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Reduction of Petroleum Hydrocarbons and Toxicity in Refinery Wastewater by Bioremediation

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Abstract The aim of the study was to investigate petroleum waste remediation and toxicity reduction by five bacterial strains: *Ralstonia picketti* SRS (BP-20), *Alcaligenes piechaudii* SRS (CZOR L-1B), *Bacillus subtilis* (I′-1a), *Bacillus* sp. (T-1), and *Bacillus* sp. (T′-1), previously isolated from petroleum-contaminated soils. Petroleum hydrocarbons were significantly degraded (91%) by the mixed bacterial cultures in 30 days (reaching up to 29% in the first 72 h). Similarly, the toxicity of the biodegraded petroleum waste decreased 3-fold after 30 days. This work shows the influence of bacteria on hydrocarbon degradation and associated toxicity, and its dependence on the specific microorganisms present. The ability of these mixed cultures to degrade hydrocarbons and reduce toxicity makes them candidates for environmental restoration applications at other hydrocarbon-contaminated environments.

Keywords Biodegradation · Petroleum hydrocarbons · Toxicity · Microtox

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Numerous genera of bacteria are known as good hydrocarbon degraders. Most of them belong to *Pseudomonas*, *Sphingomonas*, *Aeromonas*, *Alcaligenes*, *Acinetobacter*, *Arthobacter*, *Brevibacterium*, *Xanthomonas*, *Mycobacterium*, *Rhodococcus* and *Bacillus* species (Atlas 1984; Bartha 1986). They tolerate high concentrations of the hydrocarbons and have a high degradation capability. It is now generally accepted that a single species of microorganism will not completely degrade any particular oil. The degradation of crude and refined oils seems to involve a consortium of microorganisms (Berry et al. 2006). Many microorganisms which are involved in crude and refined oils produce surface active compounds (biosurfactants) (Plaza et al. 2007). In recent years, the interest in microbial surfactants (biosurfactants) has increased due to their diversity, selectivity, performance under extreme conditions and potential applications in environmental protection.

In order to develop environmental technologies for crude oil degradation it is necessary to isolate and characterize specific microbial species for evaluation of their efficacy in utilization of hydrocarbons at the bench scale before field applications. Extensive work has been carried out on laboratory and field scale on the isolation of biosurfactant-producers and hydrocarbon degraders, characterization of the biosurfactants produced, and the products of biodegradation of hydrophobic compounds in soils and sediments (Bodour and Maier 2002; Christofi and Ivshina 2002; Li et al. 2006; Benincasa 2007).

The assessment of environmental hazards of remediated environments is generally based on chemical analyses. In these investigations the analyses are typically based on the site-specific contaminants of concern (COCs). However, not all COCs may be known and undetected metabolites, compounds and by-products may be formed during the degradation processes. There is increasing interest for

incorporation of toxicity tests (with a battery of different assays) for ecological assessment and for supporting management decisions for remediation (Plaza et al. 2005). The use of biological endpoints help to appropriately define acceptable cleanup standards of contaminated sites and establish the ecological soil quality assessment (Fent 2003; Breure et al. 2005; Winding et al. 2005).

Detection of residual toxicity remaining after biodegradation underscores the need to test for toxicity changes during biodegradation studies. The occurrence of contaminants in mixture like crude oil is an important problem because the removal or degradation of one component can be inhibited by other compounds or by-products in the mixture, and because different conditions may be required to treat different compounds within the mixture. Bioassays provide important information for the assessment of pollutant effects. In contrast to chemical analyses, they detect effects of multiple contaminants, by-products, metabolites, and synergistic processes. A large number of bioassays have been applied for the evaluation of bioremediation efficacy (Eisentraeger et al. 2005; Maila and Cloete 2005). Toxicity of petroleum contaminated soils, water, and sediment undergoing remediation can be a health concern due to genotoxic by-products (Płaza et al. 2005).

This paper focuses on (i) the influence of bioaugmentation by bacteria isolated from petroleum contaminated soils were tested as a consortium for their ability to degrade petroleum in wastewater, (ii) the growth of the bacteria on the waste, and (iii) the effects of the biodegradation on toxicity as measured by assays carried out with the bioaugmentation tests.

Materials and Methods

The bacterial strains (BP-20, CZOR L-1B, T-1, T′-1, I′-1a) used in this study were isolated from a 100-year-old oil refinery sludge in Czechowice-Dziedzice, Poland as described by Berry et al. (2006). The aged sludge was acidic (pH 2) and highly contaminated with polycyclic aromatic hydrocarbons. The isolates were selected based on their ability to produce biosurfactant and degrade of aliphatic and aromatic petroleum hydrocarbons as reported earlier (Płaza et al. 2006, 2007). Strains were maintained on nutrient agar (SMA, bioMerieux) slants and subcultured every 3 weeks.

The bacterial isolates were identified based on the 16S rRNA gene sequence analysis. A direct-colony PCR was set up to amplify the 16S rRNA gene in a 30-cylce PCR using universal primers 27F and 1492R. The PCR conditions used were: initial denaturation at 95°C for 8 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 1 min; followed by final elongation step at 72°C for 10 min. The amplified PCR products were purified using the Qiagen-PCR purification kit as per the manufacturer's instructions. The purified PCR products were sequenced from both ends at the DNA Sequencing Core facility of the University of Michigan at Ann Arbor. The 16S rRNA gene sequences were analysed at the Ribosomal Database Project (RDP) II (http://rdp.cme.msu.edu). The top 10 most homologous sequences were aligned using the CLUSTALW program v1.83 at the European Bioinformatics site (http://www.ebi.ac.uk/clustalw). The similarity matrix was prepared using the DNAdist program in the PHYLIP package (Felsenstein 1989) with the Jukes Cantor corrections. Isolates were identified as that genus/species to which they showed highest 16S rRNA gene sequence similarity in the RDP database. The characterization of the bacterial isolates was also carried out by traditional microbiological methods.

The raw petroleum-containing wastewater for experiments with the isolates was obtained from the Czechowice-Dziedzice oil refinery (CzOR), Poland. No treatments were done before the sampling of the wastewaters. Physico-chemical and microbiological characterization of the wastewaters included the following: pH-7.4, BOD-155 g/m^3, COD-275 g/m^3, oxygen consumption-64 g/m^3, TPH-802 g/m^3, number of mesophilic bacteria-945 CFU/mL, number of psychrophilic bacteria-2730 CFU/mL, total number of fungi-102 propagules/mL.

To evaluate of the growth of bacterial strains on petroleum waste, 500 μL of 24 h cultures of each strain (10^4–10^5 CFU/mL) growing on liquid medium contained (g/L): peptone – 8; yeast extract – 2.5; glucose – 1 were transferred aseptically to 100 mL of combined petroleum wastewater and mineral medium (1:1 v/v). Composition of mineral medium (MM) was as described by Abu-Ruwaida et al. 1991 (g/L): Na_2HPO_4 – 2.2; KH_2PO_4 – 1.4; $MgSO_4{\cdot}7H_2O$ – 0.6; $(NH_4)_2SO_4$ – 3; yeast extract – 1; NaCl – 0.05; $CaCl_2{\cdot}7H_2O$ – 0.02; $FeSO_4{\cdot}7H_2O$ – 0.01. The medium was supplemented with 1 mL of the trace elements solution (Gerhardt 1981) (mg/L): $ZnSO_4{\cdot}7H_2O$ – 50; $MnCl_2{\cdot}4H_2O$ – 400; $CoCl_2{\cdot}6H_2O$ – 1; $CuSO_4{\cdot}5H_2O$ – 0.4; H_3BO_2 – 2; $Na_2MoO_4{\cdot}2H_2O$ – 500. The cultures were grown aerobically at 30°C for 7 days with constant shaking (150 rpm). Total viable counts (CFU/mL) were determined to monitor aerobic bacterial growth. The experiments were carried out using three replicates.

The biodegradation of petroleum waste by the bacterial consortium was tested by adding the consortia to a wastewater and mineral media solution in microcosms. The microcosms were developed with 1 mL of bacterial strain consortia, initial concentrations 10^4–10^5 CFU/mL, aseptically transferred to 250 mL Erlenmayer flasks (five replicates) containing 50 mL each of sterile MM and petroleum waste contained 1.9 mg TPH/mL. The

microcosms were incubated at 30°C with continuous shaking (150 rpm) for 30 days. An uninoculated microcosm with petroleum wastewater and mineral medium served as a control. Samples were aseptically taken at 0, 3, 8, 15, 20 and 30 days interval for both total petroleum hydrocarbon (TPH) analysis and toxicity assays. The residual TPH was extracted with CCl_4 from the liquid cultures and analysed by FT-IR after passing the extract through a Florisil column. The extract was quantitatively measured after calibration with a standard mixture (v/v) of n-hexadecane (37.5%), isooctane (37.5%) and benzene (25%). The spectrum was recorded between the 3,100–2,800 cm^{-1} range. The absorbance value was measured at 2,926 cm^{-1} with an IR spectrophotometer (UNICAM SP1000, UK). The TPH content was related to the CH_2 group number.

Microtox® toxicity assay (SDI Europe) was carried out on microcosm samples following the basic test protocol (Microbics Corporation 1998). The assay is based on the analysis of light emission reduction of luminescent bacteria (*Vibrio fischeri)* under toxic stress and was carried out in triplicates on a Microtox Model 500 Analyzer as per the manufacturer's instructions. The luminescence inhibition after 15 min exposure was taken as endpoint. The obtained data was used to calculate EC50 (concentration effect causing 50% toxic effect) and TU (toxicity unit = 1/EC50 × 100).

Statistical analysis of the results was performed using STATISTICA 5.1 a Windows program.

Results and Discussion

The five bacterial strains were identified as: *Ralstonia pickettii* SRS (BP-20), *Alcaligenes piechaudii* SRS (CZOR L-1B), *Bacillus subtilis* (I′-1a), *Bacillus* sp. (T-1), *Bacillus* sp. (T′-1). 16S rRNA gene sequencing could not clearly assign isolates T-1 and T′-1 to any species in the genus *Bacillus* as both these isolates showed >99% similarity to two distinct species of the genus (*B. subtilis* and *B. licheniformis* for T-1 and *B. subtilis* and *B. amyloliquefaciens* for T′-1). The bacteria were isolated from sludge samples obtained from 100-year-old oil refinery in Czechowice-Dziedzice (Poland).

Figure 1 presents the growth of mixture bacterial cultures in the petroleum wastewater microcosms for 9 days. The consortium achieved maximum growth in the second day of the incubation. During the next days of the experiment duration the growth of bacterial consortium stabilized at 11–12 log CFU/mL (Fig. 1).

The degradation of petroleum waste as measured by total petroleum hydrocarbons (TPH) was determined over 30 days (Fig. 2). The bacterial consortium effectively degraded the petroleum waste as demonstrated by the

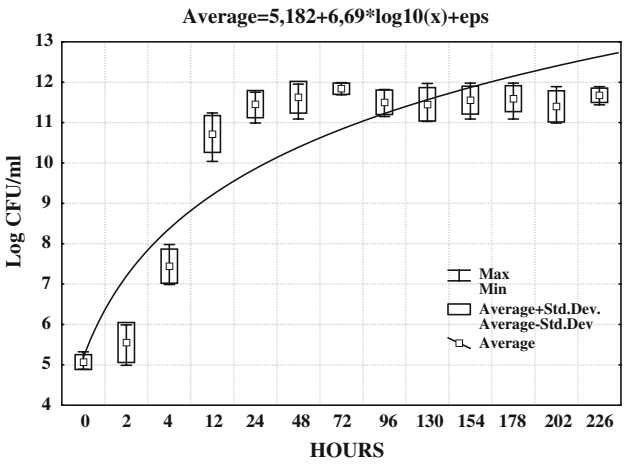

Fig. 1 Growth of mixed bacterial cultures in the petroleum waste medium as measured by CFU/mL (three replications were made)

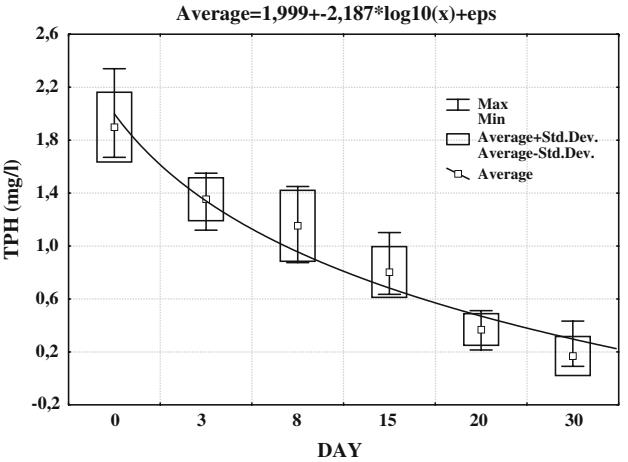

Fig. 2 Changes in total petroleum hydrocarbons (TPH) concentrations during 30 days experiment with the five isolated strains (five replications were made)

10-fold reduction in TPH (Fig. 2 and Table 1). The TPH concentration in petroleum waste was 1.9 mg/mL at the beginning which decreased to 0.17 mg/mL (91% of TPH removal) after 30 days of incubation. In the first 3 days of microcosm incubation TPH decrease was the highest, and reached ~29% removal. The rapid increase in bacteria densities during the first days of microcosm incubation was correlated with high degradation of petroleum hydrocarbons (Figs. 1, 2). Bacterial strains used in the experiment had the ability to remediate the hydrocarbons under these test conditions. Control flasks with no microbial amendments showed no significant changes in TPH over 30 days.

The changes of toxicity as a function of petroleum biodegradation activity were also determined over 30 days (Table 1). At the beginning, the toxicity indicator Toxicity Units (TU) was high (14.2) which decreased by ~43% to 8.07 in three days and reaching 33% of the original (4.55)

Table 1 Concentrations changes of Total Petroleum Hydrocarbons (TPH) and toxicity during the 30-day test with the microbial consortia

Incubation time (days)	TPH (mg/mL) (mean values ± Std. Dev.)[1]	Removal (%)	Toxicity indicators (mean values)[2]	
			EC50 (mean values ± Std. Dev.)[2]	TU
0	1.90 ± 0.26	0.00	7.02 ± 1.2	14.2
3	1.35 ± 0.16	28.66	12.4 ± 2.8	8.07
8	1.15 ± 0.27	39.00	21.9 ± 1.8	4.56
15	0.80 ± 0.19	57.66	20.1 ± 0.9	4.96
20	0.37 ± 0.12	80.55	21.0 ± 1.1	4.77
30	0.17 ± 0.15	91.10	22.0 ± 0.7	4.55

[1] Five number of replications;
[2] three number of replications

toxicity by 30 days. This decrease was due to the efficient conversion of the toxic raw-petroleum material to less or non-toxic intermediates and by-products during biodegradation. Detection of this activity or decrease in residual after biodegradation underscores the need to test for toxicity changes during environmental restoration studies.

It has been demonstrated that ecotoxicity bioassays should be used as supplementary tools for monitoring the effectiveness of remediating petroleum contaminated sites (Płaza et al. 2005). In this way ecological relevant criteria for estimating risk assessment can be combined with monitoring data.

There are many ecotoxicity tests with varying sensitivities and applications, however short-term assays are often required to offer a fast and simple responses. In this context, Microtox® toxicity assay using the luminescent bacteria, *Vibrio fischeri* as test organism seems to be an effective tool in detecting toxicity, and has been used as a toxicity surveillance system in many specific cases. This method has been undergoing with large applications (DeZwart and Sloof 1983; Nohava et al. 1995; Froehner et al. 2000; Araujo et al. 2005). In presented study, Microtox® was used to evaluate the residual toxicity of the petroleum waste during the biodegradation.

The ability to simultaneously degrade petroleum compounds and reduce toxicity makes the investigated strains potential candidates for bioremediation. The major bacterial genera reported previously as biosurfactant producers include *Pseudomonas*, *Acinetobacter*, *Bacillus*, *Rhodococcus*, *Arthobacter*, *Staphylococcus* and *Flavobacterium* species (Banat et al. 2000; Bodour and Maier 2002; Ron and Rosenberg 2002; Singh and Cameotra 2004). The present study investigated the bacterial isolates from a hydrocarbon-contaminated site belonging to the genus *Ralstonia*, *Alcaligenes* and *Bacillus*. The isolates *Ralstonia picketti* SRS and *Alcaligenes piechaudii* SRS, their surface active and biodegradation properties were described previously (Płaza et al. 2006, 2007). The capacity of these natural bacterial strains to produce biosurfactants and degrade petroleum hydrocarbons is promising for environmental restoration applications. The potential application of these cultures in conjunction with other methods including biopiles or bioreactors could be of great use and benefit to remediation efforts and in reducing long-term restoration costs.

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