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Biodegradation of oil tank bottom sludge using microbial consortia

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Abstract We present a rationale for the selection of a microbial consortia specifically adapted to degrade toxic components of oil refinery tank bottom sludge (OTBS). Sources such as polluted soils, petrochemical waste, sludge from refinery-wastewater plants, and others were used to obtain a collection of eight microorganisms, which were individually tested and characterized to analyze their degradative capabilities on different hydrocarbon families. After initial experiments using mixtures of these strains, we developed a consortium consisting of four microorganisms (three bacteria and one yeast) selected in the basis of their cometabolic effects, emulsification properties, colonization of oil components, and degradative capabilities. Although the specific contribution each of the former parameters makes is not clearly understood, the activity of the four-member consortium had a strong impact not only on linear alkane degradation (100%), but also on the degradation of cycloalkanes (85%), branched alkanes (44%), and aromatic and sulphur–aromatic compounds (31–55%). The effectiveness of this consortium was significantly superior to that obtained by individual strains, commercial inocula or an undefined mixture of culturable and non-culturable microorganisms obtained from OTBS-polluted soil. However, results were similar when another consortium of four microorganisms, previously isolated in the same OTBS-polluted soil, was assayed.

Keywords Bioaugmentation · Biomarker · Microbial consortia · Oil tank bottom sludge · Priming

Abbreviations

OTBS	oil tank bottom sludge
NSO	nitrogen, sulphur, oxygen compounds in oil
CDS	cyclodextrin–diesel agar synthetic medium

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Introduction

Oily wastes in crude storage tank bottoms are periodically removed, posing difficulties for disposal (Ferrari et al. 1996). In the past, these wastes were disposed of after mixing them with soil and partially stabilizing them with additives such as magnesite; in contrast, nowadays, they are generally detoxified using expensive physical–chemical processes. Therefore, biotreatments should be considered as an alternative, not only to decrease the volume of waste, but to reduce their toxicity. Bioreactors, bioslurry techniques, or landfarming treatments comprise bioremediation alternatives for oil tank bottom sludge (OTBS) and for hydrocarbon wastes in general (Riser-Roberts 1998). In all cases, the application of a microbial mixture (generally termed ''consortium''; Hurst 2002) specialized in degrading or tolerating compounds included in this sort of sludge (aliphatic and aromatic hydrocarbons, NSO (nitrogen, sulphur, oxygen) compounds, asphaltene fractions, and heavy metals) (Speight 2001) can be contemplated. In fact, individual microorganisms can metabolize only a limited array of substrates (Wackett and Hersberger 2001); therefore, mixed populations with wide-ranging enzymatic capacities and synergistic–cometabolic relationships are required. However, not only metabolic abilities and cometabolism should be considered when selecting individual strains to be included in effective consortia. In this sense, the production of surface-active compounds (biosurfactants or bioemulsifiers) increase the bioavailability of hydrophobic water-insoluble substrates, thereby enhancing the growth of bacteria and the rate of bioremediation (Ron and Rosenberg 2002).

Evidence for the cooperation of mixed cultures in biodegradation has been previously reported in natural environments (Bouchez et al. 2000; Mishra et al. 2001; Viñas et al. 2002). Specifically, two good examples in hydrocarbon degradation studies are increased degradation of polycyclic aromatic hydrocarbons (PAHs) in the presence of alkane compounds (Yuste et al. 2000) and the selection of a consortium growing in diesel capable of mineralizing benzo[a]pyrene (Kanaly et al. 2000). Nevertheless, our understanding of the interactions and synergistic effects is still incomplete and new knowledge regarding the dynamics of mixed cultures involved in treatment of heterogeneous substrates may contribute to future advances (Van Hamme et al. 2000; Carvalho et al. 2002).

The aim of this work was to select a simple microbial consortium to degrade the OTBS in order to identify specific microbial interactions favoring hydrocarbon biodegradation. This was accomplished by screening different hydrocarbon pollution sources for promising microorganisms followed by evaluation of the chemical changes in the OTBS during degradation experiments with different strain combinations. Finally, we tested commercial inocula and ''primed'' inocula (i.e., previously predisposed to OTBS degradation; Singer et al. 2005), by using OTBS-polluted soil conceivably enriched with microorganisms highly competent in degrading this contaminant.

Materials and methods

Sampling, isolation, and storage of microorganisms

Different hydrocarbon-polluted environments such as soils and groundwater affected by recent and old spills (most of them having been bioremediated at the time of sampling), petrochemical waste, sludge from refinery-wastewater plants, and others were sampled to obtain a large variety of hydrocarbon-degrading microorganisms.

Samples of liquid waste and hydrocarbon-polluted groundwater were taken in sterile conditions and representative subsamples were cultivated in cyclodextrin-diesel agar synthetic medium (CDS) (wt/vol): 0.15% NH_4NO_3; 0.05% $MgSO_4 \cdot H_2O$; 0.02% $CaCl_2 \cdot 2H_2O$; 0.5% KH_2PO_4; 0.5% K_2HPO_4; 1% β-cyclodextrin. Plate-growth experiments with β-cyclodextrin as the only carbon source revealed that it was not used by any of the isolated hydrocarbon-degraders, as stated in previous reports (Schwartz and Bar 1995); however, its inclusion in the medium aids in homogenizing diesel distribution in plates (Bardi et al. 2000). Diesel was sterilized by filtration and added to the former medium as the sole carbon

source (2% wt/vol). Serial dilutions of 0.1 ml were spread over the surface of duplicate agar plates and incubated directly at 30°C for 1 week. Only growing microorganisms (bacteria and yeast) with $>10^5$ CFU/ml were isolated and selected.

Enrichment methods were also used to isolate microorganisms from solid samples. One-gram samples of hydrocarbon-polluted soils or oily solid wastes were added to 10 ml of a 0.1% wt/vol $Na_2HPO_4{\cdot}12H_2O$ solution and vortexed vigorously for 10 min. After standing for 2 min, 5 ml of suspension were centrifuged at 27,200×*g* for 10 min and the sediment was resuspended in 2 ml of sterile water. This was inoculated in a 250-ml flask containing 100 ml of the following synthetic medium (wt/vol): 0.15% NH_4NO_3; 0.05% $MgSO_4{\cdot}H_2O$; 0.02% $CaCl_2{\cdot}2H_2O$; 0.5% KH_2PO_4; 0.07% K_2HPO_4, plus 1% heavy crude oil (Maya), light crude oil (Arabian light), or diesel, depending on the contaminants present at the sampling sites. Incubation took place in a rotary shaker at 250 rpm and 30°C. After 10 days, 0.1 ml of successive dilutions of the culture were spread in duplicate CDS-plates. Only growing microorganisms with $>10^5$ colony forming units per ml (CFU/ml) were isolated.

To maintain the strains and preserve metabolic capabilities, samples were frozen at –70°C in 1.7% wt/vol trehalose and 25% vol/vol glycerol medium and/or lyophilized (1.7% wt/vol trehalose, 2% wt/vol skimmed milk, and 1.25% vol/vol MOPS medium; García López and Uruburu 2001). In addition to the initially isolated pool of microorganisms, different hydrocarbon-degrading bacteria and yeast strains available from previous studies (Gallego et al. 2001a, b, and other unpublished data), were also included in the analysis.

Microorganism identification

Initial characterization of individual strains consisted of Gram staining, oxidase test, morphology observations, and biochemical tests (API strips; bioMérieux). Sequence analysis of 16S bacterial ribosomal DNA (16S rDNA) was performed on selected isolates after DNA extraction using a commercial kit (Chemagic Bac 100 Kit, Chemagen). The 16S rRNA was amplified by PCR in a Minicycler™ (MJ Research) using the conserved Eubacterial primers pA (5′ AGAGTTTGATCCTGGCTCAG 3′) and pH (5′ AAGGAGGTGATCCAGCCGCA 3′) (Bruce et al. 1992). Amplification products (1,500 bp) were detected after gel electrophoresis of a 5 µl aliquot in 0.8% agarose gel, followed by ethidium bromide staining and visualization under UV light. PCR products were purified using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences). Sequencing was conducted in an ABI Prism™ 3100 Genetic Analyzer (Perking Elmer) and sequences were compared with the gapped BLAST database search program at the National Centre for Biotechnology Information (NCBI) (Altschul et al. 1997).

Restriction patterns generated from the region spanning the internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene were used to identify yeast species (Esteve-Zarzoso et al. 1999). Yeast cells were directly collected from a fresh colony and suspended in 50 µl PCR reaction mix containing 0.5 µM primer ITS1 (5′ TCCGTAGGTGAACCTGCGG 3′), 0.5 µM primer ITS4 (5′ TCCTCCGCTTATTGATATGC 3′), 10 µM deoxynucleotides (Amersham Biosciences), 1.5 mM $MgCl_2$, 10× buffer, and 0.2 µl *Eco* Taq DNA Polymerase (Ecogen S.R.L). After amplification, the PCR products were purified as above and 0.5–1.0 µg were digested with the restriction endonucleases *Cfo*I, *Hae*III, and *Hin*fI (Takara Bio Inc.). Endonucleases *Alu*I, *Dde*I, *Scr*FI, or *Taq*I were additionally used in specific cases. The restriction fragments were separated on 3% wt/vol agarose gels. After electrophoresis, gels were stained with ethidium bromide and visualized under UV light. Sizes were compared with those obtained for known yeasts (Esteve-Zarzoso et al. 1999).

Cultures in OTBS medium

For routine, rapid preparation of inocula, bacteria were preincubated in 3% wt/vol-TSB (tryptic soy broth, Merck) supplemented with 0.1% wt/vol yeast extract for 24 h in orbitary shakers at 250 rpm and 30°C. For yeasts, the preincubation medium consisted of 0.5% wt/vol micopeptone

(Oxoid) supplemented with 4% wt/vol glucose and 0.5% wt/vol yeast extract. These pure cultures were centrifuged, washed, and resuspended in sterile distilled water; suspensions were adjusted using optic density (660 nm), up to 10^9 cells/ml for bacteria and 10^8 cells/ml for yeasts. Microbial degradation of oil tank bottom sludge was tested in 100 ml OTBS medium (wt/vol): 0.6% NH_4NO_3; 0.5% KH_2PO_4; 0.07% K_2HPO_4; 0.05% $MgSO_4 \cdot H_2O$; 0.02% $CaCl_2 \cdot 2H_2O$; 0.2% oligoelements, and OTBS at 4% as the only carbon source; OTBS was obtained from crude oil tanks at the Puertollano refinery (Spain, Repsol-YPF). After inoculating the above medium with 1 ml of the bacterial suspensions, it was incubated for 10 days in orbitary shakers at 250 rpm and 30°C. The above conditions were used irrespective of the number of strains. Populations were periodically determined by plate-counting; concretely for the two-member and four-member consortia experiments detailed in Results, this was carried out by direct observation of colony morphologies in duplicate plates and by means of microscopy observations when necessary. A commercial product (Alken Clear-Flo® 7036, Alken-Murray Corp.) containing different hydrocarbon-degrading bacteria (predominantly *Bacillus* and *Pseudomonas*) was also tested in triplicate experiments with the above OTBS medium, following the manufacturer's recommendations.

Emulsifying activity

At 3 days, emulsifying activity in the cultures was estimated by a variation of the method proposed by Barathi and Vasudevan (2001). Five milliliter of a previously centrifuged culture fluid plus 1 ml diesel were vortexed for 5 min to promote hydrocarbon–water emulsion at the top of the graduated cylinder used for the determinations. The mixture was allowed to stand for 24 h after which emulsion was measured. Volume increases greater than 50% versus control (diesel plus water) were considered positive for emulsifying activity.

Priming approaches

Priming experiments were performed with OTBS-polluted soil (natural soil in which OTBS wastes had been routinely disposed of in the past). An initial approach consisted of isolating an undefined mixture of culturable and non-culturable microorganisms as follows: a soil sample (10 g) was resuspended in 100 ml of 0.1% wt/vol $Na_2HPO_4 \cdot 12H_2O$ solution and vortexed vigorously for 10 min; after standing for 2 min, 50 ml of the suspension were centrifuged at 27,200×g for 10 min and the sediment was resuspended in sterile water; an inoculum of approximately 5×10^8 cells of culturable bacteria was then taken and added to OTBS medium. A second approach was based on a defined inoculum of culturable microorganisms obtained after CDS-plate isolation of the predominant four microorganisms present in the undefined inoculum.

Chemical analysis and biotransformation indices

At the end of each experiment, OTBS cultures were collected in appropriate bottles, sealed, and stored in the dark at 4°C. Subsequent extraction consisted of circulation through a SPE cartridge (solid phase extraction—EPA Method 3535) and washing with 20 ml of dichloromethane. For soil samples and pure product analysis, hydrocarbons were extracted with a Soxhlet apparatus and purified by liquid chromatography (EPA Method 3540C). Both SPE extracts and liquid chromatography purified extracts were injected into a gas chromatograph GC system HP 6890 Series equipped with a capillary column (AT5 Alltech, 25 m length and 0.25 mm internal diameter); the column oven temperature was raised from 60°C to 300°C at a rate of 6°C/min and the injector temperature was 275°C. This equipment was attached to a mass detector MSD HP 5973 Series which allowed peak area determinations in Selective Ion Mode (SIM) using the Wiley275 database (EPA Method 8270C). Additionally, TPH determinations (EPA Method 8015) were performed with selected samples. For initial characterization of tank bottom sludge, multielemental inorganic composition was obtained with ICP-AES after digestion in aqua regia and with Leco Analyzer CHN-600.

Hopane normalization has been reported as the best method to quantify hydrocarbon biodegradation, specifically the use of 17α (H), 21β

Table 1 Families of compounds abundant in OTBS, biotransformation ratios, and ions used for peak area determinations (ion 191 for 17α(H), 21β(H) hopane)

Compound families		Ratio	SIM ion/s
Saturate fraction	Linear alkanes	$\sum C_{11}$–C_{15}/17α(H), 21β(H) hopane	57
		$\sum C_{16}$–C_{20}/17α(H), 21β(H) hopane	57
		$\sum C_{21}$–C_{25}/17α(H), 21β(H) hopane	57
		$\sum C_{26}$–C_{33}/17α(H), 21β(H) hopane	57
	Branched alkanes	$\sum$(alkyl-C_{11}, alkyl-C_{12}, alkyl-C_{17}, alkyl-C_{19})/17α(H), 21β(H) hopane	71
	Isoprenoids	$\sum$(Farnesane, Nor-pristane, Pristane, Phytane)/17α(H), 21β(H) hopane	183
	Cycloalkanes	$\sum$(alkylcyclohexanes)/17α(H), 21β(H) hopane	55, 83
Aromatic fraction	Alkyl-PAHs	$\sum$(dimethylnaphthalenes)/17α(H), 21β(H) hopane	156
		$\sum$(methylanthracenes-phenanthrenes)/17α(H), 21β(H) hopane	192
		$\sum$(dimethylanthracenes-phenanthrenes)/17α(H), 21β(H) hopane	192
	Sulphur compounds	$\sum$(dimethyldibenzothiophenes)/17α(H), 21β(H) hopane	212

(H) hopane as an internal conserved standard (Bragg et al. 1994). Moreover, it is the most abundant pentacyclic terpene in the OTBS (see Results) and its extremely low solubility, biodegradability, and volatility guarantee persistence. Therefore, different ratios between peak areas of 17α(H), 21β(H) hopane, and selected compounds were determined (Table 1). Selection criteria consisted of abundance in OTBS, easy location and identification in SIM chromatograms (Peters and Moldowan 2004), and representativity of the main oil components (Speight 2001). To facilitate understanding, biotransformation indices were finally created (Pollard et al. 1999) as percentages of degradation for the different families previously listed. For example, the term 'dimethyldibenzothiophenes biotransformation index' is expressed as:

$$\text{Biotransformation}(\%) = 100 - \left(\frac{\left(\sum \text{C2-DBT}/17\alpha 21\beta\text{hopane}_{\text{sample}} \right)}{\left(\sum \text{C2-DBT}/17\alpha 21\beta\text{hopane}_{\text{abiotic control}} \right)} \bullet 100 \right)$$

Results and discussion

Tank bottom sludge analysis

Multielemental analysis of OTBS revealed concentrations of 78% C, 13% H, 2% S, 0.5% N, and trace element concentrations of 220 ppm of Ni and 130 ppm of V, highly suggestive of porphyrine compounds corresponding to heavy oils (Speight 2001). Moreover, 0.5% Fe levels revealed pollution from steel tank walls. GC-MS determinations of OTBS (Fig. 1) showed a heavy oily mixture rich in *n*-alkanes with chain lengths of 11–36 atoms. Branched alkanes, alkylcycloalkanes with long alkyl groups, and biomarkers such as isoprenoids, hopanes, and steranes also abound in the saturate fraction. The aromatic fraction presented abundant two-ring and three-ring compounds (specially their methyl, dimethyl, and trimethyl derivatives) and sulphur compounds such as benzothiophenes and their derivatives. Light *n*-alkanes and most BTEX derivatives were not present, as they probably volatilized after years of OTBS permanence in tanks. All of these data supported the selection of the biotransformation indices previously listed in Table 1.

Isolation of hydrocarbon-degrading microorganisms

Initial screening isolated some thirty strains (bacteria and yeasts), mainly belonging to genera with documented hydrocarbon-degradative capabilities: *Pseudomonas*, *Acinetobacter*, *Bacillus*, *Rhodotorula*, among others (Timmis and Pieper 1999; Furukawa 2003). Many of these strains were

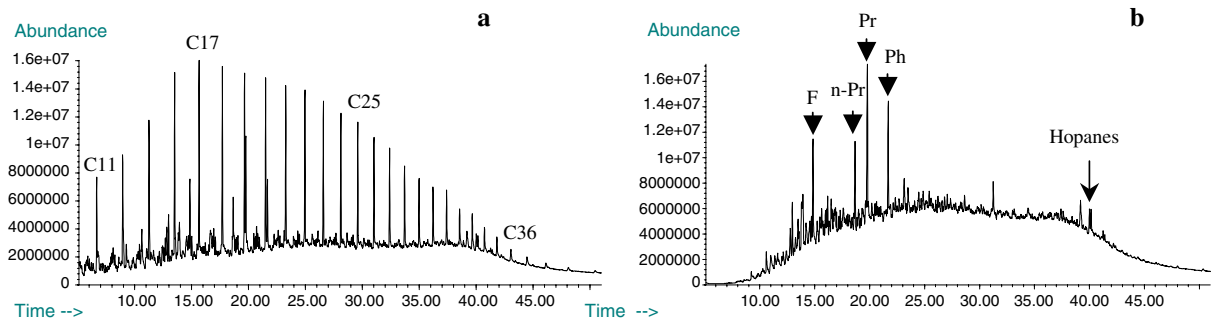

Fig. 1 Total ion chromatograms after 10 days of abiotic cultivation in OTBS medium (**a**) and cultivation in the presence of *Nocardiodes simplex* (B4 strain) (**b**). Predominant, regularly spaced peaks in (**a**) represent the linear alkanes. Only peaks belonging to isoprenoids (arrows, left to right: Farnesane, nor-Pristane, Pristane, Phytane) and hopanes can be easily distinguished in the overall chromatogram of (**b**) after complete depletion of *n*-alkanes

ruled out for several reasons, such as difficulties in obtaining appropriate microbial mass in the media used for preincubation, inconclusive results when tested in OTBS medium, possible pathogenic properties (certain *Pseudomonas* species; Alonso et al. 1999), and slow growth kinetics. A group of eight strains (six bacteria and two yeasts) was finally selected for further analysis and testing in OTBS medium (Table 2). Most of these microbes were able to metabolize *n*-alkanes, as well as some branched alkanes and cycloalkanes (Table 3). In this sense, short- and medium-chain alkanes are less hydrophobic and generally degrade more easily than long-chain ones (Ghazali et al. 2004); despite this, the results obtained showed that the best performing strains completely degraded all the linear alkanes regardless of chain-length. This could be related to their capability of producing surface-active compounds (bioemulsifiers or biosurfactants), which increase bioavailability of the more hydrophobic compounds (Southam et al. 2001). Levels attained in aromatic degradation (Table 3) were negligible with the exception of B6, B8, and Y3 strains, which is consistent with numerous reports on the dominance of aliphatic- over aromatic-biodegrading isolates (Yuste et al. 2000; Medina-Bellver et al. 2005). Moreover, consortia have been seen to be more efficient aromatic degraders than single strains (Foght et al. 1998).

Of note, the actinomycete *Nocardiodes simplex* (strain B4) demonstrated a special ability to colonize and degrade hydrocarbon aggregates, probably due to the hydrophobic nature of the

Table 2 Strains selected for inclusion in the consortia

Strain	Identification (*16S and 26S rDNA*)	Domain, Phylum (Class)	Origin
B2	*Acinetobacter calcoaceticus*	Bacteria Proteobacteria	Natural soil polluted with diesel fuel
B3	*Acinetobacter lwoffi*	Bacteria Proteobacteria	Groundwater polluted with diesel fuel (Gallego et al. 2001a, b)
B4	*Nocardiodes simplex*	Bacteria Actinobacteria	Natural soil affected by an old spillage of crude oil
B6	*Stenotrophomonas* sp.	Bacteria Proteobacteria	Sludge from a refinery wastewater plant
B8	*Pseudomonas alcaligenes*	Bacteria Proteobacteria	Soil polluted with fuel oil being treated by landfarming
B13	*Aeromonas hydrophila*	Bacteria Proteobacteria	Sludge obtained at an urban wastewater plant
Y3	*Rhodotorula graminis*	Eukarya Fungi (Basidiomycetes)	Lixiviates from toxic residues at a landfill
Y4	*Rhodotorula mucilaginosa*	Eukarya Fungi (Basidiomycetes)	Lixiviates from toxic residues at a landfill

Table 3 Biotransformation indices of hydrocarbon families (GC-MS) obtained after 10 days of incubation of OTBS medium inoculated with individual strains

Compound families		Strain							
		B2	B3	B4	B6	B8	B13	Y3	Y4
Linear alkanes	C_{11}–C_{15}	100	74	100	11	100	67	100	41
	C_{16}–C_{20}	100	58	100	< 5	100	68	97	15
	C_{21}–C_{25}	100	63	100	< 5	100	72	87	18
	C_{26}–C_{33}	100	61	100	< 5	100	77	76	4
Branched alkanes		10	29	15	7	17	19	39	18
Isoprenoids		< 5	7	< 5	< 5	< 5	< 5	< 5	6
Cycloalkanes		45	58	61	11	35	59	19	35
Dimethylnaphthalenes		10	< 5	6	46	15	< 5	24	< 5
Methyl anthracenes/phenanthrenes		< 5	< 5	9	20	11	< 5	19	< 5
Dimethyl anthracenes/phenanthrenes		< 5	< 5	15	17	16	< 5	26	11
Dimethyldibenzothiophenes		< 5	< 5	< 5	23	< 5	< 5	5	< 5

Emulsifying potential was positive only for strains B2, B3, and B4. All data are expressed as the average of duplicate experiments (errors are within 5%)

cell wall material. The growth rate of B4 in OTBS medium showed a rapid increase during the first 50 h, followed by a 10-day stationary phase (data not shown). Bulk TPH determinations revealed approximately 85% hydrocarbon reduction after 10 days of growth in OTBS medium, which is consistent with the growth rates and biotransformation ratios obtained by GC-MS (Table 3). The overall behavior of this strain seemed to suit for the purposes of the work and hence, B4 was selected as a reference strain for consortia design.

Analysis of OTBS degradation by two-member consortia

Seven binary combinations of *Nocardiodes simplex* (B4) with one of the strains previously selected (Table 2) were tested in OTBS medium. In some mixtures, antagonism and/or competitive inhibition was suggested by immediate decline or relatively low numbers of one of the two microorganisms (data not shown). Conversely, synergistic effects were promoted in cases such as B4-B2 (*Nocardiodes simplex* plus *Acinetobacter calcoaceticus*) and, remarkably, B4-Y3 (*Nocardiodes simplex* plus *Rhodotorula graminis*) mixtures, leading to improved degradation of several hydrocarbon families with respect to single strain results (Table 4). Specifically, degradation of branched alkanes, cycloalkanes, and dimethyl-anthracenes/phenanthrenes was better in the cases of B4-B2 and B4-Y3 than that obtained with individual members. Additionally methyl-anthracenes/phenanthrenes were significatively better degraded by the B4-Y3 consortium than by B4 alone or any other two-member consortia (Table 4). In light of the good results obtained with the contribution of *Rhodotorula graminis*, the B4-Y3 mixture was considered a good starting point for the final consortium design.

Four-member, eight-member, and commercial consortia for OTBS degradation

The above two-member consortia could have been potentially extended by combining three or more strains by means of a factorial experiment design. However, a more rational selection procedure was attempted by considering potential physical, metabolic, and community interactions contributing to the behavior of hydrocarbon-degraders (Van Hamme and Ward 2001). In this sense, cometabolic and synergistic reactions probably take place in mixed microbial populations acting on complex mixtures such as OTBS (Schink 2002). Also, biosurfactant and bioemulsifier production contribute to the ability of the microorganisms to colonize oil droplets, an aspect that can be directly observed by light microscopy and scanning electron microscopy (Southam

Table 4 Biotransformation indices of hydrocarbon families (GC-MS) obtained after 10 days of incubation of OTBS medium inoculated with mixed binary cultures of *Nocardiodes simplex* (B4 strain) and another seven strains (comparison with B4 individual culture is also shown)

Compound families		Consortia							
		B4	B4–B2	B4–B3	B4–B6	B4–B8	B4–B13	B4–Y3	B4–Y4
Linear alkanes	C_{11}–C_{15}	100	100	100	100	100	100	100	100
	C_{16}–C_{20}	100	100	100	100	100	100	100	100
	C_{21}–C_{25}	100	100	100	100	100	100	100	100
	C_{26}–C_{33}	100	100	100	100	100	100	100	100
Branched alkanes		15	28	< 5	< 5	< 5	12	32	38
Isoprenoids		< 5	< 5	< 5	< 5	< 5	< 5	< 5	8
Cycloalkanes		61	84	< 5	34	47	21	82	32
Dimethylnaphthalenes		6	3	< 5	< 5	< 5	< 5	15	< 5
Methyl anthracenes/ phenanthrenes		9	14	< 5	< 5	16	< 5	22	< 5
Dimethyl anthracenes/ phenanthrenes		15	30	< 5	< 5	18	< 5	35	< 5
Dimethyldibenzothiophenes		< 5	< 5	< 5	< 5	< 5	< 5	29	< 5

Emulsifying potential was always positive except in B4-B3 and B4-B6 experiments. All data are expressed as the average of duplicate experiments (errors are within 5%)

et al. 2001). Therefore, a four-member consortium (strains B4, B8, B2, and Y3) was selected on the basis of individual effectiveness for hydrocarbon degradation (Table 3), the observed complementary OTBS-degrading capabilities tested in the two-member consortia (see above and also Table 4), and physical accessibility to oil components as analyzed by confocal laser-scanning microscope observations (data not shown).

As discussed above, *Nocardiodes simplex* (B4) was initially selected because of its emulsification ability. On the other hand, *Pseudomonas alcaligenes* (B8) is a free-living mobile bacteria which maintained reasonably growth levels and acceptable degradation results when combined with B4 (Table 3). In addition, this species has been previously reported as a xylene and polycyclic aromatic hydrocarbon (PAH) degrader (Gordon and Dobson 2001) and has been used for bioaugmentation purposes in phenanthrene-contaminated soil experiments (O'Mahony et al. 2006). *Acinetobacter calcoaceticus* (B2) is also a well-known hydrocarbon-degrader (Lal and Khanna 1996; Van Hamme et al. 2000) capable of producing an active polymeric bioemulsifier. Finally, as happens with other Rhodotorula species (MacGillivray and Shiaris 1993; Romero et al. 2002), *Rhodotorula graminis* degrades aromatic compounds (Durham et al. 1984), a property that has been corroborated in the present work (see data for Y3 dimethylnaphtalenes and dimethylanthracenes/phenanthrenes degradation in Table 3). In spite of the fact that numerous yeasts have been reported as capable of metabolizing hydrocarbons (Fickers et al. 2005; Yan et al. 2005, and references herein), only a few studies adress about the inclusion of eukaryotic microorganisms, specifically yeasts, in biodegradative consortia (Kim et al. 2004).

Therefore, the four-member consortium was tested in OTBS medium (Fig. 2). Growth of *Acinetobacter calcoaceticus* increased notably from the beginning of the experiment and this bacterium was numerically predominant in the culture during the first 50 h; at that point, its population started to decline, whereas *Nocardiodes simplex* (B4) maintained a high number of CFU/ml during most of the cultivation period (similar to previous observations when grown alone; data not shown). In the case of *Pseudomonas alcaligenes* (B8) and *Rhodotorula graminis* (Y3), growth was seen to decrease earlier but to resume after 70 h, followed by a second growth phase until about 200 h and 150 h, respectively. After this, growth decreased remarkably, especially in the case of *Rhodotorula graminis*.

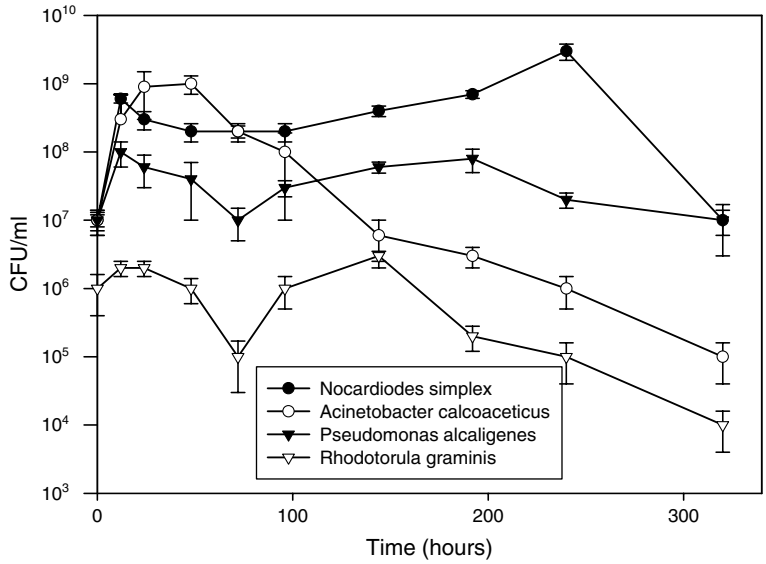

Fig. 2 Growth curves in OTBS medium of the different microorganisms of the consortium comprised of *Acinetobacter calcoaceticus* (B2), *Nocardiodes simplex* (B4), *Pseudomonas alcaligenes* (B8), and *Rhodotorula graminis* (Y3)

Biotransformation indices were noticeably better with this four-member consortium than with any two-strain mixture including B4-Y3 (Table 5). In this regard, the results obtained for branched alkanes, and especially for the two-ring and three-ring aromatic compounds studied, were particularly striking (Table 5 and Fig. 3).

Consortia with a higher number of crude-oil degrading members have been described. For example, a six-member consortium, obtained from a collection of petroleum-degrading bacteria (two *Mycobacterium* species, two *Pseudomonas* species, one *Corynebacterium* species, and one *Xanthomonas* species) was assayed to test freshwater oil spill bioremediation agents (Foght et al. 1998). In that case, the rationale for the selection of these bacteria was the choice of three aliphatic-degrading bacteria and three-aromatic degrading bacteria, in order to preserve hypothetical losses of some of the members during consortium manipulation. Similarly, we tried to enhance the metabolic abilities of our four-member consortium by studying the effect of adding the eight strains initially selected in the work (Table 2; see

Table 5 Biotransformation indices of hydrocarbon families (GC-MS) obtained after 10 days of incubation of OTBS medium inoculated with different consortia (comparison with B4-Y3 coculture is also shown)

Compound families		Consortia			
		B4–Y3	B4–B2–B8–Y3	B4–B2–B3–B6–B8–B13–Y3–Y4	Commercial consortium
Linear alkanes	C_{11}–C_{15}	100	100	100	100
	C_{16}–C_{20}	100	100	100	100
	C_{21}–C_{25}	100	100	100	100
	C_{26}–C_{33}	100	100	100	100
Branched alkanes		32	44	29	28
Isoprenoids		<5	10	<5	<5
Cycloalkanes		82	85	66	63
Dimethylnaphthalenes		15	55	43	41
Methyl anthracenes/phenanthrenes		22	33	31	31
Dimethyl anthracenes/phenanthrenes		35	45	36	40
Dimethylbenzothiophenes		29	31	9	7

Emulsifying potential was positive in all experiments. All data are expressed as the average of duplicate experiments (errors are within 5%)

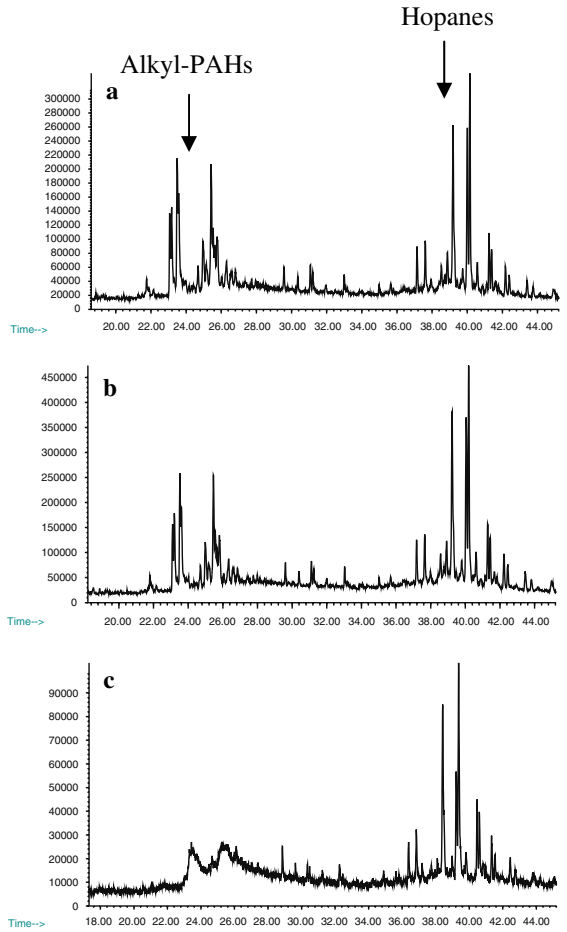

Fig. 3 Chromatograms of ion 191 (SIM mode) of OTBS extracts after 10 days: sterile control (**a**), cultivation in the presence of *Nocardiodes simplex* (**b**), and cultivation with the four-member consortium formed by *Acinetobacter calcoaceticus* (B2), *Nocardiodes simplex* (B4), *Pseudomonas alcaligenes* (B8), and *Rhodotorula graminis* (Y3) (**c**). Note the advanced alkyl-PAHs degradation in (**c**)

above): those from the four-member consortium (B2-B4-B8-Y3), plus *Acinetobacter lwoffi* (B3), *Stenotrophomonas* sp. (B6), *Aeromonas hydrophila* (B13), and *Rhodotorula mucilaginosa* (Y4). When this mixture was tested in OTBS medium, results revealed that the extra four strains were superfluous and even detrimental, given that the biotransformation indices attained (except those for linear alkanes) were lower that those obtained with the four-member consortia (Table 5). Finally, we also analyzed the behavior of a commercial product (see Methods) in OTBS degradation experiments (Table 5). The results were very good as regards linear alkane degradation; nevertheless degradation yields of other saturate and aromatic compounds were worse than those obtained with the four-member consortium. This is probably due to the suitability of the members of our consortium that were selected for their specific abilities and growth compatibility in the OTBS medium. In any case, commercial products do not seem to degrade hydrocarbons much better than natural microbial communities (Thouand et al. 1999).

Experiments with OTBS-polluted soil consortia (priming)

A difficult and as yet unresolved problem in bioaugmentation experimentation is the inability to cultivate all potentially relevant biodegradative microorganisms (Vogel and Walter 2002). Therefore, ''priming'', conceived as the use of inocula from soil samples previously enriched with the contaminants (for instance in microcosms), might attenuate this problem and constitutes a potentially very practical and environmentally acceptable method (Singer et al. 2005). Thus, as described in Methods, we tested a similar approach by using an undefined inoculum of microorganisms extracted from OTBS-polluted soil. A second experiment with a pre-defined inoculum of four culturable microorganisms (belonging to the genera *Pseudomonas*, *Acinetobacter*, *Mycobacterium*, and *Rhodotorula*) from the OTBS-polluted soil was also carried out. The objective of the first experiment was to include all the culturable and non-culturable microorganisms present in the OTBS-polluted soil; however, the degradation yields did not improve previous results obtained with the B2-B4-B8-Y3 consortium (Table 6). Conversely, the second approach was more efficient overall than the previous one; this was specifically evident in the case of long-chain linear alkanes and non-linear (branched and cyclic) alkanes, and particularly relevant considering the isoprenoid degradation achieved (25%) (usually recalcitrant hydrocarbons). Nevertheless, efficiency in degrading aromatic compounds was less than that obtained by the B2-B4-B8-Y3 consortium (Table 6), albeit greater than that obtained with the first priming approach. These differences are probably due to the particular

Table 6 Biotransformation indices of hydrocarbon families (GC-MS) obtained after 10 days of incubation of OTBS medium inoculated with two different priming consortia and comparison with the results obtained with the consortium B4-B2-B8-Y3

Compound families		Consortia		
		Priming approaches		B4–B2–B8–Y3
		Undefined inoculum after extraction	Four-member consortia from OTBS-polluted-soil	
Linear alkanes	C_{11}–C_{15}	100	100	100
	C_{16}–C_{20}	100	100	100
	C_{21}–C_{25}	89	100	100
	C_{26}–C_{33}	75	100	100
Branched alkanes		25	57	44
Isoprenoids		<5	25	10
Cycloalkanes		66	100	85
Dimethylnaphthalenes		36	40	55
Methyl anthracenes/phenanthrenes		15	15	33
Dimethyl anthracenes/ phenanthrenes		24	31	45
Dimethyldibenzothiophenes		16	25	31

Emulsifying potential was positive in all experiments. All data are expressed as the average of duplicate experiments (errors are within 5%)

metabolic interactions within the consortia members while growing in the OTBS medium and also might suggest that the number of microorganisms utilized is critical, although this was not addressed in this study.

In any case, all things being equal (the same number of microorganisms and their similar characteristics, given that three genera coincide), the slightly better degradation yields for aromatic compounds (usually the most toxic) would make the B2-B4-B8-Y3 four-member consortia a more suitable choice for pilot or full-scale designs in bioreactors.

Conclusion

Microbial consortia interactions that promote hydrocarbon biodegradation have been studied in order to select a consortium specifically adapted to degrade components of oil refinery tank bottom sludge (OTBS). A four-member consortium of three bacteria plus one yeast was therefore selected on the basis of biotransformation indices for specific hydrocarbon families, analyses of metabolic interactions, microscopy examinations to determine accessibility to oil components, and emulsifying activities of the individual strains. This consortium achieved 100% degradation of linear alkanes, 85% degradation of cycloalkanes, and values ranging from 30% up to 55% for branched alkanes, alkyl-PAHs, and sulphur-compounds. A priming experiment with an undefined culture from OTBS-polluted soil, aimed at testing the non-culturable microorganisms' contribution to hydrocarbon degradation, did not improve the results attained by the four-member consortium, which also yielded better outcomes than commercial mixtures. In contrast, another four-member consortium comprised of the predominant culturable microorganisms directly isolated from OTBS-polluted soil showed similar effectiveness on OTBS biodegradation as that achieved by the four-member consortium designed in this study.

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