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Technical Note

The efficacy of an oxidation pond in mineralizing some industrial waste products with special reference to fluorene degradation: a case study

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Abstract

The efficacy of the oxidation pond on the outskirts of the 10th of Ramadan, the main industrial city, in Egypt was examined. Samples of wastewater collected from the inlet and the outlet were screened for some priority pollutants. Acenaphthene and fluorene were the most frequently detected polycyclic aromatic hydrocarbons, while dimethyl phthalate was the most frequently detected phthalate ester. The spectrum of pollutants, their concentrations and frequencies were similar in the inlet and the outlet, indicating an inferior mineralization capability of the pond. Several degradative bacterial strains were isolated from the pond and grown on M56 minimal media supplemented with different pollutants as the carbon source. The efficacy of pure and mixed cultures to break down fluorene, the most frequently detected pollutant was examined. Fluorene degradation was fast in the first 10 days, then followed by a slow phase. Mixed culture had a higher rate of fluorene degradation in comparison to pure cultures. High performance liquid chromatography analysis of fluorene degradation showed three degradative metabolites. But GC/MS analysis detected one compound, identified as acetamide. The present work has indicated the poor efficacy of the pond. Lack of primary treatment of industrial effluent at factory level, coupled with shock loads of toxicants that may damage the microorganisms and their degradative capabilities are presumably main factors behind such inferior performance. Moreover, the type of pollutants discharged into the pond tend to fluctuate and change depending on the rate from the factories discharge and work shifts. Such irregular feeding of persistent pollutants may have led to a wash out of specialized strains of bacteria capable to degrade such persistent pollutants.

Keywords: Oxidation pond; PAH; Fluorene; Degradation; Bacterial isolates; Industrial waste; HPLC; GC/MS

1. Introduction

Industrial waste is a major cause of concern in developing countries. Limited experience, coupled with lack of public awareness are major constraints on waste management practices in these countries. Waste products that resist mineralization in conventional biological waste treatment systems are potential pollutants whose permanent or transitory accumulation in the environment have an adverse effect on man and his environment.

Microorganisms have a remarkable ability to degrade a wide array of organic compounds. Such capabilities have created a wide interest in the use of microorganisms in pollution abatement and mitigating environmental damage. Various reports have provided ample details of the microbial degradation of many priority pollutants. Dawson and Chang [1] reported the involvement of microbial communities in the degradation of various xenobiotics and the significance of genetic exchange between populations of different organisms in the evolution of novel biodegradative pathways. Heitkamp et al. [2] demonstrated the ability of bacteria in water, soil or sediment to degrade polycyclic aromatic hydrocarbons (PAH) a class of refractory pollutants with potential mutagenic and carcinogenic effect [3]. Mineralization of PAH was studied by Smith [4] and Marks et al. [5], who also indicated the relationship between PAH molecular weight and their microbial

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degradability. Similarly, Ribbons et al. [6] highlighted the role of bacteria in the break down of phthalate esters, PE, a main constituent of plastics of proven toxicity to man [7]. Nevertheless, Leisinger and Brunner [8] suggested that shock loads of toxic chemicals may damage microorganisms and affect their degradative capabilities. Moreover discontinuous feeding of persistent chemicals may also lead to a wash out of specialized strains that mineralize poorly degradable compounds.

The present study was conducted at the 10th of Ramadan, one of the newly established satellite cities around Cairo. The city was planned to encompass a wide range of industries and to accommodate a considerable sector of industry-related citizens to help manage the over-population Cairo suffers from.

Industrial and domestic waste effluents are directed to an oxidation pond at the outskirts of the city without any primary treatment. Mixing domestic and industrial effluents was thought of as a method to catalyze the mineralization of major recalcitrant pollutants [6].

In this paper, the inlet and outlet of the oxidation pond are screened to ascertain major pollutants, their frequencies and concentrations as an indicator for the efficacy of the pond. The occurrence of bacteria tolerating organic pollutants in the pond and their ability to degrade fluorene, the most frequently detected pollutant, was studied and the main fluorene degradative metabolite was identified.

2. Material and methods

2.1. Wastewater sampling

Water samples were collected from inlet and outlet of the oxidation pond. Samples of 2 l each were collected in acid washed dark bottles. Sampling was conducted on a monthly basis for 6 consecutive months.

2.2. Pollutants extraction

Water samples were filtered to remove solid particles using Whatman no.1 filter paper. A sub sample of 125 ml was mixed with 250 ml dichloromethane in a separatory funnel. The funnel was shaken vigorously for 3 min, then phases were allowed to separate and each sample was extracted twice more. Solvent extracts were combined and evaporated to near dryness using a rotary evaporator. Residues were quantitatively transferred on top of a glass column packed with activated silica gel and topped with anhydrous sodium sulphate. The column was eluted with 20 ml dichloromethane. The eluent was concentrated to near dryness and quantitatively transferred in 3 ml acetonitrile to a glass vial ready for chromatography analysis.

2.3. High performance liquid chromatography

A Beckman, model 342 high performance liquid chromatograph equipped with two pumps, model 112, a solvent programmer, model 210 and a fixed wave length detector, model 160 was used in this study. For PAH detection an Ultrasphere, C18 analytical column (25 cm×4.6 mm i.d.) preceded by a stainless steel pre column (4.5 cm×4.6 mm i.d.) was used for reversed phase chromatography. Mobile phase was made of a mixture of acetonitrile and water (87 + 13%V/V), running at a flow rate of 0.7 ml/min. PAH were detected by monitoring the UV absorbance of the column elute at 254 nm. The retention time of acenaphthene, fluorene, anthracene and pyrene were 3.6, 8.4, 9.6 and 11.9 min, respectively. Reversed phase chromatography was also employed to separate and detect phthalate esters using an Ultrasphere C8 analytical column (15 cm×4.6 mm i.d.) preceded by a stainless steel pre-column (1.5 cm ×4.6 mm i.d.). The mobile phase was made of acetonitrile and water (70 + 30%V/V) running at flow rate of 0.7 ml/min. PE were detected by monitoring the UV absorbance of the column elute at 254 nm. The retention times of di *n*-butyl phthalate, dimethyl phthalate and diethyl phthalate were 2.5, 4.3, and 5.3 min, respectively.

2.4. Quantitation

A standard curve of each compound was constructed by plotting peak area against concentrations (external standard). Peak areas were linearly related to the concentrations of injected PAH and PE compounds over the range of 50 folds. A Spectra Physics computing integrator (Data Jet) was used to measure peak areas.

2.5. Isolation of degradative bacteria

Bacterial strains tolerant to selected organic pollutants were isolated from the oxidation pond. Water samples, each of 1 l, taken from the pond were collected in sterile glass bottles and taken to the laboratory. Bacterial characterization and bacterial count were conducted using direct plating and enrichment techniques [9]. The identification system API-20 NE was used to identify the non-enteric, gram negative bacteria.

2.6. Biodegradation of fluorene

The efficacy of the bacteria isolated from waste water to degrade fluorene was studied using pure and mixed cultures.

2.7. Pure culture

Inocula of single bacterial strains were grown on nutrient broth 100 ml in a 250 ml conical flask and

incubated at 37°C on an orbital shaker at 200 rpm. An aliquot of 0.5 ml was inoculated into 50 ml M 56 minimum medium [4] in a 250 ml conical flask and supplemented with 0.1% w/v 100 mg kg^{-1} fluorene. Control flasks were prepared with the same contents but without inoculation.

2.8. Mixed culture

One ml of the liquid enrichment was used to inoculate 250 ml conical flasks containing 50 ml M 56 minimal medium and supplemented with 0.1% 100 mg kg^{-1} fluorene. Flasks were incubated on a rotary shaker at 20°C and 200 rpm. Control flasks were prepared with the same contents but without inoculation.

2.9. Extraction of fluorene

Samples, each of 2 ml, were withdrawn from enrichment flasks at different time intervals and extracted with 5 ml dichloromethane with vigorous shaking for 3 min. Phases were allowed to separate,then the lower organic phase was removed and transferred into clean vials. Dichloromethane was evaporated at room temperature. The extract was redissolved in 1 ml acetonitrile and filtered through a Millipore membrane filter, 0.45 μm and kept in new glass vials ready for HPLC, GC/MS analysis.

2.10. Rate of fluorene degradation

High performance liquid chromatography was used to study the rate of fluorene disappearance in pure and mixed cultures. Chromatography operational and separation conditions of fluorene were reported by Diab [9].

2.11. Gas chromatography–mass spectrometry (GC/MS)

GC/MS analysis was conducted to identify degradative products of fluorene. A Hewlett Packard gas chromatograph model 5890+coupled with a mass spectrometer model 5971 A equipped with a HP 91030 chemostation was used in this study. The mass spectrometer was used in two modes of monitoring, total ion monitoring and selected ion monitoring. In the total monitoring mode the MS continuously scanned a wide mass range from 20 to 350 amu. Each scan was recorded and stored where data can be retrieved for inspection and interpretation. A plot of this total voltage (ionization) vs time would correspond to the output of the flame ionization detector of the gas chromatograph. In the selected ion monitoring (SIM) mode the mass spectrometer scans only certain pre-selected masses and the computer stores the intensity of each mass. Gas chromatography was performed on BP 25 m×0.25 mm i.d. bound phase capillary column. Helium was used as a carrier gas at a flow rate of 0.8 ml/min. Temperature was programmed from 35 to 250°C at 10°C/min. Injector temperature was 250°C and detector temperature was 280°C. Split ratio was 10:1.

3. Results and discussion

Polycyclic aromatic hydrocarbons and phthalate esters detected in the inlet and outlet of the oxidation pond, their concentrations and frequencies are shown in Table 1. Acenaphthene and fluorene were the most frequently detected pollutants, while pyrene, dimethyl phthalate and di-butyl phthalate were the least. The spectrum of pollutants detected in the inlet and the outlet and their concentrations are similar. This result indicated that pond minerilazation of these compounds is low. It is likely that the lack of primary treatment at factory level has led to a state where the oxidation pond is receiving a higher organic load than it can cope with. Moreover, the type of pollutants discharged in the pond tend to fluctuate and change depending on the rate of discharge from the factories and work shifts. Such irregular feeding of persistent pollutants may have led to a wash out of specialized strains of bacteria capable of

Table 1
Priority pollutants detected in the outlet of the oxidation pond, their frequencies, upper and lower concentrations[a]

Compound	Inlet			Outlet		
	Frequency	Higher concentration (ng l^{-1})	Lower concentration (ng l^{-1})	Frequency	Higher concentration (ng l^{-1})	Lower concentration (ng l^{-1})
Acenaphthene	+ + +	100	35	+ + +	85	50
Fluorene	+ + +	70	40	+ + +	60	25
Antheracene	+ +	55	35	+ +	35	20
Pyrene	+	n.c.	n.c.	+	n.c.	n.c.
Dimethyl phthalate	+ +	60	45	+ +	30	10
Dimethyl phthalate	+	47	20	+	50	17
Di *n*-butyl phthalate	+	65	40	+	55	40

[a] + + + detected during all screening (six times); + + detected during most screening (four times); + detected only once; n.c., not counted.

degrading such persistent pollutants. The present results have also indicated that mixing domestic and industrial effluent did not enhance the mineralization process of persistent pollutants.

Bacteriological studies showed that a total of 89 pure unknown cultures were isolated from the pond on M 56 minimal medium supplemented with different pollutants as the carbon source. Table 2 shows the numbers and percent of bacterial isolates. All the isolated strains were gram negative except ANIL 16,18,20,38,40, DEP 56,61,62,FL 24 and DMP 42, which were gram positive.

The ability of strains B7, B8, T15, DMP2 and a consortium to degrade or utilize fluorene as a sole carbon source is shown in Figs. 1–5 respectively. Strains B7 and T15 have caused a sharp decrease in fluorene concentration after 10 days of incubation in comparison to the control. On the other hand, strain B8 had caused a 50% reduction in fluorene concentration after 4 weeks of incubation. Similar rate of fluorene disappearance was observed with DMP2. The ability of a consortium to degrade fluorene (Fig. 5) was much higher than pure cultures isolated from the same consortium. The relatively sharp rate of fluorene disappearance observed in the first 10 days, followed by a constant concentration may be explained by the production of some compounds that prohibit or prevent any further degradation of fluorene. Such compounds are called dead-end rout.

Table 2
Number of bacteria isolated on minimal medium supplemented with different pollutants as carbon source

Pollutants	No. of isolates	% of total (89)
Toluene	8	7.9
Benzene	5	6.8
Aniline	6	6.8
Anthracene	18	20.5
Dimethyl phthalate	10	11.4
Diethyl phthalate	6	6.8
Di *n*-butyl phthalate	13	14.7
Fluorene	12	13.8
Pyrene	9	10.2
Acenapthene	11	12.5

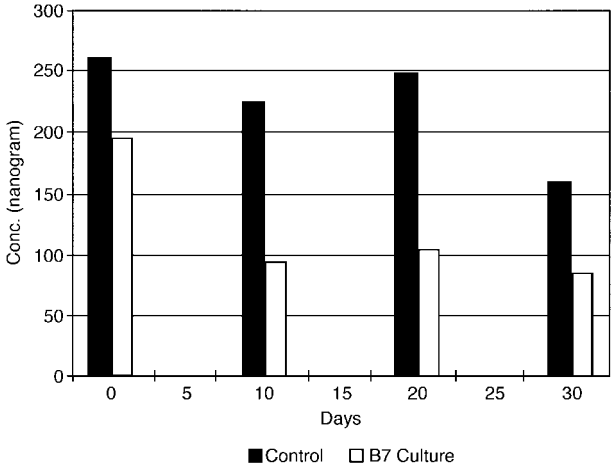

Fig. 1. Rate of fluorene disappearance by B7 culture.

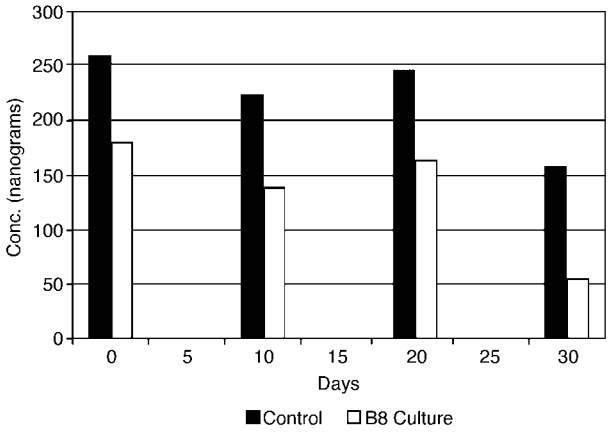

Fig. 2. Rate of fluorene disappearance by B8 culture.

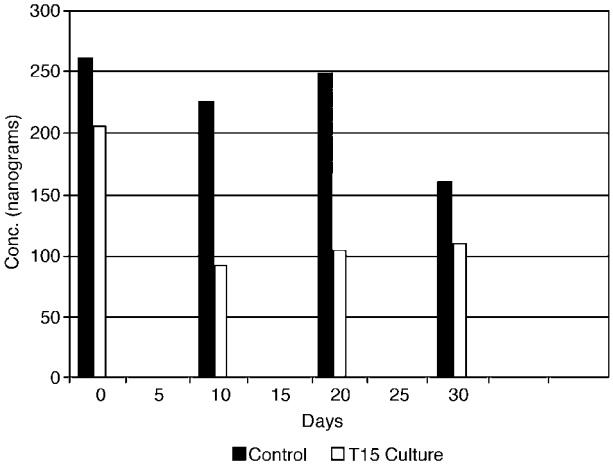

Fig. 3. Rate of fluorene disappearance by T15 culture.

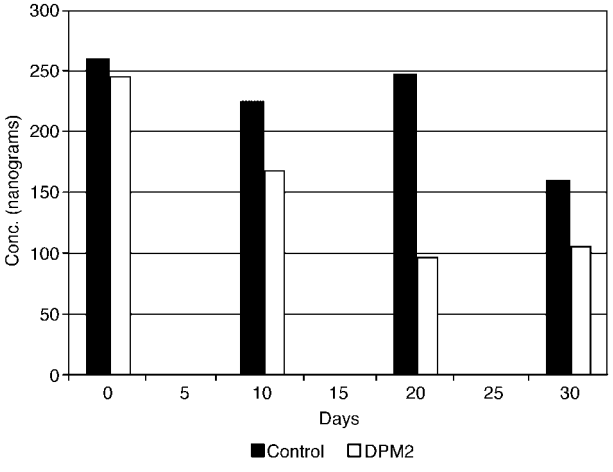

Fig. 4. Rate of fluorene disappearance by DMP2 culture.

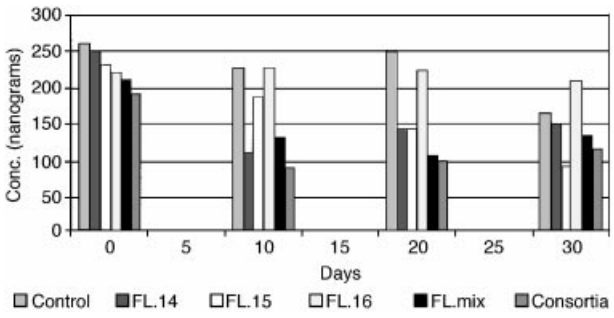

Fig. 5. Rate of fluorene disappearance by various culture.

This suggestion was brought about by the study of Grifoll et al. [10] who discussed the monooxidation of fluorene by Arthobacter to 9 hydroxyl fluorene (9-fluorenal) and subsequent dehydration to form 9-fluorenoal as a dead-end rout. Alternatively, such pattern of fluorene degradation could be interpreted in view of its inefficiency as a sole carbon source as previously suggested by Boldrin et al. [11]. The higher efficacy of the consortium to degrade fluorene can also be ascribed to the wide range of enzymes a mixed culture would possess in comparison to the limited enzyme pool of a pure culture [1].

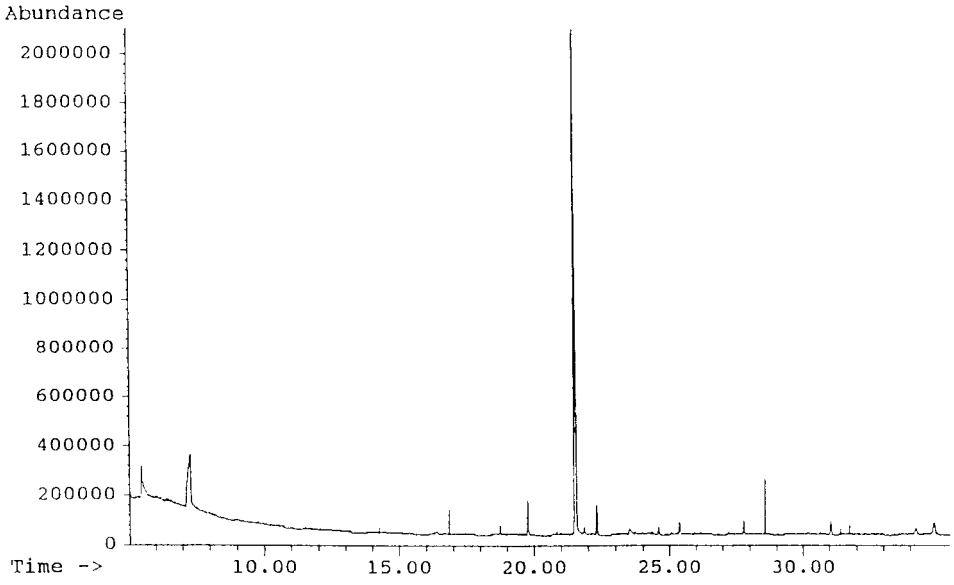

Fig. 6. GC/MS chromatograph of acetamide.

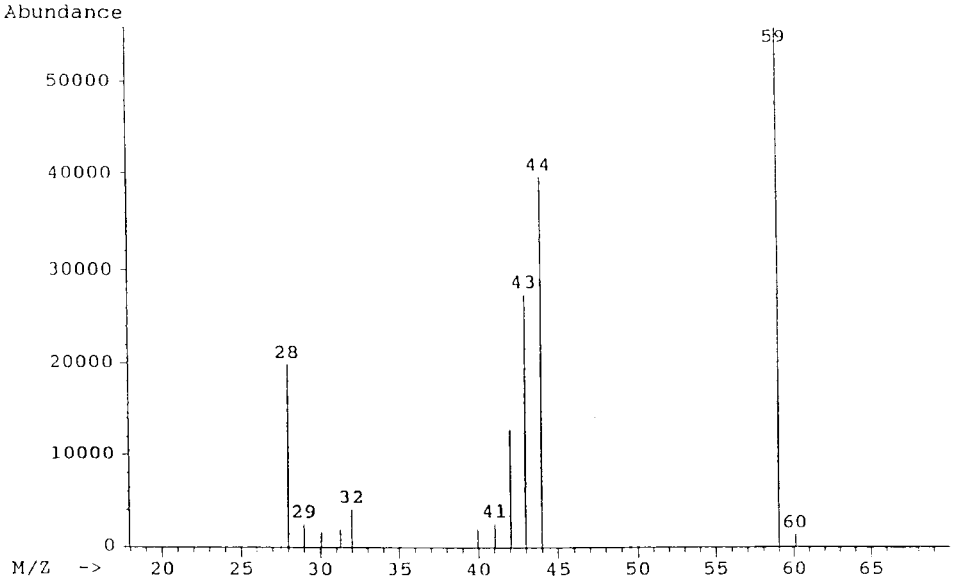

Fig. 7. Electron impact mass spectrum of acetamide.

High performance liquid chromatography analysis has revealed a range of fluorene metabolites detected in the consortium extract but not in any of the pure culture extracts. One unidentified metabolite was detected after 10 days of incubation, while three unidentified metabolites were detected in the sample extracted 20 days after incubation. All detected metabolites were eluted before the fluorene peak, indicating a more polar nature in comparison to fluorene. On the other hand GC/MS analysis has detected only one metabolite in the consortium sample extracted after 20 days of incubation. The GC/MS chromatogram of that metabolite is shown in Fig. 6 and its electron impact mass spectrum is shown in Fig. 7. The compound was identified as acetamide, a suspected carcinogen [12]. The failure of GC/MS analysis to show the full spectrum of fluorene metabolites shown in HPLC analysis is probably because of the thermo-labile nature of some of these metabolites that could not stand the high temperature of GC operation.

4. Conclusion

The present work has indicated the low efficacy of the oxidation pond on the outskirts of the 10 th of Ramadan city in mineralizing some industrial pollutants. Lack of primary treatment at factory level, shock loads of toxicants and irregular discharge of industrial waste are factors behind the poor performance of the pond. Meanwhile, mixing industrial with domestic effluent did not seem to improve the degradation of industrial pollutants as it was thought. Bacterial strains isolated from the pond showed degradative capabilities of various pollutants. Fluorene degradation with pure and mixed cultures was studied under laboratory conditions Both type of cultures showed a remarkable degradation ability of fluorene, but mixed culture was more efficient than single isolates. Acetamide was identified as the main degradative metabolite of fluorene.

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References

[1] Dawson TD, Chang F. Screening test of the biodegradative capability of a new strain of *Pseudomonas glodioli* (BSU 45124) on some xenobiotic organics. Bull Environ Contam Toxicol 1992;49:10.
[2] Heitkamp AM, Freeman PJ, Miller WD, Cerniglia BC. Pyrene degradation by a *Mycobacterium* sp.: identification of ring oxidation and fission products. Appl and Environ Microbiol 1988; October: 556.
[3] International Agency for Research on Cancer (IARC). Monograph on the evaluation of the carcinogenic risk of chemicals to humans: polycyclic aromatic hydrocarbons, vol. 32. Lyons (France) WHO, 1983.
[4] Smith RM. The biodegradation of aromatic hydrocarbons by bacteria. Vol. 1, Biodegradation. Kluwer, Academic Press, 1990. p. 191–206.
[5] Marks ER, Field DS, Wojtanawiez AC, Britenbeck AG. Biological treatment of petrochemical wastes for removal of hazardous polycyclic aromatic hydrocarbon constituents. Wat Sci Tech 1992;25(3):213.
[6] Ribbons DW, Keyser P, Kutiz DA, Taylor BF, Eaton RW, Anderson BN. Microbial degradation of phthalates. In: Gibson DT, editor. Microbial degradation of organic compounds. New York: Marcel Dekker Inc., 1984.
[7] Nomura Y, Harashmia S, Oshiina Y. PHT, a transmissible plasmid responsible for phthalate utilization in *Pseudomonas putida*. J of Fermentation and Biogen 1989;70(5):295–300.
[8] Leisinger T, Brunner W. Poorly degradable substances. Biotechnology, Microbial degradation 1986;8:475.
[9] Diab AM. Biological degradation of some industrial waste. M.Sc. thesis, Suez Canal University, Egypt, 1995.
[10] Grifoll M, Casellas M, Bayoma MJ, Solanas J. Isolation and characterization of a fluorene-degrading bacteria: Identification of ring oxidation and ring fission products. Appl Environ Microbiol 1992;58:2910.
[11] Boldrin B, Tiehem A, Fritzsche C. Degradation of phenanthrene, fluorene, flouranthene and pyrene by a *Mycobacterium sp*. Appl. and Environ. Microbiology 1993; June: 1927–30.
[12] Weisburger HJ. Chemical carcinogensis. In: Cassarett I, Doull J, editors. Toxicology the basic science of poisons. New York: Macmillan Publishing Co. Inc., 1975.