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ISOLATION OF HYDROCARBONOCLASTIC BACTERIA FROM OILY WASTES AND THEIR PILOT APPLICATION FOR WATER AND SOIL DECONTAMINATION

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Abstract: Excessive consumption of petroleum products carries the risk that these toxic chemicals enter and accumulate in the environment via transportation, usage or improper storage, thus hazarding natural habitats or human health. Bioremediation is a cost-effective and environmentally friendly technique that involves the use of microorganisms or plants in order to neutralize environmental pollutants. Considering that bacteria occur not only in aqueous but even in oil phases, intermediates, by-products or wastes can pose hidden reservoirs of effective microbial degraders with potential application in oil bioremediation. Using mazut (a residual fuel oil from atmospheric distillation of crude oil) as an origin matrix, thirteen bacterial strains were isolated. The best performing strains, identified as *Rhodococcus* sp. PAE1 and *Rhodococcus* sp. PAE8, were able to degrade structurally variant hydrophobic compounds (including hexadecane, cooking oil, mazut or lubricant oil) in aqueous systems. Thus, they were used in further small-scale soil/groundwater experiments in order to model the bioremediation process of a local ares exposed to years lubricant oil pollution. Our study represents a targeted tool for the bioremediation of oil-polluted aquatic and terrestrial environments and revealed that oily wastes can be considered as valuable sources of new hydrocarbon-utilizing isolates.

Keywords: *bioremediation, mazut, hexadecane, hydrocarbon-utilizing*

1. INTRODUCTION

Since hydrophobic organic compounds pose serious risks to natural communities or human health, pollution of soils and waters by petroleum products or other oil-related compounds are still among the major environmental concerns that humankind must cope with. Petroleum products can enter into the environment through accidental oil spills or reckless human activity [1–5]. Areas close to vehicle traffic or where handling and maintenance operations of vehicles take place are considered to be particularly vulnerable, since the probability of contamination inevitably increases [6].

Mazut is a residual fuel oil from the atmospheric distillation process of crude oil [7]. It is a viscous mixture of hydrocarbons with high carbon number, PAHs, resins and cycloalkanes, often having complex structure with high heavy metal and sulfur content. These properties make mazut cumbersome for both biodegradation and further refining [7–8]. Nonetheless, mazut can be further processed by vacuum distillation in order to produce diesel oils, base oils, lubricants and heavy fuel oils [9]. Lubricant oils (LOs) are widely used for reducing friction in the engines of motorized vehicles such as cars, motorcycles or locomotives. Therefore, used lubricant oils (ULOs), containing long-chain hydrocarbons, additives and heavy metals, are considered widespread, hazardous pollutants and hence potential targets for environmental rehabilitation processes [10–12]. Several physicochemical and biological waste management techniques are available for neutralizing oil-related pollutants in the environment but most of these methods still need further developments [13–15]. Bioremediation utilizes the degradative capability of plants and/or microorganisms for the decontamination of polluted environments [16–19]. As an environmentally sound and cost-effective approach, it is considered to be one of the most promising rehabilitation technologies [16–18].

Bacterial communities occur in aqueous and even in oil phases [20]. Thus, isolation and examination of bacterial strains with the ability to degrade hydrocarbons from these oily environments can provide a promising tool for biological remediation and also a better understanding of the microbial community structure and in oil-polluted niches.

The aim of this study was to provide useful tools for the bioremediation of aqueous and terrestrial environments polluted by petrochemical products. To this end, isolation from mazut was carried out to gain new isolates with the ability to degrade petrochemicals and hydrophobic organic compounds, even if they have as high structural complexity as mazut and LOs do.

2. EXPERIMENTAL

2.1. Bacterial strains

Hydrocarbonoclastic bacteria were isolated from mazut using liquid minimal medium. Pure strains were selected, characterized [21] and tested for hydrocarbon biodegradation. The two most effective strains – PAE1 and PAE8 – were identified according to their 16S rDNA gene homology [22].

2.2. Biodegradation tests in aqueous systems

The best performing *Rhodococcus* sp. PAE1 strain was used in subsequent biodegradation tests performed in aqueous systems. Pollution of hydrophobic compounds was modelled by hexadecane, representing easily biodegradable *n*-alkanes; cooking oil, representing a wide-spread contaminant in municipal sewage; and mazut for a complex hydrocarbon mixture that is hard to biodegrade. All vials were capped and respiration activities were monitored by gas chromatography (GC). At the end of

the experiments, the remaining contaminants were extracted with diethyl ether or chloroform and then bioconversion (B%) values were calculated using gas chromatography coupled to mass spectrometry (GC-MS) and/or gravimetric data and applying the following equation [23]: $B\% = [(Contaminants_{cell\text{-}free\ samples} - Contaminants_{inoculated\ samples})/Contaminants_{cell\text{-}free\ samples}]x100$. Data are expressed as mean ± SE (standard error). Statistical significance was analyzed using one-way analysis of variance (ANOVA) followed by Duncan's test.

2.3. Small-scale *ex situ* soil bioremediation tests

The most effective hexadecane-degrader *Rhodococcus* sp. PAE1 and *Rhodococcus* sp. PE8 strains were tested for LO biodegradation and then used in small-scale soil experiments in order to model the bioremediation process of a long-time ULO-polluted area. Soil samples from a local ULO-polluted site were used to construct *ex situ* soil microcosms in order to model various bioremediation approaches. Rehabilitation treatments included biostimulation (BS, 30% soil moisture was set with the addition of minimal medium, which contained soluble, inorganic nutrients, such as nitrogen and phosporus) and bioaugmentation combined with biostimulation (BAS, in addition to biostimulation, oil-degrader *Rhodococcus* sp. PAE1 or *Rhodococcus* sp. PAE8 strains were introduced into the polluted soil at an inoculation level of 10^9 cells per gram soil). Non-treated control (NTC) samples represented a natural loss in the ULO-concentration. All samples were incubated for 40 days. All ULO-polluted soil microcosms were closed and respiration activities were monitored by gas chromatography (GC) for 30 days. The headspaces of the vials were refreshed every two days to maintain proper aeration. At the end of the experiments, the remaining ULOs were extracted with carbon disulfide as solvent. The extracts were analyzed with an infrared oil-measuring equipment to determine the concentration of total petrol hydrocarbons (TPHs). Bioconversion (B%) was calculated applying the following equation: $B\%=[(TPH_{non\text{-}treated\ soil} - TPH_{treated\ soil})/TPH_{non\text{-}treated\ soil}]x100$. Data are expressed as mean ± SE (standard error). Statistical significance was analyzed using one-way analysis of variance (ANOVA) followed by Duncan's test.

3. RESULTS AND DISCUSSION

3.1. Bacterial strains

Using mazut as an origin matrix, thirteen pure bacterial strains were isolated (one of them was pathogenic, thus, it was omitted from further experiments). The most important physiological and biochemical characteristics of all strains were determined. Data are summarized in *Table 1*.

Preliminary experiments revealed that eleven strains out of thirteen were able to utilize hexadecane as sole carbon and energy source (data not shown). After sequencing the 16S rDNA gene of the best hexadecane-utilizing strains PAE1 and PAE8, both of them were identified as members of the genus *Rhodococcus*, so in

further experimental works, the name *Rhodococcus* sp. PAE1 and *Rhodococcus* sp. PAE8 were used. *Rhodococci* play an important role in environmental and industrial biotechnology [23–27].

Table 1

Physiological and biochemical characteristics of the newly isolated bacterial strains

Characteristics	PAE1	PAE3	PAE4	PAE5	PAE6	PAE7	PAE8	PAE9	PAE10	PAE11	PAE12	PAE13
Gram staining	+	+	+	+	+	+	+	+	+	+	+	+
Cell length (μm)	2.48 ±0.3	2.09 ±0.33	1.94 ±0.26	1.95 ±0.27	0.77 ±0.12	1.91 ±0.13	1.87 ±0.24	2.09 ±0.19	2.08 ±0.22	1.96 ±0.20	1.97 ±0.24	1.90 ±0.16
Indole production	–	–	–	–	–	–	–	–	–	–	–	–
Hemolytic activity	–	–	–	–	–	–	–	–	–	–	–	–
Catalase activity	+	+	+	+	+	+	+	+	+	+	+	+
Casease activity	–	–	–	–	–	–	–	–	–	–	–	–
Lipase activity	+	+	+	+	+	+	+	+	+	+	+	+
Urease activity	–	–	–	–	–	–	–	–	–	–	–	–
Beta- galactosidase activity	–	–	–	–	–	–	–	–	–	–	–	–
Nitrate/nitrite reduction	w	w	w	w	–	w	w	w	w	w	w	w
Startch hydrolysis	–	–	–	–	–	–	–	–	–	–	–	–
Methyl red-test	–	–	–	–	–	–	–	–	–	–	–	–
Oxidation/ fermentation (OF) test	w	w	w	w	+	w	w	w	w	w	w	w
Tween 80 hydrolysis	+	+	+	+	–	+	+	–	–	–	+	+

3.2. Biodegradation tests in aqueous systems

Respiration activity of *Rhodococcus* sp. PAE1 was investigated in liquid mineral medium, artificially contaminated with 1% (m v^{-1}) of various hydrophobic pollutants. Three hydrophobic compounds were used as sole carbon and energy source: hexadecane represented the *n*-alkanes, cooking oil was used as a common contaminant in municipal sewages, and mazut represented the original isolation matrix of the new strain. According to the obtained results *(Figure 1)*, microbial activity decreased with the increasing structural complexity of the available substrates.

At the end of the incubation, the remaining amount of hydrophobic organic substrates was evaluated and bioconversion values were calculated for each carbon sources *(Figure 2)*. Coinciding with CO_2 measurements, bioconversion also decreased when a structurally more complex carbon source was available. Our results suggest that this newly isolated strain can be a targeted tool for the biodegradation of petroleum products in polluted waters.

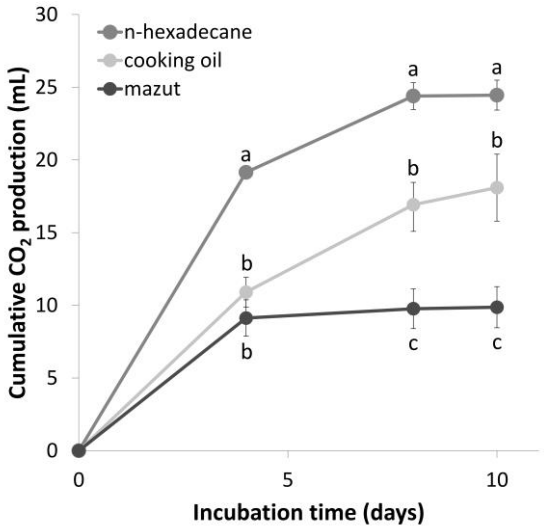

Figure 1

Cumulative CO_2 production of Rhodococcus sp. PAE1 in liquid mineral medium artificially contaminated with 1% (m v^{-1}) of various hydrophobic compounds. Different letters in the same incubation time represent a significant difference at $P \leq 5$ (n = 3).

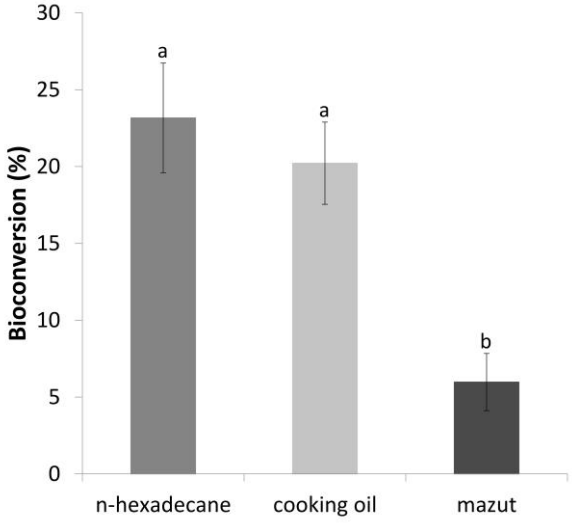

Figure 2

Bioconversion of 1% (m v^{-1}) n-hexadecane, cooking oil and mazut by Rhodococcus sp. PAE1 in liquid mineral medium. Different letters represent a significant difference at $P \leq 5$ (n = 3).

Since *Rhodococcus* sp. PAE1 and *Rhodococcus* sp. PAE8 exhibited the highest respiration activities in preliminary experiments (data not shown) and *Rhodococcus* sp. PAE1 proved to be able to utilize structurally diverse hydrophobic compounds *(Figure 2)*, both strains were tested for lubricant oil biodegradation in liquid mineral medium. 1% (m v^{-1}) fresh LO was used as sole carbon and energy source. Aerobic biodegradation of hydrocarbons by hydrocarbonoclastic bacteria consumes oxygen alongside with the release of carbon-dioxide [28]. Thus, increasing relative CO_2 content was considered as an indirect measure of the microbial degradation of LO in the closed vials *(Figure 3)*.

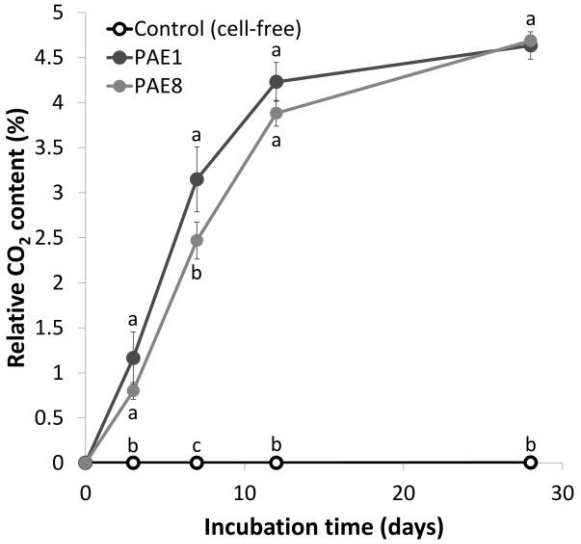

Figure 3

Relative CO_2 content in the headspaces of the closed vials containing liquid mineral medium artificially contaminated with 1% (m v^{-1}) fresh lubricant oil. Different letters in the same incubation time represent a significant difference at $P \leq 5$ (n = 3).

Based on our results, *Rhodococcus* sp. PAE1 and *Rhodococcus* sp. PAE8 were able to utilize fresh LO as their sole carbon and energy source at a similar rate, thus, both strains can be potentially applied not only for water decontamination but even for modelling the bioremediation of a local ULO-polluted area.

3.3. Small-scale *ex situ* soil bioremediation tests

Respiration activity of ULO-polluted soil microcosms was followed with gas chromatography. According to the CO_2 production *(Figure 4)*, even NTC samples were active in respiration, indicating the presence of metabolically active microbiome in ULO-polluted soil. Respiration activity and thus microbial activity could be

increased by the supplementation of inorganic nutrients in BS samples. The most active respiration was observed in the soil samples inoculated with *Rhodococcus* sp. PAE1 (BAS_PAE1) and *Rhodococcus* sp. PAE8 (BAS_PAE8). Nevertheless, evolution of CO_2 in a hydrocarbon-polluted soil cannot be considered as sole evidence of hydrocarbon biodegradation due to the plentiful availability of organic matters and the compositional complexity of the soil matrix.

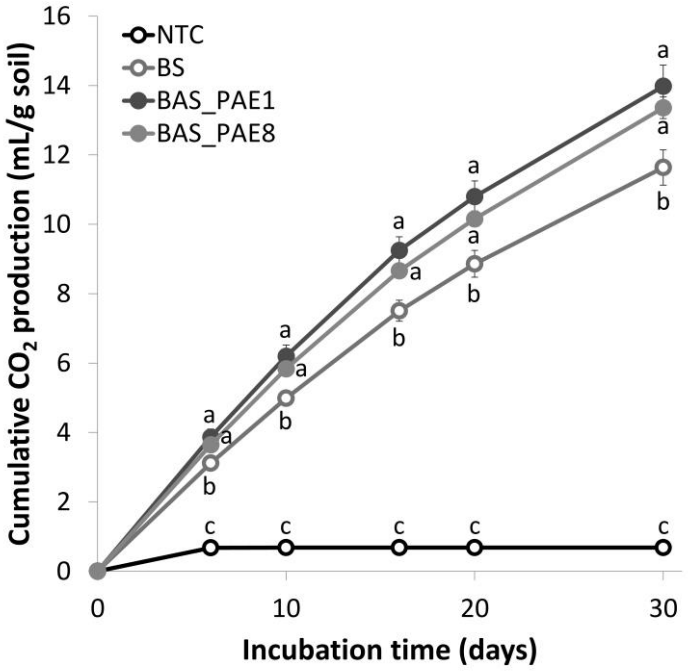

Figure 4

Cumulative CO_2 production in ULO-polluted soil microcosms (NTC: non-treated control soil, BS: biostimulation, BAS_PAE1: biostimulation+bioaugmentation using Rhodococcus sp. PAE1, BAS_PAE8: biostimulation+bioaugmentation using Rhodococcus sp. PAE8). Different letters in the same incubation time represent a significant difference at $P \leq 5$ ($n = 3$).

At the end of the experiment, remaining ULOs were extracted and TPH concentrations were evaluated in order to calculate TPH bioconversions in soil microcosms *(Figure 5)*. A considerable level of TPH bioconversion was observed in the biostimulated samples (BS), indicating the natural occurrence of ULO-degrading microorganisms even in heavily contaminated environments. Moreover, introduction of the newly isolated *Rhodococcus* sp. PAE1 and *Rhodococcus* sp. PEA8 significantly enhanced the TPH bioconversion to 38% and 40%, respectively.

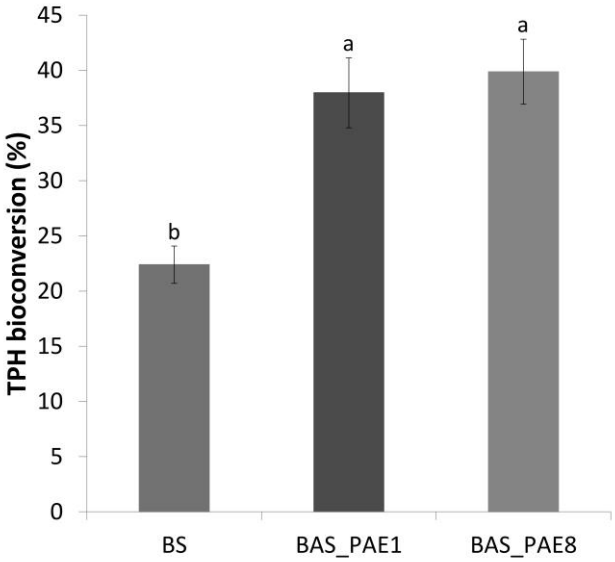

Figure 5
TPH bioconversion in ULO-polluted soil microcosms after 40 days of incubation (BS: biostimulation, BAS_PAE1: biostimulation+bioaugmentation using Rhodococcus sp. PAE1, BAS_PAE8: biostimulation+bioaugmentation using Rhodococcus sp. PAE8). Different letters represent a significant difference at $P \leq 5$ ($n \geq 15$).

4. CONCLUSION

Since bacterial communities occur in aqueous and even in oil phases, we hypothesized that oily wastes or by-products can provide undiscovered microbial degraders. Based on this assumption, 13 bacterial strains were isolated from mazut. The best performing hydrocarbon-utilizing isolates were identified as members of the genus *Rhodococcus* and assigned as *Rhodococcus* sp. PAE1 and *Rhodococcus* sp. PAE8. Despite the structural differences and complexities, *Rhodococcus* sp. PAE1 was able to utilize *n*-hexadecane, cooking oil and even mazut in liquid minimal medium. Further testing of *Rhodococcus* sp. PAE1 and *Rhodococcus* sp. PAE8 in aqueous systems showed that both strains were capable of LO biodegradation, and potentially applicable for ULO-polluted soil decontamination. Thus, ULO-polluted soil microcosms were constructed and then submitted to various biological treatments in order to model and evaluate options for the bioremediation of a long-term ULO-polluted site. Bioaugmentation with *Rhodococcus* sp. PAE1 or PAE8 significantly decreased the pollutant concentration compared to biostimulation. Although optimal conditions for the biodegradation are barely revealed and still need further

development, our results represent a targeted tool for the bioconversion of petroleum contaminants in aqueous and terrestrial environments. Additionally, this work highlights the fact that oily wastes and by-products can be potential sources of yet-to-be isolated hydrocarbonoclastic bacteria.

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