International Journal of Progressive Sciences and Technologies (IJPSAT)
ISSN: 2509-0119.

Vol. 34 No. 1 August 2022, pp. 376-386

Evaluation Of Biodegradation Ability Of Bacterial Consortium In Comparison With Combination Of It With Plant Species For Their Phytoremediation Potential

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Abstract – Oil and its derivatives spills have been a major issue across decades due to transport, import, Export and accidents with its wider use in industry and it is difficult to be biodegraded. Many techniques were developed to clean up petroleum contaminated soil, among all of them, the biological treatments are more efficient and economic compared to chemical and physical ones. Bioremediation is applied as a treatment technology that is cost-effective, ecologically friendly and efficient for the decontamination of hydrocarbon pollution. In this study, crude oil contaminated soil samples were collected from oil extraction fields in Libya. The bacterial strains were isolated using selective media (agar containing crude oil). Isolated bacteria were identified using microbial features and selective media and then used in biodegradation experiment of 0.5% and 1% of crude oil contaminated soil. The bacterial concentration was adjusted to 1.5 x 10^8 scf/ml before supplementing in the soil. Three plants were chosen to perform experiment (*Malva punilora*, *Ricinus communis* and *Triticum repens*) on 0.5% and 1% crude oil contaminated soil. The chosen species were implanted directly in the contaminated soil together with prepared bacterial consortia. Soil sample (triplicate) was taken from each experiment at zero time, after 15 days, and 30 days of experiment, hexane was added to the soil samples and the absorbance was measured using spectrophotometer at 360 nm. As a result, two bacterial strains proved to be oil biodegrades were isolated and identified as *Escherichia coli* and other bacillus bacteria. The percentage of crude oil removal by bacteria was more than 60% in the soil contaminated by 0.5% of crude oil after 15 and 30 days of experiment, while it was 30% at 1% crude oil contaminated soil after 15 days and increased to be 75% after 30 days of experiment. The experiment included combination between plants and bacteria resulted in the 90% of removal by *Triticum repens* and bacterial mixture at 0.5 crude oil after 30 days, and 80% of removal for 1% contaminated soil using the same plant. In general, both techniques were effective in crude oil removal. *Triticum repens* showed good results in crude oil removal. This suggests more field application of this plant on crude oil contaminated oil

Keywords – Bioremediation, Phytoremediation, Crude Oil, Bacteria, Hydrocarbon.

I. INTRODUCTION

Petroleum oil is the most important strategic resource that all countries compete (Sun, 2009). Environmental deterioration can be resulted from petroleum use (Xue et al., 2015). Spills and discharges of petroleum hydrocarbons occurs during petroleum production, storage and transportation, refining and processing, in addition to blowout accidents during development of oilfield, oil pipelines leakage and storage tanks, oil tanker and tanker leakage accidents, oil well waxing, and during overhauls of refineries and petrochemical production equipment (Chaerun et al., 2004; Chen et al., 2015; Wang C. et al., 2018). Large spills should be treated as much as possible, by recycling or elimination but in some cases, it is difficult to recover the spilled materials. As a result remains in the affected area, posing persistent environmental risks.

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Petroleum hydrocarbon-degrading bacteria have evolved as a result of their presence in naturally occurring petroleum environment, these microorganisms are good candidates for oil pollutants treatment (Lea-Smith et al., 2015; Ron and Rosenberg, 2014; Margesin et al., 2003). For these reasons, many bacterial species have been tested and used to degrade chemical and pharmaceutical industries, food and agricultural waste products. Recent, utilization of bacteria to treat environmental pollutants has become a promising technology due to its economical and eco-friendly nature (Guerra et al., 2018). Continuous evolution and development of microbial bioremediation procedures has also provided a new techniques for bioremediation of petroleum hydrocarbon pollution, which has captivated much attention (Dombrowski et al., 2016). Certain bacterial species have the ability to metabolize specific alkanes, while other species can degrade hydrocarbons aromatic or resin fractions. This is due to petroleum hydrocarbon components chemical structure. Recent researchers have identified bacteria from more than 79 genera which was able to degrade petroleum hydrocarbons (Tremblay et al., 2017); many of these bacteria Including *Achromobacter*, *Acinetobacter*, *Alkanindiges*, *Alteromonas*, *Arthrobacter*, *Burkholderia*, *Dietzia*, *Enterobacter*, *Kocuria*, *Marinobacter*, *Mycobacterium*, *Pandoraea*, *Pseudomonas*, *Staphylococcus*, *Streptobacillus*, *Streptococcus*, and *Rhodococcus* were found to have essential roles in degradation of petroleum hydrocarbon (Chaerun et al., 2004; Jin et al., 2012; Margesin et al., 2003; Nie et al., 2014; Varjani and Upasani, 2016; Sarkar et al., 2017; Varjani, 2017; Xu et al., 2017).

Phytoremediation come up with economical friendly substitute to soil remediation by acceleration of contaminants removal from soil by plant and microbial activities at plant roots (Cunningham et al. 1996). The utilization of local plant species in phytoremediation is favorable as local plant species adapt well to local environment and conditions of soil, therefore, having higher expectation of success in growing and propagating on contaminated soil (Anh et al. 2017). Phytoremediation studies focused mainly on plant species that are local in the study areas. This study aimed to test the ability of plant species common in Libyan environment to remediate crude oil present in contaminated. In bioremediation process, seven mechanisms were identified. Phyto-extraction which known also as phyto-accumulation, contaminants are absorbed by roots of the plant and then translocated to other parts (Rascio N and Navari-Izzo F 2010). Phyto-extraction which involve extraction of contaminants in high concentrations from contaminated soil (Rascio N and Navari-Izzo F 2010). Phytostabilization that minimize contaminants leaching from soil by binding the contaminants to the plant's roots (Sarma H 2011). Phytodegradation which depends on microbes attached to and secreted enzymes by the roots to degrade contaminants (Pilon-Smits E 2005). Phytostimulation, involves stimulation of activity of soil microorganisms presents at the rhizosphere (Pilon-Smits E 2005). Phytovolatilization removes soil contaminants by volatilizing them (Limmer M and Burken J 2016). Rhizofiltration which removes contaminants via the roots action (Surriya O et al. 2015). Phytodesalination, reduces salt concentration of soil by halophytes to enhance soil fertility (Ali H et al. 2013).

Phytoremediation studies are often localized, focusing on species of regional plant that are capable of particular contaminants treatment. Chekol et al. examined sericea lespedeza, alfalfa, flatpea, deertongue, switchgrass reed canarygrass and tall fescue commonly found in the North America, for their potential of polychlorinated biphenyl (PCB) contaminated soil treatment. The study found that the plants significantly reduced PCB in the vegetated pots to various extents, in comparison to unplanted control pots (Chekol et al. 2004). Huang et al. revealed in a similar study, that the plant growth promoting rhizobacteria associated with Kentucky blue grass, tall fescue and wild rye enhanced phytoremediation (Huang X et al. 2004). Two plant species commonly found in Vietnam were identified by Anh et al. ias arsenic hyperaccumulators and four grasses with potential for treating lead and zinc contaminated soil (Anh et al. 2017).

II. Materials And Methods

Soil Sample Collection

Contaminated soil samples by crude oil, were collected from oil fields in Libya, using (ASTM, 1998) sampling of soil. Samples were collected randomly from superfacial soil layers (0-15 cm). then labeled and stored in sterilized ethylene bags at 4 ºC.

Microbial medias

Solid media

microbial media was prepared by mixing (15.0 g agar, 0.2 g disodium hydrogen phosphate (Na_2HPO_4) and 0.5 g ammonium sulfate ($(NH_4)_2SO_4$) and 1000 mL demineralized water). Mixture was autoclaved for 20 minutes at 120 °C, then cooled to 55°C,

10 mL of crude oil was added. Nutrient agar (23 g) was added to 1000 mL of demineralized water, then autoclaved for 20 minutes at 120 °C. All media were obtained from Faculty of pharmacy, University of Tripoli.

Liquid media

Nutrient broth was prepared by adding 8 g of nutrient broth to 1000 mL of demineralized water, autoclaved for 20 minutes at 120 °C. Media was obtained from Biotechnology Research Centre.

Plant collection

Three plant species (*Ricinus communis*, *Malva parviflora* and *Triticum repens*) were collected and used in this study depending on their phytoremediation effect in previous study (Saadawi et al. 2015).

Microorganisms and their Isolation

Microorganisms were isolated by selective enrichment technique from Libyan regions. According to modified procedure of Ilyina et al. (2003), microbial isolation was carried out using selective medium (agar containing crude oil). Each nutrient broth (1 mL) containing individual soil sample was serially diluted by adding 1 mL of the mixture to 9 mL of demineralized sterilized water, solution was mixed well, then this was repeated to the dilution 10^{-12} (chart 1), 1 mL of each dilution was added to the media and spread well then incubated at 27 °C for up to 21 days. Biggest colonies were selected at the end of the first week, two weeks and three weeks, then all colonies were inoculated on fresh agar media and incubated at 27 °C for one week. Pure stocks were prepared for the pure colonies for further tests. Selected single colonies were restreaked on agar plate and incubated for one week at 27 °C. This procedure was repeated several times to develop pure colony. colonies purity was confirmed under microscope when all the microbial cells had same Gram staining and morphological structure then transferred into a tube or bottle of fresh agar medium (pure stock culture). The pure isolated microorganisms were labeled (Ilyina et al. 2003).

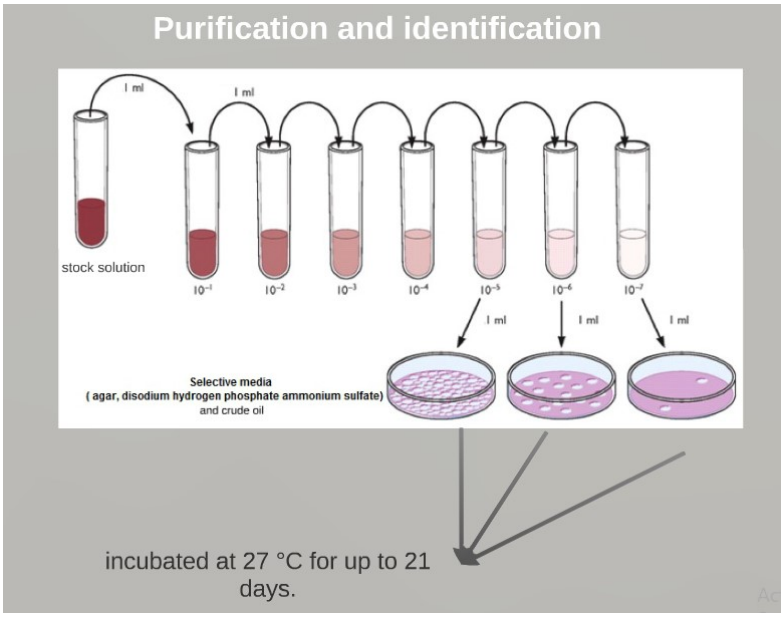

Chart 1 Serial dilutions of bacterial mixture isolated from crude oil contaminated soil.

Identification of Microorganisms

Isolated bacteria were identified according to the general principles of microbial classification, using Gram staining, macro- and microscopic examination of morphological characters (Sharma, 2007). Isolated bacterial strains from contaminated soil were

Gram stained in the lab, then a pure culture was identified in the Microbiology Laboratory, Department of Microbiology, Faculty of Pharmacy according to Bergey and Breed (1957) manual for bacteria identification.

III. Rate Of Biodegradation By Isolated Microorganisms

Microorganism Preparation

Each contaminated soil sample (10 g) was suspended in 100 mL of Nutrient broth (Alfreda & Ekene 2012), incubated at 37 Cº for 24 hrs. Each nutrient broth (10 mL) were added to 70 mL nutrient broth to obtain a microbial consortium then incubated at 37 Cº for 24 hrs. Bacterial count was carried out by measuring absorbance using (6505 UV/VIS) spectrophotometer at 560 nm wavelength, until a cell concentration of 1.5 x 10^8 colony forming unit (CFU)/mL (1 McFarland Standard) was achieved, 5 mL culture was transferred into 1 Liter nutrient broth. 375 mL of (1.5 x 10^8 CFU/mL) was added to 3 kg of soil in each pot. Two groups (duplicates) of pots were used (chart 2 & 3). The first group was for the effect of bacterial bioremediation (bacterial mixture + crude oil (0.5% and 1%w/w)). The second group contained mixture of both plant and bacteria (bacteria + plant (*Ricinus communis*, *Malva parviflora* and *Triticum repens* individually) + crude oil (0.5% and 1%w/w)).

All pots in the experiment were left under a shade, at 10-20 °C, humidity was maintained by daily spraying with distilled sterilized water to avoid dryness. Sampling were performed at zero time, 15 days, and 30 days of the experiment. Percentage of hydrocarbon degradation was determined.

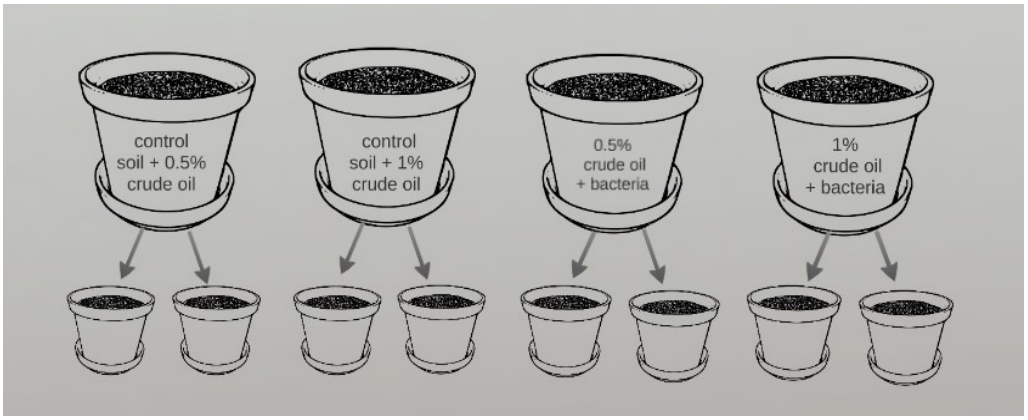

Chart 2 Experimental design for the effect of prepared bacterial consortia on 0.5% and 1% crude oil contaminated soil.

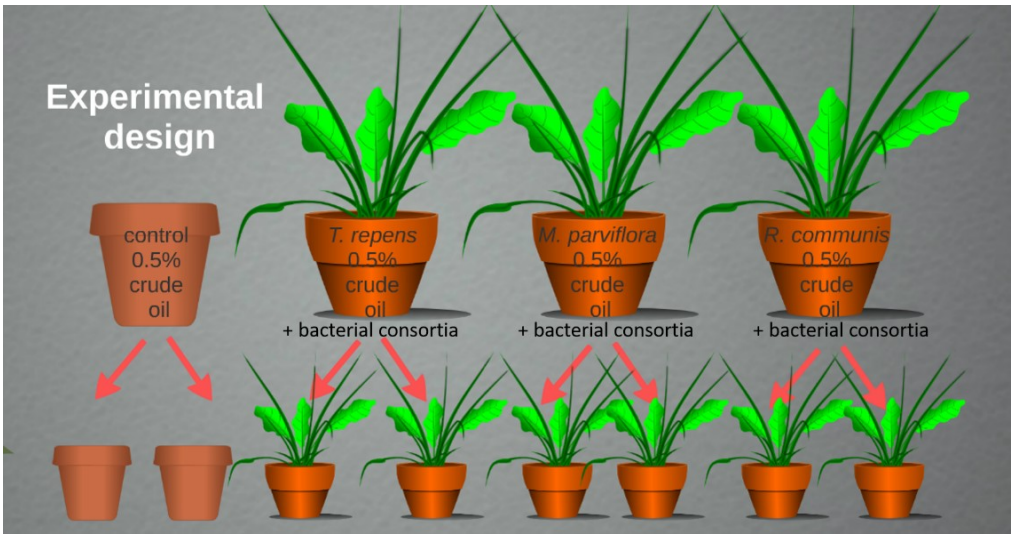

Chart 3 Experimental design for the effect of bioremediation and phytoremediation at 0.5% and 1% crude oil contaminated soil.

Measurement of Total Extractable Hydrocarbon Content

At zero time, triplicates (1 g) of soil in control pots (contain 0.5% and 1% w/w of crude oil) was weighed and transferred to dry, clean test tubes. Then 10 mL of hexane was added, vortexed and allowed to settle down for 30 min. Absorbance of hexane-oil extracts was determined using spectrophotometer (UV-Visible spectrophotometer 6505 UV/VIS. (JENWAY), at 360 nm wavelength for crude oil. After screening of several dilutions of crude oil in the spectrophotometer, the wave length was chosen. The best absorbance was detected at 360 nm. All pots were sampled in triplicate by the same way at 15 days and 30 days period (chart 4).

Concentration were calculated by comparing the resultant absorbance of samples with standard curve which was obtained by measuring absorbance of dilute standard solution of crude oil. Total hydrocarbon content was calculated after reading the absorbance of extracts from the spectrophotometer, exploiting from calibration curve and multiplying by an appropriate dilution factor (Osuji et al., 2006).

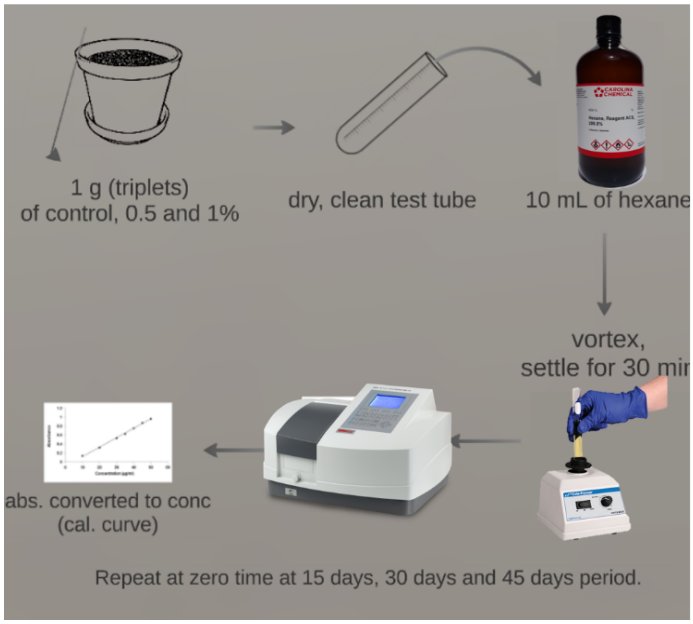

Chart 4 Measurement of Total Extractable Hydrocarbon Content

Calculation of Percentage of Oil Samples Degradation

Crude oil degradation percentage was calculated by comparing the concentration results of the test with those of the control using the following formula:

$$\text{Percentage degradation of oil samples} = \frac{(\text{Concentration of control} - \text{Concentration of test})}{\text{Concentration of control}} x\ 100$$

IV. Results And Discussion

Isolation of microbial strains

Soil samples were collected from oil fields in Libyan South desert, two microbial strains were isolated and identified on their macro- and microscopic features and Gram staining results. Their cultivation was performed on mineral salt media and agar media.

Identification of Isolated Microorganisms

Pure isolated microbial strains were inoculated on agar plate and incubated at 37 °C for one week. Macro- and microscopic examination of morphological characters were performed in Department of Microbiology, Faculty of Pharmacy. One strain was identified at the genus levels, the other isolate was identified morphologically (Table 1).

Table 1: Microbial isolates from Libyan oil fields on mineral salt media with crude oil as a carbon source.

Area	Mineral salt media
Soil sample from oil fields	E. coli
	Bacillus bacteria

It was found that the microbial growth occurred mostly on agar media, showing that the growth on mineral salt media containing crude oil was not preferable by microorganisms. This might be due to the presence of hydrocarbon degraders but in limited number. This can be interpreted due to the complex hydrocarbons that the microbes needed longer time to degrade.

Rate of biodegradation of oil samples

Soil triplicates in all pots in two groups (0.5% and 1% w/w of crude oil) was added to 10 mL hexane. The absorbance of hexane-oil extracts was determined using spectrophotometer at 360 nm wavelength for crude oil. Sampling was at zero time, 15 days and 30 days. Absorbance was converted to concentration by comparing it with standard curve, total hydrocarbon content was calculated.

Percentage of removal of 0.5 % and 1 % of crude oil by the prepared mixture of isolated bacteria (Figure 1), was 66 % of removal for 0.5 % crude oil concentration, while it was just 32 % of removal for 1 % concentration after 15 days. Percentage of removal increased to 67 % for 0.1 % crude oil after 30 days.

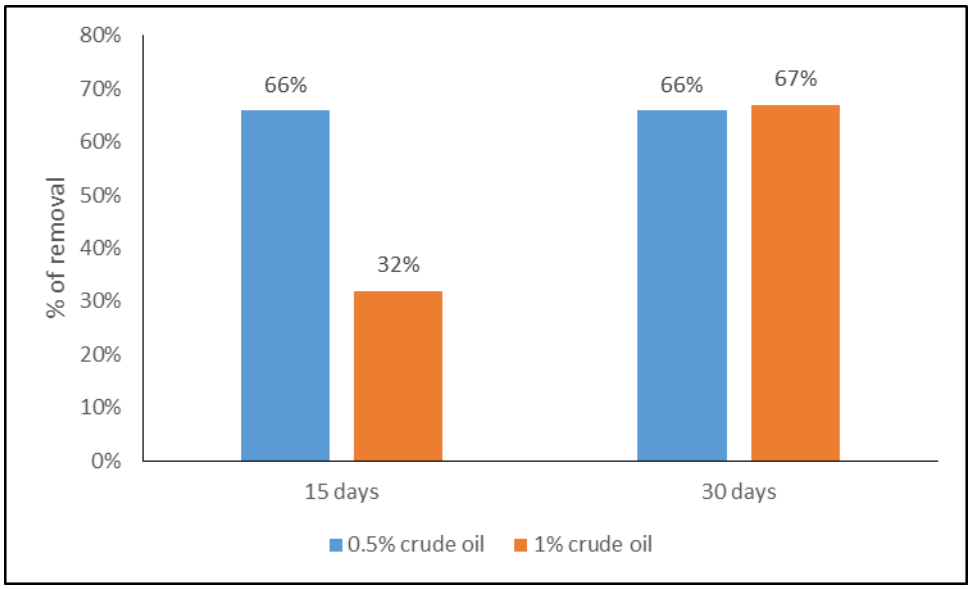

Figure 1. Percentage of removal of 0.5 % and 1 % crude oil contaminated soil by prepared isolated bacterial mixture at 15 days and 30 of experiment.

The result indicated that bacteria can degrade low percentage of crude oil easily in short period of time, increasing concentrations, led to lower percentage of removal. This can be explained that bacteria were adapted to the higher concentration of crude oil that may affected growth and replication after 15 days to reach higher percentage of removal after 30 days.

When the bacterial mixture of isolated bacteria was added to the soil that the plant species were vegetated individually, the percentage of removals of 0.5 % of crude oil (Figure 2), were the best among all used groups. The percentage of removal was 92 % for bacterial mixture and *Ricinus communis* for 0.5 % crude oil after 15 days only, followed by bacterial mixture and *Triticum repens* (79 %) then bacterial mixture and *Malva punilora* (71 %) at the same period of time.

It was noted that *Malva parviflora* and bacterial mixture had the lowest percentage of removal after 30 days, while the percentage of removals of other pots were improved. This is might be due to the competition between plant and bacteria on the nutrition sources, or due the accumulation of toxic metabolites that the plant couldn't handle. In group of pots contained bacterial mixture of isolated bacteria with the 1% crude oil contaminated soil that the plant species were vegetated in (Figure 3), the highest percentage of removal was for bacteria and *Ricinus communis* mixture after 15 days, while the highest percentage after 30 days was for bacteria and *Triticum repens* mix. The lowest percentage of removal was for *Malva parviflora* and bacteria mix (6 %) after 15 days.

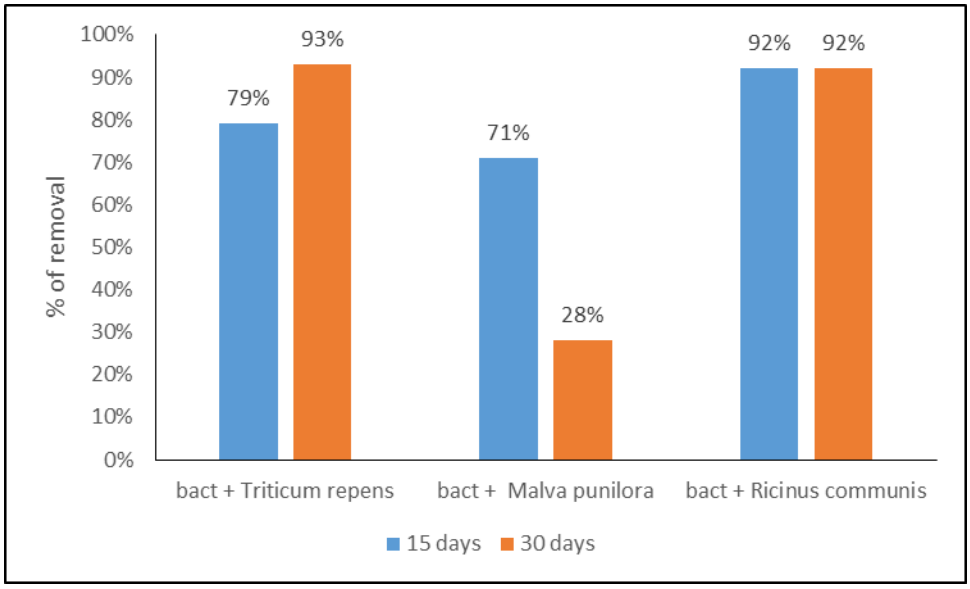

Figure 2. Percentage of removal of 0.5 % crude oil contaminated soil by isolated bacterial mixture and Ricinus communis, Malva parviflora and Triticum repens at 15 days, 30 days of experiment.

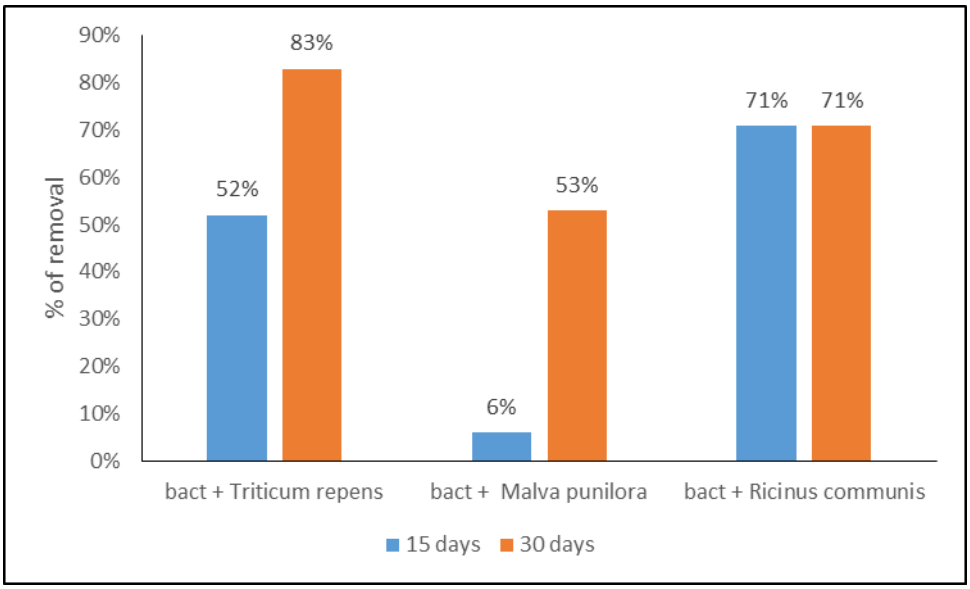

Figure 3. Percentage of removal of 1 % crude oil contaminated soil by isolated bacterial mixture and Ricinus communis, Malva parviflora and Triticum repens at 15 days, 30 days of experiment.

It was noted that *Malva parviflora* and bacterial mixture had the same pattern in 0.5 % and 1 % of crude oil. This can be explain for the same above reasons. *Ricinus communis* and bacterial mixture showed clear improvement in percentage of removal of crude oil both concentration groups, this was due to the effect of bacteria, because it was very clear that the plant couldn't handle crude oil and showed to be dead and dry during the experiment. This increment in removal percentage after applying the mix between plant and isolated bacteria may be due to potentiation of crude oil degradation by techniques used by plants and bacteria leading to more degradation than each of them individually.

In all tested pots that contained selected plants, percentage of crude oil removal and crude oil removal rates were higher than the control which contained no plants. This is compatible with previous results which showed that phytoremediation increased

removal of crude oil from contaminated soil (Liao C et al. 2016; Surriya O et al. 2015). Removal of crude oil occurred in unvegetated soil because of natural-occurring soil microbial activities but the presence of plants enhanced these activities and supported the degradation or absorption of the contaminants (Juck D et al. 2000). Roots provide large surface area for crude-oil degrading microorganisms and for absorption, breakdown and removal of crude oil. It is also likely that plant root exudates promote the growth of soil microorganisms as they contain nutrients and energy sources (Ma Y et al. 2016).

N-alkenes based on their chemical nearness combination are divided into six classes: < C_{13}, C_{13}-C_{16}, C_{17}-C_{21}, C_{22}-C_{25}, C_{26}-C_{29}, C_{29}-C_{36}. The first class includes normal alkanes which are smaller than C13. As these hydrocarbons evaporate in normal conditions, so they cannot be measured (Chorom et al., 2010). This was the reason might explain hydrocarbon contents loss in control group compared. Time effect on petroleum degradation was significant during study period.

Sang-Hawn et al. (2007) found that hydrocarbon degrading bacterial populations increased rapidly during the first four weeks of 14 weeks testing period. They proposed this finding that it may be considered as an indicator for the feasibility of oil-contaminated soils bioremediation. However, with increasing of time, due to the oil-resistant components with high chain and within less remaining nutrients, bacterial growth as well as oil degradation were decreased (Schaefer and Juliane, 2007). Van Gestel et al. (2001) reported a significant increment of the oil-polluted soil bioremediation in bacteria population.

V. CONCLUSION

The ability of various indigenous microorganisms, especially those isolated from polluted sites, to metabolize hydrocarbons is well known. In this study, microorganisms that are able to grow in the presence of crude were isolated from the contaminated soil sites in Libyan South Desert. Selective mediums (agars containing crude oil) was used to select oil samples degraders. Growth of the isolated microorganisms on crude oil containing media indicated the presence of hydrocarbon and oil-degrading activities in isolated microbial strains. research goals were individually evaluated and successfully executed. The following conclusions have been reached: of the 2 microorganisms isolated from soil of different oil fields areas selected for studying hydrocarbon biodegradation, the two bacterial species found, *E. coli* spp., and bacillus bacteria, were the effective degraders for crude oil in 0.5 % and 1 % concentration. In addition. The highest percentage of crude oil removal was obtained by *Triticum repens* and bacterial mixture.

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