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*Research Article*

# Degradation of Asphaltenic Fraction by Locally Isolated Halotolerant Bacterial Strains

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Three halotolerant bacterial species were isolated from locally oil-polluted water sample for their ability to utilize asphaltene (Asph) fraction as sole carbon and energy source. These bacteria degrade 83–96% of 2500 mg/L asphaltene within 21 d at 30°C and pH7. They were identified as *Bacillus* sp. Asph1, *Pseudomonas aeruginosa* Asph2, and *Micrococcus* sp. Asph3. A statistically significant difference at 95% confidence level for cell growth and percentage biodegradation (%BD) was observed in all biotreatment flasks relative to noninoculated (–ve control) flasks. Regression analysis estimated a quadratic polynomial equation for Asph biodegradation as a function of cell growth. Gel permeation chromatographic (GPC) and Fourier transform infrared (FT-IR) analysis revealed decrease in Asph average molecular weights and significant alternations in functional groups after biotreatment, respectively.

## 1. Introduction

Petroleum is a complex mixture of many thousands of compounds mainly consisting of carbon and hydrogen. These can be divided into four major groups: alkanes, aromatics, resins, and asphaltenes. In general, the alkane fraction is the most biodegradable. The aromatic compounds, especially the polycyclic aromatic hydrocarbons (PAHs), are of intermediate biodegradability whereas the polar fractions (i.e., resins and asphaltenes) are resistant to biological degradation [1, 2].

Asphaltenes (Asph) are petroleum fraction containing nitrogen, sulfur, and oxygen. Asph molecular weights can range between 600 and 2,000,000. Their molecular structures are extremely complex; several aromatic, acyclic, and heterocyclic structures are bonded by aliphatic hydrocarbons [1, 3–5]. These complex molecular structures of asphaltenes make them resistant to biodegradation and cause their accumulation in ecosystems where petroleum and its refining byproducts are spilled either in accidental or purposeful ways [6, 7].

Microbial biodegradation processes have long been known to be important in the remediation of polluted habitats through the destruction of large numbers of compounds [8–12]. It has been reported that microorganisms, exposed to asphaltenes during long periods, could have the ability to degrade these compounds [1, 13].

In Egypt, most bioremediation efforts have been focused on the degradation of aliphatic and aromatic fractions rather than the heavy fractions, that is, resins and asphaltenes pollutants [10–12, 14, 15].

In this study, halotolerant bacterial isolates from oil-polluted water sample were obtained and identified by enrichment cultures, using the recalcitrant asphaltenic fraction as sole carbon and energy source. The bacterial growing capabilities on Asph were evaluated along with their degradation effect.

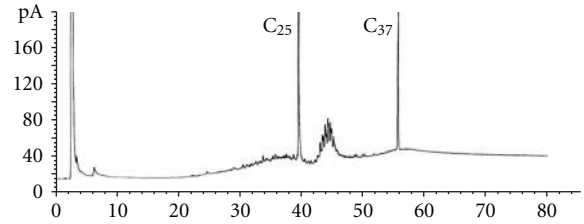


Figure 1: GC chromatogram of the extracted hydrocarbons from the collected water sample.

## 2. Materials and Methods

*2.1. Materials*

(1) Asphaltene used for enrichment and isolation of asphaltene degrading bacteria (ADB) was extracted from Belayim Mix crude oil. IP 143 method was used in the deasphalting of crude oil.

(2) Tetrahydrofuran (THF) used for gel permeation chromatographic (GPC) analysis was of HPLC grade.

(3) All other chemicals were of analytical grades.

Basal salts medium (BSM) used for enrichment, isolation of bacteria capable of degrading asphaltene (ADB), and studying their degradation efficiencies towards asphaltene, was prepared according to El-Gendy [16]. Asph dissolved in methanol were added to sterile BSM in a final concentration of 2500 mg/L under aseptic conditions. Isolation, purification, and monitoring of ADB (cells/mL) were done on BSM/Asph/agar plates where BSM free of any carbon source with 2% agar was sterilized at 121°C for 15 min, cooled, poured in plates, left to solidify, and then sprayed under aseptic conditions with 500 $\mu$L of 2500 mg/L Asph dissolved in methanol to form thin film.

Trypton glucose yeast extract medium (TGY) used for obtaining biomass and maintenance of bacterial isolates, was prepared according to Benson [17]. TGY/agar plates were used for monitoring the total viable count, TCFU (cells/mL).

Water sample used for isolating ADB was collected from the API separator of Sumid Corporation, Suez terminal, Egypt. Water pH and temperature were determined immediately after sampling using portable pH-meter with temperature sensor (Jenway, model 3071). Samples were then transferred in ice box to the laboratory. Water salinity was determined by ion chromatography, IC (Dionex, 600) according to the method reported by El-Gendy [16]. The total petroleum hydrocarbons (TPHs) were extracted from the water sample according to the method described by Moustafa [18] and the TPH concentration in the extract was determined gravimetrically according to El-Tokhi and Moustafa [19]. To identify the source of pollution and degree of weathering, the extracted TPH was analyzed using gas chromatography-flame ionization detector GC/FID (Agilent, 6890) equipped with capillary column HP-1 (100% methyl silicon siloxane, 30 m $\times$ 0.25 mm ID $\times$ 0.25 $\mu$m film). Oven temperature: 80–260°C (3°C/min), detector temperature: 325°C, injector temperature: 300°C, carrier gas: $N_2$ (2 mL/min), and sample size: 0.5 $\mu$L. Identification of n-paraffins peaks was established by chromatographing a reference mixture of n-paraffins of known composition under the same operating conditions.

*2.2. Fourier Transform Infrared (FT-IR) Spectroscopy.* Analytical FT-IR Perkin Elmer, instrument was used to identify and determine the asphaltene structures before and after biotreatment. The asphaltenes were measured as KBr discs by mixing the sample with KBr (spectroscopic grade) where the solid samples were transferred into the cell after melting using an infrared lamp. The spectra of all studied samples were measured in the range of 400–4000 $cm^{-1}$ with suitable scan resolution 4 $cm^{-1}$ and scan rate 16 cm/min.

*2.3. Gel Permeation Chromatographic (GPC) Analysis.* Analytical GPC waters 600 E was used for the determination of average molecular weights of the asphaltenes before and after biotreatment, using the following conditions:

Column: set of styragel columns; HR4, HR5E, 7.8 $\times$ 300 mm.

Solvent: tetrahydrofurane (THF), HPLC grade.

Flow rate: 1 mL/min.

Time: 60 min.

Detector: refractive index, model Waters 4110.

*2.4. Enriching, Detecting, and Assessing the Size of Both Indigenous Culturable Bacterial Population (TCFU) and Asph Degrading Bacteria (ADB) in the Collected Oil-Polluted Water Sample.* 10 mL of the water sample was added to 100 mL sterile saline solution (8.5 g NaCl/L distilled water) in 500 mL conical flask, under aseptic conditions, then incubated at 30°C for 1 h in a rotatory shaking incubator (150 rpm). Serial dilutions ($10^{-1}$–$10^{-7}$) of the suspension were inoculated on TGY/agar and BSM/Asph/agar plates and then incubated at 30°C for 48 h and 168 h, to enumerate TCFU and ADB, respectively, before enrichment.

Series of 500 mL Erlenmeyer conical flasks with 100 mL of enrichment (En) medium (BSM containing Asph in a final concentration of 2500 mg/L) were inoculated with 10 mL aliquots of the above bacterial suspension. The cultures were incubated at 30°C for 7 d in a rotatory shaking incubator (150 rpm). Then 10 mL of the bacterial suspension from each flask was transferred separately to other flasks each containing fresh 100 mL En-medium and the procedure

Table 1: TCFU and ADB before and after enrichment.

| Source of isolation | TCFU/mL | ADB/mL | ADB% |
|---|---|---|---|
| Before enrichment | $3 \times 10^5$ | $2 \times 10^3$ | 0.67 |
| After enrichment | | | |
| First subculturing | $2 \times 10^{12}$ | $8 \times 10^6$ | $4 \times 10^{-4}$ |
| Second subculturing | $2.8 \times 10^{11}$ | $9.7 \times 10^6$ | $3.5 \times 10^{-3}$ |
| Third subculturing | $1.5 \times 10^{10}$ | $3.6 \times 10^7$ | 0.24 |
| Fourth subculturing | $2 \times 10^9$ | $5.3 \times 10^8$ | 26.5 |

was repeated, in a total of three transfers. Serial dilutions ($10^{-1}$–$10^{-13}$) of each transfer were inoculated on TGY/agar and BSM/Asph/agar plates and then incubated at 30°C for 48 h and 168 h to enumerate TCFU and ADB, respectively. Separated colonies from BSM/Asph/plates were picked and purified by subculturing on fresh BSM/Asph plates. The purification step was successively repeated for four times to assure the potency of the selected isolates for Asph biodegradation. All steps were in duplicates.

*2.5. Biodiversity and Screening of Asphaltene Degrading Bacterial Isolates.* All isolates before and after enrichment were differentiated morphologically (size, color, shape, elevation, margin, surface, and transparency) and microscopically (Gram staining) to eliminate apparently similar strains. The first selection of the most promising ADB was based on their presence after the fourth En-cycle. The selected most promising bacterial isolates were preliminary characterized from their morphological and biochemical properties according to Bergey's Manual of Systematic Bacteriology [20, 21] and Bergey's Manual of Determinative Bacteriology [22]. The selected ADB were preserved in microtubes containing (1 : 1 v/v) glycerol : pure bacteria in TGY broth media at −20°C.

*2.6. Asphaltenes Biodegradation Assay in Liquid Culture.* Enrichment culturing selected only those indigenous microorganisms that have been especially acclimated to asphaltenes. The second selection was based on studying the ability of the preselected bacterial isolates to degrade asphaltenes in liquid cultures. Each bacterial isolate was incubated at 30°C in TGY medium for 24 h in a shaking incubator (150 rpm), cells were harvested by centrifugation at 3000 rpm for 15 min, supernatant was decanted and pellets were washed twice with sterile BSM and resuspended in fresh BSM free from any C-Source. Washed cells were inoculated under aseptic conditions into sterile 250 mL Erlenmeyer conical flasks containing 50 mL BSM of pH7, with Asph in a final concentration of 2500 mg/L. The inocula were adjusted so that the initial viable count was ~$10^5$ cells/mL. The cultures were incubated at 30°C for 21 d, in a shaking incubator (150 rpm). The growth was monitored at prescribed time intervals by total viable count (cells/mL) on TGY plates. Noninoculated flasks subjected to the above conditions were used as negative controls. pH of cultures was also monitored using pH-meter (Digimed, DM-23). After the biodegradation phase, asphaltenic fractions were subjected to liquid-liquid extraction using 50 mL of dichloromethane and the total contents of the culture media. Organic phases were then separated, dried to constant weight, and degradation was determined by weight difference to calculate the percentage biodegradation (%BD). Extracted Asph before and after biodegradation were subjected to GPC and FTIR analysis to determine the changes in Asph average molecular weights and changes in functional groups, respectively. All steps were in duplicates.

*2.7. Identification of the Selected Potent Bacterial Strains.* This was done using the 16S ribosomal DNA (rDNA) amplification and sequencing, which was determined by direct sequencing of polymerase-chain-reaction (PCR-) amplified 16S rDNA. Sequencing was done directly by MicroSeq500 V 5.3 kit (applied biosystems). The electrophoresis and data gathering were done automatically by ABI prism 310 genetic analyzer (Applied Biosystems/Hitache), in National care for Scientific Research (NCSR) Company, Cairo, Egypt. Blast program (http://www.ncbi.nlm.nih.gov/blast/) was used to assess the DNA similarities.

*2.8. Statistical Analysis.* This was performed for cell growth and %BD results using MATLAB version 7.0.0. A multiple comparison test of means was done to determine the significance of Asph biodegradation process using the obtained bacterial isolates, relative to the −ve control flasks. Also regression analysis was performed to estimate a correlation for Asph biodegradation (%BD) as a function of cell growth (logarithm of cells/mL).

## 3. Results and Discussion

*3.1. Physicochemical Characteristics and Hydrocarbons Assessment of the Collected Water Sample.* The physical and chemical analysis of the water sample revealed that the pH value was (7.86), its temperature immediately after sampling was 27.7°C, and its salinity was 42,000 mg/L. In the preparation of En-medium, 42 g/L NaCl was added before sterilization to the constituents of the En-medium to mimic the salinity of the collected water sample.

Characterization of pollutants contamination is environmentally and microbiologically significant in terms of assessing pollution status. Analysis of TPH from water sample revealed that the TPH concentration was 40 mg/L. It can be considered as oil-polluted water according to the Egyptian Environment Law Number 4 of 1994.

The gas chromatogram of the extracted oil is shown in Figure 1. By studying the general characteristic features, it is clear that the sample is contaminated by mixed petrogenic (the presence of unresolved complex mixture UCM hump) with biogenic hydrocarbons (the presence of $nC_{25}$ and $nC_{37}$ as predominant peaks). It is also characterized by a profile typical to highly weathered crude oil. Its n-paraffin peaks are shown to be greatly affected by weathering processes (natural and biochemical weathering). The unusually high

Table 2: Biodiversity on BSM-Asph plates before and after enrichment.

| Isolates | Before En | After En | | | |
|---|---|---|---|---|---|
| | | 1st En | 2nd En | 3rd En | 4th En |
| Asph1<br>Gram +ve bacillus shape, straight rods with rounded ends, opaque, smooth, convex, entire, and mutt creamy white | (+++) | (+++) | (+++) | (+++) | (++) |
| Asph2<br>Gram −ve rod-shaped, straight or slightly curved rods, irregular margin, opaque, smooth, low convex, entire, and shiny beige | (+++) | (+++) | (+++) | (++++) | (++++) |
| Asph3<br>Gram +ve cocci shape, small, circular, opaque, smooth, convex, entire, and yellow canary color | (++) | (++) | (++) | (++) | (+++) |
| Asph4<br>Gram +ve short-rod-shaped, small, straight, or slightly curved rods, opaque, smooth, convex, entire, and orange color | (+) | (+) | (+) | (−) | (−) |

CPI value (16.00) may be due to the addition of the biogenic hydrocarbons which were obvious in the two predominant odd peaks ($nC_{25}$ and $nC_{37}$) that disturb the chromatographic profile. Biogenic hydrocarbons could be derived from zooplankton, phytoplankton, benthic algae, and/or microorganisms [23–25]. From the GC chromatographic calculations, the percentage of total resolvable peaks TRP (iso and n-alkanes) is 21% and the percentage of UCM (naphthenes and aromatics) is 79%. The high UCM value and low TRP values are indicative of chronic degraded and highly weathered petroleum contamination. The two isoprenoids, pristane Pr (2, 6, 10, 14-tetramethylpentadecane) and phytane Ph (2, 6, 10, 14-tetramethylhexadecane), are not detected in the sample indicating also high degree of weathering. Similar observation was reported by Wang and Fingas [26], Medeiros and Caruso Bícego [27], and El-Gendy and Moustafa [25].

*3.2. Enumeration of the Total Colony-Forming Units and Asphaltene Degrading Bacteria in the Collected Water Sample.* In the present study, the total indigenous bacteria (TCFU) as well as the culturable bacteria able to grow on Asph as a sole source of carbon and energy (ADB) were enumerated on TGY plates and BSM-Asph plates, respectively, directly after collection and after four weeks of enrichment on enrichment (En) medium (Table 1).

Total viable count on TGY plates (TCFU) and the count of ADB on BSM/Asph plates directly after collection showed good microbial population of $3 \times 10^5$ cells/mL and $2 \times 10^3$ cells/mL, respectively.

According to Madigan et al. [28], contaminants are often potential energy sources for bacteria, and according to Ilyina et al. [29] and Dong et al. [30], microorganisms survive in contaminated habitats because they are metabolically capable of utilizing its resources and can occupy a suitable niche.

It is obvious from the data listed in Table 1 that TCFU/mL and ADB/mL after enrichment are higher than those before enrichment. But TCFU/mL decreases with successive enrichments, while ADB/mL increases. This may be due to the adaptation of indigenous microbial population in the sample to the Asph used for enrichment. This also indicates that not all indigenous bacterial population can tolerate successive enrichment on Asph as sole carbon and energy source. The presence of yeast extract in the En-media could have also accelerated the adaptation of strain(s) with high rate and capacity of degradation [31].

Konishi et al. [32], Kishimoto et al. [33], El-Gendy [16], and Gaskin and Bentham [34] reported similar observations during isolation of different biodegrading microorganisms using different substrates for enrichment. Chen et al. [35] reported that repeated exposures to a compound usually increase the adaptative capabilities of microorganisms, their enzymes activity, and their metabolic rate with a new exposure to the compound.

Youssef et al. [36] reported that the ratio of hydrocarbons degrading bacteria to total bacterial count appears to be more consistent and valid indicator than the absolute number of bacterial counts.

The frequency of occurrence of ADB relative to the total bacterial population (TCFU), ADB%, is given in Table 1. Directly after water sampling, it recorded 0.67% then sharply decreased after first En-cycle, recording $4 \times 10^{-4}$%. It was then increased with successive En-cycles, recording 26.5%, after the fourth En-cycle. These results coincide with Atlas [37] and Adebusoye et al. [38], who reported that populations of hydrocarbon degraders are normally less than 1% of the total microbial communities but successive exposure to oil pollutants increases these hydrocarbon degrading populations, reaching to more than 10% of the community.

Indigenous bacteria isolated in this study were selected by enrichment culturing technique. The results confirmed the fact that repeated exposure to petroleum products usually increases the adaptive capabilities of the microorganisms and though increases the rate of degradation with a new exposure. There is a high degree of variability in the ability of

Table 3: Biochemical characteristics of the three most promising bacterial isolates.

| Parameters | Asph1 | Asph2 | Asph3 |
|---|---|---|---|
| Gram staining | + | − | + |
| Spore former | + | − | − |
| motility | + | + | − |
| Growth aerobically | + | + | + |
| Growth anaerobically | − | − | − |
| Growth at 20°C | + | + | + |
| Growth at 30°C | + | + | + |
| Growth at 40°C | + | + | + |
| Growth at 45°C | + | + | + |
| Growth at 50°C | + | + | − |
| Growth at 55°C | − | − | − |
| NaCl 0% | + | + | + |
| NaCl 5% | + | + | + |
| NaCl 7.5% | + | + | + |
| NaCl 10% | − | + | + |
| NaCl 12.5% | − | − | Doubtful |
| NaCl 15% | − | − | − |
| Biochemical tests | | | |
| 3% KOH | − | + | − |
| Catalase | + | + | + |
| Oxidase | + | + | − |
| Relation to oxygen | + | + | + |
| Sodium malonate utilization | − | + | − |
| Aesculin hydrolysis | + | Doubtful | − |
| KCN resistance | + | + | Doubtful |
| Nitrate reduction | + | CR | + |
| Nitrite reduction | + | CR | − |
| Methyl red (MR) test | − | − | − |
| Voges-Proskauer (V.P.) test | − | − | − |
| Oxidation-fermentation (O/F) test | +/+ | +/+ | −/+ |
| Indole production | − | − | − |
| Citrate utilization | + | Doubtful | − |
| $H_2S$ production | − | − | − |
| Levan formation | + | − | + |
| Carbohydrate fermentation | | | |
| D (−) Glucose | (+ve) acid production | (+ve) acid production | (+ve) acid production |
| D (+) Arabinose | − | (+ve) acid production | − |
| Cellobiose | (+ve) acid production | − | − |
| Dextrine | (+ve) acid production | − | − |
| D (−) Fructose | (+ve) acid production | − | (+ve) acid production |
| D (−) Galactose | − | (+ve) acid production | − |
| D (−) Lactose | − | − | − |
| Maltose | (+ve) acid production | − | (+ve) acid production |
| D (−) Mannitol | − | − | (+ve) acid production |
| D (+) Mannose | (+ve) acid production | (+ve) acid production | − |
| D (−) Raibose | (+ve) acid production | (+ve) acid production | − |
| Rhamnose | − | − | − |
| Starch | (+ve) acid production | − | − |
| Sorbitol | − | − | − |
| D (+) Sucrose | (+ve) acid production | − | − |

Table 3: Continued.

| Parameters | Asph1 | Asph2 | Asph3 |
|---|---|---|---|
| D (+) Trehalose | (+ve) acid production | − | (+ve) acid production |
| D (+) Xylose | − | (+ve) acid production | − |
| D (+) Raffinose | − | − | − |
| Inulin | − | − | − |
| Extracellular enzyme production | | | |
| Amylase | + | − | Doubtful |
| Gelatinase | + | + | − |
| Lipase | + | − | − |
| Pectinase | + | − | − |
| Urease | + | + | + |
| Cellulase | + | − | + |
| Growth on blood | + | + | + |
| Blood haemolysis | Beta haemolysis | Beta haemolysis | Gamma haemolysis |
| Growth on agar medium | | | |
| King's (A) agar | − | + | − |
| King's (B+) agar | − | + | − |
| Mannitol salt agar (MSA) | + | − | + |
| MacConkey agar (MA) | − | + | − |

+ve: positive; −ve: negative; CR: complete reduction.

a microbial community to adapt to compounds. Adaptation depends on many factors, such as the induction or derepression of enzymes specific for the degradation pathways of a particular compound or an adaptation of existing catabolic enzymes to the degradation of novel compounds. The larger and more complex the structure of hydrocarbons, the more slowly their oxidation is. This may depend upon the type of organism involved and the medium, in which it was developed. For this reason, the longer enrichment period as well as fresh medium and decantation of toxic cometabolites apparently enhances the proliferation of bacteria capable of utilizing more complex compounds in the investigated water sample.

According to MacNaughton et al. [39], biodiversity may be influenced by the complexity of chemical mixtures present and the length of time the populations have been exposed to the contaminants.

Table 2 illustrates the morphological characteristics and diversity of ADB in the water sample before and after enrichment.

Results from the visual and microscopical examination demonstrate that successive enrichment significantly affects not only the concentration of microbial population (Table 1) but its abundance as well (Table 2).

One of the objectives of this study is to isolate as many culturable strains as possible in order to determine their hydrocarbon biodegradation potential in standardized culture conditions. For this reason, a first screening of strains was done after Gram staining and microscopic examinations for bacteria to eliminate apparently similar strains.

It was found that only four bacterial strains were found in the collected water sample: Gram +ve Asph1, Asph3, Asph4, and a Gram –ve Asph2. Asph1 and Asph2 were the most abundant isolates in the water sample before enrichment followed by Asph3 and Asph4 in a decreasing order. Asph4 was lost in the third En-cycle. After successive En cycles, Asph2 expressed the highest abundance on BSM-Asph plates which may indicate its high biodegradation potential followed by Asph3 and Asph1 in a decreasing order. These three bacterial isolates may have enzymatic system characterized by good utilization of Asph as sole source of carbon and energy due to their adaptation to this complex compound, obtained overtime through the continuous exposure to Asph in successive En-cycles.

The three bacterial isolates, Asph1, Asph2, and Asph3, were further streaked on fresh BSM-Asph plates for four successive subculturing to assure their potency for asphaltene biodegradation. These three most promising bacterial isolates Asph1, Asph2, and Asph3 were being preliminary characterized from their morphological and biochemical characteristics (Table 3).

They are characterized by being nonspore former except Asph1 which is spore former, exclusively aerobic, commonly catalase positive, oxidase positive except Asph3 which is oxidase negative. They can be considered halotolerant bacteria as they can survive at high concentrations of NaCl; Asph2 and Asph3 tolerate up to 10% NaCl while Asph1 can tolerate up to 7.5% NaCl. All of the three isolates can tolerate high temperature and can be considered thermophilic. Asph1 and Asph2 tolerate up to 50°C while Asph3 tolerate up to 45°C. The data illustrated in Table 3 suggest that the three bacterial

Table 4: Growth and degradation efficiencies of different bacterial isolates on Asph fraction.

| | Parameters | | | | | |
|---|---|---|---|---|---|---|
| | 7 d | | 14 d | | 21 d | |
| | %BD | Cells/mL | %BD | Cells/mL | %BD | Cells/mL |
| Negative control | 0.15 | Nil | 0.4 | Nil | 0.5 | Nil |
| Asph1 | 50.98 | $9 \times 10^7$ | 82.2 | $2.5 \times 10^9$ | 82.97 | $1 \times 10^9$ |
| Asph2 | 82.97 | $5 \times 10^8$ | 89.3 | $9.5 \times 10^{11}$ | 95.6 | $3.1 \times 10^{13}$ |
| Asph3 | 18.54 | $8 \times 10^7$ | 68.3 | $9 \times 10^8$ | 87.94 | $4 \times 10^9$ |

Table 5: Regression parameters and correlation coefficients for the three treatments.

| | $a$ | $b$ | $c$ | $R^2$ |
|---|---|---|---|---|
| Asph1 | −22.52 | 412.4 | −1804 | 1 |
| Asph2 | 0.47 | −7.70 | 114.7 | 1 |
| Asph3 | −10.03 | 216.4 | −1065 | 1 |

isolates Asph1, Asph2, and Asph3 are members of the genera *Bacillus*, *Pseudomonas,* and *Micrococcus,* respectively.

*3.3. Studies on the Biodegradation of Asphaltenes in Liquid Cultures.* First selection of the most promising ADB (i.e., Asph1, Asph2, and Asph3) was based on their appearance after the fourth En cycle and their potency after the fourth purification step. Even though enrichment culturing selected only those indigenous microorganisms that have been especially acclimated to asphaltene, it was necessary to characterize the biodegradation potential for each individual isolate towards Asph in liquid culture.

In this study, the three presented bacterial isolates *Bacillus* sp. Asph1, *Pseudomonas* sp. Asph2, and *Micrococcus* sp. Asph3 were assayed for their ability to grow on asphaltenic fractions of petroleum in liquid culture.

Results listed in Table 4 reveal that all tested isolates grow very well on asphaltene as sole carbon and energy source but with different efficiencies. Similar observation was reported by Connan [40], Pineda et al. [1], and Tavassoli et al. [5].

*Bacillus* sp. Asph1 recorded continuous increment of growth, reaching $9 \times 10^7$ and $2.5 \times 10^9$ cells/mL after 7 and 14 d of incubation, respectively, and it remains nearly sustained thereafter till the end of incubation period recording $1 \times 10^9$ cells/mL after 21 d. *Pseudomonas* sp. Asph2 expressed the highest growth, where it showed sharp increase after 7 d of incubation recording $5 \times 10^8$ cells/mL with a continuous increase thereafter, recorded $9.5 \times 10^{11}$ and $3.1 \times 10^{13}$ cells/mL after 14 and 21 d of incubation, respectively. *Micrococcus* sp. Asph3 expressed the lowest growth during the first 14 d of incubation, recording $8 \times 10^7$ and $9 \times 10^8$ cells/mL after 7 and 14 d of incubation, respectively, while it increases thereafter reaching $4 \times 10^9$ cell/mL at the end of incubation period of 21 d. A statistically significant difference at ($\alpha = 0.5$) 95% confidence level was found for cell growth (cells/mL) in all biotreatment flasks relative to the –ve control flasks ($p = 3.4e - 5$).

There was no significant loss in Asph concentration in the negative control flasks, recording ≈0.5% decrease after 21 d of incubation.

There was also no significant change in pH values that occurred in all cultures (ranging between pH 6.96–pH 6.99). The relatively high contents of mono- and dibasic phosphates in the BSM provided good pH buffering capacity and this could explain the unchanged pH values.

The results also reveal the possibility of using these microbes to decrease the asphaltenes in ecosystems. The three bacterial isolates show good biodegradation potentials on the asphaltenes fraction, which are in correlation to the trend of the bacterial growth. Generally the three bacterial isolates showed continuous increments in biodegradation percentages allover the incubation periods. *Pseudomonas* sp. Asph2 recorded the highest biodegradation efficiencies ≈83%, 89%, and 96% after 7, 14, and 21 d of incubation, respectively. However *Bacillus* sp. Asph1 recorded biodegradation efficiencies of ≈51% after 7 d of incubation which was then increased up to ≈82% after 14 d of incubation and remains nearly sustained thereafter recording ≈83% at the end of incubation period, 21 d. *Micrococcus* sp. Asph3 expressed the lowest degradation potential during the first 14 days of incubation ≈19% and 68% after 7 and 14 d of incubation, respectively, then sharply increased, reaching ≈88% at the end of incubation period 21 d. A statistically significant difference at ($\alpha = 0.5$) 95% confidence level was found for Asph biodegradation (%BD) in all biotreatment flasks relative to the –ve control flasks ($p = 3.5e - 3$).

The highest previous biodegradation report of asphaltene in the literature is 35% [41, 42] and 46% [5].

The variations in bacterial efficiencies for asphaltene biodegradation may be due to different enzyme systems utilized by each strain to degrade asphaltene and/or may be due to other factors, for example, kinetics, physical, and chemical parameters required by each isolate to utilize asphaltene. Several other factors can also be responsible for this behavior, such as substrate mass transfer through the cell wall, the pathway through which they could utilize asphaltene and/or inhibition of activity by the intermediates and byproducts produced from asphaltene metabolism.

A quadratic polynomial correlation was estimated for Asph biodegradation (%BD) as a function of cell growth (logarithm of cells/mL):

$$f(x) = ax^2 + bx + c \quad (1)$$

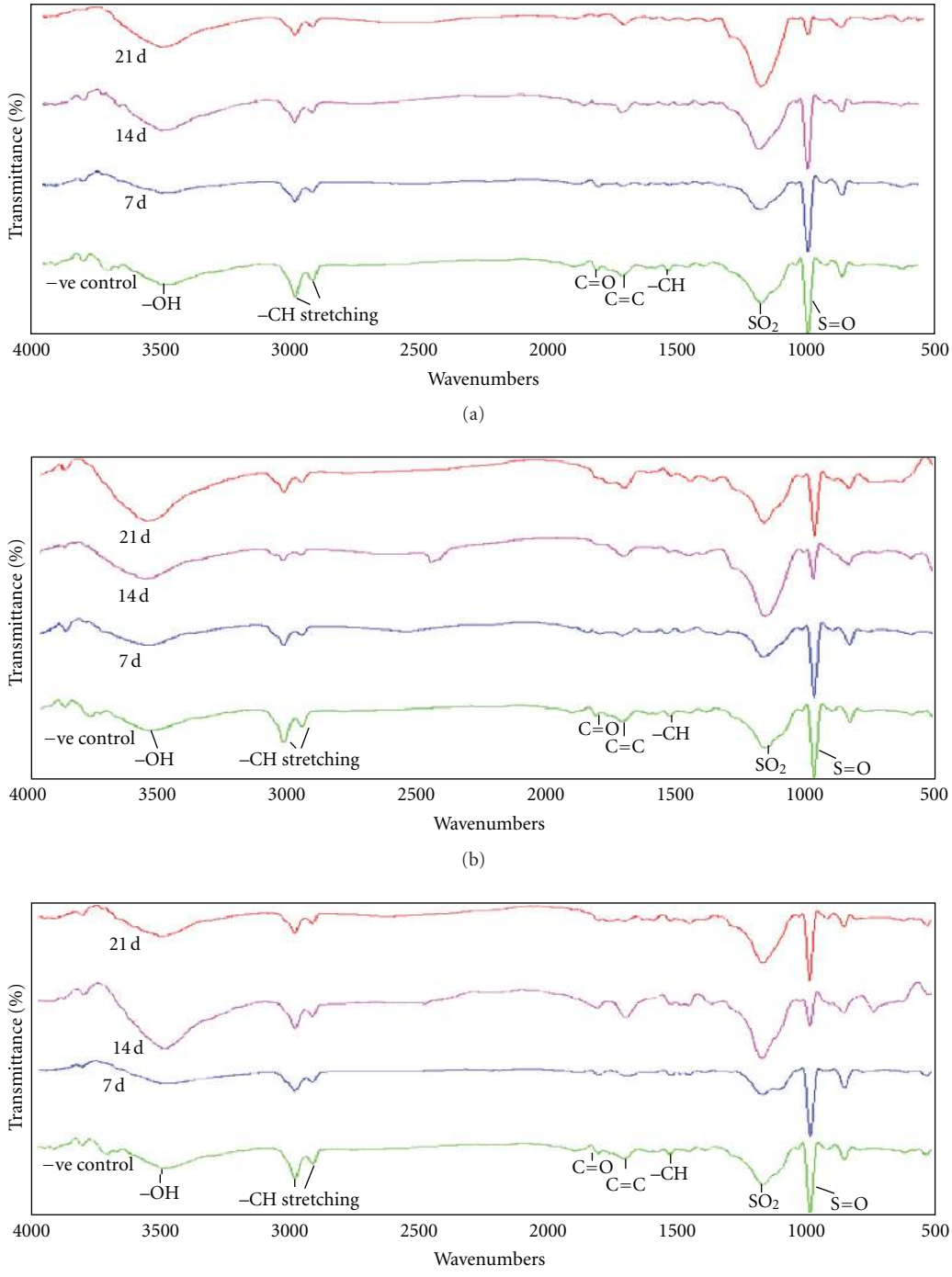


Figure 2: FT-IR spectra of asphaltenes before and after biotreatment with (a) *Bacillus* sp. Asph1, (b) *Pseudomonas aeruginosa* Asph2, and (c) *Micrococcus* ssp. Asph3.

$f(x)$ is the %BD (dependent variable) and $x$ is the logarithm of cell growth/mL (independent variable). Values of the parameters $a$, $b$, and $c$, together with correlation coefficient $R^2$, for the three treatments Asph1, Asph2, and Asph3 are given in Table 5.

*3.4. Fourier Transform Infrared Spectroscopic and Gel Permeation Chromatographic Analysis.* The analysis of the asphaltenic fractions by FTIR can permit the identification of those absorption bands that suffer modifications due to the biological treatment. Degradation pathways of asphaltenes

have not been defined in this study due to the complex structure of these compounds and the scarce information available on that subject.

The FTIR spectra obtained from the biodegradation experiments displays relatively simple patterns (Figure 2). After biodegradation the spectra reflect more pronounced alterations than the blank (−ve control). The bands that suffered major modifications in the macromolecular structure of asphaltene are as follows. (I) Absorption bands of C–H stretching (2929–2856 $cm^{-1}$) and C–H deformation (1457–1378 $cm^{-1}$) of aliphatic $CH_2$ and $CH_3$ groups. (II) Absorption band of C=C aromatic ring in the range of 1500 $cm^{-1}$. (III) Absorption typical for C=O bonds of carboxylic acids, esters, or ketones can be detected at about 1725–1735 $cm^{-1}$. (IV) IR spectra showed also an increase in the intensity of the hydroxyl peak, at the range of (3443 $cm^{-1}$), indicating some kinds of biodegradation. The formation of such oxygenated functional groups which are clearly more abundant after biodegradation as compared with the –ve control experiments can be the result of oxidative alterations of the macromolecular structures induced by the bacteria. (V) The IR spectra after biodegradation exhibit distinct changes at the bands for sulfones and sulfoxide in the range of 1110 $cm^{-1}$ and 1030 $cm^{-1}$, respectively. These data suggest that the bacteria are capable of oxidizing the abundant thioether linkages of macromolecular structures to sulfoxide and sulfone functions. Similar results were obtained by Ayala et al. [13].

Moustafa et al. [43] reported that, for the biomodification of asphaltenes, the reactions with organosulfur moieties could be very significant, because sulfur is the most abundant element in Asph besides carbon and hydrogen; sulfur can form up to 8% wt. of Asph and has an important role in its molecular structure. Asph is considered to be the product of complex heteroatomic aromatic macrocyclic structures polymerized through sulfide linkages [44].

The increase in biodegradation of asphaltenes observed after 21 d may be due to the microbial attack on the polysulfide linkages, which leads to biodepolymerization of the asphaltene fraction. This is associated with a decrease in the average molecular weight as shown clearly by GPC average molecular weight determinations. Average molecular weight recorded percentage decrease of ≈34.08%, 42.45%, and 34.66% for Asph1, Asph2, and Asph3, respectively. Similar observations were reported by Lin et al. [45] and Moustafa et al. [43].

Identification of Asph2 which is characterized by the highest asphaltenes biodegradation capabilities as illustrated in Table 4 was confirmed using 16SrDNA sequence. It was found to belong to genus *Pseudomonas* with 99.9% similarities to *Pseudomonas aeruginosa.*

## 4. Conclusions

This study characterizes three halotolerant bacterial strains isolated from oil-polluted sea water, for their ability to grow on high concentration of asphaltene as sole source of carbon and energy. To our knowledge, this is the first report of bacteria expressing high biodegradation of asphaltenes (83–96%, %BD) within relatively short incubation period (21 d), at high salinity (≈42,000 mg/L). Our study of biodegraded asphaltene fraction, based on GPC analysis and FT-IR spectra, suggested that biodegradation can influence both molecular weight and structure of asphaltenes. These bacterial isolates can be recommended as good candidates, for bioremediation of petroleum-contaminated saline ecosystems.

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