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Isolation of some benzo(a)pyrene- degrading bacteria from contaminated area in Kirkuk city, Iraq

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Abstracts

Benzo(a)Pyrene is one of the most important environmental contaminants belongs to high molecular PAHs and listed by USEPA as priority pollutant. It has attracted wide interest due to its carcinogenic activity. In order to isolate and diagnose bacterial species that possess the ability to utilise BaP in Mineral Basal Salt Medium (MBSM), Soil samples were collected from the oil contaminated soils from automobile workshop (washing and greasing cars) sites in Kirkuk city. Iinitial diagnosis of bacterial isolates was done rotine method in addition of Vitek 2 compact system to get quick results and prescription in bacterial isolates identification. The results showed eight bacterial species as follows: Pseudomonas mendocina, Stenotrophomonas maltophilia, Enterobacter cloacae, Cupriavidus pauculus, Klebsiella pneumoniae, Pantoea spp, Acinetobacter haemolyticus, Comamonas testosterone. Anew bacterial genera were recorded in the present study including Cupriavidus pauculus, Pantoea sp, Cupriavidus pauculus and Comamonas testosteroni have the ability to utilize BaP as the only source of carbon and energy. The results of experimental culture to determine CFU/ml of each bacterial isolate referred to highest CFU/ml was recorded for Enterobacter cloacae (2.96×1026 CFU/ml) indicating the highest growth followed by Klebsiella pneumonia (2.92×1024 CFU/ml). There was linear increase in number CFU of cells with increase the incubation time (0, 2, 4, 8, 16 and 32 days). On the day 32 incubation about 74.67% and 67.48 % of BaP were utilize by both E. cloacae and K. pneumonia respectively.

Key words: Contaminated area;BaP; Bacteria; Biodegradation

.Introduction

Industrial development has resulted many impacts on the environment, such as increasing the petroleum and petrochemical industries produce large quantities of aromatic hydrocarbons [1]. The polycyclic aromatic hydrocarbons (PAHs) are widespread environmental pollutants distributed into all ecosystem components and have sources from natural and anthropogenic activities [2, 3]. Among the sixteen PAHs compounds listed by USEPA as priority pollutants, five ring – PAHs, Benzo(a)Pyrene (BaP) is most important and more considered due to its toxicity to human and other organisms. Benzo (a)Pyrene is a highly recalcitrant, with high genotoxicity and carcinogenicity. It is produce due to incomplete combustion of fossil fuel and various anthropogenic activities including cigarette smoke and automobile

exhausts [4]. Many studies describe the ability of numerous genera of bacteria and fungi in soils and sediments to bioremediation of high molecular PAHs like B(a)P [5]. They serve as better clean biotechnology for bioremediation of PAH contaminated sites and removal of PAHs including BaP from the environment. Bacterial species, including *Mycobacterium* sp, *Sphingomonas paucimobilis* [6-8], and *Strenotrophomonas maltophilia* [9] are capable of degrading BaP co-metabolically. It is found that *Bacillus subtilis* from automobile contaminated soil utilise BaP in basal salt mineral (BSM) medium as a sole source of carbon and energy [4]. The isolates of *Bacillus cereus* and *Bacillus vireti* can remove about 58.98% of BaP in mineral salt medium and produce metabolites as Cis-4-(7- hydroxypyrene-8-yl)-2-oxobut-3enoic acid and recorded as a new bacterial resource for biodegrading BaP [10]. The principal aim of this study is to isolate species of bacteria from contaminated area that have the ability to utilise BaP from cultural media as source of carbon and energy and may serve as the biotechnology for BaP bioremediation.

Material and Methods

Soil samples

Soil samples were collected from the oil contaminated soils from automobile workshop (washing and greasing cars) sites in Kirkuk city. A composite sample of the soil from depth (2-10 cm) were taken and placed in sterile plastic bags and, and transported to the laboratory for next study.

Isolation of Benzo(a)Pyrene - degrading bacteria

About one gram of composite soil sample was added to sterile flask containing Basal Salt Medium BSM [(0.38) g (KH2PO4), (0.6) g (K2HPO4), (0.2) g (MgSO4.7H2O), (1.0) g (NH4Cl) and (0.05) g (FeCl3 dissolved in 1.0L of distilled water (Mohandass *et al.*, 2012) [10]. The medium was supplemented with 5 mg/liter of benzo (a) pyrene (dissolved in dimethylformamide before adding) as the only source of carbon and energy. The flasks were incubated in dark shaker for 14 days at 30°C and 200 rpm, and after incubation time, about 0.1 ml of liquid culture was plated and spread on Basal Salt Agar medium supplemented with BaP and incubated at 37°C for 48 hours. The clear colonies were purified, characterised and identified in the next step.

Identification of BaP – degrading bacteria

Identification of bacterial isolates were done depending on cell and colony morphology, , various staining reactions, growth characteristics & various biochemical tests including oxidase test, catalase, indole, vox Broschor, Red instance, Gelatin, Urease, Citrate and motility, as described by Benson, 2002 [11]. To obtain more information's about the isolates, Vitek 2 compact system were used in which colorimetric reagent cards that are incubated and interpreted automatically were used. Bacterial suspensions were prepared by transfer sufficient number of colonies to sterile saline for inoculated identification cards and after incubation at 35.5$\pm$1.0ºC, data were collected at 15-minute intervals during the entire incubation period [12, 13].

Comparison the efficiency of bacterial species for BaP degradation

In order to compare the efficiency of Bacterial isolates in consuming BaP as the only source of carbon and energy, experimental culture was conducted by incubation each species on basal salt media containing BaP for one week incubation time at 37˚C in shaking incubator at 150 rpm and the efficient isolates were selected according to highest CFU/ml [4]. For activation bacterial cultures, ten ml of nutrient broth was inoculated with each isolates, incubated at 37˚C for 24 h in shaking incubator at 150 rpm. After incubation time, centrifugation was done for each bacterial culture at 8000 rpm for ten minutes to obtain cell pellets. Bacterial suspension (by washing cell pellets with Basal Salt Medium) of each isolate with 1.5×10^8 cells per ml was prepared by taking the optical density. About ml of bacterial suspension of each isolate was transferred to tube containing 10 ml Basal salt medium supplemented with BaP as a source of energy and carbon and incubated at 37˚C for one week. About 0.1 ml of bacterial suspension was taken and series of dilution up to 10^{10} was done for each bacterial suspension and 0.1m was spread on BSM containing glucose 10g/ L) and incubated for 12-24 h at 37˚C. The colonies were counted and CFU/ml was determined for each isolate [14].

BaP degradation, Extraction and Analytical procedure

The best bacterial culture in the previous step capable of degrading and consuming BaP as the only source of carbon and energy was activated for the next experiment by inoculation 10ml of Nutrient broth and after incubation at 37 for 24 h in shaking incubator at 150, bacterial suspension was obtained with 1.5 cell/ml .Series of sterile tubes containing BSM supplemented with 0.05 mg /ml were prepared and inoculated with 1 ml of each bacterial suspension ,incubated at 37 in shaking incubator at 150 for 32 days with maintain tubes as control without BaP. The number of bacteria was counted for incubation times (0, 2, 4, 8, 16 and 32 days). BaP was extracted for each bacterial culture tubes after each incubation time using 10 ml of Dichloromethane with three replication times. The organic layers were collected and

evaporated by rotary evaporator with special condition. The residue then dissolved in 1 ml of acetonitrile to be ready for analytical procedure. BaP in the extracts was analyzed by YL9100 HPLC system(The ministry of Science and Technology). Twenty µl of sample was injected into a stationary phase capillary column with a dimension of (15cm×4.6mm ID) and determined with UV detector at 254nm. The flow rate of mobile phase (water and acetonitirile) was kept at 1.5 ml/min. The peaks in the chromatogram were identified by comparing of the retention time and spectra of control.

Results and Discussion

Identification of BaP – degrading bacteria

Eight bacterial isolates obtained from the oil-contaminated soils in Kirkuk city were screened for their ability to grow on Basal salt Agar medium supplemented with Benzo(a)pyrene as a sole source of carbon and energy. Identification were done depending on microscopic examination of cells, characteristics of colony grown on BSAM containing BaP as colour , appearance and shape as resulted in Figure 1 and Table 1. Microscopic examination showed that all isolates were motile, gram negative; the isolates were rod shape, while both isolates 4 and 7 showed coccus cells and short coccobacilli in shape respectively. Colony characteristic of bacterial isolates showed irregular, spherical in shape, creamy colonies for isolates 1 to 8 with creamy to yellow for isolate 8. The colonies of all bacterial isolates appear to be mucilage (table1). About nine biochemical tests (table 2) were done for bacterial isolates depending on Collee *et al*., 1996 and Konemann *et al*., 1997 [15, 16]. Oxidase test revealed that isolates 1, 4 and 8 showed positive results with appearance of violet colour, while the rest isolates gave no change in colour indicating negative results. The isolates 1-7 showed positive result for catalase test with appearance of air bubbles indicating the presence of catalase enzyme in bacterial cells, while the isolate 8 showed negative result. The results of Indol test showed the positive results for isolates 4 and 6 with appearance of red ring indicating the ability of isolates to produce tryptophanse enzyme that converts tryptophan to pyruvic acid and ammonia to be used by bacterial as a source of energy and nutrition.

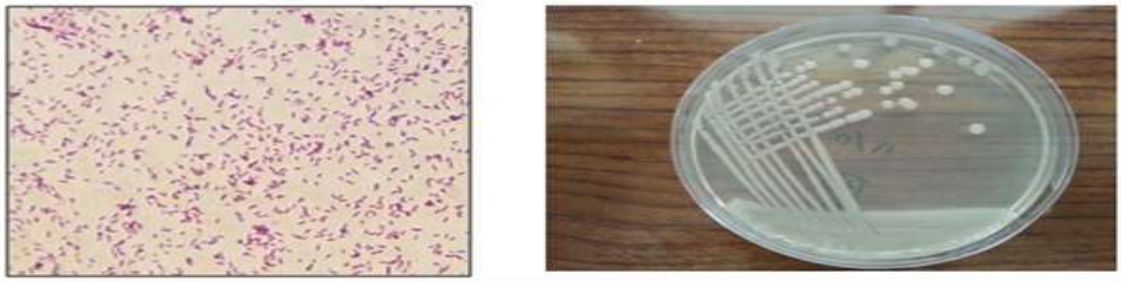

Figure1. Microscopic examination and colony on Basal Salt Agar Medium BSAM supplemented with Benzo(a)Pyrene

Table1. Colonies characteristics on Basal Salt Agar Medium BSAM supplemented with Benzo(a)Pyrene.

Bacterial isolates	Colony on Basal Salt Medium BSM supplemented with Benzo(a)Pyrene		
	Appearance	colour	Shape
1	Mucilage	Creamy	Irregular
2	mucillage	Creamy	Small spherical
3	Mucilage	Creamy	spherical
4	Mucilage	Creamy	spherical
5	Mucilage	Creamy	spherical
6	Mucilage	Creamy	spherical
7	Mucilage	Creamy	spherical
8	Mucilage	Creamy- yellow	spherical

For methyl red test, the isolates 2 and 6 gave positive results with convert of medium to red color while the other isolates gave negative results with yellow color indicating no acid remains in the medium. For Voges proskauer test, the presence of acidic products as Acetyl-methyl carbinol in the medium gave rose or pink colour indicating positive result for (2, 3, 5, 6), while the presence of acidic products resulted negative test for (1, 4, 7, 8).The convert of medium colour from green to blue indicating positive result for isolates(3, 5, 6, 7) and utilisation of citrate by bacteria as a source of carbon and energy for isolates , while negative result was recorded for isolates(1, 2, 4, 8). The isolates (1, 4, 5) gave positive result for urease test indicating the production of urease by bacteria that convert urea to ammonia and CO_2, while the isolates (2, 3, 6, 7, 8) gave negative result. Kligler iron agar (KIA) test was done for bacterial isolate to show the ability of bacterial isolates ferment ate lactose and dextrose and production of CO_2 and H2S (Table 2). The isolates (2, 6, 7) gave positive result for gelatinase test indicating the ability of bacteria to convert gelatine to amino acid by gelatinase enzyme.) API 20 E test System((bioMérieux) was used to identify enterobacteriaceae and gram negative rod bacteria .The kit consists of twenty microtubules containing substratesfor 21 biochemical tests that react with bacterial isolate after inoculation. The results indicated that the isolate 3 is Enterobacter *cloacae* with the isdentification ratio of 99% and the isolate 5 is *Klebsiella pneumoniae* with 97%, while isolate 1 gave 38.7% identification ratio to be either *Bordetella* or *Alcaligenes* or *Moraxella.*

Table 2. The results of biochemical tests for bacterial isolates grown on BaP – Basal salt medium.

Isolates no.	Oxidase Test	Catalase Test	Indole Production Test	Methyl Red Test	Vogues Proskauer Test	Citrate Test	Urease Test	Kligler Iron Agar				Gelatin Hydrolysis
								slant	butt	gas	H_2S	
1	+	+	-	-	-	-	+	red	red	-	-	-
2	-	+	+	+	+	-	-	red	red	-	-	+
3	-	+	-	-	+	+	-	yellow	yellow	+	-	-
4	+	+	-	-	-	-	+	red	red	-	-	-
5	-	+	-	-	+	+	+	yellow	red	+	-	-
6	-	+	+	+	+	+	-	red	red	-	-	+
7	-	+	-	-	-	+	-	yellow	yellow	-	-	+
8	+	-	-	-	+	-	-	red	red	-	-	-

In order to get quick results and prescription in bacterial isolates identification, the Vitek 2 compact system (bioMérieux) were used. The system detects bacterial growth and metabolic changes in the microwells of thin plastic cards by using a fluorescence-based technology. The results recorded in table 3 indicated that 96 - 99% of identification ratio was obtained within 4 to 10 h (table 3). A new genera were recorded in the present study including *Cupriavidus pauculus*, *Pantoea sp*, *Cupriavidus pauculus* and *Comamonas testosteroni*. It is found that more than 60 bacterial genera have the ability to biodegrade the hydrocarbons (Both aliphatic and aromatic hydrocarbons) compounds in the soil under aerobic and un-aerobic condition [17, 18]. The use of efficient microorganism proved to be the important way to bioremediation of pollutant in the environment and few studies have focused on the biodegradation of high molecular PAHs like BaP. Sun *et al*., 2010 [19] screen the community structure of bacteria from HMW-PAHs contaminated soil using construction of 16S rRNA gene libraries, among these *Pseudomonas, Stenotrophomonas maltophilia and Acinetobacter haemolyticus*. There are several bacterial species capable of benzo(a)pyrene degradation

including *Sphingomonas paucimobilis* [6] and *Stenotrophomonas maltophilia* [9]. Juhaz *et al*., 2000 [20] found that *S. maltophilia* VUN 10,003 can degrade benzo(a)pyrene by 22% for 14 days incubation. The best hydrocarbon-degrading bacteria, including *Arthrobacter sp., Enterobacter sp., Sphingomonas sp., Pseudomonas koreensis, Pseudomonas putida and Pseudomonas plecoglossicida*, were isolated directly from the soil [21].

Table 3. The results of Vitek 2 compact system for identification of BaP- degrading bacteria isolated from contaminated soil.

Bacterial isolates	Bacterial species	Diagnosis time (hours)	The proportion of diagnosis
1	*Pseudomonas mendocina*	7:00	99%
2	*Stenotrophomonas maltophilia*	5.25	99%
3	*Enterobacter cloacae*	6:00	99%
4	*Cupriavidus pauculus*	7:00	99%
5	*Klebsiella pneumoniae*	4:00	99%
6	*Pantoea spp*	5:75	98%
7	*Acinetobacter haemolyticus*	8:00	97%
8	*Comamonas testosteroni*	10:00	98%

The efficiency of bacterial species for BaP degradation

In order to check bacterial growth in the BaP-BSA medium and compare between bacterial species for their ability to utilize BaP in the culture medium as the only source of carbon and energy , CFU/ml of each bacterial isolate was determined after week incubation at 37 c(Table 4). The results referred to highest CFU/ml was recorded for *E. cloacae* (2.96×10^{26} CFU/ml) indicating the highest growth followed *by K. pneumonia* (2.92×10^{24} CFU/ml), while the lowest CFU/ml 3.96×10^{14} CFU/ml was recorded for *Comamonas testosterone*.

Table 4. Log10 of CFU/ml of bacterial species after week incubation.

No.	Bacterial isolates	Log10 of CFU/ml after week incubation	No. of bacterial cells after week of incubation
1	*Pseudomonas mendocina*	18.58	3.4×10 18
2	*Stenotrophomonas maltophilia*	20.57	3.8×10 20
3	*Enterobacter cloacae*	26.47	2.96 ×10 26
4	*Cupriavidus pauculus*	15.51	3.24×10 15
5	*Klebsiella pneumoniae*	24.46	2.92×10 24
6	*Pantoea spp*	16.44	2.76× 10 16
7	*Acinetobacter haemolyticus*	18.53	3.44× 10 18
8	*Comamonas testosteroni*	14.59	3.96×10 14

To assess the ability of *E. cloacae* and *K. pneumonia* to utilise BaP, Growth experiment was done under different incubation times(0, 2, 4, 8, 16 and 32 days)(Table 5 and 6) and determine the mineralization of BaP by high pressure liquid chromatography HPLC. At the beginning of the experiment about 3.04 x 10^{8} CFU/ml bacterial cells of *E. cloacae* was added to the BSM containing BaP , the CFU/ml was increase to 2.91×10^{15} after 8 days of incubation to mineralize about 56.33% of BaP . For *K. pneumonia*, the CFU number was increase from 2.68 x 10^{8} at the beginning to 3.64×10^{24} after 8 days incubation to mineralize about 41.15 %.

Table 5. The number of live bacterial cells (*E. cloacae*) and (K. *pneumoniae)* during different periods of incubation.

Number of bacterial cells						Bacterial species	No.
32 days	16 days	8 days	4 days	2 days	Zero time		
2.72×10^{4}	1.72×10^{19}	3.8×10^{28}	2.4×10^{18}	3.36×10^{14}	3.04×10^{8}	*E. cloacae*	1
3.52×10^{6}	3.28×10^{20}	3.64×10^{24}	3.84×10^{16}	2.76×10^{16}	2.68×10^{8}	*K. pneumoniae*	2

Table 6. BaP degradation percentage in culture medium by (*E. cloacae*) and (K. *pneumoniae*) during different periods of incubation.

Degradation percentage% of BaP during incubation times						Bacterial species	No.
32 days	16 days	8 days	4 days	2 days	Zero time		
74.67	67.13	56.33	23.42	10.34	0.0	*E. cloacae*	1
67.48	58.34	41.15	17.09	8.47	0.0	*K. pneumoniae*	2

As shown from the results there was linear increase in number CFU of cells with increase the incubation indicating the ability of bacteria to utilize BaP in the medium. After 8 days incubation the logCFU of both bacterial species start to decrease due to accumulation of toxic metabolites or due to exhaustion of BaP as source of carbon and energy as explained by Lily *et al*., 2013 [14] regarding the degradation of anthracene by *Brachybacterium paraconglomeratum*. On the day 32 incubation about 74.67% and 67.48 % of BaP were utilize by both *E. cloacae* and *K. pneumonia* respectively. Many studies have been done regarding degradation of some PAHs compound. About 44.07% of BaP was degraded by a mixture of different types of bacteria are (*chrobactrum sp., Stenotrophomonas maltophilia & Pseudomonas fluorescens*) after 14 days of incubation (Luo *et al*., 2012) [22]. Lily *et al.*, 2009 [4] found that the novel strain *Bacillus subtilis* BMT4i (MTCC 9447) showed increasing in biomass within 7 days and about 84.66% of BaP was degraded through 28 days of incubation.. Lily *et. al*., 2012 [23] also used *Bacillus subtilis* but after a genetic mutation of bacteria by ultraviolet rays and was able to get (100%) degradation through 28 days of incubation. Mohandass *et al*., 2012 [10] found that the isolates of *Bacillus cereus* and *Bacillus vireti* have the ability to remove about 58.98% of BaP in Mineral salt medium and may be more efficient to be used for bioremediation of heavily contaminated area).A mixture of *Stenotrophomonas maltophilia* and *Penicillium janthinellum* have the ability to remove about 25% of BaP through (45) days [24]. About 44.07% of BaP was degraded by a mixture of different types of bacteria are (*chrobactrum sp., Stenotrophomonas maltophilia & Pseudomonas fluorescens*) after 14 days of incubation [22].

Conclusion

Few studies were done to focus on the degradation of high molecular weight PAHs .In our present study , eight bacterial isolates (*Pseudomonas mendocina, Stenotrophomonas maltophilia, Enterobacter cloacae, Cupriavidus pauculus, Klebsiella pneumoniae, Pantoea spp, Acinetobacter* haemolyticus, *Comamonas testosterone*) were detected from BaP – contaminated area have the ability to remove BaP and utilize it as the only source of carbon and energy. Anew genera were recorded in the present study including *Cupriavidus pauculus, Pantoea sp, Cupriavidus pauculus* and *Comamonas testosterone*. On the day 32 incubation about 74.67% and 67.48 % of BaP were removed from growth culture supplemented with BaP by *E. cloacae* and *K. pneumonia* respectively. The best regarded bacteria were *E. cloacae* and *K. pneumonia* and may be used for clean of BaP- contaminated area in bioremediation technology.

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