Morphological, biochemical and molecular identification of petroleum hydrocarbons biodegradation bacteria isolated from oil polluted soil in Dhahran, Saud Arabia

Fahad A. Al-Dhabaan

Department of Biology, Science and Humanities College, Shaqra University, Alquwayiyah, Saudi Arabia

ARTICLE INFO

Article history:
Received 20 March 2018
Revised 20 May 2018
Accepted 31 May 2018
Available online 31 May 2018

Keywords:
Khurais oil field
Hydrocarbon components
Biodegradation
Morphological features
Biochemical tests
Universal 16S rDNA primers
Degradation %

ABSTRACT

Accumulation of petroleum hydrocarbon residual considered a major environmental problem in the kingdom of Saudi Arabia due to intensive efforts for oil detecting. Until now, in situ biodegradation considered the most effective method for petroleum hydrocarbon residual biodegradation. The aim of this study is isolation and identification biodegradable capability bacteria from contaminated sites in Khurais oil field, Dhahran, Saud Arabia via Different morphological and biochemical and molecular methods. Furthermore, degradation level in contaminated liquid medium and soil were evaluated. Three bacterial strains were selected from petroleum-contaminated soils of Khurais oil field depending on their capacity to grow in the existence of hydrocarbon components and identified according to morphological, biochemical. Interestingly, 16S rDNA sequencing fingerprinting results confirmed our bacterial identification as *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Bacillus cereus*. Phyllogenetic tree was constructed and genetic similarity was calculated according to alignments results. Biodegradation patterns for different three isolates were reflected varied degradation ability for three isolates regarding incubation time. Different features were studied for three biodegrading bacterial strains and identified as *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Bacillus cereus*. Remarkable degradation rate % patterns for hydrocarbons residual were recorded for all three isolates with varied.

1. Introduction

Petroleum (crude) oil are compounded of thousands compounds mixture. 50–98% of crude oil is Petroleum hydrocarbons which considered a major component depending on the source of the oil. Different microorganisms could be applied for Petroleum hydrocarbons biodegradation. However, bacteria considered important biodegradable microorganisms which play a critical role in hydrocarbon degradation (Udgire et al., 2015).

One of the initiative efficient, economical and environmental treatment mechanisms for petroleum biodegradation is In situ biodegradation through degrade petroleum and other hydrocarbons from culture via widely distributed microorganisms and applied for varied hydrocarbon-contaminated soils and waters (Margesin and Schinner, 1997; Whyte et al., 1997). Thus, continuous evaluation for biodegradation rate considered a critical needed for different biodegradable microorganisms (Alquati et al., 2005). Petroleum hydrocarbons biodegradable illumination depends on the indigenous microorganisms to transform or mineralize the organic contaminants (Fig. 1). Many different factors of contaminated soil characterize influence petroleum hydrocarbons bacterial biodegradation such as pH, electrical conductivity, total nitrogen and heavy metal which are important indicators of soil quality, fertility and productivity. Eight hydrocarbon degrading bacteria were specifically detected as *Alcaligen* sp, *Bacillus* sp, *Chromobacterium* sp, *Corynebacterium* sp, *Pseudomonas* sp, *Aeromonas* sp, *Serratia* sp, and *Flavobacterium* sp, (Gayathiri et al., 2017).

Recently, many advanced molecular culture-dependent techniques (like library clone, TGGE/DGGE, LH-PCR, RISA, RT-Q-PCR, FISH, RAPD and RFLP) were developed and considered a helpful tool for isolation and identification new bacterial strains with degradation capabilities (Stancu, 2018).

Powerful points for Molecular especially, rDNA-dependent methods to identify microorganisms are rapidly, precisely and
reliability analysis of microbial cultures comparing with traditional, biochemical culture-dependent techniques, thus molecular technique has great potential for bacterial identification in new era. 16S rRNA specific molecular marker has been constructed to identify specific bacterial genes (Wang et al., 1996; Wheeler et al., 1996) which dedicated to two methods. Firstly, designed to amplify a wide spectrum of bacterial sequences (Teske et al., 1996; Marchesi et al., 1998).

This investigation was carried out to identify isolated microbial strains from oil-contaminated soils through extensive study consisting of morphological, biochemical and molecular fingerprinting method. Furthermore, hydrocarbons biodegradation capabilities were evaluated for identified isolates through estimated degradation rate % during incubation time.

2. Materials and methods

2.1. Soil samples collection

In this investigation, approximately 10 g of oil-contaminated soil samples were collected from various locations of Khurais oil field (Fig. 2) with an area of 2890 km², 250 km southwest of Dhahran and 150 km east-northeast of Riyadh, 25.0715°N, 48.0556°E, Saudi Arabia in 2018. After taking out 5 cm of perfunctory soil were collected to a depth of 20 cm. Sterile polyethylene bags were used to samples reserving and stored under −20°C. One gram of soil was liquefied in ten ml of double distilled water to prepare soil suspensions.

2.2. Bacterial isolation from contaminated oil soil

To isolate crude oil degrading bacteria, Enrichment technique was used via added 5 g of oil-contaminated soil to 500 ml of mineral salts media in sterilized Erlenmeyer flasks. Sole carbon source was applied as 2000 μl of crude oil and incubated with shaking for 7 days at room temperature. Then, ten-fold serial dilutions were performed for enrichment sample suspension and one millilitre of each dilution was poured into oil agar plates to isolate crude oil utilizing bacteria and incubated at 30°C for 3–7 days. Sub-culturing repeated for selected pure colonies on oil agar plates and transferring on Nutrient agar slants for morphological, biochemical and molecular identification.

2.3. Bacterial isolates identification and characterization

2.3.1. Morphological characterization

Colour, shape, transparency and margin were examined and recorded as colony morphological characteristics according to Cheesbrough (1991). Microscopic features were recorded for all isolates via Gram stain protocol.

2.3.2. Biochemical characterization

2.3.2.1. Oxidase test. Tested bacterial colony was smeared on the filter paper previously saturated with freshly prepared oxidase reagent. Positive oxidase test was recorded as the development of a blue-purple colour within 10 s (Cheesbrough, 1991).
2.3.2.2. Catalase test. Gas bubbles detecting within 10 s after added purified bacterial culture to 5 ml of hydrogen peroxide solution, considered as a positive catalase test (Cheesbrough, 1991).

2.3.2.3. Urease test. Slanted two millilitres of urea medium which placed in bijou bottles applied for the incubated bacterial colony at room temperature. Red-pink colour in the medium was considered as a positive test for urease induction (Cheesbrough, 1991).

2.3.2.4. Indole test. Appearance of bright red and yellow color which composed after added 0.5 ml of Kovac's reagent to incubated bacterial culture at 35 °C for 24 h on SIM media indicated a positive and negative results respectively (Cheesbrough, 1991).

2.3.2.5. Simmons Citrate test. Simmons Citrate test was performed via inculcate Simmons Citrate Agar plates (TSBA, Himedia) surface with bacterial cultures then, incubated at 37 °C up to 48 h. changing media colour from green to bright blue indicate positive reaction.

2.3.2.6. Methyl red (MR) test. After adding methyl red indicator solution (TSBA, Himedia) to inoculated culturing media and incubation at 35 °C for up to 4 days, changing color to red indicate MR test positive- appearance of tested bacteria (Color Atlas and Textbook of Diagnostic Microbiology, 2016).

2.3.2.7. Gelatin hydrolysis. Nutrient gelatin stab method was applied according to Edison et al. (2012). Heavy inoculums of a test bacterium inoculated into tubes containing nutrient gelatin, gelatin liquefaction is the positive results for bacterial gelatin hydrolysis.

2.3.3. Molecular fingerprinting as a confirmatory identification technique

E.Z.N.A.® Bacterial DNA Kit (Omega Bio-Tek, D3350-01, USA) was applied to extract total bacterial genomic DNA. Universal 16S rDNA primers (27 F, 5-AGAGtttGAtcAtGctcAG-3 and 1492 R, 5-tAcGG ttAccttGttAcGActt-3) were applied for bacterial identification through PCR technique with targeting fragment size 1400 bp. Thermal cycler conditions was designed as 94 °C for 5 min, 3 cycles at 94 °C for 45 s, 57 °C for 50 s, 72 °C for 120 s; 3 cycles at 94 °C for 45 s, 56 °C for 30 s, 72 °C for 120 s; 3 cycles at 94 °C for 45 s, 55 °C for 30 s, 72 °C for 120 s; 26 cycles at 94 °C for 45 s, 53 °C for 30 s, 72 °C for 120 s; and a final step at 72 °C for 5 min (Linderman, 1998). 1.5% of Agarose gel was applied for detecting amplified amplicon of 16S rDNA fragment which eluted (E.Z.N. A.® Gel Extraction Kit, Omega Bio-Tek D2500-01, USA), sequenced and alignment through comparing with other sequences from gene bank database using Blast (www.ncbi.nlm.nih.gov/Blast) for bacterial identification (Stach et al., 2001).

2.4. Biodegradation studies

2.4.1. Degradation in liquid medium

Broth (LB) liquid medium containing 1% crude oil was applied for culturing and incubating three investigated isolated at 37°C. Degradation rate % was evaluated spectrophotometrically according to the described method by Odu (1972).

2.4.2. Degradation of contaminated soil

For obtaining soil sample with 1% concentration of crude oil, five grams of drying, sieving and sterilizing polluted soil, was mixed with contaminated oil solution and 1% inoculum was added and cultured at 30 °C for a month. Oil degradation rate was evaluated weighting method weekly and germ-free soil was considered as the control.

Fig. 3. Morphological characters and gram stain for three bacterial strains A, B and C isolated from Khurais oil, Dhahran, Saudi Arabia.
3. Results and discussion

3.1. Strain isolation and identification

Three different oil degrader’s bacteria were selected and isolated from contaminated soil samples from Khurais oil field, 250 km southwest of Dharan and 150 km east-northeast of Riyadh, Saudi Arabia and initially labelled as A, B and C. Fig. 3 and Table 1 illustrated morphological characteristics for isolates. Isolate A was distinguished with rod, Creamy white, Circular flat and entire margin. By contrary, B isolates characterized by cylindrical rods size, white, Irregular large form, negative gram stain and undulate margin. Rods size, Creamy white, Circular flat, entire margin and positive gram stain features characterize isolate C.

Based on biochemical assays (as shown in Table 2) and previous morphological examination, three targeted isolates reflected different biochemical features. Positive for Oxidase test and negative for Indole and Urease tests identified isolate A as Bacillus sp. On the other hand, positive for Oxidase and Catalase tests and negative for Indole and Urease tests refer to Pseudomonas sp. negative oxidase, Indole and Urease tests and positive Catalase test remarked third isolate as Bacillus cereus. Performed morphological colony characteristics examination (Gram stain, mobility and motility, shape and color) and biochemical tests (catalase, urease, oxidase activities, nitrate reduction, Idol production, acid/gas production from carbohydrates and fermentation of sugars) to identify our Hydrocarbons degradation isolates were considered a traditionally identification methods (Ventosa et al., 1982; Smibert and Krieg, 1994; Claus and Berkeley, 1986; Udgire et al., 2015).

16S rDNA fingerprinting method which performed to identify hydrocarbons degradation bacteria is based on highly conserved regions, which help in the analysis. Almost 1.4 Kb of targeted amplicons were amplified, eluted and sequenced (Fig. 4). Regarding morphological, biochemical and molecular features for three obtained hydrocarbon-degrading isolates (as shown by Table 3), strain A was identified as Bacillus subtilis with 99% of homology percentage and B strain was identified as Pseudomonas aeruginosa with 100% and of homology percentage. Third isolate (C) was identified as Bacillus cereus with 99% of homology percentage. As shown by a Fig. 5, Neighbour-joining phylogenetic tree based on 16S rRNA sequence alignments results. The highly genetic similarity was detected among our bacterial isolates (A), (B) and (C) which identified as Bacillus subtilis, Pseudomonas aeruginosa and Bacillus cereus and different strains for Bacillus subtilis, Pseudomonas aeruginosa and Bacillus cereus which indicated our identification results. Our obtaining results for applied 16S rRNA sequences for hydrocarbonoclastic bacteria (HCB) was in accordance of. They compared the limited capability of PCR-based technique such as cell lysis biases ability, a variation of rRNA copy number or different templates amplification efficiency with PCR-based studies which considered as an important procedure to classify microbial diversity. Based on previous findings, 16S rRNA genes considered an effective method to get an association between taxonomic identification with degrading hydrocarbons capability. More support was added to our sequenc-

Table 1
Morphological colony features for three bacterial isolates.

| Isolates | size        | Gram stain | Color     | Form          | Margin   |
|----------|-------------|------------|-----------|---------------|----------|
| A        | Rods        | +          | Creamy white | Circular flat | Entire   |
| B        | Cylindrical rods | –        | white      | Irregular large | Undulate |
| C        | Rods        | +          | Creamy white | Circular flat | Entire   |

Table 2
Three bacterial isolates biochemical tests results.

| Isolates                      | Oxidase test | Catalase test | Simmons Citrate test | Indole test | Methyl Red (MR) test | Urease test | Gelatin Hydrolysis |
|-------------------------------|--------------|---------------|----------------------|-------------|---------------------|-------------|---------------------|
| Bacillus subtilis (A)         | Variable     | (+)           | (+)                  | (−)         | (−)                 | (−)         | (+)                 |
| Pseudomonas aeruginosa (B)    | (+)          | (+)           | (+)                  | (−)         | (−)                 | (−)         | (+)                 |
| Bacillus cereus (C)           | (−)          | (+)           | (+)                  | (−)         | (−)                 | (−)         | (−)                 |

Table 3
16S rDNA sequencing data of the isolated strains A and B from contaminated soils in Khurais oil field; Saudi Arabia.

| Strain number | Total length (bp) | Gene bank accession no. | Identification      | Identity % |
|---------------|-------------------|-------------------------|---------------------|-----------|
| A             | 1440              | KC197028.1              | Bacillus subtilis   | 99        |
| B             | 1417              | MGR18964.1              | Pseudomonas aeruginosa | 100       |
| C             | 1500              | KR071870.1              | Bacillus cereus     | 99        |
ing identification data based on findings of Wieckowicz (2009) via clearing efficiency of a sequencing technique for bacterial identification through sequences alignments lower than 3%. More light was added to our findings by Subathra et al. (2013) through applied both biochemically and phylogenetically methods to identify 3 crude oil biodegradation bacteria isolates as *Bacillus subtilis* I1, *Pseudomonas aeruginosa* I5 and *Pseudomonas putida* I8. Furthermore, our obtaining results for applying biochemical tests and 16S rRNA sequences to identify Petroleum-degrading bacteria were in agreement of Godini et al. (2018). They identified *Brevibacillus* sp., *Microbacterium oxydans*, *Staphylococcus arlettae*, *Staphylococcus warneri*, *Methylobacterium persicinum*, and *Achromobacter xylosoxidans* as degrading bacteria in the light of biochemical and molecular identification results.

**Fig. 5.** Phyllogenetic tree for three degradation of petroleum hydrocarbons bacterial isolates based on 16S rDNA sequence.

**Fig. 6.** Degradation dendogram of petroleum hydrocarbons of 7, 14, 21 and 24 days for *Bacillus subtilis* in liquid medium and in polluted soil.

**Fig. 7.** Degradation dendogram of petroleum hydrocarbons of 7, 14, 21 and 24 days for *Pseudomonas aeruginosa* in liquid medium and in polluted soil.

**Fig. 8.** Degradation dendogram of petroleum hydrocarbons of 7, 14, 21 and 24 days for *Bacillus cereus* in liquid medium and in polluted soil.
Degradation % of petroleum hydrocarbons of isolates Bacillus subtilis, Pseudomonas aeruginosa and Bacillus cereus in liquid medium and in polluted soil after 7, 14, 21 and 28 days of incubation.

| Incubation days | Degradative samples | Degradation % |
|-----------------|---------------------|---------------|
|                 | Bacillus subtilis    | Pseudomonas aeruginosa | Bacillus cereus |
| 7 days          | Liquid medium 20     | 15             | 19             |
|                 | Polluted soil 25     | 16             | 25             |
| 14 days         | Liquid medium 38     | 20             | 20             |
|                 | Polluted soil 30     | 32             | 27             |
| 21 days         | Liquid medium 50     | 30             | 55             |
|                 | Polluted soil 55     | 38             | 68             |
| 28 days         | Liquid medium 52     | 56             | 46             |
|                 | Polluted soil 70     | 68             | 50             |

3.2. Evaluation of hydrocarbons degradation rate %

Different patterns were monitored, recorded and detected for hydrocarbons degradation rate in liquid media and soil for Bacillus subtilis, Pseudomonas aeruginosa and Bacillus cereus, as shown by Figs. 6–8 and Table 4. Generally, degradation rate in polluted soil was superior compared with liquid media. Hydrocarbons degradation rate of Bacillus subtilis was increasing gradually by incubation time. Degradation rate % was 20, 38, 50, 52 and 25, 30, 55, 70 after 7, 14, 21 and 28 days of Hydrocarbons incubation with liquid media and contaminated soil respectively. Interestingly, incubation Hydrocarbons in liquid media for 14 days was superior for biodegradation rate % comparing with Hydrocarbons incubation for contaminated soil. Pseudomonas aeruginosa remarked with low Hydrocarbons degradation rate % after 21 incubation days comparing with Hydrocarbons degradation rate of Bacillus subtilis. Unique Hydrocarbons degradation rate distinguished Bacillus cereus with highest degradation rate % after 21-days of incubation and sudden decrease after 28 days of incubation. Our findings of compared residual crude oil quantitatively with control sample were reflected varied biodegradation ability for different bacterial genera for varied incubation time (Mirdamadian et al., 2010).

4. Conclusion

Three bacterial strains isolate from the contaminated soil of Khurais oil field Dhahran, Saudi Arabia were isolated and characterized for hydrocarbons degradation capability. Bacterial identification was carried out though morphological and biochemical methods and confirmed via 16S rDNA sequence fingerprinting method as Bacillus subtilis, Pseudomonas aeruginosa and Bacillus cereus. Distinguishable capability for hydrocarbons degradation was evaluated for three isolates which reflected varied degradation rate % patterns.

References

Allen, S.D., Jard, W.M., Schreckenberger, P. C., Winn W.C., 2016. Color Atlas and Textbook of Diagnostic Microbiology. In: Koneman, Elmer W. (Ed.), 4th ed. AplacIni, C., Papacchini, M.R., Spicaglia, C., Bestetti, G., 2005. Diversity of naphthalene-degrading bacteria from a petroleum contaminated soil. Ann. Microbiol. 55, 237–242.

Cheesbrough, M., 1991. Medical Laboratory Manual for Tropical Countries. University Press, Cambridge.

Claus, D., Berkeley, R.C.W., 1986. Genus Bacillus Chon. In: Sneath, P.H.A., Mair, N.S., Sharpe, M.E., Holt, J.G. (Eds.), Bergey’s Manual of Systematic Bacteriology. The Williams & Wilkins Co., Baltimore, Md, pp. 1105–1129.

Edison, T.E., Cruz, D., Martin, J., Torres, O., 2012. Gelatin hydrolysis test protocol. Am. Soc. Microbiol.

Gayathiri, E., Bharathir, B., Selvadas, S., Kalaikandhan, R., 2017. Isolation, identification and molecular characterization of hydrocarbon degrading bacteria and its associated genes - a review. Int. J. Pharm. Bio. Sci. 8 (2), 1010–1019 (B).

Godini, K., Samarghandi, M.R., Zafari, D., Rahmani, A.R., Akhami, A., Arabestani, M. R., 2018. Isolation and identification of new strains of crude oil degrading bacteria from Kharg Island, Iran. Pertol. Sci. Technol. 36 (12).

Linderman, R.G., 1998. Mycorrhiza interactions with the rhizospheric microflora: the mycorrhizosphere effect. Phytopatology 78, 366–371.

Marchesi, J.R., Saro, T., Weightman, A.J., Martin, T.A., Fry, J.C., Hiom, S., Wade, W.G., 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl. Environ. Microbiol. 64, 795–799.

Margesin, R., Schinner, F., 1997. Efficiency of indigenous and inoculated cold adapted soil microorganisms for biodegradation of diesel oil in Alpine soils. Appl. Environ. Microbiol. 63, 2660–2664.

Mirdamadian, S.H., Emtriizi, G., Golabi, M.H., Ghavanati, H., 2010. Biodegradation of petroleum and aromatic hydrocarbons by bacteria isolated from petroleum-contaminated soil. J. Pet. Environ. Biotechnol. 1, 102. https://doi.org/10.4172/2157-7463.1000102.

Odu, C.T.I., 1972. Microbiology of soil contaminated with petroleum hydrocarbons. Extent of contamination and some soil microbial properties after contamination. J. Inst. Petrol. 58, 201–208.

Smibert, R.M., Krieg, N.R., 1994. In: Phenotypic characterization Methods for general and molecular bacteriology. American Society for Microbiology, pp. 611–651.

Stach, J.E., Bathe, S., Clapp, J.P., Burns, R.G., 2001. PCR-SSCP comparison of 16S rDNA sequence diversity in soil DNA obtained using different isolation and purification methods. FEMS Microbiol. Ecol. 36 (2-3), 139–151.

Stancu, M.M., 2018. Bacterial degradation of petroleum and petroleum products. J. Mol. Microbiol. 2 (1), 2.

Subathra, M.K., Immanuel, G., Suresh, A.H., 2013. Isolation and Identification of hydrocarbon degrading bacteria from Ennore creek. Bioinformation 9 (3), 150–157.

Teske, A., Sigalievich, P., Cohen, Y., Muyzer, G., 1996. Molecular identification of bacteria from a coculture by denaturing gradient gel electrophoresis of 16S ribosomal DNA fragments as a tool for isolation in pure cultures. Appl. Environ. Microbiol. 62, 4210–4215.

Udgire, M., Shah, N., Jadhav, M., 2015, Enrichment, isolation and identification of hydrocarbon degrading bacteria. Int. J. Curr. Microbiol. Appl. Sci. 4 (6), 708–713. ISSN: 2319-7708.

Ventosa, A., Quesada, E., Rodriguez-Valera, F., 1982. Numerical taxonomy of moderately halophilic Gram-negative rods. J. Gen. Microbiol. 128 (9), 1959–1968.

Wang, R.F., Cao, W.W., Cerniglia, C.E., 1996. PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. Appl. Environ. Microbiol. 62, 1242–1247.

Wheeler, A.E., Oerther, D.B., Larsen, N., Stahl, D.A., Raskin, L., 1996. The oligonucleotide probe database. Appl. Environ. Microbiol. 62, 3557–3559.

Whyte, L.G., Bourbonnière, L., Greer, C.W., 1997. Biodegradation of petroleum hydrocarbons by psychrotrophic Pseudomonas strains possessing both aalkane (alk) and naphthalene (nah) catabolic pathways. Appl. Environ. Microbiol. 63, 3719–3723.

Węckowicz, M., 2009. Molecular methods for the identification of microorganisms in complex communities. Postepy Mikrobiolii 48 (1), 67–73.