Bioresource Technology 102 (2011) 11003–11010

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Biodegradation potential of oily sludge by pure and mixed bacterial cultures

Vanessa S. Cerqueira [a,*], Emanuel B. Hollenbach [a], Franciele Maboni [b], Marilene H. Vainstein [b], Flávio A.O. Camargo [c], Maria do Carmo R. Peralba [d], Fátima M. Bento [a,*]

[a] Department of Microbiology, Federal University of Rio Grande do Sul, Sarmento Leite, 500, CEP 90050-170, Porto Alegre, RS, Brazil
[b] Department of Molecular Biology and Biotechnology, Federal University of Rio Grande do Sul, Bento Gonçalves, 9500, CEP 91500-970, Porto Alegre, RS, Brazil
[c] Department of Soils, Federal University of Rio Grande do Sul, Bento Gonçalves, 7712, CEP 91540-000, Porto Alegre, RS, Brazil
[d] Department of Inorganic Chemistry, Federal University of Rio Grande do Sul, Bento Gonçalves, 9500, CEP 91500-970, Porto Alegre, RS, Brazil

ARTICLE INFO

Article history:
Received 15 July 2011
Received in revised form 17 September 2011
Accepted 18 September 2011
Available online 22 September 2011

Keywords:
Biodegradation
Biosurfactants
Oily sludge
Consortium
Hydrocarbons

ABSTRACT

The biodegradation capacity of aliphatic and aromatic hydrocarbons of petrochemical oily sludge in liquid medium by a bacterial consortium and five pure bacterial cultures was analyzed. Three bacteria isolated from petrochemical oily sludge, identified as *Stenotrophomonas acidaminiphila*, *Bacillus megaterium* and *Bacillus cibi*, and two bacteria isolated from a soil contaminated by petrochemical waste, identified as *Pseudomonas aeruginosa* and *Bacillus cereus* demonstrated efficiency in oily sludge degradation when cultivated during 40 days. The bacterial consortium demonstrated an excellent oily sludge degradation capacity, reducing 90.7% of the aliphatic fraction and 51.8% of the aromatic fraction, as well as biosurfactant production capacity, achieving 39.4% reduction of surface tension of the culture medium and an emulsifying activity of 55.1%. The results indicated that the bacterial consortium has potential to be applied in bioremediation of petrochemical oily sludge contaminated environments, favoring the reduction of environmental passives and increasing industrial productivity.

1. Introduction

Petroleum industry is one of the main sectors of the world economy, and recognizes Brazil as a developing country of huge economic and environmental potential. According to the Brazilian National Agency of Petroleum, Natural Gas and Biofuels (ANP, 2010), in 2009 Brazil reached the 14th place amongst the largest world petroleum producers (2 million barrels/day).

Petroleum industries bring innumerous benefits to society but are globally recognized as an economic activity with great environmental impacts. Petrochemical industries and refineries generate a large amount of solid waste oily sludge, classified according to the Brazilian Association of Technical Standards – NBR 10004 as Class I or Hazardous Waste (residues that cannot be reutilized or recycled, and may present characteristics such as flammability, corrosivity, toxicity or pathogenicity). Estimates indicate that a petrochemical industry processing 200–500 oil barrels per day generates nearly 10,000 m^3 of sludge (Gafarov et al., 2006).

The oily sludge is a recalcitrant residue characterized as an emulsion of water, oil, fats, solids, organic compounds and metals. Among the organic compounds, the most common are alkanes, cycloalkanes, benzene, toluene, xylenes, phenols and polycyclic aromatic hydrocarbons (HPAs) (Kriipsalu et al., 2008). Many components of the oily sludge are listed as priority pollutants by the United States Environmental Protection Agency due to their toxicity and their mutagenic and carcinogenic potential towards humans (Dhote et al., 2009; Janbandhu and Fulekar, 2011).

Considering the pollution and toxicity of oil hydrocarbons, the development of an effective and environmentally friendly strategy to reduce it still remains a challenge. The increasing pressure by environmental regulatory agencies has motivated the companies to develop clean technologies, highlighting the bioremediation technology. Bioremediation can be defined as the use of microorganisms to remove environmental pollutants in water and soil, aiming the complete degradation of hydrocarbons into carbon dioxide and water (Fernández-Luqueño et al., 2011). In order to increase the bioremediation process yield, the bioaugmentation strategy (addition of microorganisms exogenous and/or endogenous with effective degradation capacity in the contaminated local) has been proposed and evaluated (Bento et al., 2005; Janbandhu and Fulekar, 2011; Mukred et al., 2008; Verma et al., 2006). The employment of a mixed bacterial culture has demonstrated to be more advantageous in comparison with pure cultures due to synergistic interactions among members of the associations, which may lead to the complete degradation of the product (Mukred et al., 2008). The utilization of microorganisms with proven degradation potential and survivability in the contaminated environment is crucial for a successful bioaugmentation. In view of this, the present paper aims to study the degradation capacity of petrochemical oily sludge by

* Corresponding authors. Tel.: +55 (51) 33084497; fax: +55 (51) 3308 3665 (V.S. Cerqueira).
E-mail addresses: vanescerqueira@yahoo.com.br (V.S. Cerqueira), fatimabento@yahoo.com (F.M. Bento).

0960-8524
doi:10.1016/j.biortech.2011.09.074

pure and mixed bacterial cultures isolated from oily sludge and *Landfarming* soil, for bioremediation process applications.

2. Methods

2.1. Sample collection

The oily sludge and soil samples were collected from a petrochemical industry situated in the city of Triunfo, Rio Grande do Sul State, Brazil. The oily sludge samples were collected aseptically from treatment and disposal ditches (VTD's) and the soil samples from *Landfarming* cells which have received petrochemical residues for 24 years. The samples were collected in 5 random and distinct sites, homogenized and aliquots were removed for microorganism isolation.

2.2. Bacterial isolation

The bacteria were isolated through the enrichment technique. The technique consisted of incubating, in 125 mL Erlenmeyer flasks containing 50 mL of MM1 sterile mineral medium [composition in g/L: KCl, 0.7; KH_2PO_4, 2.0; Na_2HPO_4, 3.0; NH_4NO_3, 1.0; micronutrients solution, 1 m/L ($MgSO_4$, 4.0; $FeSO_4$, 0.2; $MnCl_2$, 0.2; $CaCl_2$, 0.2)] (Richard and Vogel, 1999), 1% of oily sludge for isolation of bacteria from the oily sludge, and 1 g of humid soil with 1% of oily sludge for isolation of bacteria from the soil. The flasks were kept in a rotary incubator at 100 rpm and 30 °C. An aliquot (1 mL) was transferred every 5 days to a new MM1 medium and incubated under the same conditions. After 5 transferences, the surface spreading and streak plate techniques in nutrient agar were performed. The purified isolates were pre-selected according to their ability to use different hydrocarbons as carbon sources, their growth capacity and tolerance to different concentrations of oily sludge and their biosurfactant production (data not shown).

2.3. Bacterial identification

The isolates were identified based on the partial sequencing of the 16S rRNA gene. The bacteria were grown in 10 mL nutrient broth at 37 °C for 24 h. The genomic DNA of each culture was extracted from 1.5 mL, according to Sambrook and Roussell (2001), using the CTAB (cetyltrimethylammonium bromide) method with minor modifications. Cell lysis was performed using SDS 10% and 20 mg/mL proteinase K, followed by CTAB 1% incubation and extraction with phenol–chloroform.

The polymerase chain reaction tests (PCR) for the amplification of the 16S rRNA gene of each studied isolate were conducted using universal oligonucleotides corresponding to the 27F (5′ AGA GTT TGA TCM TGG CTC AG 3′) and 1492R (5′ GGT TAC CTT GTT ACG ACT T 3′) positions. The reactions were prepared using 1.5 mM $MgCl_2$, 0.2 mM dNTP, 10 pmol of each oligonucleotide, 1.25 U Go Taq DNA polymerase (Promega) and 1 μl genomic DNA, resulting in 25 μL of reaction. The negative control was prepared in parallel, differing from the other reactions by the absence of genomic DNA. The amplifications were carried out as follows: one cycle at 95 °C for 5 min, followed by 35 cycles at 95 °C for 1 min, 65 °C for 1.3 min, 72 °C for 1 min and a final extension step at 72 °C for 5 min. The fragments generated from the reactions were precipitated with transfer RNA (tRNA–Invitrogen). For this purpose, 1 μg tRNA and 2.5 volumes of absolute ethanol (4 °C) were added to each PCR reaction and incubated at -20 °C for 16 h. After centrifugation for 30 min of and washing with ethanol 70%, the DNAs were resuspended in 18 μL of ultrapure water and stored at −20 °C until the sequencing began. The fragments amplified and purified by tRNA were subjected to automatic sequencing using the Dyenamic ET Dye Terminator Cycle Sequencing Kit (Amersham Biosciences), in the automatic sequencer MEGABACE 1000, as described in the user manual. For confirmation of nucleotide sequences of the 16S gene, each amplification product was subjected to sequencing in triplicate. The sequencing results were processed using the Staden et al. (2003) software package, and analyzed with the Blastn algorithm from the BLAST/NCBI software (Altschul et al., 1997).

Morphological and biochemical characterization of the isolates were also carried out and compared to the Bergey manual (Garrity et al., 2005).

2.4. Bacterial inoculum

The bacterial inoculum was prepared in 50 mL sterile nutrient broth for 24 h at 30 °C at 100 rpm in a rotary shaker. Subsequently, the cells were centrifuged at 4 °C, at 9000 rpm for 15 min and washed in sterile MM1 mineral medium (this step was repeated three times). The obtained pellet was resuspended in MM1 mineral medium and kept in the rotary incubator for 24 h, at 100 rpm and 30 °C in order to deplete energetic reserves (starvation).

2.5. Biodegradation of oily sludge in liquid medium

The oily sludge biodegradation capacity by bacterial isolates was evaluated individually and in consortium through a submerged culture system in Bartha biometer flasks (Bartha and Pramer, 1965). The Bartha respirometric method, prescribed in technical standard L6.350 by CETESB and in NBR 14283/99, aims to directly determine the microbial activity by measuring the carbon dioxide produced during microbial respiration and, indirectly, the biodegradation of organic contaminants.

The experiments were performed in triplicate using 125 mL biometer flasks. The isolates were inoculated into a $10^5 - 10^6$ cells/mL initial concentration in MM1 medium (50 mL) containing 1% of oily sludge as the sole carbon source, and kept in a rotaty shaker at 100 rpm, and 30 °C during 40 days. Every 5 days, the biometer flasks were aerated for 5 min through ascarite filters (soda lime). Control tests were used, consisting of mineral medium and oily sludge without microorganism inoculation, as well as blank tests, consisting of empty flasks.

2.6. Analyses

2.6.1. Evaluation of bacterial growth and microbial activity

The heterotrophic microorganisms total during 40 days was estimated using the Most Probable Number (MPN) method in microtiter plate according to Bento et al. (2005). The microbial population was determined using MPN tables (APHA, 1995).

The carbon dioxide produced during the microbial activity was captured by a 0.20 M KOH solution (10 mL) located by the side of the biometer flasks. Periodically, the KOH solution was removed and 1 mL of 0.5 M barium chloride solution and 3 drops of 1% phenolphthalein indicator were added. The residual KOH was titrated with HCl 0.1 M standardized solution. The amount of carbon dioxide produced was obtained through the equation CO_2 generated (mg) $= (V_B - V_A) \cdot (M_{CO_2}/2)$. M_{HCl}. CF, where V_B and V_A are the volume of HCl 0.1 M used to titrate the blank and the treatment in mL, respectively; M_{CO_2} is the molar mass of carbon dioxide in g/mol; M_{HCl} is the molar concentration of HCl standard solution in mol/L; and CF is the correction factor for acid/base molarity (M_{HCl}/M_{KOH}).

2.6.2. pH Measurement and biosurfactant production

The pH of the supernatant was measured with a digital pH meter (Digimed, MD-22). The cells were removed from the culture medium by centrifugation at 9000 rpm for 15 min at 4 °C.

The emulsifying activity was determined in the presence and absence of cells. A 2 mL aliquot was vortexed with kerosene (2 mL) in flat-bottomed glass tubes for 2 min. The emulsifying activity was determined after 24 h by the height of emulsified oil and total height ratio (Cerqueira and Costa, 2009). Surface tension measurements were carried out in the absence of cells, using a digital surface tension meter (Gibertini, Milan, Italy), employing the Wilhelmy plate method. Distilled water (72.1 mN m^{-1}) and ethanol (24 mN m^{-1}) were used as standards.

2.6.3. Biodegradation assays

The remaining oily sludge in the liquid medium at the end of the 40 day period was removed by liquid–liquid extraction. Initially, the culture medium was acidified to pH 2.0 and subjected to three extractions with dichloromethane (CH_2Cl_2) using a separating funnel. Excess water in the mineral medium after extractions was removed by adding anhydrous sodium sulfate (Na_2SO_4). The extract was concentrated in a rotary evaporator. The separation of saturated and aromatic fractions was performed by Preparative Liquid Chromatography in glass columns, using as stationary phases silica gel 60 (SiO_2) and aluminum oxide 90 (Al_2O_3) and as mobile phases n-hexane and n-hexane/toluene mixture (12:8). The purified fractions were concentrated by rotary evaporation and further brought to dryness under a light nitrogen flow.

The fractions were analyzed by gas chromatography in a gas chromatograph (AGILENT 6890 Series GC System) with a mass selective detector (AGILENT 5973 Network Mass Selective Detector). Quantitative analysis was performed by internal standardization, adding a known concentration of *n*-alkane C_{20} deuterated standard to the saturated fraction, and known concentrations of naphthalene, phenanthrene, and chrysene deuterated standards to the aromatic fraction. The analysis conditions were: fused silica column, type 5% phenyl and 95% dimethylpolysiloxane (30 m × 0.25 mm × 0.25 mM); injector temperature: 290 °C; initial oven temperature: 40 °C for 1 min followed by a temperature increase of 6 °C/min up to 300 °C, maintaining this temperature for 20 min; sample amount: 1 μL in splitless mode; transfer line temperature of 290 °C and gas flow set at 1 mL/min.

2.7. Statistical analysis

The statistical analysis of the results was carried out through the Tukey test (comparison between means) with a confidence level of 95%.

3. Results and discussion

3.1. Bacterial identification

Bacterial isolates were obtained from petrochemical oily sludge samples and *Landfarming* soils which had been subjected to 24 years of petrochemical waste disposal. Through the enrichment technique in liquid mineral medium, using oily sludge as the sole carbon source, it was possible to isolate 21 soil bacteria and 13 oily sludge bacteria. Previous studies of the isolates growth capacity and tolerance to varying contaminants concentration and biosurfactants production (data not shown) allow selecting three oily sludge bacteria and two soil bacteria as potential candidates to oily sludge biodegradation processes. Morphological and physiological

Table 1
Morphological, biochemical and physiological analyses of bacteria isolated from *Landfarming* soil and petrochemical oily sludge.

Characteristic	*Stenotrophomonas acidaminiphila*	*Pseudomonas aeruginosa*	*Bacillus megaterium*	*Bacillus cibi*	*Bacillus cereus*
Gram's staining test	Gram negative	Gram negative	Gram positive	Gram positive	Gram positive
Morphology	Rods	Rods	Rods	Rods	Rods
Endospore			Central	Central	Central
MacConkey agar growth	+	+			
Oxidase	–	+	–	–	+
Catalase	+	+	+	–	+
Oxidation/fermentation	–	–	–	–	F
Motility	+	+	+	+d	+
Indole production	–	–	–	–	–
H_2S production	–	–	–	–	–
Citrate utilization	–	+	–	–	–
Gelatin hydrolysis	–	+	–	–	–
Arginine dehydrolase	–	+			
Lecithinase	–	–	–	–	+
Lipase		–			
Aesculin hydrolysis	+	–			
Urease	–	+d	–	–	+
Starch hydrolysis	–	–	+	+	+
Methyl red test	–	–	–	–	+
Voges Proskauer test	–	–	–		+
Nitrate reduction	–	+	–d	–	+
Acid production from glucose	+d	–	+	–	+
Arabinose	–	–	+	+d	+
Xylose	–	–	–	–	–
Mannitol	–	–	+	–	–
Trealose	+	–	+	+	+
Inositol	–	–	–	–	–
Lactose	–	–	–	–	–
Growth at 41 °C		+			
Growth at 50 °C			–	+	–
Casein hydrolysis			+	+	+
Phenylalanine			–	–	–
DNAse	+	–			
Growth 7.5% (w/v) NaCl	–	–	–	+	–
Anaerobic growth	–	–	–	Facultative	Facultative

+, positive reaction; –, negative reactions; d, dubious.

Table 2
Molecular identification of bacteria isolated from *Landfarming* soil and petrochemical oily sludge.

Isolate	Origin	Identification	Identity (%)
BB05	Oily sludge	*Stenotrophomonas acidaminiphila*	99
BB06	Oily sludge	*Bacillus megaterium*	99
BB07	Oily sludge	*Bacillus cibi*	98
BS11	Soil	*Pseudomonas aeruginosa*	98
BS20	Soil	*Bacillus cereus*	98

analyses of the isolated strains are summarized in Table 1. The analyses of the partial sequence of the 16S rRNA gene showed that three bacteria belong to the *Bacillus* genus, one belongs to the *Pseudomonas* genus, and one belongs to the *Stenotrophomonas* genus (Table 2).

There are few reports in the literature regarding the isolation of bacteria directly from oily sludge. It is far more common to find references on bacteria isolated from hydrocarbon-contaminated soils. Several strains of the genera *Pseudomonas*, *Stenotrophomonas* and *Bacillus* isolated from hydrocarbons-contaminated environments are able to grow and degrade aliphatic and aromatic hydrocarbons (Assih et al., 2002; Fernández-Luqueño et al., 2011; Thavasi et al., 2011). In studies conducted by Assih et al. (2002), *Stenotrophomonas acidaminiphila* was isolated from anaerobic sludge in a lab-scale upflow anaerobic sludge blanket (UASB) reactor treating petrochemical effluents. *Pseudomonas aeruginosa* has been isolated from hydrocarbon-contaminated soil and are biosurfactant producers (Kumar et al., 2008). Studies regarding the ability of *Bacillus megaterium* and *Bacillus cereus* strains to degrade hydrocarbons and produce biosurfactants have also been reported (Thavasi et al., 2011; Tuleva et al., 2005). However, few reports were found in the literature regarding hydrocarbon degradation capacity and studies on biosurfactant production by *Bacillus cibi* were not found.

The enrichment technique with oily sludge and *Landfarming* soil contaminated with oily sludge allowed the isolation and selection of bacteria tolerant to this residue. In order to treat contaminated areas, it is important to search for microorganisms which are native to the contaminated environment and provide excellent degradation potential, considering their advantages when compared to exogenous microorganisms. These microorganisms present among others greater adaptability, greater resistance to changes in environmental conditions and lower susceptibility to genetic variation caused by environmental stress.

3.2. Bacterial growth

Bacterial growth throughout 40 days of oily sludge cultivation was estimated by the Most Probable Number (MPN) technique (Fig. 1).

An increase in bacterial population was observed over time, reaching a maximum of 10^{10} MPN/mL for isolates *B. megaterium* (13 and 33 days), *B. cibi* (33 days), *P. aeruginosa* (40 days), and the bacterial consortium (28, 33 and 40 days), as well as a maximum of 10^{8} MPN/mL for isolates *S. acidaminiphila* (33 days) and *B. cereus* (23 days). The bacterial population increase indicated that the oily sludge, used as the sole carbon source, stimulated the growth and metabolism of different isolates.

Among the experiments, only the bacterial consortium showed a reduction of microbial population in the initial period of the process. This reduction can be related to the impact on the bacteria caused by the oily sludge addition to the medium, the adaptability period or the competition among the strains. After 13 days, the consortium yielded bacterial population growth, reaching its maximum population between 28 and 40 days, indicating that, during this period, there was no inhibition or competition of microbial activity.

3.3. Microbial activity (CO_2) and pH measurements

Microbial respiration is one of the oldest parameters for quantification of microbial activity. In aerobic conditions, respiratory activity can be easily evaluated by the amount of carbon dioxide generated by microbial activity under controlled conditions or in the field over a given period (Moreira and Siqueira, 2006). The microorganisms are capable of assimilating hydrocarbons as a carbon source, liberating carbon dioxide.

Fig. 2 shows cumulative CO_2 production throughout 40 days of experiment in Bartha respirometers. The highest carbon dioxide generation occurred in 8 days of processing for all the tests, reaching a maximum production of 15.1 mg with isolate *P. aeruginosa*. Highest productions of CO_2 were observed within initial process periods (up to 13 days). A similar result was observed by Mariano et al. (2008) in studies on biodegradability of pure diesel (B0), biodiesel (B100) and blends (B2, B5 and B20) in water during 50 days, in which the maximum daily production of CO_2 was obtained in the first days of processing for all tested compounds and concentrations.

From the microbial growth behavior and CO_2 production, it was inferred that the isolates demonstrated a rapid utilization of oily

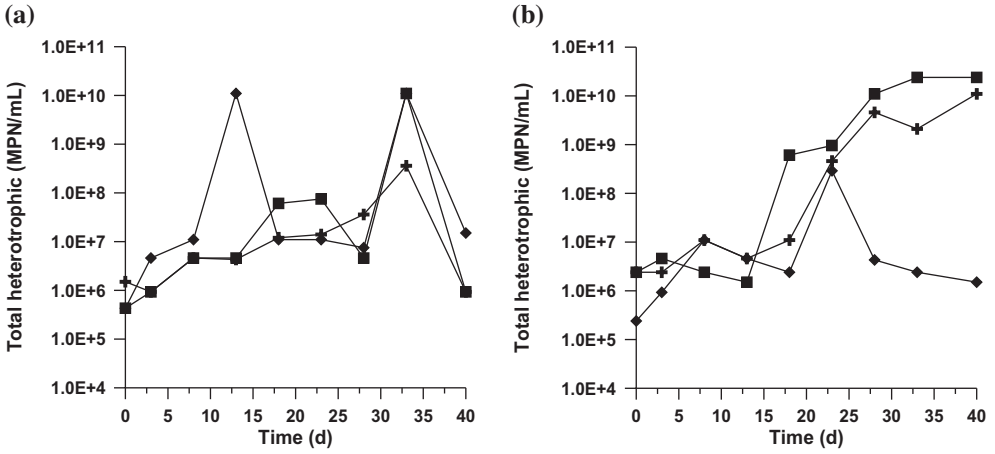

Fig. 1. Estimated number of total heterotrophic microorganisms throughout 40 days of experiment. (a) Bacteria isolated from oily sludge ✚ BB5, ♦ BB6, ■ BB7. (b) Bacteria isolated from *Landfarming* soil and bacterial consortium ✚ BS11, ♦ BS20, ■ bacterial consortium.

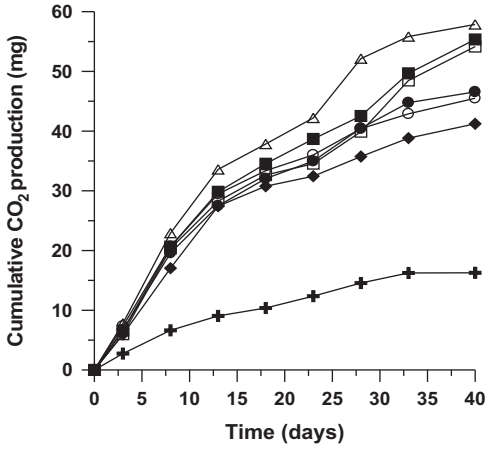

Fig. 2. Cumulative CO_2 production (mg) during 40 days of cultivation. ✚ Control, ♦ *S. acidaminiphila* BB5, ■ *B. megaterium* BB6, ● *B. cibi*, △ *P. aeruginosa*, ○ *B. cereus*, □ Bacterial consortium.

sludge as a carbon and energy source, without an adaptation phase. This is probably due to the use of starvation prior to inoculation, since in a previous study (Cerqueira et al., submitted article), it was observed that the addition of this stage to the process has led to a faster use of oily sludge as carbon source. The early high carbon dioxide production in the process means that the oily sludge was readily used in the first weeks. The aliphatic fraction, which is more labile when compared to the aromatic fraction, was probably more rapidly utilized by the microorganisms.

The highest CO_2 production over time was obtained with isolate *P. aeruginosa*, demonstrating a cumulative production of 57.9 mg. Similar values were obtained for isolate *B. megaterium* BB6 (55.4 mg) and for the microbial consortium (54.2 mg). The CO_2 production result, which measures microbial activity, was directly proportional to bacterial growth. The isolates *P. aeruginosa, B. megaterium* BB6 and the bacterial consortium showed higher microbial growth over time and higher cumulative CO_2 production. A similar result was also observed on isolates *B. cereus* and *S. acidaminiphila*, which showed the lowest cumulative CO_2 productions and lowest microorganism concentrations when compared to the others. The mixture of isolates in the consortium formulation demonstrated positive synergistic effects, once it presented optimum microbial growth, as well as high CO_2 production. The use of microbial consortia may result in metabolic complementarity, since metabolites produced through incomplete degradation by a specific microorganism can be used as carbon source by other microorganisms, increasing the possibility of a complete elimination of hydrocarbons from the environment.

The growth and microbial activity in mineral medium resulted in a pH decrease in all tests. *B. megaterium*, which presented high CO_2 production and high microbial population, had the greatest pH reduction. As shown in Table 3, the pH decrease of isolate *B. megaterium* differs only statistically from isolate *S. acidaminiphila* which presents the lowest pH reduction ($p < 0.05$) and the lowest cumulative production of CO_2. The pH reduction during cultivation can be attributed to the release of organic acids as a consequence of hydrocarbon degradation or to the production of extracellular polymers (Verma et al., 2006). Similar results were observed in studies conducted by Janbandhu and Fulekar (2011), which found that the growth of a microbial consortium formed by *Sphingobacterium* sp., *B. cereus* and *Achromobacter insolitus* MHF growing for 14 days in mineral medium and containing 100, 250 and 500 mg/L of phenanthrene caused a pH reduction from 7.0 to 5.6, 5.3 and 5.2, respectively. A pH reduction was also observed in studies by

Table 3
Surface tension (ST), emulsifying activity in the absence (EAac) and in the presence (EApc) of bacterial cells and pH for the different isolates and the microbial consortium on the 40th day of cultivation.

Isolate	ST (mN m^{-1})	EAac (%)	EApc (%)	pH
S. acidaminiphila	41.0 ± 0.2^{a}	40.0 ± 0.0^{a}	40.0 ± 4.0^{a}	6.83 ± 0.18^{a}
B. megaterium	41.0 ± 0.1^{a}	23.3 ± 0.0	20.0 ± 0.0	6.26 ± 0.03^{b}
B. cibi	42.6 ± 0.1^{b}	$44.0 \pm 3.8^{a,b}$	$46.7 \pm 3.3^{a,b}$	$6.73 \pm 0.13^{a,b}$
P. aeruginosa	$41.8 \pm 0.0^{a,b}$	40.0 ± 1.6^{a}	$48.0 \pm 0.0^{b,c}$	$6.74 \pm 0.01^{a,b}$
B. cereus	41.4 ± 0.3^{a}	33.3 ± 0.1	$44.0 \pm 4.0^{a,b}$	$6.72 \pm 0.10^{a,b}$
Consortium	36.6 ± 0.1	47.8 ± 1.9^{b}	55.1 ± 1.7^{c}	$6.74 \pm 0.16^{a,b}$

Identical letters indicate that the tests did not present significant differences ($p > 0.05$) for each studied response.
Surface tension of the medium in the control test in 40 days = 60.4 mN m^{-1} and initial medium pH = 7.2.

Verma et al. (2006) when *Bacillus* sp., *Pseudomonas* sp. and *Acinetobacter* were cultivated in a mineral medium containing oily sludge as the sole carbon source for 7 days.

The pH is a selective environmental factor affecting the diversity and microbial activity, controlling enzyme activities, transport processes and nutrient solubility (Dhote et al., 2009).

3.4. Biosurfactant production

One of the main factors affecting the biodegradation efficiency of complex oily compounds is the low availability of contaminants for microbial attack. An alternative to expand bioavailability and contaminant metabolism is increasing substrate solubilization by using biosurfactants. Biosurfactants are characterized as organic molecules containing a hydrophobic and a hydrophilic portion, giving them the ability to act in the interface of different compounds (Cerqueira and Costa, 2009; Nitschke and Pastore, 2002).

In the treatment of areas contaminated with complex compounds such as oily sludge, which are difficult to degrade, it is both economically and environmentally interesting the use of microorganisms that present excellent degradation capacity together with the production of biosurfactant from the contaminant as a carbon source. This alternative has great advantage over the use of bacteria which only present the degradative capacity (Cameotra and Singh, 2008). Table 3 presents the results of biosurfactant production after 40 days of experimentation.

Analyses of surface tension and emulsifying activity demonstrated that all isolates and the microbial consortium were able to produce biosurfactants in mineral medium containing oily sludge as the sole carbon source. The greatest surface tension reduction was achieved with the microbial consortium, which reached a value of 36.6 mN/m in 40 days of cultivation, showing a significant difference when compared to other experiments ($p < 0.05$). The microbial consortium presented an emulsifying activity of 47.8% in the absence of cells, and 55.1% in the presence of cells.

High emulsifying activity in the presence of cells was also observed with *P. aeruginosa*. Several studies have shown the potential of *P. aeruginosa* in the production of biosurfactants. Rhamnolipids are one of the most common groups of glycolipids produced by *P. aeruginosa*. This isolate produces mainly two families of rhamnolipids containing one or two rhamnose residues (mono- or di-rhamnolipids, respectively). Currently, rhamnolipids represent one of three commercially available biosurfactants, along with sophorolipids and surfactin, and is the only biosurfactant approved by the United States Environmental Protection Agency (USEPA) for use in food, cosmetics and pharmaceutical products (Toribio et al., 2010).

The isolates demonstrated an emulsifying activity of up to 55.1% and a reduction of up to 39.4% in surface tension. Studies conducted by Dhote et al. (2009) reported surface tension reduc-

tions from 42 to 30 mN m^{-1} and from 51 to 34 mN m^{-1}, as well as emulsifying indexes of 46% and 57% for two bacteria isolated from petrochemical oily sludge. The bacteria were tentatively identified as *Bacillus* sp. and *Pseudomonas* sp., when cultivated in a mineral medium containing chrysene as the sole carbon source. Verma et al. (2006) reported that the bacteria identified as *Bacillus* sp. SV9, *Pseudomonas* sp. SV17 and *Acinetobacter* sp. SV4, isolated from an oily sludge-contaminated soil, were capable of reducing the surface tension in 59%, 51%, and 32%, respectively, after 5 days of cultivation in mineral medium containing 1% of oily sludge as the sole carbon source.

3.5. Oily sludge degradation

The oily sludge degradation at the end of 40 days was evaluated through chromatographic analysis. The degradation percentage of saturated and aromatic fractions is shown in Table 4.

The oily sludge is a complex mixture of aliphatic, aromatic, resins and asphaltene compounds. In the present study, only fractions of alkanes and aromatics were determined. The initial sample characterization showed amounts of approximately 90% of aliphatic and 10% of aromatics. According to Toledo et al. (2006), the relative proportions of these fractions vary from oil to oil, and the susceptibility of specific oil to microbial degradation can be predicted from this composition. According to Mohamed et al. (2006), the biodegradability of petroleum compounds follows a decreasing preferential order: *n*-alkanes > branched-chain alkanes > branched chain alkenes > monoaromatic > cycloalkanes > polyaromatic > asphaltenes.

Fractions containing n-alkanes are usually more susceptible to biodegradation, considering that the saturated fractions containing branched alkanes are less vulnerable to microbial attack (Toledo et al., 2006). This was evidenced in this study since, in the final days of cultivation, the isolates efficiently degraded the aliphatic compounds (*n*-C11 to *n*-C28), presenting only significant amounts of the pristane and phytane compounds. Pristane ($C_{19}H_{40}$) and phytane ($C_{20}H_{42}$) are two isoprenoid alkanes present in fuels. According to Paudyn et al. (2008), due to their branched nature, isoprenoids are relatively resistant to biodegradation and are more slowly degraded when compared to linear ones.

All bacterial isolates and the consortium presented higher degradation rate for the saturated fraction when compared to the aromatic compounds. Higher degradation values of saturated fraction were achieved with the microbial consortium (90.7%) and *S. acidaminiphila* (91.7%), a difference which is not statistically significant ($p > 0.05$). In relation to the aromatic fraction, higher values were obtained in the experiments with bacterial consortium (51.8%) and *B. cibi* (64.3%). Studies carried out by Das and Mukherjee (2007) observed that a *Bacillus subtilis* strain and a consortium formed by two *P. aeruginosa* strains presented higher degradation rates of the n-alkane fraction than the aromatic fraction during petroleum degradation in soils. Verma et al. (2006) verified the oily sludge degradation capacity of three pure cultures cultivated separately in liquid mineral medium after 5 days. *Bacillus* sp. SV9 presented the highest efficiency, reaching 89% for the aliphatic fraction and 75% for the aromatic fraction, followed by *Acinetobacter* sp. SV4, which degraded 78% of aliphatics and 58% of aromatics and *Pseudomonas* sp. SV 17 which degraded approximately 60% of both fractions.

When compared to other isolates, *S. acidaminiphila* showed a higher degradation rate for aliphatic and aromatic compounds, lower cumulative CO_2 production rate and lower maximum microbial population. This isolate probably gives preference to aliphatic compounds as a carbon and energy source, developing specific enzymes which are necessary for the degradation of these hydrocarbons. Due to the lower CO_2 release rate, it can be inferred that its metabolic activity was also slower when compared to other isolates, reaching maximum growth in 33 days of processing. Presumably, the high degradation of the aliphatic fraction led to the exhaustion of the more easily degradable hydrocarbons and to the intake of nutrients, causing a delay in the catabolic activity of the aromatic fraction. As a consequence, the degradation of the aromatic fraction may have been hampered due to a lack of energy required for the expression and production of specific enzymes to attack the aromatic molecules.

Another possibility concerns catabolic repression, in which a compound cannot be degraded in the presence of another compound, acting as a repressor to the synthesis of a great number of enzymes involved in several metabolic paths. Due to their structure, aromatic compounds are less likely to be attacked and consumed as a carbon source by the microorganisms. According to Mohamed et al. (2006), primary consumption or depletion of alkanes (saturated fraction) can result in high contents of polyaromatic fraction and asphaltenes (unsaturated fraction with a high C/H rate), which are more resistant to biodegradation.

The isolates showed aromatic fraction degradation rates ranging from 33.2% to 64.3%. In all experiments it was verified that, among the aromatic fractions, naphthalene presented the highest degradation, followed by methylnaphthalene. The isolates demonstrated a preferential order of degradation for aromatic compounds, with those of lower molecular mass presenting higher degradation rates. Aromatic fractions are more difficult to biodegrade and their susceptibility decreases with the number of aromatic or cyclic rings in the molecule.

Several studies on the degradation of polyaromatic hydrocarbons by bacteria demonstrated a tendency towards an inverse relationship between the biodegradation rates and the number of aromatic rings and molecular mass (Molina et al., 2009). According to the Vitte et al. (2011), under permanent oxic condition, the lighter PAH (i.e., with 2 or 3 aromatic rings) present in oily sludge were almost completely removed after 10 days of incubation. In contrast, the concentration of fluoranthenes, pyrenes and crysenes (heavier PAH) started to decreased only after 10 days of incubation.

Regarding the aromatic fraction, isolate *B. cibi* and the microbial consortium showed the highest degradation rate ($p < 0.05$). It is important to highlight that, in the present study, isolate *B. cibi* showed an optimum biodegradation capacity for aromatic compounds, as well as an optimum biosurfactant production capacity, reaching an emulsifying activity of 46.7%. This is the first report of biosurfactant production capacity and oily sludge biodegradation by *B. cibi*.

It is also important to note that the microbial consortium demonstrated the highest biosurfactant production, showing an emulsifying activity of 55.1% and surface tension reduction to 36.6 mN/m. All the bacteria in the consortium, when cultivated in isolation, were able to produce biosurfactants. However, this activity increased when the consortium form was used. The fact

Table 4
Degradation of saturated and aromatic fractions of oily sludge for different isolates and the microbial consortium.

Isolate	Biodegradation (%)	
	Saturated fraction	Aromatic fraction
S. acidaminiphila	91.7 ± 3.0	33.2 ± 1.1[a]
B. megaterium	89.0 ± 2.2	39.6 ± 3.8[a,b]
B. cibi	89.7 ± 1.0	64.3 ± 1.3[c]
P. aeruginosa	86.7 ± 0.8	39.5 ± 2.6[a,b]
B. cereus	88.4 ± 0.6	40.3 ± 6.7[a,b]
Consortium	**90.7 ± 1.1**	**51.8 ± 2.1[b,c]**

Identical letters indicate that the tests did not present significant differences ($p > 0.05$) for each studied response.

that these isolates present a higher capacity for aromatic degradation may be closely related to their biosurfactant production capacity. Biosurfactants can increase the aqueous dispersion of poorly soluble compounds, including polyaromatic hydrocarbons, by many orders of magnitude, changing the affinity between the bacterial cells and hydrocarbons by increasing cell surface hydrophobicity (Abalos et al., 2004). Consequently, biosurfactants increase the accessibility of microorganism to lipophilic compounds, accelerating the biodegradation of such compounds in different media (Fernández-Luqueño et al., 2011). Wang et al. (2011) suggested that the production of emulsifying agents or biosurfactants from the degradation of short chain hydrocarbons by bacteria *Dietzia* sp. DQ12-45-1b led to the initial solubility of crude oil and favored the growth of the cells and degradation of hydrocarbons. They reported that study is needed to determine whether *Dietzia* sp. DQ12-45-1b could produce different emulsifying agents that preferentially dissolve short-chain (<C25) and long-chain (>C28) alkanes, or if it produces a special emulsifying agent that preferentially dissolves both short-chain (<C25) and long-chain (>C28) alkanes, thereby leading to the preferential degradation of hydrocarbons <C25 and >C28.

Considering the sum of total fractions of saturated and aromatic compounds remaining by the end of process, the isolates and the microbial consortium were capable of achieved values of 83.1–87.4% of oily sludge degradation related to the initial content. Lower results were obtained by Zhang et al. (2007), who verified an increase in the biodegradation rate of refinery oily sludge from 69.4% to 77.4% after optimizing the fermentation conditions of *Bacillus* HJ-1 growing in liquid medium for 7 days. Lower results were also obtained in studies carried out by Verma et al. (2006) in which the isolate *Bacillus* sp. SV9 growing for 5 days in mineral liquid medium was more efficient for oily sludge degradation, reaching 59%.

The lowest aromatic fraction degradation rates were obtained with isolates *S. acidaminiphila*, *B. megaterium* and *P. aeruginosa*. However, the use of isolates in the form of consortium increased the biodegradation rate. This result was also observed in the saturated fraction. Although several pure cultures of hydrocarbon-degrading bacteria can readily use hydrocarbons as a carbon source, satisfactory degradation results can be obtained if a mixed bacterial culture is used. Moreover, biodegradation processes which use pure cultures generally do not represent the actual behavior of environmental microorganisms during bioremediation in natural environments contaminated with hydrocarbons, given that, in nature, the bioremediation process depends on cooperative metabolic activities of mixed microbial populations (Janbandhu and Fulekar, 2011).

The advantage of using microbial consortia can be attributed to the multiple metabolic capacities and the synergetic effects between the association members, causing the increase of bioremediation efficiency. The mechanisms through which the degrading bacteria benefit from synergistic interactions can be complex. It is possible for a species to remove the toxic metabolites of the preceding species (which might otherwise hamper microbial activity). It is also possible for other species to degrade compounds which the first species is capable of partially degrading, promoting cometabolism processes (Mukred et al., 2008). Given that the oily sludge is a complex mixture of hydrocarbons and other compounds, the wide range of degradation substrates by the studied isolates presents a significant advantage for bioremediation of contaminated sites.

4. Conclusion

This study showed that the microbial consortium composed by five bacteria (*S. acidaminiphila, B. megaterium*, *B. cibi*, *P. aeruginosa* and *B. cereus*) degraded, in liquid medium, high concentration of saturated and aromatic compounds contained in the oily sludge producing biosurfactant efficiently. Therefore, this microbial consortium is suitable to be applied in bioremediation process in sites contaminated with oily sludge. Considering the high production and their toxicity and carcinogenic potential towards humans, the biotreatment of oily sludge utilizing competent degraders of hydrocarbons is an interesting ecofriendly strategy since it contributes to the minimization of process time and environmental impacts.

Acknowledgements

The authors would like to thank FAPERGS (Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), for their financial support.

References

Abalos, A., Viñas, M., Sabaté, J., Manresa, M.A., Solanas, A.M., 2004. Enhanced biodegradation of Casablanca crude oil by a microbial consortium in presence of a rhamnolipid produced by *Pseudomonas aeruginosa* AT10. Biodegradation 15, 249–260.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped blast and psi-blast: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Agência Nacional do Petróleo Gás Natural e Biocombustíveis (ANP), 2010. Anuário Estatístico Brasileiro do Petróleo, Gás Natural e Biocombustíveis, Rio de Janeiro, Inss 1983–5884.

APHA, 1995. Standard Methods for the Examination of Water and Wastewater, 19th ed. Washington.

Assih, E.A., Ouattara, A.S., Thierry, S., Cayol, J.L., Labat, M., Macarie, H., 2002. *Stenotrophomonas acidaminiphila* sp. nov., a strictly aerobic bacterium isolated from an upflow anaerobic sludge blanket (Uasb) reactor. Int. J. Syst. Evol. Microbiol. 52, 559–568.

Bartha, R., Pramer, D., 1965. Features of flask and method for measurement of the persistence and biological effects of pesticides in soil. Soil Sci. 100, 68–70.

Bento, F.M., Camargo, F.A.O., Okeke, B.C., Frankenberger, W.T., 2005. Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. Bioresour. Technol. 96, 1049–1055.

Cameotra, S.S., Singh, P., 2008. Bioremediation of oil sludge using crude biosurfactants. Int. Biodeter. Biodegr. 62, 274–280.

Cerqueira, V.S., Costa, J.A.V., 2009. Biodegradação de tolueno e óleo de pescado em solos impactados utilizando surfactantes químico e biológico. Quím. Nova 32, 394–400.

Das, K., Mukherjee, A.S., 2007. Crude petroleum-oil biodegradation efficiency of *Bacillus subtilis* and *Pseudomonas aeruginosa* strains isolated from a petroleum-oil contaminated soil from North-East India. Bioresour. Technol. 98, 1339–1345.

Dhote, M., Juwarkar, A., Kumar, A., Kanade, G.S., Chakrabarti, T., 2009. Biodegradation of chrysene by the bacterial strains isolated from oily sludge. World J. Microbiol. Biotechnol. 26, 329–335.

Fernández-Luqueño, F., Valenzuela-Encinas, C., Marsch, R., Martínez-Suárez, C., Vázquez-Núñez, E., Dendooven, L., 2011. Microbial communities to mitigate contamination of PAHs in soil – possibilities and challenges: a review. Environ. Sci. Pollut. Res. 18, 12–30.

Gafarov, A.B., Panov, A.V., Filonov, A.E., Boronin, A.M., 2006. Change in the composition of a bacterial association degrading aromatic compounds during oil sludge detoxification in a continuous-flow microbial reactor. Appl. Biochem. Microbiol. 42 (2), 160–165.

Garrity, G., Brenner, D.J., Krieg, N.R., Staley, J.T., 2005. Bergey's Manual of Systematic Bacteriology, second ed. Springer-Verlag, New York.

Janbandhu, A., Fulekar, M.H., 2011. Biodegradation of phenanthrene using adapted microbial consortium isolated from petrochemical contaminated environment. J. Hazard. Mater. 187, 333–340.

Kriipsalu, M., Marques, M., Maastik, A., 2008. Characterization of oily sludge from a wastewater treatment plant flocculation–flotation unit in a petroleum refinery and its treatment implications. J. Mater. Cycles Waste Manag. 10, 79–86.

Kumar, M., León, V., Materano, A.D.S., Ilzins, O.A., Luis, L., 2008. Biosurfactant production and hydrocarbon-degradation by halotolerant and thermotolerant *Pseudomonas* sp. World J. Microbiol. Biotechnol. 24, 1047–1057.

Mariano, A.P., Tomasella, R.C., Oliveira, L.M., Contiero, J., De Angelis, D.F., 2008. Biodegradability of diesel and biodiesel blends. African J. Biotechnol. 7 (9), 1323–1328.

Mohamed, M.E., Al-Dousary, M., Hamzah, R.Y., Fuchs, G., 2006. Isolation and characterization of indigenous thermophilic bacteria active in natural attenuation of bio-hazardous petrochemical pollutants. Int. Biodeter. Biodegr. 58, 213–223.

Molina, M.C., González, N., Bautista, L.F., Sanz, R., Simarro, R., Sánchez, I., Sanz, J.L., 2009. Isolation and genetic identification of PAH degrading bacteria from a microbial consortium. Biodegradation 20, 789–800.

Moreira, F.M.S., Siqueira, J.O., 2006. Microbiologia e bioquímica do solo. Editora Ufla, Lavras.

Mukred, A.M., Hamid, A.A., Hamzah, A., Yusoff, W.M.W., 2008. Development of three bacteria consortium for the bioremediation of crude petroleum–oil in contaminated water. J. Biol. Sci. 8 (4), 73–79.

Nitschke, M., Pastore, G.M., 2002. Biossurfactantes: propriedades e aplicações. Quím. Nova 25, 772–776.

Paudyn, K., Rutter, A., Rowe, R.K., Poland, J.S., 2008. Remediation of hydrocarbon contaminated soils in the Canadian Arctic by *Landfarming*. Cold Reg. Sci. Technol. 53, 102–114.

Richard, J.Y., Vogel, T.M., 1999. Characterization of a soil bacterial consortium capable of degrading diesel fuel. Int. Biodeter. Biodegr. 44, 93–100.

Sambrook, J., Roussell, D.W., 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Staden, R., Beal, K.F., Bonfield, J.K., 2003. The Staden package. Methods Mol. Biol. 132, 115–130.

Thavasi, R., Jayalakshmi, S., Banat, I.M., 2011. Effect of biosurfactant and fertilizer on biodegradation of crude oil by marine isolates of *Bacillus megaterium*, *Corynebacterium kutscheri* and *Pseudomonas aeruginosa*. Bioresour. Technol. 102 (2), 772–778.

Toledo, F.L., Calvo, C., Rodelas, B., González-Lopez, J., 2006. Selection and identification of bacteria isolated from waste crude oil with polycyclic aromatic hydrocarbons removal capacities. Syst. Appl. Microbiol. 29, 244–252.

Toribio, J., Escalante, A.E., Soberón-Chavez, G., 2010. Rhamnolipids: production in bacteria other than *Pseudomonas aeruginosa*. Eur. J. Lipid Sci. Technol. 112, 1082–1087.

Tuleva, B., Christova, N., Jordano, B., Nikolova-Damyanova, B., Petrov, P., 2005. Naphthalene degradation and biosurfactant activity by *Bacillus cereus* 28BNZ. Z. Naturf. 60c, 577–582.

Verma, S., Bhargavaa, R., Pruthi, V., 2006. Oily sludge degradation by bacteria from Ankleshwar, India. Int. Biodeter. Biodegr. 57, 207–213.

Vitte, I., Duran, R., Jézéquel, R., Caumette, P., Cravo-Laureau, C., 2011. Effect of oxic/anoxic switches on bacterial communities and PAH biodegradation in an oil-contaminated sludge. Environ. Sci. Pollut. Res. 18, 1022–1032.

Wang, X.B., Chi, C.Q., Nie, Y., Tang, Y.Q., Tan, Y., Wu, G., Wu, X.L., 2011. Degradation of petroleum hydrocarbons (C6–C40) and crude oil by a novel *Dietzia* strain. Bioresour. Technol. 102, 7755–7761.

Zhang, X., Wu, W., Zhang, Y., Wang, J., Liu, Q., Geng, C., Lu, J., 2007. Screening of efficient hydrocarbon-degrading strains and study on influence factors of degradation of refinery oily sludge. Ind. Eng. Chem. Res. 46, 26.