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# Identification and biodegradation potential of tropical aerobic hydrocarbon-degrading microorganisms

Frédéric Chaillan [a], Anne Le Flèche [b], Edith Bury [a], Y-hui Phantavong [a], Patrick Grimont [b], Alain Saliot [c], Jean Oudot [a,*]

[a] *Muséum National d'Histoire Naturelle, USM 505 Ecosystèmes et Interactions toxiques, 12 rue Buffon, 75231 Paris Cedex 5, France*
[b] *Institut Pasteur, Unité de Biodiversité des Bactéries pathogènes émergentes, U389 INSERM, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France*
[c] *Université Pierre et Marie Curie, Laboratoire de Biogéochimie et Chimie marines, 4 place Jussieu, 75252 Paris Cedex 5, France*

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**Abstract**

Screening of aerobic culturable hydrocarbon (HC)-degrading microorganisms isolated from petroleum-polluted soils and cyanobacterial mats from Indonesia resulted in the collection of 33 distinct species. Eight bacteria, 21 fungi and 4 yeasts were identified to the specific level by molecular and phenotypic techniques. Bacterial strains belonged to the genera *Gordonia, Brevibacterium, Aeromicrobium, Dietzia, Burkholderia* and *Mycobacterium*. Four species are new and not yet described. Fungi belonged to *Aspergillus, Penicillium, Fusarium, Amorphoteca, Neosartorya, Paecilomyces, Talaromyces* and *Graphium*. Yeasts were *Candida, Yarrowia* and *Pichia.* All strains were cultivated axenically in synthetic liquid media with crude oil as sole carbon and energy source. After incubation, the detailed chemical composition of the residual oil was studied by gravimetric and gas-chromatographic techniques. Thirteen parameters for assessing the biodegradation potential were defined and computed for each strain. Maximum degradation was observed on the saturated HCs (*n*- and isoalkanes, isoprenoids), whereas aromatic HC degradation was lower and was related to the structural composition of the molecules. A principal components analysis (PCA) permitted grouping and classifying the strains as a function of their degradative capacities. It was shown that the most active strains produced polar metabolites which accumulated in the resins and asphaltene fractions. These fractions are highly resistant to microbial metabolism. No taxonomic trend could be defined between microbial phyla in terms of HC biodegradation activity.


*Keywords:* Hydrocarbon; Biodegradation; Indonesia

## 1. Introduction

The extensive use of petroleum products leads to the contamination of almost all compartments of the environment. Biodegradation of hydrocarbons by natural populations of microorganisms is the main process acting in the depuration of hydrocarbon-polluted environments. The mechanism has been extensively studied and reviewed [3,16,27].

Numerous microorganisms, including bacteria, fungi and yeasts are known for their ability to degrade hydrocarbons (HC). Anaerobic microorganisms have been isolated from petroleum reservoirs [17,23] whereas predominantly aerobic HC degraders have been isolated from HC-contaminated ecosystems, even in extreme habitats. In environmental studies, the identification of active strains is not always ascertained to a sufficient degree, and misidentifications or incomplete identifications are sometimes reported. Molecular techniques for the identification of hydrocarbon-degrading bacteria have been only rarely used in environmental studies [8,24]. Furthermore, the biodegradation activity is not always confirmed by chemical analyses of the degraded HC.

The purposes of this work were to (i) collect a number of aerobic culturable HC-degrading microorganisms from petroleum polluted terrestrial sites in Indonesia, (ii) identify the isolates to species level using molecular techniques for bacteria and yeasts and morphological characters for fungi, (iii) investigate the biodegradation activity of each strain

* Corresponding author.
*E-mail address:* oudot@mnhn.fr (J. Oudot).

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by detailed chemical analyses of petroleum residues after cultivation on petroleum-based media, and (iv) describe the population of HC degrading strains by principal components analysis (PCA) using 13 biodegradation parameters.

## 2. Materials and methods

The zone under study was located in S.E. Kalimantan (Borneo island, Indonesia) in a petroleum activity site. Samples were taken from soils contaminated for several years by Indonesian crude oil, and from cyanobacterial mats present in freshwater oil-polluted ponds. Soil and water samples were frozen and transported by air to the laboratories.

### *2.1. Isolation of strains*

The objective of the study was to isolate as many culturable strains as possible in order to determine their HC biodegradation potential in standardised culture conditions. In this way, we did not analyse the total diversity of the microbial community by molecular techniques applied to extracted genetic material [1]. Strains were isolated according to already published methods [20]. Serial dilutions of soil or mats were made in 10 ml sterile distilled water, 0.1 ml of each dilution then being spread on Petri dishes that contained different culture media according to the microbial phylum. Bacterial strains were isolated on two media, a medium M1 for the isolation of total culturable heterotrophic bacteria (isolates referenced as BHT) and a medium M2 for the isolation of hydrocarbon-adapted bacteria (BAH). M1 was composed of trypticase-soy (Bio-Mérieux, Marcy l'Etoile) 30 $g\,l^{-1}$. M2 composition was: $KH_2PO_4$, 0.68 $g\,l^{-1}$; $Na_2HPO_4$, 1.79 $g\,l^{-1}$; $MgSO_4$, 0.35 $g\,l^{-1}$; $NO_3NH_4$, 1 $g\,l^{-1}$; $CaCl_2$, 0.2 $g\,l^{-1}$; $FeSO_4$, 0.4 $mg\,l^{-1}$ and 0.1 ml of a solution containing 100 $mg\,l^{-1}$ of $H_3BO_4$, $MnSO_4$, $ZnSO_4$, $CuSO_4$ and $CoCL_2$. A reference Arabian light crude oil topped at 150 °C (BAL 150) was added at a concentration of 1 $g\,l^{-1}$. After steam sterilisation, the lightest compounds of the oil up to nC15 were lost by volatilisation. This implies that only microorganisms growing on C15 + compounds could be isolated, while the few microorganisms that grew exclusively on light HC could not be collected with this method.

Similarly, fungi were isolated on two different media: M3 for total fungi (CT) and M4 for hydrocarbon-adapted fungi (CAH). M3 was composed of malt 20 $g\,l^{-1}$ and chloramphenicol 100 $mg\,l^{-1}$. M4 contained KCl, 250 $mg\,l^{-1}$; $NaH_2PO_4$, 1 $g\,l^{-1}$; $MgSO_4$, 0.5 $g\,l^{-1}$; $NO_3NH_4$, 1 $g\,l^{-1}$, chloramphenicol, 100 $mg\,l^{-1}$, and BAL 150, 1 $g\,l^{-1}$. Some hydrocarbon-adapted yeasts grew on the M3 and M4 media and were also selectively isolated on Sabouraud medium composed of yeast extract (Sigma, Steinheim), 10 $g\,l^{-1}$; pectic-peptone (Merck, Briare le Canal), 10 $g\,l^{-1}$; glucose, 20 $g\,l^{-1}$. Yeasts were referenced as LAH. The four media were supplemented with 20 $g\,l^{-1}$ agar for plate utilisation. The organic source of carbon and energy was trypticase soy and malt for total culturable bacteria and fungi, respectively, and crude oil for hydrocarbon-adapted microorganisms. The BAL 150 crude oil was composed after steam sterilisation of 38% saturated HC, 38% aromatic HC, 11% resins and 13% asphaltenes. The complete and oil-based media were used with a view to obtaining a specific diversity as high as possible [7]. After inoculation, the plates were kept at $28 \pm 1$ °C for 8 days for total populations and 30 days for hydrocarbon-adapted populations. At the end of the incubation, individual colonies were picked out and streaked on solid media M1 (bacteria) or M3 (fungi and yeasts) for conservation, selection and further identification.

A first screening of strains was done after Gram staining and microscopic examination for bacteria and macro- and microscopic examination for fungi to eliminate apparently similar strains. This resulted in the collection of 94 BHT, 49 BAH, 39 CT, 41 CAH and 5 LAH. All strains were submitted to a preliminary test for utilisation of hydrocarbons. Each isolate was cultured in 20 ml liquid M2 (bacteria) or M4 (fungi and yeasts) medium in $20 \times 200$ mm test tubes, to which 0.1 ml of BAL 150 was added. The tubes were incubated without shaking for 30 days at $28 \pm 1$ °C. The strains that caused visible turbidity or development of mycelium were scored as potentially able to use HC as a carbon and energy source. At the end of the preliminary test, the number of presumably active strains was 27 BHT, 29 BAH, 36 CT, 38 CAH and 5 LAH. A second morphological screening for the elimination of identical isolates reduced this number to 12 BHT, 19 BAH, 12 CT, 14 CAH and 4 LAH, which corresponded to 31 bacterial strains, 26 fungal strains and 4 yeast strains. All isolates were identified and submitted to quantitative HC biodegradation cultures.

### *2.2. Identification of strains*

Bacteria were cultured in trypticase-soy agar at 30 °C. Gram-positive bacteria were lysed according to [12]. DNA was extracted and purified with the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). The almost complete 16S rDNA sequences of strains were compared to all bacterial sequences available from the GenBank database by using the BLAST program (National Center for Biotechnology Information). All 16S rDNA sequences were aligned with CLUSTAL V, and neighbour-joining trees [25] were constructed (not shown).

Fungi were identified according to general principles of fungal classification, using selective media and macro- and microscopic examination of morphological characters [19]. CETEP (Orgères, France) achieved the molecular identification of yeasts [14] by sequencing the 25S rRNA gene, D1–D2 region (280–326 bp) and comparison with sequence alignments from a database (GenBank, NCBI) containing over 1500 yeast sequences. Phylogenetic trees were constructed (not shown).

Table 1
Parameters measured on BAL 150 cultures for the estimation of petroleum biodegradation

| Parameter number | Description |
|---|---|
| P1 | Total biodegraded oil (GR) |
| P2 | Total GC biodegraded oil (GC: total area of the chromatogram) |
| P3 | Total saturated hydrocarbons (GR) |
| P4 | Sum of GC-resolved saturated HC (GC) |
| P5 | *n*-Alkanes, sum (GC) |
| P6 | Isoalkanes, sum (GC) |
| P7 | Pristane + phytane (GC) |
| P8 | Saturated unresolved complex mixture UCM (GC): cyclic and branched alkanes |
| P9 | Total aromatic hydrocarbons (GR) |
| P10 | Sum of GC-resolved aromatic hydrocarbons (GC) |
| P11 | Aromatic unresolved complex mixture UCM (GC): polycyclic aromatics |
| P12 | Resin fraction (GR) |
| P13 | Asphaltene fraction (GR) |

GR: gravimetric data, GC: gas-chromatographic data.

### 2.3. Determination of biodegradation activity

The determination of the biodegradation potential was made according to already published methods [18]. Each active strain was grown in duplicate without shaking in 250 ml Erlenmeyer flasks containing 150 ml of M2 (bacteria) or M4 (fungi and yeasts) medium and 200 µl (179 mg) of BAL 150 crude oil. The activity of a mixed culture CM including the five most active bacteria (BAH 18, BAH 23, BAH 6, BAH 2, BAH 11) was also monitored. The mean difference between the biodegradation rate of the two replicates was less than 2%. Sterile controls were used to quantify the abiotic losses due especially to evaporation. The volatilisation of the light compounds up to nC15 during steam sterilisation and incubation led to an abiotic loss of 34% of the initial oil. All results of biodegradation were obtained in reference to sterile controls. At the end of an incubation time of 30 days at 28 °C, the residual petroleum was recovered by dichloromethane extraction. After solvent evaporation, the total biodegradation rate was determined as $\%B = 100((WI - WC)/WI)$, in which WI is the mass of the residue in the sterile control and WC is the mass of the fraction in the culture. The total residual oil was fractionated into main molecular classes. After precipitation of asphaltenes in hexane and filtration on GF/A Whatman filters, the maltenes were separated into saturates, aromatics and resins by successive elution with, respectively, hexane, hexane-dichloromethane (3/2, v/v) and methanol on an activated silica gel (60–100 mesh) column. The solvents were evaporated, and the biodegradation rate (%B) of each fraction was determined as above.

A detailed analysis of the saturated and aromatic fractions was made by computerized capillary gas chromatography GC [20] after addition of an internal standard (n-eicosene). The Hewlett Packard HP 4890 chromatograph was equipped with a split injector (split ratio 30/1) and a flame ionisation detector FID both set at 330 °C; carrier gas was helium under 110 kPa; the column was a CP Sil 5 CB (Varian) capillary column (50 × 0.32 mm, film thickness 0.25 µm); temperature programming was 80–320 °C, 3 °C $min^{-1}$. Acquisition and further numerical treatment of data were performed using custom-made computer programs. In the aromatic fraction, identification of molecular types was achieved through gas-chromatography/mass spectrometry analyses. For each strain, 5 gravimetric and 8 chromatographic parameters of biodegradation activity were quantified relative to sterile controls (Table 1). The 13 parameters of biodegradation activity were compared amongst the 33 active microorganisms by principal components analysis PCA using the Matlab (Mathworks) software.

## 3. Results and discussion

### 3.1. Identification of HC-degrading strains

The identification of HC-degrading strains is given in Table 2 which lists a single isolate per taxon and also indicates the origin of the isolate (polluted soil or cyanobacterial mat). A total of 12 bacterial isolates were shown to degrade HC. Four isolates were precisely identified to named species (*Gordonia terrae, Aeromicrobium erythreum, Gordonia hydrophobica* and *Burkholderia thailandensis*); 5 isolates were precisely identified to an unnamed *Dietzia* species (referred to as E9-2); isolate BHT 14 corresponds to a new *Brevibacterium* species close to *B. epidermidis, B. iodinum* and *B. linens*; isolate BAH23 corresponds to a new species of *Mycobacterium*; and isolate BAH 6b may correspond to a new genus close to *Actinomyces* and *Thermoleophilum*.

A total of 19 isolates did not significantly assimilate the hydrocarbons of the BAL 150 petroleum; they belonged to the genera *Bacillus* (*B. megaterium, B. firmus, B. cereus, B. sphaericus, B. subtilis, B. pumilus, B.* group *aquaemaris), Brachybacterium* sp., *Brevibacterium* (group *B. epidermidis*), *Microbacterium* sp., *Cellulosimicrobium cellulans, Paenibacillus glycanolyticus.* It was already observed that bacterial strains that were scored as positive after the preliminary visual test did not confirm significant HC biodegradation potential in definitive cultures [20]. This can be explained either because they were false-positives growing at the expense of organic carbon traces resulting from the transfer of M1 medium used for conservation of the strains at the time of inoculation, or because they immediately lost their HC degradation capacities. This is particularly clear for *Bacillus* members that are sometimes cited as hydrocarbon degraders. Under our strictly controlled experimental conditions where HC degradation is chemically monitored, this activity is not confirmed, and the 7 *Bacillus* species that were tested did not assimilate HC, although some strains were isolated on medium M2 and were positive after the preliminary test. In fact, *Bacillus* spp. are considered as secondary degraders, i.e., they assimilate the metabo-

Table 2
Identification of HC-degrading strains of microorganisms

| Strain number | Identification and GenBank accession number | % identity | Origin |
|---|---|---|---|
| Bacteria | | | |
| BHT 14 | *Brevibacterium* sp. (new species) | | S |
| BAH 2 | *Gordonia terrae* X 81922 | 99.4 | M |
| BAH 5 | *Aeromicrobium erythreum* AF 005021 | 98.4 | M |
| BAH 6b | New taxon, close to *Actinomyces/Thermoleophilum* | | M |
| BAH 11 | *Dietzia* sp. group E9-2 | | S, M |
| BAH 13 | *Gordonia hydrophobica* X 87340 | 96.9 | S |
| BAH 18 | *Burkholderia thailandensis* U 91838 | 98.4 | S |
| BAH 23 | *Mycobacterium* sp. (new species) | | S |
| Yeasts | | | |
| LAH 1 | *Yarrowia lipolytica* AF 335977 | 99 | S |
| LAH 2 | *Candida viswanathii* CVU 45752 | 99.7 | S |
| LAH 3 | *Candida palmioleophila* CPU 45758 | 98 | S |
| LAH 5 | *Pichia guilliermondii* AF 257270/*Candida fukuyamaensis* CFU 62311 /*C. xestobii* CXU 45707 | 99.6 | S |
| Fungi | | | |
| CT 1 | *Aspergillus fumigatus* Fresenius | | S |
| CT 2 | *Fusarium oxysporum* Desmazières | | S, M |
| CT 4 | *Penicillium oxalicum* Currie & Thom | | S |
| CT 8 | *Penicillium simplicissimum* (Oudemans) Thom | | S |
| CT 9 | *Penicillium verruculosum* Peyronel | | S |
| CT 11 | *Aspergillus clavatus* Desmazières | | S |
| CT 16 | *Penicillium montanense* M. Christensen & Backus | | S |
| CT 19 | *Penicillium pinophilum* Hedgcock | | S |
| CT 99 | *Aspergillus sydowii* (Bainier & Sartory) Thom & Church | | S |
| CAH 2 | *Amorphoteca resinae* Parbery | | S |
| CAH 3 | *Neosartorya fischeri* (Wehmer) Malloch & Cain | | S |
| CAH 4 | *Penicillium janthinellum* Biourge | | S |
| CAH 5 | *Aspergillus niger* Van Tieghem | | S |
| CAH 7 | *Paecilomyces variotii* Bainier | | S |
| CAH 9 | *Talaromyces flavus* (Klöcker) Stolk & Samson | | S |
| CAH 11 | *Graphium putredinis* (Corda) Hughes | | S |
| CAH 13 | *Cunninghamella echinulata* (Thaxter) Thaxter | | S |
| CAH 22 | *Penicillium restrictum* Gilman & Abbott | | S |
| CAH 33 | *Aspergillus terreus* Thom | | S |
| CAH 44 | *Eupenicillium ochrosalmoneum* Scott & Stolk | | S |
| CAH 55 | *Fusarium decemcellulare* Brick | | S |

Origin: S, polluted soil, M, cyanobacterial mat.

lites produced by true primary HC degraders [10]. In other taxa like *Brevibacterium* spp., some isolates were HC degraders when close strains were not. In the bacterial active strains (Table 2), *Brevibacterium* BHT 14 was isolated on the complete medium M1, while all other BAH were isolated on synthetic M2 medium. Six HC-degrading strains originated from the soil and 4 from the cyanobacterial mats. Seven species of the 8 identified were Actinomycetales. Two were *Gordonia* (*G. terrae* BAH 2 and *G. hydrophobica* BAH 13). *G. terrae* was formerly identified as *Rhodococcus terrae* [11]. *G. hydrophobica* is phylogenetically close to *G. alkanivorans* which is known to be an HC degrader [13]. *Aeromicrobium erythreum* BAH 5 was previously identified as *Arthrobacter* [11]. *Dietzia* sp. group E9-2 (BAH 11) was the most frequently isolated species (5 isolates); it is related to *Rhodococcus* and members of the genus are HC degraders [28]. The pseudomonad *Burkholderia thailandensis* BAH 18 is close to species *B. pseudomallei*, *B. multivorans*, *B. cepacia*, already known to be HC degraders [5]. Strains of *Mycobacterium* are known to be HC degraders [4,9], like BAH 23. Four strains (BHT 14, BAH 6b, BAH 11, BAH 23) are new and not yet described species. Then a number of species identified in this work are newly cited as HC degraders, but similar bacterial groups may have been identified previously under different names due to evolving bacterial nomenclature and classification concepts. In this way, the frequently previously cited genera *Rhodococcus*, *Arthrobacter*, *Corynebacterium* and *Pseudomonas* may have been similar or very close to the strains presently identified. Due to molecular techniques, the identifications reported here are much more precise than in most previous works.

Concerning fungi, all 26 isolates showed HC degradation activity. However, 5 were replicates. Thus the identification of 21 isolates is reported in Table 2. All the genera identified have already been cited as HC degraders. As currently observed, members of the genera *Aspergillus* and *Penicil-*

*lium* are largely dominant [19]. Some species of these genera (*P. oxalicum, P. montanense*) as well as *Cunninghamella echinulata, Eupenicillium ochrosalmoneum, Fusarium decemcellulare* and *Neosartorya fischeri* are newly cited HC-degrading fungi. The active fungal strains were isolated almost equally from the complete (CT) and synthetic (CAH) media. Only one strain (*F. oxysporum*) was isolated from cyanobacterial mats (and from the soil as well), while all others originated from the soil.

Of the 5 yeast isolates, 2 were *Yarrowia lipolytica*. With the exception of *Candida viswanathii*, the species listed (*Y. lipolytica, C. palmioleophila* and *P. guilliermondii*) are known to be HC degraders [2,26]. Except for *P. guilliermondii* which cannot be separated from *C. fukuyamaensis* and *C. xestobii* by 25S rRNA sequencing, all yeast species were unambiguously identified by molecular methods.

The screening of HC-degrading microorganisms in oil-polluted Indonesian sites permitted selecting 33 active species. The specific diversity of bacteria is relatively low (8 species) although over 300 isolates were initially collected. On the other hand, the specific diversity of fungi is high, with 21 distinct species.

### 3.2. Biodegradation potential

The results of the biodegradation potential of the bacterial strains are reported in Fig. 1 which also includes results of the mixed culture. Data for fungi are given in Fig. 2 and in

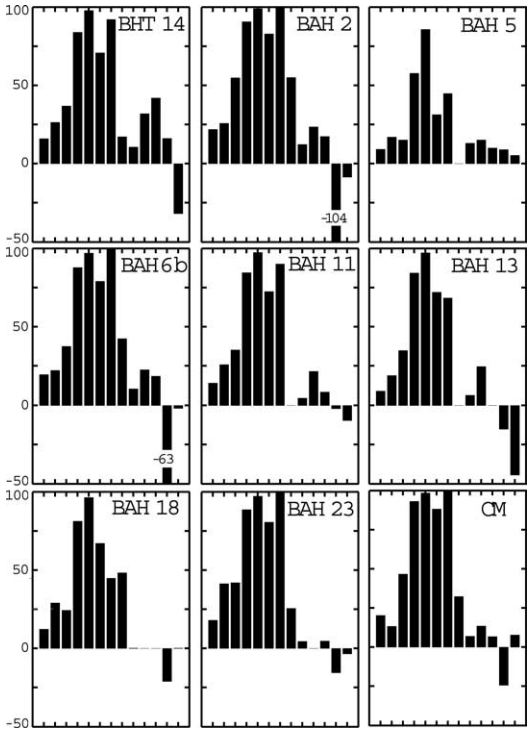


Fig. 1. Biodegradation potential of bacteria. The bars of the histograms represent percent biodegradation as compared to sterile controls. See Table 1 for the 13 parameters and Table 2 for identification of strains. CM mixed culture.

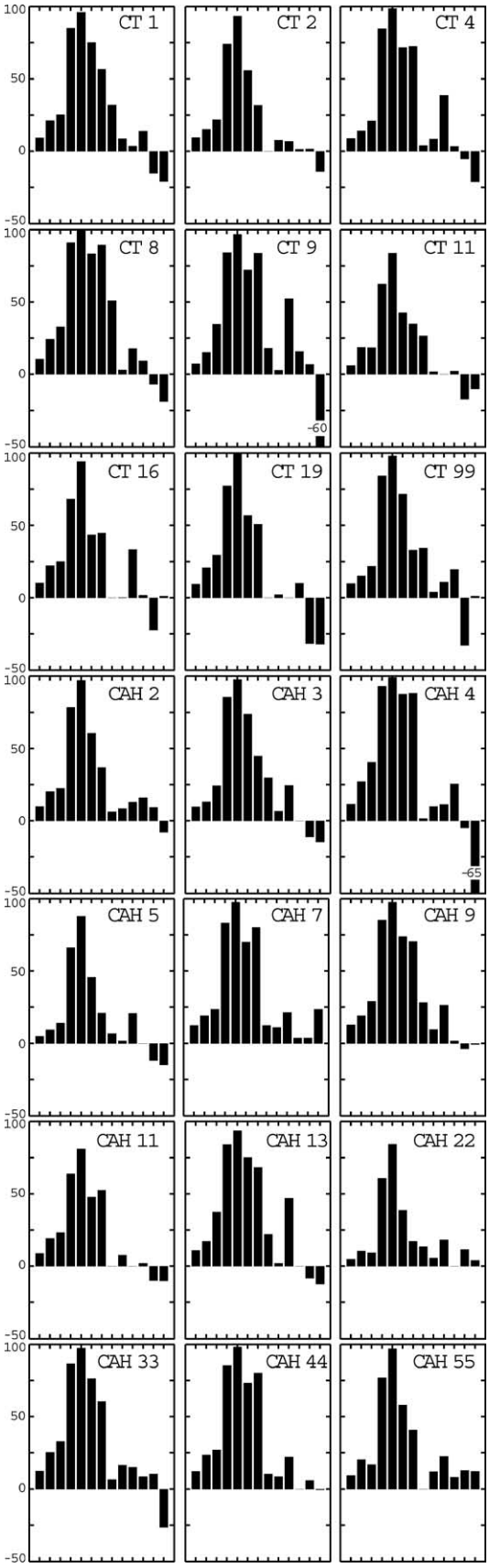


Fig. 2. Biodegradation potential of fungi. See Table 1 for parameters and Table 2 for identification of strains.

Fig. 3 for yeasts. The bars of the histograms represent the biodegradation rate %B for each of the 13 biodegradation parameters listed in Table 1. The %B were calculated with reference to sterile controls. The values of the minimum, maximum and mean values %B for the 13 biodegradation parameters are given in Table 3. Fig. 4 reports the gas chromatograms of the saturated and aromatic fractions of the sterile controls; the chromatographic traces obtained, respectively, for moderately active and highly active strains on the two fractions are also illustrated in the figure. All compounds below nC15 were lost by volatilisation during steam sterilisation of the culture flasks.

The biodegradation rate (gravimetric) for total oil (parameter P1) is relatively low, ranging from 4.9 to 22%. The data for total GC biodegradation (P2) are almost twice as high, from 9 to 41%. The difference from P1 values comes from the fact that the two methods do not quantify the same petroleum compounds. GC data do not include the heaviest compounds of the oil nor the resins (P12) and asphaltenes (P13) that cannot be resolved by GC and are very resistant to biodegradation. The saturated fraction (P3 and P4) is the most strongly degraded, with the *n*-alkanes (P5) and isoalkanes (P6) being extensively degraded (up to 100%). The isoprenoids pristane and phytane (P7) are slightly attacked by some strains, while they are completely metabolised by others. The polycyclic alkanes contained in the unresolved complex mixture (UCM) of the chromatograms of the saturated fraction (P8) are much less degraded (mean 18%) than the aliphatic compounds. This confirms a number of observations [18]. The persistence of biomarkers of the hopane series (Fig. 4) is verified as in laboratory and field observations [21,22].

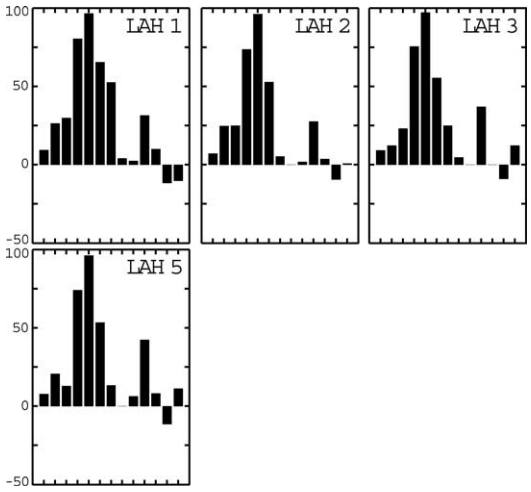


Fig. 3. Biodegradation potential of yeasts. See Table 1 for parameters and Table 2 for identification of strains.

The aromatic fraction of the BAL 150 crude oil (P9) is less degraded than the saturated fraction (0–17%). Such values for total aromatic biodegradation are on the low side of what was already observed for this parameter. This can be explained in part by the fact that the chemical composition of the reference crude oil BAL 150 used in this study may be slightly different from previously used reference oils. Also, the Indonesian crude oils that contaminate the site under study have a low content in aromatic HC (15–25%) and do not contain the whole spectrum of molecules that are present in Arabian light crude oils which are composed of over 40% aromatic HC. The pressure selection for aromatics in the field could be lower than with Arabian oils, which could explain the relatively low aromatic metabolism observed. The GC-resolved aromatic peaks (P10) are degraded to a mean of 20%, and the aromatic UCM which contains most of the polycyclic aromatic HC to a mean of 10%. Considering the molecular types of aromatic HC (Fig. 4), it is observed that light compounds (alkyl naphtalenes and fluorenes) are more degraded than heavy ones (alkyl chrysenes) and that the biodegradability of the molecular classes decreases when the number of methyl substitutions increases. This confirms already known data [6,18].

The %B values for resins (P12) and asphaltenes (P13) are often negative, as can be seen in Figs. 1, 2 and 3. This is explained by the fact that polar metabolites resulting from the biooxidation of alkanes and aromatics are extracted and pooled with the polar fractions (i.e., resins and asphaltenes) of the residual oil during the fractionation protocol [20]. These polar metabolites are predominantly alicyclic and aliphatic organic acids and ketones [15], some of which can hamper subsequent biodegradation due to their toxicity that represses microbial metabolism. In fact, resins and asphaltenes which are composed of highly condensed and polymerised macromolecules are known to be extremely resistant or refractory to microbial metabolism [18]. However, some bacteria (BAH 5), fungi (CAH 7, CAH 55) and yeasts (LAH 3, LAH 5) demonstrated a slight biodegradation activity (10–15%) on these fractions, as previously observed [19].

The biodegradation rates reported here do not reflect the highest values that can be obtained. Biodegradation rates have been shown to be higher in continuous cultures in which toxic metabolites do not accumulate [18]. However, such techniques cannot be conveniently used when handling

Table 3
Minimum, maximum and mean values (%) for the 13 biodegradation parameters listed in Table 1

| Parameter | P1 | P2 | P3 | P4 | P5 | P6 | P7 | P8 | P9 | P10 | P11 | P12 | P13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Minimum | 5 | 9 | 10 | 58 | 81 | 31 | 5 | 0 | 0 | 0 | 0 | −104 | −65 |
| Maximum | 22 | 41 | 55 | 93 | 100 | 88 | 100 | 55 | 17 | 52 | 42 | 16 | 24 |
| Mean | 11 | 20 | 28 | 80 | 96 | 65 | 59 | 16 | 6 | 20 | 8 | −11 | −11 |

See Figs. 1–3 for details.

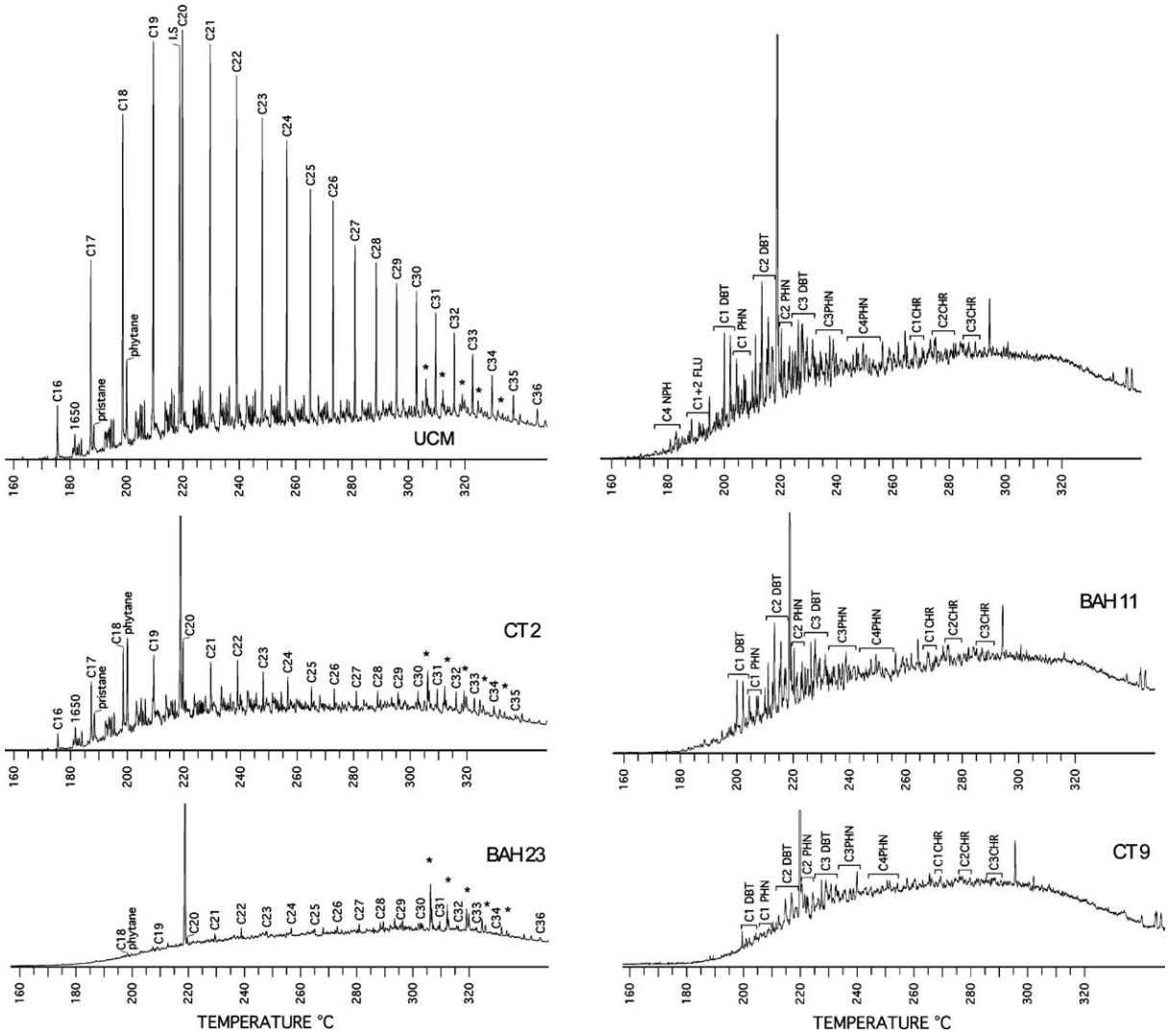


Fig. 4. Gas chromatograms of BAL 150 residual oil. Left: saturated fraction; right: aromatic fraction (vertical scale × 2.5 as compared to saturates). Top: sterile control; mid: moderately active strains; bottom: highly active strains. I.S internal standard (n-1 eicosene), UCM unresolved complex mixture. Saturated fraction: Ci number of carbon atoms in the *n*-alkane chain, intermediate peaks are isoalkanes, * hopane biomarkers. Aromatic fraction: NPH naphtalenes, FLU fluorenes, DBT dibenzothiophenes, PHN phenanthrenes, CHR chrysenes. C1–C4 number of methyl substitutions.

a high number of cultures. In any case, the protocol of this study allows accurate intercomparison between strains cultivated under the same standardised experimental conditions.

### *3.3. Principal components analysis (PCA)*

The results of PCA are reported in Fig. 5 which illustrates the distribution of the 34 cultures in the PC1–PC2 plane. Fig. 6 shows the correlation of the 13 biodegradation parameters with the two first PCA axes. The variation along the two first axes represents 56.6% of the total variation. The predominant variables along PC1 are GC-resolved isoalkanes (P6), total saturated GC-resolved peaks (P4), gravimetric total saturate (P3), isoprenoids pristane + phytane (P7) and total gravimetric residual oil (P1). In this way, the strains are classified along PC1 from lowest biodegradation activity (negative values ) to the highest activity (positive values). Resins (P12), GC-resolved aromatic peaks (P10), and asphaltenes (P13) are predominant along PC2. The most globally active strains BAH 2, BAH 23, BAH 6b and mixed culture CM are included in one cluster in which an increase in the resin fraction is observed. The mixed culture is as active (but not more) as the most active individual strains. The second group CT 9, CAH 4 and BHT 14 is dominated by the asphaltene augmentation. It can be noted from PCA that the most efficient strains produce the highest amounts of metabolites. The chemical structure of the metabolites was not studied here; it is likely that their distribution between resins and asphaltene fractions is governed by the relative polarity of the metabolites produced. The group showing the lowest biodegradation activity (CAH 22, CAH 5, BAH 5, CAH 11, CT 11) is located on the negative values of PC1. The excentric position of BAH 18 is explained by its total inactivity on aromatic HC. Amongst the three most active

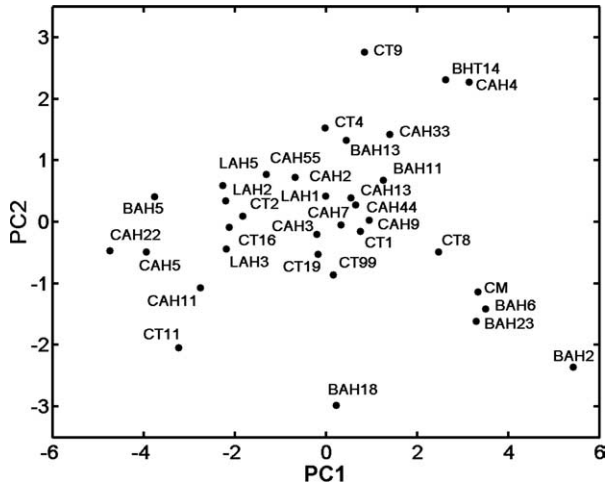


Fig. 5. Principal components analysis of HC-degrading strains versus biodegradation parameters. Data projection on the first two principal axes. See Table 2 for identification of strains. CM mixed culture.

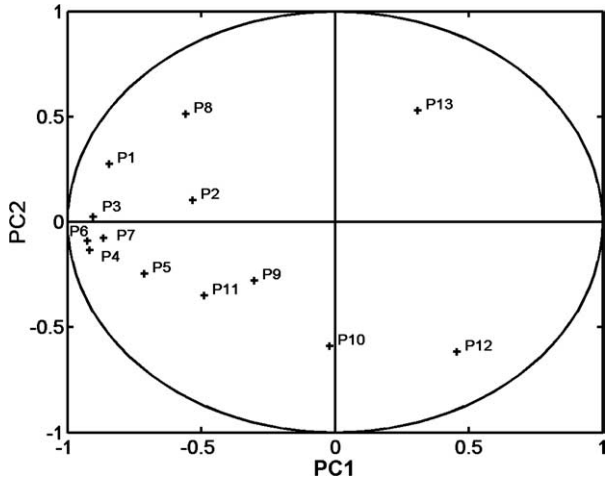


Fig. 6. Correlation of biodegradation parameters with the first two principal axes. See Table 1 for parameter definition.

bacterial strains of the first cluster, two were isolated from the cyanobacterial mat: *Gordonia terrae* BAH 2 and the new taxon BAH 6b. No significant trend can be inferred concerning the taxonomic position of the strains, and the bacterial, fungal and yeast phyla are not clearly separated, although the genes involved and metabolic pathways are different among organisms [3]. This confirms that hydrocarbon biodegradation potential is not a useful taxonomic characteristic. It can be noted, however, that this metabolic capacity is widespread among species of a limited number of ubiquitous genera.

In conclusion, thirty-three distinct species of HC-degrading microorganisms (8 bacteria, 21 fungi and 4 yeasts) were isolated from petroleum-contaminated terrestrial tropical environments located in Indonesia. With the use of powerful molecular methods, only 4 of 12 HC-degrading bacterial isolates corresponded to named species. The other isolates represented new species. By contrast, yeasts and fungi were identified with named species. Most of the strains were already known as HC degraders, although under a different nomenclature. Using a highly detailed chemical analysis of the biodegradation potential and treatment of data by principal components analysis, it was possible to characterise the microorganisms as a function of their degradative capacities. Biodegradation followed a general similar scheme, with the same compounds being totally degradable and others resistant or refractory to microbial metabolism, independently of the taxonomic position of the strain. No clear taxonomic trend related to HC degradation was visible between the phyla, thus confirming that the biodegradation of petroleum compounds is more closely related to their intrinsic biodegradability than to the peculiar enzymatic capacities of the microorganisms involved. The fact that a high number and diversity of HC-degrading microorganisms were isolated from the tropical site under study confirms that microbial biodegradation is a widespread process involved in the depuration of petroleum-contaminated environments.

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