Bioremediation of Paraffinic and Polynuclear aromatic hydrocarbons Using Laser irradiated *Bacillus amyloliquefaciens*

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Abstract: River Nile is considered as one of the most important rivers in the world, it suffers from severe increase in pollutants' levels due to the direct discharge of industrial effluents into its water body. In an effort at developing active microbial strains that could be relevance in the bioremediation of these pollutants, eighteen hydrocarbon degrading bacterial isolates were purified from five sites along the river's main stream. The most potent bacterial isolate was identified according to the BioLog [TM] microplate assay as *Bacillus amyloliquefaciens*. In a trial to promote its degradation potentiality it was subjected to different He-Ne laser (7.3 mW, 632.8nm) irradiation doses. The chromatographic analysis of the residual substrates revealed that the irradiated isolate was able to degrade 98.12% of the paraffinic fraction and completely degrade the polynuclear aromatic fraction after 30hrs of incubation, these results supports its future usage in the bioremediation strategy of industrial effluents. [Journal of American Science 2010;6(10):661-670]. (ISSN: 1545-1003).

Key Words: *Bacillus amyloliquefaciens*, He-Ne laser radiation, Paraffinic hydrocarbons, polynuclear aromatic hydrocarbons, Bioremediation.

1. Introduction:

Egypt faces a rapidly increasing deterioration of its surface water due to the increasing discharge of heavily polluted domestic and industrial effluents into its water ways. The Egyptian industries use 638M.m^3/yr of water, of which 549M.m^3/yr is discharged into the drainage system. The river Nile supplies 65% of the industrial water needs and receives more than 57% of its effluents (Wahaab and Badawy, 2004).

Aliphatic and polycyclic aromatic hydrocarbon (PAH) fractions of petroleum have received a lot of attention due to their ubiquitous distribution in water and sediments (Wang et al., 2006)**.**They induce a number of adverse effects such as immunotoxicity, genotoxicity and carcinogenicity (Sahinkaya and Dilek, 2005, Qiao et al., 2006 and Haritash and Kaushik, 2009).

Biodegradation is the process by which organic substances are broken down by the enzymes produced by living organisms (Marinescu et al., 2009***).*** It 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 conditions as temperature, pH, oxygen, nutrients and salinity (Abalos et al., 2004).

The inadequate bioavailability of the hydrocarbons to microorganisms is a limiting step in biodegradation. So, it must be found a method to increase the microbial degradation activity (Molnar et al., 2005).

The activation of microorganisms by laser radiation "Biostimultion" is of the most interest. The most commonly used sources of laser radiation are the helium-neon laser (He-Ne,λ=632.8nm), the gallium-aluminum laser (Ga-Al ,λ=630-685nm), the helium-neon-arsenate laser (He-Ne-As , λ=780-870nm) and the gallium arsenate laser (Ga-As, λ=904nm) (Geweely et al., 2006). It was found that the dynamics of cell bioenergetic processes are known to increase after the exposure of the microbial population to laser light of specific wave length (Hilszczanska et al., 1999). Vladimirov et al., (2004) reported that low power laser radiation can brought about acceleration in the cell division and enhanced the protein synthesis in various microorganisms. However higher doses are inhibitory to the microbial growth.

The aim of the present work was to isolate and identify a hydrocarbon degrading bacteria and to investigate the effect of He-Ne laser radiation on stimulating its biodegradation potentiality to be used in clearing up hydrocarbon contaminated water.

2. Materials and Methods:

I-Sampling area:

Surface water samples were collected from five locations extending for about 60Km along the main stream of the river Nile in greater Cairo

segment. The sampling sites were at Kafr Elelw , El Maasara, El Giza , Embaba and El Galatma.

II-Determination of hydrocarbon pollutants:

II.1-Gravimetric estimation of hydrocarbon:

One liter of each water sample was acidified to pH 2using HCl and poured into a separating funnel, Hydrocarbon extracted with chloroform then gravimetrically estimated according to ASTM-2007(1999) standard method.

II.2-Fractionation of the extracted hydrocarbons via capillary gas chromatography (CGC):

The extracted hydrocarbons were subjected to chromatographic analysis to determine iso and normal paraffins as well as the unresolved mixture (UCM). The analysis was performed according to IP-318(1995) standard method using Agilent 6890 plus gas chromatograph equipped with flame ionization detector (FID) and fused silica capillary column Hp-5 of 30m length, 0.35 mm internal diameter and 0.5um film thickness. The column was heated isothermally at 100 °C for separation of the individual components, while for the paraffinic hydrocarbons it was heated at temperature programming started at 80°C and ended at 250°C with a rate of 5°C min^{-1} and final time 20 min till the end of the program. The injector and detector temperatures are 250°C and 300°C respectively. Nitrogen was used as a carrier gas at a flow rate 2 ml min^{-1}. Degradation was estimated by the integration of the area under the resolved chromatographic profile.

III-Determination of the most potent hydrocarbon degrading bacterial isolate:

III.1- Isolation:

The hydrocarbon degrading bacterial isolates were isolated by plating 0.1ml of the water samples on the surface of basal mineral salts (BMS) agar medium [composition (g/L) $NaNO_3$, 3; KH_2PO_4, 1; $MgSO_4$. $7H_2O$, 0.5; KCl, 0.5; yeast extract, 1 and agar, 20 (pH 4.5)] supplemented with 0.5% V/V paraffinic oil as a sole carbon source. After 48 hrs of incubation at 30°C all the hydrocarbon degrading bacterial isolates were purified (April et al., 2000).

III.2-Screening:

The screening of the most potent bacterial isolate was monitored by recultivation of all the purified isolates (10^4 CFU/ml) on BMS agar medium seeded with normal octane (n C_8) and ethyl benzene (EB) (0.5% V/V) individually as carbon sources. After 24 hrs of incubation at 37°C the isolate with the maximum colony forming unit (CFU) count percentage on both substrates was selected for further studies.

III.3-Identification:

The selected bacterial isolate was purified and identified using the Biolog™ micro-plate system (Biolog, Inc. 3938 Trust way, Hayward, CA 94545, USA) which is based on the simultaneous examination of utilization of 95 different carbon sources , this utilization was demonstrated by the formation of purple color as compared to a negative control (Winding,1994).

IV-Stimulation of the degradation potentiality using He-Ne laser radiation:

The laser used was He-Ne Laser (NEC, Japan) with an output power of 7.3 mW, λ=632.8nm and beam diameter of 1.3mm.

The bacterial suspension was prepared and subjected to different radiation doses by varying the exposure durations for 1, 3 and 6 mins. Non irradiated suspensions were used as control (Geweely et al., 2006). Inocula of cell density 10^4 CFU/ml were seeded in 250ml screw capped bottles each containing 100ml BMS broth medium adjusted at pH 6 and supplemented with n C_8 and EB individually and in combination as sole carbon sources. The residual substrates were extracted and chromatographically analyzed after 4, 8 and 12 hrs of incubation at 37°C under shaking conditions (150 rpm).

V- Assessment of the degradation ability of paraffinic and polynuclear aromatic mixtures:

The paraffinic and the polynuclear aromatic fractions used were obtained from the distillation cuts of crude oil using the proper solvents (Central Analytical Lab, Egyptian Petroleum Research Institute, EPRI). They were added to the BMS broth individually at the level of 0.5% V/V; the media were inoculated with the selected isolate before and after its exposure to He-Ne laser for 3 mins. After incubation for 12 hrs and 30 hrs under shaking conditions (150 rpm) the residual substrates were extracted and chromatographically analyzed. The CGC conditions were the same as described for hydrocarbons, while the PAH fraction was determined by high performance liquid chromatography (HPLC) according to the ASTM-4657(1992) standard method using HPLC model Waters 600E equipped with auto sampler Waters 717 plus and dual wave length absorbance detector Waters 2487 set at 254 nm. The condition of operation is as follows: column: LC-PAH of 15cm length, 4.6mm internal diameter, 5mm particle size. The mobile phase is acetonitrile (Water HPLC

grades), gradient from 50:50 to 100% acetonitrile. Flow rate: gradient program, 0-2 min., 0.2ml min^{-1}. A reference sample of PAHs was injected under the same conditions, the retention times and areas of its peaks were used to determine quantitatively each compound in the residue.

3. Results and Discussion:

I-Estimation of the hydrocarbon pollutants in the collected water samples:

The hydrocarbon content in each water sample was estimated gravimetrically, the obtained results (Table, 1) indicated that their concentrations were ranging between 34.4ppm and 41.5ppm. These values gave an indication that the studied sites at the time of samples collection were highly polluted according to law 4/1994, which illustrates the characteristics of fresh water bodies that should remain within certain standards and specifications after the discharge of treated industrial effluents (EEAA, 1998). The chromatographic profiles (Fig.1) show a series of homologous iso and normal paraffins imposed over a hump comprising the UCM which is formed mainly of naphthenes, aromatics and cyclo alkanes. This configuration indicates petrogenic hydrocarbon contamination (Readman et al., 2002 and DeOteysa et al , 2006).

The predominance of n-C_{25} peak indicates biogenic hydrocarbon contamination from living organisms (Medeiros et al., 2005).

The higher values of the weight percentages of UCM (from 65.27% to 84.15%) compared with that of the resolved peaks (from 15.85% to 34.73%) observed in fig. 2 were due to that the later are more naturally degradable also they gave an indication of a chronic petrogenic pollution (De Oteysa et al ,2006). These obtained results indicated that river Nile receives huge amounts of industrial effluents resulting in an increase in the concentration of pollutants levels in greater Cairo segment (Abd El Shafy and Aly, 2002, Wahaab and Badawy, 2004 & El- Sayed and Ouf, 2009).

II-Isolation, purification and screening of the most potent hydrocarbon degrading bacterial isolate:

Eighteen paraffinic oil degrading bacterial isolates were isolated and purified from the studied five locations. All the isolates were recultivated on BMS agar medium supplemented with nC_8 and EB. The isolate No 4 from Embaba site was the most efficient one, as the results in Table (2) showed that it has the ability to grow on the two studied substrates with the highest CFU count percentage and this was an indication of its ability to utilize the paraffinic and aromatic hydrocarbon pollutants present in the river Nile water (Sood and Lal, 2009).

Table (1): Concentrations of the hydrocarbon pollutants in the collected water samples

Sampling sites	Concentration of hydrocarbon pollutants (ppm)
Kafr Elelw	40.3
El-Maasara	38.3
El-Giza	37.5
Embaba	34.7
El-Galatma	41.5

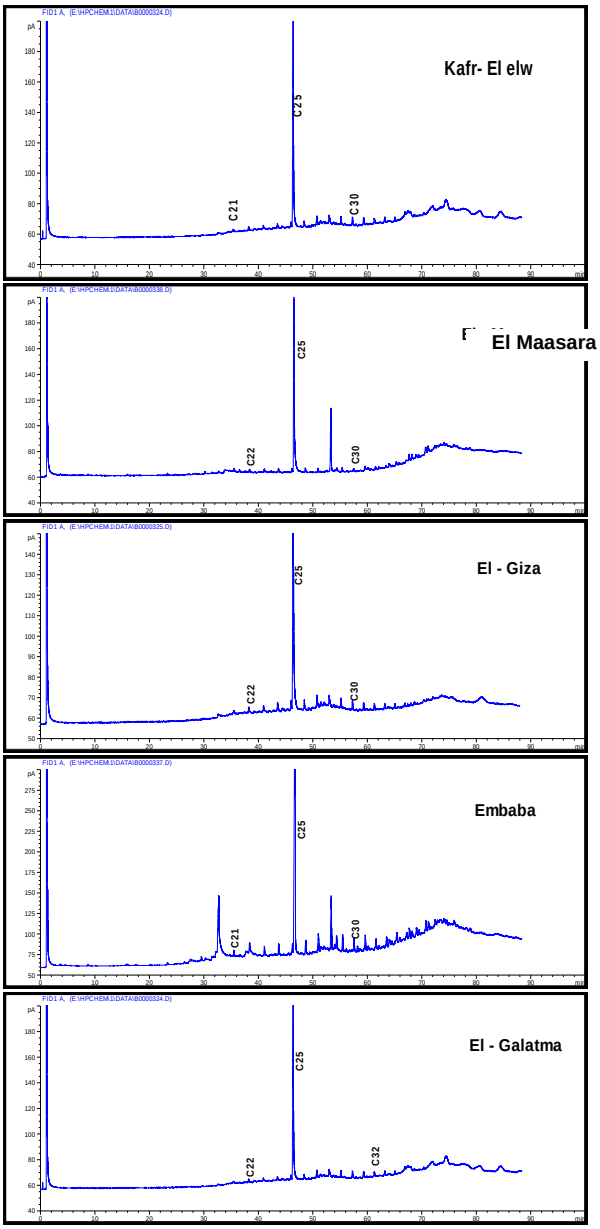

Fig. (1): Gas chromatographic tracings of the extracted hydrocarbons in the collected water samples.

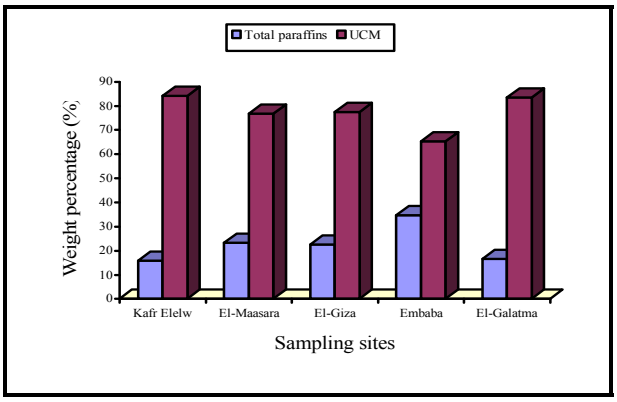

Fig. (2): Weight percentages of the total paraffinic hydrocarbons and the UCM in the collected water samples.

Table (2): Count Percentage of nC_8 and EB degrading bacterial isolates:

Isolate No.	Kafr Elelw		El Massara		El Giza		Embaba		El Galatma	
	CFU count percentage (%)									
	n C_8	EB	n C_8	EB	n C_8	EB	n C_8	EB	n C_8	EB
1	89.0	84.9	89.5	84.9	87.3	83.1	87.6	81.4	88.9	52.8
2	87.6	80.1	89.8	77.8	92.0	89.2	88.6	82.5	88.6	84.0
3	85.8	83.6	88.6	81.4	91.6	88.6	59.0	58.1	88.0	88.3
4	89.5	88.9			89.8	88.6	93.1	90.8		

III- Identification of the bacterial isolate:

The most potent bacterial isolate was identified as *Bacillus amyloliquefaciens* according to the Biolog^{TM} microplate assay (Winding, 1994), which gives a characteristic reaction pattern called the metabolic finger print that is then keyed into the Biolog's identification data base.

IV-Stimulation of the degradation ability of *B. amyloliquefaciens* using He- Ne laser radiation:

As the focus has turned to enhance the rate and efficiency of the biodegradation technology. It was found that the bioenergitic processes of the microbial cells are known to increase after their exposure to laser light of specific wave length. This in turn results in the increase in the rate of cell division and protein synthesis, also the enhancement of the production of extracellular enzymes related to biodegradation (Vladimirov et al., 2004, Geweely et al., 2006 and Jiang et al., 2007). In this regard the cell suspensions of the bacterial isolate was subjected to different doses of low intensity He-Ne laser. From Table (4) it was found that 1 min of exposure had induced a significant increase in the percentage of degradation of the studied substrates. So after 12 hrs of incubation complete degradation was achieved. Extending the exposure duration to 3 mins resulted in an increase in the degradation potentiality of the bacterial isolate .Complete degradation of the studied substrates was achieved only after 8 hrs of incubation. On the other hand the percentage of degraded substrates had decreased after their inoculation by bacterial suspensions irradiated for 6 mins, this is due to that large doses of laser can evoke damage in cell structures and morphological deformations associated with enzymatic suppression (Popov et al., 2007).

Table 3: Weight percentage of degraded substrates using the bacterial suspensions after their exposure to different durations of He-Ne laser radiation.

	Weight Percentage of degraded substrate (%)											
	n-C_8	n-C_8 in mix	EB	EB in mix	n-C_8	n-C_8 in mix	EB	EB in mix	n-C_8	n-C_8 in mix	EB	EB in mix
Incubation time / *Exposure Time (mins)*	**4 hrs**				**8 hrs**				**12 hrs**			
Zero	31.43	26.17	15.07	21.40	42.11	34.33	19.31	23.36	55.01	43.24	25.75	30.02
1	70.63	64.88	51.57	57.51	79.58	70.29	70.86	74.54	100	100	100	100
3	81.37	73.49	71.90	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	19.02	26.13

V-Degradation of paraffinic hydrocarbons:

Paraffinic hydrocarbons are considered the most wide spread organic contaminants related to soil and water (Margesin and Schinner, 2001). However *Bacillus* is one of the most predominant genera associated with the degradation of paraffinic pollutants in fresh water (Queiroga et al., 2003 and Zhao et al., 2007). The CGC profile of the paraffinic hydrocarbon mixture before treatment exhibits iso and normal paraffins which ranged from undecane C_{11} to octatricontane C_{38}. After its microbial inoculation and incubation for 12hrs, complete degradation of both heavy n-paraffinic fraction (C_{34} to C_{38}) and nearly the corresponding iso paraffinic fraction (C_{29} to C_{37}) was achieved and this was reflected on the increase of the concentration of the light paraffinic hydrocarbons. The residual iso-paraffins were found to be higher than that of the control sample; this may be due to the degradation of the heavy paraffinic hydrocarbons to produce other components of lower molecular weights. The percentage of degradation increases as a function of incubation period, so after 30hrs complete degradation of the most heavy iso components (>C_{20}) was achieved.

Irradiation of the bacterial cells leads to more increase in enzyme activity. So the weight percentage of residual paraffins was decreased to 12.36% after only 12hrs of incubation. Increasing the incubation period to 30hrs results in degradation of 98.12% of the paraffinic hydrocarbon mixture leaving trace values of 1.33% and 0.55% for normal and iso paraffins respectively (Table, 4 and Fig.3).

Carbon preference index (CPI) is expressed numerically as the ratio between the summation of the weight percentages of odd carbons and even carbons ((Medeiros et al., 2005). It is a useful indicator of odd/even carbon selectivity (Ali, 2006). From the results it was found that the CPI value was around unity, indicating that *B.amyloliquefaciens* (before and after irradiation) had the ability to degrade odd and even carbon number compounds by nearly the same ratio.

The geochemical markers n-C_{17}/pr and n-C_{18}/ph ratios were used as indicators for biodegradation (Tehrani et al., 2006 and Adebusoye et al., 2007). It was found that the ratios n-C_{17}/pr and n-C_{18}/ph were decreased after the two studied incubation times, these implied that the isolated bacterial strain before and after irradiation had mianly high ability to degrade paraffinic hydrocarbons and especially normal paraffins.

From the previous results it was clear that, the irradiated bacterial suspension was efficient to cause nearly complete degradation of the paraffinic hydrocarbon mixture after 30hrs of incubation. Also, the potentiality of the irradiated bacterial suspension on the degradation of the paraffinic hydrocarbon mixture after 12hrs of incubation was higher than that of the non-irradiated bacterial suspension after 30hrs of incubation.

Table, 4: **Weight percentage of the residual paraffins in the control sample and the treated samples with *B.amyloliquefaciens* (before and after irradiation).**

Compound name	***Weight percentage of residual components (%)***									
	Control		**12hrs**				**30hrs**			
			Before irradiation		*After irradiation*		*Before irradiation*		*After irradiation*	
	Iso-	Normal	Iso-	Normal	Iso-	Normal	Iso-	Normal	Iso-	Normal
Nonane C_9	0.00	0.00	2.58	2.09	0.00	0.00	0.00	0.00	0.00	0.00
Decane C_{10}	0.00	0.00	1.73	3.17	0.00	0.01	0.00	0.16	0.00	0.00
Undecane C_{11}	0.00	0.26	2.28	5.39	0.17	0.42	0.78	0.15	0.00	0.00
Dodecane C_{12}	0.36	1.16	1.64	4.10	0.27	0.58	0.53	1.28	0.00	0.03
Tridecane C_{13}	1.22	4.69	2.69	4.38	0.54	0.69	0.96	2.05	0.10	0.07
Tetradecane C_{14}	1.18	5.88	1.90	4.51	0.30	0.74	0.76	2.42	0.02	0.11
Pentadecane C_{15}	4.24	7.36	3.56	4.30	0.73	0.60	1.15	2.72	0.07	0.13
Hexadecane C_{16}	2.68	7.01	0.86	3.76	0.29	0.69	1.78	2.62	0.05	0.12
Heptadecane C_{17}	3.32	10.22	1.38	3.16	0.39	0.74	1.40	1.78	0.04	0.12
Pristane (pr)	3.61		3.83		0.91		2.36		0.17	
Octadecane C_{18}	1.80	5.83	0.52	2.65	0.03	0.54	4.72	1.49	0.00	0.11
Phytane (ph)	1.28		0.86		0.19		0.82		0.08	
Nonadecane C_{19}	1.09	5.69	2.58	0.33	0.03	0.51	0.06	1.30	0.02	0.10
Eicosane C_{20}	1.00	4.59	0.20	2.01	0.01	0.44	2.16	0.92	0.00	0.09
Heneicosane C_{21}	0.34	3.46	1.93	0.21	0.01	0.41	0.00	1.51	0.00	0.08
Docosane C_{22}	0.43	3.17	0.15	1.50	0.04	0.34	0.00	1.04	0.00	0.07
Tricosane C_{23}	0.36	2.99	0.19	1.42	0.01	0.35	0.00	0.83	0.00	0.06
Tetracosane C_{24}	0.25	2.44	0.07	0.98	0.01	0.29	0.00	0.53	0.00	0.05
Pentacosane C_{25}	0.30	2.18	0.10	0.81	0.01	0.26	0.00	0.36	0.00	0.04
Hexacosane C_{26}	0.24	1.69	0.07	0.56	0.01	0.19	0.00	0.21	0.00	0.03
Heptacosane C_{27}	0.16	1.53	0.04	0.45	0.00	0.17	0.00	0.16	0.00	0.03
Octacosane C_{28}	0.22	1.06	0.05	0.27	0.00	0.12	0.00	0.10	0.00	0.02
Nonacosane C_{29}	0.18	0.90	0.00	0.20	0.00	0.10	0.00	0.08	0.00	0.02
Triacontane C_{30}	0.16	0.63	0.00	0.10	0.00	0.06	0.00	0.04	0.00	0.01
Entriacontane C_{31}	0.06	0.98	0.00	0.14	0.00	0.10	0.00	0.06	0.00	0.02
Dotriacotane C_{32}	0.08	0.61	0.00	0.02	0.00	0.02	0.00	0.01	0.00	0.01
Tritriacontane C_{33}	0.09	0.44	0.00	0.05	0.00	0.04	0.00	0.00	0.00	0.01
Tetratriacontane C_{34}	0.15	0.16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pentatriacontane C_{35}	0.04	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Hexatriacontane C_{36}	0.01	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Heptatriacotane C_{37}	0.01	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Octatriacotane C_{38}	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Total	**24.86**	**75.14**	**29.21**	**46.56**	**3.95**	**8.41**	**17.48**	**21.82**	**0.55**	**1.33**
CPI		**1.19**		**0.97**		**1.09**		**1.02**		**1.10**
n-C_{17}/pr ratio	**2.83**		**0.83**		**0.81**		**0.75**		**0.71**	
n-C_{18}/ph ratio	**4.55**		**3.08**		**2.84**		**1.82**		**1.38**	

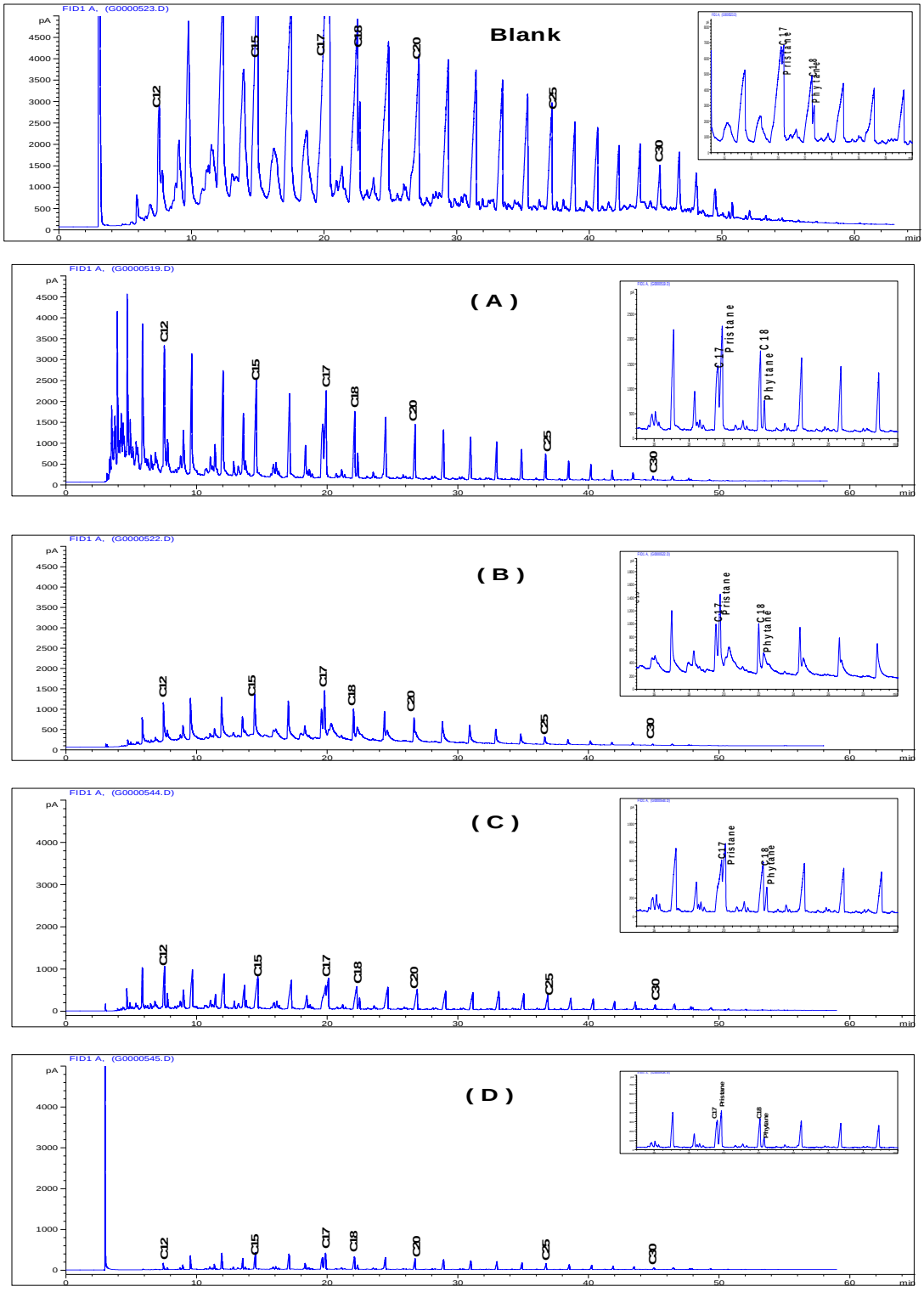

Fig. (3): **Gas chromatographic tracings of the residual paraffins in the control sample and the treated samples using irradiated (A and C) and non irradiated (B and D) bacterial suspensions.**

VI-Degradation of poly nuclear aromatic hydrocarbons (PAHs):

PAHs are fused ring aromatic compounds (2-7 rings). They are of intermediate biodegradability, but of the most concern owing to their toxicity and tendency to bioaccumulation (Lily et al., 2009).Adverse group of bacterial strains belonging to the genus *Bacillus* were identified as they can partially degrade or mineralize some high molecular weight PAHs (Neves and Durrant, 2004 and Cunha et al., 2006).

In the present work nine polynuclear aromatic hydrocarbons (Fig. 4), have been selected for studying the effect of the selected bacterial isolate.

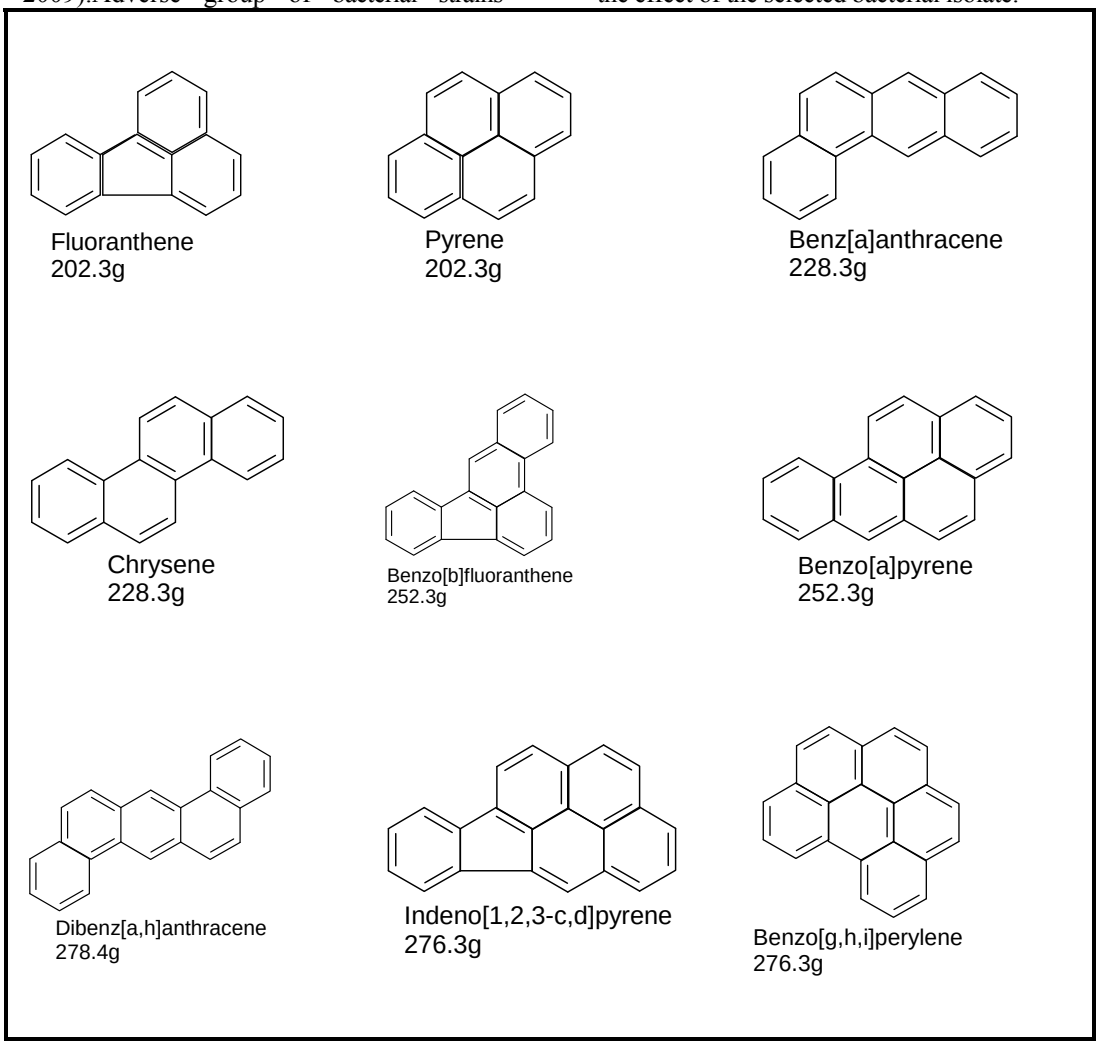

Fig. (4): Chemical structure and molecular weights of the studied nine polynuclear aromatic hydrocarbons.

The residual aromatics before and after degradation by *B.amyloliquefaciens* were extracted and analyzed using HPLC. The results given in (Table, 5) revealed that, *B.amyloliquefaciens* was able to attack four; five and six member rings polynuclear aromatic hydrocarbons. This in turn reflects its broad oxidative capacity.

A remarkable decrease in the concentration of the studied compounds was observed on increasing the incubation time to 30hrs. The irradiated bacterial suspension exhibited higher degradation power than the non irradiated one at the two studied incubation periods. After 30hrs of incubation complete degradation capacity (100%) was achieved on treating the mixture of PAHs with the irradiated bacterial suspension.

(Table, 5): Concentrations of the undegraded (control) and degraded polynuclear aromatic hydrocarbons using *B.amyloliquefaciens* (before and after irradiation).

Polynuclear aromatic hydrocarbons	*Concentration of residual polynuclear aromatic compounds (μg/ml)*				
	Control	*Incubation time12hrs*		*Incubation time30hrs*	
		Before irradiation	After irradiation	Before irradiation	After irradiation
Fluoranthene	0.76	0.42	0.20	0.22	Nil
Pyrene	0.16	0.09	0.06	0.07	Nil
Benz (a) anthracene	1.36	0.57	0.44	0.50	Nil
Chrysene	0.99	0.42	0.30	0.32	Nil
Benzo (b) fluoranthene	0.17	0.10	0.07	0.09	Nil
Benzo (a) pyrene	0.19	0.11	0.06	0.09	Nil
Dibenz (a,h) anthracene	0.67	0.39	0.19	0.19	Nil
Benzo (g, h, i) perylene	0.27	0.13	0.07	0.09	Nil
Indeno (1, 2, 3-c, d) pyrene	0.66	0.56	0.12	0.21	Nil
Weight percentage of degraded polynuclear aromatic mixture (%)		**46.65**	**71.13**	**65.97**	**100**

4. Conclusions

From the previous results it was concluded that:

1- The surface water of the river Nile in greater Cairo is subjected to severe hydrocarbon pollution of mixed petrogenic and biogenic origins, so application of a cleaning technology is a must.

2-*B.amyloliquefaciens* was considered as the most promising hydrocarbon degrading bacterial isolate within the area under study.

3-The degradation potentiality of the bacterial suspension was increased by its exposure to low power He-Ne laser (7.3mW, λ=632.8 nm) for 3mins.

4-The irradiated isolate causes degradation of 98.12% of the paraffinic fraction and 100% of the polynuclear aromatic fraction only after 30 hrs of incubation, this reveals its superiority and supports its future use in the bioremediation strategy

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