Use of rhizosphere microorganisms for bioremediation of oil contaminated soils

L A Belovezhets¹, M S Tretyakova², Yu A Markova² and A A Levchuk¹

¹Institute of Chemistry, Siberian Branch SB RAS, Russia
²Siberian Institute of Plant Physiology and Biochemistry SB RAS, Russia

E-mail: lyu-sya@yandex.ru

Abstract. Microbial soil remediation is one of the most promising methods for soil cleaning from oil and oil products. In order to search for promising oil destructors from the rhizosphere of plants growing in oil-polluted soils, bacteria that effectively destroy oil within a short time and belong to the genera Rhodococcus and Acinetobacter have been determined. Their ability to survive and decompose oil at its high and extremely high concentrations as well as at low positive temperatures has been shown. Microbial decomposition of oil model compounds (naphthalene, anthracene and phenanthrene) has been studied. All the studied strains have been established to be able to utilize these compounds with the formation of a group of metabolites, among which phthalic, gentisic and protocatechuic acids have been identified. The model compounds decrease ranged from 30 to 70% within 10 days.

1. Introduction and Background
The study of chemical mechanisms of oil biodegradation by microorganisms is a very promising issue since the chemical nature of oil products largely determines not only the scale of pollution but also the ways to eliminate them. Moreover, the problem of soil contamination with oil products is very important for oil producing and refining industries. Oil biodegradation by microorganisms is one of the ways to eliminate oil pollution [1-5]. Oil is a very complex and diverse chemical substrate, certain fractions of which are used by many microorganisms as a nourish source. Each of the fractions is processed by microorganisms differently. Thus, the ways of oil destruction are different and depend not only on the type of microorganism that consumes a specific fraction but on the chemical nature of the fraction, too. The use of individual compounds metabolized by specific strains of microorganisms makes it possible to establish the relationship between the method of a specific fraction destruction and its composition [6].

Thus, the purpose of this research was to study the biodegradation process and the oxidation kinetics of naphthalene, anthracene and phenanthrene in bacteria Rhodococcus sp. (108) and Acinetobacter sp. (112) and identify the obtained oxidation products qualitatively and quantitatively.

2. Materials and Methods
To study the oil degradation mechanism, Rhodococcus sp. (108) and Acinetobacter sp. (112) microorganisms were selected. They were previously isolated from the rhizosphere of couch grass (Elytrigia repens), selected on the oil-contaminated territory of the Irkutsk region (Tyret village) where there was a large-scale oil spill in 1993 [7]. Microorganisms were grown in mineral medium 8E with...
the composition: NaNO$_3$ – 3.0 g; K$_2$HPO$_4$ · 3 H$_2$O – 1.0 g; MgSO$_4$ · 7 H$_2$O – 0.5 g; KCl – 0.5 g; FeSO$_4$ · 7 H$_2$O – 0.01 g; D-glucose – 5 g, and tap water up to 1000 ml at a temperature of 26°C on a with stirring in the dark. To study the destruction, 2% (V/V) model compound was added to the liquid nutrient medium instead of glucose. Sampling was carried out after 3, 6, 9 days. 50 ml of the culture liquid was acidified with 2N HCl to pH 3.0 and extracted with ethyl acetate 1:1 (V:V). The solvent was then removed in a stream of cold air, and the residue was redissolved in 0.2 ml of methanol. The mineral medium without the addition of bacteria was control.

3. Experimental Section
HPLC was performed using the Agilent 1260 chromatographic system on the Zorbax SB-C18 5 μm column, 4.6 x 250 mm, with the Zorbax SB-C18 precolumn 5 μm, 4.6 x 12.5 mm. The column thermal regulation was 25 ºС. Spectra were recorded with a UV detector with a wavelength of 280 nm. Eluent – 25/75 ACN / 2% acetic acid, delivery rate – 1.3 ml/min, loop volume – 20 μL. Authentic commercial samples were applied as standard markers: protocatechuic (Serva, USA); benzoic (Reahim, Ukraine); phthalic (Reahim, Russia); gentisic acid (Sigma-Aldrich, United States); as well as pyrocatechin (Reahim, Russia).

4. Results and Discussion
Using enzymes, naphthalene and phenanthrene undergo transformation through two different mechanisms (pyrocatechin and protocatechuic acid) with the formation of mononuclear phenols, and then of acyclic hydrocarbons which are able to dissolve in water [8]. Determination of the oil degradation mechanism can be accomplished by detecting certain reference compounds characteristic only of this oxidation pathway. During HPLC, the presence of these reference compounds in the analyte composition indicates that the hydrocarbon has undergone an appropriate transformation mechanism. If both protocatechuic and pyrocatechuic acids are detected in analytes, it can be concluded that there are simultaneously two oxidation mechanisms [9].

The obtained data show (Table 1) that naphthalene degradation proceeds differently in the 108 and 112 strains. The 108 culture proceeds stepwise degradation, and there are 2 ways of its realization:

1) With the formation of gentisic acid through 1,2-dihydroxynaphthalene, salicylic aldehyde and salicylic acid;

2) With the successive formation of 1-hydroxy-2-napthoic acid, 2-carboxybenzaldehyde, o-phthalic acid and the formation of protocatechuic acid.

|       | 0 days | 3 days | 6 days |
|-------|--------|--------|--------|
| 108   | naphthalene 319.7 | 70 | 36 |
|       | protocatechuic acid - | 137.9 | 95.8 |
|       | phthalic acid - | 2.7 | - |
|       | gentisic acid - | 8.5 | - |
|       | naphthalene 319.7 | - | - |
| 112   | protocatechuic acid - | 136.1 | 150.2 |
|       | phthalic acid - | - | - |
|       | gentisic acid - | - | - |

The number of intermediates formed during the cultivation of 108 with naphthalene decreases with increasing the cultivation time. This suggests that, in addition to naphthalene transformation, the intermediates are also oxidized. The fact that protocatechuic acid is still detected in the culture medium on day 6 (despite the smaller quantities than on day 3) and phthalic and gentisic acids are not detected also confirms the hypothesis put forward.
The 112 culture is also capable of oxidizing naphthalene through two metabolic pathways. However, as the results of HPLC show, the main difference between the studied cultures is that the 112 one does not possess enzymes for further oxidation of protocatechuic acid, which accumulates in the culture medium, as its amount increases on day 6. From all of the above, it can be concluded that decomposition through protocatechuic acid is optional and is not fully accomplished.

At the same time, the authors do not detect salicylic acid peaks in chromatograms. That indicates its rapid transformation to other intermediates. On the contrary, earlier studies on crude oil decomposition revealed the presence of salicylic acid in all samples and at all stages of the experiment [10]. This is due to differences in an individual compound decomposition and in a complex mixture of aromatic substances. The inhibition of enzymes by oil destruction intermediates by the type of negative feedback is also possible [10].

The case of pyrocatechin absence among naphthalene decomposition intermediate compounds is of interest. The naphthalene metabolism in these two cultures is perhaps to occur not entering into the stage of pyrocatechin formation.

There are 2 metabolic pathways in the case of phenanthrene degradation (Table 2):

1) With the formation of gentisic acid as a result of the sequential oxidation of phenanthrene to 1-hydroxy-2-naphthoic acid, then to 1,2-dihydroxynaphthalene, salicylic aldehyde and salicylic acid;

2) With the formation of o-phthalic and protocatechuic acids as intermediates as a result of phenanthrene oxidation of to 1-hydroxy-2-naphthoic acid and 2-carboxybenzaldehyde [11–13].

| Table 2. Sum of phenanthrene degradation intermediates. |
|---------------------------------------------------------|
|              | 0 days | 3 days | 6 days | 9 days |
| phenanthrene  | 225.4  | 136.7  | 60.3   | 37.6   |
| protocatechuic acid | 0     | 81.7   | 26.5   | 3.9    |
| phthalic acid  | 0      | 60.5   | 31.5   | 4.3    |
| gentisic acid  | 0      | 87.7   | 44.2   | 6.4    |
| phenanthrene  | 225.4  | 60.2   | 71.3   | 60.8   |
| protocatechuic acid | 0     | 122.7  | 128.9  | 302.1  |
| phthalic acid  | 0      | 3.6    | 4.1    | 50.9   |
| gentisic acid  | 0      | 2.6    | 9.6    | 61.3   |

Protocatechuic acid should be remembered to undergo further transformation through the mechanism of ortho- or meta-cleavage [14]. In its turn, gentisic acid is metabolized to intermediates of the tricarboxylic acid cycle (pyruvate and fumarate) [15].

The amount of phenanthrene in the cultivation medium of the studied strains sharply decreased on day 3. However, during phenanthrene decomposition by Rhodococcus, its amount continued to decrease throughout the experiment, whereas in the case of Acinetobacter sp. (112), the residual phenanthrene amount remained at the level of day 3. This is perhaps due to the inhibition of decomposition by the accumulating intermediate, primarily by protocatechuic acid. The decomposition dynamics of the intermediates of the studied strains differed significantly. Rhodococcus was characterized by a decrease in the number of all identified intermediates throughout the experiment. At the same time, the accumulation of these substances in the culture medium Acinetobacter sp. (112) was marked.

Anthracene oxidation, as in the case of naphthalene and phenanthrene, proceeds in 2 ways [16]:

1) With the formation of protocatechuic acid from o-phthalic one through 1,2-dihydroxynaphthalene, cis-4-(2-hydroxynaphth-3-yl)-2-oxobutyric-3-enoi acid, 6,7-benzocoumarin and 3-hydroxy-2-naphthoic acid;
2) With the formation of gentisic acid through anthracene-1,2-dihydrodiol, 6,7-benzocoumarin, 3-hydroxy-2-naphthoic acid, 2,3-dihydroxyanthraquinone and salicylic acid. According to HPLC, it is difficult to quantify and qualitatively describe the products of anthracene oxidation since there are no visible changes throughout the experiment. However, developed diagrams view using the HPLC software revealed difficult identifiable peaks indicating the presence of protocatechic and gentisic acid in the culture medium approximately in the same amount. These results can be explained by the complexity of anthracene chemical structure. As the result, bacterial attack and enzymatic metabolism of this compound are complicated.

5. Summary and Conclusion
The research resulted in the study of the process of biochemical enzymatic oxidation of model oil compounds, such as naphthalene and phenanthrene, in Rhodococcus sp. (108) and Acinetobacter sp. (112). HPLC method confirmed the available theoretical data on the possible chemical mechanisms which are the basis for oil biodegradation. The oxidation kinetics, indicating that naphthalene is more accessible to oxidation by both strains than phenanthrene, was investigated. If to speak about phenanthrene, bacteria need an adaptation period due to the chemical structure complexity.

Acknowledgment
The study was supported by a grant from RFBR and the Government of Irkutsk region, project № 17-45-388078 p.a.

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