



Original Research Article

Studies on crude oil degrading Bacteria isolated from Libyan desert

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ABSTRACT

A laboratory scale study was conducted to indicate that four bacteria isolates from Libyan Desert were identified as *Cellulosimicrobium cellulans*, *Brevibacterium liquefaciens*, *Brevibacterium mcbrellneri* and *Enterococcus saccharolyticus*, respectively. Optical density (OD), total petroleum hydrocarbon (TPH) and gas chromatograph (GC) as evaluation experiments were used as the major indicator of microbial degradation of crude oil at concentration of 1% (v/v). Growth rate of isolates *C. cellulans* and *B. mcbrellneri* have shown an increase within short time (5 days), whereas, *E. saccharolyticus* and *B. liquefaciens* grown within longer time (up to the 10 days). The concentration of the TPH in the cultures of selected isolates was decreased to 18.86, 26.16 and 39.04% for *C. cellulans*, *E. saccharolyticus* and *B. liquefaciens*, respectively. Results of GC indicated that the highest degradation efficiency of crude oil was observed with *B. liquefaciens* culture, whereas moderate degradation of crude oil was performed by *C. cellulans* and *E. saccharolyticus* after 15 days incubation. They concluded that *C. cellulans*, *E. saccharolyticus* and *B. liquefaciens* have the potential to be used in the bioremediation of crude oil.

Keywords

C. cellulans,
E. saccharolyticus
and
B. liquefaciens
Libyan Desert,
Bioremediation

Introduction

Since the industrial revolution, and in particular, over the last 100 years, human technological endeavor has resulted in massive changes and sociological benefits. Hand in hand with these benefits have come particularly dangerous and deleterious impacts on our physical environment. As a result of accumulated industrial activity, our atmosphere has deteriorated, our waterways and oceans are polluted and a significant portion of our land is contaminated with

toxic chemicals (1). Bioremediation is an attractive approach that can be used to clean up petroleum hydrocarbons because it is simple to maintain, applicable over large areas, cost-effective and leads to complete destruction of the biodegradable organic contaminants (2). It is one of the newest and fastest growing applications of environmental biotechnology is bioremediation (3). The biological treatment of contaminated soil and water has become a useful strategy, especially for remediation of

sites polluted with petroleum hydrocarbons (4 & 5). Bioremediation is defined as the process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state, or to levels below concentration limits established by regulatory authorities (6). It uses naturally occurring bacteria and fungi or plants to degrade or detoxify substances hazardous to human health and/or the environment (7). Most petroleum hydrocarbons are considered biodegradable (8 & 9). Biodegradation of organic matter is achieved by assisting the microbial growth and by creating optimum environmental conditions for them to degrade the contaminants into carbon dioxide and other gases, inorganic substances, water, and to produce microorganism biomass (8, 10 & 11). Petroleum hydrocarbons can be degraded by microorganisms such as bacteria, fungi, yeast and microalgae (9 & 12). However, bacteria play the central role in hydrocarbon degradation. The driving force for petroleum biodegradation is the ability of microorganisms to utilize hydrocarbons to satisfy their cell growth and energy needs. A large number of studies report that low molecular weight alkanes are degraded most rapidly, moreover mixed cultures carry out more extensive biodegradation of petroleum than pure cultures (13, 14 & 15). Almost a century has passed since the first hydrocarbon-degrading bacteria were isolated and described, and the most recent list includes almost 200 bacterial, cyanobacterial, algal and fungal genera, representing more than 500 species and strains (16 & 17). In many ecosystems there is already an adequate indigenous microbial community. None of the microorganisms are able to utilize all hydrocarbons; so each organism can utilize only a certain range of compounds (18). The ability to degrade petroleum hydrocarbons is not restricted to a few microbial genera; a

diverse group of bacteria and fungi have been shown to have this ability, and in review noted that more than 100 species representing 30 microbial genera had been shown to be capable of utilizing hydrocarbons (19). There are differences in the relative ability of the *Acinetobacter*, *Bacillus* and *Pseudomonas* strains to degrade hydrocarbon and the ability of these strains to degrade hydrocarbons in oily sludge suggests that they could be used for the treatment of other oil-wastes. *Flavobacterium* sp. and a *Brevibacterium* sp. were able to mineralize *n*-paraffins which found as the best substrates for both organisms, but *Flavobacterium* sp. exhibited higher rates of mineralization. However, branching prevented utilization by *Flavobacterium* sp. but not by *Brevibacterium* sp. (21). Many reports reported that the bacterial strains belonged to the genera *Gordonia*, *Brevibacterium*, *Aeromicrobium*, *Dietzia*, *Burkholderia* and *Mycobacterium* are new and not yet described which were isolated from petroleum-polluted soil (22). The goal of this study is to isolate and identify crude oil degrading bacteria and to study laboratory scale bioremediation using liquid media contaminated with crude oil by a potential isolates.

Materials and Methods

Isolation and identification

Among forty (40) bacterial isolates isolated from libya desert around 700 Km south of Benghazi, fourteen had the ability to grow up to 10% on crude oil. Four of them were chosen for further studies, the selected bacterial were identify at the Agricultural Research Center, Cairo, Egypt. The BioLog3 microbial identification system was applied to identify the bacterial strains which included incubator, analyzer,

computer, printer, and software to read the Biolog MicroPlates.

Efficiency of the identified bacterial isolates for the degradation of crude oil

To estimate the ability of identified bacteria to degrade the crude oil, the experiment details are as follow: Four identified bacteria isolates were streaked onto nutrient agar plates and then incubated at 30°C for 18-24h for preparation of bacteria inoculation. Sterilized Erlenmeyer flasks (250ml capacity) containing 100 ml of sterilized Basal medium supplemented with 1% (v/v) crude oil were prepared. Two to three colonies of identified bacteria were inoculated to Erlenmeyer flasks and incubated at 35°C ± 2°C under shaking condition (120 rpm) for 15 days. To evaluate the biodegradation rate of identified bacteria different kinds of tests were applied as following:

Optical Density (OD)

Optical density (OD) of cultures was measured to monitor the growth rate of biodegradation at a wavelength of 540nm, and samples were taken at fifth, seventh, tenth and fifteenth days incubation.

pH

Samples of bacteria cultures were taken for pH readings at first and fifteenth day of incubation.

Total Petroleum Hydrocarbon (TPH)

The Gravimetric method (filtrate) EPA standard method was used to extract hydrocarbon in the sample to measure total petroleum hydrocarbon (TPH), each treated samples were filtrated with a filter paper (Waterman No. 1) and a fixed volume of the

solution was collected. After that, 50 ml of dichloromethane was added and mix thoroughly and leave it to separate. The lower layer was collected in cleaned-dried beaker, as well upper layer extracted 3 times with dichloromethane. To remove water from extracted solution, Sodium Sulphate (anhydrous) was used, and to measure TPH, Pre-weighed cleaned dishes (placed in a moisture-free environment) were used to collect the lower layer and allowed to evaporate solvent (dichloromethane) completely, then weight the dishes; calculate the % of extractable organic material, as follows:

$$\% = [\text{Difference in weight} / \text{volume of the pre-treated filtrate}] \times 100$$

The residue was washed with an equal volume of dichloromethane and the presence of hydrocarbon is detected.

Extraction of Residual Crude Oil

The residual crude oil was extracted from the Basal culture after 15 days of incubation by washing three times with dichloromethane in 1:2 ratio, as described by (23). Briefly, 10ml of culture were mixed vigorously with 20ml dichloromethane and centrifuged for 15 minutes at 18,000g. The organic layer (upper layer) was taken, and the aqueous layer was washed twice with dichloromethane. The three separated organic layers were combined and the solvent was evaporated by a rotary evaporation under vacuum at 37°C. The residual crude oil was analyzed by Gas Chromatography (GC).

Gas Chromatographic analysis

Analysis was performed by gas chromatography (Varian CP-3800) using 007 series Methyl Silicon (capillary column

a 30m x 0.25m, ID 0.25u Film Thickness). The flow rates of hydrogen and air for the FID detector were 30 and 400ml/min, respectively. Injector and detector temperature were 300°C. The initial oven temperature was 80°C and programmed to increase at 6°C per min to 280°C for 30 minutes and helium was used as the carrier gas. Analysis of TPH and GC were carried out in the laboratories of Libyan Petroleum Institute (LPI), Tripoli, Libya.

Result and Discussion

Bioremediation is the use of biological systems to destroy or reduce the concentrations of hazardous wastes from contaminated sites. Such systems have the potential site applications include ground water, soils, lagoons, sludge and process waste-streams. Many studies articles have documented the potentials of microorganisms to degrade oil both in the laboratory and field trials. A number of scientific papers including several review articles covered aspects of the biodegradation process as well as results from controlled field experiments designed to evaluate degradation rates in various environments (26 & 27). Crude oil is a complex but biodegradable mixture of hydrocarbons, and the observation that hydrocarbon degrades can be enriched in many, if not most, types of environments have contributed to the development of oil bioremediation techniques (28). The fate of petroleum hydrocarbons in the environment is largely controlled by a biotic factors which influence the rates of microbial growth and enzymatic activities that determine the petroleum hydrocarbon utilization. The ability to isolate high numbers of certain oil-degrading microorganisms from oil-polluted environment is commonly taken as evidence that these microorganisms are the active

degraders of that environment (24). Isolation, identification, and genetic manipulation of a vast number of indigenous bacterial species for bioremediation of pollutants have been the focus of many investigations worldwide (25 & 13). The present work deals with the biodegradation of crude oil by Forty bacteria isolates which were isolated from the Libyan desert contaminated with crude oil. Bacterial isolate D2, S3, SI1 and SI2 were identified as *Cellulosimicrobium cellulans*, *Brevibacterium liquefaciens*, *Brevibacterium mcbrellneri* and *Enterococcus saccharolyticus* respectively (Table,1).

The data of these experiments showed promising hydrocarbon degrading ability with bacteria isolates. Because our ultimate goal was to select isolates with the ability to utilize hydrocarbons, eventually many complex substrates such as crude oil and industrial organic waste, we chosed four of the most successful isolates for further investigation. *C. cellulans*, *B. liquefaciens*, *B. mcbrellneri* and *E. saccharolyticus* isolates were selected due to their increased ability to degrade hydrocarbons in batch culture experiments. These isolates were further tested in Basal medium containing 1% (v/v) of crude oil. During aerobic oxidation of crude oil, the pH of the cultures was lowered by 2.72, 1.01, 1.71 and 1.91 by *C. cellulans*, *B. liquefaciens*, *B. mcbrellneri* and *E. saccharolyticus* after 15 days of incubation, respectively (Table,2). Since, the pH of the culture is important in determining the course of a metabolic sequence and increase or decrease in pH of the culture indicated the production of basic or acidic metabolites. Other studies have detected fatty acid production from hydrocarbons using the fall in pH as a criterion of acid formation (29 & 30).

Table.1 The Growth (calculated as Optical Density) and pH changes of identified bacteria strains grown in 1 %(v/v) crude oil at 23 °C

Bacteria	Optical Density at 540 nm						
	Zero Day		First Day	Fifth Day	Tenth Day	Fifteenth Day	
	OD	pH				OD	pH
<i>Cellulosimicrobium cellulans</i> D2	0.028	7.4	0.120	0.225	0.126	0.101	4.68
<i>Brevibacterium liquefaciens</i> S3	0.026	7.3	0.285	0.344	0.610	0.131	6.29
<i>Brevibacterium mcbrellneri</i> S11	0.039	7.41	0.596	0.635	0.599	0.503	5.7
<i>Enterococcus saccharolyticus</i> S12	0.042	7.36	0.416	0.482	0.512	0.488	5.45

Figure.1 TPH concentration in the cultures of identified bacteria with 1% (v/v) crude oil after 15 days of incubation

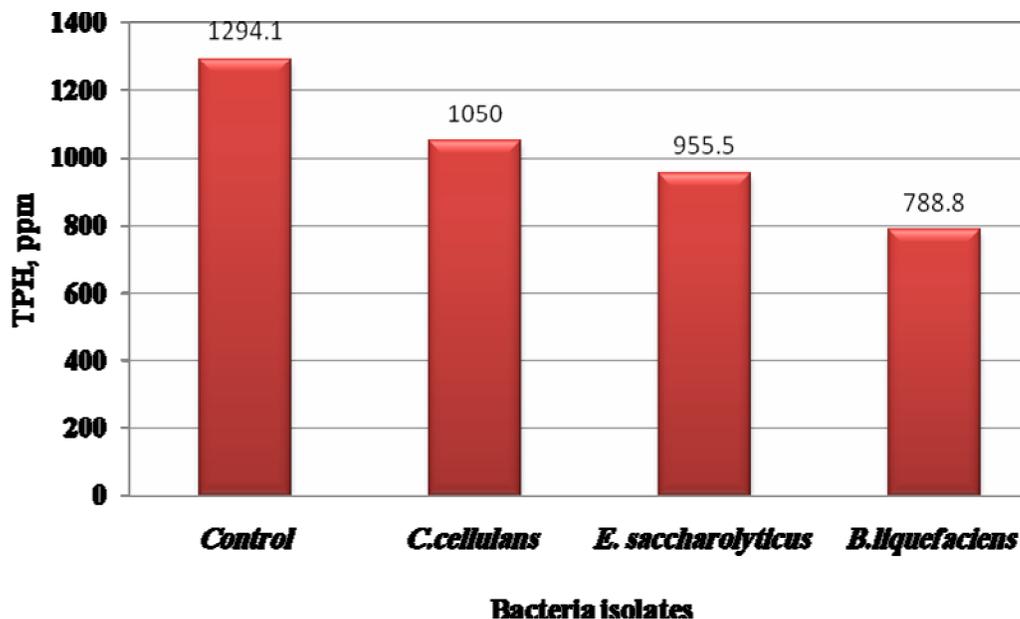
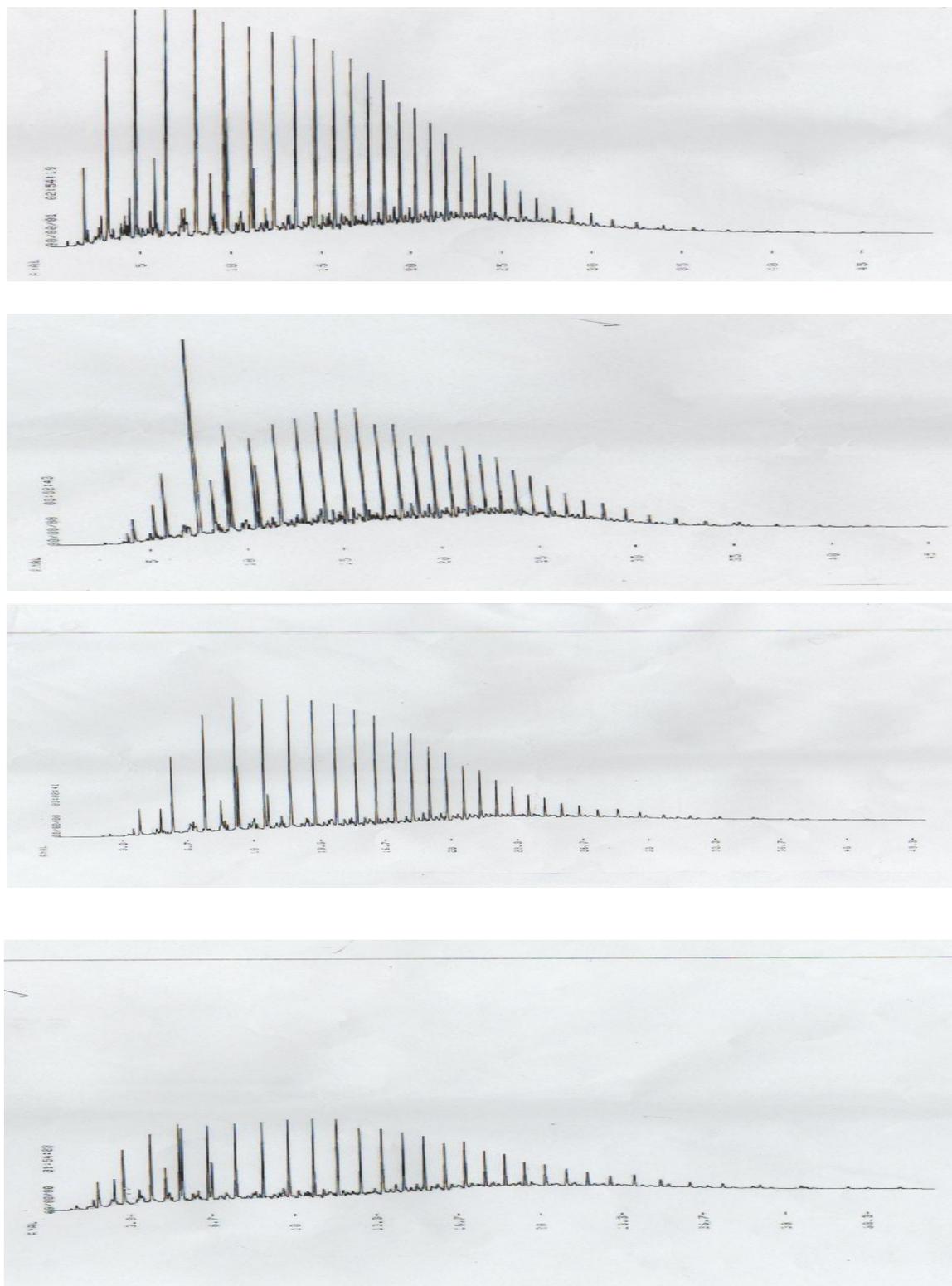


Figure.2 Gas chromatographic (GC) analyses of crude oil before and after inoculation
a.) no bacteria (control); **b.)** *C. cellulans* (D2); **c.)** bacteria isolate *E. saccharolyticus* (SI2);
d.) bacteria isolate *B. liquefaciens* (S3) after 15 days of incubation



References

- 1) Lucas J. A., Wibberley L. J., Kennedy E. M. and Dlugogorski B. Z. (2001). Hydrocarbon contaminated soils and the development of an innovative thermal process. ANZ Geo.Environment. November: 28-30 November, Newcastle, Australia.
- 2) Lee S., Oh B. and Kim J. (2008). Effect of various amendments on heavy mineral oil bioremediation and soil microbial activity. *Bioresource technology*. 99 :2578-2587.
- 3) Rittmann B. E. and McCarty P. L. (2001). Environmental biotechnology: principles and applications. McGraw-Hill.
- 4) Sims R. C. (1990). Soil remediation techniques at uncontrolled hazardous waste sites-a critical review. *Journal of the air and waste management association* 40:704-732.
- 5) Bouwer E., Mercer J., Kavanaugh M. and DiGiano F. (1988). Coping with groundwater contamination. *Journal water pollution control federation*. 60 :1415-1427.
- 6) Mueller J. G., Cerniglia C. E. and Pritchard P. H. (1996). Bioremediation of Environments Contaminated by Polycyclic Aromatic Hydrocarbons. In *Bioremediation: Principles and Applications*. Cambridge University Press, Cambridge.pp.125-194.
- 7) Vidali M. (2001). Bioremediation. An overview. *Pure applied Chemistry*. 73:1163-1172.
- 8) Eweis J. B., Ergas S. J., Chang D. P. Y. and Schroeder E. D. (1998). *Bioremediation Principles*. McGraw-Hill International.
- 9) Riser-Roberts E. (1998). *Remediation of Petroleum Contaminated Soils: Biological, Physical, and Chemical Processes*. Lewis CRC Press, Boca Raton, Florida.
- 10) Suthersan S. (1997). *Remediation Engineering Design Concepts*. Lewis Publishers, CRC Press, Boca Raton, Florida.
- 11) Kriipsalu M., Marques M., Nammaria D.R. and Hogland W. (2007). Bioremediation of oily sludge: The contribution of amendment material to the content of target contaminants, and the biodegradation dynamics. *Journal of hazardous materials*. 148 :616-622.
- 12) Bundy J. G., Paton G. I. and Campbell C. D. (2004). Isolation of a *Pseudomonas stutzeri* strain that degrades *o*-xylene. *Applied and environmental microbiology*.53 :2129-2132.
- 13) Ghazali M. F., Zaliha N. R., Abdul R. N., Salleh A. B and Basri M. (2004). Biodegradation of hydrocarbons in soil by microbial consortium. *International biodeterioration and biodegradation*.54 :61-67.
- 14) Trindade P. V. O., Sobral L. G., Rizzo A. C. L., Leite S. G. F. and Soriano A.U. (2005). Bioremediation of a weathered and recently oil-contaminated soils from Brazil: a comparison study. *Chemosphere* :58:515-522.
- 15) Gerdes B., Brinkmeyer R., Dieckmann G. and Helmke E. (2005). Influence of crude oil on changes of bacterial communities in Arctic sea-ice. *FEMS. Microbiology ecology*. 53 :129-139.
- 16) Prince R. C., Lessard R. R. and Clark J. R. (2003). Bioremediation of marine oil spills. *Oil gas science and technology*. 58:463-468.
- 17) Head I. M., Jones D. M. and Roling W. F. (2006). Marine microorganisms make a meal of oil. *Nature reviews microbiology*. 4:173-182.
- 18) Alvarez H. M. (2003). Relationship between β -oxidation pathway and the hydrocarbon-degrading profile in actinomycetes bacteria. *International biodeterioration and biodegradation*. 52 :35-42.

- 19) Atlas R. M. (1981). Microbial Degradation of Petroleum Hydrocarbon: an Environmental Perspective *Microbiological Reviews*.1:180-209.
- 20) Verma S., Bhargava R. and Pruthi V. (2006). Oily sludge degradation by bacteria from Ankleshwar, India. *International biodeterioration and biodegradation*.57 :207-213.
- 21) Bartha R. and Atlas R.M. (1973). Biodegradation of oil in seawater: Limiting factors and artificial stimulation. In: The microbial degradation of oil pollutants. D.G. Ahearn and S.P. Meyers. Baton Rouge, LA.
- 22) Chaillan F., Le Flèche A., Bury E., Phantavong Y., Grimont P., Saliot A. and Oudot J. (2004). Identification and biodegradation potential of tropical aerobic hydrocarbon-degrading microorganisms. *Research in microbiology*.155 : 587-595.
- 23) Kokub D., Shafeeq M., Khalid Z. M. and Maik K.A. (1990). Comparative studies on Emulsification and biodegradation of indigenous crude oils by enriched bacteria culture. *Biorecovery*. 2: 55-68.
- 24) Ojo O. A. (2006). Petroleum-hydrocarbon utilization by native bacterial population from a wastewater canal Southwest Nigeria. *African journal of biotechnology*. 5: 333-337.
- 25) Van Hamme J. D., Singh, A. and Ward O. P. (2003). Recent advances in petroleum microbiology. *Microbiology and molecular biology review*, 67:503-549.
- 26) Wang Chun-Ming, Li Da-Ping and Liu Shi-Gui. (2007). Screening and Identification of Four PAHs-biodegrading Strains. 13-4. 546-550.
- 27) Gunkel W. and Gasmann G. (1980). Oil, oil dispersants and related substances in the marine environment. *Helgoland marine research*. 33:164-181.
- 28) Margesin R. and Schinner F. (1997). Bioremediation of diesel-oil contaminated alpine soils at low temperature. *Applied microbiology and biotechnology*. 47: 462-468.
- 29) Bird C.W. and Molton P.W. (1970). Production of fatty acid from hydrocarbons by microorganisms. *Topics in lipid chemistry*. 3:125-169.
- 30) Abbot B. J. and Gledhill W. E. (1971). The extracellular accumulation of metabolic products by hydrocarbon degrading microorganisms. *Advances in applied microbiology*.14 :249-388.
- 31) Pelczar M., Reid R. and Chan E. (1982). *Microbiology*. McGraw-Hill publishing company Ltd. New York.
- 32) Fidorak P. M. and Westlake D. W. S. (1981). Microbial degradation of aromatic and saturates in Prudhoe crude oil as determined by glass capillary gas chromatography. *Canadian journal of microbiology*. 27:432-443.