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The Investigation of Nutrient Addition Impact on Bioremediation Capability of Gasoil by *Alcaligenes faecalis*

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Oil and Gas well excavation, frequent extraction and its pumping to refineries and translocation of oil products to consuming sites, cause ecosystem contamination, especially soil contamination. This issue make inevitable the application of efficient methods which are either environmental friendly or appropriate for any sites. The bioremediation technology which is biological remediation of these contaminants, is much more efficient and safe than other clean-up technologies. Therefore, the aim of this study was to investigate hydrocarbon (Gasoil) removal rate from soil by *Alcaligenes faecalis* with the addition of nutrient in a bioremediation experiment. This study was carried out as factorial in a completely randomized design. The factors were two levels of bacteria (with inoculation by *Alcaligenes faecalis* and without inoculation) plus two levels of nutrient (with the addition of nutrients and without nutrients addition). There were three replication for each treatment. Therefor the experiment was establish with 12 experimental unites in plastic containers which had soils with 4% of Gasoil. The experiment was carried out in laboratory conditions (addition of nutrients, moisture as 70%-80% FC and daily aeration) for 40 days. After this time the biological removal rate of Gasoil in soils were measured using EPA 413.1 and EPA 9071 methods. Results showed that regarding mentioned condition, *Alcaligenes faecalis* could remediate in average 78% of Gasoil contamination. Furtheremore, the addition of nutrients could increase the efficiency of bioremediation of Gasoil by 11% in comparison with condition no nutrient addition.

Key words: *Alcaligenes faecalis,* Bioremediation, Gasoil, Petroleum contaminant.

Modern society primarily relies on hydrocarbons of crude oil for energy production. Despite recent advances in technology, soil contamination caused by accidental spills of crude oil and refined products it occurs repeatedly during routine operations, mining, transportation, storage, refining and distribution occurs (zhu *et al*., 2001). Therefore, water pollution and soil with this material is facing world with a serious threat. The most common environmental contaminants are oil, gasoline, solvents (chlorinated solvents) and PAHs (parker *et al*., 1987). Crude oil, with more than 340 products, one of the main driving force of the global economy and Iran possesses 9% of energy resources of total world. Crude oil is a complicated complex of various compounds including hydrocarbons, nitrogen, sulfur and vanadium that the hydrocarbons including aromatic compounds, aliphatic and asphaltene (Dorfer, 1992). These compounds are dissolved in fat and accumulate in the fatty tissues of the

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human body. It has carcinogenicity, mutagenicity and toxicity fetal disorders that are likely to enter the food cycle. Cheney) *et al*, 2009; Shojaosadati and HashemiNajafabadi, 2002). Thus concerns of twenty-first century society about collapse of the natural cycle of life and earth and human health, living organisms and ecosystems and loss of soil and water resources has increased (Mirsal, 2004). Little attention to the environmental consequences of oil extraction and refining caused pollution of water and soil resources, especially in the southern parts of the country's petroleum hydrocarbons. . Therefore, adopting a proper strategy to eliminate this problem and achieve sustainable development in this field is essential. No doubt this strategy should be based on accurate understanding of the possibilities and conditions as much as possible and minimize the risk of disrupting the balance of the environment. Oil pollution is decontaminated in three ways: physical, chemical and biological. . Physical methods are such as burning, increased ventilation, heat absorption and chemical methods are such as precipitation, extraction by solvents, oxidation and restore. . The use of physical and chemical methods to clean up the pollutants, especially in soil that is a bearing environment of solid mineral, always faced with problems of variety of reasons such as low solubility, non-polar and hydrophobic (Yaghmaei, 2001). But for reasons such as low cost of living and low equipment to be used in a large area and the lack of adverse effects on the physical and chemical properties and biological soil seems effective. Plant Purification methods involving the use of plants to purify the contaminated soil with the release of nutrients and secrete acids and organic compounds such as sugar compounds and delivering them to the roots in the soil cause stimulate and increase microbial activity in oil pollution degradation. . The success of the Plant Purification to a large extent depends on the age and species, the availability of nutrients and pollutants (Liste and Felgentrea, 2006). Meanwhile bio-purification method seems to be less harmful than the above methods. Bio-purification is a spontaneous process in which biological catalysts, act on contaminant compounds and treat them. The goal of Oil bio-purification is degradation of hydrocarbons into water and carbon dioxide by microorganisms. The process is safe and effective, eco-friendly and economical and to remove contaminants from the soil uses all living microorganisms to reduce organic contaminants and therefore cleaning environment, 2008; Kolomytseva et, 2009; Keane *et al.*, 2008). Chen *et al*). Generally, environmental restoration is done in three ways: biodegradation, mineralization, transformation. In general, this technology optimizes environment that specific microorganisms can grow and damage the maximum amount of pollution. So bio- purification is a method of choice for the elimination of hydrocarbon pollution from the environment. In recent decades, many studies were done on the use of bio- purification technology for the purification of oil-polluted soil. In this context, the aim of this study was to determine the rate of hydrocarbon contamination decline by bacteria Alcaligenes faecalis. It should be noted that in the discussion of purification of oil-contaminated soils various chemical and physical factors such as the cleaning of, soil properties, pH, nutrients, temperature, pollutants in the soil, moisture are effective. Therefore, in this study to increase the speed of bio-purification of soils contaminated with oil, gas and increase the efficiency of removal of containments by the bacteria Alcaligenes faecalis, nutrient factors were examined. Based on previous research, scientists used from certain nutrient with ratios of C: N: P = 1: 5: 100 (USEPA, 2001; Atagana *et al.*, 2003).

MATERIALS AND METHODS

Stages of this research are as follow

1. Prepare and measure some physical and chemical parameters of the soil used in the tests; for this purpose, sandy loam soil were sampled from around the southeast of Ardabil and dried at the laboratory, crushed and passed through a sieve of 2 ml. Then, a series of tests and measurements to determine soil physical and chemical properties was conducted. These tests include determination of pH, EC, soil organic carbon content (by Walcott Black), Field capacity moisture, Kjeldahl nitrogen content , the percentage of phosphorus

(Olsen method), and plaster in soil. These tests were performed according to standard methods (Page *et al*, 1982). Based on the standards set for the ratio of C: N: P in the soil for optimum growth of bacteria in the bio- purification (1: 5: 100) the lack of these elements in soil estimated and was compensated by the addition of di potassium hydrogen phosphate (K2HPO4) and ammonium nitrate (NH4NO3) (USEPA, 2001; Atagana *et al*., 2003).

2. Preparation of inoculums of Alcaligenes faecalis bacteria for bio- purification of contaminated soil with hydrocarbon contaminants: Alcaligenes faecalis bacteria were prepared in form of lyophilized from Scientific and Industrial Research Organization of Iran center of collection of fungi and bacteria. To enable ampoules containing lyophilized bacteria nutrient broth media was used. Maintaining sterile conditions inside laminar, lyophilized injections door open and some distilled water was added to the injections. Bacteria samples and distilled water were stirred well. Then were inoculated in erllen containing the nutrient media broth. Cultured erllen were laid in vitro for 24 hours in a temperature range of 27-29 ° C on Shaker with rotational speed of (rpm) 120. After 24 hours, the bacteria in nutrient media broth reached to log phase population (Bacteral number / ml) 108 × 4/1 (according to McFarland). McFarland standard, including 10 tubes in each tube by mixing a certain amount of barium chloride, 1% and 1% sulfuric acid, followed by deposition of barium sulfate, certain opacity is created. Each opacity is indicative of a certain number of bacteria that are presented in the table of McFarland (Table 1). Lowest opacity is for Tube1 and the highest opacity is for Tube = 10.

t should be noted that the standard McFarland, is an approximate estimation for number of bacteria, But since the purpose of estimating the bacterial population in the study, only express a relative value comparisons between treatments, the use of this method is sufficient. To estimate the population of bacteria based on absorption spectrophotometry at a wavelength of nm830 by standard table McFarland we did as follows: First opacity of the 10 standard pipes were read at the same wavelength and by the same spectrophotometer. The table shows the absorption device for Each 10 standard.

As can be seen, as absorption machine shows higher number, the number of bacteria per ml increases.

Figure 1 shows the relationship between absorption spectrophotometer and the number of bacteria per milliliter.

Chart equation above and R2 is as follows:

$Y=748314\ X+ 5.37 \quad R^2= 0.737$

According to Figure 1 and the resulting linear equations, for the opacity of Alcaligenes faecalis bacteria suspensions resulting from bacteria, we can approximately estimate bacterial population (Cappochino et.al, 1987)).

Measure the biological removal of oil and gas by the bacteria

This research was conducted in factorial completely randomized design (CRD) In other words, the treatment was launched in two bacterial treatment (experimental units “inoculated with bacteria Alcaligenes faecalis “and test units “without inoculation with bacteria” at two levels “by adding nutrients” and “without adding nutrients”, in three replication, in a total of twelve experimental units. So at this point, twelve plastic containers of the same dimensions were selected and then over 600 grams of soil was poured. And then test platform was contaminated by gas oil (at a rate of 4% by weight) (Since the density of the gas oil is 89/0, so 4% weight of gas oil for 600 grams testing platform, which is equivalent to 27 ml was sprayed to the soil). Finally, platforms were prepared by 10 ml of seminal fluid and in the second stage were inoculated with population (Bacteria number / ml) 108 × 4/1. And litter moisture was reached to FC 0/7 - 0/8 and was retained the same at test duration. The environmental factors such as “temperature” and “humidity” of samples were controlled daily.

And to aerated and create aerobic conditions of soils under bio-purification operations they were stirred by hand daily. After a period of 40 days, 5 grams of platform experimental units were weighed and the remaining the gas oil was measured in test units.

. In this study, the n-hexane was used as a solvent to extract oil and gas. . For every 5 grams of platform, 25 ml of n-hexane were added. The resulting suspension was placed on Shaker device for 2 hours (200 rpm) and then was centrifuged for 15 minutes (3500 rpm) to deposit the soil. The amount of the gas oil remaining in the samples was measured by 1/413 EPA and 9071EPA (Eaton and Franson, 2005; USEPA, 2001). According to this method, the upper solution in centrifuge tubes were removed and transferred to glass jars and were placed under the hood for one hour so that hexane solvent evaporate normally. After this period, what was left over, was weighed and remaining TPHs were determined according to milligrams on five grams of soil. Data from the study were analyzed using SPSS software and mean comparison was done with Duncan's multiple range tests at the level of 01/0.

RESULTS AND DISCUSSION

As mentioned in the Materials and Methods a series of tests and measurements to determine some physical and chemical characteristics of the soil was conducted in this study, and results are shown in Table 3. After measuring the "the gas oil" remained in the experimental units we can estimate "bio-purification efficiency of Alcaligenes faecalis in every single experimental unit. Since 5 g of platform experimental unit is weighed and the amount of the gas oil left in it is measured, so the initial contamination (4% by weight) for 5 grams of platform, is equal to 2.0 grams. We expect that in experimental units inoculated with bacteria, the amount of the gas oil remaining is less than 2.0 grams, and in control treatment (without bacteria), the amount is equal to 2.0 grams or close to this value. A small reduction of Oil pollution in treatment without bacteria is probably due to spontaneous decomposition of the gas oil during this period. The results of the gas oil remaining in the experimental units are given in Figure 1.

The results of analysis of variance between the residual gas oil treatment inoculated with bacteria Alcaligenes faecalis and treatments without inoculation with bacteria (control) has significant differences at 1% (Table 4).

Duncan comparison at 05/0 showed that the lowest gas oil remains was related to treatments containing Alcaligenes faecalis. (Table 5)

Two independent samples T-test results show that the two conditions, the addition of nutrients (NP) and without the addition of nutrients (C) of the amount of the gas oil remaining in the different treatments tested there are significant differences at the level of 01/0. In The experimental units (without added nutrients) after 40 days, more gas oil remaining emissions can be seen, Indeed, it suggests the addition of ammonium nitrate as a source of nitrogen and potassium hydrogen phosphate, as a source of phosphorus in (NP) treatments and bringing C: N: P to 100: 5: 1, can have a positive impact on increasing the bioavailability and biodegradation. In other words, bacteria in experimental units

Table 1. McFarland barium sulfate standard

The number of bacteria per ml	Sulfuric acid 01/0	Barium Chloride 01/0	tube
3×10^8	9/9	1/0	1
6×10^8	8/9	2/0	2
9×10^8	7/9	3/0	3
$2/1 \times 10^9$	6/9	4/0	4
$5/1 \times 10^9$	5/9	5/0	5
$8/1 \times 10^9$	4/9	6/0	6
$1/2 \times 10^9$	3/9	7/0	7
$4/2 \times 10^9$	2/9	8/0	8
$7/2 \times 10^9$	1/9	9/0	9
3×10^9	9	1	10

Table 2. Results of opacity assessment of McFarland standards (numbers are based on absorption of spectrophotometer)

Absorption at830 nm	The number of bacteria per milliliter	tube
3/80	3×10^8	1
139	6×10^8	2
207	9×10^8	3
267	$2/1 \times 10^9$	4
280	$5/1 \times 10^9$	5
366	$8/1 \times 10^9$	6
400	$1/2 \times 10^9$	7
440	$4/2 \times 10^9$	8
494	$7/2 \times 10^9$	9
519	3×10^9	10

Table 3. Results of the physical and chemical tests for soil

pH	75/7
EC (ds/m)	25/2
O.C (%)	79/1
F.C (%)	6/16
N (%)	189/0
P (mg/kg)	4/24
Gypsum (%)	0
Sandi(%)	50
Clay(%)	18
Silt (%)	32

containing nutrients have been able to be more efficient and increase the level of bio-purification (Table 6).

Nutrients are causing more bio-degradable of hydrocarbon emissions, therefore use of food in bio-purification operation, in order to have a positive impact on the growth of oil-eating microbes activity and thus have a positive impact on biological contaminant removal, it seems very effective. So impact of the presence and positive role "of nitrogen and phosphorus" as a nutrient in oil polluted areas is approved, so that greater efficiency resulting from the failure of the hydrocarbon contaminants in the soil also confirms that. The role of the nutrients nitrogen and phosphorus for growth of bacteria with the ability to remove hydrocarbon contaminants has been proven in a wide range of scientific studies and is consistent with the results of this study: Facundo *et al* in 2000 in a study found that 13 days after inoculation with bacteria and application of fertilizer ammonium nitrate (NH4NO3), soil pollution to gasoline decreased 90 percent. These results were obtained by comparing the area under the curve peak gas chromatography (GC) which represents gas consumption by the population of bacteria and soil microbial activity in the contaminated soil

Table 4. Summary of Analysis of variance for residual gas oil

The source variations	The sum of squares(SS)	Degree of freedom	Mean square (MS)	F	Level
Bacteria	085/0	1	085/0	4E285/3	000/0
level	001/0	1	001/0	036/336	000/0
Level* bacteria	000/0	1	000/0	750/211	000/0
error	5-E067/2	8	6-E583/2		

Table 5. Comparison of the mean of residual the gas oil

Bacteria	number	mean	Standard deviation
Alcaligenes faecalis	6	0255/0	01268/0
Without bacteria	6	1933/0	00234/0

Table 6. Statistical Indicators for two terms (NP) and (C)

Level	Number	Mean	Standard deviation
C	6	1160/0	08654/0
NP	6	1028/0	09733/0

inoculated with bacteria and the use of fertilizers. Ayotamuno *et al* in 2006 in Environmental Impact Studies for the bio- purification of soil contaminated with Crude oil in Nigeria concluded that application of chemical fertilizer, increased biodegradation in oil contaminated soils and

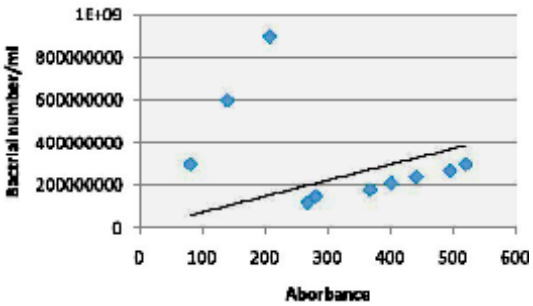

Fig. 1. Diagram of relationship between absorption spectrophotometer and the population of bacteria

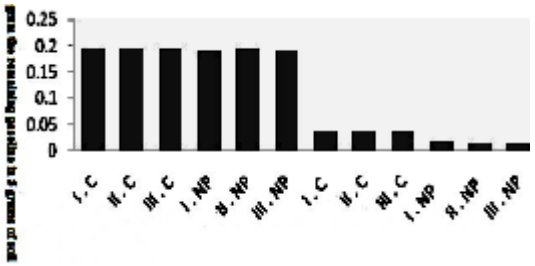

Fig. 3. The gas oil remaining in all of experimental units after 40 days

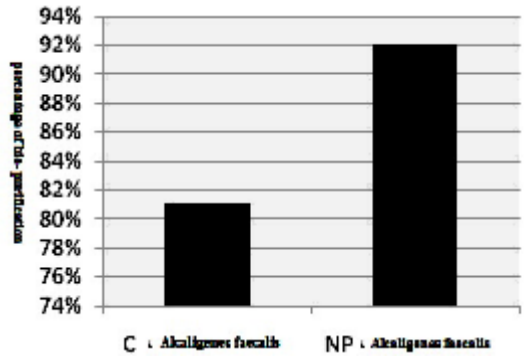

Fig. 3. The percentage of bio- purification of oil gas by bacteria Alcaligenes faecalis under two conditions (C, NP)

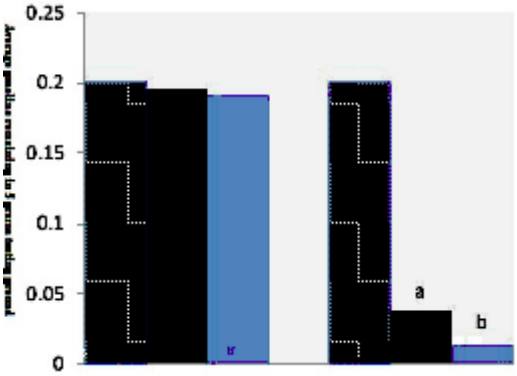

Fig. 4. The amount of the gas oil remaining in the treatments inoculated with the bacterial species " Alcaligenes faecalis " and control treatments (without inoculation) after 40 days

caused 95-50 percent removal of hydrocarbon contaminants. The results of this study show according to the environmental conditions (nutrients, temperature 29- 27 ° C, 7 / 0-8 / 0% of water-holding capacity as well as a daily conditioning) bacteria Alcaligenes faecalis in the contaminated experimental units to gas oil within 40 days were able to remove 81% to 92% of hydrocarbon pollution (the gas oil) In treatments without nutrients (C) and contains nutrients (NP) respectively. In fact, the addition of ammonium nitrate as nitrogen source and di potassium hydrogen phosphate as a source of phosphorus has caused an increase in the percentage of removal of pollutants from the soil by bacteria. . According to the results of research, applying nutrients treatments has increased percentage of bio-purification by 11%. That Due to the time of performance it seems to be satisfactory. (Figure 2).

The soil used in this study for the test platform is sandy loam soil and due to medium texture and proper ventilation it can provide conditions for the growth of bacteria. So far, several species of bacteria and fungi have been identified for biodegradation and cleaning of hydrocarbons areas. That in aerobic conditions or anaerobic, it can use oil hydrocarbon as a source of carbon and energy. In this study, Alcaligenes faecalis bacteria have been identified as a microorganism capable of depredating hydrocarbons and to clean up environment. Alcaligenes genus is a common bacterium that often there are reports of biodegradation, and presence of them in most circumstances, is an important factor that makes it possible to use them in most environments. A brief look at the study of bioremediation using strains of Alcaligenes, it is determined that in case of the optimal conditions for the growth and activity of bacteria and there will be more than 90% clearance. Alcaligenes are the most powerful bacteria in degradation of hydrocarbon, and high potential of this bacterium comes from two

factors: a good catabolic enzymes and ability of metabolic adjusting. In a study conducted by Hill *et al* in 2002 indicated that Alcaligenes are the most powerful species of bacteria capable of biodegradation or elimination of biological contaminants hydrocarbon with high concentration in the shortest time. Similar results were obtained in the investigation of Baxi *et al* in 2002 and Bharali *et al* in 2011. Bio- purification technique is simple, affordable and effective and cause complete elimination of pollution in areas contaminated by petroleum products and at the same time has the least environmental pollution. Favorable results of the tests show that during the bio- purification process significant percentage of hydrocarbon contaminants could be removed from the environment with the least damaging effect on the environment left. Because now, environmental cleanup of hydrocarbon pollution, especially Crude oil pollution resources, are new approaches to the environment and taking into account the critical point that "soil and water contamination are national capital of the country" was considered therefore, protection and clearance soil and water contaminated again, is one of the main concerns of environmental organizations and all lovers of the environment.

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