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Original Research

Biodegradation of Crude Oil and Distillation Products by Biosurfactant-Producing Bacteria

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Abstract

Biodegradation of crude oil and 7 different distillation products by *Ralstonia picketti* SRS and *Alcaligenes piechaudii* SRS was investigated. Crude oil degradation was above 80% after incubation of 20 days for both strains and their mixture. Removal of short/long alkanes and BTEX investigated was different, from 97% (for hexane degraded by the mixture of the isolates) to 22% (for tridecane degraded by *Alcaligenes piechaudii* SRS). Hexadecane, pristane and cyclohexane were degraded with high efficiency between 72-91%. Among BTEX, toluene was degraded very well by both strains and their mixture. Benzene and m+p-xylenes were efficiently degraded by the mixture of the isolates, 78% and 87%, respectively.

Degradation of light boiling fractions achieved 80-100%. On the other hand, degradation removal of heavy, high boiling fractions was lower, and reached between 10-81%.

The mixture of the isolates enhanced slightly the degradation efficiency of crude oil and its distillation products.

Changes in crude oil toxicity as a function of biodegradation activity were also undertaken. Slight luminescence inhibition as a measure of toxicity was found during the experiment time. The samples were not found to be toxic to *Vibrio fisheri* during the crude oil biodegradation. The luminescence inhibition could be due to the presence of toxic intermediates produced during biodegradation.

Keywords: biodegradation, crude oil, hydrocarbons, distillates, toxicity

Introduction

Oil pollution is a great hazard to soil and aquatic environments. Crude oil is a complex mixture of many compounds such as alkanes, aromatics, resins and asphaltenes.

Many microorganisms have been reported to use various petroleum hydrocarbons, including BTEX and PAHs as their sole carbon and energy substrate, despite their extreme insolubility in the aqueous phase. Numerous genera of bacteria are known as good hydrocarbon degraders. They tolerate high concentrations of the hydrocarbons and have a high capability for their degradation. Most of them belong to *Pseudomonas*, *Sphingomonas*, *Aeromonas*, *Alcaligenes*, *Acinetobacter*, *Arthobacter*, *Brevibacterium*, *Xantomonas*, *Mycobacterium*, *Rhodococcus* and *Bacillus* species [1, 2].

Biological degradation of pure alkanes and aromatic hydrocarbons by isolated bacterial cultures and mixed

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bacterial population has been reported [3-10]. However, reports on the utilization of complex hydrocarbon mixtures like crude oil by isolated microbial species are few [11-15]. Shailubhai *et al*. [13] reported the degradation of oil sludge by pure strains of *Rhodotorula rubra* and *Pseudomonas aeruginosa*. Foght *et al*. [14] reported the utilization of crude oil by pure bacterial strains of *Acinetobacter calcoaceticus* RAG-1 and *Pseudomonas sp*. HL7b. Margesin and Schinner [15] investigated biodegradation of diesel oil by two psychotrophic bacteria that were assigned to the genera *Pseudomonas sp*. and *Arthobacter sp*. To obtain an efficient oil-degrading bacterial consortium and monocultures, knowledge of the diversity of the microbial community present in soils contaminated with crude oil, their metabolic features and capacity to degrade crude oil are of paramount importance. One of the factors that limits biodegradation of oil pollutants is their limited availability to microorganisms. However, most of the hydrocarbon degraders produce surface active compounds named biosurfactants (BS) [16-18]. Biosurfactants have several potential advantages which are important for bioremediation applications in contaminated environments. These advantages include their biodegradability, low toxicity, better specificity for some applications, the potential for *in situ* production, the ability to be produced from cheap raw materials, effectiveness at extreme conditions of temperature, pH and salinity, and the organisms producing them can be modified genetically to overproduce these compounds [19, 20]. Recently biosurfactants or microorganism-produced biosurfactants have been widely used in environmental protection, including enhanced oil recovery (EOR), oil spill control, biodegradation and detoxification of oil-contaminated industrial effluents and soils [21-27].

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 before application to field conditions.

The aim of our paper is to describe the ability of two bacterial strains, *Ralstonia picketti* SRS and *Alcaligenes piechaudii* SRS, to degrade crude oil and its refined products under laboratory conditions. Both bacteria investigated produce biosurfactants belonging to the major class of glycolipides – rhamnolipids as previously described [28]. The outcome of this microbiological work can be used for research on remediation of petroleum-contaminated environments.

Experimental Procedures

Characterization of Crude Oil and Its Distillation Fractions

Crude oil was obtained from Czechowice-Dziedzice oil refinery, which is located in the Katowice Voivodship in southern Poland. Physico-chemical properties of obtained crude oil were as follows: density at 20°C – 0.726g/cm^3; viscosity at 20°C – 0.7731cSt; viscosity at 30°C – 0.7047 cSt; water content – 0.1% (w/v); H_2S content – < 0.3 mg/dm^3; salt content – 1.1 mg/dm^3; vapour pressure at 37.8°C – 55.4 kPa; vapour pressure at 50°C – 78.3 kPa; sulfur content – 0.24% (w/v). In Table 1 some characteristics

Table 1. Characterization of distillates used in the experiment.

Fractions	Density (15°C)	Initial distillation (temp. °C)	Final distillation (temp. °C)	Sulfur content (%)	Viscosity (cSt)
A0	0.68-0.74	27-31	138-142	0.09-0.13	-
A1	0.724-0.729	44-49	155-158	0.08-0.14	-
A2	0.773-0.778	142-151	203-204	0.06-0.11	-
A3	0.792-0.798	181-187	232-238	0.07-0.11	-
A4	0.821-0.827	202-209	318-320	0,.1-0.19	2.4-2.6
A5	0.826-0.845	160-183	350	0.24-0.40	5.0-5.3
P1	0.847-0.850	-	-	0.32-0.44	5.1-5.8
P2	0.864-0.878	-	-	0.31-0.48	2.0-2.4
P3	0.875-0.884	-	-	0.29-0.47	6.4-6.7
P4	0.887-0.894	-	-	0.31-0.50	9.9-10.9
P5	0.895-0.910	-	-	0.39-0.54	17.5-20.7
P6	-	-	-	0.38-0.68	10.5-11.2

Fractions A0 – A2 – components of petrol; Fractions A3 – P1 – components of diesel oils and light fuel oils; Fractions P2 – P6 – components of high fuel oils (raw material to mazout)

A – distillation under atmospheric conditions, P – distillation under vacuum pressure

of distillates collected from the Czechowice-Dziedzice oil refinery are presented.

Isolation and Identification of Bacterial Strains

The bacterial strains used in this study were isolated by Berry et al. [29] from the soil contaminated by petroleum hydrocarbons. Some physicochemical and microbiological parameters to characterize soil were as follows (mean range): pH – 5.5 in distilled water (1:1, w/v); humidity – 17.5%; microbial activity measured by TTC method – 25 mg TPF/g d.w.; TPH – 32 g/kg d.w.; PAHs – 7.8 mg/kg d.w.

Screening of bacteria-producing biosurfactants and their identification are described by Płaza et al. [28, 30]. Evidence of biosurfactant production by *Ralstonia picketti* SRS and *Alcaligenes piechaudii* SRS was demonstrated by both microbial and biochemical characterization, and further characterization is planned. Both isolates showed the type of pigmentation on CTAB medium indicated rhamnolipid production [31]. The percentage of 3-hydroxy numbered fatty acids has been correlated with biosurfactant activity [32].

Growth of *Ralstonia picketti* SRS and *Alcaligenes piechaudii* SRS in the Liquid Medium with Crude Oil as Carbon and Energy Source

The bacterial strains from overnight culture (10^4–10^5 cells/ml) were transferred to 100 ml of sterile mineral medium (MM) described by Abu-Ruwaida et al. [33] with 0.1% (v/v) of crude oil as carbon and energy source. The cultures were grown aerobically at 30°C by 7 days with shaking (150 rpm). Bacterial growth was estimated by two parameters: colony forming unit (CFU/ml) and optical density of the cultures at 600 nm using a CECIL CE 2031 spectrophotometer. Growth curves were marked.

Crude Oil Biodegradation

The bacterial strains from overnight culture (10^4–10^5 cells/ml) were transferred to 250 ml Erlenmayer flasks, each containing 100 ml of sterile mineral medium (MM) and 0.1% (v/v) of crude oil. The flasks were incubated at 30°C with continuous shaking (150 rpm) for 3 weeks.

The residual total petroleum hydrocarbons (TPH) after bacterial degradation was extracted with carbon tetrachloride (CCl_4) from the liquid cultures, and analyzed by FT-IR. The interfering substances were removed by passing the extract through a column filled with Florisil. The standard substance was a mixture of 37.5% (v/v) n-hexadecane, 37.5% (v/v) isooctane and 25% (v/v) benzene, and the spectrum was recorded between the 3100-2800 cm^{-1} range. The absorbance value was measured at 2926 cm^{-1} with an IR spectrophotometer Unicam SP1000 (UNICAM, UK). The TPH content was related to the CH_2 group number.

An uninoculated medium served as control. Samples were taken for analysis after 4, 8, 14 and 20 days of incubation. The experiment was carried out in three replications.

Measurements of Short/Long Alkanes and BTEX Concentrations During Crude Oil Degradation

The experiment was performed using 125 ml serum bottles containing 25 ml medium as described by Abu-Ruwaida *et al*. [33]. 500 µl of 24–hour bacteria culture (10^4–10^5 cells/ml) as inoculum and 0.1% (v/v) of crude oil were added to the bottles. The vials were sealed with a Teflon-coated rubber and aluminium septum-cap. Headspaces of the bottles were flushed with oxygen. Incubation was carried out at 30°C for 20 days. Measurements of BTEX were done after 1, 2, 5, 10, and 20 days of incubation time. Sterile controls were prepared to evaluate hydrocarbon evaporation. The experiment was done in triplicate.

Hydrocarbon concentrations were determined according to the method described by Wypych and Mańko [34]. HS-SPME-GC/MS technique (Head-Space Solid Phase Microextraction-Gas Chromatography/Mass Spectrometry) was used. The analysis was carried out with Star 3400 Cx gas chromatograph; it was coupled to a Saturn 3 mass spectrometer and Autosampler 8200 Cx (Varian) with 10 ml autosampler vials. The chromatographic column with the phase DB624 and length 30 m x 0.32 mm ID (1.8 µm film thickness) was used. Helium was used as the carrier gas: purity 99.999%, flowing capacity of 1.0 ml/min (at 40°C). The gas chromatographic conditions were as follows: oven temperature was held at 40°C for 10 minutes, then increasing to 250°C by 10°C/min, and finally increasing by 5°C/min to 270°C. Total analysis time was 45 minutes. Injector temperature was 250°C.

Measurements of Residual Toxicity During Crude Oil Degradation

Microtox® toxicity assay was used to evaluate the residual toxicity. The method is based on the analysis of light emission reduction of luminescent bacteria (*Vibrio fischeri)* under toxic stress. All materials were purchased from SDI Europe. The tests were carried out in a Microtox M500 analyzer according to the Microtox Manual (1992). The samples were centrifuged (11,000 x g) for 5 min. Results of toxicity are presented as a percent of luminescence reduction. If luminescence reduction is ± 20%, the samples are not found to be toxic to *Vibrio fischeri*; if luminescence reduction is between 20–50%, the samples have low toxicity; if luminescence reduction reaches above 50%, the samples are toxic, and EC50 should be calculated.

Biodegradation of Distillation Products

The bacterial strains from overnight culture (10^4–10^5 cells/ml) were transferred to 100 ml of sterile mineral medium (MM) with 0.1% (v/v) of refined petroleum products as a carbon and energy source. Removal of petroleum products was done after 20 days of incubation time. Sterile controls were prepared to evaluate hydrocarbon evaporation. The experiment was done in triplicate.

The residual refined petroleum products after bacterial degradation was extracted with 15 ml of carbon tetrachloride (CCl_4) from the liquid cultures, and analyzed by GC-FID method. The analysis was carried out with a gas chromatograph HP 6890 GC (Hewlett-Packard) equipped with flame-ionization detector (FID). Measurement conditions were as follows: capillary column HP-5 (5% Phenyl Methyl Siloxane), size: 30m x 320µm i.d. x 0.5µm; as carrier gas nitrogen was used at flowing capacity of 30 ml/min; split 1:4; temperature of injector: 280°C, temperature of detector: 310°C; programme of temperature: 80°C (2 min, 12°C/min) to 280°C, 12 min final time).

Results and Discussion

In this work two bacterial strains *Ralstonia picketti* SRS and *Alcaligenes piechaudii* SRS were used to degrade crude oil and its distillation products. The results making the investigated strains interesting for bioremediation is their ability to simultaneously degrade petroleum compounds and produce biosurfactants. Their properties to produce biosurfactant and simultaneously degrade BTEX hydrocarbons were reported earlier [28, 30]. The isolates were selected based on their ability to produce biosurfactants while growing in culture media with aliphatic and aromatic hydrocarbons.

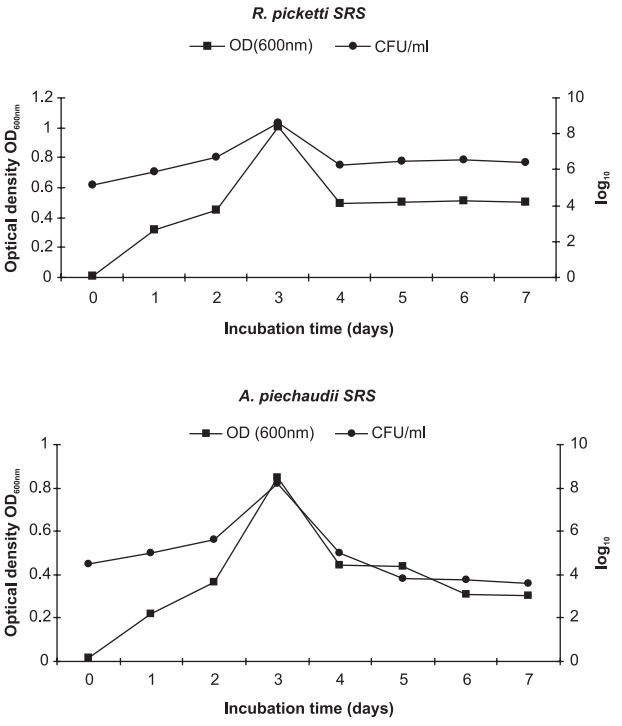

Fig. 1. Growth characterization of isolates in the mineral medium with crude oil as carbon and energy source. Two parameters were measured: colony forming unit (CFU/ml) and optical density.

Fig. 1 presents the growth of bacteria strains in the liquid medium with crude oil as energy and carbon source. Both strains grew very well in this medium. However, *Ralstonia picketti* SRS growth was found to be better than that of *Alcaligenes piechaudii* SRS. The growth curves were typical for the batch culture. Crude oil emulsification was observed during the incubation period.

Changes of TPH concentrations during the crude oil degradation by both isolates and their mixture are presented in Fig. 2. Degradation of crude oil were above 80% after incubation of 20 days for both bacterial strains and their mixture. The maximum degree of removal was achieved after 1-4 days (above 50%). Afterwards removal values decreased progressively and finally approached above 80%. Under the experimental conditions *Alcaligenes piechaudii* SRS was a better degrader than *Ralstonia picketti* SRS. The mixture of the isolates enhanced slightly the degradation efficiency of crude oil.

The removal of short/long chain alkanes (from C6 to C19), cyclohexanes and BTEX during the crude oil degradation are presented in Table 2. All measured hydrocarbons were significantly biodegraded by both bacteria and their mixture. Removal of investigated hydrocarbons was different, from 97% (for hexane degraded by the mixture of the isolates) to 22% (for tridecane degraded by *Alcaligenes piechaudii* SRS). Hexadecane, pristane and cyclohexane were degraded with high efficiency between 72-91% by both isolates and their mixture. Among BTEX, toluene was found to be degraded very well by both strains and their mixture. Benzene and m+p-xylenes were efficiently degraded by a mixture of the isolates, and removal values were 78% and 87%, respectively.

The bacterial cultures also were used to study changes in crude oil toxicity as a function of biodegradation activity (Table 3). Inhibition of luminescence in all tested

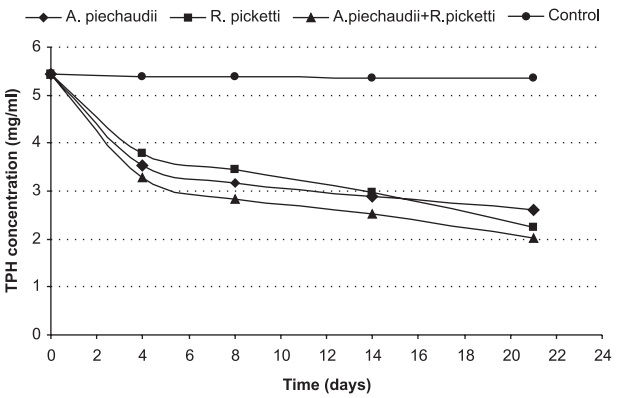

Fig. 2. Changes of TPH concentrations during crude oil degradation (mean values; SD ± 0.1-0.5 mg/ml).

Table 2. Removal of different hydrocarbons (mean values) by *Ralstonia picketti* SRS, *Alcaligenes piechaudii* SRS and their mixture after 21 days of crude oil biodegradation in the mineral medium at 30°C.

Hydrocarbons	Removal values (%)		
	R. picketti SRS	*A. piechaudii* SRS	*R. picketti S*RS + *A. piechaudii* SRS
Hexane C-6	93	86	97
Heptane C-7	87	90	96
Nonane C-9	63	68	66
Decane C-10	59	64	60
Undecane C-11	49	54	51
Dodecane C-12	54	52	49
Tridecane C-13	56	22	52
Hexadekane C-16	91	90	89
Pristane C-19	72	74	72
Cyclohexane	81	91	94
Benzene	52	64	78
Toluene	82	82	87
Ethylobenzene	54	44	45
m+p-xylenes	46	87	87
o-xylene	56	26	58

SD ± 0.5-3%

Table 3. Residual toxicity measured during crude oil degradation in liquid medium at 30°C (mean values).

Incubation time (days)	Residual toxicity (% of luminescence inhibition)			
	R. picketti	*A. piechaudii*	*R.picketti* + *A. piechaudii*	Control
5	10	8	7	5
14	25	20	11	9
21	38	23	27	6

SD ± 1- 4%

samples were comparable to the background level in the abiotic control that caused from 6 to 9% of luminescence reduction. During the time of the incubation luminescence inhibition slightly increased. This inhibition could be due to the presence of toxic intermediates produced during biodegradation of petroleum hydrocarbons. The samples were not to be found toxic to *Vibrio fischeri*. Detection of this activity or residual toxicity remaining after biodegradation underscores the need to test for toxicity changes during biodegradation studies. The occurrence of contaminants in mixtures 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. Bioassys provide important information for the assessment of pollutant effects. In contrast to chemical analyses, they detect effects of multiple contaminants, by-products and metabolites. A large number of bioassays have been applied for the characterization of contaminated soils and for the evaluation of bioremediation efficacy [35-37].

Table 4 presents removal of distillation products. Both bacteria had the ability to degrade refinery products with different boiling range. Degradation of light boiling fractions was between 80-100%. Heavy, high boiling fractions were also degraded by tested isolates. Their removal values reached between 10-80%. The reasons of poor degradability of the high boiling distillates used were their molecular size and physico-chemical parameters (especially water solubility).

Two bacteria strains isolated have special properties for petroleum hydrocarbon remediation, e.g. they produce

Table 4. Removal of distillation products (mean values) by *Alcaligenes piechaudii* SRS, *Ralstonia picketti* SRS and their mixture after biodegradation of 21 days in mineral medium at 30°C. Different distillates were used as carbon and energy sources.

Distillates	Removal values (%)		
	R.picketti SRS	*A.piechaudii* SRS	*R.picketti* SRS+ *A.piechaudii* SRS
A1	83	80	84
A3	100	100	100
A5	83	88	81
P1	81	66	66
P2	10	34	47
P3	64	67	69
P5	60	75	54

SD ± 0.2- 4.5%

biosurfactant and degrade petroleum hydrocarbons. Probably, the uptake and transport of hydrocarbons, and consequently the speed and degree of their degradation, were mostly improved by produced biosurfactants. However, there is no data on biosurfactant concentrations or mode of action. The beneficial effect of biosurfactants on the biodegradation process has not been evaluated.

The petroleum hydrocarbons degradation experiment demonstrated that isolated bacteria are useful to assess the potential for natural attenuation of hydrocarbon-contaminated environments. *Ralstonia* and *Alcaligenes* species have been found in petroleum-contaminated soils [1, 2]. While biodegradation of specific hydrocarbons and biosurfactant production by bacteria such as *Pseudomonas sp*., *Acinetobacter sp*., *Bacillus sp*., *Rhodococcus sp*., and *Arthobacter sp*. have been well studied; not found were reports on the ability of *Ralstonia picketti* and *Alcaligenes piechaudii* to degrade crude oil and produce biosurfactants [38]. *Alcaligenes denitryficans* has been observed to degrade some PAHs: naphthalene, fluoranthene and pyrene [5, 39]. Phenanthrene degradation genes were found in plasmids in *Comamonas testosterone*, *Beijerinckia sp*. and *Alcaligenes faecalis* AFK2 [5, 39].

Both bacteria investigated in this paper produce biosurfactants belonged to the one major class of biosurfactants – rhamnolipids as previously described [28]. It is currently known that the type of biosurfactant made is dictated by the producing microorganisms. Up to now the production of rhamnolipides by *Pseudomonas aeruginosa* is well documented [19, 40]. There are a few reports that other *Pseudomonas sp*. produce rhamnolipids; however, these isolates were not accurately identified to the species [19, 20].

As shown by Bodour et al. [41] biosurfactant-producing organisms were found in at least three major divisions of the *Eubacteria*, including *Proteobacteria*, *Firmicutes* and *Cytophaga-Flexibacter-Bacteroides*, as well as the two divisions of the *Archea*, i.e. *Crenarchaeota* and *Euryarchaeota*.

The capacity of these natural microorganisms to produce biosurfactants and their petroleum degradation is promising for environmental restoration applications at hydrocarbon-contaminated sites.

Conclusions

In our experiment crude oil and its distillation products were efficiently degraded by investigated strains and their mixture. The mixture of the isolates enhanced slightly the degradation efficiency of crude oil and its distillation products. The short alkanes as well as long alkanes and BTEX were well degraded during crude oil degradation. The cultures of the bacteria and their mixture were not found to be toxic to *Vibrio fischeri* during crude oil degradation. As described before, *Ralstonia picketti* SRS and *Alcaligenes piechaudii* SRS produce biosurfactants. However, there is no evidence that biosurfactants produced by the strains were indeed involved in the degradation of crude oil and its distillates. Multifunction of biosurfactants is known. Because their chemical structures and surface properties are so different, their role in mechanisms of assimilation/uptake, and in hydrocarbon biodegradation is difficult to generalize.

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