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HYDROCARBON OXIDIZING MICROORGANISMS: THEIR ISOLATION AND STUDY OF COLONIZATION CAPACITY FOR THE USE IN RHIZOREMEDIATION PROCESSES OF CONTAMINATED SOILS

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Abstract:

Developing methods to clean up the environment from oil and oil sludge contaminations is a priority area of the environmental biotechnology. Despite significant advances in the field of studies, the issue of expanding the methods of soil purification from oil contamination remains open. In our studies, we, first of all, obtained a hydrocarbon oxidizing bacteria capable of the active colonization of plant rhizosphere and the oil degradation at the same time. Among the isolated strains, the strains belonging to the *Pseudomonas* genus had the greatest capacity to colonise plant roots and oil degradation. Identification of the main strains with high colonization capacity via biotyper and 16S rRNA gene analysis has shown that *Pseudomonas putida* and *Pseudomonas fluorescens* relate to the species that constitute a high priority for rhizoremediation. These microorganisms colonized the rye (*Secale cereale*) roots with an average density of $1.1-2.0 \times 10^6$ cells per centimetre of root length. Furthermore, at inoculation of germinated rye seeds and rye seeds treated with hydrocarbon bacteria, distribution of the bacteria degrading oil was observed over the entire area where the plant roots are growing. The total number of bacteria on the plant roots did not only maintain, but increased. Therefore, the isolated and identified strains of *Pseudomonas putida* and *Pseudomonas fluorescens* bacteria can be recommended for the use in rhizoremediation methods.

Key words: microorganisms, oil degradation, rhizoremediation, rye, colonization.

Introduction

Active development of the petrochemical industry leads to environmental contamination at all stages of oil production, transportation and refining. In this case, the main anthropogenic attack is primarily made on the soil. The physico-chemical methods to remove such contamination often cause irreversible damage to the soils of contaminated areas and they are expensive and difficult to use. On the other hand, the biotechnological approaches

are cheap, more reliable and safe. They are reduced to the degradation of oil components by microorganisms - bioremediation and land recultivation using plants - phytoremediation. But there are a currently method of biotechnological recultivation of the oil contaminated soil: – rhizoremediation - combines both of these approaches. It provides for the preparation and use of rhizospheric microorganisms that can simultaneously stimulate the plant growth and effectively degrade the xenobiotics that enter the soil when oil spills occur. The rhizosphere is a soil area nearest to the plant roots and, therefore, it is under the direct influence of the root system. Plants provide microorganisms with root exudates consisting of sugars, alcohols, acids, enzymes, which are sources of carbon and energy ensuring their development. It has been established that due to exudates, the number of cells in the microbial population of rhizosphere is 5-100 times higher than in the soil not contacting with the plant roots [1,2]. On other hand, a plant with an increased microbial population is capable of active degradation of organic contaminants in the rhizosphere. The root secretions are a link between plants and microorganisms, which leads to the rhizosphere effect. However, the exudation type and amount depend on the type and stage of plant growth, soil type and other factors that directly affect the diversity of microorganisms growing in the immediate vicinity of the plant roots. Microorganisms with the ability to degradate of organic contaminants play an important role in cleaning up the environment from oil contaminations. Sadly, there is a very small amount of published works related to the development of specific methods and techniques for isolation and study of microorganisms in the root zone and rhizosphere of plants.

These studies on the development of rhizoremediation methods for oil contaminated soils have been aimed at obtaining active colonizers of plants capable of using oil hydrocarbons as the sole carbon and energy source.

Materials and Methods

The rhizospheric microorganisms isolated from oil contaminated soils in the Almetyevsk District of the Republic of Tatarstan were chosen as the objects of study. The sterility-based samples were taken at the level of 10-25 cm from the surface. The samples were transported at 4°C.

The microorganisms were isolated by the method proposed by Markarov and Archegova [3]. The selected sample of oil contaminated soil (100 g) was filled with the sterile Muntz mineral medium (1 l) and incubated with shaking flasks in a thermostat at 28°C. After 7 days of cultivation, for the purpose of further selection of microorganisms and producing enrichment culture of hydrocarbon oxidizing bacteria the obtained soil slurry was subcultured on the Muntz medium with the addition of crude oil at a concentration of 1 volume percent. Then the subcultivation

procedure was repeated several times and completed with inoculation and many-fold dilution of samples on the meat infusion agar-agar. The isolated colonies of the microorganisms grown on the surface of nutrient agar were identified with MALDI Biotyper using cells of 96-well steel target plate (MSP 96 target ground/polished steel, Bruker Daltonics GmbH, Germany) and Biotyper matrix solution. The colonization capacity of microorganism strains was detected by means of inoculation of sterile, germinated rye seeds using the microorganisms capable of the hydrocarbon biodegradation [4, 5]. Sterile seeds were cultivated on filter paper soaked with water for 3 days. After the formation of sprouts, they were placed into vials, which contained the bacterial suspension, and stirred for 10 minutes. Then inoculation was made on the conventional gnotobiotic system (7 mL of quartz sand + 5% plant nutrient solution) [6]. The incubation lasted 7 days at room temperature. After the cultivation had been over, a part of root was cut off and aseptically triturated in a mortar; 10 ml of sterile saline was added [8]. Then, the resulting suspension was subsequently diluted (10^{-2} , 10^{-3} , 10^{-4}) and plated on the meat infusion agar-agar. The plating was cultured for 3 days at 28°C. On expiry of the cultivation period, grown colonies were counted, and isolates showing a different morphology were recovered. The initial density of colonizing mixture amounted to 1×10^6 cells/ml. The species affiliation of bacteria was determined by the PCR method - amplification and sequencing of the 16S rRNA gene using standard molecular biology methods (polymerase chain reaction, isolation of DNA fragments from agarose gel, identification and analysis of nucleotide sequences). To develop segment of the 16S rRNA gene, forward (BD) (AGAGTTTGATCCTGGCTCAG) and reverse (FD) primer (AAGGAGGTGATCCAGCCGCA) [9] was used. The stages of polymerase chain reaction included initial denaturation of the DNA strands at 94°C during 1 min; 30 cycles, each consisting of 30 seconds at 94°C, 30 seconds at 55°C and 1 min at 72°C; the final stage lasted 5 minutes at 72°C. The amplified DNA segments were detected by means of horizontal gel electrophoresis (BioRad, USA).

To conduct the RFLP analysis (restriction fragment length polymorphism), RsaI restriction enzymes were used. Restriction fragments were detected by electrophoresis in 12.5% polyacrylamide gel under the denaturing conditions.

Results and Discussion

We searched microorganisms with the ability to biodegrade petroleum hydrocarbons were searched in soil samples contaminated with oil and taken from natural sources. Using a medium containing the mineral composition allowed obtaining an enrichment culture, which was able to use petroleum hydrocarbons as carbon nutrition.

25 strains of bacteria were determined in the composition of this culture; they have different morphological properties. Identification of colonies in the individual strains by means of the biotyper helped reduce a diversity of

isolated strains up to 9 bacterial species. Among them, the largest number of strains related to the *Pseudomonas* genus. The isolated and identified bacteria belonged to the species: *Pseudomonas putida*, *Pseudomonas aureginosa*, *Burkholderia cepacia*, *Aeromonas hydrophylla*, *Serratia marcescens*, *Delftia thsuruhatensis*, *Pseudomonas fluorescens*, *Aeromonas salmonicida*, *Pantoea aglomerance*. In order to use these bacteria in the rhizoremediation processes, it was necessary to check the colonization capacity of plant roots. To this end, the germinated rye seeds were treated with a suspension of individual isolated strains (Table 1).

Table 1. Colonization capacity of hydrocarbon oxidizing bacteria.

Strains	Apical part of the root in CFU/cm after a 7-day incubation
<i>Pseudomonas putida</i>	$6.50 \pm 0.2 \times 10^5$
<i>Pseudomonas aureginosa</i>	$2.80 \pm 0.2 \times 10^5$
<i>Burkholderia cepacia</i>	$2.60 \pm 0.2E \times 10^4$
<i>Aeromonas hydrophylla</i>	$1.40 \pm 0.2 \times 10^4$
<i>Pseudomonas fluorescens</i>	$3.50 \pm 0.3 \times 10^5$
<i>Aeromonas salmonicida</i>	$1.40 \pm 0.2 \times 10^2$

When treating with isolated strains of *Serratia marcescens*, *Delftia thsuruhatensis*, *Pantoea aglomerance*, single colonies were observed at the apical part of the rye root.

The studies have showed that only 6 strains of isolated hydrocarbon oxidizing microorganisms were able to colonize an apical part of the rye roots. The strains of *Serratia marcescens*, *Delftia thsuruhatensis*, *Pantoea aglomerance* had mild colonization capacity.

Additional study of species affiliation of the strains having colonization capacity was carried out using molecular genetic methods, namely the 16S rRNA gene fragments treated with the *RsaI* restriction enzyme (Figure 1).

Analysis of Isolates

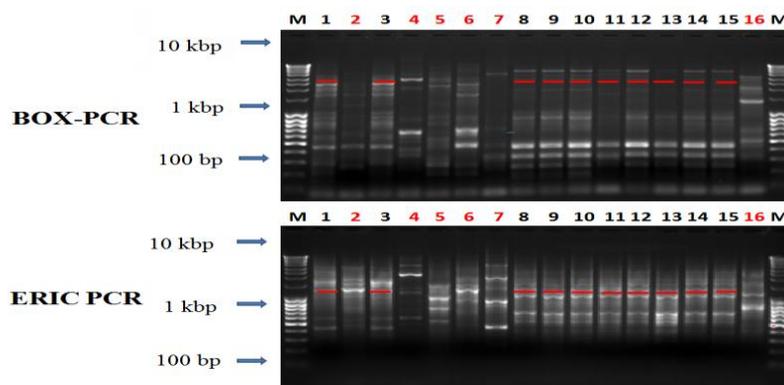


Figure 1. Identification of bacteria capable of biodegradation of petroleum hydrocarbons.

***Pseudomonas putida*(2), *Pseudomonas aureginosa*(4), *Burkholderia cepacia*(5), *Aeromonas hydrophylla*(6), *Pseudomonas fluorescens*(7), *Aeromonas salmonicida*(16).**

The results obtained by the PCR confirmed the species affiliation of six studied strains to the species listed above.

However, their comparative colonization capacity varied over a wide range (Figure 2). Only 2 strains of bacteria, namely *Pseudomonas putida* and *Pseudomonas fluorescens*, had the most active colonization capacity. The strain of *Pseudomonas aeruginosa* had colonization capacity but it was two times less than that of *Pseudomonas putida*.

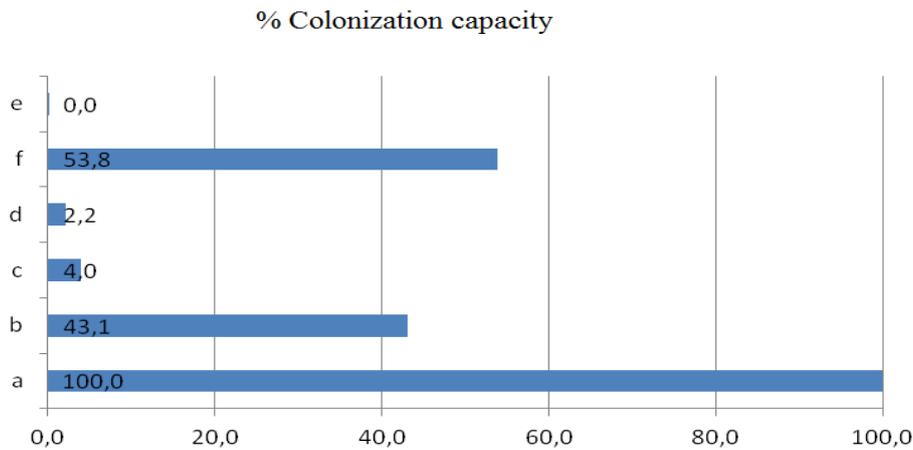


Figure 2. Colonization capacity: a) *Pseudomonas putida* b) *Pseudomonas aureginosa* c) *Burkholderia cepacia* d) *Aeromonas hydrophylla* e) *Aeromonas salmonicida*, f) *Pseudomonas fluorescens*.

Colonization capacity of other strains was much lower. In addition, to use the isolated microorganisms as biotechnological agents for rhizoremediation, it was necessary to identify those strains among them, which had pathogenic properties. Check of the obtained strains in the handbook The Technical Rules for Biological Agents [TRBA, 2006] has shown that 3 strains, namely *Pseudomonas aureginosa*, *Burkholderia cepacia*, *Aeromonas hydrophylla*, relate to pathogens and therefore, they were removed from further experiments.

Deeper study of the strains of *Pseudomonas putida* and *Pseudomonas fluorescens* according to their colonization capacity of different parts of the rye root has revealed that *Pseudomonas putida* colonizes the middle part of the root an order of magnitude higher and the apical part of the rye root - 1.8-2.0 times higher, than the strain of *Pseudomonas fluorescens*. On the other hand, *Pseudomonas fluorescens* strain rather actively colonizes the root base and other root parts; it allows recommending it for rhizoremediation as well (Table 2).

Table 2. Determination of colonization properties of *Pseudomonas putida* and *Pseudomonas fluorescens* in different parts of the root.

Strains of bacteria	Apical part of root, CFU/cm	Middle part of root, CFU/cm	Root base, CFU/cm	Total CFU/cm of root
<i>Pseudomonas putida</i>	$6.5 \pm 0.2 \times 10^5$	$3.5 \pm 0.5 \times 10^5$	$8.0 \pm 0.3 \times 10^5$	$1.80 \pm 0.3 \times 10^6$
<i>Pseudomonas fluorescens</i>	$3.5 \pm 0.3 \times 10^5$	$5.5 \pm 0.4 \times 10^4$	$8.5 \pm 0.6 \times 10^5$	$1.25 \pm 0.3 \times 10^6$

The total content of microorganisms on the rye roots after a 7-day cultivation of the germinated seeds, after they had been treated with the strain of *Pseudomonas putida*, increased by 1.5-2.0 times, and *Pseudomonas fluorescens* - by 1.1-1.5 times in comparison with the colonizing mixture's initial density of 1.0×10^6 CFU/ml. The results obtained indicate not only the colonization capacity of the strains, but also that there are quite favorable conditions for their reproduction on the plant roots.

Summary

Using standard methods for isolation of hydrocarbon oxidizing microorganisms, we have isolated and identified 6 bacterial strains that are capable of colonization of the plant roots. However, according to the information contained in the handbook *The Technical Rules for Biological Agents*, 3 strains from among the colonizers of plant roots were classified as pathogenic bacteria, which use is unacceptable for biotechnological purposes. The rest of the strains had various degrees of colonization properties. The most active strains, namely *Pseudomonas putida* and *Pseudomonas fluorescens*, colonized rye roots with a total density of $1.5-2.0 \times 10^6$ CFU/cm and $1.1-1.5 \times 10^6$ CFU/cm, respectively. These values were higher than the colonizing mixture's initial density in treating the germinated seeds, which indicates high colonization capacity of the strains. Furthermore, all strains colonized well all root parts; this demonstrates a possibility of their long-term preservation and reproduction with the plant growth and especially with the growth of roots.

Conclusions

The strains *Pseudomonas putida* and *Pseudomonas fluorescens* isolated and identified from oil contaminated soils are not pathogenic strains; they have high colonization capacity and can be recommended for developing a rhizoremediation method for the soils contaminated with xenobiotics.

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