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INVESTIGATION OF HYDROCARBON BIO-REMOVAL BY THE INDIGENOUS BACTERIA ISOLATED FROM CRUDE OIL CONTAMINATED SOILS

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ABSTRACT

The current study reports hydrocarbon degrading capacity of two bacterial strains isolated from soils contaminated by crude oil. Two indigenous bacterial strains (Bs1 and Bs2) were isolated from polluted soils around oil reservoirs, Ahvaz, Iran. Their abilities to degrade different hydrocarbons, including olive oil, crude oil and coal tar were investigated, using measuring total organic carbon (TOC) supplemented as sole carbon source in the mineral medium. To identify bacterial isolates, morphology and biochemical characteristics along with 16S rDNA analysis were considered. The results showed strain Bs1 was able to consume more than 34 % of coal tar, 61% of crude oil, and 81% of olive oil during 14 days incubation. The hydrocarbon degrading ability of Bs2 was 24, 39, and 68% in the presence of coal tar, crude oil and olive oil, respectively as sole carbon source. Additionally, their mixed culture of two isolates led to a 5% increase in their biodegradation effectiveness. The subsequent morphological and biochemical characterization and phylogenetic analyses showed the close relationship of Bs1 and Bs2 (about 98 and 99%) to *Pseudomonas aeruginosa* strains of CMG581 and BM8, respectively.

Keywords: Bioremediation, Biodegradatin, *Pseudomonas*, Crude oil, Coal tar

INTRODUCTION

The petroleum industry is the backbone of Iran economy, recognizes it as one of the few countries with a huge amount of underground oil reservoirs. Hence, activities relating with oil exploration, refining and transportation give rise to spillage and accumulation of large amounts of crude oil and other petroleum contaminants on the marine and terrestrial environments **(Hassanshahian *et al.*, 2012; Zolfaghari-Baghbaderani *et al.*, 2012)**. Additionally, the unpredictable repercussions of oil pollutants, impact of these hazardous wastes on human health is complicated due to their incomplete decomposition to harmless end products, acquiring toxic, carcinogenic potentials **(Sathishkumar *et al.*, 2008).**
Considering the detrimental impact of oil hydrocarbons on the living aspects of ecosystems, a global worry has risen not only among environmental protection organizations, but also among the whole population **(Silva-Castro *et al.*, 2013; Zolfaghari-Baghbaderani *et al.* , 2012)**. This issue urges oil associated industrial companies to develop clean technologies, and eco-friendly strategies highlighting the bioremediation. The bioremediation is defined as the use of microorganisms to eliminate environmental contaminants from water and soil, through the complete degradation of different hydrocarbons into carbon dioxide and water or conversion of them to harmless byproducts. However, it is pointed out that oil contaminated areas contains a wide variety of structurally different compounds, with supportive or otherwise effect on the local micro-flora, depending on their individual concentrations and nature **(Das and Chandran, 2011)**. The oily sludge is a very complicated recalcitrant residue, composed of different oils, fats, solids, organic compounds and metals. There is structurally a large diversity among organic compounds, from simplest readily degradable forms such as short-chain linear alkanes (known as aliphatics) to most complex and less-degradable ones with one or more cyclic rings (aromatics) like cycloalkanes, benzene, toluene, xylenes, phenols, and at the top, polycyclic aromatic hydrocarbons **(Bamforth and Singleton, 2005; Xu and Obbard, 2004).** Regarding the very complex structure of some oily sludge constituents, the synergistic interactions among the member of the micro-flora associations has been demonstrated and favored over pure cultures in bioaugmentation strategy **(Alvarez *et al.*, 2011; Domde *et al.*, 2007; Maqbool *et al.*, 2012)**.The present paper aims to study the degradation capacity of the two most efficient isolates, including Bs1 and Bs2, which were investigated in pure and mix form inoculation to mineral media supplemented with different organic hydrocarbons.

MATERIAL AND METHODS

Culture medium

Nutrient agar (Oxoid) and mineral media compositions from Merck, Germany were exploited for respective bacterial enrichment and experimental steps. The mineral medium consisted of the following constituents: NH_4Cl (1.95g/l); NaCl (0.85 g/l); Na_2HPO_4 (0.24g/l); KCl (0.05 g/l); $MgSO_4$ (0.01 g/l); Calcium chloride $CaCl_2$ (0.01 g/l); $FeSO_4$ (0.01g/l); and trace elements (**Monteiro et al., 2009).**

Sample collection and bacterial isolation

The oily sludge and soil samples were collected from oil exploration sites desert areas around Ahvaz, south of Iran; and transferred to laboratory in sterile bottles. To isolate the bacterial community, 10 g of soil samples were mixed with 100 ml aseptic distilled water in 250 ml flasks and then shaken at 100 rpm for 10 min. After sedimentation of the soils, supernatants were serially diluted from 10^{-1} to 10^{-10}. Of each serial dilution, 100 µl was streaked on nutrient agar plates by an L-shape glass rod. All the plates then, were incubated at 32 °C for 72 h. The colonies appeared on agar plates were tested to possess ability to hydrocarbon degradation through culturing on 100 ml mineral media supplemented with 500 mg of either crude oil or coal tar as the sole carbon and energy source. Culture conditions for first step were adjusted as temperature 32 °C, pH 8 and orbital shaking 100 rpm for two weeks incubation period.

Optimization of biodegrading efficiency

To assay biodegradability of the respective hydrocarbons of coal tar, crude oil and olive oil, 100 ml of mineral medium in the 250 ml flasks containing 500 mg of each hydrocarbon, was prepared. Subsequently, 1 ml of each isolate, separately and both as co-culture with the turbidity of 0.5 McFarland (1.5×10^8 cells/ml) were added to the flasks as initial inoculums. The control prepared with the equal conditions, consider for the bacterial experiments, but without initial inoculum. Simultaneously, all the flasks were incubated at 32 °C for two weeks on orbital shaker with 100 rpm. All of the experiments were performed in triplicate.

Biomass growth and substrate reduction measurement

The total protein assay was employed as the bacterial growth indicator through Bradford protein method every other day during the incubation period. Briefly, 1 ml of the samples was centrifuged at 10000 rpm for 10 min. The cell pellets were then re-suspended in 1 ml distilled water and digested by NaOH (0.3 M) with subjecting in the bath water about 60 ºC for 90 min. Finally, spectrophotometric absorbance for each sample were recorded at 595 NM. Total protein content via comparing standard curve prepared with bovine serum albumin was determined. Hydrocarbon-consuming rate for each single colony or mixed culture was measured using total organic carbon assay according to **Walkley and Black (1934)** with little modifications. The test was modified as that remaining carbon content, which represents the subtracted hydrocarbon in the free cell liquid media, was oxidized with a certain amount of potassium dichromate concomitant of sulfuric acid 98%, then, with adding ferrous solution 0.5 M (as titrating agent) and ferrozin(as indicator) non-reacted amount of potassium dichromate was indirectly determined. In fact that, organic carbon catabolism was indirectly assessed by monitoring total organic carbon reduction in the supernatants simultaneous with the total protein assay.

Identification of oil-degrading colonies

The morphological and biochemical characterization were determined with referring to Bergey's manual of determinative bacteriology **(Holt *et al.*, 1994)**. Phylogenetic identification was performed using 16S rDNA analysis. In this order, genomic DNA was extracted through the phenol/chloroform/isoamyl alcohol according to **Sambrook *et al.* (2001)** method. 16S rDNA gene fragment was amplified by polymerase chain reaction (PCR) with a set of universal primer including Fd1 (5'-AGAGTTTGATCCTGGCTCAG-3') and Rd1 (5'-AGGAGGTGATCCAGCC-3'). The PCR amplification mixture of 16 µl, contained the bacterial DNA (of about 200 ng), 1 µl (3 units) of Taq DNA polymerase, 5 µl of Taq buffer (TAPS, pH 8.8, 3mM MgCl2, 50 mM KCl), 5 µl of 2 mM dNTP mix and 5 µl of each primer (10 pM/ µl). The amplification program was set for 30 cycles; each consisted of a denaturation at 96 ºC for 10 s, an annealing at 50 ºC for 10 Sec and an extension step at 60 ºC for 4 min, followed by a final extension step at 72 ºC for 5 min. Subsequently, PCR products were electrophoresed on a 1% agarose gel and visualized using ethidium bromide. DNA fragments were sequenced by a sequencer (SEQLAB, Germany). Finally, all the sequences were compared with reference sequences of the NCBI databases (http://WWW.ncbi.nlm.nih.gov/Genbank) using Blast software. Phylogenetic analysis was done using MEGA software version 4 and genetic relationship was showed based on the 16S rDNA gene sequence by neighbor-joining tree with bootstrap value of 1000 replication.

RESULTS AND DISCUSSION

Bacterial identification

Among 6 colonies isolated as oil biodegrading bacteria two colonies which have high potential biodegradability were selected for further studies. The morphological and biochemical characteristics for two isolates are summarized in tab 1, which corresponds to those of *Pseudomonas* genus. In addition, their 16S rDNA sequences' alignments with reference sequences present in NCBI indicated their close relationships to Pseudomonas aeruginosa. As shown in Figure 1, Bs1 and Bs2 strains had 98% similarity with *P. aeruginosa* CMG581 and BM8 strain and our respective corresponded bacterial strains (Bs1 and Bs2).

Table 1 Morphological, biochemical and physiological analyses of bacteria isolated from oil contaminated areas

Characteristic	BS1	BS2
Gram's staining test	Gram negative	Gram negative
Morphology	Coccobacillus	Coccobacillus
Anaerobic growth	-	-
McConkey agar growth	+	+
Growth in mannitol salt agar	-	-
Mobility	+	+
Indole production	+	+
H_2S production	-	-
Citrate utilization	-	+
Starch hydrolysis	-	-
Gelatin hydrolysis	+	+
Catalase	+	+
Oxidase	+	+
Urease	-	-
Nitrification	-	+
Denitrification	+	+
Nitrite to nitrate reduction	+	+
Nitrogen fixation	-	-
Lysine decarboxylase test	+	+

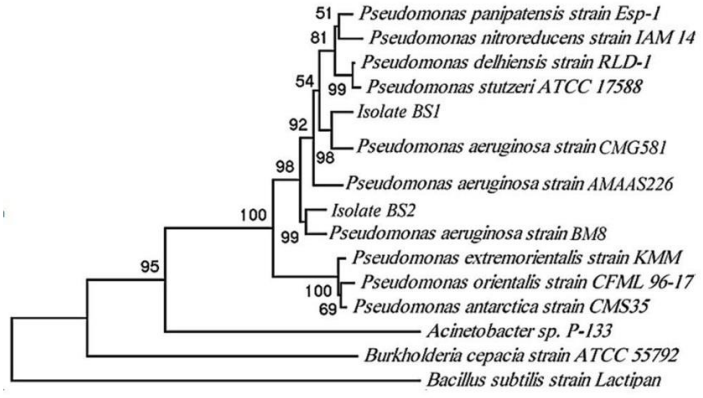

Figure 1 Phylogenetic neighbor-joining tree based on the 16S rDNA gene sequence alignment of our bacterial strains with *Pseudomonas aeruginosa* CMG581 and BM8 as well as others. Bootstrap values (1000 replicates) are shown as percentages at each node.

Biomass production and substrate reduction assessment

Figures of 2, 3 and 4 show the TOC removal capacity of the single isolates and their mix culture within 14 days. In general, the TOC reduction in the liquid media culture which inoculated with Bs1, Bs2 and their co-culture, is highest for olive oil supplemented media, while it diminishes for crude oil and then coal tar supplements in the efficiency order. As Figure 4 shown, up to 80% of olive oil was consumed by the both Bs1 and Bs2 during less than 10 days incubation, concomitant of ascending the biomass value until it reached a stationary phase. However, percent removal of TOC for the media supplemented with coal tar and crude oil decreases when the respective Bs2, Bs1 and their mixture cultivations were used.

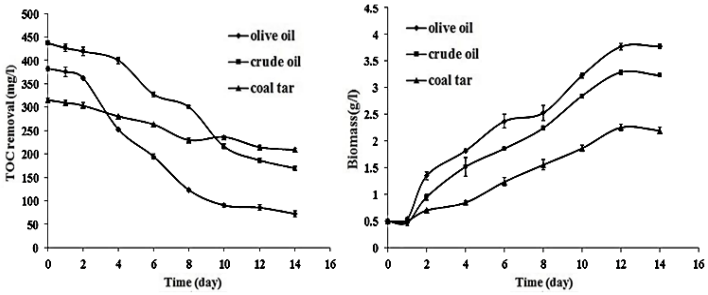

Figure 2 Biomass production (right) and TOC removal profiles (left) for Bs1 isolate

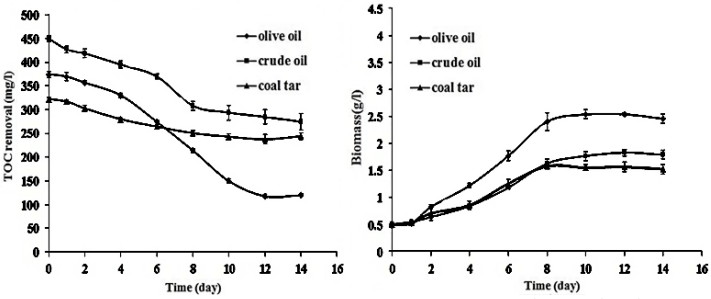

Figure 3 Biomass production (right) and TOC removal profiles (left) for Bs2 isolate

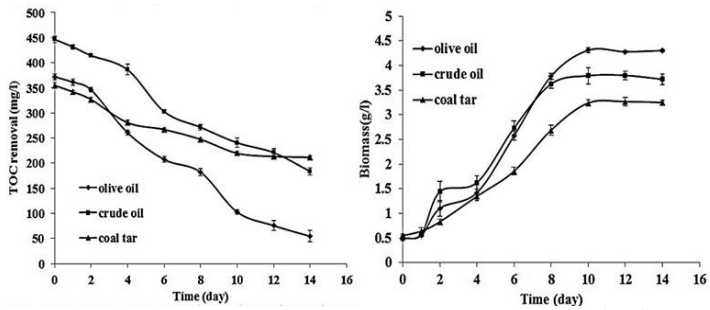

Figure 4 Biomass production (right) and TOC removal profiles (left) for co-culture of Bs1 and Bs2 isolates

There are many reports in the literature regarding the highly effective potential of members of *Pseudomonas* genus to degrade various hydrocarbon pollutants, including aliphatic, aromatic and polycyclic aromatic compounds in various environments, from marine ecosystem with strict condition to those with very unsteady harsh climates such as salterns and even extreme environments **(Emtiazi *et al*., 2005; Enontiemonria *et al*., 2012; Silva *et al*., 2006).** Their ubiquitous presence in almost every environment is reasonable cause that they possess strong enzymatic pathways involve in the metabolism of various hazardous materials such as compounds derived from petroleum or organic pesticides. However, the excess spillage of petroleum products in the environment led to be exhausted metabolic capacity of microorganisms and ultimately accumulation of hydrocarbons with toxicity and carcinogenicity, which raised concern over their hazardous problems to both human and ecosystem **(Plaza et al., 2005; Voll *et al*., 1978).** Therefore, it is both economically and environmentally necessary to screen these sites in the hope of finding single microorganism or compatible consortia with the greater degradation capacity to eliminate the contaminated locals. Our data showed that 5 to 10 % increase in hydrocarbon biodegradability when Bs1 and Bs2 are employed as mixed culture **(Arulazhagan *et al*, 2010).** Many reports that contribution of bacteria involve in hydrocarbon compounds have approved **(Daane *et al*, 2001; Shahriari *et al*, 2014).**

The use of microbial consortia may result in metabolic complementary, as metabolites produced through partial degradation by a specific microorganism can be utilized as carbon source by other organisms, increasing the chance for complete elimination of hydrocarbons from the environment **(de Morais and Tauk-Tornisielo, 2009; Joint *et al*., 2010; Sathishkumar *et al*. , 2008; Singh and Fulekar, 2007)**. This is especially more significantly necessary to eliminate pollutants with more complex compounds, e.g. polycyclic aromatic compounds (PAH), than those are with linear aliphatic structure (in our case, olive oil), since the metabolic pathways for PAHs are too long and complicated thus, needs numerous enzymes and several metabolic pathways, to be accommodated in single organism **(Alquati *et al*., 2005; Arulazhagan and Vasudevan, 2009)**. On the other hand, it is probable that some species remove the toxic metabolites of the preceding species, which might otherwise hamper microbial activity **(Das and Chandran, 2011; Peixoto *et al*., 2011).**

CONCLUSION

Overall, it can be suitable to enrich these strains through fermentation for bioaugmentation and/or adjust the chemical composition of their pollutant sites by addition of some bulk useless compounds from other activities, to optimize the condition for their growth, together with increase in the local decontamination. Usage of their bulk mixture with high catabolic capacity is favored over other strains with the equivalent or even higher biodegradation potential for following reasons: these strains were attained from the local, that is, their native habitats, meaning higher adaptation with the given environmental condition. They would readily be survive and grow in the locals more efficiently without any conditional or little adjustment. After all, they are more easily accessible than other strains for bioremediation in Ahvaz, Iran.

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