from the surface into the water layer, where they are intensively decomposed by oil-degrading microorganisms due to the large contact surface.[13,15]

Thus, on the basis of humic substances of reed fen peat and hydrocarbon-oxidizing microorganisms *Rhodococcus erythropolis S67*, it is possible to develop an efficient system for stabilization, binding and recirculating of oil spills from aqueous environments.

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