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Liquid Paraffin Degrader- Bacteria Isolated from Several Soils of East- Azarbayjan, Iran

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ABSTRACT
Biodegradation is an efficient, economic and environmentally safe treatment technique. By regarding that microflora of soils are very diverse and it differ from one site to others.The objective of this study was to isolates the microorganisms with liquid paraffin degradation potential from different soil samples that were collected from different cities of Iran. For this purpose, 55 colonies of different bacteria were isolated from soil samples that collected from Jolfa, Tabriz, Azarshar,Meyaneh, Marageh, and Sarab cities of Iran. The ability of degradation in all isolates was studied and the produced metabolites were identified with GCmass analysis. The 8 (14.5%) different colonies degrade more than 70% of liquid paraffin and different metabolites were produced. So, research might be to optimize the process for molecular identification of strains of microorganisms which are found to have degradative capabilities and assay their abilities in field.

INTRODUCTION

In 1946, Claude E. Zobellreviewed the action of microorganisms on hydrocarbons (1).On the other hand,petroleum is an extremely complex mixture of hydrocarbons (2). Crude oils are naturally occurring, exceedingly complex mixtures, which consist predominantly of hydrocarbons, and contain sulfur, nitrogen, oxygen and metals minor constituents. The petroleum industry, which holds a prime position in the modern world economy, is, in the main centered on crude oil (3). Hydrocarbons within the saturate fraction include n-alkanes, branched alkanes, and cycloalkanes. The n- alkanes are generally considered the most readily degraded components in a petroleum mixture (4). Biodegradation is an efficient economic and environmentally safe treatment technique; its quantitative and qualitative aspects depend on the type of hydrocarbons present and also the environmental condition as temperature, pH, oxygen, nutrients and salinity (5). Hydrocarbon-utilizing fungi belonged to the genera *Candida*, *Penicillum*, *Aspergillus*, *Rhizopus* ,*Cladosporium*, *Mucor* and *Fusarium*.The bacteria based on their 16S rRNA gene were affiliated to*Actinobacterium*sp.,*Micrococcus luteus*, *Rhodococcus erythropolis*, and *Rhodococcusopacus*. Individual pure fungi and bacterial isolates grew on a wide range of individual pure aliphatic (n-alkanes with chain lengths between C (9) and C (40) and aromatic (benzene, biphenyl, anthracine, naphthalene and phenanthrane) hydrocarbons as sole sources of carbon and energy (6).The aim of the present work was to isolate hydrocarbon degrading bacteria (liquid paraffin) and to investigate the main producedmetabolites from biodegradation by bacteriafrom soils of East-Azarbijan,Iran.

MATERIALS AND METHODS

Sampling

Sampling was conducted from arid soils of different parts of Azarshahr,Meyaneh, Marageh, Jolfa, Sarab and Tabriz cities in Iranwith an especial geographic location (table 1). For this purpose, some digs were made with 30 cm depth and width. About 400 g soil was collected in a plastic packet (7). All of samples were transferred to microbiological laboratory of medical science faculty of Tabriz Islamic Azad University, Iran as soon as possible.

Isolation and purification of isolates:

All of soils' samples were diluted with saline in serial dilutions of 10^{-1} to 10^{-6}. By using surface plate count method, 100 µl of any dilution was cultured in starch casein agar and incubated at 28°C for a week. The same isolated colonies from one geographic location were transferred to yeast glucose malt agar

forpurification and growth fortification (8). The characters of isolate were recorded and purified strains were kept in refrigerator at -70°C.

Extraction method

Three or four colonies from each of isolated bacteria was inoculated into Trypticase soy broth(TSB) and incubated at 28°C until bacterial suspension equivalent to standard 0.5 McFarland (OD 0.8 -1 in 625 nm) has been prepared. Then, 0.5 ml of bacterial suspension was placed into Falcon tubes which contained 25 ml of Muller Hinton Broth and 1 ml liquid paraffin. The tubes were incubated in 120 rpm shaker incubator at 28°C for a week (9, 10). On the other hand, in order to determine λ_{max} for complete paraffin extraction, different solvents such as benzene, chloroform, dichloromethane, hexane, and toluene were examined. Therefore, solutions with 1% to 0.125% of liquid paraffin were prepared in mentioned solvents and absorption spectrum, on the range of 200 to 500nm, of each solution was recorded using double beam spectrophotometer (Shimadzu UV 1700). Maximum peak of absorption spectrum was considered as λ_{max}; and it was used for evaluating the absorption of treated samples on that wavelength. In order to extract the remaining paraffin, contents of Falcon tube were purred in Decantation funnel and 5 cc of chemical solvent (N-Hexane) was used.Optical absorption of understudied samples was determined in pre-assigned wavelength by means of double beam spectrophotometer; also, the percentage of paraffin degradation was calculated as follows:

$$\text{Degradation percentage} = \frac{A_1 - A_2}{A_1} \times 100$$

Where

A_1= paraffin absorption before its degradation by microorganisms

A_2= remaining paraffin absorption after its degradation by microorganisms (8).

GC mass analyses for determination of metabolites

GC mass analyseswere preformed onShimadzu GC 2010 equipped with spilt (rate=100) as injection mode inorder to determine the metabolites resulted from bacterialdegradation. The CPED1-M25-025columnwas used. The length ofcolumn 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

RESULTS

From every soil sample of cities 8 – 10 different colonies were selected and purified. The general character of them with the geographic location of isolated colonies showed in table1. All of colonies which showed more than 70% liquid paraffin degradation tested were done in duplicate. The results in second process were the same of first test. So, their samples were tested for metabolites by GC mass analyze. From 55 different isolates, 8 (14.5%)colonies degrade more than 70% of liquid paraffin that table 2 wereshown the most probable metabolites.

Table1; General especialcharacteristics of isolates

Sample code	Geographic location	Gram Stain	% degradation	city	Sample code	Geographic location	Gram Stain	% degradation	city
A35	38°0′26/04″ N 46° 8′ 9/28″E	BG-	73.78	Tabriz	A5	37°21′46/54 ″N 46 9′ 50/7″ E	BG+F	56.41	Marageh
D32	38°125′/12N 22° 6′ 7/32″E	BG-	57.99	Tabriz	M43	37°17′44/52 ″N 44° 9′ 48/5″E	BG+F	36.74	Marageh
E15	37°58′7/18″ N 46° 3′ 57/4″E	BG-	68.09	Tabriz	M47	37°25′12/44 ″N 46°8′38/4″E	BG+F	66.10	Marageh
E3	37°44′5/42″ N 22°7′25/51″E	BG-	95.70	Tabriz	D33	37°22′11/35 ″N 46°10′54″ E	BG-	61.68	Marageh
A11	37°40′8/22″ N 34°8′18/41″E	BG+F	66.53	Tabriz	E10	37°42′44/54 ″N 38°12′24″ E	BG+F	66.46	Marageh
M17	38°27′39″N 45°34′0″E	BG+F	40.79	Tabriz	M10	37°19′56/72 ″N	BG+F	25.36	Marageh

						46°19'39/16 ″E			
M18	38°27′39″N 45°34′0″E	BG+F	17.13	Tabriz	M46	37°18′44/76 ″N 46°21′44/37 ″E	BG+F	52.54	Marageh
M29	38°10′1/66″ N 46°9′39/68″E	BG+ Coryneform	16.34	Tabriz	A1	37°56′22/87 ″N 47°44′15/82 ″E	BG-	14.58	Sarab
M30	38°10′27/76″ N 46°10′58/34″ E	BG+F	51.75	Tabriz	A12	37°57′53/41 ″N 47°29′11/99 ″E	BG+F	31.68	Sarab
D34	38°27′39″N 45°34′0″E	BG+ Coryneform	50.78	Tabriz	M15	37°56'40/84″ N 47°41′38/53 ″	BG+ Coryneform	48.42	Sarab
E19	37°42′10/26″ N 44°42′16/22″ E	BG-	23.54	Meyaneh	M24	37°44′52/35 N 36°22′18/14 ″E	BG-	34.48	Sarab
D39	37°25′12/38″ N 26°40′36/47″ E	BG+F	34.68	Meyaneh	M33	37°56′45/40 ″N 47°28′47/80 ″E	BG-	12.59	Sarab
F20	37°51′44/26″ N 54°36′47/33″ E	BG+F	65.43	Meyaneh	E18	37°48′36/32 ″N 38°25′12/42 ″E	BG-	76.99	Sarab
F9	37°28′51/44″ N 38°34′26/41″ E	BG+F	62.40	Meyaneh	M40	37°56′45/40 ″N 47°28′47/80 ″E	BG+F	22.57	Sarab
A16	37°35′62/52″ N 22°43′64/28″ E	BG+ Coryneform	43.52	Meyaneh	A25	38° 17′ 11/44″ N 64° 7′ 33/6″ E	BG+ Coryneform	72.94	Jolfa
A8	37°32′44/42″ N 38°44′64/26″ E	BG+ Coryneform	51.39	Meyaneh	A3	38°41′16/36 ″N 55°6′ 28/4″ E	Coccus G+	70.58	Jolfa
E14	37°24′38/54″ N 43°52′38/44″ E	BG+ Coryneform	57.08	Meyaneh	A10	38° 32′ 14/25″ N 38° 2′ 66/3″ E	BG+F	65.92	Jolfa
E16	37°26′45/40″ N 43°45′66/34″ E	BG-	73.24	Meyaneh	A31	38° 12′ 25/39″ N 46° 5′ 39/6″ E	BG+F	76.93	Jolfa
A32	37°62′38/24″ N 36°62′54/33″ E	BG+F	61.74	Meyaneh	E17	38° 48′ 33/50″ N 28° 6′ 33/5″ E	BG+ spore forming	84.56	Jolfa
A4	37° 43′ 16″ N 45° 56′ 14″ E	BG+F	39.16	AzarShahr	F14	38° 12′ 48/46″ N 12° 7′ 43/8″ E	BG+F	78.87	Jolfa
M53	37° 54′ 41″ N	BG+F	46.16	AzarShahr	M16	38 °15′	BG+	51.51	Jolfa

	45° 59′ 46″ E					22/41″ N 13° 8′ 55/9″ E	Coryne form		
M51	37° 43′ 16″ N 45° 56′ 14″ E	BG+F	49.51	AzarShahr	M6	38° 14′ 47/26″ N 12° 5′ 33/3″ E	BG+ Coryne form	18.40	Jolfa
A2	37° 42′ 54″ N 45° 54′ 54″ E	BG+F	66.64	AzarShahr	F12	38° 27′ 44/39″ N 43° 8′ 29/4″ E	BG+F	52.23	Jolfa
F24	37 55′ 54″ N 45° 54′ 12″ E	BG+F	60.53	AzarShahr	A38	38° 58′ 25/39″ N 12° 5′ 43/6″E	BG+ Coryne form	89.34	Jolfa
F10	37° 52′ 55″ N 45° 56′ 42″ E	BG-	90.73	AzarShahr	E20	37° 44′ 14″ N 45° 46′ 42″ E	BG+F	35.53	AzarShahr
M67	37° 52′ 55″ N 45° 56′ 42″ E	BG+F	53.99	AzarShahr	M11	37° 47′ 28″ N 45° 57′ 47″ E	BG+F	43.82	AzarShahr
E12	37° 43′ 16″ N 45° 54′ 14″ E	Coccus G+	92.85	AzarShahr	–	–	–	–	–

Table 2: the probable Produced Metabolites in GC mass column

Sample Code	Time(') In GC column	Probable Produced Metabolites	Case #	Phonotypic identification
A3	21.17	Tetradecane	#113293	Micrococcus sp.
	23.68	Cycloheptasiloxanetetradecamethyl	#371831	
	26.04	Hexadecane	#154901	
	27.67	Dibutanoylmorphine	#344975	
A25	23.68	Cycloheptasiloxanetetradecamethyl	#371831	Diphteriod
	27.66	6-Aza-5,7,12,14-tetrathiapentacene	#302266	
A35	23.70	Cycloheptasiloxane, tetradecamethyl	#371831	Pseudomonas sp.
	27.67	Silane, [[4-[1,2-bis[(trimethylsilyl)oxy]ethyl]	#357373	
	30.36	Cyclononasiloxane, octadecamethyl-	#386248	
E3	23.68	Cycloheptasiloxane, tetradecamethyl	#371831	Pseudomonas sp
	27.65	Dibutanoylmorphine	#344975	
	31.09	4-(3,4-Dimethoxybenzylidene)-1-(4-nitrophenyl)-3-	#346855	
E17	23.68	Cycloheptasiloxane, tetradecamethyl-	#371831	Bacillus sp.
	27.66	#344975: Dibutanoylmorphine	#344975	
	31.09	Cyclononasiloxane, octadecamethyl-	#386248	
E18	23.67	Cycloheptasiloxane, tetradecamethyl-	#371831	Pseudomonas sp.
	26.04	Hexadecane (CAS) $$ n-Hexadecane	#154903	
	27.66	6-Aza-5,7,12,14-tetrathiapentacene	#302266	
	31.09	Cyclohexasiloxane, dodecamethyl	#352523	
F10	4.93	Acetic acid, butyl ester	#16517	Achromobacter sp.
	23.67	Cycloheptasiloxane, tetradecamethyl-	#371831	
	27.66	6-Aza-5,7,12,14-tetrathiapentacene	#302266	
	31.09	Cyclohexasiloxane, dodecamethyl	#352523	
F12	20.71	Cycloheptasiloxane, tetradecamethyl-	#371831	Streptomyces sp.

	23.68	Cycloheptasiloxane, tetradecamethyl-	#371831	
	27.11	Dibutanoylmorphine	#344975	
	27.66	6-Aza-5,7,12,14-tetrathiapentacene	#302266	
	30.96	4-(3,4-Dimethoxybenzylidene)-1-(4-nitrophenyl)-3-...	#346855	

DISCUSSION AND CONCLUSION

Regarding with inscriptions successes on bioremediation and biodegradation research to optimize the process is ongoing with isolation of strains of microorganisms which are found to have enzyme systems which degrade and utilized oils as source of carbon and hydrogen (11). So the purpose of thisstudy was to isolate strains with liquid paraffin degrade potential from different arid soils collected from 6 cities in East- Azarbayjan, Iran. The results showed microbial populations of arid soils in East- Azarbyjan, Iran had paraffin- degrader potential. In this study, 2 isolates from Tabriz, 4 isolates from Jolfa, 1isolate from Sarab and one another from Azarshar were isolated which has degradation rate more than 70% (Table 2). These strains were identified by phenotypic characters and must be to test by molecular methods in later research.

A similar study was carried out in Boucherville, Quebee, for demonstrate the potentials of hydrocarbons of contaminated soil. The summary of the results is that on bioremediation the site recorded a removal rate of 72% within 48 weeks (12).In Liado et al. study, in all bioremediation assays achieved between 30% and 50% total petroleum hydrocarbon biodegradation, after 200 day with the *Trametesversicolor* inoculation degrading it the most (13).In Guo-Liang study, in summary more than 58% of crude oil was degraded after addition small amount of rhamnolipids or glycerol when incubated in crude oil together with 1g/L glecerol, *Pseudomonas aeruginosa* produced rhamnolipids in two stages, possibly reflecting that microbial began to use petroleum when glycerol was used up in medium (14).In Komaet al. study (15), paraffin degrading bacteria has been studied byusing a mineral medium containing 1% of n-paraffin. From 50 isolates, Strain ODDK7 showed more cellular density with maximum efficiency for consumption of n-paraffin, so, this strain identified as *Acintobacter*sp ODDK7. In that study, n-paraffin peak was decreased gradually and approximately were done after 96 hours incubation.

In this study, the isolates from Jolfa were more powerful than others which need to continue the research in fields and in minimal media for assay their degradation potential.

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