New strains of oil-degrading microorganisms for treating contaminated soils and wastes

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Abstract. Two new strains Achromobacter marplatensis101n and Acinetobacter sp. S-33, capable of degrading 49 and 46% of oil within 7 days were isolated, identified, and characterized. The application of A. marplatensis 101n in combination with ammonium nitrate (100 mg·kg⁻¹) for 30 days of cultivation resulted in the degradation of 49% of the initial total petroleum hydrocarbon content (274 g·kg⁻¹) in the original highly acid (pH 4.9) oil-contaminated waste. Up to 30% of oil sludge added to a liquid mineral medium at a concentration of 15% was degraded after 10 days of cultivation of A. marplatensis 101n. Application of yellow alfalfa (Medicago falcata L.) plants with Acinetobacter sp. S-33 for bioremediation of oil-sludge-contaminated soil improved the quality of cleanup in comparison with the bacterium- or plant-only treatment. Inoculation of Acinetobacter sp. S-33 increased the growth of both roots and shoots by more than 40%, and positively influenced the soil microflora. We conclude that the new oil-degrading strains, Acinetobacter sp. S-33 and A. marplatensis 101n, can serve as the basis for new bioremediation agents for the treatment of oil contaminated soils and waste.

1. Introduction
In the modern world, hydrocarbons are the most common and demanded raw material and energy source, and, as a result, the worst pollutants of the environment, especially in the regions where they are extracted, transported, and processed. As a result of the activities of oil and gas enterprises, there is a need to clean up soil and water bodies affected by oil pollution, as well as the problem of utilization of processing wastes containing significant amounts of hydrocarbon pollutants [1]. A modern approach to the purification of oil-contaminated objects is the application of bio- and phytoremediation technologies based on the use of vital activity of living organisms. Plant-microbial associations and symbiosis with a flexible metabolism and unique enzyme systems have great potential for survival under extreme conditions of oil-polluted ecosystems, and their survival is caused not only by increased tolerance to xenobiotics but also by the active removal of toxic substances from the environment [2, 3]. Microorganisms that utilize hydrocarbons as the sole carbon and energy source are the basis of such bioremediation technologies as biostimulation, bio-augmentation, and rhizoremediation [4-6]. In the past decades, numerous bioremediation agents have been developed that contain oil-degrading bacteria based on monocultures and microbial consortia, with fertilizers, structuring agents, and emulsifiers for improving the quality of remediation [7]. The use of microbial oil-degrading agents over the years has also revealed some problems, among which are the lack of equal effectiveness of existing bioremediation agents under different climatic conditions; gradual loss...
of specific activity of the microbial strains forming the basis of a particular bioremediation agent; and the frequent discrepancy between commercial products and the declared characteristics of bioremediation agents, not only in oil-degrading activity, but also in microbial composition. At the same time, it has been noted that the bioremediation agents based on natural hydrocarbon oxidizing microorganisms isolated in a specific climatic zone are the most effective, because the introduced microflora that is not characteristic of a particular ecosystem can be suppressed by indigenous microbial populations [8,9]. All the above-mentioned indicates that the search for new strains of oil-degrading microorganisms and the development of alternative bioremediation agents based on them remain relevant, because their application in environmental biotechnology is still in demand with environmental organizations.

New oil-degrading microbial strains isolated from oil-sludge and oil-contaminated rhizosphere soil were studied in this work for use in bio-and phytoremediation technologies.

2. Material and methods

2.1. Microorganisms and cultivation conditions
We used 80 oil-hydrocarbon-degrading microorganisms: 26 strains from the Collection of Rhizosphere Microorganisms of the IBPPM RAS and 54 new isolates from oil contaminated rhizosphere and nonrhizosphere soil samples.

Samples of oil sludge and oil-contaminated soil, and methods of direct plating and enrichment cultivation were used to isolate microorganisms able to degrade petroleum hydrocarbons. For the cultivation of microorganisms, beef-extract agar, LB broth and Bushnell-Haas’s mineral medium for hydrocarbon-oxidizing microorganisms were used [10].

The total number of culturable heterotrophic microorganisms (THMs) in the samples was determined by the standard plating dilutions method. The number of hydrocarbon-oxidizing microorganisms (HOMs) was determined by the method of membrane filters and by cultivation of immobilized microbial cells on Bushnell-Haas’s agar medium, as described previously [11]. Inoculated plates were incubated at 29 °C for 7 days, after which the CFU was calculated.

2.2. Study of oil-degrading activity of microbial isolates
The degradative activity of the isolated microbial strains was studied by culturing them in tubes or flasks with Bushnell-Haas’s liquid medium containing 1% (w v⁻¹) of crude oil as the sole source of carbon and energy. Cultivation was carried out under aeration conditions on a rocking shaker (160 rpm) for 7 days. The initial optical density of the microbial suspension was D = 0.5 (λ = 590 nm). The degradative activity of the microorganisms was determined by the loss of oil from the medium after cultivation and expressed as a percentage.

2.3. Identification of microbial isolates
The taxonomic affiliation of the isolated microorganisms was determined on the basis of a study of the cultural-morphological and physiological-biochemical features, as well as 16S rRNA gene sequence analysis, by using the EzTaxon server (http://www.ezbiocloud.net/eztaxon) [12] and using Bergey’s Manual of Systematic Bacteriology [13,14].

2.4. Analyses of oil content
The residual oil content in the medium after cultivation was determined by preliminary extraction with nonpolar solvents, gas and liquid chromatography, and gravimetric analysis. For the primary evaluation of the degradative activity of microorganisms, the content of oil remaining in the medium was determined by gravimetric analysis after preliminary trichloromethane extraction of oil hydrocarbons from the culture medium [15]. Gas and liquid chromatography with selective extraction of oil fractions was used to characterize the range of microbial degradation of hydrocarbons. For gas chromatographic analysis, the extraction of oil from the culture medium was carried out with carbon
tetrachloride, and the extracts were analyzed according to [15] on a Shimadzu 2010 gas chromatograph fitted with an Equity-1 non-polar capillary column (Supelco, USA) with a flame ionization detector (40 ms), makeup gas, He; H2 flow, 40 ml·min⁻¹; air flow, 400 ml·min⁻¹; makeup gas flow 30 ml·min⁻¹; carrier gas, He. The sample volume was 1 μl in all cases. Fractional analysis of the contaminant remaining after the cultivation of microorganisms was carried out by separating the oil into fractions by liquid column chromatography, selective extraction of various hydrocarbon classes with different solvents, and a gravimetric method for determining their share [16]. Residual oil was divided into the following fractions: paraffins, naphthenes, mono- and bicyclic aromatic hydrocarbons (MBCAs), polycyclic aromatic hydrocarbons (PAHs) and alcohol-benzene tars (ABTs). In actual waste samples the concentration of total petroleum hydrocarbons was determined by gravimetric analysis by using the standard procedure [17]. All samples were analyzed in triplicate.

2.5. Bioremediation of original oil-contaminated soils and wastes

Two types of wastes were used to test the activity of selected oil-degrading strains: oil contaminated soil and oil sludge.

The oil-contaminated soil contained 24.6% of oil sludge and 2.1% of mechanical impurities; the pH value was 4.9. For studying the possibility of bioremediation, oil-contaminated soil was thoroughly mixed, a sample of soil (200 g) was taken for preliminary analyses, and 600-g portions were placed in 2-liter containers. There were four experimental treatments: 1) untreated soil (control); 2) treatment with a mineral nitrogen fertilizer; 3) treatment with oil-degrading microorganism; 4) joint use of mineral fertilizer and a microorganism. The soil was treated according to the following scheme: loosening, daily; moistening of the soil up to 40% of the total moisture capacity, once a week; introduction of the oil-degrading microorganism *A. marplatensis* 101n (up to a final cell concentration of not less than 10⁷ CFU·g⁻¹ of soil), every 2 weeks; application of nitrogen fertilizer (NH₄NO₃, 100 mg·kg⁻¹), every 3 weeks. The bioremediation experiment lasted 30 days.

Oil sludge was a thick viscous liquid containing 8.2% of water, 1.6% of mechanical impurities, and 90.2% of oil hydrocarbons; the pH value was 6.4. Oil sludge was treated by cultivating *A. marplatensis* 101n in a flask with liquid Bushnell-Haas’s medium containing 15% (w·v⁻¹) of oil sludge. The initial optical density of the microbial suspension was \( D = 0.42 \) (λ = 590 nm), which approximately corresponded to not less than 10⁷ cells per ml. Cultivation was carried out under aeration conditions on a circular shaker at 140 rpm, at 28° C for 10 days. At the end of the cultivation, the residual oil content in the medium was analyzed.

To test the effectiveness of application of the oil-degrading microorganisms alone and in combination with plants, we used the original oil-sludge-contaminated soil (pH 8.3; petroleum hydrocarbons, 14.3 g·kg⁻¹; P₂O₅ 150 mg·kg⁻¹; N-NO₃ 10 mg·kg⁻¹; N-NH₃ - 25.9 mg·kg⁻¹), *Acinetobacter* sp. strain S-33 and yellow alfalfa plants (*Medicago falcata* L.). The soil experiment was carried out in 2-l pots containing 1 kg of soil in three replicates: 1) untreated soil (control), 2) treatment with *Acinetobacter* sp. S-33 to a final cell concentration in the soil of at least 10⁷ CFU/g soil (bioaugmentation); 3) plant cultivation (phytoremediation); 4) cultivation of the plant and inoculation with *Acinetobacter* sp. S-33 (phytoremediation with bioaugmentation). To compensate for nitrogen deficiency, which can limit the biodegradation of oil in the soil, ammonium nitrate (NH₄NO₃) in concentrations of 200 mg·kg⁻¹ was added every 3 weeks to 2-4 treatments of the experiment. Experimental conditions were: light period, 16/8 h (day:night⁻¹); humidity, 40%; watering by weight; temperature, 21-24 °C; duration, 2.5 months. At the end of the experiment, loss of oil hydrocarbons, microbial counts, and plant biomass were estimated.

2.6. Statistics

All experiments and analyzes were performed at least in triplicates. Data were subjected to statistical processing with Microsoft Office Excel 2003.
3. Results and discussion

3.1. Oil-degrading activity of selected microbial strains

As a result of screening of 80 strains of rhizosphere and non-rhizosphere microorganisms, two most active oil degraders were selected: strain S-33 was isolated from the rhizosphere of *Medicago falcata*, and strain 101n was isolated from an oil sludge sample. A study of the degradative activity of these microorganisms toward oil (1%) showed that strain S-33 was capable of destroying 49%, and strain 101n 46%, of oil for 7 days. As shown by gas chromatography, these microorganisms destroyed 78.4 and 85.2% of oil, respectively. Hydrocarbons with an average length of the carbon chain (C12-C-28) were the most degraded (figure 1).

Fractional analysis of residual oil indicated that all main fractions were subjected to microbial degradation: paraffins, naphthenes, mono-, bi- and polycyclic aromatic hydrocarbons, as well as alcohol-benzene tars (figure 2). Strain 101n better degraded aromatic fractions, whereas the rhizosphere strain S-33 degraded saturated oil hydrocarbons the most.

![Figure 1](image1.png)

**Figure 1.** Degradation of oil hydrocarbons by strains 101n (*a*) and S-33 (*b*) according to gas chromatography data: ■, initial untreated control; □, after cultivation of microorganisms.

![Figure 2](image2.png)

**Figure 2.** Degradation of oil fractions by strains S-33 and 101n grown in 1% (v·v⁻¹) oil-containing mineral medium for 7 days: ■, initial untreated control; □, final untreated control;□, strain S-33; ■, strain 101n; *, significant differences between treatments and initial control (P≤ 0.05); ***, significant differences between treatments and untreated control (P≤0.05).
Comparison of the selected microorganisms with the previously described biological agents showed that the degradative activity of the new strains toward oil was in the range of activity reported for other microbial strains used, for example, in such biological agents as Ekoil [18] and Roder [19], other compositions [20].

On the basis of a study of cultural-morphological, physiological-biochemical and genetic characteristics, new oil-degrading strains S-33 and 101n were identified as Acinetobacter sp. and Achromobacter marplatensis, respectively.

3.2. Testing of oil-degrading activity of A. marplatensis 101n with original oil-containing wastes

The oil oxidizing activity of A. marplatensis strain 101n with the use of original oil-containing soil showed that the most successful bioremediation approach was the combined use of the oil-degrading microorganism and nitrogen fertilizer, in which the degradation of petroleum hydrocarbons was 49\% (table 1).

Table 1. Changes in characteristics of oil-contaminated soil during remediation

| Experimental treatments | pH   | Microbial count (CFU g\(^{-1}\)) | Oil hydrocarbons (g kg\(^{-1}\)) | Degradation (%) |
|-------------------------|------|----------------------------------|----------------------------------|-----------------|
|                         |      | THMs                             | HOMs                             |                 |
| Oil-contaminated soil   |      |                                  |                                  |                 |
| Initial (0 days)        | 4.9  | \(5.4 \times 10^6\)              | \(1.2 \times 10^4\)              | 274             |
| Untreated control (30 days) | 3.5  | \(1.0 \times 10^6\)              | 0                                | 5.2             |
| Agrotechnics – loosening, watering, fertilization (30 days) | 3.1  | \(1.1 \times 10^7\)              | \(2.4 \times 10^5\)              | 244             |
| A. marplatensis 101n (30 days) | 3.0  | \(5.0 \times 10^6\)              | \(1.7 \times 10^5\)              | 216             |
| A. marplatensis 101n + Agrotechnics (30 days) | 3.0  | \(2.5 \times 10^6\)              | \(3.9 \times 10^5\)              | 140             |

Oil sludge

|                         |      |                                  |                                  |                 |
|-------------------------|------|----------------------------------|----------------------------------|-----------------|
| Initial                 | 6.4  | \(2.5 \times 10^6\)              | \(-^a\)                          | 858             |
| Abiotic control (0 days) | 7.2  | –                                | –                                | 150             |
| A. marplatensis 101n (10 days) | 7.1  | \(\geq 10^b\)                    | 105                              | 29.9            |

\(^a\) HOMs were not detected by direct plating of the oil sludge sample, but they were detected as a result of enrichment culture.

\(^b\) the initial seed dose of the microbial suspension of A. marplatensis 101n.

This is a very good result, with account taken of high acidity of the soil treated. The effectiveness of microbial degradation of hydrocarbons by A. marplatensis strain 101n was confirmed by cleaning the soil using a single microorganism by 21\%. The stimulation of oil-oxidizing activity of the indigenous microflora was not effective enough for bioremediation of this soil – only 11\%. The application of the microorganism with nitrogen fertilizer led to distinct changes in the soil as early as the first days after treatment. The soil became more friable, the lumps of pollutant were more easily destroyed during loosening, presumably due to the emulsifying activity of the introduced oil-degrading microorganism, which was clearly visible in the first laboratory experiments. However, the dynamics of such changes slowed down with time, which may be due to a decrease in soil pH and to inhibition of the strain’s activity. By the end of the experiment, there was considerable acidification of the soil in all experimental treatments, which indicated its low buffer capacity. A decrease in pH to 3.0-3.5 inhibited the development of native bacterial populations but promoted the growth of fungi, which was observed in the untreated control, in which micromycetes did not compete with the bacteria.
introduced into other experimental treatments. In all variants of the experiment, with the exception of the untreated control, in comparison with the baseline, there was an increase in the number of HOMs, including micromycetes, which should also facilitate the cleanup.

*A. marplatensis* strain 101n successfully degraded oil hydrocarbons as part of oil sludge. As can be seen from Table 1, the degradation of oil sludge by *A. marplatensis* 101n was 30%. A comparison of the degradative activity of strain 101n tested for the treatment of oily wastes with previously reported data on other biological agents [21] allowed us to conclude that the microbial bioremediation agents developed on the basis of this microorganism can be promising for treating oil-containing wastes.

### 3.3. Application of *Acinetobacter* sp. S-33 and *M. falcata* for remediation of oil-contaminated soil

The prospects of using alfalfa for phytoremediation of oil-contaminated soil were shown previously [16, 22], and the additional application of a microbial inoculant can improve the remediation efficiency. As a result of a 2.5 months’ soil experiment on bioremediation of the original oil-sludge-contaminated soil (14.3 g·kg⁻¹) by using *Acinetobacter* sp. strain S-33 and yellow alfalfa, it was shown that the use of the oil-degrading microorganism for purification both as an monoculture biogent and in combination with the plant significantly increased pollutant loss (Figure 3a). Soil cleanup in the control was 20%, and the application of yellow alfalfa yielded 34%. Application of *Acinetobacter* sp. S-33 only allowed cleaning of the soil by 35%; and the use of this microorganism in combination with alfalfa, by 39%. Fractional analysis of the residual contamination showed that the plant-microbial association was the most effective in remediation of soil from aromatic hydrocarbons, significantly degrading the MBCA and PAH fractions (figure 3b).

![Figure 3. Bioremediation of original oil-sludge-contaminated soil by different approaches: a, TPH content in soil; b, oil fraction content in soil; , initial oil-contaminated soil; , untreated control; , phytoremediation with *M. falcata*; , bioaugmentation with *Acinetobacter* sp. S-33; , phytoremediation (*M. falcata*) with bioaugmentation (*Acinetobacter* sp. S-33); *, significant differences between treatments and initial control (*P* ≤ 0.05); **, significant differences between treatments and untreated control (*P* ≤ 0.05).](image-url)

Introduction of *Acinetobacter* sp. S-33 into soil had a significant effect on the soil microflora. It reduced the number of THMs by 46%, the planting of alfalfa increased eightfold (table 2). Additional application of *Acinetobacter* sp. S-33 in combination with alfalfa resulted in 10-fold increase in the THMs in comparison with the control and had a marked effect on the population of HOMs, reducing it by 20 and 24% in treatments without the plants and with alfalfa. Such quantitative changes in the oil-
degrading microbial populations could reflect a competition between the introduced and the indigenous microorganisms.

**Table 2.** Effect of the introduced strain *Acinetobacter* sp. S-33 on the soil microflora.

| Experimental treatments | Microbial count, $\times 10^3$ (CFU g$^\text{−1}$) |
|-------------------------|-----------------------------------------------|
|                         | THMs  | HOMs             |
| Untreated control       | 11.67 $\pm$ 3.04 | 2.52 $\pm$ 0.18 |
| *Acinetobacter* sp. S-33| 6.30 $\pm$ 2.10 | 2.04 $\pm$ 0.41 |
| *M. falcata*            | 88.66 $\pm$ 31.64 | 29.93 $\pm$ 1.86 |
| *M. falcata* +*Acinetobacter* sp. S-33 | 111.22 $\pm$ 51.61 | 22.94 $\pm$ 6.26 |

Inoculation of alfalfa plants with *Acinetobacter* sp. S-33 increased the accumulation of shoot and root biomass by 44 and 47%, respectively. Thus, in our soil experiment, the rhizobacterium *Acinetobacter* sp. S-33 exhibited plant-growth-promoting activity and, thus, can be considered to be a promising agent for the phytoremediation of oil-contaminated soil.

**Conclusion**

From the results of our experiments, we conclude that the new oil-degrading microbial strains *Acinetobacter* sp.S-33 and *A. marplatensis*101n can serve as the basis for microbial bioremediation agents for the treatment of oil contaminated soils and wastes.

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