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Original Article

Isolation, identification and assessment of bioremediation potential of oil-degrading bacteria from oil-polluted sites of south of Iran

M. R. Sarikhani[1]*,
M. Ebrahimi[2], A. R. Fallah[3]

[1]*Department of Soil Science, Faculty of Agriculture, University of Tabriz, Tabriz, Iran*
[2]*Department of Soil Science, Faculty of Agriculture, Bu Ali Sina University, Hamedan, Iran*
[3]*Department of Soil Biology, Institute of Soil & Water research, Karaj, Iran*

*Corresponding Author:
rsarikhani@yahoo.com

ABSTRACT

The pollution of the environment with oil is spreading throughout the world along with industrial progress. Scientists are looking for many years to find solution for removing contaminants from the soil and water environments. Today, the use of microorganism, for removing crude oil pollution from contaminated sites (bioremediation) is considered by scientists. The present study deals with isolation, identification and characterization of oil-degrading bacteria was isolated from oil-polluted sites of south of Iran (Bushehr province). Initially, a total of 90 isolates were screened from oil-polluted soils in Carbon Free Minimal Medium enriched by gas oil. The 19 isolates were selected based on high level of growth in solid medium. On the basis of morphological, biochemical, 16S rDNA gene sequencing and phylogeny analysis revealed that, the isolates were authentically identified as *Stenotrophomonas maltophilia*, *Ralstonia* sp., *Vibrio* sp., *Sphingobacterium* sp., *Zymomonas* sp., *Pseudomonas aeruginosa*, *Paracoccus* sp., *Pantoea* sp., *Achromobacter xylosoxidans*, *Acinetobacter johnsonii*, *Serattia odorifera*, *Pseudomonas alcaligenes*, *Entrobacter cloacae*, *Pseudomonas stutzeri* and *Chryseobacterium* sp.. In order to determine the efficiency of these bacteria in hydrocarbon-degrading an inoculum of bacteria containing 108 cfu/ml was used in plate and liquid assays, which were performed in factorial experiment based on completely randomized design with 3 replications. The increasing of diameter of colony and the growth turbidity (OD600nm) as indicators for utilization of hydrocarbon was measured in solid and liquid assays, respectively. Results showed that in CFMM plate assay, highest diameter of colony in presence of toluene and phenanthrene were achieved by *Chryseobacterium* sp. and *Sphingobacterium* sp., while in liquid assay and in presence of three compounds *Serattia odorifera* and *Entrobacter cloacae* were efficient. Moreover, among three compounds highest degradability in bioremediation was related to gas oil, and it's followed by phenanthrene and toluene, respectively.

KEYWORDS: Bioremediation, Oil-degradation bacteria, Gas oil, Toluene, Phenanthrene, Minimal.

INTRODUCTION

Environmental pollution was increased by increasing the industry development all over the world. Increment of this pollution caused many hazards for all organisms, even for humans such as carcinogenicity and toxicity. Crude oil is a complex mixture of aliphatics, aromatics, resins and asphaltenes [1]. Hydrocarbon products contain a mixture of different components and differ in their susceptibility to microbial attack and generally degrade in the following order of decreasing susceptibility: n-alkanes >branched alkanes >low-molecularweight aromatics >cyclic alkanes [2].

Biological and non-biological approaches are being used for remediation of oil-pollution. Bioremediation is one of principal strategies for remediation, wherein the pollutants can be removed by use of microorganism or any biological process that uses microorganisms or their enzymes to return the environment altered by contaminants to its original condition [3;4]. Many species of microorganisms including bacteria, yeasts and fungi obtain both energy and tissue-building material from petroleum. Crude oil is composed of complex mixture of Aliphatic and Aromatic hydrocarbons [5]. Hydrocarbons are organic compounds whose structures consist of hydrogen and carbon. Hydrocarbons can be seen as linear linked, branched or cyclic molecules.

Fortunately, bacteria are able to feed upon the wide variety of compounds found in petroleum. The ability of many microorganisms in order to biodegradation of hydrocarbons has been studied [6; 7; 8]. Rashid Ashmasg and coworkers [9] in a study isolated *Acinetobacter* from oil-polluted soil and investigated its ability in degradation of PAH in liquid media. They report biodegradation of phenanthrene by isolated bacterium.

Isolation of 12 different bacterial species from polluted marine sites was reported by Kayode-Isola et al [10]; they found that *Alcaligenes paradoxus*, *Aeromonas* sp, *B. licheniformis* and *P. fluorescens* were efficient in biodegradation of diesel oil. However, isolation, identification and assessment of biodegradation of different hydrocarbons have been studied and reported [11; 12; 13; 6]. Among bacteria *Pseudomonas* is well known as one of bacteria with high potentiality to remediation of different types of hydrocarbons [14]. Leahy and Colwell [2] have reported biodegradation of petroleum oil by *Achromobacter*, *Arthrobacter*, *Acinetobacter*, *Alcaligenes*, *Bacillus*, *Flavobacterium*, *Nocardia*, *Pseudomonas* and *Rhodococcus*.

With regards to the importance of bioremediation of oil-polluted soils the aim of this study was to isolate and identify the bacterial strains which able to degrade such pollutants.

MATERIALS AND METHODS

SOIL SAMPLING

Soil samples were collected from oil-polluted sites of Bushehr province, south of Iran. The depth of sampling was 0-30 cm.

BACTERIAL ISOLATION

Soil samples (1 g) were added into 25 ml Carbon Free Minimal Medium (CFMM). The composition of the CFMM used in this study was as follow (g/l): NH4NO3 3.0, CaCl2.7H2O 0.005, KH2PO4 0.8, Na2HPO4 2.2, MgSO4.7H2O 0.01. The initial pH was adjusted to 7. (4). The medium containing the soil was incubated at 28 ºC under shaking 150rpm for 24 h. In order to make an enrichment culture of bacteria and isolation, 5 ml of soil suspension was added to 45 ml of CFMM containing 2% gas oil as source of carbon. After one week of enrichment, 100 µl of prepared serial dilutions (10-5-10-8) were spread on plates. Plates were prepared with CFMM that had been solidified with 2% agar and sprayed with 2% gas oil. The plates were incubated at 28°C temperature. The colonies were grown on the plates were picked and streaked on new minimal agar plates. Colonies differing in morphological characteristics were selected and used for further studies.

PURIFICATION, IDENTIFICATION AND CHARACTERIZATION

Isolation and purification of grown colonies on CFMM solid plates were performed according to the phenotypic traits of bacterial colonies. NA medium was used to purify colonies. Selected isolates were grown on NA media. The shape and colors of the colonies were examined under the microscope after Gram staining. Isolates were biochemically analyzed for the activities of oxidase, catalase. Furthermore other biochemical features of isolates were tested by Himedi bacterial identification Kit (HiMedia, India). The tests were used to identify the isolates according to Bergey's Manual of Systematic Bacteriology [15].

16S RDNA GENE AMPLIFICATION AND SEQUENCE ANALYSIS

Molecular identification of bacteria was done without extraction of genomic DNA via application of colony-PCR method. A part of bacterial single colony was used as source of DNAg in PCR reaction. Bacterial 16S rDNA was amplified by using the universal bacterial 16S rDNA primers, 27F (5 '- AGA GTT TGA TCC TGG CTC AG – 3') and 1492R (5'- GGT TAC CTT GTT ACG ACTT - 3'). PCR was performed with a 20 µl reaction mixture containing bacterial colony as a template of DNA, 0.1 pmol of each primer, 2 mM MgCl2 and dNTPs at a concentration of 0.2 mM, as well as 0.2 U of Taq polymerase and buffer used as recommended by the manufacturer (Fermentas, Hanover, Germany). After the initial denaturation for 5 min at 94oC, there was 35 cycles consisting of denaturation at 94oC for 1 min, annealing at 53oC for 1 min, extension at 72oC for 1 min and final extension at 72oC for 10 min. PCR was carried out in a Flexigene thermocycler. PCR products were analyzed by 1% (w/v) agarose gel electrophoresis in 1x TBE buffer. A DNA fragment (approximately 1.5 kb) was eluted by using QIAgen Gel Extraction Kit. PCR products were sequenced and sequences were matched with previously published bacterial 16S rDNA sequences in the NCBI databases using BLASTn [16].

ASSESSMENT OF BIODEGRADATION IN LIQUID AND SOLID MEDIA

The grown cells of bacteria in CFMM containing 1% gas oil were recovered by centrifugation after one day of growth, washed and re-suspended in CFMM. An inoculum of bacteria containing 108 cfu/ml was used for assessment of biodegradation of gas oil, toluene and phenanthrene in plate and liquid assays. Phenanthrene was dissolved in DMSO (5% w/v) prior to incorporation into CFMM. Detection of hydrocarbon-degrading activity was first performed using the CFMM plate assay containing 1% toluene and 0.05% phenanthrene, which was measured based on the diameter of bacterial colonies. Whereas in plate assay, 10 µl of inoculums was inoculated on the agar and the inoculated plates were sealed with parafilm. Diameter of colony formed on the agar was measured daily in 3 weeks of incubation. All the carbon sources used were filter-sterilized using cellulose-nitrate membrane filter before addition into the tubes. Utilization of hydrocarbon sources were again detected in CFMM broth supplemented with 2% gas oil, 1% toluene and 0.05% phenanthrene, where the mean viable cell count recovered from hydrocarbon-supplemented broth cultures were measured at different times of 0, 72, 144, 216h. In broth assay, the inoculation was

performed in 250 ml flasks which containing 50 ml CFMM and each flask was inoculated with 1.5 ml of bacterial inoculum (108 cfu/ml). In this study the bacterial growth as an indicator for utilization and bioremediation of toluene and phenanthrene was measured. The microbial count was monitored by standard serial dilution technique using agar spread plate method. A sample from each flask at 0, 72, 144 and 216h after inoculation was taken to determine the number of bacterial colony. Flasks were incubated at 28 °C for a period of 9 days. However gas oil degradation was determined by monitoring the change of turbidity of culture (OD600nm). Finally to have a comparison among the results of three different compounds MacFarland Table has been used [17].

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

The plate and broth assay were performed in a factorial experiment based on completely randomized design with 3 replications, the experiment was done in sterile condition. Growth of bacteria reflects the bacterial growth upon utilization (degradation) of the tested hydrocarbon types as the sole carbon source [18]. Analysis of variance and mean comparison by Duncan's Multiple Range Test were carried out using MSTATC software.

RESULTS AND DISCUSSION

ISOLATION OF BACTERIA

In the present study we identify and characterize oil-degrading bacteria isolated from oil-polluted sites. More than 90 different colonies were screened from initial level of gas oil supplemented CFMM medium. Finally 19 isolates were selected in the secondary screening.

ALIGNMENTS AND PHYLOGENETIC ANALYSIS

The selected isolates were identified by sequencing of the PCR amplified 16S rDNA gene. The obtained sequences were submitted to the BLAST in order to find a homology with other 16S rDNA sequences. Comparing the sequence of the 16S rDNA gene of the isolates with the sequences in GenBank revealed that the isolates are similar to *Stenotrophomonas maltophilia*, *Ralstonia* sp., *Vibrio* sp., *Sphingobacterium* sp., *Zymomonas* sp., *Pseudomonas aeruginosa*, *Paracoccus* sp., *Pantoea* sp., *Achromobacter xylosoxidans*, *Acinetobacter johnsonii*, *Serattia odorifera*, *Pseudomonas alcaligenes*, *Entrobacter cloacae*, *Pseudomonas stutzeri* and *Chryseobacterium* sp.

MEASUREMENT OF BACTERIAL COLONY DIAMETER IN SOLID MEDIA CONTAINING PHENANTHRENE AND TOLUENE

Selection of efficient bacteria by measurement of bacterial colony diameter in solid media is a conventional method to isolate bacteria [19; 20; 12]. Malboobi and colleagues [19] applied this method to isolate high efficient phosphate solubilizing bacteria by monitoring the diameter size of bacterial colony and halo zone in Sperber medium which lack of soluble phosphate. Furthermore, the formation of clearing zones on Bushnell-Hass agar plates supplemented with paraffin, mineral oil or petrol have been successfully used by Ting et al. [20] in order to isolate *Pseudomonas lundensis* UTAR FPE2 as an efficient oil-compound biodegrader. Employing a spray-plate technique for isolation phenanthrene-utilizing bacteria via formation of a clear zone around the colonies was used by Shafiee et al. [12]. In this study, the biodegradation activity of 19 isolates on CFMM-plate supplemented with phenenathrene and toluene were investigated during 3 weeks. ANOVA analysis on colony diameter showed that all main effects (bacteria, hydrocarbon type and time) and some interaction effects (such as bacteria and time, bacteria and hydrocarbon type) were significant ($p<0.01$). In CFMM-plate assay, the mean diameter of colony was largest on agar supplemented with toluene with 0.37cm while with phenanthrene was 0.34cm. Colony diameter increased by increasing time of incubation. Largest mean of colony diameter (0.39 cm) was observed after three weeks of incubation. In CFMM-plate assay, the mean diameter of colony was larger on agar supplemented with toluene than phenanthrene. Higher utilization of toluene compared with phenanthrene might be explained by their structure and susceptibility to microbial attack, a single benzene ring (toluene) versus three benzene ring (phenanthrene) [2; 21; 18]. Ting et al. [20] reported that the mean diameter of clearing zone produced by *P. lundensis* UTARFPE2 was largest on agar supplemented with paraffin, followed by mineral oil and petrol, respectively. Largest colony diameters were obtained by *Chryseobacterium* sp. and *Sphingobacterium* sp. with 0.63 and 0.60 cm and had a significant difference with other isolates. *Paracoccus* sp. didn't have any growth and was one of the poorest bacteria in this study. Interaction effect of hydrocarbons and isolates showed that largest colony diameter was obtained by *Sphingobacterium* sp. in presence of phenanthrene (0.66 cm) and *Chryseobacterium* sp. in presence of toluene (0.65 cm) and phenanthrene (0.61 cm).
Interaction effect of isolates and incubation time mean comparison revealed that highest bacterial growth and colony diameter was related to the third week of incubation by *Sphingobacterium* sp. (0.73 cm) whereas lowest growth was observed by *Paracoccus* sp. and Vibrio sp. In a study, the potential of isolate *P. lundensis* UTAR FPE2 as a hydrocarbon degrader was established by Ting et al. [20]. Its biodegradation activity was first detected with the formation of clearing zones on

Bushnell-Hass agar plates, with the largest diameter observed on plates supplemented with paraffin, followed by mineral oil and petrol.

GROWTH BEHAVIOR OF ISOLATED STRAINS IN CFMM CONTAINING GAS OIL, TOLUENE AND PHENANTHRENE

ANOVA analysis revealed that all main and interaction effects were significant ($p<0.01$). Mean comparison by Duncan's method showed that highest number of recovered bacteria was related to *Serattia odorifera* (5.1×108 CFU/ml) and it didn't show any significant difference with the other bacteria such as *Stenotrophomonas maltophilia*, *Entrobacter cloacae*, *Pseudomonas aeruginosa*, *Paracoccus* sp., *Pseudomonas alcaligenes* and *Vibrio* sp. Among bacteria lowest mean 6.2×106 CFU/ml and 1×107 CFU/ml were obtained by *Pseudomonas stutzeri* and *Sphingobacterium* sp., respectively (Figure 1). Furthermore, hydrocarbons from the point of bacterial growth support were not the same. Mean comparison of different levels of hydrocarbon factor showed that gas oil was easily used by bacteria and it supported bacterial population in liquid assay to 6.6×108 CFU/ml while lowest number of viable recovered bacteria (1.1×107 CFU/ml) was gained in presence of toluene. Weissenfels and coworkers [21] mentioned that oil-compounds consist of a complex mixture of different hydrocarbons such as aliphatic and aromatic, and bacterial species has only limited capacity to degrade all the fractions of hydrocarbons. Some hydrocarbons included aliphatic such as gas oil is easily degraded by oil-utilizing bacteria while the others aromatic compound e.g. phenanthrene and toluene are resistant to biodegradation [21; 12]. Usually, in biodegradation of aromatic hydrocarbons by increasing of benzene rings the rate of degradation decreases [22]. In our experiment we found that utilization of phenanthrene by bacteria was more than toluene and it could be explained by its lower concentration (0.05% compared with 1%). Walker and Claus [23] reported the isolation and degradation of toluene by bacteria. They isolated two different genuses *Pseudomonas* and *Achromobacter* from toluene-polluted sites and showed that these bacteria are able to use toluene as source of carbon and energy. In other experiment, the degradation of five PAHs (phenanthrene, anthracene, pyrene, fluorene and fluoranthene) by aerobic mixed bacterial culture was investigated by Shafiee et al. [12]. The degradation experiment was conducted in liquid culture and PAH concentration was 100 mg/L. After ten days incubation, the mixed culture was capable of degrading phenanthrene completely while lowest degradation was achieved in presence of fluoranthene (composed of four fused benzene rings) [12]. Degradation of phenanthrene by mixed culture or individual strains isolated from soil in refinery fields in Korea was examined and a significantly positive relationship was observed between the microbial growth and the rate of phenanthrene degradation [24].

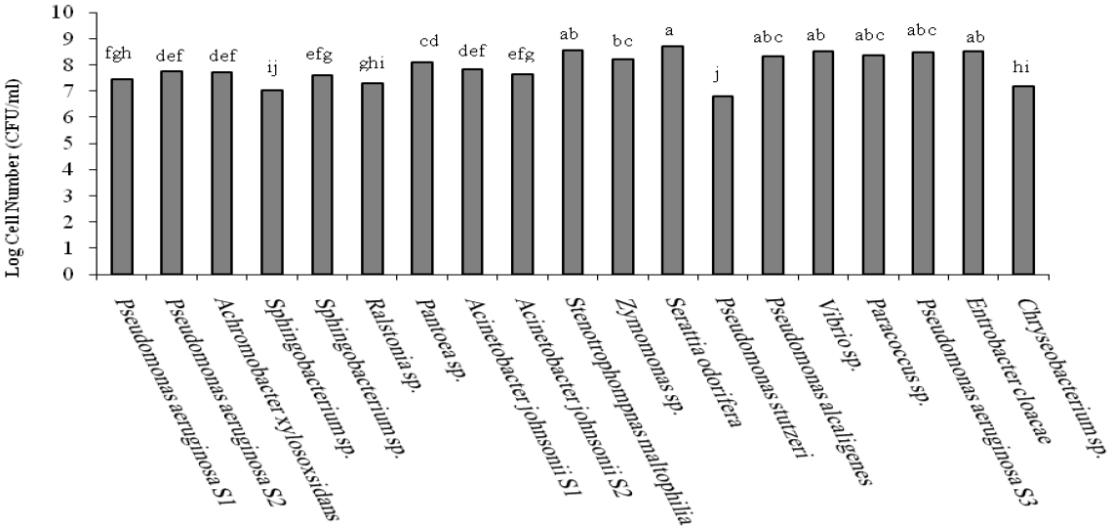

Fig. 1. Mean total number of bacterial viable cells recovered from liquid media supplemented with various hydrocarbons

In mean comparison of interaction effect of isolates and hydrocarbons, highest support from bacterial growth was observed by *Serattia odorifera* (1.2×109 CFU/ml) and *Pseudomonas aeruginosa* (8.7×108 CFU/ml) when gas oil was added to CFMM liquid medium (Figure 2). Bacterial growth was followed by *Chryseobacterium* sp. (8.5×108 CFU/ml) in presence of gas oil but when toluene and phenanthrene were used as source of carbon in media, their degradability were very low in comparison of gas oil (4.4×105 and 9.3×106 CFU/ml, respectively). As shown in Figure 2 bacterial growth as an indicator of hydrocarbon biodegradation was increased by adding aliphatics (gas oil) in comparison aromatics (phenanthrene and toluene). Ting and coworkers [20] in an

experiment by using *P. lundensis* UTAR FPE2 found that utilization of paraffin and mineral oil in comparison naphthalene is easier. The preference on aliphatics over aromatics is attributed to the predominance of aliphatics over aromatics in bacterial mineralization [21; 18]. As a result, the degradation of the aliphatic over aromatic fraction is naturally faster as also observed in this study. Furthermore, aromatic compounds (phenanthrene and toluene) are not easily degradable because of its complexity in structural composition of the molecules [25].

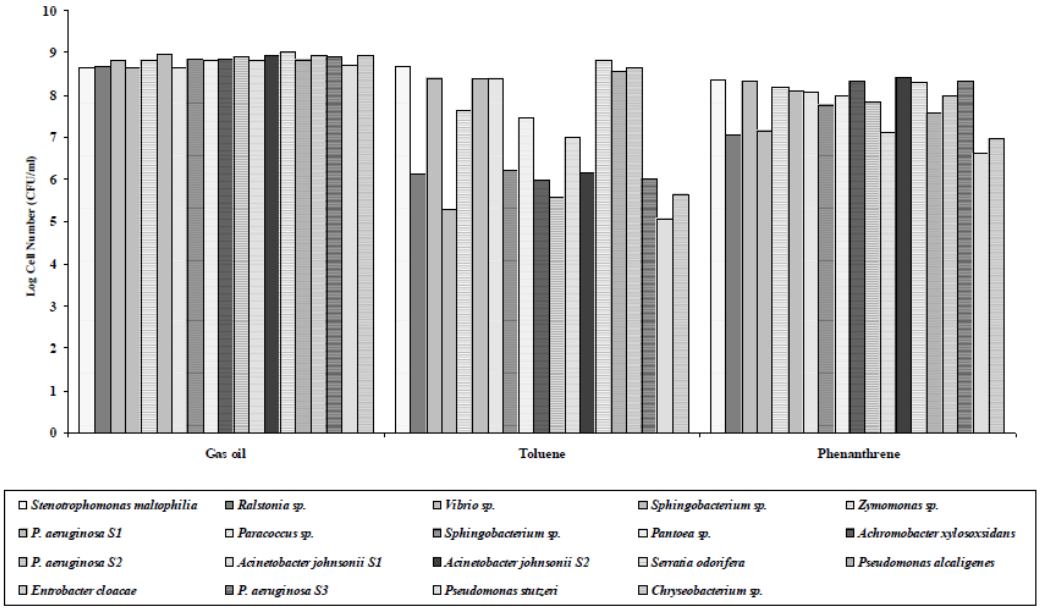

Fig. 2. Mean of different bacterial cells recovered from liquid media supplemented with various hydrocarbons (gas oil, phenanthrene and toluene).

Bacterial growth and hydrocarbon degradation were fluctuated by increasing of incubation time (Figure 3, 4). Increasing and decreasing of viable recovered bacterial number was related to the kind of hydrocarbons and also to the bacterial species. Decreasing of bacterial population in some cases may be explained by composition of hydrocarbons and its concentration which at initial step bacteria faced with a shock and stress and gradually adaptation mechanism in bacteria and induction of some mechanism which help to survive and growth in new condition [26] cause an increasing trend in bacterial number. Reversely in some condition such as high concentration of hydrocarbons and its toxicity furthermore limitation of oxygen and nutrition cause to inhibit biodegradation [2].

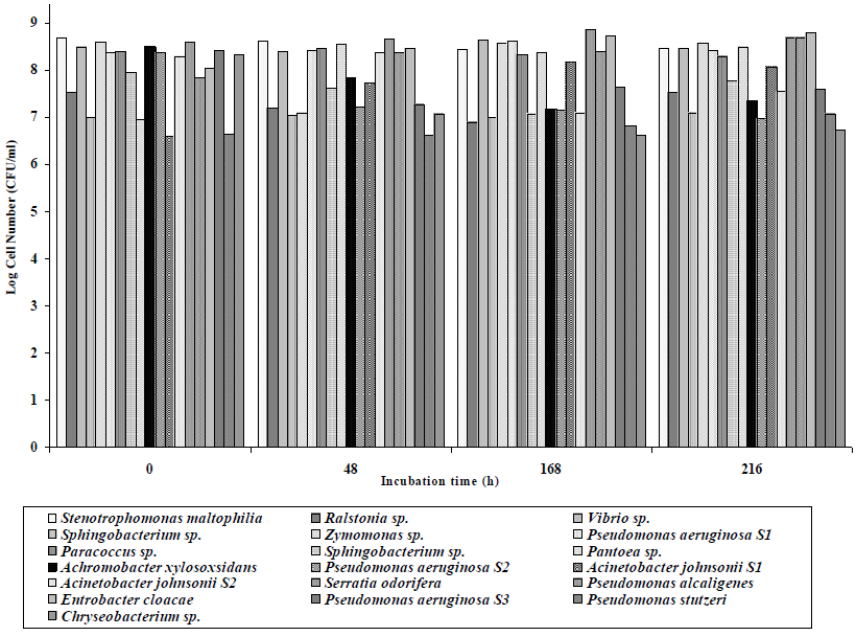

Fig. 3. Mean of different bacterial cells recovered from liquid media supplemented with various hydrocarbons at incubation time.

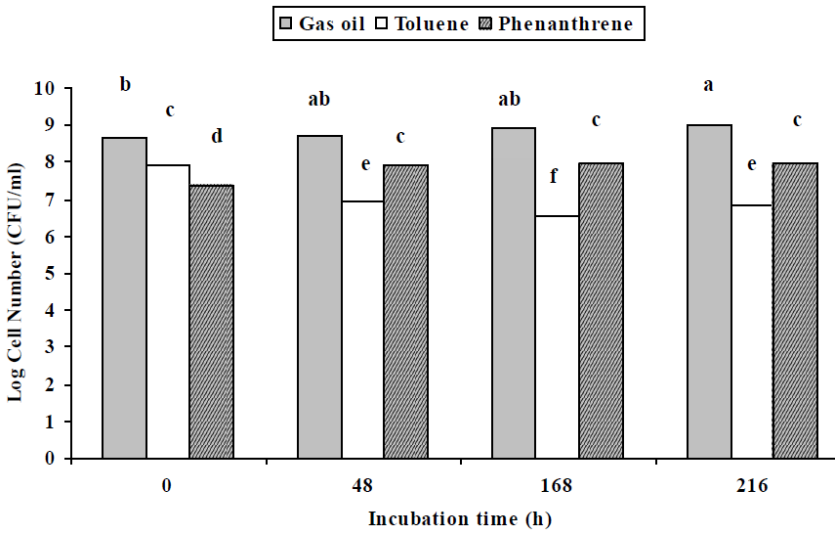

Fig. 4. Mean of bacterial cells recovered from liquid media supplemented with hydrocarbons (gas oil, phenanthrene and toluene) at different time of incubation.

According to the results exhibited in Figure 3, *Serattia odorifera* (7.2×108 CFU/ml) after 168 h of incubation and *Entrobacter cloacae* (6.3×108 CFU/ml) after 216 h had highest bacterial number. Lowest bacterial growth was obtained by *Chryseobacterium* sp. after 168 h (4.1×106 CFU/ml). Increasing time of incubation usually cause to increase of bacterial growth [26; 12; 24] while in other experiments at the end of incubation period were reduced the number of microorganisms [27].

Figure 4 present mean comparison data of interaction effect of hydrocarbon and incubation time. The number of bacteria, as assessed in CFMM liquid media supplemented with gas oil after 216 h incubation was 9.7×108 CFU/ml and it didn't have any significant difference at second and third incubation time. While lowest number of bacteria was 3.4×106 CFU/ml in presence of toluene after 168 h of incubation. Results showed that by increasing time of incubation bacterial number was increased when gas oil and phenanthrene were applied as sole source of carbon, but microbial number at the start of experiment with toluene was high, then reduced. These results are similar with the findings of other studies. Among hydrocarbons n-alkenes more easily degrade, and aromatics with low molecular weights are in the next order [2]. Onifade and Abubakr [28] investigated the potential role of hydrocarbon-degrading bacteria e.g. *Lactobacter* sp., *Arthrobacter* sp., *Bacillus* sp., *Pseudomonas* sp. and *Micrococcus* sp. in crude oil-contaminated soil and reported increasing of bacterial number from 5×103 CFU/ml to 7.6×104 CFU/ml after 18 weeks of incubation.

Finally, mean comparison of interaction effect of three factors (bacteria, incubation time and hydrocarbons) showed that highest number of bacteria was obtained by *Serattia odorifera* (2.7×108 CFU/ml) in presence of gas oil after 216 h of incubation time (data not shown). When gas oil was the source of carbon in CFMM media highest bacterial growth was achieved and in presence of toluene lowest number was obtained. After one week of incubation there weren't any significant difference among *Serattia odorifera*, *Entrobacter cloacae*, *Acinetobacter johnsonii* and *Chryseobacterium* sp. in biodegradation of gas oil which its degradation support increasing of bacterial number. Emtiazi et al. [29] in a study assessed utilization of petroleum hydrocarbons by *Pseuomonas* sp. and transformed *Escherichia coli*. They monitored the change of bacterial growth turbidity (OD600nm) at nine days of incubation in liquid media. *Pseudomonas* sp. was able to use different hydrocarbons as source of carbon and energy while highest optical density (OD=0.8) was obtained when octadecan and dodecan has been used in media but when octan was added bacterial growth just increased to OD=0.28. Isolation and characterization of a phenanthrene degrading *Sphingomonas* sp. strain P2 and its ability to degrade of eight PAHs was reported by Supaka et al. [30].

Kirimura and colleagues [31] were able to isolate *Sphingomonas* sp. CDH-7 from polluted sites. This bacterium in biodegradation of carbazole and n-hexadecan was efficient. They used optical density of bacterial growth as indicator for biodegradation. In other study efficiency of *Burkholderia cepacia* RQ1 in biodegradation of heavy crude oil was investigated, and bacterial number in presence of crude oil as sole source of carbon was increased from 105 CFU/ml to 108 CFU/ml during 15 days of incubation [32].

CONCLUSION

In this study the utilization or degradation potential of three hydrocarbons by isolated bacteria from oil-polluted sites were studied in solid and liquid assay. In solid assay highest bacterial colony diameter was

measured by *Chryseobacterium* sp. and *Sphingobacterium* sp. and in liquid assay highest support from bacterial growth was achieved by *Serattia odorifera* and *Entrobacter cloacae*. According to results from liquid assay degradation of gas oil was more than two other kind of hydrocarbons (phenanthrene and toluene) and in presence of gas oil *Chryseobacterium* sp. was one of the efficient bacteria. With respect to the results in solid and liquid media it can be concluded that *Serattia odorifera*, *Entrobacter cloacae* and *Chryseobacterium* sp. are profound bacteria in biodegradation.

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