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BIOLOGICAL METHODS OF WATER TREATMENT

Impact of Microbial *Nocardia Vaccinii* IMB B-7405 Surfactants on Oil Destruction in Water

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Abstract—The degradation degree of oil and complex oil pollutions with heavy metals in water in the presence of microbial *Nocardia vaccinii* IMB B-7405 surfactants has been studied. The destruction of oil in water (at concentrations of 2.6–6.0 g/dm^3) 25–30 days after the treatment with post-fermentative culture liquid (5–10 vol %) containing the surfactants amounted to 76–94%.

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INTRODUCTION

Oil is currently the main source of energy all over the world. At the same time, the probability of this xenobiotic getting into the environment tends to increase that is accompanied by negative consequences [1]. Since 1992 more than 20 emergency oil spills have occurred in the world that resulted in a considerable economic damage and disruption of the ecological balance. The liquidation of consequences of such accidents generally involves the use of physical and mechanical methods; however they are not always effective. According to data of the US Technology Assessment Department, the mechanical methods make it possible to remove no more than 10–15% of oil after a large-scale accident [1]. Biological techniques, including the direct introduction of oil oxidizing microorganisms (bioaugmentation) or the use of different substances stimulating the natural (autochthonous) microbiota (biostimulation), e.g. microbial surfactants, are considered promising for the liquidation of oil pollutions [2, 3]. The possible use of microorganisms for oil biodestruction in marine sediments was first described in paper [4].

In recent years the interest to microbial surfactants has been steadily growing that can be explained by a considerable progress in reducing the cost of their production processes and swift development of trends towards the conservation of environment [3, 5, 6]. Microbial surfactants can find wide application in nature protection technologies due to their ecological safety, ability to emulsify hydrophobic compounds, form complexes with heavy metals and enhance the efficiency of destruction of xenobiotics. In addition, the uniqueness of these metabolites consists in the fact that they can be obtained from industrial wastes and hereafter used for the destruction of pollutants, i.e., the effect of dual treatment of environment is achieved in production and application of microbial surfactants [3, 5, 6].

It is known that in the presence of heavy metals the efficiency of oil destruction can decline, therefore an important task is the search for the methods of ecosystem treatment from such complex pollutions [7]. One of the techniques for reducing the toxic impact of metals on cells-destructors is their binding by calcium carbonate, phosphates, chelates, clay minerals, and surfactants [8, 9].

Earlier [10] we reported about the separation of oil oxidizing bacteria *Nocardia vaccinii* K-8 (IMB B-7405) and the use of cells immobilized on keramzit (expanded clay) for water treatment from oil (100 mg/dm^3). Subsequent investigations revealed the ability of *N. vaccinii* IMB B-7405 to synthesize surfactants [11].

The purpose of this study is to investigate the effect of *N. vaccinii* IMB B-7405 surfactants on the destruction of oil in water and also complex oil pollutions with heavy metals.

EXPERIMENTAL

The main target of research is strain *Nocardia vaccinii* K-8 registered under number IMB B-7405 at the Depository of microorganisms at the Danylo Zabolotny Institute of Microbiology and Virology (NAS of Ukraine).

Strain *N. vaccinii* IMB B-7405 was cultivated in the following synthetic culture medium, g/dm^3: $NaNO_3$—0.5, $MgSO_4 \cdot 7H_2O$—0.1, $NaCl_2 \cdot 2H_2O$—0.1, KH_2PO_4—0.1, $FeSO_4 \cdot 7H_2O$—0.001, and autolyzed yeast—0.5 vol %. The source of carbon and energy was glycerin at concentration 1.0 vol %. The culture at the exponential growth phase cultivated in the medium of the specified composition containing 0.5% of glycerin was used as an inoculum. The amount of inoculum (10^4–10^5 colony-forming units (CFU/cm^3)) amounted to 5% of the culture medium volume. The cultivation of bacteria was performed in 750 cm^3 flasks containing 100 cm^3 of medium placed on a shaker (320 rev/min) at temperature 28–30°C during 120 h.

Post-fermentative culture liquid and supernatant were used as preparations of surfactants. For obtaining the supernatant the culture liquid was subjected to centrifuging (5000 g) during 30 min.

The simulation of water bodies polluted with oil and metals was performed by introducing the pumproom water in the amount of 2 dm^3 into a plastic tank. Next, we added the suspension of cells ((4.9–9.8) $\times 10^7$ CFU/cm^3) or surfactant preparations at concentration 5–15 vol % and also 0.05–1.0 mM Cu^{2+}, Cd^{2+}, and Pb^{2+} individually or in different combinations in the form of 1M solutions of salts $CuSO_4 \cdot 5H_2O$, $CdSO_4 \cdot 8H_2O$, and $Pb(CH_3COOH)_4$, respectively. Diammonium phosphate (0.01%) was used as a source of biogenic elements. Next, after six days one of the experiments involved the repeated treatment of water bodies with suspension of cells of strain IMB B-7405.

The total number of living cells in pumproom water during the experiment (7–30 days) was determined by Koch's method on meat-and-peptone agar (MPA).

The amount of oil was determined by the gravimetric method. To this end, the three-fold extraction of oil was performed by hexane (with ratio 1 : 1). The organic extract was evaporated to the constant weight on a rotary evaporator IR-1M2 (Russia) at 55°C and absolute pressure 0.04 MPa. This study was performed by using the oil with density 0.85 g/cm^3 from the oil field at Dolina, Ivano-Frankivsk Region.

In determining the protective properties of surfactants, the culture liquid after cultivating strain IMB B-7405 in liquid mineral medium to the middle of exponential and stationary phase was centrifuged (10000 g, 5 min). The cell sediment was double washed for the removal of medium residues by the sterile tap water and subjected to centrifuging (10000 g, 5 min). Next, the resultant sediment was resuspended in the initial volume of sterile tap water for obtaining the cells devoid of surfactants. The culture liquid (cells and surfactants) in the amount of 1.5 cm^3 and the cell suspension in the absence of surfactants (1.5 cm^3) were put into Eppendorf-type flasks. The initial (prior to introduction of metal cations) concentration of cells in the culture liquid and the cell suspension free of surfactants were determined by the Koch method on meat-and-peptone agar. Ions Cu^{2+}, Cd^{2+}, and Pb^{2+} (0.05–0.5 mM) were added to the culture liquid and cell suspension in the form of 1M solutions of salts $CuSO_4 \cdot 5H_2O$, $CdSO_4 \cdot 8H_2O$, and $Pb(CH_3COOH)_4$, respectively; the resultant fluid was held at thermostat (30°C) during one hour, and then the amount of living cells was determined by the Koch method on meat-and-peptone agar.

All tests were conducted with triple repetition; the number of parallel determinations in experiments varied from 3 to 5. Statistical processing of experimental data was conducted by the Lakin method as was described earlier [10, 11]. The differences of average indicators were considered authentic at the significance level $p < 0.05$.

RESULTS AND DISCUSSION

The mechanism of action of microbial surfactants is related to processes of desorption, solubilization of organic pollutants and, consequently, enhancing their bioavailability for microorganisms [3, 4, 12, 13]. Different variants of applying microbial surfactants for the treatment are proposed. They include the use of microorganisms-producers of surfactants for utilization of oil and its refinery products; treatment of most polluted areas by surfactant solutions in bioreactors; treatment of contaminated zone by surfactant solutions for solubilization of hydrocarbons that stimulates the growth of natural oil oxidizing microbiota (biostimulation) [12].

The literature data [13] reveal that the most effective removal of hydrocarbons can be achieved by using microorganisms capable of assimilating oil with simultaneous synthesis of surfactants. In this connection, for the main preparations of oil removal we used the culture liquid containing both the cells of oil oxidizing bacteria *N. vaccinii* IMB B-7405 and the surfactants produced by the latter.

The results of quantitative determination of residual oil in water (30 days after the treatment with surfactant preparations of strain IMB B-7405) are presented in Table 1.

Table 1. Indicators of the water treatment from oil by surfactant preparations of strain *N. vaccinia* IMB B-7405

Surfactant	Concentration		Degree of oil destruction, %
	Surfactant, %	Residual oil, g/dm^3	
Culture liquid	5	0.15 ± 0.007	94.2
	10	0.29 ± 0.014	88.8
	15	0.40 ± 0.020	84.6
Supernatant	5	0.45 ± 0.022	82.8
	10	0.54 ± 0.027	79.0
	15	0.55 ± 0.027	79.5

Note. Initial oil concentration in water amounted to 3.0 g/dm^3. The degree of oil destruction in control (not treated with surfactants) variant was 3.5%. In Tables 1–4 the error of determining the degree of oil destruction was within 5%; and the exposure in Tables 1, 2 and 4 amounted to 30 days.

In the presence of culture liquid and supernatant, the degree of oil destruction in water amounted to 79–94%. The maximum degradation of oil (94%) was observed in the case of using low concentrations (5%) of surfactant preparations in the form of culture liquid. Lower indicators of oil degradation in water in the presence of supernatant (79–83) as compared with those in the case of using the culture liquid (85–94%) can be evidence of the fact that producer cells take part in oil destruction.

In order to confirm this assumption, at the next stage we studied the impact of cells of strain IMB B-7405 on oil destruction in water (see Table 2).

Table 2. The impact of cell concentration of *N. vaccinia* IMB B-7405 on the degree of oil destruction in water

Cell concentration in suspension, CFU/cm^3	Number of treatment procedures	Concentration of residual oil, g/dm^3	Degree of oil destruction, %
$(9.8 \pm 0.5) \times 10^7$	1	0.14 ± 0.007	95.0
	2	0.45 ± 0.023	83.1
$(4.9 \pm 0.2) \times 10^7$	1	0.56 ± 0.028	79.2
	2	0.78 ± 0.14	70.8

Note. In Tables 2 and 4, the initial oil concentration in water amounted to 2.6 g/dm^3.

As can be seen from data presented in Table 2, the maximum degree of oil degradation (up to 95%) was achieved while using the suspension with a higher concentration of cells.

At the same time, a sufficiently high indicator of oil destruction after the treatment of polluted water by the supernatant of culture liquid of *N. vaccinii* IMB B-7405 (see Table 1) makes it possible to suggest that the main mechanism ensuring an active degradation of oil in the presence of surfactant preparations is their activation of natural oil oxidizing microbiota of water. It is also noted [14] that biostimulation of autochthonous microbiota of oil-polluted ecosystems was observed in the presence of supernatant of surfactant-containing culture liquids.

In view of the above, at the next stage we analyzed quantitative changes in the composition of water microbiota during the experiment. It was established that before the pollution with oil and the treatment by surfactant preparations, the cell concentration in water was 3.6×10^4 CFU/cm^3. The microbiota of such water was represented by four morphotypes of colonies. The total amount of water microbiota by the end of experiment increased by one-two orders of magnitude for different variants. The obtained data testifies in favor of our assumption about the activation of water microbiota by surfactant preparations of *N. vaccinii* IMB B-7405.

As was shown in paper [15], after the introduction of surface-active rhamnolipids and biogenic elements, oil degradation (at concentration 0.8 g/dm^3) in seawater amounted to 59% after five days, while the main mechanism of destruction was the stimulation by rhamnolipids of autochthonous sea microbiota.

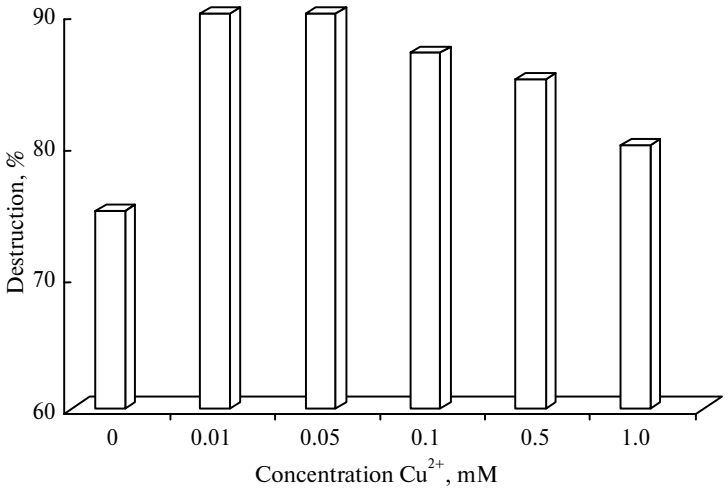
Our earlier investigations [16, 17] revealed the possibility of using surfactant preparations of *Acinetobacter calcoaceticus* K-4 (IMB B-7241) and *Rhodococcus erythropolis* IMB Ac-5017 for water treatment from oil. After 30 days, the degree of oil degradation (2.6–3.0 g/dm^3) in the presence of 5–30 vol % of surfactant preparations in the form of post-fermentative culture liquid or supernatant amounted to 81–95%. The intensifi-

cation of oil destruction was determined by the activation of natural oil oxidizing microbiota under the impact of surfactants of strains IMB Ac-5017 and IMB B-7241.

Further experiments showed that with the oil concentration in water increasing to the level of 6 g/dm^3, the degree of oil destruction in the presence of culture liquid (5 vol. %) of *N. vaccinii* IMB B-7405 reduced insignificantly (by 2–3%) as compared to the indicators of water treatment containing 2.6–3.0 g/dm^3 of oil.

Heavy metals are among the main pollutants of ecosystems [8], and they negatively affect microorganisms, thereby violating the biological balance of biosphere. The cations of the following metals: Ag, As, Be, Cd, Cr, Cu, Hg, Ni, Pb, Sb, Ti, and Zn are most often found in soil and water.

Data on oil destruction in water containing different concentrations of copper cations after its treatment with culture liquid of *N. vaccinii* IMB B-7405 is presented in the figure. In the presence of cation Cu^{2+}, the oil degradation after 20 days was much higher than without copper cations. The analysis of water microbiota during the experiment revealed the rise of cell number, however in the presence of copper cations and surfactant preparations the number of cells was 1.5–1.7 times as high as that without cation Cu^{2+}.



The impact of copper cations on oil destruction in water (3.0 g/dm^3) in the presence of culture liquid of *N. vaccinia* IMB B-7405 (5 vol %).

Next, we investigated the possible use of surfactant preparations of strain IMB B-7405 in the form of culture liquid for the treatment of water containing oil and cations of several toxic metals (see Table 3).

Table 3. The impact of culture liquid of *N. vaccinia* IMB B-7405 (10 vol %) on oil destruction (6.0 g/dm^3) in water in the presence of cations Cu^{2+}, Cd^{2+} and Pb^{2+}

Cation concentration in water, mM			Oil destruction, %	Number of cells, CFU/cm^3
Cu^{2+}	Cd^{2+}	Pb^{2+}		
0	0	0	76	$(4.9 \pm 0.24) \times 10^5$
0	0.5	0.5	65	$(2.1 \pm 0.10) \times 10^5$
0.5	0	0.5	78	$(6.5 \pm 0.32) \times 10^5$
0.5	0.5	0	81	$(7.1 \pm 0.35) \times 10^5$
0.5	0.5	0.5	82	$(7.2 \pm 0.36) \times 10^5$

Note. The number of cells in initial water (before the introduction of oil, surfactants, and metal cations) amounted to $(2.4 \pm 0.12) \times 10^3$ CFU/cm^3. The degree of oil destruction in the water that was not treated with surfactants and metal cations was 2.0%, while the exposure constituted to 25 days.

It was established that the lowest efficiency of oil destruction (65%) was observed in oil-polluted water in the presence of the mixture of cadmium and lead cations. Note that in the presence of copper cations and the mixture of metals the degree of oil degradation in water increased. The analysis of water microbiota showed that in the presence of copper cations and surfactants the total number of cells was several times as high as that without cation Cu^{2+} (see Table 3). We can assume that one of the mechanisms stipulating the rise of oil destruction in the presence of low concentrations of copper ions can be the stimulation of Cu^{2+} activity of

alkane hydroxylases (first enzymes of catabolism of hydrocarbons) of both strains-producers of surfactants and the natural oil oxidizing microbiota. Such an assumption is based on literature data regarding the fact that activators of monooxygenases are copper cations [18].

The display of protective functions of surfactants can be another mechanism forming the basis of sufficiently high efficiency of oil destruction by surfactant preparations of *N. vaccinii* IMB B-7405. It is well known [3, 9] that microbial surfactants are characterized by their ability to form stable complexes with different metals. Our experiments showed that the removal of surfactants from suspension led to the death of all *N. vaccinii* IMB B-7405 cells in the presence of Cu^{2+}, Cd^{2+}, and Pb^{2+} (0.05–0.5 mM). At the same time, in the presence of surfactants under the similar conditions, the number of surviving cells constituted 60–70%. Subsequent investigations showed that surfactants of strain IMB B-7405 displayed protective functions with respect to the native microbiota of water. Hence, the cell survival after treatment with cations of heavy metals (0.01–0.05 mM) in suspension with and without surfactants constituted 80–100 and 0–59%, respectively.

At the final stage we compared the efficiency of the Devoroil preparation consisting of five hydrocarbon-oxidizing bacteria and yeast with surfactant preparations of strain IMB B-7405 in the form of culture liquid (see Table 4).

Table 4. Biodestruction of oil in water, while using the Devoroil preparation and the culture liquid of *N. vaccinia* IMB B-7405

Preparation	Exposure, days	Concentration of residual oil, g/dm^3	Degree of oil destruction, %	Number of cells, CFU/cm^3
Devoroil	7	2.28 ± 0.11	12	$(2.2 \pm 0.11) \times 10^4$
	14	1.76 ± 0.09	32	$(7.2 \pm 0.36) \times 10^4$
	28	0.83 ± 0.04	68	$(2.0 \pm 0.1) \times 10^5$
Culture liquid of strain IMB B-7405	28	0.16 ± 0.01	94	$(3.0 \pm 0.15) \times 10^5$

Note. The number of cells in initial water (before the introduction of oil and treatment with preparations) constituted $(3.2 \pm 0.16) \times 10^3$ CFU/cm^3.

The selection of the Devoroil preparation among many other preparations was stipulated by the fact that it was one of the first that appeared on the market, and now it is best known and studied. The figures presented in Table 4 indicate that in the case of using this preparation after 28 days the degree of oil destruction in water amounted to 68%.

Note that under the similar conditions in the presence of surfactant preparations of *N. vaccinii* IMB B-7405 we could observe the oil degradation in the amount of 94%, while the number of cells of natural microbiota of water was higher than that after the treatment with Devoroil.

CONCLUSIONS

Thus, the high efficiency of applying low (5–10 vol %) concentrations of surfactant preparations of *N. vaccinii* IMB B-7405 in the form of the culture liquid for water treatment from oil (2.6–6.0 g/dm^3), in particular, in the presence of cations of toxic metals has been shown. It is assumed that the intensification of destruction of complex oil pollutions in the presence of surfactants and Cu^{2+} is determined by the stimulation of autochthonous microbiota as a result of oil solubilization, the activation by copper cations of alkane hydroxylases of both strains-producers of surfactants and the natural oil oxidizing microbiota, and protective functions of surfactants.

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