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The Biodegradation of Crude Oil by *Bacillus subtilis* Isolated from Contaminated Soil in Hot Weather Areas

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ABSTRACT: Using Environmental-friendly methods in order to remove or reduce oil pollutants and their derivations in the environment are developing. In this study, the biodegrading ability of *Bacillus Subtilis* .sp which has been separated from oil-polluted soil was examined. It was revealed that it can reduce surface tension of growth Medium and produce Bio-Surfactant at 37 and 20 Degrees centigrade. Also, it has the ability to biodegrade oil hydrocarbons. A reduction in surface tension from 58 Nm/m to 31.2 Nm/m at different percentages of crude oil at 37 degrees centigrade is one of the important results.

Key words: Biodegradation, Crude oil, Bacillus Subtilis, Bio-surfactant, Environment

INTRUDUCTION

Crude Oil is mostly composed of hydrocarbons. Hydrocarbons include some groups whose molecular structures only include hydrogen and carbon. Different types of hydrocarbons are Paraffin hydrocarbons (CnH2n+2), oil hydrocarbons such as cyclo paraffins (CnH2N), and aromatic hydrocarbons such as Benzoates (H2n-6). The second group is Heterocompounds (EPA, 2004; Margesin *et al.*, 2003). This group includes elements other than hydrogen and carbon. They include elements such as oxygen, nitrogen, sulfur. Metallic atoms such as Nickel might be combined with them (Meintanis *et al.*, 2006; EPA, 2004; Das & Mukherjee, 2007). Environmental pollutions related to crude oil and its derivations have been caused by leakage from drilling wells, transportation tankers, oil tanks, pipelines and accidents during oil transportations (Jalilzadeh *et al.*, 2013; García-Flores, 2013). Generally, physical, chemical, biological, or a combination of these methods is used to remove or reduce crude oil and its derivations in the environment. Because environmental methods such as bioremediation are environmental-friendly, they are developing on a daily basis (EPA, 2004; Oluseyi, 2011).Micro-organisms which are existent in the soil use oil compounds as the sources of carbon and energy. They produce bio-surfactant and reduce surface tension, which causes biodegradation (Mesdaghinia *et al.*, 2007; Akhavan Sepahi *et al.*, 2008; Ta-Chen Lin *et al.*, 2008). Numerous studies have been conducted to investigate oil-related pollutions and their impact on water and soil. In one study on oil-polluted soils, the efficiency of biopile and landfarming method was investigated in Taiwan in 2008.The researchers managed to remove 60 to 70 percent of pollutants by bacteria such as *Pseudomonas* and *Acintobacter* (Ta-Chen Lin *et al.*, 2008). In one research conducted in Karachi in 2012, some bacteria were studied which produce bio-surfactant and degrade the

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oil-related pollutions caused by repair shops. A group of these bacteria were called DGEF 01-08. In this study, the degree of bacteria's growth was determined by Tensiometry (Shoeb *et al.,* 2012). In another study on three populations of microbes which degrade oil, it was revealed that biodegrading bacteria can biodegrade oil compounds completely and produce n-alkanes and branched alkanes, while most of isopernoid alkanes remained (Viñas *et al.,* 2002). Jalilzadeh and colleagues studied the bioremediation of MTBE, which is one the oil derivations, by *Bacillus* sp. It was revealed that a species of Bacillus, called *Bacillus cereus,* can degrade the mentioned substance at 28 and 37 degrees centigrade in different concentrations throughout 120 days (Jalilzadeh yengejeh *et al.,* 2013). Other research which can be referred to include studies conducted by Margzin in 2003 (Margesin,R.*et al.,* 2003), Carmela and colleagues in 2005 (Carmela *et al.,* 2005), Kishordas in 2007 (Das & Mukherjee, 2007), and Akhavan Sepahi in 2008 (Akhavan Sepahi *et al.,* 2008). In this study, samples of oil-polluted soils were collected from south western Iran and biodegrading ability of one of the most important species was investigated. It was revealed that the separated *Bacillus Subtilis* in a hot area can degrade crude oil, especially oil hydrocarbons.

MATERIALS & METHOD

This study included several phases, including sampling in the area, identifying and separating bacteria, measuring the activitess level of bacteria's surface tension, and finally chemical analysis in order to be sure about biodegradation of identified bacteria. Collecting samples from the soil close to the drilling wells and oil-polluted areas was done. Soil samples were taken to the laboratory in standard condition and below 4 degrees centigrade. In this study, two groups of mediums were used, including organic medium such as Nutrient Agar and Mineral Salt Medium (MSM) (Mesdaghinia *et al.,* 2007). The main components included 7H20. 0.25-MgSO4, 0.5-KH2PO4, 0.5-K2HPO4, 1-NaCl, 0.009-CaCl2.2H2O, 0.5-KNO3. The minor components included 0.1-Mn Cl2.4H2O, 0.07-ZnCl2, 0.015-CuCl2.2H2O, 0.025-Ni Cl2.6H2O, 0.12-COCl2.6H2O, 0.025-Na2MO4.2H_2O (g/l). In this stage, after making suspension from the soil and culturing it in a suitable environment in the incubator for 24 hours and observing grown colonies in Mineral Salt Medium, re-culturing (4-phases) of microbial consortium was done and continued until solo-colonies were observed (jalilzadeh yengejeh *et al.,* 2013). In the next stage, the initial identification of bacteria genus was done by biochemical tests such as Catalase, Starch Hydrolysis, IMVIC, Arabinose Fermentation, Manitol Fermentation, and also growth at different percentages of salt concentration. After extracting Deoxyribonucleic acid (DNA), final identification of molecule was made, including sequence determination analysis by polymerase chain reaction (PCR) and 16s r RNA (Shahidi Rizi *et al.,* 2012; Jalilzadeh yengejeh *et al.,* 2013; Weisburg *et al .,* 1991).

In order to measure surface tension, Tensiometer and (du Nouy) ring method were used (Akhavan Sepahi, *et al.,* 2008; Tabatabaee *et al.,* 2005; Shahidi Rizi *et al.,* 2012). 25 ml of 48-hours samples of culture were kept in the set. The temperature of the sample was 25 degrees centigrade before measuring surface tension. The experiment was conducted three times for each sample. For each measurement, surface tension of distilled water and bacteria-free culture environment were taken as control samples. Three 250-ml Erlenmeyers which included 100 ml sterilized Mineral Salt Medium and %1 East extract and %1 sterilized crude oil. Also, a sample was taken as control sample and the amount of oil compound reduction (Biodegradation) by bacteria was measured within 48 hours, 72 hours, and 96 hours by using High-performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) (Viñas, *et al.,* 2002).

RESULTS & DISCUSSION

After culturing in Mineral Salt Medium, the stains identified by *Bacillus Subtilis sp*. and *Bacillus Cereus sp.* were put at different pH levels. *Bacillus* and separated species are the main microbial groups which degrade hydrocarbon compounds. Carmela and colleagues (2005) studied the biodegrading ability of aromatic hydrocarbons (PAH5) by Bacillus (Carmela et al. 2005). The results related to Optical Density (OD_{600}), which is one of the indicators of microbial growth, after 48 hours at 37 and 20 centigrade with a wavelength of 600 Nanometer have been shown in fig.s 1 and 2.

Considering the above Figures (Fig. 1 to 2), it can be said that the optimum pH level is 7 and the maximum amount of microbial growth occurred at 20 and 37 degrees centigrade. On the whole, bacterial growth in the range of 6-8 was observable clearly and *Subtilis* had the best performance. Because of the favorable results obtained by *Bacillus Subtilis* compared to that of *Bacillus Cereus* in optimization, the following experiments were made by *Bacillus Subtilis* (Table 1). Due to similar growth of selected strains in terms of growth rate in Mineral Salt Medium at different percentages of oil (%1, %3, %5) and the maximum of concentration reduction at %1, the study was continued with this percentage of oil.

It can be seen in table 1 that as the optimum pH level is 7-8, *Bacillus Subtilis* at 20 æ%C and 37 æ%C is

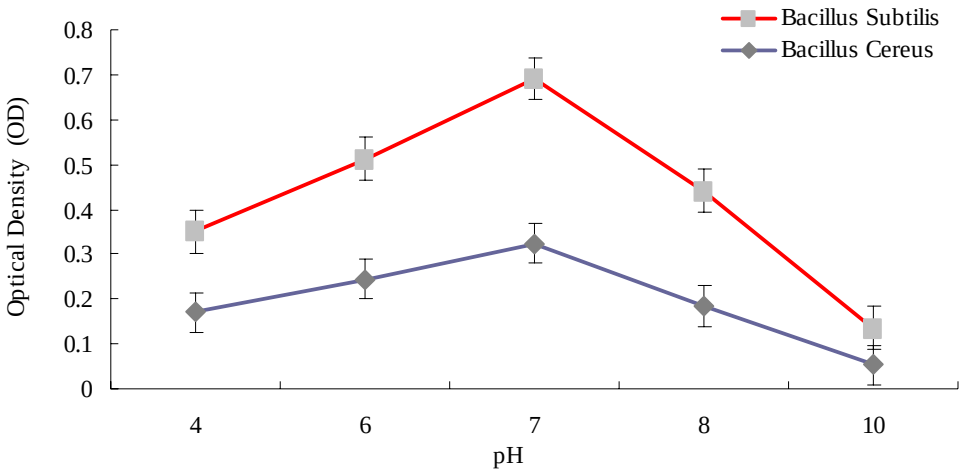

Fig. 1. The OD mean of selected strains at different pH levels at 20 degrees centigrade in 48 hours

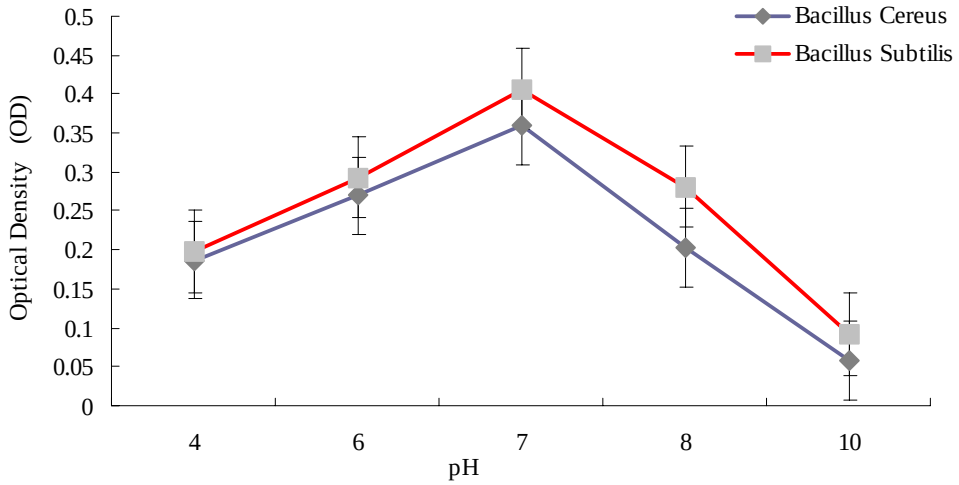

Fig. 2. The OD mean of selected strains at different pH levels at 37 degrees centigrade in 48 hours

Table 1. The amount of Bacillus Subtilis surface tension (mN/m) at 20 and 37 Centigrade in 48 hours

Temperature (^{o}C)	pH	4	6	7	8	10
	Blank	58	56	57	58	**58**
20	*Bacillus Subtilis*	6.32	8.31	31	1.32	**32**
37	Bacillus Subtilis	1.31	31	5.30	4.31	**2.31**

able to reduce surface tension to 31 and 30.5 (mN/m). The study conducted by Tabatabaee and colleagues in 2005 showed that Bacillus' strains are able to reduce surface tension to below 40 mN/M (Tabatabaee, *et al.*, 2005). Also, the study made by Akhavan and colleagues in 2008 showed that two types of Bacillus can reduce surface tension from 60 to 31 and 38 mN/m (Akhavan Sepahi *et al.*, 2008).

Then , the selected strain was cultured in liquid Mineral Salt Medium which included %1 crude oil and %1 East extract at PH=7. Then, it was put in incubator at 20 and 37 degrees centigrade for 48 hours in different shaker rotations of 100, 150, and 200. It was revealed that as the temperature increases, the Optical Density is also increased. For instance, this value at 20 and 37 degrees centigrade were at 200 rotations were 0.267 and 0.296 respectively. At next shaker rotations, the increase in optical density by the increase in temperature from 20 to 37 was completely visible. In Table 2, the average of *Bacillus Subtilis'* surface tension at 100, 150, and 200 RPM, (RPM: revolutions per minute) shaker rotations and the degree of

Table 2. The average surface tension(mN/m) of the best selected strains at various shaker rotations in %1concentration

Temperature (^{0}C)	Rotation of Shaker(RPM)	100	150	200
	Blank Sample	58	58	**58**
20	*Bacillus Subtilis*	40	34.6	**31.5**
37	*Bacillus Subtilis*	36.7	34.6	**31.2**

reduction in surface tension, which is the result of bio-surfactant production, have been shown.

In order to Measuring bio-surfactant growth at various temperatures,The selected strains in liquid Mineral Salt Medium which included %1 East extract and %1 crude oil were kept at various temperatures. After 48 hours with different shaker rotations per minute, the degree of Optical Density (DO) was measured at 600 Nanometers. The optimum results were obtained at 200 rotations per minute. Generally, temperature is one of the important environmental factors in biodegradation. Most studies have emphasized the positive and effective role of temperature, including a study made by Jalilzadeh and colleagues (2012) in which *Bacillus cereous RJ1* was examined. In this study, they found that the biodegradation of MTBE, which is one of the oil derivations, at 37 degrees centigrade is more than 28 degrees centigrade (Jalilzadeh yengejeh *et al.*, 2013). In one study of oil-polluted soils in Austria, Alpine soil bioremediation was evaluated by considering regional conditions (cold climate) (Margesin *et al.*, 2003).

After *Bacillus Subtilis* culturing in conditions similar to the pervious stages, the degree of biodegradation was measured by HPLC and GC. The

Table 3. The degree of average surface tension(mN/m) and the Optical Density of the best selected strain at different temperature in 1% concentration

Temperature (^{0}C)	20	37	40	50	60
Blank Sample	58	58	58	58	**58**
Surface Tension	32.5	31.2	34	33.4	**36**
Optical Density (OD_{600})	0.369	0.4073	0.4245	0.4135	**0.4180**

Table 4. The results of chemical analysis of samples by GC and HPLC in %1 concentration

Type of Analaysis	Sample	(Blank sample) (mg/L)	48 hours	72 hours	5 Day
GC Analaysis	C13	9.1	9	9.8	**8.9**
	C16	7.8	7.5	7.2	**7.2**
	n-C18	4.5	4.3	4.1	**4**
	n-C10	1.8	1.7	1.4	**1.4**
	n-C11	5.6	5.1	4.8	**4.6**
	C7	3.2	2.8	2.4	**1.9**
HPLC Analaysis	Naphthalene	215	180	130	**90**
	Acenaphthene	2.9	2.3	1.7	**1.5**
	Fluorene	14	10.3	8.5	**6.9**
	Phenanthrene	240	215	190	**178**
	Pyrene	12	9	7.6	**4.5**
	Chrysene	3.7	9.1	1.5	**1.2**
	Dibenz(a,h) anthracene	1.4	0.99	0.66	**0.5**
	Benzo(ghi) perylene	6.8	6.7	4.5	**4**

obtained results confirmed the biodegrading ability of identified bacteria. In tables 4, some parts of the results and the reduction of original oil compounds have been shown at 24- hour, 72- hour, and 5 –day periods. This shows the high biodegrading ability of crude oil and its transformation to simpler derivations. Also, the results showed the biodegradation of many compounds such as Naphthalene, Anthracene, and Benzo Alpha Pyrene and there was some reduction in their concentration. It showed that compared to aliphatic compounds, aromatic compounds are better biodegraded by bacteria. Several other studies have confirmed this conclusion, including a study made by Shahidi and colleagues that indicated Bacillus group can biodegrade crude oil and similar compounds (Shahidi Rizi, M., *et al.*,2012).

CONCLUSION

In the presence of oil compounds, various genera of biodegrading and environmental-friendly micro-organisms lose their ability to grow and reproduce in polluted soils (Jalilzadeh yengejeh *et al.*, 2013). Some studies have shown that the indigenous micro-organisms which have been separated from oil-polluted soils, due to their adaptability with the environment, have a greater role in the biodegradation of oil-related pollutions (Akhavan Sepahi *et al.*, 2008). Among bacteria, Bacillus, due to having spore and high endurance, can play a major role in Biodegradation (Meintanis *et al.*, 2006). In this study, among the two separated Bacteria, *Bacillus Subtilis* was able to produce bio-surfactant and reduce surface tension from 58 to 30.5 and 32.5 mN/m at 20 and 37 degrees centigrade respectively, while pH was optimized in %1 concentration of crude oil. It shows the bacterial activity and biodegrading ability of crude oil in hot areas. So, to remove or reduce oil-related pollutions, it seems necessary to examine the ability of other bacteria and various species in various Areas with hot weather in order to create a microbial bank for conducting combinatory studies by physical, biological, and chemical methods.

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