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Biodegradation of petroleum-waste by biosurfactant-producing bacteria

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Abstract

The degradation of petroleum waste by mixed bacterial cultures which produce biosurfactants: *Ralstonia pickettii* SRS (BP-20), *Alcaligenes piechaudii* SRS (CZOR L-1B), *Bacillus subtilis* (I'-1a), *Bacillus* sp. (T-1) and *Bacillus* sp. (T'-1) was investigated. The total petroleum hydrocarbons were degraded substantially (91 %) by the mixed bacterial culture in 30 days (reaching up to 29 % in the first 72 h). Similarly, the toxicity of the biodegraded petroleum waste decreased 3 times after 30 days as compared to raw petroleum waste. Thus, the mixed bacterial strains effectively clean-up the petroleum waste and they can be used in other bioremediation processes.

Introduction

In recent years, the interest in microbial surfactants (biosurfactants) has continually increased due to their diversity, environment friendly nature, possible large-scale production, selectivity, performance under extreme conditions and potential applications in environmental protection (Finnerty 1994; Banat *et al*. 2000; Bodour & Maier 2002). Biosurfactants have been widely used in environment protection, including enhance oil recovery (EOR), controlling oil spills, and biodegradation and detoxification of oil- and metal-contaminated soils (Christofi & Ivshina, 2002; Ron & Rosenberg 2002; Singh & Cameotra 2004; Mulligan 2005; Kuyukina *et al*. 2005).

Microbially enhanced oil recovery has several advantages over the conventional chemical processes for oil recovery. However, it is unclear whether microbial strains used as inocula actually grow and metabolize in the reservoir. Extensive work has been carried out on laboratory and field scale on the isolation of biosurfactant-producers, characterization of the biosurfactants

produced, and the products of biodegradation of hydrophobic compounds in soils (Finenerty, 1989; Bodour & Maier, 2002; Christofi & Ivshina, 2002). Very little is known about the use of biosurfactant-producing bacteria in the clean-up of hydrocarbon contaminated wastewaters.

In this paper, the application of biosurfactant-producing bacteria to degrade the petroleum waste is described.

Materials and Methods

Isolation, identification and characterization of bacterial isolates

The bacterial strains (BP-20, CZOR L-1B, T-1, T'-1, I'-1a) used in this study were isolated from sludge samples obtained from 100-year-old oil refinery in Czechowice-Dziedzice, Poland by Berry *et al*. (2006) and Płaza *et al*. (2006). The aged sludge was acidic (pH 2) and highly contaminated with polycyclic aromatic hydrocarbons (Brigmon *et al*. 2004). 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 ºC for 8 min, 30 cycles of denaturation at 94 ºC for 1 min, annealing at 55 ºC for 1 min and elongation at 72 °C for 1 min followed by elongation at 72 ºC 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 analyzed 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 (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 carried out by traditional microbiological methods. The biochemical characterization was based on the API ZYM test (bioMerieux S.A.) and standard procedures described by Gerhardt (1981).

Physico-chemical and microbiological characterization of crude oil and petroleum waste

The petroleum waste was obtained from wastewater treatment in the Czechowice-Dziedzice oil refinery. While the physico-chemical parameters for crude oil and petroleum waste were obtained from the Czechowice-Dziedzice oil refinery, the microbiological parameters for petroleum waste (mesophilic and psychrophilic bacterial viable counts and total fungal counts) were determined by the dilution-plate technique using Standard Methods Agar (SMA, BioMerieux) for bacteria and Malt Extract Agar (MEA, BioMerieux) for fungi. The inoculated agar plates (three replicates) were incubated at 20 ºC and 37 ºC for 2 days before the colonies were counted. The physico-chemical parameters for crude oil were: density at 20 ºC (0.726 g/cm^3), viscosity at 20 ºC (0.7731 cSt), water content (0.10 % (w/v)), H_2S content (<0.3 mg/dm^3), salts content (1.1 mg/dm^3), vapour pressure at 37 ºC (55.4 kPa) and sulphur content (0.24 % (w/v)).

Growth on crude oil and petroleum waste

The ability of the bacterial strains, BP-20, CZOR L-1B, T-1, T'-1 and I'-1a, to grow either separately or in mixed culture on the crude oil or petroleum waste as sole carbon and energy source were determined. The strains from an overnight culture (10^4–10^5 CFU/ml) were transferred aseptically to 100 ml of sterile mineral medium (MM) as described by Abu-Ruwaida *et al*. (1991) supplemented with 1 ml of the trace elements solution (Gerhardt 1981) and 1 % (v/v) of crude oil. The cultures were grown aerobically at 30 ºC for seven days with constant shaking (150 rpm). Total viable counts (CFU/ml) and A_{600} measurements on a CECIL CE 2031 were used to monitor bacterial growth.

The ability of mixed bacterial strains to grow on petroleum waste was also determined. A 100 ml mixture of petroleum waste and MM (1:1) was inoculated with 500 µl of a 24 h old culture of each bacterium (10^4 – 10^5 CFU/ml). The consortium of these five bacteria was grown aerobically at 30 ºC for a period of seven days with constant shaking (150 rpm). The bacterial growth was monitored by determining the A_{600}.

Biodegradation of petroleum waste

The biodegradation of petroleum waste by the bacterial consortium was monitored. 1 ml of bacterial strains of initial concentrations 10^4–10^5 CFU/ml were transferred aseptically to 250 ml Erlenmayer flasks (five replicates) containing 50 ml each of sterile MM and petroleum waste. The flasks were incubated at 30 ºC with continuous shaking (150 rpm) for 30 days. An uninoculated medium served as a control. Samples were taken at 0, 3, 8, 15, 20 and 30 days interval for total petroleum hydrocarbon (TPH) analysis. 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 3100-2800 cm^{-1} range. The absorbance value was measured at 2926 cm^{-1} with an IR spectrophotometer (UNICAM SP1000, UK). The TPH content was related to the CH_2 group number.

Residual toxicity during biodegradation of petroleum waste

Microtox® toxicity assay (SDI Europe) was used to evaluate the residual toxicity of the petroleum waste. The method 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 M500 analyzer as per the manufacturer's instructions. The data has been expressed as EC50 (concentration effect causing 50% toxic effect) and TU (toxicity unit = 1/EC50 x 100).

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 % 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 (Altman *et al*. 1997). The isolates were selected based on their ability to produce biosurfactant while growing in culture media with aliphatic and aromatic

petroleum hydrocarbons as reported earlier (Płaza *et al*. 2005, 2006). The biochemical characteristics as determined using the API ZYM kit (Table 1) were characteristic of the genera to which these isolates were assigned based on their 16S rRNA gene sequence similarity analyses.

The physico-chemical and microbiological parameters for the petroleum waste are given in Table 2. The concentration of total petroleum hydrocarbons was 802 g/m^3. BOD and COD were high, and reached 155 g/m^3 and 275 g/m^3, respectively. The refinery waste contained low concentration of bacteria and fungi.

All bacterial strains grew very well in the liquid medium with crude oil as energy and carbon source (Fig. 1). The bacterial growth curves were typical for batch culture. Maximal growth was obtained on the third day of the incubation period. However, after 3 days OD and CFU/ml started to significant decreased for *A. piechaudii* and *R. pickettii*. Figure 2 presents the growth of mixture bacterial cultures in the petroleum waste. The growth curve was similar as obtained for the individual isolates. However, the consortium achieved maximum culture density on the second day of growth. The increasing density of bacteria during the first days of the incubation was accompanied with the high degradation of petroleum waste.

The bacterial consortium effectively degraded the petroleum waste (Fig. 3 and Table 3). 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 three days of incubation TPH decrease was the highest, and reached ~29 %. Bacterial strains used in the experiment had the ability to clean-up the petroleum waste.

The changes of toxicity as a function of petroleum biodegradation activity were also determined (Table 3). At the beginning, the toxicity indicator TU was high (14.2) which

decreased by ~43 % to 8.07 in three days and reaching one/third of the original (4.55) at the end of the 30 day incubation period. This decrease was due to the efficient conversion of the toxic raw-material to less or non-toxic intermediates during the biodegradation. Detection of this activity or residual toxicity remaining after biodegradation underscores the need to test for toxicity changes during biodegradation studies.

The ability to simultaneously degrade petroleum compounds and produce biosurfactants 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*. (Banat *et al*. 2000; Bodour & Maier 2002; Ron & Rosenberg 2002; Singh & Cameotra 2004) The present study investigated the bacterial isolates belonging to the genus *Ralstonia*, *Alcaligenes* and *Bacillus*. The isolates *Ralstonia pickettii* SRS and *Alcaligenes piechaudii* SRS, their surface active and biodegradation properties were described previously (Płaza *et al*. 2005, 2007). However, the structure of the biosurfactants produced and their physiological role are unknown. *Bacillus* spp. are known to produce lipopeptide biosurfactants (Cooper & Goldenberg 1987; Jenny *et al*. 1991; Lin *et al*. 1994). Surfactin, a cyclic lipopeptide produced by *B. subtilis* is the most effective biosurfactant discovered so far (Cooper *et al*. 1981). The authors reported that only 20 mg/l of the purified product reduced the surface tension of water from 72 mN/m to 27 mN/m. The production of biosurfactants by *B. licheniformis* has also been reported in batch cultures under both aerobic and anaerobic conditions (McInerney *et al*. 1990).

How biosurfactants influence hydrocarbon degradation at different sites can depend on the type of microorganisms present (Al-Tahhan *et al*. 2000). Biosurfactant production has been

demonstrated as a microbial mechanism to increase petroleum and other hydrocarbon biodegradation by increasing the bioavailability of the hydrocarbons (McInerney *et al*. 2005). The capacity of these natural microorganisms to produce biosurfactants and their hydrocarbons degradation is promising for environmental restoration applications at hydrocarbon-contaminated sites.

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Table 1. Biochemical characteristics of the isolated bacterial strains

Enzymes/reactions	T-1	T'-1	I'-1a	BP-20	CZOR L-1B
Control	-	-	-	-	-
Phosphatase alkaline	+	+	+	+	+
Esterase (C_4)	+	+	+	+	+
Esterase lipase (C_8)	+	+	+	+	+
Lipase (C_{14})	-	-	+	+	+
Leucine arylamidase	+	-	+	-	-
Valine arylamidase	-	-	+	+	+
Cysteine arylamidase	-	-	-	+	+
Trypsin	-	-	-	+	+
α-chymotrypsin	-	-	-	-	+
Phosphatase acid	+	+	+	-	+
Naphthol-phosphohydrolase	+	+	+	+	+
α-Galactosidase	-	-	+	+	+
β-Galactosidase	-	-	+	-	-
β-Glucuronidase	-	-	+	-	-
α-Glucosidase	-	-	-	-	-
β-Glucosidase	-	-	-	+	-
N-Acetyl- β-glucosaminidase	-	-	-	-	-
α-Mannosidase	-	-	-	-	-
α-Fucosidase	-	-	-	-	-

Catalase test	+	+	+	+	+
Oxidase test	+	+	+	+	+
Indol production test	+	+	+	+	+
Gelatin (hydrolysis)	+	+	+	+	+
Glucose (assimilation)	+	+	+	+	+
Arabinose (assimilation)	+	+	+	+	+
Mannose (assimilation)	+	+	+	+	+
Mannitol (assimilation)	+	+	+	+	+
Utilization of pectin	+	+	++	+	+
Utilization of cellulose	-	-	-	-	-
Utilization of sodium acetate	+	+	++	+	+
Tween 80	++	++	++	+	+
Tween 20	++	++	++	+	+

+: positive reaction; -: no reaction

Table 2. Physico-chemical and microbiological analysis of refinery waste

Parameters	**Mean value**	**Unit**
Waste volume	2832	m^3/day
pH	7.4	
BOD_5 (biochemical oxygen demand)	155	g/m^3
	439	kg/day
COD (chemical oxygen demand)	275	g/m^3
	778	kg/day
Suspension	94	g/m^3
	265	kg/day
Total Petroleum hydrocarbons	802	g/m^3
(TPH)	2271	kg/day
Oxygen consumption	64	g/m^3
Mesophilic bacterial number	2730	CFU/ml
Psychrophilic bacterial number	945	CFU/ml
Total number of fungi	102	propagules/ml

Table 3. Changes of petroleum hydrocarbons concentrations and toxicity during the experiment time

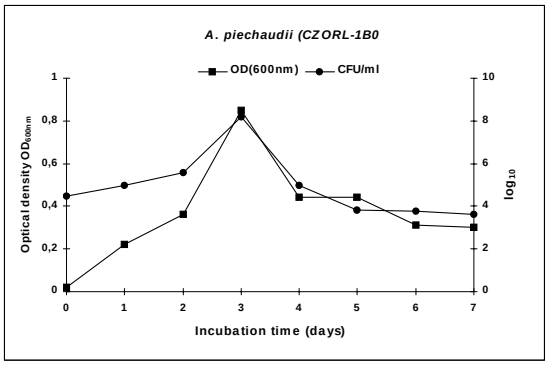
Incubation time (days)	TPH (mg/ml) (mean values)	Removal (%)	Toxicity indicators (mean values)	
			EC50	TU
0	1.90	**0.00**	7.02	14.2
3	1.35	**28.66**	12.4	8.07
8	1.15	**39.00**	21.9	4.56
15	0.80	**57.66**	20.1	4.96
20	0.37	**80.55**	21.0	4.77
30	0.17	**91.10**	22.0	4.55

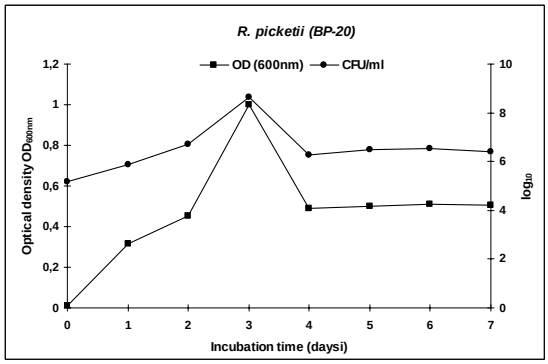
Figure legends

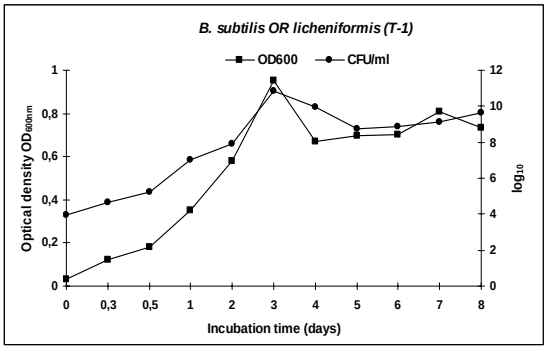
Fig. 1. Growth characterization of isolates in mineral medium with crude oil as the sole carbon and energy source. Two parameters were measured: cell forming unit (CFU/ml) and optical density

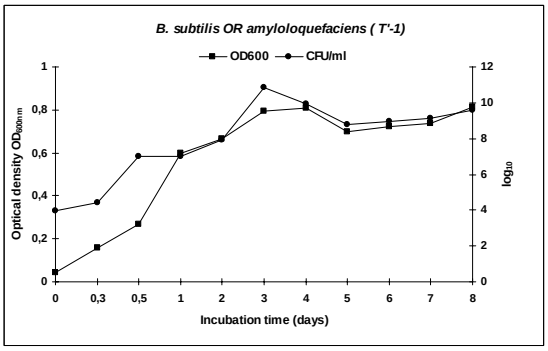
Fig. 2. Growth of mixed bacterial cultures in the petroleum waste medium

Fig. 3. Changes of TPH concentrations during the petroleum waste degradation


A. piechaudii (CZORL-1B0
OD(600nm)
CFU/ml
Optical density OD600nm
log10
Incubation time (days)


R. picketii (BP-20)
OD (600nm)
CFU/ml
Optical density OD600nm
log10
Incubation time (daysi)


B. subtilis OR licheniformis (T-1)
OD600
CFU/ml
Optical density OD600nm
log10
Incubation time (days)


B. subtilis OR amyloloquefaciens (T'-1)
OD600
CFU/ml
Optical density OD600nm
log10
Incubation time (days)

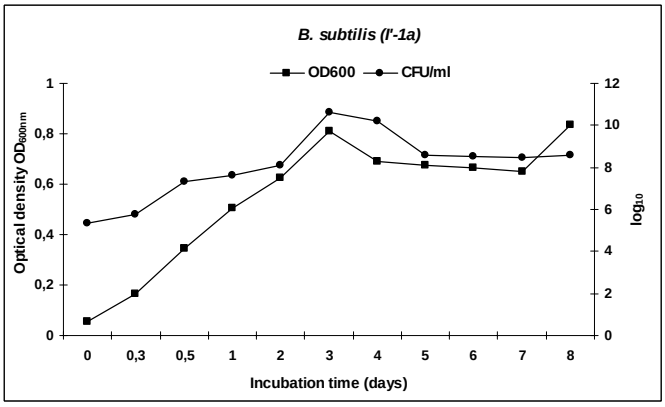
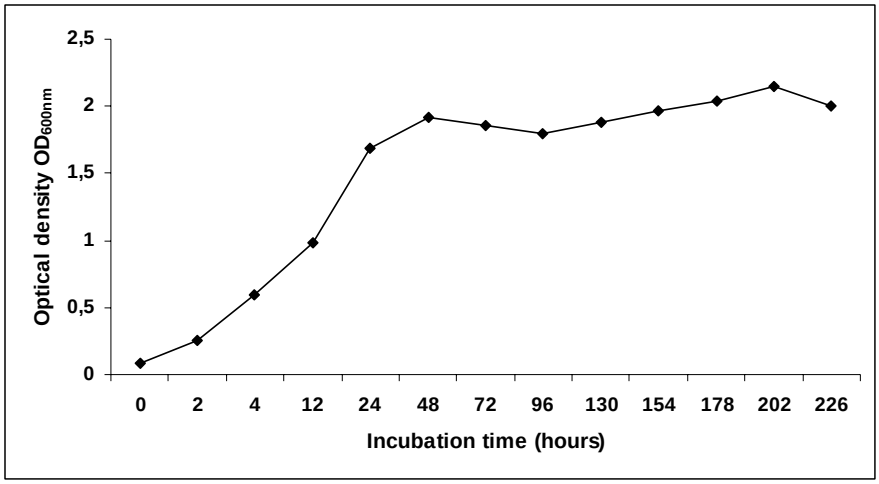

B. subtilis (l'-1a)
OD600
CFU/ml
Optical density OD600nm
log10
Incubation time (days)

Fig. 1.


Optical density OD600nm
2,5
2
1,5
1
0,5
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2
4
12
24
48
72
96
130
154
178
202
226
Incubation time (hours)

Fig. 2.

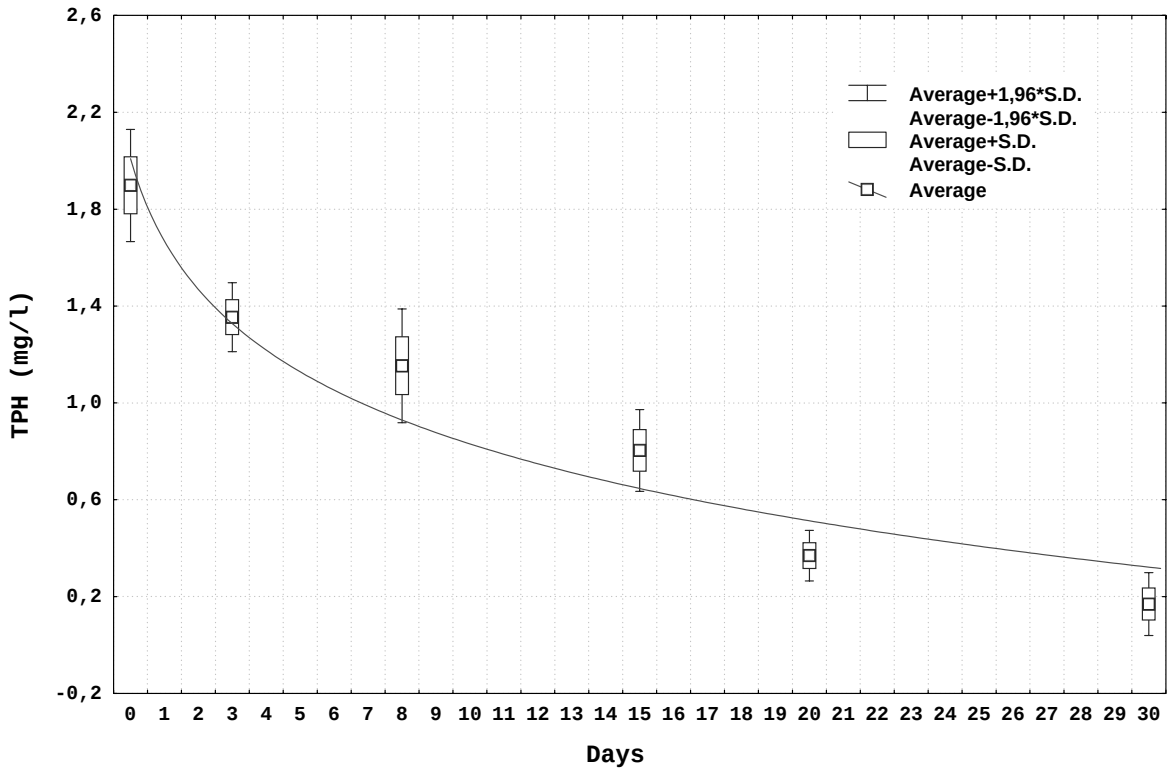
2,6
2,2
1,8
1,4
1,0
0,6
0,2
-0,2
TPH (mg/l)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Days
Average+1,96*S.D.
Average-1,96*S.D.
Average+S.D.
Average-S.D.
Average

Fig.3.