African Journal of Biotechnology Vol. 12(14), pp. 1636-1643, 3 April, 2013
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.2879
ISSN 1684–5315 ©2013 Academic Journals

Full Length Research Paper

Isolation of four hydrocarbon effluent-degrading *Bacillaceae* species and evaluation of their ability to grow under high-temperature or high-salinity conditions

Saïd Ben Hamed[1,2]*, Abderrazak Maaroufi[1], Abdeljelil Ghram[1], Ben Ali Gam Zouhaier[2] and Marc Labat[2]

[1]Laboratory of Microbiology, Bioprocess Group, Institute Pasteur of Tunisia (IPT), Bp 74, 13 place Pasteur, Belvédère-1002 Tunis, Tunisia.
[2]Laboratoire de Microbiologie IRD, Biotechnologie Microbienne des Environnements Chauds, UMR D180, IRD ESIL MIO, Aix-Marseille Université, 163 avenue de Luminy, 13288 Marseille cedex 9, France.

Accepted 1 March, 2013

Four bacterial strains belonging to the family *Bacillaceae* were isolated from a polluted site and tested for their efficiency in degrading a refinery effluent highly polluted with hydrocarbons. Among 13 strains isolated, four were selected for their efficiency. Each of these four strains demonstrated a strong ability to grow as a single strain on a hydrocarbon effluent (HCE) as sole carbon source. In batch assays using clarified wastewater as diluent, the strains achieved high-percentage decreases in total hydrocarbon concentration within 18 days: 98% with *Bacillus licheniformis* STK08, 87% with *Geobacillus stearothermophilus* STM04, 80% with *Lysinibacillus sphaericus* STZ75 and *72*% with *Bacillus firmus* STS84. The decreases were greater during the first three days of treatment, with 73, 66, 39 and 47% recorded for *Bacillus licheniformis* STK08, *G. stearothermophilus* STM04, *L. sphaericus* STZ75 and *B. firmus* STS84, respectively. Growth assays run under different conditions showed that *B. licheniformis* STK08 and *G. stearothermophilus* STM04 were able to grow at salinities of up to 120 g/L and at 55°C. Potential biosurfactant production tested using two methods namely modified drop collapse (MDC) and blue agar plate (BAP) demonstrated that the four *Bacillaceae* species are biosurfactant producers.

Key words: Hydrocarbons, biodegradation, pure culture, high salinity, high temperature.

INTRODUCTION

Petroleum oil is a major driver of worldwide economic activity, but it has also created major pollution problems due to repeated accidental spills during petroleum component transport and storage and during refining process. Many physical and chemical methods can remove toxic petroleum components, such as incineration, chlorination, ozonation and combustion, but they unfortunately require elaborate facilities and substantial amounts of additional fuels (Mrayyan and Battikhi, 2005). Moreover, these processes, especially the chemical ones, produce intermediates that may present toxicity levels similar to the original substances (Tomei et al., 2003). Thus, some studies tend to look for other process which seems to be more efficient and less costly. In fact, several studies have demonstrated that biological approaches are better suited to the degradation of these pollutants due to their low cost, their ability to convert contaminants to harmless end-products (Rahman et al., 2002) and the proven high sensitivity of the technologies employed. Efficient treatments hinge on precise knowledge of the polluting load, the physical and chemical properties of the pollutant, the environmental conditions and the specific competencies of the micro-

*Corresponding author. E-mail: benhamed_med@yahoo.fr. Tel: (+216) 71. 873 .366 / Fax (+216) 71.872.055.

organisms involved (Rahman et al., 2002; Hui et al., 2007; Das and Chandran, 2011).

Studies of biodegradation processes have demonstrated that various species belonging to different genera such as *Achromobacter, Alcaligenes, Azospirillum, Bacillus*, *Cycloclasticus*, *Micrococcus*, *Pseudalteromonas, Pseudomonas*, *Sphingomonas*, *Staphylococcus, Vibrio* and *Xanthomonas* (Hedlund et al., 2001; Zhuang et al., 2003; Viñas et al., 2005; Alonso-Gutiérrez et al., 2009) are able to use one or more polycyclic aromatic hydrocarbons (PAHs) as sole carbon source. These genera are capable of degrading crude oil in pure and mixed cultures with natural or constructed bacterial consortia. However, the degradation rate usually depends on the composition of the crude oil used and its bio-accessibility. Also, compounds such as saturates, aromatics and polar compounds present in different crude oil samples are degraded to different degrees by the same organisms (Tzarkova and Groudeva, 2000).

Hydrocarbon degradation has been widely reported in laboratory-scale batch studies. Indeed, Chavan and Mukherji (2008) demonstrated that green algae and cyanobacteria can remove 98.99% of total petroleum hydrocarbon. Likewise, using bacterial consortium isolated from oil contaminated site, Malik and Ahmed (2011) revealed percentages of hydrocarbon removal of 94.64 and 93.75 of aliphatic and aromatics hydrocarbons, respectively. Conclusions of all biodegradation studies show that bacterial efficiency is usually related to the acclimation period and growth conditions, mainly temperature and salinity.

In fact, hydrocarbon biodegradation in high salt medium was slow, because high NaCl in medium disrupts cell membrane, denaturates some proteins such as enzymes, or changes osmotic forces; these factors could be lethal for microorganisms (Minai-Tehrani et al., 2006). Nevertheless, temperature improves hydrocarbon degradation and enhances its bio-accessibility for bacteria (Margesin and Schinner, 2001). Regarding the influence of environmental conditions on the hydrocarbon degradation rate, it seems interesting to seek for efficient bacterial strains, which could be explored for the bioremediation of hydrocarbon-polluted sites in terrestrial or marine environments, especially under high temperature or high salinity conditions. Within this framework, four species belonging to the family *Bacillaceae* were isolated from sites highly polluted with hydrocarbons. The isolates were tested to evaluate their ability to grow at different temperatures and salt concentrations, to produce biosurfactant and to degrade a refinery effluent in pure culture efficiently.

MATERIALS AND METHODS

Sampling site and characterization of the hydrocarbon effluent

Samples of a hydrocarbon effluent (HCE) were collected from a rejection mud pit run by the Tunisian Refining Industries Company (STIR) located at Bizerte in Northern Tunisia. The samples collected from the STIR were transferred to pre-sterilized glass bottles and analyzed by measuring Total Hydrocarbon Concentration (THC), Chemical Oxygen Demand (COD), Biochemical Oxygen Demand (BOD_5), pH and salinity (Ben Hamed et al., 2010).

Isolation of hydrocarbon-degrading bacteria

Samples from highly polluted wastewater were collected and serially diluted and plated onto basal medium amended with 1% (v/v) HCE as the sole carbon source. The plates were incubated at 30°C for three days. Individual colonies were streaked successively onto the same medium (basal medium with 1% (v/v) HCE) for isolation and purification (Auffret et al., 2009).

The basal medium used for strain isolation contained per liter: 0.5 g KH_2PO_4, 1.5 g K_2HPO_4, 1.0 g NH_4Cl, 0.5 g $MgSO_4$. $7H_2O$, 5.0 g NaCl and 1.0 ml of trace metal solution. The trace metal solution contained, per liter: 1.5 g nitrilotriacetic acid, 5.0 g $MnSO_4.2H_2O$, 0.01 g $FeSO_4$, 0.1 g $CaCl_2.2H_2O$, 0.1 g $ZnSO_4.7H_2O$, 0.01 g $CuSO_4.5H_2O$, 0.1 g $CoCl_2$, 0.01 g $Al_2(SO_4)_3.16H_2O$, 0.01 g H_3BO_4, 0.01 g $Na_2MoO_4.2H_2O$, 18 g/L agar (Van Hamme and Ward, 2001). The pH of the medium was adjusted to 7.5, and incubation was run at 37°C for 48 h.

Morphological and biochemical characterization of isolated bacteria

Strains were identified using morphological and biochemical characteristics (colony size and shape, pigmentation, cell shape, cell arrangement, cell motility, Gram staining, oxidase and catalase tests). The main characteristics of each strain were checked using an API 50CH system gallery and its associated software (APILAB 3.3.3, Biomérieux Corporation) (Hedi et al., 2009).

DNA extraction and 16S rRNA gene amplification and sequencing

DNA was extracted from bacterial cultures using a Wizard Genomic DNA Purification Kit from Promega. The 16S rRNA gene of the isolate strain was amplified by adding 0.5 µL of extracted DNA to a thermocycler microtube containing 10 µL of taq polymerase q Biogène, 0.5 µL of 50 nM Fd1 and Rd1 primers and 38.5 µL of sterilized distilled water. The taq polymerase q Biogène contained the taq buffer, $MgCl_2$ $6H_2O$, dNTPs and the taq polymerase. The universal primers Fd1 and Rd1 (Fd1, 5'-AGAGTTTGATCCTGGCTCAG-3' and Rd1, 5'-AAGGAGGTGATCCAGCC-3') were used to obtain a PCR product of almost 1.5 kb corresponding to base positions 8-1542 based on *Escherichia coli* numbering of the 16S rRNA gene (Winker and Woese, 1991). The sample was placed in a hybrid thermal reactor thermocycler (BIOMetra, Leusden, The Netherlands), denatured for 1 min at 95°C and subjected to 30 cycles for 20 s at 95°C, 30 s at 55°C, and 1 min and 30 s at 72°C. This was followed by a final elongation step lasting for 5 min at 72°C. The PCR products were analyzed on 1% (w/v) agarose gels and sent to GATC Company (GATC Biotech Ltd., Germany) for sequencing. Multiple alignment analyses with CLUSTAL X (Thompson et al., 1997) were run, and all alignment gaps were treated as missing data. The obtained sequences then underwent a BLAST search (version 2.2.23 available on line at Http://blast.ncbi.nlm.nih.gov/blast.cgi) to determine the closest related species registered in the GenBank database (Figure 1).

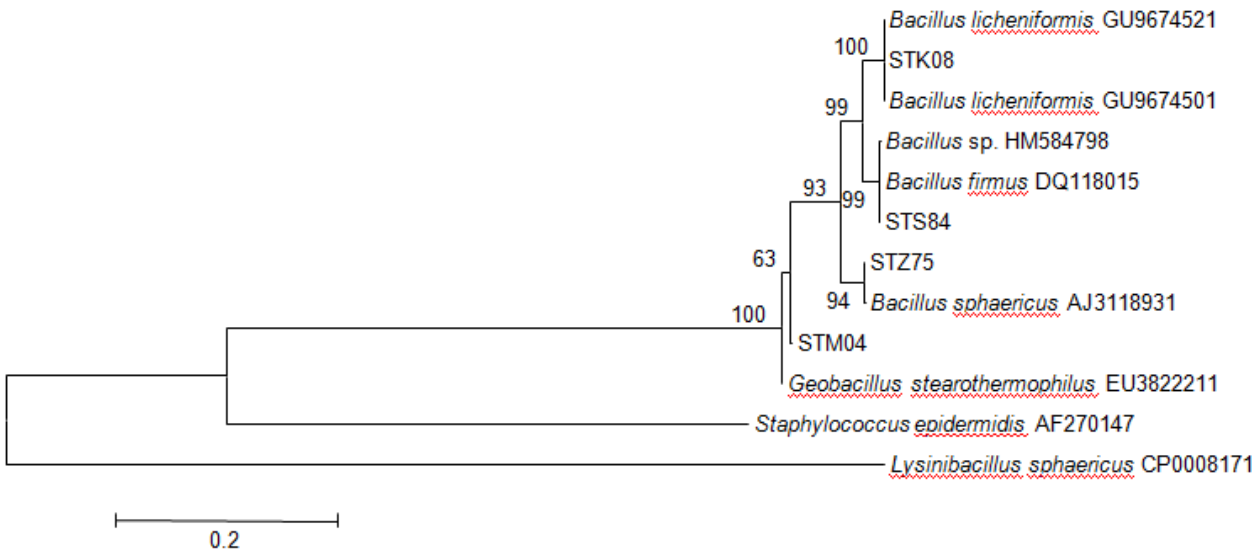

Figure 1. 16rRNA gene sequence-based dendrogram obtained by neighbour-joining method, showing the phylogenetic positions of STK08, STS84, STZ75 and STM04 *Bacillaceae* isolates. Bootstrap confidence levels greater than 50% are indicated at the internodes.

Biosurfactant production screening using the modified drop collapse method (MDC)

To prepare the assay, plates were rinsed successively with hot water, 75% ethanol, distilled water and dried with air. After preparation, plates were equilibrated and coated with a thin layer of crude oil. The preparation was left for 24 h to ensure a uniform oil coating. Bacterial suspensions of all isolated strains were prepared and OD (600 nm) was adjusted to 0.8 for each strain. A volume of 0.5 µl of each bacterial suspension was transferred on the thin oil layer.

The shape of the drop was inspected after 1 min; if the drop remained beaded, the result was scored as negative (-). If the drop collapsed, the result was scored as positive (+) (Bodour and Miller-Maier, 1998).

Biosurfactant production screening using the blue agar plate method (BAP)

This method shows an ionic biosurfactant producing strains by color reaction. Mineral salts agar medium (MSA) (Siegmund and Wagner, 1991) supplemented with carbon sources (glycerol: 2%), cetyltrimethylammonium bromide (CTAB: 0.5 mg/ml), methylene blue (MB: 0.2 mg/ml) were prepared. Plates was streaked with bacteria of interest and incubated at 30°C for 24 h. A dark blue halo around the culture was considered as positive for biosurfactant production. Sodium dodecyl sulfate (SDS: 1 mg/l) and sterile distilled water were used respectively as positive and negative controls.

Bacterial growth conditions

To check the potential use of these strains for biodegradation in biotechnological processes under extreme conditions, strain performances were tested under halophilic and thermophilic conditions. Bacterial growth assays were carried out on liquid basal medium with 1% (v/v) of HCE as sole carbon source, with 2, 60 or 120 g/L of salt, a temperature of 30 or 55°C, or simultaneous combinations of these different conditions of temperature and salinity (Table 1).

The increase in biomass was quantified by measuring the OD at a wavelength of 600 nm. A reading of OD_{600} is frequently used in studies characterizing the physiology of hydrocarbon utilization (Ciric et al., 2010).

Pre-culture of the isolated strains for biodegradation study

Each of the four isolated strains was cultivated in a 1 L flask bottle containing 800 ml of sterile clarified wastewater (CWW) as diluent, low COD (120 mg/l), and trace hydrocarbons (THC <10 mg/l). Each flask bottle was inoculated with 20% (v/v) of a single strain in pure culture, and the HCE was added at 1% (v/v). The initial COD and initial THC of the hydrocarbon effluent were high, at 312 and 71 g/l, respectively, while the COD and THC concentrations of the inoculum were low, at 0.625 and 0.15 g/L, respectively. Thus the inoculum represented about 5% of total COD whereas HCE represented about 95% of total COD of the culture medium in each assay. Consequently, the HCE degradation run in these 1 L flask bottles were tested as main rather than sole carbon source. After incubation at 30°C for 48 h in a reverse shaker (130 rpm), biomasses of each single strain of these pre-grown cultures (20% v/v) were used to inoculate each bioreactor.

Bioreactor design and biodegradation conditions

In-bioreactor biodegradation assays were conducted in a batch system at 30°C and in 2 g/L of NaCl, in view of the bacterial growth conditions previously obtained (Table 1). As done earlier, CWW was used as diluent, with HCE as main carbon source. The use of CWW as culture medium diluent in these in-bioreactor biodegradation assays was justified by the potential exploitation of this by-product combination (CWW and HCE) in industrial processes, as this would avoid the need for amendment of costly nutrients, essentially nitrogen and phosphorus organic or inorganic compounds. The HCE was added to obtain a final THC of 750 mg/l. Salinity was adjusted to 2 g/l and pH was adjusted to 7.2. Four bioreactors were trialed. Two one-liter flask bottles of each pre-grown single strain were necessary to inoculate each 10-L

Table 1. Bacterial growth under different conditions of temperature and salinity and biosurfactant production

Strain	T: 30°C; NaCl: 2 (g/L)	T: 30°C; NaCl: 60 (g/L)	T: 30°C; NaCl: 120 (g/L)	T: 55°C; NaCl: 2 (g/L)	T: 55°C; NaCl: 60 (g/L)	T: 55°C; NaCl: 120 (g/L)	Biosurfactant production (using MDC and BAP)
Bacillus licheniformis STK08	+++	+++	++	++	++	+	+
Bacillus stearothermophilus STM04	+++	+++	++	++	++	+	+
Bacillus sphaericus STZ75	+++	+	-	+	-	-	+
Bacillus firmus STS84	+++	+	+	-	-	-	+

Growth at different conditions of temperature and salinity is considered: - if $OD_{600nm} \leq 0.1$; + for $0.1 \leq OD_{600nm} \leq 0.4$; ++ for $0.4 \leq OD_{600nm} \leq 0.6$; +++ for $OD_{600nm} \geq 0.7$.

bioreactor filled up to 8 L (final volume) and fed with a 5 L min^{-1} airflow. Each bioreactor was inoculated with 20% (v/v) of a single strain in pure culture, and the HCE was added at 1% (v/v). As seen earlier, for each essay, the effluent (HCE) represented the main percentage of COD and THC in the bioreactor (93.4 and 95%, respectively). The inoculum represented a minor percentage of COD and THC (3.7 and 4.0%, respectively), while CWW represented less than 3% of total COD and less than 1% of total THC of each in-bioreactor batch assay.

The initial THC concentration of each bioreactor batch assay was adjusted to 750 mg/l (CWW + HCE + inoculum) giving an initial COD mix concentration of about 3300 mg/l. Infeeds and outfeeds of each bioreactor were equipped with a 0.22 µm filter to maintain sterile conditions. Homogenization was ensured with a bubble diffuser and a magnetic stirrer. Biodegradation assays were carried out at ambient temperature (30°C). A negative in-bioreactor control assay with similar COD (2680 mg/l) but without inoculation was run in parallel with inoculated assays at the same final adjusted volume of 8 L.

Assessment of hydrocarbon degradation in batch assays

Each assay was analyzed periodically (at three-day intervals). HCE degradation was monitored by measuring THC and COD coupled with total suspended solids (TSS). Viable cell count was made on agar plates by decimal dilution, spreading 0.1 ml of samples taken from each bioreactor (final volume 8 L). Inoculated agar plates contained basal medium supplemented with HCE as sole carbon source (1% v/v).

COD and BOD_5 were measured by standard methods (McGraw, 1985). In the initial effluent sample, and in samples collected periodically from the bioreactor, THC was analyzed on a gas chromatography (GC) system equipped with a flame ionization detector (FID) according to French standard NF EN ISO 9377-2. Each sample was acidified (pH = 2) and centrifuged at 10000 rpm for 15 min with hexane (5:1). After a separation phase, the organic phase was cleaned with Florisil® to remove the most polar substances.

After adding the internal standard (IS) (5-α-androstane, 2 mg/l), 2 µl of each extracted sample was injected under constant flow and analyzed by GC (Perkin Elmer-Auto system XL) coupled with FID and a capillary column (length 30 m; ID 0.53 mm; thickness 3 µm). The carrier gas was helium (He) (12.5 psi, flow rate 35 ml min^{-1}). A control assay under the same conditions but without hydrocarbon was also run in parallel. The oven temperature program was as follows: initial temperature (50°C) with a 1 min hold, ramped up at 10°C min^{-1} up to 300°C with a 25 min hold. Peak areas of hydrocarbon components were quantified using Perkin Elmer software.

Statistical analysis

Statistical analysis was performed using the SPSS 10.0 software. Data underwent a one-way ANOVA test, and means were compared using Duncan's multiple range tests at 5% significance level.

RESULTS AND DISCUSSION

The analysis of the initial HCE demonstrated an important concentration of hydrocarbon (THC 28.8 $\times 10^3$ mg/l), DCO (96×10^3 mg/l) and BOD_5 (37.4×10^3 mg/l). These analyses showed also that the HCE is alkaline (pH: 12) and holding an important salt concentration (NaCl concentration: 90 g/l).

Assays realized on mineral agar plates supplemented with HCE as a sole carbon source allowed the isolation of 13 bacterial strains. Among these isolates, four showed a strong ability We first studied the morphological and biochemical characteristics of these isolated bacterial strains using the API 50CH gallery system (Biomérieux Corporation). The four most efficient strains were all identified as rod-shaped, Gram-positive, oxidase-positive and motile. The API system evaluated these four strains as affiliated to *Bacillus* genus, and tentatively identified them as *Bacillus licheniformis* STK08, *Bacillus stearothermophilus* STM04*, Bacillus sphaericus* STZ75 and *Bacillus firmus* STS84 (Table 1). Phylogenetic affiliation with DNA extraction and 16S rRNA gene amplification and sequencing gave similar affiliations for the four strains but the genera names of two strains were transferred as new combinations. *B. sphaericus* was transferred to *Lysinibacillus sphaericus* comb. nov. (strain STZ75) (Nazina et al*.,* 2001) and *B. stearothermophilus* was transferred to *Geobacillus stearothermophilus* (strain STM04) (Ahmed et al.,

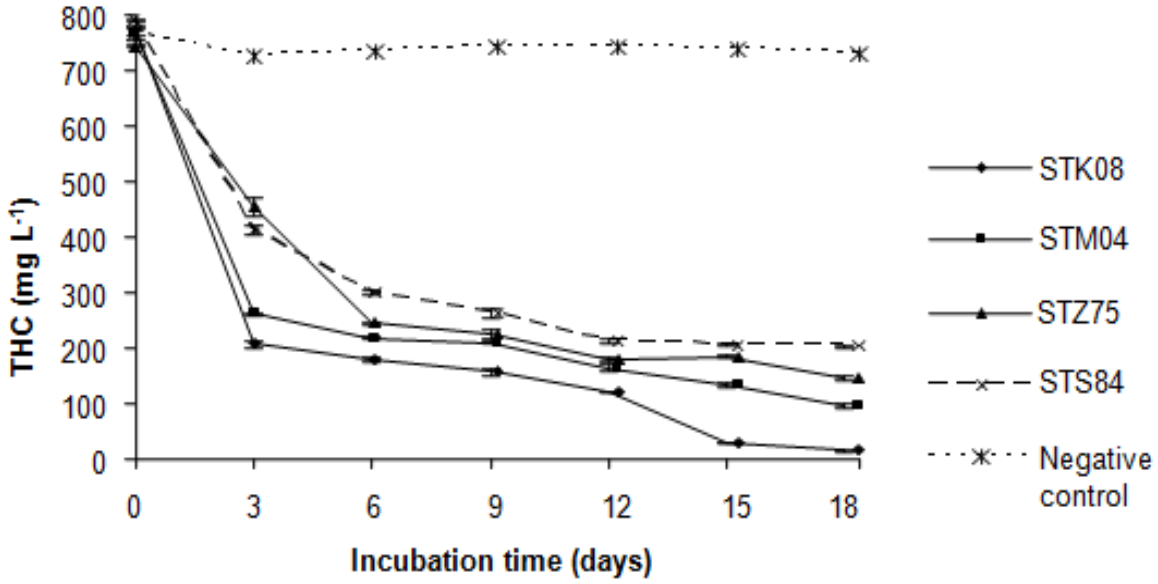

Figure 2. Total hydrocarbon concentration (THC) variation during the treatment time. The data are mean values (n = 3). The bars indicate the SDs (standard deviations). Some bars are smaller than the symbols. STK08: *Bacillus licheniformis;* STM04*: Geobacillus stearothermophilus*; STZ75: *Lysinibacillus sphaericus*; STS84*: Bacillus firmus.*

2007). These new names were not taken into consideration when the API system was utilized.

Bacterial growth under high salt concentrations and high temperature

The performances of these four strains at high salinity and high temperature were studied on basal medium supplemented with 1% (v/v) of HCE as sole carbon source (no CWW).

Bacterial growth results (Table 1) demonstrated that *B. licheniformis* STK08 and *G. stearothermophilus* STM04 strains could tolerate up to 120 g/L of NaCl and a temperature up to 55°C, but bacterial growth proved more efficient at 60 g/L of NaCl at 55°C. The *L. sphaericus* STZ75 strain was able to grow at 60 g/L of NaCl up to 30°C, and at high temperature (55°C) under low salinity (2 g/L NaCl), but was sensitive to high salinity combined with high temperature. The *B. firmus* STS84 strain did not grow at high temperature even with only 2 g/L of NaCl, but grew at 30°C and salt concentrations reaching 120 g/L.

Given that all four strains were capable of strong growth in both solid and liquid media at 30°C and 2 g/L of NaCl with hydrocarbon as sole carbon source, we tested the performance of each of these strains separately via bioreactor batch assays under these conditions.

Hydrocarbon biodegradation in bioreactor batch assays

THC variation in batch assays during the treatment period indicated that hydrocarbon concentration in each bioreactor inoculated with the four strains decreased within the 18 days of incubation (Figure 2). High-percentage decreases in THC were reached in 18 days, that is, 98% with *B. licheniformis* STK08*,* 87% with *G. stearothermophilus* STM04*,* 80% with *L. sphaericus* STZ75 and *72%* with *B. firmus* STS84 (Figure 2). This decrease was greater during the first three days of treatment, where THC removal was 73, 66, 39 and 47% with *B. licheniformis* STK08, *G. stearothermophilus* STM04*, L. sphaericus* STZ75 and *B. firmus* STS84, respectively. The THC values of the non-inoculated bioreactor remained fairly constant (Figure 2).

In addition, these four strains were all able to produce biosurfactants following the two methods used (MDC and BAP) (Table 1). COD also showed a decrease starting from the first day of treatment, and recorded COD removals were 78, 75, 78 and 82% (data not shown) with *B. licheniformis* STK08, *B. firmus* STS84, *G. stearothermophilus* STM04 and *L. sphaericus* STZ75, respectively. The main phase of both THC and COD removal by all four strains was achieved in the first three days of treatment. These percentages also demonstrated a high level of HCE biodegradability by each of these four strains performed separately.

In these bioreactor batch assays, the highest THC removal (98%) was recorded with *B. licheniformis* STK08. This strain showed a second marked decrease in THC between days 12 and 15 (Figure 2). Similarly, the colony forming unit (CFU) variation of this strain demonstrated that peak bacterial proliferation was also recorded within these first three days (3.2×10^9 CFU mL^{-1}), decreasing progressively thereafter. The TSS value recorded with

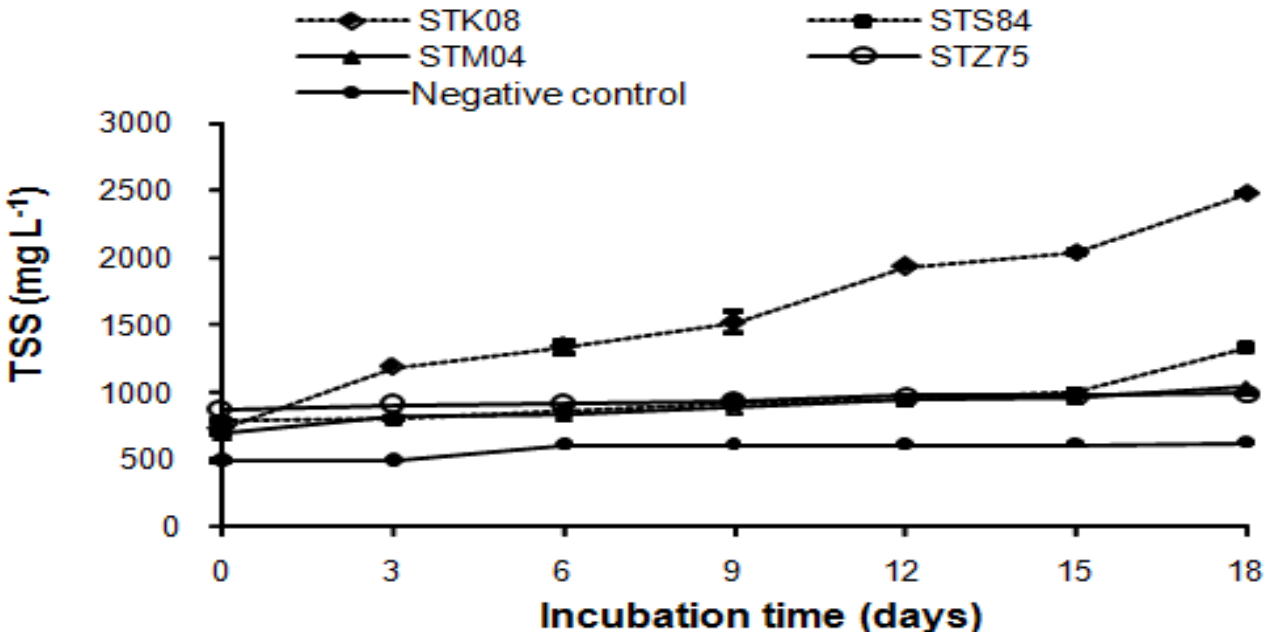

Figure 3. Variation in total suspended solids (TSS) variation during the treatment. The data are mean values (n = 3). The bars indicate the SDs (standard deviations). Some bars are smaller than the symbols. STK08*: Bacillus licheniformis;* STM04*: Geobacillus stearothermophilus;* STZ75*: Lysinibacillus sphaericus;* STS84*: Bacillus firmus.*

this strain, which was 735 mg/l on day 1, reached 1190 mg/l after three days and then doubled to 2476 mg/l between days 3 and 18 of treatment (Figure 3).

In the batch assay inoculated with *B. stearothermophilus* STM04, THC value decreased sharply (66%) during the first three days of treatment, then slowed progressively, reaching a final biodegradation rate of 87% after 18 days of treatment. Unlike strain STK08, strain STM04 showed a moderate increase in TSS during the treatment period, from 694 mg/l at day 1 to 955 mg/l at day 18 of treatment.

Figure 2 shows that THC decrease was weaker in assays carried out with *L. sphaericus* STZ75 and *B. firmus* STS84 strains, reaching about 145 and 203 mg/l, respectively, at day 18. Nevertheless, TSS values remained almost constant with these two strains, even after 18 days of treatment. Like strain STM04, strains STS84 and STZ75 showed a moderate increase in TSS, from 790 mg/l STS84 and 882 mg/l for STZ75 at day 1 to 1332 mg/l for STS84 and 990 mg/l for STZ75 at day 18 (Figure 3).

The different results under these conditions (30°C and 2 g/L NaCl) confirmed those found during isolation steps in agar plates, demonstrating that all four selected strains were able to degrade hydrocarbons contained in the HCE. The strong decrease in THC recorded mainly in the first days of treatment demonstrated that all these four strains were well acclimated to this HCE pollutant.

Time-course plots of THC variation revealed that the four strains selected in this work were able to degrade the HCE used as a main carbon source. Although all strains performed well on agar plates, there was clear variability between biodegradation rates of different strains in pure culture cultivated in liquid medium.

After 18 days of treatment, the highest biodegradation rate was recorded by *B. licheniformis* STK08 (98%) followed by *G. stearothermophilus* STM04 (87%) then *L. sphaericus* STZ75 (80%) and *B. firmus* STS84 (73%). The highest biodegradation rate for all the strains was essentially recorded in the first three days of treatment, as these highly adapted strains enabled fast biodegradation of the HCE. This fast decrease in both COD and THC, starting from day 1, disagrees with an efficient strain acclimation period in pre-culture. By contrast, Lotfabad and Gray (2002) showed that the presence of some PAH, such as phenanthrene, introduced a lag phase of 4.5 to 5 days in the degradation of other aromatic compounds.

The high THC removal recorded with our strain of *B. licheniformis* STK08 confirmed results obtained by Bayoumi et al. (2009) who report that *B. licheniformis* APIS473 has a high capacity to completely degrade anthracene and to reduce concentrations of some PAH compounds including acenaphtylene, acenaphtene, benzo(a)anthracene, benzo(b)fluoranthene, benzo(g,h,i)perylene and indeno(1,2,3-c,d)pyrene. Our study is the first to demonstrate that this species grow efficiently at high salt concentrations (up to 120 g/L), at high temperature (55°C) and under both conditions simultaneously.

As reported by Farinazleen et al. (2004), species *G. stearothermophilus* tolerates high temperatures (60°C)

and is able to degrade 80 to 89% of crude oil (5 g/L), but this study proves the capacity of *G. stearothermophilus* strain STM04 to also grow under high salinity (120 g/L) simultaneously with high temperature (55°C). Our results showed strong bacterial growth with *B. licheniformis* STK08 and *G. stearothermophilus* STM04 up to 55°C. High temperature is often a limiting factor for strains, but also has potentially positive effects since high temperatures decrease the viscosity of hydrocarbons and so increase their solubility and accessibility and favor their metabolism (Joseph and Colwell, 1990). Nevertheless, Sorkhoh et al. (1993) demonstrated that *B. stearothermophilus* grew best on pentadecane (C15), hexadecane (C16), heptadecane (C17) but it is less efficient for other hydrocarbon compounds with shorter and longer chains such as *n*-Alkanes, *n*-alkenes and aromatic hydrocarbons.

Although high salinity is known to decrease hydrocarbon degradation ability (Minai-Tehrani et al., 2006; Mille et al., 1991; Riis et al., 2003) by decreasing the number and types of substrates utilizable by microorganisms (Oren, 1988; Oren et al*.,* 1992; Riis et al., 2003), both *B. licheniformis* STK08 and *G. stearothermohilus* STM04 demonstrated a capacity to grow under high salt concentrations (120 g/L). Similarly, Obuekwe et al. (2005) reported that microorganisms cultured under stress conditions expended more energy on maintenance requirements. Therefore, provision of extra energy sources such as supplemental crude oil in the rich medium not only potentially satisfied the increased maintenance needs but also explained the increased biomass development observed.

B. firmus STS84 and *L. sphaericus* STZ75 grown on agar plates with HCE as sole carbon source appeared to show less competent biodegradation than when grown in liquid medium. The moderate biodegradation rate recorded at the end of the treatment period (73 and 80%, respectively) prompted us to conclude that these two strains were unable to degrade complex compounds present in the effluent. Mancera-Lopez et al. (2007) reported that a strain of *B. firmus* B05PR isolated from highly-polluted sites (60,000 and 500,000 mg of total petroleum hydrocarbon per kg of dry soil) was able to remove 90% of aliphatic hydrocarbons but only 80% of total hydrocarbons. These two species were previously known for their ability to degrade some hydrocarbon components and to grow at 60°C (Mohamed et al., 2006). In our study, the more moderate performance of *B. firmus* STS84 and *L. sphaericus* STZ75 compared with *G. stearothermophilus* STM04 and *B. licheniformis* STK08 might be due to difficulties in transporting a fraction of the hydrocarbon pollutant into cells. Haner et al. (1994) reported that a major fraction of dissolved organic carbon could not be directly transported into cells because of their high molecular weights or lack of transport systems.

Using the two qualitative methods (MDC and BAP), results demonstrated that the four *Bacillaceae* strains selected in this study were biosurfactant producers. This can explicate the THC degradation performance obtained with these strains in pure culture. Knowing that biodegradation depends strongly on hydrocarbon emulsion, the use of biosurfactant producer strains in bioremediation technology seems to offer more potential than chemical surfactant, due to their structural diversity, biodegradability and biocompatibility relative to synthetic surfactant (Abalos et al., 2004).

In conclusion, this study isolated four *Bacillaceae* strains that all demonstrated a high capacity to degrade heterogeneous HCE derived from a hydrocarbonic refining process. The high THC removal obtained in batch assays with these four strains indicated that clarified wastewater could be used as HCE diluent in the biotechnological process to degrade the main hydrocarbon compounds. In addition, the ability of these four strains, particularly *B. licheniformis* STK08 and *G. stearothermophilus* STM04 to grow at high temperature (55°C) and high salt concentrations (120 g/L) simultaneously and to produce biosurfactant, supports the use of these *Bacillaceae* strains for the bioremediation of hydrocarbon-polluted sites in terrestrial or marine environments, especially under high temperature or high salinity conditions.

ACKNOWLEDGEMENTS

The authors thank the Tunisian Ministry of Scientific Research, Technology and Competency Development for the financial support, the Chemical Analysis Laboratory at the Tunisian Society of Refining Industry (STIR), Bizerte, Tunisia. Special thanks to the laboratory of the Office National des Assainissements (ONAS), Bizerte, Tunisia, for their technical support.

REFERENCES

Abalos A, Vinas M, Sabaté J, Manresa MA, Solanas AM (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.

Ahmed I, Yokota A, Yamazoe A, Fujiwara T (2007). Proposal of *Lysinibacillus boronitolerans* gen. nov., sp. nov., and transfer of *Bacillus fusiformis* to *Lysinibacillus fusiformis* comb. nov. and *Bacillus sphaericus* to *Lysinibacillus sphaericus* comb. nov. Int. J. Syst. Evol. Microbiol. 57:1117-1125.

Alonso-Gutiérrez J, Lekunberri I, Teira E, Gasol JM, Figueras A, Novoa B (2009). Bacterioplankton composition of the coastal upwelling system of 'Ría de Vigo' NW Spain. FEMS. Microbiol. Ecol. 70:493-505.

Auffret M, Labbe D, Thouand G, Greer CW, Fayolle-Guichard F (2009). Degradation of a mixture of hydrocarbons, gasoline, and diesel oil additives by *Rhodococcus aetherivorans* and *Rhodococcus wratislaviensis*. Appl. Environ. Microbiol 75:7774-7782.

Bayoumi RA (2009). Bacterial bioremediation of polycyclic aromatic hydrocarbons in heavy oil contaminated soil. J. Appl. Sci. Res. 5:197-211.

Ben Hamed S, Rezgui R, Halleb A, Ghram A, Oueslati R, Labat M, Maaroufi A (2010). Efficiency of refinery sludge biodegradation using municipal wastewater and activated sludge and effect of hydrocarbon concentration on culturable bacteria community. Ann. Microbiol. 60:747-755.

Bodour AA, Miller-Maier R (1998). Application of a modified drop collapse technique for surfactant quantitation and screening of biosurfactant producing microorganisms. J. Microb. Methods 32:273-280.

Chavan A, Mukherji S (2008). Treatment of hydrocarbon-rich wastewater using oil degrading bacteria and phototrophic microorganisms in rotating biological contactor: effect of N:P ratio. J. Hazard Mater. 15:63-72.

Ciric L, Philp JC, Whiteley AS (2010). Hydrocarbon utilization within a diesel-degrading bacterial consortium. FEMS Microbiol. Lett. 303:116-122.

Das N, Chandran P (2011). Degradation of petroleum hydrocarbon contaminants: An Overview. Biotechnol. Res. Int. 2011:1-13.

Farinazleen MG, Abdul Rahman RN, Salleh AB, Basri M (2004). Biodegradation of hydrocarbons in soil by microbial consortium. Int. Biodeterior. Biodegrad. 54:61-67.

Haner A, Mason CA, Hamero G (1994). Death and lysis during aerobic thermophilic sludge treatment characterization of recalcitrant products. Water Res. 28:863-869.

Hedi A, Sadfi N, Fardeau ML, Rebib H, Cayol JL, Ollivier B, Boudabous A (2009). Studies on the biodiversity of halophilic microorganisms isolated from El-Djerid Salt Lake (Tunisia) under aerobic conditions. Int. J. Microbiol. pp. 1-17.

Hedlund BP, Staley JT (2001). *Vibrio cyclotrophicus* sp. nov., a polycyclic aromatic hydrocarbon (PAH)-degrading marine bacterium. Int. J. Syst. Evol. Microbiol. 51:61-66.

Hui LI, Zhang Y, Kravchenko I, Hui X, Zhang C (2007). Dynamic changes in microbial activity and community structure during biodegradation of petroleum compounds : A laboratory experiment. J. Environ. Sci-China. 19:1003-1013.

Joseph GL, Colwell RR (1990). Microbial degradation of hydrocarbons in the environment. Microbiol. Rev. 9:305-315.

Lotfabad S, Gray M (2002). Kinetics of biodegradation of mixtures of polycyclic aromatic hydrocarbons. Appl. Microbiol. Biotechnol. 60:361-366.

Malik ZA, Ahmed S (2012). Degradation of petroleum hydrocarbons by oil field isolated bacterial consortium. Afr. J. Biotechnol. 11:650-658.

Mancera-Lopez ME, Rodriguez-Casasola MT, Rios-Leal E, Esparza-Garcia F, Chavez-Go♌mez B, Rodriguez-Vazquez R, Barrera-Cortés J (2007). Fungi and bacteria isolated from two highly polluted soils for hydrocarbon degradation. Acta. Chim. Slov. 54:201-209.

Margesin R, Schinner F (2001). Biodegradation and bioremediation of hydrocarbons in extreme environments. Appl. Microbiol. Biotechnol. 56:650-663.

Mc Graw Hill (1985). Standard methods for the examination of water and wastewater. 16th ed., American Public Health Association (APHA). New York, USA.

Mille G, Almallah M, Bianchi M, Van Wambeke F, Bertrand JC (1991). Effect of salinity on petroleum biodegradation. Fresen. J. Anal. Chem. 339:788-791.

Minai-Tehrani D, Herfatmanesh A, Azari-dekhordi F, Minooi S (2006). Effect of salinity on biodegradation of aliphatic fractions of crude oil in soil. Pak. J. Biol. Sci. 9:1531-1535.

Mohamed ME, Al-Dousary M, Hamzah RY, Fuchs G (2006). Isolation and characterization of indigenous thermophilic bacteria active in natural attenuation of bio-hazardous petrochemical pollutants. Int. Biodeterior. Biodegrad. 58:213-223.

Mrayyan B, Battikhi MN (2005). Biodegradation of total organic carbon (TOC) in Jordanian petroleum sludge. J. Hazard. Mater. 120:127-134.

Nazina TN, Tourova TP, Poltaraus AB, Novikova EV, Grigoryan AA, Ivanova AE, Lysenko AM, Petrunyaka VV, Osipov GA, Belyaev SS, Ivanov MV (2001). Taxonomic study of aerobic thermophilic bacilli: descriptions of *Geobacillus subterraneus* gen. nov., sp. nov. and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus*, *Bacillus thermocatenulatus*, *Bacillus thermoleovorans*, *Bacillus kaustophilus*, *Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus*, *G. thermocatenulatus*, *G. thermoleovorans*, *G. kaustophilus*, *G. thermoglucosidasius* and *G. thermodenitrificans*. Int. J. Syst. Evol. Microbiol. 51:433-446.

Obuekwe CO, Badrudeen AM, Al-Saleh E, Mulder JL (2005). Growth and hydrocarbon degradation by three desert fungi under conditions of simultaneous temperature and salt stress. Int. Biodeterior. Biodegrad. 56:197-205.

Oren A (1988). Anaerobic degradation of organic compounds at high salt concentration. Antoine van Leeuwenhoek 54:267-277.

Oren A, Gurevich P, Azachi M, Henis Y (1992). Microbial degradation of pollutants at high salt concentrations. Biodegradation 3:387-398.

Rahman KS, Rahman T, Lakshmanaperumalsamy J, Banat P (2002). Towards efficient crude oil degradation by a mixed bacterial consortium. Bioresour. Technol. 85:257-261.

Riis V, Kleinsteuber S, Babel W (2003). Influence of high salinities on the degradation of diesel fuel by bacterial consortia. Can. J. Microbiol. 49:713-721.

Siegmund I, Wagner F (1991). New method for detecting rhamnolipids excreted by *Pseudomonas* spp. during growth on mineral agar. Biotechnol. Tech. 5:265-268.

Sorkhoh NA, Ibrahim AS, Ghannoum MA, Radwan SS (1993). High-temperature hydrocarbon degradation by *Bacillus stearothermophilus* from oil-polluted Kuwaiti desert. Appl. Microbiol. Biotechnol. 39:123-126.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997). The CLUSTALX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res**.** 24:4876-4882.

Tomei MC, Annesini MC, Luberti R, Cento G, Senia A (2003). Kinetics of 4-nitrophenol biodegradation in a sequencing batch reactor. Water Res. 37:3803-3814.

Tzarkova EK, Groudeva VI (2000). Bioremediation of petroleum polluted soils by adsorptive immobilized *Corynebacterium* sp. RB-96. Biotechnol. Biotechnol. Equip. 14:72-75.

Van Hamme JD, Ward OP (2001). Volatile hydrocarbon biodegradation by a mixed-bacterial culture during growth on crude oil. J. Ind. Microbiol. Biotechnol. 26:356-362.

Viñas M, Sabaté J, Espuny MJ, Solanas A M (2005). Bacterial Community Dynamics and Polycyclic Aromatic Hydrocarbon Degradation during Bioremediation of Heavily Creosote-Contaminated Soil. Appl. Environ. Microbiol. 71:7008-7018.

Winker S, Woese CR (1991). A definition of the domains Archaea, Bacteria and Eucarya in terms of small subunit ribosomal rRNA characteristics. Syst. Appl. Microbiol. 13:161-165.

Zhuang WQ, Tay JH, Maszenan AM, Kumholz LR, Tay, STL (2003). Importance of Gram positive naphtalène-degrading bacteria in oil-contaminated tropical marine sediments. Lett. Appl. Microbiol. 36:251-257.