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Effects of Indigenous Microbial Consortium in Crude Oil Degradation: A Microcosm Experiment

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ABSTRACT: Microbial biodegradation is a bio-treatment method for attenuating crude oil contaminated soils. The purpose of present study was to select and recognize indigenous bacteria and to investigate the capability of the bacterial consortium to remove crude oil in a planned microcosm. Capability of the bacterial growth in agar plates containing crude oil was used to select the isolated crude oil eating bacteria. Soil samples were collected from different contaminated sites nearby the exploitation unit of Karoon-3, Ahvaz, Iran, and incubated by individual and mixed bacterial consortium at 30°C for a 90 day period time. Two indigenous isolates were selected and designated as S_{10} and S_{31}. According to 16S rRNA sequencing, S_{10} was identified as a new strain of Bacillus subtilis and S_{31} as Bacillus licheniformis. The results showed that the highest cell mass was in 10^{th} day of incubation time with about 1.7×10^7 CFU/g, for bacterial consortium. The highest total petroleum hydrocarbons degradation (C%) in the first 10 days of incubation time raised up to about 77.5% for bacterial consortium, and also residual C:N ratio deleted about 73%. Results emphasized that the planned microcosm in laboratory with controlled conditions using consortia of the selected indigenous bacteria can make the crude oil biodegradation close to the optimal conditions and have a good potential for application in bioremediation of crude oil contaminated soils, as compared to the individual strains. This is the first report on planned microcosm and microbial consortium of Karoon soil contaminated with crude oil.

Key words: Bacterial consortia, Bioremediation, Microcosm, Petroleum, Soil

INTRODUCTION

Nowadays crude oil pollution has become a global problem particularly in industrialized and developing countries which results in significant decline in soil quality and chronic sub-acute toxicological effects within ecosystem (Tanee and Albert, 2015). Soil and ground water are often contaminated due to inevitable spillage during oil exploration, transportation, extraction, refining and also from leaking underground storage tanks and pipelines (Xiong et al., 2015). There are several clean-up techniques for removing petroleum from the contaminated area. Among them, bioremediation methods are low cost, high efficiency, environmental friendliness and simplicity technology for long term restoration of crude oil contaminated sites (Rodríguez et al., 2015; Elmahdi et al., 2014).

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Several investigations have been performed on bio-fuel production such as bio-ethanol (Tofighi et al., 2010) and bioremediation of total petroleum hydrocarbons (TPH) (Xiong et al., 2015) as well as monitoring the ability of the soil intrinsic microbial community as an approach to decrease the contaminant (Tofighi et al., 2014; Xiong et al., 2015). However, there are some different conditions such as crude oil composition, its concentrations, redox conditions and nutrition which can distinct crude oil biodegradation (Xiong et al., 2015). Little is known about the bio-systems involved in and responsible for degradation under these conditions. Therefore, a comparative study on crude oil degradation under different conditions can help to explain the bacterial communities involve the crude oil degradation to

improving the procedure and moving to in situ bioremediation (Xiong et al., 2015; Santisi et al., 2015). In this study microcosm experiments were performed to close the biodegradation process to the optimal conditions. In the planned microcosm, the crude oil biodegradability of individual and mixed bacterial consortium were evaluated in 90 days, aiming to compare the bacterial community and their crude oil degradability under sterile and unsterile conditions.

MATERIALS & METHODS

All the media and chemicals were purchased from Merck (Germany). Urea was used as nitrogen, KH_2PO_4 and K_2HPO_4 as phosphate sources. Crude petroleum-oil, which was used as the sole source of carbon in the biodegradation experiments, obtained from exploitation unit of Karoon-3 located in Ahvaz, Iran. Fig. 1 shows geographical position of places where the samples were taken.

In this study three crude oil degrading bacteria were used as followed:

Bacillus megaterium (PTCC no: 1530) as standard strain and two indigenous isolates which had been kindly donated by laboratory of environmental biotechnology and bio-safety, department of Biotechnology, IROST. The isolates had been designated as S_{31} and S_{10} which identified morphologically and biochemically as Bacillus licheniformis and B. subtilis, respectively. The bacteria had been stored in 20% glycerol at -75ºC and deposited at Persian Type Culture Collection (PTCC). Further investigation continued by collecting soil samples (10 Kg) from the top surface soil (0-15 cm) of ten different crude oil contaminated sites nearby the exploitation unit of Karoon-3 (Ahvaz, Iran) (Fig. 1). All the soil samples were mixed, homogenized by passing through a 2-mm (pore size) sterile stainless steel sieve and stored in sterile polyethylene bags for further analysis. Physico-chemical characteristics were performed as described in Agarry et al. (Agarry et al., 2013).

Total genomic DNA from the bacterial isolates was extracted (Hosseini Abari et al., 2013). The bacteria were further identified based on 16S rRNA gene. 16S rRNA gene was amplified by primers (5-CCAGCAGCCGCGGTAATACG) as forward and (5'-ATCGGTACCTTGTTACGACTTC) as reverse primer. Polymerase chain reaction (PCR) was performed in a final volume of 25 µM containing 1× Buffer, 1.5 mM $MgCl_2$, 200 mM (each) dNTP mix, 1 U Taq Polymerase, 0.132 µM of each primers and 20 ng/µL of the extracted DNA. Amplification was performed for 30 PCR cycles with denaturing at 94°C for 2 min, annealing at 55°C for 1 min, and extending at 72°C for 45 sec, with the final extension for 2 min (Hosseini Abari et al., 2013; Korenblum and Seldin, 2009). Polymerase chain reaction products were separated by 1% (w/v) agarose gel electrophoresis in 1× TAE buffer with ethidium bromide (10 mg/mL) (Adesodun and Mbagwu, 2008). Purification and sequencing were performed by the Iranian Biological Resource Center (IBRC). Research for DNA similarity was performed by the National Center of Biotechnology Information Gene-Bank.

The total petroleum hydrocarbons content was extracted from 10 g of soil samples using 20 mL of n-hexane as solvent (Milic et al., 2009). The mixture was shaken vigorously for 30 min. The extracted hydrocarbons were measured spectrophotometrically at 400 nm. A standard curve was performed to estimate the amount of TPH (Agarry et al., 2013).

The ability of the selected indigenous strains to remediate the crude oil was investigated by carrying out the bioremediation experiments using a microcosm system. The microcosm system was planned with Solidworks software (version 2007). A 5 litter glass box

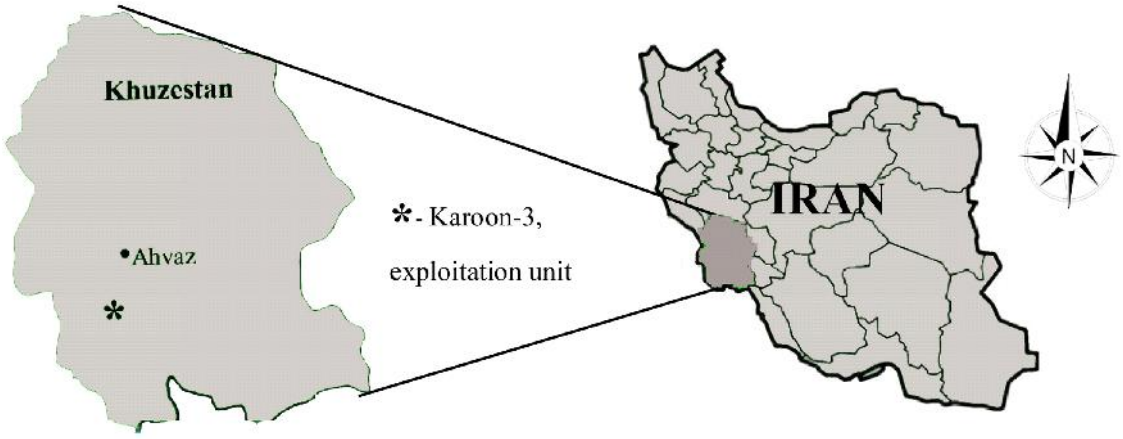

Fig. 1. Geographical position of studied area and sampling point

was used for microcosm construction. An aluminum sheet was put on the box floor and three supporter shafts were placed on it. Ten movable arms were made on each of the shafts. Natural aeration was stimulated by an air pump and a system of perforated pipes (Mittal and Singh, 2009). In order to set up the bioremediation experiments, the C: N: P ratio of the soil sample was artificially adjusted to 100:10:1 by adding the sterile Ahvaz crude oil as C, urea as N and K_2HPO_4 and KH_2PO_4 as P sources. The carbon content of the soil samples was provided at the final concentration of 2% (w/w). The crude oil was sterilized by filtration, Millipore size, 0.25 mm (Hassanshahian, 2014; Millioli et al., 2009). Microcosms were incubated for 90 days at 30 ± 2°C using 3 L/h airflow (Das and Mukherjee, 2006). The experiments were performed with 10 g enriched homogenized soil samples in each glass plates. The samples were sprayed with sterilized distilled water to hold 20 ± 5% humidity to create appropriate conditions for growth of crude oil degrading bacteria. The humidity was controlled through gravimetric method. Bioremediation ability was assessed by determining the cells growth and residual crude oil in the soil samples during 90 day incubation time (Das and Mukherjee, 2006; Jokari et al., 2013).

Soil samples were inoculated with individual and mixed bacterial consortium. To prepare the bacterial consortium, pure strains were grown separately at 30°C for 24 h, in a 20 mL nutrient broth medium containing 20 µL sterilized crude oil. Prior to the inoculation the cells were being washed for three times with normal saline and 1×10^4 CFU/mL of the bacterial strains in ratio of 1:1 were inoculated the treated soil samples (Vyas and Dave, 2010; Tebyanian et al., 2013). Each experiment was conducted in triplicates. Periodic sampling was carried out at 2 day intervals for 90 days to determine the TPH degradation and bacterial growth. The total microbial population was estimated through the pour plate technique using plate count agar. To determine the total cell counts, the residual hydrocarbons and impurities were removed by suspending, centrifugation at 5000 rpm for 5 min and re-suspending of 0.5 g of the soil samples (Rodríguez et al., 2015; Das and Mukherjee, 2006).

Analysis of Variance (SPSS version 20.0) was used to evaluate the significance of apparent differences between the TPH degradation abilities and cells growth under sterile and non-sterile conditions. The data were subjected to one-way analysis of variance (ANOVA) at 5% probability. The mean value of the triplicates were tested for level of significant differences at $P<0.05$.

RESULTS & DISCUTIONS

Molecular identification of the S_{10} and S_{31} isolates according to the 16S rRNA sequencing were analyzed. Molecular analysis exhibited 100% homology among strain S_{31} and Bacillus licheniformis ATCC14580^T (AE017333). Gene analysis of S_{10} strain showed 98.5% phylogenetic relationships among strain S_{10} and Bacillus subtilis subsp. spizizenii NRRL B-23049^T (CP002905) and it was submitted to the Gene-Bank database as Bucillus subtilis AA1350 under accession number: KT763332. Phylogenetic tree of S_{10} strain was constructed using the neighbor-joining method and illustrated in fig. 2.

Depicted results of the soil sample (Table 1) showed that the C: N: P ratio was not good enough for microbial degradation and hence the soil sample was treated by additional C, N, P and water sources. Growth of the bacteria in soil samples were estimated by measuring CFU/g of soil. All the experiments were proceeded in triplicate. Control samples were used without adding any microorganisms. C, N and P sources were added to evaluate the effects of enrichment on growth of the dominant bacteria in the control samples. Ventilation and moisturizing were continued at 30 ± 2°C for 90 days. Fig. 3 shows the effect of the enrichment on growth of the soil existing bacteria.

As shown, the dominant cells growth in control sample (Fig. 3) raised up to 8.5×10^4 CFU/g in 14th day of incubation time in the treated soil samples with 100:10:1 ratio of C:N:P sources.

Bioremediation process under sterile and unsterile conditions was conducted using individuals and mixed cultures of the crude oil eating bacteria in enriched soil samples at about 30°C in a planned microcosm for 90 days. Fig. 4, shows an outline of the planned microcosm.

The growth of the standard strain, S_{10} and S_{31} isolates and their consortium in microcosm system was illustrated in fig. 5. As represented, the population of the individual and mixed bacterial cultures in the treated soil samples has increased with time. The highest cell mass (CFU/g) under sterile and unsterile conditions was obtained in 10th day, for bacterial consortia, which was followed by S_{10} isolate, standard strain and S_{31} isolate, respectively. The biomass accumulation of all the tested bacteria entered dead phase after about 30th day of incubation time.The TPH degradability of the studied bacteria in the treated soil samples was presented in fig. 6. As illustrated, the TPH degradation raised up to about 77.5%, for bacterial consortium in the first 10 days of incubation time, in the sterile and unsterile conditions.Similarly, the residual C: N ratio shown in figure 7, with about 73% deletion, received up to 27:10. As expected, the TPH degradation (C%) and C:N ratio were significantly affected by the microbial activities in the treated soil samples (Fig. 6 and 7).

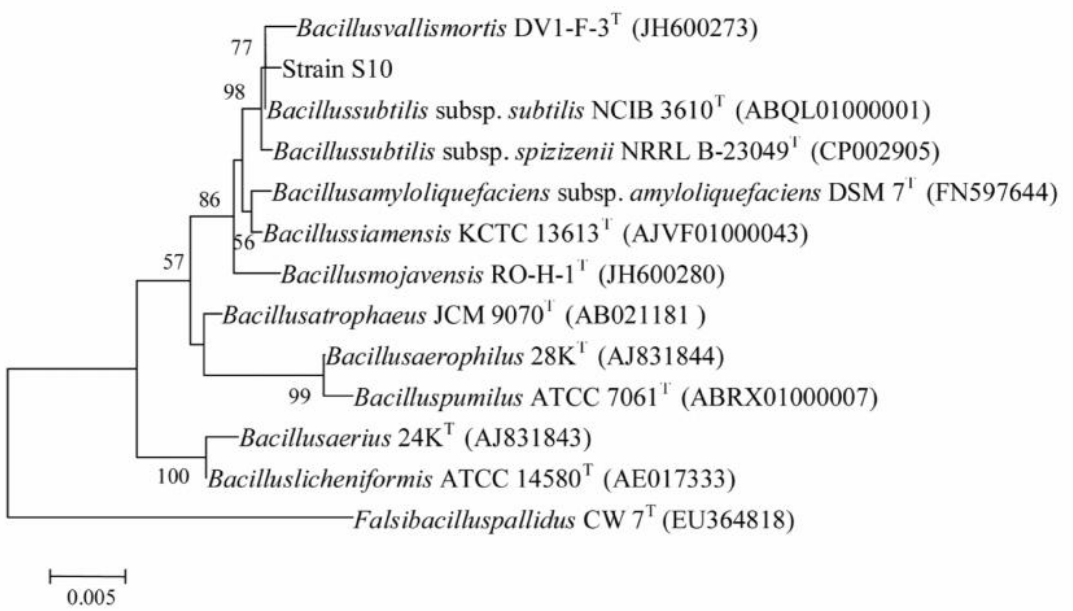

Fig. 2. Neighbor-Joining tree of the S_{10} isolate and type strains of related genera based on 16S rRNA gene sequence showing the phylogenetic relationships among strain S_{10} and type strains of related genera. Numbers at branch nodes are bootstrap values (percentages of 1000 replicates). Bar 5 substitutions per 1000 nucleotide positions

Table 1. Physico-chemical parameters of the soil samples, before and after C, N, P and water treatment

Soil Property	Amount	
	before	After
pH	6.83	6.71
WHC%*	16.1	16
EC (ms)*	1.25	1.31
C (g%)	5.9	7.8
H (g%)	0.18	0.25
N (g%)	0	0.77
S (g%)	0.05	0.052
Available P (PPM)	6.403	774.3
Humidity (g%)	9.3	23
*Water Holding Capacity **Electrical Conductivity		

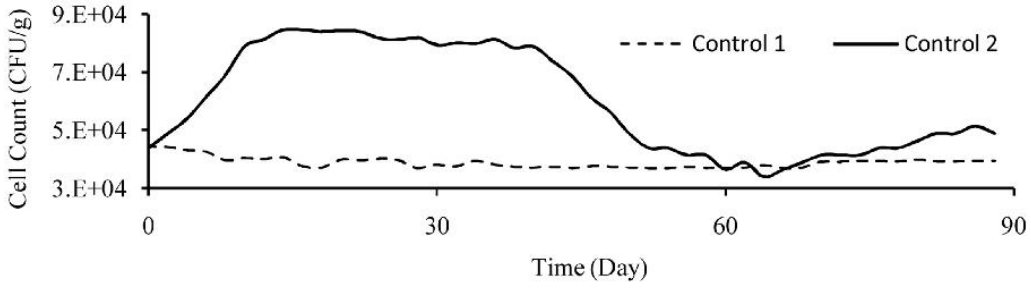

Fig. 3. Growth kinetics of two control samples under unsterile conditions during 90 days of incubation time. Control 1: The control sample containing Karoon-3 soil with aeration and moisturizing; Control 2: The control sample containing Karoon-3 soil with aeration, moisturizing enriched with 2% of Karoon-3 crude oil, urea and potassium phosphate. All the control samples were without any added microorganisms

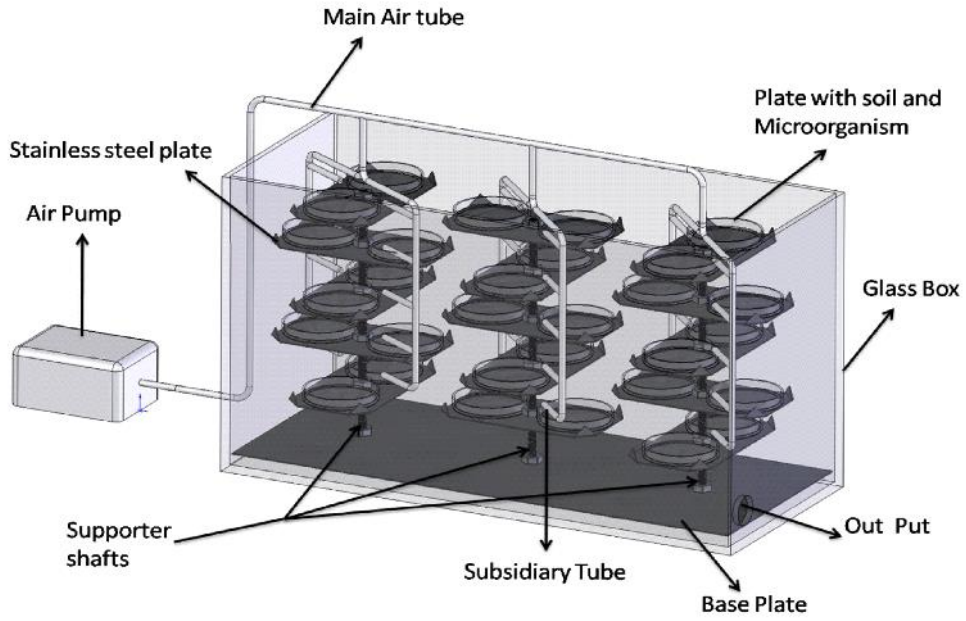

Fig. 4. Outline of the crude oil biodegradation microcosm with controlled aeration

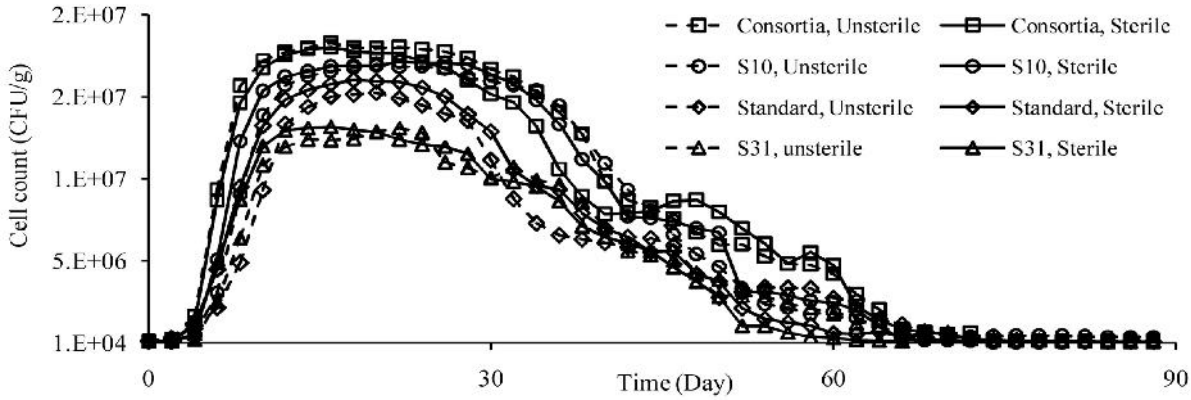

Fig. 5. Growth kinetics of standard strain, S_{10} and S_{31} isolates and their consortia in the microcosm containing enriched soil samples with 2% Karoon-3 crude oil, urea, KH_2PO_4 and K_2HPO_4, under sterile and unsterile conditions, during 90 day of incubation time

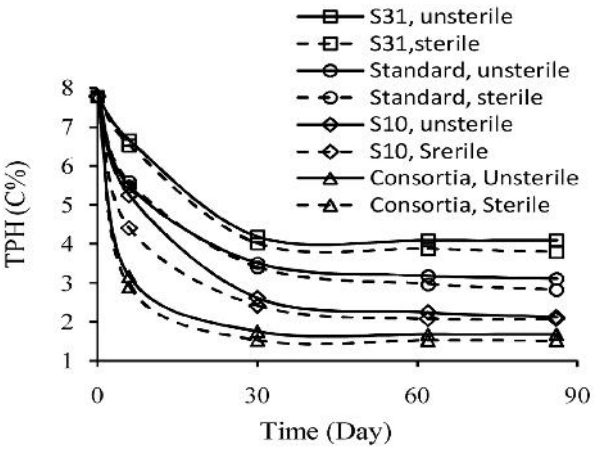

Fig. 6. Total petroleum hydrocarbons (C%) biodegradation of soil samples treated with 2% of crude oil, by S_{10} and S_{31} isolates, standard strain and the consortium of the two isolates, in the microcosm, under sterile and unsterile conditions, during 90 day of incubation time

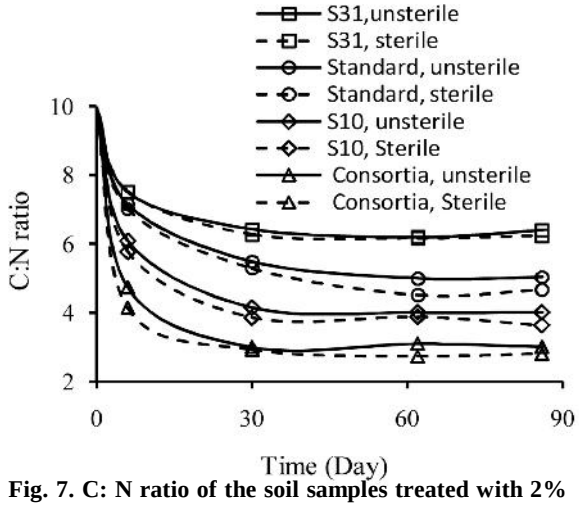

Fig. 7. C: N ratio of the soil samples treated with 2% of crude oil, by S_{10} and S_{31} isolates, standard strain and the consortium of the two isolates, in the microcosm, under sterile and unsterile conditions, during 90 day of incubation time

Petroleum hydrocarbons are the most common environmental pollutants (Tanee, and Albert, 2015). Like most studies we focused on the soil and sediment crude oil biodegradation. Microbial biodegradation has been proved to be an efficient and sustainable crude oil clean-up technique by many authors (Santisi et al., 2015; Agarry et al., 2013; Millioli et al., 2009). Different environmental properties and conditions are the most important obstacles, so that, successful crude oil biodegradation of contaminated areas remains a challenge (Xiong et al., 2015). Crude oil is toxic for many microorganisms but there are some crude oil eating bacteria which can use the crude petroleum oil as sole source of carbon and reduce the negative impacts of the crude oil contaminated areas (Macaulay, 2015). Crude oil eating microbes were found to degrade specific hydrocarbon components due to the specific metabolic pathway utilized by individual microbes. While the use of single strains to degrade hydrocarbon contaminants has been widely reported, the consortia of effective crude oil eating bacteria appear to be the biocatalysts of choice as biodegradation agents and degrade a wider range of hydrocarbon compounds (Hassanshahian, 2014; Xiong et al., 2015). As our results showed (table 1), amending the environmental and nutritional factors and providing favorable conditions such as soil pH, oxygen concentration, humidity, nutrient availability and its ratio were some of the important factors which can generally influence to improve the bioremediation strategies by accelerating microbial activity and affect nutrient availability, which were comparable with Xiong et al. as well as Vyas and Dave reports (Vyas and Dave, 2010; Xiong et al., 2015).

As compared in figure 3, the growth of existing cells raised up to 82% (CFU/g) in nutrient treated soil samples which showed the influence of the nutrition, humidity and aeration on growing the native bacteria. Enhancement of crude oil bioremediation in different experiments has been achieved through the addition of nutrient supplements (Vyas and Dave, 2010; Syafruddin et al., 2010). However, Maliji et al. and Vionothini et al. noted that, excessive nutrient concentration can impact the biodegradation of hydrocarbons negatively (Maliji et al., 2013; Vionothini et al., 2015).

Inter or intra- specific competition influences the rate of bioremediation. The interdependence of microbial consortia plays an important role in the successful application of bioremediation, which makes competition a limiting factor (Xiong et al., 2015).

In this study, microcosm system (Fig. 4) were planned to close the biodegradation process to the optimal conditions. Crude oil biodegradability of the individual and mixed bacterial consortium were evaluated in 90 days, to compare the bacterial community in crude oil degradability under sterile and unsterile conditions. Two crude oil eating isolates which had been designated as S_{10} and S_{31} were used as individual and consortium. The selected bacterial consortium in 10^{th} day of incubation time represented a higher growth (Fig. 5) up to 40%, 19% and 12% compared to the S_{31} isolate, standard strain and S_{10} isolate, respectively, with no significant differences between sterile and non-sterile conditions ($P<0.05$) which indicated a positive inter and intra challenges between the selected bacterial consortia and other native microorganisms in the treated soil samples.

It has been well established that the presence of microbial consortia and the synergistic relationships amongst them will enhance crude oil bioremediation, therefore, to continue the development of crude oil bioremediation the synergistic potential of the selected crude oil degrading consortium must be encouraged (Hassanshahian, 2014; Xiong et al., 2015).

The highest crude oil degradation was achieved through the first 10 days of incubation time with about 77.5%, 68%, 54% and 43% for microbial consortium, S_{10} isolate, standard strain and S_{31} isolate, respectively. Beneficial synergistic activities and the effects of environmental and nutritional factors on crude oil degradation could be seen in the results. There was no considerable difference in the efficiency of TPH degradation and cells growth in sterile and non-sterile conditions ($P<0.05$). As depicted in figure 6, reduction of TPH (C%) in the treated soil samples was associated with increasing and decreasing of the cells mass (CFU/g) (Fig. 5) and C: N ratio (Fig. 7), respectively. These results suggested the limiting effect of the nutritional especially C: N ratio on biodegradation of petroleum crude oil. Dong et al. pointed out that imbalance in the C: N ratio is one of the most important factors and can move the process to a sudden depletion and unsuccessful bioremediation (Dong et al., 2015).

CONCLUSIONS

As a sequence, the observations indicated that, using planned microcosm with controlled levels of aeration, temperature, and water can make the experimental conditions close to the optimized crude oil degradation. The S_{10} and S_{31} isolates were found as selected autochthones g^+ bacilli with an acceptable crude oil eating potential that belongs to Bacillus subtilis and B. licheniformis, respectively. Using S_{10} and S_{31} isolates as microbial consortium achieve more aggressive option for the microbial crude oil degradation than the use of individual microbial isolates.

According to the data, we would like to highlight the S_{10} and S_{31} consortia as a highly desirable and promising treatment option for crude oil polluted soil as compared to the individual cells. Bioremediation of crude oil-field especially, Ahvaz contaminated soils might be accomplished by enhancing their activity.

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