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# Effect of different bioremediation techniques on petroleum biomarkers and asphaltene fraction in oil-polluted sea water

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ABSTRACT

The present study has clearly demonstrated that seeding a petroleum hydrocarbon polluted sea water with a halotolerant *Pseudomonas aeruginosa* Asph2 and corn steep liquor as a cheap and readily available source of nutrients significantly enhances the progress of biodegradation BD of petroleum hydrocarbon pollutants. There was a statistically high significant difference between natural weathering NW and both bioaugmentation BA ($p = 2.92e\text{-}14$) and biostimulation BS ($p = 3.56e\text{-}13$) at 95% confidence interval ($\alpha = 0.05$). BA significantly improved the efficiency of total petroleum hydrocarbon TPH and the recalcitrant asphaltene degradation by $\approx$ 23 and 17% compared to BS process ($p = 2.0693e\text{-}5$ and $p = 1.4e\text{-}3$, respectively). The BD of biomarkers; pristane Pr, phytane Ph, and terpanes were very apparent. The BD of C28 tricyclic terpane, C27 13β, 17α- diasterane (20S), and C30 17α, 21β-hopane were higher than that of C35 17α,21β-homohopane. The BD of C27 18α(H)-22,29,30-trisnorneohopane (Ts) was higher than that of C27 17α(H)-22,29,30-trisnorhopane (Tm). The BD of sterane was higher than that of hopane. C30 18α-Oleanane was more resistant to degradation than other compounds.

*Keywords:* Bioremediation; Oil-polluted sea water; Petroleum biomarkers; Asphaltene fraction; *Pseudomonas aeruginosa* Asph2; Statistical analysis

## 1. Introduction

The incidence and threat of aquatic pollution by petroleum hydrocarbons in Egypt has resulted in extensive research [1–4]. Although mechanical and chemical techniques are the commonly applied methods in Egypt for getting rid of oil spills, the Egyptian oil industry views bioremediation of oil-polluted sites as a top-priority research field [5–12].

Hydrocarbons are weak sources of nutrients such as nitrogen and phosphorus which are essential for micro-organisms as building blocks of structural macromolecules like proteins and nucleic acids as well as enzymes and coenzymes. These nutrients are therefore highly limiting in petroleum-polluted environments. Consequently, the growth of hydrocarbon-degrading bacteria and hydrocarbon degradation process can be enhanced by adding some inorganic nutrients [13]. Corn steep liquor CSL is a liquid mixture consisting entirely of the water soluble

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components of corn steeped in water. It has been shown to be very rich in nutrients required by most micro-organisms. Its composition depends on the corn variety, steeping condition, and age of the corn kernel [13]. According to Kaplan et al. [14] and El-Gendy and Farah [15], CSL can serve as a good source of nutrients and acts as an emulsifier thereby increasing the amount of bioavailability during metabolic degradation process. In addition, CSL has an excellent balance of the majority of essential factors; lactic acid constitutes an easily assumable carbon source, the amino acids and polypeptide constitutes not only sources of nitrogen and carbon, but also sources of buffering agents, and the ashes constitute a source of mineral elements [16,17].

Biomarker compounds are highly biodegradation resistant and have been widely used in geochemistry for studying ancient sediments, thermal maturity of petroleum, and oil–oil correlations [18–21]. In recent years, they have been increasingly used for the purpose of source identification and differentiation of oils, and for monitoring the weathering and degradation processes of oils under a wide variety of environmental conditions. However, under severe weathering conditions, biomarkers are also biodegradable. Terpanes are found in nearly all oils. These bacterial terpanes include several homologous series, including bicyclic, tricyclic, tetracyclic, and pentacyclic (e.g. hopanes) compounds. Hopanes with the 17 αβ-configuration in the range of C27 to C35 are characteristics of petroleum because of their greater thermodynamic stability compared to other epimeric (ββ and βα) series. The steranes are a class of biomarkers containing 21 to 30 carbons that are derived from sterols, and they include regular steranes, rearranged diasteranes, and mono- and tri-aromatic steranes. Among them, the regular C27–C28–C29 homologous sterane series are the most common and useful steranes because they are highly specific for correlation [22].

In Egypt, most bioremediation studies have been focused on the degradation of aliphatic and aromatic fractions rather than recalcitrant asphaltenes and biomarkers [23–28].

The present study was carried out to assess the capability of treatment of oil-polluted sea water with special emphasis on biodegradation of asphaltene and different biomarkers through different bioremediation processes: natural weathering NW; natural ability to degrade the contaminant, biostimulation BS; through acceleration of bioremediation capacity of the naturally occurring micro-organisms by addition of CSL as local, commercial (1 kg of CSL costs 1.3 Egyptian pounds), and readily available source of nutrients, and bioaugmentation BA; by adding CSL and augmenting indigenous community with halotolerant *Pseudomonas aeruginosa* Asph2 previously isolated from the oil-polluted sea water under study, for its ability to degrade asphaltenes [12]. Statistical multiple comparison tests were performed to determine to what extent treatments have significance differences. Numerical investigation was performed to find the relationships between different studied parameters.

## 2. Materials and methods

Polluted water sample was purchased from Sumid Cooperation, Suez Terminal, Egypt.

Trypton glucose yeast extract TGY medium [29] was used for obtaining the biomass. TGY/agar plates were used for monitoring the microbial growth (TCFU/mL).

Corn steep liquor CSL used in this study was provided from National Company for Maize Products in 10th of Ramadan City, Egypt. It is with C:N:P:K content of 6.3:7:3.5:2.5 (%w/w), respectively. It also contains reducing sugars, lactic acid, ash, and proteins contents of 2, 15, 20, and 44 (wt.%), respectively.

### 2.1. Bacterial strain

*P. aeruginosa* Asph2 used in this study was previously isolated from the oil-polluted sea water under study [12].

### 2.2. Inoculum preparation

*P. aeruginosa* Asph2 was grown for 24 h in TGY broth, at 30˚C, and 150 rpm. Centrifuged at 5,000 rpm for 5 min, the biomass was harvested and washed twice with sterile saline (10 g NaCl/L distilled $H_2O$) and resuspended in fresh sterile saline.

### 2.3. Bioremediation treatments

Batch bioremediation study of twelve 500-mL Erlenmeyer flasks with 100 mL contaminated water took place over a period of one month at room temperature and 150 rpm. It was divided into four groups: first group is for −ve control, without any treatment but sterilized by autoclaving at 121˚C and 1.5 bar for 15 min; second group is for NW, the polluted water without any treatment or sterilization; third group is for BS, 1 g CSL was added for each flask; and fourth group is for BA, 1 g CSL and 5 mL of enriched bacterial culture were inoculated in each flask.

### 2.4. Monitoring of bioremediation process

#### 2.4.1. Bacterial count

Briefly, 1 mL aliquot culture was added to 10 mL of sterile saline, vortexed, and then serially diluted to $10^{-10}$. Serial dilutions of these suspensions were inoculated on TGY/agar plates and incubated at 30˚C for 48 h to enumerate TCFU (cells/mL). Biodiversity of bacterial population was done after Gram staining and microscopic examinations for bacteria to eliminate apparently similar strains. The identification of bacteria was done using 16S ribosomal DNA amplification and sequencing. Blast program (http:www.ncbi.nlm.nih.gov/blast) was used to assess the DNA similarities.

#### 2.4.2. TPH concentration and liquid column chromatography

The pH of the cultures was brought to pH 2 with 1 M HCl to halt biological activity. The oil phase was then extracted and its concentration was determined gravimetrically according to the method reported by Moustafa et al. [30]. After the addition of 50 mL n-Heptane to the extracted TPH, the resulting suspension was separated into soluble (maltenes) and insoluble (asphaltenes) fractions and quantified gravimetrically. Maltene was loaded onto activated alumina gel columns ($50 \times 2$ cm), mesh size 60–120, activated at 300˚C for 24 h. The column was eluted first with 120 mL n-Hexane followed by benzene with the same volume at a rate of 30–40 drops/min to collect in preweighed evaporating dishes, the saturates and aromatics fractions, respectively. The methanol was then added to collect resins. Solvents were evaporated to constant weights and the percentage degradation of saturates, aromatics, resins, and asphaltenes fractions was determined gravimetrically.

### 2.5. Analytical methods

Gas chromatography (Perkin Elmer Clarus 500, USA) with mass-selective detector-mass spectrometry GC-MS was used for the evaluation of biodegradation of biomarkers in saturates fraction, ionization mode; EI eV 70; the GC equipped with fused silica capillary column of bonded DB-5 column (5%-phenyl) methyl poly siloxane (capillary 30 m × 0.25 mm ID × 0.25 μm film). The carrier gas was helium. Run conditions were as follows: injection temperature was 280˚C, start temperature 100˚C held for 1 min, followed by 3˚C/min ramp rate to a final temperature of 310˚C which was held to another 20 min. Possible structural assignments were confirmed using the online G1035A Wiley PBM library (John Wiley and Sons, New York). Analytical Gel Permeation Chromatography GPC (Waters 600 E, USA) and Fourier Transform Infrared FTIR (Perkin Elmer Spectrum One, USA) were used to determine changes in average molecular weight and significant alternations in functional groups of asphaltene fraction after biotreatment, according to Ali et al. [12].

### 2.6. Statistical analysis

This was performed using MATLAB version 7.0.0. One-way analysis of variance (ANOVA1) for comparing the means of results obtained in the three biotreatments processes (NW, BS and BA) at $\alpha = 0.05$ level (95% confidence interval) was performed. A multiple comparison test using the information in the states structure from ANOVA1 was done to obtain pair wise comparison results. Regression analysis was also performed to estimate the correlations between some of the studied parameters.

## 3. Results and discussion

### 3.1. The physico-chemical characteristics of the collected water sample

The physical and chemical analysis of the water sample revealed pH 7.78, temperature immediately after sampling was 27.9˚C, and salinity was 42,000 mg/L with total petroleum hydrocarbons TPH content of ≈10 g/L. The total viable count TCFU on TGY plates directly after collection showed good microbial population of $1.2 \times 10^4$ cells/mL, with low biodiversity; Gram +ve *Bacillus* sp. Asph1 and *Micrococcus* sp. Asph3 and Gram –ve *P. aeruginosa* sp. Asph2. The GC chromatogram of oil extracted from the water showed regularly spaced resolved n-alkane peaks ranging from $nC_{15}$–$nC_{35}$ superimposed on an unresolved complex mixture UCM containing naphthenes and aromatics, indicating that the source of contamination is mainly petroleum hydrocarbons.

### 3.2. Monitoring of bioremediation process

During different biotreatments; there was no significant change in pH values occurred in all cultures, ranged between 6.25–6.65. CSL can act as a self-buffering agent. There was considerable increase in viable cells count (cells/mL) after 30 d of incubation reaching $6 \times 10^{14}$ and $5 \times 10^{15}$ cells/mL for BS and BA flasks, respectively. While for NW flasks, there was no significant increase in TCFU recording, $9 \times 10^4$ cells/mL. It

was noticed that the bacterial strain Asph2 was the predominant isolate in all biotreatment flasks. This may indicate the synergism between the augmented bacterium; *P. aeruginosa* Asph2 and indigenous microbial population, and the tolerance and well adaptation of Asph2 to high concentration of TPH.

Visual observations of all the tested biotreatment methods showed that in spite of the absence of a chemical surfactant, the metabolic degradative cultures are accompanied by the formation of strong emulsions. This is an indirect evidence for the production of a biosurfactant or more likely a mixture of biosurfactants. In this study, biosurfactant production was only detected in the cultures by measuring the surface tension of the medium using a DU-Nouy ring-type tensiometer, Kruss type K6. At –ve control, the flasks without CSL recorded surface tension of 70 dyne/cm while that of sterile flasks with CSL recorded 65 dyne/cm, indicating the self-emulsion effect of CSL. At the end of the incubation period, the surface tension recorded 59, 41, and 38 dyne/cm in NW, BS, and BA flasks, respectively. The results can indicate that the highest biosurfactant production is recorded in BA flasks which might be due to the synergetic effect of *P. aeruginosa* Asph2 with the indigenous microbial population that consequently increased their activities and biosurfactant production.

The NW flasks showed a low reduction in TPH concentration reaching ≈10% at the end of incubation period. This indicates that the indigenous microbial populations can biodegrade petroleum hydrocarbons in the polluted water sample but the process is very slow. This might be attributed to the lack of adequate nitrogen, phosphorus, and potassium supporting optimal microbial activities. BS and BA flasks showed significant reduction in TPH recording ≈57 and 80%, respectively, after 30 d of incubation. The recorded high biodegradation percentage in BS flasks indicates that CSL can act as a good and sufficient nutrients source. While in BA flasks the increase of TPH degradation by ≈23% compared to BS flasks indicates synergism between the augmented *P. aeruginosa* Asph2 and the indigenous microbial population and the well adaptation of Asph2 for biodegradation process as it was previously isolated from the same contaminated water under study [12].

Although there was no statistically significant difference recorded for microbial growth or decrease in surface tension in BS and BA flasks, there was statistically high significant difference for decrease in TPH concentration ($p = 2.0693\text{e-}5$). It was noticed that there was obvious correlation between the decrease in surface tension, growth potential (cells/mL), and the amount of TPH consumed (BD%). This correlation is found to be described by the following equation:

$$Z = -0.375 + 17.3\ e^{\left(-\left(\frac{X-52.3}{32.8}\right)^2\right)} + 3.608Y^{0.4} \quad (1)$$

where $Z$ is ln(TCFU/mL) while $X$ and $Y$ are the percentage decrease in surface tension and TPH, respectively. The validity of the above model equation was tested by calculating the following parameters measuring the goodness of fit; sum of square error SSE = 9.997, root mean square error RMSE = 3.162, correlation coefficient $R^2 = 0.9734$, and adjusted correlation coefficient $R^2_{adj} = 0.9468$. Fig. 1(A) represents the

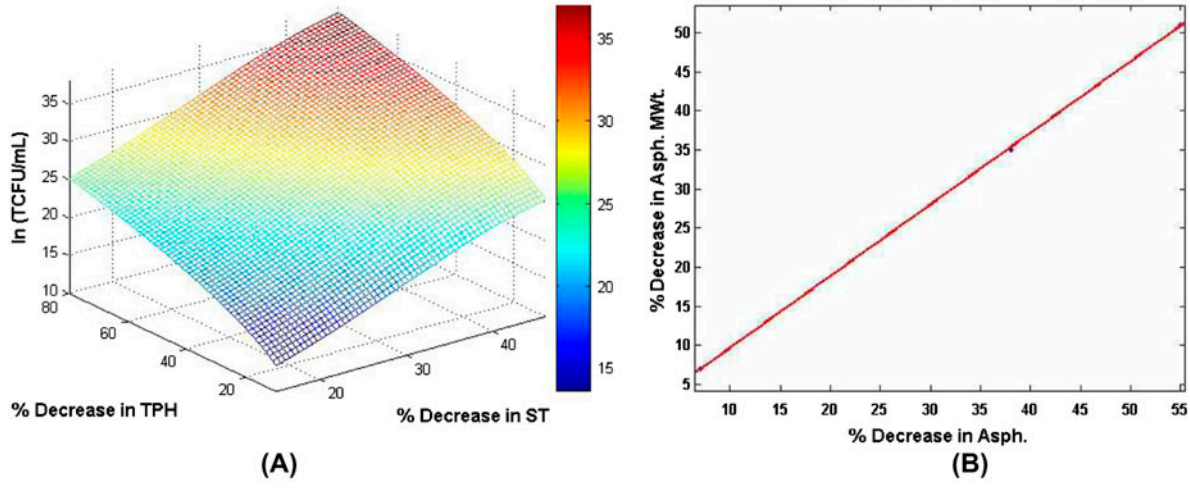


Fig. 1. Correlations between some studied parameters (A) Increase in microbial growth with decrease in surface tension and TPH (B)% decrease of Asph MWt as a function of BD% of Asph.

3-D plot of this correlation, where maximum growth occurred in BA flasks with maximum decrease in surface tension and TPH content.

### 3.2.1. Biodegradation of petroleum fractions

The BD% of maltene was higher than that of asphaltene in all biotreatment flasks. NW, BS, and BA flasks recorded BD of ≈16, 58, and 81% for maltene, and ≈7, 38, and 55% for aspahaltene, respectively. The highest recorded BD% in all biotreatment flasks was that of saturates fraction followed in decreasing order by aromatics > asphaltene. NW, BS, and BA flasks recorded BD of ≈14, 69, and 84% for saturates and ≈6, 55, and 82% for aromatics, respectively. The resin fraction showed nearly no biodegradation in all biotreatment flasks except for BA flasks, BD% of resin recorded ≈87%. There was a statistically high significant difference between BS and BA flasks in BD% of aromatics and maltene ($p = 1.1834e\text{-}5$ and 1.3143e-5, respectively), while there was statistically significant difference in BD% of saturates and asphaltene ($p = 0.02$ and 0.0014, respectively). Liao and Geng [31] reported that asphaltenes can be mainly changed into resin fraction after oxidative degradation. Chaineau et al. [32] and Ragheb et al. [33] reported the phenomenon of resin enrichment as a result of partial hydrocarbon degradation. Tavassoli et al. [34] reported that bacterial mixed culture including five selected strains of *Pseudomonas* sp. and *Bacillus* sp. exhibited 48% of asphaltene biodegradation during 60 d, and attributed this to the higher degradability of bacterial mixed culture compared to pure cultures, because the members of the mixed culture have a synergetic activity. It was reported by Pineda-Flores and Mesta-Howard [35] that when micro-organisms develop their full metabolic potential, they might have the capacity of using asphaltene as a source of carbon and energy, or degrading them by co-metabolism mechanisms. This is being possible because asphaltene contains carbon, hydrogen, sulfur, nitrogen, and oxygen, which are the necessary elements for the development of any organism.

In addition to the above gravimetric analysis of asphaltene fraction, FTIR spectra were used to follow up the biodegradation of asphaltene fraction in all biotreatment flasks. FTIR spectra, Fig. 2 displayed a relatively simple pattern and showed alternation in the band intensities of the macromolecular structure of asphaltene after different biotreatment methods; (1) Absorption bands 2,922 and 2,852 $cm^{-1}$ for asymmetric and symmetric –CH stretching, respectively. (2) Absorption bands around 1,600 and 1,455–1,376 $cm^{-1}$ for C=C aromatic and conjugated alkane and amine components, respectively. (3) Absorption band of the hydroxyl peak, at the range of 3,441 $cm^{-1}$. (4) The IR spectra after biotreatments exhibit distinct changes at bands around 1,110 and 1,030 $cm^{-1}$ for sulfone $SO_2$ and sulfoxide –S=O, respectively. This suggests that different biotreatments oxidize the abundant thioether linkages of macromolecular structures to sulfoxide and sulfone functions. The change in the above bands indicates that some kind of biodegradation has occurred in asphaltene fraction.

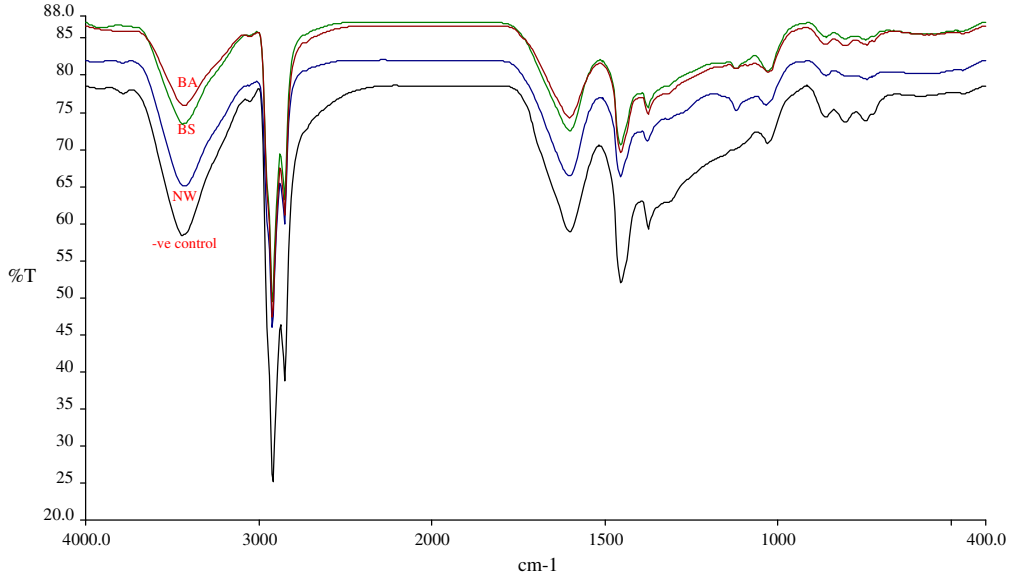


Fig. 2. FTIR spectra of asphaltene before and after different biotreatment techniques.

Lin et al. [36], Becker [37] and Moustafa et al. [38] reported that asphaltene is considered to be the product of complex heteroatomic aromatic macrocyclic structures polymerized through sulfide linkages. So, biodegradation of asphaltene can be occurred by the microbial attack on the polysulfide linkages. This is associated with a decrease in the average molecular weight of the asphaltene fraction. In this study, GPC analysis revealed a decrease in the average molecular weight MWt of asphaltene fraction after different biotreatment processes recording ≈ 7, 35, and 51% in NW, BS, and BA flasks, respectively, with statistically significant difference between BS and BA flasks ($p = 0.0031$). These results were in good correlation with the gravimetrical analysis of asphaltenes degradation data. The following power equation describes this correlation:

$$f(x) = ax^b \quad (2)$$

where $x$ is % BD of asphaltene, $f(x)$ is % decrease in asphaltene molecular weight, and $a$ and $b$ are constants = 1.002 and 0.9796, respectively. The calculated parameters measuring the goodness of fit were; sum of square error SSE = 0.2381, root mean square error RMSE = 0.488, correlation coefficient $R^2 = 0.9998$, and adjusted correlation coefficient $R^2_{adj} = 0.9995$. These parameters indicated the excellent agreement between experimental and calculated values. Fig. 1(B) represents this correlation, where maximum decrease in Asp MWt with maximum Asph BD occurred in BA flasks.

The GC profile of the saturates extracted from the biotreatments flasks express changes in the total resolvable peaks TRP (linear and branched alkanes) and unresolved complex mixture UCM (napthenes and cycloalkanes) but with different efficiencies which can be ranked in the following decreasing order BA>BS>NW. The percent BD for TRP recorded ≈15, 95, and 97%, while UCM percent BD recorded ≈5, 71, and 78% for NW, BS, and BA flasks, respectively, with no statistically significant difference between BS and BA flasks. There is a clear selective degradation of n-alkanes and isoprenoids including Pristane Pr (2,6,10,14-tetramethylpentadecane) and Phytane Ph (2,6,10,14- tetramethylhexadecane), with decreasing values of Pr/Ph, nC17/Pr, and nC18/Ph in all biotreatment flasks. Percent BD of n-alkanes was higher than that of isoprenoids, recording ≈18, 97, and 98% for n-alkanes and ≈3, 93, and 97% for isoalkanes in NW, BS, and BA flasks, respectively, with no statistically significant difference between BS and BA flasks. In NW flasks, there was no effect on Pr and Ph, but there was a higher significant effect in all other biotreatment flasks, although they have been used as conservative biomarkers and reported to be recalcitrant for biodegradation. BA flasks expressed higher BD efficiencies on Pr ≈ 97% and Ph ≈ 90% relative to that of BS, which recorded ≈80% and 76% for Pr and Ph, respectively, with statistically significant difference ($p = 0.0034$ and 0.0365, respectively). In NW, BS, and BA flasks, nC17/Pr recorded a decrease of ≈6, 96, and 97%, respectively, with no statistically significant difference between BS and BA flasks. nC18/Ph recorded a decrease of ≈2, 68, and 97%, respectively, with statistically high significant difference between BA and BS flasks ($p = 3.22e\text{-}6$). Pr/Ph recorded a decrease of ≈14, 53, and 67%, respectively, with statistically significant difference between BA and BS flasks ($p = 0.0126$). Biodegradation of Pr and Ph was also reported by Xu et al. [39], Farahat and El-Gendy [40], El-Gendy and Farah [15]. Atlas [41] and Diaz et al. [42] reported that bacteria can readily degrade n-alkanes, whereas the isoprenoidal alkanes are relatively resistant to microbial degradation.

### 3.2.2. Biodegradation of petroleum biomarkers

GC-MS analysis of other biomarkers in these residues revealed that although there was no significant effect on biomarkers of NW flasks, there was a remarkable degradation of biomarkers; steranes, hopanes, and terpanes in BS and BA flasks, with the superiority of BA flasks. This indicates the ability of *P. aeruginosa* Asph2 for biodegradation of biomarkers and its good synergetic activity with indigenous microbial population. Fig. 3(A) and Table 1 show the mass fragmentograms at $m/z = 191$ of the extracted saturates fraction; –ve control flasks are characterized by distribution in a wide range from $C_{19}$ to $C_{35}$ terpanes with C29 18α(H)-norneohopane and C30 α,β-hopane being the most prominent. The degradation of biomarker terpanes, however, is very apparent recording percent BD of ≈ 59% and 94% for BS and BA flasks, respectively, with a statistically high significant difference ($p = 6.8563e\text{-}7$). The $m/z = 191$ chromatograms, Fig. 3(A) reveal extensive degradation of both the R and S epimers of the C28 tricyclic terpane in the following decreasing order, BA>BS flasks. Similar observation reported by Bosta et al. [43], based on molecular volumes and surface areas, considers that the C26-C29 tricyclic terpanes more readily biodegraded by a proposed C-10 demethylation. A process similar to that was hypothesized in the hopanes. In this study, however, no epimer specificity was observed in the degradation of the C28 tricyclic terpane in all

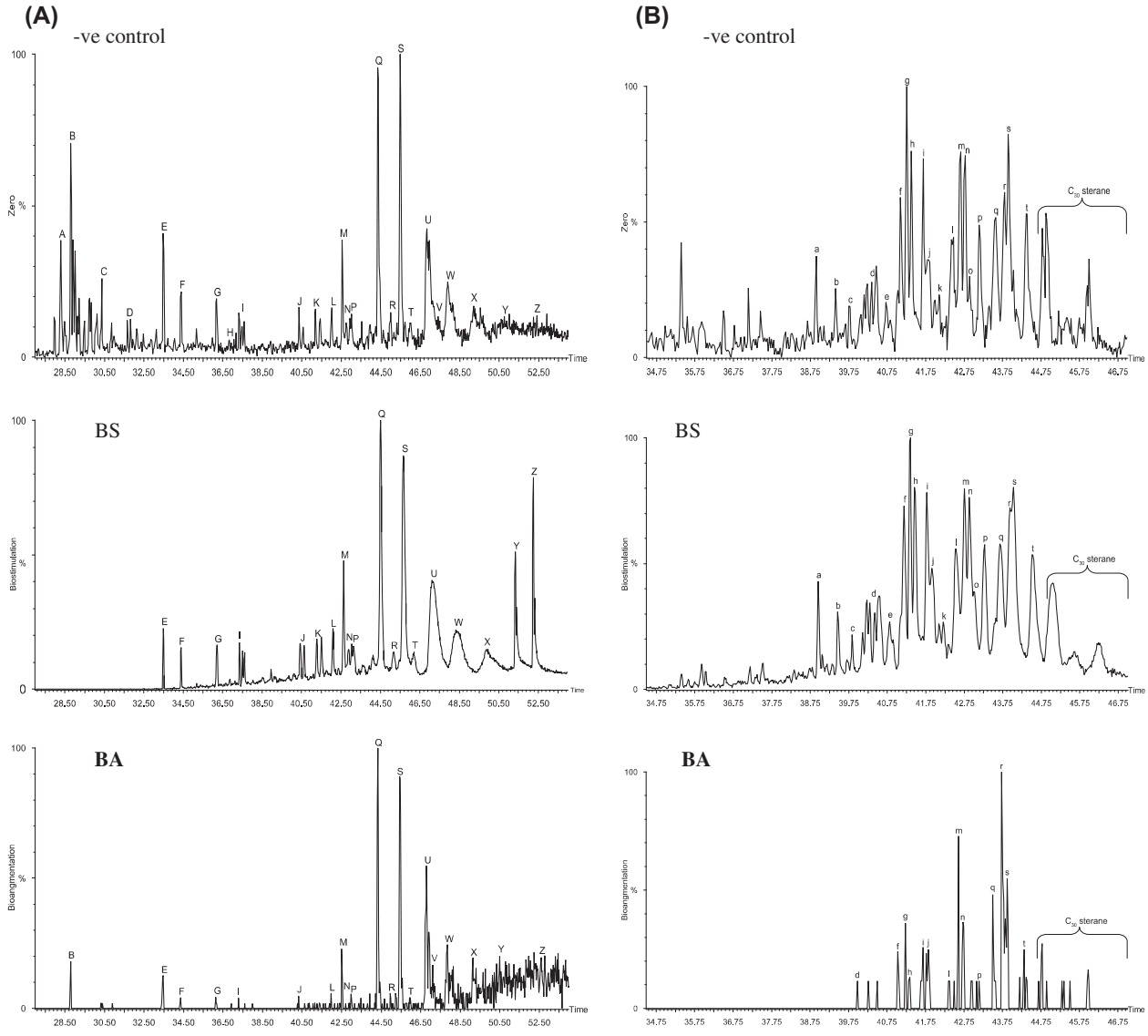


Fig. 3. (A) GC-MS chromatograms ($m/z = 191$) and (B) GC-MS chromatograms ($m/z = 217$) of extracted oils before and after biotreatments. Peaks identification is listed in Tables 1 and 3, respectively.

biotreatment flasks, the ratio of the C28 tricyclic terpane to C35 17α,21β-homohopane (Table 2) had decreased by ≈ 69% and 94% in BS and BA flasks, respectively, with a statistically high significant difference ($p = 4.2979e\text{-}5$). A similar observation was reported by da Cruz et al. [44]. The ratio of C27 13β, 17α- diasterane (20S) to C35 17α, 21β-homohopane had decreased by ≈ 77 and 100% in BS and BA, respectively, with a statistically high significant difference ($p = 9.5123e\text{-}5$). The ratio in all biotreatments is lower than 1 indicating the higher biodegradability of C27 over that of C35. C30 18α-Oleanane was more resistant to degradation than other compounds. Oleanane index remained unchanged in NW and BA flasks but yielded a 1.6-fold increase in the BS flasks. The homohopane index increased from 10.4 to 18, 60, and 81% for NW, BS, and BA, respectively, after 30 d of incubation, with a statistically high significant difference between BA and BS flasks ($p = 4.1325e\text{-}4$). A similar observation was reported by da Cruz et al. [44]. The steranes recorded percent BD higher than that of hopanes ≈ 64 and 83% for BS and BA, respectively, with a statistically significant difference ($p = 0.0075$). The percent BD of hopanes recorded ≈ 42% and 68% for BS and BA, respectively, with a statistically high significant difference ($p = 5.9298e\text{-}6$). This was obvious

Table 1
Peaks identification for the *m/z* 191 mass fragmentograms

| Peak no. | Compound name | Peak no. | Compound name |
|---|---|---|---|
| A | C19 Tricyclic terpane | N | C30 Tricyclic terpane (22S) |
| B | C20 Tricyclic terpane | P | C31 Tricyclic terpane (22R) |
| C | C21 Tricyclic terpane | P | C31 Tricyclic terpane (22S) |
| D | C22 Tricyclic terpane | Q | C29 18α(H)-norneohopane (29Ts) |
| E | C23 Tricyclic terpane | R | C30 18α(H)-oleanane |
| F | C24 Tricyclic terpane | S | C30 17α(H), 21β(H)-hopane |
| G | C25 Tricyclic terpane (22R) | T | C30 17β(H), 21α(H)-moretane |
| G | C25 Tricyclic terpane (22S) | U | C31 17α(H), 21β(H)-30 homohopane (22S) |
| H | C24 Tetracyclic terpane | | C31 17α(H), 21β(H)-30 homohopane (22R) |
| I | C26 Tricyclic terpane (22R) | V | C30 Gammacerane |
| I | C26 Tricyclic terpane (22S) | W | C32 17α(H), 21β(H)-30 bishomohopane (22S) |
| J | C28 Tricyclic terpane (22R) | | C32 17α(H), 21β(H)-30 bishomohopane (22R) |
| J | C28 Tricyclic terpane (22S) | X | C33 17α(H), 21β(H)-30 trishomohopane (22S) |
| K | C29 Tricyclic terpane (22R) | | C33 17α(H), 21β(H)-30 trishomohopane (22R) |
| K | C29 Tricyclic terpane (22S) | Y | C34 17α(H), 21β(H)-30 tetrakishomohopane (22S) |
| L(Ts) | C27 18α(H)-22,29,30-trisnorneohopane | | C34 17α(H), 21β(H)-30 tetrakishomohopane (22R) |
| M(Tm) | C27 17α(H)-22,29,30-trisnorhopane | Z | C35 17α(H), 21β(H)-30 pentakishomohopane (22S) |
| N | C30 Tricyclic terpane (22R) | | C35 17α(H), 21β(H)-30 pentakishomohopane (22R) |

from the decrease of sterane/hopane ratio, recording ≈ 22% and 33% in BS and BA flasks, respectively, with a statistically significant difference ($p = 0.0082$). Bosta et al. [43] reported that more complex saturated molecules, such as the hopanes, may also be transformed by a co-metabolic activity, as has been suggested by (1) demethylation to form norhopanes, or (2) β-oxidation of the alkyl side chain. Huesemann et al. [45] reported the complete removal of the pentacyclic triterpane C30 17α(H),21β(H)-hopane in a biotreatment experiment and reported that the use of microbial enrichment cultures speeds up the process of bioremediation.

In this study, the degradation of two norhopane species, C27 18α(H)-22,29,30-trisnorneohopane (Ts) and C27 17α(H)-22,29,30-trisnorhopane (Tm), was observed on the *m/z* = 191 chromatogram Fig. 3(A). This was obvious from the decrease of Ts/Tm ratio, recording ≈ 47% and 88% in BS and BA flasks, respectively, with a statistically high significant

Table 2
Biomarker ratios of different biotreatment techniques after 30 d of incubation period

| | C28TT/ C35H[a] | C35H/ C30H | C27D/ C35H[a] | Ts/ Tm[b] | Oleanane index[e] | Sterane/ hopane[d] | Homohopane index[c] |
|---|---|---|---|---|---|---|---|
| −ve control | 4 | 0.57 | 2.75 | 0.33 | 0.05 | 0.8 | 10.4 |
| NW | 5.2 | 0.55 | 2.74 | 0.3 | 0.05 | 0.79 | 18 |
| BS | 1.23 | 1.6 | 0.625 | 0.175 | 0.08 | 0.63 | 60 |
| BA | 0.26 | 4.5 | 0 | 0.04 | 0.05 | 0.54 | 81 |

[a]Calculated from *m/z* 191 chromatogram peak areas of C28 through C30 tricyclic terpanes (TT; 22R + 22S), C35 17α,21β(H)-homohopane (C35H; 22R + 22S) and C30 17α,21b(H)-hopane (C30H), along with the peak areas for the C27 13β,17α-diasterane (20S) (C27D) from the *m/z* = 217 mass chromatograms. [b]Calculated from *m/z* 191 chromatogram peak areas of C27 17α(H)-22,29,30-trisnorhopane (Tm) and C27 18α(H)-22,29,30-trisnorneohopane (Ts). [c]Calculated from *m/z* 191 chromatogram peak areas of [C35 (22R + 22S)/(C31–C35)(22R + 22S) homohopanes] X100. [d]In this ratio, the C27, C28 and C29 ααα(20R + 22S) and αββ (20R + 22S) regular sterane peak areas from the *m/z* 217 chromatograms and C29–C33 17α(H)-hopanes (20R + 22S) from *m/z* 191 chromatograms were used. [e]Calculated from *m/z* = 191 mass chromatogram peak areas for 18a(H)-oleanane and C30 17a,21b(H)-hopane.

Table 3
Peaks identification for the *m/z* 217 mass fragmentograms

| Peak no. | Compound name | Peak no. | Compound name |
|---|---|---|---|
| A | C20 5α(H), 14α(H), 17α(H)-Sterane | M | C27 20R-5α(H),14β(H),17β(H)-cholestane |
| B | C21 5α(H), 14β(H), 17β(H)-Sterane | N | C27 20S-5α(H),14β(H),17β(H)-cholestane |
| C | C22 5α(H), 14β(H), 17β(H)-Sterane | O | C27 20R-5α(H),14α(H),17α(H)-cholestane |
| D | C27 20S-13β(H), 17α(H)-diasterane | P | C28 20S-5α(H),14α(H),17α(H)-ergostane |
| E | C27 20R-13β(H), 17α(H)-diasterane | Q | C28 20R-5α(H),14β(H),17β(H)-ergostane |
| F | C27 20S-13α (H), 17β(H)-diasterane | R | C28 20S-5α(H),14β(H),17β(H)-ergostane |
| G | C27 20R-13α (H), 17β(H)-diasterane | S | C28 20R-5α(H),14α(H),17α(H)-ergostane |
| H | C28 20S-13β(H), 17α(H)-diasterane | T | C29 20S-5α(H),14α(H),17α(H)-stigmastane |
| I | C28 20R-13β(H), 17α(H)-diasterane | | C30 sterane |
| J | C29 20S-13β(H), 17α(H)-diasterane | | |
| K | C29 20R-13α (H), 17β(H)-diasterane | | |
| L | C27 20S-5α(H),14α(H),17α(H)-cholestane | | |

difference ($p = 1.8196e\text{-}7$). This suggests that Ts is more biodegradable than Tm. Similar observation was observed by da Cruz et al. [44]. A significant increase in both the Oleanane index and C35 17α, 21β-homohopane: C30 17α, 21β-hopane ratio reveals a more extensive degradation of C30 17α, 21β-hopane compared to C35 17α, 21β-homohopane or oleanane Table 2. The preferential degradation of the R isomer over S isomer was observed in the C31-C34 17α, 21β-homohopane degradation, Fig. 3(A). A similar observation was reported by Goodwin et al. [46] and Martin [47]. Huesemann et al. [45] also reported that hopane is not necessarily conserved during a bioremediation study; the uncritical normalization of hydrocarbon concentrations using this biomarker can lead to incorrect estimates of biodegradation rates and extents.

It is obvious from m/z 217 fragmentogram, Fig. 3 (B) and Table 3 that the oil pollutant sample (-ve control flasks) is characterized by less abundance of steranes than the terpane compounds, Fig. 3(A), and essentially consist of ααα and βββ C27-cholestanes, C28-ergostanes, and C29-stigmastanes with the quite prominent presence of lower molecular mass C20,C21, and C22 steranes and C27 diasterane. In summary, the degree of biodegradation of biomarker compounds was strongly correlated with the biotreatment method. The degradation trends of biomarkers may be proposed as follows: NW flasks showed no significant changes. The highest biodegradation was recorded in BA flasks followed by BS flasks. In BA flasks, complete removal of steranes occurred followed by the degradation of diasterane, cholestane, stigmastane, and ergostane in decreasing order; while in BS flasks, the biodegradation can be ranked in the following decreasing order: ergostane > diasterane > cholestane ≈ sterane ≈ stigmastane.

## 3. Conclusion

Since the goal of any bioremediation protocol is the destruction of the pollutants using cheap and readily available nutrients sources, encouraging results from this study would present CSL as a powerful and effective source of nutrients for optimizing biodegradation BD of petroleum contaminants in the aquatic environment. The results obtained in this work clearly demonstrate that there are micro-organisms that are able to metabolize and mineralize asphaltenes and resins, which are considered the most recalcitrant petroleum fraction. The recorded high BD percentage of asphaltene and resin in bioaugmentation BA flasks encourage the superiority of using the BA technique with previously isolated strains over biostimulation BS. The final and the most important conclusion obtained from this work is that all the suggested and studied biomarkers are proved to be less specific than previously thought, so cannot be applied safely as they may give somewhat contradicted results since they can be affected by different bioremediation techniques in various levels and values. Hence, this limits their use in ecological and paleoenvironmental investigations except where prevalent and other complementary evidence is available.

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