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Gas Chromatographic Assessment for Bioremediation of Hydrocarbons pollutants Using *Bacillus Amyloliquefaciens*

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ABSTRACT

The bioremediation of n-Octane and Ethyl benzene singly and in combination as representative solutes for aliphatic and aromatic hydrocarbons using Bacillus amyloliquefaciens was studied. The studied B. amyloliquefaciens was selected from one hundred and sixteen bacterial isolates collected from five different sites along River Nile, Egypt. The determination of the minor amounts of the residual selected solutes was obtained via capillary gas chromatography (CGC) using normal hexane as internal standard. The effects of different pH values, incubation temperatures and the exposure of the bacterial cells to 7.3mW He-Ne laser radiation on the bioremediation were investigated. PH 6 and incubation temperature 37 ^{o}C are the optimum conditions giving maximum degradation of both aliphatic and aromatic hydrocarbons. Complete degradation through incubation time 12 hours was obtained by irradiating the bacterial cells to a laser dose for one minuet. The irradiation for three minuets exhibits complete degradation through incubation time 8 hours only. Irradiation of the bacterial suspension for more than three minuets accompanied with bad effect on the bioremediation process.

Key Words:- Bioremediation, Aliphatic and aromatic hydrocarbons, Bacillus amyloliqueifaciens, capillary gas chromatography, pH values, incubation temperature and laser radiation.

INTRODUCTION

One of the major problems facing the industrialized world today is the contamination of soils, ground water, surface water and air with hazardous and toxic chemicals. In Egypt, River-Nile which is considered as the main source of water suffers from pollution due to the direct discharge of 3.8 billion m^3/years of waste water into it. In addition the river receives large discharges of pesticides, nutrients, heavy metals and hydrocarbons form industrial activities [1]. So application of biotechnology for the clean up of industrial pollutants is a must [2]. Bioremediation is a less expensive, treatment effective and favorable clean up technology that uses naturally occurring or genetically engineered microorganisms such as yeast, fungi and bacteria [3, 4].

Most heterotrophic bacteria favor a pH near neutrality, extremes in pH would be expected to have negative effect on the ability of microorganisms to degrade hydrocarbons [5]. In fresh water bacteria are the predominant type of decomposers and there are more than 25 hydrocarbon degrading genera [6]. *Baker and Herson, (1994)* [7] reported that the optimal pH for microbial activity is usually between 5.5 and 8.5. *Roberts, (1998)* [8] reported that the

bacterial isolates which grow better at a neutral pH would carryout the dioxygenation of aromatic compounds to form cis-glycol.
Temperature plays a significant role in controlling the nature and the extent of microbial metabolism of hydrocarbon, which is of special significance for in situ bioremediation. Bioavailability and solubility of less soluble hydrophobic substances, such as aliphatic and polyaromatic hydrocarbons are temperature dependent [9]. Most bacteria are mesophilic and are active between 5 and 40°C. The optimal activity generally occurs at 35°C. A few mesophilic bacteria can survive up to 60°C, but only thermophilic microorganisms can grow above this temperature [7].

It was found that some strains of the genus *pseudomonas* are capable of utilizing chlorobenzoate as the unique source of carbon and energy [10] **,** also it was found that mixed and pure cultures of *Bacillus sp.* and *Thermus sp.* were able to degrade alkane mixtures at 65°C [11]. Moreover, mono aromatic hydrocarbons such as benzene, toluene, ethyl benzene and xylene (BTEX) can be degraded an aerobically using iron reducing bacteria [12]**.**

A number of studies have been carried out to investigate the use of different types of radiation to increase the enzyme activity of microorganisms [13]. Several types of Low-intensity Laser radiation, including Helium-Neon laser (He-Ne), Gallium-Aluminum Laser (Ga-Al), Helium-Neon-Arsenate laser (He-Ne-As) and the diode laser were tried against different microorganisms to stimulate their growth [14, 15] . However, higher doses of laser are inhibitory in particular when the radiation is in the presence of a photosensitizer [16]. As a result the laser radiation was used in the present study to increase the hydrocarbon degrading power of the selected bacterial isolate.

MATERIALS AND METHODS

II.1 Isolation, purification and characterization of the most potent hydrocarbon degrading bacterial isolate:
Preliminary screening of the most potent degrading isolate:
0.1ml of water samples which were collected from five different locations along River-Nile [Kafrelelw, El-Maasara, El-Giza, Embaba and El Galatma] were plated on the surface of basal mineral salts (BMS) agar medium (3gm $NaNO_3$, 1gm KH_2PO_4, 0.5gm $MgSO_4$. $7H_2O$, 0.5hm KCl, 1gm yeast extract and 20gm agar per liter distilled water, pH 7) [17], containing normal octane (n-C_8) and ethyl benzene individually as a sole carbon sources.

After 24hrs of incubation the two isolates which achieve the maximum growth percentages on the two substrates were selected.

Quantitative screening of the most potent isolate:
250ml capacity bottles each containing 100ml BMS both medium supplemented with n-C_8, EB individually and incompination at the level of 0.5% v/v were inoculated by the selected two isolates individually, the bottles were incubated at 37°C in a shaking incubator for 48hrs (150 rpm). After incubation the residual substrates were extracted from the culture medium using chloroform (3 sample: 1 chloroform)

II-2 Gas chromatographic analysis [18]
The studied n-Octane and Ethyl benzene singly and in combination before bioremediation and after degradation using *Bacillus amyloliquefaciens* and different working conditions were analyzed using Agilent 6890 plus HP gas chromatograph, equipped with flame ionization detector (FID), using the fused silica capillary column HP-1 of 30 mete in length and 0.35 mm int. diameter, The separation of the selected solutes samples was achieved with temperature programming from 80 to 250 ^oC at a temperature rate 3 ^oC min^{-1}. Nitrogen (oxygen-free) was used as carrier gas, at flow rate 2 ml min^{-1} that measured from the end of the column with a soap bubble flow rate. Methane as an unreturned marker was used to correct the dead volume in the column in the case of FID; the quantitative analysis of the solutes samples was achieved using normal hexane as internal standard of known concentration and according to the standard ASTM method[18]. The injector and detector temperatures are 250 and 300 ^oC respectively. Degradation was estimated by integration of the area under the resolved chromatographic profile, using the HP computer of software chemistation.

Identification of the most potent isolate
The bacterial isolate which achieve maximum degradation was purified and identified according to the Biolog™ micro-plate system (Biology, Inc. 3938 Trust way, Hayward, CA 94545, USA) [19].

II-3 Selection of the most optimum conditions for biodegradation

(A) Effect of pH

Bacterial suspensions (10^4CFU/ml) were inoculated into BMS broth media adjusted at different pH values (5, 6, 7, 8) and seeded by n-C_8, EB individually and their (1:1) mixture. After 24hrs of incubation at 37°C the residual substrate was extracted and analyzed using CGC.

(B) Effect of temperature

Bacterial suspensions (10^4CFU/ml) were inoculated into BMS broth media adjusted at pH 6 and incubated for 24hrs at different temperature (22, 30, 37, 44°C) the residual substrate was extracted to be analyzed by CGC.

(C) Effect of He-Ne laser radiation [20]

Bacterial suspension (10^5CFU/ml) was prepared; the suspension was irradiated with He-Ne laser (NEC/ Japan) 632.8nm with an out put power of 7.3mw and beam diameter 1.3mm for 1, 3 and 6mins. Non irradiated bacterial suspension was used as a control. 0.1ml of bacterial suspension was cultivated in 250ml screw capped bottles containing 100ml BMS broth medium containing n-C_8, EB individually and their (1:1) mixture (0.5% v/v) the percentage of degraded substrates were determined after 4, 8, and 12hrs of incubation.

II-4 Application of the most potent bacterial isolate on a mixture of paraffinic and aromatic hydrocarbons

Irradiated bacterial suspension was incubated in BMS broth medium supplemented with a mixture of paraffinic and aromatic hydrocarbons (0.5% v/v) and incubated under optimum conditions for 8hrs. After the incubation period, the hydrocarbons were extracted and analyzed using CGC.

RESULTS AND DISCUSSION

III-1 Isolation and identification of the most promising bacterial isolate:

116 bacterial isolate were isolated from the five selected sites along the River-Nile, from the results recorded in Table (1) it was found that both the isolate No 5 from El-Giza (BZ_5) and the isolate No 7 from Embaba (BE_7) were chosen as they have the highest ability to utilize the two used substrates.

Table 1: Percentage of hydrocarbon degrading bacterial counts from the selected sites on n-C_8 and EB

Isolate No.	Site K		Site M		Site Z		Site E		Site G	
	Count % on n-C8	Count % on EB	Count % on n-C8	Count % on EB	Count % on n-C8	Count % on EB	Count % on n-C8	Count % on EB	Count % on n-C8	Count % on EB
1.	88.6	87.3	89.2	87.6	88.3	86.9	88.9	86.6	87.3	84.9
2.	89.5	80.8	89.8	88.6	89.8	88.9	89.2	88.6	87.6	55.1
3.	93.1	52.0	89.5	71.7	88.9	65.6	80.8	77.8	61.2	57.0
4.	89.0	84.9	89.5	84.9	87.3	83.1	87.6	81.4	88.9	52.8
5.	87.6	80.1	89.8	77.8	92.0	89.2	88.6	82.5	88.6	84.0
6.	85.8	83.6	88.6	81.4	91.6	88.6	59.0	58.1	88.0	88.3
7.	89.5	88.9	61.6	57.0	89.8	88.6	93.1	90.8	88.3	87.6
8.	65.1	63.0	89.8	89.2	89.8	87.3	89.8	88.6	88.6	85.4
9.	89.5	87.3	90.3	90.0	64.5	61.9	89.2	88.0	65.8	64.5
10.	86.2	83.1	90.0	89.2	86.9	84.9	88.3	58.6	63.9	61.6
11.	78.7	76.0	90.6	89.5	78.7	0.0	60.8	58.6	61.2	59.0
12.	83.1	82.0	90.3	88.6	86.9	59.9	80.8	0.0	78.7	0.0
13.	79.4	76.0	88.6	87.3	85.4	58.1	87.3	86.9	86.9	86.9
14.	77.0	0.0	89.2	88.0	63.6	58.6	86.6	85.4	60.4	59.0
15.	86.2	83.1	88.9	88.3	89.5	87.3	61.2	59.0	86.2	84.9
16.	87.3	86.2	80.1	78.7	58.1	52.0	78.7	77.0	85.8	59.0
17.	65.6	61.2	84.0	52.0	88.9	86.6	52.0	0.0	77.0	54.4
18.	56.4	0.0	60.8	58.1	65.6	62.3	83.1	82.0	58.1	0.0
19.			58.1	56.4	58.6	55.1	57.0	51.0	84.0	56.4
20.			82.0	80.8	83.1	82.0	63.6	62.3	62.3	58.1
21.			87.3	85.0	57.0	52.8	52.8	52.0	59.0	57.0
22.			86.2	84.0	51.0	52.8	87.6	84.0	86.6	82.0
23.			88.3	60.0	60.8	59.0	60.4	58.1		
24.			77.8	0.0	88.3	59.9				
25.			64.2	62.6	60.8	58.6				
26.			56.4	55.8	56.4	55.8				
27.					58.1	55.8				

The two selected isolates were subjected to a quantitative screening using CGC, the results given in Table (2) indicate that the isolate BE_7 was considered as the most promising one as it has the ability to degrade n-C_8, EB

individually and in combination by a higher percentages than BZ_5. The isolate BE_7 was identified as *Bacillus amyloliquefaciens* according to Biology^{TM} microplates which perform 95 discrete tests simultaneously and gives a characteristic reaction pattern called the metabolic finger print which is then keyed into the Biolog's Microbiology computer program.

Table 2.: Determination of the most potent degrading isolate using n-C_8, EB and their combination

Code of microbial isolate	Percentage of degraded substrate			
	n-C_8	n-C_8 (mix)	EB	EB (mix).
BZ_5	76.12	62.65	58.06	60.04
BE_7	90.62	80.66	73.51	78.79

III-2 Parameters controlling the biodegradation potential of B.amyloliquefaciens

(A) Effect of PH

The analysis and evaluation of the degraded solutes at different PH values was given in Table 3. The results indicate that the degradation of solutes decreases with increasing pH values. Also, *B.amyloliquefaciens* degraded normal octane which represents the paraffinic hydrocarbons individually than in mixture but with respect to ethyl benzene the opposite was occurred . pH 6 is the preferred one exhibiting maximum degradation for the studied substrates. The more efficient pH value 6 that obtained in this work lies between the pH regions given by Atlas, 1988.

Table (3): Effect of different pH values on the degradation of n-C_8, EB singly and in mixture.

pH values	Percentage of degraded substrate			
	n-C_8	n-C_8 (mix)	EB	EB (mix)
6	85.43	76.36	63.19	69.60
7	65.38	55.42	38.04	46.07
8	55.51	45.53	34.36	38.11
9	35.94	30.71	23.00	25.00

(B) Effect of Temperature

With respect to temperature the data was recorded in table 4. it has been revealed that as well as the temperature increases the percentage of degraded substrate increases till it reaches its maximum value at 37°C which is considered as the most optimum temperature for degradation. This result was agreed with that concluded by *Margesin and Schinner, (2001)* reported that bioavailability of aliphatic and polyaromatic hydrocarbons are temperature dependent. Also most bacteria are mesophilic and are active between 5 and 40°C *(Baker and Herson, 1994).*

normal octane was degraded individually than in mixture but with respect to the Ethyl benzene the opposite is occurs. This may be due to the behavior of genetic enzymes. At the optimum temperature 37°C, maximum degradation for individual aliphatic hydrocarbons (85.43) and aromatic hydrocarbons in mixture (69.00) was occurred.

Table (4): Effect of different incubation temperature on the degradation of n-C_8, EB individually and in combination

Temperature values (^{o}C)	Percentage of degraded substrate			
	n-C_8	n-C_8 (mix)	EB	EB (mix)
22	76.74	69.01	50.92	61.00
30	80.87	70.00	56.06	65.67
37	85.43	76.36	63.19	69.00
40	82.12	74.00	58.06	67.33

(C) ***Effect of He-Ne laser radiation on the degrading activity of Bacillus amyloliquefaciens***

The irradiation for different time intervals at three incubation periods was given in Table 5. The bioremediation of the studied substrates increases as a function not only of exposure time but also of incubation period.

It has been showed that the radiation exposure of the bacterial cells to 1min was enough for complete degradation at incubation time 12 hrs. The complete degradation of paraffinic and aromatic hydrocarbons was achieved at incubation time 8 hrs only at exposure time 3 min due to the more increase in enzyme activity. On the other hand, irradiation of the bacterial suspension for 6 min showed inhibition of growth followed by decreasing in the percentage of degraded substrates.

Table 5.: Effect of irradiation for different time intervals on the degradation efficiency of *Bacillus amyloliquefaciens*

Incubation time (hrs)	4				8				12			
Percentage of degraded substrate												
Exposure time (min)	n-C8	n-C8 mix	EB	EB mix	n-C8	n-C8 mix	EB	EB mix	n-C8	n-C8 mix	EB	EB mix
Zero	41.43	36.17	15.07	21.4	46.11	41.33	19.31	23.36	56.01	51.24	25.75	30.02
1	70.63	64.88	51.57	57.51	85.58	77.29	70.86	75.52	100	100	100	100
3	85.1	80.49	76.9	79.39	100	100	100	100	-	-	-	-
6	26.03	19.62	10.11	13.22	33.72	28.81	13.07	19.01	40.13	33.62	19.04	26.13

From the studied factors affecting the bioremediation process it has been concluded that pH 6 and temperature 37 ^{o}C are the optimum conditions giving maximum degradation of n-octane and ethyl benzene as a model for their families. Also, the complete degradation of paraffinic and aromatic hydrocarbons was obtained by exposing the bacterial suspension for three minutes to He-Ne laser radiation

***III-3 Application of Bacillus amyloliquefaciens* on a mixture of paraffinic and aromatic compounds:**

After the determination of the optimum biodegrading parameters for the most potent isolate, its application under these conditions was carried out on a mixture of paraffinic and aromatic hydrocarbons.

Table 6. Application of *B.amyloliquefaciens* on a mixture of paraffinic andaromatic compounds

Carbon No.	Control	*Wt % of residual components*
		B
5.	0	0.003
6.	0	0.103
7.	0	0.188
8.	0	0.097
9.	0	0.036
10.	0	0.079
11.	0	0.036
12.	0	0.061
13.	0.06	0.042
14.	0.17	0.097
15.	0.16	0.127
16.	1.61	0.327
17.	3.09	0.400
18.	5.64	0.345
19.	9.64	0.430
20.	15.05	1.411
21.	17.72	1.466
22.	15.28	0.902
23.	12.80	1.078
24.	8.19	2.307
25.	5.39	2.114
26.	3.07	1.357
27.	1.15	1.338
28.	0.53	0.908
29.	0.24	0.805
30.	0.14	1.023
31.	0.07	1.211
32.	0.03	0.375
33.	0	0.606
34.	0	0.509
35.	0	0.012
36.	0	0.048
37.	0	0.127
Total P	100%	19.97
UCM	100%	44.21

The ability of utilizing the mixture as a carbon source was achieved quantitatively by CGC, and the efficiency of degradation was indicated by calculating the weight percentages of the of the residual paraffines as well as the UCM after 8 hrs of incubation .Each carbon represents the summation of The corresponding normal and iso paraffines. The unresolved complex mixture (UCM) appears as hump and represents the poly aromatic hydrocarbons, naphthenic hydrocarbons and resins which can not be eluted by GC. From Table 6. It was shown that the carbon number distribution of the control sample exhibits paraffines from C_{13} to C_{32}, there are many maxima representing paraffines from C_{20} to C_{23}. C_{21} has the highest weight percentage in the mixture (1.47%).

The weight percentage of the residual UCM was decreased to 44.21% and this was reflected in the appearance of light paraffinic fractions (C_5 to C_{12}), heavy paraffinic fractions (C_{33} to C_{37}) and there is an the increase in the weight percentages of some carbons than the control may be due to the degradation of the heavy unresolved complex mixture.

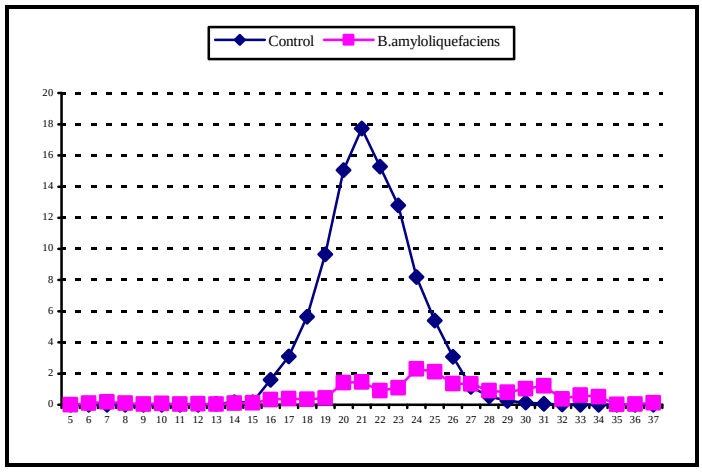

Fig. (1): Carbon number distribution of hydrocarbon compounds before and after degradation.

CONCLUSION

- *B.amyloliquefaciens* was considered the most promising bacterial isolate as it has high efficiency to degrade normal Octane, ethyl benzene individually and in combination.
- PH 6 and temperature 37 ^{o}C are the optimum conditions exhibiting maximum degradation for the studied substrates individually and in combinations after 24 hrs.
- The complete degradation of the studied substrates after 8 hrs of incubation was obtained by exposing the bacterial suspension for three minutes to He-Ne laser radiation
- High degradation of normal octane was obtained individually than in mixture with ethyl benzene but with respect to the ethyl benzene the opposite was occurred.
- Using *B. amyloliquefaciens*, the degradation of paraffinic hydrocarbons reaches about 80 % and that of unresolved complex mixture including the heavy aromatics reaches about 55 %

REFERENCES

[1] Egyptian Environmental Affairs Agency (EEAA) (**1992**): Environmental action plan of Egypt.
[2] Atlas RM: *Microbiological Reviews*, **1981**,45, 180-209.
[3] Alexander M (**1999**): Biodegradation and bioremediation, 2nd edn. Academic Press; London.
[4] Margesin R and Schinner F: *Biotechnol*, **2001**, 56, 650-663.
[5] Atlas RM, Microbiology-fundamentals and applications, 2nd (ed.) Macmillan Publishing Co. NY, **1988**, 352-353.
[6] Leahy J and Colwell RR, *Microbiol Rev*; **1990**, 45, 305-415.
[7] Baker KH and Herson DS (**1994**): Bioremediation. Published by McGrow Hill, Inc.
[8] Roberts RE (1998): Remediation of petroleum contaminated soils; Lewis Publishers CRC Press LLC, BoCa Raton.
[9] Whyte LG; Bourbonnière L; Bellerose C; and Greer CW: *Bioremediation* J; **1999**, 3, 69-79.
[10] Gallego A; Gomez CE; Fortuunato MS; Cenzano L; Rossi S; and Pagilla M (2001): Factors affecting biodegradation of 2-chlorophenol by *Alcaligens sp* in aerobic reactors. John Wiley and sons Inc.
[11] Feitkenhauer H; Müller R and Märkl H, *Biodegradation*, **2003**, 14, 367-372.
[12] Jahn MK; Haderlein SB and Meckenstock RU: *Applied and Environmental Microbiology*; **2005**, 71 (6), 3355-3358.
[13] Geweely NS; Ouf SA; Eldesoky MA and Elally AA, *Archives of Microbiology*; **2006**, 186: 1-9.
[14] Karu TI; Tiphlova OA and Fedoseyeva GE, *Laser Chem*, **1984**, 5, 19-25.
[15] Kim KS and Anhn JY: *Laser Therapy*; **1995**, 7 (4), 151-156.
[16] Karu TI: *Photochem. Photobiol*, **1990**, 52, 1089-1998.
[17] Al-Doory Y (1980): Laboratory medical mycology lea and febiger, Philadelphia.
[18] ASTM Designation: Analysis of natural gas by gas chromatography; 1945-96, 1996, 51-65.
[19] Garland JL and Millis AL: *Appl Environ Microbiol*, **1991**, 57, 2351-2359.

[20] A.Y. El Naggar, M.M.Kame, A.A.Aladly and N.A. Ismail, *Journal of American Science* **2010**, 6 (10), 661-670.