ORIGINAL PAPER

Microbial removal of engine oil from polluted media (the case study: Hamadan City)

Fariba Mohsenzadeh✉, **Sanaz Safaie**

Laboratory of Microbiology, Department of Biology, Faculty of Science, Bu-Ali Sina University, Hamedan, Iran

Date of submission: 19 Mar 2016, ***Date of acceptance:*** 25 Sep 2017

ABSTRACT

Discharging industrial wastes into the soil causes accumulation of pollutants, especially petroleum hydrocarbons and used engine oil, in the environment. This study was done to find some bacterial strains capable of removing the engine oil from contaminated soils. Soil samples contaminated by engine oil were collected from some polluted area of Hamadan City, and then their bacterial strains were isolated and classified on the basis of morphological keys and biochemical tests. The efficiency of these bacterial strains in removing the pollutant was evaluated in minimal salt medium (MSM) containing 1% engine oil. The optical density (OD) of the media was measured as an indicator of bacterial growth and degradation of the engine oil during day 0, 5, 10, and 15 at 600 nm wavelength. The bacterial strain responsible for the highest OD was chosen as the effective one, and the efficiency of oil removing was evaluated for this bacterial strain. In this study, eight bacterial strains called $EODB_{1-8}$ were isolated. The results of the OD determination showed that the four bacterial strains caused more turbidity in the samples. The effects of time, type of bacteria, and their interaction effects were also significant in all samples. The turbidity of the samples was increased with increasing exposure time. The maximum turbidity was caused by the bacterial strain $EODB_4$ and it was introduced as the best engine oil degrading bacterial strain. Its engine oil removal efficiency was evaluated to be 62.85% and it was identified as *Pseudomonas alcaligenes* using morphological keys and biochemical identification methods.

Keywords: Bioremediation, Engine oil, *Pseudomonas alcaligenes*, Soil pollution

Abbreviations: OD, optical density; EODB, engine oil degrading bacterium; NA, nutrient agar; MSM, minimal salt medium

Introduction

Soil is the basis of life, productivity, and a source of raw materials; it plays an important role in human's life. Soil protection as a task in environmental policy is essential due to its important role for all organisms.[1,2] Problems of oil pollution and its derivatives as well as its detrimental effects on living organisms and ecosystems are not negligible.[3]

Lubricant oil (engine oil) is one of the petroleum-derived soil contaminants. Engine oil as a multipurpose compound plays an important role in the safe operation of car engines.[4] Due to the existence of a wide variety of dangerous pollutants and high levels of such wastes in industrial regions, systematic management of wastes containing used engine oil is a serious challenge for industrial societies.[2,5]

Bioremediation is now one of the common technologies used to clean and remediate petroleum hydrocarbon-contaminated soils.[6] Bioremediation is a method that uses the ability of existing organisms and microorganisms to increase the amount and rate of pollutant degradation. It is also an important method for decreasing environmental pollution.[7]

Microorganisms can grow and multiply in engine oil-polluted media due to their high metabolic capacity for using the pollutant as a nutrient and energy source. They reduce the contamination rate of the soils considerably.[8] There are significant differences between the

✉ *Fariba Mohsenzadeh*
fmohsenzadeh@gmail.com

Citation: Mohsenzadeh F, Safaie S. Microbial removal of engine oil from polluted media (the case study: Hamadan City). J Adv Environ Health Res 2017; 5(2): 114-122

petroleum-degrading bacteria regarding their ability in degradation of petroleum compounds.[9] Therefore, isolation and purification of different kinds of microbial strains capable of degrading petroleum compounds can be useful for bioremediation of polluted areas.[10, 11]

There are several studies available showing that different bacterial strains exist in the soils contaminated by petroleum compounds that can be used as sources of carbon.[12] Oil pollution not only is incapable of inhibiting the bacterial growth but also causes to improve growth of the resistant bacteria.[4,12] Khan et al.[13] isolated four bacterial strains and one of which was MJH_{1101} which has the most ability in degrading petroleum compounds and decreased the thickness of an oil layer from 6 to 1 mm in 7 days of incubation. In another research by Emtiazi et al.,[14] they succeeded in removing the petroleum compounds using *Pseudomonas* strains in a period of 9 days. Onifade and Abubakr[15] investigated the potential role of hydrocarbon-degrading bacteria, e.g., *Lactobacter* sp., *Arthrobacter* sp., *Bacillus* sp., *Pseudomonas* sp., *Micrococcus* sp., in crude oil-contaminated soils and reported an increase in bacterial number from 5×10^3 to 7.6×10^4 CFU/ml after 18 weeks of incubation. Degradation of phenanthrene by a mixed culture or individual strains isolated from soil in a refinery area in South Korea was examined and a significantly positive relationship was observed between the microbial growth and the rate of the phenanthrene degradation.[1,16]

Due to a global increase in environmental pollution by petroleum and its derivatives as well as the differences between the ability of bacterial strains for degradation of petroleum compounds under different climate conditions, we need to find native bacterial strains for use in local bioremediation.[17] The aims of this study were to isolate and identify native bacterial strains in the soils contaminated by engine oil in Hamadan, and then to evaluate their biodegradation ability in removing engine oil contamination.

Materials and Methods

Sampling places

Some engine oil-contaminated areas in Hamadan were randomly selected. Heavy machinery repair centers and engine oil spots around and beside the roads were selected as sampling sites. The soil samples were collected in spring 2014 and in depths of 0–20 cm from the contaminated areas. Collected soil samples were placed in disposable bags and transferred to the laboratory for further process.

Preparation of samples

After the separation of roots and debris, soil samples were passed through a 2 mm mesh and then homogenized. Then the samples were kept at 4 ºC for further process.

Culture of samples

To make different dilutions, 1 g of the soil sample was initially weighed and then it was dissolved in 10 ml of sterile physiological salt solution for preparation of 10^{-1} dilution of the sample. Successive dilutions of 10^{-2} to 10^{-6} were prepared similarly.[18] Nutrient Agar (NA) was prepared as a growth medium and for enrichment, yeast extract was added. It was sterilized in an autoclave for 15 min at 121 ºC and 15 lb/in^2. After cooling down to 44 ºC, it was distributed between sterilized plates. After freezing the media, the plates were incubated for 24 h at 30 °C.[19]

One milliliter of each abovementioned soil dilution was added to each group containing three sterilized plates. Then 10 ml of the nutrient agar medium containing sterilized yeast extract was added to each plate and it was mixed with the sample. The plates were then incubated for 48 h at 25 ºC. After this period, the colonies formed on the plates were visible and ready for isolation.[13] The purification of the isolated microorganisms was done using linear cultivation.[18,19]

Characterization and determination of the isolated bacteria

The isolated bacterial colonies were characterized based on morphological characteristics such as their color, size, edge, height, and the consolidation of the colony as well as biochemical tests such as catalase and oxidase activities, gram staining, indole

production, citrate consumption, etc., according to laboratory manuals and taxonomical keys.[20]

Studying the potential of contamination elimination by the bacterial strains
Preparation of minimal salt medium containing 1% engine oil

This medium contained the minimal essential salts for bacterial growth (Table 1). After preparation of the medium, it was poured into an Erlenmeyer flask up to 99 ml and then 1 ml of engine oil was added to the medium. Erlenmeyer flask caps were covered by cotton and then sterilized in an autoclave for 15 min at 121 ºC and 15 lb/in^2.[13]

Table 1. Salt composition in the used MSM medium

Material	Nacl	KCl	$CaCl_2$	Na_2HPo_4	$MgSO_4$	$FeSO_4$	Glucose	NH_4Cl
Concentration (g/l)	0.8	0.8	0.1	2.0	0.2	0.1	8	2.0

Treatment of engine oil contained media by the bacterial strains

One colony of each isolated bacterial strain was initially selected as a representative. The bacteria were grown in NB to increase the population of bacteria in the inoculum. After achieving OD = 1, bacterial sediments were separated using a centrifuge (at 4000 rpm) for 10 min. The sediment was inoculated in flasks containing sterilized MSM (minimal salt medium) containing 1% engine oil prepared previously. All the flasks were shaken for 2 weeks at 24 ºC on a shaker.[4]

Assessment of the sample turbidity

The growth rate of the microbial biomass in the treated samples was measured using turbidity of the samples. For this purpose, the light absorbance was measured for the samples through the turbidity by spectrophotometry at wavelength 600 nm within 15 days including day 5, 10, and 15.[14,18,21]

Study of pollutant removal rates by bacteria

To measure the amount of removed engine oil from the sample, the samples were poured into separate vials under a sterile hood. 100 µl of tetracosane was added to each vial. The vials were centrifuged (at 4000 rpm) for 10 min to isolate the engine oil from the salt solution.[19] The engine oil was separated from the solution and then weighed. The amount of removed engine oil was determined using the following formula:[13]

$$R = \left(\frac{P_0 - P_e}{P_0}\right) \times 100 \quad (1)$$

In this equation, R is the percentage of engine oil removal by each bacterial strain, P_0 is the weight of the engine oil in the blank flask (mg), and P_e is the final weight of the engine oil in the flasks treated by the bacterial strains after 2 weeks (mg).

Since there is an evaporation possibility for engine oil from the samples, due to their volatility, control samples were also tested for pollutant removing. The control samples were prepared like the experimental ones but without bacterial inoculation.

Statistical analysis of the results

Data analysis were performed using SPSS version 18.0. Variance analysis was performed for significance data. The least significant difference (LSD) used was $P \leq 0.01$ for separating means. A one-way analysis of variance (ANOVA) was performed for the various bacterial strain indexes.[19]

Identification of the bacteria with a potential of engine oil degradation

The bacteria that were capable of degrading engine oil compounds in MSM media were chosen and their biochemical characteristics were determined (Table 3). Finally, the bacterium with the highest ability of engine oil removal efficiency was introduced as the most powerful consumer bacterium for engine oil.

Results and Discussion

Environmental pollution by engine oil is a common problem in most countries.[13] There are several methods for cleaning engine oil-polluted soils such as incineration, interring into the ground, etc. However, they are costly and in the long run have undesirable environmental impacts.[19] Bioremediation is the best method for removing oil contamination in aquatic and land systems.[22] Bioremediation is a flexible, cost-effective, and environmental friendly method for cleaning oil-polluted areas.[10] According to studies, using native and locally isolated bacteria from polluted areas is an effective method for bioremediation of soil hydrocarbons.[3] The metabolic rate and genetic compatibility of the microbial populations in the environment play an important role in the successful cleanup of contaminated environments.[7,19]

This study was done to find some native bacterial species capable of degrading engine oil in the cold climate (Hamadan). After culturing the oil-contaminated soils collected from the areas around the city of Hamadan and isolation of their bacteria, the bacterial strains were characterized based on morphological futures and some properties of the generated colonies (Table 2) and also biochemical tests (Table 3). According to the results, eight bacterial strains called $EODB_{1-8}$ were isolated from the engine oil-contaminated soils.

Table 2. Characterization of isolated bacterial colonies

Strains	Characters					
	Morphology	Color	Elevation	Edge of the colony	Shape	Size
$EODB_1$	Bacillus	Milky	Flat	Entire	Circular	Large
$EODB_2$	Bacillus	Milky	Flat	Entire	Circular	medium
$EODB_3$	Coccus	Light yellow	Flat	Entire	Irregular	medium
$EODB_4$	Bacillus	Dark yellow	Convex	Filamentous	Circular	medium
$EODB_5$	Bacillus	Beige	Convex	Entire	Circular	Small
$EODB_6$	Bacillus	Orange	Flat	Serrate	Circular	medium
$EODB_7$	Coccus	Cream	Raised	Entire	Circular	medium
$EODB_8$	Bacillus	Bright Red	Convex	Entire	Circular	Large

Table 3. Biochemical characterization of the isolated bacterial colonies

Species	Test							
	Citrate	H_2S production	Spore	Indole	Gram stain	Movement	Oxidase	Catalase
$EODB_1$	+	-	+	+	+	+	+	+
$EODB_2$	+	-	+	-	-	+	-	+
$EODB_3$	+	-	-	-	-	+	+	+
$EODB_4$	-	-	+	+	+	-	+	+
$EODB_5$	+	-	-	-	-	+	-	+
$EODB_6$	+	+	+	-	-	-	+	+
$EODB_7$	-	-	+	+	-	+	+	-
$EODB_8$	+	-	+	+	+	+	-	+

After treatment of MSM with 1% engine oil and different bacterial strains, the analysis of their turbidity during 15 days showed that the OD of all samples was increased with increasing time. The highest OD was observed after 15 days in the all groups (Figure 1).

In this study, day 0, 5, 10, and 15 were selected for studying the effect of exposure time on engine-oil degradation by the isolated bacteria. The results showed that the best exposure time is day 15. Some prior studies showed that 20 days of exposure had the maximal degradation of petroleum compounds.[4,13,14]

According to our results, the growth rate of the bacterial strains increased with time. This means that the bacteria produced enzymes needed to break down the pollutants due to exposure to pollutant and cause bacterial multiplication.[29, 30,31,32]

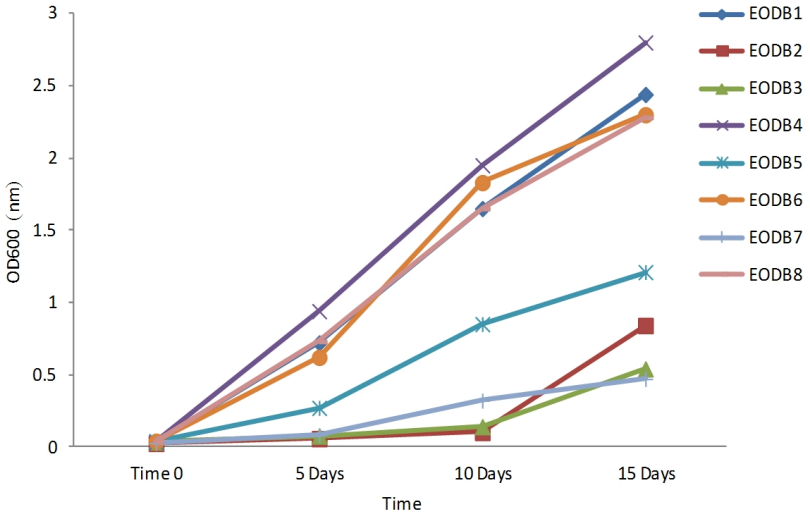

Fig. 1. Average level of turbidity of the isolated bacterial strains during the different times.Each datum represented the means of at least five samples

The average OD for different bacterial strains was compared and the results showed that there was not any significant difference between $EODB_6$ and $EODB_1$ treatments (Figure 2). It actually showed that the two collected species from the engine oil-polluted soils have the same level of performance, so these two treatments are at the same level. $EODB_4$ with a mean OD of 1.43 should be regarded as the best and most powerful bacteria with the highest growth in media containing 1% engine oil. Also, $EODB_2$, $EODB_3$, and $EODB_7$ with minimal OD values were considered as low-efficiency strains in engine oil-polluted media.

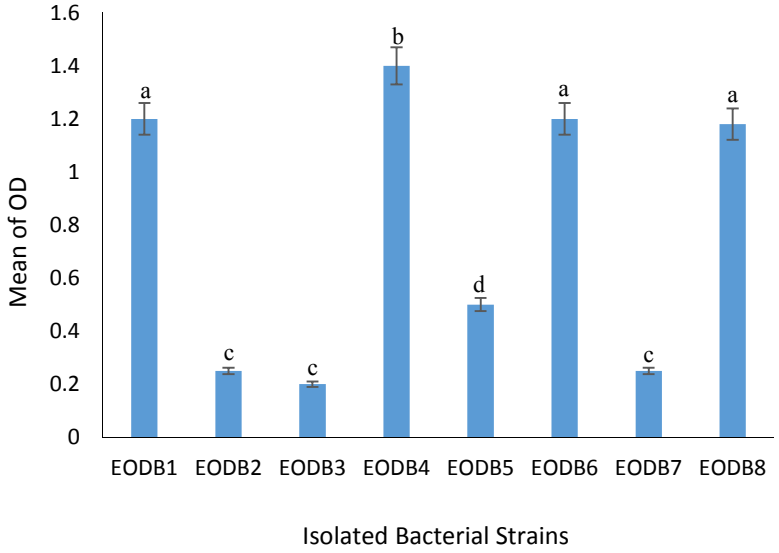

Fig. 2. Average turbidity of bacterial strains at concentration of 1% engine oil, after 15 days. Each datum represented the means ± SE of at least five samples. Different letters indicated the significant differences between the studied groups ($P \leq 0.01$).

Their efficiency and growth rate were assessed in mineral-salt broth containing 1% engine oil for 15 days at 24 ºC. The results showed that four bacterial strains including $EODB_1$, $EODB_4$, $EODB_6$, and $EODB_8$ had better growth ability under the engine-oil pollution.

To study the engine oil removal rates by the isolated bacterial strains, the remaining engine oil was measured in each experimental group after 15 days. The reduction rate and the biological removal rate of engine oil were

compared in the control and experimental groups (Figure 3). Results showed that $EODB_4$ and $EODB_3$ had the best and worst removal efficiency (64.85% and 39.33%), respectively. The decrease in engine oil in the control group was about 15.6%. The statistical analysis showed that the effect of bacterial factors, time, and their interaction (bacteria/time) were significant in pollution removing ($P \leq 0.05$).

Khan et al.[13] were able to isolate a petroleum-resistant bacterium that showed oil degradation with an efficiency of 84.41% during 7 days of incubation. Two bacterial strains, namely, TMY-2 and TMY-3, were isolated from gasoline-polluted soils that had a petroleum removing efficiency of 87% and 80%, respectively. Our isolated bacteria ($EODB_4$) showed the ability near to abovementioned reports.

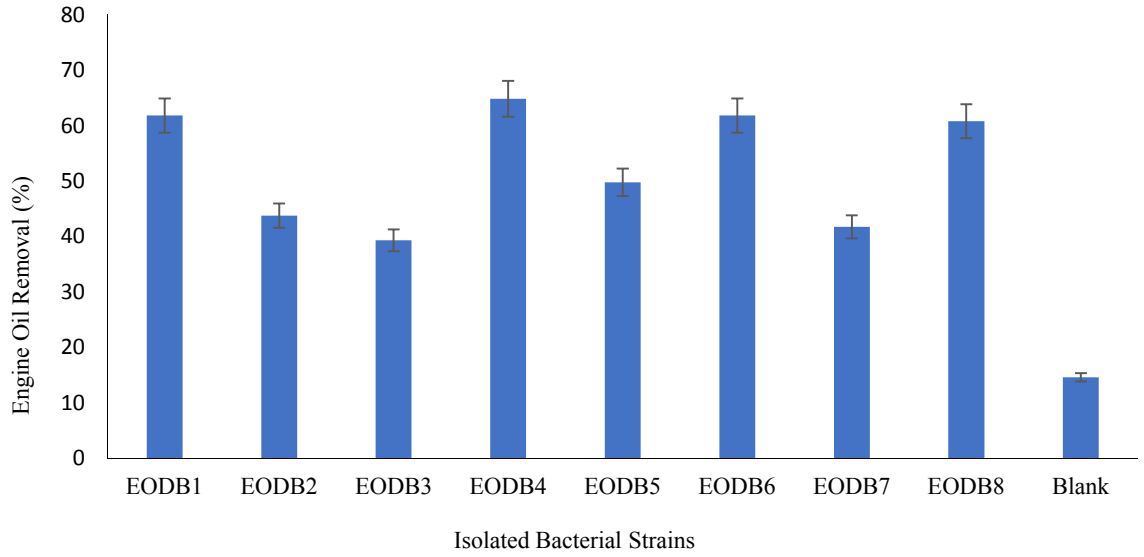

Fig. 3. Efficiency of removing engine oil by the isolated bacterial strains (%). Each datum represented the mean of at least five samples. The number of bacteria was 10^3 $CFUml^{-1}$ at the beginning of experiment. Different letter indicated significant differences between the studied groups ($P \leq 0.01$).

Finally, the bacterial strain $EODB_4$ was chosen as the best engine oil consumer due to having the most turbidity in the media and the most engine oil degrading efficiency and it was introduced as an indicator of the engine oil consumer bacteria in the contaminated soils of Hamadan. The taxonomical position of this bacterial strain was determined according to Bergey's Handbook using biochemical tests and taxonomical keys.[20] The bacterial strain was identified and reported as *Pseudomonas alcaligenes* (Tables 2, 3, and 4).

Table 4. The results of special tests for final determination of the engine oil degrading bacterial strain (the bacterium number 4)

Tests	Oxidation	Oxidase	Levan	Citrate	Starch's Hydrolyze	Casein Hydrolyze	KOH
Results	+	+	-	+	-	-	+
Tests	Methyl Red	Vosges Prosquer	Urea's Hydrolyze	Lipase	Gelatin's consumption	Phosphate	Movement
Results	-	-	+	+	-	+	+
Tests	H_2S Produce	Ketolactose Produce	Phenylalanine deaminase	Gas production from Glucose	Acid production from Glucose	Pectolytic Action	Indole
Results	+	-	+	-	+	-	-
Tests	Catalase	Growth at 4 ºC	Growth at 41 ºC	Resistance to 3% salinity	Resistance to 5% salinity	Resistance to 7% salinity	Lysine Decarboxylase
Results	+	-	+	+	+	+	-

Our results showed that all isolated bacteria from the engine oil-contaminated soils were able to grow in a medium containing engine oil. This means that the bacteria growing in the engine oil-contaminated soils have developed a metabolism not only for resistance against engine oil pollution but also for using engine oil as a carbon source due to prolonged exposure to this pollutant. This property of bacteria tends to their ability for removing engine oil pollution from the environment. Other researchers showed that a wide range of bacteria are capable to remove petroleum compounds from the environment which is in accordance with our findings.[1,4,13,14,19,23-28]

In conclusion, the results showed that the highest OD was evaluated in the treated group by the bacterium EODB4 which was a gram positive, catalase positive, and mobile bacterium producing dark yellow colonies in the nutrient agar media. This bacterium was determined as *P. alcaligenes* using the taxonomical keys and biochemical tests. The *Pseudomonas* species was reported as oil degradable bacteria by some prior researchers.[33,19] Some other reports showed the ability of *Pseudomonas* species in removing TPHs and other petroleum-derived compounds.[19,33,34,35] In another study, five *Pseudomonas* strains were isolated and reported as capable of using oil compounds.[14]

Conclusion

According to the results of this study and other prior studies, it can be concluded that using indigenous microorganisms is a very effective method for soil cleaning from engine oil pollution, without any adverse environmental impacts. On the other hand, it is the most efficient method for decomposition of pollutants within a short time. Since the growth potency of the bacterial strains and their ability for removing environmental pollutants depend on ecological conditions,[19] it can be concluded that bacterium No. 4 is the most effective one under Hamadan's climatic conditions. The bacterium was identified and introduced as *Pseudomonas alcaligenes* and it was proposed that further research should be performed for cleaning oil-polluted soils in a field study.

Acknowledgement

This research was done using a grant provided by the research council of Bu-Ali Sina University.

References

1. Norris RD, Hinchee RE, Brown RA, McCarty PL, Semprini L, Wilson JT, et al. Handbook of Bioremediation. Boca Raton, FL: CRC Press; 1994.
2. Speight J, Arjoon K. Bioremediation of Petroleum and Petroleum Products .Chichester: Wiley;2012.
3. Bento F, Camargo F, Okeke B, Frankenberger-Júnior W. Bioremediation of soil contaminated by diesel oil. Brazilian Journal Microbiology. 2003; 34: 65-68.
4. Mandari T, Lin J. Isolation and characterization of engine oil degrading indigenous microorganisms in Kwazulu- Natal, South Africa. African Journal of Biotechnology. 2007; 6: 23–27.
5. Amiri A, Ghorban A, Ghanbarzadeh LM. Life cycle inventory of used lubricating oil recycling facilities in Iran. 2011; ISWA World Congress, Daegu, Korea.
6. Baker K, Herson D. Bioremediation.New York: McGraw-Hill;1994.
7. Abdulyekeen KA, Muhammad IM, Giwa SO, Abdulsalam S. Bioremediation of used motor oil contaminated soil using elephant and horse dung as stimulants. IOSR Journal of Environmental Science, Toxicology and Food Technology. 2016; 10: 73-78.
8. Kastner M, Breuer-Jammali M, Mahro B. Impact of inoculation protocols, salinity, and pH on the degradation of polycyclic aromatic hydrocarbons (PAHs) and survival of PAH degrading bacteria introduced into soil. Applied Environmental Microbiology. 1998; 64: 359-362.
9. Baker BJ, Tyson GW, Webb RI, Flanagan J, Hugenholtz P, et al. Lineages of acidophilic archaea revealed by community genomic analysis. Science. 2006; 314: 1933-1935.
10. Brown JL, Syslo J, Lin YH, Getty S, Vemuri R, Nadeau R. On-site treatment of ontaminated soils: An approach to bioremediation of

weathered petroleum compounds. Journal of Soil Contamination. 1998; 7: 773-800.
11. Chhatre SA, Purohit HJ, Shanker R, Chakrabarti T, Khanna P. Bacterial consortia for crude oil spill remediation. Water Science Technology. 1996; 34(10):187–193.
12. Usman D H, Ibrahim AM, Abdullahi S. Potencials of bacterial isolates in bioremediation of petroleum refinary wastewater. Journal of aplied hytotechnology in Environmetal Sanitation. 2012; 1: 131-138.
13. Khan JA, Rizvi SHA. Isolation and characterization of microorganism from oil contaminated sites. Advances In Applied Science Research. 2011; 2: 455-460.
14. Emtiazi G, Shakarami H, Nahvi I, Mirdamadian SH. Utilization of petroleum hydrocarbonsby Pseudomonas sp. And transformed Escherichia coli. Africa Journal of Biotechnology. 2005; 4(2): 172–176.
15. Onifade AK, Abubakar FA. Characterization of hydrocarbon-degrading microorganisms isolation from crude oil contaminated soil and remediation of the soil by enhanced natural attenuation. Research Journal of Biological Science. 2007; 2(2): 36-40.
16. Jeing-Dong K, Su-Hyeun Sh, Choul-Gyun L. Degradation of phenanthrene by bacterial strains isolated from soil in oil refinery fields in Korea. Journal of Microbiology and Biotechnology .2005; 15(2): 337-345.
17. Mohsenzadeh F, Nasseri S, Mesdaghinia A, Nabizadeh R, Zafari D, Chehregani A. Phytoremediation of petroleum-polluted soils: Application of Polygonum aviculare and its root-associated (penetrated) fungal strains for bioremediation of petroleum-polluted soils. Ecotoxicology and environmental safety. 2010; 73(4): 613-619
18. Delille D, Bassères A, Dessommes AA. Effectiveness of bioremediation for oil-polluted Antarctic seawater. Polar Biology. 1998; 19(4): 237–241.
19. Mohsenzadeh F, Nasseri S, Mesdaghinia A, Nabizadeh R, Zafari D, Chehregani A. Phytoremediation of petroleum-contaminated soils: Pre-screening for suitable plants and rhizospheral fungi. Toxicological and Environmental Chemistry. 2009; 91(8): 1443-1453.
20. Garrity G. Bergey's manual of systematic bacteriology, 2001-2005. Springer Verlag Science and Business Media, Germany.
21. Dellagnezze BM, Pantaroto de Vasconcellos S, Soares de Melo I, Santos Neto EV, Maia de Oliveira V. Evaluation of bacterial diversity recovered from petroleum samples using different physical matrices. Brazilian Journal of Microbiology. 2016; 47(3): 712-273.
22. Leblond JD, Schultz TW, Sayler GS. Observations on the preferential biodegradation of selected components of polyaromatic hydrocarbon mixtures. Chemosphere. 2001; 42(2): 333-343.
23. Dahle H, Garshol F, Madsen M, Birkeland NK. Microbial community structure analysis of produced water from a high-temperature North Sea oil-field. Anton Leeuw. 2008; 93(1-2):37–49.
24. Bhattacharya M, Biswas D. Enhancement of waste engine oil biodegradation by optimization of media using factorial desgn study. Indian Journal of Biotechnology. 2014; 13: 193-300.
25. Tabatabaee A, Assadi MM, Noohi AA, Sajadian VA. Isolation of biosurfactant producing bacteria from oil reservoirs. Iranian Journal of Environmental Health Science & Engineering. 2005; 2(1): 6–12.
26. Udeani TKC, Obroh AA, Azubike N. Isolation of bacteria from mechanic workshops soil environment cntaminated with used engine oil. African Journal of Biotechnology. 2009; 8(22): 6301-6303.
27. Sarkar D,Ferguson M, Datta R, Birnbaum S. Bioremediation of petroleum hydrocarbons in contaminated soils: comparison of bio solids addition, carbon supplementation, and monitored natural attenuation. Environmental Pollution. 2003; 136(1): 187–195.
28. Ebrahimi M, Fallah AR, Sarikhani MR, Taheri MT. Isolation, purification and identification of oil-degrading bacteria from oil-polluted sites of bushehr. 12th Iranian Soil Science Congress. 2011; Tabriz, Iran.
29. Hong L, Jiti Z, Jing W, Weilei S, Hu T, Guangfei L. Enhanced biodecolorization of azo dyes by anthraquinone-2-sulfonate immobilized covalently in polyurethane foam. Bioresour Technol. 2010; 101(18):7185–7188.
30. Wang LY, Ke WJ, Sun XB, Liu JF, Gu JD, Mu BZ. Comparison of bacterial community in aqueous and oil phases of water-flooded petroleum reservoirs using pyrosequencing and clone library approaches. Appl Microbiol Biotechnol. 2014; 98(9): 4209–4221.

31. Boll M, Heider J. Anaerobic degradation of hydrocarbons: mechanisms of C-H-Bond activation in the absence of oxygen. In: Timmis KN, ed. Handbook of Hydrocarbon and Lipid Microbiology. Berlin, Heidelberg: Springer-Verlag; 2010:1012–1024.
32. Thapa B, Ajay Kumar KC, Ghimire A. Areview on bioremediation of petroleum hydrocarbon contamiants in soil. Kathmandu University Journal of Science, Engineering and Technology .2012; 8(1): 164-170.
33. Talaie AR. Parametric study of petroleum compounds biodegradation using microorganisms [MsC Thesis]. 2008; Islamic Azad University, Science and Research Branch, Ahvaz, Iran.
34. Butler CS, and Mason JR. Structure–function analysis of the bacterial aromatic ring–hydroxylating dioxygenases. Advanced Microbial Physiology. 1997; 38: 47-84.
35. Orji FA, Iblene AA, Dike EN. Laboratory scale bioremediation of petroleum hydrocarbon – polluted mangrove swamps in the Niger Delta using cow dung. Malaysian Journal of Microbiology. 2012; 8(4): 219-228.
36. Subarna R, Dipak H, Debabrata B, Dipa B, Ranajit K. Survey of petroleum-degrading bacteria in coastal waters of Sunderban Biosphere Reserve. World Journal of Microbiology and Biotechnology. 2002; 18(6): 575-581.