Biodegradation of Fluoranthene by Alkaliphilic and Neutrophilic bacteria

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DOI: https://doi.org/10.47372/uajnas.2016.n2.a07

Abstract

Two bacterial strains, alkaliphilic bacteria *Bacillus badius* D1 were isolated from alkaline Crater Lake, (Lonar), Buldana, M.S, India, and neutrophilic bacterial strain *Lysinbacillus sphaericus* DL8 from dye disposal area of textile industry, Ichalkaranji, India. Fluoranthene, a model four-rings polycyclic aromatic hydrocarbon was utilized as carbon and energy source by these bacteria. The degradation products of fluoranthene were isolated at every 12 hrs.. of incubation period. The generated metabolites were identified by using (GC-MS, FTIR and ^{1}HNMR). Many metabolites of fluoranthene degradation were detected in the culture such as (9-fluorenone-1-carboxylic acid, 9-hydroxy-1-fluorene-carboxylic acid, 2-carboxybenzaldehyde, benzoic acid and phthalic acid). It has been observed that these two bacterial strains have degraded fluoranthene by two different ways. Incubation of fluoranthene with these bacterial strains also resulted in the induction of biotransformation enzymes like mono oxygenases, e.g. Aminopyrine N-demethylase, acetanilide hydroxylase and the content of cytochrome P-450. The influence of some experimental parameters like concentration of fluoranthene, pH, and temperature on the degradation of fluoranthene was also studied.

Key words: Alkaliphilic, Bacillus badius, Lysinbacillus sphaericus, Fluoranthene, Biodegradation

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of the environmental hazardous chemicals attributable to a number of petrogenic and pyrogenic sources [1&2]. PAHs are ubiquitous environmental pollutant, entering the environment from natural and anthropogenic sources such as natural fires, volcanic eruptions, aluminum smelting, coke production, and creosote preservation. The anthropogenic sources typically are as a result of incomplete combustion of the organic substances that make up such substances [3]. Environments contaminated with large amounts of these compounds are considered hazardous due to potential carcinogenic, mutagenic, and teratogenic effects. Generally, higher-molecular-weight PAHs, containing four or more rings, are responsible for the common of the potential hazards to both the environment and human health [3&4]. Usually, an increase in the number of multiple rings increases the chemical stability and hydrophobicity of PAHs molecules, making them more resistant to degradation by microorganisms. Fluoranthene is a member of the non alternant polycyclic aromatic hydrocarbon (PAH) class that contains a five-member ring condensed with naphthalene and benzene rings. Like other PAHs, fluoranthene is formed during the incomplete incineration of fossil fuels or via high temperature pyrolysis of organic compounds [5&6]. Fluoranthene is a natural component of coal tar, crude oil, and fossil fuels. Fluoranthene is formed when a material, such as gasoline or wood, burns incompletely. Fluoranthene sticks to very small particles that go into the air. People and animals may breathe in the particles that contain fluoranthene and other PAHs [7]. Microbial degradation pathways of PAHs containing up to three rings have been proposed [8&9], recently, little information has been available relating to the ability of microorganisms to degrade the recalcitrant, high-molecular-weight PAHs [10]. These compounds are more resistant to biological degradation processes and persist in environment causing contamination [11]. Many bacterial cultures such as *Alcaligenes denitrificans* [12] and *Mycobacterium sp*. BB1 and PYR-1 [13,14,15], are able to mineralise fluoranthene under the natural condition, but there no reported illustrated that

ability of alkaliphilic organisms to degraded fluoranthene. Hence alkaliphilic bacteria *Bacillus badius* D1 is isolated from the alkaline Crater Lake of Lonar, Maharashtra state, India. This lake is a unique inland saline lake in Asia and only the third in the world, based on geological studies [16]. It is postulated that the lake was created as a meteorite impact crater ~50-60 thousand years ago. The Lonar crater is the only crater in basaltic rock on earth. Alkalinity of the lake is attributed to the high content of sodium carbonate [16]. Alkaliphilic organisms require an alkaline pH 9.0 or more for their growth and have an optimal growth pH of around 10. The present study has demonstrated the ability of alkaliphilic bacteria to considerably mineralize fluoranthene in alkaline culture and extensively enhance the mineralization of fluoranthene in sediments containing indigenous microorganisms, while neutrophilic bacteria *Lysinbacillus sphaericus* strain DL8 is isolated from dye disposal area of textile industry. This bacterial strain well grows at natural pH. In the present studies, attempt has been made to examine the comparative ability of both alkaliphilic and neutrophilic bacteria to degrade fluoranthene. In this report, effort has also been made to isolate and identify the metabolites/ intermediates of fluoranthene and propose the tentative degradation pathway of fluoranthene by these bacterial strains.

Materials and Methods

1. Chemicals

Fluoranthene was purchased from Acros Organics, New jersey, USA. Bacteriological media chemical from HI media, Mumbai, India and solvents were purchased from SRL Mumbai, India.

2. Microorganism and cultivation

The alkaliphiles bacteria strain *Bacillus badius* D1was isolated from Pristine Crater Lake of Lonar, Buldana, M.S. India, while neutrophilic bacteria *Lysinbacillus sphaericus* strain DL8 was isolated from dye disposal area of textile industry. Isolation of pure culture of these organisms was done by using serial dilution and plating methods under aerobic condition. These bacterial strain were grown at 37ºC in nutrient broth (NB) medium in 500 ml conical flasks, containing 100 ml of the following medium 0.5% Yeast, 0.5% Peptone,0.5% NaCl, KH_2PO_4 170 mg, Na_2HPO_4 980 mg, $(NH_4)_2SO_4$ 100 mg, $MgSO_4$ 4.87 mg, MgO 0.1mg, $FeSO_4$ 0.05 mg, $CaCO_3$ 0.20 mg, $ZnSO_4$ 0.08 mg, $CuSO_4.5H_2O$ 0.016 mg, $CoSO_4$ 0.015 mg, H_3BO_3 0.006 mg, distilled water 100 ml, drops of 0.1N NaOH to adjusted media to pH-9.0 for alkaliphilic bacterial strain.

3. Biodegradation experiments

Biodegradation was performed by adding 50 mg of fluoranthene to 100 ml of the 24 hrs grown culture (during the log phase). The conical flasks were placed on a rotary platform incubator shaker at 100 rpm at 37ºC and incubated for 12, 24, 36, 48, 60 and 72 hrs. The culture media of each flask was then centrifuged at 10,000xg for 15 min in cold centrifuge, Du Pont Instruments SORVALL RC-5B to separate the bacterial cell mass, and the resulting supernatant was preserved for the extraction of biodegradation products. The supernatant was extracted by dichloromethane (DCM) and then dried over sodium sulphate anhydrous. The solvent was evaporated at 40ºC to obtain the residue. The purified residue was subjected to thin layer chromatography (TLC) to confirm the number of metabolites. The degradation process was monitored spectrophotometricaly of JASCO V-630 analyzer.

4. Spectroscopy analysis

The extracted metabolites were subjected to TLC, using hexane and ethylacetat (9:1) as solvent phase, and the separated spots were observed under long-UV 365 nm and short-UV 254 nm light. The metabolites extract then was loaded on silica gel column to separate the metabolite by elution with dichloromethane (DCM) and concentrated by rotary evaporation at 40ºC. The recovered compounds were subjected to further analysis on Fourier transform infrared spectroscopy (FTIR) and the analysis was carried out, using the KBr pellet technique in the wavelength rang of 400-4000cm^{-1} on the shimadzu-8400 FTIR spectrophotometer. Gas Chromatography Mass (GC-MS) analysis was performed using a GB5 column with a 15-20 min runtime on a shimadzu-GC-MS-QP5050. Proton Nuclear Magnetic Resonance ^{1}HNMR studies for fluoranthene before bacterial

degradation and after the degradation was carried out in 300 MHz magnetic field, using $CdCl_3$ as a solvent on a Varian Mercury Spectrometer YH 300.

5. Enzyme activities

5.1 Preparation of cell free extract

Alkaliphilic and neutrophilic bacteria were grown in nutrient broth media containing 50mg of fluoranthene at 37°C for 24 hrs, centrifuged at 10,000 rpm for 20 min. (The cell mass of alkaliphilic bacteria was washed thrice by normal saline to bring the inner pH cell wall from pH 9.0 to pH 7.0). These cells were suspended in 50 mM potassium phosphate buffer (pH 7.4) for sonication, keeping sonicater output at 40 amplitude, maintaining temperature at 4°C and giving three strokes, each of 30 s with one min interval. The sonicated cell mass was centrifuged at 10000xg for 30 mins, the supernatant was used for enzyme assays.

5.2 Protein estimation

The cytosolic protein was estimated by the Lowry's method, using bovine serum albumin as standard [17]. The cytosolic cytochrome P450 was measured by the method of Omura and Sato [18].

5.3 Aminopyrine N-demethylase and Acetanilide hydroxylase activity

Aminopyrine-N-demethylase and acetanilide hydroxylase were assayed, using the procedure of Schenkman *et al*. [19]. Formaldehyde formed, during N-demethylation of aminopyrine, was estimated according to the procedure of Nash [20].

Results

Addition of 50 mg /100ml of fluoranthene to the 24 hr grown culture has resulted in more than 90% degradation of fluoranthene by both alkaliphilic and neutrophilic bacteria under shaking condition for 60 hrs. The growth of microorganism was steady up to 24 hrs of incubation and further it decline after 36 hrs. The UV-visible spectral analysis indicates the continuous decrease in the intensity of the parent fluoranthene molecule absorption peaks at 235nm, 277nm and 287nm. After 24 hrs of incubation, the decrease in intensity of peaks was slow up to(36 hrs.), and the peaks were disappeared after 48 hrs. **(**Fig. 1**)**

1. Identification of fluoranthene degradation products by alkaliphilic bacteria

The structural analysis of the intermediates/metabolites of fluoranthene collected at interval of each 12 hrs is determined by IR, GC-MS and ^{1}HNMR spectroscopy (Table 1). The tentative pathway for fluoranthene degradation by alkaliphilic bacterial strain *Bacillus badius* D1 (Fig. 2) has been established. The GC-MS chromatography illustrated that.

Metabolite I with retention time (Rt 13.633) and a molecular ion at an m/z of 236 and fragmentation ions 221[M+-15], 207[M+-29], 193[M+-43], 179 the losses correspond to [M+-CH_3], [M+-CHO], [CH_3CO] this compound is identified as 2, 3-dihydroxyfluoranthene-2, 3-diol. Further confirmation of this metabolite has been made by^{1}HNMR chemical shift {δ2.35 (d, H, Ar-OH, D_2O Exchangeable) 5.3-6.0 (d, H, Ar-H), 7.35-7.92 (8, H, M, Ar-H). FTIR spectra have shown the broad peak of hydroxyl group at 3435.34.

The mass spectrum of Metabolites II, with retention time (Rt 15.408) , showed a molecular ion at m/z 267, [M^++1] and fragment ions at m/z of 253 [M+ - 13], 207 [M^+ -59] and 184. The losses match to [M+ -CH], [M^+ - $C_2H_3O_2$], respectively, the ^{1}HNMR spectrum of metabolite II is in full agreement with the structure of (Z)-9-(Carboxymethylene)-9H-fluorene-1-carboxylic acid. The ^{1}HNMR chemical shifts for metabolite II is {δ10.95 (d, H, Ar-COOH, D2O Exchangeable is done) 6.20-7.42 (8H, M, Ar-H) and FTIR spectra showed the carbonyl group at 1668.48.

Metabolite III was identified as 9-fluorenone-1-carboxylic acid by GC-MS analysis of its retention time (14.592). The compound had a molecular ion at an m/z of 224 [M^+-1] and fragment ions at m/z of 205[M+-18], 193[M+-30], 180[M^+-43] and 154, matching to probable losses of [M+-H_2O], [M+-CH_2O], [M+-CH_3CO]. The structure of metabolite III was determined by FTIR, showed the carbonyl group at 1683.91.

Metabolite IV with retention time of (Rt. 19.133) and the GC-MS chromatography analysis of metabolite IV showed an apparent molecular ion [M+] at an m/z of 211, fragment ions at m/z of

183 [M+ - 28] , 166 [M+ - 45],and 155[M+-56] corresponding to probable losses of CO, COOH and C_4H_8, respectively. The molecular weight and fragmentation pattern suggested that metabolite is identified as benzene 1, 2, 3-tricarboxylic acid. FTIR spectra showed the carbonyl group at 1660.77, 1631.83.

Metabolites, V was identified as 3, 4-dihydroxybenzoic acid, the retention time for this compound was (10.450 min), and molecular weight at m/z was 155 and the fragment pattern [142,126 and 113) this compound was comfier with FTIR spectra showing that, broad peak is at 3232.80 for the hydroxyl group and carbonyl group peak is at 1666.55.

Metabolite VI with retention time (9.055 min) and molecular ion at m/z was 127 with fragmentation ions at m/z [113, 99, 85] this metabolite was detected as 2, 5-dihydro-5-oxofuran-2-carboxylic acid,**(**Fig. 3**)** by comfier with HNMR as well as FTIR was shown that analysis of HNMR spectra {δ10.53,S,H,Ar-COOH,D_2OExchangaeble)6.0-7.72 (d,H,Ar-H) and FTIR spectra showed the 1737.93,1678.13 for (COOH) and the peak at 1618.33 for the (C=O).

2. Identification of fluoranthene degradation products by neutrophilic bacteria

Fluoranthene metabolites resulting from degradation by a *Lysinbacillus sphaericus* DL8 has also been studied. Seven metabolite were detected and pathways were proposed for the metabolism of fluoranthene by neutrophilic *Lysinbacillus sphaericus* DL8 strain are summarized in (Fig. 2) and the spectroscopy analysis as shown in (Table 2).

Metabolite (I) with retention time (Rt 11.717) has a molecular ion at m/z 234 and fragmentation ions at m/z of 221, [M-CH] 205 [M-COH] 189 [COOH]. The metabolite was identified as 2, 3-dihyroxyfluoranthene. The metabolite confirmed with ^{1}HNMR spectra {δ5.35 (d, H, Ar-OH, D_2O Exchangeable) 7.28-7.90 (8H, M, Ar-H)} and the FTIR spectra illustrated the broad peak at 3228.95 for hydroxyl group.

Metabolite (II) has retention time of (14.275) with a molecular ion at m/z 266 and significant fragment ions at m/z 253[M-CH] 207[M-$C_2H_3O_2$], 193 and the metabolite was identified as (Z)-9-(carboxymethylene)-9H-fluorene-1-carboxylic acid, FTIR spectra has shown the carbonyl group at 1708.99.

Metabolite (III) have Rt 15.283 with a molecular ion at m/z of 224 and fragment ions at m/z of 212 [M-C], 206 [M-H_2O], 193 [M-CH_3O] and the metabolite was identified as 9-fluorene-1-carboxilic acid. FTIR spectra of this compound has shown the hydroxyl group at 3228.95 and carbonyl group at 1668.48.

Metabolite (IV) has Rt- 7.742 with a molecular ion 226 and fragmentation ions at m/z of 197 [M-CHO], 183 [M-CH_3CO] and169 [M-C_2HO_2], the metabolite was identified as 9-hydroxy-9H-fluorene-1-carboxylic acid. FTIR spectra has shown the broad peak for hydroxyl group at 3379.40(OH), and peak for carbonyl group at 1707.66.

Metabolite (V) with an (Rt of 4.300) the metabolite has a molecular ion at m/z of 248 and fragment ions at m/z of 233[M-CH_3], 219[M-CHO], 203[M-C_2H_5O] , this is consistent with 7-methoxyfluoranthene **(**Fig. 5**)** Confirmed this metabolite with FTIR spectra where it has been shown the broad peak for hydroxyl group at 3321.53 and peak at 1708.23 for carbonyl group. HNMR spectra has shown that {δ3.0(t.H,CH_3) 4.98 (S,H,Ar-OH,D_2OExchangaeble is done) 6.80 (d,H,Ar-H)7.40-8.25 (6H,M,Ar-H).

Metabolite (VI), with retention time (Rt-13.800) the metabolite has a molecular ion at m/z of 210 [M+] and fragmentation ions at m/z of 193[M-H_2O], 165[M-COOH], 155[C_2O_2H]. This compound is identified as benzene-1, 2, 3-tricarboxylic acid. The FTIR spectra showed carbonyl group at 1762.02.

Metabolite (VII) has Rt 11.950, molecular weight at m/z of 168[M+2H] and fragmentation ion at m/z of 150[M-H2O], 122[M-CH_3O_2], 132. The molecular was identified as phthalic acid. The FTIR spectrum of this compound has shown the broad peak at 3440.21 for hydroxyl group and carbonyl group at 1724.42.

3. Enzyme activities during degradation of fluoranthene

Fig.4 shows the effect of fluoranthene on bacterial biotransformation enzyme activity such as aminopyrine-N-demethylase and acetanilide hydroxylase and content of cytochrome P450. Incubation of 24 hr. grown alkaliphilic *Bacillus badius* D1 and neutrophilic *Lysinbacillus*

sphaericus DL8 with fluoranthene at a concentration of 50 mg/ 100 ml, has resulted in significant increase in the cytosolic protein and the content of cytochrome P450. (Table 3) similarly the activities of aminopyrine-N-demethylase and acetanilide hydroxylase were found to be increased significantly as compared to their respective controls (Table 3).

4. Factors influencing fluoranthene degradation

The effect of various parameters, like concentration of fluoranthene, pH and temperature on the degradation of fluoranthene by both alkaliphilic and neutrophilic bacteria, is reported.

4.1 Effect of Concentration

Effect of fluoranthene degradation was performed at different concentration (25, 50, 75,100 and 125mg)/100ml of media. The effect of fluoranthene concentration on degradation by alkaliphilic and neutrophilic strains has been shown in (Fig. 5), 25mg of initial concentration was complete degradation in 48hrs, while, in case of 50mg/100ml of initial concentration of fluoranthene, 90% of fluoranthene was degraded in culture by alkaliphilic and neutrophilic bacteria respectively. In case of higher concentration 125mg/100ml the degradation has been observed as 54% and 39% of fluoranthene was degraded by both alkaliphilic and neutrophilic respectively.

4.2 Effect of pH

Effect of pH on the degradation has been shown in (Fig. 6A**)** Incubation of fluoranthene at a concentration of 50 mg/100 ml in pH ranging from 6.0 to 11.0 showed the maximum degradation rate in between 9.0-10.0 (90 and 80% respectively) in case of alkaliphilic bacteria and (88%) of degradation at pH 7.00 in case of neutrophilic bacteria.

4.3 Effect of Temperature

Fig. 6B shows the effect of temperature on the degradation of fluoranthene by alkaliphilic and neutrophilic bacteria strain Incubation fluoranthene at a concentration of 50 mg/100 ml at various temperatures (25-50 °C), the biodegradation rate of this compound was (90%) at 35°C by both strains.

4. Discussion

Degradation of fluoranthene by alkaliphilic and neutrophilic bacteria has been observed. The 90% of added fluoranthene has been degraded after 60 hrs of incubation. Gordon and Dobson [21] reported that 95% of the fluoranthene had been degraded by *Pseudomonas alcaligenes* PA-10 after 168 hrs and Kelley *et al.,* [15] mentioned that in their study 78% of added fluoranthene was degraded after 5 days of incubation. In this study, we obtained the best result campier to others. The decrease in intensity of UV-visible peaks, resulted in disappearance of absorption peaks due to degradation of aromatic rings of fluoranthene to intermediates metabolites.

In the present study, the comparison of degradation pathway of fluoranthene, by alkaliphilic and neutrophilic bacterial strains, has been made.

The dioxygenation pathway appears to be the special feature of alkaliphilic and neutrophilic bacteria, the whole pathway consists of several enzymes that include ring-hydroxylating oxygenases, dihydrodiol dehydrogenase, ring cleavage dioxygenase and decarboxylase.

In case of alkaliphilic bacterial strain *B. badius* D1, the fluoranthene, degradation routes involving initial dioxygenation at C2 and C3 positions to produce 2, 3-dihydrofluoranthene 2, 3-diol (I). The metabolite (Z)-9-carboxymethylene-9H-fluorene-1-carboxylic acid (II) is produced from the dehydrogenation of 2, 3-dihydrofluoranthene 2, 3-diol and ring cleavage of one of the fused benzene rings followed by dioxygenation reaction, this metabolite was also identified during fluoranthene degradation by *Mycobacterium* sp KR20 [22], the further degradation of (Z)-9-carboxymethylene-9H-fluorene-1-carboxylic acid (II) via dioxygenation to give 9-fluorenone-1-carboxylic acid (III), the same reactions were also reported in *Mycobacterium* sp. strain KR20 [22]. In this case, alkaliphilic bacterial strain transformed 9-fluorenone-1-carboxylic acid (III) via angular dioxygenation followed by ring cleavage of five member ring of 9-fluorenone-1-carboxylic acid (III) to form benzene-1, 2, 3-tricarboxylic acid (IV). Benzene-1, 2, 3-tricarboxyylic acid was decarboxylated to form 3, 4-dihydroxybenzoic acid (V) this metabolite compound one of the main

fluoranthene central metabolites, which is transformed to 2, 5-dihydro-5-oxofuran-2-carboxylic acid, in the fluoranthene pathway would be further degraded to TCA cycle intermediates.

In the case of neutrophilic bacterial *Lysinbacillus sphaericus* DL8 strain, the biodegradation of fluoranthene produced seven metabolites: (2,3-dihydroxyfluoranthene, (Z)-9 carboxymethylene-9H-fluorene-1-carboxylic acid, 9-fluorenene-1-carboxylic acid, 9-hydroxy-9H-fluorenene-1-carboxylic acid, benzene-1,2,3-tricarboxylic acid ,7-methoxy-8-hydroxyfluoranthene, and phthalic acid, , were identified.

On the basis of identification of metabolites formed during the degradation of fluoranthene by neutrophilic bacterial strain *Lysinbacillus sphaericus* DL8, two routes pathway has been proposed. Initiate dioxygenase attack at C2, C3 positions to form 2, 3-dihydroxyfluoranthen, others dioxygenation reaction attack at C7, C8 positions to form 7, 8-dihydroxyfluoranthen.

The dioxygenation reaction attack C-2 and C3 to produce 2, 3-dihydroxyfluranthene (I). The metabolite (Z)-9-carboxymethylene-9H-fluorene-1-carboxylic acid (II) is resulted from the dehydrogenation of 2, 3-dihydroxyfluoranthene (I) and subsequent intradiol ring cleavage. The produce 9-fluorenene-1-carboxylic acid (III), is similar to the degradation of fluoranthene by alkaliphilic bacteria, and also reported in *Mycobacterium* sp strain KR20 [22].

9-fluorenene-1-carboxylic acid leads to the formation of metabolite 9-hydroxy-9H-fluorenene-1-carboxylic acid (IV). The presence of 9-hydroxy-9H-fluorenene-1-carboxylic acid indicates an alternative pathway which occurs simultaneously with the 9-fluorenene-1-carboxylic acid. These metabolites also reported in Mycobacterium sp. strain AP1 [23] and strain PYR-1 [15]. This dioxygenation route via 9-fluorenone-1-carboxylic acid was degraded to benzene-1, 2, 3-tricarboxylic acid (VI) via dioxygenation reaction. Benzene-1, 2, 3-tricarboxylic acid was decarboxylated to phthalic acid (VII).

Phthalic acid is one of the main fluoranthene central metabolites that would be further degraded to TCA cycle intermediates via phthalate degradation pathway.

In addition, the 7,8-dioxygenation route with the detection of ring cleavage products for the degradation of fluoranthene by neutrophilic bacterial strain, The detection of 8-hydroxy-7-methoxyfluoranthene also suggests that dioxygenation of the single benzene ring on the fluoranthene molecule occurred at positions C7 and C8. It was initially proposed in both *M. vanbaalenii* PYR-1 and *A. denitrificans* WW1 [24]. The formation of -7-methoxy-8-hydroxy fluoranthene by catechol-O-methyltransferase was reported in mycobacterium strain PYR-1 [15].

The formation of 7, 8-dihydroxyfluranthene with O-methylation at carbon 7 would form 7-methoxy-8-hydroxylfluranthene, this metabolite was detected in our strain neutrophilic bacterial but not in alkaliphilic bacterial strain and also reported in strain PYR-1 [15].

The oxidative biodegradation of fluoranthene is attributed to mono and di oxygenases. This has been further supported by increase in the activities of enzymes, like aminopyrine N-demethylase and acetanilide hydroxylase, and increase in the content of cytochrome P-450 when the bacterial strains were incubated in presence of fluoranthene.

Environmental factors affecting biodegradation of fluoranthene were conducted at different parameters. At higher concentration of fluoranthene, the observed rate of degradation was slow; this may be due toxic effect of liberated metabolites on cell growth. It has been also observed that the rate of fluoranthene degradation was higher in pH between 8-9 in case of alkaliphilic bacterial strain and pH 7.0 in case of neutrophilic bacteria at 35 ºC.

The ability of these microorganisms to degraded fluoranthene at 60 hrs of incubation given evidence of the usefulness of alkaliphilic *Bacillus badius* D1 and neutrophilic *Lysinbacillus sphaericus* DL8 strain for bioremediation of some PAHs and cleaning up the contaminated sites.

5. Conclusions

This study is the first published document to demonstrate alkaliphilic bacteria *Bacillus badius* D1and neutrophilic *Lysinbacillus sphaericus* DL8 to grow and degraded fluoranthene and utilised it as carbon source, 90% of fluoranthene was degraded, and this result is the best when campier to other studied. According to the metabolites identified in this study, the same fluoranthene

biodegradation pathway was used by both bacterial strains. Dioxygenase activity was found to be present in this study during degradation of Fluoranthene, as well as the oxidation, reduction, dehydrogenation and decarboxylation reactions by alkaliphilic, and neutrophilic bacteria were observed via the fluoranthene degradation pathways.

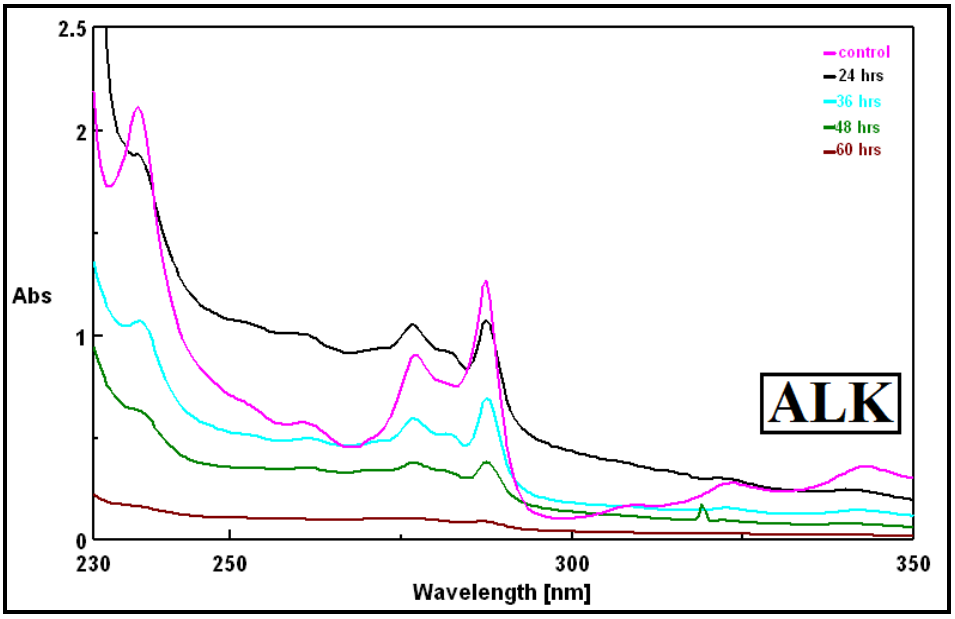

Fig.1A. The UV-Visible Overlap of Fluoranthene degradation by Alkaliphilic bacteria *Bacillus badius* D1

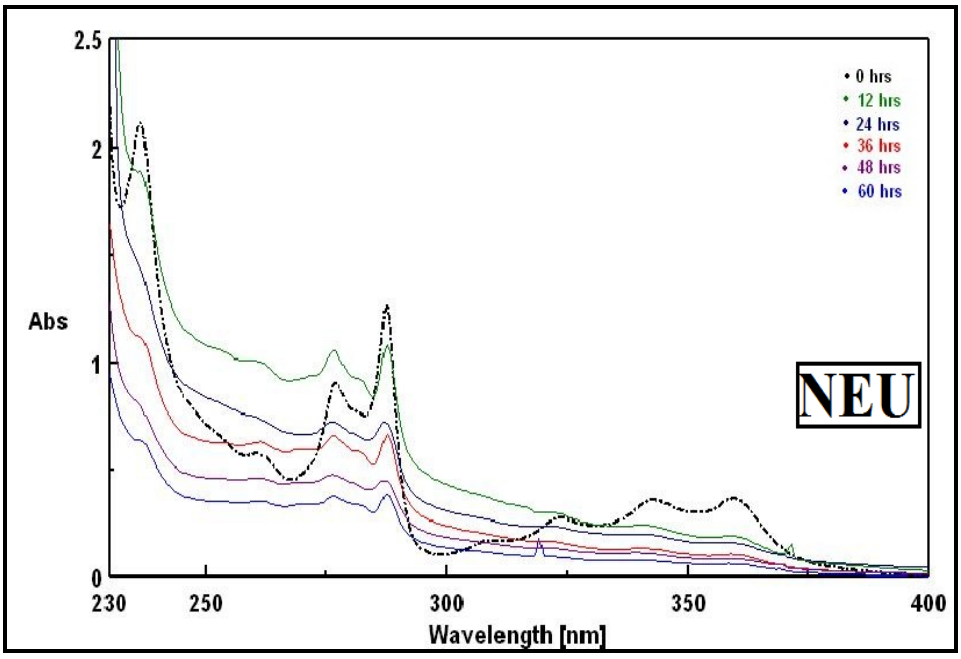

Fig.1B. The UV-Visible Overlap of Fluoranthene degradation by Neutrophilic bacteria *Lysinbacillus sphaericus* DL8

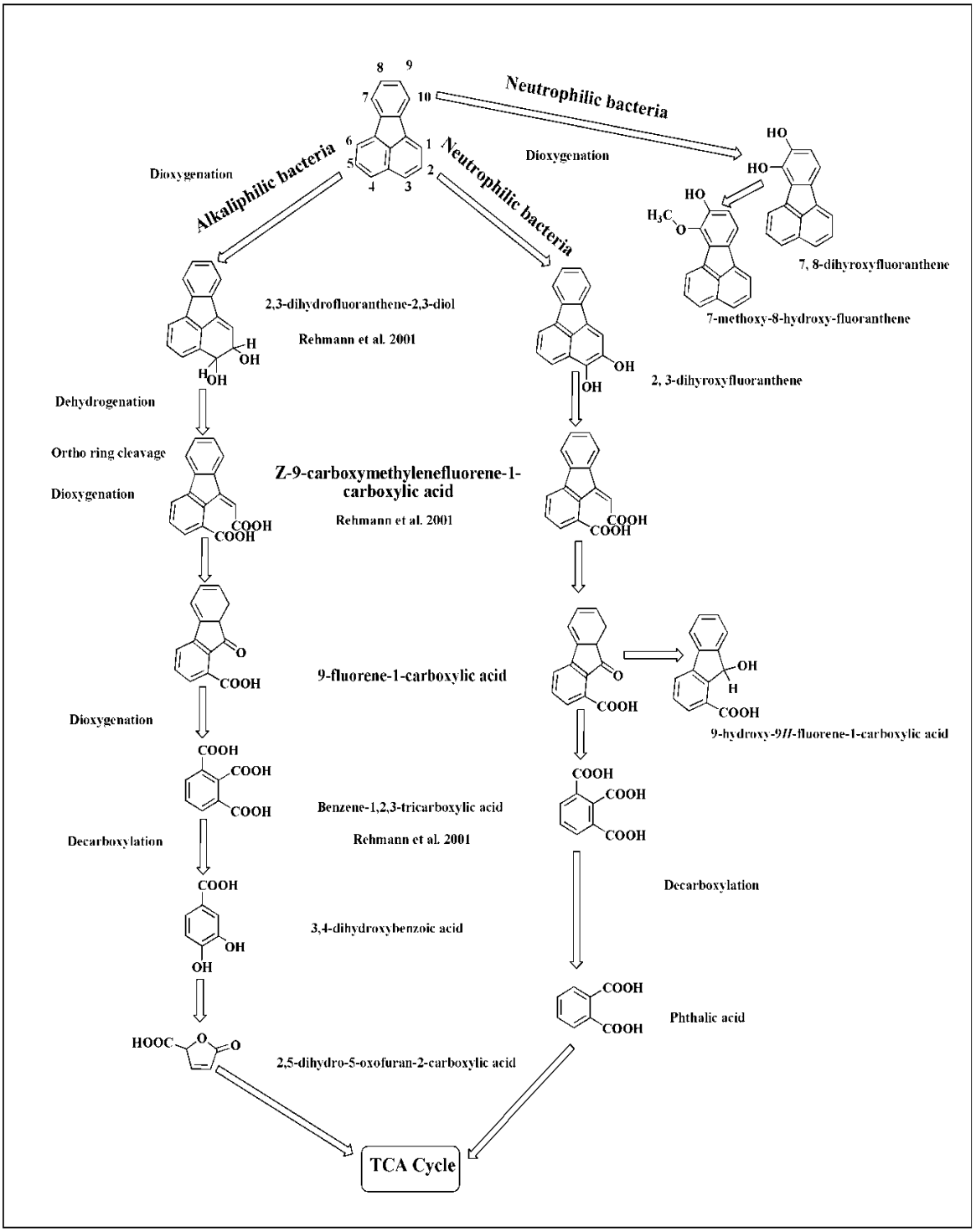

Fig.2. Proposed pathway of degradation of fluoranthene by alkaliphilic *Bacillus badius* D1 and neutrophilic Lysinbacillus *sphaericus* DL8.

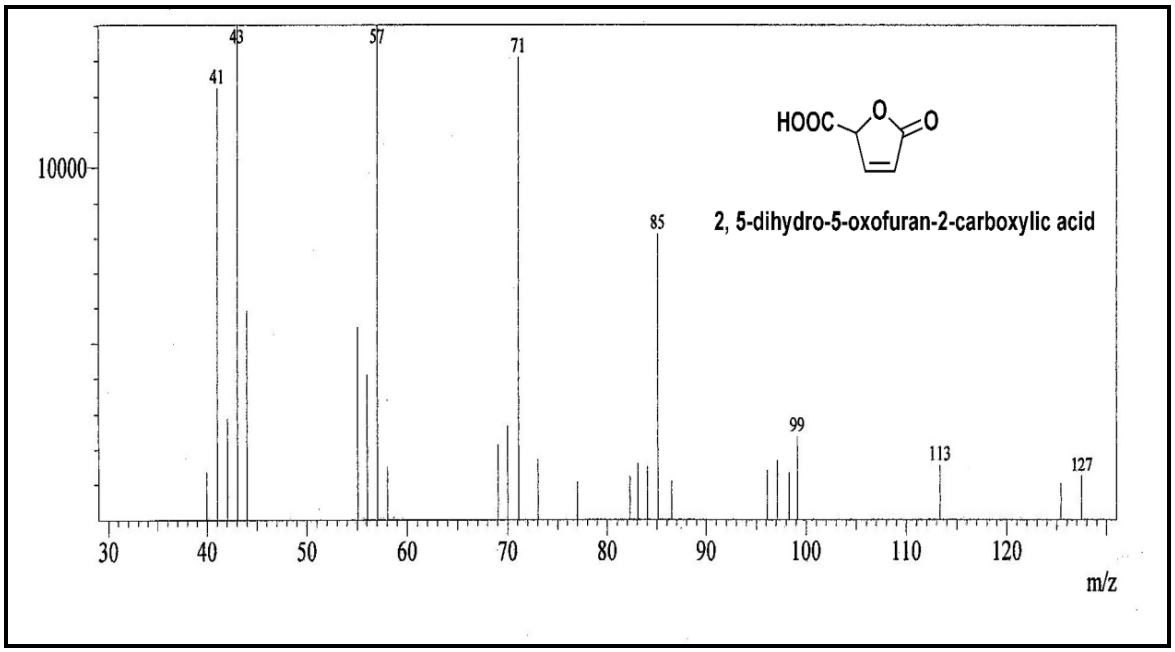

Fig.3A. GC-MS chromatography shown 2, 5-dihydro-5-oxofuran-2-carboxylic acid during the degradation of fluoranthene by alkaliphilic bacterial Bacillus *badius* D1 strain and neutrophilic bacterial *Lysinbacillus sphaericus* DL8 strain

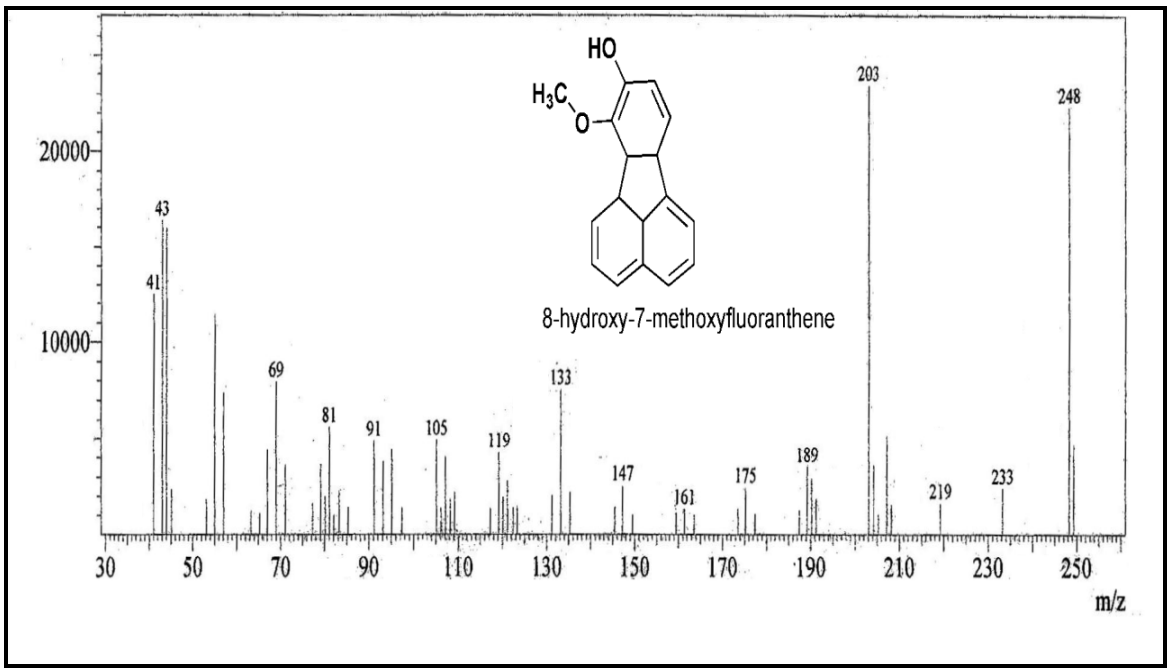

Fig.3B. GC-MS chromatography shown 8-hydroxy-7-methoxyfluoranthene during the degradation of fluoranthene by alkaliphilic bacterial Bacillus *badius* D1 strain and neutrophilic bacterial *Lysinbacillus sphaericus* DL8 strain

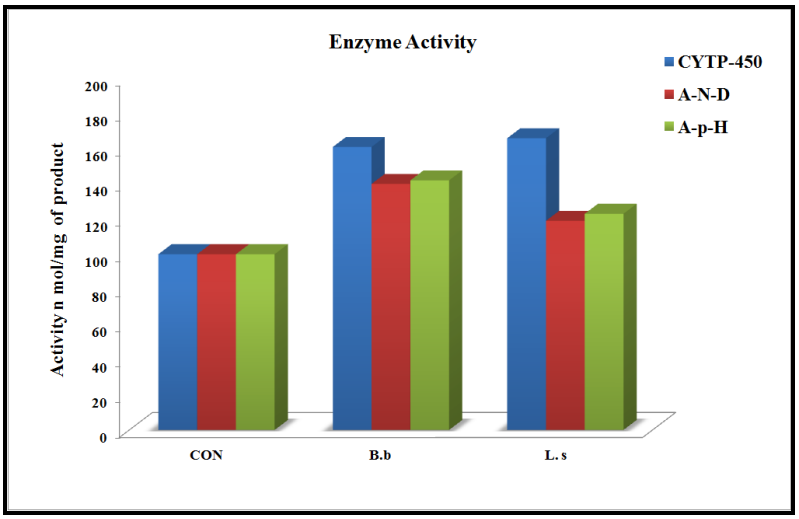

Fig.4. Effect of fluoranthene on enzyme activity by Alkaliphilic bacteria *Bacillus badius* D1 and Neutrophilic bacteria *Lysinbacillus sphaericus* DL8

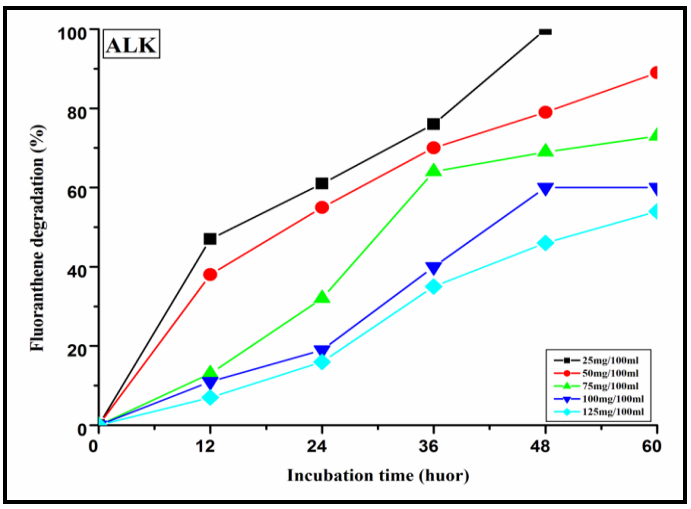

Fig.5A. The influence of initial concentration of fluoranthene on the degradation by alkaliphilic (ALK) .

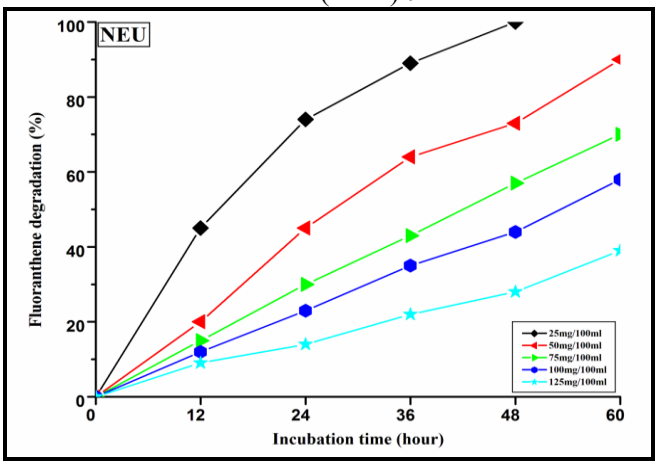

Fig.5B. The influence of initial concentration of fluoranthene on the degradation by neutrophilic bacteria (NEU).

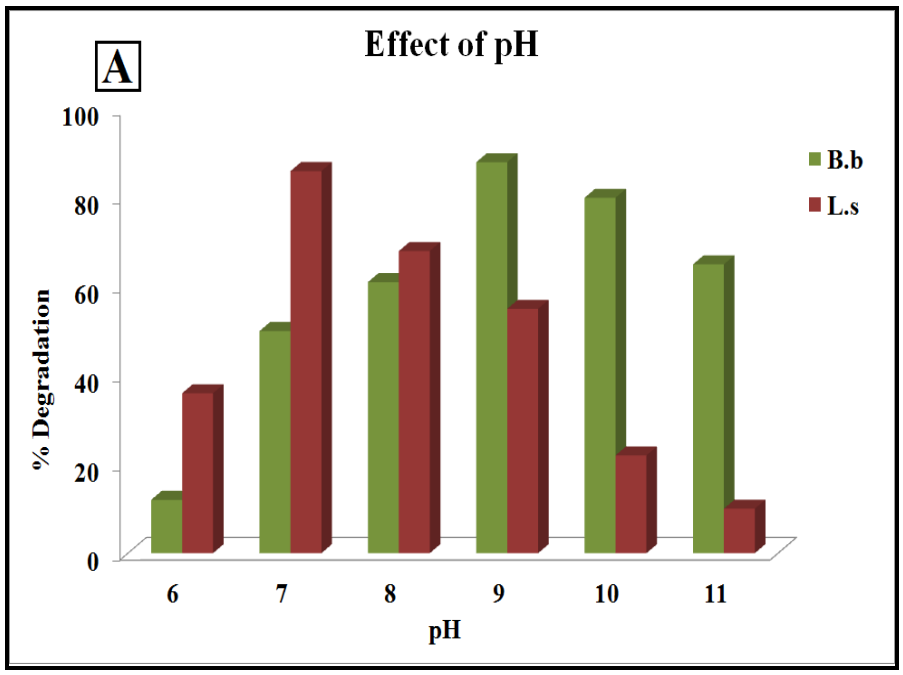

Fig.6A. Effect of pH on the fluoranthene degradation by Alkaliphilic *Bacillus badius* D1 and Neutrophilic bacteria *Lysinbacillus sphaericus* DL8

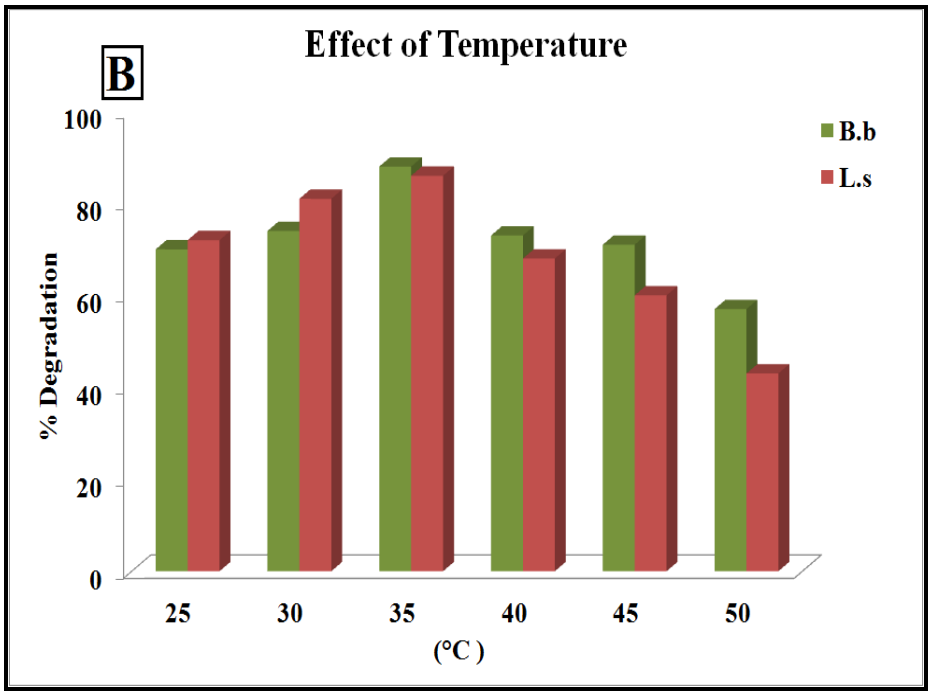

Fig.6B. Effect of temperature on the fluoranthene degradation by Alkaliphilic *Bacillus badius* D1 and Neutrophilic bacteria *Lysinbacillus sphaericus* DL

Table 1. Spectroscopic analysis of all intermediates during degradation of fluoranthene by alkaliphilic *Bacillus badius* D1

Metabolites	Form	Rt/min	M.w	Fragment ions	FTIR spectra cm	HNMR
2,3dihydrofluoranthene2, 3- diol	$C_{16}H_{12}$	13.633	236	221,207,193	3435.34, OH	{δ2.35(d,H,Ar-OH, D2OEX)5.3-6.0 (d,H,Ar-H),7.35-7.92 (8,H,M,Ar-H)
(Z)-9-(Carboxymethylene) -9H-fluorene-1-carboxylic acid	$C_{16}H_{10}$	15.408	266	253,207,184	1668.48, COOH	{δ10.95(d,H,Ar-COOH, D_2OEX)6.20-7.42 (8H,M,Ar-H)
9-fluorenone-1-carboxylic acid	$C_{14}H_{10}$	14.592	224	205,193,180	1683.91, COOH	ND
Benzene1,2,3-tricarboxylic acid	C_9H_6O	19.133	211	183,166,155	1660.77,1631.83, COOH	ND
3, 4-dihydroxybenzoic acid	C_7H_6O	10.450	155	142,126,113	3232.80,OH, 1666.55,COOH	ND
2,5-dihydro-5oxofuran-2-carboxylic Acid	C_5H_4O	9.055	127	113,99,85	1737.93, COOH,1712.85 C=O	{δ10.53,S,H,Ar-COOH, D_2OExchangable) 6.0-7.72 (d,H,Ar-H)

Table 2. Spectroscopic analysis of all intermediates during degradation of fluoranthene by neutrophilic *Lysinbacillus sphaericus* DL8

Metabolites	Formula	Rt/min	M.w	Fragment ions	FTIR spectra	HNMR
2, 3-dihyroxyfluoranthene	$C_{16}H_{10}O_2$	14.500	234	221,206,189	3228.95,OH	{δ5.35(d,H,Ar-OH, D2OEX)7.28-7.90 (8H,M,Ar-H)}
(Z)-9-(carboxymethylene)-9H-fluorene-1-carboxylic acid	$C_{16}H_{10}O_4$	14.275	266	253,207,193	1708.99,COOH	ND
9-fluorene-1-carboxilic acid	$C_{14}H_8O_3$	15.283	224	212,206,193	228.95,OH,1668.48,COOH	ND
9-hydroxy-9H-fluorene-1-carboxylic acid	$C_{14}H_{10}O_3$	7.742	226	197,183,169	379.40,OH,1707.66,C=O	ND
8-hydroxy-7-methoxy fluoranthene	$C_{17}H_{12}O_2$	4.300	248	233,219,203	321.53,OH,1708.23,C=O	{δ3.0(t.H,CH3)4.98(S,H, Ar-OH,D2OEX) 6.80 (d,H,Ar-H) 7.40-8.25 (6H,M,Ar-H)
Benzene-1,2,3-tricarboxylic acid	$C_9H_6O_6$	13.800	210	193,165,155	448.84,OH1762,COOH	ND
Phthalic acid	$C_8H_6O_4$	11.950	168	150,132,113	440.21,OH.1724.42,COOH	ND

ND*: No detected

Table 3. Effect of fluoranthene on the enzyme activity by alkaliphilic bacteria *Bacillus badius* D1 and neutrophilic bacteria *Lysinbacillus sphaericus* DL8

Bacterial strain	**Alkaliphilic bacteria *Bacillus badius* D1**		**Neutrophilic bacteria *Lysinbacillus sphaericus* DL8**	
	Control	Fluoranthene	Control	Fluoranthene
Cell Mass	0.6885	0.98	0.6722	1.015
Protein Concentration	0.391	0.4896	0.542	0.829
Cytochrome P450	100%	161%	100%	166%
Aminopyrine-N-Demethelayze	100%	140%	100%	142%
Acetanilide-p- Hydroxylase	100%	119%	100%	123%

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التحلل الحيوي لمركب الفلورانثين بواسطة البكتيريا القلوية والمتعادلة

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DOI: https://doi.org/10.47372/uajnas.2016.n2.a07

الملخص

تم عزل اثنين من السلالات البكتيرية، البكتيريا العصوية القلوية Bacteria *Bacillus* التي تم عزلها من بحيرة لونار القلوية، مقاطعة بولدانا، ولاية مهراشترا، الهند، والبكتيريا الكروية المتعادلة *Lysinbacillus sphaericus* من نفايات مخلفات صناعة الغزل والنسيج، اتشالكارانجي، الهند. مركب الفلورانثين استعمل مصدر للطاقة لهذه الأنواع من البكتيريا، والفلورانثين من الهيدروكربونات العطرية المتعددة الحلقات (أربع حلقات). تم عزل نواتج (المركبات الوسطية) تحلل مركب فلورانثين كل 12 ساعة من فترة الحضانة. وقد تم التعرف على نواتج الأيض باستعمال (GC-MS، FTIR وHNMR1). تم الكشف عن العديد من نواتج الأيض من تحلل فلورانثين مثل: (9-فلورينون-1-حمض كربوكسيل، 9-هيدروكسي-1-فلورين حمض كربوكسيل، 2-كربوكسي بنزالدهيد ، حمض البنزويك وحمض الفثاليك). وقد لوحظ أنَ هذه البكتيريا عملت على تحلل مركب الفلورانثين بطريقتين مختلفتين. أدى حضانة الفلورانثين مع هذه السلالات البكتيرية أيضاً في حث نشاط أنزيمات التحويل الحيوية مونوأوكسيجينيز مثل: الامينوبيرين N – ثنائي ميثيلاز، أسيتانيليد هيدروكسيلاز ومحتوى السيتوكروم P-450. كذلك تمت دراسة تأثير بعض المعاملات التجريبية (تركيز فلورانثين، درجة الحموضة، ودرجة الحرارة) على تحطم فلورانثين.

الكلمات المفتاحية: محبة للقلوية، البكتيريا العصوية، البكتيريا الكروية، فلورانثين، التحطم الحيوي.