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ORIGINAL ARTICLE

Biodegradation Potential of Soils in Tabriz Petroleum Refinery for Removing Solid Polycyclic Hydrocarbons

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are the most toxic and carcinogenic pollutants which cause to sever damages in soil, water and creatures due to wastes discharge of oil and petrochemical industries. In order to remove these pollutants, biological methods and using local microorganisms' potential of oil contaminated soils are preferred because of cheapness and availability.Existing soil microorganisms use these hydrocarbons as carbon and energy sources and finally, to produce water, CO_2, biomass, and harmless materials. In the present study, the sampling was conducted from different soils of Tabriz and oil-polluted soils of Tabriz Petroleum Refinery. The soil suspensions were cultured in YGM and Starch casein agar media and 100 microbial colonies and isolates were obtained. At a rate of 1000 mg/L hydrocarbons (Naphthalene) were added to the Muller Hilton broth, and then fixed amounts of these bacteria were added separately. They were incubated in shaker with 130 rpm, at 28 ˚C for one week. The rate of naphthalene destruction was evaluated by spectrophotometer and determined reliability of primary aromatic compounds by TLC method.Ninety six naphthalene reductive bacteria were isolated which their destroying rates were 3.5-92.9%.Some samples from secondary metabolites of each hydrocarbon which showed the most destroying percentage, were subjected to the GC-Mass analysis in order to their identification. Some non- toxic mediatory substances were obtained as a result of naphthalene biological degradation. By improving the growth conditions and proliferation of effective bacteria it might be possible to remediate polluted soils from PAHs in industrial pilots.

***Keywords**: Aromatic toxins, Bioremediation, Polycyclic hydrocarbons, Soil contamination, Soil microorganisms*

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INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) composed of two or more rings of six members. Aromatic hydrocarbons are among oil-pollutants which enter water and soil ecosystems by different sources like petrochemical, industrial and household sewages, oil exploitation, pharmaceutics, color, plastic, insecticide and transmitted to human being directly so cause some problems such as cancer. Naphthalene, anthracene, phenanthren, fluorine, chrysene, fluoranthene, pyrene, and their derivatives are the most important [15]. Naphthalene is an organic compound with formula $C_{10}H_8$. It is the simplest polycyclic aromatic hydrocarbon, and is a white crystalline solid with a characteristic odor that is detectable at concentrations as low as 0.08 ppm by mass [16]. The main ways of absorption of Aromatic hydrocarbons to human body are inhalation and skin. The aim organs for aromatic compounds are nervous system, liver, kidneys, skin, lungs, mucous membrane of respiratory tract, and eyes [17, 19]. Bioremediation is one of the main ways for environmental clearance. In this method living creatures specially bacteria, fungi

and plants are used in order to reduce environmental contaminants as well as to change them to nontoxic compounds. These microorganisms changes hydrocarbon compounds to carbon dioxide, biomass or other productions. Efficiency and the rate of reduction process of hydrocarbons depend on the kind of pollutant compounds, the nature of polluted material, environmental condition and microbial population properties [14].

Various microorganisms have a role in this process, most important of which are *Bacillus, Pseudomonas, Proteus, Clostridium, Staphylococcus, Acinetobacter, Mycobacterium, Rhodococcus, Micrococcus*. No microorganism, essentially, is able to reduce completely the oil-hydrocarbons to carbon dioxide and water as final products [18].This study was conducted in three main stages as follows: Pure bacterial strains obtained from soil samples Bacteria treated with the desired concentration of certain hydrocarbons Extraction and determination of residual hydrocarbons decomposed [13].

The aim of the present study is to evaluating the ability of soil-isolated organisms from different regions of Tabriz to reduce naphthalene as a polycyclic aromatic hydrocarbon.

MATERIALS AND METHODS

Sampling

Different region's soil of Tabriz and outside as well as contaminated soil of Tabriz refinery were sampled in order to isolate effective bacteria. After digging a cavity by depth of 30 cm in a specific area, approximately 400 g soil was sampled. The sample was purred in unclosed plastic bags then transferred to the laboratory [12].

Isolation and purification of isolates

The information about any sample including sampling location, altitude and latitude was attached on any packet [9]. Following preparing 10^{-1} to 10^{-4} concentrations from samples in physiologic serum, 100 µl of concentrations was cultured in a plate containing starch casein agar and incubated at 28 °C for one week. Then, grown colonies were fixed according to incubation condition in yeast glucose malt agar for fortification and purification. 25 mg of pure naphthalene (Merck) was weighted and added to 25 ml Muller Hilton broth in capped Falcon tubes in sterile condition. Then, a suspension equal to 05 -Mac Farland standard was obtained from purred bacteria in Tryptic Soy Broth; then, 0.5 ml of the suspension was added to Falcon tubes. Falcon tubes were fixed in shaker incubator (made by Pars Azma Co – Iran) at 28°C and 130rpm for one week to reduce naphthalene by bacteria [6].

Extraction method

After the mentioned period, contains of Falcon tubes was transferred to a 100 ml decantation funnel in a sterile condition. Organic solvent of toluene was used in order to isolate the remaining naphthalene. Two phases were formed by adding 10cc toluene to the funnel and mixing it. Lower phase consisted of culture medium and bacteria, and upper phase consisted of remained toluene and metabolite [19, 14] The upper phase was collected in a capped bottle and was kept in a refrigerator at 4 °C until reading the rate of OD. In order to evaluate OD at first naphthalene λ_{max} must be determined. For this purpose, various concentrations standards in toluene were obtained and their OD were determined using double beam spectrophotometer [made by Shimadzu, Japan] compared with blank solution and λ_{max} was determined. Then, the samples' OD was evaluated at this wavelength. Considering drawn curve, the rate of naphthalene reduction in different samples was observed [6,15].The percentage of naphthalene destruction by bacteria was calculated by the following relationship: Destruction percentage = $\frac{A_1 - A_2}{A_1} \times 100$

A_1: hydrocarbon absorption before destruction

A_2: hydrocarbon absorption after destruction by microorganism.

GC mass analyses for determination of metabolites

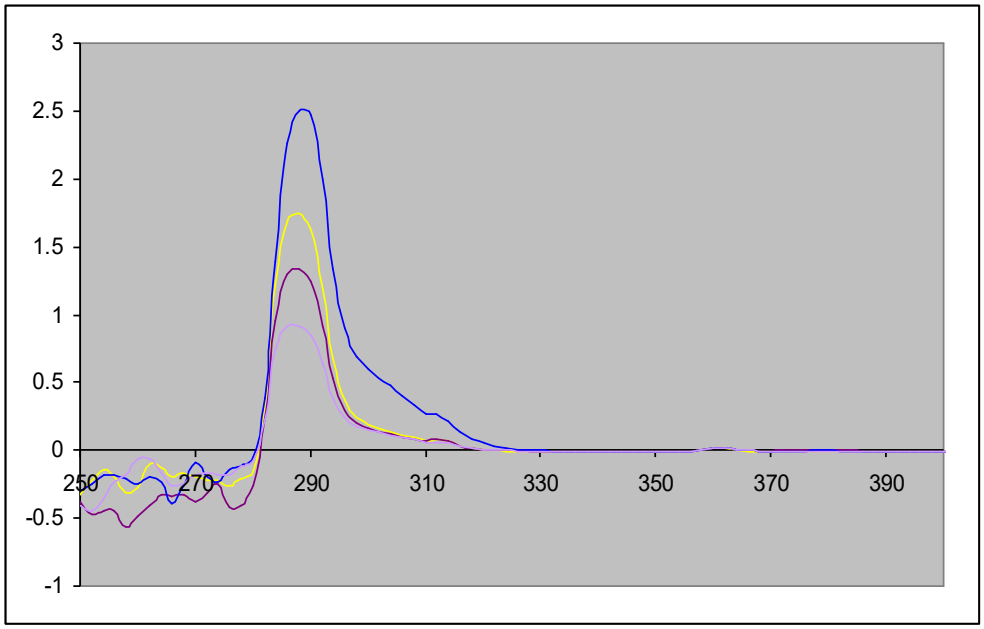
GC mass analyses were preformed on Shimadzu GC 2010 equipped with spilt (rate: 100) as injection mode in order to determine the metabolites resulted from bacterial degradation. The CPED1-M25-025 column was used. The length of column was 24.9 m. Temperature within column8°Cfor 9 minutes, and increased by 150°C. Maximum temperature of 325°C (temperature program begins from 80°C) and system conditions were as follows:

Hold time = 1 min, Detection system FID= 310°C, Injection system STL= 300°C [11].

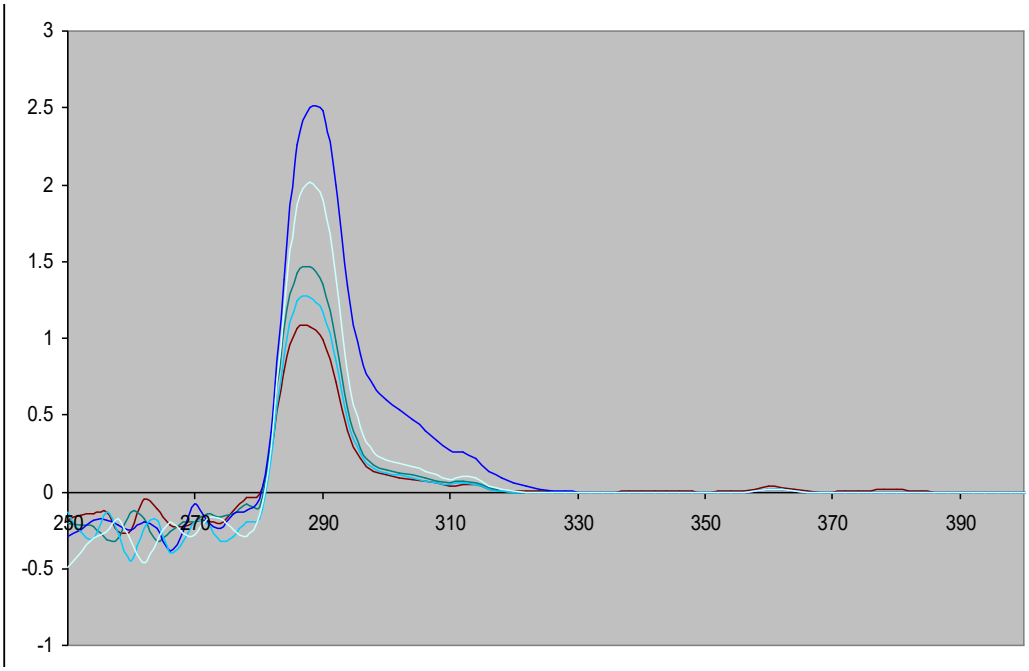
RESULTS AND DISCUSSION

Due to culturing different soils, 100 stubs of purred bacteria were obtained. 14 microbial stubs associated to Tabriz refinery with the codes of g_1 to $g_{14,}$ and 86 isolated stubs are from different regions of Tabriz. 96

bacterial stubs had the ability to reduce and destruct naphthalene in in-vitro condition following treatment the isolated bacteria. λ_{max} was determined 288nm by measuring naphthalene standard OD against blank solution in 250-500nm wavelength (curve I). Absorbed spectra by samples in wavelength of 288nm were recorded following the treatment of specific amount of naphthalene with different bacteria and exploitation of remained naphthalene (curve II). The percentage of naphthalene destruction by different microorganisms was obtained by $\frac{A_1 - A_2}{A_1} \times 100$. Findings suggest that different destruction percentages of 3.5 to 92.6 have primary naphthalene (table I).



Curve I: Absorbed spectra associated with naphthalene standard C concentration :(N_1=25, N_2=16.6 , N_3=12.5, N_4=8.3 $_{mg}$/25 $_{ml}$) OD :(N1=2.429, N_2=1.747, N_3=1.42, N_4=0.913)



Curve II: Absorbed spectra associated with naphthalene treated samples. OD: (G_6=1.071, G_7=1.722 , G_8=1.67, G_9=1.262, G_{10}=2.011)

Table I: the percentage of naphthalene destruction

№	Bacteria Stubs	Destruction%	№	Bacteria Stubs	Destruction%
1	G_1	41.4	51	M_{31}	36
2	G_2	35.3	52	M_{58}	34
3	G_3	30.1	53	M_{60}	38.1
4	G_4	24.1	54	M_{61}	0
5	G_5	46.6	55	M_{65}	39.8
6	G_6	57.1	56	M_{66}	74.8
7	G_7	41.3	57	C_2	20.4
8	G_8	0	58	C_7	31.1
9	G_9	49.5	59	C_{15}	10.18
10	F_1	19.6	60	C_{17}	29.4
11	F_2	16.6	61	C_{18}	24.8
12	F_3	36.8	62	B_2	39.2
13	F_4	50.4	63	B_7	37.6
14	F_5	9.2	64	B_{19}	27.6
15	F_6	32	65	B_{20}	87.1
16	F_9	43.6	66	B_{25}	12.3
17	F_{10}	63	67	B_{27}	69.8
18	F_{11}	54.3	68	B_{33}	31.3
19	F_{12}	34.3	69	L_1	0
20	F_{13}	43.3	70	L_2	46.2
21	F_{14}	51.5	71	L_3	35.8
22	F_{19}	33.7	72	L_4	53.5
23	F_{20}	41.2	73	L_5	30.4
24	F_{22}	38	74	L_6	24.4
25	F_{24}	43.1	75	L_7	37.6
26	E_1	71.3	76	L_8	19.9
27	E_2	36.2	77	L_9	0
28	E_3	50.3	78	L_{10}	44.2
29	E_4	33.4	79	L_{11}	37.2
30	E_5	33	80	L_{12}	24.6
31	E_6	45	81	L_{13}	24
32	E_7	36.4	82	A_{20}	34.2
33	E_8	47.4	83	A_{21}	46.7
34	E_9	39.5	84	A_{22}	48.4
35	E_{10}	48.2	85	A_{23}	92.9
36	E_{11}	3.5	86	A_{24}	53.5
37	E_{12}	55.1	87	A_{25}	48.8
38	E_{13}	40	88	A_{27}	31.8
39	E_{14}	37	89	A_{28}	36.6
40	E_{15}	37.8	90	A_{29}	21
41	E_{16}	28.1	91	A_{30}	71.8
42	E_{17}	38.2	92	A_{31}	40.9
43	E_{18}	24.9	93	A_{32}	50.9
44	E_{19}	20.4	94	A_{33}	53.1
45	E_{20}	36.9	95	A_{34}	39.4
46	D_6	37.2	96	H_1	56.8
47	D_{23}	24.8	97	H_2	47.8
48	D_{26}	24.4	98	H_3	44
49	D_{40}	21.7	99	H_4	50
50	M_2	48.2	100	H_5	16.1

Obtained metabolites from biologic reduction of hydrocarbons

Following to determine reduction percentage, the obtained metabolite from biodegradation was under Gas chromatography reduction and GC-Mass, and then they obtained results, consisted of the rate of remained percentage, type and composition of products, demonstrated according to images I, II and table II.

Area Percent Report

Data File : C:\MSDCHEM\1\DATA\SADG910G.D Vial: 1
Acq On : 1 Dec 2009 9:16 Operator:
Sample : a23n Inst : Instrumen
Misc : 0.5 MultiplIr: 1.00
Sample Amount: 0.00
MS Integration Params: autoint1.e

Method : C:\MSDCHEM\1\METHODS\TESTSAMA.M (Chemstation Integrator)
Title :

Signal : TIC

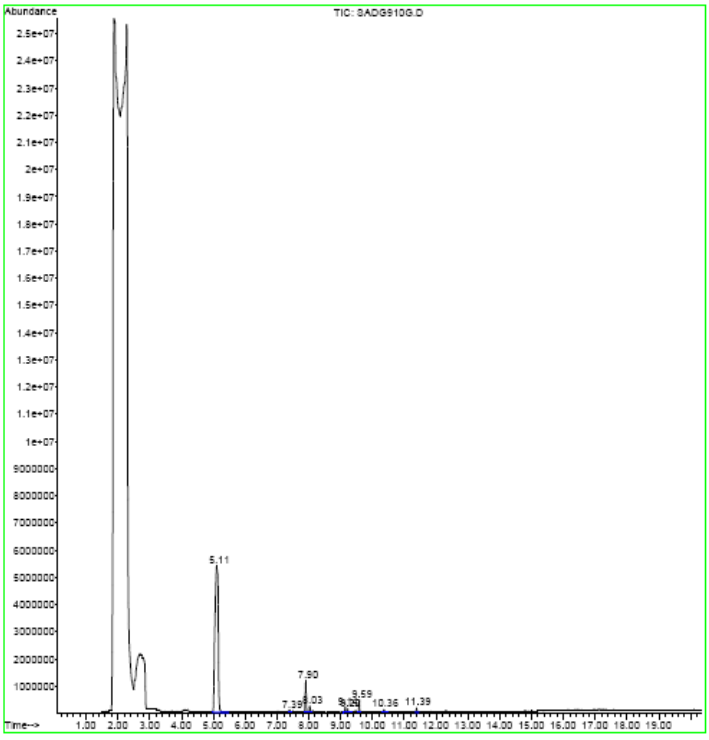
peak #	R.T. min	first scan	max scan	last scan	PK TY	peak height	corr. area	corr. % max.	% of total
1	5.113	441	454	487	VV	5373967	379304936	100.00%	89.613%
2	7.388	656	659	663	VV	93565	2016950	0.53%	0.477%
3	7.899	701	705	711	VV	1126085	21396692	5.64%	5.055%
4	8.032	711	717	721	VV	218661	4409575	1.16%	1.042%
5	9.141	813	817	821	BV	177321	2708698	0.71%	0.640%
6	9.219	821	824	834	VV	126645	2691633	0.71%	0.636%
7	9.585	853	857	863	PV	403399	5871328	1.55%	1.387%
8	10.362	923	927	938	PV	103712	2849636	0.75%	0.673%
9	11.394	1015	1020	1025	BV	139654	2022364	0.53%	0.478%

Sum of corrected areas: 423271812

Image I: Report 1, naphthalene GC-Mass

Area Percent Report

Data File : C:\MSDCHEM\1\DATA\SADG910G.D Vial: 1
Acq On : 1 Dec 2009 9:16 Operator:
Sample : a23n Inst : Instrumen
Misc : 0.5 MultiplIr: 1.00
Sample Amount: 0.00
MS Integration Params: autoint1.e
Method : C:\MSDCHEM\1\METHODS\TESTSAMA.M (Chemstation Integrator)
Title :



SADG910G.D TESTSAMA.M Wed Dec 02 14:28:58 2009 Page 2

Image II: report 2, naphthalene GC-Mass

Table II: the probable Produced Metabolites in GC mass column

Sample Code	(Time)' In GC column	Probable Produced Metabolites	Case #	Phonotypic identification
A_{23}	5.11	Naphthalene	#000091	*Pseudomonas sp.*
	7.38	Diphenylmethan	#000101	
	7.89	Benzen,1,1.ethylidenebis	#000612	
	8.03	Butylated Hydroxy toluene	#000128	
	9.14	Alpha Cadinol	#000481	
	9.21	2,5,6-dihydro-5,6-dimethylbenzo	#065990	
	9.58	Cyclohexane1-ethyl-1-methyl	#004926	
	10.36	Phenanthrene	#000085	
	11.39	Retinoic acid, methyl ester	000339	

Bioremediation is a natural process by which pollutants are recycled rather burying them. Furthermore, from public point of view, Bioremediation is more desirable and most of world organizations disseminate this method for remediating damaged regions by environmental contaminants. One of the best bioremediations is biologic methods and using microorganisms. Bacteria have the most importance compared with other microorganisms because of their different reductive enzymes [14]. Considering the results of the present study and conducted studies, soil bacteria, more or less, have the potential of reduction and destruction of polycyclic aromatic hydrocarbons.

Jussara P. Del Arco et al. demonstrated in their studies that the power of natural soil micro flour for destruction of oil-hydrocarbons increases by adding nitrogen and phosphorous resources, from 11.9% to 42.9% under incubation conditions in 28 days. In these conditions the rate of destruction of different compounds are as follow: dodecane 100%, tridecane 89%, tetradecane 79%, pentadecane 68%, hexadecane 47%, heptadecane 46%, octadecane 82%, nonadecane 60%, icosane 56% [10].

C.H.Chaineau *et al*. in their research named "The effects of nutrition's on crude oil biological destruction by soil microbial population in a farm soil" increased the rate of these materials' reduction from 47% to 62% [5].

Zhang.H.Kallimanis *et al*. demonstrated that a species of *Pseudomonas* is able to solve 35 mg/l phenanthren at presence of surfactant produced by that bacterium and finally caused to phenanthren destruction [20].

In the study conducted by our colleagues on reductive microorganisms of PAHSs succeeded to identify a yeast [AH70] isolated from oil-polluted soils which is determined as a 100% alkaline sequence homologue in determining genomic sequence of 26S rRNA. The organism is able to destruct naphthalene at the rate of 89.76%, phenanthren 77.21%, pyrene 60.77%, and benzopyrene 55.53% during 10 days [1].

Andrea R.clement et al. reported that two yeast species ere identified among studied soil microorganisms that the species 984 was able to destruct anthracene at the rate of 64%±10 and species of 870 was able to destruct Naphthalene at the rate of 69%±10 [2].

Farinazleen Ghazali et al. evaluated the rate of oil-hydrocarbons in the soil using Bacillus and *Pseudomonas*. They observed that the remained concentrations of crude oil reached to 74.34% and 19.34% after 30 and 60 days, respectively [8].

In the present study, isolation of some of soil bacteria (100 samples) from some regions of Tabriz and polluted soil of Tabriz refinery was conducted; then, treatment was done and finally the destruction of naphthalene hydrocarbon by these microorganisms was performed, and different percentages of destruction were observed and reported. Considering the results and findings of the present study, isolated bacteria from soil have the potential to reduce the oil hydrocarbons in in-vitro condition. Rich farmlands which have significant resources of phosphorous, nitrogen and sulfur, also, confirm this finding.

In the present study, stable hydrocarbons with high toxicity were used and it was observed that from 100 isolated stubs 92 bacteria had the destruction power between 3.5–92.9 % and among them, 19 stubs had over 50% destruction power. The most naphthalene destruction percentage by isolated bacterium from Tabriz refinery was 92.9% that the results of similar studies confirm that. In the present study, also, polluted soil bacteria of Tabriz refinery were used that 14 stubs of bacteria were isolated. By evaluating their results about the destruction of hydrocarbons one can understand the destructive potential of polluted soil bacteria. Bhattacharya, *et al*.[4] isolated 150 stubs of oil-hydrocarbons destructive bacteria from India oil-polluted soils and demonstrated that *Ps.citronellolis* are dominant considering the destructive ability of aromatic and aliphatic compounds. S.Barati and N. Vasudevan [3] in their research named "using oil-hydrocarbons by isolated fluorescence *Pseudomonas* from oil-polluted soils" demonstrated that the microbial stub had the significant power to destruct short and long-chain alkaline.

Eder C. Sntos *et al*. isolated a stub of *Pseudomonas* from oil-polluted soil of refinery which had the 72% destruction power by producing surfactants [7].

Considering the mentioned studies, the present study has had similar and confirmative results and had innovation aspect due to use native and wild microorganisms of Tabriz regions and refinery soils.

Considering the biological and physico-chemical potential of soil and the presence of effective microorganisms in destructing of oil-hydrocarbons the following studies are suggested:

Study and isolation of microbes of mentioned soils widely and determining the identity of isolated bacteria. Richening and optimizing of cultivation and growth conditions of the mentioned bacteria using nutritional and salty environment. Determining and providing desirable pH for soil samples for continuing bacterial growth.

Preparing biofilms from considered stubs or disseminating bacteria with high destruction power along with nutritional materials to oil-polluted soils. Exploitation and determination of resulted metabolites from biological destruction of naphthalene and other oil aromatic hydrocarbons in order to understand the rate of toxicity or healthiness of products. Designing and preparing bioreactor for improving bioremediation operations or obtaining the products of destruction such as various acids and alcohols.

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